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 \(V_{\text{max}}\) of Na\(^{+}\)-dependent taurocholate transport with an unaffected \(K_{\text{m}}\) 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 TNF\(\alpha\) 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**

[^3H(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 puncture. 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 overlaid 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 MgSO$_4$, 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 CaCl2 and 10 mM MgSO4 , bovine serum albumin (BSA) 1 mg/ml , [3H]-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 CaCl2 and 10 mM MgSO4 ) 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 NaHPO4, 1 mM Na2EDTA 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, Switzerland) 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'-GGTTCTCATTCCTTGCCGCA-3', bp 535-554; 5'-GCATCTTCTGTGCACTGACGACG-3', bp 1026-1007) were linked to a 600 bp sequence derived from the neomycin gene by PCR using the following primers (5'-GGTTCTCATTCCTTGGCCACCCCTGATGACTGACCAAC-3' forward; 5'-GCATCTTCTGTGCACTGACGACG-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, 0.4μM of each Ntcp specific primer, 10 mM KCl, 10 mM(NH₄)₂SO₄, 2mM MgSO₄, 20 mM 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 and ALT activities are markedly higher in the knockout mice than in controls. A smaller, but significant increase was found for serum bilirubin.

<table>
<thead>
<tr>
<th></th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
<th>Bilirubin (µM)</th>
<th>Bile Salts (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mdr2(+/-)</td>
<td>78 ± 24</td>
<td>29 ± 6</td>
<td>3.7 ± 0.5</td>
<td>21 ± 4.2</td>
</tr>
<tr>
<td>Mdr2(-/-)</td>
<td>203 ± 64a</td>
<td>217 ± 68a</td>
<td>5.7 ± 0.7a</td>
<td>45 ± 25.6a</td>
</tr>
</tbody>
</table>

Data are given as means ± SD, n=9 in each group.

*: significantly different from control p<0.01
**Physiological functions of biliary lipid secretion**

**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 μmol/ 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.*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mdr2(+/+)</th>
<th>Mdr2(−/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺/K⁺ ATPase (μmol·mg⁻¹·h⁻¹)</td>
<td>3.12 ± 0.44</td>
<td>1.84 ± 0.41&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>alkaline phosphatase</td>
<td>0.46 ± 0.05</td>
<td>1.10 ± 0.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>succinate cytochrome C reduct.</td>
<td>6.89 ± 0.48</td>
<td>6.64 ± 0.57</td>
</tr>
</tbody>
</table>

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

<sup>a</sup>: 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.*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mdr2(+/+)</th>
<th>Mdr2(−/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺/K⁺ ATPase</td>
<td>14.8 ± 2.1</td>
<td>16.5 ± 3.7</td>
</tr>
<tr>
<td>alkaline phosphatase</td>
<td>13.5 ± 1.6</td>
<td>16.3 ± 3.0</td>
</tr>
<tr>
<td>succ. cyt. C reduct.</td>
<td>0.15 ± 0.01</td>
<td>0.35 ± 0.10</td>
</tr>
</tbody>
</table>

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 μM 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.

**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.

**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 TNFα and IL1β are able to down-regulate Ntcp \textit{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 \textit{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.

\textit{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 \textit{Mdr2\textsuperscript{(-/-)}} 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 \textit{Mdr2\textsuperscript{(-/-)}} mice. By infusing increasing amounts of taurocholate it has been shown that \textit{Mdr2\textsuperscript{(-/-)}} mice transport bile salts at or near their maximal secretory capacity (\textit{S_{RM}}) \textit{in vitro} and \textit{in vivo} under normal circumstances [17]. This is in marked contrast to the situation in control mice in which the \textit{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 \textit{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 \textit{Mdr2\textsuperscript{(+/-)}} and \textit{Mdr2\textsuperscript{(-/-)}} 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\textsuperscript+ 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 (33 μM) [34] and the bile duct ibst (43 μM) [35] are in the same order of magnitude, as that of Ntcp (25-46 μM) [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 an 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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