Expression of liver plasma membrane transporters in gallstone-susceptible and gallstone-resistant mice

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We tested the hypothesis that differential expression of liver plasma membrane transporters might account for variations in biliary lipid secretion rates between gallstone-susceptible C57L/J and gallstone-resistant AKR/J mice. Plasma membrane fractions and total RNA isolated from livers of mice fed with a control or lithogenic (15% fat/1.25% cholesterol/0.5% cholic acid) diet were used for measurements of steady-state gene expression of hepatobiliary transport systems for bile salts (*Ntcp1*/*Slc10a1*, *Oatp1*/*Slc21a1* and *Bsep*/*Abcb11*), phospholipids (*Mdr2*/*Abcb4*), organic anions (*Mrp2*/*Abcc2*) and organic cations (*Oct1*/*Slc22a1*). Irrespective of the diet, the steady-state gene expression of hepatobiliary transporters did not differ significantly between the two strains. Despite a higher basal bile flow and bile-salt secretion in C57L mice, *Mrp2* (*Abcc2*) and *Bsep* (*Abcb11*) expression did not differ between the two strains. Elevated biliary phospholipid secretion in response to the lithogenic diet was linked to increased Mdr2 (Abcb4) protein expression, whereas the induction of *Oct1* (*Slc22a1*) might reflect an enhanced uptake of choline for augmented phospholipid synthesis. In response to the lithogenic diet, Bsep (Abcb11) protein expression was up-regulated only marginally and bile salt secretion did not increase. The down-regulation of Ntcp1 (Slc10a1) protein expression might protect hepatocytes from high intracellular bile-salt loads. We conclude that variations in protein function rather than in the gene expression of liver plasma membrane transporters might account for variations in biliary lipid secretion rates. Our findings support the concept that the formation of lithogenic bile is caused by the hypersecretion of bile salts as a result of augmented availability of canalicular membrane cholesterol, possibly amplified by bile-salt–phospholipid uncoupling due to the increased bile flow.

Key words: biliary lipids, *Lith* genes, organic anions, organic cations.

INTRODUCTION

Bile flow and biliary lipid secretion depend in part on the concerted action of canalicular and sinusoidal liver plasma membrane transport proteins [1]. Traditionally, bile flow has been divided into a bile-salt-dependent and a bile-salt-independent fraction. The multidrug-resistance-associated protein Mrp2 (Abcc2) in the canalicular membrane is responsible for the biliary secretion of glutathione conjugates, generating the bile-salt-independent bile flow [1]. The secretion of bile salts into the canaliculus is mediated by the ATP-dependent action of the bile-salt export pump Bsep (Abcb11), which is localized exclusively to the canalicular membrane [2]. Whereas biliary phospholipid secretion requires the canalicular expression of the multidrug resistance protein Mdr2 (Abcb4) [3], the identification of a canalicular cholesterol-transporting protein responsible for biliary cholesterol secretion, if present, is still awaited.

At the sinusoidal membrane of the hepatocyte, the Na^+-dependent taurocholate co-transporting protein Ntcp1 (Slc10a1) accounts for most of the hepatocellular uptake of conjugated bile salts [4]. A second sinusoidal membrane protein, the organic-anion-transporting polypeptide Oatp1 (Slc21a1), belongs to the organic anion family of solute carriers [1] and mediates the Na^+-independent bidirectional transport of bile salts [5]. Organic cations, including choline, which is required for phosphatidylcholine synthesis, are cleared from blood by the organic-cation-transporting protein Oct1 (Slc22a1) [6]. Finally, lipoprotein cholesterol uptake into hepatocytes might involve the low-density and high-density lipoprotein receptors Ldlr [7] and Srb1 [8] and a chylomicron remnant receptor [9].

When inbred AKR/J and C57L/J mice were fed with a lithogenic diet for several weeks, cholesterol gallstones formed only in the C57L strain [10]. Combining quantitative trait locus analysis with mouse chromosome mapping results identified *Bsep* (*Abcc11*) and *Mrp2* (*Abcc2*) as putative gallstone (*Lith*) genes [11]. Phenotypic characterization revealed that susceptibility to gallstones in C57L mice is associated with higher bile flow and bile-salt secretion rates [12,13]. Moreover, biliary cholesterol secretion is elevated out of proportion to the increased phospholipid secretion, thereby contributing to cholesterol-supersaturated bile and the formation of cholesterol gallstones [13,14]. It is still unknown whether differences in bile flow and biliary lipid secretion rates between gallstone-susceptible C57L and gallstone-resistant AKR mice are related to alterations in the expression or function of sinusoidal and canalicular membrane transporters. We therefore attempted to help in the achievement of convergence of mouse genotype and phenotype by investigating the gene expression of liver plasma membrane transporters

Abbreviations used: Bsep (Abcb11), bile-salt export pump; DPP IV, dipeptidyl peptidase IV; *Gapdh*, gene encoding glyceraldehyde-3-phosphate dehydrogenase; Mdr2 (Abcb4), multidrug resistance 2 P-glycoprotein; Mrp2 (Abcc2), multidrug-resistance-associated protein 2; Ntcp1 (Slc10a1), Na^+-dependent taurocholate co-transporting protein 1; Oatp1 (Slc21a1), organic-anion-transporting polypeptide 1; Oct1 (Slc22a1), organic-cation-transporting protein 1; RT–PCR, reverse-transcriptase-mediated PCR.

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relevant for bile flow and biliary lipid secretion during lithogenesis in AKR and C57L mice.

EXPERIMENTAL

Materials

Taq DNA polymerase was purchased from Boehringer Mannheim (Germany) and primers were obtained from MWG Biotech (Ebersberg, Germany). Polyclonal NTCP1 (K4) and OATP1 (K10) antibodies were kindly provided by B. Stieger (University Hospital Zurich, Zurich, Switzerland); they are described elsewhere [15,16]. An antibody directed against a C-terminal peptide of the deduced mouse Bsep protein [17] was a gift from R. M. Green (University of Chicago, Chicago, IL, U.S.A.). The polyclonal Mdr2 and dipeptidyl peptidase IV (DPP IV) antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). A monoclonal mouse anti-rabbit Na/K-ATPase a-1 antibody was obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Horseradish peroxidase-labelled secondary antibodies were from Dako Chemicals (Hamburg, Germany). Unless indicated otherwise, materials of the highest purity grade commercially available were obtained from Sigma Chemicals (Deisenhofen, Germany) or Biometra (Göttingen, Germany).

Animals

AKR/J and C57L/J mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) and bred to generate our own mouse colony. The mice were maintained under constant light–dark cycles with free access to water and standard Altromin 1314 chow (less than 0.02 % cholesterol; Altromin GmbH, Lage, Germany). Age-matched 12-week-old male animals received either a control or a lithogenic diet consisting of 15 % fat, 1.25 % cholesterol and 0.5 % cholic acid (ICN, Eschwege, Germany) for 28 days. Although the diet contained a significant amount of cholic acid, none of the animals developed diarrhoea. With the use of liver histology and measurements of liver enzymes, we excluded cholestasis and significant liver disease. Throughout the experimental period, all animals received human care in accordance with the criteria for the care and use of laboratory animals. Protocols were approved by the Institutional Animal Care and Use Committee. The killing method was consistent with recommendations of the American Veterinary Medical Association.

Isolation of liver plasma membranes

Fasted mice of each strain, fed with either a control or a lithogenic diet, were anaesthetized with pentobarbital intraperitoneally (35 mg/kg body weight). Livers were harvested immediately and put into ice-cold buffer, rinsed and weighed. A small part of each liver was snap-frozen in liquid nitrogen for storage at −80 °C until use for RNA isolation. The remaining livers of four mice were pooled and plasma membranes enriched with sinusoidal and canalicular membrane fractions were isolated as described [18], then stored in liquid nitrogen for no longer than 4 weeks. Protein concentrations were measured as described previously [19]. The relative enrichment of plasma membranes with sinusoidal and canalicular membrane fractions was assessed by measurements of Na/K-ATPase and alkaline phosphatase activities respectively. In all membrane fractions prepared, the marker enzymes were enriched 11–14-fold, indicating a similar degree of purity to that described previously [18].

Western blot analysis

Equal quantities of mouse liver plasma membranes (50–100 μg of protein) were subjected to reducing SDS/PAGE [14]. Proteins were transferred electrophoretically to nitrocellulose membranes (pore size 0.45 μm) and equal protein transfer was documented with Ponceau S staining. The membranes were blocked for 3 h in Tris-buffered saline (pH 7.4) with 5 % (v/v) skimmed milk and 0.1 % (v/v) Tween. Antigen detection was performed with dilutions of the indicated antibodies (Ntcp1, 1:1000; Oatp1, 1:5000; Mdr2, 1:1000; Bsep, 1:500). Detection of immunoreactive protein was accomplished with a chemiluminescence kit (NEN Life Science) after incubation with horseradish peroxidase-labelled secondary antibodies (Dako Chemicals). To correct for variations in protein enrichment in basolateral and canalicular membranes, the blots were stripped and re-probed with a 1:1000 dilution of a mouse anti-rabbit Na/K-ATPase a-1 antibody or a goat anti-mouse DPP IV antibody respectively. Semiquantitative analysis was performed with a GS-700 Imaging Densitometer (Bio-Rad, Munich, Germany).

Table 1 Sequences of PCR primers

Gene symbols listed follow standard nomenclature for mouse genes as defined by the International Committee on Standardized Genetic Nomenclature in Mice.

Gene	Primer pair	Primer sequence	Cycles	PCR product	PCR GenBank® accession no.
Bsep/Abcb11	5′	5′-GAGGTTACTTAATAGCCTACG	29	414bp	AF133903
	3′	5′-CATCTATCATCACAGTTCCC			
Mrp2/Abcc2	5′	5′-CCTAGACAGCGGCAAGATTGT	23	277bp	AF227274
	3′	5′-TTACAGGGTGGTTGAGACCAG			
Mdr2/Abcb4	5′	5′-AAGAATTTGAAGTTGAGCTAAGTGA	29	138bp	NM_008830
	3′	5′-TGGTTTCCACATCCAGCCTAT			
Nctp1/Slc10a1	5′	5′-GGTTCTCATTCCTTGCGCCA	21	492bp	U95131
	3′	5′-GCATCTTCTGTTGCAGCAGC			
Oatp1/Slc21a1	5′	5′-AATGCCCAAGAGACAAATGC	22	450bp	AB031959
	3′	5′-CAGGTTCTGGGTTTCCTTCA			
Oct1/Slc22a1	5′	5′-GCGGTGTGGCTGGAGCCCGGCAGAGGA	27	589bp	AF010259
	3′	5′-GGCCACCCCAGCAAGCCCCACTAGCC			
Gapdh	5′	5′-CCATCATCATCTTCCAGGAG	–	576bp	NM_008084
	3′	5′-CCTGCTTCACCACCTTCTTG			

RNA isolation and quantification of mRNA

Total mouse liver RNA was isolated by extraction with TRIzol reagent (Life Technology, Munich, Germany). Reverse transcription of total RNA was performed with the SuperScript II preamplification system (Life Technology). The reverse transcriptase reaction was diluted and an aliquot was subjected to amplification by PCR with the gene-specific primers listed in Table 1. To compensate for variations in mRNA quality and quantity as well as random tube-to-tube variation in reverse transcription and PCR, an 'competimer'/primer mix specific for 18 S ribosomal RNA (Ambion, Austin, TX, U.S.A.) was employed [12]. The ratio of 18 S 'competimers' to primers was adjusted in accordance with the manufacturer's instructions such that the amount of 18 S rRNA product was within the same range as that of the mRNA species of interest. If both amplified transcripts were of similar size, the *Gapdh* gene, encoding glyceraldehyde-3-phosphate dehydrogenase, served as an internal control. Control experiments demonstrated that the protocol employed allowed quantification within the linear range of amplification of both transcripts. Amplified samples were subjected to densitometry after agarose-gel electrophoresis and staining with ethidium bromide. Semiquantitative measurements were corrected by division of the gene specific signal by the signal of the 18 S or *Gapdh* amplicon. In initial experiments we studied steady-state Ntcp1 (Slc10a1) and Mdr2 (Abcb4) mRNA levels by Northern blotting to cross-validate our reverse-transcriptase-mediated PCR (RT–PCR) protocol. Because steady-state mRNA levels differed in our hands by only 10–15 % under our experimental conditions, the accuracies of both methods seemed to be similar.

Statistics

Data were analysed by the unpaired, two-tailed Student *t*-test. Results are expressed as the means ± S.D.; $P < 0.05$ was considered to be statistically significant.

RESULTS

Expression of canalicular membrane transporters

We recently reported 2-fold higher bile-salt and 1.5-fold higher biliary cholesterol and phospholipid secretion rates in male C57L mice compared with AKR mice [12]. To investigate whether differences in biliary lipid secretion rates under our experimental conditions resulted from the strain-specific hepatic gene expression of canalicular membrane transporters, we isolated total liver RNA and plasma membrane subfractions of C57L and AKR mice. We employed a semiquantitative RT–PCR protocol that permitted the simultaneous amplification of the specific transporter transcript and *Gapdh* as internal standard. In Figure 1, RT–PCR products are shown from a typical experiment with RNA samples from AKR and C57L mice. Steady-state Bsep (Abcb11), Mdr2 (Abcb4) and Mrp2 (Abcc2) mRNA levels were similar in both strains fed with the control diet. Interestingly, and despite the difference in bile-salt and biliary phospholipid secretion rates [12], we did not find significantly higher Mdr2 and Bsep steady-state protein levels in C57L mice (Figure 2). Stripping and re-probing the blots with DPP IV, a canalicular marker protein, demonstrated that the membrane preparations under study had a similar degree of enrichment to that of canalicular membrane proteins.

When the mice received the lithogenic diet, bile-salt secretion did not increase in either strain. Biliary phospholipid secretion was elevated 1.8-fold and 2.2-fold in AKR and C57L mice respectively; biliary cholesterol secretion increased more markedly in C57L mice [12]. We next examined whether these findings were associated with altered gene expression of canalicular membrane transporters. As illustrated in Figure 1, significant ($P < 0.001$) 4-fold and 5-fold increases in steady-state Bsep (Abcb11) mRNA levels occurred in response to the lithogenic diet in AKR and C57L mice respectively. In contrast, the steady-state expression of Mdr2 (Abcb4) and Mrp2 (Abcc2) mRNA remained similar (Figure 1). Despite this substantial hepatic up-regulation of Bsep (Abcb11) mRNA levels, steady-state Bsep (Abcb11) protein levels increased only slightly by 45 % ($P > 0.05$) and 30 % ($P > 0.05$) in AKR and C57L mice respectively. This is in line with the transcriptional regulation of *Bsep* (*Abcb11*) in both mouse strains, whereas 1.8-fold ($P < 0.05$) and 2.5-fold ($P < 0.001$) elevated Mdr2 (Abcb4) levels in AKR and C57L mice indicated post-transcriptional regulation.

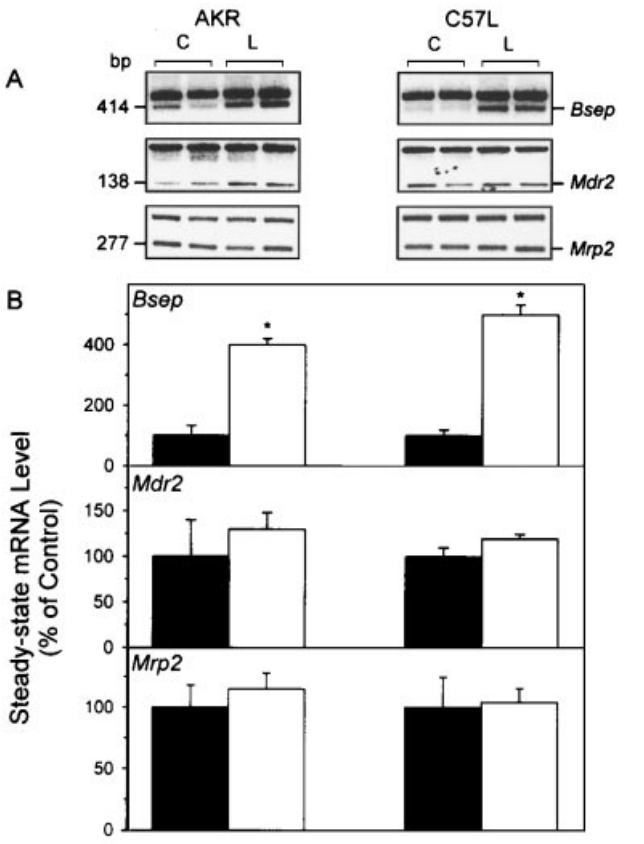

Figure 1 Effect of the lithogenic diet on steady-state mRNA levels of canalicular membrane transporters

Steady-state mRNA levels of Bsep (Abcb11), Mdr2 (Abcb4) and Mrp2 (Abcc2) in AKR and C57L mice fed with a control (C) or a lithogenic (L) diet were determined by RT–PCR. Relative levels of expression on control diet for AKR and C57L mice respectively were as follows: *Bsep/Abcb11* (0.03 compared with 0.02); *Mdr2/Abcb4* (0.27 compared with 0.22); *Mrp2/Abcc2* (0.84 compared with 0.71). (**A**) Representative data for two animals. The mRNA levels were quantified by densitometric analysis, related to *Gapdh* levels (the upper band of each pair) and calculated as percentages of the control value (mice fed with the control diet). (**B**) Black (control diet) and white bars (lithogenic diet) are means ± S.D. ($n = 6$ per group). $^*P < 0.001$ compared with control diet.

Expression of sinusoidal membrane transporters

We next investigated whether diminished steady-state Ntcp1 (Slc10a1) and Oatp1 (Slc21a1) mRNA levels might affect the

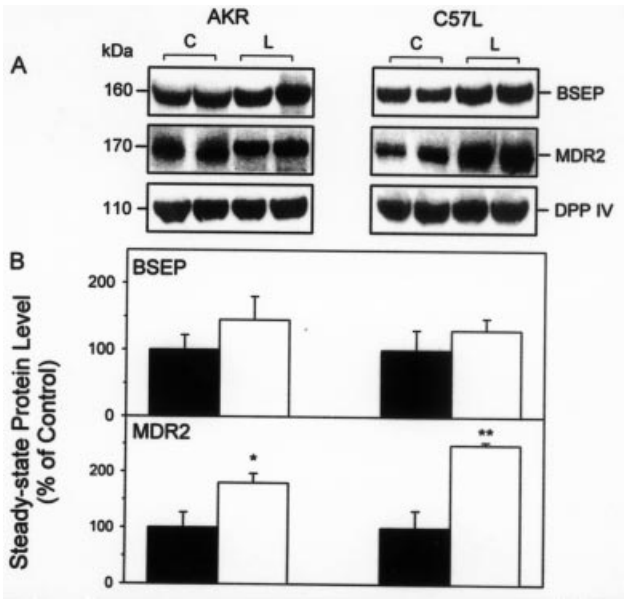

Figure 2 Effect of the lithogenic diet on steady-state protein levels of canalicular membrane transporters

Steady-state protein Bsep (Abcb11) and Mdr2 (Abcb4) levels from AKR and C57L mice fed with a control (C) or a lithogenic (L) diet were analysed by Western blotting. (**A**) Representative data for two separate membrane preparations ($n = 4$ animals per group). To correct for equal enrichment of membrane preparations with canalicular membrane proteins, blots were stripped and re-probed with an antibody against DPP IV as canalicular marker protein. (**B**) The steady-state protein expression of four separate membrane isolations is expressed as a percentage of the mice that received a control diet. Black (control diet) and white bars (lithogenic diet) are means $\pm$ S.D. $^{*}P < 0.05$, $^{**}P < 0.001$, compared with control diet.

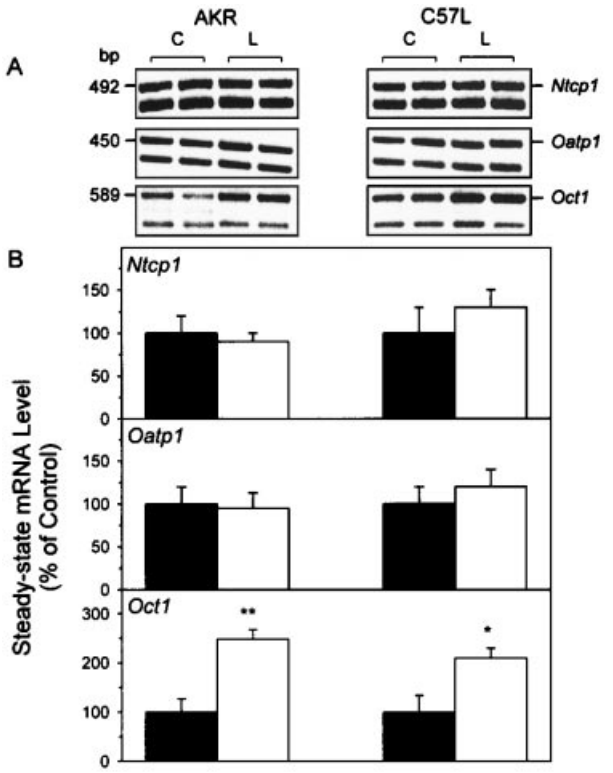

Figure 3 Effect of the lithogenic diet on steady-state mRNA expression of basolateral membrane transporters

Steady-state mRNA levels of Ntcp1 (Slc10a1), Oatp1 (Slc21a1) and Oct1 (Slc22a1) in AKR and C57L mice fed with a control (C) or lithogenic (L) diet were determined by RT–PCR. Relative levels of expression on control diet for AKR and C57L mice respectively were as follows: *Ntcp1/Slc10a1* (0.63 compared with 0.54); *Oatp1/Slc21a1* (1.08 compared with 0.94); *Oct1/Slc22a1* (0.98 compared with 1.38). (**A**) Representative data for two animals. The mRNA levels were quantified by densitometric analysis, related to 18 S rRNA levels (the lower band of each pair), and calculated as percentages of control value (mice fed with the control diet). (**B**) Black (control diet) and white bars (lithogenic diet) are means $\pm$ S.D. ($n = 6$ per group). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control diet.

hepatic uptake of bile salts, thereby contributing to the higher bile-salt secretion rates in C57L mice fed with the control diet. This was achieved with a semiquantitative RT–PCR protocol that permitted the simultaneous amplification of the specific transporter transcript together with 18 S rRNA as internal standard [12]. Figure 3 shows RT–PCR products from a typical experiment of RNA samples from AKR and C57L mice. When the animals were fed with the control diet, steady-state Ntcp1 (Slc10a1) and Oatp1 (Slc21a1) mRNA levels did not differ between the two strains studied. In contrast, steady-state Oct1 (Slc22a1) mRNA levels were slightly (41 %; $P > 0.05$) higher in C57L mice. A representative Western blot with a similar enrichment of isolated membrane preparations with basolateral membrane proteins such as Na/K-ATPase is shown in Figure 4 and indicates that there was no significant difference in steady-state Ntcp1 (Slc10a1) and Oatp1 (Slc21a1) protein levels between C57L and AKR mice.

Substantial amounts of cholic acid present in the lithogenic diet might have altered the steady-state gene expression of sinusoidal bile-salt transporters. When challenged with the lithogenic diet, the levels of Ntcp1 (Slc10a1) and Oatp1 (Slc21a1) mRNA (Figure 3) remained unchanged. In contrast, a 2.5-fold ($P < 0.01$) and 2.1-fold ($P < 0.05$) increase in steady-state Oct1 (Slc22a1) mRNA levels occurred in AKR and C57L mice respectively. As shown in Figure 4, steady-state Ntcp1 (Slc10a1) expression decreased in both mouse strains by 32 % ($P < 0.05$), indicating post-transcriptional down-regulation. In contrast with Ntcp1 (Slc10a1), no significant change was observed for steady-state Oatp1 (Slc21a1) levels in both mouse strains studied.

DISCUSSION

Variations in bile flow and biliary lipid secretion rates in gallstone-susceptible C57L mice compared with gallstone-resistant AKR mice [12,13] might be related to a differential expression of liver plasma membrane transporters. Therefore we studied steady-state mRNA and protein levels of basolateral and canalicular membrane transporters with relevance to bile formation in C57L and AKR mice during cholesterol gallstone formation.

Bile flow in C57L mice was approximately double that in AKR mice [12,13] and reflected an elevated secretion of glutathione into bile. We confirmed earlier observations of similar steady-state Mrp2 (Abcc2) mRNA levels in C57L and AKR mice [20]. Because regulation of the *Mrp2* (*Abcc2*) gene in mice apparently occurs at the transcriptional level [21], we would expect similar steady-state Mrp2 (Abcc2) protein levels; indeed, these have been reported recently [20]. Elevated bile flow without hepatic up-regulation of Mrp2 (Abcc2) protein expression seems to exclude *Mrp2* (*Abcc2*) gene mutations with impaired protein maturation or impaired apical sorting such as that found in patients with Dubin–Johnson syndrome [22–24]. One can speculate that a gain-of-function mutation in the *Mrp2* (*Abcc2*) gene

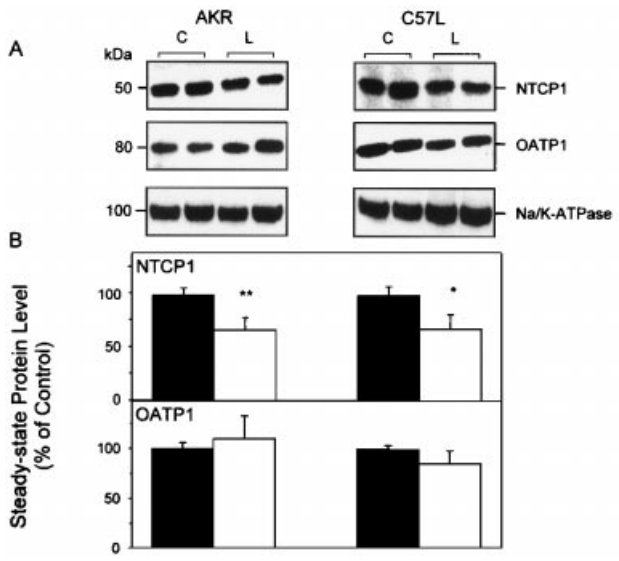

Figure 4 Effect of the lithogenic diet on steady-state protein levels of basolateral membrane transporters

Steady-state protein levels of Ntcp1 (Slc10a1) and Oatp1 (Slc21a1) in AKR and C57L mice fed with a control (C) or lithogenic (L) diet were analysed by Western blotting. (**A**) Representative data for two separate membrane preparations ($n = 4$ animals per group). To correct for equal enrichment of membrane preparations with basolateral membrane proteins, blots were stripped and re-probed with an antibody against Na/K-ATPase as a basolateral marker protein. (**B**) The steady-state protein expression of four separate membrane isolations is expressed as the percentage of mice that received a control diet. Black (control diet) and white bars (lithogenic diet) are means $\pm$ S.D. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with control diet.

of C57L mice is related to increased transport activity. Indeed, preliminary studies found a polymorphism in the *Mrp2* (*Abcc2*) gene resulting in an amino acid substitution (Arg $\rightarrow$ Gln) within the first nucleotide-binding domain [20]. Because arginine residues might be critical for protein function [25,26], further studies will need to explore whether this polymorphism in C57L mice is of physiological relevance.

Similar basal expressions of the *Ntcp1* (*Slc10a1*) and *Oatp1* (*Slc21a1*) genes in both mouse strains suggest that basolateral uptake and transhepatic flux of bile salts might not contribute to the differences in bile-salt secretion rates. In the light of the 2-fold higher bile-salt secretion rates and bile-salt pool sizes in C57L mice fed with a control diet [12,13], we expected higher steady-state Bsep (Abcb11) protein levels in the gallstone-susceptible strain. Interestingly, we not only found similar Bsep (Abcb11) protein levels but also similar Bsep (Abcb11) mRNA levels in AKR and C57L mice. It has been shown that *Bsep* (*Abcb11*) gene mutations impair bile-salt secretion rates [27,28]. It is therefore possible that a gain-of-function mutation in the *Bsep* (*Abcb11*) gene of C57L mice with increased transport activity might account for the difference in bile-salt secretion rates. However, preliminary sequencing data failed to identify a mutation in the coding region of the *Bsep* (*Abcb11*) gene [29]. Although it is unlikely in the light of similar steady-state Bsep (Abcb11) protein levels, we cannot exclude altered recruitment of Bsep (Abcb11) to the canalicular membrane [30,31]. Instead, a second but as yet unidentified canalicular bile-salt transporter might contribute to the different bile-salt secretion rates. Support comes from a recent study demonstrating that targeted disruption of the *Bsep* (*Abcb11*) gene failed to inhibit bile-salt secretion by more than 60 % [32].

Choline, an essential substrate for phospholipid synthesis, is cleared from blood by the action of Oct1 (Slc22a1) [33,34]. Similar steady-state Oct1 (Slc22a1) mRNA levels in AKR and C57L mice fed with the control diet are indirect evidence that elevated biliary phospholipid secretion rates in C57L mice might not be related to increased phospholipid synthesis. Similar steady-state Mdr2 (Abcb4) mRNA and Mdr2 (Abcb4) protein levels in both mouse strains studied suggest that increased biliary phospholipid secretion rates in C57L mice [12,13] reflect elevated bile-salt secretion rates in these animals [35].

In response to the lithogenic diet containing large amounts of cholic acid, bile-salt secretion rates remained constant, whereas biliary phospholipid and cholesterol secretion rates increased [12,13]. Elevated steady-state Bsep (Abcc11) mRNA levels together with slightly enhanced Bsep (Abcb11) protein expression is in line with transcriptional regulation [36] involving a bile-salt-responsive element and the farnesoid-X receptor/retinoid-X receptor [37]. The obvious discrepancy between substantially increased Bsep (Abcb11) mRNA and only slightly elevated Bsep (Abcb11) protein levels implicates additional post-transcriptional regulation, at least under our experimental conditions. A profound inhibition of bile-salt synthesis in response to the lithogenic diet [12,38] might also have contributed.

The hydrophobic circulating bile-salt pool in mice fed with the lithogenic diet [13] determines the quantity of phospholipids secreted into bile [35] and probably explains the elevated biliary phospholipid output [12,13]. Unexpectedly, Mdr2 (Abcb4) mRNA levels were unchanged despite elevated steady-state Mdr2 (Abcb4) protein levels. This is surprising, because a transcriptional up-regulation of *Mdr2* (*Abcb4*), possibly mediated by a bile-salt activation of protein kinase C [39], has been reported for mice receiving a diet enriched with 0.1 % cholic acid [40]. Interestingly, dietary cholesterol might decrease Mdr2 (Abcb4) mRNA levels [39], involving sterol regulatory elements [41]. Because the lithogenic diet contains both cholic acid and cholesterol, opposite effects of these dietary components on transcriptional regulation of *Mdr2* (*Abcb4*) might explain our finding of unchanged steady-state Mdr2 (Abcb4) mRNA levels. Hepatic up-regulation of Oct1 (Slc22a1) mRNA levels during lithogenic bile formation most probably reflects an increased uptake of choline [33,34], thereby providing sufficient substrate for phospholipid synthesis under conditions of biliary phospholipid hypersecretion.

In response to the lithogenic diet, steady-state Oatp1 (Slc21a1) protein expression remained unchanged, whereas Ntcp1 (Slc10a1) levels decreased without a concomitant decline in Ntcp1 (Slc10a1) mRNA levels. Although a down-regulation of Ntcp1 (Slc10a1) protein levels implicates cholestasis [42], the animals were not cholestatic, as judged by bile flow and biliary lipid secretion rates [12,13], serum liver enzymes and liver histology. This observation is reminiscent of the situation in *Mdr2* (*Abcb4*)-deficient mice [18] and might represent an adaptive response to protect the liver from high loads of intracellular hydrophobic bile salts [13].

Quantitative trait loci analysis demonstrated that *Bsep* (*Abcc11*), *Oct1* (*Slc22a1*) and *Mrp2* (*Abcc2*) constitute three candidate *Lith* genes on chromosomes 2, 17 and 19 respectively [11]. On the basis of our findings together with the available literature, we suggest a pathophysiological model that might allow cholesterol gallstone formation in C57L mice (Figure 5). The canalicular membrane of C57L mice fed with the lithogenic diet receives cholesterol continuously in abundance. This is a result of several mechanisms, which include (1) augmented intestinal cholesterol absorption and hepatic uptake of chylomicron remnants [43], (2) an increased uptake of high-density

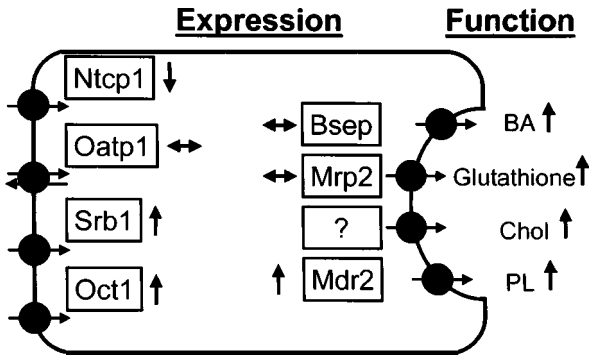

Figure 5 Liver plasma-membrane transport systems during gallstone formation in C57L mice fed with a lithogenic diet

Elevated steady-state protein Bsep (Abcb11) and Mdr2 (Abcb4) but not *Mrp2* (*Abcc2*) expression is associated with the biliary hypersecretion of bile salts (BA) and phospholipids (PL) and with increased bile flow rates. A canalicular transport protein responsible for cholesterol (Chol) release into bile remains to be identified. At the basolateral membrane, the down-regulation of Ntcp1 (Slc10a1) but not Oatp1 (Slc21a1) might protect the liver from toxic intrahepatic bile-salt concentrations. Increased *Oct1* (*Slc22a1*) gene expression might reflect an increased uptake of choline required for elevated phospholipid synthesis under conditions of biliary phospholipid hypersecretion. As shown recently [12], hepatic up-regulation of Srb1 is associated with biliary cholesterol hypersecretion.

lipoprotein via scavenger receptor BI [12], (3) decreased bile-salt synthesis [12,38], (4) enhanced intracellular cholesterol transport via sterol carrier protein 2 [14] and (5) failure to suppress cholesterol synthesis sufficiently [10,12]. A greater bile-salt pool with normal enterohepatic cycling reflected by increased *Bsep* (*Abcb11*) gene expression promotes the hypersecretion of cholesterol from the canalicular membrane into bile. Because the lithogenic diet causes the bile-salt pool to become more hydrophobic with time [13], this further augments biliary cholesterol hypersecretion. A *Mrp2* (*Abcc2*) gene mutation in C57L mice related to increased transport activity [20] might explain elevated bile flow rates, which in addition might promote biliary cholesterol hypersecretion by bile-salt–phospholipid secretory decoupling due to the binding of anions to bile salts [35]. Under such conditions, vesicles might be attached to the canalicular membrane for longer [44], thus acting as sinks for the increased cholesterol pool of the canalicular membrane. Although *Oct1* (*Slc22a1*) co-localizes with *Lith3* on chromosome 17 [11], we do not yet know how it might facilitate cholesterol gallstone formation under the current experimental conditions.

In summary, variations in the protein function of liver plasma membrane transporters rather than in their gene expression might explain, at least in part, biliary lipid secretion rates during lithogenesis in an established mouse model of cholesterol gallstone formation. Because this model might reflect molecular mechanisms responsible for cholesterol gallstone formation in obese subjects [45–47], studies of gene expression as well as protein function of liver plasma membrane transporters in humans are awaited to see whether these concepts are applicable to humans.

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