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Abstract

Oxidative stress is a common feature in most hepatopathies. Accumulating evidences indicate that reactive oxygen species (ROS) induce a number of functional changes either deleterious or adaptive in the capability of the hepatocytes to produce bile and to secrete exogenous and endogenous compounds. This review is aimed to describe the mechanisms involved in these changes. For this purpose, we will summarize:

1. The current evidence that acutely induced oxidative stress is cholestatic, by describing the mechanisms underlying the hepatocyte secretory failure, including the disorganization of the actin cytoskeleton and its most noticeable consequences, that is, the impairment of tight-junctional structures and the endocytic internalization of canalicular transporters relevant to bile formation.
2. The role for oxidative-stress-activated signalling pathways in the pathomechanisms described above, particularly those involving Ca^{2+} elevation and its consequent activation via Ca^{2+} of "classical" and "novel" PKC isoforms.
3. The mechanisms involved in the adaptive response against oxidative stress mediated by ROS-responsive transcription factors, such as upregulation of GSH synthesis pathway, antioxidant enzymes, and hepatocellular efflux pumps.
4. The consequences on hepatocellular secretory function when this adaptive response can be surpassed by the sustained/high production of ROS. This deleterious effects include transcriptional and posttranscriptional changes in the expression of transporters relevant to bile formation, as has been shown to occur, for example, after long-term administration of aluminum to rats, in the Long-Evans Cinnamon rat (a model of chronic hepatic copper accumulation mimicking Wilson's disease), and in ischemia-reperfusion injury.

Keywords (separated by "-")

Actin - Bile secretion - Calcium - Cholestasis - Oxidative stress - Protein kinases - Signalling - Tight junctions

Radical Oxygen Species and Bile Secretion

140

Cecilia L. Basiglio, Flavia D. Toledo, Enrique A. Sanchez Pozzi, and Marcelo G. Roma

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Introduction

Due to its multiple energy-dependent functions, liver has a high mitochondrial metabolic rate and is heavily engaged in detoxification mechanisms that involve redox-enzyme systems. Since these are major sources of endogenous radical oxygen species (ROS), production of these highly reactive, cytotoxic compounds is higher in liver as compared with most organs. Hence, hepatocytes are rich in antioxidant defenses to counterbalance this oxidative challenge (Cesaratto et al. 2004). However, this borderline equilibrium makes liver highly susceptible to the pro-oxidant injury induced by pathological conditions (Kaplowitz and Tsukamoto 1996). Oxidative stress (OS) is a common feature in most hepatopathies including hepatic ischemia-reperfusion injury following hepatectomy or liver transplantation (Czubkowski et al. 2011; Galaris et al. 2006); obstructive cholestasis (Vendemiale et al. 2002); chronic cholestatic liver diseases (Copple et al. 2010; Salunga et al. 2007); sepsis-induced cholestasis (Sakaguchi and Furusawa 2006); viral (Simula and De V 2010), toxic (Stehbens 2003), and autoimmune (Pemberton et al. 2004) hepatitis; alcoholic (Wu and Cederbaum 2009) and nonalcoholic (Koek et al. 2011) steatohepatitis; and pathologies leading to hepatic accumulation of heavy metals, such as iron (hemochromatosis, iron-loading anemia) (Alla and Bonkovsky 2005) or copper (Wilson's disease) (Dalgic et al. 2005).

In recent years, evidence has accumulated that OS is cholestatic. Functional changes involve impairment of biliary secretion through both direct oxidative damage of cellular structures involved in this process or, more significantly, via modification of intracellular signal transduction pathways sensitive to changes in the intracellular redox state. We will summarize here the mechanisms involved in these alterations.

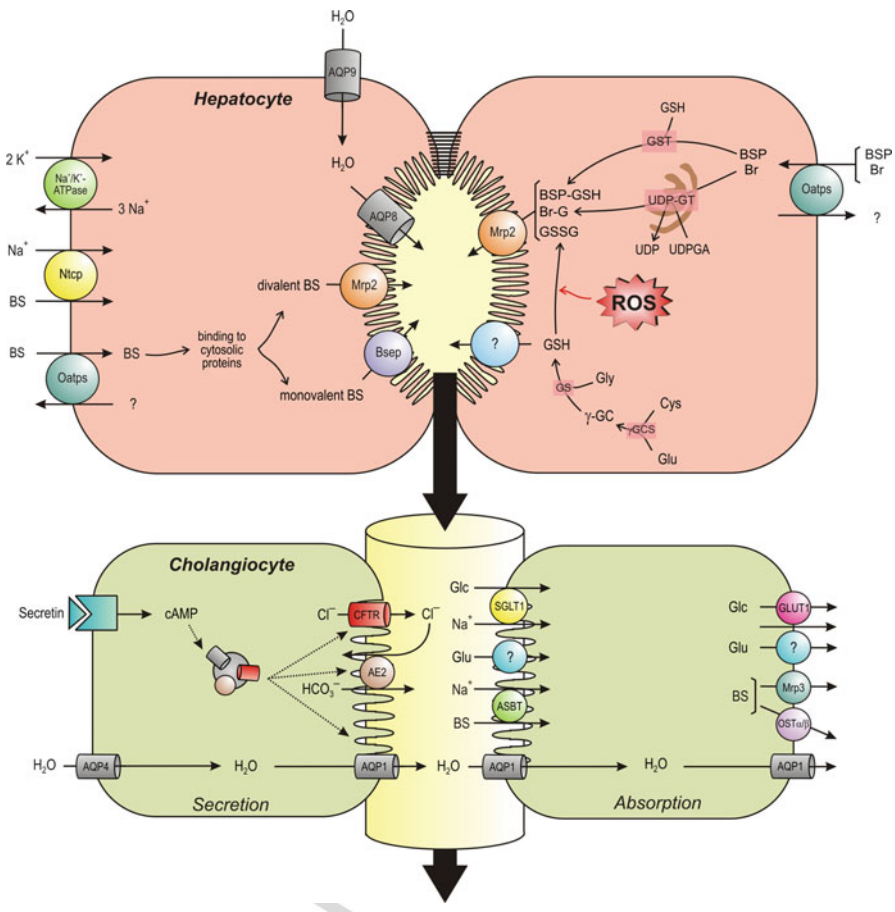

Fig. 140.1 Main transport systems and metabolic events involved in bile flow formation and the hepatic handling of the endogenous and exogenous cholephilic organic anions bilirubin and sulfobromophthalein (BSP), respectively. For details, see section "Normal Mechanisms of Bile Formation"

Normal Mechanisms of Bile Formation

Bile formation is an osmotic process driven by the vectorial transport of certain osmotic solutes into bile, mainly bile salts and both oxidized (GSSG) and reduced (GSH) glutathione (see Fig. 140.1). For these solutes to drive blood-to-bile water transport, they need to be concentrated and retained into a confined space (the bile canaliculus), sealed by tight-junctional structures localized in the paracellular pathways. Once secreted, these solutes induce osmotic water movement, mainly through aquaporins type 9 and 8, located at the basolateral and apical membranes, respectively (Marinelli et al. 2011).

This primary (canalicular) secretion is further modified by cholangiocytes during its transit along bile ducts, as a result of a balance between hormone-dependent water and electrolyte secretion and, on the other hand, the obligatory absorption of water, electrolytes and organic solutes (Elsing et al. 1996; Marinelli and LaRusso 1996).

Bile salts are the predominant organic solutes in bile. The main sinusoidal transport system for bile-salt uptake is the *Na^+-taurocholate cotransporting polypeptide*, which has been cloned from both rat (Ntcp, *Slc10a1*) (Hagenbuch et al. 1991) and human liver (NTCP, *SLC10A1*) (Hagenbuch and Meier 1994). Ntcp/NTCP is driven by a transmembrane Na^+ gradient maintained by the Na^+/K^+-ATPase pump, which is also localized in the sinusoidal membrane (Bohan and Boyer 2002). Ntcp/NTCP accounts for the transport of more than 80 % of amidated bile salts (the major circulating bile salts) and only 40 % of their unconjugated, parent compounds (Kouzuki et al. 1998). The remaining fraction of circulating bile salts is taken up by a non-electrogenic, Na^+-independent transport system, formed by a family of transporters collectively named *organic anion-transporting polypeptides* (Oatps/OATPs for rat and human, respectively) (Kullak-Ublick et al. 2000). Apart from bile salts, Oatps/OATPs accept a wide range of amphipathic, organic compounds, including bilirubin, bilirubin glucuronides, leukotrienes, estrogens, "type II" organic cations, and several exogenous organic anions, the cholephilic dye sulfobromophthalein (BSP) being a prototypical example of the latter one (Hagenbuch and Meier 2003).

After traversing the cell by Fick's diffusion bound to high-affinity cytosolic proteins, monoanionic bile salts (C24 amides conjugated with glycine or taurine) are excreted in the canalicular pole by the *bile-salt export pump* (BSEP/Bsep; *ABCB11*/*Abcb11*), an ATP-binding cassette transporter (Suchy and Ananthanarayanan 2006). In contrast, canalicular efflux of divalent, bipolar sulfated or glucuronidated bile salts is mediated by the *multidrug resistance-associated protein 2* (MRP2/Mrp2; *ABCC2*/*Abcc2*). This carrier also transfers endogenous and exogenous non-bile-acid organic anions conjugated with glutathione and glucuronic acid, including bilirubin glucuronides and BSP both in its unconjugated and conjugated forms (Nies and Keppler 2007).

The bile-salt-independent fraction of the bile flow depends on glutathione excretion, mainly in its reduced form (~80 %) (Ballatori and Truong 1992). Hepatocellular glutathione transport is poorly understood. The liver is the main site of glutathione synthesis, through a pathway involving two consecutive steps, catalyzed by the enzymes γ-glutamyl-cysteinyl synthetase (γ-GCS) and glutathione synthetase (GS). The tripeptide is then exported into both blood and bile, and all biliary glutathione comes from this intracellular source (Garcia-Ruiz et al. 1992). However, a high-affinity, electrogenic carrier has been functionally characterized, but not cloned as yet (Ballatori and Dutczak 1994), which exports actively GSH into bile, and can transfer with low-affinity GSSG and GSH conjugates as well. Another transporter likely involved in glutathione canalicular transport is Mrp2. However, this carrier bears low affinity towards GSH, although it can transfer GSSG and GSH conjugates with high affinity (Yang and Hill 2001).

Canalicular bile flow is further modified during its transit along bile ducts by both secretory and absorptive processes (Bogert and LaRusso 2007). Ductular fluid secretion is mainly driven by the secretin-regulated, cAMP-dependent output of a HCO_3^--rich fluid secreted via the Cl^-/HCO_3^--exchange system, *anion exchanger 2* (Ae2/AE2). Exchange is dependent on the out-to-in Cl^--concentration gradient, which is maintained by the Cl^- efflux across the apical membrane via the ATP-dependent, secretin-activated, *cystic fibrosis transmembrane regulator* (CFTR). Blood-to-bile water movement at the ductular level is facilitated by constitutive AQP4 in the basolateral membrane and secretin-stimulated AQP1 in the apical membrane (Marinelli et al. 2011). On the other hand, absorption of ductular water and electrolytes is driven by the osmotic gradients created by bile-to-plasma transport of electrolytes and organic solutes. They comprise (*i*) glutamate, transported by as yet unidentified carriers; (*ii*) glucose, transported by SGLT1 and GLUT1 at the apical and basolateral domains, respectively; and (*iii*) bile salts, taken up by the *apical Na^+-dependent bile-salt transporter*, ASBT/Asbt (*SLC10A2*/*slc10a2*), and extruded by both the basolateral export pump, MRP3/Mrp3 (*ABCC3*/*Abcc3*), and the heterodimeric *organic solute transporter*, OSTα-OSTß/Ostα-Ostß (Marinelli and LaRusso 1996; Xia et al. 2006).

Changes in Hepatobiliary Secretory Function Induced by OS

Compelling evidence in the literature indicates that oxidative challenge affects the hepatocyte secretory machinery by impairing both bile flow (hepatocellular cholestasis) and the biliary excretion of both endo- and xenobiotics.

OS-induced impairment of bile flow generation has been demonstrated to occur soon after exposure to a number of pro-oxidant agents, including *tert*-butylhydroperoxide (*t*BOOH) (Ballatori and Truong 1989; Schmitt et al. 2000), hydrogen peroxide (Akerboom et al. 1984; Ballatori and Truong 1989), menadione (te Koppele et al. 1991), allyl alcohol (te Koppele et al. 1991), ethylhexanol (te Koppele et al. 1991), chloro-dinitrobenzene (Schmitt et al. 2000), CCl_4 (Eipel et al. 2007), ethacrynic acid (Ji et al. 2004), and lindane (Barros et al. 1988), among others. Some pharmacological agents, such as cyclosporine A (Bramow et al. 2001), dapsone (Veggi et al. 2002, 2005), and nitrofuran derivatives (Hoener 1988), also induce cholestasis due, at least in part, to their pro-oxidant properties. Finally, maneuvers leading to hepatic OS, such as hepatic (Accatino et al. 2003; Bowers et al. 1987; Lee et al. 2000) and intestinal (Turnage et al. 1991) ischemia-reperfusion or aluminum intoxication (Gonzalez et al. 2004, 2007), also induce bile flow impairment.

The classical view to interpret the cholestasis associated to pro-oxidant conditions is based upon the following pathomechanisms:

i. Reduction of the number of living parenchymal liver cells by necrosis and apoptosis depending on the severity of the oxidative injury (Czaja 2007).
ii. Impairment of the bile-salt-dependent fraction of the bile flow due to competitive inhibition of bile-salt transport by the intracellular GSSG formed in excess

during the oxidative challenge (Akerboom et al. 1984; Ballatori and Truong 1989). Indeed, GSSG *cis*-inhibits the transport bile salts in liver canalicular membrane vesicles (Griffiths et al. 1987), and mirror curves showing an inverse relationship between GSSG and bile-salt biliary excretions have been obtained when different pro-oxidizing compounds were administered in the isolated rat perfused liver, such as hydrogen peroxide (Akerboom et al. 1984), menadione (Akerboom et al. 1988), and *t*BOOH (Akerboom et al. 1984; Ballatori and Truong 1989).

iii. Impairment of the bile-salt-independent bile flow due to a decrease in the biliary excretion of total glutathione (GSH plus GSSG). This occurs due to depletion of the hepatic levels of glutathione due to the sustained plasmatic and biliary exportation from the cell as GSSG to maintain the GSH/GSSG ratio (Koeppel et al. 1998).

The above-mentioned mechanisms may be predominant under strong oxidizing conditions. However, under mild or even low oxidizing conditions, bile secretory failure still occurs. For example, upon administration of different pro-oxidant compounds to isolated perfused rat livers, drop in bile flow and/or decrease in bile-salt secretion occurs *before* leakage of cytosolic hepatocellular enzymes or increments in intracellular GSSG become apparent (Ballatori and Truong 1989; Ji et al. 2004). Likewise, in isolated rat hepatocyte couplets, the apical secretion of fluorescent bile-salt analogues was impaired by low concentrations of the pro-oxidant compounds *t*BOOH and 2,3-dimethoxy-1,4-naphthoquinone, even when cell viability and intracellular GSSG levels remained unaffected (Pérez et al. 2006a). Overall, these results suggest that more subtle changes in the machinery involved in bile formation occur under mild OS conditions. Among them, the actin-cytoskeletal disruption, which occurs even at very low OS levels, seems to be a crucial causal factor, as discussed next.

Cytoskeletal Integrity and Hepatocanalicular Function

The actin cytoskeleton is a dynamic network of filamentous actin (F-actin), formed by the reversible assembly of monomeric actin (G-actin), spatially distributed as a belt around the bile canaliculus.

Actin cytoskeleton is one of the primary targets of ROS. The oxidative challenge promotes the oxidation of actin at a sulfhydryl group of a cysteine in position 374 (Dalle-Donne et al. 2001). This induces conspicuous changes in actin-spatial distribution, resulting in marked changes in the cellular topology (plasma membrane blebbing) (Dalle-Donne et al. 2001; Mirabelli et al. 1988).

Most of the above-mentioned roles of actin in cellular biology apply to hepatocytes, and many of them are involved in the biliary secretory processes. It is therefore not surprising that disorganization of the actin cytoskeleton induced by ROS has several deleterious effects on hepatobiliary function.

The interrelationship between ROS, Ca^{2+} elevations, actin-cytoskeletal integrity, and hepatocanalicular secretory function was exhaustively investigated in the

1990s by our group, using the hepatocyte couplet model. These studies revealed that, under mild OS conditions with preserved hepatocellular viability, a close relationship exists between the disarrangement of the pericanalicular actin cytoskeleton and the impairment in the capability of the couplets to accumulate apically and retain in their canalicular vacuoles fluorescent bile-salt analogues induced by the oxidizing compounds *t*BOOH (Ahmed-Choudhury et al. 1998; Pérez et al. 2006a, b; Roma et al. 1997) and menadione (Stone et al. 1994, 1996). These two independent tests indicated that both the apical secretion of bile salts and their further tight-junctional-dependent retention in the bile canaliculus are impaired early under OS conditions. Impairment of tight-junctional permeability was also observed in isolated perfused rat livers exposed to *t*BOOH (Ballatori and Truong 1989).

These functional alterations to secrete and retain bile salts in the biliary space seem to have a structural correlate. *t*BOOH induces disorganization of the tight-junctional complex in hepatocyte couplets, as suggested by the redistribution of the tight-junctional-associated protein, ZO-1 (Pérez et al. 2006a); F-actin is anchored to *zonula occludens*-associated proteins thus regulating paracellular permeability (Anderson and Van Itallie 1995). In addition, the canalicular bile-salt transporter Bsep suffers endocytic internalization into intracellular vesicles in hepatocyte couplets (Pérez et al. 2006b), which reduces dramatically the density of transporters properly located at the membrane domain. A similar phenomenon has been described for the canalicular transporter Mrp2, which suffered endocytic internalization after exposure of isolated perfused rat livers to pro-oxidant insult, such as exposure to *t*BOOH (Schmitt et al. 2000), chloro-dinitrobenzene (Schmitt et al. 2000), ethacrynic acid (Ji et al. 2004; Sekine et al. 2006), and lipopolysaccharide (LPS) (Sekine et al. 2010), or after hepatic ischemia-reperfusion (Yu et al. 2007). Mrp2 relocalization has a clear-cut functional correlate. Experiments in isolated perfused rat livers indicated that a high, sustained exposure to ethacrynic acid has an inhibitory effect on the excretion of both unchanged and conjugated forms of the model cholephilic dye and Mrp2 substrate BSP (James and Ahokas 1992). Mrp2 relocalization under OS conditions is reversible in nature. When the OS induced by *t*BOOH in isolated perfused rat livers was reverted by replenishment of GSH with the cell-permeable form, GSH-ethyl ester internalized Mrp2 was relocalized back to the canalicular membrane in a microtubule-dependent manner (Sekine et al. 2008).

The exact mechanisms that link OS-induced actin disorganization with tight-junctional impairment and transporter internalization are unknown, but previous studies in the literature provide some clues. Hepatic tight-junctional permeability increases following administration of the actin-disrupting agent phalloidin (Elias et al. 1980). F-actin is anchored to tight-junctional-associated proteins (e.g., ZO-1), and it is likely that F-actin disorganization induces relocalization of *zonula occludens* intermediary proteins or even proteins forming the tight-junctional strands, such as occludin and claudin.

Phalloidin-induced F-actin disorganization also induces internalization of canalicular transporters, such as Mrp2 (Rost et al. 1999). The retrieval of canalicular transporters under OS conditions (Ji et al. 2004; Pérez et al. 2006b; Schmitt et al.

2000; Sekine et al. 2006) is therefore also likely due to the simultaneous F-actin disarrangement. The molecular bases to understand this causal relationship are just emerging. Mice lacking radixin, which cross-links actin filaments and plasma membrane proteins, develop conjugated hyperbilirubinemia associated to retrieval of Mrp2 (Kocher et al. 1999), and the same holds true for obstructive and estrogen-induced cholestasis, where a disturbed colocalization of Mrp2 and radixin is associated with Mrp2 endocytic internalization (Kojima et al. 2008). Interestingly, Au3
the internalization of Mrp2 that occurs after hepatic ischemia-reperfusión is coincident with a virtual loss of radixin expression (Shu et al. 2007), and ROS-mediated dephosphorylation and relocalization of radixin has been proposed to account for LPS-induced Mrp2 internalization (Saeki et al. 2011). This latter phenomenon has been associated with the OS induced by this cytokine (Sekine et al. 2010) and seems to involve a decrease in the total amount of the active, phosphorylated form radixin and its degree of interaction with Mrp2 (Saeki et al. 2011).

Mediation of Signal Transduction Pathways in OS-Induced Acute Hepatocanalicular Dysfunction

Cytosolic Ca^{2+} elevations occur under OS conditions, due to both the entry of extracellular Ca^{2+} via plasma membrane receptor-operated Ca^{2+} channels and the release of Ca^{2+} from intracellular Ca^{2+} storages, particularly in the endoplasmic-reticulum (calciosome) (Reed 1990). Ca^{2+} elevations are a major determinant of the impairment in bile secretion following the oxidative injury. The intracellular Ca^{2+} chelator BAPTA/AM fully prevents the impairment induced by low levels of *t*BOOH in the capability of the hepatocyte couplets to accumulate and retain in their canalicular vacuoles bile-salt-fluorescent analogues (Stone et al. 1994). Suggestively, the associated actin-cytoskeletal disarrangement is also prevented by BAPTA/AM, further supporting a causal relationship between both phenomena. Furthermore, Ca^{2+}-elevating agents, such as the Ca^{2+} ionophore A23187 (Stone et al. 1994) or the inhibitor of endoplasmic-reticulum Ca^{2+}-ATPase thapsigargin (Ballatori and Truong 1989), mimic the deleterious effects of ROS on both actin-cytoskeleton integrity and hepatocanalicular function.

A number of signal pathways downstream of Ca^{2+} are involved in this phenomenon. Activation of Ca^{2+}-dependent, "classical" protein kinase C isoforms (cPKCs) seems to be one of the most important ones. Our group demonstrated that the pro-oxidant agent *t*BOOH induces cytosolic-Ca^{2+} elevations and translocation of the cPKC isoform, PKCα, from the cytosol to the plasma membrane in isolated hepatocytes, even at concentrations low enough to only affect the biliary secretory machinery (Pérez et al. 2006a). Furthermore, several previous findings showed strong similarities between the effect of ROS and those induced by Ca^{2+} and PKC agonists, namely: (*i*) Both cytosolic Ca^{2+} elevations (Nathanson et al. 1992a) and PKC activation (Corasanti et al. 1989) impair bile flow generation in the isolated perfused rat liver (Corasanti et al. 1989), in part by increasing paracellular permeability (Kan and Coleman 1988; Llopis et al. 1991); we and others further

characterized these effects in the hepatocyte couplet model and showed that cytosolic Ca^{2+} elevations impair the couplet capability to secrete and retain in their canalicular vacuoles fluorescent bile-salt analogues by activating cPKC (Roma et al. 1999) and that PKC activation by vasopressin and phorbol esters reproduced these effects (Nathanson et al. 1992b; Roma et al. 1997, 1998). (*ii*) PKC agonists induce F-actin-cytoskeletal disarrangements (Roma et al. 1998), and Ca^{2+}-elevating agents reproduced these effects by a PKC-dependent mechanism (Roma et al. 1999).

Final confirmation of a crucial role for cPKC activation in actin disorganization and hepatocanalicular dysfunction induced by ROS was provided by recent studies in hepatocyte couplets. ROS-mediated actin-cytoskeleton disarrangements were fully prevented by both PKC-pan-specific and cPKC-specific inhibitors (Pérez et al. 2006a). More relevant from the therapeutic point of view, both cytoskeleton disruption and canalicular dysfunction were reversed within 1 h by these inhibitors (Pérez et al. 2006a).

The retrieval of the bile-salt transporter Bsep from the canalicular membrane was also fully prevented by PKC antagonists (Pérez et al. 2006b). The same holds true for the impairment of the tight-junctional-retentive properties, another possible consequence of the actin disassembly induced by exposure to pro-oxidant agents (Pérez et al. 2006a). The mechanisms that explain the harmful effect of PKC on actin integrity and, by extension, on the hepatocanalicular function as a whole remain unclear. PKC phosphorylates and/or disorganizes several actin-cytoskeletal components, including actin itself, actin-associated proteins, and membrane-cytoskeletal cross-linked proteins (Keenan and Kelleher 1998; Larsson 2006).

The kind of canalicular protein that is internalized under oxidative-stress conditions and the signalling molecule involved seem to depend on the pro-oxidant agent employed and on the magnitude of the oxidative damage. Low concentrations of the oxidizing compound, ethacrynic acid, do not translocate cPKC but "novel" PKC isoforms (nPKC). Under these conditions, Mrp2 but not Bsep is internalized, by a mechanism probably involving Ca^{2+}-dependent activation of inducible nitric oxide (NO) synthase (iNOS), followed by NO-mediated cGMP increase and further cGMP-activated nPKC (Sekine et al. 2006). However, higher ethacrynic acid doses, capable of activating cPKC isoforms as well, induce internalization of both Bsep and Mrp2 (Sekine et al. 2006).

As summarized in Fig. 140.2, a picture is emerging on the effect of acutely induced OS on the hepatobiliary function. Under mild ROS challenge not affecting hepatocellular viability, Ca^{2+} elevations induce cPKC and/or nPKC activation. This brings on a number of alterations in both function and localization of structures relevant to bile formation, such as actin cytoskeleton, canalicular transporters, and tight-junctional components. This impairs, in turn, the biliary secretion and the further retention of solutes that provide osmotic driving force for bile formation. Other factors such as GSSG-induced *cis*-inhibition of bile-salt transport, reduced biliary excretion of glutathione due to intracellular glutathione depletion, and hepatocellular death may become contributing factors, depending on the magnitude of the pro-oxidant condition.

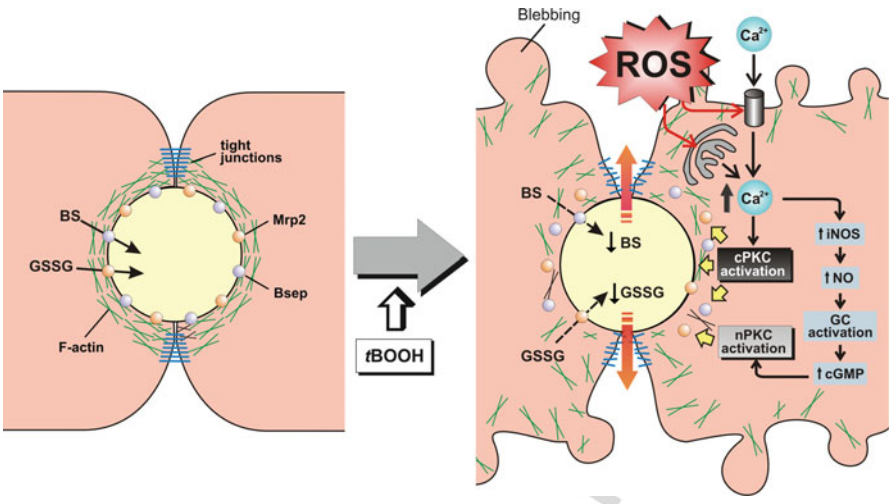

Fig. 140.2 Effect of the model oxidizing compound *tert*-buthylhydroperoxyde (*t*BOOH) on the canalicular transport of bile salts via Bsep and of oxidized glutathione (GSSG) via Mrp2. In normal cells, the pericanalicular localization of F-actin allows for the normal localization of the canalicular transporters in their membrane domain and the proper barrier function of the tight-junctional structures. The acute exposure to *t*BOOH induces mobilization of Ca^{2+} across the plasma and from the calciosome membranes and the further activation of Ca^{2+}-dependent, "classical" PKC isoforms (cPKC). This activation leads to relocalization of F-actin to the cell body, which in turn induces blebbing, Bsep/Mrp2 internalization, and tight-junctional disorganization; these two latter events explain the impairment of bile-salt (BS) and GSSG biliary excretion and their further canalicular retention. Cytosolic Ca^{2+} elevations may also activate inducible nitric oxide (NO) synthase (iNOS), which leads to NO-mediated activation of guanylate cyclase (GC) and further cyclic guanosine monophosphate (cGMP)-mediated activation of "novel" PKC isoforms (nPKC); nPKC activation internalizes selectively Mrp2

The Antioxidant Adaptive Hepatic Response and Bile Secretion

Hepatocytes develop an adaptive response against ROS when the oxidative insult is sustained (Fig. 140.3). This response involves induction of antioxidant enzymes such as catalase (Sen et al. 2005) and manganese superoxide dismutase (Kwak et al. 2001), as well as increments of glutathione synthesis via induction of γ-glutamyl-cysteinyl synthetase (Yamane et al. 1998). In addition, hepatic adaptation integrates the distinctive metabolizing and secretory capacity of the organ to reinforce these antioxidant mechanisms. In this context, it is crucial the OS-mediated induction of Au4 the phase-II-detoxifying enzymes glutathione-*S*-transferase (GST) (Kohle and Bock 2007) and UDP-glucuronosyltransferase (UGT) (Kwak et al. 2001).

GST induction enhances the coordinated inactivation, via GSH conjugation, of DNA hydroperoxides and lipid hydroperoxides formed as secondary metabolites during OS (Ketterer and Meyer 1989). GST also catalyzes GSH conjugation of highly reactive, toxic α,ß-unsaturated lipid aldehydes, 4-hydroxy *trans*-2-nonenal

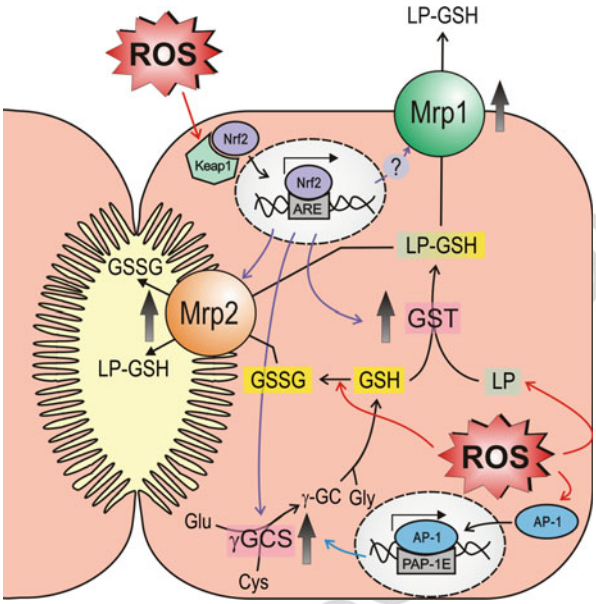

Fig. 140.3 Main hepatocellular adaptive changes induced by sustained OS in the expression of enzymes, and transporters involved in: (*i*) glutathione (GSH) synthesis via γ-glutamylcysteine synthetase (γ-GCS) and GSH synthetase (GS), (*ii*) GSH conjugation of lipid peroxides or their aldehydic derivatives (LP) via GSH *S*-transferase (GST), and (*iii*) plasmatic and biliary extrusion of conjugated LP and oxidized glutathione (GSSG) via both Mrp1 and Mrp2, respectively. This adaptive response in mainly governed by the redox-sensitive transcription factor Nrf2, which escapes from its cytosolic repressor Keap1 and translocates to the nucleus under OS conditions. Once in there, it binds to the *antioxidant response element* (ARE) and activates ARE-dependent gene transcription, including that of γ-GCS, GST and Mrp2; Mrp1 activation is instead independent on Nrf2. The effect of Nrf2 on γ-GCS is reinforced by the activation of the transcription factor AP-1, via a proximal AP-1 element (PAP-1E). For further details, see section "The Antioxidant Adaptive Hepatic Response and Bile Secretion"

(HNE) being the most abundant (Renes et al. 2000). These lipid peroxides combine spontaneously with cysteine, histidine, and lysine residues of proteins, which modifies protein function and leads eventually to cellular toxicity.

UDP-glucuronosyltransferase induction improves glucuronidation of pro-oxidant toxicants, such as benzo(*a*)pyrene (Byczkowski and Gessner 1987), pentachlorophenol (Umemura et al. 2006), acetaminophen (Clement and Williams 2005), aliphatic alcohols (Ebner and Burchell 1993), and manadione (Liu et al. 1993). These phase-II products are then extruded from the cell via the hepatocellular efflux pumps MRP1, MRP2, MRP3, and MRP4 (ABCC4) and *breast cancer resistance protein* (BCRP, ABCG2), all of which are also upregulated by ROS (Adachi et al. 2007; Aleksunes et al. 2008; Vollrath et al. 2006). Glutathione conjugates are substrates of MRP2 and MRP1 (Geier et al. 2007). Since these transporters also transfer GSSG, MRP1/2-mediated GSSG extrusion helps to maintain low

intracellular GSSG levels, when GSSG reduction back to GSH via GSSG reductase becomes rate limiting. Unlike MRP1, the basolateral carriers MRP3, MRP4, and BCRP transport glucuro- and sulfoconjugates and bile salts (Geier et al. 2007). MRP1, MRP3, and MRP4 are normally expressed at very low levels in the basolateral membrane of the hepatocytes. Upregulation of basolateral extrusion pumps during a sustained oxidant insult is expected to shift the transfer of substrates normally excreted into bile towards blood, to permit urinary excretion. As an untoward effect of this adaptive response, this phenomenon might decrease the biliary excretion of bile salts, which would contribute to the cholestatic phenomenon.

All these adaptive mechanisms are transcriptional in nature and involve the activation of a number of redox-sensitive transcription factors, such as Nrf2, NF-κB, and AP-1. The transcription factor activated depends on the magnitude of the oxidant insult. Low OS induces Nrf2, whereas higher levels trigger an inflammatory response through the activation of NF-κB and AP-1 (Halliwell and Gutteridge 1999).

Nrf2 is a key transcription factor of the hepatic adaptation to sustained OS. Its induction has been linked to different oxidant agents such as the cancer chemoprotective agent 3H-1,2-dimethiole-3-thione (Kwak et al. 2001), alcohol (Gong and Cederbaum 2006), *tert*-butylhydroquinone (Adachi et al. 2007), acetaminophen (Aleksunes et al. 2008), and bile salts (Tan et al. 2007). The action of Nrf2 depends on its accumulation in the nucleus, where it interacts with the antioxidant response element (ARE) (Nguyen et al. 2003). This is a *cis*-acting enhancer sequence that contains the 5′-TGAC-3′ tetranucleotide present in the genes of enzymes associated with glutathione biosynthesis, redox proteins with active sulfhydryl moieties, drug-metabolizing enzymes, and transporters. Nrf2 induces the transcription of γ-glutamylcysteine synthetase, the rate-limiting enzyme responsible for glutathione synthesis (Kwak et al. 2001). The effect of Nrf2 on this enzyme is reinforced by AP-1. A proximal AP-1 element (−263 to −269) has been identified to be critical in mediating the effect of OS-induced increase in the transcription of the human catalytic subunit of this enzyme (Rahman et al. 1996). Nrf2 also induces GST and UDP-glucuronosyltransferase (Kohle and Bock 2007; Kwak et al. 2001; Yueh and Tukey 2007), as well as the hepatocellular transporters MRP2 (Vollrath et al. 2006), MRP3 (Aleksunes et al. 2008), MRP4 (Aleksunes et al. 2008), and BCRP (Adachi et al. 2007). Nrf2-induced coordinated GST and Mrp2 expression increases the biliary excretion of conjugated BSP and possibly other glutathione-conjugated compounds, such as DNA and lipid hydroperoxides (Reisman et al. 2009). Furthermore, Nrf2 constitutes a defense system against oxidative stress generated in the liver by experimental models of both extrahepatic (Okada et al. 2009) and intrahepatic (Tanaka et al. 2009) cholestasis. Finally, Nrf2 is required for the upregulation of basolateral bile-salt efflux pumps that counteract the deleterious effects of hepatocellular build up of bile salts in cholestasis, as part of the adaptive response against this condition (Tanaka et al. 2009).

Impairment of Hepatobiliary Function Induced by Sustained OS

The adaptive, spontaneous mechanisms that take place in hepatocytes to minimize the deleterious effects of ROS are, however, not always sufficient to prevent hepatocellular oxidative damage. When ROS production is maintained with time at high levels, alterations occurs in the capability of the hepatocyte to produce bile and to secrete cholephilic compounds, mainly because of changes in the expression of transporters.

The Long-Evans Cinnamon rat, an animal model of Wilson's disease, is a prototypical model of high, chronic hepatic OS. These rats have a genetic defect in *Atp7b* gene, which is homologous to the human Wilson's disease gene, resulting in inability to mobilize copper from the liver (Harada et al. 2000). Apart from copper, these rats also have high hepatic iron levels (Kato et al. 1993). Chronic copper/iron accumulation increases lipid peroxidation by 50 %, presumably due to the capability of these metals to induce OS via both mitochondrial dysfunction (Sternlieb et al. 1995) and Fenton-type, copper/iron-catalyzed Haber-Weiss reaction (Yamamoto et al. 2001).

These mutant rats have histological features of cholestasis (Du et al. 2004) and exhibit a number of alterations in hepatic transporter expressions. They have a reduced basal bile-salt biliary excretion due to a posttranscriptional impairment in Bsep expression (Chiba et al. 2007; Levy et al. 2007). On the other hand, mRNA levels of the bile-salt uptake systems Ntcp and Oatp (isoforms Oatp1a1 and Oatp1a4) are decreased, although this has not been confirmed at the protein level (Chiba et al. 2007). Apart from alterations in bile-salt hepatic handling, Long-Evans Cinnamon rats have both hyperbilirubinemia (Du et al. 2004; Yamamoto et al. 2001) and impairment in the excretion of the Mrp2 substrate BSP (Itagaki et al. 2004). If these alterations involve changes in the expression of Mrp2 at a protein level is unknown, but Mrp2 mRNA levels are normal (Chiba et al. 2007). Alternatively, the above-mentioned transcriptional downregulation of Oatps, which also transport non-bile-salt organic anions such as BSP and bilirubin, may be a contributing factor.

Another experimental model of metal-induced chronic OS is that afforded by long-term aluminum (Al^{3+}) exposure to rats (Gonzalez et al. 2007). When administered intravenously for 1–2 weeks, Al^{3+} reduces bile flow, and this impairment correlates directly with Al^{3+} hepatic content; this was associated with elevations of serum bile salts, suggesting impaired hepatic handling of bile salts (Klein et al. 1988). An even more chronic exposure to Al^{3+} (3 months), which doubles the lipid-peroxidation levels, also reduced bile flow and the biliary output of bile salts (Gonzalez et al. 2004). Compartmental analysis of the plasma decay of BSP revealed that both sinusoidal uptake and canalicular excretion of the dye are decreased, the latter phenomenon being associated with a decrease in Mrp2 protein expression (Gonzalez et al. 2004). All these alterations were prevented by administration of the antioxidant vitamin E, suggesting that OS was the main, if not the only, mechanism (Gonzalez et al. 2007).

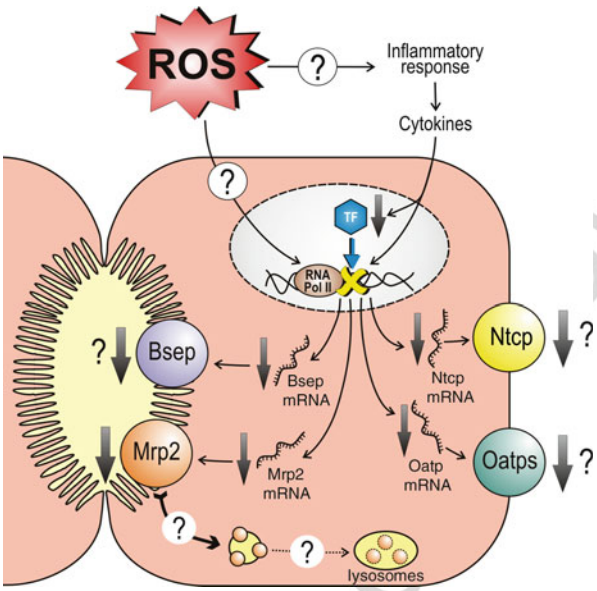

Fig. 140.4 Changes in the expression of transporters relevant to bile flow generation in hepatic injury induced by ischemia-reperfusion. An ischemic period of 60 min, followed by 1 day of reperfusion, decreases the mRNA levels of the basolateral transporters Ntcp and OATP, as well as the canalicular export pumps Mrp2 and Bsep (only confirmed at the protein level for Mrp2). It is unknown whether ischemia-reperfusion exerts these transcriptional effects via ROS-induced changes in degradation/function of RNA polymerase II (RNA Pol II) or, indirectly, by promoting the release of proinflammatory cytokines, which may both downregulate transcription factors (TF) that function as transactivators of hepatocellular transporters and reduce their DNA binding activity. Since downregulation of Mrp2 protein is more severe than that of its mRNA, an additional posttranscriptional mechanism is proposed, which involves OS-induced transporter internalization, followed by lysosomal degradation. For further details, see section "Impairment of Hepatobiliary Function Induced by Sustained OS"

Hepatic ischemia-reperfusion injury is another prototypical OS-mediated hepatopathy associated with both cholestasis (Lee et al. 2000; Lemasters and Thurman 1997) and changes in expression of hepatocellular transporters (Tanaka et al. 2006, 2008) (Fig. 140.4).

The impact of this maneuver on both bile flow generation and the expression of transporters depends on the duration of the ischemia. A 30-min ischemia decreases both bile flow and biliary bile-salt output after 1 day of reperfusion, but no changes occur in mRNA and protein levels of the main basolateral and canalicular bile-salt transporters, Ntcp and Bsep, respectively (Accatino et al. 2003); in this case, changes in localization/intrinsic activity of these transporters or impairment in the expression of other bile-salt transporters, such as Oatps, may explain bile-salt-secretory failure. Similarly, Mrp2, mRNA, and protein expressions are unaffected, in agreement with the absence of changes in the maximum-secretory rate of the Mrp2 substrate ceftriaxone (Accatino et al. 2003). Unlike a 30-min ischemia,

a 60-min ischemic period followed by 1 day of reperfusion decreases the mRNA levels of the basolateral transporters Ntcp and Oatp (all isoforms), as well as those of the canalicular export transporters, Mrp2 and Bsep (Tanaka et al. 2006); this has been confirmed at the protein level only for Mrp2 (Tanaka et al. 2008).

The mechanisms by which these transcriptional alterations occur are far from being understood. First, we have to bear in mind that high levels of OS globally inhibit gene transcription by inducing RNA polymerase II degradative ubiquitination and decrease in histone H3 and H4 acetylation; histone acetylation dissociates DNA from the histone complex, allowing transcription to proceed (Berthiaume et al. 2006). However, at least part of these transcriptional alterations may be due to the ROS-dependent hepatic inflammatory response, which leads to release of cholestatic, proinflammatory cytokines (e.g. TNF-α and IL-1ß) with capability to transcriptionally impair transporter expression (Geier et al. 2007). This phenomenon involves downregulation of the ubiquitous heterodimerization partner retinoid X receptor (RXRα), leading to impairing of the binding activity of nuclear receptor heterodimers that requires RXRα for their transcriptional activity or, in particular, for Ntcp and Mrp2, respectively, and to reduction of the nuclear levels of the monomeric transcription factors hepatocyte nuclear factor-1α (HNF-1α) (Geier et al. 2007) and interferon regulatory factor 3 (IRF3) (Hisaeda et al. 2004).

In ischemia-reperfusion injury, downregulation of Mrp2 protein is more profound than that of mRNA, suggesting additional posttranscriptional mechanisms (Tanaka et al. 2008). Although the causes underlying the latter phenomenon are presently unknown, the early OS-induced transporter internalization, sustained with time, may lead to delivery of the endocytosed transporters to the lysosomal compartment, followed by degradation, as was suggested to occur late in LPS-induced cholestasis (Kubitz et al. 1999) and in obstructive cholestasis (Paulusma et al. 2000) in rats, two cholestatic models exhibiting OS. Apart from alterations in transporter expression/function, the tight-junctional barrier is impaired in ischemia-reperfusion injury, as shown in 24- or 48-h-cold-stored isolated perfused rat livers subjected to reperfusion (Almada et al. 2003).

Taken together, these models of long-lasting OS show consistently that cholestasis and/or impairment of the constitutive expression of transporters relevant to bile formation is a common feature in prolonged OS and that both transcriptional and posttranscriptional mechanisms are involved. Proinflammatory cytokines released by the inflammatory response to the oxidative liver damage may be key mediators.

Future Directions

Cholestasis is a common feature under OS conditions, even at OS levels far lower than those affecting hepatocellular viability. Despite considerable progresses have been made in the characterization of the effects of ROS on the biliary secretory machinery, the characterization of the molecular mechanisms underlying these

effects is in its infancy. A bridge needs to be built between early events and late consequences of OS on bile secretion, in order to reconstruct the cascade of events leading to the posttranscriptional changes in transporter protein expression observed eventually during the sustained oxidative challenge. Also, we need to distinguish direct ROS-mediated effects from secondary consequences of the oxidative injury (e.g., inflammatory response, accumulation of biliary solutes). In addition, we must fully characterize the redox-sensitive signalling pathways involved in these effects; a more complete picture should provide more selective therapeutic strategies to interfere with ROS-mediated harmful pathways or to enhance the protective ones. Studies on the impact of OS on transport function of biliary epithelial cells are also eagerly awaited; cholangiocytes may contribute to bile secretory failure, as they are the main target of cholangiopathies associated with periductal inflammation and OS. And finally, it remains to be ascertained the actual contribution of OS in both transcriptional and posttranscriptional changes in liver transporter expression occurring in chronic cholestatic liver diseases in humans (Geier et al. 2007).

Genomic and proteomic tools are accelerating the discovery of new ROS-responsive genes and the molecular targets of ROS action. These approaches are expected to greatly help to achieve the goals above. Meanwhile, we hope this preliminary information contributes to draw attention about the convenience of limiting OS in hepatopathies with cholestatic features. Some short-scale clinical studies using co-adjuvant, antioxidant therapies have shown encouraging results (Vendemiale et al. 2002), but multicentric and long-term clinical trials are needed to determine whether this strategy holds promise for the future.

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