



# Bile Acid Toxicity and Protein Kinases

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## Abstract

If the bile acids reach to pathological concentrations due to cholestasis, accumulation of hydrophobic bile acids within the hepatocyte may result in cell death. Thus, hydrophobic bile acids induce apoptosis in hepatocytes, while hydrophilic bile acids increase intracellular adenosine 3',5'-monophosphate (cAMP) levels and activate mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) pathways to protect hepatocytes from apoptosis.

Two apoptotic pathways have been described in bile acids-induced death. Both are controlled by multiple protein kinase signaling pathways. In mitochondria-controlled pathway, caspase-8 is activated with death domain-independent manner, whereas, Fas-dependent classical pathway involves ligand-independent oligomerization of Fas.

Hydrophobic bile acids dose-dependently upregulate the inflammatory response by further stimulating production of inflammatory cytokines. Death receptor-mediated apoptosis is regulated at the cell surface by the receptor

expression, at the death-inducing signaling complex (DISC) by expression of procaspase-8, the death receptors Fas-associated death domain (FADD), and cellular FADD-like interleukin 1-beta (IL-1 $\beta$ )-converting enzyme (FLICE) inhibitory protein (cFLIP). Bile acids prevent cFLIP recruitment to the DISC and thereby enhance initiator caspase activation and lead to cholestatic apoptosis. At mitochondria, the expression of B-cell leukemia/lymphoma-2 (Bcl-2) family proteins contribute to apoptosis by regulating mitochondrial cytochrome c release via Bcl-2, Bcl-2 homology 3 (BH3) interacting domain death agonist (Bid), or Bcl-2 associated protein x (Bax). Fas receptor CD95 activation by hydrophobic bile acids is initiated by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent reactive oxygen species (ROS) signaling. However, activation of necroptosis by ligands of death receptors requires the kinase activity of receptor interacting protein1 (RIP1), which mediates the activation of RIP3 and mixed lineage kinase domain-like protein (MLKL). In this chapter, mainly the effect of protein kinases signal transduction on the mechanisms of hydrophobic bile acids-induced inflammation, apoptosis, necroptosis and necrosis are discussed.

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## Keywords

Bile acid · Hydrophobic bile acids · Glycochenodeoxycholic acid (GCDCA) · Na<sup>+</sup>/taurocholate (TC) cotransporter (NTCP) · Apical sodium bile acid cotransporter (ASBT) · Cholangiocytes · Canalicular bile salt export pump (BSEP) · Ursodeoxycholic acid (UDCA) · Tauroursodeoxycholic acid (TUDCA) · Transmembrane G-protein-coupled receptor (TGR5)

## 1 Introduction

Bile acids induce cytotoxic effects, depending on their hydrophobic properties. The retention and accumulation of hydrophobic bile acids in hepatocytes during cholestasis is a major cause of liver damage. Hydrophilic bile acids may induce apoptosis and interleukin-8 (IL-8) synthesis, but not the cytolysis (Araki et al. 2001; Attili et al. 1986). Therefore, the bile salt-induced liver damage is thought to be depend on the total amount, and the hydrophobic-hydrophilic balance of bile salt species (Morita et al. 2011). The cytotoxicity of hydrophobic bile acids may be ameliorated by hydrophilic bile acids under certain conditions. Nevertheless, the cytotoxicity caused by bile salts in hepatocytes is largely determined by bile salt species, bile salt concentrations, cholesterol and cell membranes phospholipids, increased membrane fluidity and permeability (Araki et al. 2003; Ikeda et al. 2017; Morita et al. 2019). Biliary cholesterol solubilization is dependent not only on the concentration of cholesterol itself but also on the phospholipid/bile salt ratio. Increase in the cholesterol/phospholipid ratio makes them more resistant to the solubilizing action of bile salts (Ikeda et al. 2017). The relative cytotoxic effect of bile salts is likely due to both amounts of bile salts accumulated in the cell and direct toxic effects of bile salts including the abilities to disrupt cell membranes and to alter intracellular signaling (Ikeda et al. 2017). In addition, the bile salt molecules modulate canalicular cell membrane phospholipids and membrane fluidity by

penetrating into the phospholipid bilayers, related with their hydrophobic properties (Asamoto et al. 2001). Eventually, hydrophobic bile acids stimulate reactive oxygen species (ROS) generation, release of cytochrome c and apoptosis-inducing factor. Thereby, in human hepatic mitochondria exposed to excess hydrophobic bile acids in cholestasis, ROS generated the mitochondrial permeability transition (MPT) induction leads to mitochondrial pathway-related cell death (Rodrigues et al. 1998; Sokol et al. 2005). In fact, MPT induction is a critical intracellular event that triggers both the apoptotic and necrotic forms of cell death in hepatocytes (Botla et al. 1995; Lemasters et al. 1998; Sokol et al. 2005; Yerushalmi et al. 2001). Hydrophobic bile acids induce hepatocyte apoptosis by activating the death receptor via extrinsic pathway. This pathway involves ligand-independent oligomerization of Fas, recruitment of Fas-associated death domain (FADD), activation of caspase 8, and subsequent activation of all effector proteases, including downstream caspases (Faubion et al. 1999). Furthermore, induction of apoptosis through tumor necrosis factor (TNF)-related apoptosis-inducing ligand- receptor 2 (TRAIL-R2)/death receptor 5 (DR5) expression by bile acids is Fas-independent but it is dependent on death receptors (Higuchi et al. 2001). After death receptor activation and death-inducing signaling complex (DISC) formation, caspase 8 is activated and the pro-apoptotic protein, BH3 interacting domain death agonist (Bid) is cleaved and translocated to the mitochondria. Subsequently, MPT pores open and the cytochrome c is released. A caspase cascade then initiates and, finally, activation of the effector caspases, leads to irreversible hepatocyte death (Higuchi et al. 2001; Reinehr et al. 2004; Scaffidi et al. 1998; Yang et al. 2007).

In intrinsic pathway of apoptosis, bile acids are also able to induce apoptosis through the mitochondrial pathway, in which intracellular oxidative stress causes mitochondrial dysfunction and the subsequent release of proapoptotic factors. ROS generation, MPT induction, and cytochrome c release from mitochondria are critical steps in the induction of apoptosis by bile acids. Antioxidants may reduce liver injury

caused by low levels of bile acids by preventing the generation of oxidant stress (Rodrigues et al. 1998; Yerushalmi et al. 2001). This chapter debates the control mechanisms of protein kinases that are effective on opposite signaling pathways leading to cell death and survival via hydrophobic and hydrophilic bile acids, respectively.

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## 2 Bile Acid Biosynthesis and Transporters

Human liver synthesizes about 200 to 600 mg bile acids per day. A total bile acid pool is approximately 3 g, and consists of approximately 40% cholic acid (CA), 40% chenodeoxycholic acid (CDCA), 20% deoxycholic acid (DCA), and trace amount of lithocholic acid (LCA). All these are recycled 4–12 times a day (Chiang 2013). Primary bile acids, CA and CDCA are synthesized from cholesterol in the liver via two multistep biosynthetic pathways (Gonzalez 2012). In the neutral bile acid pathway (or classic pathway), steroid ring modification precedes side-chain cleavage, whereas in the acidic pathway (or alternative pathway) side-chain cleavage precedes steroid ring modifications (Chiang 2004). Both the classical and the acidic pathways are responsible for the production of at least 95% of the bile acids, and these two pathways contribute about equally to bile acid synthesis in humans (Pellicoro and Faber 2007). The sequential conversion of cholesterol molecules to bile acids via classical neutral synthesis pathway is accomplished by seventeen enzymes, which are located in the cytosol, endoplasmic reticulum (ER), mitochondria, and peroxisomes (Russell 2003). This pathway is responsible for synthesis of approximately 80% of bile acids in human livers and their formation starts with a  $7\alpha$ -hydroxylation of cholesterol by the microsomal cholesterol  $7\alpha$ -hydroxylase (cytochrome P450 7A1; CYP7A1) enzyme. Thus,  $7\alpha$ -hydroxylase (CYP7A1) is the only rate-limiting enzyme in bile acid synthesis, and synthesizes two primary bile acids, CA and CDCA in human liver. The alternative pathway starts with a hepatic or extra-

hepatic sterol 27-hydroxylation by CYP27A, which is a mitochondrial cytochrome P450 enzyme, and widely found in most tissues and macrophages (Duane and Javitt 1999; Norlin and Wikvall 2007). Transcription of CYP7A1 is downregulated by the bile acid-activated farnesoid X receptor (FXR). When bound to bile acids, FXR represses transcription of the gene encoding CYP7A1 and activates the gene encoding intestinal bile acid-binding protein (IBABP) (Makishima et al. 1999; Parks et al. 1999). The acidic pathway may contribute to relatively little, the amount of bile acid synthesis that takes place via the 27-hydroxylation pathway in healthy humans is about 9% of total bile acid synthesis in human hepatocytes (Duane and Javitt 1999). Conjugated bile acids are secreted into bile via the canalicular bile salt export pump (BSEP), forming mixed micelles with cholesterol and phosphatidylcholine in the gallbladder to prevent precipitation of cholesterol and to protect gallbladder epithelium cells from bile acid toxicity. In humans, most bile acids are amino conjugated at the carboxyl group (amidation), with the ratio of glycine-to-aurine-bile acid conjugates in bile is about 3.5:1, dependent on dietary intake of amino acids. At first step, the rate-limiting microsomal synthetase catalyzes the formation of the bile acid-coenzyme A (CoA) thioester, followed by conjugation with taurine or glycine catalyzed by the bile acid-CoA:amino acid N-acyltransferase in cytosol (Hardison and Grundy 1983; Solaas et al. 2000). Once formed, bile acids undergo extensive enzyme-catalyzed taurine and glycine conjugation to form four different “amidated” bile acids. Eventually, taurocholic acid (TCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), and taurochenodeoxycholic acid (T-CDCA) are synthesized. This mixture is actively transported out of the liver into the bile. Glucuronidated and sulphated bile acids contribute to the serum bile acids by 14–32%, 16–44%, to urine bile acids by 4–11% and 61–82% and biliary bile acids by 0.2–1% and 0.3–2%, respectively (Takikawa et al. 1985). GCDCA is the most abundant bile acid of the total pool in human liver with the concentration of 31% and represents a notable differ-

ence from rodents. Conjugated forms with glycine and taurine of CDCA, a primary bile acid in humans, represent about 50% of the total bile acid pool in the human liver, whereas the CA and muricholic acid (MCA) forms are more than 80% of the total volume in rodents. These interspecies differences in the hepatic bile acid pool composition should be taken into consideration in pathological conditions occurred due to specific bile acid accumulation (García-Cañaveras et al. 2012; Humbert et al. 2012). About 90% of the bile acids secreted into the intestine are reabsorbed into portal blood and are then efficiently taken up back by hepatocytes. The ileum is the major site of reabsorption. Bile acids continue to cycle between the intestine and the liver, creating an enterohepatic cycle (Ballatori et al. 2009). In the distal intestine, bacterial  $7\alpha$ -dehydroxylases convert the conjugated-CA and CDCA from CA and CDCA to DCA and LCA, respectively. DCA and LCA are the secondary bile acids. LCA induces its own detoxification by activating nuclear receptors to promote sulfotransferase, which conjugate the LCA by sulfation in the enterocyte. Subsequently, conjugated LCA is effluxed back into the intestinal lumen. In fact, sulfation is the major pathway for detoxification of hydrophobic bile acids in humans (Hofmann 2004). The excretory pole of the hepatocyte forms the border of the bile canaliculus. The basolateral and canalicular membranes differ in their biochemical composition and functional characteristics (Boyer 1980). Basolateral bile salt transport systems are essential for bile formation since major portion of bile salts excreted into bile by the liver are reabsorbed on each pass through the intestine and undergo an “enterohepatic circulation”. Bile salts are taken up by human hepatocytes via the basolateral membrane  $\text{Na}^+$ /taurocholate (TC) cotransporter (NTCP) and organic anion transporting proteins (OATPs) (Hagenbuch and Meier 1994; Hofmann 1999). The bile salt enterohepatic circulation is mainly regulated by three different bile salt transport proteins. These are the canalicular BSEP (ABCB11), the ileal  $\text{Na}^+$ -dependent bile salt transporter ISBT (SLC10A2) and the hepatic sinusoidal  $\text{Na}^+$ -TC cotransporting polypeptide

NTCP (SLC10A1). Other additional transporters include the OATPs (SLC21A) and the multidrug resistance-associated proteins 2 and 3 (MRP2,3: ABCB2,3) (Kullak-Ublick et al. 2000; Trauner and Boyer 2003). During bile secretory failure (cholestasis), bile salt transport proteins undergo adaptive responses that serve to protect the liver from bile salt toxicity. They facilitate extrahepatic routes of bile salt excretion (Trauner and Boyer 2003). Firstly; small amounts of bile acids may escape into the systemic circulation, reabsorbed when passing through the renal tubules in the kidney, and are then circulated back to the liver through systemic circulation. In the renal tubule the apical sodium bile acid cotransporter (ASBT) acts as a salvage mechanism to prevent urinary excretion of bile acids that undergo glomerular filtration (Barnes et al. 1977; Christie et al. 1996). On the other hand, dianionic conjugated bile salts are secreted into bile with the MRP2. In bile ductules, a minor portion of protonated bile acids and monomeric bile salts are reabsorbed by non-ionic diffusion and the ASBT transports back into the periductular capillary plexus by MRP3 or a truncated form of Asbt (tAsbt), and subjected to cholehepatic shunting (Trauner and Boyer 2003). In cholehepatic shunt, bile acids are absorbed in the biliary tract, secreted into the periductular capillary plexus, and carried directly back to the hepatocyte for secretion. Alternatively, ASBT functions to sense bile acid concentration in bile. Thereby, intracellular signaling systems in cholangiocytes is activated via bile acid transport (Xia et al. 2006). Canalicular efflux of divalent sulfated or glucuronidated bile salts is mediated by the MRP2, which is strongly decreased in cholestasis in human. Decreased MRP2 expression leads to compensatory increases in the basolateral expression of MRP1 and MRP3 in liver (Borst et al. 1999; Kullak-Ublick et al. 2000). Intestinal epithelial cells reabsorb majority of the secreted bile acids through both ASBT and sodium independent OATPs. Cytosolic IBABP mediates the transcellular movement of bile acids to the basolateral membrane across which they exit the cells via organic solute transporters (OST). They enter portal venous circulation by a  $\text{Na}^+$ -independent mechanism. Subsequently,

majority of conjugated bile acids are efficiently reabsorbed from portal venous blood into hepatocytes, mainly via the NTCP (Alrefai and Gill 2007). In humans, three liver-specific OATPs (OATP-A, OATP-C, and OATP-8) transport bile acids (Kullak-Ublick et al. 2001). For the hepatic uptake of bile acids, OATP-C is the most relevant isoform and is exclusively localized in liver. Within the hepatocyte, bile acids are bound to cytosolic proteins and then traverse by diffusion. Transport across the canalicular membrane is the rate-limiting step in overall hepatocellular bile acid excretion. This is mediated by the BSEP (Kullak-Ublick et al. 2000; Tamai et al. 2000). The major portion of biliary bile acids are reabsorbed by five different ways, which are included apical OATP3, the ASBT, cytosolic intestinal bile acid-binding protein (cIBABP), and basolateral Mrp3/MRP3 and tAsbt (Meier and Stieger 2002). Intracellular trafficking and membrane insertion of bile salt transporters is regulated by lipid, protein, and extracellular signal-related kinases (ERK) in response to physiologic stimuli such as cyclic adenosine 3',5' monophosphate (cAMP) or TC (Kullak-Ublick et al. 2004). cAMP activates protein kinase C zeta (PKC- $\zeta$ ) in the phosphatidylinositol 3-kinase (PI3K)-dependent manner without inducing translocation of PKC- $\zeta$  to the plasma membrane. Inhibition of cAMP-induced PKC- $\zeta$  activity results in inhibition of cAMP-induced increases in TC uptake and NTCP translocation. Neither dominant-negative protein kinase B (PKB, Akt) nor constitutively active PKB affects cAMP-induced activation of PKC- $\zeta$ . Consequently, cAMP-induced NTCP translocation involves the activation of the PI3K/PKC- $\zeta$  signaling pathway. This is followed by the activation of the PI3K/PKB signaling pathway (McConkey et al. 2004).

Size and function of cholangiocytes are variable according to their location along the biliary tract. Conjugated bile acids are taken up at the apical domain of cholangiocytes via the ASBT. The apical Na<sup>+</sup>-dependent bile acid transporter (ABAT) and the cIBABP messenger RNAs are detected in large cholangiocytes. These proteins mediate bile acid uptake from the duct lumen in large ducts. Bile acid uptake by ABAT and the

PI3K pathway are important for bile acids to signal cholangiocyte proliferation. Thus, in bile duct obstruction, increased biliary bile acid concentration and ABAT expression initiate increased cholangiocyte proliferation and secretion (Alpini et al. 1997, 2002b; Kip et al. 2004; Lazaridis et al. 1997).

A specific BSEP is present at the level of the hepatocyte canalicular membrane. This member of the adenosine triphosphate (ATP)-binding cassette (ABC transporter) family of proteins is the primary transporter of bile acids from the hepatocyte to the biliary system. Transport of bile salts is dependent on ATP hydrolysis (Kubitiz et al. 2012; Soroka and Boyer 2014). The activity of this transporter regulates the formation of the "bile acid-dependent bile flow". One of the important functions of bile acids is their feedback regulation on their own synthesis in the hepatocytes according to their specific concentration in the cell by inhibiting CYP27A1. This mechanism is obtained by direct regulation of FXR by bile acids in hepatocytes. In fact, increased level of bile acids in the hepatocytes activates FXR, which is a strong inhibitor of CYP27A1, the rate limiting enzyme in bile acids synthesis (Makishima et al. 1999). Canalicular ABC (ATP-binding cassette) transporters consist of multidrug resistance gene 1 (MDR1), MDR2, and sister of P-glycoprotein (SPGP). BSEP is exclusively expressed in the liver on the hepatocyte apical, canalicular membrane, evenly distributed throughout the lobular domains. BSEP traffics out of the Golgi directly into a subapical, vesicular compartment where it can reside before moving to the apical plasma membrane. Whereas, there is a direct route from Golgi to the canalicular membrane for trafficking of MDR1, which is an ATP-dependent transporter of organic cations. In hepatocytes, PI3K regulates bile acid secretion and intracellular trafficking of MDR1 (Kipp and Arias 2000; Sai et al. 1999). The half-life of BSEP in the apical membrane is approximately 4–6 days and constitutively recycles between the plasma membrane and subapical vesicles (endosomes). Polarization of hepatocytes requires recruitment of rab11a and myosin Vb to intracellular membranes that

contain apical ABC transporters (Lam et al. 2007; Wakabayashi et al. 2005). Hepatocytes take up bile acids and secrete them again into bile for ongoing enterohepatic circulation. Uptake of bile acids into hepatocytes occurs largely in a sodium-dependent manner by the NTCP. BSEP transports primarily monovalent bile salt species, including taurine and glycine conjugates of primary bile salts, CA and CDCA, and the secondary bile salt, DCA, as well as ursodeoxycholic acid (UDCA). BSEP constitutes the rate limiting step of hepatocellular bile salt transport and drives enterohepatic circulation of bile salts (Stieger 2011). In this context, the intestine also plays an active role in bile acid-mediated suppression of bile acid synthesis in liver. FXR induces the fibroblast growth factor intestine 19 (FGF19). This growth factor activates the cell-surface receptor tyrosine kinase, fibroblast growth factor receptor 4 (FGFR4) signaling, thus inhibiting CYP7A1 mRNA expression levels in human hepatocytes through a c-Jun N-terminal kinase (JNK)-dependent pathway (Holt et al. 2003). Regulation of canalicular bile salt efflux through BSEP, basolateral elimination through organic solute transporters alpha and beta (OST $\alpha$ /OST $\beta$ ) and inhibition of hepatocellular bile salt uptake through basolateral NTCP provides critical steps in protection from bile salt toxicity of hepatocyte (Baghdasaryan et al. 2014). When intracellular bile acid concentration is less than 10  $\mu\text{mol/L}$  in both hepatocytes and stellate cells, it functions as intracellular signaling molecule triggering wide variety of protein kinases. The expression of bile acid transporter is required for bile acid signaling, and the degree of bile acid transporter expression determines the sensitivity of cells to bile acid signaling. UDCA and tauroursodeoxycholic acid (TUDCA)-inhibited ABAT expression is dependent on Ca<sup>2+</sup>-induced PKC $\alpha$  activation in cholangiocytes. Bile acids alter Ca<sup>2+</sup>, cAMP, PKC and PI3K intercellular signaling systems in cholangiocytes. Reduced cholangiocyte ABAT expression decrease endogenous bile acid stimulation of cholangiocyte growth and secretion. Furthermore, TUDCA increases membrane translocation of the Ca<sup>2+</sup>-dependent PKC $\alpha$  and inhibits the activity of mitogen-

activated protein kinase (MAPK), but this response is independent from Raf proteins and MAPK p38 and JNK/stress-activated protein kinases (Alpini et al. 2001, 2002a, 2004; LeSage et al. 2001). Both solute carrier (SLC) and ABC drug transporters can be regulated by PKCs-related signaling pathways (Mayati et al. 2017). Moreover, p38 MAPK and PKC signal transduction pathways are important in the stimulated exocytosis of BSEP by TUDCA from a vesicular compartment to the plasma membrane. In this context, p38 MAPK regulates BSEP trafficking from the Golgi to the canalicular membrane. The Golgi serves as a BSEP pool in cholestasis, when p38 MAPK activity is inhibited. Thereby, activation of p38 MAPK by tauroursodesoxycholate (TUDC) recruits Golgi-associated BSEP. TUDC-induced stimulation of TC excretion is accompanied by a p38 MAPK-dependent insertion of subcanalicular BSEP into the canalicular membrane. Thereby, TUDC induces a transient and concentration-dependent activation of p38 MAPK and of ERK-2 (Kubitz et al. 2004; Kurz et al. 2001). Both ERK and p38 MAPK activation is required for the choleric effects of both TUDC and hypo-osmotic cell swelling (Kurz et al. 2001). G protein- and tyrosine kinase-dependent, PKC-independent activation of MAPKs are involved in the regulation of TC excretion by liver cell hydration changes. Hyperosmolarity induces retrieval of BSEP from the canalicular membrane, correlating with cholestasis, whereas, hypo-osmolarity is accompanied by a rapid recruitment of intracellular BSEP to the canalicular membrane. Hypotonic swelling of cells acts through activation of ERK-1/2 and p38 MAPK, and results in stimulated bile acid excretion. In this procedure, BSEP- and MRP2-specific vesicles participate in the short-term osmoregulation of canalicular secretion. This is due to a cause-effect relationship between bile salt excretion and transporter localization (Häussinger et al. 2000; Noé et al. 1996; Schmitt et al. 2001).

ABC transporters located in the hepatocyte canalicular membrane of mammalian liver are critical players in bile formation and detoxification. Direct Golgi-to-apical membrane

trafficking of ABC transporters provides specific physiological regulation of transporter. However, the PI3K/PKB (AKT) signaling pathway contributes to the biliary secretory failure through the internalization of canalicular transporters endocytosis via classical PKC (cPKC) in cholestasis (Boaglio et al. 2010; Kipp and Arias 2002; Roma et al. 2000). Whereas, PI3K pathway is shown to be involved in exocytosis of ATP-dependent canalicular transporters. PI3K inhibition results in decreasing protein levels in canalicular membrane vesicle and sinusoidal membrane vesicle fractions. PI3K is required for intracellular trafficking of itself, as well as of ATP-dependent canalicular transporters (Misra et al. 1998). Bile acid secretion induced by cAMP and TC is associated with recruitment of several ABC transporters to the canalicular membrane. However, trafficking of BSEP and MRP2 to the canalicular membrane in response to cAMP is independent of PI3K activity (Misra et al. 2003). Hepatocellular canalicular network formation, an important component of hepatocyte polarization, requires activation of the liver kinase B1 (LKB1) and adenosine monophosphate (AMP)-activated protein kinase (AMPK). The serine/threonine kinase LKB1, which is activated by the bile acid TC and, in turn, activates AMPK1/2 has emerged as a key determinant of hepatic polarity. AMPK and LKB1 also participate in network maintenance through preventing low-Ca<sup>2+</sup>-mediated disruption of the canalicular network and tight junctions (Fu et al. 2010; Treyer and Müsch 2013). In fact, LKB1 exerts its effects by phosphorylating and activating 14 different protein kinases, all related to the AMPK. LKB1 is required for establishment of a fully polarized canalicular network. Maintenance of polarity may require subsequent activation of other kinases (Alessi et al. 2006). In brief, LKB1 is an upstream kinase that is activated by bile acids and facilitates the establishment of apical polarity in the hepatocyte. Additionally, its activity is required for microtubule-dependent trafficking of canalicular ABC transporter, ABC subfamily B member 11 (ABCB11) to the canalicular membrane (Fu et al.

2010; Homolya et al. 2014). Thus, LKB1 plays a critical role in bile acid homeostasis. Lack of LKB1 in the liver results in cholestasis (Woods et al. 2011). As mentioned above, TC affects hepatocyte polarity by activating the cAMP-Epac-Rap1-mitogen-activated protein extracellular signal-regulated kinase (MEK)-LKB1-AMPK pathway. TC accelerates canalicular network formation and concomitantly increases cAMP. Similarly, activation of exchange protein directly activated by cAMP (Epac), (cAMP downstream kinase), accelerates canalicular network formation, similar to the effects of TC. Inhibition of Epac downstream targets, Ras-related protein 1 (Rap1) and mitogen-activated protein kinase kinase (MEK), block the TC effect. TC rapidly activates MEK, LKB1, and AMPK, which are prevented by inhibition of adenyl cyclase or MEK (Fu et al. 2011). DCA stimulate ASBT gene expression acting on the down-stream activated protein-1 (AP-1) response element via the epidermal growth factor (EGF) receptor and MEK cascade (Duane et al. 2007).

On the other hand, bile salts as signaling molecules, activate nuclear receptors in the hepatocyte and ileal enterocyte, as well as an increasing number of G-protein coupled receptors (Hofmann and Hagey 2008). Transmembrane G-protein-coupled receptor (TGR5) is activated by bile acids. TGR5 is present in several subcellular locations within the cholangiocyte, including cilia, plasma membranes, intracellular space, vesicles, and ER. TGR5 is expressed on diverse cholangiocyte compartments, including a primary cilium, and its ciliary localization determines the cholangiocyte functional response to bile acid signaling (Masyuk et al. 2013). Hepatocytes do not express TGR5. However, TGR5 is an important mediator of bile acid-induced cholangiocyte proliferation as well as protects cholangiocytes from death receptor-mediated apoptosis via TGR5-dependent CD95 receptor serine phosphorylation. These mechanisms protect cholangiocytes from bile acid toxicity under cholestatic conditions (Reich et al. 2016).

### 3 Protein Kinases-Bile Acids Crosstalk

As mentioned above, subsets of both conjugated and unconjugated bile acids have been shown to activate multiple kinase signaling pathways such as PKC, ERK1/2, MAPK, p38 MAPK, JNK, and/or PI3K/AKT. Bile acid receptors are not only important in the regulation of bile acid synthesis and their metabolism, but also regulate glucose homeostasis, lipid metabolism, and energy expenditure. In addition to their well-known function as dietary lipid detergent, bile acids have emerged as important signaling molecules that regulate energy homeostasis. Bile acids modulate the activity of transmembrane and nuclear receptors by binding to the TGR5 and the FXR, respectively (Trabelsi et al. 2017). Bile acids may mediate a liver/ $\beta$ -cell axis by maintaining the glucose competence of these cells through an FXR-dependent mechanism. Consequently, bile acids or FXR increases glucose-stimulated insulin secretion in primary islets (Seyer et al. 2013). Insulin induces the CYP7A1 gene expression in primary human hepatocytes, while it is repressed by glucagon. However, insulin has dual effects on human CYP7A1 gene transcription. Thus, physiological concentrations of insulin rapidly inhibit FoxO1 activity leading to stimulation of the human CYP7A1 gene, whereas prolonged insulin treatment induces sterol regulatory element-binding protein-1c (SREBP-1c), which inhibits human CYP7A1 gene transcription. Glucagon and cAMP strongly repress CYP7A1 mRNA expression in human primary hepatocytes. In this case, cAMP and PKA inhibit human CYP7A1 gene transcription at PKA-responsive region located within the hepatic nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ) binding site in the human CYP7A1 promoter (Li et al. 2006b; Song and Chiang 2006). Glucose stimulates bile acid synthesis by increasing ATP levels to inhibit AMPK, while inducing HNF4 $\alpha$  to stimulate CYP7A1 gene transcription (Li et al. 2010). Glucose and insulin rapidly induce CYP7A1 gene expression and bile acid synthesis leading to an enlarged bile acid pool and elevated circulating bile acids (Li et al. 2012b). Insulin

signaling activates AKT, and possibly also the MAPK/ERK1/2 pathway to inhibit CYP7A1 gene transcription. On the other hand, glucagon and cAMP strongly inhibit CYP7A1 expression via activation of PKA, which phosphorylates HNF4 $\alpha$  to abolish its DNA-binding activity. Consequently, both insulin and glucagon effects result in inhibition of CYP7A1 expression in human hepatocytes (Song and Chiang 2006). Indeed, bile acids activate specific nuclear receptors (FXR, pregnane X receptor, and vitamin D receptor), TGR5, and JNK1/2/AKT/ERK1/2/MAPK cell signaling pathways in the liver and gastrointestinal tract (Dent et al. 2005; Hylemon et al. 2009). Unconjugated bile acid, DCA activates the Raf-1/MEK/ERK and AKT signaling cascades in primary hepatocytes via an epidermal growth factor receptor (EGFR)/Ras-dependent mechanism. DCA and TDCA cause ROS generation in hepatocytes that is dependent on metabolically active mitochondria. The generation of ROS is essential for protein tyrosine phosphatase (PTPase) inactivation, receptor tyrosine kinase activation, and enhanced signaling down the ERK1/2 and AKT pathways (Fang et al. 2004; Rao et al. 2002). LCA, which is a hydrophobic secondary bile acid produced by colonic microflora, is the most toxic bile acid. The basal as well as inducible glutathione (GSH) levels are important determinants of cellular resistance to LCA toxicity. The activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) induces the major hydroxylation enzymes CYP3As and antioxidative genes. Thereby kelch-like ECH-associated protein 1 (Keap1)-Nrf2-antioxidant responsive element (ARE) signaling represents an important adaptive mechanism of cellular defense against toxic LCA. In this case, Nrf2 provokes GSH biosynthesis (Kensler et al. 2007; Tan et al. 2007). Whereas, UDCA increases the GSH synthesis through an activation of the PI3K/Akt/Nrf2 pathway in hepatocytes (Arisawa et al. 2009). On the other hand, TUDCA increases the expression levels of the bile acid transporters and Nrf2, decreases the expression levels of glucose-regulated protein 78 (GRP78), protein kinase R (PKR)-like ER kinase (PERK), activating transcription factor 4 (ATF4), and CCAAT/



enhancer-binding protein (C/EBP)-homologous protein (CHOP) in hepatocytes (Zhang et al. 2017). FXR-TC-JNK axis is a novel mechanism to protect hepatocyte against toxicant-induced acute hepatitis. In this cycle, disruption of FXR in hepatocytes impairs BSEP function and increases TC levels. It is thought that TC-induced JNK activation is mediated by sphingosine-1-phosphate receptor 2 (S1PR2) like hydrophobic bile acids. Thereby JNK may promote hepatocyte cell death via S1PR2 in FXR-disrupted hepatocytes (Takahashi et al. 2017; Webster and Anwer 2016). Furthermore, TC activates the signaling pathways of AKT by 9-fold and ERK1/2 by 3 to 5-fold, whereas it downregulates the mRNA levels of phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G-6-Pase) (Cao et al. 2010). In fact, AKT phosphorylates FoxO1 and inhibits PEPCK and G-6-Pase in gluconeogenesis. In addition, glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) activity is inhibited by phosphorylating via AKT pathway and glycogen synthesis enhances in hepatocytes. Bile acids may mimic the insulin action in regulating glucose metabolism by stimulating glycogen synthesis and inhibiting gluconeogenesis (Fang et al. 2007). Activation of the PI3K/PDK-1/PKC- $\zeta$  pathway is required for the optimal activation of FXR by TC, which induces the small heterodimeric partner (SHP). SHP, as a nuclear receptor protein interacts with HNF4 $\alpha$ , FOXO1, CEBP $\alpha$  transcription factors and inhibits their functions (Cao et al. 2010). Activation of the nuclear FXR induces the membrane G protein-coupled receptor TGR5, which alters bile acid composition, and both FXR and TGR5 may coordinately stimulate GLP-1 secretion from intestinal L cells to improve hepatic glucose and lipid metabolism (Pathak et al. 2017). In brief, bile acid-activated FXR and signal transduction pathways are also involved in the regulation of hepatic gluconeogenesis, glycogen synthesis and insulin sensitivity (Trauner et al. 2010). The activated FXR-SHP pathway regulates the enterohepatic recycling and biosynthesis of bile acids and underlies the downregulation of hepatic fatty

acid and triglyceride biosynthesis. Indeed, SHP inhibits the activity of several nuclear receptors. Thereby, SHP induction negatively regulates biosynthesis and uptake of bile acids. The Bile acid-TGR5-cAMP the cAMP-dependent thyroid hormone activating enzyme type 2 iodothyronine deiodinase (D2) signaling pathway increases energy expenditure (Wei et al. 2009). Conjugated bile acids activate the ERK1/2 and AKT signaling pathways via unidentified G protein alpha i (G $\alpha$ i) protein coupled receptor(s) in primary hepatocytes, while unconjugated bile acids activate the ERK1/2 and AKT pathways by at least two different mechanisms. Unconjugated bile acids activate hepatocyte receptor tyrosine kinases and intracellular signaling pathways in a ROS-dependent manner. In contrast, conjugated bile acids primarily activate receptor tyrosine kinases and intracellular signaling pathways in a G protein-coupled receptors (GPCR) (G $\alpha$ i)-dependent and as well as ROS-dependent manner. Expression of dominant-negative G $\alpha$ i reduces TCA-induced activation of AKT and of glycogen synthase (GS) in intact hepatocytes (Dent et al. 2005; Fang et al. 2007). The generation of ROS is essential for protein tyrosine phosphatases inactivation, receptor tyrosine kinase activation, and enhanced signaling down the ERK1/2 and AKT pathways. Thus, DCA activates the ERK1/2 and AKT pathways by stimulating the synthesis of superoxide ions, which is shown to inactivate phosphotyrosine phosphatase(s) resulting in the activation of the EGFR (Fang et al. 2004). In addition, DCA, CDCA and T-CDCA can activate matrix metalloproteinase(s) (MMPs) that generate transforming growth factor  $\beta$  (TGF- $\beta$ ), an EGFR ligand in cholangiocytes (Werneburg et al. 2003). The GPCRs-activated by S1P have been linked to the activation of various cell signaling pathways, including ERK1/2 and AKT. Sphingosine kinase 2 (SphK2) is primarily located in the nucleus and is activated by phosphorylation by phospho ERK1/2 (pERK1/2) to produce S1P, a powerful inhibitor of histone deacetylase 1 and 2 (Hait et al. 2009).

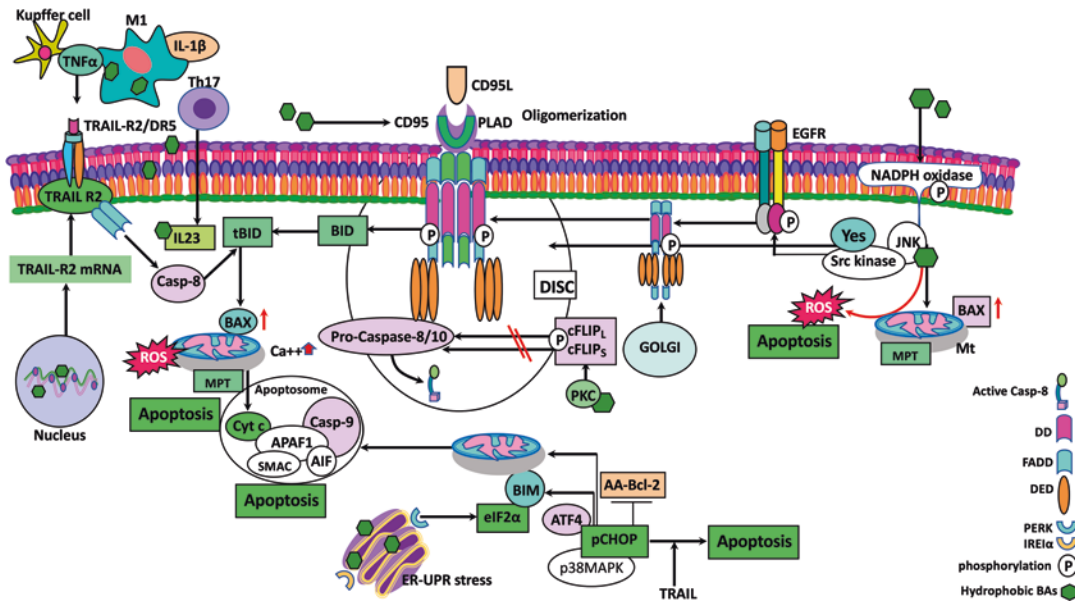
## 4 Bile Acids and Cell Death

At pathophysiologic concentrations, bile acids induce proinflammatory cytokine expression in human hepatocytes, but not in nonparenchymal cells or cholangiocytes. This liver-specific inflammatory response requires bile acid-entry into hepatocytes via basolateral transporter, NTCP. Pathophysiologic levels of bile acids induce the markers of ER stress and mitochondrial damage (Cai et al. 2017). Not all bile acids are toxic. In contrast to hydrophilic bile acids, accumulation of hydrophobic bile acids within the hepatocyte induces cell death. Inhibition of BSEP causes hepatic accumulation of toxic bile acids, CDCA, glyco-deoxycholic acid (GDCA), and DCA, leading to hepatocyte death (Oizumi et al. 2017). Toxic bile acids promote death receptor-mediated cell death signaling. Therefore, they induce oligomerization of cell surface death receptors either by ligand-independent or -dependent mechanisms. The cytotoxic signals downstream of the DISC differ between cell types and have been characterized as “type I” and “type II” cellular responses. In type II cells, overexpression of B-cell leukemia/lymphoma-2 (Bcl-2) or B-cell lymphoma-extra large (Bcl-xL) blocks caspase-8 and caspase-3 activation as well as apoptosis. In type I cells, induction of apoptosis is accompanied by activation of large amounts of caspase-8 within the DISC, whereas in type II cells DISC formation is strongly reduced and activation of caspase-8 and caspase-3 are independent to DISC (Scaffidi et al. 1998). Current concepts suggest that bile acid-associated hepatocyte apoptosis is death-receptor dependent. Glycochenodeoxycholate (GCDC)-induced hepatocyte apoptosis involves ligand-independent oligomerization of Fas, recruitment of FADD, activation of caspase 8, and subsequent activation of effector proteases, including downstream caspases and cathepsin B (Faubion et al. 1999; Higuchi and Gores 2003; Miyoshi et al. 1999) (Fig. 9.1). On the other hand, mitochondrial generation of ROS, and increase in mitochondrial free  $\text{Ca}^{2+}$  promotes the MPT. In fact, MPT is a causative factor in necrotic cell death. Therefore, onset of the MPT to increasing num-

bers of mitochondria within a cell leads progressively to autophagy, apoptosis and necrotic cell death (Lemasters et al. 1998).

## 5 Bile Acid-Induced Hepatic Inflammation

Bile acid accumulation in the liver causes cholestatic liver injury that often leads to progressive liver damage and subsequent liver failure. Hepatocytes release inflammatory cytokines that stimulate neutrophil chemotaxis. This inflammatory response involves mitochondrial damage and toll like receptor 9 (TLR9) activation in hepatocytes. Bile acid-stimulated chemokine expression in hepatocytes is mediated by nonparenchymal cells. Deleterious effects of TCA on mitochondria and the ER are both dose- and time-dependent. As evidenced by cytochrome c, apoptosis-inducing factor (AIF) and 78-kDa glucose-regulated protein (Grp78) leakage into the cytosol, is positively correlated with bile acid induction of [C-C motif] chemokine ligand 2 (Ccl2) and chemokine (C-X-C motif) ligand 2 (Cxl2) mRNA expression. NTCP plays a critical role in the pathogenesis of cholestatic liver injury since it is required for conjugated bile acid to be taken up into hepatocytes. By blocking of NTCP functions, hepatocytes injury is reduced (Cai et al. 2017). In response to cholestasis, activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and MAPK signaling, production of inflammatory cytokines, and recruitment of neutrophils occur. In acute cholestatic liver injury, loss of cellular FADD-like interleukin 1-beta (IL-1 $\beta$ )-converting enzyme (FLICE) inhibitory protein (cFLIP) in hepatocytes promotes a rapid release of proinflammatory and chemotactic cytokines (IL-6, IL-1 $\beta$ , CCL2, CXCL1, and CXCL2), an increased accumulation of CD68+ macrophages and an influx of neutrophils. All these result in apoptotic and necrotic hepatocyte cell death in the liver (Gehrke et al. 2018). ER stress or the unfolded protein response (UPR) contributes to hepatocyte cell death together with the alterations of lipid and fatty acid metabolism. Receptor-interacting pro-



**Fig. 9.1** Induction of death transduction pathways by hydrophobic bile acid in hepatocyte. Hydrophobic bile acids promote both Fas- and TRAIL-R2/DR5-mediated cell death signaling by activating ligand-dependent or -independent death receptor oligomerization. Moving of Fas receptor to the plasma membrane by JNK- and PKC- activation results in death receptor oligomerization and DISC formation. Hydrophobic bile acid-induced NADPH oxidase-JNK pathway triggers the EGFR activation, through Src kinase Yes. Following CD95 tyrosine phosphorylation and DISC formation, tBid/Bax-dependent mitochondrial dysfunction and accompanied excessive ROS generation lead to either apoptosis or necrosis. Furthermore, hydrophobic bile acids may directly target mitochondria, either through induction of the MPT and ROS production or activation of pro-apoptotic Bcl-2 family members. (Abbreviations: AA-Bcl-2: Antiapoptotik Bcl-2, AIF: Apoptosis-inducing factor, APAF1: Apoptosis peptide activating factor 1, ATF4: activating transcription factor 4, BA: Bile acid, BAX: BCL-2 associated X apoptosis regulator, BCL-2: antiapoptotic B-cell leukemia/lymphoma 2 protein, BID: BH3-domain-only pro-apoptotic factor, BIM: Bcl-2 interacting mediator of cell death, Casp: Caspase, CD95: death

receptor, APO-1, Fas receptor, CD95L: CD95 with its natural ligand (CD178), cFLIP: The antiapoptotic caspase 8-homolog cellular FLICE inhibitory protein, CHOP: C/EBP-homologous protein, Cyt c: Cytochrome c, DD: Death domain, DED: Death effector domain, DISC: Death-inducing signaling complex, DR: Death receptor, EGFR: Epidermal growth factor receptor, eIF2 $\alpha$ : The alpha subunit of the translation initiation factor 2, ER: Endoplasmic reticulum, FADD: Fas (The receptor CD95)-associated death domain, Fas: The receptor CD95, IL-1 $\beta$ : Interleukin-1 $\beta$ , IL23 Interleukin-23, IRE1 $\alpha$ : Inositol-requiring enzyme 1  $\alpha$ , JNK: c-Jun N-terminal kinase, MAPK: Mitogen-activated protein kinase, M1: Macrophage type 1, MPT: Mitochondrial permeability transition, Mt.: Mitochondria, NADPH: Reduced nicotinamide adenine dinucleotide phosphate, PERK: PKR-like endoplasmic reticulum kinase, PKC: Protein kinase C, PLAD: Pre-ligand binding assembly domain, ROS: Reactive oxygen species, SMAC: Second activator of mitochondrial apoptosis, tBid: Truncated BID, Th17: T helper 17, TNF $\alpha$ : Tumor necrosis factor- $\alpha$ , TRAIL: Tumor necrosis factor-associated apoptosis-inducing ligand, UPR: Unfolded protein response)

tein (RIP) kinases have an important function in necrotic cell death in hepatocyte injury (Malhi et al. 2010). It is suggested that the death receptors Fas, TNF receptor 1 (TNF-R1), and tumor necrosis factor-related apoptosis inducing ligand (TRAIL) receptor 1/2 (TRAIL-R1/2) are major mediators of the apoptotic pathway. Despite this idea, bile acids do not appear to enhance TNF- $\alpha$ /TNF-R1 cytotoxicity, because, hepatocytes can-

not express TNF- $\alpha$  following GDCD treatment (Higuchi et al. 2001). Indeed, enhanced expression and oligomerization of this death receptor has been described in GDCD-induced apoptosis in Fas-deficient cell lines. Aggregation of TRAIL-R2/death receptor 5 (DR5) is observed following GDCD treatment of these cells. Bile acid increases expression of TRAIL-R2/DR5 mRNA and protein 10-fold while expression of

TRAIL-R1 is unchanged (Higuchi et al. 2001). The death receptors oligomerize and recruit different adaptor proteins which activate the initiator caspase 8 and likely caspase 10. Active caspase 8 cleaves the BH3-only protein, BH3 interacting domain death agonist (Bid) and generates truncated Bid (tBid), following N-myristoylation (Zha et al. 2000). Although Bcl-2 associated protein x (BAX)-induced cytochrome c release is not dependent on tBid, the two proteins could function synergistically. However, Bid induces cytochrome c release through a mechanism independent of mitochondrial permeability transition pore and Bax (Kim et al. 2000). Thus, an activation cascade of proapoptotic proteins from BID to Bcl-2 antagonist killer (BAK) or BAX integrates the pathway from surface death receptors to the irreversible efflux of cytochrome c (Korsmeyer et al. 2000). In brief, activated Bid is translocated to mitochondria and induces cytochrome c release, which in turn activates the downstream caspases. This Bid-mediated pathway is critical in hepatocyte apoptosis induced by Fas/TNF-R1 engagement (Yin 2000). Since death receptors are ubiquitously expressed in the liver, hepatocytes are subject to death receptor-induced Bid cleavage with subsequent BAX/BAK activation and cell death. Adequate cellular levels of the antiapoptotic proteins myeloid cell leukemia 1 (Mcl-1) and/or Bcl-xL are, therefore, necessary to counteract the deleterious consequences of constitutive Bid activation (Malhi et al. 2010).

Elevated concentrations of bile acids during cholestasis increase expression of the transcription factor early growth response factor-1 (Egr-1) in hepatocytes. Egr-1 then upregulates proinflammatory mediators that causes neutrophils to accumulate in the liver and become activated to damage hepatocytes (Kim et al. 2006). Exposure of hepatocytes to bile acids increases levels of numerous mediators, including cytokines (IL-1 $\beta$ , IL-10), chemokines (keratinocyte chemoattractant; KC), interferon- $\gamma$ -inducible protein 10 (IP-10), interferon-inducible T cell alpha chemoattractant (I-TAC), monocyte chemoattractant protein-1 (MCP-1), regulated upon activation, normal T cell expressed and pre-

sumably secreted (RANTES), MCP-3, macrophage inflammatory protein-1 alpha (MIP-1 $\alpha$ ), MIP-2, MIP-3 $\alpha$ , lipopolysaccharide-induced CXC chemokine (LIX), scavenger receptor for phosphatidylserine and oxidized low density lipoprotein (SR-PSOX), MCP-3, B lymphocyte chemokine (BLC), adhesion molecules (intercellular adhesion molecule 1(ICAM-1), vascular cell adhesion molecule 1 (VCAM-1)), enzymes in arachidonic acid metabolism (cyclooxygenase-2; COX-2), and other proteins that influence immune cell levels and function (Allen et al. 2011). Activation of MAPK signaling by bile acids is required for upregulation of Egr-1 in hepatocytes. Indeed, bile acids modulate gene expression in hepatocytes through activating the FXR-MAPK signaling. Thus, expression of Egr-1 in hepatocytes is upregulated by bile acids through FXR and MAPK-dependent mechanisms during cholestasis (Allen et al. 2010, 2011).

CHOP is a key component in ER stress-mediated apoptosis. Thus, cholestasis induces CHOP-mediated ER stress and triggers hepatocyte cell death. Over-expression of CHOP and BAX are downstream target in the CHOP-mediated ER stress pathway in cholestasis. However, in acute period of cholestasis, necroptosis mediates hepatic necroinflammation. In this case, the PERK/eukaryotic initiation factor 2 alpha (eIF2 $\alpha$ ) signaling pathway plays a role in the regulation of CHOP by insulin-like growth factor type 1 receptor (IGF-1R). IGF-1R contributes to cholestatic liver injury, promoting the involvement of both CHOP and BAX in this process (Afonso et al. 2016; Cadoret et al. 2009; Tamaki et al. 2008). Conjugated bile acids can activate the S1PR2 in cholangiocyte plasma membranes which initiates an AKT and ERK1/2 signaling pathway that elicits an inflammatory and fibrotic response (Wang et al. 2017). In fact, neutrophil accumulation is the principal cause of hepatocyte toxicity in the early stages of cholestatic liver injury and inflammation. Neutrophils are activated and recruited to the liver by the pro-inflammatory mediators induced by high levels of bile acids (Li et al. 2017). Monocytes and macrophages express TGR5, the

G-protein coupled bile acid receptor, that can be activated by both conjugated and unconjugated bile acids. Among these, LCA, DCA, CDCA, and CA are the most potent activators in a descending order (Perino and Schoonjans 2015). TGR5 activation leads to an increase in cAMP and PKA activation and then modulates bile acid homeostasis and inflammation (Guo et al. 2016a; Perino and Schoonjans 2015; Thomas et al. 2008). TGR5 has been detected in cholangiocytes, Kupffer cells, and sinusoidal endothelial cells but not hepatocytes (Keitel and Häussinger 2012). Activation of TGR5 in macrophages reduces pro-inflammatory cytokines while maintaining anti-inflammatory cytokine expression thus promoting the development of an anti-inflammatory macrophage phenotype (Reich et al. 2017). The anti-inflammatory effect of TGR5 in macrophages is mediated by inhibiting the NF- $\kappa$ B and JNK signaling pathways. TGR5 activation alone induces the expression of IL-1 $\beta$  and TNF- $\alpha$  in macrophages. The TGR5-mediated increase in pro-inflammatory cytokine expression is suppressed by JNK inhibition. The induction of pro-inflammatory cytokine expression in Kupffer cells by TGR5 activation is correlated with the suppression of cholesterol 7 $\alpha$ -hydroxylase (Cyp7A1) expression in hepatocytes. These results suggest that TGR5 mediates the bile acid-induced pro-inflammatory cytokine production in Kupffer cells through JNK-dependent pathway (Lou et al. 2014; Perino and Schoonjans 2015). Recent studies suggest that TGR5 is required for bile acid induced cholangiocyte proliferation and the activation of TGR5 protects cholangiocytes from death-receptor-mediated apoptosis by TGR5-dependent CD95 receptor serine phosphorylation in cholestasis (Keitel and Häussinger 2013; Reich et al. 2016).

IL-1 $\beta$  and CDCA reduce CYP7A1 but induce c-Jun messenger RNA expression in human primary hepatocytes. However, IL-1 $\beta$  inhibits human CYP7A1 reporter activity via the HNF4 $\alpha$  binding site. HNF4 $\alpha$  is one of the two major transcription factors, which are driving CYP7A1 promoter activity in hepatocytes. IL-1 $\beta$  and CDCA inhibit HNF4 $\alpha$  but induces c-Jun, which

in turn blocks HNF4 $\alpha$  recruitment of peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) to the CYP7A1 chromatin and results in inhibition of CYP7A1 gene transcription. Thereby, the JNK/c-Jun signaling pathway protects the hepatocytes against the toxic effect by inhibiting bile acid synthesis (Li et al. 2006a). On the other hand, IL-17A signaling is a prerequisite for hepatic neutrophil accumulation during obstructive cholestasis (Meng et al. 2012). Bile acids activate ERK1/2 in hepatocytes, which stimulate up-regulation of Egr-1. Egr-1 then regulates production of MIP-2. The synergistic enhancement of MIP-2 expression by IL-17A and TCA is important for obstructive cholestasis (O'Brien et al. 2013). In fact, bile acids stimulate the production of IL-23 and IL-17A by hepatocytes and by T-helper 17 (Th17) cells, respectively. IL-17A enhances the bile acid-induced production of IL-23 and other inflammatory mediators by hepatocytes. In cholestatic liver, bile acid accumulation triggers the production of inflammatory mediators, which are induced by the IL-23/IL-17A axis in Kupffer cells. Thereby, IL-17A promotes hepatic inflammation during cholestasis by synergistically enhancing bile acid-induced production of proinflammatory cytokines by hepatocytes (Meng et al. 2012; O'Brien et al. 2013). CDCA, as the major hydrophobic primary bile acid, dose-dependently induce nucleotide oligomerization domain (NOD)-, leucine-rich repeat (LRR)- and pyrin domain-containing protein 3 (NLRP3) inflammasome activation and secretion of pro-inflammatory cytokine-IL-1 $\beta$  in macrophages by promoting ROS production. CDCA is an endogenous danger signal to activate NLRP3 inflammasome and initiates liver inflammation during cholestasis (Gong et al. 2016). Bile acids are endogenous danger-associated molecular patterns (DAMPs) that can activate both signal 1 and 2 of the NLRP3 inflammasome in inflammatory macrophages (Hao et al. 2017). Receptor-interacting serine/threonine-protein kinase 1 (RIPK1)-RIPK3-MLKL-mediated necroptosis has been attributed to cause inflammatory response through the

release of DAMPs (Moriwaki and Chan 2016). mRNA and protein expression of RIPK3 and MLKL, as well as MLKL phosphorylation, strongly increase in the cholestatic hepatocytes due to bile acid toxicity. RIP3 deficiency prevents necroinflammation induced by bile acids accumulation (Afonso et al. 2016). DAMPs that are released from dead hepatocytes may activate inflammatory responses in immune cells and hepatic stellate cells. DAMPs result in the assembly of a cytosolic protein complex termed the inflammasome, which activates the serine protease caspase-1, resulting in activation and secretion of IL-1 $\beta$  and other cytokines (Kubes and Mehal 2012). Furthermore, inflammasomes sense DAMPs and pathogen-associated molecular patterns (PAMPs) via nucleotide-binding oligomerization domain NOD-like receptors (NLRs). DAMPs and PAMPs activate caspase-1 which then proteolytically activates IL-1 $\beta$  and IL-18. IL-1 $\beta$  amplifies the inflammatory response by further stimulating production of inflammatory cytokines. In cholestasis, bile acids inhibit NLRP3 inflammasome activation in macrophages via the TGR5-cAMP-PKA axis and phosphorylation of NLRP3 on Ser 291 (Guo et al. 2016b; Li et al. 2017). Interestingly, while the NLRP3 inflammasome aggravates inflammatory liver injury by inducing IL-1 $\beta$ , on the other hand, affects the epithelial integrity of cholangiocytes by inducing the production of pro-inflammatory cytokines. In addition, bile acids, including DCA and CDCA, can activate the NLRP3 inflammasome in macrophages (Wang et al. 2019). In hepatocytes, inflammasome activation induces hepatocyte death via pyroptosis (Schroder and Tschopp 2010).

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## 6 Bile Acid-Induced Apoptosis

Accumulation of bile acid within hepatocytes results in cell injury and death. The exposure to low concentration of bile acid leads to apoptosis, while high concentration exposure causes necrosis. The percent of cells undergoing apoptosis increase in a time- and concentration-

dependent manner in cells exposed to bile acid (Perez and Briz 2009; Sokol et al. 1995). Cholestasis is characterized by disruption of bile flow, resulting in intrahepatic and systemic retention of bile acids, with a concomitant toxic response in liver parenchymal cells. Cholestatic liver injury may arise from genetic disorders, drug toxicity, hepatobiliary malignancies or obstruction of the biliary tract. Inflammation and necrosis have an equal impact or one of them has more prevalent role (Castro and Rodrigues 2017). The accumulation of bile acids causes defective autophagic clearance, shown by the accumulation of insoluble p62 and ubiquitinated proteins and cell death accompanied by caspase-3 processing. Hepatocytes exposed to bile acids lead to the accumulation of autophagosomes due to suppressed autophagy flux (Kim et al. 2018). Apoptosis is activated late after extrahepatic cholestasis in parallel with development of liver fibrosis, whereas it is widely accepted that apoptosis may trigger hepatic fibrogenesis (Luedde et al. 2014). Two apoptotic pathways are described as the mitochondrial pathway and the classical death receptor pathway. These two apoptotic pathways interact between caspase activation, mitochondrial dysfunction, and cellular distribution of Bcl-2-related proteins in cholestatic hepatocyte injury. GCDCA induces apoptosis in a mitochondria-controlled pathway in which caspase-8 is activated in a Fas-associated protein with death domain-independent manner. However, bile acid-induced apoptosis in cholestasis is limited. This is explained by cytokine-induced activation of NF- $\kappa$ B-regulated antiapoptosis genes like A1 and cellular inhibitor of apoptosis 2 (cIAP2) (Maher 2004). Bile acid-associated hepatocyte apoptosis occurs through the death receptors Fas. Glycochenodeoxycholate (GCDCA)-induced hepatocyte apoptosis involves ligand-independent oligomerization of Fas, recruitment of FADD, activation of caspase 8 (Faubion et al. 1999). Toxic bile acids cause cell death, partly due to the activation of a protease cascade. The proximal signaling protease caspase 8 appears to be activated by toxic bile acids in a Fas-receptor-dependent manner. However,

both play a critical role in bile acid-induced apoptosis (Faubion et al. 1999). CD95 ligand (CD95L)-induced apoptosis involves a sphingomyelinase- and PKC- $\zeta$ -dependent activation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase isoforms. It has been demonstrated that bile acid-induced oxidative stress may trigger the activation of JNKs and PKC (Reinehr et al. 2005a). CD95L triggers activation of the Src kinase family member Yes, which requires NADPH oxidase-mediated oxidative stress as an upstream event. Yes-activation is also responsive to hydrophobic bile acids. CD95L-induced CD95 activation and DISC formation are dependent upon Yes signal. Hydrophobic bile acids activates Yes and the CD95 system and induces apoptosis (Reinehr et al. 2004).

Hydrophobic bile acids, such as GCDCA and T-CDCA, could induce cytotoxicity by acting as detergents on cell membranes. However, serum bile acid levels in cholestatic patients are insufficient to produce a significant detergent effect. Therefore, bile acid-induced cell death occurs by either necrosis or apoptosis. In contrast to T-CDCA and TUDCA, GCDCA-induced apoptosis occurs by a mitochondria-controlled pathway in hepatocytes (Maher 2004). Indeed, pathophysiological concentrations of bile acids induce apoptosis by directly activating death receptors. However, GCDCA-induced hepatocyte apoptosis involves ligand-independent oligomerization of Fas, recruitment of FADD, activation of caspase 8, and subsequent activation of effector proteases, including downstream caspases and cathepsin B (Faubion et al. 1999) and inducing oxidative damage and mitochondrial dysfunction. The percent of cells undergoing apoptosis increase in a time- and concentration-dependent manner in cells exposed to GCDCA, and when compared to GCA is much lesser extent. ROS generation precedes the onset of apoptosis. MPT blockers, caspase-8 inhibition, and antioxidants prevent apoptosis. ROS generation is reduced in hepatocytes (Rodrigues et al. 1998; Yerushalmi et al. 2001). Toxic bile acids have been shown to induce apoptosis in a Fas-dependent manner. Suppression of PKC prevents

bile acid-stimulated cFLIP-L and -S phosphorylation, restores cFLIP binding to glutathione S-transferase (GST)-FADD, and attenuates bile acid-induced apoptosis (Faubion et al. 1999; Higuchi et al. 2003). cFLIP is a phosphoprotein and that toxic bile acids potentiate phosphorylation of both cFLIP-L and -S by a PKC-dependent mechanism (Higuchi et al. 2003). The phosphorylation of cFLIP decreases binding of cFLIP to FADD, resulting in reduced recruitment to the DISC. Decreased cFLIP results in enhanced activation of pro-caspase-8/10 (Higuchi and Gores 2003). Recruitment of the FADD involves the activation of caspase-8 and Bid, as well as downstream effector caspases. Activation of death receptors by bile acids results in induction of their transport from the Golgi (Sodeman et al. 2000). Oxidative stress due to bile acid accumulation ultimately leads to Fas activation, involving NADPH oxidase activation. Thus, interaction of bile acids with mitochondria directly generates ROS (Reinehr et al. 2005a). More recently, G-protein-coupled receptor TGR5 activation has also been implicated in Fas translocation and oligomerization. Bile acid-induced JNK activation is dependent on bile acid activation of TGR5. JNK formed complexes with caspase 8, are reduced following bile acid treatment, but this reduction is prevented when TGR5 or JNK is inhibited. In conclusion, bile acids activate TGR5, which leads to JNK activation and reduced complex formation of JNK with caspase 8, thus facilitating caspase 8 recruitment to DISC (Yang et al. 2007). Fas expression at the cell surface via transport from the Golgi complex induce Fas-FADD binding (Bennett et al. 1998). In addition, p53 contributes to cyclin kinase inhibitor-enhanced, bile-acid-induced apoptosis via Fas. Blockade of DCA-induced ERK1/2 and AKT activation, with inhibitors of RAS, PI3K, or MEK1/2, increases apoptosis approximately 10-fold. In this case, apoptosis is dependent on bile acid-induced, ligand-independent activation of the CD95 death receptor. Increased expression of CD95 in hepatocytes overexpress p21 or p27. Overexpression of p21 or p27 in a p53-dependent fashion enhances basal plasma membrane levels of CD95 but do not further enhance bile

acid stimulated CD95 activation. Increased expression and nuclear localization of p53 correlates with increased expression of multiple proapoptotic gene products (Zhang et al. 2008). Hydrophobic bile acids are known to induce apoptosis in hepatocytes, in contrast, hydrophilic bile acids increase intracellular cAMP levels and activate MAPK and PI3K pathways to protect hepatocytes from apoptosis. Overexpression of p21 enhances p53 protein levels. p53 overexpression is evident in the enhanced apoptotic response. Thus, overexpression of p21 leads to elevated sensitivity to proapoptotic stimuli. Taurolithocholate-3-sulfate (TLCS) induces a JNK-dependent EGFR/CD95 association in the cytosol and moving of this protein complex towards the plasma membrane. Although inhibition of EGFR tyrosine kinase activity allows for cytosolic EGFR/CD95 association, it prevents targeting of the EGFR/CD95 complex to the plasma membrane. Furthermore, hydrophobic bile acids activate NADPH oxidase isoforms. Resultant oxidative stress triggers activation of the CD95 system, and apoptosis (Amaral et al. 2009; Qiao et al. 2002a; Reinehr et al. 2005b). Accumulation of hydrophobic bile acids in the liver contributes to cholestatic liver injury. They dose-dependently induce NLRP3 inflammasome activation and secretion of pro-inflammatory cytokine-IL-1 $\beta$  in macrophages by promoting ROS production and K<sup>+</sup> efflux. Hydroperoxide generation precedes the hepatocyte injury, and thiobarbituric acid-reacting substances (TBARS) are accumulated, thereby mitochondrial pathways of cell death are stimulated. In this respect, CDCA triggers ROS formation in part through TGR5/EGFR downstream signaling, including PKB/AKT, ERK and JNK pathways (Gong et al. 2016; Sokol et al. 1995). On the other hand, bile acids inhibit NLRP3 inflammasome activation via the TGR5-cAMP-PKA axis. TGR5 bile acid receptor-induced PKA kinase activation leads to the ubiquitination of NLRP3, which is associated with the PKA-induced phosphorylation of NLRP3. Furthermore, PKA-induced phosphorylation of NLRP3 serves as a critical brake on NLRP3 inflammasome activation and subsequent apoptosis of hepatocytes (Guo et al.

2016b). Intracellular ROS generation by mitochondria appears to be an early event in hydrophobic bile acid-induced hepatocyte toxicity (Sokol et al. 1995). Bile acids also induce apoptosis through the mitochondrial or intrinsic pathway, in which intracellular stress causes mitochondrial dysfunction and the subsequent release of proapoptotic factors. Whereas, hydrophobic bile acids can induce the MPT. Thus, the GCDG-mediated hepatocyte necrosis via MPT induction is inhibited by a hydrophilic bile salt, UDCA. The critical common steps of apoptosis by bile acids involve ROS generation, MPT induction, cytochrome c release and decrease of antioxidants (Botla et al. 1995; Rodrigues et al. 1998; Yerushalmi et al. 2001). The E3 ligases inhibitor of apoptosis (cIAP1/2) and the linear ubiquitin chain assembly complex (LUBAC) ubiquitinate RIP1 in the TNFR1 signaling complex (TNF-RSC). Polyubiquitinated RIP1 then engages downstream adaptors such as nuclear factor-kappa B essential modulator (NEMO) to activate IKK to promote NF- $\kappa$ B transcriptional activity, leading to cell survival, proliferation, and differentiation (Walczak 2011). When RIP1 ubiquitination is blocked by removal of the E3 ligases cIAP1 and cIAP2 RIP1 forms a secondary complex in the cytosol with FADD and caspase-8 termed the "Ripoptosome", which contains RIP1, FADD, caspase-8, caspase-10, and caspase inhibitor cFLIP isoforms. cFLIPL prevents ripoptosome formation to initiate apoptotic cell death. Ripoptosome is necessary but not sufficient for cell death (Feoktistova et al. 2011; Tenev et al. 2011). Bile acids not only initiate ligand-independent death-receptor oligomerization but also modulate the signaling pathway, causing a strong sensitization of the cells to death receptor-mediated apoptosis. Death receptor-mediated apoptosis is regulated at the cell surface by the receptor expression, at the DISC by expression of procaspase-8, FADD, or apoptosis inhibitor cFLIP. At mitochondria, the expression of proapoptotic or antiapoptotic Bcl-2 family proteins contribute to apoptosis. In this regard, Bcl-2, Bcl-xL, Bid, or Bax, regulate mitochondrial cytochrome c release. Bile acids promote death-receptor oligomerization by either ligand-



dependent or -independent mechanisms. In the absence of Fas, toxic bile acids increase the TRAIL-R2/DR5 on the cell surface. Whereas in the presence of Fas, bile acids induce the cell surface trafficking of Fas mRNAs, and they accumulate on the plasma membrane (Higuchi and Gores 2003). At the DISC level, bile acids do not alter expression of procaspases, FADD, or cFLIP. However, bile acids appear to prevent cFLIP recruitment to the DISC and thereby enhance initiator caspase activation. Both caspase 8 and caspase 10 recruitment and processing within the TRAIL-DISC are greater in GCDCA-treated cells whereas recruitment of cFLIPL and cFLIPS is reduced (Higuchi et al. 2003). Loss of cFLIP in hepatocytes promotes acute cholestatic liver injury early after extrahepatic cholestasis, which is characterized by a rapid release of proinflammatory and chemotactic cytokines, TNF, IL-6, IL-1 $\beta$ , CCL2, CXCL1, and CXCL2, an increased CD68+ macrophages and an influx of neutrophils into liver. These result in apoptotic and necrotic hepatocyte cell death (Gehrke et al. 2018). Due to bile acid toxicity, expression of Th1-related cytokines increases, and TNF- $\alpha$  stimulates the TRAIL-R2/DR5 in hepatocytes (Gomez-Santos et al. 2012). In Fas-independent apoptosis in Fas deficient cells, aggregation of death receptor, TRAIL-R2/DR5 mediates apoptosis in bile acids-exposed cells (Higuchi et al. 2001).

TGR5 expression and its responsiveness to bile acids are confirmed in human hepatocytes. TGR5 inhibition attenuates bile acid-induced caspase 8 activation, which results from reduced bile acid-induced caspase 8 recruited to a DISC. Bile acid-induced JNK activation is dependent on bile acid activation of TGR5. JNK forms complexes with caspase 8, which are reduced following bile acid treatment, but this reduction is prevented when TGR5 or JNK is inhibited (Yang et al. 2007). JNK activation may be important for bile acid-induced apoptosis by triggering ligand-independent CD95 surface trafficking and activation of apoptosis. Inhibition of JNK1 or PKC prevented tauro lithocholic acid-3 sulfate (TLCS)-induced CD95 membrane trafficking and blunted the apoptotic response

(Graf et al. 2002). Induction of apoptosis by hydrophobic bile salts involves EGFR activation and EGFR-dependent CD95 tyrosine phosphorylation, which triggers CD95 membrane targeting and Fas-associated death domain/caspase-8 recruitment. The latter step is apparently controlled by PI3K (Reinehr et al. 2003). CD95 activation by hydrophobic bile acids is initiated by NADPH oxidase-dependent ROS signaling. Consequences of TLCS-induced oxidative stress is JNK activation and Src kinase family member Yes-dependent activation of the EGFR. EGFR-catalyzed CD95 tyrosine phosphorylation and formation of the death-inducing signaling complex result in apoptosis. JNK-dependent EGFR-CD95 association in the cytosol allows transport of this protein complex to the plasma membrane (Reinehr et al. 2005b). EGFR-dependent CD95 tyrosine phosphorylation, triggers CD95 membrane targeting and Fas-associated death domain/caspase-8 recruitment. The latter step is apparently also controlled by PI3K (Reinehr et al. 2003). The Src kinase Yes as an upstream target of proapoptotic bile acids, triggers EGFR activation (Reinehr et al. 2004). EGFR associates in a JNK-dependent pathway with CD95 and catalyzes CD95 tyrosine phosphorylation. Eventually, membrane trafficking of CD95 and subsequent DISC formation leads to apoptosis (Reinehr et al. 2003). Bile acid-induced NADPH oxidase activation not only triggers CD95 activation, but also hepatocyte shrinkage. Hydrophobic, proapoptotic bile salts induce hepatocyte shrinkage largely through NADPH oxidase-derived oxidative stress. A vicious cycle between oxidative stress and cell shrinkage propagates CD95 activation and apoptosis (Becker et al. 2007).

Detached from their natural extracellular matrix, hepatocytes enter apoptosis (Pinkse et al. 2004). Hepatocyte adherence to the extracellular matrix is mediated by integrins. Integrins have cytoprotective effect through the activation of non-receptor dual tyrosine kinase complex, which consists focal adhesion kinase (FAK) and c-Src (Mitra and Schlaepfer 2006). Hydrophobic bile acid induced apoptosis is associated with

augmented JNK and decreased FAK phosphorylation. cAMP-guanine exchange factor (cAMP-GEF) activation in hepatocytes increases the PI3K dependent phosphorylation of FAK. Phosphorylation of this protein kinase is necessary for cytoprotective effect in hydrophobic bile acid-induced apoptosis via cAMP-GEF/PI3K/FAK survival pathway in hepatocytes (Usechak et al. 2008). Indeed, hepatocytes express three catalytic PI3K Class I isoforms (p110 $\alpha$ / $\beta$ / $\gamma$ ). PI3K p110 $\gamma$  (p110 $\gamma$  is associated with detrimental effects) is activated by hydrophobic, but not by hydrophilic bile salts. Bile salt-induced hepatocyte apoptosis is partly mediated via a PI3K p110 $\gamma$  dependent signaling pathway, potentially involving JNK (Hohenester et al. 2010). PKC- $\delta$  silencing increases GCDC-induced JNK phosphorylation, decreases GCDC-induced AKT phosphorylation, and increases expression of Bcl-2 interacting mediator of cell death (BIM). GCDC translocate BIM to the mitochondria in hepatocytes. PKC- $\delta$  does not mediate GCDC-induced apoptosis in hepatocytes. Instead PKC- $\delta$  activation by GCDC stimulates a cytoprotective pathway that involves JNK inhibition, AKT activation, and downregulation of BIM (Webster et al. 2014). GCDC, the predominant circulating bile acid in humans, paradoxically elicits prodeath as well as prosurvival signals in hepatocytes (Dent et al. 2005; Hohenester et al. 2010; Rust et al. 2000, 2009; Schoemaker et al. 2004). Prodeath pathways converge on sustained phosphorylation of JNK. The role of JNK in hepatocyte death is supported by results that show that chemical or genetic inhibition of this kinase. JNK attenuates bile acid-induced apoptosis (Hohenester et al. 2010; Kluwe et al. 2010; Reinehr et al. 2005b). Bile acid-induced prosurvival signaling involves activation of PI3K and AKT. Inhibition of either kinase results in increased cell death in response to GCDC (Cullen et al. 2004; Schoemaker et al. 2004). Sustained JNK activation is a prodeath signal in bile acid-induced apoptosis in hepatocytes (Hohenester et al. 2010; Reinehr et al. 2005b), whereas activation of PI3K/AKT is prosurvival (Hohenester et al. 2010; Webster et al. 2002). Our studies show that genetic silencing of

PKC- $\delta$  results in an increase in GCDC-induced phosphorylation of JNK and apoptosis while decreasing GCDC phosphorylation of AKT. These opposing effects would both promote hepatocyte cell death (Webster et al. 2014). DCA-induced JNK2 signaling is cytoprotective whereas DCA-induced JNK1 signaling is cytotoxic. DCA-induced ERK1/2 activation was responsible for increased DNA binding of C/EBP $\beta$ , cAMP response element-binding protein (CREB), and c-Jun/AP-1. Inhibition of C/EBP $\beta$ , CREB, and c-Jun function promotes apoptosis following DCA treatment, and the level of apoptosis is further increased in the case of CREB and c-Jun, but not C/EBP $\beta$ , by inhibition of MEK1/2. The combined loss of CREB and c-Jun function or of C/EBP $\beta$  and c-Jun function enhance DCA-induced apoptosis above the levels resulting from the loss of either factor individually; however, these effects are less than additive. Loss of c-Jun or CREB function correlates with increased expression of FAS death receptor and p53 upregulated modulator of apoptosis (PUMA) and decreased expression of c-FLIPL and c-FLIPS, proteins previously implicated in the modulation of the cellular apoptotic response. Collectively, these data demonstrate that multiple DCA-induced signaling pathways and transcription factors control hepatocyte survival (Qiao et al. 2003). Cholestatic liver disorders are accompanied by the hepatic accumulation of cytotoxic bile acids that induce cell death. Increases in cAMP protect hepatocytes from bile acid-induced apoptosis by a cAMP-guanine exchange factor (GEF)/PI3K/AKT pathway. The hydrophobic bile acid-induced phosphorylation of the proapoptotic kinase JNK is inhibited by GSK inhibition or cAMP-GEF activation. The hydrophobic bile acids-induced apoptosis is accompanied by phosphorylation of the ER stress markers pIEF2 $\alpha$  and IRE-1, and cAMP-GEF analog or GSK inhibitors prevent this phosphorylation. cAMP/cAMP-GEF/Rap1/PI3K/AKT/GSK $\beta$  survival pathway in hepatocytes inhibits bile acid-induced JNK phosphorylation (Johnston et al. 2011). Bile salt-induced intracellular signaling pathways play important roles in modulating hepatocyte apoptosis. GCDCA induces ER-related calcium

release. Significant increases in activities of calpain and caspase-12 are observed after GCDCA treatment. Bip and CHOP mRNA expressions are increased with the dose- and incubation time-dependently. Cytochrome c release from mitochondria suggest that ER stress is induced by GCDCA (Tsuchiya et al. 2006). GCDCA-induced activation of JNK, ERK, and AKT, relies on the presence of the bile salt transporter NTCP. In order to achieve this, either GCDCA enters the cell to modulate intracellular kinase phosphorylation or binds to NTCP, which is necessary to induce kinase phosphorylation (Webster and Anwer 2016). GCDCA leads to activation of sphingosine kinase (SphK) and then generation of sphingosine-1-phosphate (S1P). The S1P is transported out of the cell by an ABC transporter and binds to S1PR2, leading to activation of G $\alpha$ i and inhibition of cAMP production. S1PR2 is the predominant S1P receptor expressed in cholangiocytes. Intracellular mediators of inflammatory damage, induced by S1PR2 stimulation, are the ERK1/2/AKT/NF- $\kappa$ B axis, with increased COX-2 activity at the biliary level (Baiocchi et al. 2019; Salomone and Waeber 2011). The hepatotoxic bile acid GCDCA modulates hepatocyte cell death through activation of JNK, AKT, and ERK. The nonhepatotoxic bile acid TC activates Akt and ERK through the S1PR2. S1PR2 inhibition attenuates GCDCA-induced apoptosis and inhibits and augments GCDCA-induced JNK and AKT phosphorylation, respectively. S1PR2 activation is proapoptotic in GCDCA-induced cell death (Webster and Anwer 2016). Transfer of calcium to mitochondria is required for SphK2-induced apoptosis, as cell death and cytochrome c release is abrogated by inhibition of the mitochondrial Ca<sup>2+</sup> transporter. Targeting SphK1 to the ER converts it from anti-apoptotic to pro-apoptotic state (Maceyka et al. 2005). Thus, SphK1 plays a pivotal role in mediating bile salt-induced apoptosis in hepatocytes in part by interfering with intracellular [Ca<sup>2+</sup>] signaling and activation of S1PR1 (Karimian et al. 2013; Maceyka et al. 2002). The S1PR2 inhibition attenuates GCDCA-induced apoptosis and inhibits and augments GCDCA-induced JNK and Akt phosphorylation, respectively. In addition, GCDCA must enter hepatocytes

to mediate cell death or activate kinases. These results suggest that S1PR2 activation is proapoptotic in GCDCA-induced cell death but that this effect is not due to direct ligation of the S1PR2 by the bile acid (Webster and Anwer 2016).

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## 7 Antiapoptotic Effect of Bile Acids

Bile acids are known to be involved in hepatocyte cell survival pathways. In this regard, hydrophobic bile acids have been reported to be cytotoxic and to induce apoptosis in hepatocytes (Amaral et al. 2009). In contrast, hydrophilic bile acids such as UDCA and conjugates have been reported to prevent apoptosis possibly via the activation of the AKT and ERK1/2 signaling pathways (Qiao et al. 2002b; Schoemaker et al. 2004). It is noted that TLR4 is not required for the initiation of acute inflammation during cholestasis. Bile acids do not directly cause cell toxicity but increases the expression of numerous proinflammatory mediators, including cytokines, chemokines, adhesion molecules, and other proteins that influence immune cell levels and function in cholestasis. Bile acids activate Erk1/2 in hepatocytes which stimulates up-regulation of Egr-1. Egr-1 then regulates production of inflammatory mediators (Allen et al. 2011).

Since its ability to reduce the apoptotic threshold in several cell types, UDCA, is an endogenous hydrophilic bile acid in clinical use for the treatment of certain liver diseases (Beuers et al. 1998; Lazaridis et al. 2001). UDCA and its amidated conjugates, TUDCA and glycoursoodeoxycholic acid, are shown to modulate mitochondrial membrane perturbation, pore formation, Bax translocation, cytochrome c release, caspase activation, and subsequent substrate cleavage. UDCA inhibition of apoptosis involves an interplay of events in which both depolarization and channel-forming activity of the mitochondrial membrane are inhibited (Rodrigues et al. 1999). In addition, TUDCA can directly stabilize mitochondrial membranes, having a profound effect on Bax channel formation in isolated mitochondria. TUDCA is a potent

inhibitor of Bax association with mitochondria. Thus, TUDCA modulates apoptosis by suppressing mitochondrial membrane perturbation (Rodrigues et al. 2003). TUDCA significantly decreases tauro litholic acid (TLCA)-induced upregulation of cFos and JunB. Furthermore, TUDCA inhibits TLCA-induced AP-1 transcriptional activity and reduces TLCA-induced apoptosis. Thus, TUDCA modulates apoptosis, which is induced by toxic bile acids, through inhibition of the human transcription factor AP-1 (Pusl et al. 2008). AP-1 is a dimer consisting of proteins belonging to Jun, Fos, ATF, or musculoaponeurotic fibrosarcoma (MAF) families of transcription factors, which are involved in the regulation of cell proliferation, transformation, and death (Shaulian and Karin 2002). The p38/ERK/MAPK and PI3K pathways are involved in protection of TUDCA against GCDCA-induced apoptosis in hepatocytes (Schoemaker et al. 2004). The NTCP is crucial for conjugated bile salt-induced hepatocyte apoptosis. TUDCA protection against GCDCA-induced apoptosis is dependent on activation of p38/ERK/MAPK, and PI3K pathways, but independent of competition on the cell membrane, NF- $\kappa$ B activation, and transcription (Schoemaker et al. 2004). In addition, UDCA inhibits TGF- $\beta$ 1-induced degradation of NF- $\kappa$ B and its inhibitor inhibitor kappa B (IkappaB). In conclusion, these results demonstrate that UDCA inhibits E2F-1 transcriptional activation of hepatocyte apoptosis, thus modulating p53 stabilization, NF- $\kappa$ B degradation, and expression of Bcl-2 family members. Moreover, UDCA modulates transcription factors, E2F-1 and p53, independent of its effect on mitochondria and/or caspases (Sola et al. 2003). UDCA reduces p53 transcriptional activity, thereby preventing its ability to induce Bax expression, mitochondrial translocation, cytochrome c release, and apoptosis in hepatocytes. Interestingly, p53 is a key molecular target in UDCA prevention of cell death, where the finely tuned, complex control of p53 by mouse double minute 2 homolog (Mdm-2) represents a prime target for UDCA modulation of p53-mediated cell death (Amaral et al. 2007). Indeed, UDCA improves liver function by three major mecha-

nisms of action, including protection of cholangiocytes against the cytotoxicity of hydrophobic bile acids, stimulation of hepatobiliary secretion, and inhibition of liver cell apoptosis (Solá et al. 2006). Similar to glucocorticoid receptors (GR), mineralocorticoid receptors (MR) contribute to the protective effect of UDCA against the E2F-1/Mdm-2/p53 apoptotic pathway induced by TGF- $\beta$ 1 in hepatocytes (Solá et al. 2004).

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## 8 Bile Acid-Induced Necroptosis and Necrosis

In addition to apoptosis, different regulated necrotic cell death forms may emerge in bile acid-exposed cells. These are defined as genetically controlled cell death processes with morphological hallmarks of oncotic necrosis. Although RIPK1- and RIPK3-MLKL-mediated necroptosis is the most understood form of regulated necrosis, cell death mechanisms known as parthanatos, oxytosis, ferroptosis, NETosis, pyronecrosis and pyroptosis can be observed (Vanden Berghe et al. 2014). Necroptosis, the most well-studied pathway of regulated necrosis, depends on RIPK3 activity. RIP1 and RIP3 engage in physical interactions upon activation of death receptors. The expression of RIP3 in different cell lines correlates with their responsiveness to necrosis induction. The kinase activity of RIP3 is essential for necrosis execution. Upon induction of necrosis, RIP3 is recruited to RIPK1 to form a necrosis-inducing complex (He et al. 2009), creating a filamentous amyloid protein complex called necrosome (Li et al. 2012a). The MLKL is a functional RIP3 substrate that binds to RIP3 through its kinase-like domain but lacks kinase activity of its own. Upon phosphorylation by active RIP3, MLKL oligomerizes and translocates to cellular membranes, hence compromising their ability to preserve ionic homeostasis. The membrane localization of MLKL is essential for Ca<sup>2+</sup> influx, which is an early event of TNF-induced necroptosis (Cai et al. 2014; Wang et al. 2014). In agreement with a role of necroptosis in cholestatic liver injury, combined ablation of hepatocyte-specific caspase-8 and NF- $\kappa$ B essen-

tial modulator results in spontaneous massive liver necrosis and cholestasis, with a concomitant formation of necrosome complexes in the foci of necrotic areas (Liedtke et al. 2011). Activation of necroptosis by ligands of death receptors requires the kinase activity of RIP1, which mediates the activation of RIP3 and MLKL, two critical downstream mediators of necroptosis. Blocking the kinase activity of RIP1 inhibits the activation of necroptosis and allows cell survival and proliferation in the presence of death receptor ligands. Necroptosis is suppressed by caspase-8/FADD-mediated apoptosis (Zhou and Yuan 2014). Cholestasis results in necroptosis, concomitant with progressive bile duct hyperplasia, multifocal necrosis, fibrosis and inflammation. MLKL phosphorylation increases and insoluble aggregates of RIP3, MLKL and RIP1 form in hepatocytes (Afonso et al. 2016). In fact, although RIP3 and MLKL progressively increase in both liver soluble and insoluble protein fractions, the phosphorylated MLKL/MLKL ratio is particularly increased in insoluble fraction (Afonso et al. 2016). RIP3 deficiency attenuates development of jaundice and cholestasis in severe chronic hepatitis induced by conditional deletion of TGF- $\beta$ -activated kinase-1 in liver parenchymal cells (Vucur et al. 2013). Hepatocytes are protected from cell death by only two anti-apoptotic proteins, Bcl-xL and Mcl-1. ER stress or the UPR contributes to hepatocyte cell death during alterations of lipid and fatty acid metabolism. Finally, the current information implicating RIP kinases in necrosis provides an approach to more fully address this mode of cell death in hepatocyte injury (Malhi et al. 2010).

## 9 Conclusion

The effect of bile acids on cell viability depends on the ratio between the hydrophobic and hydrophilic bile acids, and as well as the protein kinase pathways that provide signal transduction. However, prevention of hydrophobic bile acid-induced apoptosis by hydrophilic bile acid is not due to a competition of both bile salts. TUDC

induces the dual specificity MAP kinase phosphatase 1 (MKP-1), which prevents GDC-induced phosphorylation of MKK4 and JNK activation. Thereby, TUDC exerts its anti-apoptotic effect via preventing hydrophobic bile acids-induced CD95 activation at the levels of JNK activation and CD95 serine/threonine phosphorylation (Sommerfeld et al. 2015). Some protein kinases mediate both the prevention and stimulation of bile acids related cell death. Fyn activation mediates cholestasis, Src kinase Yes triggers CD95 activation and apoptosis (Reinehr et al. 2013). Hydrophobic bile acid-activated Yes triggers EGFR activation, subsequent CD95 tyrosine phosphorylation, and apoptosis. In contrast, cAMP exerts antiapoptotic effect via protein kinase A-dependent inhibition of Yes/EGFR pathway (Reinehr et al. 2004).

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