REGULATORY NETWORKS OF PXR, CAR AND LXR IN CHOLESTEROL AND BILE ACID METABOLISM

by

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The orphan nuclear receptors Pregnane X Receptor (PXR) and Constitutive Androstane Receptor (CAR) have been proposed to play an important role in the detoxification of xeno- and endobiotics by regulating the expression of detoxifying enzymes and transporters. We showed that the combined loss of PXR and CAR resulted in a significantly heightened sensitivity to bile acid toxicity in a sex-specific manner. The increased sensitivity in males was associated with genotype-specific suppression of bile acid transporters and loss of bile acid-mediated down regulation of small heterodimer partner, whereas the transporter suppression was modest or absent in the female DKO mice.

The liver X receptors (LXRs), including the α and β isoforms were identified as sterol sensors that regulate cholesterol and lipid homeostasis and macrophage functions. We found that activation of LXRα in transgenic mice or with LXR ligands confers a female-specific resistance to lithocholic acid (LCA)-induced hepatotoxicity and bile duct ligation (BDL)-induced cholestasis. In contrast, LXR α and β double knockout mice (LXR DKO) exhibited heightened cholestatic sensitivity. The LCA and BDL resistance in transgenic mice was associated with an increased expression of bile acid detoxifying sulfotransferase 2A (SULT2A) and selected members of the bile acid transporters.

We also showed that genetic or pharmacological activation of the orphan nuclear receptor liver X receptor (LXR) sensitized mice to cholesterol gallstone disease (CGD) induced by a high cholesterol lithogenic diet. LXR-promoted CGD was associated with increased expression of several canalicular transporters that efflux cholesterol and phospholipids, leading to higher

biliary concentrations of cholesterol and phospholipids. The biliary bile salt concentration was reduced in these mice, resulting in increased cholesterol saturation index (CSI). Interestingly, the lithogenic effect of LXR was completely abolished in the low-density lipoprotein receptor (LDLR) null background or when the mice were treated with Ezetimibe, a cholesterol-lowering drug that blocks the intestinal dietary cholesterol absorption. We propose that LXRs have evolved to have dual function in maintaining cholesterol and bile acid homeostasis.

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FOREWORD

"Om Bhoor Bhuvah Swah Tat Savitur Varenyam
Bhargo Devasya Dheemahi Dhiyo Yo Nah Prachodayat"

May my intellect be steady without agitations. May it be clear without anger or passion. May the brilliant light of the sun beam through my intellect so that my perception of the world may be clear, my discrimination may be subtle, my judgment correct and quick, my comprehension of situations and persons be precise and wise.

Dedicated to my parents, to my family
and to the fond memory of Pankaj

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This thesis is part of the research work, which I did while I worked for almost five years in Dr Wen Xie's research group in the Center for Pharmacogenetics, University of Pittsburgh. During this time, I have worked with a great number of people and their contribution in different ways to my research deserves special mention. I would like to convey my gratitude to them all in my humble acknowledgment.

I would like to convey my first and foremost thanks to my advisor Dr Wen Xie. Dr. Xie as a mentor has been like a trusted counselor. He has made a distinct impact on my life by motivating and challenging me, as well as by the kindness, compassion and sincerity that he has shown. I have learnt a lot from him and he has guided me expertly through the years of graduate school. He has given me the freedom to pursue my interests while simultaneously overseeing my work to make sure that it is on the right track. I feel that Dr. Xie's most incredible talent as a mentor stems from his unwavering optimism in my work and his support for me both at work as well as personally. He has provided me with a tremendous amount of enthusiasm and motivation for the future!

I greatly indebted to my thesis committee Dr's Donald DeFranco, Raman Venkataramanan, Shivendra Singh and Allan Zhao for their positive and enthusiastic support of my work during my Ph.D. Everyone has different areas that need to be nourished, my committee has been superb at identifying those areas and prodding me to focus on those that need to be strengthened. There have of course been let down and pitfalls in my work, but my committee has always had an ability to put a positive spin on my projects, and this has really been inspirational. I believe that this will greatly help me to be fully prepared for the challenges ahead and to excel in my career.

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The time spent during my PhD in Pittsburgh has given me some treasures, some cherished moments- the friends I have made. Friends have come and gone, but a precious few have built bonds and changed my life. Dileep, Kanishka, Saket, Jameel, Ramalinga, Brian- I value your friendship, and I feel blessed having you as friends.

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Words fail me to express my appreciation to my wife Poonam and my children Prateek and Joshua whose dedication, love and persistent confidence in me, was the strength during my years in Pittsburgh. Without the love and caring of my wife and children and without their sacrifice and support, my PhD would have been a selfish motive. The Pittsburgh experience was an extraordinary time in our lives. My most important legacy is my two children Prateek & Joshua. Through my experiences, I want them to build character, recognize that accomplishments require perseverance, and realize that loving is the most critical element of life itself. Thus, my realization of the Pittsburgh dream is the inheritance they gain.

Finally, I would like to thank everyone who was important to the successful realization of my research work, and would like to express my apology to friends and family whom I could not mention individually.

My life's experiences thus far have been inspiring and transformational; my greatest strength is my humility in knowing that I owe every success to my family, friends, coworkers, and community. I am grateful for the opportunities that have been given to me. For me success is more than a heart-pounding race to the finish; it is an all-encompassing life full of commitment, passion and optimism. This pursuit of success as it helps me live life from inside out and inspires me to serve others is my lifelong motivation.

PREFACE

Two chapters of this thesis have been published:

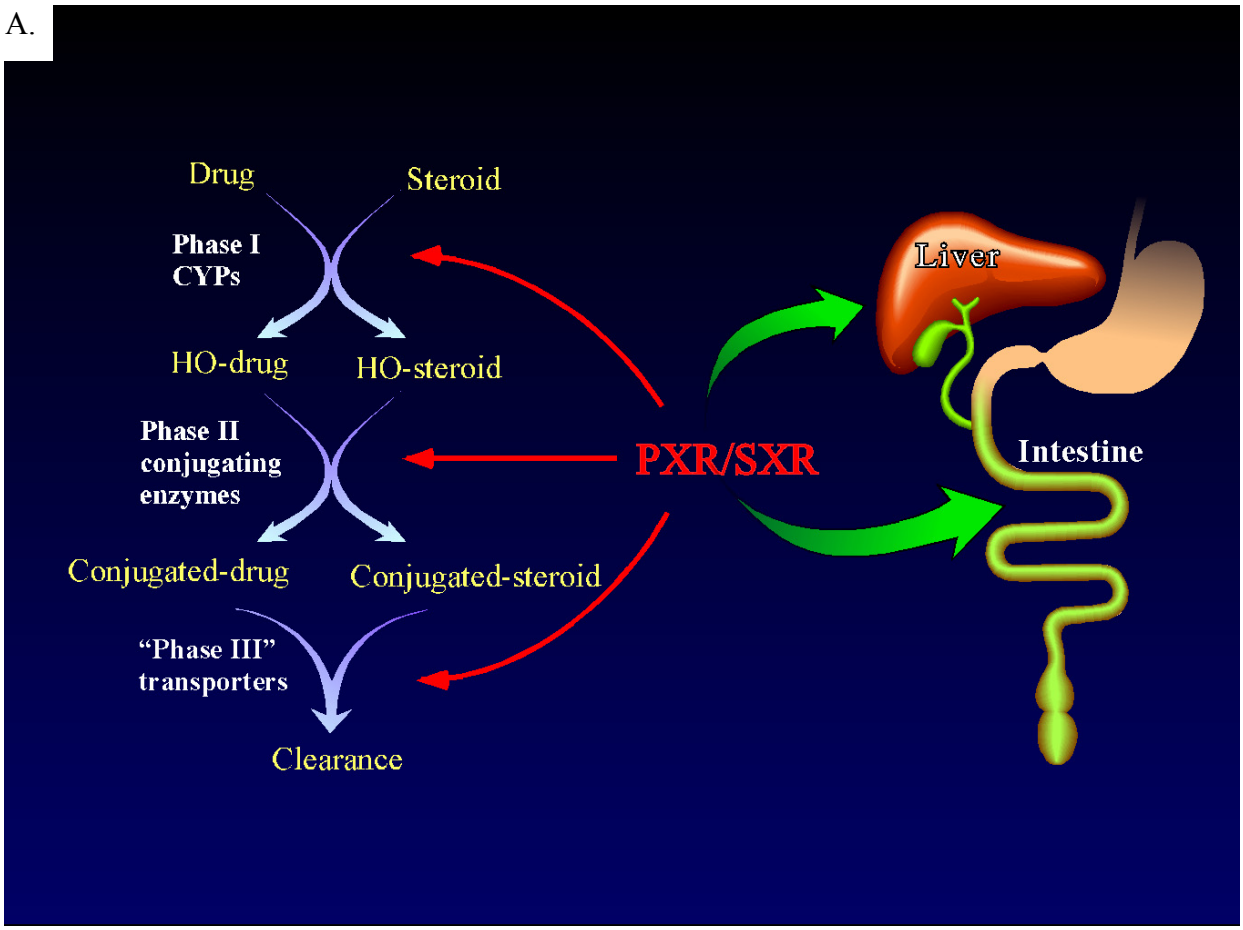
Uppal H, Saini SP, Moschetta A, Mu Y, Zhou J, Gong H, Zhai Y, Ren S, Michalopoulos GK, Mangelsdorf DJ, Xie W. Activation of LXRs prevents bile acid toxicity and cholestasis in female mice. (2007). ***Hepatology***. 45(2):422-32.

Uppal H, Toma D, Saini SP, Ren S, Jones TJ, Xie W. Combined loss of orphan receptors PXR and CAR heightens sensitivity to toxic bile acids in mice. (2005). ***Hepatology***. 41(1):168-76.

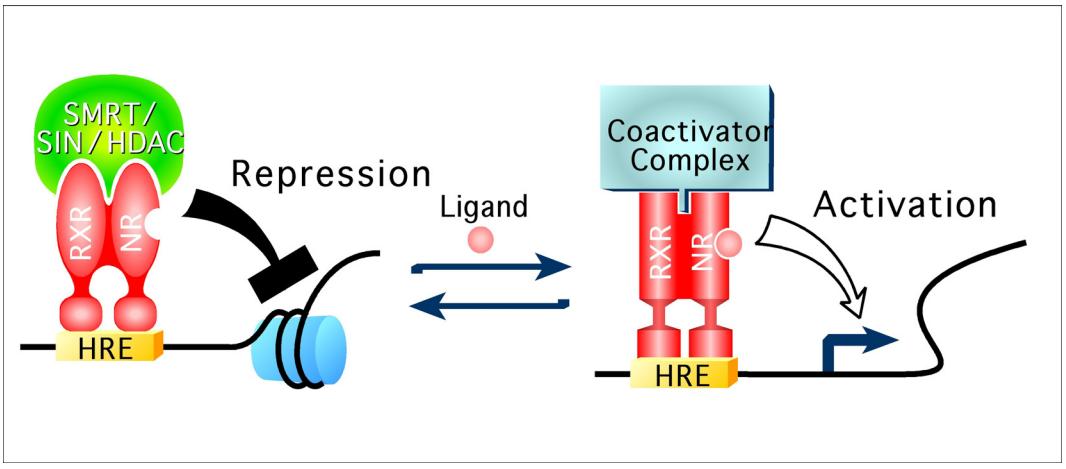
1.0 INTRODUCTION

Drug metabolizing enzymes and transporters play an essential role in the metabolism and detoxification of numerous xeno- and endobiotics (Fig 1 A). The nuclear receptors are bound to specific sequences in the promoters of the genes they activate or repress. When they activate gene expression upon ligand binding, the repressors are removed and the activators bind to the nuclear receptors and activate the gene expression (Fig 1 B). As a defensive mechanism, mammals often launch inducible expression of these enzymes and transporters in response to xeno- and endobiotic chemical challenges (Fig. 1 C). Cloning and characterization of the orphan nuclear receptors pregnane X receptor (PXR, NR1I2) and constitutive androstane receptor (CAR, NR1I3) led to major breakthroughs in studying the transcriptional regulation of drug-metabolizing enzymes and transporters. The Liver X receptors (LXR, NR1H2/3) are sterol responsive transcription factors that regulate expression of genes involved in cholesterol and lipid metabolism and homeostasis. More recently, additional roles for CAR, PXR and LXR have been discovered. As examples, CAR and PXR have been shown to be involved in the homeostasis of cholesterol, bile acids, bilirubin, and other endogenous molecules in the liver. LXRs have recently been focused for their roles in cholesterol, lipid, and carbohydrate metabolism. In addition, LXRs have been found to regulate inflammatory responses. The focus of the study is to work towards understanding the gene regulation mediated by PXR, CAR and LXR and the implications of this regulation in physiology and pathophysiology of the liver (1, 2).

A.


Drug
Steroid
Phase I
CYPs
HO-drug
HO-steroid
Phase II
conjugating
enzymes
Conjugated-drug
Conjugated-steroid
"Phase III"
transporters
Clearance
PXR/SXR
Liver
Intestine

B.


SMRT/
SIN/HDAC
RXR
NR
HRE
Repression
Ligand
Coactivator
Complex
RXR
NR
HRE
Activation

C.

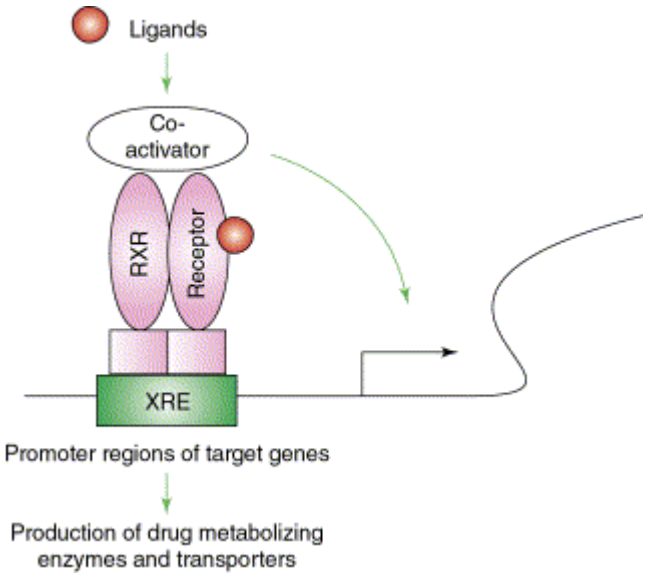


Figure 1: Xenobiotic receptor-mediated regulation of drug-metabolizing enzymes and transporters.

(A) Drug metabolizing enzymes and transporters play an essential role in the metabolism and detoxification of numerous xeno- and endobiotics and are regulated by nuclear receptors in the liver and intestine.

(B) Repression and Activation state of nuclear receptors.

(C) Activation of xenobiotic receptors, such as pregnane X receptor (PXR) and constitutive androstane receptor (CAR), induces phase I and II enzymes and drug transporters. This transcriptional activation requires: (1) binding of ligands and recruitment of co-activators; (2) formation of heterodimers with retinoid X receptor (RXR); and (3) binding of the heterodimers to the xenobiotic response elements (XRE) in the target gene promoters (Adapted from Xie *et. al.* (3).

1.1 BILE ACID MEABOLISM

1.1.1 Cholesterol metabolism and bile acid synthesis

The formation of bile acids occurs with the breakdown of cholesterol in the body. This process of breakdown of cholesterol to form the bile acids can occur by two different pathways, a classical or neutral and an alternative or acidic pathway (reviewed in (4-7)) (Fig. 2 A, B & C; (8)). The breakdown of cholesterol by the classical pathway leads to the formation of cholic acid and this pathway comprises of about 90% of bile acid synthesis. The key component of the classical pathway is the hydroxylation of cholesterol at the 7α and 12α positions via the cytochrome P450 (CYP) enzymes CYP7A1 and CYP8B1, respectively, further followed by

hydroxylation by the mitochondrial CYP27A1 enzyme (9, 10). The alternative pathway is primarily responsible for the formation of chenodeoxycholic acid (CDCA). Cholesterol is first converted to oxysterols by hydroxylation of the side chain by three distinct enzymes: sterol 27-hydroxylase (CYP27) in mitochondria, cholesterol 25-hydroxylase and cholesterol 24-hydroxylase (CYP46) in the endoplasmic reticulum (11). The 25- and 27-hydroxycholesterol intermediates are substrates for oxysterol 7alpha-hydroxylase (CYP7B1) (12), whereas 24-hydroxycholesterol is the substrate for oxysterol 7alpha-hydroxylase, CYP39A1 (13). The bile acid composition in majority of the healthy human beings is composed of cholic acid and cholic acid metabolites (70%), whereas CDCA comprises the minor pool of bile acids (30%) (14). After their synthesis, bile acids are conjugated with glycine and taurine and are present as anionic salts under physiological pH conditions, and are therefore called bile salts. This helps to improve their solubility in the bile fluid and in the intestinal lumen.

1.1.2 Enterohepatic recirculation of bile acids and the bile acid pool

After bile acids have been synthesized, they are rapidly secreted from the liver into the bile duct, and via the bile they go into the lumen of the gastro-intestinal tract, where they become part of a resident pool of bile acids. The bile acid pool present in the GIT and in the entero-hepatic recirculation pathway usually remains constant throughout life (11, 15, 16). Several different bile acid transporters located in the intestinal cells and hepatocytes function to make certain that the size of the bile acid pool is preserved during its enterohepatic recycling (7, 17-19). Each cycle of bile acid recirculation results in a small loss of bile acids via the fecal excretion. The loss in the pool is replenished in the liver by de-novo synthesis of bile acid from cholesterol. The intestinal bile acid pool size and composition plays a critical role in the absorption of essential lipids, fat-soluble vitamins, and intestinal cholesterol in the body. The bile acid recirculation pathway is one of the major pathways by which the body can control the cholesterol levels in the body by regulating the efficiency with which dietary and biliary cholesterol are absorbed (11, 20-24).

1.1.3 Excretion of bile acids from the liver

The conjugated bile salts are excreted into the bile canaliculus via membrane transporters, which are expressed at the canalicular membrane of hepatocytes. The excretion of bile salts into the bile canaliculi is the rate-limiting step in bile formation and is the first step in the enterohepatic re-circulation of bile salts. The concentration of bile acids in the hepatocytes is in the micro molar range and the concentrations across the canalicular membrane are a thousand fold higher (25). Therefore, the process of transport of bile salts from the hepatocytes to the canaliculi cannot rely on a passive mechanism. The transport occurs as active transport and the main membrane transporters that are primarily responsible for the transport of bile salts to the canaliculi for bile formation have largely been characterized and belong to different members of the superfamily of ABC transporters (6, 7, 17, 18, 22, 26). In the liver, this family includes members of the multidrug resistance (MDR) protein family, (ABCB-gene family), the multidrug resistance associated (MRP) protein family (ABCC-gene family) as well as of the family of ABC-half transporters (ABCG-gene family) (26) (Fig. 3). The major transporters involved in the canalicular transport include the bile salt export pump (BSEP), MRP2, MDR1, ABCG2 or the breast cancer related protein (BCRP) and some non-ABC transporters like the familial intrahepatic cholestasis-1 (FIC-1) (6, 7, 17, 18, 22, 26). Bile salts are important for the solubilization of canalicular phospholipids to form mixed micelles. The micelles are further stabilized by the integration of cholesterol. The mixed micelles serve an important function as a medium for stabilize the cholesterol in the bile, as cholesterol is unable to solublize in the bile by itself for excretion. This helps to protect the cells in the canaliculi and bile duct from the detergent properties of bile salts and is an important mechanism to cholesterol secretion from the liver (27).

Fig. 2A.

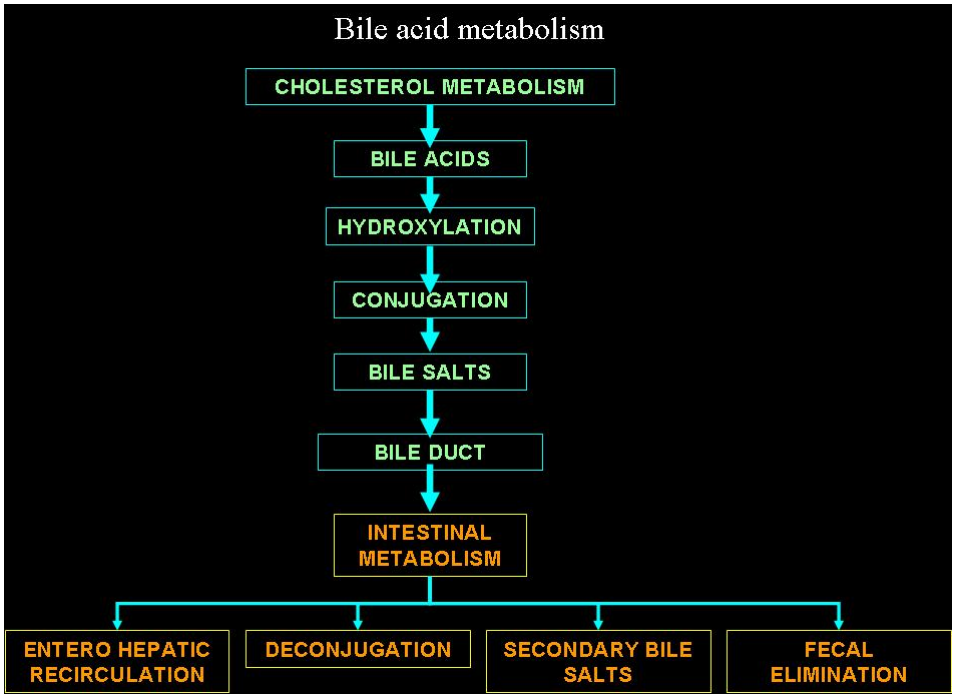

Bile acid metabolism
CHOLESTEROL METABOLISM
BILE ACIDS
HYDROXYLATION
CONJUGATION
BILE SALTS
BILE DUCT
INTESTINAL METABOLISM
ENTERO HEPATIC RECIRCULATION
DECONJUGATION
SECONDARY BILE SALTS
FECAL ELIMINATION

Fig. 2B.

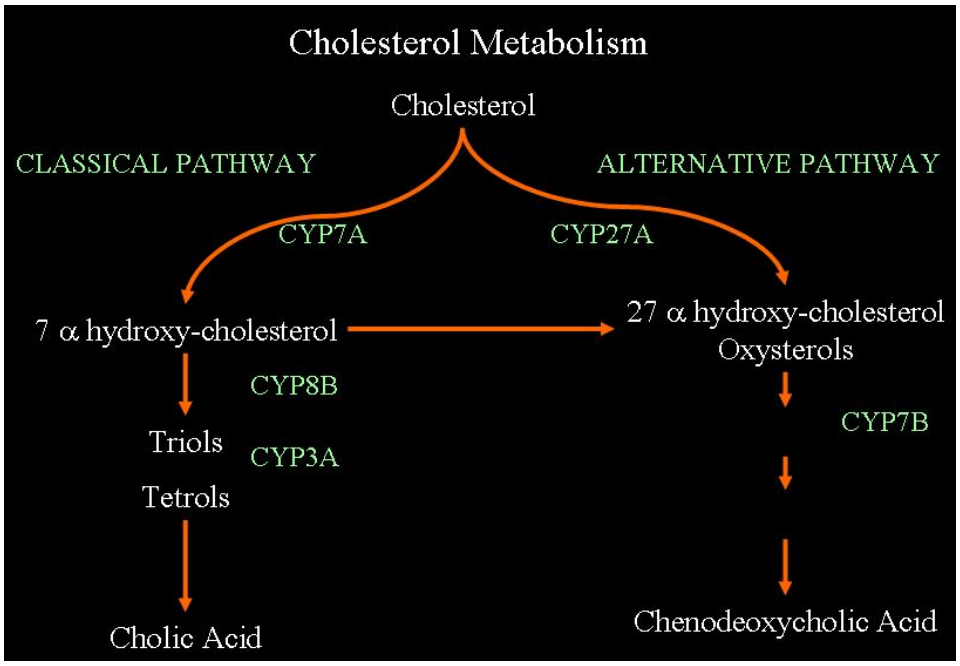

Cholesterol Metabolism
Cholesterol
CLASSICAL PATHWAY
ALTERNATIVE PATHWAY
CYP7A
CYP27A
7 α hydroxy-cholesterol
27 α hydroxy-cholesterol
Oxysterols
CYP8B
CYP7B
Triols
CYP3A
Tetrols
Cholic Acid
Chenodeoxycholic Acid

Fig. 2C.

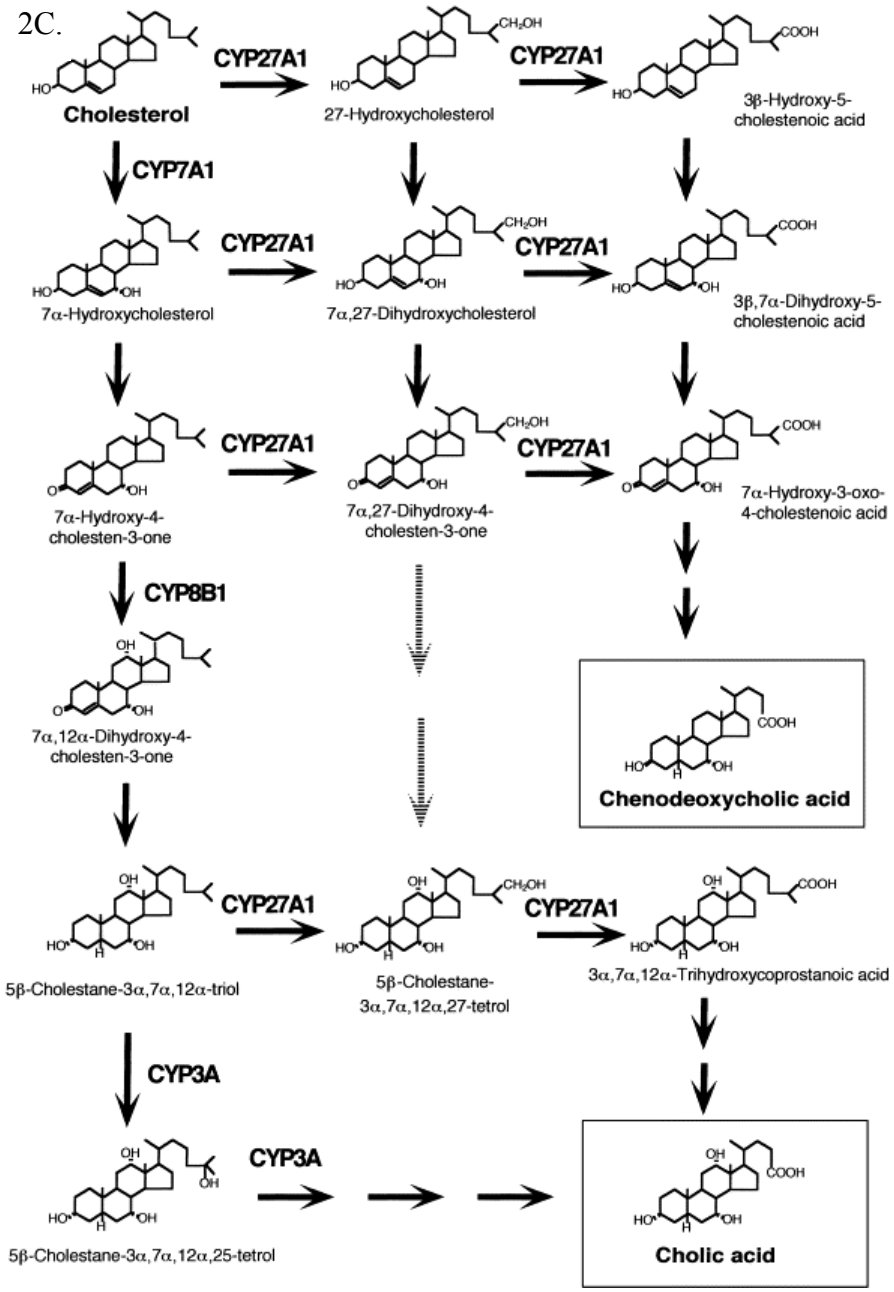


Figure 2. Bile acid biosynthetic pathways.

A & B. Overview of the cholesterol breakdown and bile acid metabolism process in the body.

C. Bile acid biosynthesis from cholesterol initiated either by a series of ring modifications (classic pathway) or by side chain hydroxylation (alternative pathway). Solid arrows represent known enzymatic steps while broken arrows represent putative reactions that have yet to be confirmed. "Reprinted from Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease Volume 1588, Issue 2, 20 November 2002, Pages 139-148. Matsuzaki *et. al.* Selective inhibition of CYP27A1 and of chenodeoxycholic acid synthesis in cholestatic hamster liver. Copyright (2002), with permission from Elsevier Science B.V. (8)"

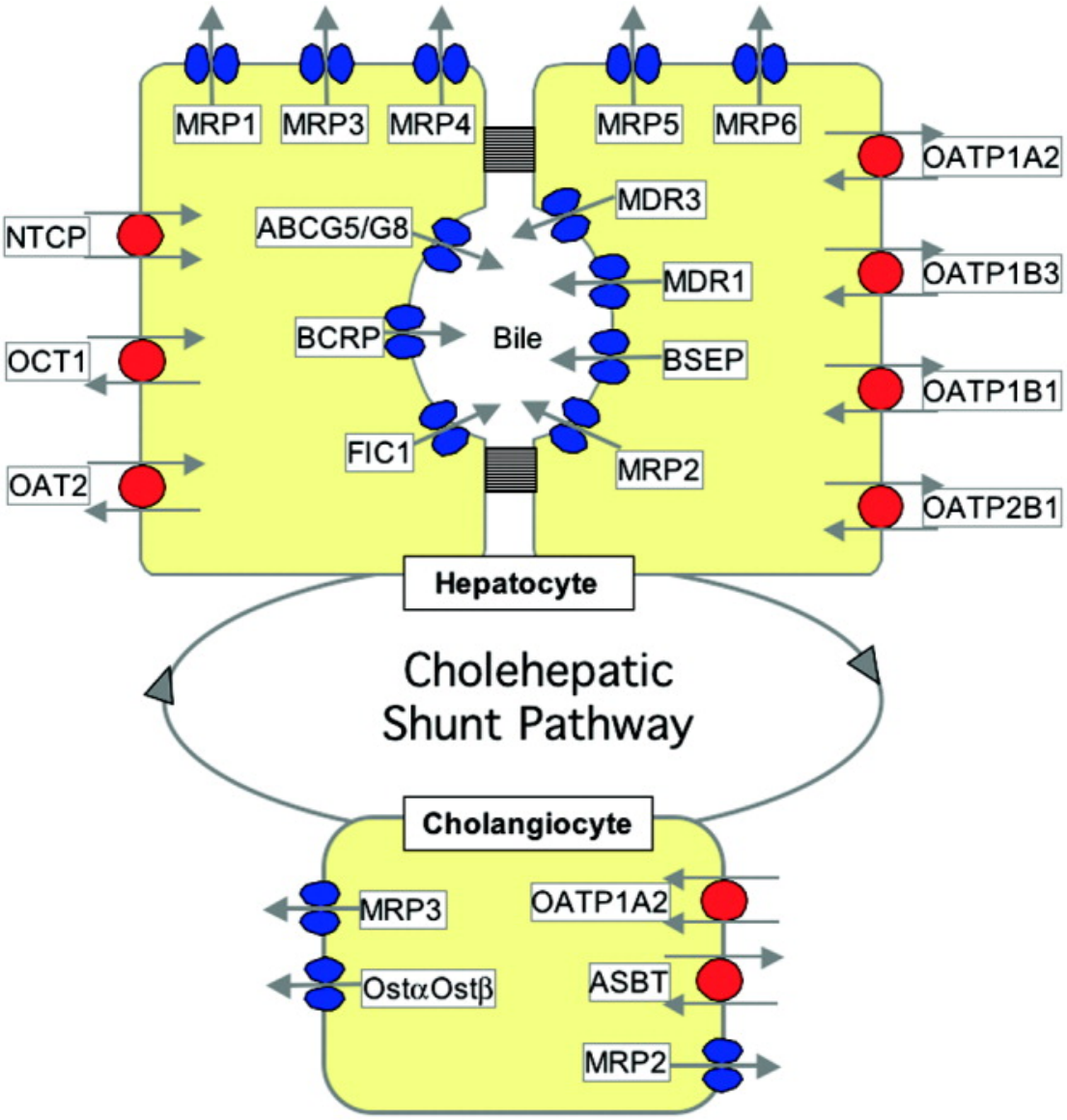


Figure 3. Bile salt transporters in human liver, cholangiocytes and small intestine and transcription factors involved in their regulation.

Bile salt transporters in human liver and cholangiocytes. Efflux transporters (blue symbols): BSEP, bile salt export pump; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; ABCG5/8; BCRP, breast cancer resistance protein; Ost/Ost. Uptake transporters (red symbols): ASBT, apical sodium dependent bile salt transporter; NTCP, sodium taurocholate cotransporting polypeptide; OATP, organic anion-transporting polypeptide; OCT, organic cation transporter; OAT, organic anion transporter. "Reprinted from Hepatology. 2006 Oct; 44(4):778-87. Pauli-Magnus C, Meier PJ. Hepatobiliary transporters and drug-induced cholestasis; with permission from The American Association for the Study of Liver Diseases (26)."

1.1.3.1 Bile salt export pump (BSEP)

The bile salt export pump BSEP (ABCB11) and is a highly conserved member within the family of multidrug resistance proteins. It is involved in the secretion of bile acids from the hepatocyte into the bile canaliculus (reviewed in: (6, 7, 28-30)). BSEP is the major bile salt efflux transporter of hepatocytes and is involved in the excretion of a large number of conjugated bile salts, such as taurine- or glycine-conjugated cholate, chenodeoxycholate and deoxycholate from the hepatocytes (28, 31). Genetic mutations in the BSEP gene can cause BSEP dysfunction and result in severe liver disease , cirrhosis and chronic liver failure (32). High levels of bile salts in the hepatocytes can upregulate the nuclear receptor FXR, which in turn can increase the expression of BSEP to remove the harmful bile salts from the liver (33).

1.1.3.2 The multidrug resistance protein 3 (MDR3, ABCB4)

The multidrug resistance protein 3 (MDR3, ABCB4) is also a highly conserved member within the family of multidrug resistance proteins and is involved in the secretion of lipophilic compounds from the hepatocyte into the bile canaliculus (reviewed in: (6, 7, 28-30)). MDR3 is a ATP-dependent phospholipid transporter, which helps to transport phospholipids like phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane (reviewed in: (19, 34)). Defects of the phospholipid export pump MDR3 result in impaired biliary excretion of phosphatidylcholine and a variety of cholestatic syndromes ranging from progressive familial intrahepatic cholestasis in neonates to biliary cirrhosis in adults (35). Patients have been identified with a high level of biliary phospholipid deficiency due to a missense mutation in the MDR3 gene, which results in low phospholipid associated cholelithiasis. The patients exhibit intrahepatic and bile duct gallstones at a young age and a high risk of recurrent biliary symptoms (36).

1.1.3.3 Multi-drug resistance associated protein 2 (MRP2)

MRP2 is a member of multidrug resistance associated protein family that is present on the canalicular side of the membrane. It plays a role in the transport of glucuronidated and sulfated bile salts into the bile canaliculi. MRP2 also plays a major role in the excretion of divalent bile salts out of the hepatocytes into the bile along with the transport of reduced glutathione. MRP2

also transports a large number of organic anions, including bilirubin, glutathione-conjugates, divalent bile salt conjugates, anionic conjugates of lipophilic compounds and drugs, like chemotherapeutic agents and antibiotics (reviewed in (7, 34, 37)). Since MRP2 plays a major role in hepato-biliary elimination of many structurally diverse xenobiotics, including organic anions and drug conjugates, it most likely contributes to pharmacokinetic parameters of these compounds. The over expression of MRP2 has been shown to confer a multidrug resistance phenotype to tumor cells (38). The MRP2 levels and activity can also be affected by activating the nuclear receptor PXR, CAR and FXR (39). Deficiency of MDR2 inhibits the hepatic excretion of conjugated bilirubin, which in turn leads to Dubin-Johnson syndrome, characterized by the accumulation of glucuronidated bilirubin in the body (40-44).

1.1.3.4 Cholesterol efflux transporters

The heterodimeric transporter ABCG5 and ABCG8 have been identified as the apical transport system involved in the hepatobiliary excretion of plant sterols and cholesterol (34, 45, 46). ABCG5/ABCG8 are twin sterol half-transporters and function as heterodimers during the cholesterol transport process. Mutations in either of the half-transporters ABCG5/G8 have been shown to cause sitosterolemia; a disorder manifested by increased intestinal absorption and decreased biliary excretion of dietary sterols. This can lead to hypercholesterolemia and early onset of atherosclerosis (47). Over expression of ABCG5/ABCG8 in transgenic mice have been shown to increase biliary cholesterol secretion and reduce the intestinal absorption of dietary cholesterol. This work confirmed the role of ABCG5/ABCG8 in the hepatocellular secretion and intestinal efflux of cholesterol (45). In mice having targeted deletions of the ABCG5 and ABCG8 transporters, there was an increase in the absorption of dietary plant sterols and a considerable increase in the plasma and hepatic cholesterol levels after cholesterol feeding (48, 49). The cholesterol secretion into the bile along with the acid and phospholipids helps to form the micelles, which results in efficient removal of both the bile acids as well as the cholesterol. Activation of LXR causes a higher efflux of cholesterol from the liver and intestine by upregulating the ABCG5 and ABCG8 transporters (50).

1.1.4 Basolateral excretion of bile salts

In addition to the canalicular excretion, the efflux of bile acids and salts also takes place from the basolateral or sinusoidal membrane of the hepatocytes. This occurs via the family of multidrug resistance associated proteins. Five members of this family MRP1 (ABCC1), MRP3 (ABCC3), MRP4 (ABCC4), MRP5 (ABCC5) and MRP6 (ABCC6) are located on the basolateral surface of the hepatocytes (7). It has been shown that the levels of MRP1 and MRP3 are low in the normal liver, and the levels are upregulated in cholestasis (34, 51). MRP4 is expressed to a greater level in the liver. The MRP1 transporter is involved in the cellular efflux of various organic anions including drugs, glutathione, glucuronide and sulfate conjugates. MRP3 and MRP4 function as ATP dependent transporters to efflux the mono anionic bile salts and bile salt conjugates, the transport of nucleoside analog drugs, the cyclic nucleosides etc. MRP4 also helps to efflux the bile salts by cotransport of reduced glutathione or S-methyl-glutathione along with the bile salts (52). MRP4 and MRP5 have also been shown to be involved in efflux of the purine analogs (34). The basolateral efflux pathway helps in export of the bile salts and other compounds to the blood and this might be an alternative pathway for the elimination of toxic metabolites in the event of canalicular transport being affected by various pathologic conditions.

1.1.5 Intestinal bile salt absorption

The intestinal absorption of bile salts, cholesterol and phospholipids takes place from the intestinal lumen of the terminal ileum. Intestinal reabsorption of bile salts is primarily localized to the terminal ileum and is mediated by a 48-kd sodium-dependent bile acid cotransporter (SLC10A2 or ASBT). ASBT is also expressed in renal tubule cells, cholangiocytes, and the gallbladder (53). In addition to ASBT, another transporter the OATP-B (OATP2B1) is a pH-sensitive transporter expressed in the apical membranes of small intestinal epithelial cells. The OATP2B1 mediates the transport of taurocholic acid at acidic pH (54). Sodium independent intestinal uptake of glycine-conjugated bile salts in small intestinal cells is also mediated via OATP1A5 in rats or OATP1A2 in human (55).

After the absorption of bile salts into the enterocytes, the bile salts have to be transported to the basolateral membrane for excretion to the portal circulation. The intracellular transport of bile salts occurs via the ileal bile acid binding protein (I-BABP) which is a14 kilo-Dalton ileal protein attached to ASBT in the cytoplasmic side (56). MRP3 and OSTα/OSTβ are the two transporters, which further help to transport the bile salts across the ileocyte basolateral membrane into the portal circulation. MRP3 has been shown to have a high expression in terminal ileum and functions in bile salt transport into portal circulation (57). MRP3 was recently shown not to have a major role in bile salt physiology, but is involved in the transport of glucuronidated compounds, which could include glucuronidated bile salts in humans (58). The heteromeric organic solute transporter OSTα/OSTβ were discovered as basolateral bile salt carriers in mice and might be responsible for bile salt efflux in ileum and other ASBT-expressing tissues (59). The selective localization of OSTα and OSTβ to the basolateral plasma membrane of epithelial cells and the substrate selectivity of the transporter responsible for bile acid and sterol reabsorption indicate that OSTα/OSTβ is a key basolateral transporter for the reabsorption of sterols and bile salts (60, 61).

1.1.6 Hepatocellular uptake of bile salts

In the small intestine, 95% of the bile salts from the biliary secretion pathway are reabsorbed in they re-migrate into portal circulation (7). Therefore, in the recirculation pathway the sinusoidal uptake of bile salts is a very important step in the bile acid metabolism pathway. The two transporter mechanisms mediating the hepatic uptake of xenobiotics and endobiotics from the circulation consist of the sodium-dependent and sodium-independent transporter mechanisms (7, 26). Bile salts circulate in plasma tightly bound to albumin and lipoproteins such as high-density lipoprotein (62). More than 80% of conjugated bile salts undergo single-pass extraction by the liver, predominantly via the sodium dependent transport via the sodium-taurocholate cotransporting polypeptide NTCP (SLC10A1) (53). The NTCP is exclusively expressed on the basolateral membrane of hepatocytes and is primarily responsible for the transport of conjugated bile salts and certain sulfated steroids. NTCP accounts for more than 80% of conjugated (i.e. taurocholate and glycocholate) but of less than 50% of unconjugated (i.e. cholate) bile salt uptake (7, 26, 53). NTCP takes advantage of the membrane inward directed sodium ion gradient

which is maintained by the Sodium/Potassium ATPase of the hepatocytes (17, 19). It has been observed that decreases in the NTCP expression in cultured rat hepatocytes and in regenerating livers are accompanied by a decreased sodium dependent taurocholate uptake by the hepatocytes, indicating that the NTCP is the major intake transporter for the bile salts (51).

The sodium-independent transporters belong to the superfamily of organic anion transporting polypeptides (OATP/SLCO) (63), which has nine members in the human beings and three members in rats (64). The members of this family have overlapping substrate affinity and mediate the sodium-independent uptake of unconjugated bile salts. The OATPs also mediate the uptake of a large number of other compounds having a variety of structure like bilirubin conjugates, hormones, neutral steroids and xenobiotics (17, 30, 53, 63). Unlike the NTCP, the OATPs are also expressed in extra-hepatic tissue like the kidney, intestine and brain (17). Some human OATPS may not have high homology with the rat and mouse OATPs, but they seem to have similar function in the liver. Four of the human members have a high expression in the liver, and the highest expression is for the OATP1B1 (SLCO1B1) and OATP1B3 (SLCO1B3). OATPs transport a large variety of albumin-bound amphiphatic organic compounds (reviewed in (26)). The uptake function of the OATP is bidirectional involving anion exchange with reduced glutathione and bicarbonate (17).

It has been seen that there are alterations in the hepatobiliary transport systems in experimental animal models of cholestasis, which involves the downregulation of NTCP and the OATPs. This down regulation of NTCP and OATPS has also been seen in inflammatory hepatocellular cholestasis, primary biliary cirrhosis and primary sclerosing cholangitis (19). It has been seen that the activation of the FXR results in an upregulation of the biliary secretion of bile acids via upregulating the BSEP, MRP2 and UGT2B4. In addition the FXR upregulation results in the upregulation of the Short Heterodimer Partner (SHP) which is a negative regulator of transcription and this results in downregulation of NTCP, CYP7A1 and CYP8B1 (19).

The pathways involved in the intracellular movement of bile salts across hepatocytes are not fully understood compared to other transport mechanisms occurring at the canalicular and basolateral surface. Under physiological conditions, the majority of bile salts are bound to

intracellular binding proteins, while a smaller fraction of unbound bile salts rapidly diffuses through the hepatocyte (reviewed in (7, 65)). The major proteins involved in this pathway are the 3α-hydroxysteroid dehydrogenase and the hepatic bile acid binding protein in the humans and the glutathione-s-transferases and the hepatic fatty acid binding protein in the rodents (25). The hydrophobic bile salts may be sequestered into intracellular organelles such as endoplasmic reticulum and Golgi apparatus, especially during the presence of high bile salt concentrations (7, 25, 65). It has also been suggested that passive diffusion and vesicular transport or intracellular trafficking play a role in the bile salt movement in the cytoplasm (25).

1.2 PXR AND CAR, TWO PROTOTYPIC XENOBIOTIC ORPHAN NUCLEAR RECEPTORS

1.2.1 Orphan nuclear receptors

Orphan nuclear receptors belong to the nuclear receptor (NR) superfamily of transcriptional factors. In most cases, these receptor proteins were identified without knowing their endogenous and/or exogenous ligands, so they were called "orphan" receptors. Most, if not all, NRs share two essential functional domains that include the N-terminal DNA binding domain (DBD), and a C-terminal ligand-binding domain (LBD) (66). The conserved DBD consists of two DNA-binding zinc fingers. The LBD folds to form a hydrophobic pocket into which the ligand binds.

1.2.2 Cloning of PXR and CAR

In 1998, the rodent orphan nuclear receptor PXR (pregnane X receptor) (67) and its human homolog hPXR/SXR (steroid and xenobiotic receptor)/PAR (68-70) were isolated as candidate xenobiotic receptors postulated to regulate CYP3A gene expression. As pregnenolone and its derivative, pregnenolone 16 -carbonitrile (PCN), can activate PXR, it was so named. Another orphan receptor CAR (constitutive androstane receptor) was cloned several years earlier (71) but its identity as a xenobiotic receptor was not appreciated until the discovery that the constitutive

activity of CAR can be inhibited by selective androstane metabolites (72). The role of CAR in positive xenobiotic regulation of CYP2B genes was first shown in 1998 (73).

1.2.3 Regulation of xenobiotic gene expression by PXR and CAR for xenobiotic

Xenobiotic receptors, such as PXR and CAR, regulate gene expression by forming heterodimers with the retinoid X receptor (RXR). The regulation is achieved by binding of the PXR/RXR or CAR/RXR heterodimers to the specific xenobiotic response elements (XREs) present in the promoter regions of drug metabolizing enzymes and transporters. PXR is activated by a variety of xenobiotics including drugs known to induce hepatic and intestinal CYP3A activity (68-70). While CAR exhibits relatively high basal activity to transactivate genes without ligand ("constitutive"), its activity can be inhibited by antagonists, such as androstane metabolites (72), and potentiated by agonistic phenobarbital (PB) and TCPOBOP (73-75). The respective regulation of CYP3A and 2B by PXR and CAR has been firmly established via the creation of mice deficient in PXR and CAR (76-78). Disruption of the mouse PXR locus by homologous recombination abolishes the CYP3A induction in response to PCN and dexamethasone (76, 78). Similarly, CYP2B induction in response to both PB and TCPOBOP was completely eliminated in the CAR null mice (77). Subsequent functional analysis has revealed a much broader role of PXR and CAR in xenobiotic regulation. It became evident that both receptors can function as master regulators in regulating additional Phase I and Phase II enzymes, as well as drug transporters. The mechanism of this broad regulation is the presence of PXR and CAR response elements in the promoter regions of many of these enzyme and transporter genes (for a review, see (79)). These include the Phase I enzymes CYP2C8/9/19 (80, 81) and CYP3A7, Phase II enzymes glutathione S-transferases (GST) (82), UDP-glucuronosyltransferases (UGT) (83-85) and sulfotransferases (SULT) (86, 87), and the transports multidrug resistance protein 1 (MDR1) (81, 88), MDR2 (89), multidrug resistance-associated protein 2 (MRP2) (39), and organic anion transporter polypeptide 2 (OATP2) (76). A broad role of PXR and CAR in xenobiotic regulation was further confirmed by several gene profiling analyses performed in wild type, transgenic and knockout mouse models (90, 91).

1.2.4 Overlapping and coordinated gene expression regulation by PXR and CAR

Another unique functional feature of PXR and CAR is the overlap in the genes regulated by these receptors (Fig 4). For instance, PXR can regulate CYP2B genes and CAR can regulate CYP3A genes. The mechanism of cross-regulation has been shown to be due to shared response elements between receptors as revealed by receptor-DNA binding analysis and transient transfection and reporter gene assays (75, 91-95)). The creation of transgenic mice that bear hepatic expression of activated receptors allowed evaluation of potential cross-regulation *in vivo*. Sharing similar DNA binding specificities with their wild type counterparts, the activated VP-PXR and VP-CAR were created by fusing the VP16 activation domain of the herpes simplex virus to the amino terminal of the receptors. Genetic activation of PXR *in vivo* caused sustained induction of both CYP3A and 2B (75, 78). In contrast, in the VP-CAR transgenic mice, while CYP2B was induced as expected, the expression of CYP3A was largely unchanged or even slightly suppressed (86). The lack of CYP3A11 induction in the VP-CAR mice was not due to the unresponsiveness of CYP3A11 in this transgenic line, since the expression of CYP3A11 in the VP-CAR mice remained inducible in response to the CAR ligand TOPOBOP (86).

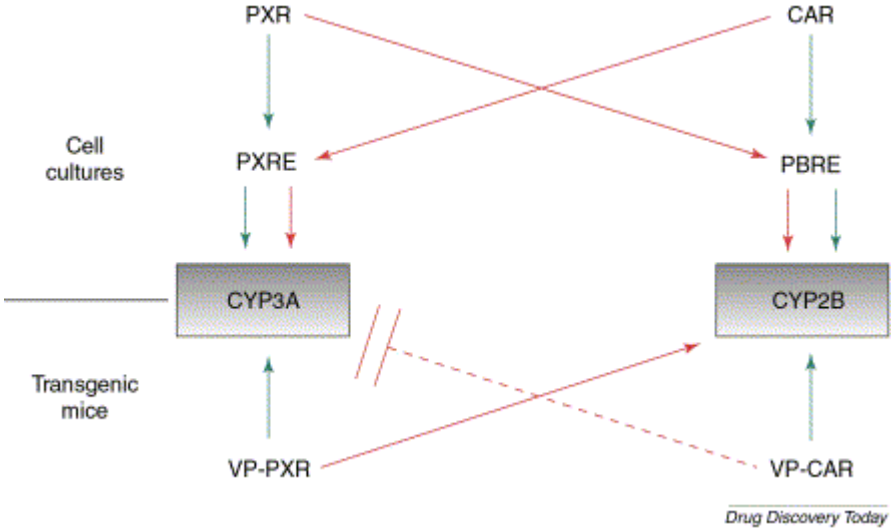


Figure 4: Cross-talk in xenobiotic nuclear receptor-mediated regulation of cytochrome P450 (*CYP*) genes.

The reciprocal activation of xenobiotic response genes by pregnane X receptor (PXR) and constitutive androstane receptor (CAR) has been shown in cell cultures. In transgenic mice, activation of PXR induces *CYP3A* and *CYP2B*. In contrast, expression of activated CAR (VP-CAR) in mice induces *CYP2B* but not *CYP3A*. The green and red colored arrows indicate the direct and cross-regulation, respectively. Abbreviations: PXRE, PXR response element; PBRE, phenobarbital response element. (Adapted from Xie *et. al.* (3).

1.2.5 Endogenous PXR ligands and role of PXR in Endobiotic Metabolism

Even though PXR has been identified as a "xenobiotic receptor," emerging evidence has pointed to an equally important role of PXR as an "endobiotic receptor" that responds to a wide array of endogenous chemicals, i.e., endobiotics. Moreover, the activation of PXR by endogenous ligands has implications in several important physiological and pathological conditions. One family of endogenous PXR ligands identified shortly after the cloning of PXR are bile acids. Bile acids are catabolic end products of cholesterol metabolism. Despite some beneficial function, excess accumulation of bile acids, such as the secondary bile acid lithocholic acid (LCA), has been shown to cause cholestasis in experimental animals and has long been suspected of doing the same in humans. Xie *et. al.* and Staudinger *et. al.* showed that PXR acts as a LCA sensor and plays an essential role in detoxification of cholestatic bile acids (76, 96). Activation of PXR by bile acids or other xenobiotic inducers causes the induction of CYP3A, an enzyme that facilitates the detoxification of bile acids. Pretreatment of wild-type mice, but not the PXR null mice, with PCN reduced the toxic effects of LCA. Moreover, genetic activation of PXR by expressing the activated PXR in the liver of transgenic mice was sufficient to confer resistance to the hepatotoxicity of LCA (96). Consistent with the notion that activation of PXR facilitates bile acid detoxification, increased serum levels of bile acids have been suggested to be a factor in the development of pruritis and studies in humans have shown that PXR activator RIF can be used to treat cholestasis-associated pruritis (97, 98).

More recently, the bile acid intermediates formed during cholesterol catabolism have been shown to function as PXR agonists. The sterol 27-hydroxylase (CYP27A1) is an important enzyme in regulating the production of bile acids from cholesterol. In humans, mutations in the CYP27A1 gene were responsible for the cerebrotendinous xanthomatosis (CTX), a genetic disease manifested by the accumulation of 25-hydroxylated bile alcohols, such as 25-tetrol, several 25-pentol isoforms, and possibly hexols and heptols. The clinical hallmarks of the disease include a marked deposit of sterols in a variety of tissues, a decrease in chenodeoxycholic acid production and associated mental retardation, premature atherosclerosis and tendon and brain xanthomas (99). Surprisingly, the CYP27 null mice did not develop the clinical manifestations of CTX (16, 100). This may be due to a dramatic increase in the

expression of CYP3A in the CYP27 null mice with a resultant increase in the CYP3A-mediated hydroxylation and clearance of bile acid intermediates (101, 102). The increase in CYP3A enzyme production in the CYP27A null mice has been reasoned to be due to the activation of mouse PXR by these bile acid intermediates, among which are three potentially toxic sterols, 7α-hydroxy-4-cholesten-3-one, 5β-cholestan-3α, 7α.12α-triol, and 4-cholestan-3-one. Interestingly, these intermediates are more potent inducers toward mPXR than hPXR, which may explain, at least in part, why humans lacking functional CYP27A1 do not display a compensatory increase in CYP3A activity (101, 102). These reports establish the existence of a feed-forward regulatory or salvage pathway in which potentially toxic bile acid intermediates activate PXR and induce their own metabolism and clearance to avoid accumulation.

1.2.6 Implication of PXR and CAR in bilirubin and bile acid metabolism

The implication of PXR- and CAR-mediated gene regulation in drug metabolism and drug interaction has been recognized since the first cloning of these xenobiotic receptors. Consistent with the notion that these enzymes and transporters are also implicated in the biotransformation and homeostasis of many endogenous chemicals that can influence physiological and pathological processes, accumulating evidence has pointed to a role of orphan receptor-mediated xenobiotic regulation both in normal physiology and in disease states.

Bilirubin is the catabolic byproduct of heme proteins, such as β-globin and CYP enzymes. Accumulation of bilirubin in the blood is potentially hepato- and neuro-toxic. For example, an insufficiency in expression of UGT1A1, a key enzyme for the conjugation of bilirubin, in the Crigler-Najjar syndrome and Gilbert's diseases results in severe hyperbilirubinemia. Deficiency of MDR2, a transporter protein responsible for the hepatic excretion of conjugated bilirubin, leads to Dubin-Johnson syndrome, characterized by the accumulation of glucuronidated bilirubin. Both PXR and CAR have been shown to induce the expression of UGT1A1 (83-85) and this has been proposed to explain why the transgenic mice expressing a constitutively active form of hPXR had twice the bilirubin clearance of the wild type mice (85). While it remains to be confirmed in transgenic mice, it is possible that PXR and CAR promote the clearance of

bilirubin by increasing the expression of multiple key components in the clearance pathway (103). In addition to UGT1A1, PXR and CAR have been shown to induce the expression of OATP2, GSTA1 and 2 and MRP2. OATP2 facilitates bilirubin uptake from blood into hepatocytes (104). GSTA1 and 2 reduce bilirubin back efflux from hepatocytes into blood. Interestingly, Huang et al. showed that CAR expression is low in human neonates, this functional deficit may be a factor in neonatal jaundice seen in almost 60% of infants and explain the effectiveness of PB for the treatment of this condition (83).

Bile acids are the major products of cholesterol catabolism in the liver. Despite their beneficial role in solublizing biliary lipids and promoting their absorption, accumulation of bile acids can cause irreversible liver damage resulting in cholestasis (impaired bile flow) (105). PXR has been shown to be protective against bile acid hepatotoxicity. Both pharmacological (using PCN) and genetic activation of PXR in mice was sufficient to confer resistance to toxicity by LCA (76, 96). In contrast, mice deficient in PXR exhibited heightened LCA toxicity. The PXR-mediated protection was originally thought to be due to the induction of CYP3A (96). Subsequent studies suggest that the induction of hydroxysteroid sulfotransferase (SULT), another PXR target gene, might also play a role in the protection (87, 106). More recently, Saini et al. reported a novel CAR-mediated and CYP3A-independent pathway of bile acid detoxification (86). Using transgenic mice bearing conditional expression of the activated CAR, Saini et al. demonstrated that activation of CAR is both necessary and sufficient to confer resistance to the hepatotoxicity of LCA (86). Surprisingly, the CAR-mediated protection is not due to the expected and previously characterized CYP3A pathway, but rather associated with a robust induction of SULT gene expression and increased LCA sulfation. Interestingly, activation of CAR was also associated with an increased expression of the 3'-phosphoadenosine 5'-phosphosulfate synthetase 2 (PAPSS2), an enzyme responsible for generating the sulfate donor PAPS (86). However, it is not clear whether PAPSS2 is a direct transcriptional target of CAR. Analysis of gene knockout mice revealed that CAR is also indispensable for ligand-dependent activation of SULT and PAPSS2 *in vivo*. Therefore, CAR has been established to play an essential and unique role in controlling the mammalian sulfation pathways and to facilitate bile acid detoxification. It is important to note that several other orphan receptors, such as Farsenoid X Receptor (FXR) and SHP, also play a critical role in the homeostasis of bile acids (107-109).

1.3 THE LIVER X RECEPTOR

Liver X receptors (LXRs) are nuclear oxysterol receptors and metabolic sensors initially found to regulate cholesterol metabolism and lipid biosynthesis. LXR has two isoforms, LXR α (or RLD-1) (110, 111) and LXR β (UR, NER, RIP15, and OR-1) (112-115). LXRα and LXRß (also known as NR1H3 and NR1H2, respectively) were cloned more than a decade ago based on sequence homology with other receptors. The LXRs are ligand-dependent transcription factors that form heterodimers with the retinoid X receptor (RXR) and the complex can be activated by ligands of either partner and in an independent manner e.g. 9-cis retinoic acid and oxysterols. LXR/RXR heterodimers bind to LXR-responsive elements (LXREs) in DNA consisting of the direct repeats (DRs) of the hexameric core sequence AGGTCA separated by 4 nucleotides (DR-4) (116). Like most other nuclear receptors that form heterodimers with RXR, LXRs reside within the nucleus, bound to LXREs and in complex with corepressors such as silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) (117) and nuclear receptor corepressor (N-CoR) (118). In the absence of ligand, these corepressor interactions are maintained and the transcriptional activity of target genes is repressed. Binding of ligand to LXR results in a conformational change that facilitates coactivator-for-corepressor-complex exchange and transcription of target genes (119). Ligand activation of LXRs also inhibits transcription from promoters of certain genes (e.g., proinflammatory cytokines) that do not contain LXREs, a phenomenon referred to as trans-repression (120) which occurs by recruiting corepressor proteins such as the NCoR & SMRT (117, 118, 121, 122).

1.3.1 Activation of LXR

The two LXRs share sequence homology and respond to similar ligands, however their tissue distribution differs considerably. LXRα is expressed in the liver, spleen, adipose tissue, lung, and pituitary, whereas LXRß is expressed ubiquitously (123). The ligands for the LXRs were originally unknown, and hence they were included in the family of orphan nuclear receptors (110, 111). The endogenous oxidized cholesterol derivatives i.e. the oxysterols were found to bind to and activate these receptors at physiological concentrations (111, 124, 125). The most

potent LXR activators are the naturally occurring oxysterols, 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol, and 24(S), 25-epoxycholesterol, and they can induce LXR transcriptional activity at physiological concentrations. 24(S), 25-epoxycholesterol is abundant in the liver where the levels of LXR are high and it participates actively in the cholesterol metabolism pathway (126). In addition, 27-hydroxycholesterol is an abundant oxysterol in circulation, which has been shown to be a LXR ligand and may be the more relevant natural LXR ligand (127). The 6α-hydroxy bile acid analogs and cholestenoic acid have been also been identified as the selective ligands of LXRα (128, 129). Upon activation of PXR, the upregulation of CYP3A catalyzes the 4β-hydroxylation of cholesterol leading to increase of the circulating 4β-hydroxycholesterol levels (130, 131). This cholesterol metabolite further activates LXR (125). In contrast to the oxysterols, the poly unsaturated fatty acids have shown to be inhibitors of the LXR function, probably by antagonizing the LXR activator binding, and preventing activation at the LXREs (132-134).

1.3.2 Role of LXR in lipid metabolism

LXRs play an important role in hepatic fat metabolism. In LXRα knockout mice, SREBP-1 stearoyl-CoA desaturase (SCD-1), fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC) mRNA are reduced, implicating LXR in regulating the triglyceride synthesis (135). These mice also show defects in cholesterol metabolism (135). The regulation of SREBP-1 by LXR was confirmed by the identification of an LXR response element in SREBP-1c gene (136). Endogenous oxysterols activate LXRα and induce SREBP-1c, which stimulates lipogenesis by transcriptional induction of many lipogenic genes and leads to hypertriglyceridemia (136-138). LXRs bind two functional LXREs in the promoter region of the SREBP-1c gene, which are required for both the basal and inducible expression of SREBP-1c protein (136, 139). In wild-type mice, cholesterol feeding or administration of a synthetic ligand for LXR resulted in increased SREBP-1c expression and fatty acid synthesis. This increase in SREBP-1c levels and fatty acid synthesis was abolished in LXR-null mice, which does not respond to both cholesterol and insulin induced expression of SREBP-1c (136). In addition to mediating lipogenesis via the SREBP-1c upregulation pathway, LXR also seems to target some lipogenic genes directly.

Some lipogenic genes like the as fatty acid synthase (FAS) (140) and Phospholipid transfer protein (PLTP) (141, 142) have been reported to be direct LXR targets and might also contribute to the hypertriglyceridemia (140, 143). Further more LXR has also been shown to activate the expression of angiopoietin-like protein 3 (Angptl3) (144, 145) and is also involved in the insulin mediated induction of lipogenesis (146). Through these direct and in-direct mechanisms, LXR contributes to the induction of the lipogenic process and can lead to severe hepatic steatosis (147).

1.3.3 Role of LXR in cholesterol metabolism

Oxysterol were first identified as the natural ligands for LXR and this indicated that LXR might have a role in cholesterol metabolism (124, 125). When the LXRα knockout mice were fed a high cholesterol diet, they showed high level of cholesteryl ester accumulation in their livers (135). This provided convincing evidence that LXR had an important role in cholesterol metabolism. The increased cholesterol accumulation in the LXR knockout mice was associated with failure to induce the CYP7A1, a cytochrome P450 family member and the rate-limiting enzyme in the classical pathway of bile acid synthesis. The inability of LXRα knockout mice to induce hepatic CYP7a1 expression resulted in a diminished ability to metabolize cholesterol to bile acids, and the accumulation of cholesteryl esters. Thus CYP7A1 was identified as the first direct target gene of the LXRs. The human gene of CYP7A1 did not show the same LXR response element as present in the mouse gene was found to be mutated, so CYP7A regulation may not be LXR dependant in the humans (148).

An exciting phase in LXR research was the implication of a number of LXR target genes in the reverse cholesterol transport pathway. The role of LXR in the cholesterol metabolism pathway led to the identification of its role in several key pathway regulating the cholesterol levels in the body (126, 149). An important discovery was the role of LXRs in the control of ABCA1 and ABCG1 expression and cholesterol efflux (150-154), members of the ATP-Binding Cassette (ABC) superfamily of membrane transporters (34). ABCA1 facilitates the efflux of free-cholesterol and phospholipids to lipid-poor lipoproteins (e.g., apoA-I) and cholesterol-poor nascent high density lipoproteins and its induction may contribute to the increase in plasma HDL

levels seen with LXR ligand treatment (150, 154-158). Homozygous mutations in the ABCA1 gene lead to Tangier disease, a rare genetic disorder characterized by almost a complete absence cellular cholesterol efflux and HDL in plasma of patients (159-161). In these patients there is an accumulation of cholesterol in tissue macrophages, and an increased incidence of cardiovascular disease (159-161). However studies in mice lacking the ABCA1 gene have shown that these mice do not generally bear a risk for higher arthrosclerosis (162).

The induction of two other ABC transporters, ABCG5 and ABCG8 (50, 163), which promote cholesterol efflux from the liver into the bile, appears to be mediated by LXR. The activation of these two receptors by LXR ligands leads to decrease intestinal absorption of cholesterol (152). These transporters form a heterodimer that functions to limit cholesterol and plant-sterol absorption in the gut, and to mediate cholesterol efflux from hepatocytes into bile (164, 165). Mutations in either of the heterodimer transporters causes a genetic disease which is characterized by increased absorption of plant sterols, increased cholesterol absorption leading to sitosterolemia and premature atherosclerosis (166, 167).

In addition to the traditional components of the cholesterol metabolism pathway, LXR also targets some other genes that also contribute towards the cholesterol homeostasis. LXR upregulates the expression of ApoE in a tissue specific manner (168), and ApoE being a principal component of the very low density lipoprotein levels (VLDL) as well as the chylomicron remnants, helps in their hepatic uptake. ApoE is also the extracellular acceptor for cholesterol in the ABCA1 efflux pathway, and helps in the reverse cholesterol transport (169). LXR also helps regulate the expression of lipoprotein remodeling enzymes including the cholesterol ester transfer protein (CETP) (170), lipoprotein lipase (LPL) (171), and the PLTP (141, 142). These three components help in the HDL metabolism and contribute to LXR's role in the cholesterol homeostasis process.

LXRβ is also expressed in the liver, but LXRβ mice unlike their LXRα counterparts do not display an obvious hepatic phenotype even when challenged with a high-cholesterol diet (172), indicating that LXRα is the primary player in hepatic lipid metabolism. However, it is still possible that a functional overlap occurs between the two LXRs.

1.4 CHOLESTEROL GALLSTONE DISEASE

The incidence of cholesterol gallstone disease (CGD) has become relative high in recent times, and it has a incidence of 10-15% in Europe and the United States (173, 174). The majority of the gallstone patients do not exhibit any symptoms, however in the event of complication patients require the surgical removal of the gallbladder, usually by laparoscopic cholecystectomy (175). The most common treatment for CGD is cholecystectomy, with over 700,000 operations performed each year in the United States (174, 176-178). The procedure is effective and remains the standard line of treatment for this disease (176, 177). However the procedure is invasive and may cause surgical complications in some patients and due to the concern of co-morbidity, elderly patients may not be suitable for surgical intervention (178). Mortality rates following cholecystectomy in the US are about 3,000 deaths (0.12% of all deaths) per year are attributed to complications of cholelithiasis and gallbladder disease (179). Non-surgical approaches, including gallstone dissolution by ursodeoxycholic acid and extracorporeal shock-wave lithotripsy are performed only for uncomplicated symptomatic cholecystolithiasis in small number of patients (177, 178). Oral administration of ursodeoxycholic acid is used to treat CGD but is not always successful because of the long duration of treatment and its low efficacy. There is a frequent relapse of gallstone formation following termination of therapy (176, 177). In the US, CGD is next to gastroesophageal reflux disease, in being the second most expensive digestive disease associated with annual medical expenses of US $6.5 billion (180). The development of effective, noninvasive therapies can help to overcome the toxicity and low efficacy of other non-invasive treatments and may help to reduce the costs of health care associated with this disease.

1.4.1 An overview of gallstone disease

Bile composition is determined by the combination of water, lipids, electrolytes and proteins. The major lipid based fluxes are represented by the bile acids, phospholipids and cholesterol and this process involves secretion from the liver into the bile duct by active membrane transport systems (181). In its native form, cholesterol is insoluble in aqueous solutions, however in the bile a large amount of cholesterol is stored (~ 20mM) (27). The increased solubility occurs due

to the formation of mixed micelles in which the bile acids combine with phospholipids and cholesterol creating a lipid-rich core and a water-soluble surface (27, 182). Bile secretion is important for the excretion of many endogenous and exogenous amphipatic compounds like drugs and toxins. In addition the movement of bile acids to the intestine via the bile is essential for the emulsification of dietary lipids (181, 182). Development of cholesterol gallstones happens when the balance between cholesterol, bile salt, and phospholipid levels in the bile is altered. The cholesterol gets supersaturated when the amount of cholesterol in bile increases; or when the levels of the solubilizing bile salts or phospholipids decrease compared to the cholesterol (181, 182). In human beings having gallstones, there is either an excess of biliary cholesterol as compared to bile salts and phospholipids due to the hypersecretion of cholesterol (179), or from hyposecretion of bile salts or phospholipids (173, 183) leading to decreased gallbladder motility and increased mucin production (183). Cholesterol hypersecretion is the most common cause of supersaturation resulting from increased hepatic uptake or synthesis of cholesterol, decreased hepatic synthesis of bile salts, or decreased hepatic synthesis of cholesteryl esters for incorporation in VLDL (179). The ability of bile salt and phospholipid mixed micelles to maintain cholesterol solubility can be surpassed leading to the rapid precipitation of cholesterol crystals (173, 183). In due course of time, the cholesterol crystals aggregate to form the pathogenic gallstones. Due to the role of cholesterol hypersecretion in the gallstone pathogenesis the enzymes, transporters or regulator of this process of hepatic cholesterol metabolism can potentially affect the formation of cholesterol gallstones (184).

1.4.2 Molecular Mechanisms of cholesterol gallstone disease

The secretion of biliary lipids i.e. cholesterol and phospholipids and the bile salts takes place via an intricate network of ABC transporters (177, 181, 182). The efflux of free cholesterol from the hepatocyte occurs through direct transport to the bile via ABCG5 and ABCG8. In the hepatocytes the cholesterol is also broken down to form the bile acids through the classic bile acid synthesis pathway, of which CYP7A1 is the rate-limiting step (50, 135, 163). Accumulation of intracellular bile acid levels through uptake of circulating bile acids via NTCP (53) and the bile salt and phospholipid elimination occurs via the induction of ABCB11 and ABCB4. Under normal conditions, these pathways interact to achieve a homeostasis of

cholesterol and bile acid levels in the cell while providing sufficient cholesterol solubility in the bile (reviewed in (177, 181, 182)).

The CYP7A catalyzes the conversion of cholesterol into bile acids and in the mouse its activity is mediated by ligand-driven activation of two members of the nuclear receptor superfamily, LXR and FXR (4, 185). LXR acts as an cholesterol sensor, plays a role in the elimination of intracellular cholesterol by inducing the transcription of CYP7A1 and stimulating expression of the ABCG5 and ABCG8 cholesterol efflux transporters (50, 135, 163). On the other hand, FXR acts as an bile acid sensor (reviewed in (4-6)), and it plays a role in the bile acid and phospholipid export through induction of the ABCB11/BSEP (33) and ABCB4/MDR3 (186, 187) export pumps, and represses the CYP7A1 transcription (188) as a feedback mechanism to shut down bile acid synthesis to prevent intracellular bile acid levels from becoming toxic.

Recently, Moschetta *et. al.* (185) reported that FXR-null or gallstone-susceptible C57L/J mice secrete proportionally less bile acids and phospholipids relative to cholesterol. This leads to an imbalance in bile lipids resulting in cholesterol precipitation, stone formation and inflammation in the gallbladder. In these studies, the bile salt and phospholipid levels were found to be significantly lower in the FXR null mice due to a lack of FXR-mediated expression of ABCB11 and ABCB4. The treatment of the gallstone-susceptible C57L/J mice with the FXR activator GW4064 lead to increased bile acid and phospholipid secretion, and helped to correct the imbalance in bile lipids and prevented the formation of cholesterol gallstones. GW4064 treatment prevented CGD onset in the C57L mice through FXR-mediated upregulation of ABCB11 and ABCB4 resulting in the increased transport of bile salts and phospholipids to the bile. This helped to reduce the cholesterol saturation index and protected from cholesterol monohydrate crystal formation. Importantly, the cholesterol levels were not significantly altered, because regulation of the cholesterol transporters ABCG5 and ABCG8 through LXR occurred independently of FXR.

1.5 RESEARCH GOAL AND HYPOTHESIS

Our goal is to better understand the role of CAR, PXR and LXR in the transcriptional regulation of their target genes in cholesterol and bile acid metabolism and the regulation's implication in physiological, pharmacological, and pathological processes. We hypothesize that these nuclear receptors participate in maintaining the homeostasis of cholesterol, bile acid and lipid homeostasis (Fig 5). Altered cholesterol, bile acid or lipid physiological profiles can affect the regulatory network of these receptors that can initiate/downregulate transcription of metabolizing enzymes, transporters or other genes encoding enzymes of the cholesterol, bile acid or lipid metabolism pathways. To test this hypothesis, we have created mouse models with compromised (knockout) or heightened (transgenic) CAR, PXR and LXR receptor activity. The effect of altered receptor activity on the regulation of enzymes and transporters is being evaluated by using molecular, genomic, pharmacological, and proteomic approaches. More importantly, the effects of receptor action and inactivation on hepatic functions are evaluated.

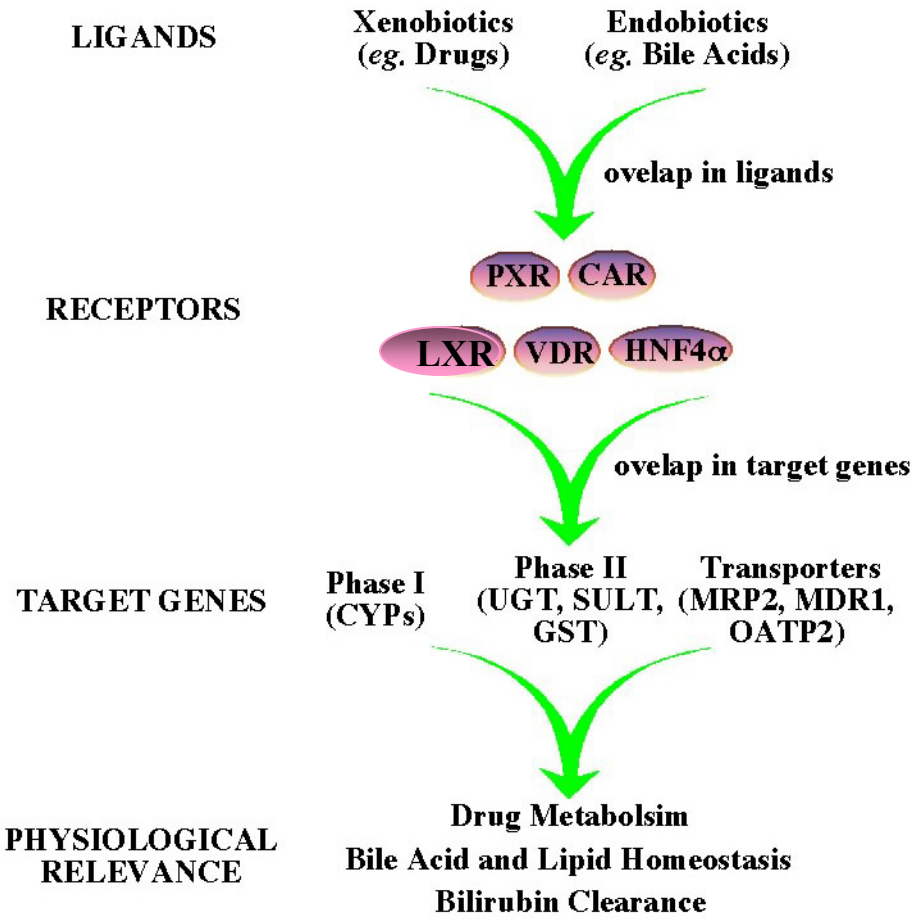
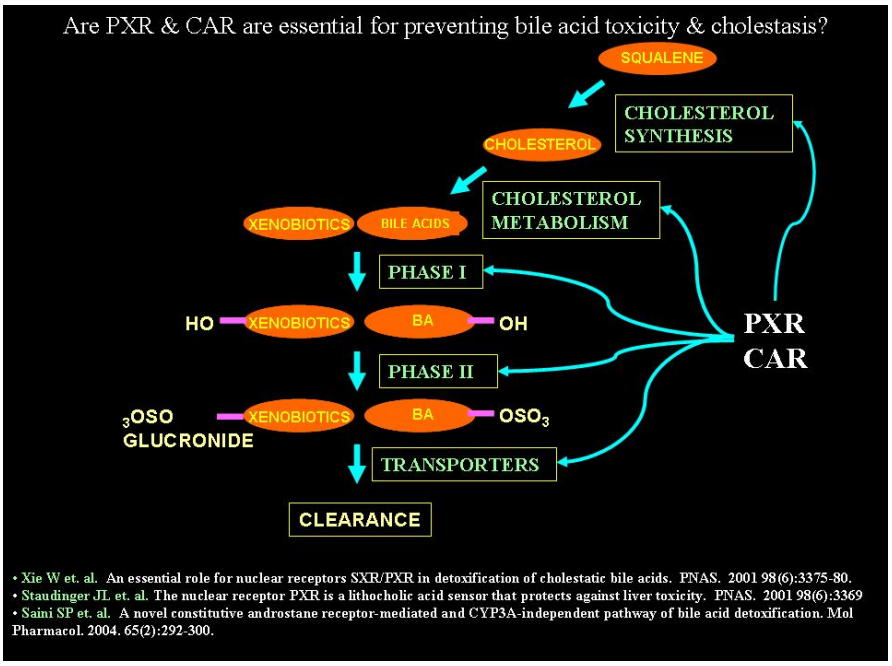


Figure 5: The complexity of mammalian xenobiotic response and its regulation by xenobiotic nuclear receptors.

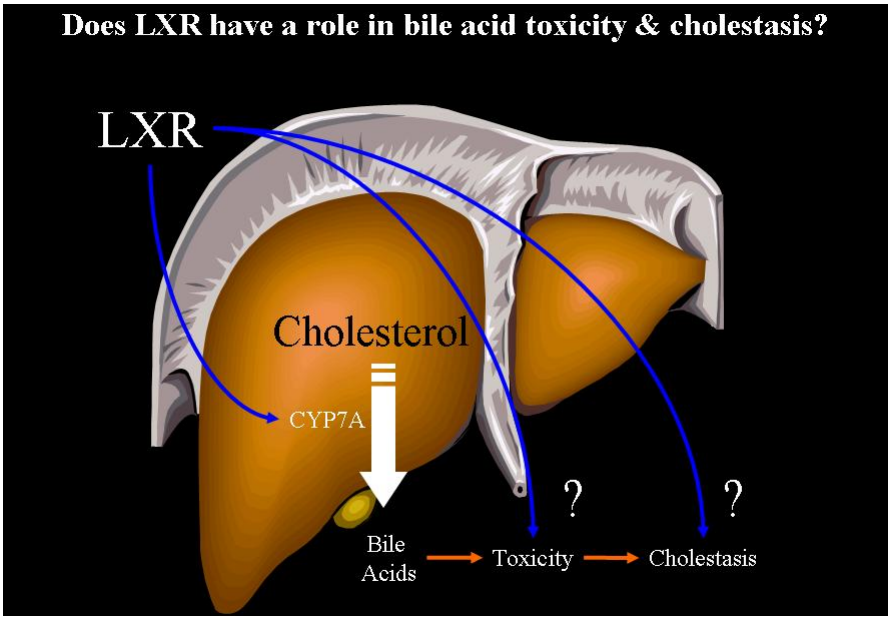
The activation of nuclear receptors by xenobiotic and endobiotic ligands, and subsequent regulation of phase I and phase II enzymes and drug transporters will eventually affect many physiological and pharmacological responses, such as drug metabolism and the homeostasis of bile acids, lipids and bilirubin. Abbreviations: PXR, pregnane X receptor; CAR, constitutive androstane receptor; LXR, Liver X receptor; VDR, vitamin D receptor; HNF4α, hepatic nuclear factor 4α; CYPs, cytochrome P450 family; UGT, UDP-glucuronosyltransferases; SULT, sulfotransferase; GST, glutathione S-transferases; MRP2, multidrug resistance-associated protein 2; MDR1, multidrug resistance protein 1; OATP2, organic anion transporter polypeptide 2. (Adapted from Xie *et. al.* (3).

Based on various studies it is well established that nuclear receptors contribute significantly to the regulation of xeno- and endobiotic metabolism in the body. Based on the various roles of PXR, CAR and LXR in metabolism in the body, we wanted to work on three specific hypotheses:

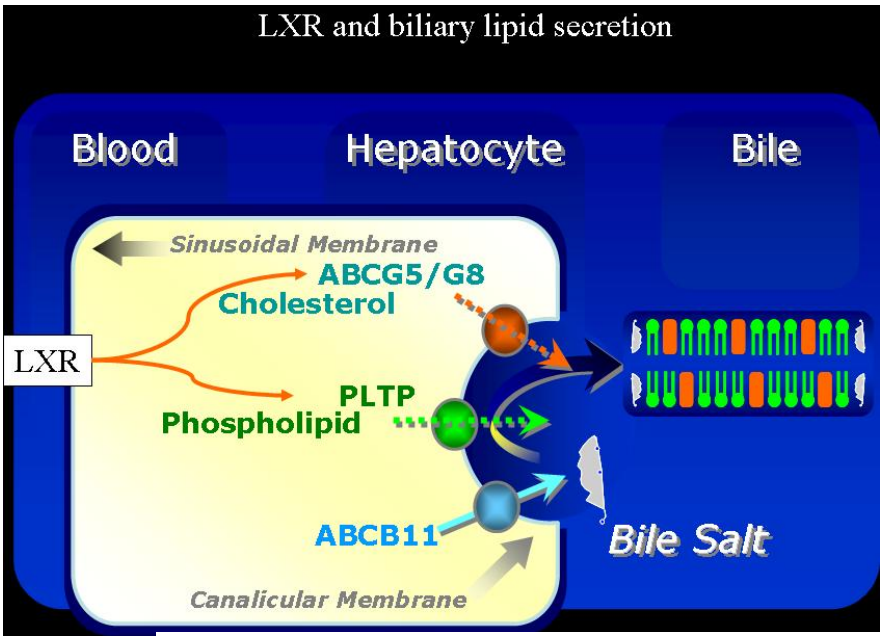
1. Although activation of PXR or CAR has been shown to promote bile acid clearance, it has not been systemically examined whether an individual or combined loss of PXR and CAR will compromise bile acid detoxification. What is the combined role of PXR & CAR in preventing bile acid toxicity and cholestasis?



2. LXR plays a role in the breakdown of cholesterol and the formation of bile acids. Does the activation of LXR prevent bile acid toxicity and cholestasis?



3. LXR plays a role in the excretion of cholesterol and phospholipids from the liver. What is the role of LXR in cholesterol gallstone disease?



Adapted from lipidsonline.org(Baylor College of Medicine)

2.0 COMBINED LOSS OF ORPHAN RECEPTORS PXR AND CAR HEIGHTENS SENSITIVITY TO TOXIC BILE ACIDS IN MICE

2.1 INTRODUCTION

Bile acids are the end products of cholesterol catabolism. Despite their beneficial function in lipophilic nutrient absorption, accumulation of bile acids is potentially toxic and can impair liver function and induce cholestasis. (189) An example of this can be seen with lithocholic acid (LCA), a secondary bile acid that is not only a potent cholestatic agent but has also been shown to promote colon cancer. (190)

Hepatointestinal bile acid trafficking plays an important role in bile acid detoxification (reviewed by Kullak-Ublick et al.; Meier and Stieger; and Trauner and Boyer (18, 25, 30)). The hepatic uptake of bile acid is mediated by the Na+-taurocholate cotransport proteins (NTCPs) and the Na+-independent organic anion-transporting polypeptides (OATPs) that include OATP1, OATP2, and OATP4/solute carrier (SLC) 21A6 (rodents)/OATP-C (humans). The uptake is further enhanced through the binding of bile acids to intracellular proteins, such as glutathione-S-transferases (GSTs) A1 and A2. The export of bile salts from hepatocytes into the bile is facilitated by several canalicular membrane export transporters, such as the bile salt export pump (BSEP) and multidrug resistance-associated proteins (MRPs) 2 and 3. The majority of biliary bile salts is aggregated into micelles and transported into the intestine, where bile salts can be reabsorbed by OATP3,(55) sodium-dependent bile acid transporter, (191) intestinal bile acid-binding protein,(192) MRP3, and truncated sodium-dependent bile acid transporter (reviewed by Trauner and Boyer(18)). Impairment of transporter expression or function may cause cholestasis. (193) In addition to bile salt trafficking, the Phase I and Phase II drug metabolizing enzymes also play a role in bile acid homeostasis. The Phase I cytochrome P450 (CYP) 3A enzymes catalyze

the hydroxylation of LCA, promoting its elimination. (76, 96) The Phase II sulfotransferases (SULTs), such as SULT2A9 (also called hydroxysteroid SULT), are also important for detoxification. (194-196)

Pregnane X receptor (PXR) and constitutive androstane receptor (CAR) have both been implicated in bile acid detoxification. (76, 86, 87, 96, 197) Expression of activated PXR (VP-PXR) in transgenic mice was sufficient to confer resistance to LCA hepatotoxicity. This protection may be due to a combined induction of CYP3A and SULT2A9. (87, 96) More recently, we showed that the expression of an activated CAR (VP-CAR) was both sufficient and necessary to confer LCA resistance. (86) Interestingly, the CAR-mediated protection was independent of CYP3A induction but was associated with SULT2A9 induction. (86) In addition to their ability to regulate Phase I and II enzymes, PXR and CAR have also been shown to stimulate the expression of drug transporters, such as MRP2, OATP2, and SLC21A6. (39, 76, 197) The coordinated regulation of detoxifying genes by PXR and CAR suggest that both receptors might function as master regulators to control bile acid homeostasis. Although activation of PXR or CAR has been shown to promote bile acid clearance, it has not been systemically examined whether an individual or combined loss of PXR and CAR will compromise bile acid detoxification.

In addition to bile acid detoxification, the liver also plays a role in bile acid formation, a process that influences the homeostasis of both bile acids and cholesterol. The catabolism of cholesterol to bile acids is controlled by several CYP enzymes, including CYP7A, CYP8B1, and CYP27A. (198) Bile acids themselves are signaling molecules that activate the FXR. (199-201) When bound to and activated by bile acids, FXR represses the transcription of CYP7A, the rate-limiting enzyme in bile acid synthesis, thereby repressing the conversion of cholesterol to bile acids. (199-201) FXR null mice had elevated levels of bile acid, further supporting the role of FXR in bile acid homeostasis. (108) Several other hepatic factors, including the small heterodimer partner (SHP), liver receptor homolog 1, and hepatic nuclear factor 4, were subsequently found to be involved in FXR-mediated CYP7A repression. (188, 202) The hepatic expression of SHP was induced by bile acids or the synthetic FXR agonist GW4064, and this expression was inversely related to CYP7A1 and CYP8B1 expression. (5, 107, 203) In contrast, a loss of SHP

in mice caused increased synthesis and accumulation of bile acids because of a de-repression of CYP7A1 and CYP8B1. (204) These studies suggest that SHP functions as a negative regulator by interacting with liver receptor homolog 1 and hepatic nuclear factor 4, (5, 107, 203) two positive regulators of CYP7A and CYP8B1.

In this report, we showed that the combined - but not individual - loss of PXR and CAR resulted in a robust sensitivity to LCA hepatotoxicity in male mice. The increased sensitivity in the double knockout males was associated with a profound decrease in the expression of bile acid transporters. We propose that PXR and CAR function in concert to regulate bile acid homeostasis.

2.2 MATERIALS AND METHODS

2.2.1 Animals, drug treatment, and histological evaluation

The creation of mice with individual or combined loss of PXR and CAR has been described. (77, 78, 86) All mice were of a mixed background of SVJ129 and C57B and were maintained on Prolab RMH3000 diet (PMI Nutrition International, St. Louis, MO). In pilot experiments, mice were given daily gavage of LCA (8 mg/d) and killed 24 hours after the last dose. (96) For subsequent LCA treatments, mice were given two intraperitoneal doses of LCA each day for a total daily dose of 250 mg/kg. The LCA treatment lasted for 4 days, and mice were killed 24 hours after the last dose. Liver tissues were fixed in 4% formaldehyde, embedded in paraffin, sectioned at 5 m, and stained with hemotoxylin-eosin. Frozen sections (10 m) were used for Oil Red O staining. When necessary, mice were subjected to a single intraperitoneal injection of clotrimazole (50 mg/kg), dexamethasone (50 mg/kg), and phenobarbital (40 mg/kg) 24 hours before tissue harvest. The use of mice in this study has complied with relevant federal guidelines and institutional policies.

2.2.2 Northern blot analysis and reverse-transcriptase polymerase chain reaction.

Total RNA was prepared from tissues using the TRIZOL reagent (Invitrogen, Carlsbad, CA). Northern hybridization was performed as described previously. (78) The complementary DNA (cDNA) probes for CYP3A11, CYP2B, UGT1A1, SULT2A9, and CYP7A have been described. (75, 78, 85, 87) All other cDNA probes were cloned via reverse-transcriptase polymerase chain reaction using primers designed based on cDNA sequences in GenBank. Reverse-transcriptase polymerase chain reaction for MRP2 and MRP3 was performed as described. (107)

2.2.3 Measurement of serum chemistry and hepatic lipid profiles.

Upon completion of the LCA treatment, the mice were killed and blood was collected. Serum levels of bile acids, bilirubin, and aspartate aminotransferase (AST) were measured by ANTECH Diagnostics (Lake Success, NY). (86) The triglyceride and cholesterol levels in liver lipid extracts were measured using the Stanbio Laboratory assay kits (Boerne, TX). GraphPad Instat Software (San Diego, CA) was used for statistic analysis.

2.3 RESULTS

2.3.1 PXR and CAR double knockout mice exhibited heightened sensitivity to LCA-induced histological liver damage.

To evaluate the effect of PXR and CAR, single and double knockout mice (77, 78, 86) were gavaged with vehicle or LCA (8 mg/mouse) for 4 days, a regimen shown to be survived by wild-type and PXR null mice. (96) Pilot experiments showed that all double knockout males died after 2 to 4 days of LCA gavage. Subsequent experiments showed that a regimen of 250 mg/kg intraperitoneal LCA for 4 days was survived by most of the double knockout males. This regimen was then chosen to compare toxicity in wild type and knockout mice, except those indicated in Table 1 and Fig. 6.

Liver histology was evaluated via hemotoxylin-eosin staining. In vehicle-treated males, no significant alteration in liver histology was found in PXR and/or CAR knockout mice when compared with wild-type mice (Fig. 6A-D). Following intraperitoneal LCA, the wild-type liver showed minimal histological changes (Fig. 6E). Liver histology of the PXR null and CAR null mice also indicated no or low toxicity (Fig. 6F, H), suggesting the single knockout mice had sufficient LCA detoxification to prevent hepatotoxicity. In a sharp contrast, the livers of LCA-treated double knockout males showed massive liver damage, which was scored by the appearance of areas of saponification and coagulative necrosis (Fig. 6I). Interestingly, the responsiveness in PXR null mice was administration route dependent, because gavage of a similar dose of LCA (8 mg/mouse) resulted in expected liver damage, as we have described previously (Fig. 6G; Table 1). (196) LCA sensitivity in double knockout mice was subject to sex effect. The incidence of histological liver damage was less than half in females compared with males. Table 1 summarizes the genotype-, route-, and sex-dependent penetrance of LCA hepatotoxicity.

Table 1: LCA-Induced histological liver damage in mice with an individual or combined loss of PXR and CAR

Males								Females	
WT		**PXR Null**		**CAR Null**		**Double KO**		**Double KO**	
i.p.	**p.o.**	**i.p.**	**p.o.**	**i.p.**	**p.o.**	**i.p.**	**p.o.**	**i.p.**	**p.o.**
1/7 (14.3%)	7/12 (58.3%)	0/11 (0%)	10/10 (100%)	3/9 (33.3%)	ND	11/13 (84.6%)	NA	2/6 (33.3%)	ND

Abbreviations: i.p., intraperitoneal injection; p.o., gavage; ND, not determined; NA, not applicable due to lethality.

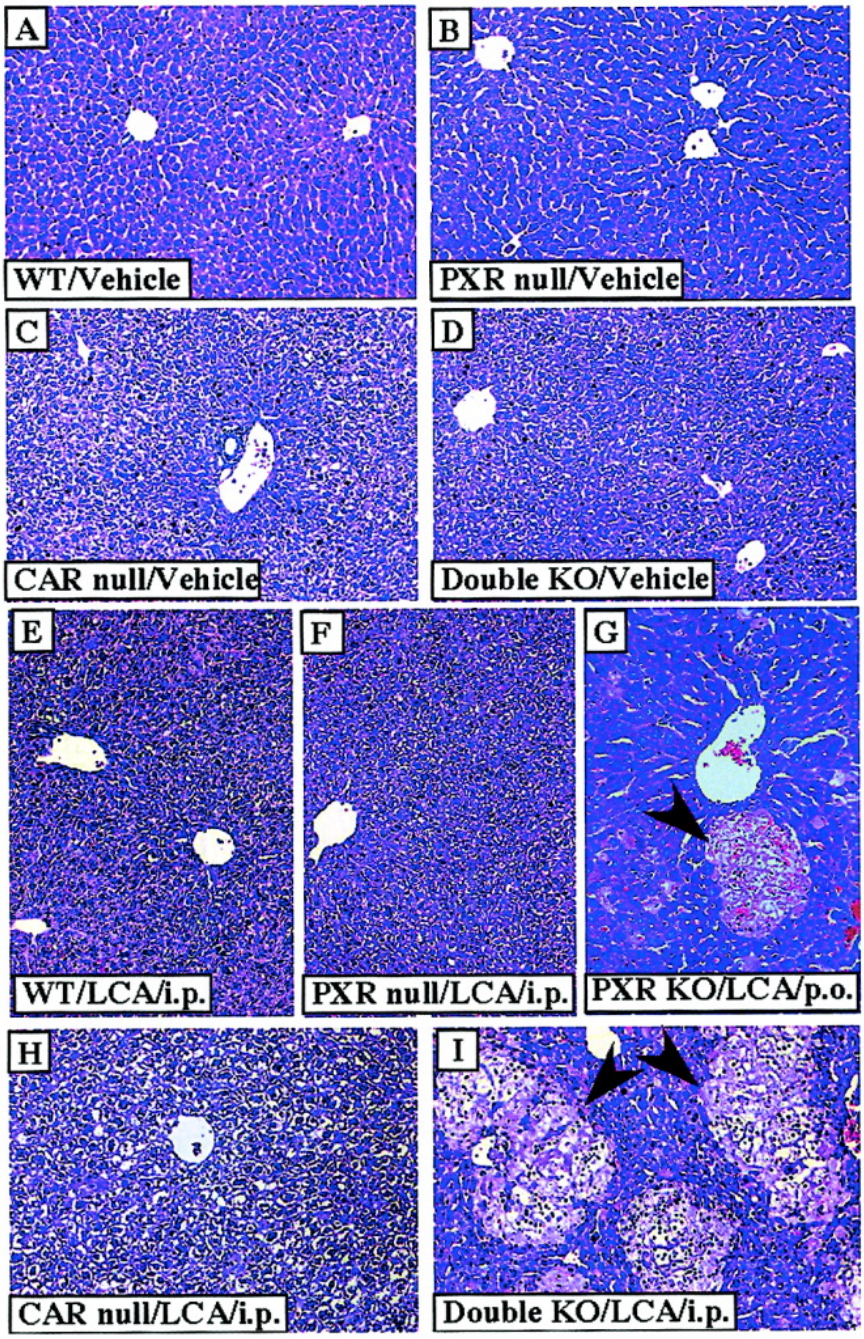


Figure 6: Mice deficient in both PXR and CAR exhibited heightened sensitivity to LCA-induced histological liver damage.

Male mice of the indicated genotypes were given a daily treatment of (A-D) vehicle or (E-I) LCA for 4 days before liver harvesting. The drug administration route was intraperitoneal administration except for panel G (oral gavage). Liver paraffin sections were stained with hemotoxylin-eosin. Areas of necrosis are marked by arrowheads in panels G and I. The magnification is ×200 for all panels. WT, wild-type; PXR, pregnane X receptor; CAR, constitutive androstane receptor; KO, knockout; LCA, lithocholic acid; i.p., intraperitoneal injection; p.o., gavage.

2.3.2 Defects in bile acid clearance and serum chemistry in double knockout males.

Consistent with the histological damage, changes in serum chemistry were seen in LCA-treated double knockout males. The most notable change was a massive accumulation of serum bile acids. The serum levels of total bile acids in individual knockout mice were similar to those in the wild-type mice after LCA treatment (Fig. 7A). In contrast, double knockout males had a significant accumulation despite some individual variation, suggesting the combined loss of PXR and CAR led to impairment of biliary excretion. Serum levels of several other chemical parameters indicative of hepatotoxicity, such as bilirubin and AST, were also increased in LCA-treated double knockout males. No significant difference in serum bilirubin was seen in vehicle-treated mice regardless of genotype (Fig. 7B). The CAR null mice had a modest increase in serum bilirubin, although it was less than that seen in the double knockouts (Fig. 7B). Similar serum levels of AST, ranging from 65 to 82 U/L, were seen in all of the vehicle-treated mice (data not shown). LCA treatment resulted in elevated AST levels - a sign of hepatotoxicity - in all genotypes, with the most dramatic increase in double knockouts (Fig. 7C). Consistent with their lower incidence of histological liver damage (Table1), the double knockout females had a serum chemistry profile similar to the wild-type mice.

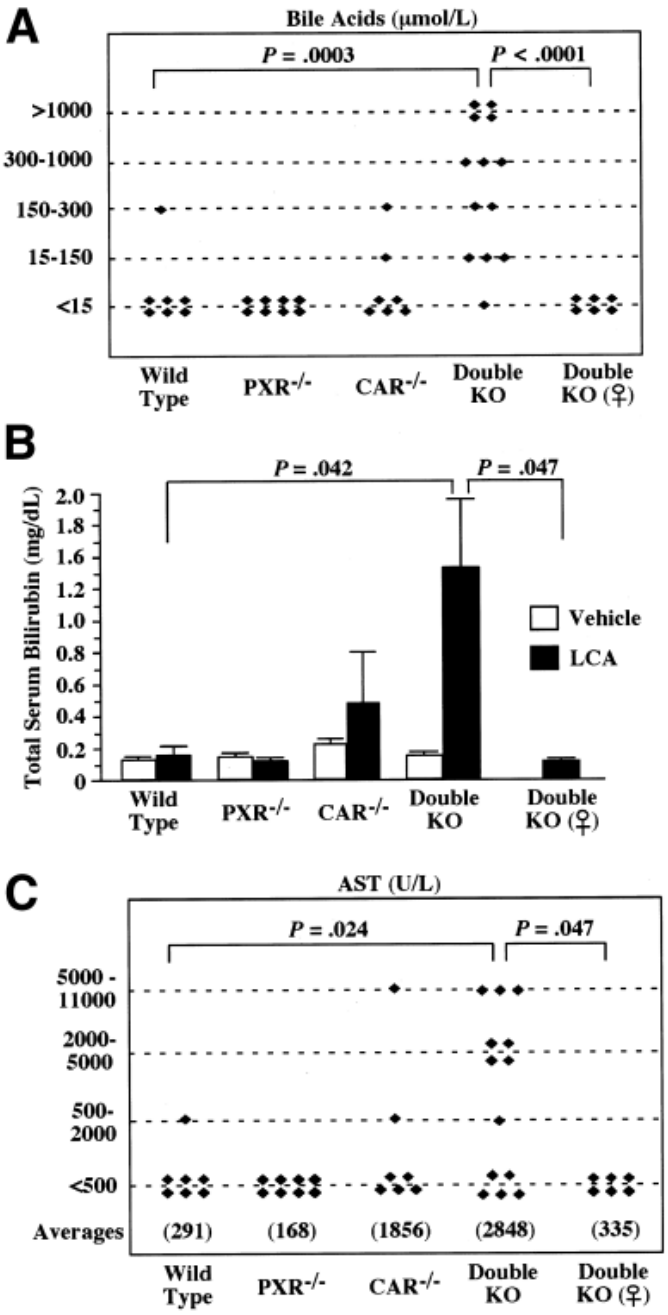


Figure 7: Defects in bile acid clearance and serum chemistry in double knockout mice.

The serum collected from LCA-treated mice of the indicated genotypes and sexes were evaluated for (A) total bile acid, (B) total bilirubin, and (C) AST. Serum bilirubin levels in vehicle-treated mice are also shown in panel B. Mice in the first four columns are all males. Each spade () in panels A and C represents one animal (wild type, n = 7; $PXR^{-/-}$, n = 8; $CAR^{-/-}$, n = 7; double knockout male, n = 13; double knockout female, n = 6). PXR, pregnane X receptor; CAR, constitutive androstane receptor; KO, knockout; LCA, lithocholic acid; AST, aspartate aminotransferase.

2.3.3 Increased hepatic lipid accumulation in LCA-treated CAR null and double knockout mice.

Oil Red O staining revealed that LCA induced genotype-specific hepatic lipid deposition in CAR null and double knockout males (Fig. 8). Livers of the vehicle-treated CAR null and double knockout mice exhibited slight increases in lipid staining compared with wild type and PXR null mice (Fig. 8A-D). Upon LCA treatment, although the wild type and PXR null mice still had minimal staining (Fig. 8E-F), the CAR null mice showed markedly increased lipid staining (Fig. 8G), and the increase was even more profound in the double knockouts (Fig. 8H). Tissue lipid measurements revealed that the hepatic triglyceride (Fig. 9A) but not cholesterol (Fig. 9B) levels were significantly increased in the LCA-treated double knockout mice. Surprisingly, the triglyceride level in CAR null mice was not elevated despite the increased Oil Red O staining.

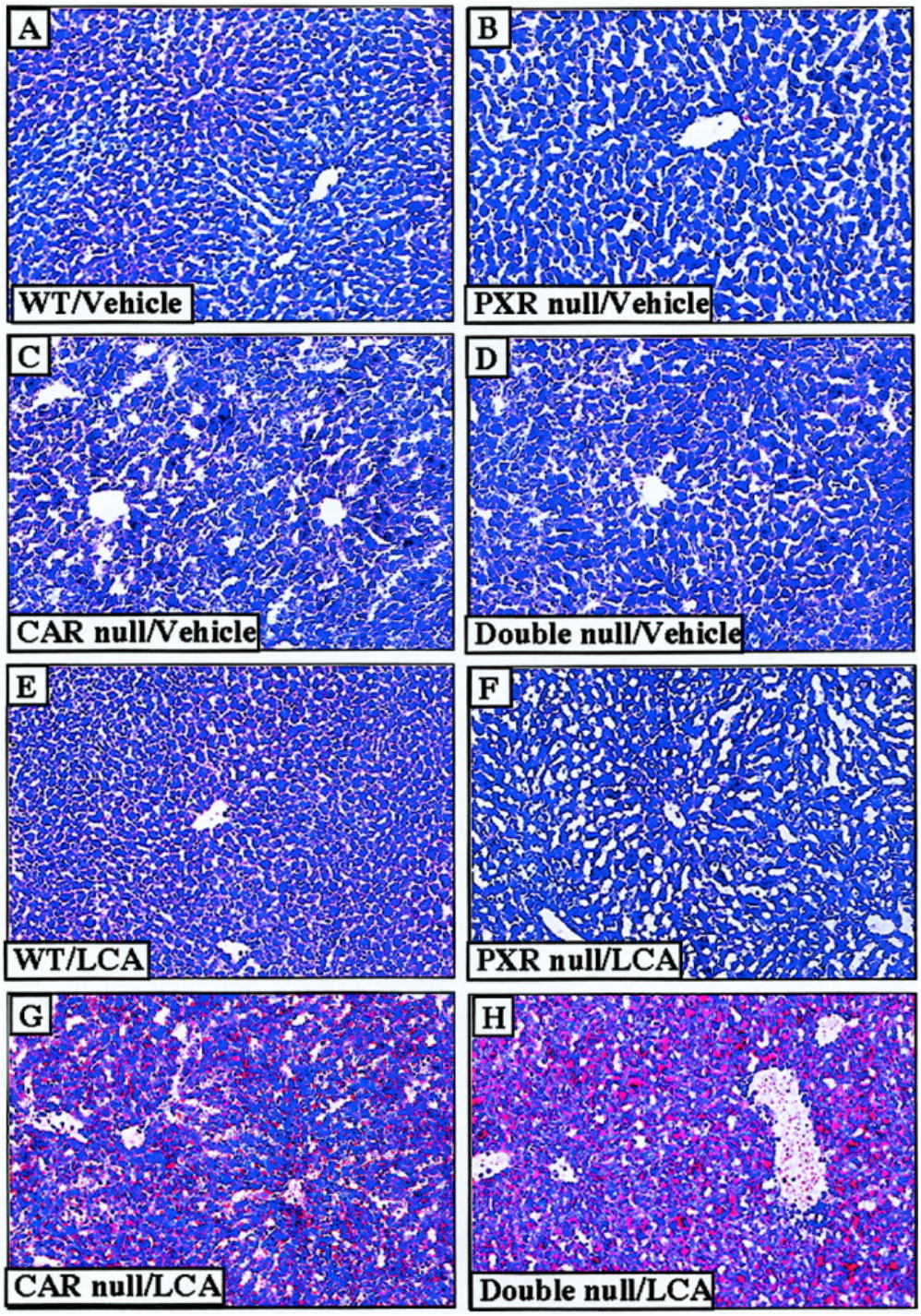


Figure 8: Increased hepatic lipid deposit in LCA-treated CAR null and double knockout male mice.

Mice of the indicated genotypes were given a daily treatment of (A-D) vehicle or (E-H) LCA for 4 days before liver harvest. Frozen liver sections were stained with Oil Red O; lipid droplets stained red. The magnification is ×200 for all panels. WT, wild-type; PXR, pregnane X receptor; CAR, constitutive androstane receptor; LCA, lithocholic acid

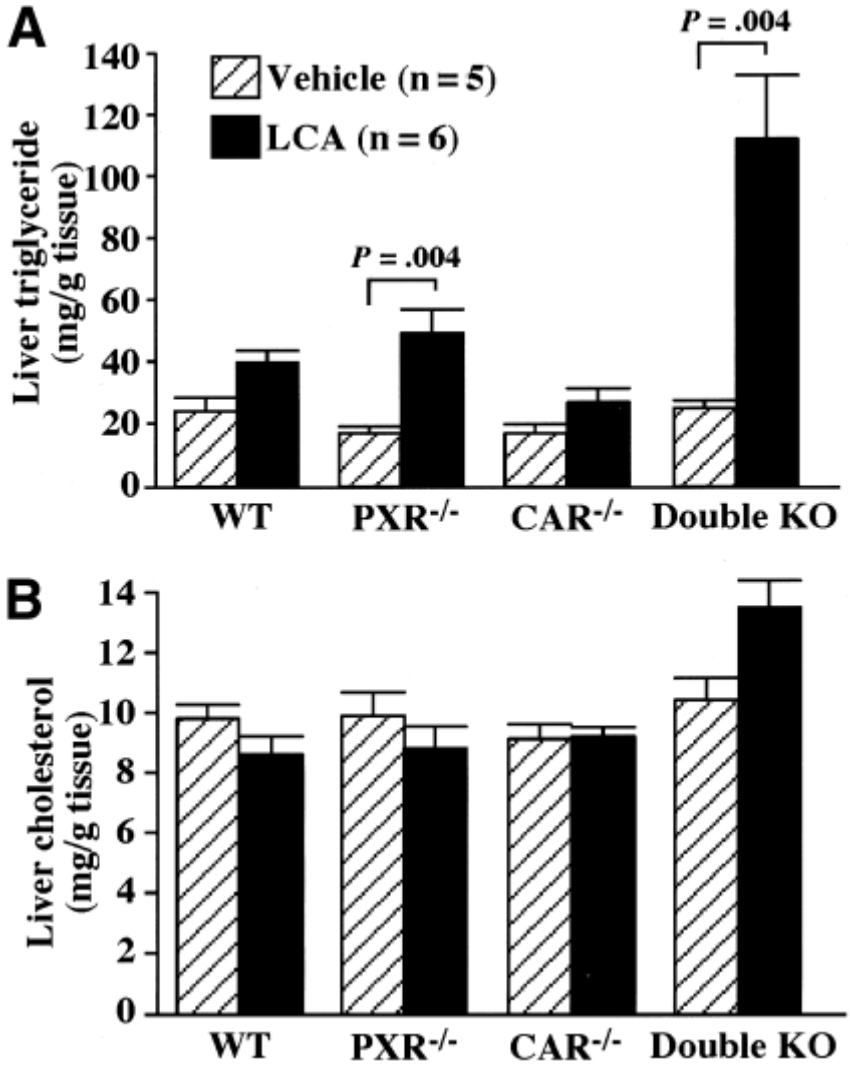


Figure 9: Increased hepatic levels of triglyceride but not cholesterol in LCA-treated double knockout males.

Liver lipids were extracted from vehicle-treated (n = 5 per genotype) and LCA-treated (n = 6 per genotype) mice and were subject to measurements of (A) triglyceride and (B) cholesterol levels. LCA, lithocholic acid; WT, wild type; PXR, pregnane X receptor; CAR, constitutive androstane receptor; KO, knockout**.**

2.3.4 Marked suppression of bile acid transporter expression in LCA-treated double knockout males.

To understand the mechanisms of heightened LCA sensitivity in double knockout males, hepatic messenger RNA (mRNA) expression of the major bile acid transporters was evaluated using Northern blot analysis. LCA treatment greatly and specifically reduced the expression of these transporters in the double knockout mice. The most notable change was the markedly reduced expression of BSEP, OATP1, OATP4/SLC21A6 (Fig. 10A), and NTCP (Fig. 10B). The intestinal expression of these transporters was barely detectable with Northern blot analysis (data not shown). Among other transporters, reverse-transcriptase polymerase chain reaction revealed that MRP2 and MRP3 mRNA expression was elevated in vehicle-treated PXR null mice, but their expression was upregulated by LCA regardless of genotype (Fig. 10C), consistent with the notion that MRPs are upregulated in severe liver disorders. (205, 206)

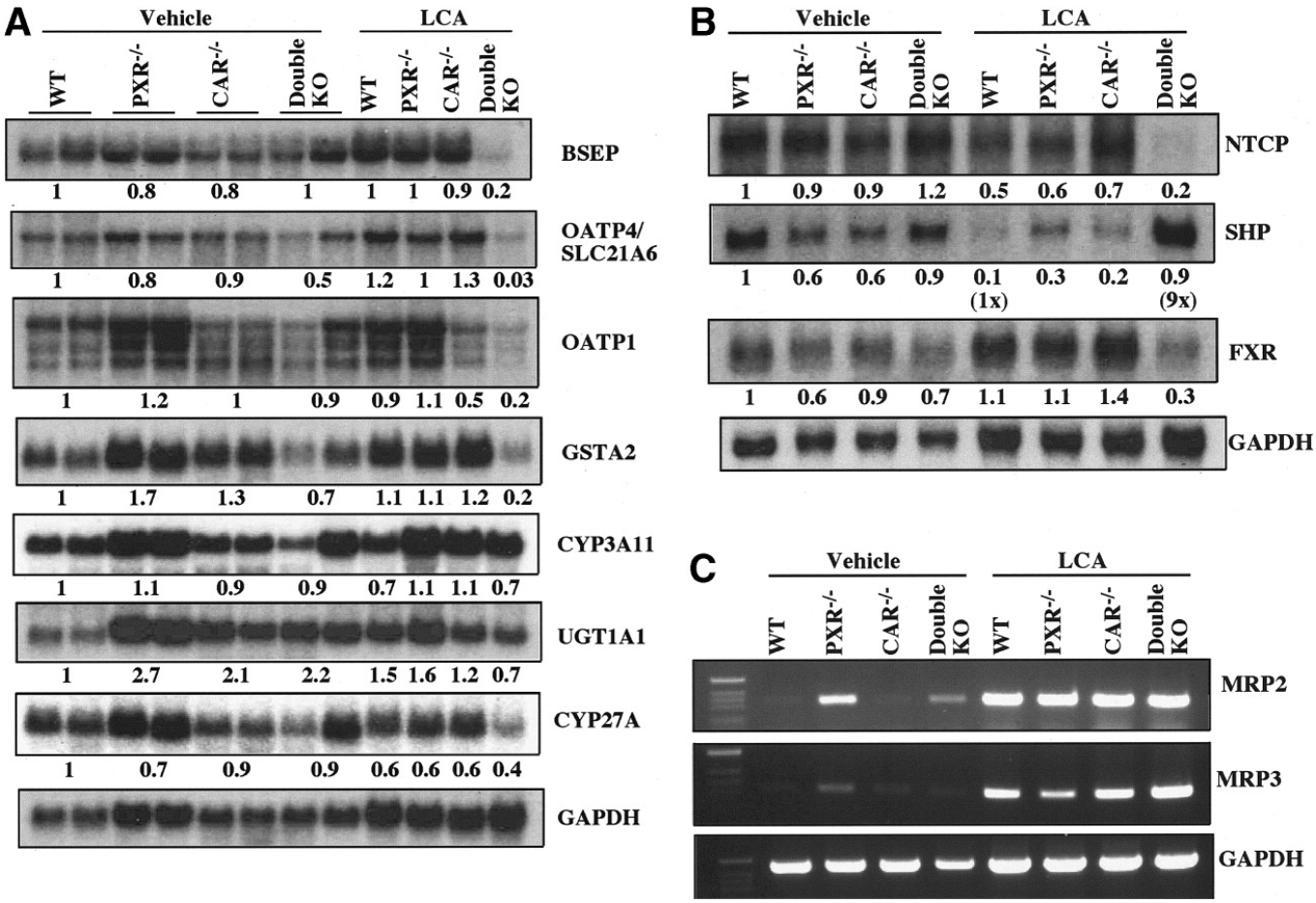


Figure 10: Marked suppression of transporter gene expression in LCA-treated double knockout male mice.

(A) Mice of the indicated genotypes were given a daily treatment of vehicle or LCA for 4 days before tissue harvest. Total liver RNA was subjected to Northern blot analysis. The membranes were hybridized with the indicated 32P-labeled cDNA probes. Two individual mouse samples are shown for the vehicle-treated groups, whereas liver RNA was pooled from 6 animals per LCA treatment group. The signals were normalized against glyceraldehyde-3-phosphate dehydrogenase, and fold inductions over the vehicle-treated wild-type mice are shown. The averages of the fold induction are shown for the vehicle-treated groups. (B) Northern blot analysis as in panel A, except that liver RNA was pooled from 5 animals per vehicle-treated group. (C) Semi quantitative reverse-transcriptase polymerase chain reaction evaluation of hepatic MRP2 and MRP3 mRNA expression in vehicle- and LCA-treated mice. LCA, lithocholic acid; WT, wild-type; PXR, pregnane X receptor; CAR, constitutive androstane receptor; KO, knockout; BSEP, bile salt export pump; OATP, organic anion-transporting polypeptide; GST, glutathione-S-transferase; CYP, cytochrome P450.

We also profiled the expression of Phase I and II enzymes and nuclear receptors, because these gene products are also implicated in bile acid detoxification. The most notable was the profound reduction in GSTA2 expression in LCA-treated double knockout males (Fig. 10A). The hepatic expression of CYP3A11 was not significantly altered by intraperitoneal LCA injection (Fig. 10A), which is in contrast to a modest CYP3A11 induction in LCA-gavaged mice (see Discussion). (96) In the intestine, however, LCA injection caused measurable CYP3A11 induction regardless of genotype (data not shown). The intestinal CYP3A induction might have been mediated by the vitamin D receptor, a bile acid sensor and positive CYP3A regulator. (207, 208) The expression of UGT1A1 was increased in vehicle-treated PXR knockout mice. Upon LCA treatment, the UGT1A1 mRNA levels were modestly reduced in individual and double knockout mice compared with their vehicle-treated counterparts (Fig. 10A). The expression of SULT2A9 was not detectable in male livers (data not shown). The phenotype of hepatic lipid accumulation prompted us to examine the expression of CYP27A and CYP7A, two enzymes that play roles in cholesterol catabolism of and bile acid homeostasis. Bile acids are known to suppress the expression of these two genes. (107, 188, 202) As expected, the expression of CYP27A was reduced in all genotypes, but a greater suppression was seen in the double knockout mice (Fig. 10A). The expression of CYP7A was also suppressed by LCA (data not shown).

The expression of other orphan receptors known to regulate transporter and enzyme gene expression was also examined. LCA treatment was found to dramatically suppress the expression of SHP in the wild-type mice, and to a lesser degree in individual knockout mice. This suppression was consistent with the notion that a sustained bile acid presence represses SHP gene expression. (5) In contrast, LCA failed to suppress SHP expression in the double knockout mice, resulting in a 9-fold expression of SHP in this genotype compared with LCA-treated wild-type mice (Fig 10B). The expression of FXR was lower in LCA-treated double knockout mice.

2.3.5 Molecular mechanisms of lower LCA sensitivity in double knockout females

The expression of transporters and enzymes was profiled in vehicle- and LCA-treated double knockout females (Fig. 11). The expression of NTCP, GSTA2, and CYP3A11 was also reduced in LCA-treated double knockout females, but to a lesser degree than that achieved in their male counterparts (Fig. 10). In contrast, LCA had little effect on the expression of OAPT1, OATP4/SLC21A6, and BSEP (Fig. 11). SHP was induced by LCA in double knockout females (Fig. 11). The bile acid-detoxifying SULT2A9 was abundantly expressed in females, and this expression was sustained in LCA-treated animals.

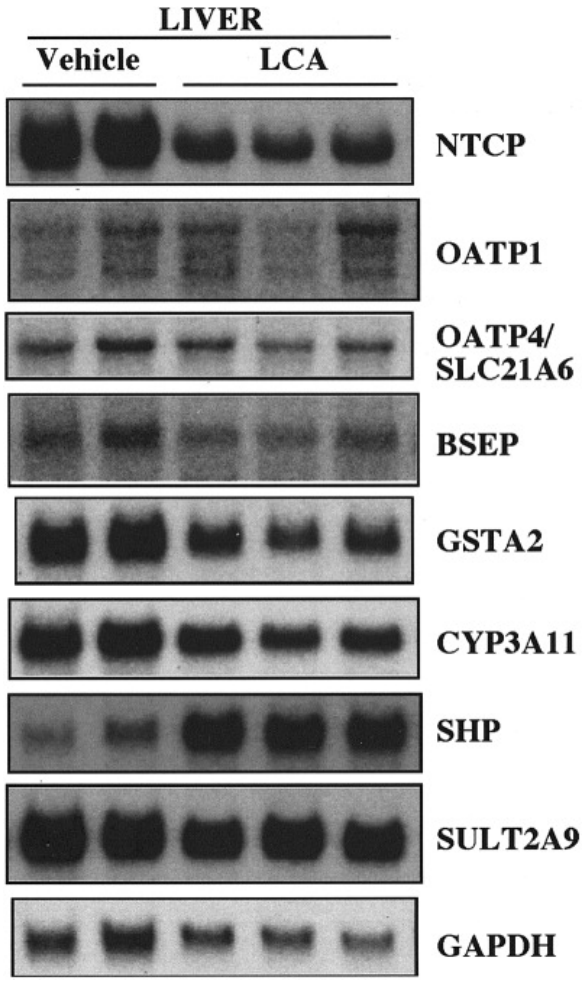


Figure 11: Molecular mechanisms of lower LCA sensitivity in double knockout female mice.

Double knockout females were given a daily treatment of vehicle or LCA for 4 days before tissue harvest. Total liver RNA (20 g) was subjected to Northern blot analysis. The membranes were hybridized with the indicated 32P-labeled cDNA probes. OATP, organic anion-transporting polypeptide; BSEP, bile salt export pump; GST, glutathione-S-transferase; CYP, cytochrome P450; SHP, small heterodimer partner.

2.3.6 Additional dysregulation of PXR and CAR target genes in double knockout males.

The unique LCA-dependent gene regulation in the double knockout mice prompted us to examine drug-induced transporter and enzyme expression in this genotype. Clotrimazole (CTZ) activates PXR and CAR *in vivo*, (78, 95) dexamethasone (DEX) at high doses activates PXR, and phenobarbital (PB) results in CAR nuclear translocation and increased gene expression because of its constitutive activation (for a review, see Swales and Negishi (209)). As shown in Fig. 12, among transporters, CTZ was found to inhibit the hepatic mRNA expression of OATP1, NTCP, and BSEP, but not OATP4/SLC21A6. DEX inhibited OATP1, OATP4/SLC21A6, and BSEP but had little effect on NTCP. PB treatment had little effect on any of these transporters. Interestingly, the selective repression of the transporters by CTZ and DEX was not associated with increased SHP expression. Among Phase II enzymes, GSTA2 was suppressed by CTZ and DEX but not by PB. UGT1A1 was suppressed by DEX in the liver and by both CTZ and DEX in the intestines. Among Phase I enzymes, CTZ and PB are known to induce CYP3A11 that is sustained in the PXR null mice. (75, 78) In contrast, the induction of CYP3A11 by DEX and PB was absent in the PXR null and CAR null mice, respectively. (75, 77) In LCA-treated double knockouts, the expression of CYP3A11 was suppressed by CTZ in the liver and by PB in the intestine. To our surprise, the intestinal expression of CYP3A11 was robustly induced by DEX in the double knockout mice, in contrast to the loss of induction in PXR single knockout mice. (78) In the liver, CYP2C29 and CYP2A4 were suppressed by CTZ and DEX but not PB. In summary, the double knockout mice exhibited gene- and tissue-specific regulation of xenobiotic transporters and enzymes in response to PXR and CAR agonists. Moreover, many of the regulatory profiles were distinct from those observed in individual knockout mice.

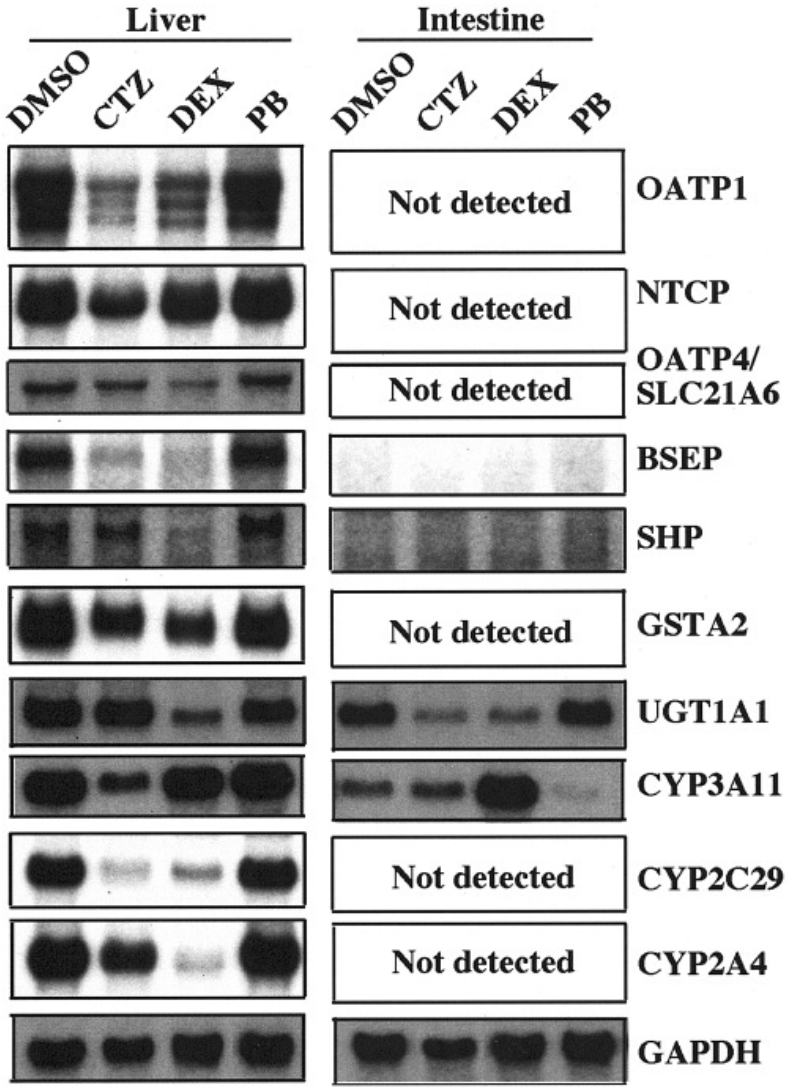


Figure 12: Additional dysregulation of PXR and CAR target genes in double knockout male mice.

Double knockout males were given a single intraperitoneal injection of vehicle or indicated drugs 24 hours before tissue harvesting. Total liver and intestine RNA was subjected to Northern blot analysis. The membranes were hybridized with the indicated ^{32}P-labeled cDNA probes. CTZ, clotrimazole; DEX, dexamethasone; PB, phenobarbital; OATP, organic anion-transporting polypeptide; BSEP, bile salt export pump; SHP, small heterodimer partner; GST, glutathione-*S*-transferase; CYP, cytochrome P450.

2.4 DISCUSSION

In this report, we show that mice deficient in both PXR and CAR exhibited a markedly heightened LCA hepatotoxicity compared with wild type and individual knockout mice. We suggest the accumulation of serum bile acids and associated hepatotoxicity were likely due to a genotype- and sex-specific suppression of hepatic transporter expression. The reduction of hepatic efflux transporters, such as BSEP, is known to aggravate hepatic damage in cholestasis. On the other hand, the reduction of uptake transporters, such as NTCP, OATP1, and OATP2, is suggested to be a protective mechanism to reduce liver damage. Blockade of uptake, however, may contribute to the accumulation of circulating bile acids and associated systemic toxicity.

Our results show that LCA sensitivity in mice is subject to a sex effect and can be affected by the route of drug administration. The lower LCA sensitivity found in double knockout females compared with their male counterparts is likely due to the sustained expression of transporters and SULT2A9 (Fig. 12). This female-specific LCA resistance was also reported for FXR null mice and was found to be associated with an increased hepatic SULT2A expression and LCA sulfation. (106) The notion that an oral dose of LCA is more toxic than an intraperitoneal injection was supported by the observation that gavage, but not intraperitoneal injection, caused histological liver damage in PXR null mice and was lethal in double knockout males. The drug route may also affect CYP3A11 gene regulation. LCA gavage has been shown to modestly induce CYP3A11 in both wild-type and PXR null mice, (96) but intraperitoneal LCA injection failed to induce hepatic CYP3A11 (Fig. 10A). The first pass effect through the liver may account for heightened LCA hepatotoxicity associated with the oral route. We noticed that a regimen of intraperitoneal LCA had been reported to cause liver damage in another independently created PXR null line. (76) Discrepancy in the effect of PXR on the basal expression of CYP3A11 has also been reported between these two lines. (76, 78)

Both PXR and CAR play important roles in the regulation of drug-metabolizing enzymes and transporters. (67-69, 77, 78) Interestingly, although some inducible expression was lost, basal and selective inducible expression of many enzymes and transporters remained intact in PXR- or CAR-deficient mice. (76, 78, 86) This has been speculated to be due to continued CAR (in PXR

null mice) or PXR (in CAR null mice) expression and signaling. The current study represents the first systemic profiling of xenobiotic gene expression in double knockout mice. Our results show that the basal expression of many xenobiotic genes was maintained in double knockouts, suggesting the involvement of additional nuclear receptors or other transcriptional regulators. In contrast, the combined loss of PXR and CAR had a gene-, tissue-, and sex-specific effect on the expression of transporters and Phase I and II enzymes in response to xeno- or endobiotic challenges, such as bile aids.

The failure of LCA-mediated downregulation of SHP may contribute to the suppression of transporter expression in double knockout mice. SHP has been shown to negatively regulate the expression of several transporters, such as NTCP. (210) Although, SHP failed to directly inhibit the OATP-C/SLC21A6 promoter; it may repress the expression of this transporter by inhibiting other coregulators. (211) The mechanisms for the sustained SHP expression in LCA-treated double knockout mice have yet to be elucidated. SHP is known to function as a negative transcriptional regulator by interacting with other nuclear receptors, such as liver receptor homolog 1, hepatic nuclear factor 4, PXR, (212) and LXR. (213) The expression of SHP must be under stringent control to prevent untoward suppression of nuclear receptor activities. Our results suggest that PXR and CAR play a crucial role in regulating the LCA-regulated hepatic SHP expression in that the suppression of SHP by LCA is dependent on PXR and CAR. We notice SHP was also induced in double knockout females, although the females were less sensitive to LCA and had a smaller transporter suppression. The creation of PXR, CAR, and SHP triple knockout mice, if viable, will shed further light on the role of SHP in transporter regulation. However, we cannot exclude the potential involvement of additional cellular factors in mediating transport suppression. (33, 214)

Among Phase I and Phase II enzymes, the decreased expression of GSTA2, a bile acid uptake facilitator, may contribute to hepatotoxicity. The males have minimal expression of SULT2A9, suggesting the increased sensitivity could not be explained by a reduced SULT2A9 expression in this sex. The double knockout females, on the other hand, have high levels of SULT2A9 expression that may account for their lower sensitivity to LCA toxicity. (106) The regulation of

CYP3A11 does not seem to play a major role in LCA sensitivity in the double knockout mice despite the appreciated significance of this Phase I enzyme in bile acid detoxification. (76, 96)

The increased lipid accumulation in the double knockout mice, also seen in CAR null mice, was intriguing. Although the long-term dietary supplement of bile acids has been shown to cause lipid accumulation in wild-type mice, (215) the current regimen of LCA treatment did not (Fig. 9). Despite the increased lipid deposit and higher levels of hepatic triglyceride, neither the serum triglyceride nor cholesterol levels were significantly altered in these two genotypes. The mechanism of LCA-induced hepatic lipid accumulation has yet to be determined. Nevertheless, our results provide evidence that the loss of CAR alone or in combination with the loss of PXR is sufficient to increase hepatic lipid deposition upon bile acid exposure.

3.0 ACTIVATION OF LXRS PREVENTS BILE ACID TOXICITY AND CHOLESTASIS

3.1 INTRODUCTION

The liver X receptors (LXRs), including the α and β isoforms, were defined as sterol sensors as they can be activated by cholesterol derived oxysterols, such as the 22(*R)*-hydroxycholesterol (216, 217). LXRs can also be activated by the synthetic TO1317 (143).

LXR was first shown to be anti-atherosclerotic by increasing hepatic cholesterol catabolism and inhibiting cholesterogenesis (116). In rodents, LXRα increases cholesterol catabolism to bile acids by inducing cholesterol 7α-hydroxylase (CYP7A1) (135), but this induction does not occur in humans (148, 218). Despite their beneficial functions, excess bile acid accumulation is harmful and may lead to cholestasis. Orphan nuclear receptors, such as PXR, CAR, FXR and SHP, have been shown to prevent bile acid toxicity and cholestasis through various mechanisms (2, 76, 86, 96, 187, 215, 219). However, it is not known whether LXR also plays a role in cholestasis. In the intestine and macrophages, LXRs increase the expression of ATP-binding cassette (ABC) superfamily of transporters, leading to cholesterol efflux (152). LXRs inhibit macrophage inflammatory response and impact antimicrobial responses (220-222). LXRs have also been shown to promote lipogenesis by activating SREBP-1c, a transcriptional factor known to regulate the expression of a battery of lipogenic enzymes (136, 143, 223, 224).

In this report, we showed that activation of LXR prevents bile acid toxicity and cholestasis, a phenotype that was associated with LXR-mediated regulation of bile acid detoxifying enzymes and transporters. LXRs may also increase the oxysterol levels by inhibiting CYP7B1 expression. We propose that LXRs have a dual function in promoting cholesterol catabolism and preventing cholestasis from excess bile acid accumulation.

3.2 MATERIALS AND METHODS

3.2.1 Mouse models.

To create FABP-VP-LXRα transgene, the VP-LXRα cDNA was placed downstream of the rat fatty acid binding protein promoter (225). The transgenic mice were produced at the University of Pittsburgh Transgenic Core Facility. The LXR DKO mice have been described previously (135). The C57BL/6J background transgenic mice were created by backcrossing to C57BL/6J mice for 7-8 generations. All other mice have a mixed background of C57BL/6J and 129/SvImJ. The use of mice in this study has complied with all relevant federal guidelines and institutional policies.

3.2.2 Animal drug treatment, histological evaluation, and plasma and urine chemistry.

The LCA treatment (86), bile duct ligation (226), and double-blinded liver histology analysis were performed as previously described. In LCA and BDL models, when necessary, daily gavage of TO1317 (50 mg/kg) was given three days prior to the LCA and BDL procedures and continued throughout the experiments. For gene expression analysis in Fig. 15, mice were treated with a single i.p. dose of TO1317 or 22(*R*)-hydroxycholesterol (50 mg/kg each) 24 h before the animals were sacrificed. Plasma chemistry was measured by ANTECH Diagnostics (Lake Success, NY). Urine samples were collected by using metabolic cages.

3.2.3 DNA-binding analysis and transient transfections.

EMSA was performed as previously described (86). CV-1 and HepG2 cell transfections were described previously (75, 86, 227). CV-1 cells were chosen as LXR naïve cells, whereas HepG2 cells were chosen for the responsiveness of the natural SULT2A9 promoter. When necessary, cells were treated with drugs in media containing 10% charcoal stripped serum for 24 h prior to luciferase assay. The transfection efficiency was normalized against the β-gal activity from the co-transfected CMX-β gal vector.

3.2.4 Northern blot analysis and real-time PCR.

Northern hybridization was carried out as described (86). When necessary, the signals were quantified with the NIH Image software. Real-time PCR using pre-designed Assay-On-Demand TaqMan reagents or SYBR Green-based assays was performed with the ABI 7300 Real-Time PCR System.

3.2.5 Sulfotransferase assay.

Sulfotransferase assay was carried out using [^{35}S]-PAPS (Perkin Elmer) as previously described (86). In brief, 5 μg/ml of total liver cytosolic extract was used with 2 μM LCA or pNP substrate. After the reactions, free [^{35}S]-PAPS was removed by extracting with ethyl acetate. The aqueous phase was then counted in scintillation counter for radioactivity.

3.2.6 Statistical analysis

Experiments presented were repeated at least three times with at least four samples per group. Unpaired Students *t*-test or the Mann-Whitney test was used to calculate the statistical differences.

3.3 RESULTS

3.3.1 Activation of LXR prevents bile acid toxicity and cholestasis.

We recently created transgenic mice expressing the activated LXRα (VP-LXRα) in the liver and intestine under the control of the fatty acid binding protein (FABP) promoter (225) (Fig. 13). Created by fusing the VP16 activation domain of the herpes simplex virus to the amino-terminal of mouse LXRα, VP-LXRα shares the same DNA binding specificity as its wild type

counterpart (data not shown) and co-transfection with VP-LXRα activated the LXR responsive reporter gene in the absence of an agonist (Fig. 13B). Northern blot analysis showed the transgene was expressed in the liver and all segments of the small intestine (Fig. 13C), tissues that express the endogenous LXRα. The expression of several known LXR-responsive lipogenic enzyme genes was induced in the transgenic mice as expected (Fig. 13D).

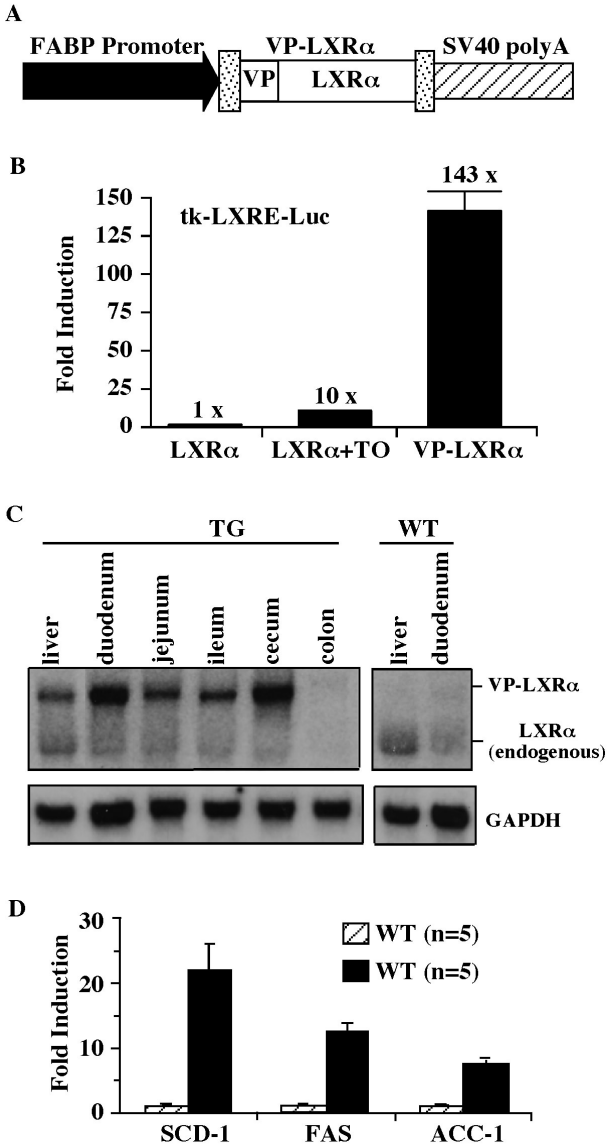


Figure 13: Creation of FABP-VP-LXRα transgenic mice

A) Schematic representations of the transgene construct. **B)** VP-LXRα activates the LXRE reporter gene in the absence of a ligand in CV-1 cells. **C)** Total RNAs were subjected to Northern blot analysis. The membrane was hybridized with a LXRα cDNA probe that detects both transgenic and endogenous LXRs. **D**) Induction of LXR-responsive lipogenic genes in transgenic mice. SCD, stearoyl CoA desaturase; FAS, fatty acid synthase; ACC, acetyl CoA carboxylase.

Table 2: LCA-induced histological liver damage in mice of indicated genotypes.

	Females					Males		
	WT	**TG**	**WT***	**TG***	**WT*+TO**	**WT**	**TG**	**DKO**
LCA-Induced Histological Liver Damage	7/7 (100%)	0/7 (0%)	10/10 (100%)	0/6 (0%)	1/5 (20%)	14/23 (60.9%)	5/10 (50.0%)	5/5 (100%)

Asterisks indicate mice of C57B background. All other mice are in C57BL/6J and 129/SvImJ mixed background. The histological liver damage is defined by the appearance of necrotic foci.

We used both VP-LXRα transgenic mice and LXR DKO mice (135) to evaluate the role of LXR in lithocholic acid (LCA) hepatotoxicity and bile duct ligation (BDL) cholestasis. The histological liver damage was scored by the appearance of necrotic foci (2, 86, 96). As summarized in Table 2, in the LCA model, while the wild type females were 100% sensitive, the transgenic females showed a complete resistance to LCA-induced liver damage. Surprisingly, the transgenic males remained as sensitive as their wild type counterparts did, although the penetrance was not as high as the wild type females. The initial transgenic phenotype was observed in the C57BL/6J-129/SvImJ mixed background but similar results were seen in mice of C57BL/6J background. The protective effect was also seen in wild type C57BL/6J females that were treated with TO1317. LXR DKO males, on the other hand, were 100% sensitive. Fig. 14 shows the representative histology of the vehicle- or LCA-treated mice (Fig. 14A-K). The same patterns of histological changes were seen in the BDL model (Fig. 14L-R). The BDL toxicity in the LXR DKO females was so severe that the experiments had to be terminated two days earlier to prevent the loss of animals. The hepatic toxicity was supported by plasma bile acid accumulation and increased liver enzyme activities (Table 3).

Table 3: Plasma chemistry in female VP-LXRα transgenic mice and male and female LXR DKO mice.

	Bile Acids (μmol/L)	ALT (U/L)	AP (U/L)
WT/Female/Vehicle	11.8±0.3	61.2±14.5	49.2.8±26.1
WT/Female/LCA	23.9±6.07	1503.6.2±493.8[a]*	153.6±56.0
TG/Female/Vehicle	10.0±4.6	53.2±8.4	60.4±24.1
TG/Female/LCA	5.2±1.4	48.0±14.5	36.0±14.6
WT/Female/BDL	582.9±85.5	650.7±205.3	1360.0±314.9
TG/Female/BDL	380.6±68.0[b]**	208.4±64.2[b]**	563.6±173.7[b]**
WT/Female/Sham	11.6±2.8	41.3±2.8	13.0±3.5
DKO/Female/Sham	12.4±1.5	63.0±33.6	16.8±3.7
WT/Female/BDL	404.0±24.8	322.6±70.1	204.2±38.6
DKO/Female/BDL	648±84.7[c]*	157.2±48.2[c]*	625.3±246.3
WT/Male/Vehicle	4.9±0.5	31.2±7.0	9.6±4.1
DKO/Male/Vehicle	9.7±2.5	57.0±15.3	13.6±6.8
WT/Male/LCA	13.1±3.8	581.8±421.7	24.0±9.6
DKO/Male/LCA	133.7±21.2[d]**	1843.6±634.3	136.6±57.5[d]**

All values are presented as averages and standard error. ALT, alanine aminotransferase; AP, alkaline phosphatase; LCA, lithocholic acid; BDL, bile duct ligation. [a]*$P<0.05$ compared to WT vehicle; [b]**$P<0.05$ compared to WT BDL; [c]*$P<0.05$ compared to WT BDL; [d]**$P<0.05$ compared to WT LCA.

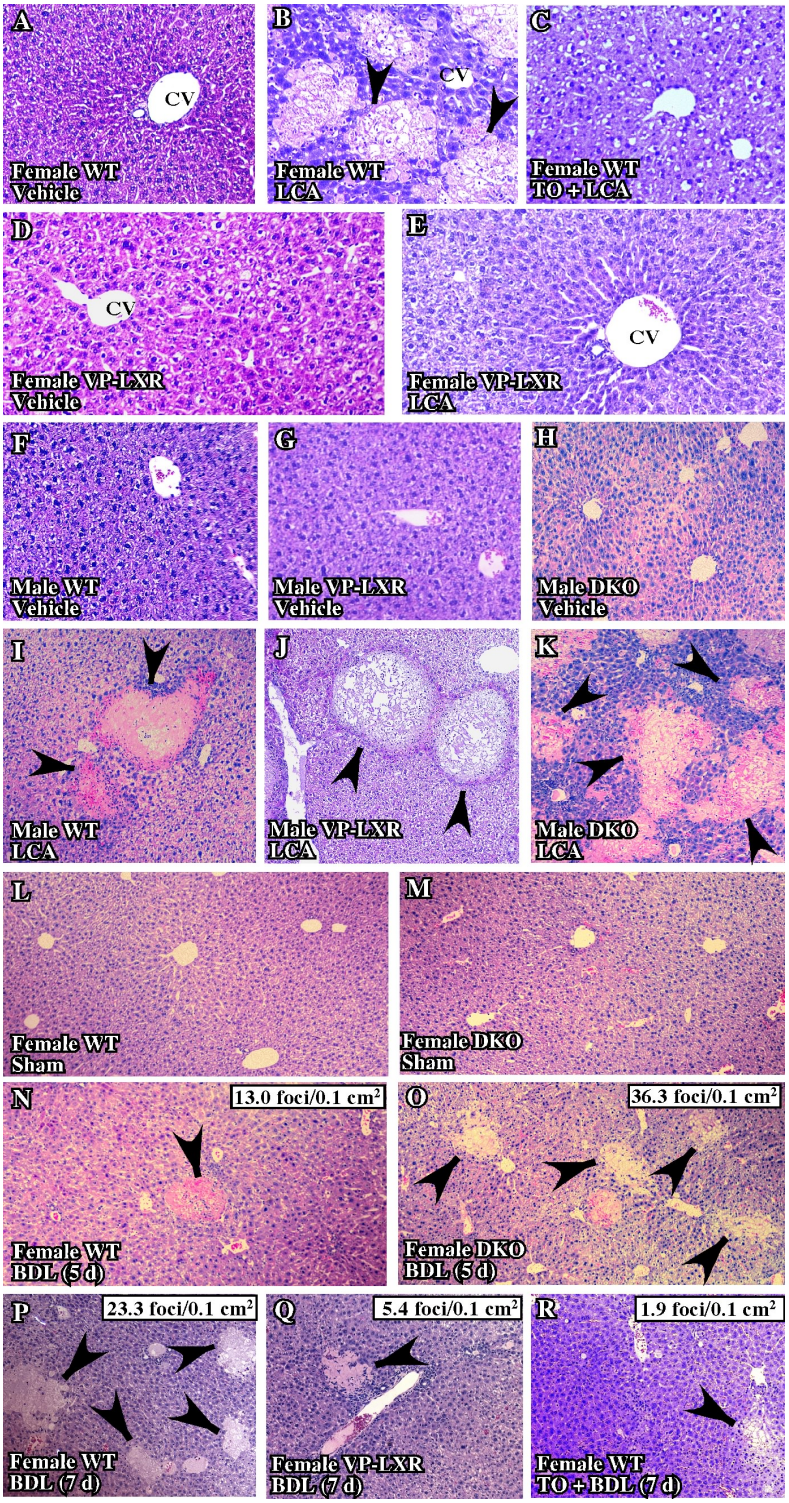


Figure 14: Effects of altered LXR activity on LCA- and BDL-induced histological liver damage

A-K) Liver histology of the LCA model. Mice of indicated sexes were given a daily treatment of vehicle, or LCA for 4 days before histological examination by H&E staining. Areas of necrosis are marked by arrowheads. **L-R**) Liver histology of the BDL model. Female mice were subjected to sham or BDL and sacrificed 5 or 7 d after the procedure. Areas of necrosis are marked by arrowheads and the density of the necrotic foci was quantified. Mice in (**C and R**) were treated with TO1317 as described in "Methods." The magnification is 200x for all panels.

3.3.2 Molecular mechanisms by which LXR regulates bile acid and cholestatic sensitivity.

The expression of bile acid detoxifying enzymes and transporters was profiled by Northern blot analysis and real-time PCR. The most notable change in the untreated transgenic mice was a marked up-regulation of SULT2A9 in the livers of both sexes although the females had higher basal expression of this enzyme. Interestingly, upon LCA treatment, the SULT2A9 induction was abolished in transgenic males but this induction was sustained in transgenic females (Fig. 15A), providing a plausible explanation for the female-specific protection. In wild type females, LCA treatment suppressed the basal expression of SULT2A9, which may account for their higher cholestatic sensitivity than their male counterparts. The regulation of SULT2A9 was confirmed by real-time PCR and extended to the BDL model (Fig. 15B) and LXR DKO mice (Fig. 15C). The basal expression of SULT2A9 in the sham treated LXR DKO females was reduced by approximately 60% compared to the wild types, suggesting that LXRs are critical for maintaining the basal expression of this SULT isoform.

LXR also affects the basal and cholestatic responsive expression of bile acid transporters. In vehicle-treated females, expression of the transgene resulted in significant induction of BSEP and suppression of OATP1. Upon LCA treatment, the expression of uptake transporters NTCP, SLC21A6 and OATP1 was significantly suppressed in the wild types but this suppression was absent in the transgenics. The expression of the efflux transporters BSEP and MRP4 was also significantly higher in the transgenic mice than the wild type mice in the presence of LCA (Fig. 15D). The sustained and/or induced expression of transporters may have contributed to the LCA resistance in female transgenics. In males, the overall pattern of transporter expression was similar to that observed in the females, except that BSEP was suppressed rather than being induced in vehicle-treated transgenics (data not shown). In LXR DKO males, both the basal and LCA-responsive expression of transporters was reduced (Fig. 15E). Despite its intestinal expression, the transgene had little effect on the expression of intestinal bile acid uptake transporters ASBT and organic solute transporter (OST) α and β (59, 61) (data not shown).

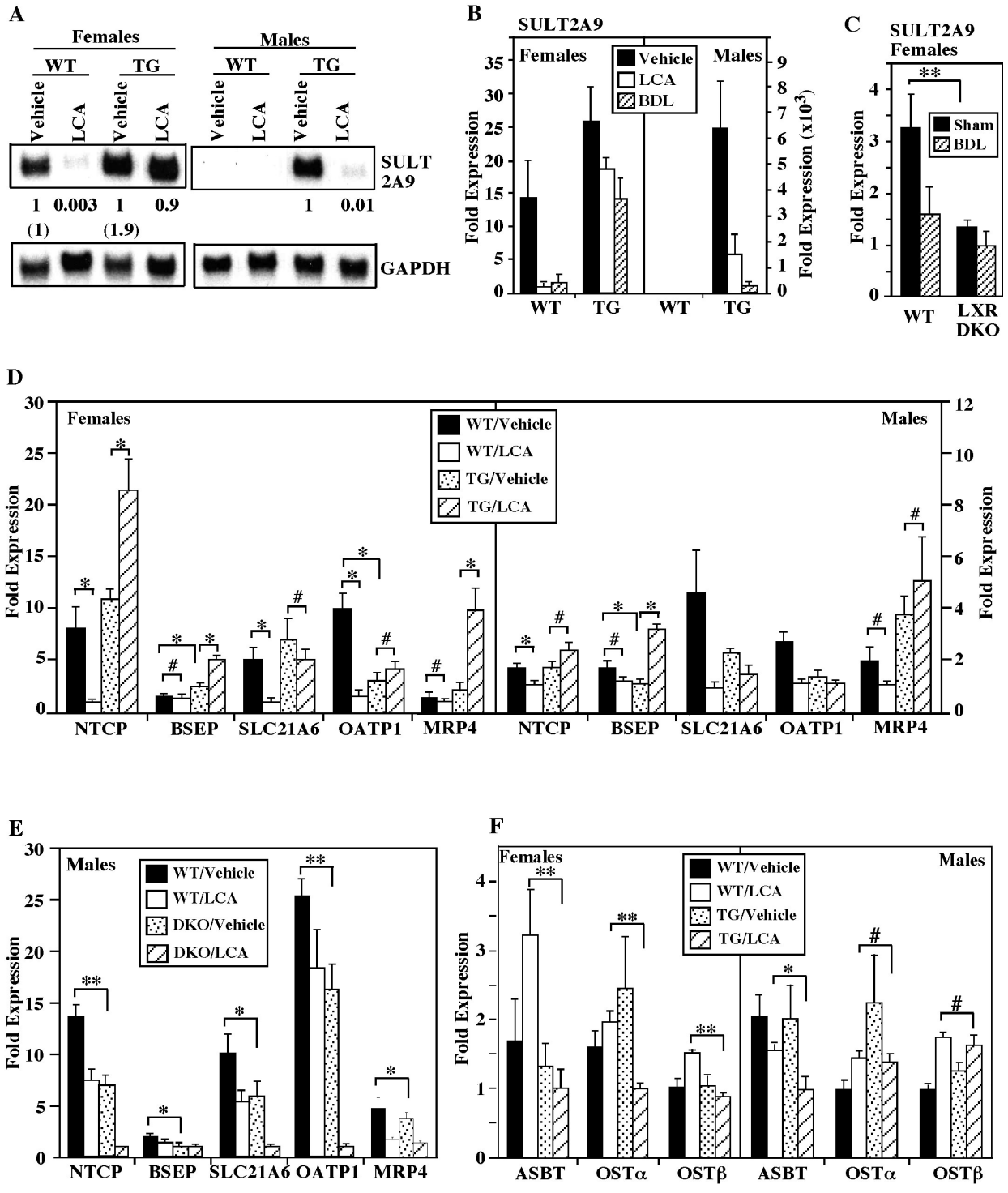


Figure 15: Molecular mechanisms by which LXR regulates cholestatic sensitivity

A) Mice were given a daily treatment of vehicle or LCA for 4 days before liver harvesting and Northern blot analysis. All lanes represent pooled samples from 4-6 mice. B-E) Real-time PCR analysis on liver RNA derived from LCA- or BDL-treated mice. Results are presented as averages and standard error from 4-6 mice per genotype and treatment. In (B), the fold expression is only comparable within the same sex due to the basal differences and results of the "Sham" groups were identical to those of the "Vehicle" groups (data not shown). *P<0.05; **P<0.01; #P>0.05.

3.3.3 Activation of LXRα caused female-specific increase in urinary bile acid elimination.

Consistent with the notion that sulfation increases the solubility and renal secretion of bile acids, the average urinary output of bile acids was 30-50% higher in the female transgenic mice than their wild type counterparts throughout the LCA regimen (Fig. 16). In contrast, there is little difference in the urinary bile acid output in the male transgenic mice. These observations are in agreement with the female-specific LCA resistance and lower plasma bile acid concentrations in the transgenic mice.

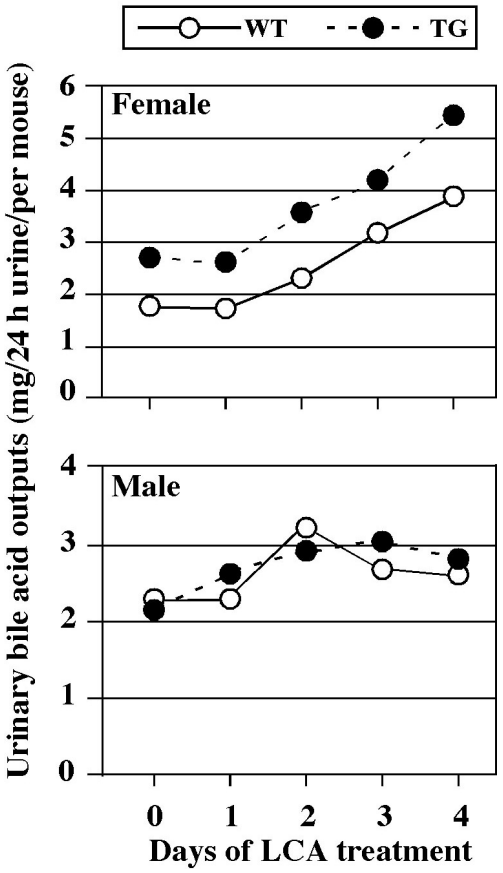


Figure 16: Activation of LXR α increased urinary output of bile acids

24-h cumulative urine samples were collected using metabolic cages before and during the LCA treatment. Urine of five mice per genotype was pooled and measured for total bile acids. Results represent the averages from triplicate assays whose variations are less than 5% (data not shown). Results in females were repeatable when using urine samples from individual female mice (n=6 per group) (data not shown).

3.3.4 SULT is a likely transcriptional target of LXR.

In addition to its induction in the transgenic mice, SULT2A9 was also induced in wild type mice treated with TO1317 and 22(*R*)-hydroxycholesterol (Fig. 17A), and the TO1317 effect was abolished in the LXR DKO mice (Fig. 17B). The transgenic mice also exhibited increased hepatic sulfation activity toward LCA and p-nitrophenol (pNP) (Fig. 17C), two known SULT2A9 substrates (86). To understand the regulatory mechanism, we found that both LXRα/RXR (Fig. 17D) and LXRβ/RXR (data not shown) heterodimers can bind to IR-0 (inverted repeats without a spacing nucleotide) in the SULT2A9 gene promoter, a response element known to bind to CAR (86), PXR (87), and FXR (196). This binding can be efficiently competed by excess unlabeled wild type IR-0 or SREBP-1c/DR-4 (136), but not by the mutant IR-0. Consistent with the EMSA results, the SULT/IR-0 containing synthetic (Fig. 17E) and natural (Fig. 17F) SULT2A9 promoters were activated by LXRα in the presence of TO1317. The expression of SULT2A1, the human homolog of SULT2A9, was induced by TO1317 in primary cultures of human hepatocytes (Fig. 17G), suggesting that the human homolog may also be regulated by LXR.

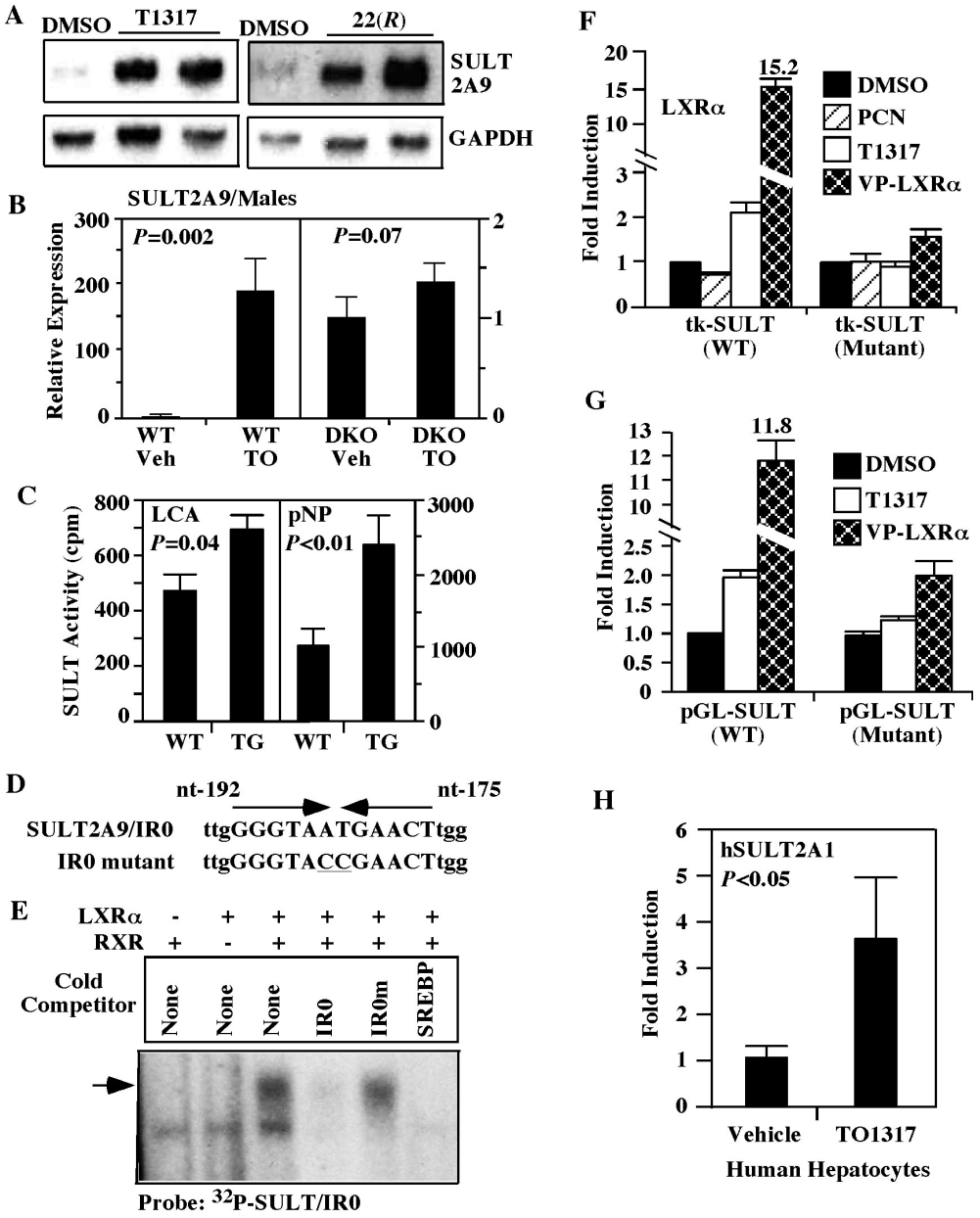


Figure 17: SULT is a likely transcriptional target of LXR

A) Activation of SULT2A9 in wild type male mice treated with TO1317 or 22(*R*)-hydroxycholesterol as revealed by Northern blot analysis. **B)** Loss of TO1317-mediated SULT2A9 induction in LXR DKO mice as revealed by real-time PCR. **C)** Increased SULT activity in VP-LXRα mice. Cytosolic liver extracts from transgenic males or control littermates were subjected to sulfation assay using LCA and p-NP as the substrates. WT, n=4; TG, n=5. **D)** LXRα/RXRα heterodimers bind to IR-0 as revealed by EMSA. The IR-0 element sequence and its mutant are capitalized. **E)** The synthetic tk-SULT/IR0-Luc reporter or its mutant variants were transfected into CV-1 cells in the presence of LXR expression vectors. Cells were treated with indicated drugs for 24 h before luciferase assay. **F)** Transient transfections similar to those in **(E)** but with the SULT2A9 natural promoter reporter and HepG2 cells being used. **G**) The human hepatocytes from four patients (all Caucasians, including a 3-year old male, a 16-year old male, a 29-year old female, and a 31-year old female) were mock treated or treated with TO1317 for 24 h before RNA harvesting and real-time analysis. The concentration of ligand in (**E-G**) is 10 μM.

3.3.5 Activation of LXR suppressed CYP7B1 expression.

LXR has been shown to activate CYP7A1, which was confirmed in our VP-LXRα transgenic mice (data not shown). Interestingly, the expression of the oxysterol 7α-hydroxylase (CYP7B1) was suppressed in both VP-LXRα transgenic mice (Fig. 18A) and TO1317-treated wild type mice (Fig. 18B). The TO1317 effect was abolished in the LXR DKO mice (Fig. 18B). We propose that LXR has a dual function in promoting cholesterol catabolism and preventing bile acid toxicity (Fig. 18C, and see Discussion).

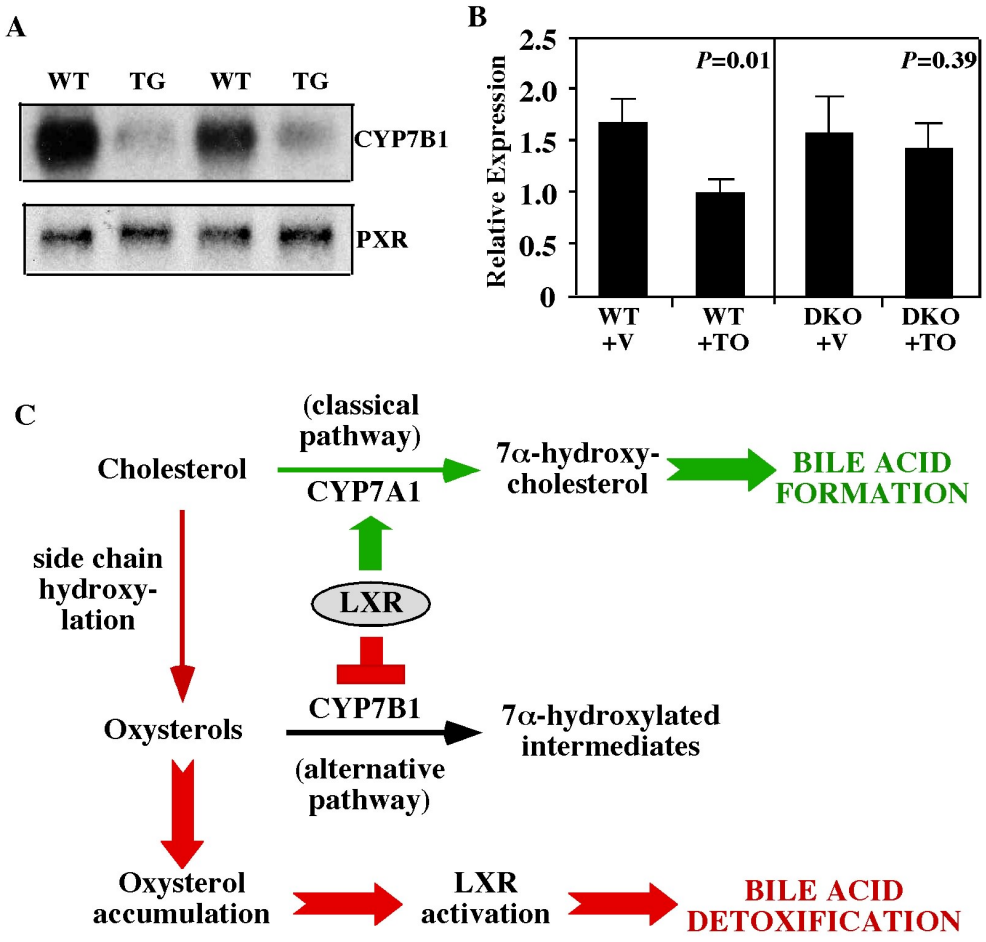


Figure 18: Activation of LXR suppressed CYP7B1 expression

A) Inhibition of CYP7B1 expression in the VP-LXRα transgenic mice as revealed by Northern blot analysis. PXR is included as a non-target gene control. **B)** Treatment with TO1317 inhibits CYP7B1 expression in wild type but not in LXR DKO mice as revealed by real-time PCR. **C)** Model of the dual role of LXR in promoting cholesterol catabolism and preventing bile acid toxicity. CYP7A1 and CYP7B1 catalyze the classical and alternative pathways of cholesterol metabolism, respectively.

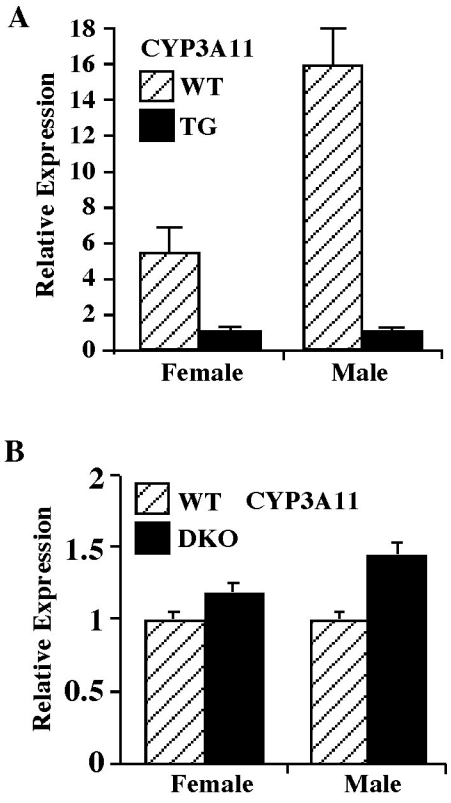


Figure 19: Expression of CYP3A11 in VP-LXRα transgenic and LXR DKO mice

The liver expression of CYP3A11 in the VP-LXRα transgenic (**A**) and LXR DKO (**B**) mice was evaluated by real-time PCR. Results are presented as averages and standard deviation from 5 mice per group.

3.4 DISCUSSION

In this report, we showed that LXRs prevent LCA toxicity and cholestasis, which is associated with their positive regulation of SULT2A9. Since this SULT isoform is expressed in the liver but not in the intestine (2) and many of the evaluated transporters have liver-specific expression patterns, it is likely that the liver is primarily responsible for the phenotypes. However, we cannot exclude the possibility that the intestine may have also contributed to the phenotypes.

The Phase I CYP3A enzymes also play a role in bile acid detoxification. Unlike PXR, the LXR effect appears to be CYP3A11-independent, since the expression of CYP3A11 was not induced but rather reduced in the transgenic mice (Fig. 19A). The expression of CYP3A11 in LXR DKO

mice was also largely unchanged (Fig. 19B). Although the association between SULT2A9 induction and cholestatic resistance is strong and the SULT2A9-mediated sulfation may be a prerequisite for the subsequent transporter-mediated bile acid elimination (228), we cannot exclude the possibility that regulation of genes other than SULT2A9 may have also contributed to the phenotypes.

The gender-specific effect of LXR on LCA and cholestatic sensitivity is intriguing. In the transgenic mice, the induction of SULT2A9, seen in untreated mice of both sexes, was specifically lost in the LCA-treated males, and this may have contributed to the failure of protection in this sex. Interestingly, both the male and female LXR DKO mice are more sensitive to cholestasis, presumably due to the decreased basal expression of both SULT2A9 and bile acid transporters. The mechanism and human relevance of this gender-specificity remain to be determined. In human primary biliary cirrhosis, an immune-mediated disorder that often is associated with cholestatic symptoms, 90% of the patients are women (229). CYP7A is believed to be regulated by LXRs in mice, but not in humans (148, 218). SULT2A1 was induced by TO1317 in human hepatocytes, but it remains to be determined whether the ligand effect is mediated by LXR or PXR (230).

LXR was previously shown to promote the catabolism of cholesterol to bile acids by activating CYP7A1, the cholesterol 7α-hydroxylase. In this report, we showed that CYP7B1, the oxysterol 7α-hydroxylase, was markedly suppressed by LXR activation. Disruption of CYP7B1 in mice is known to lead to an accumulation of oxysterols (12), the LXR agonists. We propose that, at least in rodents, LXRs have evolved to have dual function in maintaining cholesterol and bile acid homeostasis by increasing cholesterol catabolism and, at the same time, preventing toxicity from bile acid accumulation (Fig. 18).

4.0 ACTIVATION OF LXR SENSITIZES MICE TO GALLSTONE DISEASES

4.1 INTRODUCTION

Gallstone disease is a clinical disorder due to an imbalanced biochemical composition of the gallbladder bile. Cholesterol gallstone diseases (CGD) account nearly 80% of the gallstone diseases and affect as much as 10% of the population of the United States (174). CGD is manifested by cholesterol precipitation/crystallization, increased bile acid hydrophobicity and gall bladder epithelial inflammation (177, 231, 232). Other clinical symptoms of CGD include abdominal pain, especially when CGD is accompanied by inflammation of the gallbladder. Cholecystectomy, the most common surgical treatment for CGD (233), although effective, is limited by its invasive nature and risk of side effects, especially in elderly patients. The standard treatment of laparoscopic cholecystectomy has also made gallstone disease the second most costly digestive disorder in most Western countries. It is therefore necessary to better understand the pathogenesis of CGD and to develop effective and non-invasive methods to prevent and treat CGD.

CGD results from disrupted balances between cholesterol, bile salts and phospholipids (mainly phosphatidylcholine, or lecithin) in the bile (27). Cholesterol crystals form when the bile salt and phospholipids fail to maintain cholesterol solubility. The cholesterol crystals will eventually aggregate to form pathogenic gallstones. Thus, disruptions in the biliary biochemical composition and the resultant increases in bile salt hydrophobicity are key pathogenic factors for CGD. In addition to biliary biochemistry, the structure and function of the gallbladder also play a role in CGD. For example, impaired gallbladder emptying in the fasting state could promote cholesterol crystallization and gallstone formation by allowing time for progressive bile concentration (for a review, see (234)).

The biliary lipid and bile salt secretion is controlled by a network of hepatic transporters, including those responsible for transport of cholesterol, phospholipids and bile salts (27, 177, 231, 232). The transcriptional regulation of these transporters has been shown to be mediated by orphan nuclear receptors, such as the farsenoid X receptor (FXR) (108, 199, 200) and liver X receptor (LXR) (111, 125). FXR has been reported to be preventive from CGD (185). Deficiency of FXR sensitizes mice to CGD, whereas a synthetic FXR agonist prevented sequelae of the disease. The protective effects were thought to be mediated by FXR-dependent increases in biliary bile salt and phospholipid concentrations, which restored cholesterol solubility and thereby prevented gallstone formation. The increased bile salt and phospholipid efflux is due to the positive regulation of bile salt export pump (*Bsep/Abcb11*) and multi- drug resistance protein 2 (*Mdr2/Abcb4*) by FXR. Consistent with the litho-preventive effect of FXR, hydrophilic bile acids, such as ursodeoxycholic acid (UDCA), has been shown to be effective to treat gallstone diseases in both patients and rodent models of CGD (235, 236). In addition to increase bile salt pool size, other proposed therapeutic mechanisms of UDCA include decreasing biliary secretion and intestinal absorption of cholesterol (237-239), both of which could contribute to maintain proper cholesterol saturation in the bile.

LXRs, including the α and β isoforms, can be activated by endogenous cholesterol derivative oxysterols, as well as synthetic drugs, such as T0901317 (TO1317) (143). LXRα is highly expressed in the liver and is also found to be expressed in adipose, intestine, kidney, and macrophages, whereas LXRβ expression is detectable in most tissues. LXRs were initially characterized as sterol sensors that affect cholesterol and lipid homeostasis and inflammation (216, 217). In rodents, LXRα promotes bile acid synthesis in the liver by activating *Cyp7a1*, a rate-limiting enzyme to convert excess cholesterol to bile acids (135) (reviewed in (5, 6, 240)). In addition to its role in bile acid formation, we have recently reported that LXR promotes bile acid detoxification and elimination, and thus alleviates cholestasis in mice (241). The anti-cholestatic effect of LXR is reasoned to be due to the positive regulation of bile acid detoxifying enzyme and transporter genes by this receptor (241). In addition to their effect on bile acids, LXRs are also known to induce the expression of several transporters that efflux cholesterol and phospholipids. These include the cholesterol efflux transporters *Abcg5* and *Abcg8* (50), as well as *Abca1*, another ABC transporter that effluxes both cholesterol and phospholipids (242, 243).

The potential effect of LXR on bile acid, cholesterol and phospholipids, three key components of the bile, prompted us to evaluate whether or not LXRs play a role in the pathogenesis of CGD.

In this report, we revealed an important metabolic role of LXR in sensitizing mice to lithogenic diet-induced CGD. This lithogenic effect may have resulted from LXR-mediated increases in biliary efflux of cholesterol and phospholipids, as well as a reduced bile salt pool size in the gallbladder. Inhibition of hepatic cholesterol uptake (by deletion of the low-density lipoprotein receptor (LDLR) gene) or intestinal cholesterol absorption (by using the cholesterol absorption inhibitor Ezetimibe) was sufficient to abolish the lithogenic effect of LXR. We propose that development of LXR antagonists may represent a novel strategy to treat and prevent CGD.

4.2 MATERIALS AND METHODS

4.2.1 Animals and diet and drug treatment.

The creation FABP-VP-LXRα has been described (1). The LDLR null mice (244) were purchased from the Jackson laboratory (Bar Harbor, Maine). FABP-VP-LXRα/ LDLR null mice were created by cross-breeding. All mice are in a mixed background of C57BL/6J and 129/SVJ and maintained in Prolab RMH3000 diet (PMI Nutrition International, St. Louis, MO). In animal experiments, the wild type littermates were used as the appropriate controls. When necessary, mice were subjected to lithogenic diet (Harlan Teklad TD-02189) that contains 21% butterfat, 1.5% cholesterol, 0.5% cholic acid, 23% casein and essential minerals and vitamins. For drug treatment, daily gavage of TO1317 (10 mg/kg) or Ezetimibe (5 mg/kg) was given three days (for TO1317) or one week (for Ezetimibe) prior to the beginning of lithogenic diet and continued until the completion of the experiments. Both TO1317 and Ezetimibe were prepared in 0.75% methylcellulose and applied by gavage. Plastic-coated feeding needles were used to minimize tissue damages. The use of mice in this study has complied with relevant federal guidelines and institutional policies.

4.2.2 Microscopic examination of cholesterol crystals.

The animals were sacrificed and cholecystectomies were performed, and gallbladder bile was harvested, spread on glass slides, and examined using a Leica DM5000 polarized microscope for the presence of cholesterol gallstone crystals.

4.2.3 Histological evaluation, measurement of hepatobiliary chemistry, and cholesterol saturation index estimation.

Gallbladders were fixed in 4% formaldehyde, embedded in paraffin, sectioned at 5 μm and stained with H&E. Biliary and hepatic lipids were extracted according to a slightly modified procedure of the Folch Method (245) as we described previously (246). The hepatic and biliary phospholipids and cholesterol, plasma and liver triglycerides were measured using assay kits from Stanbio Laboratory (Boerne, TX). Total bile salts were quantified using the 3α-hydroxysteroid dehydrogenase enzymatic method (247). The cholesterol saturation index of bile was calculated according to Carey's critical tables for calculating the cholesterol saturation of bile (248) using a software program. Urine and fecal samples were collected by using mouse metabolic cages.

4.2.4 Quantitative real-time PCR.

Total RNAs were extracted using the TRIZOL Reagent (Invitrogen, Carlsbad, CA). Real-time PCR using SYBR Green-based assays was performed with the ABI 7300 Real-Time PCR System as previously described (246). All primers are designed by our lab and custom made by Integrated DNA Technologies (Coralville, IA).

4.3 RESULTS

4.3.1 Activation of LXR sensitizes mice to cholesterol gallstone crystal formation and gallbladder inflammation.

We first used the FABP-VP-LXRα transgenic mice to investigate the role of LXRα in cholesterol gallstone formation. This transgenic line bears the hepatic and intestinal expression of the constitutively activated mouse LXRα (VP-LXRα) under the control of the rat fatty acid binding protein (FABP) gene promoter (241). The constitutively activated VP-LXRα was created by fusing the viral protein 16 (VP16) activation domain of the herpes simplex virus to the amino-terminal of mouse LXRα. VP-LXRα shares the same DNA binding specificity as its wild type counterpart and activates known LXR target genes in cell cultures and in mice in the absence of LXR agonists (241).

The transgenic mice and their wild type littermates were fed with a standard lithogenic diet that contains 1.5% of cholesterol and 0.5% of cholic acid (185). The treated mice were then analyzed for CGD by microscopic examination of the cholesterol crystals in the bile (249) and evaluation of the appearance and histology of the gallbladders. A markedly increased cholesterol crystal formation was observed in the transgenic mice. As summarized in Table 4, while the wild type male mice were largely resistant to CGD, the transgenic males showed a 77-86% penetrance of crystal formation after 1-3 weeks of lithogenic diet treatment. Increased crystal formation was also seen in wild type males that were simultaneously treated with the LXR agonist TO1317 (Table 4). Increased lithogenic diet-induced gallstone formation was also seen in female transgenic mice (data not shown). Figure 20 shows the cholesterol crystal formation in the wild type, transgenic and TO1317-treated wild type mice. The cholesterol gallstones in the transgenic mice or ligand-treated wild type mice are not only large in size but also have a multi-color feature (Fig. 20B, D, and F). The gallbladders of the transgenic mice (Fig. 20H) and TO1317-treated wild type mice (Fig. 20I) turned dark and turbid compared to the yellow and clear appearance of those of the vehicle-treated wild type mice. H&E histology analysis revealed that the gallbladders of the transgenic mice showed signs of inflammation, a hallmark of gallstone diseases (250). The inflammatory features include thickening of the epithelial

layers, infiltration of granulocytes in the stromal layer, progressive fibrosis, edema, and epithelial cell indentation (Fig. 20K). (Fig. 20).

Table 4: Incidence of lithogenic diet-induced gallstone formation.

	Duration of Lithogenic Diet	
	1 week	**3 weeks**
Wt	0% (0/5)	33.3% (2/6)
Tg	85.7% (6/7)	76.5% (13/17)
Wt+Vehicle	ND	25.0% (3/12)
Wt+TO1317	ND	72.7% (8/11)
Tg/*Ldlr*$^{-/-}$	ND	0% (0/8)
Tg+Ezetimibe	ND	0% (0/6)

Wt, wild type; Tg, VP-LXRα transgenic; ND, not determined

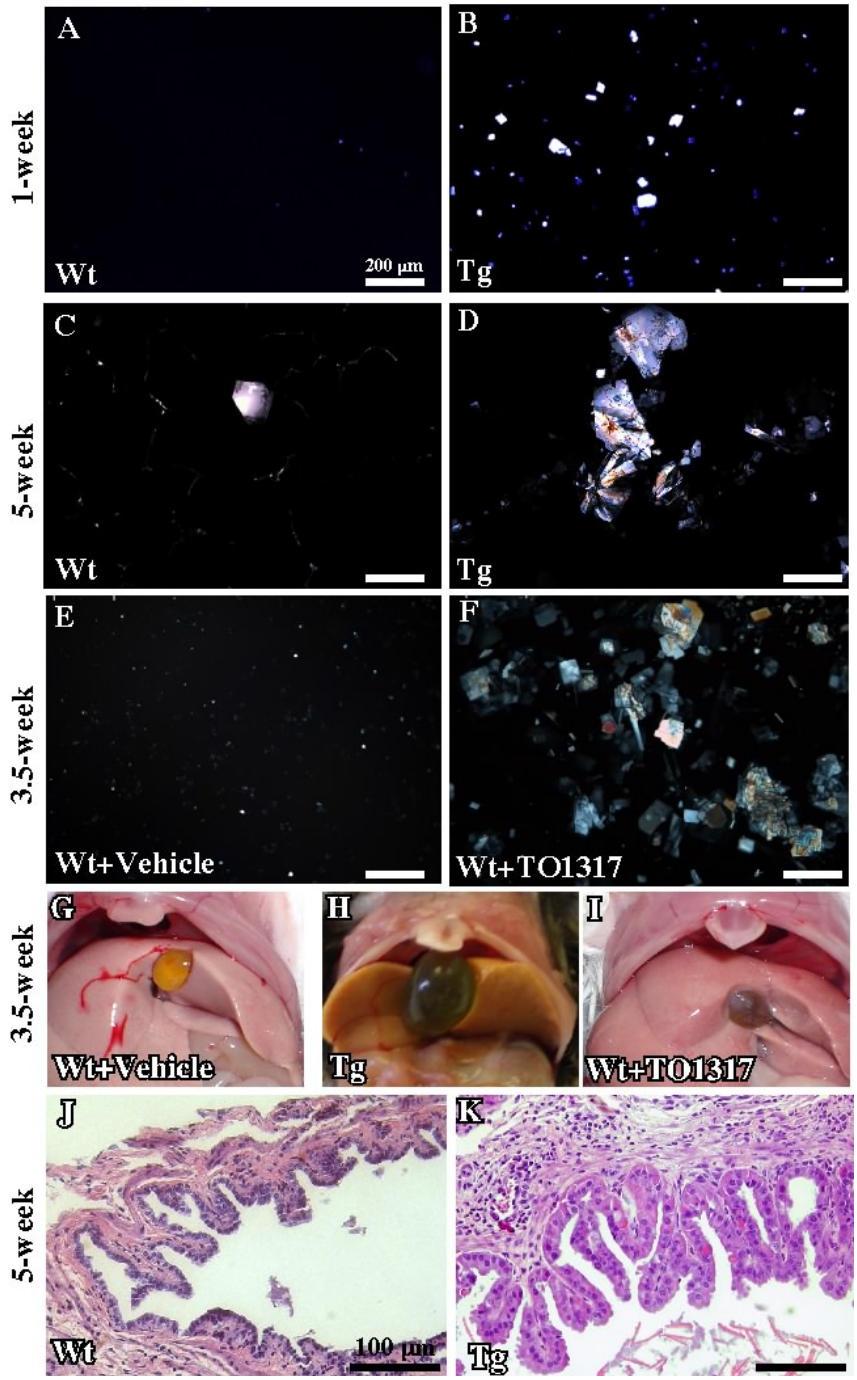


Figure 20: Activation of LXR sensitizes mice to cholesterol gallstone crystal formation and gallbladder inflammation.

All mice were subjected to lithogenic diet treatment for indicated amounts of time. **(A-F)** Polarizing light microscopic examination of cholesterol crystals from mice of indicated genotypes. Mice in **(E)** and **(F)** received daily treatment of vehicle and TO1317 (50 mg/kg), respectively, beginning three days prior to the diet treatment and continued until the completion of the experiment. Scale bars are labeled. (**G-I)** Gross appearance of gallbladders of mice with indicated genotypes and drug treatment. **(J-K)** Histological examination of the gallbladder by H&E staining. All mice shown are males. Wt, wild type; Tg, transgenic.

4.3.2 Activation of LXR alters biliary and hepatic lipid profiles and increases biliary cholesterol saturation index.

The lithogenic transgenic phenotype prompted us to analyze the biochemical composition of the bile in lithogenic diet-treated transgenic mice, as compared to their wild type counterparts. Upon the diet treatment, the biliary concentrations of cholesterol (Fig. 21A) and phospholipids (Fig. 21B) in the transgenic mice were significantly higher than those of the wild type mice. In contrast, the biliary concentration of bile salt, the anti-lithogenic detergent, was decreased in the transgenic mice (Fig. 21C). These biochemical alterations in the transgenic mice led to a significantly increased biliary cholesterol saturation index (CSI) (Fig. 21D). An increased CSI is known to compromise cholesterol solubility and therefore facilitate cholesterol precipitation and gallstone formation (234, 248, 251), providing a plausible biochemical explanation for the lithogenic phenotype of the transgenic mice. In contrast to the higher bile concentrations of cholesterol, the liver tissue concentrations of cholesterol was either significantly lower in the lithogenic diet-fed transgenic mice (Fig. 21E, left panel) or unchanged in the TO1317-treated wild type mice (Fig. 21F, left panel), presumably due to increased efflux of liver cholesterol into the bile. As controls, the liver triglyceride levels were increased in both the transgenic and TO1317-treated wild type mice (Fig. 21E and 21F, right panels).

The decreased bile salt concentration in the bile and liver of the transgenic mice prompted us to evaluate the circulating level and urinary and fecal secretion of bile acids. As shown in Fig. 21G, the plasma level of bile acid was significantly higher in lithogenic diet-treated transgenic mice than their wild type counterparts. The plasma concentrations of cholesterol and triglyceride were also higher in lithogenic diet-treated transgenic mice (Fig. 21H). The average urinary secretion of bile acid was also increased in the transgenic mice, with the 24-h urinary bile acid output in the transgenic mice nearly twice of the wild type after two weeks of lithogenic diet treatment (Fig. 21I). The average fecal secretion of bile acids was higher in the transgenic mice within the first two weeks of lithogenic diet treatment. After two weeks, while the fecal bile acid secretion in the transgenic had little further increase, the secretion in the wild type mice increased dramatically, resulting in a higher secretion in the wild type mice than the transgenic mice (Fig. 21I).

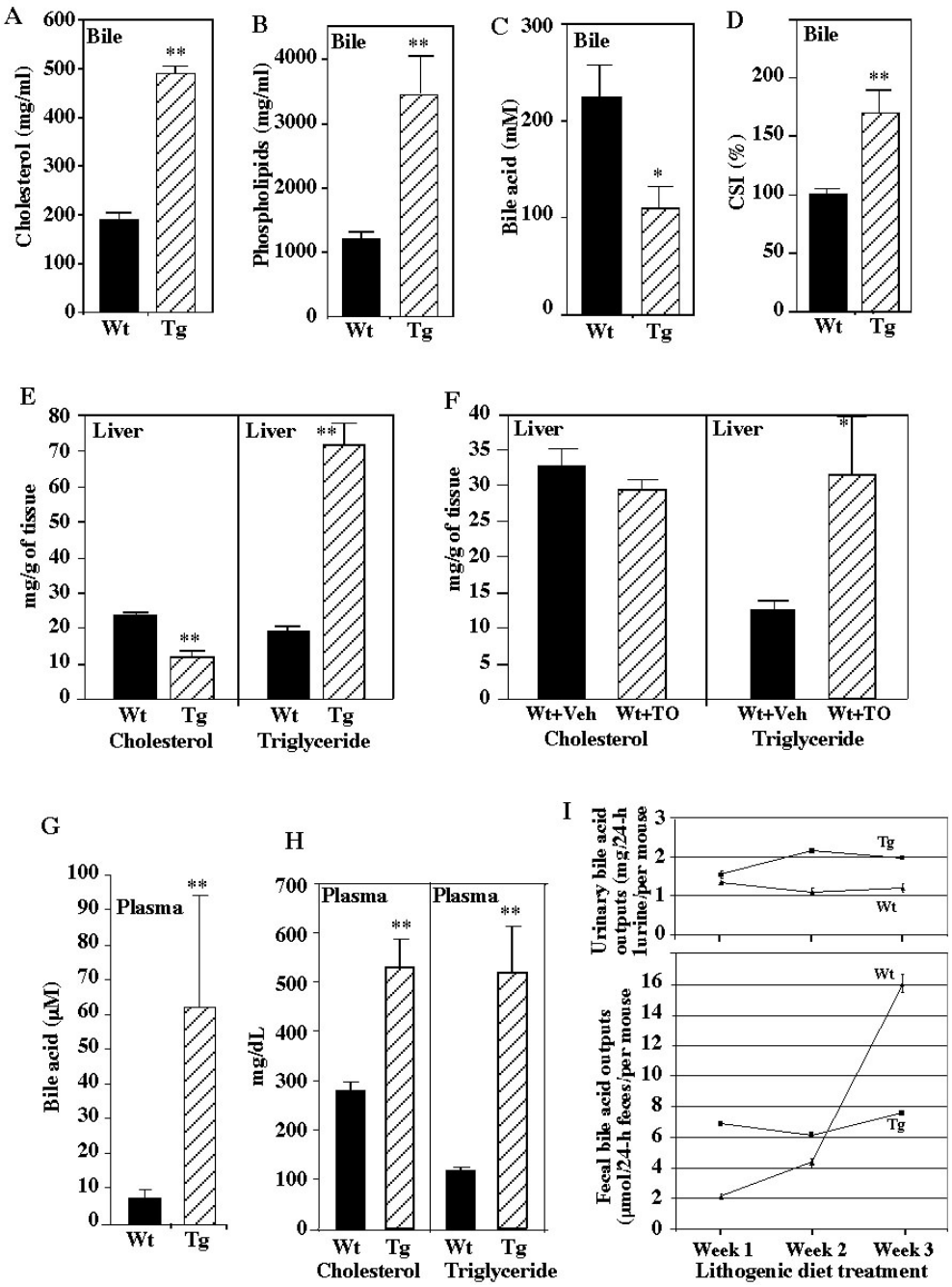


Figure 21: LXR alters biliary and hepatic lipid profiles and increases biliary cholesterol saturation index.

(A to C) Biliary concentrations of cholesterol **(A)**, phospholipid **(B),** and bile acid **(C)** in the wild type (Wt) and VP-LXRα transgenic mice (Tg) (n = 6 each genotype) after five weeks of lithogenic diet treatment. **(D)** Cholesterol saturation indices calculated using parameters in **(A to C)**. **(E and F)** Liver tissue levels of cholesterol and triglyceride in mice of indicated genotypes. Mice in **(E** and **F)** were treated with lithogenic diet for 3 and 3.5 weeks, respectively. Mice in **(F)** received daily treatment of vehicle (Veh) or TO1317 (TO). N=5-6 for each group. **(G and H)** Plasma levels of bile acid (**G**), cholesterol and triglyceride (**H**) derived from mice in (**A**). (**I**) 24-h cumulative urine and feces samples from 5-6 mice per group were pooled and measured for total bile acid. Results represent the averages and standard deviation from triplicate assays. **P*<0.05; ***P*<0.01, compared to Wt (**A-E, G and H**) or Wt+Veh **(F)**.

4.3.3 The LXR-responsive gene expression profile favored lithogenesis.

The canalicular efflux of cholesterol, bile salts, and phospholipids is known to be respectively mediated by Abcg5/g8, Bsep/Abcb11, and phospholipid transporter (Pltp) and Abcb4/Mdr2 (252) (for reviews, see (17, 18, 27, 178, 253)). In addition, Abca1, another ABC transporter and a target gene of LXR, effluxes both cholesterol and phospholipids (242, 243). Mrp2 also contributes to bile acid canalicular efflux. Mrp3 and Mrp4 play an important role in the cellular export of conjugated bile acids and thus promoting the renal secretion of bile acids (254, 255).

To understand the molecular mechanisms by which LXR promotes lithogenesis, the expression of these transporters, in the absence or presence of lithogenic diet, was profiled by quantitative real-time PCR. Among cholesterol and phospholipids transporters, both the basal and lithogenic diet-responsive mRNA expression of *Abcg5*, *Abcg8*, *Abca1*, and *Pltp* were increased (Fig. 22A and 22B), consistent with their identities as LXR target genes. The basal expression of *Abcb4*/*Mdr2* was increased in the transgenic mice but there was little further induction upon lithogenic diet treatment (Fig. 22B). Among the bile acid transporters, the transgene had little effect on the basal expression of the bile salt efflux pump (*Bsep*) and the bile acid uptake transporter Na^+-taurocholate cotransport protein (*Ntcp*), but the transgene conferred a *Bsep* induction in the presence of lithogenic diet (Fig. 22C). Lithogenic diet caused a suppression of *Ntcp* expression in the wild type mice but this suppression was absent in the transgenic mice (Fig. 22C). The expression of *Mrp2* and *Mrp4*, but not *Mrp3*, was increased in both the regular chow- and lithogenic diet-fed transgenic mice (Fig. 22C). A similar pattern of transporter regulation in the presence of lithogenic diet treatment was seen in wild type mice that were treated with TO1317, although the overall magnitude of regulation was not as dramatic as their transgenic counterparts (Fig. 22D).

Since the transgene was also targeted to the small intestine (241), we also evaluated the expression of several intestinal cholesterol and bile acid transporters. These include cholesterol efflux transports *Abcg5* and *Abcg8*, cholesterol absorption transporters Niemann-Pick C1 like 1 (*Npc1l1*) (256), and bile acid absorption transporters sodium-dependent bile acid transporter (*Asbt*), organic solute transporter α (*Ostα*), and *Ostβ*. The expression of microsomal triglyceride transfer protein (*Mtp*), a protein essential for the assembly of chylomicrons in enterocytes and

thus facilitating dietary cholesterol absorption (257), was also evaluated. Compared to the wild type mice, the basal expression of *Abcg5*, *Abcg8*, *Npc1l1*, and *Ostα* was significantly increased in the transgenic mice (Fig. 22E). However, the expression difference between the wild type and transgenic mice became not significant in the lithogenic diet-treated animals, mainly because the lithogenic diet increased the expression of these transporters in the wild type mice. *Mtp* expression was not affected by the transgene and its expression was equally induced by the lithogenic diet in both wild type and transgenic mice (Fig. 22E). The transgene had little effect on the basal expression of *Asbt*, but it significantly suppressed the lithogenic diet-responsive *Asbt* induction seen in the wild type mice (Fig. 22E). The expression of *Ostβ* was not affected by either the transgene or the lithogenic diet (Fig. 22E).

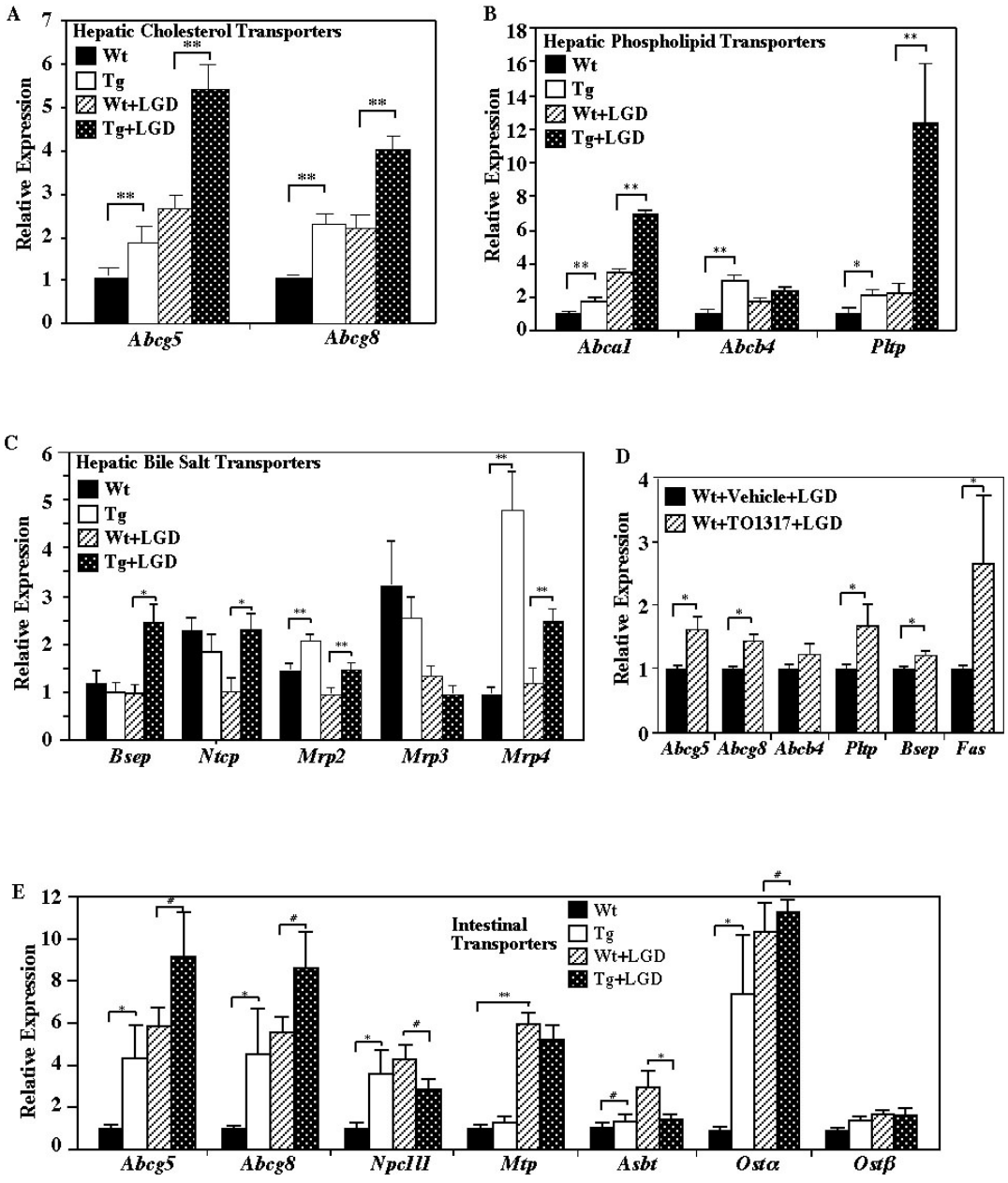


Figure 22: Gene expression profile upon LXR activation favors cholesterol gallstone formation.

The hepatic mRNA expression of cholesterol transporters **(A)**, phospholipid transporters **(B)** and bile salt transporters **(C)** was analyzed by quantitative real-time PCR. The wild type (Wt) and transgenic (Tg) mice were under regular chow or they were treated with lithogenic diet (LGD) for one week. **(D)** Wt mice treated with vehicle or TO1317 were subjected to LGD treatment for 3.5 weeks before liver harvesting and real-time PCR analysis. Drug treatment began 3 d prior to the diet treatment and continued until the completion of the experiments. Fatty acid synthase (*Fas*) was included as a positive control of LXR target gene. **(E)** Expression of intestinal transporters as revealed by real-time PCR. Samples are derived from mice in (**A-C**). Results are presented as averages and standard deviation from 4-6 mice per genotype and treatment. *Asbt*, sodium-dependent bile acid transporter; *Abc*, ATP binding cassette; *Bsep*, bile salt export pump; *Npc1l1*, Niemann-Pick C1 like 1; *Ntcp*, Na^+-taurocholate cotransport proteins; *Pltp*, phospholipid transfer protein; *Mtp*, microsomal triglyceride transfer protein; *Ostα*, organic solute transporter α. **P*<0.05; ***P*<0.01; $^{\#}$*P*>0.05, comparison as labeled.

4.3.4 The lithogenic effect of LXR is abolished in the absence of low-density lipoprotein receptor (LDLR).

Reduced biliary cholesterol secretion and gallstone prevalence have been reported in apolipoprotein E null, acetyl-coenzyme A acetyltransferase-2 (ACAT-2) null, or mice showing low efficiency of intestinal cholesterol absorption (24, 258, 259). This could be due to the decreased availability of chylomicron-derived cholesterol in the liver for biliary cholesterol secretion. The hepatic uptake of chylomicron and chylomicron remnant cholesterol and LDL cholesterol is known to be mediated by LDLR (244, 260). We therefore hypothesize that LDLR may play a role in LXR-promoted lithogenesis. To determine the effect of LDLR, VP-LXRα transgenic mice were cross-bred with the *Ldlr* null mice (244). The resultant VP-LXRα transgenic/*Ldlr* null mice were then subjected to lithogenic diet for three weeks and evaluated for gallstone formation. As summarized in Table 1 and shown in Fig. 23A, the lithogenic effect of the VP-LXRα transgene was completely abolished in the *Ldlr* null background. Introducing of *Ldlr* null background into the transgenic mice resulted in a dramatic increase of serum cholesterol level as expected (Fig. 23B, right panel), presumably due to the blockade of hepatic cholesterol uptake. Interestingly, the decreased hepatic cholesterol level seen in the transgenic mice was recovered in the Tg/*Ldlr*$^{-/-}$ mice, which may be explained by the loss of *Abcg5/8* induction in this genotype (see Fig. 23C). Gene expression analysis revealed that upon lithogenic diet-treatment, the induction of *Abcg5*, *Abcg8*, *Pltp* and *Abcb4*, seen in the VP-LXRα direct transgenic mice, was abolished in the VP-LXRα/*Ldlr* null mice (Fig. 23C). Interestingly, the loss of transporter activation appeared to be gene-specific. The activation of *Bsep* in the VP-LXRα/*Ldlr* null mice was sustained, so was the activation of lipogenic LXR target genes *Scd-1*, *Fas*, and *Acc-1* (Fig. 23C).

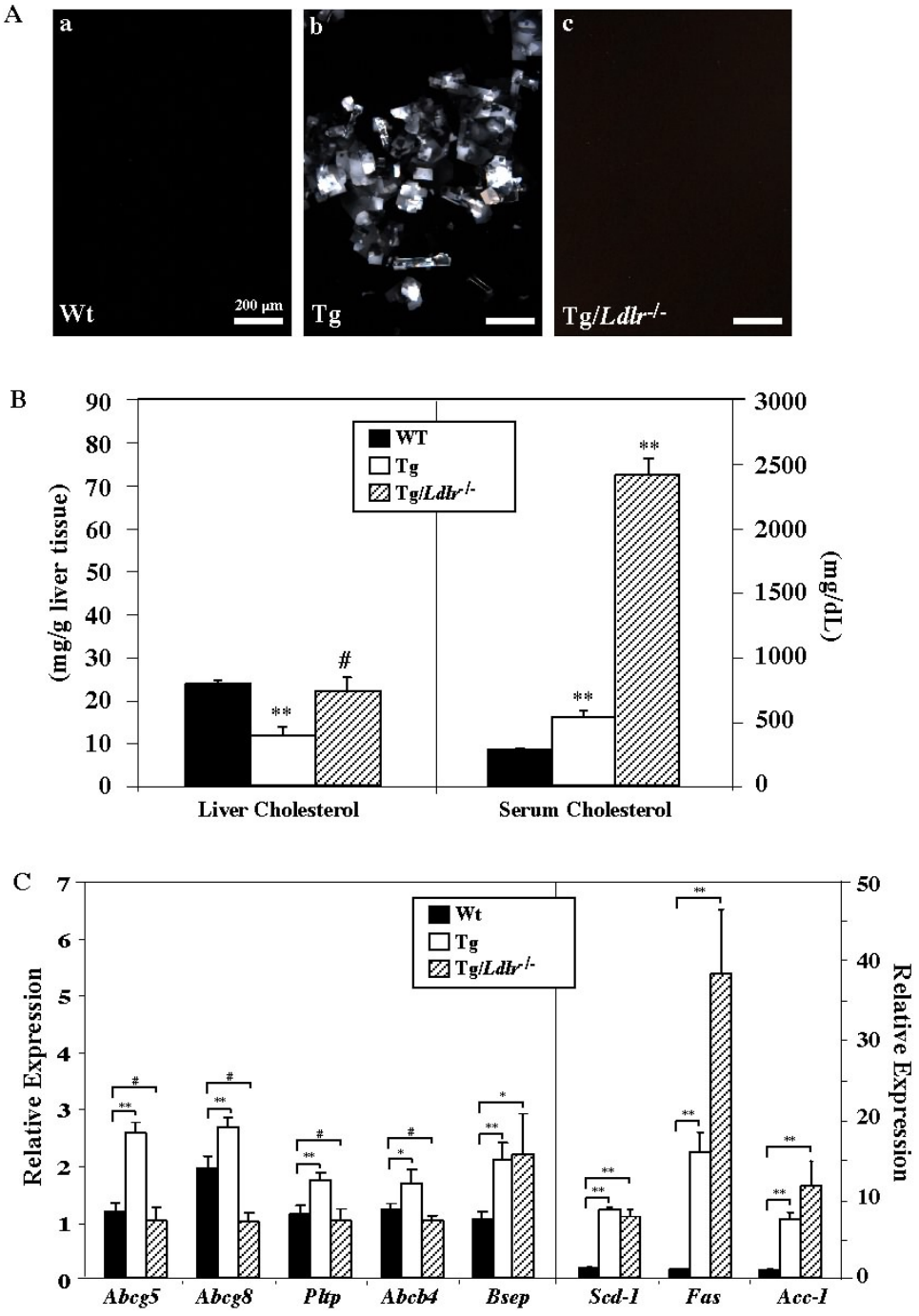


Figure 23: The lithogenic effect of LXR abolishes in the absence of low-density lipoprotein receptor (LDLR).

A) Wild type (Wt), VP-LXRα transgenic (Tg), or Tg/*Ldlr*$^{-/-}$ mice were treated with lithogenic diet for three weeks before microscopic examination of cholesterol crystals. Scale bars are labeled. **(B)** Liver and serum levels of cholesterol in Wt, Tg, or Tg/*Ldlr*$^{-/-}$ mice treated with lithogenic diet for three weeks. **(C)** Real-time PCR analysis of hepatic gene expression in Wt, Tg, or Tg/*Ldlr*$^{-/-}$ mice treated with lithogenic diet for three weeks. *Scd-1*, *Fas* and *Acc-1* are included as positive controls of LXR target genes. Results shown are presented as averages and standard deviation from 4-6 mice per genotype and treatment. *Scd-1*, stearoyl-CoA desaturase-1; *Acc-1*, acetyl-CoA carboxylase-1. **P*<0.05; ***P*<0.01; $^{\#}$*P*>0.05, comparisons are as labeled.

4.3.5 The lithogenic effect of LXR was preventable by the cholesterol lowing drug Ezetimibe.

It has been shown that the efficiency of intestinal cholesterol absorption correlates positively and significantly with the prevalence of cholesterol gallstones among inbred mice (24, 261). The intestinal cholesterol absorption in humans and rodents can be inhibited by Ezetimibe, a recently developed drug that reduces serum and LDL cholesterol levels. Ezetimibe inhibits dietary absorption of cholesterol in the small intestine by blocking the sterol transporter (NPC1L1) (262, 263). Ezetimibe has been used to lower cholesterol by itself (*e.g.,* Zetia®, an US brand) or in combination with a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor statin (*e.g.,* Vytorin®, an US brand). To evaluate the effect of dietary cholesterol absorption on LXR-promoted lithogenesis, we subjected the VP-LXRα transgenic mice to lithogenic diet, in the presence or absence of Ezetimibe. As summarized in Table 1 and shown in Fig.24, treatment with Ezetimibe prevented lithogenesis in the transgenic mice. As expected, treatment with Ezetimibe lowered the liver and serum concentrations of cholesterol in both the wild type and transgenic mice (Fig. 24B). When the gene expression was analyzed, we found that the transgenic induction of *Abcg5*, *Abcg8*, *Abcb4*, *Ntcp* and *Mrp2* was abolished in Ezetimibe-treated transgenic mice (Fig. 24C, left panel). The activation of *Bsep* and *Mrp4* in the transgenic mice was sustained, although the basal expression of these two transporters was decreased in the wild type mice upon Ezetimibe treatment (Fig. 24C, left panel). Interestingly, treatment with Ezetimibe increased *Mrp3* expression in the wild type mice but not in the transgenic mice (Fig. 24C, left panel). *Pltp* was induced in lithogenic diet-treated transgenic mice but its expression is indistinguishable between the wild type and transgenic mice upon Ezetimibe treatment (Fig. 24C, right panel). The regulation of the control lipogenic *Scd-1* gene is similar to that of *Bsep* and *Mrp4* (Fig. 24C, right panel).

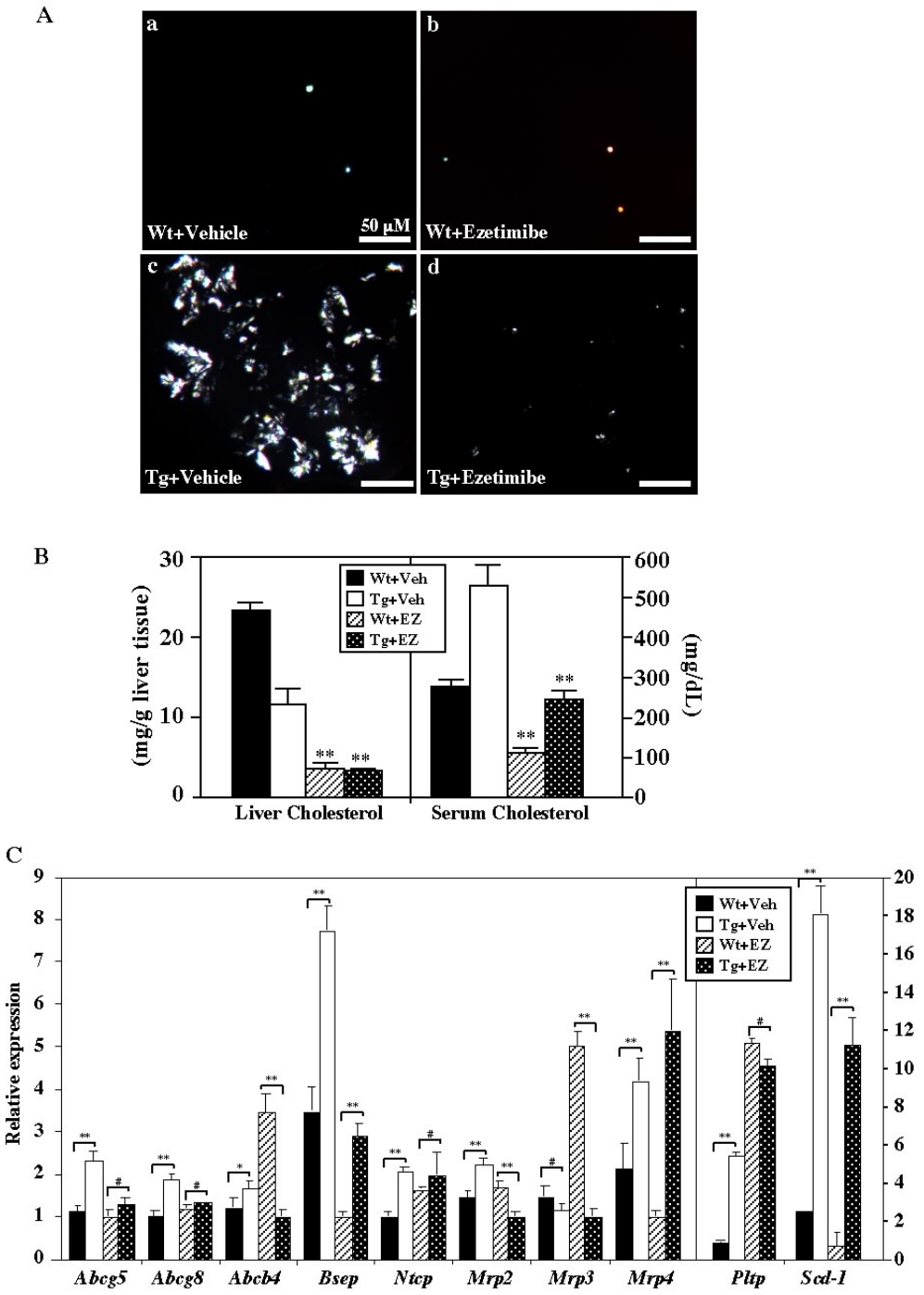


Figure 24: The lithogenic effect of LXR was preventable by the cholesterol lowing drug Ezetimibe.

(A) Wild type **(**Wt, **a and b)** and transgenic mice **(**Tg, **c and d)** were treated with lithogenic diet for three weeks before microscopic examination of cholesterol crystals. Mice in **(a and c)** received vehicle, whereas mice in **(b and d)** received a daily gavage of Ezetimibe (5 mg/kg). The Ezetimibe treatment began one week prior to the lithogenic diet treatment and continued until the completion of the experiment. Scale bars are labeled. **(B)** Treatment with Ezetimibe lowered the liver and serum concentrations of cholesterol in both Wt and Tg mice. All mice received three weeks of lithogenic diet treatment. $^{**}P<0.01$, compared to their vehicle treated counterparts. (**C**) Expression of hepatic transporter genes as revealed by real-time PCR. Samples are derived from mice in (**B**). $^{**}P<0.01$, comparisons are as labeled.

4.4 DISCUSSION

In this report, we show that genetic or pharmacological activation of LXR promotes CGD. The lithogenic effect of LXR was associated with an increased expression of hepatic transporters responsible for canalicular efflux of cholesterol and phospholipids, which may have led to their biliary accumulation. Meanwhile, the concentration of bile salt in the bile was decreased in transgenic mice, leading to an increased biliary cholesterol saturation index and formation of the cholesterol gallstones. The lithogenic effect of LXR was completely abolished by deletion of LDLR or inhibition of dietary cholesterol absorption by Ezetimibe (Fig. 25).

The lithogenic effect of LXR is opposite to the litho-preventive function of FXR (185). Deficiency of FXR sensitizes mice to CGD, whereas a synthetic FXR agonist prevented sequelae of the disease. The protective effects were thought to be mediated by FXR-dependent activation of bile salt export pump *Bsep* and phospholipids transporter *Mdr2*/*Abcb4* (252) and subsequent increases in biliary bile salt and phospholipid concentrations (185). In the LXR transgenic model, although the transgene had little effect on the basal expression of *Bsep*, a higher responsive *Bsep* expression was seen in the lithogenic diet-treated transgenic mice. It was recently shown that upregulation of *Abcb11/Bsep* in mice fed with a lithogenic diet showed an increased rate of cholesterol crystal and gallstone formation which was associated with an increase in both the hydrophobic bile salt and cholesterol content of gallbladder bile (264). The expression of *Mrp2*, another bile acid efflux transporter, was also induced in the transgenic mice. However, in our study the LXR transgenic mice had lower bile acid levels in the gallbladder bile upon feeding the lithogenic diet. Other studies have reported that LXR activation may reduce the bile acid levels in the gallbladder bile, and this may potentiate the cholesterol gallstone formation in the gallbladder (265-267). We reason the low biliary bile salt content is due to the up-regulation of *Mrp4* in the transgenic mice. Mrp4, a cholestatic protective transporter that effluxes sulfonated bile salts to the blood to promote their renal secretion (254, 268, 269), was induced in both unchallenged and lithogenic diet-treated transgenic mice. Consistent with the up-regulation of *Mrp4*, both the circulating level and the renal secretion of bile acid were increased in the transgenic mice upon lithogenic diet treatment. It remains to be determined whether *Mrp4* is a transcriptional target of LXR. The inhibition of lithogenic diet-responsive activation of *Asbt* may have compromised bile acid intestinal re-absorption and contributed to

the lower biliary bile salt contents. The later notion was supported by the higher fecal bile acid secretion in the transgenic mice in the first two weeks of lithogenic diet treatment. Increased fecal bile salt secretion has also been reported for TO1317-treated wild type mice (265, 266).

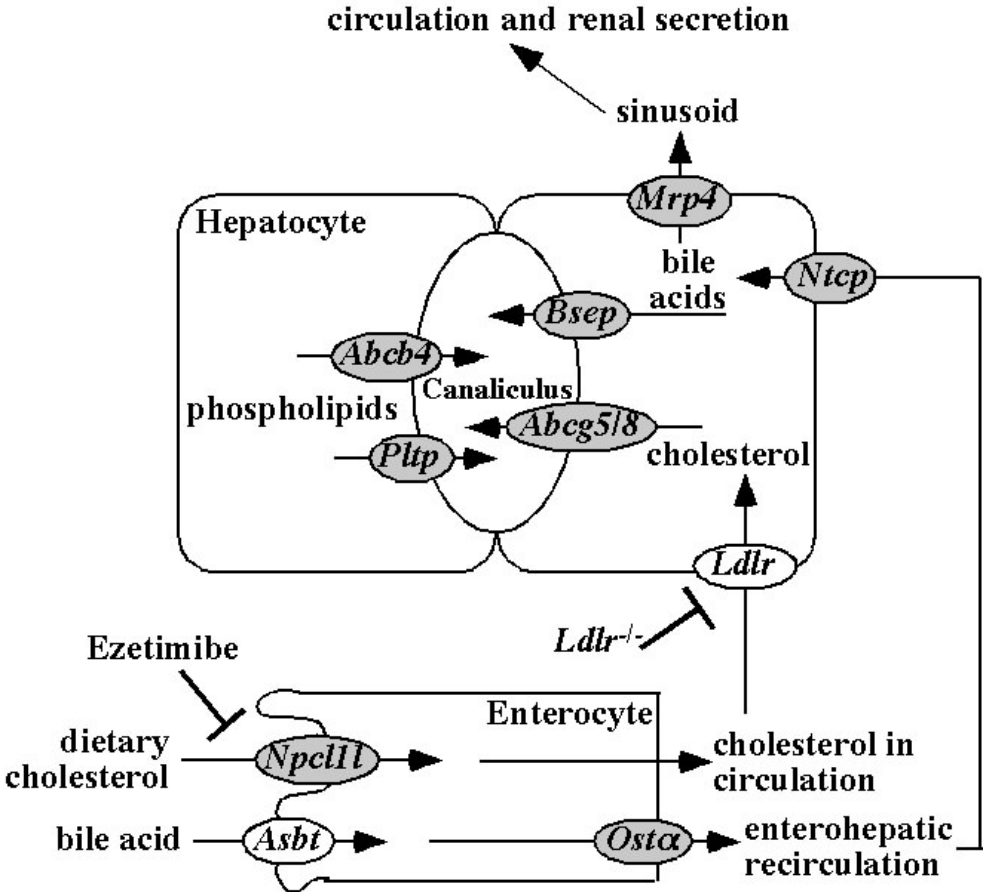


Figure 25: Model for the effect of LXR on CGD.

Transporters in shaded ovals are up-regulated by LXR activation. The net effect of LXR activation is increased biliary concentrations of cholesterol and phospholipid and decreased content of biliary bile salts. The lithogenic effect of LXR can be abolished by deletion of LDLR or inhibition of dietary cholesterol absorption by Ezetimibe.

In addition to the lower biliary bile salt content, the up-regulation of hepatic cholesterol efflux transporters may have also contributed to the LXR-promoted lithogenesis. Abcg5/8 are known LXR target genes and a low function of Abcg5/8 has been linked to resistance to gallstone disease (27, 179). Torsten and colleagues reported that mice treated with TO1317 had increased biliary cholesterol secretion (265, 266), consistent with results from our VP-LXRα transgenic mice. The notion that canalicular cholesterol efflux plays an important lithogenic role is supported by the requirement of cholesterol in the lithogenic diet and the observation that the

lithogenic effect of LXR was abolished when the hepatic LDL cholesterol uptake was blunted in the *Ldlr* null background and when the dietary cholesterol was blocked by Ezetimibe. In lithogenic diet-treated mice, introducing of the *Ldlr* null allele abolished the induction of *Abcg5/g8* by the VP-LXRα transgene. The loss of *Abcg5/g8* induction in the VP-LXRα/ *Ldlr* null mice may have resulted from the lack of hepatic cholesterol accumulation and biliary secretion due to the reduced hepatic cholesterol uptake. Similar studies evaluating the hepatic cholesterol uptake mechanisms in cholesterol gallstone disease formation via apoE and apoB expression show that this is an important pathway for regulating biliary secretions of diet-derived cholesterol leading to diet-induced cholesterol gallstone formation in mice (258, 261). Our results show that modulating the key cholesterol transport molecules involved in the hepatic uptake of chylomicron remnant cholesterol, play an important role in controlling bile cholesterol elimination and cholesterol gallstone formation induced by dietary cholesterol.

Furthermore, our results also show that the intestinal cholesterol absorption pathway is an additional mechanism for modulating the cholesterol gallstone formation. This increased cholesterol uptake in animals placed on a lithogenic diet increases biliary cholesterol hypersecretion. The higher intestinal absorption and biliary secretion of cholesterol significantly increase cholesterol saturation of bile, and gallstone formation in the susceptible LXRα mice. Recent animal studies have also shown that the increased intestinal cholesterol absorption in high cholesterol diet fed animals may play an important role in biliary cholesterol secretion and contribute to cholesterol gallstone formation (24, 261). Further work on understanding the molecular events underlying intestinal cholesterol absorption and hepatic secretion of cholesterol into the bile may lead to new insights into the mechanisms of cholesterol gallstone disease and offer a new approach to the prevention of cholesterol gallstone formation.

In summary, our results have revealed a novel function of LXR in lithogenesis. Studies in human and mouse models have shown that there is a complex genetic basis for predisposition towards cholesterol gallstone disease (179, 270-274). LXRα looks to be a promising candidate gene for the cholesterol gallstone disease process. It is of interest to determine whether the familial gallstone diseases are associated with increased expression and/or activity of LXRs. Our results suggest that the development of LXR antagonists may also represent a novel strategy to treat and prevent gallstone diseases.

5.0 SUMMARY AND CONCLUSIONS

The present study establishes a crucial and collective role for PXR, CAR and LXR in maintaining the homeostasis of cholesterol and bile acids *in vivo* (Fig. 26). We conclude that PXR, CAR and LXR mediate overlapping, yet discrete, layers of defense to prevent cholestatic hepatic damage by bile acids. The role of dual sensing of lipids and bile acids, in conjunction with the functional networking with xenobiotic receptors, establishes LXR as a critical and "smart" orphan nuclear receptor in mediating xeno- and endobiotic homeostasis. Our results have also revealed a novel function for LXR in lithogenesis. Thus, the role of these three nuclear receptors in regulating the cholesterol metabolism into bile acids, metabolism of bile acids for excretion and the coordinated export of cholesterol and bile acids from the body has been shown.

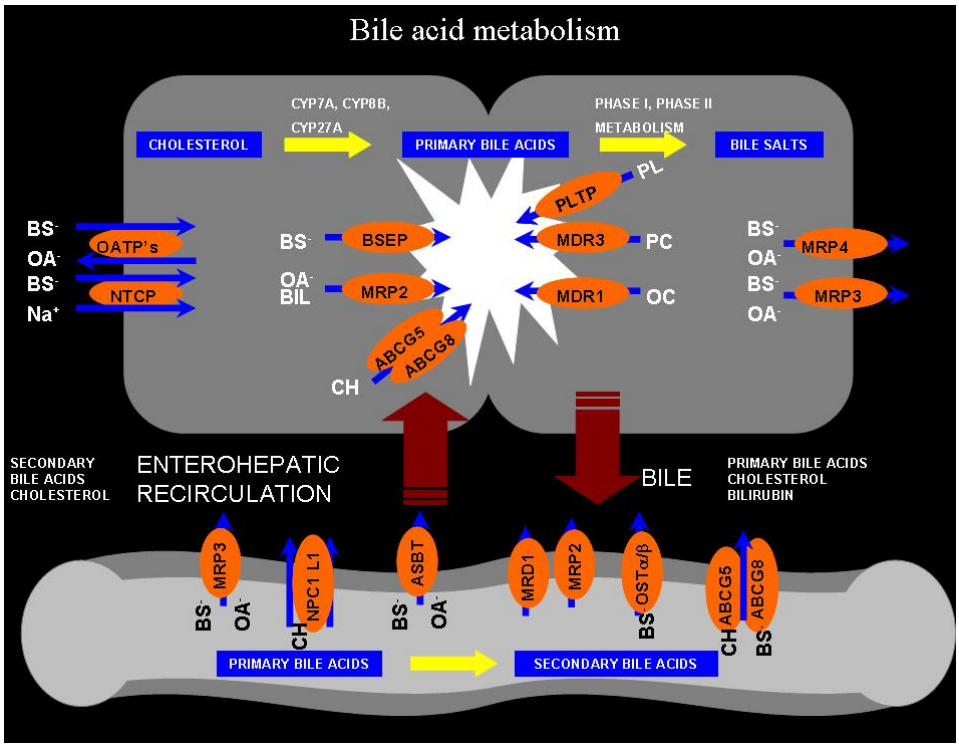


Figure 26: Role of PXR, CAR & LXR in the bile acid and cholesterol metabolism processes

Our current work with the bile acid metabolism involving the PXR and CAR as well as previous work done by other groups indicates the these nuclear receptors promote metabolism and excretion of bile acids. The pathway of PXR activation helps to detoxify bile acids such as lithocholic acid by inducing an array of bile acid metabolizing Cytochrome P450 enzymes; the phase II conjugating enzymes, primarily the sulfotranferases; as well as bile acid transporters (2, 96, 275). Similarly, the activation of CAR has an overlap in this protection, as an activation of CAR induces the SULT2A, which helps to detoxify the toxic bile acids. Therefore, both PXR and CAR help to protect against hepatotoxicity (Fig. 27). Thus our results show that both PXR and CAR are required for the bile acid detoxification and secretion pathways. The combined loss of PXR and CAR leads to a decreased bile acid secretion via the transporter down regulation as well as decreased bile acid detoxification via the phase I and phase II metabolizing enzymes. This initially leads to the development of hepatotoxicity, and is further complicated by the systemic buildup of bile acid due to loss of hepatic function i.e. loss of detoxification transporter based uptake and excretion. The systemic buildup of bile acids leads to systemic toxicity probably due to the detergent properties of the bile acids and their exposure to the various organs in the body. Therefore, the combined loss of PXR and CAR has a net effect in potentiating the bile acid toxicity over and above the individual loss of these nuclear receptors (Fig. 27).

Our current work on LXR has added another dimension to this fail-safe network to protect against bile acid toxicity (Fig. 28). It is well established that LXR has a major role in the cholesterol metabolism pathway. LXR helps in the reverse cholesterol transport from the peripheral organs to the liver, in the de novo synthesis of cholesterol, in the cholesterol breakdown by the CYP7A via the classical pathway and in the hepatic cholesterol excretion into the bile. LXR is able to help in the process of maintaining the cholesterol homeostasis in the body by coordinating all these diverse mechanisms. Through our work, we have been able to demonstrate a novel role of LXR in the cholesterol homeostasis pathways by revealing its role in detoxification of bile acids via the SULT mediated pathway. To keep the cholesterol levels in check, LXR also inhibits the CYP7B in the alternative pathway of cholesterol breakdown into bile acids. This leads to the buildup of the oxysterols, which are LXR ligands, and this in turn leads to a feedback-based activation of the LXR. The feedback activation of LXR causes the CYP7A to be upregulated to help in increased breakdown of cholesterol into bile acids, and

additionally an increased activation of the phase II SULT via the LXR results in an increased detoxification of the bile acids (Fig. 28).

We have also been able to show that LXR can also help in detoxifying against a bile acid accumulation by CYP3A independent mechanisms, which involves primarily the upregulation of SULT2A for sulfation of the bile acids. In addition, LXR can also induce an assortment of bile acid transporters in the liver and intestine, which facilitate the bile acid clearance. We believe that the higher NTCP expression on the liver upon LXR activation helps to efficiently absorb the bile acids taken from the intestine via the portal circulation as well as from the general circulation. In the liver, LXR along with PXR and CAR contribute towards the conjugation of toxic bile acids by activating the sulfation pathway. The sulfated bile acids can be excreted either to the bile or to the circulation via the MRP4 for renal secretion. This hypothesis is supported by the fact that there is a higher bile acid excretion into the urine when LXR is activated.

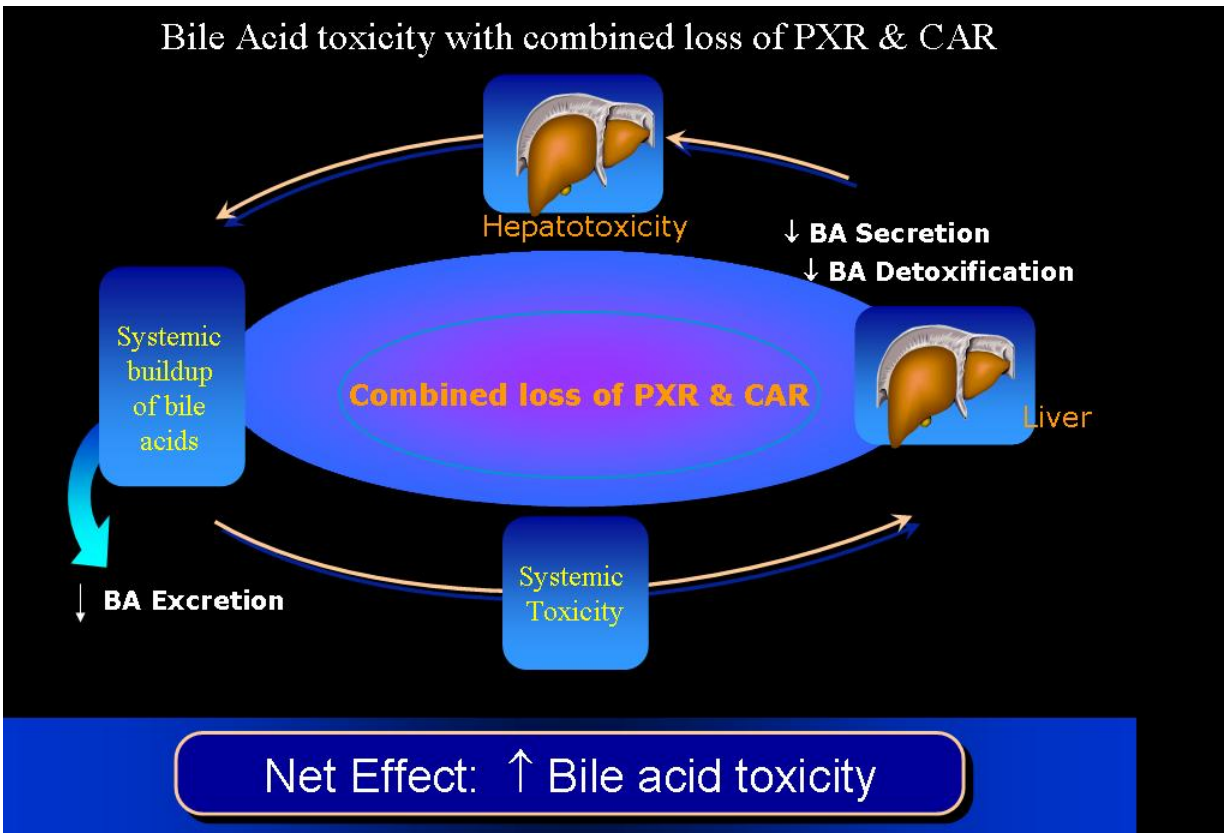


Figure 27: Overlapping roles of PXR and CAR in bile acid detoxification

Adapted from lipidsonline.org(Baylor College of Medicine)

The existence of overlap in endogenous ligands to activate PXR, CAR and LXR also suggests that there is a functional interaction between these receptors in the liver. This interaction also helps to regulate cholesterol biosynthesis and bile acid metabolism. Cholesterol biosynthesis starts from acetyl-CoA and involves a large number of enzymatic steps. Major intermediates in the pathway are acetoacetyl-CoA, hydroxy-methylglutaryl-CoA, mevalonate, squalene and lanosterol. Many of the intermediates of this pathway serve as natural ligands for the nuclear receptors. CAR and PXR are activated by isoprenoids and squalene metabolites from the cholesterol biosynthesis pathway (276, 277). If cholesterol biosynthesis is blocked, PXR and CAR might be activated by these metabolites and subsequently inhibit cholesterol metabolism to bile acids by repressing CYP7A1 as seen after activation of PXR by other ligands (76, 275). This regulation could prevent the body levels of cholesterol from becoming too low when cholesterol biosynthesis is impaired. Mevalonate, mevalonate phosphate, or a unknown derivative of this pathway act as an endogenous activator of LXRα from this pathway (278). This could on the other hand help to remove bile acid metabolites, which are formed in conditions when cholesterol levels are high by activation of CYP7A. However, geranylgeranyl-pyrophosphate, another intermediate in mevalonate metabolism to cholesterol, inhibits LXR activity and thus also results in lower CYP7A1 levels (278). The geranylgeranyl-pyrophosphate would go up in conditions when the cholesterol biosynthesis pathway is impaired and one would need to lower the CYP7A activity via LXR suppression. Thus at the level of cholesterol biosynthesis pathway the PXR, CAR and LXR help to regulated the cholesterol and bile acid metabolism.

Cholesterol biosynthesis leads to the most abundant steroid in the body, and cholesterol occurs either as a free molecule or as bound to fatty acids in the form of cholesteryl esters. Cholesterol performs a variety of functions in the body, most importantly the structural component of cell membranes and as a precursor for other steroids such as steroid hormones. Excessive cholesterol is then metabolized to bile acids. As discussed in the introduction, cholesterol is metabolized by two different pathways: the classic bile acid biosynthesis pathway resulting in the formation of the primary bile acids cholic acid and chenodeoxycholic acid; and secondly, the alternative pathway that produces the oxidized cholesterol, which is further, converted to the bile acids. The main classical pathway is highly regulated at the first enzymatic step of the CYP7A. CYP7A is upregulated in mouse and rat by the LXR by binding to a DR4 element which strongly

upregulates transcription (Fig 28) (124, 135). This element is mutated in the human CYP7A gene which prevents the LXR from binding (148). A potent product-mediated negative feedback inhibition in the regulation of bile acid synthesis from cholesterol occurs by bile acid activation of FXR, which in turn induces the expression of the SHP. Subsequently, SHP binds to the liver receptor homolog-1 (LRH-1) which is a potent activator of CYP7A1 and this leads to decreased transcriptional activity of LRH-1 and inhibition of CYP7A1 transcription (188, 202). Another pathway of CYP7A inhibition occurs via the PXR, which upon being activated by bile acids and xenobiotics has been shown to repress CYP7A transcription (76, 275).

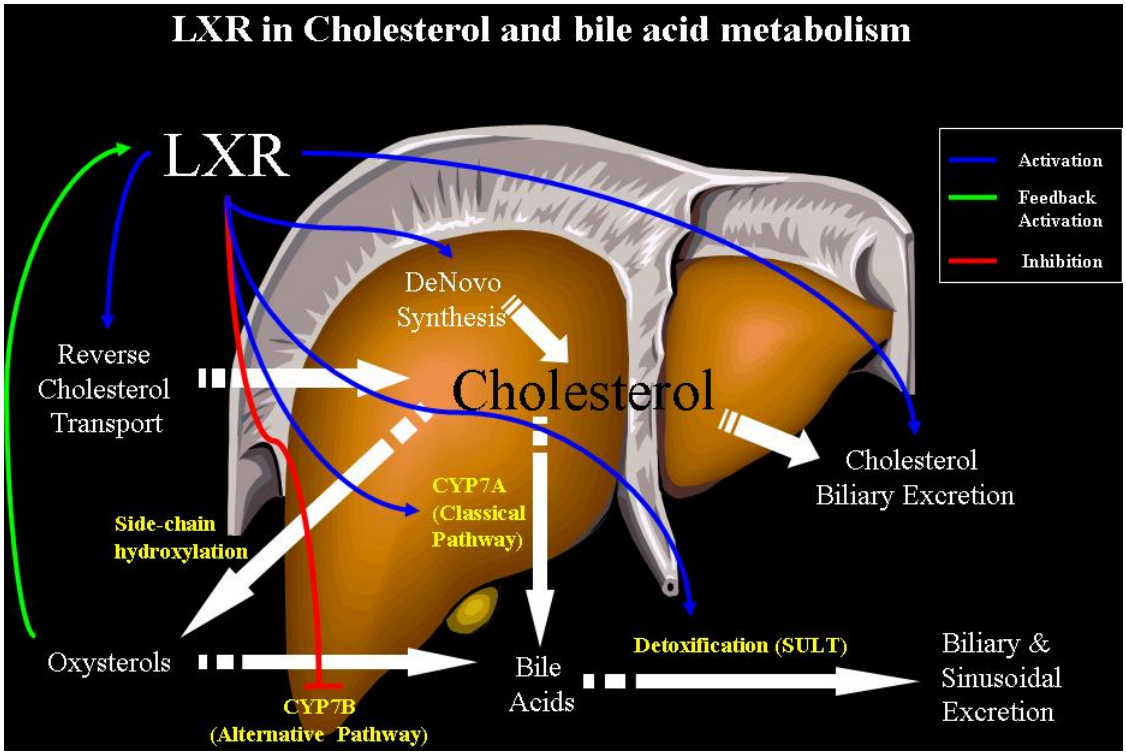


Figure 28: Role of LXR in cholesterol and bile acid metabolism

Adapted from lipidsonline.org(Baylor College of Medicine)

The cholesterol metabolism pathway yields ligands for the nuclear receptors PXR, CAR and LXR. PXR was named based on its activation by a number of different C21 steroids, including the hormone progesterone, which is a pathway derived from cholesterol (67). A further derivative of this pathway are the sex steroids, and the androstanol was found to inhibit the activity of CAR (72). Various metabolites of the CYP7A and CYP27A metabolism pathway like the 7α-hydroxycholesterol and 27α-hydroxycholesterol, and 22*(R)*-hydroxycholesterol from the sex steroid biosynthetic pathway, 24*(S),* 25-epoxycholesterol a side-chain pathway metabolites

from squalene, and 24*(S)*-hydroxycholesterol from the CYP46 metabolism of bile acids in the alternative pathway were found to be LXR activators (124, 125, 278). PXR was further shown to be activated by cholesterol metabolites *in vivo* in mice lacking the enzyme sterol 27-hydroxylase, due to elevated concentrations of bile acid precursors such as the triols and tetrols (101, 102). In addition the bile acids like the lithocholic acid were also shown to activate PXR (76, 96, 277). Thus the presence of a large number of ligands from the cholesterol synthesis and metabolism pathway does indicate that these receptors work together to regulate the balance of cholesterol and bile acids in mammals.

Our work also showed that LXR had an inhibitory effect on the CYP3A expression. On the other hand, PXR and CAR can both upregulate the CYP3A expression. CYP3A is a major enzyme which helps to hydroxylate the bile acids for detoxification (96). Thus, the contrasting roles of PXR/CAR with LXR might be important to maintain a balance of the CYP3A expression for xenobiotic and bile acid metabolism. The CYP3A also catalyzes the 4β-hydroxylation (131) as well as a 6α-hydroxylation of cholesterol (101, 102, 279, 280). Upon activation of PXR, the upregulation of CYP3A catalyzes the 4β-hydroxylation of cholesterol leading to increase of the circulating 4β-hydroxycholesterol levels (130, 131). This cholesterol metabolite further activates LXR (125), and thus the PXR and LXR are therefore able to function in a coordinate manner. Further the major cholesterol metabolism breakdown product in the liver 24*(S)*, 25-epoxycholesterol can activate both PXR and LXR (230). Increase in the levels of this metabolite could lead to a downregulation of the HMG-CoA reductase, and could lead to a balance in the CYP7A and CYP3A expression for a regulated bile acid metabolism pathway via PXR and LXR activation. The 6α-hydroxylation of cholesterol by CYP3A leads to the formation of 6α-hydroxylated chenodeoxycholic acid and 6α-hydroxylated lithocholic acid, two bile acids known to activate LXR (128, 281). It has also been shown that treatment of hepatic cells with 6α-hydroxylated bile acids reduced the drug-induced expression of CYP3A. Thus LXR negatively regulates the CYP3A mediated hydroxylation leading to accumulation of the CYP7A, CYP27A and CYP46 metabolites, which in turn are ligands for LXR and lead to LXR activation. This will also lead to protection against accumulation of hydroxylated bile acids. The accumulation of the CYP7A, CYP27A and CYP46 metabolites, will also lead to the PXR

activation via the triols and tetrols, and thus a balance between LXR and PXR activation will be maintained for cholesterol and bile acid homeostasis.

The present studies on the bile acid metabolism shed light on the fact the this process is complex and involves several key steps that are still poorly understood. Hereditary mutations or exposure to cholestatic liver injury results in altered expression and function of the hepatobiliary transport systems. It remains to be understood which of the alteration at the molecular level contribute towards or maintain cholestasis and which of these changes results from adaptive processes of the body aimed at reducing the extent of the cholestatic liver injury. The mouse and human mechanisms of cholesterol metabolism are similar; however, there still exist some fundamental differences. The primary difference is the differential regulation of the CYP7A in the classical cholesterol metabolism pathway. In addition, the sensitivity of the mouse and human beings towards the dietary cholesterol is also different. Mice require high levels of dietary cholesterol as compared to human beings to advocate any cholesterol-related perturbation in the system. It is not know what the role is and what adaptive responses for compensation exist, in the classical and alternative pathways of cholesterol breakdown; and how these two pathways differ in human and mice during normal and cholestasis conditions. This indicated that the regulation of cholesterol breakdown and bile acid export in human beings might be regulated in a different way as compared to mice. Therefore, the usefulness of the hepatocellular transport mechanisms studies in mice awaits confirmation in human studies. In addition, it has been well documented that there exist gender differences in the bile acid metabolism in various species, particularly the mouse. These could affect the interpretation of result when studying the pathways in one gender. This led us to undertake the studies in both the male and female mice. Studies have indicated that sex hormonal differences including some growth hormones significantly contribute towards the regulation of the basolateral and sinusoidal transport of bile acids (282).

In addition to this cholesterol and bile acid metabolism is regulated both in the liver as well as in the intestine. Therefore, a defect in either the bile acid efflux and uptake mechanisms in the liver or the intestine can lead to cholestasis. This implies that study of the cholestasis disease process has to be done in tandem in both the organs. The bile acid metabolism process is also affected by adaptive response including physiological and exogenous stimuli like the postprandial

increases in portal venous salt load, xenobiotic absorption from the intestine, hormonal changes during pregnancy and septicemia (51). Along with the identification and characterization of the bile salt transport mechanisms, it has also become clear that it is a process, which occurs concomitantly with the biliary lipid secretion. The study of the cholestatic disease process should therefore integrate the understanding of the function of biliary lipid secretion along with the bile salt homeostasis. In view of these open questions, further insights into this disease process will improve not only the understanding of molecular mechanisms of the disease, but also be clinically relevant and could help in the design of specific therapeutic strategies.

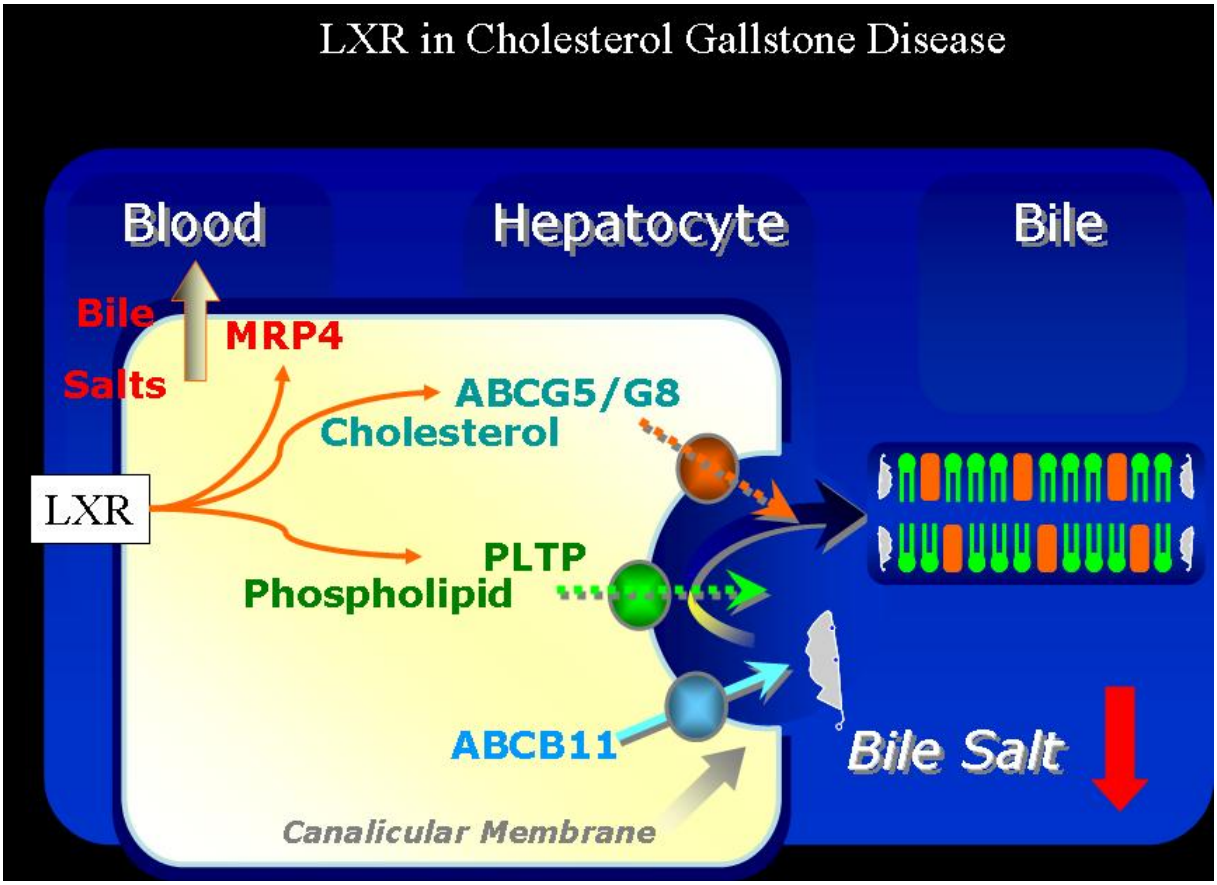


Figure 29: Role of LXR in cholesterol gallstone disease

Adapted from lipidsonline.org(Baylor College of Medicine)

The work on the role of LXR in cholesterol gallstone disease formation demonstrates that the activation of LXR results in the enhanced excretion of cholesterol into the bile. When animals have an excess of cholesterol in the system as in the cases of enhanced dietary cholesterol

uptake, an activation of LXR in the liver can result in the excess secretion of cholesterol into the bile. LXR activation also leads to increased phospholipid secretion into the bile to maintain the micellar formation. However, the LXR activation leads to decreased bile salt secretion into the bile and this results in the disturbance of the ratios of the cholesterol, phospholipids and bile salts leading to precipitation of the excess cholesterol in the bile. The bile salt excretion is enhanced towards the sinusoidal secretion via the MRP4 leading to a decreased secretion via the BSEP into the bile. The precipitation of excess cholesterol forms the cholesterol crystals, and in the long run, these crystals accumulate and nucleate to form the gallstones (Fig. 29).

A lot of work, including ours, on the cholesterol disease pathogenesis and molecular mechanisms has shown that CDG is a complex disease involving the complex regulation of whole body cholesterol homeostasis and specifically transport and metabolism pathways in the liver, intestine and the gallbladder. Our studies have help to identify the role of LXR in some of these processes, but a lot remains to be done. The physiology of the mouse compared to the humans with respect to cholesterol metabolism is different, especially with differential regulation of the CYP7A and the absence of the Cholesterol ester transfer protein in the mouse. Therefore, the molecular mechanisms of this disease in humans might be different and as a result, there might be different genetic determinants for CGD in human beings. This also is related to the fact that genetic predisposition to cholesterol gallstone disease has been observed in human beings (179, 270), and it remains to be seen if there is overlap in the human and mouse genes. Future studies warrant the precise understanding of the role of the identified genes as well as their function in the disease process. In addition, most of the studies done in mice involve acute loading of cholesterol into the system via various dietary regimens. This does not entirely mimic the situation in human beings, especially considering the fact that obesity and metabolic syndromes do play a role in this disease.

In summary, our studies have revealed a redundant role of PXR, CAR and LXR in maintaining the homeostasis of cholesterol and bile acids in the body. Further work is also necessary to understand the hierarchy of the protection mediated by the individual receptors. Nevertheless, the metabolic safety net mediated by multiple receptors appears to be the nature's way to protect mammals from xenobiotic and endobiotic chemical insults.

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