Bile Formation: a Concerted Action of Membrane Transporters in Hepatocytes and Cholangiocytes

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A large number of membrane transport mechanisms in hepatocytes and cholangiocytes serve for the secretion of bile acids, various other organic anions, organic cations, lipids, and electrolytes. After their functional characterization, some of these mechanisms’ individual transport molecules are now identified, allowing better understanding of inherited and acquired disorders of bile formation.

Bile formation is a vital process. Bile acid secretion serves the intestinal digestion of lipids and assimilation of lipid-soluble nutrients. In addition, superfluous and potentially toxic material is disposed of in bile, including cholesterol, bilirubin, and an abundance of xenobiotics such as drugs and environmental chemicals and their metabolites.

The primary site of bile production is the network of tiny bile canaliculi, formed by and embedded between adjacent liver cells. At this site, bile constituents may be synthesized within hepatocytes and secreted across their apical (canalicular) cell membranes into the bile canaliculi. Alternatively, liver cells may extract and take up “cholephils” from the sinusoidal blood stream by transport across their basolateral (sinusoidal) membranes and, after intracellular binding, sequestration, biotransformation, or conjugation, they produce metabolites that are amenable for secretion across the canalicular membrane. These primary processes result in canalicular bile formation in which secretory products such as bile acids and glutathione are excreted at concentrations that are high enough to generate osmotic water flow into the bile canaliculi. In parallel with the transport of water-soluble bile constituents, lipid vesicles are detached from the apical membrane to form biliary micelles composed of phospholipids, cholesterol, and bile salts. In addition, high molecular bile constituents such as plasma proteins are secreted by vesicular transport and by apical exocytosis. Forward movement of bile within the canaliculi is promoted by peristaltic contractions of an actin-myosin web located underneath the apical membrane of hepatocytes. Bile is then drained into the branches of intrahepatic bile ductules that converge to the common hepatic bile duct. At these latter sites, bile composition and bile volume are modified by reabsorption of some bile constituents (e.g., reuptake of some bile acids), but the predominant mechanism accomplished by cholangiocytes is the secretion of HCO₃⁻ together with additional fluid, resulting in ductular bile formation.

The experimental approaches employed to study the individual transport mechanisms involved in canalicular and ductular bile formation have become more and more sophisticated over the past three decades, mainly through adopting newly developed techniques from research in other organs such as the kidney or intestine. Initial approaches to studying the physiology of bile secretion used less refined techniques, such as bile duct cannulation in whole animal studies, the isolated perfused liver, or isolated hepatocytes and cholangiocytes. In cell culture, both these cell types form “secretory units” and enclose a lumen that expands as fluid secretion proceeds, thus providing an in vitro model to study biliary physiology. Identification and characterization of individual transport processes took advantage of the use of isolated membrane vesicles of canalicular and basolateral origin. These techniques provided information on binding affinities of individual substrates or on the interaction between substrates by mechanisms such as competitive inhibition or trans-stimulation. Furthermore, photoaffinity labeling techniques could identify some transport molecules, such as P-glycoprotein (described below). On the basis of thermodynamic considerations, these preparations also served to identify the driving forces for “uphill” transport (against a transmembrane concentration gradient). As exemplified below, these mechanisms use energy provided either by hydrolysis of ATP (transport ATPases, primary active transport), or they are energized by coupling to dissipation of transmembrane ion concentration gradients (secondary active transport; e.g., cotransport with Na⁺) or by the transmembrane electric potential (electrogenic transport). In addition, tertiary active transport mechanisms couple uphill transport of one solute to dissipation of the concentration gradient of another solute (e.g., apical SO₄²⁻ bile-acid exchange or basolateral dicarboxylic-acid/bile-acid exchange in hepatocytes) (1). Microelectrode techniques and, more recently, patch-clamp technology were used to identify ion channels that determine the physiological ion concentration and potential gradients that support these transport processes. Thus transport of organic substances [e.g., taurocholate (TC)] and bile flow are modulated by experimental alterations in electrolyte transport, e.g., by pharmacological inhibition of Na⁺-K⁺-ATPase or ion channel activity. However, it remains largely unclear how changes of electrolyte transport affect transport of organic compounds in various pathophysiological states. Until the approach of molecular biology techniques, studies on biological or pathophysiological mechanisms of transporter function also remained restricted. Identification of transport molecules for organic substrates started in 1991 with expression cloning and
functional characterization of the basolateral Na⁺-TC-cotransporting polypeptide (ntcp) (6). Other transport proteins have been identified by their homology with proteins expressed in other vertebrate organs or even in yeast. Those studies begin to provide insight into the regulation of transcription of transporter proteins and on sequences that could connect transporter function with intracellular signaling systems. Furthermore, mutations of individual genes are identified that cause inherited disorders of bile formation, such as progressive familial intrahepatic cholestasis (see below). In addition, sequential expression of transporter genes during ontogenesis reflects the development of individual liver transport functions and, in acquired cholestatic conditions, expression of transporter genes is altered (14). This review describes the role of individual transport molecules with focus on 1) bile acid secretion, 2) transport of other organic anions, 3) transport of organic cations, and 4) ductular secretion.

Canalicular bile formation is mainly driven by the active secretion of bile acids (bile salt-dependent bile flow). Bile acids are supplied either by synthesis in liver cells or, after their return from the enterohepatic circulation, are taken up from the bloodstream across the sinusoidal cell membrane (7). In accordance with the classical osmotic theory of bile formation, there exists a linear correlation between the amount of bile acid secreted into bile and the amount of water that follows (7–25 μl/μmol). This apparent choleretic activity of individual bile acids is species dependent, and it is lower for those bile acids that have a higher tendency to form micellar aggregates in bile. There are exceptions to this rule, in that some bile acids (e.g., ursodeoxycholic acid) produce hypercholeresis, whereby bile flow exceeds the rate expected from their relatively low concentration in bile, which, in compensation, is rich in HCO₃⁻. “Cholehepatic shunting” offers an explanation for this phenomenon: a hypercholeretic bile acid is initially secreted by hepatocytes and produces canalicular bile flow but is reabsorbed downstream by cholangiocytes that return the bile acid into the bloodstream in exchange for HCO₃⁻. Accordingly, several individual membrane transport steps are involved in hepatic bile acid secretion: uptake of bile acids by hepatocytes across their basolateral membranes followed by secretion across the canalicul cell membrane and reabsorption along bile ductules.

A large number of exogenous compounds is actively secreted into canalicular bile at high concentrations, mainly in the form of anionic metabolites. Cholangiographic agents represent an illustrative example. Similar to bile acids, secretion of these organic anions causes an increase of bile flow. So far, glutathione is the only endogenous anion identified that promotes bile salt-independent canalicular bile formation under physiological conditions (10). Similar to the secretion of bile acids, the rate of glutathione secretion is quantitatively related to bile flow.

The following sections will describe individual transport processes according to their localization in the hepatocyte and cholangiocyte apical and basolateral membranes. Despite their overlapping substrate specificity, Fig. 1 depicts these transporters according to their predominant role in conveying vectorial transport of the major bile constituents. Emphasis will be placed on those transport processes whose genes have been identified.

**Individual transport mechanisms: hepatocytes**

**Basolateral (sinusoidal) uptake.** BILE ACID TRANSPORT. Rodent ntcp and its human homologue (NTCP) have been identified from expression cloning as the carrier proteins that mediate high-affinity [Michaelis-Menten constant (Km) ~6 μM] Na⁺-dependent TC uptake across the basolateral liver cell membrane (6). A possible role of microsomal epoxide hydrolase (MEH) in mediating Na⁺-dependent bile acid transport could not be consistently verified. Ntcp transport is electrogenic, with a Na⁺/TC stoichiometry of 2:1. Substrates of ntcp include other conjugated bile acids, and NTCP could mediate a small fraction of cholate uptake also. As shown by expression in Xenopus laevis oocytes, some chemically unrelated compounds are transported as well, including estrogen conjugate, sulfobromophthalein (BSP), the cyclic peptide phalloidin, and drugs such as the loop diuretics furosemide or bumetanide. During development, increasing expression has been observed after birth, followed by complete glycosylation of the protein, both leading to postnatal maturation of transport function. Similar to cholesterol-7α-hydroxylase, transcription is inhibited through a bile acid-responsive element, and expression is low in experimental cholestasis (bile duct ligation) or extrahepatic biliary atresia and is downregulated by cytokines in endotoxemia. Teleologically, this downregulation could protect liver cells from bile salt toxicity.

**Organic anion transport.** Organic anion-transporting polypeptide 1 (oatp1) has been cloned from rat liver, but it is also expressed in the renal proximal tubule and choroid plexus. It was the first member of a family of organic anion-transporting polypeptides that includes oatp2 (cloned from rat brain but also expressed in rat liver and kidney), a rat kidney homologue (ROAT-K1), rat and human prostaglandin transporters, and the thyroid hormone-transporting peptide oatp3. OATP is the human homologue of oatp1, with 67% amino acid identity but with different substrate specificity (9). Oatp1 functions as a multispecific carrier that mediates Na⁺-independent transfer into hepatocytes of a variety of amphipathic compounds, including conjugated and unconjugated bile acids (e.g., taurocholic acid, taurocholic acid, TC, and cholic acid), organic anionic dyes (such as BSP), steroid conjugates, neutral steroids (ouabain, cortisol, aldosterone), glutathione conjugates, leukotriene C4, small peptides, and organic cations (azopentyldeoxy-ajmalinium, a representative type II cation; see below). The driving force for this carrier-mediated uptake has not been firmly established but may involve exchange of intracellular HCO₃⁻ or glutathione with the extracellular substrate. In ontogenesis, oatp expression precedes that of ntcp and, in contrast to ntcp, expression is enhanced by bile acids or in cholestatic liver disease (primary sclerosing cholangitis). In these latter conditions, this transporter may be more prone to operate in the reverse direction and serve for bile acid eflux into the sinusoidal blood following an intracellular substrate load. Rodent oatp2 is preferentially expressed in acinar zone 2...
hepatocytes and, besides accepting TC but not BSP, exhibits a particularly high affinity for digoxin.

BSP has been used as the test substance for identifying transporters for biliary anion secretion. Although most of the transporters listed above accept BSP as a substrate, these transporters do not provide for hepatic uptake of bilirubin. As for other substrates, several carriers could be responsible for bilirubin transport, including bilitranslocase, bilirubin-BSP-binding protein (BBBP) and organic anion-binding protein (OABP).

ORGANIC CATION TRANSPORT. Lipophilic organic cations are eliminated from the body preferentially by secretion into bile. This group of compounds includes drugs with tertiary or quaternary amine groups but also endogenous compounds such as choline or N-methylnicotinamide. A systematic analysis of transport systems for uptake into liver and biliary elimination was initially based on kinetic and substrate competition experiments in rat liver. These studies led to the definition of two categories of substrates: small, monovalent, more hydrophilic cations (type I) and bulkier, divalent, more lipophilic cations (type II). Respective examples are tri-n-butylmethylammonium or vecuronium and quinine. Other transporters with higher substrate selectivity have also been identified, e.g., for thiamine or N-methylnicotinamide. Some substrates exhibit countertransport with H+, but, with some exceptions, driving forces for concentrative uptake, such as dependency on Na+ or the membrane potential, are not firmly established. Rat organic cation transporters were initially cloned from a kidney cDNA library (oct1 and oct2), but oct1 is also expressed in hepatocytes and small intestine. This transporter provides for electrogenic uptake of type I substrates, such as tetrathymethylammonium, choline, and 1-methyl-4-phenylpyridinium. Its human homologue, OCT1, is exclusively expressed in liver and differs from rat oct1 in its substrate specificity by also accepting type II substrates (5).

Apical (canalicular) secretion. The canalicular liver cell membrane is equipped with a set of transporters that belong to the large superfamily of ATP-binding cassette (ABC) proteins with two subclusters of primary importance, the multidrug resistance (MDR) P-glycoprotein cluster and the multidrug resistance-related proteins (MRP). These transport ATPases serve a variety of excretory functions, such as multispecific export of organic cations or organic anions or for more specific transport of bile acids or phospholipids (8).

Secretion of cationic and lipophilic compounds. The first member of this group of transport ATPases identified in liver was P-glycoprotein (MDR1 in humans; mdr1a and mdr1b in mice). P-glycoprotein expression in tumor cells is one mechanism that conveys multidrug resistance, whereby the protein functions as a membrane pump that exports anticancer drugs from the cell. P-glycoprotein is constitutively expressed in liver,
intestine, kidney, and endothelial cells of the blood-brain barrier. In the canalicular liver cell membrane, MDR1 mediates the biliary secretion mainly of amphiphilic cationic xenobiotics (including anthracyclines and vinca alkaloids), a mechanism that largely determines their bioavailability (8, 12). Other substrates include steroid hormones, hydrophobic peptides, and glycolipids. Although P-glycoprotein appears to protect cells and organs mainly from accumulation of xenobiotics, a physiological substrate for this transporter is not known. P-glycoprotein may mediate release of ATP from liver cells. Although ATP is split by canalicular ectonucleotidases and adenosine is taken up by Na⁺-dependent transport, nucleotide concentrations in bile remain high enough to activate purinoceptors in hepatocytes or cholangiocytes that could affect bile formation (see below). ATP release from liver cells and purinoceptor activation have also been proposed as signals that modulate Cl⁻ channel activity in cell volume regulation. Expression of mdr1 in liver is elevated in animals treated with xenobiotics and during liver regeneration.

**Bile Acid Secretion.** Human sister protein of P-glycoprotein [SPGP, identical to bile salt export pump (BSEP)], which is homologous to MDR proteins, has recently been cloned (4). Although other transport proteins such as canalicular ecto-ATPase have been previously discussed, rat bsep now appears to be the major pump for secretion of monoanionic bile salts (e.g., TC) that sustain canalicular bile salt-dependent bile flow. The density of bile salt transporters in the apical membrane, and thus the maximal transport capacity, appears to be regulated by exocytic membrane insertion from subapical vesicles that contain this transporter. This process is stimulated by cell swelling, by cAMP, and by TC itself, and it requires intact phosphatidylinositol 3-kinase activity and an intact micellar vesicle. Expression of mdr1 in liver is elevated in animals treated with xenobiotics and during liver regeneration.

**Phospholipid Secretion.** The role of mdr2 (MDR3 for human) in bile formation was initially discovered by knock-out of the mdr2 gene in mice, which resulted in severe liver injury (feathery degeneration of hepatocytes, bile duct proliferation) associated with lack of biliary lipid secretion and reduced secretion of glutathione. Mdr2 (MDR3) is highly expressed in the canalicular membrane, in which it appears to function as a "flippase" that translocates phosphatidylcholine (PC) from the inner to the outer leaflet of the cell membrane. Thereby PC-rich microdomains are generated, into which cholesterol partitions. By the detergent environment of bile, these domains detach from the outer leaflet to form biliary unilamellar vesicles that may transform into micelles (2).

**Possible Role of HCO₃⁻ Secretion.** Transporters that regulate intracellular pH in hepatocytes exhibit a polarized distribution. Cell alkalization is accomplished by basolateral Na⁺/H⁺ exchange (NHE1) and by Na⁺-/HCO₃⁻ symport, whereas acidification is provided by apical Cl⁻/HCO₃⁻ exchange (AE2). This arrangement of transporters could provide for vectorial transport of HCO₃⁻ across the cell into bile or, after dissociation of intracellular carbonic acid, for basolateral H⁺ and apical HCO₃⁻ extrusion. Nonetheless, it is not established whether canalicular bile formation is indeed sustained by hepatoacellular secretion of HCO₃⁻. Similarly, hepatoacellular secretion of electrolytes has not been established as a mechanism to sustain canalicular fluid secretion; the tight junctions that separate the canalicular lumen from the basolateral space of Disse appear to be leaky enough to allow for equilibration of ion gradients generated by transcellular transport (1).

Besides apical pumps and carriers that serve for secretion, the canalicular membrane is also equipped with enzymes (e.g., ecto-ATPase, dipeptidylpeptidase) and with carriers that reclaim secretory products. Within the concept of glutathione-dependent and bile salt-independent canalicular secretion, it is noteworthy that split products of glutathione could be reabsorbed by Na⁺-dependent transport of its constituent amino acids.

**Individual transport mechanisms: cholangiocytes**

The primary bile produced by hepatocytes at the canalicular level is modified along the biliary tree with respect to both composition and volume. In humans, 40% of bile volume is of ductular origin, with secretion of HCO₃⁻ being the predominant mechanism. Secretion of HCO₃⁻ is promoted by secretin and also by VIP. Both hormones generate intracellular cAMP and are antagonized by somatostatin. Acetylcholine binds to M3 cholinoceptors to elevate intracellular Ca²⁺ concentration ([Ca²⁺]), and potentiates the effects of secretin. Furthermore, ATP, thought to be delivered into bile at the canalicular level or by cholangiocytes themselves, binds to apical purinoceptors (P₂Y₂) to increase [Ca²⁺] (15).

HCO₃⁻ secretion is now believed to result from the cooperation of a number of transporters; intracellular HCO₃⁻ is generated by the dissociation of carbonic acid catalyzed by carbonic anhydrase (type II in rat, pig, and guinea pig) or it is taken up across the basolateral cell membrane (via Na⁺- and Cl⁻-dependent transport in humans and Na⁺-/HCO₃⁻ symport in...
Basolateral Na+/H+ exchange (NHE1 isoform in humans and rats) expels H+ generated by carbonic anhydrase. These latter basolateral transporters are driven by the out-to-in Na+ concentration gradient established by basolateral Na+-K+-ATPase. HCO₃⁻ secretion across the apical cell membrane is accomplished by Cl-/HCO₃⁻ exchange (AE2 isoform in humans). The driving force for this electroneutral exchanger is the out-to-in concentration gradient of Cl⁻. Its low intracellular concentration is maintained by Cl⁻ efflux across apical Cl⁻ channels driven by the intracellular negative electrical potential. This, in turn, results from the presence of basolateral K⁺ channels that also serve for efflux of K⁺ that has been taken up either by Na+/K+-ATPase or by basolateral bumetanide-sensitive Na⁺-K⁺-2Cl⁻ cotransport that is also driven by the Na⁺ concentration gradient. Na⁺-K⁺-2Cl⁻ cotransport fuels the cell with Cl⁻ for apical efflux. Basolateral K⁺ efflux and apical Cl⁻ efflux appear to establish the transcellular current responsible for the lumen negative transepithelial potential (~6 mV). This, in turn, favors Na⁺ secretion across the paracellular pathway. In sum, these mechanisms lead to secretion of NaCl, with a fraction of luminal Cl⁻ being exchanged for HCO₃⁻. Secretion of NaCl and NaHCO₃ is accompanied by water flux, which is facilitated by the presence of water channels (aquaporin AQP1) in apical and basolateral cell membranes. Aquaporin density in the apical membrane appears to be upregulated by secretin through microtubule-dependent membrane insertion.

HCO₃⁻ secretion is stimulated in experimental cholostasis in rats by increased expression of secretin receptors. Together with reduced expression of MRP2 (see above), this could be responsible for the appearance of “white bile” observed during prolonged bile duct obstruction.

Apical Cl⁻ channels. Special attention has been paid to the mechanisms that couple apical Cl⁻ efflux with Cl⁻/HCO₃⁻ exchange and that are subject to hormonal regulation. The cystic fibrosis transmembrane conductance regulator (CFTR) has been localized to the apical membrane of cholangiocytes by immunohistochemistry, and it appears to be the Cl⁻ channel that is regulated by hormones that act through intracellular cAMP. Activation of CFTR will thus enhance HCO₃⁻ secretion by promoting recycling of Cl⁻ that is taken up by Cl⁻/HCO₃⁻ exchange. In addition to CFTR, Ca²⁺-activated Cl⁻ channels have been demonstrated in human and rat cholangiocytes that could be the target for hormones that promote HCO₃⁻ secretion via Ca²⁺ signaling. Furthermore, cooperation between CFTR and Ca²⁺-activated Cl⁻ channels has been suggested, in that CFTR could also operate as an ATP efflux channel that leads to stimulation of apical P₂Y₂ receptors that activate Cl⁻ channels through an increase of [Ca²⁺]. Resulting HCO₃⁻ secretion could be assisted by simultaneous activation of basolateral Na⁺/H⁺ exchange through P₂Y₂ receptor stimulation followed by an increase of intracellular HCO₃⁻ concentration (3).

Reuptake of solutes. Bile duct cells also reclaim substances that have been secreted upstream at the canalicular level. Cholangiocytes exhibit Na⁺-dependent and Na⁺-independent glucose transport, and, similar to mechanisms in hepatocyte canalicular membranes, split products of glutathione (glutamate, glycine) are reabsorbed. Certain dihydroxy bile acids, including ursodeoxycholic acid, appear to be reabsorbed in a functional (but not molecular) exchange with HCO₃⁻, a mechanism that leads to generation of HCO₃⁻-rich bile, rendering those bile acids hypercholeretic (see above). The significance of expression in cholangiocytes of the apical (intestinal) sodium-bile acid transporter [ASBT (ISBT)], a transporter that reclaims bile acids to prevent loss in the stool, remains to be elucidated.

Inherited disorders of bile formation

Recent years witnessed a fascinating development in which combined efforts from different disciplines, such as genomic screening in endogamous communities with high incidence of familial cholestasis, identification of transport proteins from their physiological function by expression cloning, mutations in animal species, knock-out studies, and even yeast genetics, led to identification of genes that are defective in inherited disorders of bile formation. Thus it was recognized early that hyperbilirubinemia in an inbred Wistar rat strain (TR rats) is due to a defect in mrp2. The homologous transporter in humans (MRP2) is mutated in Dubin-Johnson syndrome or “chronic idiopathic jaundice,” characterized by a defect in biliary secretion of conjugated bilirubin and of drug metabolites, including iodinated cholangiographic dyes (13). Byler disease was first described in the Amish community. It presents as progressive intrahepatic cholestasis, sometimes associated with unexplained diarrhea or pancreatic insufficiency, and leads to end-stage liver disease, usually before adulthood. Byler disease or progressive familial intrahepatic cholestasis (PFIC1) describes a similar disease in unrelated populations. Mutations of the gene FIC1 on chromosome 18 have been shown to be responsible for these diseases, and, independently, mutations on this same gene have been identified in benign recurrent intrahepatic cholestasis (BRIC), characterized by episodes of cholestasis but not resulting in liver damage. Although these diseases are characterized by low bile salt secretion, the precise functions of FIC1 are not known. On the other hand, two additional forms of progressive familial intrahepatic cholestasis could be ascribed to mutations in SPGP, the canalicular bile salt export pump (PFIC2), and, in MDR3, the canalicular phospholipid transporter (PFIC3) (11). In cholangiocytes, the genetic defect in CFTR in cystic fibrosis frequently (~30%) results in late-onset cholestatic symptoms.

Conclusion

Within recent years, modern experimental techniques from various disciplines, molecular biology methodology in particular, have been employed to explore the membrane transport mechanism responsible for generation of bile flow along bile canaliculi and bile ducts. Those studies led to identification of
an increasing number of individual transport proteins and their genes, their functions and interactions being explored and their defects becoming known. This article is an attempt to illuminate this widespread field. It could not properly pay tribute to those many investigators responsible for these numerous new discoveries, who lead us to a better understanding of liver transport functions.

References