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REVIEW ARTICLE

Dynamic Localization of Hepatocellular Transporters: Role in Biliary Excretion and Impairment in Cholestasis

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Abstract: Bile flow generation is driven by the vectorial transfer of osmotically active compounds from sinusoidal blood into a confined space, the bile canaliculus. Hence, localization of hepatocellular transporters relevant to bile formation is crucial for bile secretion. Hepatocellular transporters are localized either in the plasma membrane or in recycling endosomes, from where they can be relocated to the plasma membrane on demand, or endocytosed when the demand decreases. The balance between endocytic internalization/exocytic targeting to/from this recycling compartment is therefore the main determinant of the hepatic capability to generate bile, and to dispose endo- and xenobiotics. Furthermore, the exacerbated endocytic internalization is a common pathomechanisms in both experimental and human cholestasis; this results in bile secretory failure and, eventually, post-translational transporter downregulation by increased degradation. This review summarizes the proposed structural mechanisms accounting for this pathological condition (*e.g.*, alteration of function, localization or expression of F-actin or F-actin/transporter cross-linking proteins, and switch to membrane microdomains where they can be readily endocytosed), and the mediators implicated (*e.g.*, triggering of "cholestatic" signaling transduction pathways). Lastly, we discussed the efficacy to counteract the cholestatic failure induced by transporter internalization of a number of therapeutic experimental approaches based upon the use of compounds that trigger exocytic targetting of canalicular transporters (*e.g.*, cAMP, tauroursodeoxycholate). This therapeutics may complement treatments aimed to transcriptionally improve transporter expression, by affording proper localization and membrane stability to the *de novo* synthesized transporters.

Keywords: Bile salts, cAMP, cholestasis, endocytosis, hepatocellular transporters, signaling pathways, ursodeoxycholate, vesicular trafficking.

1. INTRODUCTION

Bile formation is a highly-controlled process, involving in this regulation several bile constituents, nerves and hormones [1]. Regulatory mechanisms aim to handle the physiological demand for biliary disposition of endo- and xenobiotics. This is attained by modulation of the expression, intrinsic activity, and localization of relevant transporters localized in both basolateral (sinusoidal) and apical (canalicular) hepatocellular membrane domains.

Regulation of carrier-mediated transport system expression occurs at different time scales, varying from virtually instant responses to effects taking days to manifest themselves. Long-term regulations involve modulations of transporter turnover, via changes in the synthesis-degradation balance. This balance is modified by *i)* transcriptional changes in carrier synthesis, mainly regulated by the nuclear receptor family of ligand-modulated transcription factors, *ii)* post-transcriptional changes in the stability and distribution

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of the different transcripts, or *iii)* post-translational changes in transporter degradation rate [2-4]. This latter process might involve, as a causal event, sustained carrier internalization from its plasma membrane domain, followed by intracellular lysosomal/proteosomal degradation. Finally, the intrinsic activity of the transporters can be quickly modulated without changes in their constitutive expression. This often occurs by rapid changes in the carrier phosphorylation status or modifications in the lipid microenvironment where the transporters reside; the latter may involve shift from highly condensed plasma membrane "rafts" microdomains to more loosely packed "non-rafts" microdomains [5]. Longer-term modifications of intrinsic activity may involve changes in bulk lipid fluidity, via modulation of lipid composition [6].

This review will focus on the transitory changes in carrier localization by endocytic internalization/exocytic insertion to/from an endosomal recycling compartment, which occurs as part of a physiological, short-term regulatory mechanism aimed to rapidly modulate plasma membrane transporter density [2, 7]. In addition, we will discuss here in detail the mechanisms underlying the abnormal changes in hepatocellular transporter localization that occur under several pathological conditions, which may help to account for the cholestatic manifestations in these hepatopathies [7]. Finally, we will describe new therapeutic strategies based upon counteracting this deleterious mechanism.

To reach these goals, we will summarize here the current information on *i*) the dynamics of hepatocellular carrier localization under normal and cholestatic conditions, *ii*) the structural molecular machinery involved in this process, *iii*) the intracellular signaling mechanisms involved in its regulation or dysregulation, and *iv*) the experimental therapeutic approaches in cholestasis based upon its prevention or reversion.

2. HEPATOCELLULAR TRANSPORTERS AND THEIR ROLE IN BILE GENERATION AND DRUG DISPOSITION

Hepatocytes are polarized cells that express differential multispecific transport systems in their plasma membrane domains. These carriers belong to two major superfamilies, namely *solute carrier* (SLC) and *ATP-binding cassette* (ABC) [8]. These transport systems play a crucial role in the unidirectional movement of solutes and water from sinusoidal blood into the bile canaliculus, thus contributing to bile flow generation and biliary disposition of many endo- and xenobiotics. These carriers have been identified by molecular cloning, and their transport capability and selectivity have been assessed by functional studies. Their localizations and transport functions are depicted in Fig. (**1**).

2.1. Sinusoidal Transporters

Hepatic sinusoids have a specific architecture that allows the free transit through sinusoidal fenestrae of cholephilic organic compounds bound to albumin into the Disse´s space, from where they can be taken up by hepatocytes via several basolateral transport systems [9].

Sinusoidal uptake transport systems are either Na^+-dependent or Na^+-independent in nature [1]. Na^+-dependent uptake is driven by electrochemical Na^+ gradients, generated and maintained by the Na^+/K^+-ATPase, strategically localized in this membrane domain [10]. The Na^+-independent uptake of organic anions involves their exchange with other anions, such as HCO_3^- or reduced glutathione (GSH) [11].

Bile salts are the chief organic compounds in bile, and the major osmotic driving force for bile generation [1, 12]. They are mainly taken up by the *Na^+-taurocholate cotransporting polypeptide* [NTCP (SLC10A1)/Ntcp (Slc10a1), for humans and rodents, respectively] [13, 14]. A residual fraction of bile salts is taken up in a Na^+-independent way via a family of transporters referred to as *organic anion-transporting polypeptide* [OATP (SLCO)/Oatp (Slco)] [13, 14]. OATPs/Oatps transport conjugated and unconjugated bile salts, and a wide range of cholephilic substrates, such as glucuronidated (and maybe unconjugated) bilirubin, estrogen-conjugates (*e.g.*, estrone-3-sulfate and estradiol-17β-D-glucuronide), leukotrienes, thyroid hormones, exogenous organic anions (*e.g.*, sulfobromophthalein), and mycotoxins, among many other xenobiotics [15, 16]. Four OATPs have been identified in human liver, namely OATP1A2 (SLCO1A2), OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3), and OATP2B1 (SLCO2B1), with the former being localized exclusively in cholangiocytes, and the remaining ones only in hepatocytes [14]. Three Oatps have been described in rats, namely Oatp1a1 (Slco1a1), Oatp1a4 (Slco1a4), and Oatp1b2 (Slco1b2), with the latter being the murine ortholog of both OATP1B1 and OATP1B3 [14].

Hepatocyte uptake of a broad range of structurally unrelated organic cations occurs by passive, facilitated diffusion, down their electrochemical gradients. It is facilitated by two different transport systems depending on the substrate molecular size [17]. Small ("type 1") organic cations, such as tetraethylammonium,

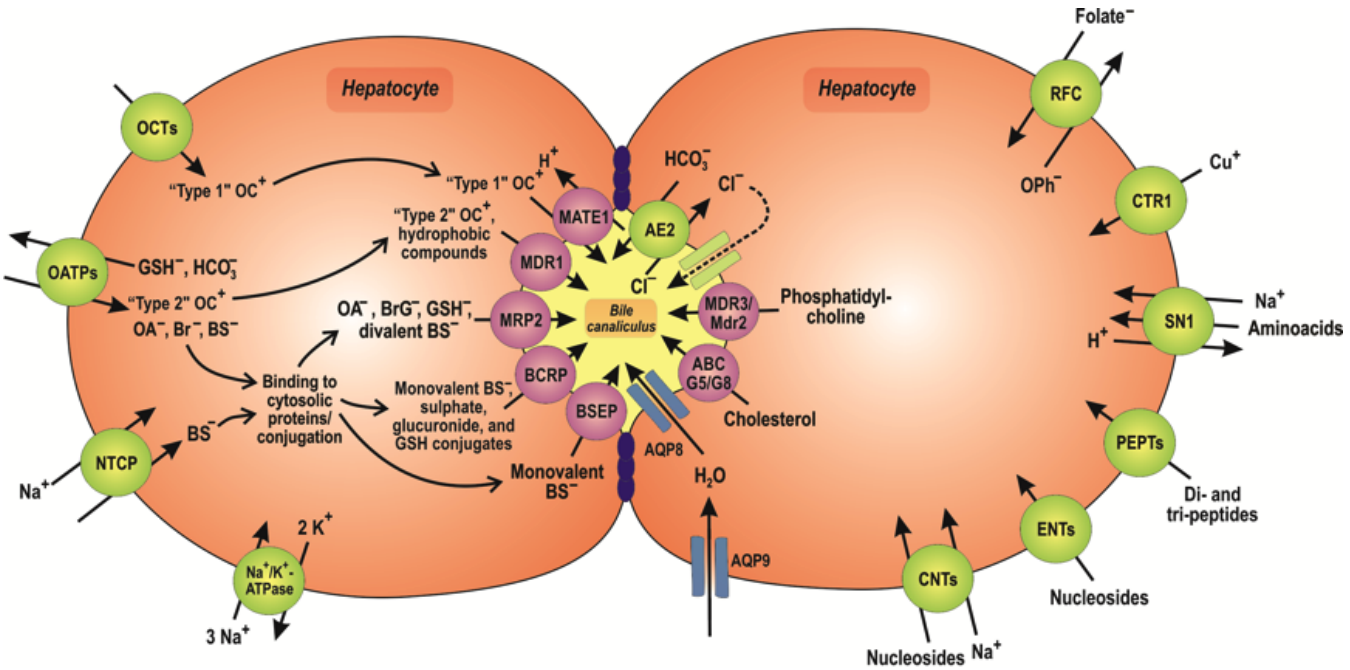

Fig. (1). Localization and transport function of the main sinusoidal and canalicular hepatocellular transporters. The Na^+-dependent basolateral uptake of bile salts (BS^-) occurs via the *Na^+-taurocholate cotransporting polypeptide* (NTCP), driven by the Na^+/K^+-ATPase-generated electrochemical Na^+ gradient. This Na^+ gradient also drives the uptake of certain amino acids (*e.g.*, glutamine, histidine, asparagine, and alanine), mediated by the *system N 1* (SN1), and nucleosides, mediated by the *concentrative nucleoside transporter* (CNT) family. The Na^+-independent uptake of a number of exogenous and endogenous organic anions (OA^-), such as bilirubin (Br^-) and bile salts (BS^-), as well as of "type II" organic cations (OC^+), is mediated by members of the *organic anion-transporting polypeptide* (OATP) family; this transfer involves anion exchange, with glutathione (GSH) and HCO_3^- being some of the supposed intracellular counter-anions. Other Na^+-independent uptake transporters includes the *equilibrative nucleoside transporter* (ENT) family for nucleosides, the *cupper transporter 1* (CTR1) for Cu^+, the *oligopeptide transporter* (PEPT) family for di- and tri-peptides, and the organic phosphate (OPh^-) countertransporter *reduced folate carrier* (RFC) for folates. Basolateral uptake of "type I" OC^+ is facilitated by members of the *organic cation transporter* (OCT) family. Transfer across the apical membrane occurs mainly via active export pumps, which belong to the superfamily *of ATP-binding cassette* (ABC) transporters. Several *multidrug-resistance proteins* (MDRs) have been identified in this membrane domain. MDR1 is involved in the canalicular excretion of OC^+ and other hydrophobic compounds, while ABCG5/G8 and MDR3 (Mdr2 in rodents) are floppases, which translocate cholesterol and phosphatidylcholine (PC), respectively, from the inner to the outer leaflet of this membrane domain. Apical excretion of BS^- is mediated by BSEP, while *multidrug-associated resistance protein 2* (MRP2) transports non-BS^- organic anions, such as glutathione (GSH^-), bilirubin glucuronides (BrG^-), and sulfated/glucuronidated BS^-. Monovalent BS^-, as well as sulphated, glucuronide, and GSH conjugates, are also transported via the *breast cancer resistance protein* (BCRP). Apical transport of HCO_3^- is facilitated by the Cl^-/HCO_3^- exchanger AE2; the out-to-in Cl^- gradient necessary for this exchange involves an as yet unidentified canalicular Cl^- channel. "Type I" OC^+ are excreted into bile via the *multidrug and toxin extrusion protein 1* (MATE1). The water channels *aquaporins* (AQP) 8 and 9 facilitate transcellular water transport in the sinusoidal and canalicular membranes, respectively. For more details, see item 2.

metformin, cimetidine, procainamide, 1-methyl-4-phenylpyridinium, nicotine, and choline, are taken up by the liver-specific *organic cation transporter 1* [OCT1 (SLC22A1)/Oct1 (Slc22a1)] and *organic cation transporters 3* [OCT3 (SLC22A3)/Oct3 (Slc22a3)]. In addition, both OATP1A2 in humans (but not other OATP family members) and Oatp2 in rodents facilitate the uptake of bulky ("type 2") organic cations, such as vecuronium, rocuronium, d-tubocurarine, and metocurine [17].

The sinusoidal membrane also contains carriers involved in the uptake of amino acids. Among them, the *system N 1* (SN1/Sn1, SNAT3/Snat3, official nomenclature: SLC38A3/Slc38a3) mediates the uptake of glutamine, asparagine, alanine, and histidine, together with the influx of two Na^+ cations and one amino acid molecule per transport cycle, coupled to the efflux of one H^+; this renders the transport mechanism electrogenic in nature [18]. In addition, di- and tripeptides are taken up by the *oligopeptide transporters 1* (PEPT1;

SLC15A1/Slc15a1) and *oligopeptide transporters 2* (PEPT2; SLC15A2/Slc15a2), which also transports several β-lactamic antibiotics, 5-aminolevulinic acid, and some anticancer drugs (*e.g.*, bestatin) [19]. Finally, many other solutes are taken up by hepatocytes, including: *i*) nucleosides, via either a Na^+-dependent, active transport or a facilitated, passive transport mediated by the *concentrative nucleoside transporter* (CNT; SLC28A/Slc28a) and the *equilibrative nucleoside transporter* family (ENT; SLC29A/Slc29a), respectively, *ii*) folate and folate derivatives (*e.g.*, the antifolate drug metothrexate), via the organic phosphate antiporter *reduced folate carrier* (RFC; SLC19A1/Slc19a1), and *iii*) monovalent copper, and potentially other metal (*e.g.*, silver) and metal-based drugs (*e.g.*, cisplatin), via *cupper transporter 1* (CTR1; SLC31A1/Slc31a1) [19].

2.2. Canalicular Transporters

After reaching the canalicular domain by Fick's diffusion bound to high-affinity cytosolic proteins, cholephilic compounds are exported into bile principally by ATP-dependent carriers of the ABC superfamily, particularly those belonging to the *multidrug-resistance proteins* (MDR/Mdr) family or to the *multidrug resistance-associated proteins* (MRP/Mrp) family.

MDRs/Mdrs were first identified in cancer cell lines, where they confer resistance to chemotherapeutics. In rodents, three gene products were identified, namely Mdr1a (Abcb1a), Mdr1b (Abcb1b), and Mdr2 (Abcb4), while two variants have been described in humans, namely MDR1 (ABCB1) and MDR3 (ABCB4); Mdr1a/b is the murine ortholog of MDR1, whereas Mdr2 is the murine ortholog of MDR3.

MDR1/Mdr1, also known as *P-glycoprotein* (P-gp), functions as an efflux pump for a broad range of amphiphilic, bulky type 2 cationic drugs, and other hydrophobic compounds; the number of solutes that interact with this carrier is well in excess of 300 [20]. This includes endogenous and exogenous metabolites or toxins, hydrophobic peptides, steroid hormones, and even glycolipids [21]. As part of its broad specificity spectrum, MDR1/Mdr1confers resistance against chemotherapeutic drugs, including vinblastine, doxorubicin, and paclitaxel, among many others. Its substrate specificity in part overlaps with that of *breast cancer resistance protein* [BCRP (ABCG2)/Bcrp (Abcg2)], another ABC transporter that plays a role in multidrug resistance; it transports glucuronide, sulphated, and GSH conjugates of sterols and several xenobiotics [22].

Smaller, type 1 cationic organic drugs are not transported by either MDR1 or BCRP, but by the electroneutral H^+/organic cation antiporter *multidrug and toxin extrusion 1* (MATE1; SLC47A1/Slc47a1) [23, 24]. This transporter can also mediate the biliary efflux of some organic anions and amphoteric compounds, such as anionic estrone sulphate, acyclovir, ganciclovir, and fluoroquinolones [25].

MDR3/Mdr2 functions as a floppase, by translocating phosphatidylcholine from the inner leaflet to the outer leaflet of the canalicular membrane [26]; this is followed by the release of phosphatidylcholine-containing vesicles from the outer leaflet into the biliary space, an event facilitated by the detergent action of luminal bile salts [27]; MDR3/Mdr2 contributes to maintain the asymmetry of the hemileaflets of the apical membrane, thus conferring resistance against the elevated concentrations of detergent bile salt in the canalicular lumen [28]. Likewise, a heterodimeric protein composed of two half ABC transporters, ABCG5 (Abcg5)/ABCG8 (Abcg8), mediates the biliary excretion of cholesterol, by flopping this lipid from the inner leaflet to the outlet leaflet of the apical membrane, followed by release of cholesterol into the biliary space, and its further incorporation into mixed micelles composed of bile salts and phosphatidylcholine [29].

Monoanionic bile salts are transported at the apical membrane by *bile salt export pump* [BSEP (ABCB11)/Bsep (Abcb11)], another MDR family member [30], and to a minor extent by BCRP [31]. On the other hand, canalicular efflux of divalent, bipolar glucuronidated or sulfated bile salts is mediated by MRP2 (ABCC2)/Mrp2 (Abcc2) [32]. This carrier is also involved in the biliary excretion of many other organic anions, including glucuronides (*e.g.*, bilirubin and estrogen glucuronides), glutathione *S*-conjugates *(e.g.*, leukotriene C4 and sulphobromophthalein), oxidized glutathione (GSSG), and reduced glutathione (GSH), the latter with lower affinity (but higher capacity) than GSSG [33, 34]. Both GSSG and GSH are chief determinants of the so called 'canalicular bile salt-independent fraction of canalicular bile flow' [35].

The apical domain also contains the electroneutral *anion exchanger 2* [AE2 (SLC4A2)/Ae2 (Slc4a2)], which extrudes HCO_3^- in exchange for Cl^- [36]. This exchanger is associated with a canalicular Cl^- channel that maintains electrochemical Cl^- gradients, but this channel has not been identified as yet [1, 36]. AE2/Ae2 also participates in hepatocyte intracellular pH recovery from an alkaline load [36]. In addition, canalicular AE2/Ae2 would play a role in bile flow generation, since

HCO_3^- excretion into the bile canaliculus is thought to be an additional primary driving force of the bile salt-independent canalicular bile flow [37]. Three transcript forms of AE2/Ae2 gene have been identified in humans and rats, namely the full-length transcript AE2a/Ae2a, expressed from the upstream promoter in most tissues, and the N-terminal variants AE2b1/ Ae2b1 and AE2b2/Ae2b2 transcribed from alternative promoters, which are expressed mainly in hepatic and renal tissue. AE2b1/2/Ae2b1/2 transcription is driven from overlapping promoter sequences within intron 2, yielding AE2/Ae2 isoforms with short N-terminal differences [38, 39].

2.3. Water Transporters

For a solute to drive vectorial blood-to-bile water transport, the resultant osmotic forces would need to be associated with *aquaporins* (AQPs). Constitutive sinusoidal expression of AQP9 and regulated expression of the N-glycosylated form of AQP8 in the canalicular membrane have been shown by both immunohistochemical and functional studies [40, 41]. Localization of AQP8 is mainly intracellular in nature, with a preferential pericanalicular localization; AQP8 colocalizes with AE2 in these intracellular vesicles, which may explain why both share similar signaling-mediated stimuli for their membrane targeting (*e.g.*, cAMP-mediated regulation) [42, 43].

Since it is targeted to the apical membrane on demand, AQP8 has been suggested play a role in bile flow generation, by facilitating the osmotic movement of water induced by choleretic stimuli [41]. AQP9 is also likely to play a role, by facilitating sinusoidal water uptake [44], but conclusive evidence has not been provided as yet.

The AQP isoforms that are involved in polarized water transport in human hepatocytes remains to be identified. Both AQP8 and AQP9 are expressed [45], and AQP9 localization is predominantly membranous, as in the rat [46]. Preliminary evidence for a role for human AQP8 in bile generation has been provided by silencing AQP8 gene expression in the human hepatoma cell line HepG2, where AQP8 has an apical localization, like in rat hepatocytes [47].

3. STRUCTURAL AND REGULATORY ASPECTS OF THE PHYSIOLOGICAL DYNAMIC LOCALIZATION OF HEPATOCELLULAR TRANSPORTERS

3.1. Basolateral Transporters

NTCP/Ntcp. Sinusoidal localization of NTCP/Ntcp depends on its traffic from the endoplasmic reticulum to Golgi, but not on its trafficking via the trans-Golgi complex, as suggested by the brefeldin A-sensitive, monensin-insensitive nature of this process [48]. The process may also involve microtubule and microfilament motors. A role for cytoskeleton in NTCP trafficking has been studied in detail in HepG2 cells, using GFP-tagged NTCP. Targeting of NTCP to the sinusoidal membrane involves two events: *i*) targeting of NTCP to the sinusoidal membrane immediacy via microtubules, and *ii*) insertion of NTCP into this membrane domain, via a microfilament-mediated process [49]; actin requirement has also been shown in isolated rat hepatocytes [50]. The latter event most likely involves targeting of NTCP from recycling endosomes [51]. This endosomal compartment functions as a reservoir of carriers for quick targeting to the sinusoidal membrane under a physiological stimulus; it is therefore expected that NTCP trafficking from this compartment is a highly regulated process. NTCP contains two N-linked glycosylation sites at asparagine residues N5 and N11; it has been shown in HepG2 cells that the presence of at least one glycan linked to N5 or N11 residue is necessary for the normal targeting of NTCP to its membrane domain [52]. Non-glycosylated NTCP remains intracellularly localized, and is rapidly degraded in lysosomes [52], unlike the glycosylated form, which is degraded by the ubiquitin-proteasome system [53]. Lipid composition of the sinusoidal membrane domain can modulate Ntcp activity. Molina *et al.* [54] found that Ntcp is mainly localized in "raft" microdomains in mouse liver, its specific membrane localization and functionality being dependent on the membrane cholesterol content.

OATP/Oatp. Sorting of human OATP1B1 to the sinusoidal hepatocyte membrane is mediated by both the Golgi complex- and the vacuolar H^+-ATPase vesicle-mediated membrane trafficking pathways [54]. cAMP accelerates the first sorting process via *protein kinase A* (PKA) activation [55]. Regarding structural requirements, interaction with the scaffold protein *postsynaptic density 95/discs large/zonula occludens-1 domain-containing 1* (PDZK1, aka: NHERF3) is necessary for proper basolateral membrane localization of rat Oatp1a1 in hepatocytes [56]. PDZK1 controls vesicular trafficking of Oatp1a1 by recruiting motor proteins to vesicles containing Oatp1a1. If PDZK1 is absent, these vesicles fail to recruit kinesin-1, and they associate with dynein as a predominant minus-end directed motor, which redirects these vesicles toward the cell interior [57]. It was also found that phosphorylation of Oatp1a1 at S^{634} and S^{635} residues, which does not modify its ability to interact with PDZK1 [58], leads to

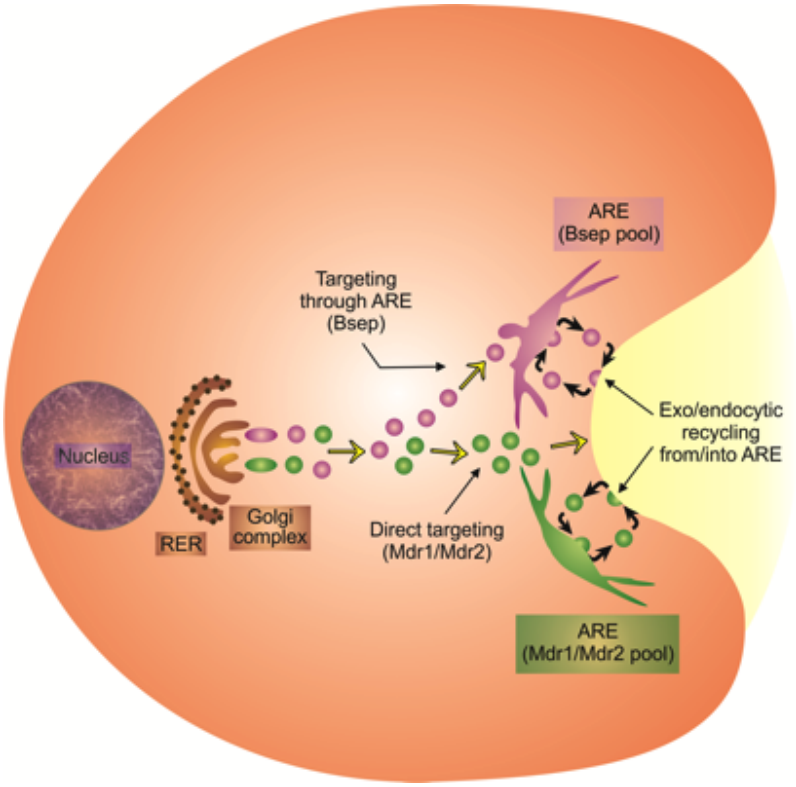

Fig. (2). Different pathways involved in trafficking of *de novo* synthesized canalicular ABC transporters. The vesicular pathways that deliver Bsep-containing vesicles (in rose) or Mdr1/Mdr2-containing vesicles (in green) from the synthesis place to the canalicular domain is different from each other. Mdr1 and Mdr2 traffic directly to the canalicular membrane, whereas Bsep is indirectly targeted to this membrane domain via the endosomal, subapical compartment; this compartment mediates the recycling (exocytic targetting/endocytic retrieval) of these transporters. Once inserted in the plasma membrane, Mdr1 and Mdr2 also recycle between the subapical compartment and the canalicular membrane. *Abbreviations*: ARE, apical recycling endosomes; RER, rough endoplasmic reticulum.

downregulation of Oatp1a1 transport activity [59], by inducing rapid internalization of the transporter into an intracellular vesicular pool [58].

3.2. Canalicular Transporters

ABC transporters. The pathways implicated in the vesicular trafficking of newly synthesized canalicular ABC transporters are depicted in Fig. (**2**). Once synthesized in the rough endoplasmic reticulum, canalicular ABC transporters belonging to either the MDR or the MRP family traffic directly to the canalicular membrane via the Golgi complex [60-62]. Studies of pulse-chase with ^{35}S-methionine followed by immunodetection of the transporters in subcellular fractions showed that these transporters are targeted directly to the apical membrane, since they were never detected at the sinusoidal membrane between the time they traverse the Golgi complex and the time they arrive at the canalicular membrane [61]. However, post-Golgi trafficking differs between the ABC transporters studied. Mdr1 and Mdr2 are targeted to the apical membrane by 30 min after ^{35}S-methionine pulse labeling [61]. This was reproduced for Mdr1 in WIF-B cells, a hybrid of human fibroblasts and rat hepatoma cells with functional bile canaliculi [63]. On the other hand, Bsep arrives to the canalicular membrane only after 2 h of ^{35}S-methionine pulse-labeling, which indicates that, unlike Mdr1/2, Bsep is retained in an endosomal compartment prior delivery to the canalicular membrane. This was confirmed by a further chase-labeling study carried out in a combined endosomal fraction, apart from the plasma membrane [62]. This large vesicular compartment also serves as a reservoir of ABC transporters, for quickly recruitment to the canalicular membrane on physiological demand (*e.g.*, increased delivery of bile salts for lipid digestion/absorption). The recycling process involves exocytic membrane targeting, followed by endocytic retrieval once demand has been satisfied [60, 62].

Once inserted into the plasma membrane, tethering to the cytoskeleton is required for proper membrane retention of canalicular transporters (Fig. **3**). To cope

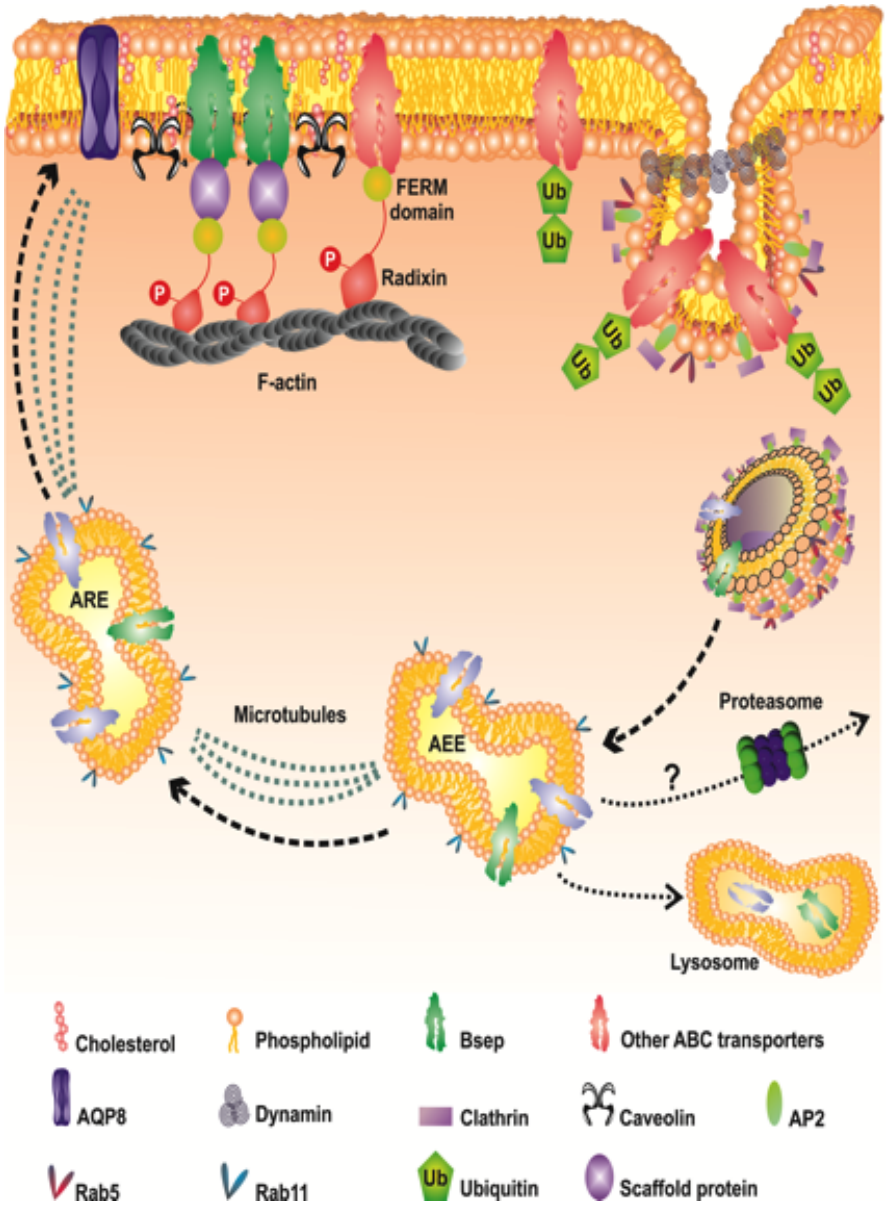

Fig. (3). Normal localization, plasma membrane stabilization, and constitutive recycling of canalicular transporters. ABC Canalicular transporters, including Bsep, Mrp2, and Mdr3, together with the water channel AQP8, are mainly localized in cholesterol- and caveolin-enriched "raft" microdomains under physiological conditions. Tethering to actin cytoskeleton is required for proper plasma membrane retention. The ERM (ezrin-radixin-moesin) protein family, with radixin being the main ERM protein in hepatocytes, plays a key role in the regulation of the structure and function of specific domains of the submembraneous actin cortex, by crosslinking the plasma membrane to the actin cytoskeleton. In its phosphorylated, active form, radixin binds membrane transporters to the F-actin cytoskeleton either directly or via adaptor proteins; interaction of Mrp2 and Mdr3 with radixin involves interaction of their PDZ domains with the membrane-cytoskeletal cross-linking protein NHERF-1/EBP50, while Bsep interaction with radixin is independent on adaptor proteins, and involves direct interaction with the radixin N-terminal FERM domain. Apical transporters are constitutively recycled from/into the plasma membrane and endosomal domains; endosomes serve as intracellular reservoirs of transporters, which are available for plasma membrane targeting on demand. This process, at least for Bsep, involves short-chain ubiquitination of the transporter prior to interaction with the adaptor protein AP2, which triggers clathrin-dependent endocytosis. Endocytosed transporter-containing vesicles traffic in a microtubule-independent fashion to apical early endosomes (AEE), from where they can either recycle in a microtubule-dependent way to the apical membrane via Rab11-positive apical recycling endosomes (ARE) or being sorted to lysosomal and/or proteasomal degradation.

with this function, filamentous actin (F-actin) interacts with, and possibly regulates, transmembrane proteins via binding to adaptor proteins of different types. The *ezrin-radixin-moesin* (ERM) protein family is relevant for this function, by regulating the structure and function of cell cortex specific domains via crosslinking between membranous proteins and F-actin [64]. Activation of ERM proteins involves phosphorylation by specific protein kinases [65]. Every ERM protein contains two *ERM association domains* (ERMADs), namely *i*) the membrane-binding domain, N-ERMAD, which is localized at the -NH2 terminus of the ERM protein, and *ii*) the F-actin binding domain, C-ERMAD, which is localized at the

-COOH terminus of the ERM protein [65]. Phosphorylation in a threonine residue at the -COOH terminal end (Thr^{558}, Thr^{567}, and Thr^{564} in moesin, ezrin, and radixin, respectively) is essential to dissociate intramolecular N-and C-ERMAD interaction, thus promoting "open", active forms of ERM protein monomers [66]; this phosphorylation is regulated via the Rho small GTPase signaling pathway [66, 67]. The main hepatocellular ERM protein is radixin [68], which is preferentially localized at the apical membrane in hepatocytes [68, 69].

Radixin connects several membrane proteins to the cytoskeleton, either directly or via protein adaptors. As for Bsep and Mrp2, Wang *et al.* [70] showed the influence of radixin expression on their localization and transport function in rat hepatocytes; the silencing of radixin expression led to retention of the transporters in subapical, Rab 11-positive compartments.

The link between ERM and transmembrane proteins can also be indirect, via scaffolding proteins containing PDZ domains, such as PDZK1 and *ERM-binding phosphoprotein of 50 kDa* (EBP50, aka: Na^+-H^+ exchanger regulatory factor 1, NHERF1) [71]. For example, binding of Mrp2 [72] and MDR3 [73] to radixin involves interaction of their PDZ domain with EBP50/NHERF-1. Additionally, it has been reported the interaction of the C-terminal domain of MRP2 with the fourth PDZ moieties of the scaffold protein PDZK1 in HepG2 cells [74]; inhibition of this interaction leads to loss of apical localization of MRP2 and intracellular accumulation. It was recently found that Bsep interaction with radixin is independent of the adaptor protein EBP50, and involves direct interaction of Bsep with radixin N-terminal FERM domain [75].

Other plasma membrane transporters lacking an obvious PDZ-interacting motifs, such as those of the MDR family MDR1, MDR2, and BSEP, can alternatively bind to actin via other cytosolic scaffold proteins, such as *HS1-associated protein X-1* (HAX-1), and the *receptor for activated C-kinase 1* (RACK1). HAX-1 is a small (34 kDa) cytosolic protein that acts as a bridge for the attachment of the members of the MDR family stated above with F-actin [76]. HAX-1 also interacts the F-actin binding protein *cortactin* [77, 78], a cytosolic protein that promotes polymerization and rearrangement of the F-actin cytoskeleton [77], and that is engaged in clathrin-induced endocytosis [79]; interestingly, expression of dominant negative cortactin in MDCK cells augmented significantly the steady-state BSEP levels in plasma membrane, suggesting a role for cortactin in BSEP endocytosis [77]. RACK1 was formerly identified by its ability to bind activated *protein kinase C* (PKC) and facilitate cellular PKC trafficking [80]. However RACK1 interacts with a broad spectrum of cellular proteins to regulate different cellular processes, including cell surface expression of several transport protein, among others [81]. All the above mentioned scaffold proteins possess different protein-binding domains, and are required for proper retention of transporters in their specific membrane domains [76, 77, 82, 83].

Another F-actin cross-linking protein that has been proposed to stabilize plasma-membrane transporters is *myristoylated alanine-rich C kinase substrate* (MARCKS), a ubiquitous, specific PKC substrate [84]. In its unphosphorylated form, MARCKS is an F-actin crosslinking protein [85]. The binding of MARCKS to the membrane requires both the electrostatic interaction between its basic (serine) residues in the effector domain and membrane acidic lipids, and the hydrophobic interaction of myristate with the membrane core [85, 86]. When the serine residues are phosphorylated by PKC, the electrostatic interaction is disrupted, resulting in MARCKS removal from the plasma membrane [87]. Although phosphorylated MARCKS remains bonded to actin, its membrane detachment results in local disruption (softening) of the F-actin with enhanced plasticity, thus affecting transporter localization.

Another factor that may determinate the probability for a protein to be either retained or endocytosed from the plasma membrane is its relative distribution in "raft" (caveolin-1-postive) and "non-raft" (clathrin-positive) membrane microdomains. "Rafts" are membrane regions enriched in cholesterol and sphingomyelin, arranged in an ordered and tight manner [88]. The main transporters/channels relevant to bile formation, including the canalicular ABC transporters Bsep, Mrp2, Mdr1, and Abcg5, as well as the HCO_3^-/Cl^- exchanger AE2 and the water channel AQP8, all mainly

reside in lipid "rafts". These regions are resistant to the solubilizing action of tensioactive bile salts [89, 90]. The selectivity of bile salts for "non-raft" microdomains is reflected in the preferential extraction of phosphatidylcholine from the canalicular membrane outer leaflet by bile salts, despite this phospholipid represents only 35% of the total canalicular phospholipids [91, 92]; in contraposition, there is a tinny removal of sphingomyelin, a major phospholipid of the canalicular domain [91]. Thus, it is postulated that these membrane microdomains protect canalicular transporters from the solubilizing effect of detergent bile salts. "Rafts" microdomains provides an adequate environment for canalicular transporter function as well. Canalicular transporters are more intrinsically active in "raft" domains because high-cholesterol positively modulates their activity [93-95].

Different kind of molecular modifications and/or interactions could be responsible for a protein to be preferentially concentrated in "raft" microdomains, namely *i*) several lipidation processes such as acylation (*e.g.*, with myristic or palmitic acids), glycosyl-phosphatidylinositol (GPI)-linkage, and covalent attachment to cholesterol, *ii*) protein-protein interactions with scaffolding proteins like caveolin (highly enriched in "rafts") or EBP50, which interacts with the *phosphoprotein associated with glycosphingolipid-enriched microdomains* (PAG); EBP50-PAG interaction was proposed to be important to connect membrane "rafts" to the F-actin cytoskeleton [96], and *iii*) presence in the aminoacidic chain of a *cholesterol recognition/ interaction amino acid consensus* (CRAC) motif; this moiety is described by the amino acid consensus pattern -L/V-(X)(1-5)-Y-(X)(1-5)-R/K-, where (X)1-5 is any sequence of amino acids from 1 to 5 residues in length [97].

Preferential "raft" association of a plasma membrane protein is not a determinant of their endocytosis via the caveolae-dependent pathway [98]. Actually, Bsep binds preferentially to cell surface "raft" domains, but it is internalized via a clathrin-dependent, AP2-mediated mechanism [99, 100] (Fig. **3**). Recent studies by Lam *et al.* [99] suggest that, under physiological conditions, Bsep undergoes clathrin-dependent endocytosis, with its C-terminal domain (conserved in all the members of the ABC superfamily) being key for this endocytic process. Additionally, studies of immunostaining, coimmunoprecipitation, pull-down, and real-time image analysis confirmed that the AP2 interaction with Bsep through a C-terminal tyrosine motif is a key mediator of Bsep endocytosis [101]. In *in vivo* studies, Hayashi *et al.* [102] demonstrated that 4-phenylbutyrate treatment, which significantly decreases expression of the AP2 subunits α and μ2 adaptin, inhibits Bsep/BSEP internalization in rat and human liver. Moreover, knockdown of AP2 by siRNA in 3xFlag-BSEP-expresing HeLa cells and in sandwich-cultured human hepatocytes inhibited internalization of BSEP, thus increasing its expression and transport function [99]; non-phenylated short-chain fatty acids (*e.g.,* propionic and butyric acids) and medium-chain fatty acids (*e.g.,* heptanoic, octanoic, and decanoic acids) have even higher BSEP-stabilizing effects than 4-phenylbutyrate [103]. Additionally, Aida *et al.* [104] demonstrated in Flp-In T-Rex 293 cell line stably transfected with 3x FLAG-BSEP that both inhibition of ubiquitination and clathrin-dependent endocytosis blocked in a non-additive manner 3x FLAG-BSEP internalization, suggesting that ubiquitination plays a role in the constitutive clathrin-dependent endocytosis of BSEP. Further supporting this mechanism, endocytosis of BSEP in MDCK cells stably transfected with the transporter was decreased by expression of the dominant-negative form of EPS15, another crucial component of the clathrin-dependent endocytic machinery [77]. Little is known about constitutive endocytosis of MRP2. Aida *et al.* [104] reported that, in Flp-In T-Rex 293 cells, MRP2 endocytosis, unlike BSEP, would be a dynamin-dependent but a clathrin-independent mechanism. It was also reported that BSEP and MRP2 are modified by short-chain ubiquitination [101, 105]. In *in vitro* experiments, this covalent modification was required for clathrin-dependent endocytosis of BSEP [104]. Similarly, blockage of ubiquitination with 4-phenylbutyrate inhibited internalization and further degradation of Mrp2 in rats, increased membrane MRP2 expression, and decreased plasma bilirubin levels in patients receiving this drug to treat ornithine transcarbamylase deficiency [101].

The existence of a subapical compartment to which endocytosed transporters are trafficked has been demonstrated, based on the following experimental evidence: *1*) immunoelectron microscopy in rat hepatocytes showed that Bsep resides in endosomal structures near the canalicular membrane [106], a localization that could also be demonstrated for Mrp2, Bsep, and Mdr1 in isolated rat hepatocyte couplets by immunofluorescence staining [107]; *2*) ^{35}S-methionine administration *in vivo* followed by the follow-up of its incorporation into proteins revealed that Bsep is distributed in both the canalicular membrane and a mixed endosomal fraction [61]; *3*) recycling between the subapical endosomes and canalicular membrane could be directly visualized for Bsep [63] and Mrp2 [108]. The nature of

this subapical compartment is under discussion, but a more complete picture is emerging (Fig. **3**). Studies by Wakabayashi *et al.* [109] demonstrated in WIF-B9 cells that Bsep is localized both in the canalicular membrane and in vesicles, either near the microtubule organization center or scattered in the cytoplasm; in these vesicular structures, Bsep colocalizes with Rab11, a marker of apical recycling endosomes (ARE). By using photobleaching techniques, they showed that Bsep resides in the canalicular membrane only transiently before being targeted to ARE. In addition, when treating cells with microtubule inhibitors, both the endocytic trafficking from the canalicular membrane to ARE and the exocytic trafficking between ARE and the canalicular membrane are interrupted, showing that recycling is a microtubule-dependent process. On the other hand, when treating cells with actin disruptors, Bsep did not traffic to ARE, indicating that the trafficking from the canalicular membrane to ARE depends on F-actin integrity as well [109]. Since the distance between ARE and the canalicular membrane is long enough to require microtubule-mediated transport, actin dependence strongly suggests that there must be an intermediate endosomal compartment between the canalicular membrane and ARE, which would be located very close to the membrane; Bsep may traffic towards this compartment via F-actin rails, as they are capable of mediating short-range vesicular trafficking via the motor protein myosin [110]. A compartment with these characteristics that has been characterized in many cell lines, including hepatocytes, is the apical early endosomes (AEE) [111]. Although there is no direct evidence for the involvement of AEE in the Bsep and Mrp2 endocytic routes, AEE is the first endosomal compartment known to receive the retrieved proteins from the apical membrane in a microtubule-independent way [111]. This endocytic process would be followed by an exocytic step, which would involve transcytosis from AEE to ARE in a microtubule-dependent manner [112], and a subsequent microtubule-dependent reinsertion into the canalicular membrane, when under demand [60]. This theory would reconcile the above-described evidence for a clathrin-dependent endocytosis of canalicular transporters (which is actin-dependent and microtubule-independent) with the finding of Wakabayashi *et al.* [109], showing the microtubule dependence for the transporters to arrive to ARE, and for their reinsertion into the canalicular membrane under demand. This postulated mechanism is further confirmed by the following evidences: *1*) Mrp2 and Bsep colocalize with Rab11, an ARE marker [112, 114, 115]; *2*) these transporters colocalize in the same vesicles with the microtubule motor protein dynein and with the polymeric immunoglobulin receptor, a plasma-membrane protein that undergoes transcytosis from the sinusoidal to the canalicular membrane through ARE [107]; *3*) the insertion of Bsep from ARE into the canalicular membrane is a microtubule-dependent process [109]; *4*) ARE are interconnected to AEE in a microtubule-dependent manner [113].

It has been postulated that the constitutive recycling of ABC transporters contributes to their relatively high half-life, which can amount up to 5 days [62]. Another possibility is that, through an endocytic process, the transporters localized in AEE traffic, via late endosomes, to lysosomes for degradation [116]; supporting evidence for this process was provided by experiments carried out in rat livers following retrograde *in situ* administration of endocytic markers; they suffered clathrin-dependent apical endocytosis, traversed subapical compartments, and finally colocalized with lysosomal markers [117]. This degradation pathway would be minimal under physiological conditions, as suggested by the high half-life of the canalicular transporters [62]. This is likely due to the fact that, once transported to ARE, the carriers cannot traffic to the lysosomal compartment for degradation [118]. However, the degradation pathway from AEE could be exacerbated under situations of massive and sustained internalization of the transporters under pathological conditions, such as cholestasis.

AE2. The canalicular Cl^-/HCO_3^- exchanger AE2 is localized in cholesterol- and caveolin-enriched "raft" microdomains in the canalicular membrane [119]. Adicionally to this functional localization, AE2 resides in subapical vesicles [38, 120], from where they can migrate to the canalicular membrane on demand. The sorting of the three AE2 variants, AE2a, AE2b1, and AE2b2, was studied using primary cultures of rat hepatocytes in collagen sandwich configuration [38]. By 72-96 h, GFP constructs from each AE2 isoform colocalized both in the canalicular membrane and in subapical vesicular structures, but they were not detected at the basolateral pole. The sorting of AE2 isoforms to the canalicular membrane was stopped by the microtubule-depolymerizing agent colchicine, suggesting that AE2 vesicular transport and exocytic insertion is microtubule dependent in nature [38]. This was confirmed further by functional studies. The Cl^-/HCO_3^- exchange mediated by AE2 is enhanced in hepatocytes exposed to a medium containing HCO_3^- or in response to cAMP, and this was blocked by colchicine [42]. Glu-

cagon, a cAMP-elevating hormone in hepatocytes, also stimulates AE2 exchange function, through a cAMP- and microtubule-dependent, PKA-mediated pathway [121].

3.3. Water Channels

AQP8. This water channel is largely internalized in vesicular structures in hepatocytes, as shown by confocal immunofluorescence [43], subcellular fractionation [43], and immunoelectron microscopy studies [122]. However, it can be quickly targeted to the apical membrane on demand [123, 124]. cAMP triggers redistribution of AQP8 to the apical membrane, and increases canalicular-membrane water permeability in a microtubule-dependent fashion [43, 125]. Further studies in isolated rat hepatocytes [126] showed that, like for AE2, the cAMP-elevating hormone glucagon can also induce AQP8 translocation to the plasma membrane, via a process involving both PKA and *phosphoinositide 3-kinase* immunofluorescence co-staining studies in WIF-B cells showed intracellular colocalization of AQP8 and AE2, suggesting that they share a common pool of pericanalicular PI3K) signaling pathways [127]. Interestingly, immunofluorescence co-staining studies in WIF-B cells showed intracellular colocalization of AQP8 and AE2, thus suggesting that they share a common pool of pericanalicular vesicles [128]. This justifies the similar behavior of both transporters when exposed to a similar regulatory stimulus (*e.g.*, glucagon), inducing insertion of both AQP8 and AE2 into "raft" microdomains of the canalicular membrane [119]. Thus, hepatocytes may not only modulate the biliary excretion of osmotically-active solutes via exocytic targetting of relevant transporters (*e.g.*, BSEP, MRP2, and AE2), but also modulate their apical membrane water permeability by inserting AQP8, to facilitate water osmotic movement under choleretic conditions.

4. ALTERATIONS OF THE LOCALIZATION STATUS OF HEPATOCELLULAR TRANSPORTERS IN CHOLESTASIS

The endocytic internalization of crucial hepatocellular carriers relevant to bile generation is a common feature in cholestasis. The acute alterations in transporter localization that occurs, for example, in several cases of drug-induced cholestasis may become a major pathogenic mechanism accounting for the initial secretory failure, by quickly decreasing carrier density at the plasma membrane (hepatocellular cholestasis). Alternatively, changes in transporter localization may occur secondarily to a primary mechanical impediment to drain away bile into the duodenum (obstructive cholestasis). In such a case, transporter mislocalization may aggravate and even perpetuate the initial secretory failure. If carrier internalization is maintained with time, as for example under chronic cholestatic conditions, the internalized transporters may be delivered to the degradation machinery of the cell for catabolism.

Next, we will outline the current bibliographic evidence supporting the contention that changes in the transporter localization status occur under both experimental and human cholestatic conditions (summarized in Table **1**).

4.1. Endocytic Internalization of Transporters in Experimental Cholestasis

4.1.1. Drug-induced Cholestasis

Administration of drugs known to induce functional cholestasis, or exposure to endogenous metabolites thought to play a role as etiological agents in cholestatic human hepatopathies, has been used as an experimental tool to study the mechanisms of the disease in laboratory animals. For example, administrations of the endogenous metabolite of estradiol estradiol-17β-D-glucuronide (E17G) [129, 130], the bile salts taurolithocholate (TLC) [131-133] and taurochenodeoxycholate (TCDC) [134], and the immunosuppressive drug cyclosporine A [135, 136] all induce acute cholestasis, associated with rapid endocytic internalization of canalicular transporters. The estrogenic compound ethynylestradiol, which induces cholestasis in a longer time scale (days) since it requires metabolic activation [137], also induces Mrp2 internalization [138, 139].

The molecular basis for the internalization of canalicular transporters by cholestatic drugs is being actively studied. Changes in the phosphorylation status of actin-bound scaffold proteins involved in carrier stabilization in the canalicular membrane by tethering it to the F-actin cytoskeleton, such as MARCKS and radixin (see below, item 5.1 for details), have been identified as putative mechanisms underlying this mislocalization [143]. Studies in rat hepatocytes and HuH-7 cells transfected with NTCP (HuH-NTCP) suggest that MRP2 retrieval by TLC involves MARCKS phosphorylation, and the consequent MARCKS membrane detachment [144]. In turn, radixin can be detached from its link with the carrier by inactivation associated with phosphatase-mediated dephosphorylation, as has been shown in estrogen-induced cholestasis in rats [138].

At the basolateral pole, changes in localization of uptake transport systems under cholestatic coonditions have been less studied. TLC induces non-competitive

Table 1. Structural and signaling factors involved in the endocytic internalization of hepatocellular carriers in experimental models of cholestasis and in human cholestatic hepatopathies.

Cholestatic Model/ Human Cholestatic Disease	Transporter Internalized	Structural Alteration	Signaling Pathways Involved
Estrogens	* Mrp2 [112, 114, 115, 130, 138, 139, 236, 238, 241, 245, 332] * Bsep [112, 114, 115, 146, 236, 238, 245, 307]	* Radixin dephosphorylation and internalization (for Mrp2) [138] * Preserved actin organization [129, 130] * Independence of microtubule integrity [252] *Bsep switch from "rafts" to "non-rafts" microdomains [223]	* cPKC [236] * PI3K/Akt (complementarity with cPKC) [112] * $p38^{MAPK}$ (downstream of cPKC) [112] * ERK1/2 (downstream of PI3K/Akt) [112] * ERα [238] * GPR30/ adenylate cyclase/PKA [245] * EGFR-Src [241]
TLC	* Mrp2 [131-133, 332]/MRP2 (in HuH-NTCP) [141, 256] * Bsep [132, 133, 307] * NTCP (in HuH-NTCP) [143]	* Preserved actin organization [132] * MARCKS phosphorylation (for MRP2 endocytosis in HuH-NTCP) [141]	* PI3K [257] * PKCε [141, 237] * $p38\beta^{MAPK}$ [256]
Oxidative Stress	* Mrp2 [154-156, 160-162, 167, 254] * Bsep [153, 156, 167, 254]	* F-actin disruption [153, 167, 254] * PP-1-dependent radixin dephosphorylation [153] * Mrp2 SUMOylation [164]	* cPKC [153] * Ca^{2+}/NO/nPKC [156] * MAPKs (ERK1/2, $p38^{MAPK}$, JNK1/2 co-activation) [254]
Hypertonicity	* Bsep [170, 171, 276] * Mrp2 [171, 177, 273] * Ntcp [274]	* Oxidative stress-induced cortactin phosphorylation leading to loss of F-actin cross-linking activity [171]	* PKC□/NOX/ROS/Fyn [171, 274]
LPS	* Mrp2 [139, 169-172, 177, 181,182, 189-191]/MRP2 [176, 190] * Bsep [179, 180]/BSEP [176] * Ntcp [178, 187]/NTCP (in HuH-NTCP) [188]	* Preserved actin organization [190] * Radixin dephosphorylation [191] * NTCP dephosphorylation and S-nitrosylation (in HuH-NTCP) [188]	* Pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) [176, 178, 181, 182, 186, 187] * ROS [189, 190] * PI3Kγ [269]
BDL	* Mrp2 [147, 192] * Bsep [192, 205]	* Portal inflammation [205]	* Pro-inflammatory cytokines (TNF-α and IL-1β) [205]
Massive hepatectomy	* Ntcp [206]		
NAFLD	* Mrp2 [208, 209]	* Radixin dephosphorylation [208] * MARCKS phosphorylation [208] * Rab11 decreased expression [208]	
Human cholestatic hepatopathies	* MRP2 [213-217] * BSEP [213]	* Disturbed expression and localization of radixin (for MRP2) [214, 215] * Disturbed expression and localization of ezrin (for MRP2) [217]	* PKCα, δ and ε [217]

Abbreviations: BDL, bile duct ligation; Bsep/BSEP, rodent/human bile salt export pump; cPKC, classical, Ca^{2+}-dependent protein kinase C; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated protein kinases 1/2; GPR30, G-protein coupled receptor 30; HuH-NTCP, HuH-7 cells stably transfected with NTCP; IL, interleukin; JNK1/2, c-Jun N-terminal kinase 1/2; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; Mrp2/MRP2, rodent/human multidrug resistance-associated protein 2; MARCKs, myristoylated alanine-rich C-kinase substrates; NO, nitric oxide; NAFLD, non-alcoholic fatty liver disease; NOX, NADPH oxidase; nPKC, novel protein kinase C; Ntcp/NTCP, rodent/human Na^{+}-taurocholate cotransporting polypeptide; PI3K,phosphoinositide 3-kinase; PKA, protein kinase A; PKC protein kinase C; PP-1; protein phosphatase-1; ROS, radical oxygen species; TLC, taurolithocholate; TNF-α, tumor necrosis factor α.

inhibition of taurocholate (TC) uptake in rat hepatocytes [142], which was explained by Ntcp internalization from the sinusoidal membrane [143]. TLC failed to induce a similar internalization in HuH-NTCP cells [143]. This difference was suggested to reflect intrinsic differences between human and rat transporters, but may also be due to absence of structural and/or regulatory mechanisms needed for NTCP translocation in this cell line, which originally lacks the transporter itself. Other potentially damaging bile salts, as for example TCDC, also internalizes Ntcp via mechanisms similar to those activated by TLC in rat liver [144]; this may represents a mechanism to regulate bile salt uptake along the acinus during enhanced portal bile salt load, which protects periportal hepatocytes from deleterious bile salt accumulation.

Our group has studied in detail the mechanisms underlying transporter endocytosis in E17G-induced cholestasis, which mimics in part pregnancy-induced cholestasis. Different experimental models have been employed, including whole rat, isolated perfused rat liver, isolated rat hepatocyte couplets, and sandwich-cultured rat hepatocytes (Fig. **4**).

Following an *in bolo*, i.v. injection of this compound *in vivo*, a biphasic pattern of bile flow is observed, with a dose-dependent initial drop during the first 20 min, followed by a spontaneously recovery, reaching normal values by 2 h post-injection [145]. The initial, cholestatic phase is associated with endocytosis of Bsep and Mrp2, while the recovery phase is accounted for by the spontaneous exocytosis of subapical, transporter-containing vesicles into the apical membrane [130, 146]. Whereas the endocytosis occurs by microtubule-independent mechanisms, the exocytosis depends on microtubules. Repetitive administration of E17G induces both a deeper internalization and the partial, ectopic localization of Mrp2 at the lateral plasma membrane [147]. The latter effect probably reflects loss of tight junctional barrier between the apical and basolateral domains [148], as described for the apical enzyme Ca^{2+}, Mg^{2+}-ATPase in rats with bile-duct ligation (BDL) [149].

Unlike canalicular solute transporters, the canalicular water channel AQP8 is not relocalized in E17G-induced cholestasis [150].

4.1.2. Oxidative Stress-induced Cholestasis

Oxidative stress is a frequent phenomenon underlying most hepatopathies [151]. Radical oxygen species (ROS) trigger hepatocanalicular transport dysfunction and cholestasis, even at very low levels, and endocytosis of apical transporters would play a crucial role [152].

Our group showed that Bsep is endocytosed in isolated rat hepatocyte couplets when exposed to low levels of the pro-oxidant agent *tert*-buthylhydroperoxyde (*t*BOOH) [153]. This was associated with impaired efficiency to accumulate fluorescent analogues of bile salts in the canalicular space. A similar effect has been reported for Mrp2 by exposing isolated perfused rat livers to the pro-oxidants *t*BOOH [154] and chlorodinitrobenzene [154], and to the GSH-depleting agent ethacrynic acid [155, 156]. Similar endocytic phenomena are induced by hepatic ischemia-reperfusion, another pro-oxidant maneuver [157], and this was apparent even prior to microcirculatory disturbances [158]. Since Mrp2 is a canalicular transporter for GSH [33, 34], and GSH is the main intracellular antioxidant [159], internalization of the transporter under oxidative stress conditions may help to preserve GSH levels, thus attenuating the deleterious effect induced by a further pro-oxidant injury. In line with this view, hypertonicity-induced endocytic internalization of Mrp2 delayed ethacrinic acid-induced ROS formation and the consequent oxidative injury in the isolated rat perfused liver model [155].

Acute Mrp2 internalization induced by *t*BOOH in this model is reversible in nature, as reverted by GSH restoration with GSH ethyl ester [160]. *t*BOOH-induced Mrp2 endocytosis is accompanied by the decrease in the content of phosphorylated (active) radixin (p-radixin), which destabilizes Mrp2 in the plasma membrane via *PKC-dependent protein phosphatase-1* (PP-1) activation [161]. On the other hand, the interaction of Mrp2 with p-radixin was enhanced by the PKA activation associated with the recovery phase after GSH replenishment [161].

Contrarily to what happens when oxidative stress is quickly reversed, sustained oxidative stress gradually leads to Mrp2 degradation, as shown in whole rats exposed for 8 h to the GSH-depleting agent buthioninesulfoximine [162]. The proteosomal pathway has been suggested to play a role in Mrp2 degradation due to the concomitant increase in the ubiquitinated-to-total Mrp2 ratio under this condition [162]. However, ubiquitinization, particularly the short-chain one, signals for lysosomal rather than for proteosomal Mrp2 degradation [101], and lysosomes are the final destination of other apical membrane proteins, such as 5'-nucleotidase and aminopeptidase N, as reported in WIF-B, polarized hepatic cells [163]. Post-translational modification of Mrp2 with *small ubiquitin-like modifier* (SUMO)

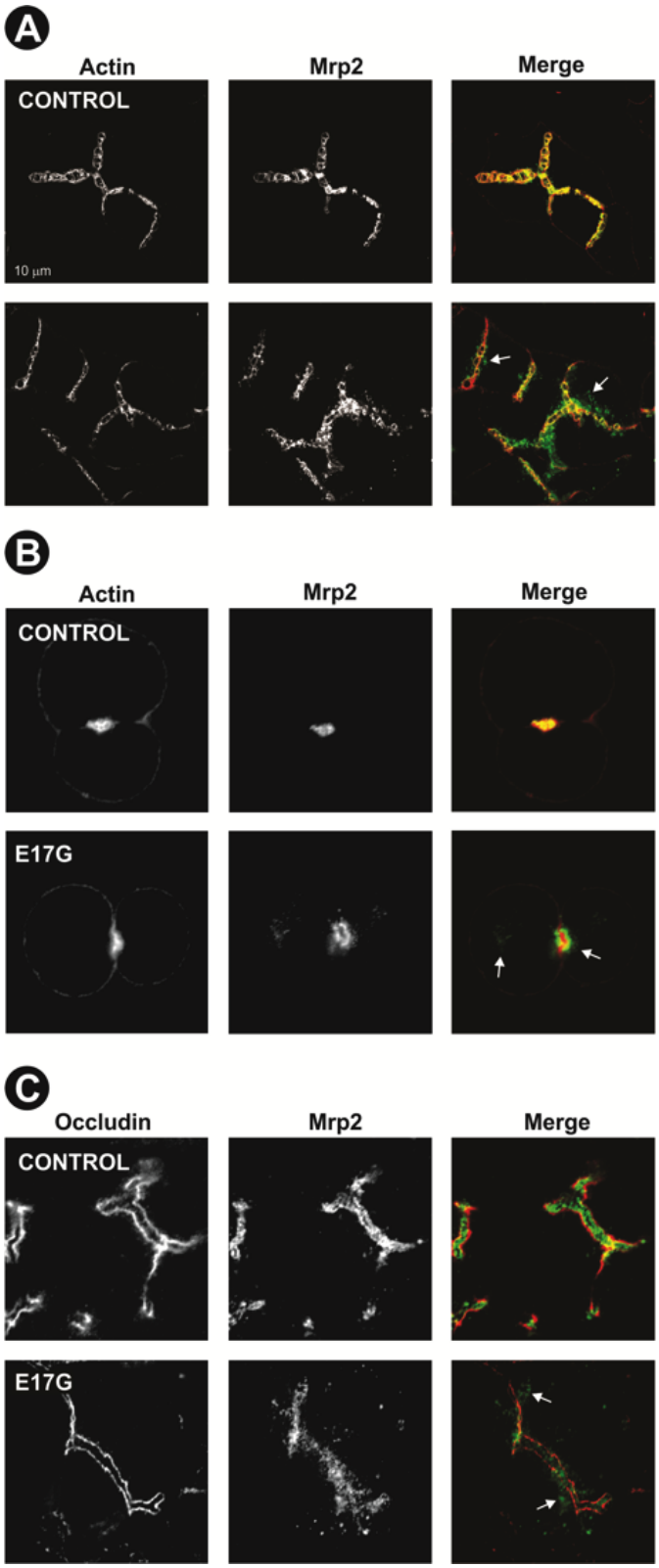

Fig. (4). Representative confocal images of Mrp2 endocytic internalization induced by E17G in different experimental models of bile secretion. Confocal laser images showing the endocytic internalization of Mrp2 induced by E17G in (*A*) sandwich-cultured rat hepatocytes, (*B*) isolated rat hepatocyte couplets, and (*C*) hepatic tissue samples from perfused rat livers. Either F-actin (*panel A* and *B*) or occludin (*panel C*) staining was used to demarcate canalicular membrane limits. In the three models, Mrp2 internalization is visualized as an increase in the occurrence of vesicular structures beyond the limits of the canalicular membrane (white arrows in merged images).

proteins, a process that enhances Mrp2 expression in cultured mammalian cells [164], is decreased as ubiquitinization is increased under sustained oxidative stress conditions [162]. This prompted the authors to propose that the SUMOylation/ubiquitination balance determines the fate of Mrp2 after internalization (*i.e.*, targeting back to the canalicular membrane *vs*. degradation) [164]. Sustained hypoxia, another condition leading to increased oxidative stress, also leads to Mrp2 internalization in WIF-B cells, with radixin being translocated from the pericanalicular area to the cytoplasmic space [165].

In spite of most hepatopathies involve either or both elevated ROS generation and impairment of antioxidant defenses, they are often non-cholestatic in nature, suggesting that hepatoprotective mechanisms against ROS-induced cholestasis exist. Bilirubin is a likely candidate to play a protective role, since it is a potent antioxidant and its levels are high inside hepatocytes [166]. Supporting this possibility, we showed that, even when exposed at physiological concentrations, unconjugated bilirubin counteracted *t*BOOH-induced bile secretory failure and canalicular transporter internalization both in isolated rat hepatocyte couplets and in perfused rat livers, by preventing F-actin disorganization [167]. This suggests that those hepatopathies with an oxidative background would have a worse cholestatic outcome if bilirubin intracellular levels are reduced.

4.1.3. Hypertonicity-induced Cholestasis

Hepatocyte hydration changes are induced by ambient aniso-osmolarity or, under isosmotic conditions, by hormones (*e.g.*, insulin, glucagon), substrates (*e.g.*, bile salts), and oxidative stress. Hepatocellular volume is a well-recognized determinant of bile formation [168]. Hypotonic liver cell swelling induces choleresis by quickly increasing bile salt excretion, whereas hypertonic shrinkage induces rapid cholestasis by impairing it [169]. To explain these phenomena, the short-term effect of aniso-osmolarity on localization of the canalicular transporters Bsep and Mrp2 [170] and the basolateral bile salt transporter Ntcp [171] was studied in perfused rat liver. Whereas hypotonicity rapidly prompted canalicular targeting of both Bsep and Mrp2 from the pericanalicular vesicular pool, hypertonicity resulted in endocytosis of both transporters; Bsep and Mrp2 colocalized in only 15% of the endocytosed vesicles, revealing that a differential pool of vesicles exists for both transporters [170]. Similarly, the hepatocyte swelling induced by hypotonicity triggers rapid targeting of the basolateral bile salt transporter Ntcp to its membrane domain [172], whereas hypertonicity-induced hepatocyte shrinkage induces Ntcp retrieval [171]. Since bile salts induce a fast drop in the hepatocyte hydration status [169] and are able to quickly stimulate internalization of Ntcp [144], this may represent a protective mechanism to avoid hepatocellular accumulation of potentially toxic bile salts.

Cell hydration and oxidative stress are casually interwined, and it is therefore not surprising that a role for *NADPH oxidase* (NOX)-dependent oxidative stress in hyperosmolarity-induced internalization of Ntcp has been shown [171]. A role for phosphorylation and further activation of cortactin in the endocytic internalization of both basolateral and canalicular transporters promoted by hyperosmotic exposure-induced oxidative stress has been suggested, but not proved [171].

4.1.4. Lipopolysaccharide (LPS)-induced Cholestasis

LPS is an endotoxin from the outer membrane of gram-negative bacteria. This endotoxin induces cholestasis by promoting the systemic release by monocytes/macrophages of pro-inflammatory cytokines, such as *tumor necrosis factor-α* (TNF-α), *interleukin-6* (IL-6) and *interleukin-1ß* (IL-1ß) [172]; in liver, these cytokines are mainly produced by Kupffer cells [173-175]. Endotoxin also promotes nitric oxide (NO) synthesis via *inducible NO synthase* (iNOS) in most hepatic cell types [176]. Administration of LPS to experimental animals represents, therefore, a valuable model of inflammatory cholestasis, not only by sepsis but also that related to hepatitis secondary to hepatotropic viruses, alcoholism, drug intake, or autoimmunity.

Post-transcriptional rather than transcriptional mechanisms play a major role in sepsis-induced down-regulation of BSEP and MRP2 in humans [176], and internalization and further degradation of transporters may play a crucial role. LPS administration to rats leads to endocytic internalization of Bsep and Mrp2 [139, 176-180]. The time-dependency of this effect has been described [178]. Three hours after LPS treatment, Mrp2 remained localized in pericanalicular vesicles, but these vesicles traffic to deeper endosomal compartments following 6-12 h of LPS administration. Further time periods have been studied by Zinchuk *et al.* [180], who showed relocalization to the basolateral domain of Mrp2, but not of Bsep, following 48 h of LPS administration. Colocalization of both transporters decreased with time, pointing to differential routes of both transporters after endocytosis [180]. Endocytic internalization of ABC canalicular transporters appears to be somewhat specific, since the canalicular enzyme

dipeptidyl peptidase IV remained localized in its membrane domain [178]. Mrp2 internalization can be reversed by exposing the liver to a hypoosmotic perfusate [178], which accelerate exocytic insertion of canalicular carriers [170, 177]. However, this "rescue" only occurs within 3 h of LPS injection [178]. It is probably that this reversibility depends on how deep Mrp2 has been internalized, since sustained internalization may allow transporter delivery for degradation.

Further support for a key role of pro-inflammatory cytokines in LPS-induced cholestasis is provided by studies showing that LPS-induced canalicular transporter internalization can be counteracted by both dexamethasone treatment [176, 178] and heat stress [181, 182], two procedures that decrease synthesis and/or release of pro-inflammatory cytokines.

Direct evidences for changes in transporter localization status at the basolateral domain level in cholestasis by LPS are lacking. However, indirect evidences support this contention. Inhibition of the gene expression of the bile salt uptake transporter Ntcp occurs late (~ 1 day) after administration of LPS to rats, leading to a subsequent decrease in Ntcp protein levels (> 90 %) [183]; a similar drop in genomic Ntcp expression was prompted by IL-6 [184, 185], TNF-α [176, 183], and IL-1β [176, 183], suggesting involvement of these pro-inflammatory cytokines in LPS cholestatic effect. However, *prior* to downregulation of Ntcp mRNA, both IL-6 [186] and TNF-α [187] induced a noncompetitive inhibition (*i.e.*, a *V*max drop) in Ntcp-mediated transport, thus suggesting an early, post-transcriptional decrease in membrane Ntcp content, most likely due to Ntcp internalization. Furthermore, exposure of HuH-NTCP to NO donors aimed to simulate LPS-induced NO intracellular synthesis selectively reduced *V*max for NTCP-mediated transport activity by 30 min, a phenomenon due to internalization of this transporter, as indicated by biotinylation studies [188]; since iNOS-induced intracellular NO elevations mediated by cytokines occurs shortly after LPS administration (by 3 h) [176], Ntcp internalization may be indeed an early event in LPS-induced cholestasis.

LPS induces oxidative stress, and this may be a main causal event in LPS-induced cholestasis and canalicular transporter retrieval. The antioxidant agent, dimerumic acid, prevents LPS-induced cholestasis and Mrp2 endocytosis soon after LPS exposure, either in the whole rat (3 h) [189] or in cryopreserved rat and human liver slices (1.5 h) [190]. On the other hand, the antioxidant failed to counteract the reduction in Mrp2 mRNA and protein expression after 12 h of LPS administration to rats, suggesting that, unlike the short-term post-translational regulation of Mrp2 expression by endocytosis, the long-term, transcriptional regulation is independent of the intracellular redox status [189]. However, the levels of oxidative stress induced by LPS seems to be low, as F-actin localization was unaltered 3 h after LPS administration [191]; this perhaps explains the selective internalization of Mrp2 that was reported in this study, since Bsep would require stronger oxidant conditions sufficient to induce F-actin disorganization to relocalize [152, 153]. Under low oxidant conditions, the mechanism of Mrp2 internalization seems to involve impairment of the interaction of radixin with Mrp2, by reducing the phosphorylation status of the former [191].

4.1.5. BDL

Obstruction of the biliary tree, either at the intrahepatic or extrahepatic level, is a common feature of a large number of liver diseases, with cholelithiasis, strictures, and tumors being the most usual causal agents. BDL is the more common experimental model to mimic obstructive cholestasis in experimental animals. BDL leads to marked alterations in the localization status of both Mrp2 and Bsep. Paulusma *et al*. [192] reported that, 48 h after BDL to rats, immunostaining of these transporters changed to a fuzzy pattern, suggesting internalization of the transporters into subapical intracellular vesicles. A similar staining pattern was reported by Trauner *et al.* for Mrp2 after 7 days of BDL [139]. These changes were associated with a significant failure in the hepatocellular transport of model Mrp2 solutes, as shown for dinitrophenyl-GSH in hepatocytes isolated from bile duct-ligated rats [192]. Endocytic internalization seems to affect transporters other than Mrp2 or Bsep, as a similar event was reported for the apical enzyme Ca^{2+}/Mg^{2+}-ATPase [149]; however, the phenomenon displays some selectivity, since AQP8 did not change its canalicular expression, even after 3 day of BDL in the rat [193].

Mislocalization of Bsep and Mrp2 may reflect an exacerbation of the secretory dysfunction associated with the concomitant decrease in the hepatocyte content of the transporters also occurring under this condition [194, 195], or even to be a causal reason for this downregulation [139, 192, 196-198]. Indeed, numerous studies reported that, unlike what happens with Mrp2 protein content, Mrp2 mRNA levels are maintained after BDL, thus suggesting post-transcriptional mechanisms [139, 192, 198]. Paulusma *et al.* [192] postulated that this endocytic internalization may be the primary

event toward the exacerbated metabolic breakdown of the endocytosed transporters.

The mechanisms underlying the endocytic internalization of Bsep and Mrp2 in rats with BDL remain to be clarified. Accumulation of potentially toxic endogenous compounds in hepatocytes secondary to the biliary blockage may represent a key causal event. Likely candidates are bile salts, whose serum levels increase aprox. 40 times after 24 h of BDL in mice [199]. In line with this, TCDC overload to rats triggers Mrp2 internalization, accompanied by disorganization of both actin and radixin [134], most likely via pro-oxidant mechanisms [200, 201]. This deleterious effect could be aggravated by systemic endotoxemia [202], which occurs in this cholestatic model due to the lack of bacteriostatic, endotoxin-neutralizing, and mucosal-trophic properties of intraluminal bile [203]; this enhances bacterial and LPS translocation into portal blood and further release of pro-inflammatory cytokines in liver, which triggers oxidative stress [204]. Supporting this view, both the zonal (periportal) downregulation and the endocytic internalization of Bsep in obstructive cholestasis is associated with portal inflammation, via a mechanism involving TNF-α and IL-1β [205].

4.1.6. Massive Hepatectomy-induced Cholestasis

Extensive liver resection with a significant reduction in the functional liver mass is a fairly common surgical practice, as for example to remove massive liver metastases. In these cases, persistent cholestasis occurs. To give an insight into the pathogenic mechanisms, localization of bile salt transporters has been examined in a rat model with massive (90%) hepatectomy, leading to a steady increment in bile salt plasma levels. Apart from suffering a decrease in constitutive expression, Ntcp was hardly detected in the basolateral membrane and relocalized to the cytoplasm from day 3 onwards [206]. This may help to protect the remnant liver from the high bile salt load to which it is exposed, which may aggravate the initial liver injury. Interestingly, at day 7, a recovery in Ntcp constitutive expression occurs, but the newly synthesized carrier still remains in cytosol, suggesting that not only endocytosis of the transporter is exacerbated, but also the targeting to the basolateral membrane is impaired [206].

4.1.7. Non-alcoholic Fatty Liver Disease (NAFLD)

Intrahepatic cholestasis is a likely outcome in alcoholic and NAFLD, particularly (but not only) in advanced stages of the disease [207]. Internalization of Mrp2 has been shown in rats fed with a methionine/choline deficient diet, which leads to non-alcoholic steatohepatitis (NASH), an advanced form of NAFLD characterized by liver inflammation [208, 209]. This resulted in decreased biliary output and hepatocyte accumulation of the Mrp2 substrate morphine-3-glucuronide [209], suggesting increased risk of toxicity with this opioid in particular, and with other drugs excreted via Mrp2 in general. Signaling-mediated inactivation by alterations in the phosphorylation status of actin-bound proteins involved in Mrp2 membrane stabilization, including radixin (inactivated by dephosphorylation) and MARCKS (inactivated by phosphorylation) [140], has been identified as a putative pathogenic mechanism [208]. In addition, there was a decrease in the expression of Rab11, a GTPase that is crucial to exocytosis by interacting with the exocytic complex for docking and fusion of recycling endosomes at the plasmalemma [210].

Since oxidative stress and inflammatory cytokines play a crucial role in the development of NASH [211], and both factors are well known mediate internalization of Mrp2 (see above, items 4.1.2 and 4.1.4., respectively), it is likely that they are involved in the onset of NASH pathogenic mechanisms.

Clinical and histological cholestasis may be present at all stages of alcoholic liver disease [212], and common mechanisms of injury underline NASH and alcoholic liver disease. It is therefore likely that a similar relocalization of hepatocellular carriers is implicated in the occurrence of cholestasis in the latter case.

4.2. Endocytic Internalization of Transporters in Human Cholestatic Hepatopathies

Mislocalization of canalicular transporter has been shown in several human cholestatic liver diseases. Since impairment in the protein expression of these transporters in human hepatopathies is often triggered via post-transcriptional mechanisms, cholestasis in humans may have endocytosis followed by degradation as a key pathomechanism of downregulation.

Shoda *et al.* [213] studied the correlation between localization and transport function of the main apical transporters in patients with extrahepatic cholestasis subjected to percutaneous transhepatic biliary drainage. They found a fuzzy staining in the vicinity of the canalicular domain in livers from poorly drained patients, which was far less marked in patients exhibiting good drainage. These results were reproduced by Kojima *et al.* [214], who also extended the finding to other cholestatic liver diseases, such as that induced by drugs [214] and primary biliary cirrhosis (PBC) [215]. In the latter case, the authors reported on changes in localiza-

tion and expression of the canalicular transporters MDR3 and MRP2, and showed that the internalization degree correlated well with the severity of the bile secretion impairment, as assessed by elevations of serum cholestasis markers.

In addition, Watanabe *et al.* [216] studied, in 26 cases of intrahepatic cholestasis induced by drugs, the correlation between the alterations in liver hystopathology or liver function tests with the degree of MRP2 relocalization. They also distinguished between cholestasis of short- and long-lasting evolution. Serum bilirubin half-life and lobular inflammation were more noticeable in patients with longer cholestasis development, and this was accompanied by MRP2 relocalization to pericanalicular vesicles.

The mechanism underlying the internalization of canalicular transporter in human cholestasis is beginning to be characterized. It has been proposed that changes in phosphorylation status and localization of anchoring proteins, such as radixin or ezrin, is a causal factor of the altered canalicular localization of MRP2 in cholestatic patients (see 5.1 section for information on the role for anchoring proteins in MRP2 membrane stability). Kojima *et al.* [215] found in stage III PBC, drug-induced liver injury [214], and poorly drained obstructive cholestasis [214] that canalicular areas of irregular MRP2 immunostaining had also a reduced radixin expression, associated with vesicular MRP2 internalization. On the other hand, immunoprecipitation studies carried out by Chai *et al.* [217] in patients with obstructive cholelithiasis identified ezrin, but not radixin, as the anchoring protein interacting with MRP2, and found that the expression levels of the phosphorylated, active form of ezrin correlate positively with the amount of co-imunoprecipitated MRP2.

Irrespective of the mechanism involved, the available information consistently support that endocytosis of canalicular carriers is associated with impairment in hepatocellular transport function in most human cholestatic hepatopathies, in full agreement with what had been reported in experimental models of cholestasis.

5. MECHANISMS OF ENDOCYTIC INTERNALIZATION OF HEPATOCELLULAR TRANSPORTERS IN CHOLESTASIS

5.1. Structural Basis

As extensively discussed in item 3, tethering of membrane proteins to F-actin, via a number of F-actin-associated, adaptor proteins, plays a key role in plasma membrane carrier stability. Hence, any situation leading to either or both actin disorganization and impairing of expression/localization of cytosolic anchoring proteins is expected to lead to transporter endocytic internalization. In agreement with this, administration of phalloidin, an F-actin poison that produces disruption of actin-cytoskeletal integrity [218], or secondary to F-actin disorganization induced by pro-oxidant compounds, such as *t*BOOH [153] or the hydrophobic bile salt TCDC [134], exacerbated canalicular carrier endocytosis. Nevertheless, this endocytosis was also observed with conserved F-actin organization, as for example in TLC- [132] and E17G-induced cholestasis [130, 146]. Under these circumstances, constituents of the cytoskeleton different from actin, but linked to it, could have been alternatively affected. Suggestively, mice deficient in radixin, the main ERM protein in the canalicular microvilli, develop jaundice associated with Mrp2 retrieval [219]. In addition, radixin downregulation in collagen sandwich-cultured rat hepatocytes using siRNA knockdown impaired canalicular biogenesis and Bsep plasma membrane stabilization, with the carrier being largely localized in Rab11-containing endosomes [70]. Furthermore, impaired colocalization of radixin and Mrp2, accompanied by endocytic internalization of the carrier, has been reported in BDL- and estrogen-induced cholestasis in rats [138], as well as in different human cholestatic liver diseases, such as drug-induced liver injury, obstructive cholestasis, primary sclerosing cholangitis, PBC (stage III), and autoimmune hepatitis [214, 215].

The phosphorylation levels of ERM proteins and other F-acting associated proteins is a crucial determinant of their function, and the activation of protein phosphatases targeting their regulatory sites under cholestatic conditions may play a key role. For example, PKC-dependent activation of PP-1 leading to radixin dephosphorylation, which turns radixin to its inactive form, explains Mrp2 internalization in oxidative stress-induced cholestasis [161] and in LPS-induced cholestasis [189]; both events can be causally linked, since LPS-induced cholestasis is associated with oxidative stress [190]. Mrp2 internalization associated with radixin dephosphorylation also was apparent in rat models of both intrahepatic cholestasis (EE treatment) and extrahepatic cholestasis (BDL) [138]. Somewhat contradictory results have been recently reported in human cholestasis. MRP2 was associated with ezrin rather than with radixin, and MRP2 internalization in obstructive cholestasis involved hyperphosphorylation rather than dephosphorylation of ezrin [217]. Interestingly, excessive phosphorylation of radixin in WIF-B cells expressing radixin-T564D mutant (a dominant-active construct that mimics constant phosphorylation) af-

fected its normal canalicular localization, with redistribution to the basolateral membrane as a result of indiscriminate binding to any (apical or basolateral) cellular F-actin pool, followed by Mrp2 endocytosis [220]; whether the same applies for ezrin, as well as for human cholestasis, remains to be confirmed.

Impairment in localization/function of F-actin interacting-partner (scaffold) proteins, such as RACK1 (for MDR3), HAX-1 (for Bsep, Mdr2, and Mrp1), and PDZK1 or EBP50 (for Mrp2), remains to be confirmed as a causal factor of cholestasis. This is indeed likely, since Oatp1a1 [56] and Mrp2 [74] retention in the sinusoidal and canalicular membranes, respectively, requires interaction with PDZK1. In addition, the stability of Mrp2 in the apical membrane depends upon EBP50 interaction [72]. Furthermore, RACK1 downregulation by siRNA knockdown in HepG2 cells induced MDR3 endocytosis [82]. Finally, there is circumstantial evidence in Madin-Darby canine kidney cells that HAX-1 is involved in clathrin-dependent BSEP endocytosis [77].

Another scaffold protein associated with clathrin-mediated endocytosis whose changes in phosphorylation may prompt canalicular transporter endocytosis is *cortactin*, as has been shown in hyperosmotic stress-induced cholestasis [171]. Cortactin is an F-actin-associated protein that binds directly to the large GTPase dynamin, and participates actively in clathrin-mediated endocytosis by regulating the pinching off of clathrin-coated pits from the plasma membrane [221]. Cortactin has an N-terminal acidic domain and six-and-a half tandem repeats, followed by an α-helix, a proline-rich region, and a C-terminal *Src homology-3* (SH3) domain [222]. Phosphorylation of cortactin by the tyrosine kinases Src or Fyn at Tyr^{421}, Tyr^{466}, and Tyr^{485} in the cortactin moiety that binds to F-actin attenuates the cross-linking interaction among them. This leads to F-actin disassembly by depolymerization and destabilization [222], which triggers clathrin-mediated endocytosis [171].

Finally, changes in the phosphorylation status of MARCKS, another F-actin cross-linking protein, have been recently proposed to be associated with cholestasis. Endocytosis of MRP2 induced by TLC in HuH-NTCP cells depends on PKCε-mediated MARCKS phosphorylation [141]. Since MARCKS is a well-recognized PKC substrate [84] and PKC is a key protein kinase involved in cholestatic events (see next item), MARCKS is a very interesting molecule to be studied in more realistic cholestatic models.

The appropriate localization of canalicular transporters in their normal plasma membrane microdomains, where they display maximal intrinsic activity and membrane stability (see item 3), could also be impaired in cholestasis. Marrone *et al.* [223] reported recently that, in EE-induced cholestasis, Bsep suffers redistribution from "raft" (caveolin-1 positive) to "non-raft" (clathrin-positive) microdomains. *In vivo* adenoviral transfer of human AQP1, which is expressed like rat AQP8 in "raft" domains of the canalicular membrane, prevents Bsep from leaving the "raft" membrane microdomain, thus improving Bsep activity and bile salt excretion in cholestatic rats [223]. Since Bsep [146], but not AQP8 [150], is internalized in estrogen-induced cholestasis, and Bsep is endocytosed via a clathrin-dependent mechanism, it is tempting to hypothesize that transfected human AQP1 has anchored Bsep in the "raft" microdomain via protein-protein interactions, thus preventing its shift to "non-raft" membrane regions, from where it can be further endocytosed via a clathrin-mediated mechanism. More studies are needed to support these assumptions.

Finally, small GTPases of the Rab family control different steps of the endocytic route in hepatocytes [224], and are therefore important functional components of the endocytic machinery that may become dysfunctional in cholestasis. Particularly, Rab4 and Rab11 are involved in the quick and slow recycling from early and late endosomal compartments to the plasma membrane, respectively [225], and are positively modulated by phosphorylation. Even though several studies have addressed the role for protein kinases in Rab4 and Rab11 activation and the further stimulation of transporter targeting to the plasma membrane [226, 227], studies exploring the impairment of Rab activity or expression in cholestasis, and its link to enhanced transporter internalization, are lacking. Only one study has shown a reduced expression of Rab11 and increased Mrp2 internalization in a rat model of NASH [208], but the causal link between both events has not been confirmed.

Although much advance has been made in the identification of putative mediators of the changes in the normal localization of transporters in cholestasis, more effort is needed to ascertain with confidence the structural basis underlying this alteration under each particular cholestatic condition.

5.2. Role for Signaling Pathways

5.2.1. Cell Signaling and Canalicular Transporter Endocytic Internalization

Cell signaling is central for the transduction of signals that start with the interaction of a cholestatic agent with one or more cellular receptors, and that end in the

post-transductional regulation (*e.g.*, by phosphorylation/dephosphorylation) of key molecules regulating transporter endocytosis/exocytosis.

Representative examples of signaling proteins linked to cholestasis are *Ca^{2+}-dependent ('classical') PKC isoforms* (cPKC), mostly PKCα in hepatocytes. Both endocytic Bsep retrieval and bile flow impairment is induced by specific activation of cPKC with thymeleatoxin in isolated perfused rat livers [228]. Similarly, redistribution of MRP2 from the apical to the basolateral membrane is also induced by the pan-specific, cPKC and *'novel' PKC isoform* (nPKC) activator phorbol-12-myristate-13-acetate in human hepatoma HepG2 cells [229]. Sinusoidal transporters seem to share a similar signaling mechanism, as cPKC activation triggers endocytic Ntcp retrieval in Ntcp-transfected HepG2 cells [230].

The different signaling pathways involved in the regulation of normal transporter recycling, or in transporter stability in the plasma membrane, could involve different targets, including the carriers themselves, or any of the structural components implicated in transporter trafficking, recycling, and/or membrane anchoring. As for the first mechanism, no evidence for alterations in phosphorylation levels of canalicular carriers in cholestasis exists. Even though direct PKCα-mediated phosphorylation of mouse Bsep has been shown in a transfected insect cell line derived from *Spodoptera frugiperda* (Sf9), this structural change increased rather than decreases Bsep intrinsic transport activity [231]. Likewise, *in vitro* MRP2 phosphorylation by cPKC activation in a baculovirus-co-expressing system was accompanied by an increase in the transfer of model MRP2 substrates [232]. Direct *in vitro* phosphorylation by either PKCα (a cPKC) or PKCε (a nPKC) was shown in MRP2 immunoprecipitated from HepG2 hepatoma cells; since phosphorylation was more pronounced when MRP2 was simultaneously exposed to both isoforms, it seems that these PKC isoforms phosphorylate the transporter at different sites [233].

The transporter phosphorylation level may also alter carrier association with anchoring proteins. *In vitro* binding experiments showed that MRP2 phosphorylation at the C-terminal domain increased its interaction with both the scaffold proteins *intestinal and kidney enriched PDZ protein* (IKEPP) and EBP50 [234]. Unfortunately, the impact of MRP2 phosphorylation on binding affinity to PDZK1, the chief MRP2-interacting PDZ protein in hepatocytes, has not been evaluated as yet. As for Bsep, no studies on the influence of phosphorylation changes in its "linker" region to HAX-1 have been carried out. However, the closest Bsep homologue, Mdr1, is phosphorylated at three serine residues of the C-terminal region by PKC, which interacts with HAX-1 [235].

The involvement of PKC in the endocytic internalization of Mrp2 and Bsep, and the consequent impairment in transport function, has been confirmed in different models of cholestasis. Our group has demonstrated a role for cPKC in Mrp2 and Bsep endocytic plasma membrane retrieval in E17G-induced cholestasis in rats [236], as well as in Bsep internalization in the cholestasis caused by *t*BOOH-induced oxidative stress [156]. Another constituent of the PKC family, specifically PKCε, has been involved in TLC-induced cholestasis [237].

In the case of E17G-induced cholestasis, several signaling proteins are known to participate apart from cPKC, namely PI3K, the *extracellular signal-regulated protein kinases 1/2* (ERK1/2) subfamily of *mitogen-activated protein kinases* (MAPKs), *epidermal growth factor receptor* (EGFR), *adenylate cyclase* (AC), and PKA, among others (Fig. **5**). Two estrogen receptors have been implicated in this cholestasis, namely *estrogen receptor-α* (ERα) and *G-protein coupled receptor 30* (GPR30). Upon activation, signaling proteins are recruited in three complementary pathways. One pathway involves ERα, and the remaining two involve GPR30. What is interesting about ERα activation by E17G (assessed by phosphorylation at Ser^{118}) is that it is preceded by PKCα activation [238]. In agreement with this, Joel *et al.* [239] showed that pan-activation of PKC with phorbol esters results in phosphorylation of ERα at this site. This could indicate that the original receptor that interacts with E17G is still missing. Alternatively, PKC itself might act as the initial binding partner of E17G, since PKC can function as a steroid receptor [240]. Inhibition of ERα partially protected against E17G-induced Mrp2 and Bsep activity impairment both in hepatocyte couplets and in perfused rat liver [238]. A similar partial protection was afforded by inhibitors of PKC [238], $p38^{MAPK}$ [112], EGFR, and Src [240]. Experiments testing the effects of combinations of these inhibitors show no additive effect [112, 238, 241], suggesting that all these signaling molecules belong to the same pathway. The sequence was confirmed by evaluating the effect of kinase or receptor inhibitors in the activation of the other kinases or receptors (*e.g.*, phosphorylation). Results indicate that the pathway is initiated in cPKC, followed by ERα, EGFR, and Src. $p38^{MAPK}$ is downstream of cPKC, but its relation to the other proteins is still unknown. The methodology

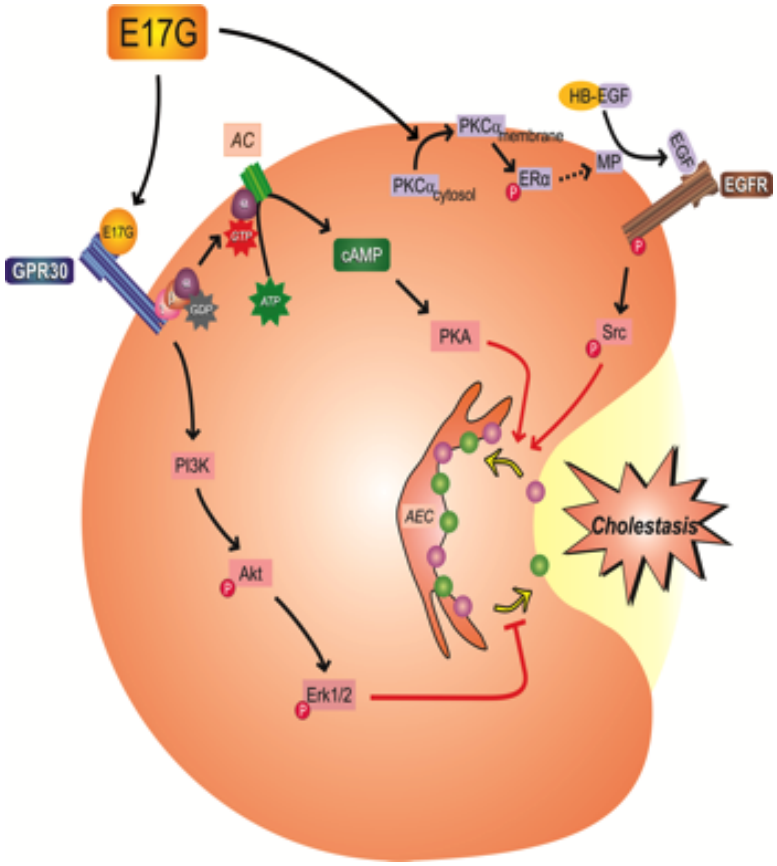

Fig. (5). Signaling pathways involved in E17G-induced colestasis. E17G activates signaling proteins from three complementary pathways; in two of them, the initial estrogen receptor is GPR30 and, in the remaining one, the initial receptor has not been identified as yet. Among the two pathways beginning in GPR30, one involves the tandem adenylyl cyclase (AC)-PKA, whereas the other one involves the PI3K-Akt-ERK1/2 signaling pathway. The first event in the third pathway is the translocation of PKCα from cytosol to the membrane, followed by the sequential phosphorylation of estrogen receptor-α (ERα), EGFR, and Src; ERα and EGFR interaction could be mediated by metalloproteinase-release of extracellular EGFR ligands. The three pathways are necessary to impair Mrp2 and Bsep activity, and the inhibition of either of them leads to partial prevention of transport impairment. Both PKCα-ERα-EGFR-Src and AC-PKA pathways are responsible for the initial endocytic internalization into the apical endosomal compartment (AEC), whereas the PI3K-Akt-ERK1/2 signaling pathway interferes with the reinsertion of vesicles containing canalicular carriers occurring spontaneously over the recovery phase of cholestasis.

employed allows placing signaling proteins in a sequence, but it does not discard the existence of intermediary proteins. For example, cPKC could phosphorylate GSK-3β [242], and the latter can in turn phosphorylate ERα in Ser^{118} [243]. Similarly, ERα and EGFR interaction could be mediated by metalloproteinase-mediated release of extracellular EGFR ligands [244].

Using similar methodologies, two pathways starting in GPR30 have been additionally described. One pathway involves the tandem AC-PKA [245], whereas the other one involves PI3K-Akt-ERK1/2 [112, 245, 246]. The three pathways are necessary to impair Mrp2 and Bsep activity, and the inhibition of either of them leads to partial prevention of transport impairment. On the other hand, either activation of GPR30 by its agonist G1 [245] or activation of EGFR by EGF [241] alone did not affect transporter activities, whereas the combination of G1 and epidermal growth factor (EGF) reduced Mrp2 activity, indicating that the simultaneous activation of the three pathway is necessary to trigger E17G-induced cholestasis.

How these three pathways act complementarily at the apical hepatocyte pole to trigger cholestasis is yet unknown. Based on *in vivo* and perfused rat liver experiments, the cPKC-ERα-EGFR-Src and AC-PKA signaling pathways appears to be responsible for the initial endocytic carrier retrieval, whereas the PI3K-Akt-ERK1/2 one acts complementarily, by delaying the reinsertion of the canalicular carrier-containing vesicles that occurs spontaneously during the recovery phase of cholestasis. The link between these pathways with the

phenomena that takes place at the canalicular pole can only be hypothesized at present. For example, Src, a kinase present in the first pathway, can phosphorylate β2-adaptin, a subunit of the clathrin adaptor protein *adaptor protein complex 2* (AP-2), in its C-terminal moiety [247]. Hence, the pathway cPKC-ERα-EGFR-Src could be involved in the activation of clathrin-mediated protein endocytosis. The inhibition of the pathway that involves AC-PKA produces the highest protection against cholestasis, even when this protection is still partial in nature; this could indicate the inhibition of a central step of the whole endocytic process, as for example the release of the transporters from the radixin-actin mesh, which may allow for both their movement from "raft" to "non-raft" membrane microdomains and their further clathrin-dependent endocytosis. Finally, as for the role of PI3K-Akt-ERK1/2 in delaying spontaneous reinsertion of transporters, different PI3K isoforms have the potential to interact with proteins linked with the endocytic process, namely *1*) PI3K-C2γ, which interacts with Rab5 [248], *2*) PI3K-C2α, which interacts with clathrin and Rab11 [249], and *3*) class III PI3K vps34, which interacts with Rab5 and Rab7 [250, 251]. Taking into account the microtubule dependency of canalicular exocytosis [246, 252] and the association of endocytosed canalicular transporters with Rab11a [115], ERK1/2 could modulate, downstream of PI3K, motor proteins responsible for microtubule-dependent exocytosis. In agreement, the binding activity of the microtubule motor, *kinesin-1*, is inhibited by ERK1/2 [253].

The knowledge on the signaling pathways activated by other cholestatic agents is more limited (Fig. **6**). In cholestasis associated with *t*BOOH-induced oxidative stress, cPKC is involved in the endocytic internalization of Bsep [151], and the MAPKs *c-Jun N-terminal kinase 1/2* (JNK1/2), $p38^{MAPK}$, and ERK1/2 all co-participate in the endocytic internalization of Bsep and Mrp2 induced by the pro-oxidizing agent [254]. cPKC could be one of the receptors that interact with ROS, since PKC contain structural traits that make it susceptible to redox-dependent activation [255]; JNK1/2 and $p38^{MAPK}$ are downstream of cPKC, whereas ERK1/2 belongs to a different pathway, initiated by the interaction of ROS with another receptor [254]. The inhibition of any of the three MAPKs partially protects from *t*BOOH-induced transport impairment, suggesting the requirement of different pathways to induce the secretory failure, similar to what happens in E17G-induced cholestasis. The molecular mechanisms by which MAPKs induce cholestasis when activated in an oxidative context is not clear as yet, but a critical role for the interaction of MAPKs with actin cytoskeleton can be hypothesized, since inhibition of any of the three MAPKs prevented actin cytoskeleton disorganization [254].

The signaling pathways involved in oxidative stress-induced cholestasis appear to depend upon the level of the oxidative challenge. Exposure to low levels of the oxidizing agent ethacrynic acid induces nPKC but not cPKC activation [148]. In these circumstances, ethacrynic acid induces selectively internalization of Mrp2 without affecting that of Bsep, via a mechanism likely involving Ca^{2+}-depending activation of iNOS, followed by NO-mediated cGMP elevations, and further cGMP-dependent activation of nPKC [156]. However, at higher doses, which are instrumental in activating cPKC as well, ethacrynic acid induces endocytosis of both Mrp2 and Bsep [156].

In TLC-induced cholestasis, circumstantial evidences in HuH-NTCP cells suggest the participation of PI3K [141], PKCε [141], MARCKs [141], and $p38\beta^{MAPK}$ [256]. PI3K inhibition by wortmannin partially prevented the cholestatic effect of TLC in the perfused rat liver [257]. Unlike E17G-induced cholestasis, PI3K was implicated in the initial decay of bile flow, but not in the recovery phase. PI3K mediates TLC-induced activation of nPKC in rat hepatocytes [257], but not in HuH-NTCP cells [141]. Using dominant negative kinase HuH-NTCP cell mutants, Schonhoff *et al.* [143] provided evidence that PKCε precedes MARCKS, so that PI3K, PKCε, and MARCKS probably belong to the same pathway. On the other hand, $p38\beta^{MAPK}$ has not been yet ascribed to any particular pathway. Although a role for MARCKS in apical transporter endocytosis remains to be confirmed, it has been proposed that the capacity of unphosphorylated MARCKS to bind to actin allows for the stabilization of transporters in the plasma membrane [86]. Therefore, MARCKS phosphorylation would weaken the binding of this protein to F-actin, which could be an important step in the complex process leading to canalicular transporter endocytosis.

The receptor that interacts with TLC to initiate the signaling cascade that leads to transporter internalization is still missing. TLC can interact with the bile salt receptor TGR5, but this receptor is absent in hepatocyte [258]. Other possible TLC receptors in hepatocyte are *sphingosine-1 receptor 2* (S1PR2) [259] and the cholinergic receptor *M3* [260]. Further studies are needed to address this matter.

In sepsis, particularly that produced by gram-negative bacteria, there is a systemic immune response

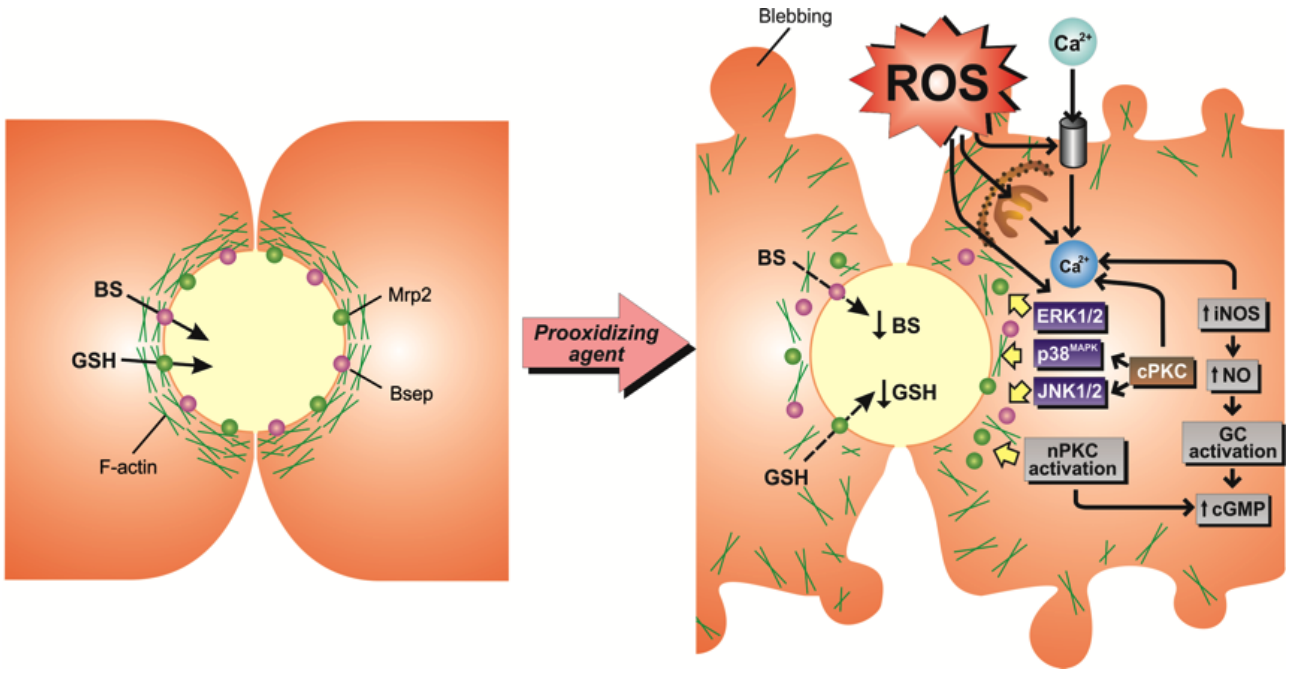

Fig. (6). Effect of oxidative stress on canalicular transporter localization. In normal hepatocytes, the pericanalicular distribution of F-actin permits the stabilization of the canalicular transporters in their membrane domain, allowing canalicular excretion of bile salts (BS) through Bsep, and glutathione (GSH) through Mrp2. The acute exposure to high doses of a prooxidizing agent promotes external Ca^{2+} entering across the plasma membrane and Ca^{2+} release from intracellular stores (calciosome). This leads to activation of Ca^{2+}-dependent, "classical" PKC isoforms (cPKC). cPKC activates the MAPKs JNK1/2 and $p38^{MAPK}$, which together with ERK1/2 activation (by a still unidentified signalling pathway) lead to relocalization of actin filaments all over the plasmalemma and to the cell periphery, with the consequent bleb formation and Bsep/Mrp2 internalization; the latter event explains the oxidative stress-induced BS and GSH secretory impairment. Lower levels of oxidative stress promote, in a Ca^{2+}-dependent manner, the sequential activation of inducible nitric oxide (NO) synthase (iNOS), the NO-induced activation of guanylate cyclase (GC), and finally, the cyclic guanosine monophosphate (cGMP)-mediated activation of "novel" PKC isoforms (nPKC); these PKC isoforms internalize selectively Mrp2, without apparent F-actin disorganization.

to LPS. This toxin is responsible for the macrophage secretion of proinflammatory cytokines, mainly IL-1β, IL-6, and TNF-α. These cytokines are the main causal agents of the biliary secretory impairment [174]. LPS induces cholestasis by altering BSEP- and MRP2-mediated canalicular transport through post-transcriptional processes [176, 261]. The nature of the events remains unknown but, based in the action of other cholestatic agents, the activation of signaling proteins by cytokines could play a key role. TNF-α activates signaling pathways/kinases that have been involved in cholestatic processes, such as PI3K-Akt [257, 262] and certain PKC isoforms (α, δ) [263, 264]. Similarly, IL-1β signaling involves MAPKs [262], cAMP [266], and probably PI3K-Akt [267]. However, these signaling events have been characterized is cell types different from hepatocyte, and hence, they were responsible for effects different from the cholestatic ones. In hepatocytes, IL-1β activates JNK1/2, which is implicated in the post-translational modification of RXRα, an event necessary to suppress transporter gene expression in LPS-induced cholestasis [268]. Recknagel *et al.* [269] linked PI3K with the recycling of MRP2 in HepG2 hepatoma cells treated with both a cytokine mixture (TNF-α, IL-1β, IFN-γ) and LPS. This treatment led to a significant decrease in MRP2 protein at the membrane level, and both AS605240 (a PI3Kγ specific inhibitor) and wortmannin (a pan-specific PI3K inhibitor) significantly prevented this effect, suggesting a key involvement of PI3Kγ in the control of cytokine-induced MRP2 internalization; suggestively, cholestatic bile salt (*e.g.*, TLC), but not the anticholestatic ones (*e.g.*, TUDC and TC), selectively activate PI3Kγ [270]. The receptors involved in cytokine-induced transporter internalization would probably be the specific cytokine receptors, *i.e.* TNFR1 or TNFR2 for TNF-α and IL-1R for IL-1β. TNFR1 and TNFR2 are expressed in low amount in hepatocytes of normal subjects, but their expressions are increased in patients with inflammatory liver diseases [271]. A similar effect occurs with IL-1R in hepatocytes [272].

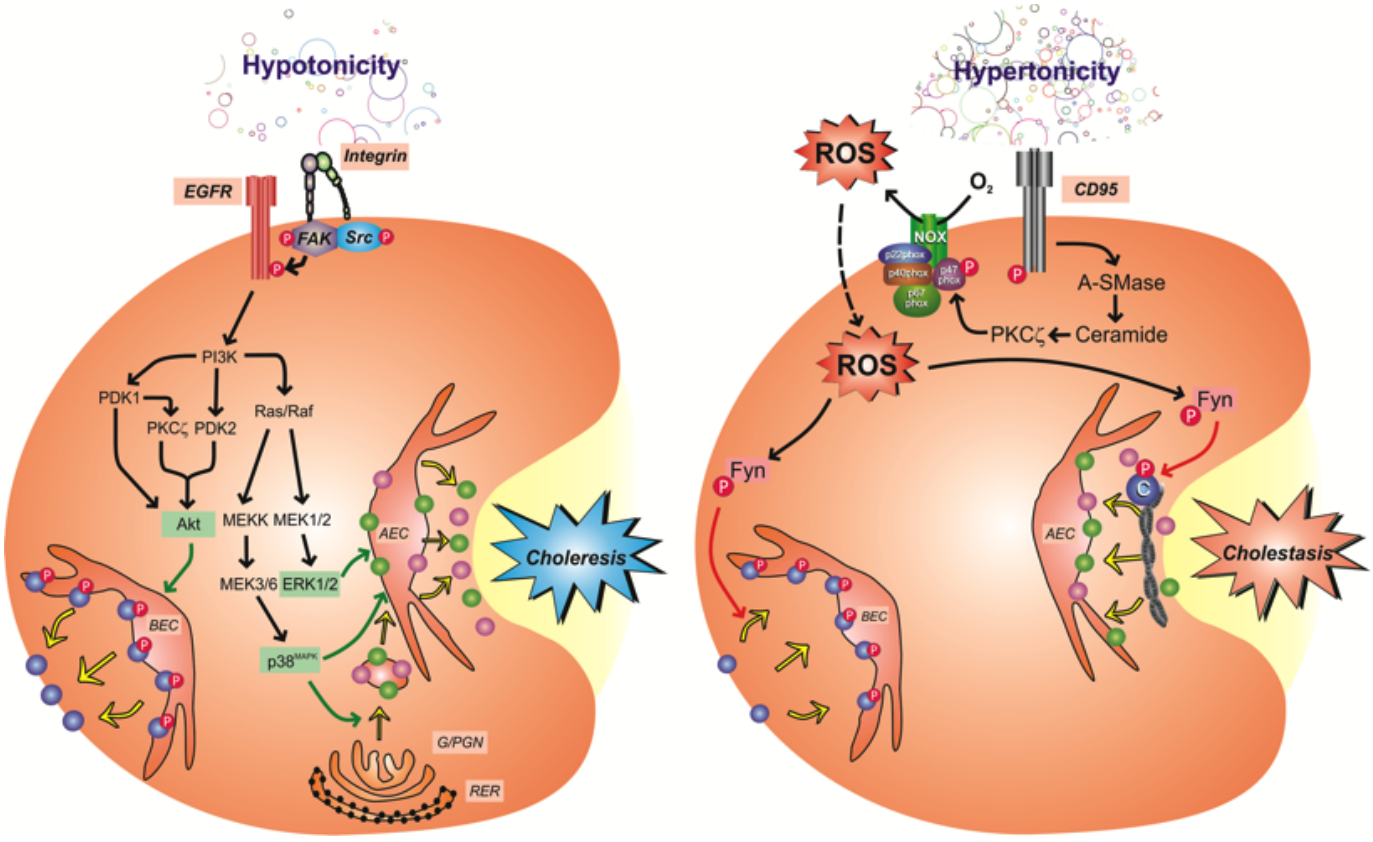

Fig. (7). Signaling pathways involved in choleretic and cholestatic effects induced by anisoosmolarity. *Left panel:* Signaling pathways involved in hypotonicity-induced choleresis. Hypotonic stress stimulates biliary excretion through integrin-dependent osmosensing, followed by sequential activation of c-Src kinase, EGFR phosphorylation, PI3K activation, and phosphorylation of ERK1/2 and $p38^{MAPK}$. These MAPKs stimulate translocation of both pre-synthesized canalicular transporters from the apical endosomal compartment (AEC) or the *de novo* synthesized ones from Golgi/trans-Golgi network (G/TGN). PI3K activation also leads to activation of Akt via PDK1, or coactivation via PDK2/PKCζ, which promotes trafficking of the sinusoidal bile salt transporter Ntcp to the plasma membrane from the basolateral endosomal compartment (AEC). *Right panel:* Signaling pathways involved in hypertonicity-induced cholestasis. Hypertonicity induces transporter endocytosis by a mechanism that involves osmosensing by the receptor CD95, which further activates NADPH-oxidase (NOX) via a signaling route implicating acidic sphingomyelinase activation, ceramide-dependent PKCζ activation, and the PKCζ-dependent phosphorylation p47phox, the NOX regulatory subunit. NOX-derived reactive oxygen species (ROS) activate Fyn, a member of the Src family which phosphorylates cortactin (C), leading to F-actin disarrangement and transporter endocytosis from the canalicular membrane.

Aniso-osmolarity affects biliary secretion by modulating the localization status of carriers involved in bile flow production, such as Bsep and Mrp2 in the apical pole [170, 273], and Ntcp in the basolateral pole [274]; whereas hypotonicity-induced swelling leads to choleresis, hypertonicity-induced shrinkage induces cholestasis (Fig. **7**). Hypo-osmotic stress stimulates biliary excretion through integrin-dependent osmo-signaling, involving further sequential activation of c-Src kinase, EGFR phosphorylation, PI3K activation, and phosphorylation of the ERK1/2 and $p38^{MAPK}$ subfamilies of MAPKs [172, 275-277]. Under similar hepatocyte swelling conditions but, in this case, induced by TUDC rather than by hypo-osmotic stress, these MAPKs stimulate targeting to the plasma membrane of aplical transporters from ARE [278, 279] or, for $p38^{MAPK}$, the whole trafficking of *de novo* synthesized canalicular transporters from Golgi [280]. PI3K activation also leads to Akt activation, which inserts Ntcp into the basolateral membrane [172, 277]. Hypertonicity, on the other hand, induces transporter internalization by a mechanism that involves the Src family kinase, Fyn [171, 274]. The role for Fyn in transporter endocytosis would depend on Fyn-mediated phosphorylation of cortactin, a dynamin/F-actin-cross-linking protein. Cortactin binds to the proline-rich domain of dynamin via its SH3 domain, and it has been suggested to regulate actin dynamics to facilitate clathrin-based endocy-

tosis by favoring vesicle invagination and scission; phosphorylated cortactin has less F-actin cross-linking activity [281], and this leads to actin filament disarrangement and transporter endocytosis. Other hypothetical mechanism is, as indicated above, that Src kinase phosphorylates β2-adaptin, a subunit of the clathrin adaptor AP-2 [247], and consequently, activates clathrin-mediated endocytosis. The proposed osmolarity sensor in hepatocytes is CD95 (*a.k.a*. Fas) [282]. Then, a chloride-driven acidification of early endosomes activates NOX through a signaling pathway involving ceramide generation by acidic sphingomyelinase activation, ceramide-dependent PKCζ activation, and the PKCζ-dependent phosphorylation of p47phox, the regulatory subunit of NOX [283]. NOX-derived ROS trigger activation of the Src family member Fyn. This mechanism was confirmed in p47phox knock-out mice; neither hypertonicity-induced Fyn activation nor Mrp2/Bsep retrieval was observed in livers from these animals [171].

5.2.2. Cell Signaling and Basolateral Transporter Endocytosis

Among the multiple basolateral transporters, NTCP/Ntcp is one of the few ones that are stored in a vesicular compartment. A pathway constituted by AC-PKA-PI3K-PKCζ-Akt inserts Ntcp in the basolateral membrane [277, 284, 285], whereas the pan-activation of PKC with phorbol esters leads to its endocytic internalization [144]. For human NTCP, its retrieval in a cholestatic context is less clear. TLC does not internalize NTCP in HuH-NTCP cells, whereas it does so in rat hepatocytes, probably by activating PCKε [143]. The differential behavior between NTCP and Ntcp could indicate divergences between species or, alternatively, limitations of the NTCP transfection model. The hydrophobic bile salt TCDC, but not the more hydrophilic one TC, induces selective endocytosis of Ntcp in rat liver, via a mechanism involving PKC and *protein phosphatase-2B* (PP2B) activation [144]; the latter finding is puzzling, since PP2B dephosphorylates Ntcp at Ser^{226}, which triggers plasma membrane retention rather than internalization [286].

In the cholestasis model induced by hyperosmolarity (Fig. **7**), Ntcp is endocytosed from the basolateral membrane of rat hepatocytes via a mechanism that depends on the Src-family kinase Fyn, but not on Yes or c-Src. Hyperosmolarity-induced NOX-dependent oxidative stress participates in Ntcp internalization, since *i*) the NOX inhibitor apocynin and the antioxidant N-acetyl cysteine prevented internalization, and *ii*) Ntcp retrieval was not observed in hepatocytes from p47phox knock-out mice [171]. As indicated above, Fyn also mediates endocytosis of Bsep and Mrp2 from the apical membrane induced by hyperosmolarity, suggesting that regulation of sinusoidal bile salt uptake and canalicular export systems may be coordinately down-regulated.

Apart from NTCP phosphorylation, *S*-nitrosylation by the covalent attachment of NO in one or more cysteine residues is another post-translational regulation that can influence NTCP localization. In HuH-NTCP cells, NO elevations induce retrieval of NTCP via this mechanism [188]. Since during sepsis-induced cholestasis there is a LPS-mediated burst of NO production in hepatocytes [287] associated with a decrease in the density of bile acid transporters in the basolateral membrane (as inferred from a reduction in *V*max for TC uptake in rat hepatocytes) [288], *S*-nitrosylation of NTCP can be indeed relevant to this cholestatic disease.

Oatps are another kind of sinusoidal transporters subjected to endosomal recycling. Phosphorylation is central for rapid internalization of Oatp1A1 [58], Oatp1A2 [289], Oatp1B1 [290], and Oatp2B1 [291] into an intracellular vesicular pool in different cellular lines stably transfected with these human OATP isoforms. These phosphorylations depend on PKC activation, and lead to internalization in a clathrin-dependent fashion [289-291]. Unfortunately, studies on Oatp signaling modulation in cholestasis and its role in hepatocyte secretory failure are still lacking.

Although it is now clear that signaling modulation underlies most, if not all, cholestatic hepatopathies, clearly, a great deal of research is still necessary to understand the intricate network of signaling pathways implicated in each of them. For this purpose, identification of signaling proteins that complete the missing links is awaited. In addition, comparison among cholestatic models is expected to help to identify common signaling mechanisms that trigger transporter endocytic internalization, which may become appealing targets for new therapeutic approaches.

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

Our now better understanding of the cellular mechanisms involved in hepatocellular carrier internalization from both the structural and regulatory point of view has opened a big deal of novel possibilities to modulate this phenomenon with a therapeutic aim.

Numerous pharmacological targets, ranging from molecules of the endocytic machinery to the numerous signaling pathways regulating the phenomenon, are emerging as promising therapeutic alternatives. Once identified, inhibition of these pathogenic molecules *before* the cholestatic insult occurs (*i.e.*, in a "preventive" therapeutic scheme) will be in advance successful, but it is expected to be also beneficial if the inhibition is carried out when cholestasis is already established (*i.e.*, in a "curative" therapeutic scheme). Indeed, blockage of signaling pathways involved in carrier endocytosis leads to a very rapid recovery to normality even in presence of the cholestatic agent, as we have shown in oxidative stress-induced cholestasis after cPKC inhibition [153]. Furthermore, after a transient cholestatic insult with E17G, a similar quick spontaneous recovery has been shown to occur concurrently with the cholestatic agent depuration in the whole rat, by the existence of the microtubule-dependent retargeting of the endocytosed transporters [252]. However, E17G activates simultaneously a signaling pathway that halts carrier re-insertion (*i.e.*, PI3K/Akt/ERK1/2) [112, 246], and therefore, a curative approach would require inhibition of this pathogenic mechanism as well. Another possible consequence of sustained cholestasis is the breakdown of the endocytosed transporters; in this case, recovery will require the contribution of *de novo* synthesized canalicular transporters to replace the degraded ones. Coincidently, ABC carrier trafficking from the synthesis place to the plasma membrane shares in part the same endosomal compartments as those used by the transiently endocytosed ones, and is regulated by the same signaling mediators [62, 292].

Based on the existence of these recovery mechanisms, several experimental therapeutic interventions have been designed to accelerate the re-insertion of transporters endocytosed by the cholestatic insult. Initially, regulatory molecules known to induce choleresis by favoring insertion of canalicular transporters have been assayed, with the “arm wrestling” rationale that exocytic events may overcome endocytic ones. However, even when many anticholestatic agents induce choleresis under normal conditions by favoring transporter trafficking to the apical membrane, this property often involves a different set of regulatory proteins from those mediating their anticholestatic effects. Therefore, "choleresis" and "anticholestasis" should be regarded as two separate phenomena.

The compounds assayed with a therapeutic aim to counteract the endocytic internalization of apical carriers and/or speed up exocytic reinsertion include cAMP-elevating compounds and the anticholestatic bile salt TUDC, and we will discuss next their therapeutic mechanisms.

6.1. cAMP

This second messenger, or other messengers further stimulated by this molecule (*e.g.*, Ca^{2+}), mediates choleresis induced by certain hormones, such as glucagon and adrenaline, as shown either or both in rats [293-295] and humans [296]. Endogenous bile salts, such as TC, also stimulate cAMP synthesis via activation of AC [258].

cAMP was among the first signaling mediator evaluated in its ability to promote vesicular trafficking in general, and exocytic targetting of basolateral and apical transporters in particular [297-299].

cAMP-induced PI3K activation, presumably by PKA-induced PI3K phosphorylation at Thr^{505}, stimulates Ntcp translocation to the basolateral membrane via Akt-dependent mechanisms [50]. PI3K-induced Akt activation may occur by two signaling pathways involving either *3-phosphoinositide-dependent protein kinase* (PDK) *1* (PDZ1) [277], which is directly activated by PI3K, or PDZ2, whose activation by PI3K requires the mediation of certain PKC isoforms, such as the novel PKCδ [296] or the atypical PKCζ [285]; activation of Akt requires PDK1 phosphorylation, followed by PDK2 phosphorylation, which activates Akt fully only when associated with PKCζ phosphorylation. Alternatively, a direct, non-Akt-mediated activating role for PKCζ in Ntcp targeting has been proposed [281]. Other likely targets of PKCζ are the microtubule motor proteins that propel the trafficking of Ntcp-containing vesicles. Most of these vesicles co-localize with PKCζ, and their motility on microtubules is blocked by inhibitors of both PI3K and PKCζ, and accelerated by PI3K products [51].

Since Ntcp occurs in its phosphorylated form in the endosomal compartment and only the non-phosphorylated form of Ntcp traffics to the plasma membrane, a previous dephosphorylation step is required. This is catalized by PP2B, a Ca^{2+}-calmodulin-dependent phosphatase that is activated by the elevations of cytosolic free Ca^{2+} induced by cAMP [300].

cAMP accelerates the trafficking of carriers to the apical membrane as well. Studies in HuH-NTCP cells suggest that this second messenger stimulates vesicular trafficking of Mrp2 localized in recycling endosomes rather than in the early ones [226]. The signaling mediators involved cAMP-induced Mrp2 vesicular transport to the apical membrane in hepatocytes are uncer-

tain, and somewhat conflicting results exist. cAMP-stimulated translocation of Mrp2 was reported to be entirely mediated by the PI3K-mediated activation of the nPKC PKCδ [301, 302]. Nevertheless, other studies reported on the selective involvement of $p38\alpha^{MAPK}$ isoform [256, 303], which was PI3K independent in nature [303]; this agree well with previous studies showing that cAMP-stimulated targeting of Mrp2 and Bsep to the apical membrane is independent of PI3K [304]. A likely candidate mediating this effect is PKA, since its specific activator, 6-Bnz-cAMP, stimulated trafficking of Bsep to the canalicular membrane in hepatocytes cultured in collagen sandwich. [305]. On the contrary, the signaling pathway involving *exchange protein activated by cAMP* (Epac)/*MAPK kinase* (MEK)/*liver kinase B1* (LKB1)/*AMP-activated protein kinase* (AMPK), which is also activated by cAMP in hepatocytes, seems not to be implicated [305].

Apart from this pro-exocytic activity, cAMP has anticholestatic properties by counteracting the endocytosis of both sinusoidal and canalicular carriers occurring in experimental models of cholestasis.

Studies in rat hepatocytes showed that TLC inhibits TC uptake associated with internalization of Ntcp, and that cAMP would counteract this alteration by promoting insertion of transporters from the endosomal pool via activation of the PKCε/PI3K/Akt pathway [143].

At the canalicular pole, we demonstrated in the whole rat that the freely permeable cAMP analogue dibutyryl-cAMP counteracted partially the rapid, initial drop in bile flow induced by TLC, and extensively shortened the restoration to normality of this parameter that occurs spontaneously during the recovery phase from cholestasis, associated with restoration of Mrp2 localization and function [132]. A somewhat similar pattern of protection by cAMP has been reported by us for both Bsep [144] and Mrp2 [130, 306] in acute E17G-induced cholestasis. This cAMP beneficial effect was reproduced in isolated rat hepatocyte couplets for the alterations in Bsep function and localization induced by both E17G [146, 307] and TLC [132, 307]. Furthermore, an analogous anticholestatic mechanism was described for silibinin, the major active constituent of the hepatoprotector silymarin [307]; silibinin inhibits cAMP phosphodiesterase, thus elevating intracellular cAMP levels [307]. Similarly, exposure to the cAMP-elevating agents, glucagon and salbutamol (a β-adrenergic agonist), both in perfused rat livers [114] and isolated rat hepatocyte couplets [115], prevented E17G-induced impairment of Mrp2 and Bsep localization/function. However, despite these compounds have similar cAMP-elevating effects, the prevention afforded by glucagon was PKA-dependent and microtubule-independent, while that afforded by salbutamol was PKA-independent, but Epac- and microtubule-dependent in nature. Since glucagon and salbutamol effects were additive, these compounds would modulate two different pools of cAMP, which may activate different anticholestatic signaling mechanisms. Furthermore, the fact that cAMP is also involved in opposite, procholestatic effects when stimulated via GPR30 (Fig. **5**) most likely reflects the well characterized compartmentalization of the cAMP signaling by which the final effects resulting from its activation depends on the agonist used to activate them [308].

A PKA-independent anticholestatic effect of cAMP similar to that of salbutamol was observed when using the cAMP analogue dibutyryl-cAMP in rat hepatocyte couplets [307]. This protective effect was inhibited by BAPTA/AM, a Ca^{2+} chelator, suggesting the participation of Ca^{2+}-dependent signaling pathways [307]. cAMP specifically enhances *type II inositol 1,4,5-triphosphate receptor* (InsP3R2)-dependent Ca^{2+} release from the calciosome, independently of PKA activation [309]; coincidently, ATP, which elevates Ca^{2+} via this mechanism, was instrumental in accelerating the targeting of Mrp2 to the canalicular membrane [310].

6.2. Tauroursodeoxycholate (TUDC)

Ursodeoxycholate (UDC) is currently the best established and most widely used drug for the treatment of cholestatic liver disease [311]. TUDC is the main UDC metabolite likely mediating its therapeutic effects. This metabolite prevented the endocytosis of the canalicular export pumps Mrp2 and Bsep in several experimental models of cholestasis, including those induced by TLC [131, 133], E17G [312], and cyclosporine A [136].

In addition to displaying anticholestatic effects, UDC and TUDC induce choleresis when administered to normal liver [313-316]. TUDC stimulates exocytic targetting of Ntcp to the sinusoidal membrane [174], as well insertion of Bsep [281, 282] and Mrp2 [226, 316] in the canalicular domain. This pro-exocytic property has been attributed to its capability to induce hepatocyte swelling [169, 317].

TUDC-induced rapid choleresis involves β1-integrin-mediated extracellular sensing and further activation of *focal adhesion kinase* (FAK)/Src [317, 318]. This is followed by Src-mediated EGFR activation, which leads to rapid activation of PI3K and Ras/Raf [319], and the subsequent dual activation of ERK1/2

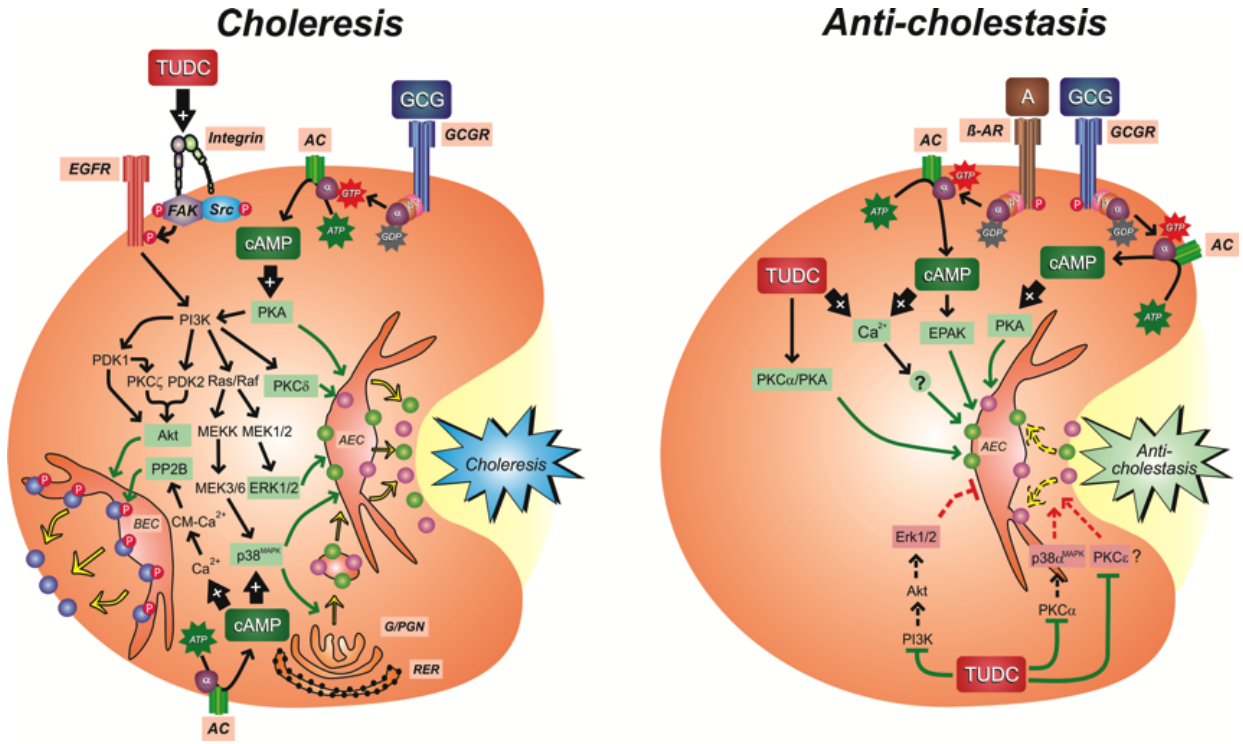

Fig. (8). Signaling pathways involved in choleretic and anticholestatic effects of tauroursodeoxycholate (TUDC) and cAMP-elevating compounds. *Left panel:* Signaling pathways implicated in the choleresis induced by the choleretic compounds TUDC and glucagon (GLC). TUDC induces choleresis via the integrin-induced activation of Src/FAK; this phosphorylates EGFR, which in turn activates PI3K. Downstream, PI3K activates PKCδ (via PDK1) and the Ras/Raf signaling, with the consequent activation of MAPKs of the ERK1/2 and $p38^{MAPK}$ types. Both MAPK types stimulate separately the trafficking of carriers from the apical endosomal compartment (AEC) to the canalicular membrane. $p38^{MAPK}$ also accelerates the trafficking of *de novo* synthesized canalicular transporters from the rough endoplasmic reticulum (RER) to the apical membrane, via the Golgi/post-Golgi network (G/PGN). On the other hand, GLC stimulates insertion of canalicular transporters via a cAMP-stimulated, PKA-dependent mechanism; cAMP is produced by adenilate cyclases (AC) after binding of GLC to its GTP-binding-coupled receptor (GLCR). cAMP also induces PI_3K activation, which leads to targeting of the basolateral transporter Ntcp, via the sequential activation of PDK and Akt, or coactivation of Akt by PDK2/PKCζ. Plasma membrane targeting of Ntcp requires the further activation of protein phosphatase 2 B (PP2B) by the Ca^{2+}/Ca^{2+}-calmodulin complex (Ca^{2+}-CM); the PP2B-mediated Ntcp dephosphorylation is a prerequisite for Ntcp to be stably inserted in the plasma membrane. Like TUDC, cAMP also activates $p38^{MAPK}$, and presumably the $p38^{MAPK}$-stimulated trafficking events described above. *Right panel:* Signaling pathways mediating the anti-cholestatic effects of TUDC and cAMP. TUDC anticholestatic effect was dependent on Ca^{2+}, but the downstream mediators are still unknown. The cooperative activation of PKCα and PKA has also been proposed. TUDC also inhibits the activation of the pro-cholestatic kinases PKCα, PI3K, and PKCε, by inhibiting its activation by phosphorylation; this prevents PKCα and PKCε from inducing retrieval of canalicular transporters, and PI3K from arresting the re-targeting of the endocytosed transporters that have been endocytosed via PKCα/PKCε. As for cAMP, the signaling pathways involved in its anticholestatic effects depend on the hormonal mechanism involved in their stimulation. Whereas the cAMP generated by the binding of β-adrenergic agonists (A) to the β-adrenergic receptor (β-AR) induces anti-cholestasis via Epac, the cAMP generated by binding of GLC to its receptor, GLCR, induces anti-cholestasis via PKA.

[279] and $p38^{MAPK}$ [278], presumably the $p38\alpha^{MAPK}$ isoform of the latter [257]. $p38\alpha^{MAPK}$ also stimulates the vesicular transfer of BSEP from Golgi to the canalicular membrane [280]. Besides, TUDC promotes targetting of Ntcp to the basolateral membrane via the β1-integrin-mediated activation of PKA. Downstream effectors of PKA are unclear, but, like happens with cAMP (see item 6.1), this kinase can activate PI3K by Thr^{505} phosphorylation [284], which in turn may lead to Akt-mediated Ntcp phosphorylation and further plasma membrane insertion via two mechanisms, namely PDZK1- [277] and PDZK2-mediated Akt phosphorylation; the latter phenomenon involves certain PKC isoforms activated by PI3K, such as PKCδ [299] and PKCζ [284].

Although UDC/TUDC choleretic mechanisms have been thought to account for the anticholestatic properties of this bile salt (*i.e.*, the "arm wrestling" hypothesis), UDC/TUDC anticholestatic mechanisms actually involves a dissimilar arrange of signaling pathways (Fig. **8**).

TUDC anticholestatic effect on TLC-induced cholestasis is independent of MAPKs [320], and unlike TUDC choleretic effect which is independent of the Ca^{2+}-dependent PKC isoform PKCα [228, 319], activation of PKCα has been suggested to be involved [131], through a cooperative PKCα/PKA-dependent mechanism [233]. This is however in disagreement with a report showing that specific activation of PKCα with thymeleatoxin induces cholestasis [228], and with our findings that PKA inhibitors failed to block TUDC protective effects against TLC-induced hepatocanalicular dysfunction in the hepatocyte couplet model [321]. Therefore, the issue remains controversial. Anticholestatic TUDC effects are also dependent on intracellular Ca^{2+} [321]; coincidently, TUDC-induced choleresis depends on Ca^{2+} as well, since it was impaired in the InsP3R2 KO mouse, which fails to release Ca^{2+} from intracellular stores [310]. Although the Ca^{2+}-dependent downstream mediators are still unknown, we have shown in the hepatocyte couplet model that trafficking of Mrp2 from intracellular vesicular compartments during hepatocyte couplet repolarization depends on both Ca^{2+} and the Ca^{2+}-calmodulin complex [310]; the latter has been implicated in both microtubule-dependent and microtubule-independent steps in transcytosis [322], via interaction with the microtubule-based and actin-based motor proteins, myosin and kinensin, respectively [109]. Finally, TUDC counteracts activation of the nPKC isoform PKCε, which has been suggested (but not proved) to be involved in TLC-induced cholestasis [131, 237, 257, 323]. Since nPKC isoforms have been involved in other cholestatic events, as for example in oxidative stress-induced cholestasis [156], and there is circumstantial evidences of its involvement from experiments in HuH-NTCP cells showing that TLC induces MRP2 retrieval through PKCε-mediated MARCKS phosphorylation [141], TUDC may indeed exert anticholestatic effects via PKCε inhibition.

Apart from stimulation of carrier exocytosis, another possible post-translational mechanism that might explain the UDC/TUDC-induced enhanced transporter expression in plasma membrane is the prevention of the carrier endocytic process. UDC and TUDC are membranotropic agents that stabilize phospholipid-rich membranes by mimicking cholesterol [324, 325], thus increasing and stabilizing "raft" structures [326]. Since a shift of Bsep from "raft" to "non-rafts" microdomains has been causally associated with ethynylestradiol-induced cholestasis [223], and UDC/TUDC have been shown to bear anticholestatic effects in this model of cholestasis [327-330], it is tempting to hypothesize that UDC stabilizes canalicular export pumps in these enlarged "raft" microdomains, thus preventing their shift to "non-raft" structures, from where they can be endocytosed via a clathrin-mediated mechanism (see item 5.1 for more details). In line with this, UDCA enhances BSEP retention in the apical membrane of MDCK II cells stably transfected with the carrier, as suggested by the improvement of its apical surface expression associated with a noticeable increase in its half-life [331]. If confirmed, enhanced retention of canalicular pumps in "rafts" by UDC/TUDC should have profound functional consequences as well. Intrinsic Bsep transport activity is much higher in "raft" microdomains, where Bsep resides under physiological conditions, since Bsep activity correlates positively with plasma membrane cholesterol content [94, 95]. Therefore, proper localization in "raft" microdomains may be critical not only to prevent carrier endocytosis, but also to maintain optimal transport activity.

CONCLUSION AND PERSPECTIVES

The wide range of information that is currently available on synthesis, trafficking, and recycling of hepatocyte transporters has rendered apparent that modulation of transporter trafficking from their synthesis place or from recycling endosomes, from where they can be sorted to the plasma membrane, are key determinants of the overall liver capability to produce bile on demand, and to dispose endo- and xenobiotics.

The alteration in the dynamic localization of hepatocyte transport systems is a common feature in both experimental and human cholestasis. In spite of the relevance of this pathological mechanism in the occurrence of the cholestatic injury and in its perpetuation via degradation of the internalized carriers, its understanding at the molecular level is its infancy. However, the extraordinary advances in the molecular biology of the hepatobiliary function have potential implications to facilitate the understanding at a molecular level of the regulatory mechanisms governing the physiological dynamic localization of hepatocellular carriers, and their impairment in cholestasis.

The recognition of the key role that transductional pathways have for these processes is the first step in the complex, long-term goal of identifying the molecular

targets of these regulations, and provides a glimmer of hope for the eventual elucidation of the mechanistic and structural basis of this pathomechanism.

Many significant questions remain, however, to be addressed, for example:

(i) Why similar signaling pathways can induce contrary outcomes (*e.g.*, cholestasis or anticholestasis) depending upon the context in which they are activated? Maybe, we have to consider signaling mechanisms as a complex control network involving "switch on" and "switch off" of signaling events, which can modulate, in a binary way, the fate of the major executor pathways.

(ii) Which are the final molecular targets whose phosphorylation status change to trigger transporter endocytosis? Many key molecules known to be determinant of the normal localization of hepatocellular carriers, including cytoskeletal components, endocytic proteins, and the transporters themselves, are modulated by phosphorylation, and the confirmation of their involvement in cholestatic hepatopathies is in progress.

(iii) At which levels empirically employed anticholestatic agents exert its beneficial effects? Do they counteract phosphorylation events that regulate the endocytic event or, alternatively, they act at an upstream level, by stabilizing the transporters anchored to their normal lipid microdomains, thus preventing endocytosis itself? Answer to these questions should prompt the design of novel therapeutic drugs with optimized properties against these cholestatic mechanisms.

(iv) Can alterations in localization of these carriers be prevented, or even better reversed by agents counteracting these dysfunctions? Experimental evidence indicates that retargeting of endocytosed carriers into their membrane domains and cytoskeletal reconstitution occurs spontaneously in a quick and regulated manner, opening the possibility of accelerating this process with a therapeutic aim.

(v) Is exacerbated endocytosis of transporter a specific pathogenic mechanism for hepatocytes or it occurs in other secretory cells? Every cell, irrespective of its type, remodels its plasma membrane protein composition either constitutively or in response to external cues, and endocytosis is a major event in this process. Therefore, it is quite possible that dysregulation of this physiological mechanism occurs under pathological conditions elsewhere; if so, the progress made in its understanding and treatment in cholestasis may be rapidly applied to other secretory disorders.

Answering to these questions would help to envisage new therapeutic approaches in cholestasis to ensure appropriate transporter localization, in an attempt to confer them fully functional activity and to preclude exacerbated degradation. In chronic cholestatic diseases where not only localization but also the whole expression of transporters is impaired, this therapeutics may complement treatments aimed to transcriptionally improve transporter expression, by affording proper localization and membrane stability to the newly synthesized carriers.

We do hope advances in experimental therapeutics based upon these findings prompts clinicians to apply this knowledge to envisage improved, innovative therapeutic options for the treatment of human cholestatic hepatopathies.

LIST OF ABBREVIATIONS

ABC	=	ATP-binding cassette
AC	=	Adenylate cyclase
AE2	=	Anion exchanger 2
AEE	=	Apical early endosomes
AMPK	=	AMP-activated protein kinase
AP-2	=	Adaptor protein complex 2
AQP	=	Aquaporin
ARE	=	Apical recycling endosomes
BCRP	=	Breast cancer resistance protein
BDL	=	Bile-duct ligation
BSEP	=	Bile salt export pump
CNT	=	Concentrative nucleoside transporter
cPKC	=	Ca^{2+}-dependent ('classical') protein kinase C isoforms
CRAC	=	Cholesterol recognition/interaction amino acid consensus
CTR	=	Cupper transporter
E17G	=	Estradiol-17ß-D-glucuronide
EBP50	=	Ezrin/radixin/moesin –binding phosphoprotein of 50 KDA
EGFR	=	Epidermal growth factor receptor
ENT	=	Equilibrative nucleoside transporter
Epac	=	Exchange protein activated by cAMP

ERK1/2 = Extracellular signal-regulated protein kinases 1/2
ERM = Ezrin-radixin-moesin
ERMADs = Ezrin/radixin/moesin association domains
ERα = Estrogen receptor-α
F-actin = Filamentous actin
FAK = Focal adhesion kinase
GPI = Glycosyl-phosphatidylinositol
GPR30 = G-protein coupled receptor 30
GSH = Reduced glutathione
GSSG = Oxidized glutathione
HAX-1 = HS1-associated protein X-1
HuH-NTCP = HuH-7 cells transfected with NTCP
IKEPP = Intestinal and kidney enriched PDZ protein
IL-1ß = Interleukin-1ß
IL-6 = Interleukin-6
InsP3R2 = type II inositol 1,4,5-triphosphate receptor
JNK1/2 = c-Jun N-terminal kinase 1/2
LKB1 = Liver kinase B1
LPS = Lipopolysaccharide
MAPK = Mitogen-activated protein kinase
MARCKS = Myristoylated alanine-rich C kinase substrate
MATE = Multidrug and toxin extrusion
MDR = Multidrug-resistance protein
MEK = MAPK KINASE
MRP = Multidrug resistance-associated protein
NAFLD = Non-alcoholic fatty liver disease
NASH = Non-alcoholic steatohepatitis
NOX = NADPH OXIDASE
nPKC = 'novel' protein kinase C isoforms
NTCP = Na^+-taurocholate cotransporting polypeptide
OATP = Organic anion-transporting polypeptide
OCT = Organic cation transporter
PAG = Phosphoprotein associated with glycosphingolipid-enriched microdomains
PBC = Primary biliary cirrhosis
PDZ1 = 3-phosphoinositide-dependent protein kinase 1
PDZK1 = Postsynaptic density 95/discs large/zonula occludens-1 domain-containing 1
PEPT = Oligopeptide transporter
P-gp = P-glycoprotein
PI3K = Phosphoinositide 3-kinase
PKA = Protein kinase A
PKC = Protein kinase C
PP-1 = PKC-dependent protein phosphatase-1
PP2B = Protein phosphatase-2B
RACK1 = Receptor for activated C-kinase 1
RFC = Reduced folate carrier
ROS = Radical oxygen species
S1PR2 = Sphingosine-1 receptor 2
Sf9 = Spodoptera frugiperda
SH3 = Src homology-3
SLC = Solute carrier
SN1 = SYSTEM N 1
SUMO = Small ubiquitin-like modifier
*t*BOOH = *tert*-buthylhydroperoxyde
TC = Taurocholate
TCDC = Taurochenodeoxycholate
TLC = Taurolithocholate
TNF-α = Tumor necrosis factor-α
TUDC = Tauroursodeoxycholate
UDC = Ursodeoxycholate

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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