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- Review Article

INTRACELLULAR SIGNALING BY BILE ACIDS

Mohammed Sawkat Anwer*

Department of Biomedical Sciences
Cummings School of Veterinary Medicine at Tufts University
200 Westboro Road, North Grafton, MA, USA

Abstract

Bile acids, synthesized from cholesterol, are known to produce beneficial as well as toxic effects in the liver. The beneficial effects include choleresis, immunomodulation, cell survival, while the toxic effects include cholestasis, apoptosis and cellular toxicity. It is believed that bile acids produce many of these effects by activating intracellular signaling pathways. However, it has been a challenge to relate intracellular signaling to specific and at times opposing effects of bile acids. It is becoming evident that bile acids produce different effects by activating different isoforms of phosphoinositide 3-kinase (PI3K), Protein kinase Cs (PKCs), and mitogen activated protein kinases (MAPK). Thus, the apoptotic effect of bile acids may be mediated via PI3K-110γ, while cytoprotection induce by cAMP-GEF pathway involves activation of PI3K-p110α/β isoforms. Atypical PKCζ may mediate beneficial effects and nPKCε may mediate toxic effects, while cPKCα and nPKCδ may be involved in both beneficial and toxic effects of bile acids.The opposing effects of nPKCδ activation may depend on nPKCδ phosphorylation site(s). Activation of ERK1/2 and JNK1/2 pathway appears to mediate beneficial and toxic effects, respectively,of bile acids. Activation of p38α MAPK and p38β MAPK may mediate choleretic and cholestatic effects, respectively, of bile acids. Future studies clarifying the isoform specific effects on bile formation should allow us to define potential therapeutic targets in the treatment of cholestatic disorders.

Key words: PI3K, PKC, MAPK, Bile formation, Cholestasis.

Introduction

Bile acids were shown to play an important role in bile formation by the liver as early as 1870(Schiff, 1870). Since then bile acids have been shown to produce diverse cellular effects, that can be beneficial as well detrimental to cells(Maillette de Buy and Beuers, 2010). The beneficial effects include stimulation of bile formation, immunomodulation, cell survival, while the toxic effects include inhibition of bile formation, apoptosis and cellular toxicity. It is becoming evident that bile acids produce many of these effects by activating intracellular signaling pathways, including cAMP, calcium, phosphoinositide 3-kinase (PI3K), Protein kinase Cs (PKCs), mitogen activated protein kinases (MAPK) and others (Amaya and Nathanson 2013, Anwer 2004,Hylemon *et al.* 2009, Maillette de Buy and Beuers 2010, Nguyen and Bouscarel 2008).

Activation of signaling pathways may, in some cases, involve activation of cell surface receptors, such as TGR5 (Pols *et al.* 2011) and spingosine-1-phosphate receptor2 (Studer *et al.* 2012) by bile acids. Interestingly, the same signaling pathways can be activated by bile acids producing opposing effects. Thus, it has been a challenge to relate intracellular signaling to specific and at times opposing effects of bile acids.

* Corresponding address: Telephone number: 508-839-8788, Fax number: 508-839-8787. *Email:* sawkat.anwer@tufts.edu

Studies in recent years have provided a better understanding of the mechanisms underlying toxic versus beneficial effects of bile acids. This review focuses on the signaling pathways postulated to be involved in bile acid mediated bile formation and cholestasis.

I. Bile formation and cholestasis

The liver, the largest gland in the body, plays a central role in the metabolism and excretion of endogenous and exogenous solutes (Anwer 1991, Anwer 2004, Nathanson and Boyer 1991). Bile is the exocrine secretion of the liver. The site of initial bile formation is the canalicular space between two hepatocytes. Thus, bile formed by hepatocytes are known as canalicular bile as opposed to ductularbile formed by bile ductular cells. Canalicular bile is modified by ductular cells before being stored in the gall bladder for animals with gallbladder. Animals without gall bladder appear to store bile in the bile duct. Bile can be further modified (i.e., concentrated) in gall bladder. Following a meal bile from the gall bladder is emptied into the duodenum. Bile provides a route of excretion for many endogenous and exogenous solutes. Bile also assists in digestion and absorption of fat by providing bile acids and phospholipids to the duodenum and plays an immunological role by delivering IgA to the intestine.

Bile being an aqueous solution (97.5% water) is more suitable for the excretion of water soluble compounds. However, the presence of micelle forming bile acids above their critical micellar concentration allows solubilization of lipids in bile. Thus, water-soluble as well as lipid-soluble compounds are excreted via the biliary route. Only solutes that are excreted into the bile directly contribute to bile formation and these solutes include like bile acids, cholesterol, phospholipid, bilirubin, proteins, inorganic ions, glutathione, drugs and toxins. Since biliary solutes are, in most part, derived from sinusoidal blood, vectorial transport of solutes from the sinusoidal space to the canaliculus provides the osmotic driving force for bile formation and is accomplished by various transporters located at the basolateral and canalicular membrane of hepatocytes and cholangiocytes(Anwer 1993, Anwer 1998, Hagenbuch and Meier 2003, Kanno *et al.* 2001,Mennone *et al.* 2001, Singh *et al.* 2001,Trauner and Boyer 2003).

Agents that increase bile formation are known as choleretic agents, while agents that decrease bile formation are known as cholestatic agents. Bile acids have been shown to produce choleresis as well as cholestasis (Anwer 2004,Maillette de Buy and Beuers 2010).The term “cholestasis” is used to describe conditions associated with decreased bile formation. It is thus easy to appreciate the paradigm that cholestasis results when the ability of the liver to transport solutes into the canaliculus is compromised.

Our present understanding of the pathogenesis of cholestasis is based on studies to define the physiological regulation of various transporters involved in bile formation (Fig. 1) and their deregulation in experimental models of cholestasis and patients with cholestatic disorders (Bohan and Boyer 2002, Elferink and Groen 2002, Jansen *et al.* 2001, Lee and Boyer 2000). In addition, studies on the expression of transporters in cholestatic diseases have provided valuable information on the role of specific transporters in the pathogenesis of some of these diseases. It is becoming clear that bile acids produce acute choleresis or cholestasis by altering signaling pathways that regulate hepatobiliary solute transporters.

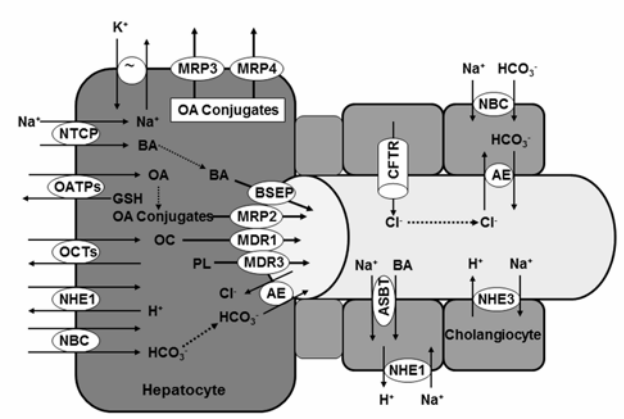

Fig.1. Transporters involved in bile formation: hepatic uptake of bile acid (BA), organic anions (OA) and organic cations (OC) is mediated primarily by Na^+/taurocholate cotransporting polypeptide (NTCP), the family of organic anion transporting proteins (OATPs) and organic cation transporters (OCTs), respectively. Na^+/H^+ exchanger (NHE1) and Na^+/HCO_3^-cotransporter (NBC) at the sinusoidal membrane of hepatocytes and basolateral membrane of cholangiocytes are involved in intracellular pH regulation and HCO_3^- excretion. NHE3 present on the apical membrane is involved in fluid absorption, and Na^+-K^+-$2Cl^-$ (not shown) may be involved in fluid secretion in cholagiocytes. Multi-drug resistance proteins (MRP3 and MRP4) mediate sinusoidal efflux of organic anions, including toxic bile acids, while MRP2 and BSEP (Bile salt export pump) mediate canalicular excretion of conjugated organic anions and bile acids, respectively. MDR1 and MDR2 (multidrug resistance gene products) are involved in biliary excretion of organic cations and phospholipids, respectively. Chloride/bicarbonate exchange is mediated by anion exchanger (AE) at canalicular as well as apical membrane of cholangiocytes. Cystic fibrosis transmembrane conductance regulators (CFTR) act as chloride channels and reabsoprtion of conjugated bile acid from the biliary tree is mediated via the apical Na^+-dependent bile acid transporter (ASBT).

II. Bile acids

Bile acids are steroid acids synthesized from cholesterol in the liver. Bile acids undergo extensive enterohepatic circulation (Hofmann 2009, Hofmann and Hagey 2008), which involves cycling of bile acids from the liver to the intestine via bile duct and from intestine to the liver via the portal vein. The first and the rate limiting step of bile acid synthesis is the hydroxylation of cholesterol by cholesterol 7-α-hydrozylase (CYP7A1). Bile acids synthesized in the liver, such as cholic acid (CA; 3α, 7α, 12α-trihydroxycholanic acid) and chenodeoxycholic acid (CDCA; 3α, 7α-dihydroxycholanic acid) are termed primary bile acids. Cholic and chenoxeodycholic acids are dehydroxylated to secondary bile acids,deoxycholic acid (DCA; 3α, 12α-dihydroxycholanic acid) and lithocholic acid (LCA; 3α-monohydroxycholanic acid), respectively, by intestinal bacteria (Fig. 2). In addition, ursodexoycholic acid (UDCA; 3α, 7β-dihydroxycholanic acid), a primary bile acid in bears, nutria and beavers,can be synthesized to a limited extent from chenodeoxycholic acid by epimerization in the liver of other species(Hofmann 2009). All bile acids can be conjugated with either taurine or glycine in the liver to form tauro-bile acids and glycol-bile acids, respectively. These conjugated bile acids are stronger acids (Hofmann and Hagey, 2008)and hence less permeable than their respective unconjugated bile acids; conjugated bile acids are deconjugated by bacterial enzymes in the intestine. The enterohepaticcirculation of bile acids (Fig. 3) are maintained by various transporters present in the liver and the intestine (Kosters and Karpen 2008).

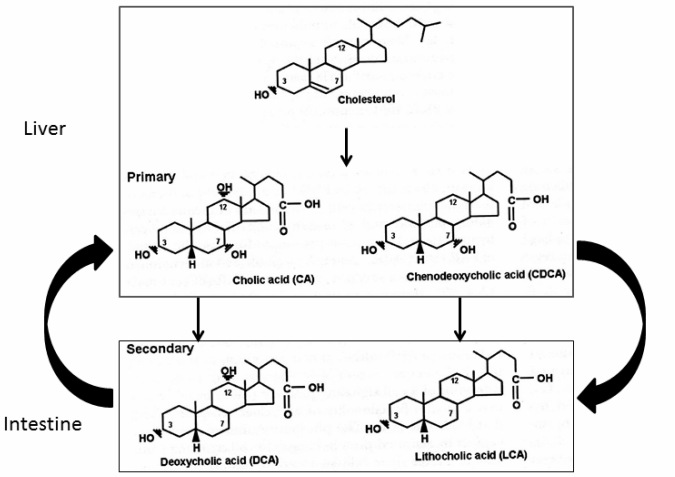

Fig.2. Primary bile acids (CA and CDCA) are synthesized from cholesterol in the liver and are converted to secondary bile acids (DCA and TCA) in the intestine. Secondary bile acids are absorbed from the intestine and taken up and secreted by the liver. Thus, bile contains primary as well as secondary bile acids (Hofmann 2009).

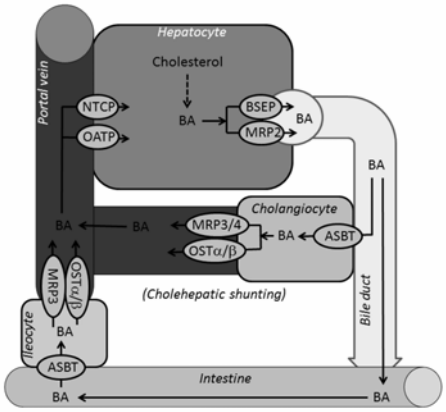

Fig.3. Enterohepatic circulation of bile acids. Bile acids are active secreted into bile canaliculi by bile acid export pump (BSEP) and multidrug associated protein 2 (MRP2). Secreted bile acids flow down the biliary tract to the gallbladder for storage and then delivered to the upper intestine following meal. During the passage through the bile duct, a small fraction of BA undergoes reabsorption through cholangiocytes into the portal blood by transporters located at the luminal side (ASBT) and basolateral side (MRP3/4 and OSTα/β) and then into the hepatocytes. This cycling of bile acids between cholangiocytes and hepatocytes is known as cholehepatic shunting. In the intestine, bile acids are absorbed across ileocytes into the portal blood by ASBT at the luminal side and MRP3 and OSTα/β on the basolateral side. Bile acids in the portal blood are transported at a high efficiency into hepatocytes by sodium-dependent NTCP as well as sodium-independent OATP1B1 and OATP1B3 present at the sinusoidal membrane of hepatocytes. BSEP = Bile acid export pump, MRP = multidrug resistance-associated protein, ASBT = apical sodium-dependent bile acid transporter , OST = organic solute transporter, NTCAP = sodium/taurocholate co-transporting polypeptide, OATP = organic anion transporting polypeptide.

Physicochemical properties & biological effects of bile acids: Bile acids, because of their beneficial as well as toxic effects, can be divided into three board groups: Bile acids that have a) beneficial effects, b) beneficial and toxic effects and c) toxic effects. The corresponding bile acids are sometimes termed the good (CA, UDCA), the bad (CDCA, DCA) and the ugly bile acids (LCA), respectively (Hofmann 1999). Bile acids are planar molecules with a hydrophobic (steroid backbone) and a hydrophilic side (hydroxyl groups). With the exception of UDCA, the hydrophobicity of a bile acid increases with decreasing hydroxyl groups. UDCA is less hydrophobic than CDCA because of beta orientation of the 7-hydroxyl group. This spatial orientation allows the bile acids to solubilize lipids by forming micelle.It is of note that the beneficial effects are observed with bile acids that are more hydrophilic, while the toxic effects are seen with bile acids that are more hydrophobic. More specifically, CA and UDCA produce beneficial effects, while LCA produces toxic effects. Thus, taurine conjugates of these bile acids (TCA, TUDCA and TLCA) have often been used to study the mechanism of bile acids induced choleresis and cholestasis.

III. Signaling pathways affected by bile acids

For a long time bile acids have been considered to be detergent molecules (also termed biological soap) involved in the solubilization of cholesterol in bile and digestion of fat in the intestine. The ability of bile acids to produce choleresis is due to osmotic force generated by active secretion of bile acids and other solutes into the bile canaliculus. Over the last two decades, this passive role of bile acids has been replaced by a more active role as regulatory/signaling molecules. Bile acids have been shown to affect a variety of signaling pathways involved in the regulation of various cellular activities. These effects are best exemplified by choleretic/cholestatic and apoptotic/antiapoptotic effects of bile acids. Thus, GCDCA and TCDCA, which predominantly accumulate in patients with cholestatic diseases, have been shown to produce apoptosis and cholestasis in experimental models (Perez and Briz 2009, Rust *et al.* 2005). In contrast, more hydrophilic bile salts, such as TUDCA and TCA, promote cell survival, produce choleresis, and reestablish normal liver function in cholestasis (Amaral *et al.* 2009, Paumgartner and Beuers 2004, Perez and Briz 2009). Despite these opposing effects, these bile acids have been shown to activate the same intracellular signaling kinases, such as PI3K, PKCs and MAPKs. What is emerging as a theme is that bile acids produce different and at time opposing effects by activating different isoforms of PI3K, PKC and MAPK, as described below.

III a . Role of PI3K pathway

PI3Ks are a family of lipid kinases (classes I, II, and III) that phosphorylate the inositol ring of phosphatidylinositides (PIs)at 3 position known as D3 phosphorylation(Cantley, 2002). The resulting phosphorylated PIs (PIPs), acting in concert with phosphoinositide-dependent kinases (PDKs), are involved in the activation of downstream kinases, such as PKCζ/λ, Akt/PKB, and $p70^{S6K}$. These kinases are involved in vesicle trafficking, cell survival, cell proliferations, cell migration, and transport of glucose and bile acids. (Anwer 1998, Rameh and Cantley 1999, Toker 2000).Among the various classes of PI3K, the class IA PI3K is primarily responsible for production of D-3 phosphoinositides in response to growth factors (Cantley 2002). The Class IA PI3K consists of a regulatory subunit, p85, and one of the three catalytic p110 subunits (p110α/p110β/p110δ). The catalytic subunit is mainly activated by binding of p85 to a phosphotyrosyl peptide in tyrosine kinases (Cantley 2002). Moreover, it has been shown that p110β can also be activated by G protein-coupled receptors (Guillermet-Guibert *et al.* 2008). The Class IB PI3K has a single catalytic isoform, p110γ, which interacts with the regulatory subunit p101, and is activated by β/γ subunits of G proteins (Cantley 2002). The in vivo substrate for Class I PI3Ks is PI-4, 5-diphosphate (PI-4,5-P2), which is converted to the signaling molecule PI-3,4,5-P3 (PIP3). PIP3 serves as a docking site for the binding and activation of downstream signaling molecules, including Akt (Cantley 2002).

It is becoming evident that different PI3K p110 isoforms mediate different cellular events. For example, activation ofp110$\alpha$is implicated in cell survival (Benistant *et al.* 2000, Gates *et al.* 2009), physiologic cardiac hypertrophy (Pretorius *et al.* 2009), and insulin signaling (Foukas *et al.* 2006). De novo DNA synthesis (Benistant *et al.* 2000)

and carcinogenesis (Jia *et al.* 2008) are associated with activation of p110β. In contrast, the induction of inflammatory responses by p110δ is primarily confined to the hemopoietic system. Although p110γ was long believed to be restricted to immunecells (Hawkins and Stephens 2007), recent studies suggest a role for p110γin the development of atherosclerotic lesions (Chang *et al.* 2007), loss of cardiac contractility (Pretorius *et al.* 2009), andacute pancreatitis (Fischer *et al.* 2007, Lupia *et al.* 2004). Thus, p110α appears to mediate beneficial effects, while p110γ may mediate toxic effects. Studies in hepatic cells suggest that the opposing effects of bile acids may also be mediated via different isoforms of class I PI3K.

A role of PI3K in bile formation is evident from a study (Folli *et al.* 1997) showing that wortmannin, a specific inhibitor of PI3K, inhibits bile formation, bile acid secretion and vesicle trafficking in isolated perfused rat liver. Since then various studies have provided evidence supporting a role for PI3K/Akt pathway in cell survival and translocation of hepatocellular transporters to the plasma membrane, indicating a beneficial role of PI3K in hepatic cells (Anwer 2004, Gates *et al.* 2009, Rust *et al.* 2005 ,Webster *et al.* 2002b). However, it is now evident that choleretic (TCA and TUDCA) as well as cholestatic (TLCA and TCDCA) bile acids activate PI3K (Beuers *et al.* 2003, Kurz *et al.* 2000, Misra *et al.* 1998, Rust *et al.* 2000). Thus, it would appear that PI3K may mediate beneficial as well as toxic effects of bile acids. In that case, the opposing effects of bile acids may be mediated via different PI3K/p110 isoforms. Indeed studies in rat hepatocytes with choleretic (TUDCA and TCA) and cholestatic (TCDCA, GCDCA, TLCA) bile acids showed that all bile acids activated PI3K-p110β, but only toxic bile acids activated PI3K-p110γ (Hohenester *et al.* 2010). Further studies showed that inhibition of p110γ attenuated GCDCA-induced apoptosis and activation of c-Jun N-terminal kinase (JNK), but did not alter TUDCA- or cAMP-induced Akt-signaling (Hohenester *et al.* 2010).Since activation p110γ is associated with toxic effects in non-hepatic cells (Chang *et al.* 2007, Fischer *et al.* 2007, Lupia *et al.* 2004, Pretorius *et al.* 2009), these results in hepatocytes would suggest that the toxic effect of bile acids may be mediated by activation of PI3K-p110γ pathway. Moreover, since Activation of JNK pathway has been linked to bile acid-induced hepatotoxicity (Graf *et al.* 2002a, Gupta *et al.* 2004a, Usechak *et al.* 2008) and PI3K-p110γ has been shown to induce JNK-mediated signaling cascades in non-hepatic cells (Go *et al.* 1998), the cytotoxic effect of bile acids may be mediated via PI3K-p110γ-JNK pathway. On the other hand, the beneficial effects of bile acids may be mediated via PI3K-p110α/β-Akt pathway (Fig. 4).

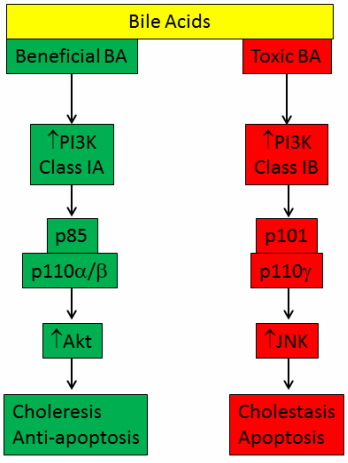

Fig.4. Postulated role of PI3K-p110 isoforms in beneficial and toxic effects of bile acids. Beneficial effects (choleresis and anti-apoptosis) of bile acids are mediated via PI3K/p110 α/β/Akt pathway, while the toxic effects (cholestasis and apoptosis) is mediated via PI3K/p110γ/JNK pathway

Such a hypothesis is consistent with the other findings that a) PI3K-Akt pathway is cytoprotective(Gates *et al.* 2009, Rust *et al.* 2005, Webster *et al.* 2002b), b) the cytoprotectiveeffect of cAMP-GEF in hepatocytes is associated with PI3Kp110α/p110β activation (Gates *et al.* 2009), and c) PI3K-p110α is necessaryfor insulin signaling in the liver(Foukas *et al.* 2006).

IIIb. Role of Protein kinase C

Protein kinase C belongs to a family of serine/threonine protein kinases that are involved in the regulation of diverse cellular functions andconsists of at least 12 isoforms (Newton 2003, Reyland 2009). These include conventional (cPKCα, β, βI, βII and γ), novel (nPKCδ, ε, η and θ), atypical (aPKCζ andλ/ι) isoforms . These isoforms differ in their dependency on Ca^{2+} and phospholipids, such that cPKCs are dependent on Ca^{2+} and diacylglycerol (DAG), nPKCs are Ca^{2+}-independent and aPKCs are independent of both Ca^{2+} and DAG (Fig. 5).Activation of most PKCs, if not all, is PI3K dependent (Newton 2003).PKCs shown to be present in rat hepatocytes include cPKCα, nPKCδ, nPKCε, and aPKCζ with the presence of cPKCβII being controversial. (Beuers *et al.* 1999,Jones *et al.* 1997, Stravitz *et al.* 1996).

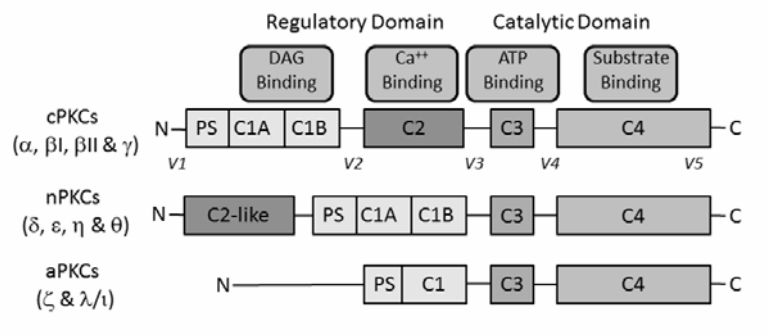

Fig.5. Primary structures of PKCs(Michalczyk *et al.* 2013,Newton 2003, Newton 2010, Reyland 2009, Tan and Parker 2003). There are four structurally conserved domains (C1-C4) in PKC isoforms divided into the N-terminal regulatory domain (C1-C2) and the C-terminal catalytic domain (C2-C4). The regulatory domain contain the binding sites for pseudosubstrate (PS), DAG (C1) and Ca^{++} (C2 or C2-like). The catalytic domain contains the binding sites of ATP (C3) and substrate (C4). C-regions (C1-C4) represent conserved domains and V-regions represent variable domains (V1-V5). The regulatory and the catalytic domains are separated by a flexible hinge domain (V3), which is cleaved by caspase-3 in apoptotic cells. Novel isozymes contain a C2-like domain which is unable to bind Ca^{++} and hence do not require Ca^{++} for activation. Atypical isozymes contain a variant of the C1 domain, which lacks the ligand-binding pocket for DAG and lacks C2 domain. As a result aPKCs are not regulated by DAG and Ca^{++}; they are regulated by protein-protein interactions.The intermolecular binding between PS and catalytic domain is highly regulated by membrane interactions, PKC conformation and phosphorylation.

Initial studies using known general activators and inhibitors of conventional and novel PKCs suggested that activation of PKCs produce cholestasis (Anwer 2004). However, both choleretic and cholestatic bile acids activate PKCs (Table 1) and PKCs have now been implicated in apoptotic, anti-apoptotic, cholestatic and choleretic effects of bile acids and other agents, (Anwer 2004, Castello *et al.* 2005, Jones *et al.* 1997, Kubitz *et al.* 2004a, Paumgartner and Beuers 2004, Perez *et al.* 2006, Rust *et al.* 2000).It is becoming evident that the opposing effects of bile acids may be mediated via different isoforms of PKCs, although the role of all PKC isoforms has not been clearly established. Studies to date would indicate that aPKCζ may mediate beneficial effects and nPKCε may mediate toxic effects, while cPKCα and nPKCδmay be involved in both beneficial and toxic effects of bile acids.

Table 1.Effect of bile acids on PKC isoforms and the reported role of PKC isoforms in hepatic cells.

PKC isoforms	TCA	TUDCA	GCDCA	TCDCA	TDCA	TLCA	Role of PKC isoforms
cPKCα	↑/–	↑	↑	↑	↑	↓	Cholestasis (Kubitz *et al.* 2004a) Apoptosis (Jones *et al.* 1997), anticholestatic (Wimmer *et al.* 2008), Ntcp retrieval (Muhlfeld *et al.* 2012), Mrp2 translocation (Beuers *et al.* 2001)
nPKCδ	↑/–	–	↑	↑	↑	–	Apoptosis (Jones *et al.* 1997) , Non-bile acid cytotoxicity (Castello *et al.* 2005, Maddox *et al.* 2003), Ntcp & Mrp2 translocation (Park *et al.* 2012, Schonhoff *et al.* 2008), Antiapoptotic (unpublished)
nPKCε	↑/–	–	↑	ND	ND	↑	Cholestasis (Beuers *et al.* 2003),Mrp2 retrieval (Schonhoff *et al.* 2013)
aPKCζ	–	ND	ND	↑	ND	–	Ntcp translocation (McConkey *et al.* 2004), Antiapoptosis (Rust *et al.* 2000), Activation of insulin signaling pathway (Cao *et al.* 2010)

TCA (Beuers *et al.* 1996, Rao *et al.* 1997, Stravitz *et al.* 1996), TUDCA (Beuers *et al.* 1996, Rao *et al.* 1997), GCDCA(Jones *et al.* 1997), TCDCA(Lali *et al.* 2000, Rao *et al.* 1997), TDCA (Rao *et al.* 1997) and TLCA (Beuers *et al.* 2001, Beuers *et al.* 2003, Schonhoff *et al.* 2013) has been shown to affect PKC isoforms activity in hepatocytes. TCA has been reported to activate (Rao *et al.* 1997, Stravitz *et al.* 1996) or have no effect on cPKCα (Beuers *et al.* 1996) in hepatocytes. The effect of TCA is dependent on PKCζ(Cao *et al.* 2010), but TCA has not been reported to activate PKCζ. Activation (↑), inhibition (↓) or no effect (–); ND= not determined.

Role of cPKC/cPKCα: Activation of cPKCα has been implicated in cardiomyocyte hypertrophy, thrombus formation, cell proliferation and apoptosis in non-hepatic cells (Konopatskaya and Poole 2010, Reyland 2009).Both choleretic and cholestatic bile acids can activate cPKCα (Table 1) and cPKCα has been suggested to mediate bile acid-induced beneficial as well as toxic effects.

TUDCA activatesc PKCα in isolated rat hepatocytes(Beuers *et al.* 1996)and cPKCα may be involved in the translocation of Mrp2 to the canalicular membrane by TUDCA (Beuers *et al.* 2001). TUDCA has been shown to reverse TLCA-induced cholestasis(Beuers *et al.* 2001, Scholmerich *et al.* 1990)and it has been suggested that TUDCA may reverse TLCA-induced cholestasis by activating cPKCα (Beuers *et al.* 2001, Beuers *et al.* 1996). This is consistent with a recent study showing that TUDCA-induced reversal of TLCA-mediated decreases in bile formation is partially inhibited by combined inhibition of cPKC and PKA (Wimmer *et al.* 2008). Interestingly, inhibition of cPKC or PKA alone did not reverse TUDCA effect. Also, neither TUDCA nor TLCA affected PKA activity in hepatocytes. It is suggested that the anticholestatic effect of TUDCA may in part be mediated via cooperative post-translational cPKCα-/PKA-dependent mechanisms. The nature of this postulated cooperative mechanism remains to be evaluated. It may be noted that acute cholestasis induced by TLCA and ethinylestradiol-17βglucuronide is associated with retrieval of Bsep and Mrp2 from the canalicular membrane and these effects are reversed by cAMP (Crocenzi *et al.* 2003a, Crocenzi *et al.* 2003b, Mottino *et al.* 2002).It is however not known whether the anticholestaticeffect of cAMP, which does not activate cPKCα in hepatocytes (Schonhoff *et al.* 2008),may also require a permissive role of cPKCα.

In contrast, cPKCα has also been implicated in effects of bile acids that are not beneficial. For example, GCDCA-induced apoptosis requires activation of cPKCα (Jones *et al.* 1997) and cPKCs have been reported to mediate cholestasis induced by drug and oxidative stress (Kubitz *et al.* 2004a, Perez *et al.* 2006). In addition, a recent study showed that activation of cPKC by TCDCA, a cholestatic bile acid, or thymeleatoxin can lead to retrieval of Ntcp from the plasma membrane and thereby decrease bile acid uptake (Muhlfeld *et al.* 2012, Stross *et al.* 2010). Conventional PKCs have also been implicated in cholestasis associated with Bsep retrieval (Kubitz *et al.* 2004a, Perez *et al.* 2006).On the other hand, TLCA induces retrieval of Ntcp in rat hepatocytes (Schonhoff *et al.* 2009), but inhibits cPKCα (Beuers *et al.* 2001). Thus, the retrieval of Ntcp by bile acids may involve mediators in addition to cPKCα.

These studies suggest that cPKCα may mediate cholestasis as well as anticholestatic effects of bile acids. The mechanism by which this is accomplished is unclear at this time. It is possible that activation of cPKCα by choleretic and cholestatic bile acids involves translocation to different subcellular membrane resulting in activation/inhibition of different downstream effectors. In that case, the opposing effects of cPKCα may be dependent on downstream effectors affected. Thus, it would be useful to know if cPKCα is targeted to different cellular sites by choleretic and cholestatic bile acids. It may be noted that the role of cPKCα in these studies were evaluated by using chemical inhibitors, which may not be as specific as currently believed. Further studies using other approaches (knockout, knockdown, constitutively active and dominant negative mutants) to activate and inhibit cPKCα are needed to establish the role of cPKCα.

Role of PKCδ: While certain bile acids have been shown to activate nPKCδ (Table 1), only limited studies have evaluated the role of nPKCδ in the effects of bile acids. One study using chemical inhibitors of PKCs suggested that GCDCA-induced apoptosis in hepatocytes requires activation of nPKCδ (Jones *et al.* 1997). However, a recent study using molecular activators and inhibitors of nPKCδ showed that activation of nPKCδ by GCDCA actually induces a cytoprotective pathway by inhibiting JNK activation and down-regulating proapototic BIM (unpublished data). Other studies suggest that nPKCδ may be involved in allyl alcohol-induced hepatotoxicity (Maddox *et al.* 2003) and 4-hydroxynonenal-induced apoptosis in hepatocytes (Castello *et al.* 2005). In contrast, cAMP, a known choleretic and anti-apoptotic agent (Webster *et al.* 2002b, Webster and Anwer 1998), activates nPKCδ and nPKCδ has also been shown to mediate cAMP-induced translocation of NTCP and MRP2 to the plasma membrane (Park *et al.* 2012, Schonhoff *et al.* 2008). This is consistent with known transporter regulatory effect of nPKCδ, which include stimulation of Na^+-H^+ exchanger in glial cells (Chen and Wu 1995), α1-adrenergic activation of Na^+-K^+-$2Cl^-$ cotransport in tracheal epithelial cells (Liedtke and Cole 1997), insulin-mediated GLUT4 translocation in myocytes and adipocytes (Braiman *et al.* 1999, Elmendorf 2002) and serotonin-mediated inhibition of Cl^-/OH^- exchange in Caco-2 cells (Saksena *et al.* 2005). Thus, it appears that nPKCδ may mediate both toxic and beneficial effects in hepatocytes. The mechanism by which this is accomplished is unclear and may be related to phosphorylation of nPKCδ.

Novel PKCδ has been reported to positively and negatively regulate apoptosis depending on sites phosphorylated by various stimuli (Brodie and Blumberg 2003, Jackson and Foster 2004). It has been suggested that the cleavage of activated nPKCδ to a catalytic fragment stimulates apoptosis (Jackson and Foster 2004). The cleavage of nPKCδ appears to be dependent on phosphorylation at Tyr^{311}(Yoshida 2007) and the phosphorylation at this site by c-Abl, a nonreceptor tyrosine kinase, promotes the apoptotic effect of nPKCδ in glioma cells (Lu *et al.* 2007). Tyrosine phosphorylated nPKCδ transiently accumulates in the nucleus, where it is cleaved by caspase-3 to generate the nPKCδ catalytic fragment (delta-CF), a constitutively activated, pro-apoptotic form of nPKCδ (Reyland 2009). On the other hand, the PI3K-dependent activation of nPKCδ by serum in HEK293 cells (Le Good *et al.* 1998) and by VEGF in HUVEC (Gliki *et al.* 2002) involves phosphorylation of Thr^{505} in the activation loop, and the

latter is involved in VEGF-stimulated angiogenesis (Gliki *et al.* 2002). These studies may suggest that activation via Tyr^{311} phosphorylation may lead to toxic effect, while Thr^{505} phosphorylation may be beneficial. Consistent with this hypothesis is the finding that activation of nPKCδ by cAMP involves Thr^{505} and not Tyr^{311} phosphorylation (Schonhoff *et al.* 2008). Further studies showing that choleretic and cholestatic effects of bile acids depends on nPKCδ phosphorylation at Thr^{505} and Tyr^{311}, respectively, will be required to confirm this hypothesis.

Role of nPKCε: A number of cellular processes have been shown to be regulated by nPKCε, including transporters, endocytosis, exocytosis and tumor progression in cell specific manner (Akita 2002, Akita 2008, Reyland 2009). Of interest to mechanism of bile formation is the postulated role of nPKCε in the regulation of transporters. Thus, PKCε may decrease chloride secretion by internalizing Na-K-2Cl cotransporter (Del I *et al.* 2005) and fluid phase endocytosis in T84 cells (Song *et al.* 1999). On the other hand, carbachol-induced secretion in lachrymal gland (Jerdeva *et al.* 2005) is enhanced by nPKCε. In hepatocytes, choleretic (TCA) as well cholestatic bile acids (GCDCA and TLCA) activate nPKCε (Beuers *et al.* 1999, Jones *et al.* 1997, Rao *et al.* 1997, Stravitz *et al.* 1996) and nPKCε has been suggested to be involved in the cholestatic effect of TLCA. The role of nPKCε in the effect of TCA and GCDCA has not been studied directly. TLCA activates nPKCε (Beuers *et al.* 1999, Schonhoff *et al.* 2009) and induces Mrp2 retrieval (Nakashima 2002)resulting in decreased solute excretion and bile formation. TUDCA, which reverses TLCA-induced cholestasis (Beuers *et al.* 2001;Scholmerich *et al.* 1990) and Mrp2 function (Beuers *et al.* 2003), inhibits TLCA-induced nPKCε activation (Beuers *et al.* 2003). Our unpublished study showed that cAMP can reverse TLCA-induced Mrp2 retrieval and nPKCε activation in hepatocytes. Thus, PKCε may mediate TLCA-induced Mrp2 retrieval, and cAMP and TUDCA may reverse this effect of TLCA by inhibiting TLCA-induced activation of nPKCε. TLCA also inhibits TCA uptake in hepatocytes (Schwenk *et al.* 1977) and in HuH-NTCAP cell (Schonhoff *et al.* 2009). However, TLCA-induced inhibition of TCA uptake is not mediated via nPKCε (Schonhoff *et al.* 2009). Thus, the canalicular membrane may be the target of nPKCε and this is consistent with the finding that TLCA translocates nPKCε to the canalicular membrane (Beuers *et al.* 1999).

Mechanism by which TLCA acting via nPKCε induces internalization of Mrp2 is still being determined. One study suggested that this may involve nPKCε-mediated phosphorylation of Mrp2 (Wimmer *et al.* 2008). This study reported that phorbolmyristate acetate, an activator of cPKCs and nPKCs, can phosphorylate Mrp2 in rat hepatocytes and this is inhibited by staurosporine, an inhibitor of cPKCs and nPKCs. In addition, recombinant cPKCα and nPKCε phosphorylated MRP2 immunoprecipitated from HepG2 cells in vitro. These results suggest a role for cPKCα and nPKCε in Mrp2 phosphorylation. It is however not known whether Mrp2 translocation is regulated by its phosphorylation as it has been suggested for Ntcp (Anwer *et al.* 2007, Anwer *et al.* 2005). A recent study (Schonhoff *et al.* 2013) showed that TLCA-induced Mrp2 retrieval may involve PKCε mediated phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS). MARCKS is a membrane-bound F-actin crosslinking protein and is phosphorylated by PKCs(Fujise *et al.* 1994, Heemskerk *et al.* 1993). MARCKS phosphorylation has been implicated in endocytosis and this may involve detachment ofphosphorylated MARCKS (pMARCKS) from the membrane (Park *et al.* 2006). Studies in hepatocytes and HuH-NTCP cells (Schonhoff *et al.* 2013) showed that TLCA, but not cAMP, increased MARCKS phosphorylation. In HuH-NTCP cells transfected with phosphorylation-deficient MARCKS, TLCA failed to increase MARCKS phosphorylationor decrease plasma membrane MRP2. It is suggested that MRP2 retrieval by TLCA involves TLCA-mediated activation of nPKCε followed by MARCKS phosphorylation and consequent detachment of MARCKS from the membrane(Schonhoff *et al.* 2013). It would appear that TLCA-induced activation of nPKCε may result in MRP2 retrieval from the canalicular membrane by at least two different mechanisms.

Role of aPKCζ: This atypical PKC isoform is a downstream effector of PI3K and plays an important role in cell survival signaling (Reyland 2009). Atypical PKCζ has been shown to play a cytoprotective role in hepatocytes (Rust *et al.* 2000). This study showed that TCDCA activated aPKCζ and inhibition of aPKCζ converted TCDCA into a cytotoxic agent. In addition, over expression of wild-type aPKCζblocked GCDCA-induced apoptosis. In addition to its effect on cell survival, aPKCζ has been implicated in cellular metabolism and solute transport in hepatocytes. aPKCζ is involved in TCA-induced activation of the insulin signaling pathway via G -protein-coupled receptors (Cao *et al.* 2010). The PI3K/aPKCζ pathway is also involved in cAMP-induced Ntcp translocation to the plasma membrane in hepatocytes (McConkey *et al.* 2004). So far only one bile acid, TCDCA, has been shown to activate aPKCζ (Rust *et al.* 2000). The activation of insulin signaling pathway by TCA is dependent on aPKCζ (Cao *et al.* 2010) and TCA activates PI3K pathway (Misra *et al.* 1998). Thus, TCA may also activate PI3K/aPKCζ pathway. However, whether aPKCζ is involved in the action of other bile acids is unknown at this time.

IIIc. Role of MAPKs:

MAPKs mediate/regulate diverse cellular functions including embryogenesis, apoptosis, immunity, proliferation, and differentiation by integrating signals from intra- and extracellular stimuli (Cook *et al.* 2007, Han and Sun, 2007, Kaminska 2005, Raman *et al.* 2007). Mammalian cells have four major types of MAPKs cascade (Fig. 6) and these include ERK1/2, JNKs, p38 MAPKand ERK5 cascades (Avruch 2007, Keshet and Seger 2010, Krishna and Narang 2008, Morrison 2012). Each of these cascades consists of a core module of three tiers of protein kinases termed MAPK, MAP2K, and MAP3K. There are seven MAP2Ks (also known as MEK, MAP/ERK kinase, or MKK) that differentially activate different MAPKs by dual phosphorylation on Thr and Tyr. Thus, ERK1/2 are activated by MKK1/2, p38 MAPKs are activated by MKK3, MKK4, and MKK6, JNKs by MKK4/7 and ERK5 by MKK5. Activated MAPKs, in turn, phosphorylate and activate transcription factors leading to expression of target genes. The deactivation of MAPKs is achieved through dephosphorylation catalyzed by MAPK-specific phosphatases (MKPs) including dual specific MAPK phosphatases (Caunt and Keyse 2013, Kondoh and Nishida 2007). Choleretic as well as cholestatic bile acids activate ERK1/2 and P38 MAPK and only cholestatic bile acids active JNK1/2 (Table 2). The postulated roles of these kinases in the effect of bile acids are summarized below.

Table 2. Effects of bile acid on MAPKs and the reported effects of MAPKs in hepatic cells

	ERK1/2	P38 MAPK	JNK1/2
TCA	↑	↑	–
TUDCA	↑	↑	–
GCDCA	↑	↑	↑
DCA	↑	↑	↑
TLCA	↑	↑	↑
TLCS	↑	↑	↑
Effects in hepatic cells	Choleresis (Kurz *et al.* 2000, Schliess *et al.* 1997)	Choleresis (Haussinger *et al.* 2003, Kubitz *et al.* 2004b)	Apoptosis (Hohenester *et al.* 2010, Park *et al.* 2007)
	Anti-apoptosis (Qiao *et al.* 2002)	Apoptosis (Grambihler *et al.* 2003)	Cytoprotection (Qiao *et al.* 2003)
	Cell polarization (Fu *et al.* 2011)	Anti-apoptosis (Schoemaker *et al.* 2004)	↓ Cyp7A1(Gupta *et al.* 2001)
		↓ Cyp7A1(Xu *et al.* 2007)	
		Proliferation (Awad *et al.* 2000)	

Both choleretic and cholestatic bile acids activate ERK1/2 and p38 MAPK, and only cholestatic bile acids activate JNK1/2. Activation of ERK1/2 is associated with beneficial effects of bile acids, while activation of JNK1/2 may result in toxic effects of bile acids. Some studies suggest that activation of JNK2 may be cytoprotective. Activation of p38 MAPK is associated with beneficial as well as toxic effects of bile acids. TLCS=Taurolithocholate sulfate

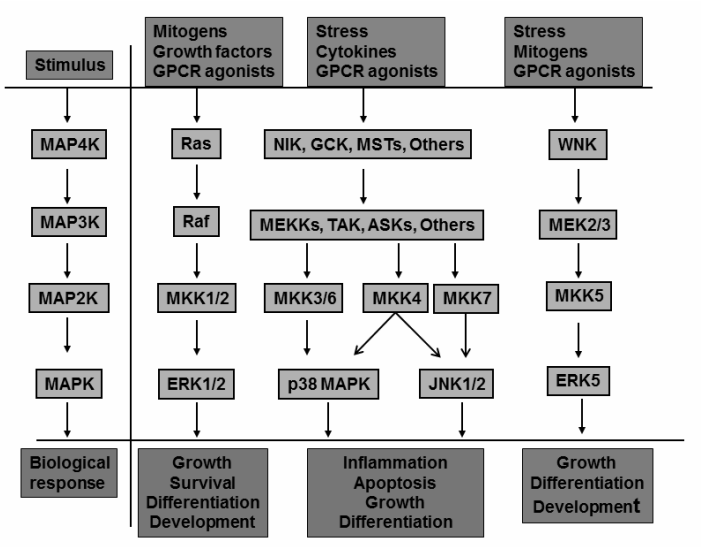

Fig.6. MAPK signaling cascades consist of a core of three sequentially activated protein kinases and these include MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K) and MAPK (Avruch 2007, Keshet and Seger 2010, Krishna and Narang 2008, Raman *et al.* 2007). In addition, there is an upstream MAPK kinase kinase (MAP4K) and a downstream MAPK activated protein kinase (MAPKAPK, not shown) in certain cells and for certain stimulations, but they are not always necessary for signaling through the cascades. Each cascade is initiated following extracellular stimulus. The extracellular stimuli include growth factors, mitogens, G-protein coupled receptor (GPCR) agonist, stress and inflammatory cytokines. Activation of MAP4K typically involves phosphorylation by protein kinases activated by interaction of an agonist with its cell surface receptor. MAP3K, which is phosphorylated and activated by MAP4K, directly phosphorylates and activates MAP2K (also known as MKK or MEK). Activation of MAPK is then accomplished by dual phosphorylation of a conserved tripeptide (Thr-X-Tyr) motif in the conserved segment by MAP2K. MAP kinases include extracellular signal-regulated kinase (ERK1/2), c-Jun amino-terminal kinases(JNK1/2/3), p38 MAPK and ERK5. Activation of each MAPK leads to a diverse array of biological response.

Role of ERK1/2: Bile acids activate the ERK1/2 signaling pathway and the mechanism of activation differs between conjugated and unconjugated bile acids (Hylemon *et al.* 2009). The activation by conjugated bile acids may involve activation of the G protein-coupled receptor, S1P receptor 2 (Dent *et al.* 2005, Fang *et al.* 2007, Studer *et al.* 2012). Activation by unconjugated bile acids may involve generation of mitochondrial superoxide ion followed by inactivation of protein phosphatases resulting in the activation of epidermal growth factor receptor (EGFR) and ERK1/2 (Hylemon *et al.* 2009).Activation of ERK1/2 by bile acids may be involved in cytoprotection from bile acid-induced apoptosis (Qiao *et al.* 2002), bile acid-mediated hepatocyte polarization (Fu *et al.* 2011) and stimulation of bile formation (Schliess *et al.* 1997).Although bile acids activate ERK1/2 and nuclear receptors in hepatocytes, a cause-effect relationship between these two effects has not been clearly established (Hylemon *et al.* 2009).Recent studies suggest that bile acids may accelerate development of hepatocyte polarity by activating a signaling pathway that involves activation of ERK1/2 and AMP-kinase (Fu *et al.* 2011). TUDCA-induced increases in bile acid excretion in perfused rat livers are dependent on ERK1/2 activation and the effect of TUDCA is inhibited by cAMP (Schliess *et al.* 1997). The effect of cAMP is consistent with a role of ERK pathway in as much as cAMP has been shown to inhibit

ERK1/2 in hepatocytes (Webster and Anwer 1999). The effect of TUDCA appears to be mediated via a PI3K-dependent activation of Ras/ERK pathway (Kurz *et al.* 2000) and this pathway is in turn activated by an interaction of TUDCA with integrins resulting in the activation of focal adhesion kinase (FAK)/Src pathway (Haussinger *et al.* 2003). ERK1/2 is not involved in TCDCA-induced retrieval of NTCP(Muhlfeld *et al.* 2012). Overall, activation of ERK1/2 pathway appears to mediate beneficial effects of bile acids.

Role of P38 MAPK:The effects of p38MAPK in the liver include regulation of proliferation (Awad *et al.* 2000), protection against hypoxic injury (Carini *et al.* 2007), gluconeogenesis (Cao *et al.* 2005), bile acid synthesis (Xu *et al.* 2007) and anti-apoptotic effect of TUDCA(Schoemaker *et al.* 2004), bile acid-induced apoptosis (Grambihler *et al.* 2003) and biliary excretion of bile acids (Kubitz *et al.* 2004b). TUDCA-induced increases in bile acid secretion and Bsep translocation to the canalicular membrane require PI3K-independent activation of p38 MAPK (Kubitz *et al.* 2004b, Kurz *et al.* 2001). Translocation of Mrp2 by cAMP is also mediated by p38 MAPK (Schonhoff *et al.* 2010). Interestingly, choleretic (TUDCA, TCA, cAMP) as well as cholestatic (GCDCA, TLCA) agents have been shown to activate p38 MAPK (Graf *et al.* 2002b, Graf *et al.* 2003, Grambihler *et al.* 2003, Kurz *et al.* 2001, Qiao *et al.* 2003, Webster *et al.* 2002a). Thus, p38MAPK appears to mediate both toxic and beneficial effects of bile acids in the liver. One possible explanation for the opposing effects mediated by p38 MAPK may be due to activation of different p38 MAPK isoforms.

There are four known isoforms (α, β, γ and δ) of p38 MAPK and only α and β isoforms are expressed in human liver (Jiang *et al.* 1997). While MKK6 activates all p38 MAPK isoforms, MKK3 does not activate p38β isoform (Enslen *et al.* 1998, Jiang *et al.* 1997). Thus, MKK3 is expected to activate p38α, and not p38β MAPK in the liver. Cyclic AMP has been shown to specifically activate p38α, but not p38β in adipocytes (Robidoux *et al.* 2005). Selective activation of p38 isoforms has been reported in other cells (Kaminska, 2005, Korb *et al.* 2006, Wang *et al.* 2002). Cyclic AMP-induced translocation of Mrp2 in hepatic cells involves activation of MKK3/P38α MAPK pathway (Schonhoff *et al.* 2010). While both cAMP and TLCA activate p38 MAPK in mouse hepatocytes, activation of p38MAPK by cAMP, but not byTLCA was inhibited in hepatocytes isolated from MKK3 knockout mice. In addition, cAMP-induced translocation of Mrp2, but not the retrieval of Mrp2 by TLCA was inhibited in hepatocytes from MKK3 knockout mice (unpublished data). These results would suggest that the effect of cAMP, but not TLCA, is mediated via MKK3/P38α MAPK. Thus, it appears that the beneficial effects of bile acids are mediated via activation of p38α MAPK. In that case, the toxic effects of bile acids may be mediated via MKK6/p38β MAPK, respectively. Further studies are needed to test the validity of this hypothesis.

Role of JNKs: There are three isoforms (JNK1, JNK2 and JNK3) of JNK;JNK1and JNK2 are ubiquitous, whereas JNK3 is relatively restricted to brain (Krishna and Narang 2008). JNK proteins are involved in cytokine production, the inflammatory response, stress-induced and developmentally programmed apoptosis, actin reorganization, cell transformation and metabolism (Krishna and Narang 2008, Morrison 2012, Weston and Davis 2007). In hepatocytes, JNK1 and JNK2 are activated by bile acids known to produce toxicity (Graf *et al.* 2002b, Gupta *et al.* 2004b, Park *et al.* 2007, Qiao *et al.* 2003) and are implicated in bile acid-induced apoptosis (Hohenester *et al.* 2010, Park *et al.* 2007, Qiao *et al.* 2003) and hepatic manifestation of metabolic syndrome (Czaja 2010). Activation of the JNK 1/2 signaling pathway in primary hepatocytes by bile acids has been shown to downregulate CYP7A1 mRNA (Gupta *et al.* 2001), and thereby decrease bile acid synthesis. Studies to date suggest that JNK1 may mediate toxic effects, while JNK2 may be cytoprotective in certain liver injury (Qiao *et al.* 2003, Schattenberg *et al.* 2006, Singh *et al.* 2009, Tuncman *et al.* 2006). However, JNK2 activation has also been shown to be involved in liver injury (Schattenberg *et al.* 2012). Whether JNKs are involved in bile acid induced choleresis and cholestasis has not been studied.

IV. Future perspective

Our understanding of the intracellular signaling pathways involved in the regulatory roles played by bile acids is evolving. Recent studies have led to a better understanding of the role of isoforms of PI3K, PKCs and MAPKs in beneficial and toxic effects of bile acids. These studies have led to plausible hypotheses that merit further confirmation and exploration. However, signaling pathways involved in the opposing effects of bile acids are incompletely understood. Thus, it is not known whether the beneficial and toxic effects of bile acids can be explained by a) differential activation of PI3K-p110α/β and PI3K-p110γ isoforms, b) different subcellular localization of aPKCα, c) differential phosphorylation of nPKCδ, and e) different isoforms of p38 MAPK. It is anticipated that further progress in these areas will allow us to identify specific signaling pathways that are activated by choleretic and cholestatic bile acids. Armed with such information we should be able to design therapeutic approaches to treat cholestasis by targeting specific pathway(s).

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