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Molecular Regulation of Sinusoidal Liver Bile Acid Transporters During Cholestasis

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Impairment of the hepatic transport of bile acids and other organic anions will result in the clinically important syndrome of cholestasis. Cloning of a number of specific hepatic organic anion transporters has enabled studies of their molecular regulation during cholestasis. The best characterized transport system is a 50-51 kDa sodium-dependent taurocholate cotransporting polypeptide (ntcp), which mediates the sodium-dependent uptake of conjugated bile acids at the sinusoidal plasma membrane of hepatocytes. Under physiologic conditions and after depletion of biliary constituents, ntcp remains constitutively expressed throughout the liver acinus. However, both function and expression of ntcp are rapidly down-regulated in rat liver in various models of experimental cholestasis, such as cholestasis induced by common bile duct ligation, estrogen, endotoxin or cytokine treatment. In addition to ntcp, the sinusoidal organic anion transporting polypeptide oatp-1 is also down-regulated at the protein and steady-state mRNA levels in estrogen-cholestasis, but does not affect sodium-independent uptake of taurocholate. The regulation of a recently cloned member of the organic anion transporter family (oatp-2), which is highly expressed in liver, remains to be studied under cholestatic conditions.

INTRODUCTION

Bile formation is an important function of the liver cell to excrete lipophilic organic anions, such as bile acids, bilirubin, steroids and xenobiotics, as well as cholesterol, either directly or by conversion of cholesterol into bile acids. Furthermore, bile plays an important role in the digestion and uptake of dietary lipids and lipophilic vitamins in the intestine [1].

Bile flow is driven by generating osmotic gradients between portal blood, hepatocytes and the bile canaliculus. Organic anions, especially bile acids and their taurine and glycine conjugates, are the most important osmotically active solutes [2]. They generate the bile acid dependent bile flow, which accounts for at least 50 percent of total bile flow in all species. In addition, bile acid independent bile flow is generated by canalicular secretion of glutathione and bicarbonate, and its proportion of total bile flow varies among different species. After synthesis by the hepatocytes, bile acids undergo an efficient enterohepatic circulation, which includes excretion into bile, delivery to the intestine, absorption in the ileum, transport to the liver via the portal blood and final reabsorption by the hepato-

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[b] *Abbreviations:* ntcp, sodium-dependent taurocholate co-transporting polypeptide; oatp, organic anion-transporting polypeptide; cMOAT, canalicular multispecific organic anion transporter; mrp, multidrug resistance protein; EE, ethinyl-estradiol; LPS, lipopolysaccharide.

cytes [3]. Only 5 percent of the total bile acid pool is daily lost by the feces and must be replaced by *de novo* synthesis in the liver.

BILE ACID TRANSPORT SYSTEMS IN THE LIVER

The current understanding of the vectorial transport of bile acids in the liver is based on the concept that hepatocytes are classical epithelial cells, which are highly polarized with respect to transport systems at the basolateral (sinusoidal) and apical (canalicular) plasma membranes [4]. The inorganic ion transporter Na^+-K^+-ATPase is exclusively localized at the basolateral plasma membrane of hepatocytes and maintains ion gradients necessary for driving secondary and tertiary active transport systems. This cation pump exchanges three intracellular sodium ions for two extracellular potassium ions, thereby generating an inwardly directed sodium gradient and an outwardly directed potassium

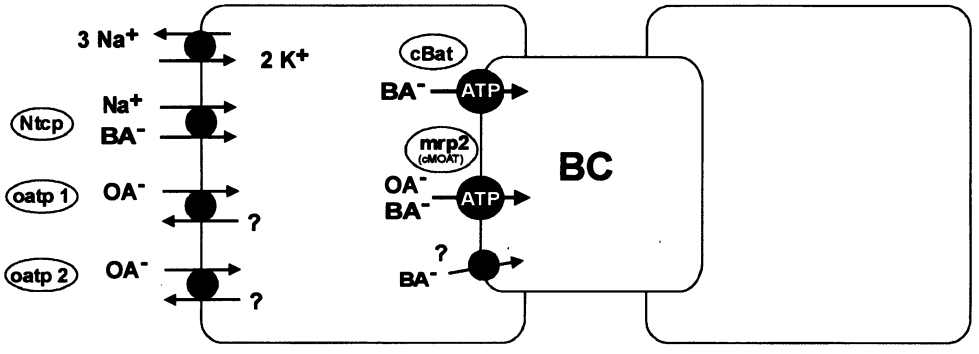

Figure 1: Schematic illustration of basolateral and canalicular bile acid and organic anion transporters in rat hepatocytes. At the basolateral plasma membrane uptake of conjugated bile acids (BA-) occurs by the sodium-dependent taurocholate cotransporting polypeptide (ntcp). At least two multispecific organic anion transporters (oatp-1, oatp-2) mediate the uptake of a variety of organic anions (OA^-) including bile acids. The physiological driving force of both oatps remains still unknown. At the canalicular plasma membrane monovalent bile acids are secreted into the bile canaliculus (BC) by the ATP-dependent bile acid transporter (cBAT), whereas divalent organic anions are transported by the canalicular multi-organic anion transporter (cMOAT). The latter has recently been shown to be identical with a canalicular isoform of the multidrug resistance protein (mrp2). The contribution of a potential driven pathway for secretion of monovalent bile acids at the canalicular membrane remains controversial.

gradient [5]. In addition to maintaining ion gradients, the Na^+-K^+-ATPase generates intracellularly a negative electrical potential, which drives uptake and secretion of positively and negatively charged solutes, respectively. Regarding bile acid transport, five different carriers have been identified at these domains [6, 7] (Figure 1).

The best characterized transporter is a secondary active, sodium-dependent bile acid uptake system at the basolateral membrane, which mediates a high affinity uptake of conjugated bile acids (K_m 10-30 μM) from the portal blood. This transport system represents a 50-51 kDa taurocholate cotransporting polypeptide (ntcp/NTCP) and the genes encoding

these proteins have recently been cloned from rat and human liver [8-11]. The second transport system belongs to a growing family of organic anion-transporters and was the first to be identified in rat liver by expression cloning [12]. This organic anion transporting polypeptide (oatp-1), with an apparent molecular weight of 80 kDa, mediates the sodium-independent uptake of a broad spectrum of amphipathic compounds, such as organic anions (e.g., bile acids and bromosulphthalein (BSP), anionic steroid conjugates (e.g., estrone 3-sulfate and estradiol 17ß-glucuronide), neutral steroids (e.g., aldosterone, ouabain) and some organic cations (e.g., N-(4,4-azo-n-pentyl)-21-deoxyajmaline) [13,14]. A cloned human OATP has also been shown to transport independent of sodium similar substrates as the rat liver oatp-1 including bile salts and BSP [15, 16]. A third member of the oatp/OATP family with a 37 percent amino acid identity with the rat liver oatp-1 may be involved in the transport of prostaglandins in rats [17]. Finally, an additional organic anion transporter (oatp-2) with high expression in rat liver mediates the uptake of taurocholate and cholate as well as digoxin after expression in *Xenopus laevis* oocytes [7].

After translocation of bile acids across the hepatocytes, two different active transport systems at the canalicular domain of the hepatocytes are involved in the secretion of bile salts into the bile canaliculus. Canalicular excretion of bile salts, which represents the rate limiting step in overall hepatic bile acid transport, generates an osmotic gradient by a more than 100-fold concentration of bile salts within the bile canaliculus over that of the liver cells and portal blood. This gradient leads to passive influx of water and electrolyte from the liver cell and via the paracellular pathway. Secretion of monovalent bile acids is mainly mediated by an ATP-dependent bile acid transporter, which has functionally been characterized [18-21]. Preliminary data suggest that the canalicular bile acid transporter might be identical to the recently cloned sister-p-glycoprotein, a member of the ATP-binding cassette superfamily [22]. Sulphated and glucuronidated bile acids are substrates of the canalicular multiorganic anion transporter (cMOAT), which has recently been shown to be identical with a canalicular isoform of the multidrug resistance-associated protein mrp2 [23-26]. *mrp2* is not expressed in TR^-/GY-rats and its protein is absent in patients with the Dubin-Johnson syndrome due to a mutation in the *mrp2/cMOAT* gene [25, 27, 28]. In addition to ATP-dependent transport, potential driven canalicular secretion of bile acids has been demonstrated in isolated perfused livers and hepatocytes couplets [5]. However, its present role in overall canalicular secretion of bile acids remains controversial, since subfractionation studies using free-flow electrophoresis have localized electrogenic taurocholate transport almost exclusively to the endoplasmic reticulum, whereas ATP-dependent transport of taurocholate was confined to the canalicular membrane and a subapical compartment [29].

Impairment of function and expression of either the Na^+-K^+-ATPase or any of the bile acid transporters might result in cholestasis characterized by reduced bile flow, retention of biliary constituents including bilirubin and bile acids, and clinically important features such as jaundice and pruritus. Although changes in transporter function have been described for some of these transporters in a variety of experimental models of cholestasis, cloning of *ntcp*, members of the *oatp/OATP*-family and *cMOAT/mrp2* has enabled for the first time studies of their molecular regulation under cholestatic conditions. Cloning of the canalicular ATP-dependent bile acid transporter will close a major gap in our current understanding of hepatic bile acid transport within the near future.

FUNCTION AND EXPRESSION OF THE NA$^+$-K$^+$-ATPASE DURING CHOLESTASIS

Although Na$^+$-K$^+$-ATPase plays an important role in maintaining the functional polarity of the hepatocytes and driving secondary and tertiary active transport systems (e.g., the sodium-dependent uptake of bile acids, amino acids and glucose), little is known about its function and expression under various cholestastic conditions. Na$^+$-K$^+$-ATPase is a transmembrane protein and consists in the liver of a large 112-kDa catalytic subunit (α_1-subunit) and a 50-kDa glycoprotein ($ß_1$-subunit) of unknown function. Changes in the activity of Na$^+$-K$^+$-ATPase correlate with similar alterations of the bile acid independent bile flow [5]. The best studied model of experimental cholestasis affecting Na$^+$-K$^+$-ATPase activity has been the cholestasis induced by synthetic estrogens in rat liver (e.g., ethinyl estradiol 5 mg/kg body weight for 5 days). Most studies demonstrate a decrease in Na$^+$-K$^+$-ATPase activity by about 50 percent within 1-5 days of treatment with estrogens [30]. Whereas expression of both α_1 and β_1-subunits remained unchanged at the protein and mRNA levels [30], decrease in Na$^+$-K$^+$-ATPase activity has been attributed to changes in membrane fluidity, which is reversible by treatment of membranes with Triton WR 1339 and can be prevented by simultaneous application of *S*-adenosyl-L-methionine [31]. Although taurocholate uptake is markedly diminished in estrogen-induced cholestasis, reduced Na$^+$-K$^+$-ATPase activity does not seem to be of any pathophysiological significance regarding bile acid transport. Normalization of fluidity in vitro does not prevent the decrease in taurocholate uptake and reduced Na$^+$-K$^+$-ATPase activity has been associated with both decreased bile flow after estrogen treatment and increased bile flow after treatment with spironolactone [30, 32-34]. In endotoxin-induced cholestasis a similar decrease in Na$^+$-K$^+$-ATPase activity by about 50 percent of controls and a reduction in bile flow and taurocholate uptake, respectively, have been reported by several investigators [35-37], but protein and mRNA expression at least of the α_1-subunit remained unchanged. In contrast to sodium-dependent uptake of taurocholate, sodium-dependent uptake of the amino acid alanine is increased in endotoxemic rats [36] suggesting that alterations of the Na$^+$-K$^+$-ATPase activity do not affect sodium-dependent transport in this model of cholestasis. Although the mechanisms of altered Na$^+$-K$^+$-ATPase activity in endotoxemia remain unclear, decreased membrane fluidity as previously reported in endotoxemic shock might play a role in the regulation of the enzyme activity [38]. In extrahepatic cholestasis induced by common bile duct ligation, Na$^+$-K$^+$-ATPase activity seems to be slightly increased after 3 days, but no detailed study has been published so far. Similar to other cholestastic models, expression of both protein and mRNA levels of the catalytic α_1-subunit of the Na$^+$-K$^+$-ATPase remained unchanged for up to 7 days of bile duct ligation [39]. In summary, Na$^+$-K$^+$-ATPase activity is reduced at least in experimental models of intrahepatic cholestasis. However, several lines of evidence argue against any role of altered Na$^+$-K$^+$-ATPase activity in the pathogenesis of reduced sodium-dependent taurocholate uptake and diminished bile flow in cholestasis, especially as major alterations occur in function and expression of bile acid transporters as "down-stream" events during cholestasis.

MOLECULAR REGULATION OF THE NA$^+$-DEPENDENT BILE ACID TRANSPORTER IN CHOLESTASIS

So far the rat liver Na$^+$/taurocholate cotransporting polypeptide (ntcp) represents functionally and at the molecular level the best characterized bile acid transport system, and serves as a paradigm for the study of other bile acid transporters under physiological and pathophysiological conditions. Function and expression of this transport system have been extensively characterized in models of extrahepatic and intrahepatic cholestasis [30,

Table 1: Effect of various experimental models of cholestasis on ntcp function and expression.

Animal model	Transport	Protein mass	Steady-state mRNA	Gene transcription
Bile duct ligation	↓↓ (30%)	↓↓↓ (< 10%)	↓ (40–50%)	↓↓ (20%)
Estrogen	↓↓↓ (10%)	↓↓ (≈ 25%)	↓ (50 %)	n.d.
Endotoxin				
3 days	↓ (50%)	↓↓ (≈ 25%)	↓↓↓ (< 15%)	n.d.
16 hr	↓↓↓ (absent)	↓↓↓ (< 10%)	↓↓↓ (< 10%)	n.d.

Data were obtained from previous publications: bile duct ligation [39], estrogen [30], endotoxin (3 days [36]; 16 hours [37]). The number of arrows correlates with the extent of down-regulation. The numbers given in parenthesis are percentage of sham-operated controls.

36, 37, 39]. In all of these experimental models of cholestasis, studies demonstrated specific down-regulation of both function and expression of ntcp in rat liver (Table 1).

In a model of extrahepatic cholestasis induced by common bile duct ligation (CBDL), ntcp protein mass virtually disappeared 3 days after common bile duct ligation, whereas other basolateral proteins such as the α_1-subunit of the Na^+-K^+-ATPase and CE-9 remained unchanged by this form of cholestasis. Down-regulation of the protein mass was accompanied by a reduction in *ntcp* mRNA levels by 50 percent and gene transcriptional activity by 80 percent of controls. These results suggest both transcriptional and posttranscriptional regulation of the ntcp gene during cholestasis. Furthermore, changes in ntcp expression resulted in a 70 percent decrease of sodium-dependent taurocholate uptake [39].

In estrogen-induced cholestasis, initial sodium-dependent taurocholate uptake was also significantly reduced. As early as 3 days after ethinyl estradiol (EE) treatment, sodium-dependent taurocholate uptake declined by 40 percent of controls and was further reduced by 90 percent after treatment had been extended for up to 7 days. Similar to sodium-dependent taurocholate uptake, ntcp protein mass significantly declined by 45 percent as compared to controls after 3 days, and was further reduced by 80 percent after 5 days of EE application. Two bands of the ntcp protein with an apparent molecular weight of 56 kDa and 51 kDa were identified in sinusoidal membrane preparation, which presumably represented two differentially glycosylated isoforms of ntcp, since deglycosylation resulted in a single band with an approximate molecular weight of 33 kDa. Whereas the higher glycosylated form of ntcp was rapidly reduced by 40 percent within 12 hr after the first EE application, the major 51 kDa band remained unchanged until 5 days of EE treatment, when its protein content declined to 35 percent of control values. In contrast to the slow decline in ntcp protein mass, steady-state mRNA levels of ntcp rapidly decreased by 50 percent of controls within 12 hr of EE treatment, and remained at similar levels for up to 5 days [30].

Induction of cholestasis by endotoxin or cytokines such as tumor necrosis factor alpha (TNF-α) or interleukin 1ß (IL-1ß) exhibited a similar effect on sodium-dependent taurocholate uptake and ntcp protein and steady-state mRNA levels as described for cholestasis induced by bile duct ligation or estrogens [36, 37]. After daily injection of endotoxin (*E. coli* 0127:B8 LPS, 1 mg/kg body weight) for 3 days, Moseley et al. found a 50 percent decrease in the V_{max} of sodium-dependent taurocholate uptake, and a reduction in ntcp protein mass by more than 70 percent and its mRNA by more than 85 percent of controls, respectively. A similar decline was observed in the initial uptake rate of taurocholate and the expression of *ntcp* mRNA 4 hours after application of TNF-α [36]. Green et al. reported that injection of a less potent form of LPS, although at a much higher dose (*E. coli*

26:B6, 15 mg/kg body weight), resulted in non-detectable sodium-dependent taurocholate uptake and a specific decline of ntcp protein mass and its steady-state mRNA levels by more than 90 percent of control levels 16 hr after LPS injection. In a mouse model, injection of the proinflammatory cytokines TNF-α and IL-1ß decreased ntcp mRNA levels by 65 percent of controls within 6 hr after application. In contrast, various doses of interleukin-6 did not affect *ntcp* mRNA *in vivo* similar to observation in isolated hepatocytes by the same authors [40].

The results of these studies in various models of experimental cholestasis suggest that liver cells in general respond to cholestasis by down-regulation of their basolateral Na^+-dependent bile acid uptake system. Clinical relevance of this finding is implicated by a recent publication of Shneider and co-workers, who reported a marked down-regulation of *NTCP*-mRNA in patients with biliary atresia, a disease, in which extrahepatic bile duct obstruction occurs [41]. Since decreased ntcp expression results in significantly reduced Na^+-dependent uptake of bile acids, which accounts physiologically for approximately 90 percent of conjugated bile acid uptake, it can be speculated that down-regulation of ntcp/NTCP represents a protective mechanism by which the hepatocytes minimize the uptake of bile acids and limit their hepatotoxicity during cholestasis.

Whereas TNF-α, IL-1ß and estrogens have to be regarded as potential regulators of the *ntcp* gene during intrahepatic cholestasis, no specific factors have been identified so far as regulators of ntcp in extrahepatic cholestasis. However, studies in various models of bile retention and depletion suggest that retention of biliary constituents plays an important role in the regulation of the *ntcp* gene. In all models of bile retention including the choledochocaval fistula and the selective bile duct ligated rat, ntcp expression was down-regulated as long as biliary constituents were increased in the serum and presumably retained within the liver [42]. In selective bile duct ligated rats, down-regulation of *ntcp* mRNA levels was confined to the obstructed liver lobes, whereas *ntcp* mRNA remained unchanged in open liver lobes in this cholestastic model. As shown by regression analysis of data combined from all surgical cholestasis and retention models, the decrease in ntcp steady-state mRNA levels correlates linearly with serum bile acid levels in the 0-100 µmol/l range [42]. Although no specific compounds in bile have been identified which regulate ntcp expression *in vivo*, preliminary studies using the cloned ntcp promoter have shown suppression of *ntcp* promoter activity by various bile acids [43, 44].

MOLECULAR REGULATION OF THE ORGANIC ANION TRANSPORTING POLYPEPTIDE OATP-1 IN CHOLESTASIS

In addition to sodium-dependent transport, bile acids and other organic anions are taken up into the liver cells by a sodium-independent transport mechanism. Although several organic anion transporters have functionally been identified, the numbers and types of transporters and their substrate specificity remain controversial [6]. The cloned *oatp-1* has been shown to mediate the sodium-independent uptake of a variety of conjugated and unconjugated bile acids [13]. Less well characterized than ntcp in cholestasis, oatp-1 protein and steady-state mRNA levels are also down-regulated after estrogen treatment, but no change was observed in the sodium-dependent uptake of taurocholate for up to 7 days [30]. Although estrogen treatment resulted in a rapid decrease in *oatp-1* mRNA by 72 percent of controls within 12 hr and a further decline by 90 percent after 5 days, oatp-1 protein mass was still detectable after 5 days of EE treatment reaching 38 percent of control levels. These data suggest that either a decrease in oatp-1 protein mass does not correlate with its transport ability within a certain range or that oatp-1 does not play an important role in the overall sodium-independent transport of bile acids. Alternatively, loss in oatp-1 transport activity due to its decreased protein expression might be com-

pensated by additional organic anion transporters (e.g., the recently cloned *oatp-4* [7]), which remain functionally unaffected by cholestasis. Preliminary studies in cholestasis induced by common bile duct ligation would support the latter hypothesis, since down-regulation of oatp-1 protein and its steady-state mRNA by bile duct ligation did not affect sodium-independent uptake of bile acids [45].

CONCLUSIONS

The cloning of sinusoidal bile acid and organic anion transporters has opened a new era in our understanding of the pathophysiology of cholestasis. The identification of the canalicular multiorganic anion transporter (cMOAT/mrp2) as a liver homologue of the multidrug resistance-associated protein and the study of its regulation in cholestasis [46] has added another piece of the puzzle. The cloning of the ATP-dependent canalicular bile acid transporter will finally close a major gap in studying hepatic plasma membrane bile salt transport under physiologic and pathophysiologic conditions. With the availability of human probes, expression of bile acid and organic anion transporters can be determined in various human liver diseases including cholestatic syndromes.

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