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Localization status of hepatocellular transporters in cholestasis

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1. ABSTRACT

Vectorial transport of osmotically active solutes from blood into bile is essential for bile flow generation. Therefore, the localization status of hepatocellular transporters involved in this function is critical. These transporters are localized either in the plasma membrane or in an endosomal, submembranous compartment, from where they undergo recycling to the plasma membrane. The balance between exocytic targeting/endocytic internalization from/to this recycling compartment is therefore a chief determinant of the liver capability to secrete bile. Furthermore, its impairment may lead to sustained endocytic internalization, eventually resulting in transporter degradation. Exacerbated internalization of hepatocellular transporters occurs in several experimental models of cholestasis, and also in most human cholestatic liver diseases. This review outlines the possible mechanisms explaining this alteration (*e.g.*, alteration of the organization of actin or actin-transporter linking proteins), and the mediators involved (*e.g.*, activation of "cholestatic" signaling pathways). Finally, several experimental therapeutic approaches based upon the administration of compounds that stimulate exocytic targeting of canalicular transporters (*e.g.*, cAMP, tauroursodeoxycholate) are described with regard to their capability to prevent cholestatic alterations resulting from transporter internalization.

2. INTRODUCTION

Bile secretion is a highly regulated process. Such a regulation is aimed to handle the physiological demand for biliary disposition of endo- and xenobiotics. This is achieved by the modulation of the constitutive expression, the dynamic localization and the intrinsic activity of relevant transport proteins located at both the sinusoidal (basolateral) and the canalicular (apical) membranes of the hepatocyte.

Modulation of carrier transport activity may occur at different time scales. Long-term regulations occur by changes in carrier turnover, leading to modifications of the synthesis-degradation balance. Altered synthesis rate involves transcriptional or translational changes in carrier expression. On the other hand, modification of the carrier degradation rate is a post-translational process. It may involve, as a triggering event, sustained internalization of the carrier protein from its plasma membrane domain, followed by lysosomal degradation.

Contrarily, transitory, reversible changes in transporter localization by vesicle-mediated insertion/internalization from/to an endosomal recycling compartment may occur as part of a short-term, physiological mechanism aimed to quickly modulate carrier density at the plasma membrane. This is a tightly regulated

Table 1. Transporter internalization under experimental cholestasis and in human cholestatic hepatopathies

Cholestatic model/ Human cholestatic disease	Transporter internalized	Structural features	Signaling mediators involved
BDL	Mrp2[38,68], Bsep[68,82]	Portal inflammation[82]	Pro-inflammatory cytokines (TNF-α and IL-1β)[82]
Estrogens	Mrp2[29,94], Bsep[30]	Radixin dephosphorylation and internalization[94], preserved actin organization[29,30], independence of microtubular integrity[37]	cPKC[101], PI3K/Akt (complementarity with cPKC)[104]
TLC	Mrp2[31], Bsep[32], Ntcp[35]	Preserved actin organization[32]	PI3K[103]/PKCε ?[102]
Oxidative Stress	Mrp2[43-46], Bsep[42]	Actin disruption[42]	cPKC[42], Ca^{2+}/NO/nPKC[45]
LPS	Mrp2[52,53,55,56,66,67,110]/MRP2[54], Bsep[55,56]/BSEP[54], Ntcp?[63,64]/NTCP[65]	Radixin dephosphorylation and internalization[110]	Pro-inflammatory cytokines (TNF-α, IL-1β, IL-6)[53,54,58,59,63,64], NO (S-nitrosylation of NTCP)[65], ROS[66,67]
PBC, PSC, obstructive cholestasis, drug-induced cholestasis, autoimmune hepatitis	MRP2[83-86], BSEP[83]	Disturbed expression and localization of radixin[84,86]	

Footnote: Summary of the hepatocellular transporters that suffer endocytic internalization under different experimental cholestatic models as well as several cholestatic hepatopathies in humans. The structural features reported in each situation and the possible intracellular and extracellular signaling pathways involved are also summarized. Superscript numbers indicate the related references. For more details, see the text, items 4 and 5. Abbreviations: BDL: bile duct ligation; TLC: taurolithocholate; LPS: lipopolysaccharide; PBC: primary biliary cirrhosis; PSC: primary sclerosing cholangitis; TNF-α: tumor necrosis factor α; IL: interleukin; cPKC: "classical", Ca^{2+}-dependent protein kinase C; nPKC: "novel" PKC; ROS: radical oxygen species.

process, and the signaling mediators involved are being actively characterized (1).

Apart from its role in biliary physiology, changes in the proper localization of hepatocellular transporters also occur in a number of pathological conditions, and they may contribute to explain the cholestatic manifestations occurring in these hepatopathies. This has prompted investigators to give an insight into the mechanisms involved in this particular pathomechanism at a molecular level, and to envisage and test in experimental models of cholestasis new therapeutic approaches based on its prevention/reversion.

This article is aimed to give an overview of this subject, by summarizing the current information available in the literature on changes in hepatocellular transporter dynamic localization in experimental and clinical cholestasis, the putative molecular machinery underlying this pathomechanism and the intracellular signaling pathways involved in its regulation (summarized in Table 1). The beneficial modulation of this phenomenon by experimental therapeutic agents known to stimulate exocytic insertion of canalicular transporters is also discussed.

3. HEPATOCELLULAR TRANSPORTERS AND THEIR ROLE IN BILE FORMATION

The hepatocyte is a polarized cell that expresses differential transport systems in its plasma membrane domains. These transporters play a key role in the vectorial transport of solutes and water from sinusoidal blood into bile, thus contributing to bile formation and the biliary excretion of many xenobiotics. Most of these transport proteins have been identified by molecular cloning, and their transport properties characterized by functional studies. Their localization and transport function are shown in Figure 1.

3.1. Sinusoidal transporters

Liver sinusoids possess a specific architecture that allows passage of organic compounds bound to albumin through endothelial fenestrae into the Disse`s space, from where they can be taken up by the sinusoidal transport systems of the hepatocyte (2).

Basolateral uptake transporters can be classified into Na^+-dependent and Na^+-independent transport systems. Na^+-dependent uptake involves cotransport of solutes with Na^+, and is driven by the electrochemical Na^+ gradient generated and maintained by the Na^+/K^+-ATPase, strategically localized at this membrane domain. The Na^+-independent uptake of organic anions is driven primarily by exchanging them with other organic anions, such as HCO_3^-, reduced glutathione (GSH) or bile salts (3).

Bile salts are the predominant organic solutes in bile, and the main determinants of bile flow (4). Bile salts are mainly taken up by the *Na^+-taurocholate cotransporting polypeptide* (NTCP (SLC10A1)/Ntcp (Slc10a1) for humans and rodents, respectively) (5). A remaining fraction is taken up in a Na^+-independent manner by a family of transporters collectively referred to as *organic anion-transporting polypeptide* (OATP (SLCO)/Oatp (Slco)) (6,7). In addition to conjugated and

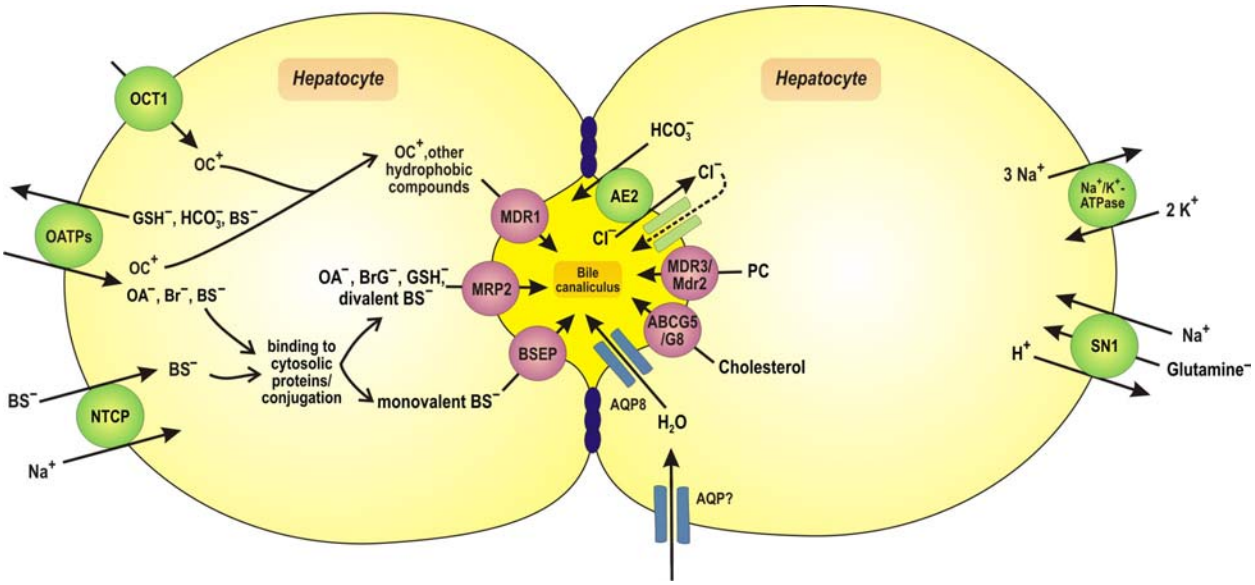

Figure 1. Localization and function of sinusoidal and canalicular transporters in hepatocytes. The Na^+-dependent sinusoidal uptake of bile salts (BS^-) is mediated by NTCP, driven by the electrochemical Na^+ gradient generated by the Na^+/K^+-ATPase. This Na^+ gradient is also the driving force for the uptake of amino acids, such as glutamine (mediated by SN1). The Na^+-independent uptake of several exogenous and endogenous organic anions (OA^-), bilirubin (Br^-), bile salts (BS^-) and "type II" organic cations (OC^+) is mediated by members of the OATP family. This transport involves anion exchange, some of the presumed intracellular counter-anions being glutathione (GSH^-), HCO_3^- and BS^-. Sinusoidal uptake of "type I" OC^+ is mediated by OCT1. Transport across the canalicular membrane is driven mainly by ATP-dependent export pumps, which belong to the superfamily of ATP-binding cassette (ABC) transporters. Several multidrug-resistance proteins (MDR) have been identified in the canalicular membrane. MDR1 mediates canalicular excretion of OC^+ and other hydrophobic compounds, while MDR3 (Mdr2 in rodents) and ABCG5/G8 function as floppases, translocating phosphatidylcholine (PC) and cholesterol, respectively, from the inner to the outer leaflet of this membrane domain, thus facilitating canalicular excretion of lipids. Apical excretion of BS^- is mediated by BSEP, while non-BS^- organic anions, such as bilirubin glucuronides (BrG^-), GSH^- and sulfated/glucuronidated BS^- are transported by MRP2. Canalicular transport of HCO_3^- is mediated by the Cl^-/HCO_3^- exchanger AE2. The out-to in Cl^- gradient required for this exchange would involve an unidentified apical chloride channel. The water channels aquaporins (AQPs) facilitate transcellular water transport from sinusoidal blood into bile both in the sinusoidal and canalicular membranes. AQP8 was demonstrated to mediate this transport at the canalicular membrane of both human and rodent hepatocytes, whereas AQP9 mediates the sinusoidal water transport, a fact only proved so far in rodents.

unconjugated bile salts, OATPs/Oatps accept a wide range of cholephilic compounds, including glucuronidated (and maybe unconjugated) bilirubin, exogenous organic anions (*e.g.* sulphobromophthalein), leukotrienes, estrogen-conjugates (*e.g.* estrone-3-sulfate and estradiol-17ß-D-glucuronide), thyroid hormones, mycotoxins, and numerous xenobiotics (5,8-10). Four OATPs have been cloned and characterized from human liver, namely OATP1A2 (SLCO1A2), OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3) and OATP2B1 (SLCO2B1). There are three Oatps identified in rats, namely Oatp1a1 (Slco1a1), Oatp1a4 (Slco1a4) and Oatp1b2 (Slco1b2). Oatp1b2 is the rodent ortholog of both OATP1B1 and OATP1B3 (11).

Hepatocellular uptake of organic cations is mediated by two separate transport systems depending on the substrate molecular size (12). Small ("type I") organic cations are taken up by the organic cation transporter OCT1 (SLC22A1)/Oct1 (Slc22a1). On the other hand, both OATP1A2 (but not the remaining members of the OATP family) and Oatp2 mediate the uptake of bulky ("type II") organic cations in humans and rats, respectively.

The basolateral membrane also contains transporters involved in the uptake of amino acids. Among them, the *system N 1* (SN1/Sn1, SNAT3/Snat3, official nomenclature: SLC38A3/Slc38a3) was shown to mediate the uptake of glutamine, histidine, asparagine, and alanine. This transporter mediates the influx of two Na^+ cations and one amino acid molecule per transport cycle coupled to the efflux of one H^+, rendering the transport process electrogenic (13).

3.2. Canalicular transporters

After traversing the cell by Fick's diffusion mostly bound to high-affinity cytosolic proteins, cholephilic compounds are excreted into bile mainly by ATP-dependent pumps belonging to the superfamily of ATP-binding cassette (ABC) transporters, in particular those of the family of *multidrug-resistance proteins* (MDR/Mdr) or to the family of *multidrug resistance-associated proteins* (MRP/Mrp).

MDRs/Mdrs were originally described in cancer cell lines, where they confer resistance to therapeutic

agents. Three gene products have been identified in rodents, namely Mdr1a (Abcb1a), Mdr1b (Abcb1b) and Mdr2 (Abcb4), whereas two have been identified in humans, namely MDR1 (ABCB1) and MDR3 (ABCB4). MDR1/Mdr1 functions as an efflux pump for a wide range of amphiphilic, bulky type II cationic drugs, together with other hydrophobic compounds, such as endogenous and exogenous metabolites or toxins, steroid hormones, hydrophobic peptides and even glycolipids (10). MDR3/Mdr2 functions as a floppase, translocating phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane; this is followed by the release of phosphatidylcholine-containing vesicles from the outer leaflet into bile, a process facilitated by the detergent properties of luminal bile salts (14). Similarly, a heterodimeric protein formed by two half ABC transporters, ABCG5 (Abcg5)/ABCG8 (Abcg8), facilitates the biliary excretion of cholesterol, by flopping this lipid from the inner to the outer leaflet of the canalicular membrane, followed by incorporation into biliary mixed micelles, together with bile salts and phosphatidylcholine (15).

Monoanionic bile salts are transported at the apical membrane by the *bile salt export pump* (BSEP (ABCB11)/Bsep (Abcb11)), another member of the MDR family (16). In contrast, canalicular efflux of divalent, bipolar sulfated or glucuronidated bile salts is mediated by the *multidrug resistance-associated protein 2* (MRP2 (ABCC2)/Mrp2 (Abcc2)) (6,17). This carrier also mediates the biliary excretion of many other organic anions, including glutathione *S*-conjugates (*e.g.*, of leukotriene C4 or sulphobromophthalein), glucuronides (*e.g.*, bilirubin and estrogen glucuronides), oxidized glutathione (GSSG), and GSH, the latter with lower affinity than GSSG (18,19). Both GSSG and GSH are major determinants of the bile salt-independent fractions of the canalicular bile flow (20).

The canalicular membrane domain contains also the electroneutral *anion exchanger 2* (AE2 (SLC4A2)/Ae2 (Slc4a2)), which extrudes HCO_3^- by exchanging it for Cl^- (21). It has been suggested that this countertransporter functions in association with an apical chloride channel that maintains favorable Cl^- gradients, although this specific channel remains to be identified (3,21). AE2/Ae2 also participates in intracellular pH regulation when hepatocytes are exposed to an alkaline load (21). In addition, AE2/Ae2 would play a role in bile flow generation, since HCO_3^- excretion is thought to be an additional primary driving force of the bile salt-independent canalicular bile flow (21,22). Both in humans and rats, three transcript variants of AE2/Ae2 have been described, namely the full-length transcript AE2a/Ae2a, expressed from the upstream promoter in most tissues, and the alternative transcripts AE2b1/Ae2b1 and AE2b2/Ae2b2, expressed in a more tissue-restricted fashion (mainly in liver and kidney). AE2b1/2/Ae2b1/2 transcription is driven from overlapping promoter sequences within intron 2, which result in AE2/Ae2 protein isoforms with short *N*-terminal differences (23,24).

3.3. Water transporters

For a solute to induce de vectorial blood-to-bile water transport, resultant osmotic forces need to be associated with aquaporin (AQP)-mediated transcellular movement of water molecules from plasma into the bile canaliculus. Both immunochemical and functional studies have demonstrated the constitutive expression of the water channel AQP9 at the basolateral membrane of rat hepatocytes, and the regulated expression of the water channel AQP8 at the hepatocellular canalicular membrane domain (25-27). Due to its property of being inserted into the canalicular membrane on demand, AQP8 was suggested to play a n important role in bile formation, by facilitating the osmotic movement of water under a choleretic stimulus (26,27). The AQP isoforms that mediates polarized water transport in human hepatocytes remains to be identified. However, evidences for a role of human AQP8 in human bile formation were recently obtained by silencing gene expression in the human hepatoma cell line HepG2, using specific small interfering RNA (siRNA) (28).

4. ALTERATIONS OF THE DYNAMIC LOCALIZATION OF TRANSPORTERS IN CHOLESTASIS

There is compelling evidence in the literature that, under cholestatic conditions, the physiological recycling of membrane transporters via vesicle-mediated insertion/internalization from/to the endosomal compartment is altered, leading to a shift towards retrieval of these transporters; this decreases their plasma membrane expression, and consequently impairs the transport of their substrates. This mechanism is schematized for canalicular transporters in Figure 2, although a similar phenomenon occurs for the basolateral ones. The molecular mechanisms and the signaling pathways involved are under active study, and will be discussed later in this review.

Endocytic internalization of hepatocellular transporters is a common feature in those hepatopathies primarily involving impairment in the hepatocyte capability to produce bile (hepatocellular cholestasis). In this case, changes in transporter localization may become a major pathomechanism explaining the secretory failure. Alternatively, changes in carrier localization can occur as a secondary consequence of a cholestatic manifestation due to mechanical impediments to deliver bile into the duodenum (obstructive cholestasis). In such an event, transporter mislocalization may aggravate and perpetuate the primary secretory halt. Below, we summarize the current evidence in the literature supporting the concept that alterations in the dynamic localization of transporters occur in both experimental and human cholestatic liver disease.

4.1. Endocytic internalization of transporters in experimental cholestasis

4.1.1. Drug-induced cholestasis

Administration to laboratory animals of drugs known to induce functional, hepatocellular cholestasis, or administration of endogenous compounds thought to be the etiological agents of cholestatic human hepatopathies, has been used as an experimental tool to study the mechanisms of the disease. Administration of either the endogenous estradiol metabolite estradiol-17ß-D-glucuronide (E17G)

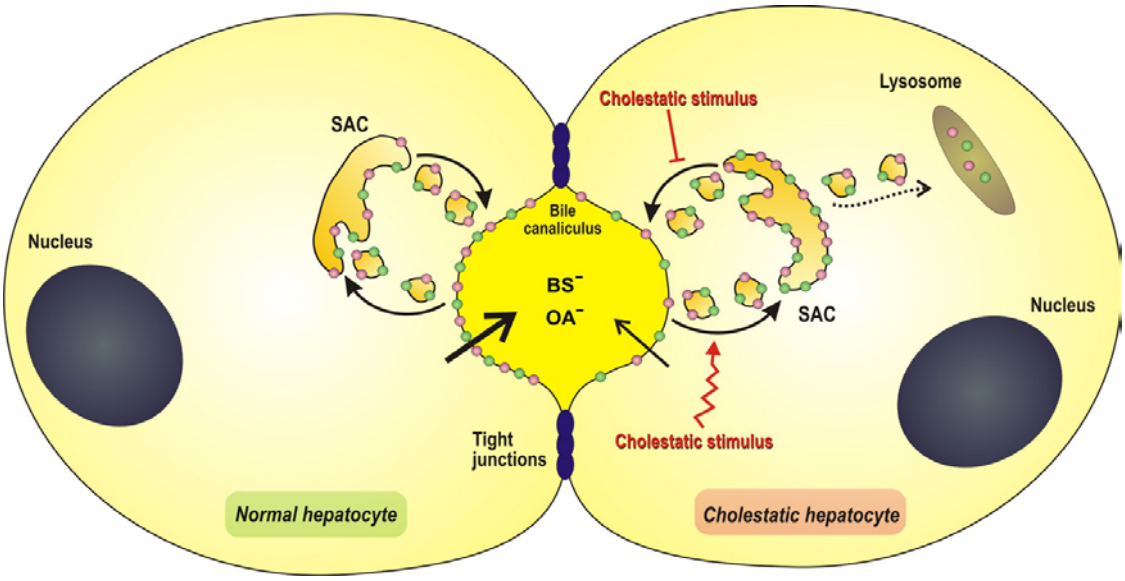

Figure 2. Dynamic localization of canalicular transporter under normal and cholestatic conditions. Under physiological conditions (Normal hepatocyte), the density of transporters at the canalicular membrane domain, responsible for the normal biliary excretion of bile-forming solutes such as bile salts (BS^-) and organic anions (OA^-), is regulated by a vesicle-mediated recycling to/from a subapical compartment (SAC), which serves as a reservoir of transporters available on demand. Cholestatic stimuli disrupt this mechanism, leading to a decrease in the expression of transporters at the canalicular membrane, with a concomitant increase in SAC; this is accompanied by a reduced biliary excretion of their substrates (Cholestatic hepatocyte). Transporter internalization may be produced either by stimulating the endocytic internalization of transporters or by inhibiting their normal exocytic insertion from SAC. Sustained cholestatic stimuli could lead to the sorting of endocytozed transporters to lysosomes, followed by degradation.

(29,30), the monohydroxylated bile salt taurolithocholate (TLC) (31,32), or the immunosuppressor drug cyclosporine A (33) induces cholestasis in a short-term fashion, accompanied by endocytic internalization of Mrp2 and Bsep. At the basolateral pole, TLC was also reported to induce a non-competitive inhibition of taurocholate uptake in rat hepatocytes (34), which was recently shown to be associated with internalization of Ntcp from the basolateral membrane (35).

Our group has characterized in detail the mechanisms of transporter internalization in E17G-induced cholestasis, an experimental model that reproduces in part pregnancy-induced cholestasis. After a single, i.v. administration of this compound, bile flow decreases in a dose-dependent fashion, with a nadir at 20 min. After that, bile flow spontaneously recovers, reaching normal levels by 2 hs post-injection (36). The cholestatic phase is associated with endocytic internalization of both Mrp2 and Bsep, whereas the recovery phase parallels the spontaneous reinsertion of subapical transporter-containing vesicles into the canalicular membrane (29,30). While the internalization is a microtubule-independent event, reinsertion is a microtubule-dependent one. The latter event is stimulated by cAMP, a signaling mediator known to stimulate vesicular trafficking and exocytic insertion of canalicular transporters (37). We also found that repeated administration of E17G to rats leads to both a deeper internalization of Mrp2 and the abnormal localization of a small fraction of this transporter at the lateral plasma membrane (38). The latter phenomenon likely reflects loss of the fence between apical and basolateral domains due to the simultaneous alteration of the tight-junctional structures (39).

4.1.2. Oxidative stress-induced cholestasis

Oxidative stress is a common feature in most hepatopathies (40). Radical oxygen species induce biliary secretory failure and cholestasis, even at low, pre-necrotic levels (41), and endocytic internalization of canalicular transporters would play a key role. We have shown that Bsep suffers endocytic internalization into intracellular vesicles in isolated rat hepatocyte couplets when exposed to low levels of the pro-oxidant compound *tert*-buthylhydroperoxyde (*t*BOOH) (42). This was accompanied by a reduced capability of the hepatocyte couplets to accumulate the fluorescent bile salt analogue cholyl-lysyl-fluorescein in their canalicular vacuoles. A similar phenomenon has been shown for Mrp2 after exposure of isolated perfused rat livers to different pro-oxidant agents, including *t*BOOH (43), chloro-dinitrobenzene (43) and ethacrynic acid (44,45). Something similar has been shown to occur in hepatic ischemia-reperfusion, a pro-oxidant event that seems to involve Kupffer cell-mediated thromboxane A2 release (46), which becomes apparent even before irreversible microcirculatory disturbance is evident (47). Mrp2 internalization induced by *t*BOOH administration to the isolated, perfused rat liver was shown to be reversible in nature, since it was reverted by replenishment of GSH with GSH-ethyl ester, a cell-permeable compound that renders GSH after hydrolysis by intracellular esterases (48).

4.1.3. Lipopolysaccharide (LPS)-induced cholestasis

LPS is an endotoxin localized in the outer membrane of gram-negative bacteria. This toxin induces cholestasis mainly by promoting the release of cytokines, such as tumor necrosis factor-α (TNFα) and interleukins (ILs) 6 and 1β; these pro-inflammatory mediators are released by monocytes/macrophages and, in the liver, by Kupffer cells (49-51). These cytokines are thought to be principal mediators of the bile secretory failure induced by LPS (50). The endotoxin also stimulates NO production by inducible nitric oxide synthase (iNOS) in all major liver cell types (50). Administration of LPS to laboratory animals represents, therefore, a good experimental model of inflammatory cholestatic diseases, not only of those caused by endotoxemia, but also those related to hepatitis of different etiologies (*e.g.*, alcoholism, drug intake, or autoimmune attack).

LPS administration leads to endocytic internalization of Mrp2 and Bsep, with these transporters being relocalized in intracellular vesicular structures (52-56). The time-dependency of LPS effect on Mrp2 internalization has been characterized by Kubitz *et al.* (53). These authors found that, 3 hs after LPS treatment, Mrp2 is localized in intracellular vesicles in the vicinity of the canalicular membrane, and that these vesicles are deeply internalized after 6 to 12 hs of LPS treatment. Further periods of time have been studied by Zinchuk *et al.* (56), who demonstrated a relocation of Mrp2 to the basolateral domain 48 hs after LPS administration. Endocytic internalization of the ABC canalicular transporters seems to be specific, as localization of the canalicular enzyme dipeptidyl peptidase IV was not affected by the treatment (53). Mrp2 internalization is reversed by perfusing the liver with a hypoosmotic buffer (53), a maneuver known to stimulate exocytic reinsertion of canalicular transporters (52,57). However, this rescue of transporters occurs within 3 hs of LPS administration, but not later on (53). It is possible that reversibility of the endocytic process depends on the degree of internalization of Mrp2, and that sustained internalization leads to delivery of the protein to the lysosomal compartment, followed by degradation. Pro-inflammatory cytokines seems to be major determinants of these alterations, since canalicular transporter internalization by LPS can be prevented by both dexamethasone─administration (53,54) and heat stress (58,59), two maneuvers known to cause a decrease in the synthesis and/or release of cytokines.

Although there is no direct confirmation of changes in transporter localization status at the basolateral membrane in endotoxemia, indirect evidences suggest that this could be the case. An inhibition at the gene expression level of the bile salt uptake transporter Ntcp occurs late (20-24 hs) after LPS administration to rats, leading to a further decrease in the protein Ntcp levels (higher than 90 %) (60). A similar decrease in Ntcp gene expression was induced by administrating IL-6 (61,62), TNF-α (54,60) and IL-1ß (54,60), suggesting mediation by these pro-inflammatory cytokines in this effect. However, *before* downregulation of Ntcp mRNA becomes apparent, IL-6 (63) and TNF-α (64) induced a noncompetitive inhibition (*i.e.*, a selective *V*max reduction) of the Ntcp-mediated uptake of taurocholate; this suggests that an early post-transcriptional decay in the content of Ntcp in basolateral membrane occurs, most likely involving Ntcp internalization. In line with this, exposure of HuH cells stably transfected with human NTCP to NO donors so as to mimic NO intracellular elevation induced by endotoxin led to a rapid inhibition of taurocholate uptake (within 30 min), a phenomenon that was suggested to be due to internalization of this transporter (65). Cytokine-mediated elevation of intracellular NO in endotoxemia is expected to occur shortly after LPS administration, since the release of proinflammatory cytokines and iNOS induction is rapid (within 3 hs) (54). Therefore, if confirmed, Ntcp retrieval would be an initial event in LPS-induced cholestasis.

LPS-induced oxidative stress seems to be a major causal factor of both LPS-induced cholestasis and canalicular transporter internalization. Dimerumic acid, an antioxidant used by the traditional Chinese medicine, prevented LPS-induced cholestasis and Mrp2 internalization after a short period of LPS exposure both in the whole rat (3 hs) (66), and in cryopreserved rat and human liver slices (1.5 hs) (67). Interestingly, in the *in vivo* model, the antioxidant was unable to compensate for the drop in Mrp2 protein content and mRNA expression following 12 hs of LPS administration, suggesting that, unlike the fast Mrp2 internalization, long-term transcriptional regulation of Mrp2 expression by LPS does not depend on the intracellular redox status (66).

4.1.4. Bile duct ligation (BDL)

Ligation of the common bile duct in the rat is a well-accepted experimental model of obstructive cholestasis. BDL leads to a marked alteration in the pattern of staining of both Mrp2 and Bsep, as detected by immunofluorescence microscopy. Paulusma *et al.* found that, 48 hs after BDL to rats, immunostaining of these canalicular transporters becomes fuzzy, in contrast to the well-delimited staining in shams (68). The authors assumed that this would represent internalization of the transporters to intracellular vesicles at a subapical compartment, next to the canaliculus. These alterations were accompanied by a severe impairment of the biliary excretion of model solutes. For example, Mrp2-mediated transport of the model substrate dinitrophenyl glutathione was substantially impaired in isolated hepatocytes from rats with BDL (68). Endocytic internalization seems not to be circumscribed to Mrp2 or Bsep, as a similar phenomenon was observed for the canalicular enzyme Ca^{2+}/Mg^{2+}-ATPase (69). Altered localization of Mrp2 and Bsep may represent an aggravation of the secretory dysfunction due to the parallel decrease in the hepatocellular content of the carriers also occurring in this disease (70,71), or even a causal factor of this downregulation (68,72-75). Indeed, several studies found that, in contrast to what was observed for Mrp2 protein content, mRNA levels are preserved after BDL, suggesting post-transcriptional downregulation of Mrp2 expression (68,72,75). Paulusma *et al.* (68) postulated that vesicular retrieval may represent the primary step toward enhanced metabolic breakdown of the endocytosed carriers. If maintained with time in chronic cholestatic conditions,

this may trigger delivery of the protein to the lysosomal compartment, followed by degradation.

The events leading to endocytic internalization of Mrp2 and Bsep in rats with BDL remain uncertain. It is likely that accumulation of endogenous, potentially toxic compounds in the liver represents a causal factor. Likely candidates are endogenous bile salts, which increase roughly 40 times in serum after 1 day of bile duct obstruction in mice (76). Supporting this view, bile salt overload to rats induces retrieval of Mrp2, associated with rearrangements of both actin and the actin-binding protein, radixin (77), most likely through an oxidative stress-mediated mechanism (78,79). The detrimental action of bile salt-induced oxidative stress on transporter localization could be reinforced by that of the endotoxemia, which occurs secondary to the lack of intestinal bile salts that occurs in this cholestatic model (80); endotoxin produces a sustained release of pro-inflammatory cytokines which trigger, in turn, further oxidative stress (81). In line with this, Donner *et al.* reported that zonal (periportal) downregulation and vesicle-mediated internalization of Bsep in obstructive cholestasis is associated with portal inflammation, and that this event is mediated by TNFα and IL-1ß (82).

4.2. Endocytic internalization of transporters in human cholestatic liver disease

Changes in canalicular export pump localization have been shown to occur in many human cholestatic hepatopathies. Since downregulation of the expression of these transporters in human cholestatic disease is mostly post-transcriptional in nature, internalization of these transporters followed by degradation may represent a crucial mechanism to explain the disease in humans.

Shoda *et al.* studied the association between expression of major canalicular transporters and their functional performance in patients with obstructive cholestasis subjected to percutaneous transhepatic biliary drainage. They reported decreased expression of BSEP and MRP2 mRNA levels, in association with fuzzy staining of the transporters at the canalicular membrane in liver specimens from patients poorly drained; these alterations were significantly attenuated in patients exhibiting good drainage (83). The possibility that major canalicular transporters suffer internalization in cholestatic liver disease conditions was confirmed by Kojima *et al.* The authors described alterations in the expression and localization of hepatocellular transporters in primary biliary cirrhosis, and showed that the degree of internalization correlated well with the severity of the alteration in biliary secretion, as suggested by elevations of serum markers of cholestasis (84).

More recently, Watanabe *et al.* (85) evaluated 26 cases of drug-induced acute intrahepatic cholestasis by assessing serum biochemical markers, including bilirubin, in association with histopathological studies of liver specimens examined by both light and electron microscopy. Major causative agents of the intrahepatic cholestasis studied were tiopronin, chlorpromazine and diclofenac. The authors additionally made a correlation of liver hystopathology and function with the relocalization of MRP2, as examined immunohistochemically. They distinguished between cholestasis of short and prolonged evolution. They found that half-life of serum bilirubin and liver injury, detected as lobular inflammation, were more prominent in patients with prolonged cholestasis, and that this was associated with loss of the normal localization of MRP2 at the canalicular level and relocalization in pericanalicular vesicles. Finally, Kojima *et al.* suggested an alteration of localization and phosphorylation of anchoring proteins to be a potential triggering factor to explain altered localization of MRP2 at the canalicular level in cholestatic patients (see item 5.1 for details) (86).

Unambiguously, these reports strongly support the concept that internalization of canalicular transporters are associated with the impairment in biliary function in human cholestic disease, in agreement with findings reported in experimental models of cholestasis in rodents.

5. MECHANISMS OF ENDOCYTIC INTERNALIZATION OF TRANSPORTERS IN CHOLESTASIS

The mechanism by which endocytosis of hepatocellular membrane transporters occurs in cholestasis remains poorly understood, in part due to its multifactorial nature. At present, this subject is under active investigation, and new mechanistic basis are emerging, both on the structural cellular biology underlying the endocytic phenomenon and on the role of signaling pathways evoked by the cholestatic agents that trigger this event. Figure 3 schematizes some of the possible cellular targets and signaling pathways involved in this pathomechanism, which will be discussed in more detail next.

5.1. Structural basis

The actin cytoskeleton is a dynamic structure which consists of actin filaments and associated actin-binding proteins. Its integrity and normal dynamics is essential to various cellular events including the maintenance of cell shape, canalicular contractility, and the distribution and stability of integral membrane proteins. Indeed, actin can interact with, and possibly regulate, transmembrane proteins via binding to proteins of the ezrin-radixin-moesin (ERM) family, which act as cross-linkers between the plasma membrane and actin filaments. Membrane proteins, such as transporters, can also bind to cytosolic scaffold proteins, such as PDZK1, HAX-1, and the receptor for activated C-kinase 1 (RACK1). These proteins possess several protein-binding domains, and were demonstrated to be required for the proper retention of membrane transporters in their specific membrane domain (87-90). Accordingly, it is not surprisingly that conditions affecting either or both, actin integrity or normal expression/localization of cytosolic anchoring proteins, leads to internalization of transporters.

In line with this, canalicular transporter internalization was reported under conditions of disruption of actin-cytoskeletal integrity by administration of the F-actin poison phalloidin (91), or secondary to the

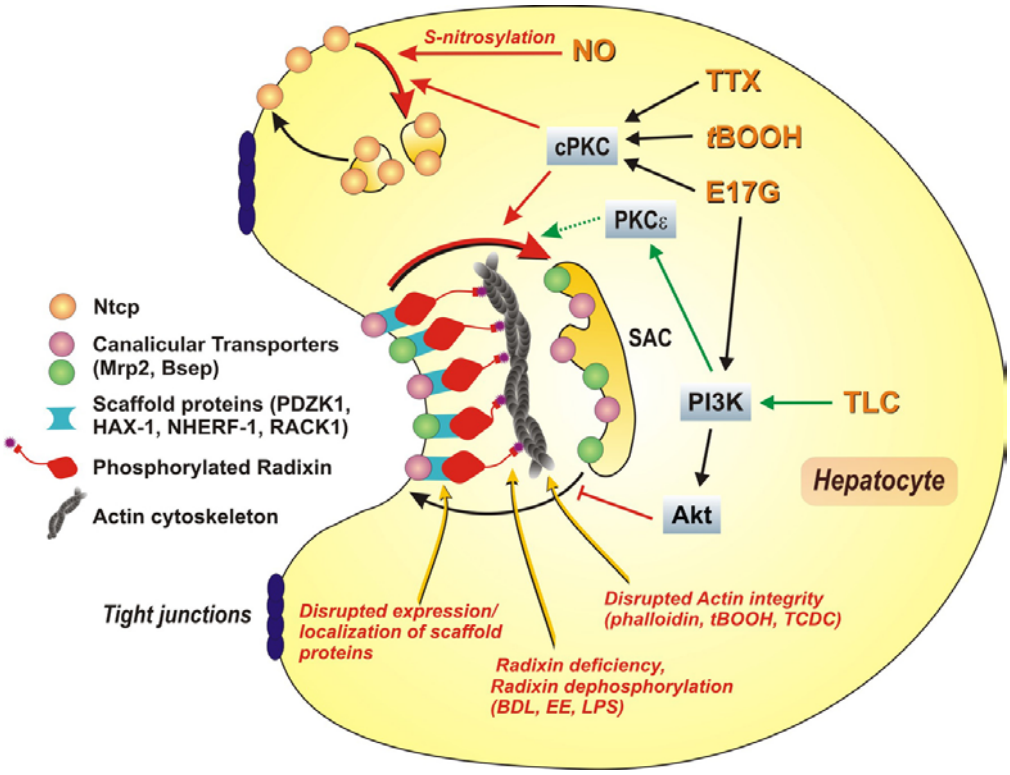

Figure 3. Schematic representation of the possible signaling pathways and cellular targets involved in the endocytic internalization of transporters under cholestatic conditions. Stability of canalicular transporters in their membrane domain depends on the integrity of the actin cytoskeleton and the normal expression and localization of actin-associated proteins. The latter includes actin-binding proteins belonging to the ezrin-radixin-moesin (ERM) family, and also cytosolic scaffold proteins (PDZK1, HAX-1, RACK1, and NHERF-1). Several cholestatic conditions and agents can act at this level, unbalancing the normal recycling of canalicular transporters between the membrane and an endosomal subapical compartment (SAC), in favor of endocytic internalization. For example, alteration of integrity of actin cytoskeleton by phalloidin, *tert*-buthylhydroperoxide (*t*BOOH) or taurochenodeoxycholate (TCDC) treatment leads to transporter internalization. Inactivation by dephosphorylation of the actin-binding radixin, the main ERM protein in hepatocytes, also leads to transporter internalization, as was described after bile duct ligation (BDL), or treatments with ethinylestradiol (EE) or lipopolysaccharide (LPS); similarly, radixin deficiency (by gene knockout or knockdown) also induces internalization of canalicular transporters. Several signaling pathways are involved in these pro-endocytic mechanisms. The monohydroxylated, cholestatic bile salt taurolithocholate (TLC) induces endocytic internalization of canalicular transporters by activating the PI3K-dependent pathway, probably via PKCε. Alternatively, endocytic internalization of these transporters could depend on activation of "classical", Ca^{2+}-dependent protein kinase C isoforms (cPKC), as was suggested by studies using the specific cPKC activator thymeleatoxin (TTX), or cholestatic agents known to activate cPKC, such as *t*BOOH or estradiol 17β-d-glucuronide (E17G). For E17G, a PI3K/Akt-dependent signaling pathway is also activated, which delays the reinsertion of the canalicular transporters previously endocytozed via cPKC. In the basolateral pole, the bile salt transporter Ntcp, which also recycles constitutively between its membrane domain and an intracellular endosomal compartment, can be retrieved via cPKC activation, or by NO-induced *S*-nitrosylation of cysteine residues.

administration of pro-oxidant compounds such as *t*BOOH (42) or the hydrophobic bile salt taurochenodeoxycholate (77). However, this internalization also occurs with preserved actin organization, *e.g.* in E17G- (29,30) or TLC-induced cholestasis (32). In these cases, components of the microfilament network other than actin, but associated with it, could have been independently affected. Suggestively, mice lacking radixin, the main ERM protein in liver, develop conjugated hyperbilirubinemia associated with retrieval of Mrp2 (92). Furthermore, down-regulation of radixin using interference RNA (siRNA) technology in collagen sandwich-cultured rat hepatocytes disturbed the normal development of canalicular structures and dissociated canalicular export pumps from their normal location at the apical membrane, with the transporter being found largely associated with Rab11-containing endosomes (93). Moreover, a disturbed colocalization of both MRP2/Mrp2 and radixin associated with endocytic internalization of the carrier was apparent in obstructive and estrogen-induced cholestasis in rats (94). The same holds true for several cholestatic hepatopathies in humans, including primary biliary cirrhosis (stage III), drug-induced liver injury, obstructive cholestasis, primary sclerosing cholangitis, and autoimmune hepatitis (84,86). On the

contrary, alteration in cholestasis of the localization/function of interacting-partner proteins, such as PDZK1 (for Mrp2), HAX-1 (for Bsep, Mdr2, and Mrp1) or RACK1 (for MDR3) remain to be confirmed. This possibility however exists, since retention of Mrp2 (95) and Oatp1a1 (96) in the apical and the basolateral membranes, respectively, requires interaction with the scaffold protein, PDZK1. Additionally, Mrp2 stability at the canalicular membrane was recently shown to be dependent on the interaction with NHERF-1 (EBP50) (97), a scaffold protein with two PDZ domains and an ERM interacting domain. Moreover, knockdown of RACK1 gene by siRNA led to internalization of MDR3 in HepG2 cells (90). Finally, there is evidence that HAX-1 participates in clathrin-mediated Bsep endocytosis from the canalicular plasma membrane (88). Clearly, much more work is needed to establish with certainty the structural basis underlying transporter internalization in each cholestatic situation.

5.2. Role for signaling pathways

Accumulating evidence in the literature indicates that changes in transporter localization occurring in cholestasis depend on activation by the cholestatic agent of critical intracellular signaling pathways regulating transporter endocytosis/exocytosis. Representative examples are Ca^{2+}-dependent (classical) protein kinase C isoforms (cPKC), mainly PKCα in hepatocytes. Selective activation of cPKC with thymeleatoxin induces both endocytic internalization of Bsep and cholestasis in the isolated perfused rat liver (98). Coincidently, the pan-specific activation of both classical and novel (nPKC) PKC isoforms with phorbol-12-myristate-13-acetate also induces redistribution of MRP2 from the canalicular to the basolateral membrane in human hepatoma HepG2 cells (99). This signaling mechanism seems to apply also for basolateral transporters, since it was recently described that activation of cPKC leads to vesicular internalization of Ntcp in Ntcp-transfected HepG2 cells (100).

In line with these findings, a critical involvement of cPKC in the endocytic internalization of Bsep and Mrp2, and the associated bile-salt and organic-anion secretory failure, respectively, was recently demonstrated by our group in E17G-induced cholestasis in rats (101), and in the cholestasis associated with *t*BOOH-induced oxidative stress, in this latter case for Bsep (42). However, under oxidative-stress conditions, the signaling molecule involved seems to depend on the magnitude of the oxidative challenge. Low concentrations of the oxidizing compound ethacrynic acid do not activate cPKC but nPKC isoforms. Under these conditions, the compound internalizes Mrp2, by a mechanism probably involving Ca^{2+}-dependent activation of inducible nitric oxide (NO) synthase (iNOS), followed by NO-mediated cGMP increase, and further cGMP-activation of nPKC (45). In contrast, higher doses of ethacrynic-acid, possibly activating not only nPKC but also cPKC, induce internalization of both Bsep and Mrp2 (45).

The nPKC isoform epsilon (PKCε) is also activated in TLC-induced cholestasis, and has been suggested to be involved in TLC cholestatic effect, although direct evidences remain to be provided (102). PKCε activation is mediated by TLC-induced activation of phosphoinositide 3-kinase (PI3K), consistent with the finding that PI3K products are potent activators of PKCε (103). Furthermore, PI3K activation was shown to play a key role in TLC-induced cholestasis, and in the internalization of canalicular transporters involved in this phenomenon (103). Coincidently, we recently showed that the PI3K signaling pathway, via activation of the PI3K downstream effector Akt, is involved in the internalization of Mrp2 and Bsep induced by the estrogen metabolite E17G (104). In this case, PI3K/Akt acts complementarily with cPKC. Whereas cPKC is responsible for the initial endocytic internalization, PI3K/Akt delays the spontaneous reinsertion of the canalicular transporter containing-vesicles occurring during the recovery phase of the cholestasis. Since PI3K has been also shown to have pro-inserting properties, this may be regarded as paradoxical. However, both pro-exocytic and pro-endocytic effects of PI3K have been inferred by using pan-specific inhibitors of PI3K, and different isoforms of this kinase may have accounted for by these differential effects; this possibility is likely, since cholestatic bile salts have been shown to activate differentially PI3K isoforms, as compared with the choleretic ones (105). Alternatively, cholestatic or choleretic agents may activate parallel signaling pathways apart from PI3K which, by operating in concert with PI3K, result in opposite final effects. These alternative routes may differentially inhibit signaling molecules acting downstream of PI3K, so that a cholestatic route (*e.g.*, that mediated by Akt) or a choleretic one (*e.g.*, that mediated by mitogen activated protein kinases – MAPKs) is selectively activated depending on the cholestatic or choleretic nature of the stimulus. Suggestively, the choleretic (and anticholestatic) compound tauroursodeoxycholate (TUDC) prevents Akt phosphorylation (103), whereas it simultaneously activates routes that mediate choleretic effects, such as the MAPKs of the p38 and Erk1/2 types (106,107). The opposite also happens: the cholestatic bile salt taurolithocholate inhibits the activation by TUDC of certain choleretic signaling pathways downstream of PI3K (*e.g.*, Erk1/2), and simultaneously activates potentially cholestatic ones (*e.g.*, Akt and PKCε) (103).

Participation of different signaling pathways in the regulation of the normal recycling, or in the permanence of transporters in their membrane domains, involves several protein kinases, likely indicating that one or more steps are regulated by changes in the phosphorylation status of specific intracellular targets. The identity of these phosphorylation targets is poorly known at present. It putatively involves the transporter themselves, or any of the cellular structures required for transporter trafficking, recycling and membrane anchoring. For instance, it has been shown in HepG2 cells that SNAT3 (SN1, SLC38A3), a basolateral transporter involved in hepatic glutamine uptake, is internalized after treatment with the pan-specific PKC activator phorbol myristate acetate, with the consequent inhibition of the cellular glutamine uptake (108). This study also shows that internalization of the transporter is a caveolin-dependent event, and that direct phosphorylation of neither the transporter nor caveolin is involved. Based on previous

evidence, the authors suggested that SNAT3 internalization requires PKCα-dependent phosphorylation of *myristoylated alanine-rich C-kinase substrate* (MARCKS), an actin filament crosslinking protein regulated by PKC and Ca^{2+}-calmodulin, leading to reorganization of the actin cytoskeleton and, finally, withdrawal of the transporter into caveolae (108). Interestingly, the main ABC transporters involved in bile formation, *i.e.* Bsep, Mrp2, Mdr2 and Abcg5, are localized in caveolin1-positive microdomains on the canalicular membrane of rat hepatocytes (109). This opens the possibility that similar signaling pathways modulating their permanence in the membrane (*e.g.*, cPKC activation) affect some of them through a similar mechanism. Another target whose phosphorylation status may be associated with canalicular transporter internalization is radixin. Several maneuvers leading to cholestasis, such as BDL (94) or administration of either ethinyl estradiol (94) or LPS (110), lead to both Mrp2 and radixin internalization. In all these cases, Mrp2 internalization was associated with a decrease in radixin phosphorylation; this dephosphorylation occurs at a C-terminal threonine residue of radixin, as has been shown for LPS (110). When dephosphorylated in this domain, radixin losses its capability to bind to the PDZ-binding motif located in the C terminus of Mrp2, and adopts an inactive conformation via intramolecular/intermolecular interactions between its N- and C-terminal domains. This also masks the N-terminus, which functions as a nexus with the actin cytoskeleton, thus decreasing stability of Mrp2 at the apical membrane and inducing eventually internalization. Multiple protein kinases and protein phosphatases are known to influence the phosphorylation status of the C-terminal region of ERM proteins, so as to regulate the balance between active and inactive forms (111-113). Protein phosphatases involved in ERM dephosphorylation may be in turn regulated by phosphorylation by other protein kinases, an event that remains poorly, if any, characterized. A deeper insight into this puzzle awaits more information on the interplay among all these factors in cholestasis.

There is accumulating evidence that NO can act as a signaling molecule inducing posttranslational modification of proteins by binding to cysteine residues, a process known as *S*-nitrosylation (114,115). Accordingly, it was recently demonstrated that elevation of NO levels by using a NO donor molecule inhibits NTCP transport by inducing *S*-nitrosylation and membrane retrieval of this transporter in stably transfected HuH cells (65).

Finally, although appealing, there is no apparent evidence of changes in the phosphorylation status of the canalicular transporters themselves in cholestasis, so as to infer an involvement in the endocytic internalization process. Although direct phosphorylation of mouse Bsep by PKCα (116), but not by PKCδ or PKCε (117), has been demonstrated in transfected *Spodortera frugiperda* (Sf9) insect cells, phosphorylation of Bsep rather enhances intrinsic transport activity. Indeed, Bsep-transfected Sf9 cells, when subjected to PKC activation, exhibited both a reduced Km and a higher Vmax value for taurocholate transport (117). Furthermore, MDR1, the closest Bsep homolog, was phosphorylated in a baculovirus expression system when coexpressed with PKCα, and this event induced an improvement in its ATPase activity (118). Consistent with these findings, Ma *et al.* have demonstrated that inhibition of MDR1 phosphorylation by using PKC inhibitors led to a significant increase in the accumulation of MDR1 substrates, and concomitant inhibition of drug efflux in resistant cells (119). In support to a role for phosphorylation as a modulatory factor of transporter activity, Ito *et al.* (120) have described that *in vitro* phosphorylation of MRP2 by activation of cPKC in a baculo virus co-expressing system was associated with increased transport of model MRP2 substrates.

Apart from intrinsic transport activity, the phosphorylation status of MRP2 may affect interaction to anchoring proteins. Indeed, *in vitro* binding experiments showed increased interaction of MRP2 to the scaffold proteins EBP50 and IKEPP when phosphorylated at the C-terminal domain (121). Unfortunately, the influence of this phosphorylation on the binding capacity towards PDZK1, the main PDZ protein interacting with MRP2 in hepatocytes, has not been evaluated in this work. Additional studies, preferentially using liver-cell based models exposed to cholestatic agents, are required to better understand the role of direct phosphorylation of canalicular transporters for a normal membrane localization status.

6. ANTICHOLESTASTIC THERAPEUTIC APPROACHES BASED UPON MODULATION OF DYNAMIC TRANSPORTER LOCALIZATION

As illustrated above for E17G-induced cholestasis (see item 4.1.1), internalization of hepatocellular transporters in cholestasis is spontaneously reversed if the cholestatic insult is transient. This spontaneous recovery occurs by the microtubule-dependent re-targeting of the endocytosed transporters to the canalicular membrane (37). Based on this phenomenon, a number of experimental therapeutic approaches have been designed to prevent transporter internalization and/or to accelerate this re-insertion; this would avoid irreversible consequences of sustained internalization, such as accelerated transporter degradation. Prevention of transporter endocytic internalization in cholestasis and, on the other hand, acceleration of their exocytic reinsertion should however be regarded as two separate mechanisms, often involving differential arrangements of regulatory signaling molecules rather than just an “arm wrestling” by which exocytic events overcome endocytic retrieval. Similarly, although most of these anticholestatic compounds are choleretic under normal conditions by favoring the mobilization of canalicular transporters from the subapical pool, this property often involves a different set of protein kinases from those regulating their anticholestatic effects. Therefore, choleresis under normal conditions and prevention of cholestasis should be regarded as two independent events.

The compounds that have been shown to prevent the endocytic retrieval of canalicular transporters and/or accelerate their reinsertion include:

6.1. cAMP

This second messenger was among the first signaling molecules tested in its capability to stimulate vesicular transport in general, and exocytic insertion of canalicular carriers in particular (1). The signaling pathways mediating cAMP-induced Mrp2 translocation in hepatic cells are unclear, and somewhat contradictory results exist. cAMP-stimulated translocation of Mrp2 was proposed to be fully mediated by the PI3K-dependent activation of the nPKC isoform PKCδ (122). On the other hand, a more recent publication reported the involvement of p38 MAPK (α isoform), which was PI3K independent in nature (123). These results in hepatocytes, however, rely on the use of chemical inhibitors of the signaling molecules studied, which may have cross-reactivity in some extent. Involvement of p38 MAPK was supported further by studies in HuH-7 cells where p38 MAPK activation was hampered by transfection with dominant negative MKK3 and MKK6, or by direct knockdown with p38α MAPK shRNA (123). However, similar approaches in hepatocytes are lacking, and should provide a clearer picture of the putative signaling mediators involved.

Apart from the pro-exocytic activity of cAMP, this second messenger was shown to have anticholestatic properties by preventing/reversing internalization of canalicular ABC transporters occurring in several experimental models of hepatocellular cholestasis. We demonstrated that, in E17G-induced cholestasis in the isolated perfused rat liver, the initial drop in both bile flow and the transport activity of Bsep (30) and Mrp2 (29) occurring in the acute phase of the cholestasis can be partially prevented by administering cAMP as its freely permeable precursor, dibutyryl-cAMP. More significantly, cAMP extensively shorted the spontaneous restoration to normality of bile flow, Mrp2 function and Mrp2 localization that occurs spontaneously during the recovery phase (29). A similar acceleration of the re-insertion of endocytosed transporters has been described by our group for Bsep in TLC-induced cholestasis (32). In isolated rat hepatocyte couplets, a preventive effect of cAMP was confirmed on both E17G- (30,124) and TLC-induced Bsep internalization, and in the apical secretion of the fluorescent Bsep substrate cholyl-lysyl-fluorescein (32,124). In this case, however, prevention by cAMP was complete. Unlike the isolated perfused rat liver, the couplet model does not allow for a dynamic recording of the cholestatic phenomenon and its reversion. Therefore, the full normalization observed in this model most likely reflects that, at the fixed end-point selected to measure the cholestatic phenomenon, a complete exocytic reinsertion of the canalicular transporters has been reached. Irrespective of this, cAMP protective effect was significantly blocked by the Ca^{2+} chelator BAPTA/AM, but not by the PKA inhibitor KT5720, suggesting a major involvement of Ca^{2+}-dependent signaling pathways. A similar anticholestatic mechanism in terms of the signaling modulators involved was afforded by silibinin, the active component of the hepatoprotector silymarin (124). This was most likely due to the capability of silibinin to inhibit cAMP phosphodiesterase, thus increasing endogenous cAMP intracellular levels (124). Coincidently, we recently showed that hormonal modulation of intracellular cAMP levels in isolated hepatocyte couplets by the cAMP-elevating agents glucagon and salbutamol, a β-adrenergic agonist, prevents E17G-induced alteration of localization and function of both Bsep and Mrp2 (E. J. Sánchez Pozzi, unpublished results). However, in spite of their similar cAMP-elevating capacity, prevention provided by glucagon was PKA-dependent and microtubule-independent, while that afforded by salbutamol was PKA-independent, and showed dependency on the microtubular network and on *exchange protein activated by cAMP* (Epac), a PKA-independent target of cAMP. Since glucagon and salbutamol effects were additive, this strongly suggests that these compounds elevate two different cAMP pools, which activate different anticholestatic signaling pathways.

6.2. Tauroursodeoxycholate (TUDC)

This taurine-conjugated bile salt stimulates exocytic insertion of canalicular export pumps as part of its choleretic effect (106). In addition, it counteracts endocytic internalization of both Bsep (125) and Mrp2 (31) in TLC-induced cholestasis. The Ca^{2+}-dependent PKC isoform PKCα has been proposed to mediate TUDC anticholestatic effect (31), via a cooperative PKCα/PKA-dependent mechanism (126). This is in apparent contradiction with findings that PKCα activation lead to cholestasis rather than hepatoprotection (98). However, the biological response evoked by the combined action of different protein kinases (PKCα/PKA) may be different from that evoked by just one of them (PKCα). In addition to these anticholestatic effects, which become obviously apparent under cholestatic conditions, TUDC has choleretic effects when administered to normal rats, in part by stimulating the exocytic insertion of canalicular transporters. Although largely thought to explain its anticholestatic effects, this phenomenon involves a different set of protein kinases. TUDC choleretic effect is mediated by the dual activation, within minutes, of MAPKs of both Erk 1/2- (127) and p38 MAPK-types (106). This activation involves integrin sensing and *focal adhesion kinase* (FAK)/Src activation as upstream events (128), followed by the sequential activation of PI3K and Ras/Raf for p38 MAPK (129). On the contrary, the anticholestatic effect of TUDC in TLCA-induced cholestasis is not mediated by MAPKs (107).

7. PERSPECTIVES

The impairment in the dynamic localization of hepatocellular transporters is a common feature in both experimental and clinical cholestasis. Despite the importance that this mechanism seems to have in the onset of the cholestatic phenomenon, and on its perpetuation by accelerating degradation of the endocytosed transporters, the characterization of the molecular mechanisms underlying this alteration is in its infancy. The overwhelming progress in the molecular biology of the hepatobiliary function has greatly facilitated the characterization at the molecular level of the regulatory mechanisms that governs the dynamic localization of canalicular transporters, and their impairment in cholestasis. The acknowledgement of the crucial importance that signaling pathways have in this processes

is the first step in a long searching for the molecular targets of these regulations, thus providing a glimmer of hope for the final elucidation of the structural and mechanistic basis of the phenomenon. Many crucial questions remain however to be answered, for example: (*i*) why the same signaling route can trigger opposite effects (*e.g.* cholestatic or anticholestatic ones) depending on the context in which it is evoked? Do we have perhaps to regard signaling mechanisms as a regulatory network involving "switch on" and "switch off" of pathways modulating, in a binary manner, the effects of main executor pathways?; (*ii*) which are the final molecular targets that change their phosphorylation status to trigger carrier internalization?; (*iii*) do anticholestatic agents exert its therapeutic effects by counteracting these phosphorylation events?; (*iv*) can changes in localization of these transporters be not only prevented but, what is more important from the therapeutic point of view, *reversed* by factors counteracting these dysfunctions? Satisfactory answers to these questions would allow for the design of new therapeutic strategies in cholestatic hepatopathies to assure proper localization of transporters in an attempt to make them fully functional, and to prevent accelerated degradation. We hope that progress in experimental therapeutics based on this information encourages clinical researchers to apply this knowledge to envisage better, innovative therapeutic alternatives for the treatments of human cholestatic liver disease.

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