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Sodium-dependent bile salt transporters of the *SLC10A* Transporter Family: More than solute transporters

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Summary

The *SLC10A* transporter gene family consists of seven members and substrates transported by three members (*SLC10A1*, *SLC10A2* and *SLC10A6*) are Na⁺-dependent. *SLC10A1* (sodium taurocholate cotransporting polypeptide or NTCP) and *SLC10A2* (apical sodium-dependent bile salt transporter or ASBT) transport bile salts and play an important role in maintaining enterohepatic circulation of bile salts. Solute other than bile salts are also transported by NTCP. However, ASBT has not been shown to be a transporter for non-bile salt substrates. While the transport function of NTCP can potentially be used as liver function test, interpretation of such a test may be complicated by altered expression of NTCP in diseases and presence of drugs that may inhibit NTCP function. Transport of bile salts by NTCP and ASBT is inhibited by a number of drugs and it appears that ASBT is more permissive to drug inhibition than NTCP. The clinical significance of this inhibition in drug disposition and drug-drug interaction remains to be determined. Both NCTP and ASBT undergo post-translational regulations that involve phosphorylation/dephosphorylation, translocation to and retrieval from the plasma membrane and degradation by the ubiquitin-proteasome system. These posttranslational regulations are mediated via signaling pathways involving cAMP, calcium, nitric oxide, phosphoinositide-3-kinase (PI3K), protein kinase C (PKC) and protein phosphatases. There appears to be species difference in the substrate specificity and the regulation of plasma membrane localization of human and rodent NTCP. These differences should be taken into account when extrapolating rodent data for human clinical relevance and developing novel therapies. NTCP has recently been shown to play an important role in HBV and HDV infection by serving as a receptor for entry of these viruses into hepatocytes.

Introduction

Currently, the *SLC10A* gene family consists of seven members (30,41). Sodium-dependent bile salt transport in rat hepatocytes was first reported in 1978 (6). The transporter was first cloned from rat using a functional expression cloning approach (61,64) and is known as the sodium taurocholate cotransporting polypeptide (NTCP). It is the founding member of this family and is named *Slc10a1*(59). Other members of the family include apical bile salt transporter (ASBT; *SLC10A2*), *SLC10A3*, *SLC10A4*, *SLC10A5*, Na⁺-dependent organic anion transporter (SOAT; *SLC10A6*) and *SLC10A7*(30,41,50). NTCP is localized at the basolateral membrane of hepatocytes, while ASBT is localized at the apical membrane of cholangiocytes, ileum and renal proximal tubules (32,41). SOAT transports sulfated

tauroithocholate and sulfated steroid metabolites (34). Neither transport activity nor specific substrates have been identified for the gene products of *SLC10A3*, *SLC10A4*, *SLC10A5* and *SLC10a7* and these are currently considered orphan transporters. Of the seven members of the *SLC10A* family, NTCP and ASBT have been extensively characterized with established role in the enterohepatic circulation of bile salts.

Bile salts are the major constituents of bile and play an important role in the intestinal digestion of fat and absorption of fat soluble vitamins (75). They may also play an important role in systemic energy homeostasis (123). Bile acids are synthesized in hepatocytes from cholesterol in a complex series of biochemical reactions (129). The resulting primary bile acids, cholic acid and chenodeoxycholic acid, are conjugated to glycine or to taurine (75). These conjugated bile salts have lower pKa values and are consequently more hydrophilic and less cytotoxic than their unconjugated forms (75). Inborn errors of bile acid biosynthesis can cause severe liver diseases (31,71,143). In hepatocytes, the newly synthesized bile salts mix with bile salts entering the cells via the basolateral membrane and are exported against a steep concentration gradient into the canaliculus. The canaliculi are the starting point of the biliary tree from where bile salts reach the duodenum to assist fat digestion. Bile salts are reabsorbed along the small intestine and transported via the portal circulation back to the liver for uptake and resecretion (76). This circling of bile salts is called enterohepatic circulation and bile salts can be used as a model for understanding the journey of drugs undergoing enterohepatic circulation (36,77,148).

NTCP and ASBT are the two members of the SCLC10 transporter family involved in maintaining uninterrupted enterohepatic circulation of bile salts. Uptake of bile salts into the hepatocytes occurs predominantly by NTCP (*SLC10A1*) and to a lesser extent by OATPs (*SLCOs*)(105,146). So far, no inherited severe diseases associated with these transporters have been reported. Export of bile salts across the canalicular membrane is mediated by the ATP-dependent canalicular bile salt export pump BSEP (*ABCB11*)(105,146). Mutations in this transporter lead to progressive familial intrahepatic cholestasis type 2 suggesting a lack of backup transporter(s) for canalicular efflux of bile salts (80,92). Uptake across the apical membrane of enterocytes is mediated by the apical bile salt transporter ASBT (*SLC10A2*) (34,36), while the efflux from enterocyte at the basolateral membrane is facilitated by the heterodimeric transporter OST α /OST β (35,36). Mutations in the gene coding for ASBT may lead to primary bile acid malabsorption (7). During the passage through the bile duct, a small fraction of bile salts undergoes reabsorption through cholangiocytes into the portal blood by transporters located at the luminal side (ASBT) and the basolateral side (MRP3/4 and OST α / β) and then into the hepatocytes by NTCP. This cycling of bile salts between cholangiocytes and hepatocytes is known as cholehepatic shunting (30,75).

This review will focus on NTCP and ASBT highlighting latest developments and post-translational regulations. Aspects of NTCP and ASBT not included in this review can be found in a number of excellent recent reviews on the family of *SLC10A* transporters (30,34,41,50,65,88).

2. Transport function of NTCP

NTCP is the only hepatocellular sodium-dependent uptake system for bile salts, at least in rats if not in all mammals and this is based on results that a rat NTCP-specific antisense oligonucleotide co-injected with total rat liver mRNA into *Xenopus laevis* oocytes blocked the expression of Na⁺-dependent taurocholate (TC) uptake by approx. 95% (62). To date, no information on mice with a disrupted *Slc10a1* gene has been published, which would allow to further support this previous finding. Also, in humans so far no patients with mutations rendering the transport activity of human NTCP (hNTCP) non-functional have been

published. Hence, a definitive answer on the number of sodium dependent transport system(s) for uptake of bile salts into hepatocytes is still lacking.

From a thermodynamic point of view, NTCP is a typical secondary active transporter and has been shown to be strictly sodium-dependent. NTCP necessitates the binding of two sodium-ions together with a bile salt molecule for the transport step. In the case of the monoanionic TC, as well as the monoanionic fluorescent bile salt cholyl-Nε-NBD-lysine, rat NTCP (rNTCP) transport activity has been shown to be electrogenic, while it is electroneutral for the dianionic fluorescent bile salt cholylglycylamidofluorescein (164). Hence, depending on the charge of the substrate, the driving force of the uptake may vary and may consist of the energy available from the chemical and/or the electrochemical gradient of sodium.

The list of substrates transported by NTCP has been extensively reviewed (30,41,146). Solutes other than bile salts including steroid hormones, thyroid hormones, drugs and drugs conjugated with bile acids can be transported by hNTCP/rNTCP (*SLC10A1/Slc10a1*)(41,44). Comparison of published K_m values for bile salts obtained from intact livers and heterologously expressed hNTCP/rNTCP (41,146) suggests that the properties of the cloned NTCP/Ntcp reflect reasonably well the affinity of NTCP/Ntcp in *in-vivo* situations. A study reported that human hepatocellular carcinomas express NTCP, which mediates high-affinity uptake of chlorambucil-taurocholate (94). Thus, cytotoxic drugs conjugated with TC could provide a potential therapeutic strategy to deliver these drugs to hepatocellular carcinomas.

In addition to endogenous substrates, drugs including statins and recently micafungin (170), an antifungal agent, have been reported to be transported by NTCP (41). Interestingly, rosuvastatin is transported by hNTCP, but not by rNtcp (73). This is one example, which demonstrates that despite a large substrate overlap among rat, mouse and human NTCP (146) transport data should be extrapolated with caution across different species.

The physiological relevance of a transporter is raised when a solute is transported by multiple transporters. For example, statins are also transported by organic anion transporting polypeptides (OATPs) (117) and this may also be true for other solutes not yet described. While the question of the physiologically relevant transporter(s) for drugs cannot be answered now, limited information is available for bile acids and bile salts as endogenous substrates. Upon heterologous expression of hNTCP/rNTCP, this transporter is capable of transporting conjugated bile salts as well as unconjugated bile acids (146). The same is observed for OATPs expressed in liver with a preference for unconjugated bile acids (63). Thus, one could conclude that NTCP might, with respect to bile salt transport, replace hepatocellular OATPs. However and interestingly, mice with an inactivated locus for the *Slc10a1/1b* subfamily display normal serum levels of conjugated bile salts, but have 13-fold elevated levels of unconjugated bile acids (157). Knocking out only *Slc10b2* in mice also leads to some elevation of unconjugated bile acids with normal bile salt levels in serum (33). These findings suggest that, at least in mice, NTCP is a very poor, if at all a transporter, for unconjugated bile acids.

In order to address the issue on transporter overlap for conjugated bile salts, data from *Slc10a1* knock-out mice would be needed.

The structure of NTCP has not yet been resolved. The current knowledge derived from modeling and mutation experiments is still very limited and summarized elsewhere (30,41). In cases where exact structural information is lacking, chemical modifications of substrates with subsequent transport/inhibition studies may yield further information on transport properties. To this end, the side chain of bile salts was identified as very important for

substrate interaction with hNTCP (9). Later, it was found by using a series of bile acids analogs with modifications at the C-3 and C-7 position that modifications at the C-7 but not at the C-3 position abolished hNTCP mediated transport with comparable results for hASBT (87). Two recent studies reported on a pharmacophore model and a structure-activity relationship for hNTCP, respectively (40,55). The first study (55) identified two hydrogen bond acceptors and three hydrophobic elements as important structural features. Thereby the distance between the hydrogen bond acceptor features as well as the presences of one or two negative charges (absent in their features) are essential for successful interaction of compounds with hNTCP. The second study (40) used inhibition studies with drugs and found that two hydrophobic elements and one hydrogen bond acceptor are of importance for the interaction with hNTCP. Hence, the two models show considerable differences. One reason could be the use of IC₅₀ values for model development. IC₅₀ values give no information on the type of inhibition (23) and hence may include substance binding distant to the substrate binding site of hNTCP.

3. Transport function of ASBT

Different from NTCP, the substrate specificity of ASBT is considerably narrower. Traditionally, bile acids and bile salts were identified as sole ASBT substrates (34,41). Lately, evidence has been presented supporting the concept that bile salt transport capacity of ASBT is phylogenetically conserved (101). Interestingly, a bacterial homologue of ASBT is also capable of mediating bile salt transport (78). New studies have expanded the list of substrates to two uncharged non-bile salt substrates containing an amide bond and an alcohol expanding the substrate spectrum of hASBT to solutes other than bile salts (87). ASBT has been proposed to be used as target for mediating intestinal absorption of drugs conjugated to bile salts to increase the bioavailability of such drugs. This “Trojan horse” approach has, however, met with limited success (8). This observation further supports the concept of ASBT having a rather narrow substrate specificity. Mechanistically, ASBT is electrogenic requiring cotransport of two sodium ions together with a bile salt molecule (34).

The structure of a mammalian ASBT has not yet been determined. However, recently a crystal structure of ASBT from *Neisseria meningitidis* was elucidated at 2.2 Å. ASBT_{NM} from *N. meningitidis* is 26 % identical and 54 % similar to human ASBT and transports TC in a sodium-dependent manner with a K_m-value of 52 μM (78). This structure, obtained in the presence of TC and sodium, revealed 10 transmembrane domains and a hydrophobic inward-facing binding cavity. This bacterial ASBT structure differs considerably from the currently favored model for mammalian ASBT thought to cross the plasma membrane 7 to 9 times (30,41). Hence, clearly much more work is needed to understand the structure of mammalian ASBT.

As ASBT is a candidate for enhancing intestinal absorption of (pro)drugs, extensive structure function studies aimed at determining a pharmacophore structure for ASBT substrates have been conducted. Based on functional studies using intestinal perfusion and everted intestine sac models, several requirements for ASBT substrates were found to be important (34) and these include a negatively charged side chain, at least one hydroxyl group at the steroid nucleus position 3, 7 or 12 and a *cis*-configuration of the steroid backbone. Extensive further studies led to the development of a first pharmacophore model, which differed considerably from the early model. This model consists of a hydrogen bond donor function, a hydrogen bond acceptor function and three hydrophobic features. Importantly, neither the 3-hydroxygroup of the steroid ring nor a *cis*-orientation of the A-ring was found to be important (9). The current state of knowledge on the chemical properties of ASBT substrates shows the following main features (87,90): 1) binding of a

bile salt to ASBT involves one hydrogen bond donor, one hydrogen bond acceptor and three hydrophobic interactions, 2) lack of a negatively charged C-24 side chain maintains some affinity for ASBT, but the negatively charged side chain is necessary for high affinity and 3) the 3-position is the preferred position for drug targeting to ASBT.

4. Role of NTCP in Liver function tests

Anionic dyes have been known to be extracted from blood by the liver and that some of them may undergo enterohepatic circulation has been known for more than 100 years (1). Bromosulfophthalein (BSP) and indocyanine green (ICG) have been used clinically, whereby the latter is still in use today (131). BSP was indeed the substrate used when the first Oatp (rat Oatp1a1) was identified by expression cloning (81). Today, the role of transport systems in liver function tests is increasingly appreciated (74,147). Rat Ntcp was found to transport BSP if expressed in *Xenopus laevis* oocytes (60) and displayed a Km for BSP of 3.7 μ M when stably expressed in HeLa cells (68). Also human NTCP transports BSP (43) and in addition mediates uptake of ICG into stably transfected cells (37). A comparative study (97) using the three heptaocellular OATPs and NTCP found that NTCP can mediate uptake, albeit weakly, of the magnetic resonance imaging contrast agent gadolinium-ethoxybenzyl-diethylenetriamine pentaacetic acid (Gd-EOB-DTPA). Hence several exogenous compounds used to assess dynamic liver functions in humans are (among other transporters) taken up by NTCP into human hepatocytes. Naturally, endogenous substrates are a viable option for dynamically assessing liver function, as there will be no issues related to potential toxicity, as for example for BSP (131). Indeed, radioactively labeled cholyglycine shows a decreased clearance in patients with liver disease in comparison to healthy controls (96). In addition, there is a good correlation between clearances of cholytaurine and ICG (119). Instead of using radioactively labeled bile salts, fluorescent bile salts could potentially be an alternative, given that NTCP does not exclusively transport bile salts. A pilot study in a small number of patients with liver cirrhosis proved this concept for choly-lysyl-fluorescein (CLF) (108). An investigation of CLF as potential substrate for organic anion transporters present in hepatocytes did however demonstrate that CLF is not a substrate for NTCP, but rather for OATP1B3 and to a lesser extent for OATP1B1 (38). The same study however found that CLF is a very good inhibitor of NTCP. This exemplifies that the modifications of NTCP substrates have to be carefully considered and support the concept that the side chain of bile salts is a critical determinant for their recognition by NTCP as a transported substrate (9). The following fluorescent bile salts have been shown to be substrates of rat or human NTCP: cholyglycylfluorescein and chenodeoxycholylysyl-NBD (22), NBD-cholytaurine (122), cholyglycylamidofluorescein and chenodeoxycholyglycylamidofluorescein (109,114).

The fact that several substances used to perform dynamic liver function tests are transported by NTCP has several implications: 1. The sodium-gradient across the plasma membrane constitutes a driving force for NTCP. This sodium gradient is maintained by (Na⁺, K⁺)-ATPase. In some forms of liver diseases, the activity of (Na⁺, K⁺)-ATPase may be impaired as shown in an animal model of endotoxin-induced cholestasis (156). Also, treatment of rats with pharmacologic doses of ethinylestradiol has been found by some to reduce the activity of (Na⁺, K⁺)-ATPase (11,107) but not by others (17,18,95) 2. NTCP expression can be up or down regulated in certain forms of human liver diseases. Thus, NTCP is down-regulated in progressive familial intrahepatic cholestasis (84), inflammatory cholestasis, primary biliary cirrhosis and chronic hepatitis C (67,115,173,174), while NTCP is up-regulated in nonalcoholic steatohepatitis (10), end-stage primary biliary cirrhosis (153) and in patients with late-stage obstructive cholestasis (25). 3. Polymorphic variants of the *SLC10A1* gene lead to the expression of NTCPs with different transport properties (72). 4. mRNA expression of NTCP is subject to considerable inter-individual variability (85) and may

translate into variable protein expression as demonstrated for hepatocellular ABC-transporters (106). 5. Certain drugs may interfere with the transport activity of NTCP. Hence, while some liver function tests may be affected by a disease and patient dependent altered expression and/or function of NTCP, clearly more information is needed to take advantage of NTCP as a molecular determinant in dynamic liver function tests.

5. Clinical implication of inhibition of NTCP/ASBT by drugs

Drugs may inhibit solute transport by NTCP/ASBT in two major ways: 1) drugs may be transported by the transporters resulting in competitive inhibition and 2) drugs may inhibit solute uptake by the transporters without being transported by the transporter. Indeed, there are drugs that inhibit hNTCP/rNTCP mediated bile salt uptake, but are not transported by hNTCP/rNTCP (41,89,146). Drugs that inhibit solute transport by transporters with or without being transported can have significant effects on the disposition of endogenous substrates and drugs. These effects are important considerations in drug development, as they may result in adverse effects or provide therapeutic strategies. Thus, inhibition of NTCP would be expected to impair disposition of bile salts resulting in higher systemic concentration of bile salts and consequent adverse effects (103,109). Similarly, inhibition of ASBT will result in disruption of enterohepatic circulation of bile salts and increased fecal loss of bile salts and their metabolites resulting in undesirable effects of bile acids in the colon, such as diarrhea (124). Inhibition of rodent ASBT also results in effects secondary to decreased intestinal bile acid absorption, such as decreased plasma cholesterol (100). Thus, an experimental drug (SC-435) inhibits TC uptake by ASBT and reduces atherosclerosis in apolipoprotein E null mice (14). In addition, inhibitors of ASBT (A3309 & 264W94) have been shown to produce beneficial effects in patients with chronic idiopathic constipation (27,144) and alleviate diabetes in Zucker Diabetic Fatty (ZDF) rats (26). A number of drugs have been shown to inhibit ASBT (40,54,172) and these include calcium channel blockers (nifedipine, isradipine, diltiazem, verapamil), HMG-CoA reductase inhibitors (simvastatin, mevastatin, lovastatin), diuretics (spironolactone, bumetadine, althazide, but not furosemide) and others (dibucaine, indomethacin, mesoridazine, quinine). However, the clinical implications of inhibition of ASBT by these drugs are yet to be determined.

The effect on drug disposition can also lead to drug-drug interaction (DDI). DDI is an important determinant of drug safety and efficacy, and is influenced by factors that affect drug disposition. It is becoming evident that transporters often work together with drug metabolizing enzymes in drug disposition. While interactions with drug metabolizing enzymes have long been considered to be important in drug disposition, a role for transporters in drug disposition, therapeutic efficacy and adverse drug reactions has increasingly been recognized (90). Some transporters are considered to be clinically important in drug absorption and disposition and therefore could mediate DDIs (51). Although a number of SLC transporters are considered to have clinical importance in the absorption and disposition of drugs (51), NTCP and ASBT are not included. This is mainly because the clinical importance of these transporters is not well established. Since drugs can be transported by NTCP and can also inhibit solute transport by NTCP, a potential role of NTCP in drug-drug interaction is very likely and is reported (69,170). Similarly, ASBT may also be involved in drug-drug interactions that may be revealed in future studies.

There appears to be a difference in the ability of drugs to inhibit NTCP and ASBT. A previous study in rabbit suggested that more drugs inhibited NTCP than ASBT (91). A recent study compared the inhibitory potentials of a number of FDA approved drugs for NTCP and ASBT (40). This study identified 27 drugs as novel NTCP inhibitors, including a variety of therapeutic classes including antifungal, antihyperlipidemic, antihypertensive, anti-inflammatory, and glucocorticoid drugs. Of the 72 drugs tested, a total of 31 drugs

inhibited NTCP, while 51 drugs inhibited ASBT with overlapping inhibition. These results suggest that ASBT is more permissive to drug inhibition than NTCP and this may be related to NTCP possessing fewer pharmacophore features. Whether the differences in the permissiveness to drug inhibition between the two transporters are due to species difference remains to be established. The possibility of species differences is consistent with the findings that rosuvastatin is a human NTCP substrate but not a substrate for rat NTCP (73) and bosentan is a more potent inhibitor for rat NTCP than human NTCP (98).

6. Post-Translational Regulation of NTCP and ASBT

Both NTCP and ASBT undergo transcriptional and post-transcriptional regulations. Transcriptional regulation involving various nuclear receptors has recently been reviewed by others (30,88) and hence the focus of this review will be post translational regulation. Plasma membrane transporters, after translation, need to be translocated to the plasma membrane to carry out their intended functions. The level of a transporter in the plasma membrane may be increased or decreased based on the need to regulate solute transport by the transporter. This is a highly regulated post-translational event requiring involvement of various cellular signaling pathways and is best exemplified by the regulation of glucose transporter (GLUT4) by insulin in adipocytes and skeletal muscle (45,99). The post-translation event also involves protein quality control to select and target dysfunctional proteins for degradation by the ubiquitin-proteasome system (29). Studies to date suggest that the post-translational regulation of NTCP as well as ASBT involves plasma membrane transport/retrieval and degradation by the ubiquitin-proteasome system. Several signaling pathways have been implicated in the post-translational regulation of these transporters and these pathways include cAMP, intracellular Ca^{2+} , nitric oxide, and activation of phosphoinositide-3-kinase (PI3K), protein kinase Cs (PKCs) and protein phosphatases. The role of these pathways is better understood for NTCP (Fig. 1) than ASBT because of limited studies with ASBT. Some of these pathways have previously been reviewed (4,20,88).

Plasma membrane level of NTCP and thereby hepatic TC uptake is regulated by cAMP and bile acids. Cyclic AMP stimulates TC uptake (19,57,118,139) and increases plasma membrane level of NTCP in rat hepatocytes and in hepatic cells stably transfected with human NTCP (42,111,139). In contrast, cholestatic bile salts (taurochenodeoxycholate (TCDC) and tauroolithocholate (TLC)) inhibit TC uptake (110,142) and decrease plasma membrane rNTCP (110,141). Thus, bile salts uptake by NTCP undergoes dynamic regulation. The increase in uptake by cAMP, increased by glucagon, may allow the hepatocytes to efficiently absorb bile salts returning from the intestine under physiological conditions, while the decrease in uptake may protect hepatocytes from further increases in intracellular bile salts observed in cholestasis (125). Functionally important polymorphisms in hNTCP may result in decreased plasma expression. Multiple single nucleotide polymorphisms in NTCP have been shown to be present in populations of European, African, Chinese, and Hispanic Americans, and the altered transport activity of Ile223 to Thr variant seen only in African Americans was due at least in part to decreased plasma membrane expression (72).

A role of PI3K in bile formation is evident from a study (46) showing that wortmannin, a specific inhibitor of PI3K, inhibits bile formation, bile acid secretion and vesicle trafficking in isolated perfused rat liver. Since then various studies have provided evidence supporting a role for PI3K/Akt pathway in cell survival and translocation of hepatocellular transporters to the plasma membrane, indicating a beneficial role of PI3K in hepatic cells (4,48,130,160). PI3Ks are a family of lipid kinases (classes I, II, and III) that phosphorylate the inositol ring of phosphatidylinositides (PIs) at 3 position known as D3 phosphorylation (24). The resulting phosphorylated PIs (PIPs), acting in concert with phosphoinositide-dependent

kinases (PDKs), are involved in the activation of downstream kinases, such as PKC ζ/λ , Akt/PKB, and p70^{S6K} (154). The PI3K/Akt pathway is involved in cell swelling and cAMP-induced increases in TC uptake and rNTCP translocation to the plasma membrane (159,163) and cAMP inhibits TLC-induced inhibition of TC uptake by activating the PI3K pathway (141). The effect of cAMP on hNTCP/rNTCP translocation is also mediated via PI3K/PKC δ (118,136) and PI3K/PKC ζ (102) pathways (see below).

NTCP translocation to the plasma membrane is regulated by phosphorylation and dephosphorylation, although kinase(s) involved in phosphorylation of NTCP has not yet been identified. Rat NTCP is a serine/threonine phosphorylated protein (112) and cAMP-induced increases in TC uptake and plasma membrane rNTCP is associated with NTCP dephosphorylation (113) at Ser²²⁶(5). Cyclic AMP increases intracellular Ca²⁺(57) and the ability of cAMP to dephosphorylate rNTCP is inhibited by a calcium chelator (112). Cyclic AMP activates protein phosphatase 2B (PP2B), a Ca²⁺/calmodulin-dependent serine and threonine protein phosphatase (86) by increasing intracellular Ca²⁺(161). PP2B directly dephosphorylates rNTCP and an inhibitor of PP2B inhibits cAMP-induced dephosphorylation and translocation of rNTCP (161). Taken together, these results suggest that cAMP activates PP2B by increasing intracellular Ca²⁺ and PP2B in turn dephosphorylates rNTCP facilitating its translocation to the plasma membrane (Fig. 1). Interestingly, inhibition of PP2B has been shown to reverse TCDC-induced retrieval of rNTCP (110). Whether TCDC-induced retrieval of rNTCP involves PP2B mediated dephosphorylation of NTCP remains to be established. It is however possible that inhibition of PP2B results in increased phosphorylation of a mediator that is involved in the retrieval of rNTCP.

Cyclic AMP-dependent increases in apical ASBT in rat cholangiocytes (3) and rat ileum (128) have been reported. Secretin, acting via cAMP, increases apical ASBT in rat cholangiocytes, which is reversible and requires intact microtubules (3). This result suggests acute stimulation of vesicular translocation of ASBT to the apical membrane and this may explain secretin induced increased cholehepatic shunting and associated increases in the choleric effect of bile acids (165). It does not appear that ASBT translocation is affected by phosphorylation. ASBT has not been shown to be phosphorylated. However, mutation of potential phosphorylation sites (S335 and T339) in the cytoplasmic tail of rASBT by non-phosphorylatable alanine results in decreased initial bile acid transport activity and increases the basolateral distribution of the mutants without significantly decreasing apical distribution (152). Thus, phosphorylation, if it occurs, may inhibit basolateral targeting of ASBT in rat cholangiocytes.

Like NTCP (93), ASBT in human cholangiocyte cell lines undergoes degradation via the ubiquitin-proteasome system (166). NTCP is shown to be degraded by the ubiquitin-proteasome system at the level of ER-associated degradation. It is speculated that dysfunction of this pathway may result in intracellular NTCP deposits in cholestatic livers (93). Since mutation of S335 and T339 to alanine in human ASBT reduces the rate of ASBT disposal under basal conditions as well as IL-1 β -dependent ubiquitination and disposal of ASBT, it is suggested that human ASBT degradation by proteasome involves phosphorylation followed by ubiquitination (166). However, role of phosphorylation has not been directly assessed.

Protein kinase C isoforms have been shown to mediate NTCP/Ntcp translocation to and retrieval from the plasma membrane (Fig. 1). Protein kinase C belongs to a family of serine/threonine protein kinases that are involved in the regulation of diverse cellular functions and consists of at least 12 isoforms (116,127). Activation of most PKCs, if not all, is PI3K dependent (116). PKCs shown to be present in rat hepatocytes include PKC α , PKC δ , PKC ϵ ,

and PKC ζ ; with the presence of PKC β II being controversial. (13,83,149). Initial studies with PMA, activator of conventional and novel PKCs, suggested that activation of PKCs inhibits TC uptake in hepatocytes (4,21,57). More recent studies suggest that TC uptake and NTCP translocation/retrieval are differentially affected by PKC isoforms. Thus, PKC δ (118,136) and PKC ζ (102) mediate cAMP-induced hNTCP/rNTCP translocation, while conventional PKCs (most likely PKC α) mediate rNTCPp retrieval (110,150). The activation of PKC δ and PKC ζ by cAMP is PI3K-dependent (102,118,136), while TCDC-induced rNTCP retrieval is enhanced when PI3K is inhibited (110). Thus, it does not appear that TCDC-induced activation of PKC α is dependent on PI3K. On the other hand, TLC induces retrieval of NTCP in rat hepatocytes (141), but inhibits PKC α (12). Thus, the retrieval of rNTCP by cholestatic bile acids may involve mediators in addition to PKC α . TLC, which activates PKC ϵ in rat hepatocytes (13,141), also inhibits TC uptake in rat hepatocytes (142) and in HuH-NTCP cell (141). However, TLC-induced inhibition of TC uptake is not mediated via PKC ϵ (141). In contrast, TLC-induced retrieval of MRP2 is mediated via PKC ϵ (140). Thus, the canalicular membrane may be the target of PKC ϵ and this is consistent with the finding that TLC translocates PKC ϵ to the canalicular membrane (13). PMA, an activator of PKCs, has been shown to inhibit TC uptake and decrease plasma membrane ASBT in Caco-2 cells transfected with ileal ASBT (133). This effect of PMA appears to be mediated via PKC ζ . It is speculated that this pathway may underlie the pathophysiology of diseases associated with disruptions in bile acid homeostasis.

There are limited studies exploring the mechanism by which PKCs affect NTCP translocation. These studies suggest a role for Rab4, a small GTPase, which cycles between GTP bound (Rab4-GTP) active form and GDP bound (Rab4-GDP) inactive form and is involved in endocytosis (2). Thus, PKC δ facilitates hNTCP translocation by activating Rab4 (118) and PKC ζ increases motility of rNTCP containing vesicles along the microtubules (132). Other studies showed that Rab4 facilitates cAMP-induced rNTCP translocation (139), rNTCP containing vesicles co-localize with Rab4 (132), cAMP induced rNTCP translocation is dependent on PI3K dependent (162) and actin cytoskeleton and microtubules (42,162), and activation of PKC δ and PKC ζ is PI3K dependent (102,118,136). Taken together, these results may suggest that activation of PI3K/PKC δ by cAMP leads to activation of Rab4 in NTCP containing Rab4 vesicles and this is followed by PI3K/PKC ζ mediated increased motility of NTCP/Rab4 containing vesicles along the microtubules to the plasma membrane (Fig. 2).

Post translation modification affecting plasma membrane localization of NTCP also involves S-nitrosylation. Treatment with thiol-binding agents inhibits TC uptake in isolated rat hepatocytes (15,16) and by NTCP and ASBT (66) indicating a role for cysteine residues in transport function. Nitric oxide has been shown to inhibit TC uptake in rat hepatocytes (145), HuH7 cells stably transfected with human NTCP (138) and human hepatocytes (53). One of the ways that NO exerts its cellular effects is through a posttranslational modification termed S-nitrosylation, which involves binding of NO to reactive thiol groups on cysteine residues (70,135). A number of proteins (transcription factors, kinases, phosphatases, membrane receptors and transporters) are regulated by S-nitrosylation (39,70) and abnormal S-nitrosylation of proteins has been implicated in a number of human diseases including sepsis, diabetes and cystic fibrosis (28,47,58,120,126,137). Human NTCP has four cysteine residues (Cys44, Cys98, Cys125, and Cys266) that are conserved in rat, mouse, and rabbit (171). Thus, inhibition of TC uptake by NO may involve S-nitrosylation of NTCP. Indeed, NO has been shown to S-nitrosylate NTCP (53,138) and this is associated with a decrease in plasma membrane NTCP (138). Moreover, dithiothreitol reverses NO-mediated inhibition of TC uptake and S-nitrosylation of NTCP (138). A follow up study showed that the inhibition of TC uptake by NO may involve S-nitrosylation of rNTCP-Cys⁹⁶(155). Such a mechanism

may be involved in LPS-induced cholestasis, which is associated with a burst of NO production by iNOS in liver cells (49).

NO also produces its cellular effects by nitration of tyrosine residues (79) and has been shown to increase tyrosine nitration of hNTCP (53). However, the effect of tyrosine nitration on TC uptake and plasma membrane NTCP has not been directly studied. It may be noted that two tyrosine residues on the cytoplasmic tail of rat NTCP appear to be involved in targeting to the basolateral membrane (151). Thus, it is possible that tyrosine nitration of NTCP by NO may also result in decreased translocation to the basolateral membrane resulting in decreased TC uptake.

There appears to be species differences in the regulation of plasma membrane localization of hNTCP and rNTCP. TLC inhibits TC uptake by hNTCP and rNTCP. However, TLC decreases plasma membrane rat NTCP but not human NTCP (141). Like Bosentan (98), TLC inhibits TC uptake by rNTCP and hNTCP non-competitively (142) and competitively (141), respectively. NO inhibits TC uptake by hNTCP/rNTCP. However, NO decreases plasma membrane level of human NTCP (138) and does not affect plasma membrane level of rat NTCP (155). In contrast, cAMP-induced increases in TC uptake are associated with translocation of hNTCP as well as rNTCP. These differences should be taken into account when extrapolating mechanisms regulating rat NTCP to human NTCP for the purpose of human drug development and also for understanding the mechanistic relevance in human diseases.

7. A novel function of NTCP

A novel function for NTCP as a receptor and transporter for hepatitis B virus (HBV) and hepatitis D virus (HDV) has recently been described (169). Both HBV and HDV contain three envelope proteins known as small (S), middle (M), and large (L). These proteins share the same C-terminal domain corresponding to the S protein, but differ at the N-terminal domains by the presence of pre-S2 domain in M protein and pre-S1 and pre-S2 domains in L protein. The entry of both HBV and HDV is determined by the pre-S1 domain of L protein after interacting with cellular receptor(s) on hepatocytes (52), as evidenced by inhibition of viral entry and infection by myristoylated N-terminal 47 amino acids of the pre-S1 domain of L protein (56,104). Myrcludex B, a drug based on myristoylated pre-S1 domain, has entered clinical trials after demonstration of efficacy against both HBV and HDV infection in an uPA-SCID mouse model (121), indicating potential clinical significance of viral entry inhibition in the treatment of HBV/HDV. Despite these advances, the identity of the receptor(s) remained elusive until the studies by Yan et al (169).

In an elegant series of experiments Yan et al showed (169) that the receptor-binding region of pre-S1 domain of L protein specifically interacts with NTCP and amino acids 157 to 165 of NTCP (KGIVISLVL) are critical for viral entry. A follow up study showed that mouse NTCP can bind pre-S1 domain of HBV L protein, but does not support HBV or HDV infection (168). However, substitution of mouse Ntcp residues 84 to 87 by human counterparts effectively supports viral infections. Thus, there may be multiple domains of NTCP involved in supporting viral infections. In addition, insensitive hepatic cell lines (HuH-7 and HepG-2) transfected with human NTCP supported HBV/HDV infection (168), although the extent of infection was much lower than in hepatocytes. Thus, human NTCP plays an important role in viral entry and consequent infection. However, viral entry is only one step in efficient viral infection and replication in human hepatocytes and other factors, such as host components, are likely to be involved (134,158,167).

These results raise interesting questions about the transport function of NTCP. One possibility may be that these viruses are transported by NTCP the same way as it transports

bile salts. In that case, it would be important to know whether the transport of HBV and HDV into hepatocytes is Na⁺ dependent and is inhibited by known substrates/inhibitors of NTCP. It is however possible that this is a novel function of NTCP with molecular mechanism of the transport function yet to be determined. A likely possibility is that NTCP is internalized by endocytosis following binding to the virus allowing the virus to enter the cell, as also suggested by others (158,167). Internalization of HBV/HDV may be initiated by protein-protein interactions between the envelop protein of the virus and NTCP leading to endocytosis. In that case, what would be the signal for internalization and how is that signal generated? Calcium dependent conventional PKCs and most likely PKC α have been shown to induce rNTCP retrieval (110,150). Whether PKC α or other signaling molecules may be involved remains to be investigated. Irrespective of the transport mechanism involved, the finding that NTCP is involved in HBV and HDV infection is expected to inspire further studies leading to a better understanding of the role of NTCP in HBV/HDV infection and development of agents to inhibit viral entry and infection.

8. Future perspectives

NTCP and ASBT were originally described to transport bile salts and play an important role in enterohepatic circulation of bile salts. It is now apparent that these transporters are capable of transporting other solutes including drugs and viruses. The physiological and clinical relevance of NTCP for drugs transported by additional transporters requires further examination. The ability of drugs to be transported or to inhibit solute transport by these transporters is likely to affect drug disposition resulting drug-drug interactions, provide strategies for novel drug development and alter disposition of endogenous substrates. Further studies are needed to fully explore the clinical relevance of such interactions. The ability of NTCP to transport HBV/HDV and thereby facilitate hepatic viral infection should lead to further studies providing better understanding of the transport mechanism and regulation of NTCP as well as mechanisms of and therapy against HBV/HVD infection. Further studies are needed to define the conditions and substrates for NTCP to be used for liver function tests. Our understanding of cellular mechanisms involved in the post-translational regulation of NTCP/ASBT has been steadily increasing. It is apparent that solute transport by these transporters is acutely regulated by translocation to and retrieval from the plasma membrane. However, signaling pathways involved are incompletely understood, especially for ileal ASBT. A better understanding of the signaling pathways regulating plasma membrane localization should allow us to target specific pathways to limit or enhance solute transport and thereby limit hepatic toxicity or enhance hepatic functions as needed. It may be noted that most of these studies are conducted in rodents. While there are some similarities (based on limited studies) in substrate specificity and regulatory mechanisms between rodents and humans, there are also differences. Thus, caution should be exercised in extrapolating rodent data to humans until findings in rodents are confirmed for the human isoforms. Most of the *SLC10A* transporters are considered to be orphan transporters and hence very little is known about the regulation of these orphan transporters. It is anticipated that substantial progress in these areas will be made in the near future.

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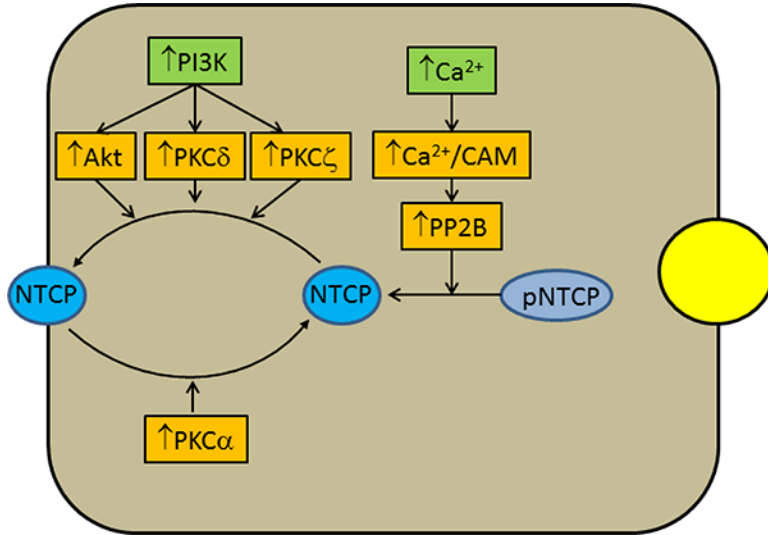


Figure 1. Postulated signaling pathways regulating NTCP insertion into and retrieval from the plasma membrane. Cyclic AMP increases hepatic uptake of bile acid by increasing plasma membrane level of NTCP. The effect of cAMP is mediated via two major pathways; activation of PI3K and increase in intracellular Ca²⁺. Activation of PI3K leads to activations of Akt, PKCδ and PKCζ, which in turn induce translocation of NTCP from intracellular compartment to the plasma membrane most likely by stimulating vesicular trafficking of NTCP containing vesicles. Rat NTCP is a phosphoprotein and the dephosphorylated NTCP undergoes translocation to the plasma membrane. Increases in intracellular Ca²⁺ by cAMP leads to activation of Ca²⁺-dependent protein phosphatase 2B (PP2B) via Ca²⁺-calmodulin kinase (Ca²⁺-CAM). PP2B dephosphorylates rat NTCP allowing translocation to the plasma membrane. On the other hand, activation of PKCα by a cholestatic bile acid, taurochenodeoxycholate (TCDC), stimulates retrieval of rat NTCP from the plasma membrane by an as yet unknown mechanism except that inhibition of PI3K and PP2B enhances the effect of TCDC.

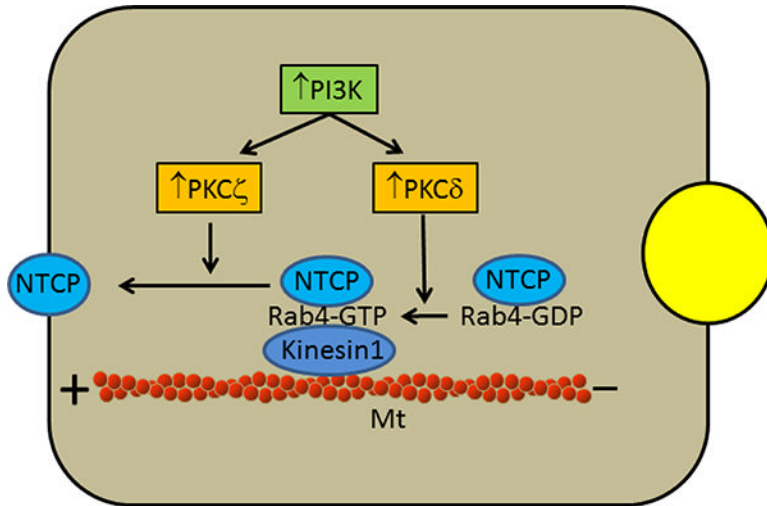


Figure 2.

Postulated role of Rab4, PI3K/PKC δ and PI3K/PKC ζ in the translocation of NTCP. NTCP containing vesicles co-localize with Rab4, which cycles between GTP bound (Rab4-GTP) active form and GDP bound (Rab4-GDP) inactive form. Activation of PKC δ leads to the conversion of inactive Rab4-GDP to active Rab4-GTP, which moves NTCP containing vesicles towards the plus end of microtubules (Mt) by interacting with kinesin-1. This movement is further facilitated by PKC ζ .