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Physiological functions of biliary lipid secretion

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CHAPTER 5

DECREASED NA^+ DEPENDENT TAUROCHOLATE UPTAKE AND LOW EXPRESSION OF THE SINUSOIDAL NA^+-TAUROCHOLATE COTRANSPORTING PROTEIN (NTCP) IN LIVERS OF MDR2 P-GLYCOPROTEIN-DEFICIENT MICE.

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ABSTRACT

Background / Aims: Ntcp-mediated uptake of bile salts at the basolateral membrane of hepatocytes is required for maintenance of their enterohepatic circulation. Expression of Ntcp is reduced in various experimental models of cholestasis associated with increased plasma bile salt concentrations. Mdr2 P-glycoprotein-deficient mice lack biliary phospholipids and cholesterol but show unchanged biliary bile salt secretion and increased bile flow. These mice are evidently not cholestatic, but plasma bile salt concentrations are markedly increased. The aim of this study was to investigate the role of Ntcp in the elevated bile salt levels in mdr2 P-glycoprotein-deficient mice. *Methods*: Plasma membranes were isolated from male wild type *Mdr2*$^{(+/+)}$ and *Mdr2*$^{(-/-)}$ mice for measurement of Na^+-dependent taurocholate transport and assessment of Ntcp protein levels by Western blotting. Northern blot analysis and competitive RT-PCR were used to determine hepatic *Ntcp* mRNA levels. *Results*: Kinetic analysis showed a 2-fold decrease in the V_{max} of Na^+-dependent taurocholate transport with an unaffected K_m in *Mdr2*$^{(-/-)}$ mice compared with *Mdr2*$^{(+/+)}$ controls. Ntcp protein levels were 4-6 fold reduced in plasma membranes of *Mdr2*$^{(-/-)}$ mice relative to sex-matched controls. Surprisingly, hepatic *Ntcp* mRNA levels were not significantly affected in the *Mdr2*$^{(-/-)}$ mice. *Conclusions*: Elevated plasma bile salt levels in mdr2 P-glycoprotein-deficient mice in the absence of overt cholestasis are associated with reduced Ntcp expression and transport activity. This is due to posttranscriptional down-regulation of Ntcp.

INTRODUCTION

Uptake of bile salts from portal blood at the sinusoidal membrane of hepatocytes predominantly takes place by a sodium-dependent process mediated by Na^+-taurocholate cotransporting protein (Ntcp). Ntcp-mediated uptake is thought to be essential for maintaining the enterohepatic circulation of bile salts. The expression and function of Ntcp is not regulated by physiological fluctuations in hepatic bile salt flux [1], but is down-regulated in various models of experimental cholestasis, including cholestasis induced by endotoxin or TNFa administration, bile duct ligation and ethinylestradiol administration [2-6]. Partial hepatectomy also leads to rapid down-regulation of Ntcp in rats [7]. Cholestatic conditions are usually associated with increased plasma bile salts levels: Ntcp expression appears to be inversely related to plasma bile salt levels, probably reflecting their intracellular concentrations, in cholestatic rat models [8].

Mice in which the gene encoding for mdr2 P-glycoprotein (Pgp) has been disrupted ($Mdr2^{(-/-)}$ mice) are unable to secrete phospholipids and cholesterol into bile, but show increased bile flow and an unchanged biliary bile salt secretion [9]. These mice are evidently not cholestatic when cholestasis is defined as “impairment or cessation of bile flow” [10-12]. Yet, these mice have elevated plasma bile salt and bilirubin levels. Furthermore, plasma levels of alkaline phosphatase, aspartate transaminase and alanine transaminase are increased when compared with (+/-) and $Mdr2^{(+/+)}$ control mice, indicating damage of liver parenchyma. Morphologically, the livers of *mdr2* knockout mice show some degeneration of hepatocytes, ductular proliferation and portal inflammation. Liver pathology in these mice has been shown to be related to the formation of lipid-free bile, exerting cytotoxic actions of bile salts towards bile duct epithelial cells [13,14].

To provide a mechanistic basis for the elevated plasma bile salt levels in the absence of cholestasis in mdr2 Pgp-deficient mice, we determined the Na^+-dependent transport of taurocholate in plasma membrane vesicles isolated from livers of these mice and we examined hepatic Ntcp protein and *Ntcp* mRNA levels.

MATERIALS AND METHODS

Chemicals

[^{3}H(G)]-taurocholate (3.47 Ci/mmol; 99% pure by HPLC and thin layer chromatography) was obtained from Du Pont/New England Nuclear (Boston, MA, USA). Unlabeled taurocholate was obtained from Calbiochem (La Jolla, CA, USA). The polyclonal Ntcp antibody (K4) and the complementary DNA used were described previously [1,15,16]. All other chemicals were of reagent grade or the highest purity grade commercially available and purchased from Sigma Chemicals (St. Louis, MO, USA) or Amersham (Little Chalfont, UK).

Animals

Homozygous $Mdr2^{(-/-)}$ mice and control $Mdr2^{(+/+)}$ mice of the FVB strain [17] used in these experiments were bred at the animal laboratory of the University of Amsterdam. Animals were used at 3-6 months of age and were kept in a light- and temperature-controlled environment. The mice had free access to lab chow and tapwater prior to the experiments. The animals received humane care and

experimental protocols complied with the local guidelines for use of experimental animals.

Prior to removal of the liver, the mice were anaesthetized with halothane and a blood sample was obtained by cardiac punction. The liver was weighed and immediately transferred to ice-cold $NaHCO_3$ buffer (see below). A small part of the liver was removed at this point and frozen quickly in liquid nitrogen for subsequent RNA isolation.

Analyses

Bile salts in plasma were determined by an enzymatic fluorimetric assay [18]. Aspartate transaminase (AST), alanine transaminase (ALT) and bilirubin in plasma were measured by standard laboratory techniques.

Isolation of liver plasma membranes and determination of enzyme activities

Plasma membranes were isolated by a procedure adapted from the one described by Emmelot et al. [19]. Five grams of liver tissue, pooled from three mice, was cut in small pieces in 25 ml 1mM $NaHCO_3$, pH 7.4 with 17 mg/l PMSF (phenylmethylsulfonylfluoride) and homogenized by seven strokes in a loose dounce (Braun, Melsungen, Germany). This homogenate was filtered through cheesecloth and diluted to a total volume of 35 ml $NaHCO_3$ per 5 grams of liver. The homogenate was then centrifuged for 10 min at 1500g. The supernatant was removed and the pellet was resuspended in 35 ml fresh $NaHCO_3$ buffer. This was centrifuged again for 10 min at 1000g. The supernatant was removed again and the remaining pellet was resuspended and centrifuged again at 1000g, for 10 min. This was repeated three times in total. The remaining pellet was resuspended in $NaHCO_3$ buffer to a total of 3.6 ml and 10 ml of 62.2% (w/w) sucrose was added under mild stirring. This suspension was divided over two ultracentrifuge tubes, and was overlayered with respectively 6 ml 44.68%, 8 ml 40.81% and 6 ml 37.02% sucrose. The tubes were filled up with 0.25 M sucrose. These gradients were centrifuged for 1.5 hr at $90.000g_{av}$ in a Centrikon TI28.38 rotor, in a Centrikon T-1080 ultracentrifuge (Kontron Instruments, Milan, Italy). The bands enriched in plasma membranes floating on the 40.8% and 44.68% layers were recovered, pooled, 4 times diluted with $NaHCO_3$ and subsequently centrifuged for 15 min at 7500g. The pellet was resuspended in 40 ml $NaHCO_3$ buffer and centrifuged for 10 min at 2500 g. The final membrane pellet was resuspended in buffer containing 300 mM sucrose, 0.2 mM $CaCl_2$, 10 mM $MgS0_4$, 10 mM Hepes pH 7.5, homogenized by 50 strokes through a syringe needle and stored immediately in liquid nitrogen.

Protein concentrations were determined according to Lowry [20]. Relative enrichments of Na^+ / K^+-ATPase as a marker enzyme for the basolateral membrane fraction, alkaline phosphatase as a marker enzyme for the canalicular plasma membrane fraction and succinate cytochrome C reductase as marker for the contamination with mitochondria, i.e. the activity of these enzymes in the isolated plasma membrane preparation divided by their activity in the homogenate, was used to determine the degree of purification of the isolated membranes in the different experimental groups. Na^+/ K^+ ATPase [21], alkaline phosphatase [22] and cytochrome C reductase [23] were measured using a Uvikon 931 spectrophotometer (Kontron Instruments, Milan, Italy).

Western Blotting

Plasma membranes equivalent to 20 μg of protein were electrophoresed through a 10% polyacrylamide gel at 100 V. The proteins were electrophoretically transferred onto a nitro-cellulose filter (Amersham, Little Chalfont, UK) by tank blotting. Ponceau S staining was performed to check equal protein transfer. The filters were blocked overnight at 4°C in a solution of Tris-buffered saline with 0.1% Tween and 4% skim-milk powder pH 7.4. The blots were incubated with the primary antibody Ntcp (K4) in a 1:10.000 dilution for 3 hrs at room temperature, washed and immune complexes were detected using horseradish peroxidase-conjugated donkey anti rabbit IgG by the ECL Western blotting kit (Amersham, Little Chalfont, UK). Protein density was determined by scanning the blots using an Image Master VDS system (Pharmacia Biotech, Upsalla, Sweden)

Transport studies.

Transport studies were carried out in plasma membrane vesicles using a rapid filtration technique [24]. Five μl membrane vesicles (15 μg protein) were preincubated at 25°C for 1 min. Uptake was initiated by addition of 20 μl prewarmed incubation medium (final concentration: 100 mM NaCl or KCl, 100mM sucrose, 10 mM Hepes pH 7.5, 0.2 mM $CaCl_2$ and 10 mM $MgSO_4$, bovine serum albumin (BSA) 1 mg/ml , [^{3}H]-taurocholate was added in different concentrations) to the membranes. Uptake was performed at 25°C. Uptake was stopped by adding 750 μl of ice-cold stop solution (100 mM sucrose, 100 mM KCl, 10 mM Hepes pH 7.5, 0.2 mM $CaCl_2$ and 10 mM $MgSO_4$) to the incubation medium. The sample was immediately filtered through a 0.45 μm Millipore filter (Millipore, Bedford, MA) that was prewashed with 1 ml stop solution containing 1 mM unlabeled taurocholate, and subsequently washed twice with 4 ml ice-cold stop solution. The filters were dissolved in Ultima gold MV scintillation fluid (Packard Instruments, Dowers Grove, IL) and counted in a liquid scintillation counter type Packard 1500 (Packard Instruments, Dowers Grove, IL).

Northern Blotting

Total RNA was isolated according to Chromczynski and Sacchi [25], separated on agarose formaldehyde gel and transferred to a nylon membrane, Hybond N (Amersham, Little Chalfont, UK), by overnight blotting. cDNA probes were labelled using a random primed labelling kit to a specific activity of 10^8-10^9 cpm/μg. Blots were prehybridized in hybridization solution (0.5 M $NaHPO_4$, 1 mM Na_2EDTA and 7 % SDS, pH 7.2) and 100 μg herring sperm DNA per ml, and hybridized at 65°C overnight at 1-2 *10^6 cpm/ml in hybridization solution. They were washed twice for 15 min in 2* SSC washing buffer (0.3 M NaCl, 30 mM Na-citrate and 1% SDS, pH 7.0) at 65°C and subsequently two times in 1* SSC washing buffer (0.15 M NaCl, 15 mM Na-citrate and 1% SDS ,pH 7.0) at 65°C. Activities were corrected for concentration differences, using 28S rRNA as an internal control

Reverse transcription

Total RNA was isolated using the Rneasy kit (QIAGEN AG, Basel, Switserland) and 1 μg samples were reverse transcribed with oligo (dT) primers and 15 units of AMV reverse transcriptase (promega, Madison WI) in a 20 μl reaction volume.

Competative PCR

primers specific for the mouse Ntcp (5'-GGTTCTCATTCCTTGCGCCA-3', bp 535-554; 5'-GCATCTTCTGTTGCAGCAGC-3', bp 1026-1007) were linked to a 600 bp sequence derived from the neomycin gene by PCR using the following primers (5'-GGTTCTCATTCCTTGCGCCACCCTGAATGAACTGCAGGAC-3' forward ; 5'-GCATCTTCTGTTGCAGCAGCAGGCGATGCGCTGCGAATCG-3' reverse). This PCR product was re-amplified with the Ntcp specific primers resulting in a 640 bp fragment which was purified and used as a heterologous competitor fragment for Ntcp. Coamplification of liver cDNA and competitor fragment yielded two PCR products of 491 bp (Ntcp) and 640 bp (competitor). Samples of the reverse transcribed reaction corresponding to 10 ng of total RNA were amplified along with competitor cDNA corresponding to 2, 1, 0.5, 0.25, 0.125 or 0.0625 amol in a 50 230l PCR reaction that contained 0.2 mM dNTPs, 0.4mM of each Ntcp specific primer, 10 mM KCl, 10 mM$(NH_4)_2SO_4$, 2mM $MgSO_4$, 20mM Tris-HCl (pH8.75), 0.1% Triton X-100, 0.1 mg/ml BSA and 2.5 U TaqPlus Long polymerase mixture (Stratagene GmbH, Heidelberg, Germany). Cycle conditions were: 2 minutes denaturation at 95°C, 30 cycles of 45 seconds at 95 °C, 45 seconds annealing at 50°C, 1 minute elongation at 72°C, and final elongation for 5 minutes at 72 °C. Ten microliters of the reaction were separated on a 1% TAE agarose gel and after ethidium bromide staining competitor and Ntcp specific bands of equal intensities were determined visually.

Statistics

Data are expressed as mean – SD for the indicated number of experiments. Statistical analysis between the experimental groups was assessed using Student's two tailed *t* - test. Statistical significance was considered at p-values of < 0.05.

RESULTS

Serum parameters

The plasma levels of bile salts, bilirubin and the liver-function markers AST and ALT are summarized in Table 1. Plasma concentrations of bile salts are elevated significantly, i.e. by 100% in *Mdr2*$^{(-/-)}$ mice compared with *Mdr2*$^{(+/+)}$ controls. As shown earlier [9,13], AST en ALT activities are markedly higher in the knockout mice than in controls. A smaller, but significant increase was found for serum bilirubin.

Table 1. Plasma parameters of liver function in male wild type and mdr2 Pgp-deficient mice.

	AST (IU/L)	*ALT (IU/L)*	*Bilirubin (mM)*	*Bile Salts (mM)*
Mdr2$^{(+/+)}$	*78 – 24*	*29 – 6*	*3.7 – 0.5*	*21 – 4.2*
Mdr2$^{(-/-)}$	*203 – 64* [a]	*217 – 68* [a]	*5.7 – 0.7*[a]	*45 – 25.6*[a]

Data are given as means – SD, n=9 in each group.
[a]*: significantly different from control p<0.01*

Enzyme activities in homogenates and enrichment of marker enzymes in isolated membranes Table 2 shows the activities of marker enzymes in the experimental groups. Absolute Na^+/K^+ ATPase activities in the homogenates are significantly lower in the *Mdr2*$^{(-/-)}$ mice than in the controls, i.e. 3.12 and 1.84 mmol/ mg protein / hr respectively. The hepatic activity of alkaline phosphatase, on the other hand, is increased in the *Mdr2*$^{(-/-)}$ mice. The activities of succinate cytochrome C reductase are not different in both groups of mice.

Enrichments of the membrane-bound marker enzymes relative to the liver homogenates are summarized in Table 3. Similar enrichments were found for the experimental groups and the contamination with mitochondria, as indicated by the loss of succinate cytochrome C reductase, is low in both groups.

Table 2. Activities of marker enzymes in liver homogenates of wild type and mdr2 Pgp-deficient mice.

	Na^+/K^+ATPase	alkaline phosphatase ($mmol \cdot mg^{-1} \cdot h^{-1}$)	succinate cytochrome C reduct.
Mdr2$^{(+/+)}$	3.12 – 0.44	0.46 – 0.05	6.89 – 0.48
Mdr2$^{(-/-)}$	1.84 – 0.41[a]	1.10 – 0.21[a]	6.64 – 0.57

Data are given as means – SD, n=3 in each group, triplicate determinations
[a]: significantly different from control $p < 0.05$

Table 3. Enrichment of marker enzymes in plasma membranes isolated from livers of male wild type and mdr2 Pgp-deficient mice.

	Na^+/K^+ATPase	alkaline phosphatase	succ. cyt. C reduct.
Mdr2$^{(+/+)}$	14.8 – 2.1	13.5 – 1.6	0.15 – 0.01
Mdr2$^{(-/-)}$	16.5 – 3.7	16.3 – 3.0	0.35 – 0.10

Data are given as means – SD, n=3 in each group, triplicate determinations

Transport studies

Km and Vmax values of taurocholate uptake were determined by measuring the initial uptake velocity over a concentration range from 10 to 160 mM in plasma membranes isolated from *Mdr2*$^{(+/+)}$ and *Mdr2*$^{(-/-)}$ mice. Taurocholate uptake is clearly decreased in *Mdr2*$^{(-/-)}$ mice (Figure 1). Because uptake in the presence of a K^+ gradient is considered to represent the passive component of transport, the difference between Na^+-mediated and K^+-mediated transport, shown in Figure 1, represents the Na^+-dependent, carrier-mediated portion of transport across the membrane. Analysis of the initial uptake rates at various concentrations of taurocholate in male *Mdr2*$^{(+/+)}$ and *Mdr2*$^{(-/-)}$ mice revealed a marked decrease in the Vmax of taurocholate uptake, i.e. from 436 – 143 in *Mdr2*$^{(+/+)}$ to 177 – 43 pmol/ mg protein/ 5 sec ($p<0.05$) in *Mdr2*$^{(-/-)}$ mice. In contrast, the Km values (51 – 17 and 41 – 15) did not show a significant difference between the groups. These results indicate a decrease in the number of functional Na^+-dependent taurocholate transporters with a similar affinity for its substrate in isolated plasma membranes of mdr2 Pgp-deficient mice.

Ntcp protein

To determine if decreased Na^+-dependent transport activity in plasma membranes of *Mdr2*$^{(-/-)}$ mice is associated with decreased levels of Ntcp, we measured Ntcp protein levels in plasma membranes isolated from *Mdr2*$^{(+/+)}$ and *Mdr2*$^{(-/-)}$ mice. Figure 2 shows a representative Western blot showing three different membrane preparations of both groups of mice. As determined by scanning the blots, the relative Ntcp protein levels in *Mdr2*$^{(-/-)}$ mice are 4-6 times lower than in *Mdr2*$^{(+/+)}$ mice.

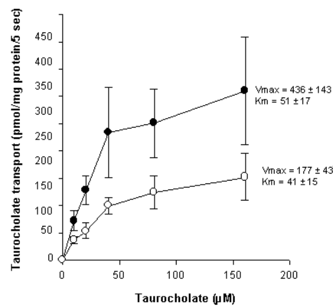

Figure 1. *Kinetics of sodium-dependent taurocholate transport in plasmamembranes isolated from Mdr2*$^{(+/+)}$*, closed symbols and Mdr2*$^{(-/-)}$*, open symbols, mice. Initial uptake velocity of taurocholate was measured over a range from 10 to 160 µM. Each point represents the mean of triplicate measurement in three different membrane preparations. * p< 0.05 compared with Mdr2*$^{(+/+)}$.

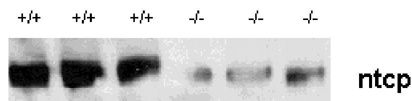

Figure 2. Ntcp protein mass in plasma membranes isolated from wildtype and mdr2 Pgp deficient mice Mdr2$^{(-/-)}$*. Three different membrane preparations of each group are shown.*

Steady-state mRNA levels

To investigate whether changes in protein expression of Ntcp can be explained by an altered gene expression, as is the case in models of experimental cholestasis, steady state mRNA levels were compared using Northern blot analysis (Figure 3a). To exclude effects of differential RNA loading onto the gel, the 28S rRNA levels were determined for normalization. Interestingly, this ratio was about one in both groups indicating that no regulation takes place at transcriptional level. Likewise, no differences in *Ntcp* mRNA levels were detected by competitive RT-PCR analysis (figure 3b). Obviously, these results indicate that posttranscriptional down-regulation of Ntcp occurs in these knockout mice.

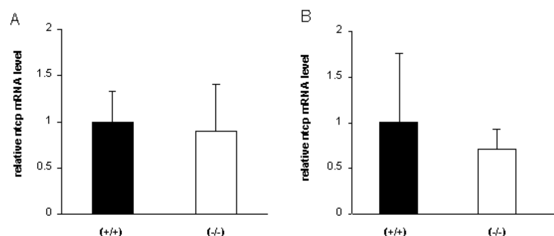

Figure 3 Relative ntcp mRNA levels in control mice and mdr2 knockout mice. Ntcp mRNA levels were determined by Northern blotting (a) or competitive RT-PCR (b). Values were normalized by using a 28S rRNA probe (a) or by setting the control mRNA amount to 1 (b). Each bar represents the mean of triplicate total RNA isolations from different livers (a). Competitive RT-PCR was performed with total RNA from 5 Mdr2$^{(-/-)}$ *and 3 Mdr2*$^{(+/+)}$ *mice.*

DISCUSSION

This study is the first to describe a clear down-regulation of Ntcp protein levels and activity in a non-cholestatic animal model. In recent years expression and activity of sodium-dependent bile salt transport has been studied in a number of

experimental models of cholestasis [2-6]. Without exception, cholestasis was found to be associated with a clear down-regulation of Ntcp, in most cases on activity, protein as well as on mRNA levels. The molecular background of these changes in Ntcp levels and activity are not yet understood. The presence of a putative BS responsive element in the promotor region of rat*Ntcp* [26,27] suggests that high intracellular bile salt concentrations as occur during cholestasis may be responsible for down-regulation. In fact, Gartung et al. [8] reported an inverse relationship between plasma bile salt levels on Ntcp protein levels in rat liver. In addition, it was shown that cytokines like TNFa and IL1b are able to down-regulate Ntcp *in vivo* and *in vitro* [3,4,28]. Both bile salt induced- and cytokine induced-phenomena can play a role in the different cholestatic models. It has been proposed that down-regulation of Ntcp in conditions associated with cholestasis protects the hepatocyte from intracellular accumulation of toxic bile salts [1,8,29]. The *mdr2* knock out mice represent a non-cholestatic model when judged on the basis of bile flow. However, these mice do present with a “cholestatic” plasma, with elevated transaminases and alkaline phosphatase, increased bilirubin and increased bile salt levels.

Mdr2 Pgp-deficiency in mice leads to formation of phospholipid- and cholesterol-depleted bile. Bile salt secretion is not affected in these mice. Bile flow is increased, which has been attributed to a higher choleretic potency of biliary bile salts in the absence of mixed micelle formation. This view is supported by the fact that glutathione secretion, which is considered to be responsible for the generation of BSIF in rodents, is diminished in *Mdr2*$^{(-/-)}$ mice [9]. The increased flow of water probably is generated at the level of the bile ducts that are strongly proliferated. The composition of the bile salt pool is not changed, but pool size appears to be increased in *Mdr2*$^{(-/-)}$ mice. By infusing increasing amounts of taurocholate it has been shown that *Mdr2*$^{(-/-)}$ mice transport bile salts at or near their maximal secretory capacity (S_{RM}) under normal circumstances [17]. This is in marked contrast to the situation in control mice in which the S_{RM} is about 2-fold higher than the normal physiological output rate [17].

A decrease in the Vmax for taurocholate transport with unchanged Km was found in plasma membranes isolated from *mdr2* knockout mice. The kinetic values obtained in the present study are in agreement with those reported before for Ntcp-mediated transport in rat liver [24,30]. However, we did find a discrepancy between transport data and Ntcp protein levels as determined by Western blotting. Whereas Ntcp protein levels were 4-6 times lower, we found the Vmax of taurocholate transport to be decreased only 2-fold. One explanation for this divergence can be the difference in sensitivity of the two methods applied. Another explanation is that there is an additional, as yet unidentified, sodium-dependent taurocholate transporter in hepatic plasma membranes of the mouse. In fact, an alternative splice variant of Ntcp, called Ntcp-2, has been found in mouse liver [31]. However, Ntcp-2 mRNA is about 25 times less abundant than that of Ntcp-1, both in *Mdr2*$^{(+/+)}$ and *Mdr2*$^{(-/-)}$ mice and therefore is probably of no physiological importance (data not shown). Alternatively, since we have used total plasma membrane preparations in our study, it may be that bile salt transporters present in bile ductuli contribute to overall Na^+-dependent transport in these studies. Recently, it was shown that the intestinal sodium-dependent bile salt transporter (ibst) is also expressed in

apical membrane of the bile duct epithelium in rats [32,33]. It was suggested [33]that uptake of bile salts by the bile duct cells may contribute to a cholehepatic shunt, in which bile salts taken up by cholangiocytes are returned to the hepatocytes via the hepatobiliary plexus. Since bile ducts are strongly proliferated in $Mdr2^{(-/-)}$ mice, it may be that ibst is highly expressed in these animals. If ibst is indeed upregulated in $Mdr2^{(-/-)}$ mice, this could partly explain the observed discrepancy between transport activity and Ntcp levels. At the moment, nothing is known about the kinetics of the ileal bile salt transporter in mice. The reported Km value of the intestinal rat bile salt transporter (33mM) [34] and the bile duct ibst (43 mM) [35] are in the same order of magnitude, as that of Ntcp (25-46mM) [15,30] in rats. Whether this is also the case for mice remains to be determined.

The decrease in Ntcp protein levels was, to our surprise, not accompanied by a similar decrease in mRNA levels, in contrast to the situation in the cholestatic models mentioned previously [2-6]. This suggests posttranscriptional mechanisms to be involved in Ntcp down-regulation in this particular model. In fact, a discrepancy between the reduction in mRNA levels and protein levels was also noted by Gartung *et al.[*5] in bile duct ligated rats, although in this case mRNA levels are clearly reduced. Together, these findings suggest that regulation of Ntcp may have both transcriptional and posttranscriptional components and that their contribution may depend on the severity of cholestasis. It is tempting to speculate that the situation in mdr2 Pgp-deficient mice represents a pre-cholestatic condition. As mentioned previously, biliary bile salt secretion appears to proceed at, or very near, its maximal capacity. In fact, we have recently shown that feeding of cholesterol associated with a compensatory increase in bile salt synthesis rapidly leads to elevations in plasma bile salt concentrations and to hyperbilirubinemia in $Mdr2^{(-/-)}$ mice [36]. This observation supports the view that these mice may be “on the edge” of developing cholestasis when the system is stressed. Contributing to, or even underlying this situation may be a constant exposure of the $Mdr2^{(-/-)}$ livers to small amounts of cytokines, due to the ongoing inflammation. As also indicated before, cytokines as well as inflammation induced by endotoxin rapidly lead to down-regulation of Ntcp as well as canalicular transporters involved in bile formation [3,4,28].

Thus, in the scenario outlined above, it may be that posttranscriptional down-regulation of Ntcp may be an initial event in cells that are almost cholestatic, perhaps aimed to protect the cells from high intracellular bile salt loads. In the case of a exacerbation, e.g. due to obstruction of the bile ducts, transcriptional down-regulation also jumps in to fully down-regulate Ntcp. The questions that remain to be answered concern the signals for both ways of regulation. Whereas there are strong indications that intracellular bile salt concentrations affect *Ntcp* gene transcription [5,8], mechanisms responsible for posttranscriptional regulation remain fully speculative at this moment.

A factor that could contribute is the Na^+/K^+ATPase activity in the sinusoidal membrane. We found in $Mdr2^{(-/-)}$ mice a two-fold decrease in Na^+/K^+ATPase activity. Simultaneously decreased activities of Na^+/K^+ATPase and Ntcp are also found in cholestasis caused by ethinylestradiol in rats [6,37]. Of course the question still remains whether decreased Na^+/K^+ATPase activity affects Ntcp levels, or *vice versa*.

In conclusion we demonstrate posttranscriptional down-regulation of Ntcp protein levels and transport activity in male mdr2 Pgp-deficient mice. We propose that this mechanism of down-regulation of Ntcp plays a role in the hepatic protection against high intracellular bile salt loads in the “near cholestatic” condition that has to be handled by these mice.

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REFERENCES

1.Koopen NR, Wolters H, Müller M, Schippers IJ, Havinga R, Roelofsen H, Vonk RJ, Stieger B, Meier PJ, Kuipers F. Hepatic bile salt flux does not modulate level and activity of the sinusoidal Na^+-taurocholate cotransporter (ntcp) in rats. J Hepatology 1997;27:699-706.
2.Bolder U, Ton-Nu HT, Schteingart CD, Frick E, Hofmann AF. Hepatocyte transport of bile acids and organic anions in endotoxemic rats: impaired uptake and secretion. Gastroenterology 1997;112:214-24.
3.Green RM, Beier DR, Gollan JL. Regulation of hepatocyte bile salt transporters by endotoxin and inflamatory cytokines in rodents. Gastroenterology 1996;111:193-8.
4.Moseley RH, Wang W, Takeda H, Lown K, Shick L, Ananthanarayanan M, Suchy FJ. Effect of endotoxin on bile transport in rat liver: a potential model for sepsis-associated cholestasis. Am J Physiol 1996;271:G137-46.
5.Gartung C, Ananthanarayanan M, Rahman M, Schuele S, Nundy S, Soroka CL, Stolz A, Suchy FJ, Boyer JL. Down-regulation of expression and function of the rat liver Na^+/Bile acid cotransporter in extrahepatic cholestasis. Gastroenterology 1996;110:199-209.
6.Simon FR, Fortune J, Iwahashi M, Gartung C, Wolkoff AW, Sutherland E. Ethinyl estradiol cholestasis involves alterations in expression of liver sinusoidal transporters. Am J Physiol 1996;271:G1043-52.
7.Green RM, Gollan JL, Hagenbuch B, Meier PJ, Beier DR. Regulation of hepatocyte bile salt transporters during hepatic regeneration. Am J Physiol 1997;273:G261-7.
8.Gartung C, Schuele S, Schlosser SF, Boyer JL. Expression of the rat liver Na^+/ taurocholate cotransporter is regulated by in vivo retention of biliary constituents but not their depletion. Hepatology 1997;25:284-90.
9.Smit JJM, Schinkel AH, Oude Elferink RPJ, Groen AK, Wagenaar E, van Deemter L, Mol CAAM, Ottenhoff R, van der Lugt NMT, van Roon MA, van der Valk MA, Offerhaus GJA, Berns AJM, Borst P. Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. Cell 1993;75:451-62.
10.Reichen J, Simon FR. ; Arias I, Boyer J, Fausto N, Jakoby W, Schachter D, Shafritz D, editors.The liver: Biolology and Pathobiology. 3rd ed. New York: Raven Press,Ltd. 1994; 68, Cholestasis. p. 1291-326.
11.Fallon MB, Anderson JM, Boyer JL. ; Schiff I, Schiff E, Lippinger J, editors.Diseases of the Liver. 7th ed. Philadelphia: J.B. Lippincott Company, 1993; 13, Intrahepatic cholestasis. p. 343-61.
12.Oelberg D, Lester R. Cellular mechanisms of cholestasis. Ann Rev Med 1986;37:279-317.
13.van Nieuwkerk CMJ, Groen AK, Ottenhoff R, van Wijland MJ, van den Berg Weerman MA, Tytgat GN, Offerhaus GJA, Oude Elferink RPJ. The role of bile salt composition in liver pathology of mdr2 $^{(-/-)}$ mice: differences between males and females. J Hepatology 1997;26:138-45.
14.Benedetti A, Alvaro D, Bassoti C, Gigliozzi A, Ferretri G, La Roza T, Di Saro A, Baiocchi L, Jezequel AM. Cytotoxicity of bile salts against biliary epithelium: a study in isolated bile ductule fragments and isolated perfused rat liver. Hepatology 1997;26:9-21.
15.Hagenbuch B, Stieger B, Foguet M, Lübbert H, Meier PJ. Functional expression cloning and characterization of the hepatocyte Na+/ bile acid cotransport system. Proc Natl Acad Sci 1991;88:10629-33.

16.Stieger B, Hagenbuch B, Landmann L, Höchli M, Schroeder A, Meier PJ. In situ localization of the hepatocytic Na+/ Taurocholate cotransporting polypeptide in rat liver. Gastroenterology 1994;107:1781-7.
17.Oude Elferink RPJ, Ottenhoff R, van Wijland MJ, Smit JJM, Schinkel AH, Groen AK. Regulation of biliary lipid secretion by mdr2 P-glycoprotein in the mouse. J Clin Invest 1995;95:31-8.
18.Murphy GM, Billing BH, Baron DN. A fluorimetric and enzymatic method for the estimation of serum total bile acids. J Clin Pathol 1970;23:594-8.
19.Emmelot P, Bos CJ, van Hoeven RP, van Blitterswijk WJ. ; Fleisher S, Packer L, editors.Methods in enzymology XXXI. New York, San Fransisco, London: Academic Press, 1974;Isolation of plasmamembranes from rat and mouse livers and hepatomas. p. 75-90.
20.Lowry OH, Rosebrough NJ, Farr AL, Randal RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75.
21.Scharschmidt BF, Keeffe EB, Blankenship NM, Ockner RK. Validation of a recording spectrophotometric method for measurement of membrane-associated Mg- and NaK-ATPase activity. J Lab Clin Med 1979;93:790-9.
22.Farrell GC, Duddy SK, Kass GEN, Llopis J, Orrenius S. Release of Ca^{2+} from the endoplasmic reticulum is not the mechanism for bile acid induced cholestasis and hepatotoxicity in the intact rat liver. J Clin Invest 1990;85:1255-9.
23.Goldberg JA, Rutenberg AM. The colorimetric determination of leucine aminopeptidase in urine and serum of normal subjects and patients with cancer and other diseases. Cancer Res 1958;11:283-91.
24.Meier PJ, Meier-Abt AS, Barret C, Boyer JL. Mechanisms of taurocholate transport in canalicular and basolateral rat liver plasma membranes. J Biol Chem 1984;259:10614-22.
25.Chromczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Ann Biochem 1987;162:156-8.
26.Karpen SJ, Sun AQ, Kudish B, Hagenbuch B, Meier PJ, Ananthanarayanan M, Suchy FJ. Multiple factors regulate the rat liver basolateral sodium dependent bile acid cotransporter gene promoter. J Biol Chem 1996;271:15211-21.
27.Karpen SJ, Ananthanarayanan M, Potselueva T, Suchy FJ. Bile acids suppress the rat liver Na^{+}-dependent bile acid cotransporter gene promoter. [Abstract] Hepatology 1995;22:(4)318a
28.Whiting JF, Green RM, Rosenbluth AB, Gollan JL. Tumor necrosis factor -alfa decreases hepatocyte bile salt uptake and mediates endotoxin-induced cholestasis. Hepatology 1995;22:1273-8.
29.Setchell KDR, Rodrigues CMP, Clerici C, Solinas A, Morelli A, Gartung C, Boyer JL. Bile acid concentrations in human and rat liver tissue and hepatocyte nuclei. Gastroenterology 1997;112:226-35.
30.Zimmerli B, Valantinas J, Meier PJ. Multispecificity of Na+-dependent taurocholate uptake in basolateral (sinusoidal) rat liver plasma membrane vesicles. J.Pharmacol.Exp.Therap. 1989;250:301-8.
31.Hagenbuch B. Alternative splicing results in two transcripts for the mouse liver Na^{+}/taurocholate cotransporting polypeptide. [Abstract] Hepatology 1997;26:(4)295A
32.Lazaridis KN, Pham L, Tietz P, Marinelli RA, de Groen P, Levine S, Dawson PA, LaRusso F. Rat cholangiocytes absorb bile acids at their apical domain via the ileal sodium dependent bile acid transporter. J Clin Invest 1997;100:2714-21.
33.Gurantz D, Hofmann AF. Influence of bile acid structure on bile flow and biliary lipid secretion in the hamster. Am J Physiol 1984;247:G736-48.
34.Wong MH, Oelkers P, Craddock AL, Dawson PA. Expression cloning and characterization of the hamster ileal sodium dependent bile acid transporter. J Biol Chem 1994;269:1340-7.
35.Alpini G, Glaser S, Rodgers R, E.D., Phinizy JL, Robertson W, Lasater J, Caligiuri A, Tretjak Z, Lesage GD. Functional expression of the apical Na^{+}-dependent bile acid transporter in large but not small rat cholangiocytes. Gastroenterology 1997;113:1734-40.
36.Voshol PJ, Koopen NR, Havinga R, Oude Elferink RPJ, Groen AK, Kuipers F. Hepatic actions of dietary cholesterol in mdr2 P-glycoprotein-deficient mice. [Abstract] Hepatology 997;26:(4)294A
37.Berr F, Simon FR, Reichen J. Ethinylestradiol impairs bile salt uptake and Na^{+}-K^{+}pump function of rat hepatocytes. Am J Physiol 1984;274:G437-43.