EFFECT OF BILE ACID FEEDING AND SEQUESTRATION

ON LIVER BILE ACID COMPOSITION AND GENE REGULATION IN MICE

BY

PEIZHEN SONG

Submitted to the graduate degree program in the Department of Pharmacology, Toxicology and Therapeutics and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Dissertation Committee

_____
Chairperson: Curtis D. Klaassen, Ph.D.

_____
Bryan Copple, Ph.D.

_____
Grace L. Guo, Ph.D

_____
Bruno Hagenbuch, Ph.D.

_____
Jeffrey Staudinger Ph.D.

Date defended: Dec, 3rd, 2010

The Dissertation Committee for Peizhen Song certifies that this is the approved version of the following dissertation:

EFFECT OF BILE ACID FEEDING AND SEQUESTRATION

ON LIVER BILE ACID COMPOSITION AND GENE REGULATION IN MICE

_____

Chairperson: Curtis D. Klaassen, Ph.D.

Date Approved: Dec, 3th, 2010

ABSTRACT

Bile acids (BAs) serve multiple functions. BA feeding has been used to study BA signaling and toxicity *in vivo*. However, the concentration of BAs in the feed that is non-hepatotoxic is not well defined in mice. Therefore, the purpose of my first study in my dissertation was to determine the non-hepatotoxic and hepatotoxic concentrations of five BAs in mouse feed, as well as the effect of feeding mice individual BAs on BA concentrations and BA composition in liver. Mice were fed five individual BAs, cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA) at 0.01, 0.03, 0.1, 0.3, 1.0, or 3.0% in their diets for 7 days. The data showed: 1) LCA, DCA, and CDCA at 1.0% in the diets were lethal, whereas CA at 1% and UDCA at 3.0% were not lethal. 2) LCA produced hepatotoxicity at 0.03%, indicated by increases in serum ALT and serum BA concentration, as did DCA at 0.1%, and CDCA and CA at 0.3% in the diet. UDCA at 0.3% in the diet might be hepatotoxic because the serum BA concentration was increased but the serum ALT did not increase. 3) Feeding BAs (CA, CDCA, DCA, or LCA) at hepatotoxic doses did not alter the total BA concentration in liver; however it altered liver BA composition, including increases in the percentage of the fed-BAs, and decreases in the percentage of MCAs (muricholic acids). In conclusion, it appears that an imbalance in BA composition in liver can contribute to BA hepatotoxicity. In addition, to study the physiological or pharmacological functions of each BA, non-hepatotoxic concentrations of BAs in the feed should be used. Our data show that to avoid hepatotoxicity, CA and CDCA

should be at 0.1% or lower, DCA at 0.03% or lower, and LCA at 0.01% or lower in the diet.

BAs can regulate their own biosynthesis and transport at the transcriptional level via activation of FXR. My first study has shown that feeding mice BAs at either non-hepatotoxic or hepatotoxic doses did not increase the total liver BA concentration, suggesting adaptation in the expression of genes involved in BA biosynthesis and transport. Thus, the purpose of the second study in my dissertation was to investigate the expression of genes involved in BA homeostasis and the possible roles of hepatic and ileal FXR signaling in regulating these genes to maintain total BA concentrations in the livers of mice fed different doses of BAs. Mice were fed five individual BAs, CA, CDCA, DCA, LCA, and UDCA, at various concentrations (0.01, 0.03, 0.1, 0.3, 1.0, or 3.0%) in the diet for one week. Messenger RNAs of genes involved in BA homeostasis were quantified using QuantiGene Plex assays. FXR in liver was activated by all the BAs and at all doses, indicated by induction of SHP mRNA in the livers. Correspondingly, the Cyp7a1 mRNA was reduced by all the BAs and at all doses. In contrast, the activation of the FXR-Fgf15 signaling pathway in the ileum is BA species dependent: the 12-OH BAs (CA and DCA) increased Fgf15 mRNA expression at all doses, whereas, the non-12-OH BAs (CDCA and LCA) increased Fgf15 mRNA expression only at high doses. Inversely to the activation pattern of FXR-Fgf15 signaling, Cyp8b1 was suppressed by the 12-OH BAs at all doses, and by the non-12-OH BAs at high-hepatotoxic doses. BAs had less of an effect on BA transporter expression; only high-

hepatotoxic doses of BAs decreased Ntcp. In addition, all doses of the BAs induced the mRNA of Abcg5/g8, whereas, only at high-hepatotoxic doses did all the BAs increased Mdr2 mRNA expression.

In summary, these results indicated that the hepatic FXR-SHP signaling pathway in the liver appears to mediate the suppression of Cyp7a1 by BAs, whereas, the FXR-Fgf15 signaling pathway in the ileum appears to mediate the suppression of Cyp8b1 by all doses of the 12-OH BAs and by the high-hepatotoxic doses of the non-12-OH BAs. The suppression of Cyp7a1 appears to be sufficient to maintain the total liver BA concentration when mice are fed BAs at non-hepatotoxic doses, whereas, the suppression of the BA uptake transporter Ntcp, in addition to the suppression of Cyp7a1, is needed to maintain the total BA concentration in livers of mice fed high-hepatotoxic doses of BAs. The suppression of Cyp8b1 results in an imbalanced BA composition which may result in hepatotoxicity. Moreover, the increased expression of Abcg5/g8 and Mdr2 may result in increased secretion of cholesterol and phospholipids that contributes to the formation of mixed micelles with BAs, and limits the hepatotoxicity of the fed-BAs.

The third study in my dissertation investigated the effects of BA sequestration by resin on the BA-receptor mediated signaling pathways. Mice were fed 2% resin in their diets for one week. The BAs in both serum and livers, as well as the mRNA involved in BA homeostasis were quantified. The data demonstrated that the resin tended to increase the total serum BA concentration, but significantly decreased β-MCA concentration 50% and increased CA and T-CA in serum. As expected,

feeding mice the resin decreased the total liver BA concentration 80%, which was due to an almost complete depletion of α-, β-, and ω-MCAs, as well as their taurine conjugates, and less of a decrease in most of the other BAs. In addition, the resin also altered the liver BA composition, featured by an increase in the percentage of the total CA and the secondary BA DCA. The mRNA of the BA-biosynthesis enzymes, Cyp7a1 and Cyp8b1, was increased 180 and 100% respectively. However, mRNA of the FXR target gene SHP in liver was not decreased. In contrast, the ileal Fgf15 mRNA expression was significantly decreased. Feeding the resin did not alter the BA uptake transporters on the sinusoidal membrane nor did it alter the efflux transporters on the canalicular membrane. In contrast, the efflux transporters on the sinusoidal membrane of hepatocytes, Mrp 3 and 4, were increased following resin treatment 22 and 150% respectively. In ileum, feeding the resin increased the uptake transporter, Asbt by 66%. Collectively, the data suggest that increased expression of Cyp7a1 and Cyp8b1 in mouse liver by resin administered through the diet appears to be due to a decrease in the ileal Fgf15 signaling pathway, rather than due to a decrease in hepatic FXR signaling.

In conclusion, my dissertation demonstrates that imbalanced BA composition without increases in total liver BA concentrations can cause hepatotoxicity. The doses used by most researchers in studying BA functions are hepatotoxic. To study physiological functions of BAs, non-hepatotoxic doses of individual BAs are recommended: 0.1% or lower in the diets for CA, CDCA, and UDCA, 0.03% for DCA, and 0.01% or lower for LCA. In addition, the altered liver BA composition after non-

hepatotoxic doses of BA-feeding are able to trigger BA receptor signaling pathways (including the hepatic FXR-SHP and the ileal FXR-Fgf15 mediated signaling pathways) which coordinately regulate the biosynthesis enzymes Cyp7a1 and Cyp8b1 to maintain the total liver BAs and to somewhat restore liver BA composition which is disturbed by administration of the exogenous BAs. The completely disturbed liver BA composition after hepatotoxic-doses of BA-feeding not only suppresses both Cyp7a1 and Cyp8b1, but also decreases the expression of Ntcp, which may contribute to the maintenance of the liver total BA concentration as well. Moreover, the decrease in BA concentration after resin-feeding interrupts the ileal FXR-Fgf15 signaling resulting in increased expression of Cyp7a1 and Cyp8b1.

ACKNOWLEDGEMENTS

I appreciate my mentor, Dr. Curtis D. Klaassen, for his guidance and support on my dissertation. His wise emphasis on the importance of dose response on functions of BAs impressed me most. In addition, his special management of variety of people provided me with special experience which will affect my life in some profound way.

I would also like to thank the members of my committee, Drs. Bryan Copple, Grace Guo, Bruno Hagenbuch, and Jeffrey Staudinger. Their intelligent suggestions and support greatly contributed to the success of my dissertation research.

I am also very grateful to professors Sara Li and Jonathan Li for their generous help in providing me the presentation skills, scientific suggestions, as well as instrumental support. I also thank faculty members, Drs. Hartmut Jaeschke, Karl K. Rozman, Sitta Sittampalam, Partha Krishnamurthy, Beth Levant, Xiaobo Zhong, and Li Wang for valuable suggestions on my research, as well as career development. I also appreciate research assistant professors, post-doctoral fellows, students, and research assistants who gave me generous help whenever I needed, including Drs. Guoli Dai, Cheryl Rockwell, Chuan Chen, Matt Dieter, Tamara Raphael Knight, Chunshan Gui, Yi Weaver, Bo Kong, Beth Honze, and Pan Wang, as well as Lin He, Noriko Esterly, and Margitta Lebofsky. I also want to express my thanks to the administrative staff, Rosa Meagher, Dorothy McGregor, Myrtle Sanders and Cody Tully for their great support during my study.

My greatest appreciation gave to my friends and my family, especially my husband Paul Wiley. Their wisdom has made me more mature. Their caring helped me go through the hardest time in my life. I show greatest respect and appreciation to all of them.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABREVIATIONS

ABC transporters: ATP-binding cassette (ABC) transporters

ACN: Acetonitrile

AF-2: Activation function-2

ALT: Alanine aminotransferase

ANIT: α-naphthyl-isothiocyanate,

AP: Alkaline phosphatase

AREs: Antioxidant responsive elements

Asbt: Apical sodium-dependent BA transporter

AST: Aspartate aminotransferase

BAL: Bile acid-CoA ligase

BAs: Bile acids

BARE: Bile acid response element

BAT: BA-CoA and BA-CoA:amino acid *N*-acyltransferase

Bsep: Bile salt export pump

CA: Cholic acid

CAR: Constitutive androstane receptor

CBP: CREB-binding protein

CDCA: Chenodeoxycholic acid

CH25H: Cholesterol 25-hydroxylase

CK-8: Cytokeratin 8

CPF/PHR/FTF: Cyp7a promoter-binding factor

CsA: Cyclosporine A

Cyp7a1: Cholesterol 7α-hydroxylase

Cyp27a1: Sterol 27-hydroxylase

Cyp46: Cholesterol 24-hydroxylase

Cyp7b1: Oxysterol 7β-hydroxylases

Cyp8b1: Sterol 12α-hydroxylase

CHD: Coronary heart disease

D2: Type 2 iodothyronine deiodinase

DCA: Deoxycholic acid

DISC: Death-inducing signalling complex

ECM: Extracellular matrix

EGFR: Epidermal-growth-factor receptor

Erk: Extracellular signal-regulated kinase 1/2

Fas: TNF receptor superfamily, member 6

FGF15: Fibroblast growth factor 15

FGFR4: Fibroblast growth factor receptor

FoxO1: Forkhead box protein O1

FXR: Farnesoid X receptor

GCA: Glycocholic acid

GCDCA: Glycochenodeoxycholic acid

GDCA: Glycodeoxycholic acid

GLCA: Glycolithocholic acid

GP: Glucogen phosphorylase

GSH: Glutathione

GUDCA: Glycine-conjugated ursodeoxycholic acid

HDCA: Hyodeoxycholic acid

HSCs: Hepatic stellate cells

Ibabp: Ileal bile acid binding protein

ICAM-1: Intercellular adhesion

IR-1: Inverted repeat motif

JNK: c-jun N-terminal kinase

LRH-1/NR5A1: Liver receptor homolog-1

LXR: Liver X receptor (LXR)

LCA: Lithocholic acid

MAPK: Mitogen-activated protein kinase

αMCA, α-muricholic acid

βMCA, β-muricholic acid

ωMCA, ω-muricholic acid

MDCA, Murideoxycholic acid

Mdr: Multidrug resistance protein

MPTP: Mitochondria permeability transition pores

Mrps: Multidrug resistance-associated proteins

Nqo1: NAD(P)H:quinone oxidoreductase

Nrf2: Nuclear factor erythroid 2-related factor 2

Ntcp: Na^+/taurocholate cotransporting polypeptide

Oatps: Organic anion transporting polypeptides

Ostα/β: Organic solute transporters alpha or beta

PBC: Primary biliary cirrhosis

PEPCK: Phosphoenopyruvate carboxykinase

PFIC1: Progressive familiar intrahepatic cholestasis type 1

PFIC2: Progressive familial intrahepatic cholestasis type-2

PGC-1α: PPAR gama coactivator-1α

PI3K: Phosphatidylinositol-3-kinase

PKC: Protein kinase C

PPP1R16A: Membrane-associated subunit of protein phosphatase 1

PXR: Pregnane X receptor

RAR: Retinoic acid receptor

ROS: Reactive oxygen species

RXR: Retinoid X receptor

SLC: Solute carrier

SHP: Small heterodimer protein

SREBPs: Sterol regulatory element-binding proteins

TCA: Taurocholic acid

TCDCA: Taurochenodeoxycholic acid

TDCA: Taurodeoxycholic acid

TLCA: Taurolithocholic acid

TUDCA: Tauroursodeoxycholic acid

TGFb1: Transforming growth factor β1

TNFα: Tumor necrosis factor α

UDCA: Ursodeoxycholic acid

VDR: Vitamin D receptor

VCAM-1: Vascular adhesion molecule

CHAPTER 1

INTRODUCTION

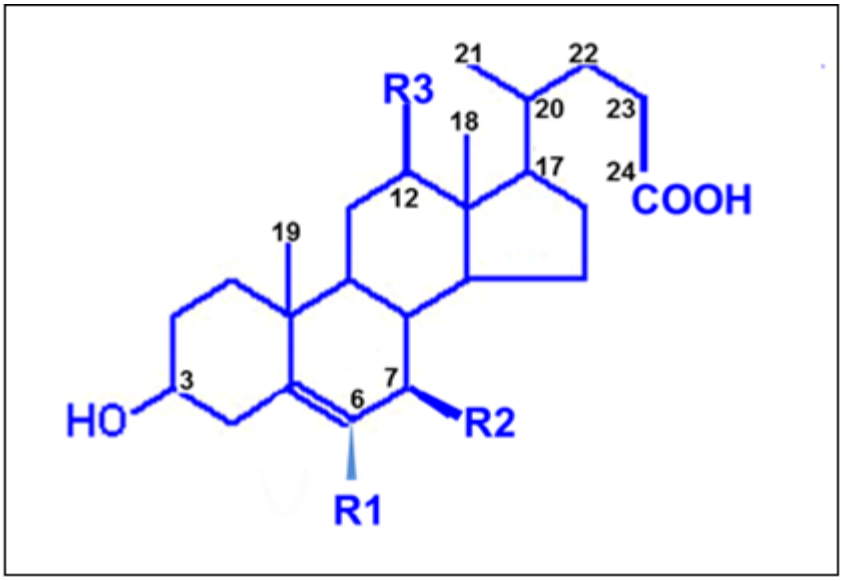
1.1 BA Biochemistry

1.1.1 BA Structures

The composition of BAs in bile varies among species. In humans and rats, the primary BAs are cholic acid (CA) and chenodeoxycholic acid (CDCA), whereas in mice, CA and muricholic acids (MCAs, including α-, and β-MCA) are the major primary BAs in bile; in bears, ursodeoxycholic acid (UDCA) is the major BA (Elias, 1999). In humans, the primary BAs can be further metabolized by intestinal bacteria producing the secondary BAs: deoxycholic acid (DCA) from CA, and lithocholic acid (LCA) from CDCA. In rodents, the primary BAs α-, and β-MCA can be further metabolized into ω-MCA. The primary BAs are relatively more water soluble, and less toxic than the secondary BAs (Salvioli et al., 1982; Botham and Boyd, 1983; Lida et al., 1989; Miki et al., 1993; Eyssen et al., 1999).

The basic structure of BAs is characterized by the steroid nucleus of four fused hydrocarbon rings and a hydroxyl group at the 3-carbon position of the steroid nucleus (Fig 1-1). Various BAs differ in the presence of additional hydroxyl groups at the 6-, 7- and/or 12-carbon positions, and the orientation of hydroxyl groups on the steroid nucleus. CA has both a 7α-hydroxyl group and a 12-hydroxyl group; whereas CDCA has only a 7α-hydroxyl group in addition to the 3α-OH group. DCA has one additional hydroxyl group at the 12-carbon position, whereas there are no hydroxyl groups at either the 7- or 12-carbon position of LCA. UDCA, an isomer of CDCA, has a β-hydroxyl group instead of α-hydroxyl group at the 7-carbon position

(Fig 1-1). In rodents, the primary BA, in addition to CA is muricholic acid (MCA) which has hydroxyl groups in the 6 and 7 carbon position (Radominska et al., 1993; Fujino et al., 2004; Thomas et al., 2008) .



BAs	R1	R2	R3
Cholic Acid (CA)	**H**	**OH(α)**	**OH**
Chenodeoxycholic Acid (CDCA)	**H**	**OH(α)**	**H**
Deoxycholic Acid (DCA)	**H**	**H**	**OH**
Hyodeoxycholic Acid (HDCA)	**OH (α)**	**H**	**H**
Lithocholic Acid (LCA)	**H**	**H**	**H**
Muri-Cholic Acid (MCA)	**OH**	**OH**	**H**
α Muri-Cholic Acid (αMCA)	**OH (β)**	**OH (α)**	**H**
β Muri-Cholic Acid (βMCA)	**OH (β)**	**OH (β)**	**H**
ω Muri-Cholic Acid (ωMCA)	**OH (α)**	**OH (β)**	**H**
Murideoxycholic Acid (MDCA)	**OH (β)**	**H**	**H**
Ursodeoxycholic Acid (UDCA)	**H**	**OH(β)**	**H**

Fig 1-1. Structure of the most common BAs (Eyssen et al., 1999; Fujino et al., 2004; Thomas et al., 2008)

1.1.2. Biosynthesis of BAs

BAs are natural end products of cholesterol catabolism. Various cytochrome-P450 enzymes are involved in the conversion of cholesterol to BAs. There are two major pathways involved in BA biosynthesis: the classic (neutral) pathway initiated by Cyp7a1, and the alternative (acidic) pathway initiated by Cyp27a1 (Norlin and Wikvall, 2007; Thomas et al., 2008).

Classic Pathway for BA Biosynthesis: Cholesterol 7α-hydroxylase Mediated Pathway

The classic BA biosynthesis pathway is also called the neutral pathway. BA synthesis through this pathway occurs uniquely in liver because the rate-limiting enzyme, cholesterol 7α-hydroxylase (Cyp7a1), is a liver-specific enzyme. In this classic pathway, the steroid nucleus is first modified by cytochrome-P450 enzymes, followed by the shortening and modification of the side chain. The first step of this pathway is the conversion of cholesterol into 7α-hydroxyl cholesterol, which is catalyzed by Cyp7a1. The intermediate product 7α-hydroxyl cholesterol can be hydroxylated by the Cyp8b1 enzyme which adds an additional hydroxyl group to the 12-carbon position, leading to production of CA. Without Cyp8b1, the intermediates will be used to produce CDCA (Fig 1.2) (Norlin and Wikvall, 2007).

Primary BAs are usually conjugated with amino acids, namely glycine and taurine, to produce either glycine or taurine conjugated BAs. These conjugated BAs are actively secreted into bile. Conjugated BAs are the major form of BAs excreted

by liver. In the intestine, bacteria convert the primary BAs into secondary BAs by deconjugation and dehydroxylation at the 7-position to produce DCA from CA, and LCA from CDCA. LCA can be further hydroxylated at the 6 carbon position to produce either hyodeoxycholic acid (HDCA) or murideoxycholic acid (MDCA). HDCA can also be formed by 7-dehydroxylation from MCA . The secondary BAs are more hydrophobic than their precursor BAs. LCA is the least water-soluble BA, and very little is reabsorbed and returned to the liver through the portal vein. Most of the other BAs, including CA, CDCA, and DCA are reabsorbed and recycled. This process is referred to as the enterohepatic circulation. In humans, the major products of the classic pathway are similar amounts of CA and CDCA.

Alternative (Acidic) Pathway: Sterol 27-hydroxylase Mediated Pathway

The alternative pathway of BA-biosynthesis is initiated in the mitochondria, either in the liver or in peripheral tissues. Cholesterol in the cytosol needs to be transported into the mitochondria by steroid-acute-response protein (StAR). Transport of cholesterol from cytosol into mitochondria is the rate-limiting step in the alternative pathway of BA biosynthesis. After cholesterol enters the mitochondria, Cyp27a1 catalyzes the hydroxylation of the side chain of cholesterol, which produces oxysterols. These oxysterols can be transferred from peripheral tissues to hepatocytes, where hydroxylation occurs at position 7 of the steroid nucleus by Cyp7b1 and at position 12 by Cyp8b1. The major product of the alternative pathway is CDCA. The CDCA produced in this pathway then undergoes further biotransformation, as in the neutral pathway, namely conjugation with glycine and

taurine, entry into enterohepatic circulation, conversion into secondary BAs by bacteria in the intestine, reabsorption and return to the liver (Norlin and Wikvall, 2007).

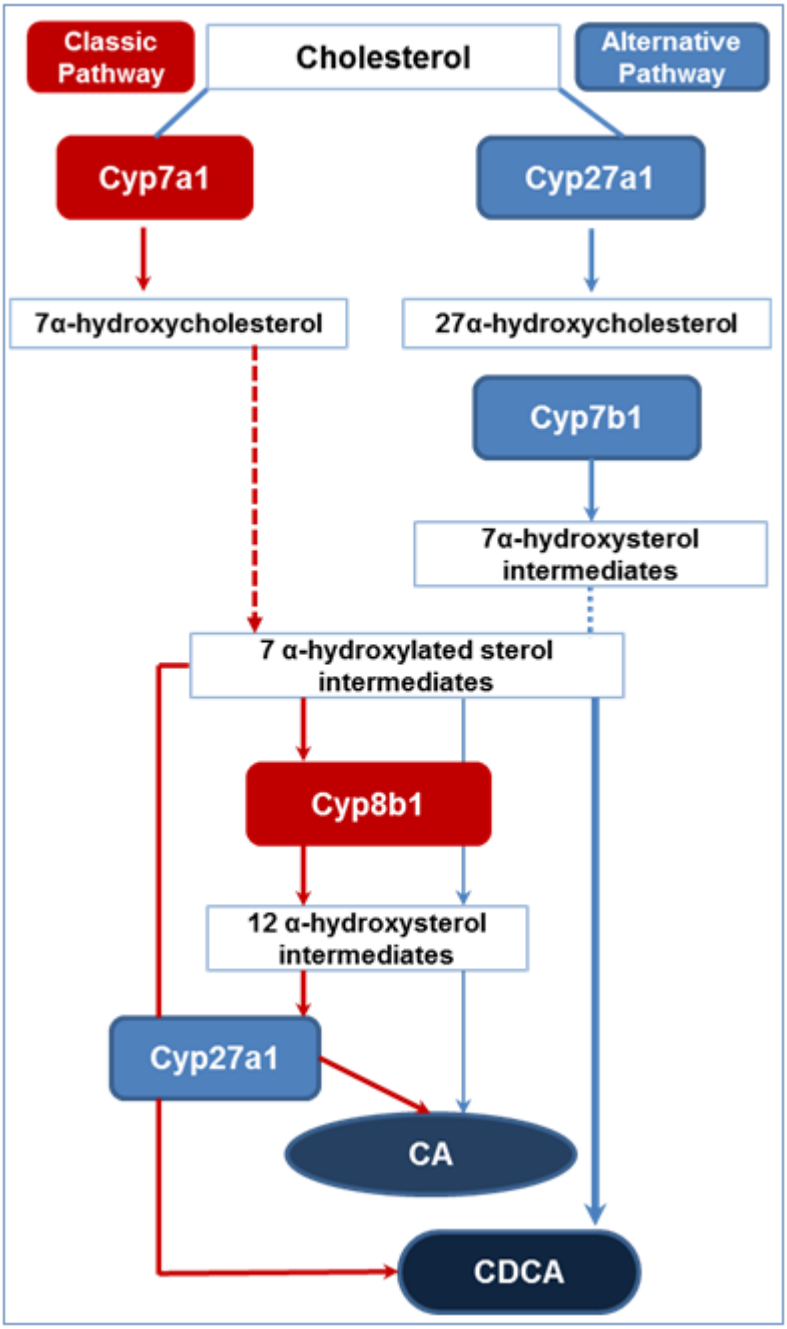


Fig 1-2. Major BA biosynthesis pathways: The classic (red arrows) and the alternative pathways (blue arrows). Cyp7a1, cholesterol 7α-hydroxylase; Cyp8b1, sterol 12α – hydrosylase; Cyp27a1, sterol 27-hydroxylase; Cyp7b1, oxysterol 7α-hydroxylase (Thomas et al., 2008).

Other Pathways

Cholesterol 25-hydroxylase and cholesterol 24-hydroxylase mediated pathways

In the classic pathway, cholesterol is initially converted in the liver to 7α-hydroxycholesterol by the rate-limiting enzyme, cholesterol 7α-hydroxylase (Cyp7a1). In the alternative pathway, cholesterol is first converted to oxysterols. In liver and extrahepatic tissues (e.g. macrophages), this involves sterol 27-hydroxylase (Cyp27a1) or cholesterol 25-hydroxylase (CH25H), whereas in brain, this step is exclusively catalyzed by cholesterol 24-hydroxylase (Cyp46). The oxysterols are then 7β-hydroxylated by oxysterol 7β-hydroxylases (Cyp7b1 and Cyp39a1) (Fuchs, 2003; Norlin and Wikvall, 2007)

In extrahepatic and intrahepatic tissues, only a very small fraction of cholesterol is converted into 25-hydroxycholesterol as a result of the very low levels of cholesterol 25-hydroxylase. A brain-specific Cyp enzyme, Cyp46, converts cholesterol into 24-hydroxycholesterol. These two oxysterols are then transferred into hepatocytes, where 25-hydroxycholesterol is catalyzed by Cyp7B1, whereas 24-hydroxycholesterol is catalyzed by Cyp39A1, producing 7α-hydroxylated oxysterols, which are further metabolized by Cyp27A1 to CA or CDCA (Fuchs, 2003; Norlin and Wikvall, 2007).

The contribution of BA products by these pathways differs among various species. In adult humans, the classic pathway contributes about 80% of CA and

CDCA, whereas the alternative pathway contributes less than 20% of BAs to the BA pool. It has been proposed that less than 5-16% of the total BAs are derived from the cholesterol 25-hydroxylase pathway. The cholesterol 24-hydroxylase pathway contributes least to the BA pool. In human infants, the percentage of CDCA in the gallbladder is very high. In addition, the Cyp7b1 enzyme is critical for fetuses to survive. The data suggest that a large contribution is made by the cholesterol 27-hydroxylase pathway in fetuses. In rats and mice, the alternative pathway of BA synthesis contributes much more than 50% of the total BAs (Fuchs, 2003).

Conjugation of BAs

Newly synthesized unconjugated BAs or deconjugated BAs from the enterohepatic circulation are extensively conjugated with either glycine or taurine in the liver. Two enzymes are critical in the conjugation process: bile acid-CoA ligase (BAL) catalyzes the formation of BA-CoA, and BA-CoA:amino acid *N*-acyltransferase (BAT) amidates BA-CoA with the amino acid glycine or taurine (Thomas et al., 2008).

BAT genes have been cloned from mouse (Falany et al., 1997), rat (Furutani et al., 1995), and human (Falany et al., 1994). The human BAT protein can conjugate BAs with both taurine and glycine (Falany et al., 1994), but the mouse BAT protein only catalyzes the conjugation of BAs with taurine (Falany et al., 1997).

1.2. Physiological Functions of BAs

1.2.1. Bile Formation and Enterohepatic Circulation of BAs

Formation of bile is an important function of liver. Bile is mainly composed of BAs, bilirubin, cholesterol, phospholipid, electrolytes, water, and various metabolites. Bile flow is driven by two forces: The active transport of BAs by hepatocytes into bile canaliculi mediates the BA dependent canalicular bile flow (Wheeler, 1972), whereas the active transport of the organic solute glutathione (GSH), and inorganic electrolyte bicarbonate (HCO_3^-) by hepatocytes or cholangiocytes mediates the BA independent bile flow. Active transport of BAs and other organic solutes generate osmotic gradients that facilitate the entrance of water and electrolytes into canalicular bile. Canalicular bile accounts for 75% of daily bile formation. Bile formed in the ductules and ducts, so-called ductualar bile, accounts for 25% of daily bile secretion (Ballatori and Truong, 1992; Tietz et al., 2003).

Canalicular bile formation depends on active hepatocellular transport systems. The hepatocytes are polarized epithelia with transporter proteins that actively take up or efflux bile components. Canalicular bile secretion is facilitated by active transport of BAs and other organic solutes via ATP-binding cassette (ABC) transporters. The bile salt export pump Bsep actively transfer glycine or taurine conjugated monovalent BAs, including conjugated CA, CDCA, DCA, and UDCA, from hepatocytes into Canaliculi (Gerloff et al., 1998). Multidrug resistance associated protein Mrp2 actively pumps the divalent BAs, such as sulfated LCA, into bile (Muller and Jansen, 1997). In addition, Mrp2 also actively transports reduced glutathione (GSH) (Trauner et al., 1998) and a variety of other anionic compounds (such as bilirubin glucuronide) conjugated with glutathione, glucuronates, or sulfate

into bile (Dubuisson et al., 1996; Cui et al., 1999; Keppler and Konig, 2000). The multidrug resistance 1 (Mdr1) mediates the canalicular secretion of cationic compounds, whereas, the Mdr2 mediates the excretion of phospholipid by transolcating the phospholipid from the inner to the outer leaflet of the membrane (Smit et al., 1993; Elferink et al., 1997). Abcg5/g8 mediates the biliary excretion of cholesterol. In addition, there are also efflux pumps located at the basolateral membrane of hepatocytes. Mrp3, Mrp4, and the heteromeric organic solute transporter Ostα/β pump BAs and other solutes from hepatocytes back into the systemic blood (Kullak-Ublick et al., 2004) .

BAs secreted into bile undergo enterohepatic circulation (EHC). EHC of BAs is important in maintaining the homeostasis of BAs. Two main processes are involved in the EHC: Liver secretion and intestinal absorption of BAs. After BAs are synthesized in liver, they are transported into bile canaliculi via various efflux transporters: Bsep and Mrps. BAs in the canaliculi enter and are stored in the gallbladder. Upon each meal, the gallbladder releases BAs into the intestine to emulsify dietary lipids and cholesterol to facilitate their absorption.

In the ileum, about 95% of the BAs are reabsorbed. The enterocytes take up BAs via the apical sodium dependent BA transporter (Asbt). Inside the enterocytes, the cytosolic ileal BA binding protein (Ibabp) mediates the transcelluar movement of BAs to the basolateral membrane of the enterocytes. The organic solute transporters (Ostα/β) pump BAs into the portal vein. BAs in the portal vein are transported back into the hepatocytes via two types of uptake transporters on the

sinusoidal membrane of hepatocytes: Na^{+}/taurocholate cotransporting polypeptide (Ntcp) and organic anion transport polypeptides (Oatps) (Alrefai and Gill, 2007). These BAs together with newly synthesized BAs then enter the next EHC cycle.

1.2.2. BA Regulation of Their Own Homeostasis

It is very important to maintain a balance between BA biosynthesis, transport and metabolism, because every aspect of BA homeostasis can affect various important physiological processes (Chawla et al., 2000; Houten et al., 2006). The genes involved in BA biosynthesis are tightly regulated. Since the discovery that nuclear receptors are involved in the regulation of BA biosynthesis pathways about ten years ago, there has been a marked increase in research in regulation of BA biosynthesis and transport (Grober et al., 1999; Downes et al., 2003)

BA Regulation of Their Biosynthesis

The most intensively studied gene in BA biosynthetic pathways is Cyp7a1 (Chiang and Stroup, 1994; Goodwin et al., 2000; Chiang et al., 2001; Kim et al., 2007), which is the rate limiting enzyme in the classic pathway of BA biosynthesis, followed by Cyp8b1 (Andersson et al., 1999; Lu et al., 2000; Sinal et al., 2000; Zhang and Chiang, 2001; Yang et al., 2002; Jahan and Chiang, 2005), which is the key enzyme determining the ratio of CA to CDCA in the BA pool, and Cyp27a1 (Araya et al., 2003; Chen and Chiang, 2003; Quinn et al., 2005), which is the key enzyme in oxysterol synthesis outside the liver. It has been known for a long time that Cyp7a1 is precisely regulated in the body. Cholesterol and its metabolites (e.g.

oxysterols) can up-regulate Cyp7a1 (Doerner et al., 1995; Lehmann et al., 1997; Menke et al., 2002; Xu et al., 2004), whereas BAs down-regulate Cyp7a1 (Pandak et al., 1994; Gupta et al., 2001; Xu et al., 2003). However, the underlying mechanisms by which Cyp7a1 is regulated were not well understood until the discovery that nuclear receptors are involved in the regulation of BA metabolism.

Introduction to Nuclear Receptors

Nuclear receptors comprise one of the superfamilies of transcription factors that regulate gene expression in a ligand dependent manner. According to their ligands, the nuclear receptor superfamily is divided into three groups (Mangelsdorf et al., 1995). The first group is the endocrine receptor subfamily whose ligands have high affinity to the corresponding receptors, for example, estrogen to the estrogen receptor. The second group is called the adopted-orphan-nuclear receptors whose ligands have low affinity to their corresponding receptors and are usually dietary lipids, for example, BAs are the ligands of farnesoid X receptor (FXR). The third group contains the so called orphan-nuclear receptors with ligands not yet identified, including SHP, LRH-1, HNF-4, etc.

Even though the nuclear receptor transcription factors regulate genes that span across a broad range, from development to physiology and homeostasis, they have similar modular structures. Generally five domains can be identified according to the conservation of DNA sequence and function of the regions: the N-terminal domain (A/B region), the DNA binding domain (DBD, C region), hinge domain (D region), ligand binding domain (LBD, E region), and C terminal domain (F region).

Among all these domains, the DBD is the most conserved domain which mediates DNA binding through two zinc-finger motifs; whereas the LBD is a less conserved domain and mediates ligand binding, dimerization, and ligand-dependent transactivation. Such ligand-dependent transactivation is called activation function-2 (AF-2), whereas the A/B region harbors a ligand-independent transactivation domain named AF-1 (Bourguet et al., 2000).

Ligands of nuclear receptors are usually molecules of small volume, low weight, and are relatively hydrophobic (Bogan et al., 1998). BAs are small amphipathic steroidal compounds, thus they have the preferred characteristics as nuclear receptor ligands. So far, several nuclear receptors have been identified as BA sensors, including farnesoid X receptor (FXR), pregnane X receptor (PXR), vitamin D receptor (VDR), and constitutive androstane receptor (CAR). These nuclear receptors mediate negative-feedback or feed-forward regulation of bile-acid biosynthesis, transport, and detoxification.

BA sensors: FXR, PXR, VDR and CAR

FXR as the BA sensor

The identification of FXR as a BA sensor in 1999 (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999) shed light on the mechanism for negative-feedback regulation of BA biosynthesis. Various BAs can bind to and activate FXR, with the ranking of binding affinity: CDCA>DCA>CA>>LCA (Lew et al., 2004). Like other class-II nuclear receptors, FXR is a heterodimer partner with retinoid X

receptor (RXR) (Mangelsdorf and Evans, 1995; Seol et al., 1995). Various models have been proposed for the negative-feedback regulation of Cyp7a1, the first and rate limiting-enzyme in the classic BA biosynthesis pathway.

Hepatic FXR-SHP-LRH-1 signaling pathway

In this signaling pathway, upon binding of BAs, FXR heterodimerizes with RXR, which recognize and bind to an inverted repeat motif (IR-1) in the promoter of SHP (Small Heterodimer Partner), and up-regulates SHP gene expression. SHP is an atypical nuclear receptor without a DNA-binding domain. It interacts with various nuclear receptors, e.g LXR, PXR, LRH-1, HNF-4, etc through its N-terminal dimerization domain, and inactivates transactivation, mediated by the corresponding nuclear receptors (Lee et al., 2000; Brendel et al., 2002; Ourlin et al., 2003). In the FXR-SHP-LRH-1 pathway, increased SHP protein binds to and inhibits LRH-1, which is necessary for activation of Cyp7a1 by LXR, resulting in decreased expression of Cyp7a1 (Goodwin et al., 2000).

FXR-mediated SHP-independent pathways involved in feedback regulation of Cyp7a1

Even though SHP is important in Cyp7a1 gene regulation (Boulias et al., 2005), SHP-knockout mice show only a slight impairment in the negative-feedback regulation of Cyp7a1 (Kerr et al., 2002). This suggests that other SHP-independent pathways may be present. Holt and colleagues found a FGF19/FGF15-dependent pathway that involves Cyp7a1 gene expression (Holt et al., 2003). In this pathway,

BA activated FXR in the enterocytes, causing increased expression of Fgf15. The Fgf15 protein enters the portal blood and travels to the liver where it activates its receptor FGFR-4 on the hepatocytes, causing decreased expression of Cyp7a1. (Holt et al., 2003; Inagaki et al., 2005). Further study using the strong FXR agonist GW4064 and tissue specific FXR null mice showed that the intestinal FXR is more important than the hepatic FXR in suppression of Cyp7a1 expression (Kim et al., 2007; Chiang, 2009).

βKlotho is also involved in the regulation of BA biosynthesis. Ito and colleagues (Ito et al., 2005) showed that mice lacking βKlotho have increased *Cyp7a1* expression and BA biosynthesis, and are resistant to the formation of gallstones. βKlotho is structurally similar to Klothos protein, which is located on cell membranes. Both proteins belong to the type-1 membrane proteins and have 2 family 1 glycosidase-like domains, but they lack enzyme activity domains (McCarter and Withers, 1994; Davies and Henrissat, 1995). The tissue distribution of βKlotho is mainly in liver, adipose tissues, and pancreas (Ito et al., 2000). βKlotho-KO and FGFR4-KO mice have similar phenotypes, including increased BA biosynthesis and small gallbladders, suggesting that these proteins may act through a common pathway (Moschetta and Kliewer, 2005). Later, it was shown that βKlotho and FGFR4 are co-receptors mediating intracellular gene regulation by Fgf15. Knock out of either FGFR4 or β-Klotho will abort the effects of Fgf15 on suppression of Cyp7a1 (Lin et al., 2007; Wu et al., 2007).

PXR as a BA sensor

PXR can be activated by a broad range of xenobiotics with dramatically different structures (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998; Savas et al., 2000; Staudinger et al., 2001b). Such ligand characteristics of PXR fit its structural features very well. PXR has a highly conserved DNA-binding domain (DBD) with more than 90% identity among all its orthologs, and low conserved ligand-binding domain (LBD) with only about 70% identity among all of its orthologs (Bertilsson et al., 1998; Blumberg et al., 1998).

Even though PXR is called the xenobiotic receptor, it can be activated by a variety of endogenous ligands, including BAs (Kliewer and Willson, 2002; Kliewer, 2003; Wagner et al., 2005). Upon activation by BAs, especially LCA, PXR regulates the expression of genes involved in BA metabolism and transport, for example, decreasing Cyp7a1 expression, as well as increasing the expression of Cyp3a enzymes and the efflux transporter Bsep (Makishima et al., 1999; Staudinger et al., 2001a; Watkins et al., 2001; Xie et al., 2001; Eloranta and Kullak-Ublick, 2005; Wagner et al., 2005). The overall effect of PXR activation is to decrease the concentration of BAs in hepatocytes and provide protective effects to the liver (Staudinger et al., 2001b; Kliewer and Willson, 2002).

It has been known for over 30 years that PCN can reduce the expression of Cyp7a1 (Mason and Boyd, 1978). PCN is an activator of mouse and rat PXR. In PXR-null mice, inhibition of Cyp7a1 by PCN treatment is disrupted (Staudinger et al., 2001a). This finding provided direct evidence that PXR mediates negative

regulation of BA biosynthesis. The finding that LCA is an endogenous activator for PXR (Staudinger et al., 2001b) suggested that PXR may play an important role in BA homeostasis. In cholestatic patients, the concentration of LCA in liver reaches 5-10 μM (Setchell et al., 1997). The IC_{50} of LCA and its 3-keto metabolite to human PXR is ~10μM (Staudinger et al., 2001b). These findings suggest that activation of PXR in cholestasis may provide an auto-protective mechanism for the liver. PXR activation by LCA inhibits the gene expression of Cyp7a1 resulting in less BA synthesis. Activation of PXR also increases gene expression of Cyp3a such that more water soluble BAs will be excreted into urine (Staudinger et al., 2001a).

VDR as a BA sensor

In addition to FXR and PXR, VDR was found to be another BA sensor, especially for LCA. Like other nuclear receptor family members, VDR also has the standard module structure of nuclear receptors and it also heterodimerizes with RXR to fulfill its function upon binding by its activator, e.g. vitamin D. VDR mediates important homeostasis with regard to calcium. In addition, VDR also affects differentiation and function of cells in the immune system (Liblau et al., 1995), and functions as a receptor for LCA (Makishima et al., 2002). They reported that at concentrations much lower than that needed for activation of either PXR (EC_{50}=100μM) or FXR (EC_{50}=7μM), LCA can activate VDR in the intestine (Makishima et al., 2002). Activated VDR can up-regulate its target gene Cyp3A (Schmiedlin-Ren et al., 1997; Thummel et al., 2001), which metabolizes LCA and many drugs. Thus the VDR in the intestine provides another protective mechanism

for the body to diminish the toxic effects of LCA, namely, activated VDR may increase the degradation of LCA in the intestine before it reaches the liver through the portal system.

CAR as a BA sensor

CAR is a nuclear receptor that resides close to the cell membrane by forming part of a protein complex with the membrane-associated subunit of protein phosphatase 1 (PPP1R16A, abbreviated as R16A) protein (Sueyoshi et al., 2008). Upon stimulation by a CAR inducer, such as phenobarbital, two R16A subunits dimerize and inhibit the activity of protein phosphatase 1β, triggering CAR translocation into the nucleus. In addition, dephosphorylation of threonine 38 of CAR is also necessary for its nuclear translocation and activation (Mutoh et al., 2009). Inside the nucleus, CAR functions to modulate the transactivation of its target genes (Sueyoshi et al., 2008). CAR is considered mainly as a xenobiotic nuclear receptor, however CAR activators also include endobiotics, such as BAs, especially toxic LCA (Guo et al., 2003; Qatanani and Moore, 2005). BAs at high concentrations can induce a CAR target gene sulfotransferase Sult2a1 (Ding et al., 2006). Sult2a1 catalyzes the sulfonation of LCA under normal conditions and other BAs under cholestatic conditions (Chen et al., 1978; Macrides et al., 1994). Increased BA sulfates contribute to enhanced urinary elimination of BAs, and thus decrease the toxicity of BAs. A recent study demonstrated that loss of both PXR and CAR in mice increases their sensitivity to BA toxicity (Uppal et al., 2005), indicating important roles of both PXR and CAR in regulating BA homeostasis.

Other factors that mediate BA regulation of BA homeostasis

BAs can activate multiple isoforms of protein kinase C (PKC) (Stravitz et al., 1995; Stravitz et al., 1996). Activated PKC leads to repression of Cyp7a1 (Stravitz et al., 1995). BAs can activate macrophages in liver, which then produce TNFα. The latter molecule can activate JNK (c-jun N-terminal kinase) (Miyake et al., 2000). It has been suggested that the JNK-mediated pathway may crosstalk with the FXR-SHP mediated pathway through activation of SHP-1 (Gupta et al., 2001).

The BARE (bile acid response element) in the Cyp7a1 promoter region has a binding motif for HNF4α (Crestani et al., 1998). Upon binding to the promoter, HNF4α recruits coactivators to the Cyp7a1 promoter region, leading to the induction of Cyp7a1. The transcriptional activity of HNF-4α on Cyp7a1 is repressed by the c-jun N-terminal kinase (JNK) signaling pathway (De Fabiani et al., 2001). The underlying mechanism for such repression is thought to be due to phosphorylation of HNF4α and subsequent dissociation of PGC-1α (PPAR gama coactivator-1α) and CREB-binding protein (CBP) from HNF4α (De Fabiani et al., 2003).

BA regulation of transport

Liver is a detoxification organ for many chemicals, and has various types of transporters in hepatocyte membranes. Numerous chemicals enter into and come out of hepatocytes. The transport of many chemicals is mediated by specific transporters located in basolateral or canalicular membranes. The transporters

involved in BA transport include: Ntcp, Oatps, Bsep, and Mrps (Meier and Stieger, 2002).

Hepatic uptake of BAs at the basolateral surface of hepatocytes:

The hepatic basolateral transporters, Na^+/taurocholate cotransporting polypeptide (Ntcp), and organic anion transporting polypeptides (Oatps), are the main uptake transporters for BAs to enter hepatocytes. Ntcp is the main sodium-dependent transporter that transports BAs together with 2 Na^+ across the hepatocyte membrane. The sodium concentration gradient produced by Na^+/K^+ ATPase is the driving force for Ntcp to transport BAs across the hepatocyte membrane (Hagenbuch and Meier, 1994; Jacquemin et al., 1994; Kouzuki et al., 2000; St-Pierre et al., 2001).

Several members of the Oatp family Na^+-independently transport BAs and other organic anions into hepatocytes. Rodent Oatp1a1 (previously called Oatp1), Oatp1a4 (previously called Oatp2) and Oatp1b2 (previously called Oatp4) are Oatps that are involved in bile-acid uptake by hepatocytes. These Oatps are located in the sinusoid membrane of hepatocytes (Jacquemin et al., 1994; Kouzuki et al., 2000; Cattori et al., 2001)

Hepatic excretion of BAs at the canalicular/apical surface of hepatocytes:

BAs are actively pumped out of hepatocytes into the canaliculi through canalicular ATP-binding cassette (ABC) transporters. Amino acid conjugated BAs

are mainly exported by the bile salt export pump (Bsep). The sulfated and glucuronidated forms of BAs are transported mainly by Mrp2 (Meier and Stieger, 2002). Mrp3 also mediates the excretion of BAs, but it mediates the transport of BAs from hepatocytes to sinusoids (Liblau et al., 1995; Hirohashi et al., 2000). Under cholestatic conditions, most transporters involved in BA transport are down-regulated, with the exception of Mrp3 which is up-regulated. The up-regulation of Mrp3 is thought to be a compensatory mechanism that the body uses to eliminate extra BAs from hepatocyte to blood, for further excretion into urine (Soroka et al., 2001).

1.2.3 BA Regulation of Lipid Homeostasis

It is well established that BAs regulate cholesterol homeostasis by facilitating its elimination into bile. In addition, BAs also affect triglyceride homeostasis. Clinically, treatment with cholestyramine resin for hypercholesterolemia causes increased VLDL (Grundy et al., 1971; Nestel and Grundy, 1976). The inhibitory effects of BAs on the biosynthesis of hepatic fatty acids and the production of VLDL are mediated by the FXR-SHP signaling pathway (Watanabe et al., 2004). SHP decreases the expression of sterol regulatory element-binding proteins (SREBPs), the transcription factors controlling cholesterol homeostasis.

1.2.4 BA Regulation of Energy Homeostasis

The effects of BAs on energy homeostasis was noticed by their inhibition of diet-induced obesity. The finding of a novel G protein-coupled cell-surface BA

receptor, TGR5, in brown fat tissue and skeleton muscle provided the mechanistic basis for BA regulating energy homeostasis (Maruyama et al., 2002; Kawamata et al., 2003; Watanabe et al., 2006). Briefly, CA can bind and activate TGR5 that triggers an intracellular signaling pathway, including increased production of cAMP (Houten et al., 2006). This signaling pathway increases the activity of type 2 iodothyronine deiodinase (D2), which increases the conversion of inactive thyroid hormone T4 to the biologically active T3, resulting in an increased metabolic rate (Watanabe et al., 2006).

1.2.5 BA Regulation of Glucose Homeostasis

Classically, BAs are identified as endogenous detergents facilitating lipid absorption and cholesterol homeostasis. Recent progress in BA research indicates that BAs may be very important in regulating glucose homeostasis. The possible regulatory effects of BAs on glucose homoestasis were noticed more than ten years ago. The hyperglucosemic condition in patients with both hyperlipidimia and type 2 diabetes was improved, after treatment with the BA sequestrant cholestyramine (Garg and Grundy, 1994). In 2003, it was found that BAs regulate gluconeogenesis in a FXR-dependent way (De Fabiani et al., 2003). In 2004, BAs were also found to be able to regulate glucose homeostasis in a SHP-dependent way (Yamagata et al., 2004).

Up to now, several mechanistic hypothesis have been proposed for BA regulation of glucose homeostasis: 1). BAs stimulate glycogen phosphorylase (GP) via mobilization of intracellular calcium, which results in an increase in the

breakdown of glucogen to glucose-1-phosphate (Bouscarel et al., 1993; Matsuzaki et al., 1997; Fang et al., 2007). 2). BAs induce the expression of phosphoenopyruvate carboxykinase (PEPCK) via activation of FXR. Activated FXR also increases insulin sensitivity resulting in decreased serum glucose levels (Ma et al., 2006; Zhang et al., 2006).

It was also reported that various BAs have different effects on gluconeogenesis. UDCA treatment seemed to be able to increase glycogen breakdown, in contrast, CA and DCA treatments seemed to be able to cause synthesis of glycogen (Zhang et al., 2006). More research is needed to address the different effects of BAs on glucose metabolism.

1.3 Hepatotoxicity of BAs

1.3.1 Cholestasis

Bile formation is a critical function of the liver. Normal bile formation depends on four factors: 1) Integral transmembrane transport systems: actively transporting BAs and other solutes to generate the osmotic gradients for bile flow, 2) intact cytoskeleton system: moving vesicles across the hepatocytes and maintaining normal bile canalicular contractions, 3) cell-cell connections: sealing off the bile canaliculi and maintaining cell polarity, and 4) normal signal transduction: regulating and coordinating the various processes involved in bile formation (Burwen et al., 1992). Any disruptions interfering the above four aspects of normal bile secretion can cause cholestasis (Popper and Schaffner, 1963). During cholestasis, retention

of hydrophobic BAs within the liver causes major pathophysiologic changes in the liver (Greim et al., 1972; Fischer et al., 1996).

Clinically, cholestatic liver diseases include a wide variety of hepatobiliary diseases of diverse etiologies that are characterized by impaired hepatocellular secretion of bile, resulting in the accumulation of BAs, bilirubin, and cholesterol. Pharmacological therapy for cholestasis is limited. Thus, to provide new therapeutic management of cholestatic liver diseases, it is critical to fully understand the mechanisms of BA toxicity. Several cholestatic liver injury models have been used to study the mechanisms of BA toxicity, including common bile duct ligation, drug induced cholestasis (e.g. α-naphthyl-isothiocyanate, ANIT, induced cholestasis), and BA over-loading induced cholestasis (Plaa and Priestly, 1976).

Concept, symptoms/signs and morphology of cholestasis

The hepatotoxicity of BAs was initially noticed under pathological conditions, namely cholestasis. Cholestasis is defined as the blockage or impairment of bile secretion and bile flow with retention of BAs and other bile components inside the liver and body. Cholestasis may arise as a functional secretory disturbance of hepatocytes and/or any obstruction occurring from the level of the liver parenchymal cells to the Papilla of Vater in the duodenum (Desmet, 1995). Based on the location of the primary injury in the bile secretory path, cholestasis can be classified as intrahepatic cholestasis or extrahepatic cholestasis. In intrahepatic cholestasis, the injured bile secretion is primarily present inside the liver, including diseases at the hepatocyte level and diseases at the intrahepatic bile duct level. In

extrahepatic cholestasis, the bile excretory blockage is present outside the liver along the extrahepatic bile ducts, thus it is also called obstructive cholestasis (Desmet, 1995).

A common consequence of cholestasis is hepatic and systemic accumulation of toxic biliary compounds, such as bilirubin, cholesterol, and BAs, especially the toxic lithocholic acid (LCA) whose level is 3-5 times higher in cholestasic than normal liver (Attili et al., 1986; Schmucker et al., 1990; Heuman et al., 1991; Trauner et al., 1998). Increased BA concentrations in liver contribute to the pathophysiological and histological changes in liver, including increased serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), and total bilirubin, hepatic necrosis, and lymphocytic infiltration mediated inflammation. In the final stage of cholestasis, liver fibrosis usually occurs, which often requires a liver transplant to save the patient's life (Li and Crawford, 2004).

In addition, cellular ultrastructural changes are observed in cholestatic liver diseases. Under the electron microscope, the canaliculi usually dilate and lose their microvilli (Schaffner and Popper, 1959). Cells around the abnormal canaliculi have characteristic ultrastructure changes: Denser cytoplasm than normal, increased smooth endoplasmic reticulum, and dilated profile of the Golgi zone. In the abnormal canaliculi, bile plugs are often seen. The bile plugs are mixtures of polysaccharide and two types of deposits: a deposit of pigment in a finely fibrillar

shape, and a deposit of liquid crystals of lecithin/cholesterol/BA mixture (Popper and Schaffner, 1963; Biava, 1964a; Biava, 1964b; Popper, 1968).

Hepatotoxicity of BAs during Cholestasis and the Mechanisms for their Hepatotoxicity

BA induced liver cell death: apoptosis and necrosis

Apoptosis as well as necrosis are common features in cholestatic liver diseases, or in experimental cholestatic disorder models, such as bile duct ligation, ANIT-induced cholestasis, or LCA feeding induced cholestasis (Desmet et al., 1968; Rodriguez-Garay, 2003; Fickert et al., 2006; Hatano, 2007). Even though cell death during cholestasis might be caused by multiple factors, it is evident that BAs, especially relatively hydrophobic BAs, are responsible for both apoptotic and necrotic cell death (Gujral et al., 2004b; Li and Crawford, 2004).

All BA molecules are comprised of two parts, the hydrophobic steroid nucleus and the hydrophilic side chain. The addition of hydroxyl groups on the steroid nucleus increases the water solubility of BAs. The amphipathic characteristics of BAs give them detergent like activities (Hofmann and Small, 1967). Such detergent-like properties of BAs make them good solvents for cholesterol under physiological conditions. However, the surfactant effects of BAs also make them toxic to cell membranes, causing cell swelling, and cell death. The more hydrophobic a BA is, the more toxic to the membrane is the BA (Scholmerich et al., 1984; Attili et al., 1986). After incubation of different BAs with primary rat hepatocytes, CA and TCA

did not cause cell damage, even at concentration of 1500 µM, whereas, CDCA and its taurine conjugates, and DCA cause cell lysis at concentrations above 500 µM. TDCA is more toxic than its unconjugated counterpart DCA in damaging the cellular membrane. Whereas, the most hydrophobic BA, TLCA, at concentrations as low as 30 µM, caused cell lysis. Accordingly, BAs during cholestasis might cause necrosis due to their detergent-like effects.

Apoptosis is programmed cell death. Apoptosis is characterized by distinctive morphological changes: shrinkage of the cells, condensation of nuclear chromatin against the nuclear membrane, DNA fragmentation, and formation of acidophilic bodies or apoptotic bodies in the liver (Searle et al., 1982). Two main signaling pathways are involved in BA-induced apoptosis: one is the death-receptor-mediated apoptosis (extrinsic pathway), the other is the mitochondria-dysfunction-mediated apoptosis (intrinsic pathway) (Jaeschke et al., 2002; Hatano, 2007).

Mechanisms for Cytotoxicity of BAs: Death Receptor Mediated Cytotoxicity

BAs cause hepatocyte apoptosis partially via activation of the death receptor, such as the TNF-α receptor, during cholestasis (Hatano, 2007). But in TNF-receptor knock-out mice, apoptosis was still present after bile-duct ligation, suggesting that other signaling pathways may be involved in BA-induced apoptosis. It was also found that the toxic effects of CDCA was partially due to its direct activation of the death receptor Fas/CD95, which leads to hepatocyte apoptosis (Faubion et al., 1999). Further research found that BAs can increase transport of FAS from cytosol

to the plasma membrane of hepatocytes to enhance the FAS-mediated apoptotic process (Faubion et al., 1999; Sodeman et al., 2000; Jaeschke et al., 2002). An *in vitro* study showed that glycine-conjugated chenodeoxycholic acid (GCDCA) induces apoptosis via activation of FAS (Patel et al., 1994). Fas activation by BAs is initiated by a NADPH oxidase derived oxidative stress signal (Reinehr et al., 2005) that activates c-Jun-N-terminal kinases (JNK) (Leppa and Bohmann, 1999) and the Src Kinase family member Yes. Activated Yes rapidly activates the epidermal-growth-factor receptor (EGFR) (Reinehr et al., 2003) that catalyzes FAS tyrosine phosphorylation in a JNK-dependent way. The phosphorylation of FAS leads to FAS-membrane trafficking, formation of the death-inducing signalling complex (DISC) and induction of apoptosis (Reinehr and Haussinger, 2007).

Except for the classic death receptor, via which BAs induce apoptosis, BAs can cause recruitment of caspase-8 into the death-inducing signaling complex (DISC), and hepatocyte apoptosis via activation of cellular surface receptor TGR5 and JNK signaling (Yang et al., 2007).

Mechanisms for Cytotoxicity of BAs: Mitochondria Medicated BA Cytotoxicity

The other mechanism via which hydrophobic BAs cause liver injury is a mitochondria-mediated pathway. Hydrophobic BAs can cause increased production of reactive oxygen species (ROS) via mitochondria, or via activation of NADPH oxidase (Sokol et al., 1995; Rolo et al., 2003a; Becker et al., 2007). ROS cause opening of mitochondria permeability transition pores (MPTP) in the inner

membrane. Non-permeable solutes that normally are unable to enter the mitochondria can freely enter the mitochondria via the MPTP, causing depolarizaqtion of the mitochondria membrane and failure to synthesize ATP. Dysfunctional mitochondria then release cytochrome *c,* which causes caspase activation, and further triggers mitochondria-mediated cell death (Kantrow et al., 2000; Halestrap and Brennerb, 2003). The generation of ROS is critical for MPT, as antioxidants and cyclosporine A (CsA) protect cultured hepatocytes from BA-induced cell death (Gores et al., 1998; Yerushalmi et al., 2001; Rolo et al., 2003a; Rolo et al., 2003b; Rolo et al., 2004).

Inflammatory liver injury during cholestasis

Neutrophil infiltration is a common feature of cholestatic liver diseases (Louis et al., 1998; Thompson et al., 1998; Gujral et al., 2003; Li and Crawford, 2004; Fickert et al., 2006). It has been concluded that neutrophil mediated inflammation contributes to liver injury during cholestasis (Jaeschke et al., 2002; Gujral et al., 2004a; Kodali et al., 2006). One chemical known for its inflammation-mediated hepatotoxicity is α-naphthylisothiocyanate (ANIT). ANIT causes hepatotoxicity in a neutrophil-dependent manner. Even though ANIT can cause direct cell death, neutrophil infiltration is thought to worsen the liver injury (Dahm et al., 1991). During cholestasis, the increased concentrations of hydrophobic BAs might initiate the liver cell injury. The injured liver cells then release various cytokines and chemokines that contribute to the neutrophil infiltration into the liver and subsequent exacerbation of liver injury. Several critical steps are involved in neutrophil

infiltration into the liver and its killing of hepatocytes: Priming or systemic activation of neutrophils and accumulation of neutrophils in the liver vasculature, extravasation, and adherence to hepatocytes are thought to execute the cytotoxic effects (Jaeschke et al., 2002; Fickert et al., 2006; Ramaiah and Jaeschke, 2007). Proinflammatory mediators, such as cytokines and chemokines are critical in neutrophil-mediated liver injury. To extravate the vasculature system into the parenchyma of the liver, the mechanically trapped neutrophils in the vasculature of the liver may interact with intercellular adhesion molecule (ICAM-1) or vascular adhesion molecule (VCAM-1) of the liver cells via integrin β1 molecules. The extravasation process of the neutrophils is also a process for full activation of the neutrophils. The superoxide and proteases produced by the activated neutrophils via NADPH oxidase and degranulation causes further release of cytokines, which promotes further inflammation and hepatocyte oncotic necrosis (Fickert et al., 2006).

Chronic liver damage usually leads to liver fibrosis. Activation of hepatic stellate cells (HSCs) is thought to be a critical step during liver fibrogenesis (Svegliati-Baroni et al., 2008). During chronic liver injury, including cholestasis, HSCs are activated by proinflammatory cytokines or growth factors, such as tumor necrosis factor α (TNFα) and transforming growth factor β1 (TGFb1) (Leyland et al., 1996; Saile and Ramadori, 2007). The activated HSCs are proliferative and are transformed from vitamin A storing cells into myofibroblasts. The activated HSC also produce more extracellular matrix (ECM), including type 1 collagen (coallagen1a) (Saile and Ramadori, 2007; Svegliati-Baroni et al., 2008).

1.3.2 Hepatotoxicity of Individual BAs

The toxicity of BAs is related to their detergent-like features. The more hydrophobic, the stronger the detergent-like effects and the membrane damaging effects of BAs (Hofmann and Small, 1967; Vyvoda et al., 1977; Desmet, 1995). According to their hydrophobicity, the ranking of cytotoxicity of individual BAs is as follows: CA<CDCA<DCA<LCA (Vyvoda et al., 1977; Attili et al., 1986).

Hepatotoxicity of CA

The toxic potency of BAs has been inversely correlated with their relative hydrophilicity. Among all major human BAs, CA has three hydroxyl groups and thus relatively hydrophilic and less toxic compared to other mono- or di-hydroxy BAs, such as CDCA, DCA or LCA. However, when CA concentration reaches a certain level, CA can be toxic. After feeding CA to mice at concentrations of either 0.5 or 1.0% in the diet for two weeks, serum ALT and AST in mice increased slightly (Delzenne et al., 1992). Another in vivo study showed that after feeding 1.0% CA for 7 days, mice lost their canalicular villi in addition to canalicular dilatation (Fickert et al., 2001). In the latter study, the mechanism of the toxic effects of CA was attributed to its effects on the cytoskeleton.

Feeding mice 0.5% and 1.0% CA for 7 days caused an increase in serum enzymes, including alanine aminotransferase (ALT) and alkaline phosphatase (AP), suggesting that at the dose of 0.5% in the diets CA caused hepatocyte and bile duct damage. Feeding CA also caused increased mRNA and protein expression of

cytokeratin 8 (CK-8). The increased expression of CK-8 is usually found in livers of bile duct ligated animals, and is considered a compensatory reaction to cholestatic liver injury (Fickert et al., 2002). In another study, feeding mice 1% CA caused an increase in serum ALT and lipid deposition in livers (Wang et al., 2003b). Livers of adult female hamsters fed 0.5% CA resulted in severe ductular/ductal proliferation and inflammatory infiltration (Siviero et al., 2008).

The hepatotoxicity of CA is also observed in *in vitro* studies. For example, at concentration of 1mM, CA progressively causes cell lysis (Delzenne, 1992). Another i*n vitro* study showed that among CA and its conjugates, the glycine-conjugated CA (GCA) is the most toxic CA (Martinez-Diez et al., 2000). At 1mM, GCA caused decreased cell viability, whereas, both unconjugated CA and taurine-conjugated CA (TCA) did not affect cell viability (Martinez-Diez et al., 2000).

Both in vivo and in vitro studies demonstrated that compared to other relatively hydrophobic BAs (CDCA, DCA, and LCA), CA is less toxic (Delzenne, 1992, Martinez-Diez et al. 2000). At a concentration of 1mM, CA caused direct cell lysis.

Hepatotoxicity of CDCA

CDCA, a primary BA in humans, has been used for the treatment of cholesterol gallstones for more than forty years. Treating rhesus monkeys or gallstone patients with CDCA caused increased serum ALT and histological

changes in the livers (Dyrszka et al., 1975; James et al., 1975). During cholestasis, the concentration of CDCA increased about 20 fold (Greim et al., 1973a).

CDCA is implicated most in cholestatic liver injury. CDCA is directly cytotoxic to hepatocytes (Miyazaki et al., 1984). *In vitro* studies showed that glycine-conjugated CDCA (G-CDCA) caused ATP depletion, and mitochondria disfunction (Spivey et al., 1993; Rolo et al., 2001). The toxic effects of CDCA have been suggested to be partially due to its direct activation of death receptor Fas, which leads to hepatocyte apoptosis (Faubion et al., 1999). Among all CDCA species, G-CDCA is the most toxic CDCA, followed by unconjugated CDCA, and the least toxic is the TCDCA (Spivey et al., 1993). The concentration used for CDCA to produce cytotoxicity was 50μM (Rolo et al., 2004).

Hepatotoxicity of DCA

DCA is the secondary BA derived from CA. Similar to CDCA, DCA is a di-hydroxy BA. DCA can cause liver necrosis and apoptosis at high concentrations (Kwo et al., 1995). In cell cultures, at 1000μM, all DCA species, including DCA, GDCA and TDCA, resulted in decreased cell viability (Martinez-Diez et al., 2000). DCA was also found to cause cell proliferation in both in vitro and in vivo studies (Cohen and Raicht, 1981).

Hepatotoxicity of LCA

LCA is the most hydrophobic BA among the four major human BAs, thus LCA is thought to be the most toxic BA and produces lithogenic effects (Palmer and

Ruban, 1966). However, in 1993, an *in vivo* study demonstrated that in rats, LCA was less toxic than the less hydrophobic BA DCA (Delzenne et al., 1992). In whole animal studies, LCA at concentrations of 0.5 or 1.0% in the diets caused liver necrosis, apoptosis, and obstructive liver damage (Fickert et al., 2006). In rats, TLCA infusion caused cholestasis, loss of canalicular microvilli, lamellar transformation of the canalicular membrane, and occasional crystalline precipitates within the canalicular lumen and in the pericanalicular region of hepatocytes (Miyai et al., 1977).

1.4 Statement of Purposes

1.4.1 General Background

BAs are endogenous derivatives of cholesterol that facilitate the digestion and absorption of lipids and lipid soluble vitamins from the intestine. During the last decade, more and more physiological importance of BAs have been revealed because of the discovery of the BA receptor FXR (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). Upon activation of FXR, BAs regulate the homeostasis of BA themselves (Ananthanarayanan et al., 2001; Chen et al., 2001; Denson et al., 2001), as well as lipids (Huang et al., 2003; Watanabe et al., 2004). One target gene of FXR is the small heterodimer protein (SHP), which is a repressor (Goodwin et al., 2000). Via activation of FXR and FXR-SHP mediated signaling pathways, BAs coordinately regulate BA biosynthesis and transport, including inhibition of the key enzyme of BA biosynthesis Cyp7a1 (Goodwin et al., 2000) and

the uptake transporter Ntcp (Denson et al., 2001), and induction of BA efflux transporter Bsep (Ananthanarayanan et al., 2001; Plass et al., 2002).

Despite their physiological importance, the detergent-like properties of BAs make them toxic to cellular membranes, and increased concentrations of free BAs can result in damage to tissues such as liver and bile duct (Delzenne et al., 1992; Wang et al., 2003c; Fickert et al., 2006). In cholestatic disorders, or BA-feeding animal models, markedly increased BA concentrations in liver are thought to contribute to liver damage (Bremmelgaard and Alme, 1980; Fischer et al., 1996; Fickert et al., 2006). Based on cholestatic disease models, conclusions have been made that increased concentrations of BAs in liver cause down-regulation of mRNA of genes involved in BA biosynthesis and BA uptake, as well as up-regulation of BA efflux transporter genes (Denson et al., 2001; Fickert et al., 2001; Zollner et al., 2003b; Ando et al., 2005). However, these internal compensatory changes could be the result of the toxic effects of BAs. Thus, knowing the difference between the effects of non-hepatotoxic doses of BAs and hepatotoxic doses of BAs is critical to proper appreciation of the physiological/pharmacological functions of BAs and the hepatotoxic effects of BAs. Therefore, the ultimate goal of the present dissertation is to identify which genes involved in BA biosynthesis and transport are regulated by BAs and possible roles of hepatic FXR and ileum FXR-Fgf15 in regulating these genes by feeding mice non-hepatotoxic doses of BAs.

1.4.2 Specific Research Hypothesis and Aims

Hypothesis 1: The doses of BAs (0.5 or 1.0% in the diet) used in most BA research in literature are hepatotoxic.

Rationale: It is thought that DCA and LCA are the most toxic BAs among the five BAs, namely CA, CDCA, DCA, LCA and UDCA. However, as the father of toxicology Paracelsus said: the dose makes a thing toxic. Therefore, in the present dissertation, it was expected that even the most hydrophilic BA CA would be toxic when administered at an appropriate dose. In addition, it was also expected that introducing one BA into the body would change the profile of other BAs in liver. However, at the present time, it is not known what dose of the major human BAs are hepatotoxic. Therefore, histopathological, biochemical and analytical methods were employed in the first study to characterize the hepatotoxicity of each of the five individual BAs and to determine the non-hepatotoxic doses and hepatotoxic doses of each BA. A central hypothesis of the present dissertation is that the non-hepatotoxic doses of BAs regulate genes differently from hepatotoxic doses of BAs because the hepatotoxic doses of BAs result in toxic effects which confound the physiological effects of BAs.

Specific Aim 1a. Characterize the lethality and hepatotoxicity of each BA in mice and determine the non-hepatotoxic and hepatotoxic concentrations of each BA in their feed using both serum ALT activity and serum BA concentration as standards.

Specific Aim 1b. Determine total liver BA concentrations and analyze the BA composition in livers of mice fed various concentrations of BAs in their feed.

Hypothesis 2. Non-hepatotoxic doses of BAs fully activate hepatic FXR as hepatotoxic doses of BAs do.

Rationale: BAs are thought to regulate their own homeostasis mainly via their sensor FXR (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). Via activating FXR, BAs up-regulate the efflux transporters Bsep and Mrp2. Via activating FXR-SHP signaling, BAs down-regulate BA synthesis enzymes and uptake transporters in liver (Goodwin et al., 2000). In addition, the intestinal FXR-Fgf15 signaling pathway is also thought to regulate the liver biosynthesis enzyme Cyp7a1 (Inagaki et al., 2005). Fgf15 in the mouse intestine has been shown to be induced by CA-feeding, and is thought to travel via the portal vein to the liver where it will activate the cell surface fibroblast growth factor receptor (FGFR) 4, triggering the intracellular signaling pathway that represses the expression of hepatic Cyp7a1.

Data generated by feeding mice hepatotoxic concentrations of BAs might not reflect the true physiological functions of BAs. Thus, the goal of this study was to determine which FXR mediated signaling pathway plays a main role in regulating genes involved in BA homeostasis in mice using all five BAs at non-hepatotoxic doses as FXR activators.

Specific Aim 2a. Determine whether FXR, FXR-SHP signaling in the liver, and FXR-Fgf15 signaling in the ileum are activated by feeding mice non-hepatotoxic doses of five individual BAs

Specific Aim 2b. Determine which genes involved in BA biosynthesis and BA transport are regulated by BAs, as well as possible roles of FXR-SHP signaling in liver and FXR-Fgf15 signaling in ileum in regulating BA regulated genes.

Hypothesis 3. Reduced total BA concentrations in the livers of mice fed the BA sequestrant cholestyramine (resin) results in diminished activation of FXR in liver and decreased signaling of FXR-Fgf15 in ileum. After resin-feeding, the mRNA expression of genes regulated by BAs will be opposite to that after BA-feeding.

Rationale: Cholestyramine, a non-absorbable ionic exchange resin, is a cholesterol lowering drug by binding BAs in the intestine and increasing their fecal excretion (Thompson, 1971). In addition, resin has been used as a BA-pool-size lowering chemical for studying the functions of BAs. The decreased enterohepatic circulation of BAs results in increased catabolism of cholesterol into BAs. The ultimate mechanism for decreasing cholesterol is the increase in mRNA expression and enzyme activity of Cyp7a1, which is the rate limiting enzyme in BA biosynthesis, after resin treatment (Thompson, 1971; Nilsson et al., 2007). Even though resin is a non-absorbable drug, it has been found to regulate glucose homeostasis and to improve atherosclerotic coronary heart disease (CHD), which is thought to have little to do with its lipid lowering effects. The binding efficiency of various BAs to resin varies (Thompson, 1971). At the pH of the intestine (about 6.0), the relatively

hydrophobic BAs (DCA and LCA) bind stronger to the resin than the hydrophilic BAs (CA and MCA) (Whiteside et al., 1966; Thompson, 1971; Story and Kritchevsky, 1976). Thus, feeding mice the resin is likely to not only decrease BA concentrations, but also alter the BA composition, as well as to change gene expression in liver and intestine. Therefore, the purpose of this study was to determine the effects of resin on BA concentrations and composition in livers of mice, as well as gene expression of enzymes, transporters, and transcription factors in both liver and ileum known to be involved in biosynthesis or transport of BAs.

Specific Aim 3a: Determine the BA concentrations in livers of mice fed 2% cholestyramine resin for one week

Specific Aim 3b: Determine the effects of feeding mice the resin on FXR signaling in the livers and the FXR-Fgf15 signaling in the ilea.

Specific Aim 3c: Determine the effects of resin on mRNA expression of genes involved in BA-biosynthesis and BA-transport in livers and ilea of mice.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Chemicals: All BAs, including CA, CDCA, DCA, LCA, and UDCA, as well as cholestyramine resin, were purchased from Sigma Chemical Co. (St Louis, MO, USA).

2.2 Preparation of BA or resin Supplemented Diets*:* Pelleted mouse feed (Teklad Rodent Diet #8604, Harlan Teklad, Madison, WI) was ground into a fine powder. CA, CDCA, and DCA were granular and thus were ground into a fine powder using a mortar and pestle. Each BA at proper weight, or resin at proper weight, was subsequently thoroughly mixed with the finely ground feed using an electronic mixer to obtain the desired concentrations in the feed

2.3 Animal Experiments: Male C57BL6 mice (22 $\pm$ 2g) at 8 weeks of age were obtained from Charles River Laboratories, Inc. (Wilmington, MA). The mice were housed and fed according to the American Animal Association of Laboratory Animal Care Guidelines. Mice were housed with a 12:12 hr light:dark cycle, and provided mouse chow and water *ad libitum*. Each mouse was housed in a single mouse cage and acclimated to the housing facility and ground control rodent diet for one week. The diet was then exchanged to those supplemented with various percentages of BAs, including CA, CDCA, and DCA at final concentrations of 0.03, 0.1, 0.3, or 1.0% (w/w), or LCA at 0.01, 0.03, 0.1, 0.3, or 1.0% (w/w), or UDCA at 0.1, 0.3, 1.0, or 3.0% (w/w), or resin at 2% in the diet. The BA or resin supplemented diets (40g) were added to a bowl in each mouse cage daily, and the remaining feed from the previous day was discarded. Cages were replaced daily to minimize contamination of feed with urine and feces. After 7 days on these diets, serum

samples were collected and stored at 4°C for biochemical analysis, whereas, livers were removed, frozen in liquid nitrogen, and stored at -80°C. Portions of livers were also fixed in 10% buffered formalin for histological studies.

2.4 Biochemical analysis: Serum ALT activity was quantified by an enzymatic-colorimetric assay using an ALT assay kit (Stanbio, Boerne, TX).

2.5 Serum BA extraction: For extraction of BAs from serum samples, 1 ml of ice-cold acetonitrile (ACN) was added to 100 µl of serum and mixed with 20 µl of internal standard (ISs: $^{2}H_4$-G-CDCA and $^{2}H_4$-CDCA at concentrations of 4 and 2 µg/ml, respectively). The mixtures were thoroughly vortexed and centrifuged at 11,000 g for 10 min. The supernatants were collected, evaporated under vacuum for 3 hrs at 50ºC, and reconstituted in 100 µl of methanol:water (1:1) (Alnouti et al., 2008).

2.6 Liver BA extraction: To extract BAs from liver samples, approximately 120 mg of liver was homogenized in 5 volumes of H_2O. Six hundred µl of liver homogenate was spiked with 10 µl of ISs, mixed, and equilibrated on ice for 10 min. Three ml of ice-cold alkaline acetonitrile (5% ammonia in acetonitrile) was added to the homogenate. The mixtures were then vortexed thoroughly and shaken continuously for 1 hr at room temperature. The mixtures were centrifuged at 12,000 x g for 10 min. The supernatant was collected. The pellet was resuspended in 1 ml of methanol, sonicated for 5 min, and centrifuged at 12,000 x g for 10 min. The two supernatants so obtained were combined and evaporated under vacuum. The

deposit was then reconstituted in 100 µl of 50% methanol, vortexed, transferred into a 0.2 µm Costar Spin-X HPLC microcentrifuge filter (Corning Inc, Corning, NY), and centrifuged at 20,000 x g for 10 min. The supernatants were collected and analyzed using UPLC-MS/MS.

2.7 BA separation and quantification by ultra performance liquid chromatography-tandum mass spectrometry (UPLC-MS/MS): A Waters ACQUITY ultra performance LC system (Waters, Milford, MA) was used to separate and to quantify individual BA in serum and liver BAs extraction samples. All chromatographic separations were performed on an ACQUITY UPLC BEH C18 column (1.7 µm, 100 X 2.1 mm I.D.). The detailed LC and mass spectrometric conditions were as described in a previous study (Alnouti et al., 2008).

2.8 H&E staining of liver sections: Livers were fixed using 10% buffered formalin overnight, dehydrated using various concentrations of alcohol, and embedded in paraffin. Paraffin embedded liver blocks were sectioned (5 µm thick). The sections were deparaffinized and rehydrated through a graded series of alcohol. Tissue sections (5 µm) were stained with hematoxylin and eosin (H&E) for general histopathology. Each liver section was visualized using an Olympus BX41 microscope and photographed using an attached Olympus DP70 digital camera.

2.9 Total RNA Extraction. Total RNA was extracted from liver and ileum tissues using RNABee reagent (Tel-test Inc., Friendswood, TX) as suggested by the manufacturer's protocol. RNA was quantified by UV spectrophotometry at 260nm and diluted to 25 ng/µl in diethyl pyrocarbanate (DEPC)-treated deionized water.

2.10 QuantiGene Plex assay. Messenger RNAs for mouse genes involved in BA homeostasis in liver and ileum were quantified using the QuantiGene Plex 2.0 assay following the manufacturer's protocol (Panomics/Affymetrix, Fremont, CA)***.*** Individual bead-based oligonucleotide probe sets were designed by Panomics/Affymetrix, Inc. using previously published NCBI gene accession numbers (www.panomics.com). Briefly, isolated RNA (10 µl /well) was hybridized overnight at 54˚C to specific oligonucleotide sequences corresponding to the genes of interest, which were bound to capture beads. The following day the hybridized RNA, oligos, and capture beads were pipetted into corresponding wells of a filter plate. The complex was then sequentially hybridized to first the pre-amplifier, followed by the amplifier, and lastly the biotinylated label probe (each for 1 hr at 50˚C). Streptavidin-PE was then allowed to bind for 30 min at room temperature and fluorescence was quantified by a Bio-plex suspension array system. The housekeeping gene GAPDH was used to normalize the data. The data are expressed as a ratio of the target mRNA relative to GAPDH mRNA.

2.11 Statistics. Differences between multiple groups were analyzed by one-way ANOVA followed by Duncan's post-hoc test. Statistical significance was considered at $p<0.05$.

CHAPTER 3

IMBALANCED BILE ACID COMPOSITION IN LIVER CONTRIBUTES TO BILE ACID HEPATOTOXICITY IN MICE

3.1 Abstract

BA feeding has been used to study BA signaling and toxicity *in vivo*. However, the concentration of BAs in the feed that is non-hepatotoxic is not well defined in mice. Therefore, the purpose of this study was to determine the non-hepatotoxic and hepatotoxic concentrations of five BAs in mouse feed, as well as the effect of feeding mice individual BAs on BA concentrations and BA composition in liver. Mice were fed five individual BAs, cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA) at 0.01, 0.03, 0.1, 0.3, 1.0, or 3.0% in their diets for 7 days. The data showed: 1) LCA, DCA, and CDCA at 0.3% or lower in the diets were not lethal, but at the highest tested dose (1.0% in the diets) were lethal, whereas CA at 1% and UDCA at 1.0 and 3.0% were not lethal. 2) LCA produced hepatotoxicity at 0.03%, indicated by increases in serum ALT and serum BA concentration, as did DCA at 0.1%, and CDCA and CA at 0.3% in the diet. UDCA at 0.3% in the diet might be hepatotoxic because serum BA concentrations were increased, but the serum ALT did not increase. 3) Feeding BAs (CA, CDCA, DCA, or LCA) at hepatotoxic doses did not alter the total BA concentrations in liver; however it altered liver BA composition, including increases in the percentage of the fed BAs, and decreases in the percentage of muricholic acids. In conclusion, it appears that an imbalance in BA composition in liver can contribute to BA hepatotoxicity. In addition, to study the physiological or pharmacological functions of each BA, non-hepatotoxic

concentrations of BAs in the feed should be used, namely, CA, CDCA, and UDCA at 0.1% or lower, DCA at 0.03% or lower, and LCA at 0.01% or lower in the diet.

3.2 Introduction

BAs are well known to facilitate the elimination of cholesterol into bile, as well as to act as emulsifiers for the absorption of lipids and lipid-soluble vitamins from the intestine. Within the last decade, BAs have been found to be important signaling molecules playing critical physiological roles in regulating the homeostasis of BAs, cholesterol, glucose, and energy via activation of a number of nuclear receptors, including the farnesoid X receptor (FXR) (Makishima et al., 1999; Parks et al., 1999; Goodwin et al., 2000), pregnane X receptor (PXR) (Staudinger et al., 2001b; Xie et al., 2001), vitamin D receptor (VDR) (Makishima et al., 2002; Wolf, 2002), as well as the membrane receptor TGR5 (Kawamata et al., 2003; Katsuma et al., 2005; Watanabe et al., 2006).

BAs are synthesized from cholesterol in the liver and secreted into the bile. In humans, the primary BAs are CA and CDCA, whereas in mice, CA and muricholic acids (MCAs, mainly β-MCA) are the primary BAs. In bears, UDCA is the major BA. Primary BAs can be dehydroxylated by intestinal flora, producing secondary BAs: deoxycholic acid (DCA) from CA and lithocholic acid (LCA) from CDCA. MCAs, including α-MCA (3α,6β,7α-trihydroxy-5β-cholanoic acid), β-MCA (3α,6β,7β-trihydroxy-5β-cholanoic acid), and ω-MCA (3α,6α,7β-trihydroxy-5β-cholanoic acid), can be 7-dehydroxylated to form murideoxycholate (MDCA) or its 6α-epimer

hyodeoxycholic acid (HDCA) by bacteria in the intestine (Lida et al., 1989; Miki et al., 1993; Eyssen et al., 1999). In humans, hyodeoxycholic acid (HDCA) is thought to be detoxification product of LCA in liver (Xie et al., 2001; Bodin et al., 2005).

Toxic potency of various BAs is different, due to the number and optical orientation of the hydroxyl groups on the steroid nucleus. The primary BAs, CA and CDCA, are relatively more water soluble and less toxic than the secondary BAs, namely DCA and LCA (Mosbach, 1972; Salvioli et al., 1982). Newly-synthesized BAs and deconjugated BAs from the enterohepatic circulation are conjugated with either glycine or taurine in liver. Two enzymes are critical in the conjugation process: BAs-CoA ligase (BAL), which catalyzes the formation of BA-CoA, and BA-CoA:amino acid *N*-acyltransferase (BAT), which amidates BA-CoA with the amino acid, glycine or taurine (Russell, 2003). The BAT enzyme in humans and rats can conjugate BAs with either taurine or glycine (Falany et al., 1994), but the mouse BAT enzyme catalyzes conjugation of BAs predominantly with taurine (Falany et al., 1997).

Even though all BAs are derived from the precursor cholesterol, they have different physiological and pathological effects. For example, CDCA is a more potent endogenous agonist of FXR compared to CA, DCA, or LCA (Makishima et al., 1999; Parks et al., 1999); CDCA and LCA can suppress LPS-induced TNF secretion in THP-TGR5 cells, whereas UDCA can boost TNFα induction (Kawamata et al., 2003). LCA is considered to be the most toxic BA and an activator of PXR (Staudinger et al., 2001b), whereas UDCA is thought to have hepato-protective

effects, and is thus used in the treatment of cholestatic liver diseases (Elias, 1999; Milkiewicz et al., 1999).

To determine the physiological effects of BAs, BA supplemented diets have often been fed to laboratory animals (Oda et al., 1990; Murphy et al., 2005). For example, mice fed 0.5 and 1.0% CA had decreased mRNA expression of the BA-uptake transporter Ntcp (Fickert et al., 2001). However, at such concentrations, CA caused liver injury, as indicated by an increase in serum ALT levels (Murphy et al., 2005). In contrast, some reports demonstrate that there is no hepatotoxicity for BAs at doses of 0.5 or 1% (Fickert et al., 2001). These divergent reports indicate that the hepatotoxicity of individual BAs requires a systematic evaluation in a strictly-designed experimental system. Nevertheless, it is unclear whether the altered gene expression reported in many studies is the result of physiological concentrations of BAs, or is a consequence of BA toxicity. Accordingly, determination of the non-hepatotoxic and the hepatotoxic doses of individual BAs becomes very critical for proper interpretation of the gene regulation functions of individual BAs.

In the present study, wild-type C57BL/6 mice were fed five individual BAs at various concentrations in their diets to investigate how individual BA-feeding influences the total liver BA concentration and liver BA composition. In addition, both the serum ALT levels and the serum BA concentrations are used as indices of liver functions to determine the non-hepatotoxic concentrations and the hepatotoxic concentrations of individual BAs in the feed.

3.3 Results

Effects of feeding BAs on survival, body weight, and liver weight. The survival of mice fed various BAs at multiple concentrations in their diets for one week is shown in table 3-1. All mice fed 0.01, 0.03, 0.1, and 0.3% of any BA (CA, CD CA, DCA, LCA or UDCA) in their diets survived. However, 1.0% CDCA, DCA, and LCA were lethal, whereas all mice fed 1.0% CA survived. Mice fed UDCA at all tested concentrations in the feed, including the highest dose of 3.0% in the diet, survived to the end of the study.

Table 3-1. Survival of mice fed bile acids at various concentrations in the feed for one week

Conc **(W/W%)**	CA	CDCA	DCA	LCA	UDCA
0.01	-	-	-	5/5	-
0.03	5/5	5/5	5/5	5/5	-
0.1	5/5	5/5	5/5	5/5	5/5
0.3	5/5	5/5	5/5	5/5	5/5
1.0	5/5	0	0	0	5/5
3.0	-	-	-	-	5/5

The BAs did not have a marked effect on body weight of mice (Fig 3-1), except 3.0% UDCA in the diet decreased body weight. The relative liver weight of mice fed the primary BAs (CA and CDCA) at concentrations lower than 0.3% was not altered, but mice fed the secondary BA, DCA, at concentrations of 0.03, 0.1 and

0.3% in the diets, had increased relative liver weights (8-32%). Mice fed UDCA at 3.0% in the diet had decreased body weight (11%), but did not demonstrate altered relative liver weight.

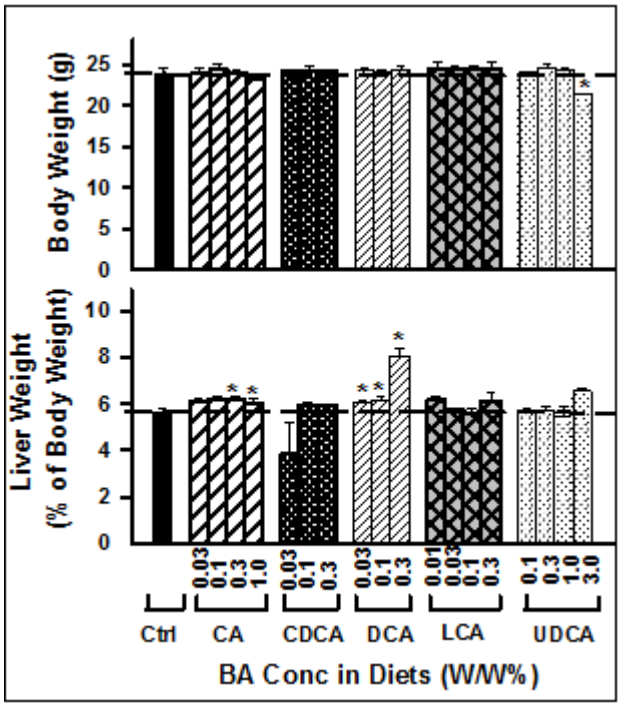


Fig 3-1 Dose response of five BAs on body weight and relative liver weight of mice treated with five BAs (CA, CDCA, DCA, LCA or UDCA) at concentrations of 0.01, 0.03, 0.1, 0.3 1.0 or 3.0% in the diets for one week. Data are presented as Mean ± S.E.M (n=5). *P<0.05

Total BA Concentrations in Serum and Liver. As shown in Fig 3-2, only feeding mice high doses of BAs, including CA and CDCA at 0.3 and 1.0%, DCA and LCA at 0.1 and 0.3%, as well as UDCA at 0.3, 1.0, and 3.0%, increased total BA concentrations in serum. Fig 3-2 surprisingly indicates that feeding CA, CDCA, DCA,

and LCA at all doses did not increase total BA concentrations in liver; however, feeding UDCA at 3% quadrupled total BA concentrations in liver.

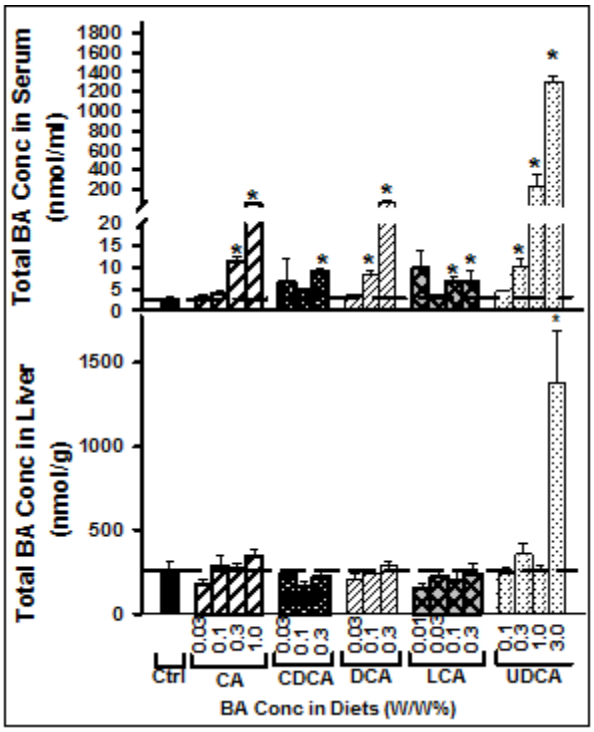


Fig 3-2 Dose response of five BAs on total BA concentration in the sera and livers of mice treated with five BAs (CA, CDCA, DCA, LCA or UDCA) at concentrations of 0.01, 0.03, 0.1, 0.3 1.0 or 3.0% in the diets for one week. Data are presented as Mean ± S.E.M (n=5). *P<0.05

Individual BA Concentrations in Serum. To determine how much individual BA concentrations were increased in serum after mice were fed the individual BAs in their diets for one week, serum was collected and analyzed for BA concentrations by UPLC-MS/MS. As shown in Fig 3-3, mice fed various concentrations of individual BAs generally had marked increases in the fed BA and

its taurine conjugate, as well as a moderate increase in its metabolite. Feeding mice CA dose-dependently increased CA and its secondary BA, DCA, as well as their taurine conjugates in sera. However, mice fed the lowest concentration of CA (0.03% in the diet) did not increase CA or DCA, nor their taurine conjugates. After mice were fed CA 0.1, 0.3, or 1.0% in their diets for one week, the serum CA concentrations increased 4-, 21-, and 42-fold, respectively. Mice fed the two higher concentrations of CA (0.3% and 1.0% in the diets) had 4- and 54-fold increases in serum T-CA, respectively. The secondary BA of CA, namely DCA, in sera of mice fed CA at 0.1, 0.3, and 1.0% in the diets increased 1.54-, 6.94-, and 16-fold, respectively. Mice fed various concentrations of CA did not have alterations in the concentrations of CDCA, LCA, or UDCA or their taurine conjugates in sera. However, feeding CA decreased α-MCA and T-MCA in sera.

Unlike the effects of CA, CDCA-feeding only increased CDCA and T-CDCA in sera, but not its secondary BA, LCA. As shown in Fig 3-3, after feeding mice CDCA at 0.03, 0.1, and 0.3% in their diets for one week, serum CDCA increased 6-, 15-, and 69-fold, and serum T-CDCA increased 46-, 0.36-, and 3-fold, respectively. Feeding CDCA at the tested concentrations did not alter LCA concentrations in sera. Mice fed 0.1 and 0.3% CDCA had a decrease in serum CA concentration, whereas only mice fed 0.3% CDCA had a decrease in serum DCA concentration; however feeding 0.3% CDCA increased α-MCA in sera (about 30%).

DCA-feeding not only increased the serum concentrations of DCA and T-DCA, but also increased the serum concentrations of the primary BA CA and its taurine

conjugate T-CA. After mice were fed 0.03, 0.1, and 0.3% DCA for one week, the serum DCA concentrations increased 3-, 19-, and 73-fold, respectively. Mice fed 0.1 and 0.3% DCA had increases in serum concentrations of T-DCA 4- and 162-fold, CA 1.5- and 7-fold, and T-CA 1- and 39-fold, respectively. Mice fed DCA did not alter the serum concentrations of CDCA, UDCA, and MCA, except that 0.3% DCA increased T-CDCA, whereas 0.03 and 0.1% DCA decreased T-MCA (about 30%).

Mice fed LCA resulted only in an increase in LCA, but not its primary BA, CDCA, in sera. Mice fed LCA at 0.03, 0.1, and 0.3% in the diets increased LCA in sera 67-, 225-, and 782-fold, respectively. Mice fed 0.3% LCA increased T-CDCA 2-fold. Mice fed 0.01% LCA in the diet had an decrease in serum concentration of T-CA, but increases in serum concentrations of DCA and UDCA. LCA at 0.1 and 0.3% in the diets increased T-DCA. Feeding mice LCA at the tested concentrations did not alter α-, β-, or T-MCA in the serum.

As shown in Fig 3-3, mice fed various concentrations of UDCA at tested concentrations not only had increases in serum UDCA and T-UDCA, but also had increases in serum CDCA, LCA, and T-LCA.

Individual BA Concentrations in Liver. As shown in Fig 3-4, mice fed individual BAs had marked increases in that particular BA and its taurine conjugate in liver. The liver concentrations of secondary BAs, namely DCA or LCA, were increased after mice were fed the primary BAs, CA or CDCA, for one week. Mice fed 0.1, 0.3, and 1.0% CA had 2-4 fold increases in liver concentrations of CA and T-CA. Mice fed these same concentrations of CA also increased liver DCA

concentrations 2-11 fold, and T-DCA 1-5 fold. However, feeding CA did not alter the concentrations of LCA, UDCA, or their taurine conjugates in liver. Feeding mice CA markedly decreased α-MCA, β-MCA, ω-MCA, and taurine conjugated MCAs as well. In addition, feeding CA also decreased HDCA, MDCA, and their taurine conjugates 30-90%.

As shown in Fig 3-4, feeding mice 0.03, 0.1, and 0.3% CDCA increased CDCA in liver 0.6-, 4-, and 17-fold, respectively. Feeding CDCA at 0.1 and 0.3% in the diets also increased T-CDCA in liver 3- and 15-fold, respectively. Feeding mice lower concentrations of CDCA did not increase the secondary BA, LCA, but the higher concentrations of CDCA (0.3% in the diet) increased LCA and T-LCA in the liver 2- and 4-fold, respectively. Feeding high concentrations of CDCA in the feed (0.1 and 0.3%) decreased CA and DCA, as well as their taurine conjugates in liver. Interestingly, feeding mice CDCA dose-dependently increased UDCA in the liver 0.5-5 fold. Feeding mice CDCA at 0.03 and 0.1% increased α-MCA 3-4 fold, whereas, the high dose of CDCA (0.3% in the diet) decreased other MCAs, including β-MCA and ω-MCA in liver. In addition, feeding mice high concentrations of CDCA in the diets decreased T-α-MCA and T-β-MCA. Interestingly, feeding CDCA also slightly increased HDCA, MDCA, and their taurine conjugates.

Mice fed the secondary BA DCA, not only had marked increases in DCA and T-DCA, but also had increases in the primary BA, CA and T-CA (Fig 3-4). Mice fed 0.03, 0.1, and 0.3% DCA had increased liver DCA concentrations 1-, 3-, and 9-fold, as well as increased T-DCA 1-, 2-, and 14-fold, respectively. Mice fed the highest

concentrations of DCA (0.3% in the diet) tended to have decreased CDCA concentrations in liver, with LCA unchanged. Feeding DCA at high dosages (0.1 or 0.3% in the diets) decreased concentrations of UDCA and T-UDCA in liver. DCA at 0.3% also decreased α- and β-MCA and taurine conjugated MCA in liver. In addition, feeding DCA also decreased other bile acid metabolites, including HDCA, MDCA, and their taurine conjugates, THDCA and TMDCA, to half of the control.

Feeding mice LCA dose-dependently increased both LCA and CDCA concentrations and their conjugates in liver (Fig 3-4). Feeding LCA decreased taurine and glycine conjugated CA, but did not alter the concentrations of DCA and its conjugates in liver. Feeding mice even low dose of LCA markedly decreased ω-MCA and T-ω-MCA in liver. Similar to the effects of CDCA, feeding LCA increased HDCA, MDCA, and their taurine conjugates.

Feeding mice UDCA increased UDCA and T-UDCA in the livers of mice. Feeding mice 0.1, 0.3, 1.0, and 3.0% of UDCA increased UDCA concentration in liver 50-194 fold and T-UDCA 28-63 fold. Feeding UDCA decreased CA and T-CA, but increased CDCA and LCA concentrations in liver. Only feeding mice high concentrations of UDCA (1.0 and 3.0% in the diets) decreased T-DCA in liver. As with other BAs, UDCA also decreased MCA, including α-MCA, β-MCA, and ω-MCA, as well as taurine conjugated MCA in liver. At the highest dose (3% in the diet), UDCA increased all MCAs (α-MCA, β-MCA, and ω-MCA) and their taurine conjugates.

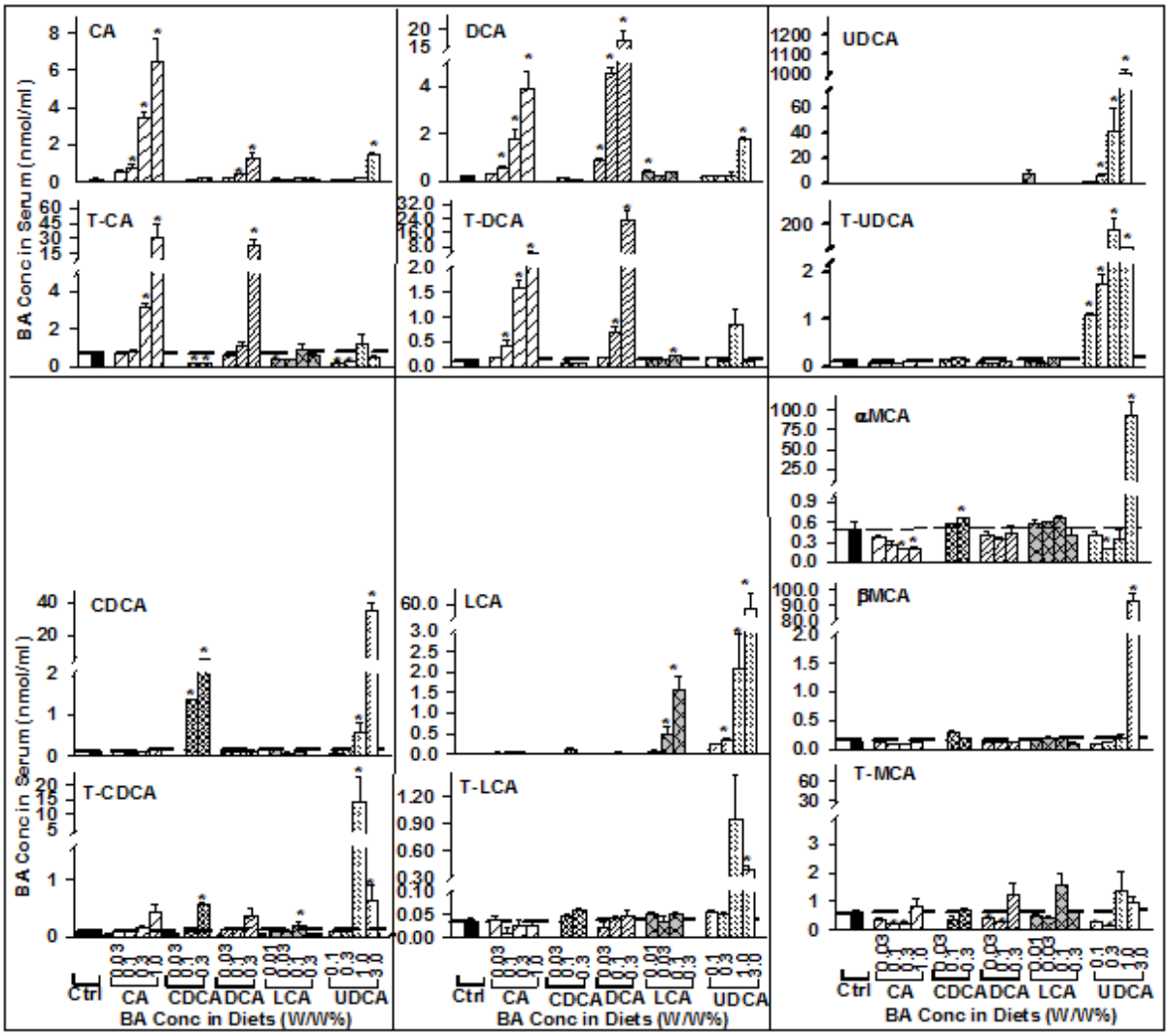


Fig 3-3 Dose response of five BAs on individual BA concentrations in the sera of mice treated with five BAs (CA, CDCA, DCA, LCA or UDCA) at concentrations of 0.01, 0.03, 0.1, 0.3 1.0 or 3.0% in the diets for one week. Data are presented as Mean ± S.E.M (n=5). *P<0.05

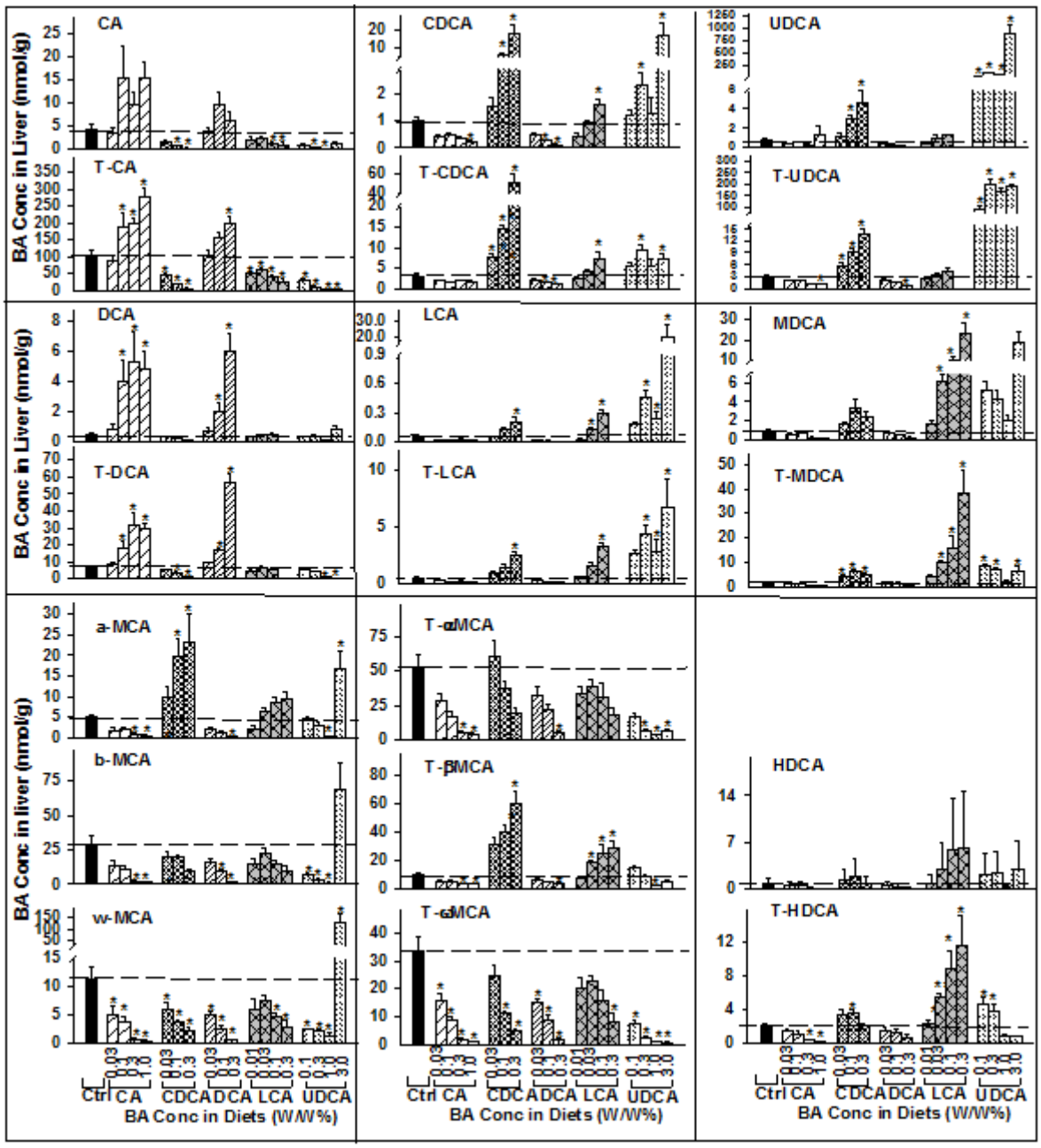


Fig 3-4 Dose response of five BAs on individual BA concentrations in the livers of mice treated with five BAs (CA, CDCA, DCA, LCA or UDCA) at concentrations of 0.01, 0.03, 0.1, 0.3 1.0 or 3.0% in the diets for one week. Data are presented as Mean ± S.E.M (n=5). *P<0.05

BA composition in liver. BA composition in the livers from control and BA-fed mice is shown in Fig 3-5. The predominant BA species in control mice were CA (CA+T-CA, 39.4%) and MCAs (MCAs+T-MCAs, 52.7%). Feeding mice various BA-supplemented diets at doses not higher than 1% in the diets did not alter the total bile acid concentrations in liver, but it altered the percentage of each fed-BA relative to the total BAs in liver (Fig 3-5). The percentage of the total CA (CA +T-CA) relative to the total BAs in the livers of mice fed CA at 0.3% in the diet increased from 39.4% to 78.6% and that of the total DCA (DCA+T-DCA) increased from 2.7 to 13.8%, whereas, the percentage of the total MCA relative to the total BAs in livers of mice fed 0.3% CA in the diet decreased markedly from 52.7% to 5.6%. In addition, feeding mice 0.3% CA also decreased the percentage of the total CDCA (CDCA+T-CDCA) from 1.5% to 0.9%, that of the total LCA (LCA+T-LCA) from 0.2% to 0.1%, that of the total UDCA (UDCA+T-UDCA) from 1.4% to 0.5%, and that of the total 6-OH BA (MDCA+T-MDCA+HDCA+T-HDCA) from 2.0% to 0.5%.

Fig 3-5 also shows that feeding mice CDCA at 0.3% in the diet increased the percentage of the total CDCA markedly from 1.5% to 30.5%. Feeding CDCA at 0.3% in the diet also increased the percentage of the total LCA and that of the total UDCA from 0.2% to 1.2%, 1.4% to 8.1%, respectively, whereas feeding CDCA at 0.3% in the diet markedly decreased the percentage of the total CA in the liver from 39.4% to 2.4% and that of the total DCA from 2.7% to 0.6%. Neither feeding CDCA at 0.3% altered the percentage of the total MCA, nor did it alter the percentage of the total 6-OH BAs in mouse livers.

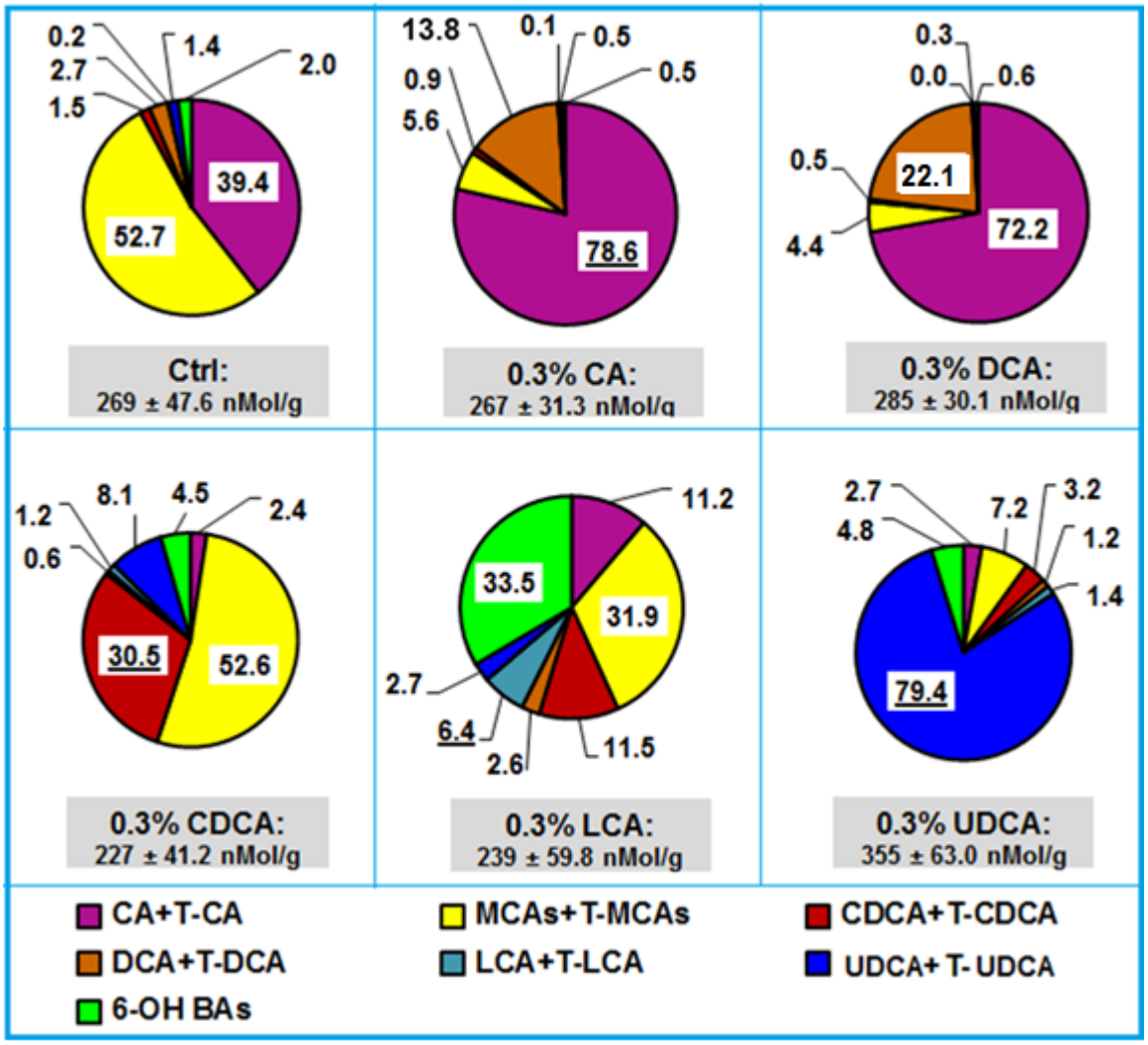


Fig 3-5. BA composition (% of total BAs) in the livers of mice fed control diet or BAs (CA, CDCA, DCA, LCA, or UDCA) at concentration of 0.3% in the diet for one week.

Feeding DCA at 0.3% in the diet increased the percentage of the total CA from 39.4% to 72.2% and that of the total DCA from 2.7 to 22.1% (Fig 3-5). However, feeding 0.3% DCA markedly decreased the percentage of the total MCA from 52.7% to 4.4%. Feeding DCA also slightly decreased the percentage of the

total CDCA, the total LCA, the total UDCA, and the total 6-OH BAs, from 1.5% to 0.5%, 0.2 to 0.03%, 1.4 to 0.3%, and 2.0 to 0.6%, respectively.

Feeding LCA at 0.3% in the diet increased the percentage of the total LCA from 0.2 to 6.4%, the percentage of the total CDCA from 1.5 to 11.5%, that of the UDCA from 1.4 to 2.7%, and that of the total 6-OH BAs from 2.0 to 33.5% (Fig 3-5). However, feeding 0.3% LCA markedly decreased the percentage of the total CA from 39.4% to 11.2 and the percentage of the total MCA from 52.7% to 31.9%. Feeding LCA had little effect on the percentage of the total DCA.

Feeding UDCA at 0.3% in the diet markedly increased the percentage of the total UDCA from 1.4 to 79.4% (Fig 3-5). However, feeding UDCA at 0.3% in the diet markedly decreased the percentage of the total CA and that of the total MCA from 39.4 to 2.7% and 52.7 to 7.20%, respectively. Feeding 0.3% UDCA slightly increased the percentage of the total CDCA from 1.5 to 3.2%, that of the total LCA from 0.2 to 1.4%, and that of the total 6-OH BAs from 2.0 to 4.8%.

Liver Histopathology. A representative histological section of liver from a control mouse and from mice fed each of the five BAs is shown in Fig 3-6. At high concentrations in the diets (CA at 1.0%, CDCA and LCA at 0.3%, or UDCA at 3.0%), none of the BAs caused liver necrosis. However, feeding DCA at 0.3%, or LCA at 0.3% in the diets caused random cell swelling and random single cell death.

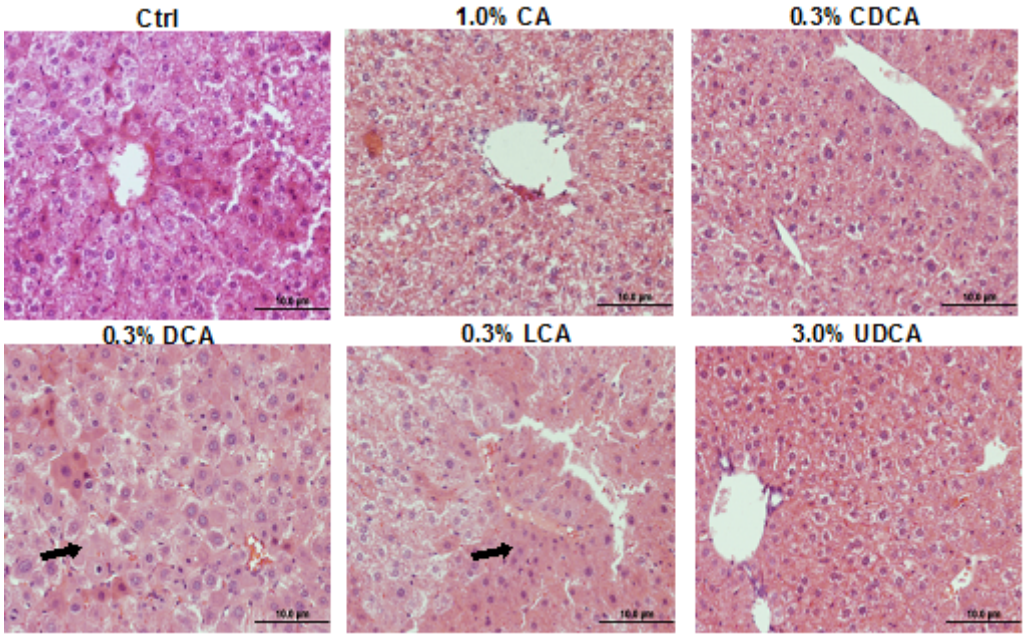


Fig 3-6 Representative microphotographs of hematoxylin and eosin (H & E) stained liver sections from control mice and mice fed individual BA supplemented diets for one week (Magnification X400). Arrows indicate focal necrosis or single cell death.

Serum ALT. To determine the doses of each individual BAs that produce hepatotoxicity, serum ALT activity was quantified. As shown in fig 7, feeding mice the primary BAs, CA and CDCA, at concentrations of 0.3 or 1.0% in the diets increased serum ALT activity. However, feeding CA and CDCA did not increase serum ALT activity at either 0.03 or 0.1% in the diets. The serum ALT activity level was increased after feeding DCA at a concentration as low as 0.1% in the diet. Feeding LCA at concentrations of 0.03, 0.1, and 0.3% in the diets increased serum ALT activity. However, there are no increases in serum ALT activity after feeding mice UDCA at any concentrations in the diets tested.

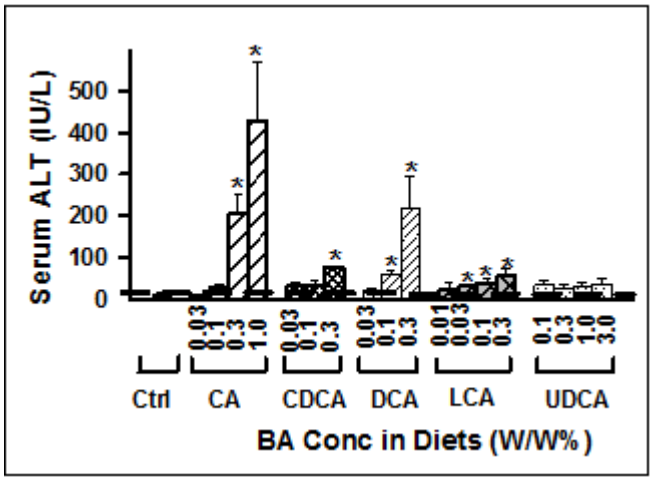


Fig 3-7 Dose response of five BAs on serum ALT after mice were fed various BAs supplemented diets for one week. Data are presented as mean ± SEM (each group, n =5 mice). Asterisks represent statistically significant differences ($p < 0.05$) between control and treatment groups

3.4 Discussion

There has been a resurgence in BA research since BAs have been found to be important signaling molecules regulating not only BA homeostasis, but also the homeostasis of cholesterol, glucose, and energy (Makishima et al., 1999; Parks et al., 1999; Redinger, 2003; Katsuma et al., 2005; Watanabe et al., 2006; Lefebvre et al., 2009). To determine the physiological functions of individual BAs, diets supplemented with various individual BAs at either 0.5 or 1.0% in the diets are often fed to mice (Zollner et al., 2003a; Miyata et al., 2006). However, it has been known that increased concentrations of BAs in liver can result in inflammation, cell death, and decreased liver function. The present study indicates that at concentrations of 0.3% or above in the diet, all BAs, except for UDCA, produced hepatotoxicity indicated by increases in serum ALT activity levels and increases in serum BA

concdentrations. Even though, UDCA at 0.3, 1.0, and 3.0% in the diet did not cause an increase in serum ALT activity levels, UDCA at these concentrations in the diet increased the total serum BA concentration and the secondary BA LCA, indicating a possible hepatotoxicity.

A novel finding in this study was that feeding exogenous BAs at doses which produce hepatotoxicity does not, as expected, increase the total liver BA concentration in mice. However, feeding exogenous BAs altered the BA composition in liver. Thus, it appears to be altered BA composition, rather than increases in total liver BA concentration, that is responsible for hepatotoxicity of the fed-BA, including CA, CDCA, DCA, and LCA.

Even though there are no reports in which the relative hepatotoxicity of the five BAs (CA, CDCA, DCA, LCA, and UDCA) were compared in the same study, it has been generalized that LCA is the most toxic BA, followed by DCA, CDCA, and CA, whereas, UDCA has hepatoprotective effects and is used for the treatment of cholestatic liver diseases (Copaci et al., 2005). In the present study, the relative hepatotoxicity of the five BAs were compared and the lowest hepatotoxic doses were determined based on the serum ALT activity, which is a traditional biochemical index of liver function and is used clinically for early diagnosis of liver injury. According to the increases in serum ALT activity, the lowest dietary concentration of CA and CDCA that was hepatotoxic in the present study was 0.3%, whereas it was

0.1% for DCA, and 0.03% for LCA. However, UDCA at any dose used in the present study did not cause an increase in serum ALT activity.

Compared to the conventional serum biochemical test, namely serum ALT, serum BA (SBA) concentration is a more sensitive and accurate indicator of liver function (Azer et al., 1997; Ambros-Rudolph et al., 2007). For example, in liver cirrhosis, the serum ALT level may be normal, however, the SBA concentration increases (Mannes et al., 1982). Moreover, SBA is used for early diagnosis of obstetric cholestasis, which can be lethal for the fetus. In some cases of obstetric cholestasis, there are no obvious symptoms, nor are there increases in serum ALT, whereas an increase in SBA concentrations usually is the only index for the diagnosis of cholestasis during pregnancy (Azer et al., 1997; Walker et al., 2002; Ambros-Rudolph et al., 2007). Thus, in the present study, SBA concentration is used as a second indicator of liver function. According to the increases in the total serum bile acid (TSBA) concentration (Fig 3-2), the lowest hepatotoxic doses for CA, CDCA, and DCA are the same as those determined based on the increases in serum ALT activity, that is, 0.3% in the diet for CA and CDCA, and 0.1% for DCA. However, the hepatotoxic doses determined according to the TSBA concentrations for LCA and UDCA are 0.1% and 0.3% respectively, which are different from those determined by serum ALT levels. The difference may be explained by the following evidence. 1) The BA composition in serum is more important than TSBAs in predicting liver dysfunction. Individual serum BAs, especially increases in hydrophobic BAs, such as LCA, are more sensitive and specific as early predictors

of liver injury (Azer et al., 1997). Even though feeding mice 0.03% LCA did not increase the TSBA concentration, the serum LCA concentration was increased more than five times (Fig 3). The increase in serum LCA is comparable to the increase in serum ALT level in mice fed 0.03% LCA. Accordingly, in the present study, the lowest hepatotoxic dose for LCA determined by increases in ALT level and serum LCA concentration is 0.03% in the diet. 2) Feeding UDCA at 0.3, 1.0 and 3.0% increased not only the TSBA, but also the concentration of LCA, suggesting liver injury. UDCA is clinically used as a therapeutic drug for various liver diseases, including primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) (Crosignani et al., 1991; Hofmann, 1994; Sinakos et al., 2010). Administration of UDCA to patients with PBC increases both the TSBA concentration and the serum LCA concentration, however, it still improved all biochemical indices of liver function in PBC patients (Crosignani et al., 1991). In addition, UDCA is known to have opposite effects to that of LCA. For example, LCA repressed LPS-induced production of TNFα, whereas, UDCA boosts LPS-induced production of TNFα in the THP-TGR5 cell line (Kawamata et al., 2003). UDCA has cytoprotective effects, whereas, LCA is cytotoxic. Direct evidence that UDCA is able to counteract the toxicity of LCA was shown in an *in vitro* study which demonstrated that UDCA can not only protect against the cholestatic effects of LCA, but also partially reversed the cholestasis caused by LCA (Milkiewicz et al., 1999). Moreover, the hepatotoxicity of UDCA is species dependent. In species that do not have the ability to sulfate and thus detoxify LCA, such as the rhesus monkey, feeding UDCA is quite toxic,

however, in humans and rodents, UDCA is relatively non-toxic (Bazzoli et al., 1982). Accordingly, even though the increases in the concentrations of TSBA and LCA in the sera of mice fed UDCA at 0.3% in the diet or above, increases the possibility of hepatotoxicity of UDCA, we could not rule out the possibility that the therapeutic effects of UDCA can counteract the toxic effects of its metabolite LCA. Collectively, the data in the present study can only conclude that 0.1% in the diet is a non-hepatotoxic dose for UDCA, however, data in the present study are not sufficient to make an absolute conclusion that 0.3% in the diet is a hepatotoxic dose for UDCA. More data should be generated in regarding to whether the therapeutic effects of UDCA under certain dose range can counteract its metabolite LCA before final conclusions are drawn on hepatotoxic doses of UDCA.

The hepatotoxic doses of the four BAs determined in the present study appears to be different from some reports in the literature that indicate that 0.5 or 1.0% CA or CDCA in the diets are not hepatotoxic in mice (Sinal et al., 2000; Guo et al., 2003; Yamagata et al., 2004; Miyata et al., 2005). However, consistent with the present findings, other reports indicate that CA at concentrations higher than 0.1% in the diet are hepatotoxic to mice (Fickert et al., 2001; Wang et al., 2003a), and others have reported that both DCA and LCA at 0.5 and 1.0% are toxic to rats (Delzenne et al., 1992). The reasons for these differences may root in differences in duration and/or cholesterol or other chemicals (Wang et al., 2003c) added with BAs in the diets, as well as different criteria for hepatotoxicity. In addition, based on our experience, the experimental procedures employed (detailed in methods), including

thoroughly mixing the finely ground BA with fine powdered diet, replacing the cages with clean ones right before feeding mice the BA-supplemented diet, and replacing the feeding bowls and cages with clean ones every day during the duration of the feeding process, may be critical in ensuring the final experimental results.

An increase in total BA concentration in liver is thought to be a major feature of cholestatic liver diseases. High concentrations of BAs in cholestatic liver cause liver inflammation and apoptosis or necrosis (Krell and Enderle, 1993; Kodali et al., 2006). Thus, it is expected that feeding mice hepatotoxic doses of BAs will increase the total BA concentrations in liver. However, the data in the present study demonstrated that feeding mice BAs, including CA, CDCA, DCA, and LCA, at hepatotoxic doses, did not increase the total BA concentration in liver, rather it markedly altered the BA composition, featured by increases in the relative percentage of the fed-BA and its corresponding metabolite, as well as a decrease in the percentage of the primary BA: β-MCA or CA (Fig. 3-5). This suggests that the balance between different BA species in liver is critical for maintaining normal liver function. An imbalance between different BA species appears to cause BA-induced hepatotoxicity. There are several possible explanations for the contribution of the imbalanced liver BA composition to the hepatotoxicity of the fed-BA: 1). Increases in hydrophobicity of BA pool: The fed-BA species are more hydrophobic than MCAs in mice. Based on the hydrophobicity index (HI) of individual BA, the relative hydrophobicity of different BA species are as follows: LCA>DCA>CDCA (HI: 0.5) >CA (HI: 0.1) >HDCA (HI: –0.30) >UDCA (HI: about –0.4) >MCAs (HI: about –

0.7) (Heuman, 1989). An increased percentage of the fed-hydrophobic-BA (CA, CDCA, DCA, or LCA) together with depletion of the hydrophilic MCAs will increase hydrophobicity of the BA pool, causing hepatobiliary injury (Attili et al., 1986; Becker et al., 2007). In certain instance, such as CDCA-feeding, the percentage of MCAs was not affected because CDCA can be easily hydroxylated at the 6β position to produce MCAs in rodents. However, the other primary BA in mice, a less hydrophobic CA (HI: 0.1) was replaced by CDCA (HI: 0.5) (Fig 5). Thus, CDCA-feeding can increase the hydrophobicity of BA pool which may also play a role in CDCA-induced hepatotoxicity. 2) Effects of the metabolites on the hepatotoxicity of the fed-BA: It is well established that the primary BAs, CA and CDCA, can be dehydroxylated at the 7α position by the flora in the intestine to produce the secondary BAs, DCA and LCA respectively (Iwamura, 1982). Meanwhile, the secondary BAs, DCA and LCA can be re-hydroxylated at the 7-postion in the liver to produce the primary BAs CA and CDCA, respectively (Greim et al., 1973b). The increased percentage of the secondary BAs after feeding mice the primary BAs, at hepatotoxic doses may contribute to the hepatoxicity of the parent compounds, whereas, the increased primary BA species (CA or CDCA) after feeding mice the secondary BA species (DCA or LCA) may account for mild toxic effects of the secondary BAs. In addition to CDCA, other notable metabolites of LCA were MDCA and HDCA which are much more hydrophilic than LCA and CDCA. The toxicity of LCA is limited through biotransformation into CDCA, MDCA and HDCA. 3) Possible contribution of altered cholesterol levels: The β-MCA is more effective than the fed-

BAs in preventing cholesterol saturation in bile (Wang and Tazuma, 2002; Wang et al., 2003a). Replacement of the hydrophilic MCAs with the fed-hydrophobic BAs (CA, CDCA, DCA or LCA, but not UDCA) will likely increase the cholesterol absorption from the intestine (Montet et al., 1982). Furthermore, feeding BA also inhibits both the mRNA expression and the enzyme activity of cholesterol 7α-hydroxylase (Cyp7a1) (Chiang, 2009), the rate-limiting enzyme in the classic BA biosynthesis pathway, resulting in cholesterol accumulation in liver. Consequently, the increased intestinal absorption of cholesterol and the decreased catabolism of cholesterol after feeding the hydrophobic BAs will result in cholesterol accumulation in liver, as a result, the balance between cholesterol and BAs is also likely to be disturbed by BA-feeding. The imbalance between cholesterol and BAs will produce so-called “toxic bile” which may damage the hepatobiliary structure (Trauner et al., 2008).

In conclusion, the present study indicates that it is the imbalanced BA composition in liver, but not the increases in the total liver BA concentration per se, that exerts major influences on the hepatotoxicity of each fed-BA. In addition, the present study also demonstrates that the lowest hepatotoxic dose of each BA is as follows, CA and CDCA at 0.3% in the diet, DCA at 0.1%, and LCA at 0.03% in the diet. UDCA at 0.3% in the diet or above might be hepatotoxic. Moreover, the present data indicate that the non-hepatotoxic dose of each fed-BA is 0.1% in the diet for CA, CDCA, and UDCA, 0.03% for DCA, and 0.01% for LCA.

CHAPTER 4

ADAPTIVE GENE EXPRESSION IN RESPONSE TO ALTERED BILE ACID COMPOSITION IN MICE FED NON-HEPATOTOXIC DOSES AND HEPATOTOXIC DOSES OF BILE ACIDS

4.1 Abstract

BAs can regulate their own biosynthesis and transport at the transcriptional level via activation of FXR. Our previous study has shown that feeding mice BAs at either non-hepatotoxic or hepatotoxic doses did not increase the total liver BA concentration, suggesting adaptation in the expression of genes involved in BA biosynthesis and transport. Thus, the purpose of this study was to investigate the expression of genes involved in BA homeostasis and the possible roles of the hepatic and the ileum FXR signaling pathways in regulating these genes to maintaining the total BA concentrations in the livers of mice fed different doses of BAs. Mice were fed five individual BAs, including CA, CDCA, DCA, LCA, and UDCA, at various concentrations (0.01, 0.03, 0.1, 0.3, 1.0, or 3.0%) in the diet for one week. Messenger RNAs of genes involved in BA homeostasis were quantified using QuantiGene Plex assays. FXR in liver was activated by all the BAs and at all doses, as indicated by induction of SHP mRNA in the livers. Correspondingly, Cyp7a1 mRNA was reduced by all doses of BAs. In contrast, the FXR in the ileum was activated by CA and DCA at all doses, as evidenced by induction of Fgf15 mRNA, whereas, only at hepatotoxic doses did CDCA and LCA activate the ileum FXR. Inversely to the activation pattern of the FXR-Fgf15 signaling pathway in the ileum, Cyp8b1 was suppressed by low-non-hepatotoxic doses of CA and DCA, but not by CDCA and LCA at same doses. In contrast, the mRNA of the major BA uptake transporter Ntcp was reduced only by high-hepatotoxic doses of the five BAs, but not by low-non-hepatotoxic doses of BAs. In addition, the cholesterol transporter

Abcg5/g8 was induced by all doses of the five BAs, whereas, the phospholipid translocator Mdr2 was induced only by high-hepatotoxic doses of the five BAs. In conclusion, the hepatic FXR-SHP signaling may mediate suppression of Cyp7a1 by all doses BAs, whereas, the ileum FXR-Fgf15 signaling may mediate suppression of Cyp8b1 by CA and DCA at all doses and by CDCA and LCA at hepatotoxic doses. In addition, suppression of Cyp7a1 by low-non-hepatotoxic doses of BAs is sufficient adaptation to maintain the total liver BA concentration, whereas, suppression of both Cyp7a1 and the major BA uptake transporter Ntcp contribute to the maintenance of the total liver BA concentration in mice fed BAs at hepatotoxic doses.

4.2 Introduction:

BAs play important roles in various physiological processes, including facilitating absorption of lipids and elimination of cholesterol, as well as acting as hormones regulating the homeostasis of BAs, cholesterol, and glucose (Lefebvre et al., 2009). However, BAs, as biological detergents, are also toxic. Thus the levels of BAs in liver must be tightly regulated. The regulation is usually through manipulation of biosynthesis, transport and detoxification of BAs.

The biosynthesis of BAs occurs mainly in liver. Two major biosynthesis pathways have been identified, namely, the classic pathway initiated by the enzyme Cyp7a1 (cholesterol 7α-hydroxylase) in the endoplasmic reticulum of the hepatocyte (Russell and Setchell, 1992), and the alternative pathway, initiated by the enzyme Cyp27a1 (sterol 27-hydroxylase) in the mitochondria of a variety of tissues (Russell

and Setchell, 1992; Bjorkhem et al., 1999). In mammals, the main products of the classic pathway are CA and CDCA. The enzyme Cyp8b1 (sterol 12-hydroxylase) is critical in determining the ratio between CA and CDCA (Pandak et al., 2001). Mice lacking Cyp8b1 do not synthesize CA (Li-Hawkins et al., 2002). With the presence of Cyp8b1, the 7α-hydroxylated intermediates from the upstream reactions in the classic BA biosynthesis pathway can be used to form a 12-OH BA, CA, whereas, without Cyp8b1, the 7α-hydroxylated intermediate will be used to form the end product CDCA, which is a non-12-OH BA. The predominant end product from the alternative pathway is thought to be CDCA. The oxysterol 7α-hydroxylase (Cyp7b1) is employed in the alternative pathway to catalyze 27α-hydroxylated intermediate from the upstream of the alternative BA biosynthesis pathway to produce 7α,27α-di hydroxylated intermediates (Bjorkhem, 1992). It is proposed that the 7α-hydroxylated intermediates of the alternative pathway can also be 12-hydroxylated by Cyp8b1 to form the end product CA, because Cyp8b1 deficiency causes complete depletion of CA (Li-Hawkins et al., 2002). In rodents, CDCA can be 6β-hydroxylated to form α-MCA, from which the dominant muri-BA, β-MCA, is derived (Botham and Boyd, 1983). Thus, in rodents, CA and β-MCA, instead of CDCA, are the primary BAs. In rodents, the alternative pathway is thought to contribute to a substantial fraction of the total BA biosynthesis under physiological conditions (Vlahcevic et al., 1997).

To ensure their physiological functions, normal enterohepatic circulation (EHC) of BAs is essential. Two main processes are involved in EHC: liver secretion

and intestinal reabsorption of BAs. Multiple active transport systems in both liver and intestine are involved in the EHC of BAs. The hepatocytes are polarized epithelia with the sinusoid membrane facing the blood and the canalicular membrane facing the bile. After BAs are synthesized in the hepatocytes, the glycine or taurine conjugated monovalent BAs are actively pumped by the BA-efflux pump, Bsep, into the canaliculi (Gerloff et al., 1998). The divalent BAs, such as sulfated LCA, are transported actively by the multidrug resistance associated protein Mrp2 into bile (Muller and Jansen, 1997; Takikawa, 2002). BAs in the canaliculi then go through various biliary ducts and are finally stored in the gallbladder. Upon each meal, BAs are released from the gallbladder into the intestine where BAs emulsify dietary lipids to facilitate their absorption. About 95% of BAs released into the intestine are reabsorbed. The Asbt (Apical sodium dependent bile salt transporter) on the apical membrane of the enterocytes takes up BAs from the lumen of the intestine and transfers BAs into the cytosol of the enterocytes. Inside the cytosol, the Ibabp (ileum BA binding protein) is thought to bind BAs and transfer the BAs across the cytosol to the basolateral membrane of the enterocytes, where the efflux transporter Ostα/β pumps BAs into the portal blood. BAs in the portal vein eventually return to the liver where they are taken up by the sodium dependent BA transporter Ntcp and sodium independent transporters Oatps. The recycled BAs together with the *de novo* synthesized BAs then go through another EHC cycle (Alrefai and Gill, 2007).

Biliary secretion of BAs must be accompanied with proportional lipid secretion, so that the mixed micelles composed of BAs, cholesterol and phospholipids can form to protect the hepatobiliary system from the toxicity of free BAs and to prevent the deposit of cholesterol in the bile. Without the lipid components, simple micelles composed solely of BAs can form, which can damage the membranes of the biliary tract (Hofmann and Hagey, 2008). Various lipid translocators in the canalicular membrane of hepatocytes are involved in lipid excretion into bile: the Abcg5/g8 mediates the translocation of cholesterol; the Mdr2 flops the phospholipid into the bile, whereas the Atp8b1 translocates the amino-phospholipids from the outer leaflet to the inner leaflet of the canalicular membrane to maintain the asymmetric distribution of phospholipids in the membrane (Eppens et al., 2001; Ujhazy et al., 2001).

It has been thought that excessive BAs can activate the nuclear receptor FXR to regulate the homeostasis of BAs (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). FXR negatively regulates BA biosynthesis enzymes Cyp7a1 and Cyp8b1 (Goodwin et al., 2000; Chiang, 2002), as well as hepatic uptake transporter Ntcp (Denson et al., 2001), while positively regulates the expression of Bsep (Ananthanarayanan et al., 2001), and Ostα/β (Boyer et al., 2006). In 2005, an intestinal signaling pathway mediated by ileum FXR-Fgf15 was identified. The intestinal FXR can be activated by CA, causing increased expression of Fgf15, which travels via the portal vein to the liver where it activates the cell surface receptor FGFR4, causing a decrease in expression of Cyp7a1 mRNA (Inagaki et al.,

2005; Kim et al., 2007). Our previous study (Chapter 3) determined the non-hepatotoxic and hepatotoxic doses of individual BAs (see summary in Table 4-1) and showed that feeding mice BAs (CA, CDCA, DCA, or LCA) at hepatotoxic doses did not increase the total BA concentration in liver, but it disturbed the liver BA composition, featured by a depletion of the primary BAs (MCAs or CA) and an enrichment of the fed-BA. These findings suggest a wide range of adaptations in BA biosynthesis and transport. Therefore, in this present study, we investigated the adaptive changes in the expression of genes involved in BA homeostasis in mice fed either low-non-hepatotoxic doses or high-hepatotoxic doses of individual BAs, as well as the possible roles of the BA receptor FXR in the regulation of these genes.

Table 4-1. List of doses of individual bile acid used in the present study.

	Non-hepatotoxic doses (W/W% in the diet)	Hepatotoxic doses (W/W% in the diet)
CA	0.03 and 0.10%	0.30 and 1.0%
CDCA	0.03 and 0.10%	0.30%
DCA	0.03%	0.10 and 0.30%
LCA	0.01%	0.03, 0.10 and 0.30%
UDCA	0.10%	0.30 and 1.0%

4.3 Results

Effect of feeding BAs at non-hepatotoxic doses on total liver BA concentration and liver BA composition. As shown in Fig 4-1, feeding each BA at low-non-hepatotoxic doses (CA, CDCA, and DCA at 0.03% in the diets, LCA at 0.01% in the diet, and UDCA at 0.1% in the diet) did not increase the total liver BA concentration, rather, the total liver BA concentration after non-hepatotoxic doses of BA-feeding tends to decrease; however the liver BA composition was slightly altered. Feeding of CA and DCA resulted in similar changes in liver BA composition. More specifically, feeding CA or DCA at 0.03% in the diet increased the percentage of the total CA (CA+T-CA) from 39.4% to about 51%, and decreased the total MCAs from 52.7% to around 38% of the total liver BAs. Interestingly, feeding both CA and DCA at 0.03% in the diet increased the percentage of the total DCA (DCA+T-DCA) from 2.7 to about 5-8%. There are no effects of feeding of CA or DCA at 0.03% in the diets on the percentage of other BA species. Feeding CDCA at 0.03% in the diet not only increased the total CDCA (CDCA+T-CDCA) from 1.5% to 4.0% relative to the total liver BAs, but also slightly increased the total MCAs (MCAs+T-MCAs) and the total 6-OH BAs (HDCA+T-HDCA+MDCA+T-MDCA) from 52.7 to 66.3% and 2.0 to 4.6% respectively. Feeding CDCA decreased the percentage of total CA from 39.4 to 19.3% relative to the total liver BAs. Feeding 0.01% LCA in the diet did not increase the percentage of the total LCA, neither did it increase the percentage of the total MCAs. However, feeding 0.01% LCA in the diet increased the percentage of its metabolites, CDCA and 6-OH BAs (MDCA plus HDCA) from 1.5 to 4.0% and

2.0 to 4.6%, respectively, of the total liver BAs. Feeding LCA also decreased slightly the percentage of total CA from 39.4 to 33.0%.

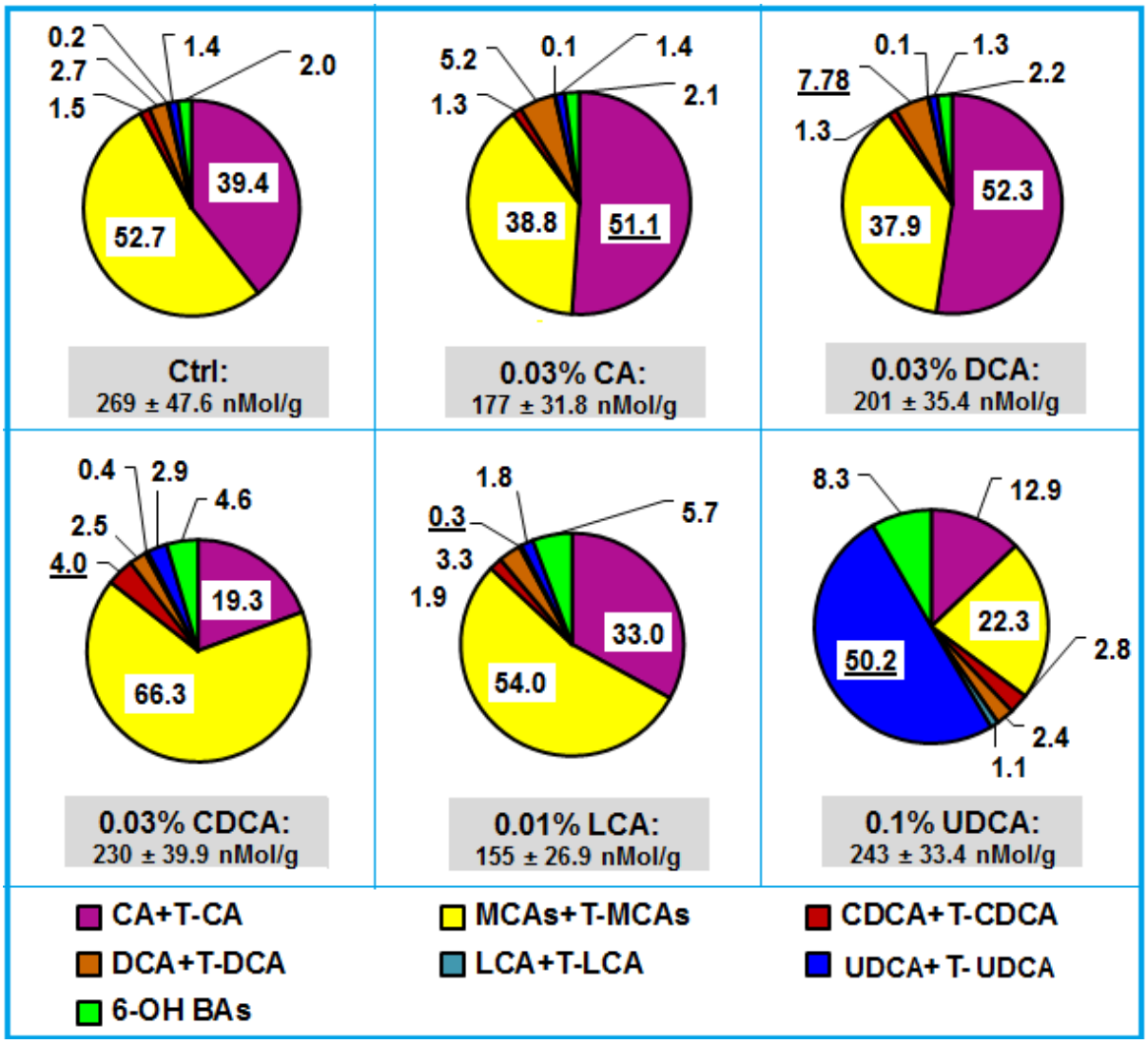


Fig 4-1 Total BA concentrations and BA composition (% of total BAs) in the livers of mice fed control diet or diets supplemented with BAs (CA, CDCA, DCA, LCA, or UDCA) at non-hepatotoxic doses for one week.

Feeding UDCA at 0.1% in the diet increased the percentage of the total LCA, the total UDCA, and the 6-OH BAs from 0.2 to 1.1%, 1.4 to 50.2%, and from 2.0 to 8.3%, respectively, whereas, feeding UDCA decreased the percentage of the total CA and the total MCAs from 39.4 to 12.9 % and 52.7 to 22.3%, respectively.

Effect of BA feeding on mRNA expression of BA-biosynthesis enzymes in liver To investigate the effects of various individual BAs on mRNA expression of BA-biosynthesis enzymes, the mRNA of Cyp7a1, 8b1, 27a1 and 7b1, were quantified. As expected, after feeding mice various BAs for one week, Cyp7a1 mRNA expression was reduced markedly (about 70-99%) by all fed-BAs at all doses (Fig 4-2). The suppression of Cyp7a1 by feeding of the 12-OH-BAs (CA and DCA) was the maximum (about 95%) even at the lowest non-hepatotoxic doses (0.03% in the diets), whereas, suppression of Cyp7a1 by feeding the non-12-OH BAs (CDCA and LCA) had an obvious dose-dependent response. Feeding the two non-12-OH BAs at the lowest dose suppressed the mRNA expression of Cyp7a1 about 70%, with doses increasing, the suppression reached the maximum at 96%. Feeding UDCA at various doses suppressed the mRNA expression of Cyp7a1 93-100%. The mRNA expression of Cyp8b1 was also down-regulated by feeding BAs, but the reduction required higher doses of BAs. The two 12-OH BAs decreased Cyp8b1 at all doses tested. However, unlike their effects on Cyp7a1 expression, the non-12-OH BAs suppressed Cyp8b1 mRNA expression only at high hepatotoxic doses. Specifically, CDCA and LCA at 0.3% in the diets, and UDCA at 0.3, 1.0, and 3.0% in the diets, decreased Cyp8b1 mRNA levels.

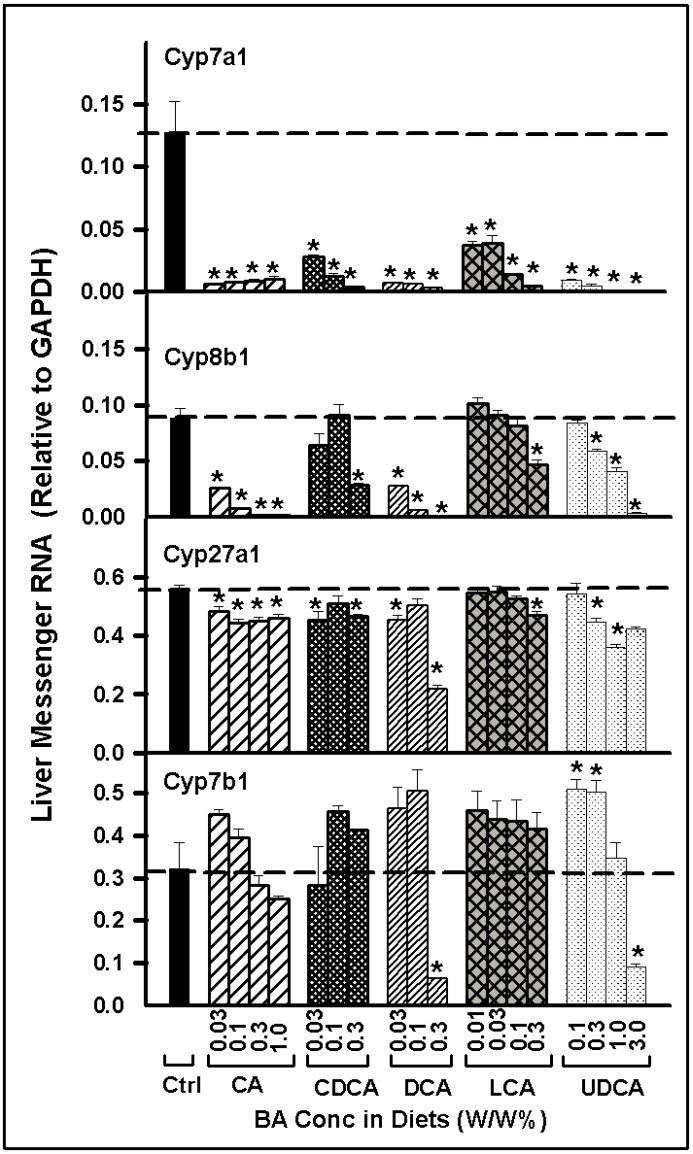


Fig 4-2 Messenger RNA expression of genes involved in BA biosynthesis in livers of mice treated with five BAs (CA, CDCA, DCA, LCA, or UDCA) at concentrations of 0.01, 0.03, 0.1, 0.3, 1.0, or 3.0% in the diets. Data are presented as Mean ± S.E.M (n=5). *P<0.05

BA feeding had relatively minor effects on Cyp27a1 mRNA expression (Fig 4-2.). Feeding CA at all doses decreased Cyp27a1 mRNA expression 12-19%. Feeding CDCA at the lowest (0.03% in the diet) and highest dose (0.3% in the diet)

decreased Cyp27a1 mRNA expression about 15%. Feeding DCA at 0.03 and 0.1% decreased Cyp27a1 mRNA expression 15% and 60%, respectively. The effect of BA feeding on Cyp7b1 mRNA was quite different than that on Cyp7a1. Whereas, BA feeding almost eliminated Cyp7a1 mRNA expression, it tended to increase Cyp7b1 mRNA expression. However, the highest doses of DCA (0.3% in the diet) and UDCA (3.0% in the diet) markedly decreased Cyp7b1 mRNA.

Effect of BA feeding on mRNA expression of hepatic uptake transporters in liver As shown in Fig 4-3, after feeding non-hepatotoxic doses of BAs, Ntcp mRNA expression was not altered, however, at higher and hepatotoxic doses, Ntcp mRNA expression was decreased. Specifically, CA and DCA (0.3 or 1% in the diet), the highest concentration of LCA (0.3% in the diet), and two intermediate concentrations of UDCA in the feed (0.3 and 1.0) produced rather small decreases (12-30%) in the expression of Ntcp. CDCA did not alter Ntcp mRNA expression at any dose.

The Oatps (Oatp1a1, 1a4, and 1b2) are considered to have a less important role in BA uptake into liver than Ntcp. As shown in Fig 4-3, Oatp1a1 mRNA was not altered markedly by BA feeding, except for the highest doses of DCA (0.3% in the diet) and UDCA (3.0% in the diet), which decreased Oatp1a1. Oatp1a4 mRNA was also not altered markedly by BA feeding, with the exception of the highest dose of UDCA (3.0% in the diet) that increased Oatp1a4 nearly 10-fold. Oatp1b2 mRNA is specifically, if not exclusively, expressed in liver; feeding BAs produced only small

increases in Oatp1b2 mRNA expression, except for the highest concentration of DCA, which decreased it about 30%.

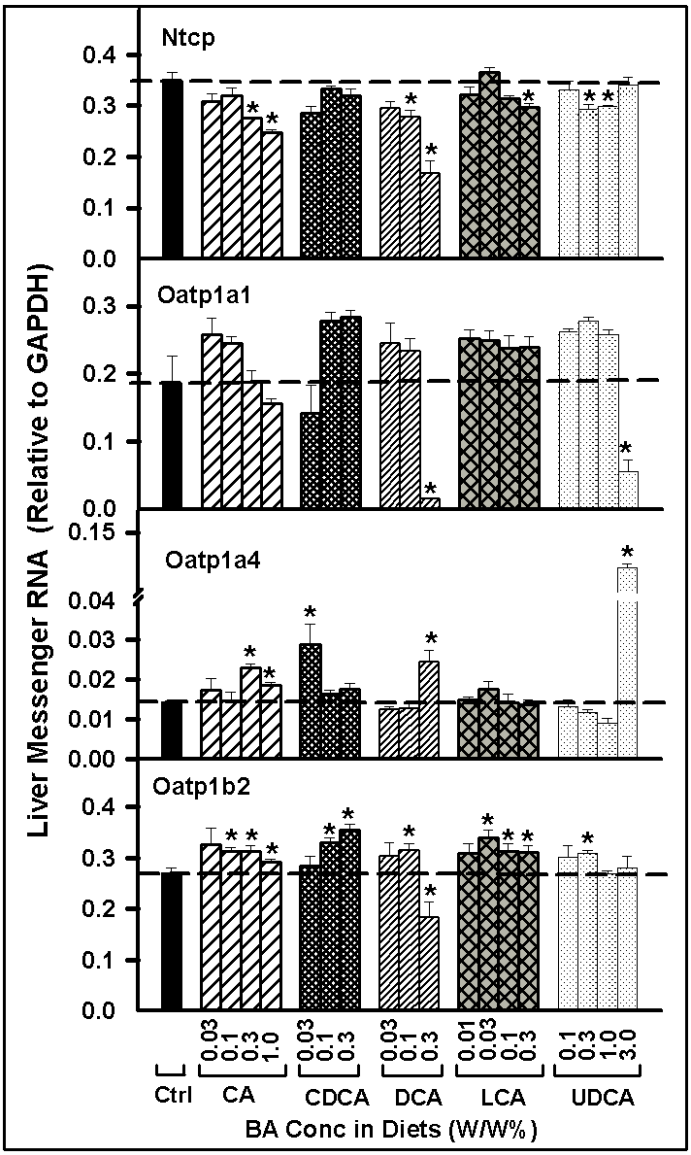


Fig 4-3 Messenger RNA expression of genes involved in BA uptake in livers of mice treated with five BAs (CA, CDCA, DCA, LCA, or UDCA) at concentrations of 0.01, 0.03, 0.1, 0.3, 1.0, or 3.0% in the diets. Data are presented as Mean ± S.E.M (n=5). *P<0.05

Effect of BA feeding on mRNA expression of hepatic efflux transporters located on the canalicular membrane of hepatocytes The effects of BA feeding on Bsep mRNA levels are shown in Fig 4-4. CA was the only BA that increased Bsep mRNA (35%), and this occurred only with the high-hepatotoxic doses of CA (0.3 and 1.0% in the diets). CDCA and LCA produced small (25-35%), but statistically significant decreases in Bsep mRNA. The three lower concentrations of UDCA also decreased Bsep mRNA.

As shown in Fig 4-4, BA feeding had relatively minor effects on Mrp2 mRNA expression.

After feeding mice various BAs, there were very little changes in Atp8b1 expression, except that the largest doses of UDCA (3.0% in the diet) decreased Atp8b1 expression about 30% (Fig 4-4).

Feeding the five BAs at all doses increased Abcg5 mRNA expression (Fig 4-4). More specifically, CA, DCA, and UDCA increased Abcg5 mRNA in a dose-dependent manner. Similarly, Abcg8 mRNA was increased by feeding all BAs. Collectively, these studies demonstrate that Abcg5 and Abcg8 are up-regulated by all five BAs at both low-non-hepatotoxic doses and high-hepatotoxic doses.

As shown in Fig 4-4, feeding of all BAs at high-Hepatotoxic doses produced small increases in Mdr2 mRNA expression in liver.

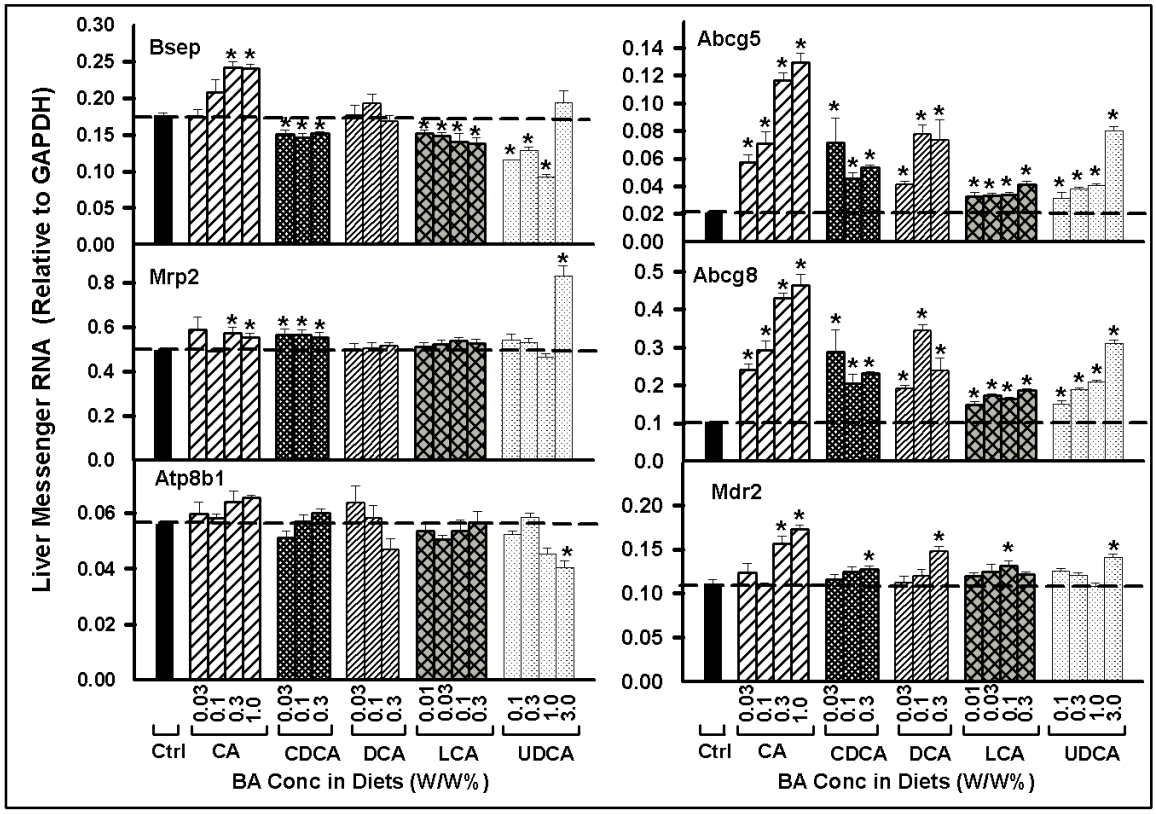


Fig 4-4 Messenger RNA expression of efflux transporters on canalicular surfaces of hepatocytes in mice treated with five BAs (CA, CDCA, DCA, LCA, or UDCA) at concentrations of 0.01, 0.03, 0.1, 0.3, 1.0, or 3.0% in the diets. Data are presented as Mean ± S.E.M (n=5). *P<0.05

Effect of BA feeding on mRNA expression of hepatic efflux transporters located on the sinusoidal membrane of hepatocytes or basolateral membrane of cholangiocytes in liver. As shown in Fig 4-5, feeding BAs (CA, CDCA, and, LCA) produced a small (about 20%) but statistically significant increase in Mrp3 mRNA. Feeding BAs had only a minor effect on the expression of Mrp4, as only feeding 3.0% UDCA in the diet increased Mrp4 mRNA.

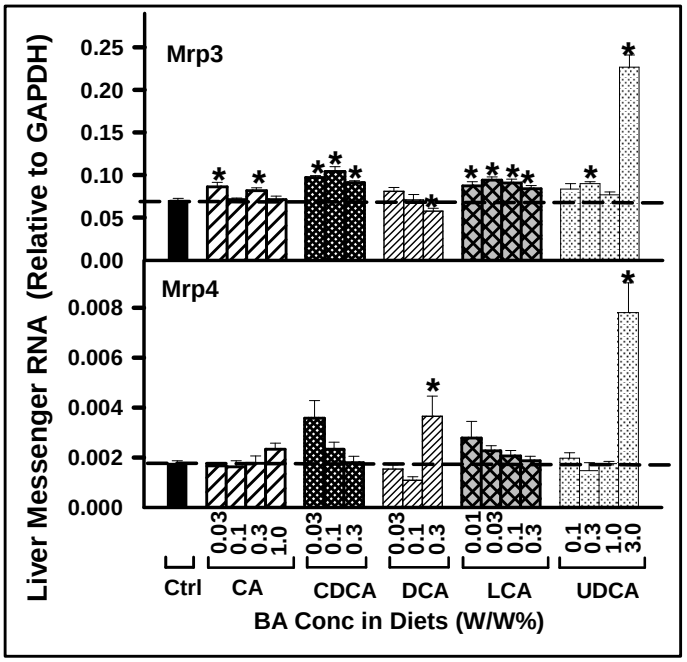


Fig 4-5 Messenger RNA expression of efflux transporters on sinusoid surfaces of hepatocytes in mice treated with five BAs (CA, CDCA, DCA, LCA, or UDCA) at concentrations of 0.01, 0.03, 0.1, 0.3, 1.0, or 3.0% in the diets. Data are presented as Mean ± S.E.M (n=5). *P<0.05

As shown in Fig S4-1, the expression of Ostα/β in liver is rather low. Ostα mRNA was increased by feeding each of the five BAs. However, Ostβ mRNA was increased dose-dependently only by CA and DCA, as well as the highest doses of UDCA. But Ostβ was not altered by either CDCA or LCA.

Effect of BA feeding on mRNA expression of nuclear receptors and their target genes in liver. As shown in Fig 4-6, FXR mRNA was not altered by any of the BAs, except for the highest dose of UDCA, which increased FXR mRNA. In

contrast, the FXR-target gene SHP was increased by all BAs and at all doses tested, suggesting that FXR is activated by all five BAs.

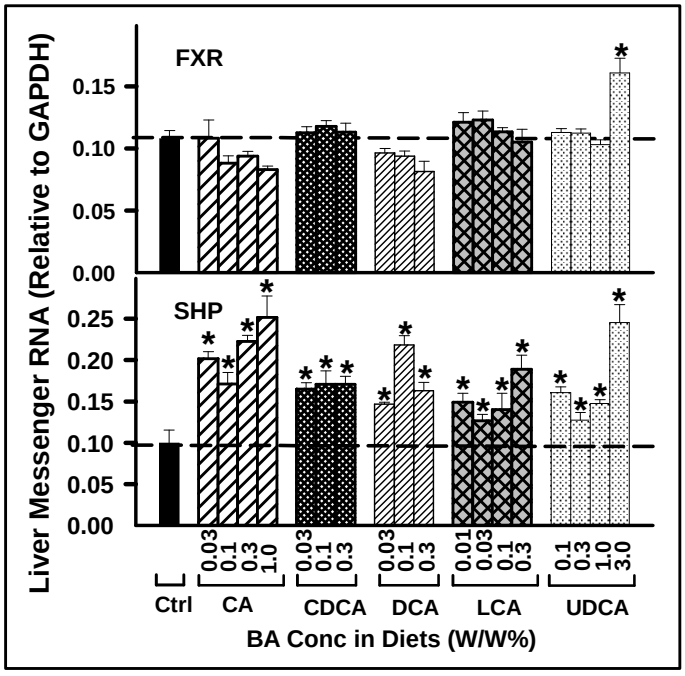


Fig 4-6 Messenger RNA expression of FXR and its target gene SHP in livers of mice treated with five BAs (CA, CDCA, DCA, LCA, or UDCA) at concentrations of 0.01, 0.03, 0.1, 0.3, 1.0, or 3.0% in the diets. Data are presented as Mean ± S.E.M (n=5). *P<0.05

PXR mRNA was only increased by feeding high concentrations of BAs (Fig S4-2), and its target gene Cyp3a11 mRNA was not increased by any of the BAs. Even though there are increases in the mRNA expression of Car (20-35% in the diets) in mice fed CA at 0.03 and 0.3%, CDCA at 0.3%, DCA and LCA at 0.1% in the diet, there are no corresponding increases in the mRNA expression of its target gene Cyp2b10. Only feeding of UDCA at 3.0% increased both Car about 130% and its target gene Cyp2b10 about 8 fold. Both Nrf2 mRNA and its target gene Nqo1

were increased by high concentrations of BAs, including CA at 0.3 and 1.0%, and DCA at 0.3% in the diets. UDCA at the highest concentration (3.0%) in the diet also increased mRNA expression of Nrf2 and its target gene Nqo1 (Fig S4-2). Surprisingly, only feeding CDCA at the lowest dose (0.03% in the diet) increased both Nrf2 and NqO1 mRNA expression.

Effects of feeding of individual BAs on mRNA expression of FXR and its target genes in ileum As shown in Fig 4-7, feeding mice BAs did not alter the mRNA expression of FXR in the ileum. However, feeding CA and DCA dose-dependently increased the FXR-target genes SHP and Fgf15 mRNA expression. Conversely, only the highest concentrations of CDCA in the diet increased SHP and Fgf15 mRNA, whereas LCA at the highest concentration and UDCA at all concentrations tested increased Fgf15 mRNA but not SHP mRNA in the ileum.

Effect of feeding individual BAs on mRNA expression of BA transporters in ileum. As shown in Fig S4-3, the effects of feeding five BAs on Asbt in ilea were not marked. Feeding CA at concentrations of 0.3 and 1.0% in the diets decreased the mRNA of Asbt 31 and 41%, respectively. Feeding CDCA and DCA had little effect on mRNA expression of Asbt. In contrast, feeding of LCA at 0.01 and 0.1% in the diets increased the mRNA of Asbt about 30%, as well as feeding 0.3% UDCA increased Asbt mRNA 23%. However, UDCA at the highest dose (3.0% in the diet) decreased Asbt mRNA expression in ileum 26%.

CA, only at the lowest dose (0.03% in the diet), increased the intestinal BA binding protein (Ibabp) mRNA 21% (Fig S4-3). At higher doses, CA did not affect

the mRNA expression of Ibabp. In contrast, only at higher doses did feeding of other BAs (CDCA at 0.3%, DCA at 0.1%, LCA at 0.1, and 0.3% in the diets) increased Ibabp mRNA 16-37%. Feeding of UDCA at 0.1, 0.3, and 1.0% in the diets increased Ibabp mRNA 50-150%.

The heterodimer transporter (Ostα/β) in ileum is essential in BA efflux from the enterocytes into blood (Rao et al., 2008). As shown in Fig S4-3, mRNA of Ostα was regulated by feeding mice various BAs. Ostα was increased by CA at 0.1, 0.3, and 1.0% in the diets about 30%, CDCA at 0.3% in the diet 31%, as well as DCA at 0.1 and 0.3% in the diets 40-46%. However, Ostα was not regulated by LCA at any concentration. UDCA at 0.1 and 0.3% in the diets also increased Ostα mRNA expression in ileum 19 and 67%, respectively. In contrast to the effect of feeding BAs on Ostα mRNA expression, feeding BAs had little effect on Ostβ mRNA expression, except that DCA at the highest concentration (0.3% in the diet) increased Ostβ mRNA in ileum 28%.

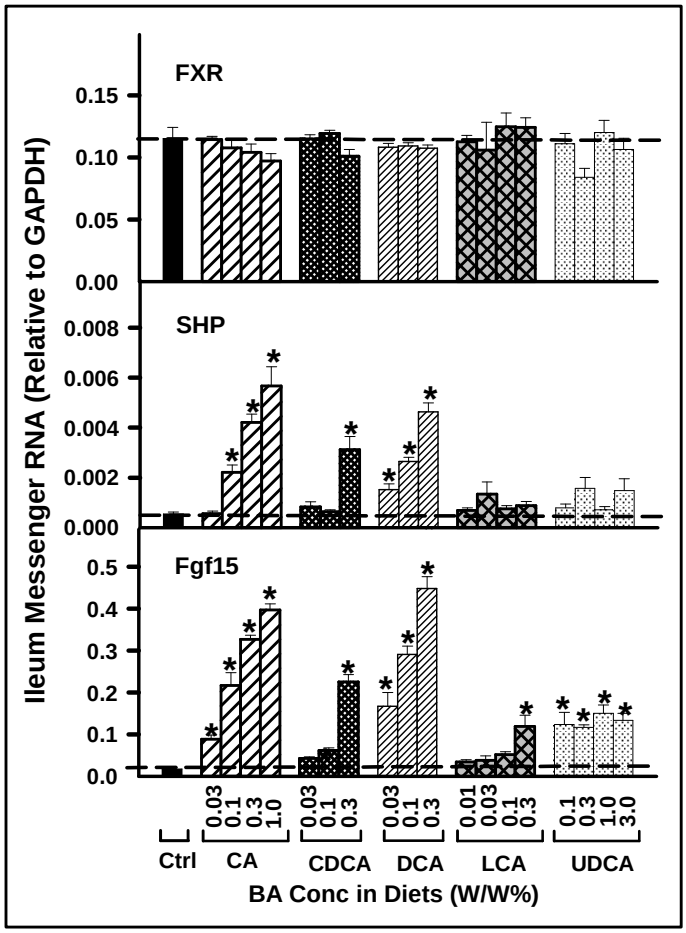


Fig 4-7 Messenger RNA expression of FXR and its target genes in ilea of mice treated with five BAs (CA, CDCA, DCA, LCA, or UDCA) at concentrations of 0.01, 0.03, 0.1, 0.3, 1.0, or 3.0% in the diets. Data are presented as Mean ± S.E.M (n=5). *P<0.05

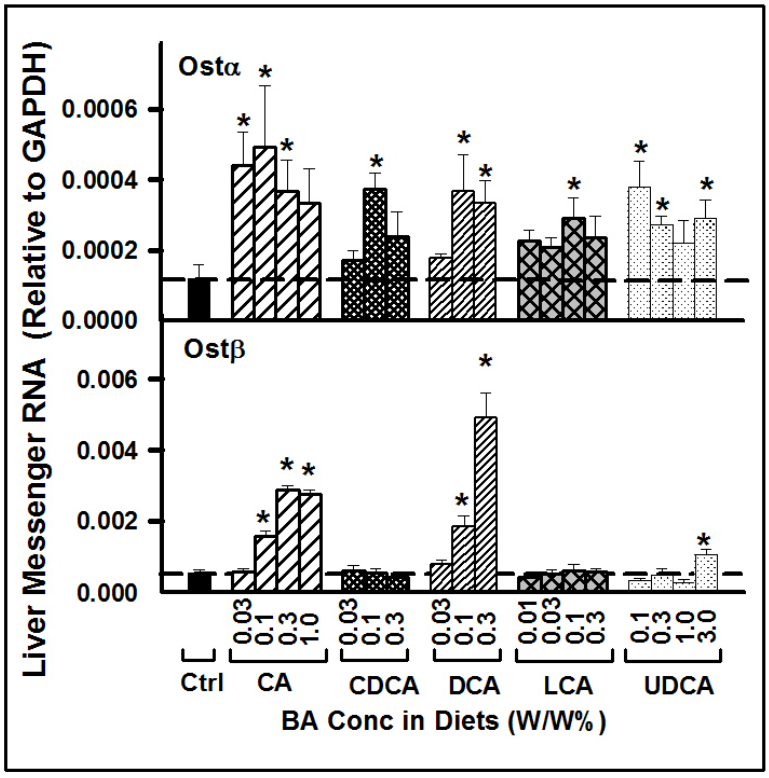


Fig S4-1 Messenger RNA expression of Ostα and β in the livers of mice treated with five BAs (CA, CDCA, DCA, LCA, or UDCA) at concentrations of 0.01, 0.03, 0.1, 0.3, 1.0, or 3.0% in the diets. Data are presented as Mean ± S.E.M (n=5). *P<0.05

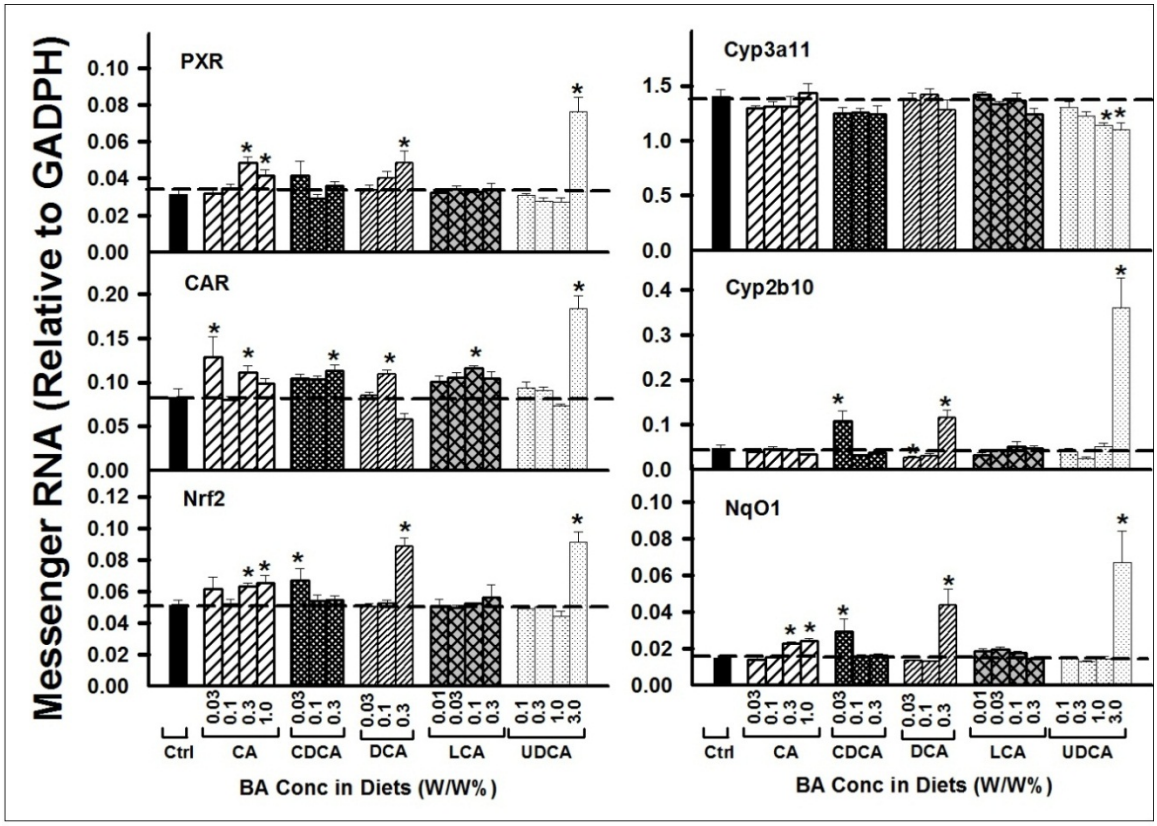


Fig S4-2 Messenger RNA expression of nuclear receptors and their target genes in the livers of mice treated with five BAs (CA, CDCA, DCA, LCA, or UDCA) at concentrations of 0.01, 0.03, 0.1, 0.3, 1.0, or 3.0% in the diets. Data are presented as Mean ± S.E.M (n=5). *P<0.05

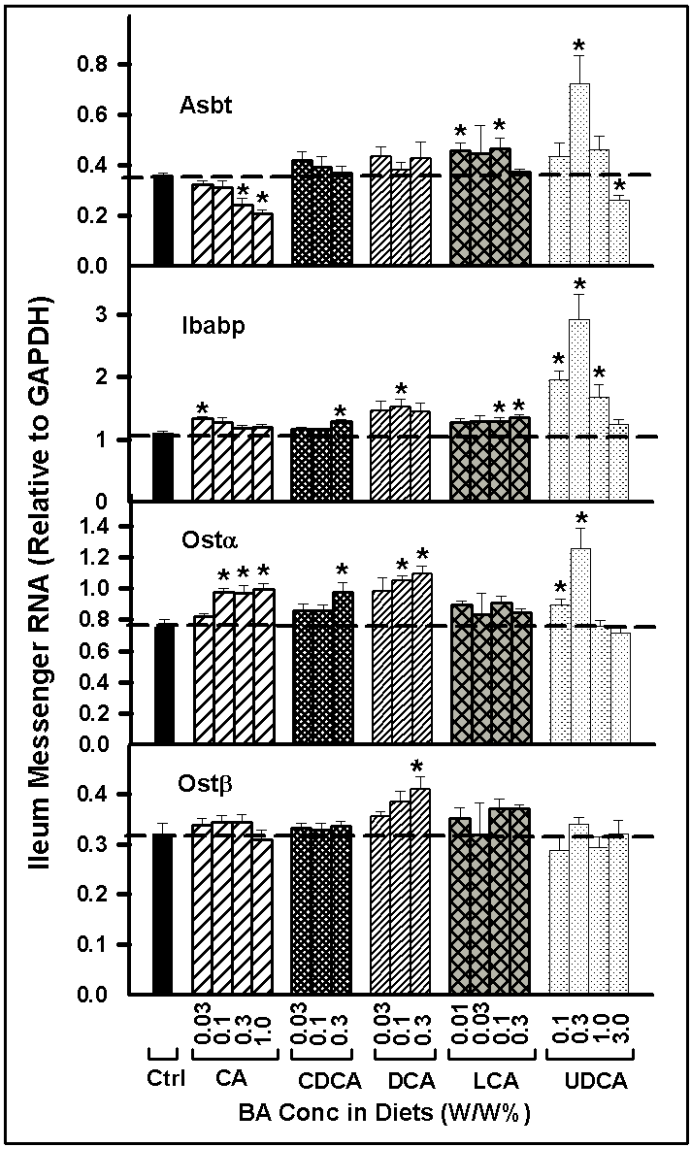


Fig S4-3 Messenger RNA expression of BA transporters in the ilea of mice treated with five BAs (CA, CDCA, DCA, LCA, or UDCA) at concentrations of 0.01, 0.03, 0.1, 0.3, 1.0, or 3.0% in the diets. Data are presented as Mean ± S.E.M (n=5). *P<0.05

4.4 Discussion

It has been thought that BAs can activate their nuclear receptor FXR to regulate genes involved in BA biosynthesis and transport at the transcriptional level. To study the roles of FXR in BA regulation of genes involved in BA homeostasis, FXR-null mouse model, bile-duct ligation mouse model, or the potent synthetic FXR activator GW4064, have been used (Goodwin et al., 2000; Inagaki et al., 2005; Kim et al., 2007). The data generated from the above-mentioned animal models are very valuable for understanding BA regulation of genes via the FXR-mediated-signaling pathway. Especially in regard to the transcriptional regulation of Cyp7a1, conclusions have been made that it is the ileum FXR-Fgf15 signaling, but not the hepatic FXR-SHP signaling, that plays the major role (Inagaki et al., 2005; Kim et al., 2007). Given the fact that the animal models used in those studies are non-physiological and that the FXR ligand used (GW4064) is 1000 times more potent than CDCA in activation of FXR (Goodwin et al., 2000), a comprehensive understanding of BA regulation of genes via the FXR-mediated pathway under normal/physiological conditions has been somewhat limited due to the lack of *in vivo* studies using intact wild-type animals fed low and non-hepatotoxic doses of individual BAs. In addition, our previous study (chapter 3) shows that the total BA concentration in the liver remains at control levels despite remarkable variations in daily input of the fed-BAs (0.01%-1% in the diet). These findings suggest that a wide range of adaptations probably occur in the *de novo* BA biosynthesis and transport.

In the present study, we demonstrate that it was hepatic FXR, but not other nuclear receptors (PXR, Nrf2, or CAR), that was activated by all five individual BAs at both low-non-hepatotoxic as well as high-hepatotoxic doses, as evidenced by increased mRNA expression of its major target gene SHP (Fig 4-6 and Fig S4-2). Herein, we characterized the adaptive changes in mRNA expression of genes involved in BA homeostasis that contribute to maintaining the total liver BA concentrations after feeding mice either low-non-hepatotoxic doses or high-hepatotoxic doses of individual BAs, and propose possible roles of FXR in liver and ileum in regulating these genes.

The data in the present study demonstrate that feeding BAs at non-hepatotoxic doses only slightly altered BA composition in the liver without affecting the total BA concentration in the liver (Fig 4-1). A surprising finding in the present study is that the FXR-SHP signaling pathway in the liver is activated even though the total BA concentration in the liver did not increase in mice fed BAs. In fact, there was a similar increase in SHP mRNA in livers of mice fed low and high concentrations of BAs in the diets, suggesting that at relatively low concentrations (0.01% or 0.03% in the diets) the BA sensor FXR in liver was fully activated by feeding any of the five BAs. In contrast, the FXR-Fgf15 signaling pathway in the ileum is activated only by feeding any dose of the 12-OH BAs (CA and DCA), but not by feeding low-non-hepatotoxic doses of the non-12-OH BAs (CDCA and LCA) (Fig 4-7). Feeding all BAs at high-hepatotoxic doses activated the ileum FXR-Fgf15

signaling, suggesting that the ileum FXR-Fgf15 signaling is more responsive to high concentrations of BAs.

Both the hepatic FXR-SHP signaling pathway and the ileum FXR-Fgf15 signaling pathway are involved in feedback regulation of BA biosynthesis. The FXR-Fgf15 signaling pathway in the ileum is proposed to play a major role in regulating Cyp7a1, whereas, the hepatic FXR-SHP signaling pathway is thought to play major roles in regulation of Cyp8b1 (Kim et al., 2007). In agreement with previous findings, the present data show that Fgf15 mRNA in the ileum was dose-dependently increased by 12-OH BAs (CA and DCA) (Fig 4-6). The increased Fgf15 may contribute to the decreased mRNA expression of Cyp7a1 in the liver by these two BAs. However, the present study also shows that feeding the non-12-OH BAs (CDCA and LCA) at low-non-hepatotoxic doses (0.1% in the diet or lower) did not increase intestinal Fgf15 mRNA expression, indicating that intestinal FXR is not activated by these two BAs at low concentrations. In contrast, the mRNA of the hepatic FXR-target gene SHP was fully induced, and the mRNA of Cyp7a1 in liver was decreased by all BAs, even at the lowest concentrations fed mice. Collectively, the present study suggests that the hepatic FXR-SHP signaling pathway in the liver is more important than the intestinal FXR-Fgf15 pathway in the basal regulation of Cyp7a1 expression by BAs, whereas the FXR-Fgf15 signaling pathway in the ileum plays more important roles in mediating feedback regulation of Cyp7a1 under BA overloading. This conclusion based on the present data is not fully in agreement with that reported in a previous study (Kim et al., 2007). There are several plausible

explanations for the difference observed in the two studies: 1). The BA concentrations (the sum of CA, CDCA, DCA and LCA) in the BA pool of mice fed the CDCA or LCA at low-non-hepatotoxic doses are lower than those in mice fed CA or DCA at same doses. Moreover, it was proposed that CA but not CDCA is the important endogenous agonist for FXR in mice (Li-Hawkins et al., 2002). Accordingly, it seems reasonable that the FXR-Fgf15 signaling will not be activated by low concentrations of CDCA or LCA. However, considering that both CDCA and LCA at low doses activated hepatic FXR to a similar level as high doses, it does not seem likely that low BA concentrations in mice fed CDCA or LCA is a reason for their not activating the FXR-Fgf15 signaling in ileum. 2). The FXR agonists used in the two studies are different. In Kim's study, the synthetic FXR activator GW4064 was used. GW4064 is 1000 times more potent than CDCA in activation of FXR (Goodwin et al., 2000). Thus, activation of FXR in the ileum by GW4064 more likely represents the effects of markedly excessive BAs, but not that of the low doses of BAs. The latter explanation can be supported by the data presented in Fig 5 and Fig 6 in Kim's report (Kim et al., 2007). The liver-specific FXR-null mice displayed a decreased expression of SHP and an increased expression of Cyp7a1, suggesting that hepatic FXR-SHP signaling pathway is important for the basal regulation of Cyp7a1. However, the ileum-specific FXR-null mice had a decreased expression of Fgf15, but did not display an increase in the expression of Cyp7a1 (Kim et al., 2007), suggesting that the FXR-Fgf15 signaling pathway in the ileum is not critical in basal regulation of Cyp7a1 in liver. All in all, both Kim's data generated from the

liver- and ileum-specific FXR-null mice and the present data generated from feeding of individual BAs at low-non-hepatotoxic doses indicate that it is the hepatic FXR-SHP signaling pathway, but not the ileum FXR-Fgf15 signaling pathway, that is playing central role in the basal regulation of Cyp7a1 by BAs, whereas, the ileum FXR-Fgf15 signaling may play a more important role when FXR is over-activated by overloaded BAs or potent activators such as GW4064.

Even though the hepatic FXR-SHP signaling pathway was fully activated by all BAs at all doses in the present study, the putative FXR-target gene, Cyp8b1, unlike Cyp7a1 that is decreased by all BAs at all doses, (Wang et al., 2006; Kim et al., 2007; Norlin and Wikvall, 2007) was only decreased by feeding the 12-OH BAs, but not by the non-12-OH BAs, except at high-hepatotoxic doses. In addition, Kim's report also demonstrated that deletion of hepatic FXR increased Cyp7a1 mRNA, but not Cyp8b1 mRNA expression (Kim et al., 2007). Accordingly, the data from both studies indicate that the hepatic FXR-SHP signaling pathway is not as critical in regulating Cyp8b1 as in regulating Cyp7a1. Instead, the regulation pattern of Cyp8b1 in liver by various BAs is conversely correlated to the pattern of Fgf15 expression in ileum, suggesting that the FXR-Fgf15 pathway in ileum might play an important role in regulating Cyp8b1. Thus, regulation of Cyp7a1 and Cyp8b1 by FXR-Fgf15 may be dependent on both concentrations and species of BAs present in the BA pool.

Compared to the effects of feeding of BAs at non-hepatotoxic doses on mRNA expression of Cyp7a1, feeding of BAs had a less effect on the mRNA expression Cyp27a1 and Cyp7b1. In mice fed non-hepatotoxic doses of BAs (including CA, CDCA, and DCA, but not LCA), there are slight decreases in Cyp27a1 mRNA expression (about 10%), and no change in Cyp7b1 mRNA expression, suggesting that the hepatic FXR-SHP signaling pathway may not play major roles in feedback regulation of the two genes. Our conclusion is supported by an *in vitro* study which demonstrates that, the hydrophobic BAs are able to suppress Cyp27a1 mRNA expression without FXR presence (Chen and Chiang, 2003).

Among all the transporters examined in the present study, only the cholesterol half transporter Abcg5 and g8 were increased by feeding the five BAs at low-non-hepatotoxic doses, other transporters, including Ntcp, Oatps, Bsep, Mrp3 or Mrp4, Atp8b1 and Mdr2, were not regulated by all five BAs at low-non-hepatotoxic doses. In contrast, in mice fed BAs at hepatotoxic doses, there were decreases in the mRNA expression of Ntcp, and increases in mRNA expression of Abcg5 and g8, as well as Mdr2. The alterations in mRNA expression of these transporters in mice fed various BAs at hepatotoxic doses may be adaptive responses to the toxic effects of the fed BAs. The decreased expression of Ntcp causes a decreased uptake of BAs, resulting in increased BA concentration in the serum (Chapter 3). In addition, the decreased expression of Ntcp may also contribute to maintaining the total liver BA concentration.

The increased mRNA expression of Abcg5 and g8 after feeding various doses of BAs could be due to an indirect effect of BAs. Feeding mice BAs at even the lowest doses fully inhibited the mRNA expression of Cyp7a1 (Fig 4-1); thus feeding mice BAs is likely to result in an accumulation of cholesterol and oxysterols (Murphy et al., 2005), which are known activators of LXR. The activation of LXR by oxysterols leads to up-regulation of Abcg5/g8 (Repa et al., 2002). Increased expression of Abcg5/g8 leads to an increase in the biliary secretion of cholesterol (Yu et al., 2002). The increased expression of Mdr2 mRNA after feeding the BAs at high-hepatotoxic doses could be an adaptive response to BA toxicity. The corresponding increase in Mdr2 may contribute to increased secretion of phospholipids into bile (Smit et al., 1993; Kneuer et al., 2007; Funk, 2008). Our previous study (Chapter 3) showed that feeding individual BAs at hepatotoxic doses altered liver BA composition and caused hepatotoxicity. The increased secretion of phospholipid together with increased secretion of cholesterol to form more mixed micelles with BAs may contribute to limiting the hepatotoxicity of BAs.

In summary, in mice fed low-non-hepatotoxic doses of individual BAs, the total liver BA concentrations were maintained via hepatic FXR-SHP mediated suppression of Cyp7a1, whereas the liver BA composition was somewhat restored via ileum FXR-Fgf15 mediated regulation of Cyp8b1 in a BA-species-dependent way. Whereas, in mice fed hepatotoxic doses of individual BAs, both Cyp7a1 and Cyp8b1 were suppressed by all BA species via activation of both the hepatic FXR-SHP and ileum FXR-Fgf15 signaling pathways, resulting in unchanged total liver BA

concentration and an imbalanced liver BA composition, which appears to contribute to the hepatotoxicity of BAs. In addition, decreases in the major BA uptake transporter Ntcp in the livers of mice fed high-hepatotoxic doses of individual BAs may also contribute to maintaining the total liver BA concentration, whereas, the increases in the lipid transporters Abcg5/g8 and Mdr2 may indicate an increased secretion of cholesterol and phospholipids, which form micelles with BAs and contribute to limiting the hepatotoxicity of BAs.

CHAPTER 5

EFFECTS OF CHOLESTYRAMINE ON LIVER BILE ACID COMPOSITION AND EXPRESSION OF GENES INVOLVED IN BILE ACID HOMEOSTASIS IN MICE

5.1 Abstract

The hypocholesterolemic drug cholestyramine (resin) functions by sequestration of BAs in the intestine. Administration of the resin increases the mRNA expression and enzyme activity of Cyp7a1, a key enzyme in the BA-biosynthesis pathway, resulting in increased conversion of cholesterol into BAs. However, the effects of resin on the liver BA concentration and composition and the subsequent alteration in BA receptor mediated signaling pathway is unknown. Therefore, mice were fed 2% resin in their diets for one week. The BAs in both the serum and the liver, as well as the mRNA involved in BA homeostasis were quantified. The data demonstrated that the resin tended to increase the serum total BA concentration, and significantly decreased β-MCA concentration 50%, but increased CA and T-CA in sera. As expected, feeding mice the resin decreased the total liver BA concentration 80%, which was due to an almost complete depletion of α-, β-, and ω-MCAs, as well as their taurine conjugates, and less of a decrease in most of the other BAs. In addition, the resin also altered the BA composition in the liver, featured by an increase in the percentage of the total CA and the secondary BA DCA. The mRNA of the BA-biosynthesis enzymes, Cyp7a1 and Cyp8b1, increased 180 and 100% respectively. However, the FXR-target gene SHP in liver was not decreased. In contrast, the ileum Fgf15 mRNA expression was significantly decreased. Feeding the resin did not alter the BA uptake transporters on the sinusoidal membrane nor did it altered the efflux transporters on the canalicular membrane, whereas, the efflux transporters on the sinusoidal membrane of

hepatocytes, Mrp 3 and 4, were increased by the resin 22 and 150% respectively. In ileum, feeding the resin increased the uptake transporter, Asbt, 66%. Collectively, the data suggest that increased expression of Cyp7a1 and Cyp8b1 in mouse liver by resin administered through the diet appears to be due to a decrease in the ileum Fgf15 signaling pathway, rather than a decrease in hepatic FXR signaling pathway.

5.2 Introduction

BAs are biosynthesized from the precursor cholesterol in liver, and the biosynthesis of BAs is a major pathway for the elimination of cholesterol from the body. BA biosynthesis is negatively regulated by BAs themselves via multiple signaling pathways. Since the discovery of the BA nuclear receptor FXR, the critical roles of the FXR mediated signaling pathways in regulation of major BA biosynthesis enzymes has been determined. FXR-mediated suppression of Cyp7a1 in mice is thought to be the predominant mechanism for the regulation of BA biosynthesis and is able to override LXR-mediated up-regulation of Cyp7a1 (Wang et al., 2006). In addition, the hepatic FXR-SHP mediated signaling pathway is proposed to play a major role in the regulation of Cyp8b1, which determines the ratio between CA (a 12-OH BA) and CDCA (a non-12-OH BA) in the BA pool, whereas, the intestinal FXR-Fgf15 mediated signaling pathway mainly plays a role in suppression of Cyp7a1 (Goodwin et al., 2000; Inagaki et al., 2005). However, our previous study showed that feeding the two 12-OH BAs (CA and DCA) activated not only the hepatic FXR-SHP signaling pathway, but also the ileum FXR-Fgf15 signaling pathway and caused a maximum suppression of the mRNA expression of Cyp7a1,

suggesting the suppression of Cyp7a1 by the two 12-OH BAs is mediated by both the hepatic FXR-SHP and the ileum FXR-Fgf15 signaling pathways. In contrast, feeding the two non-12-OH BAs (CDCA and LCA) at non-hepatotoxic doses only activated the hepatic FXR-SHP signaling, but not the ileum FXR-Fgf15 signaling pathway, resulting in a less suppression of the mRNA expression of Cyp7a1 compared to the suppression level in mice fed the 12-OH BAs at non-hepatotoxic doses. Only at high and hepatotoxic doses, did the two non-12-OH BAs activate both the hepatic FXR-SHP and the ileum FXR-Fgf15 signaling, resulting in maximum suppression of Cyp7a1. Our data suggest that the hepatic FXR-SHP signaling pathway plays major role in the basic regulation of Cyp7a1 or low doses of BAs (unpublished data).

BA composition in the enterohepatic circulation plays important roles in regulating BA biosynthesis and transport, the excretion of lipids into bile, as well as intestinal absorption of cholesterol (Heuman et al., 1989; Pandak et al., 2001). Feeding mice CA stimulates the excretion of cholesterol and phospholipids into bile, whereas other major BAs in mice, namely MCAs (α- and β-MCA), do not increase the hepatic excretion of lipids (Kubitz et al., 2005; Wagner and Trauner, 2005). BAs also vary in their ability to regulate BA biosynthesis and hepatobiliary transport. For example, the four major human BAs fed to mice at various concentrations in their diets differ markedly in their ability to regulate Cyp8b1 mRNA expression. Cyp8b1 is a 12α-hydroxylase that produces CA. Without the presence of Cyp8b1, CDCA is the final product (Vlahcevic et al., 2000). Thus Cyp8b1 determines the ratio between

12-OH BA (CA) and non-12-OH BA (CDCA) in liver (Pandak et al., 2001). Feeding the 12-OH-BAs (CA or DCA) decreased Cyp8b1 expression, whereas at the same concentrations in the diets, feeding the non-12-OH BAs (CDCA or LCA) had no effect on Cyp8b1 mRNA expression (Chapter 4). Moreover, Cyp8b1-null mice, which lack CA, have decreased expression of the FXR-target gene, SHP suggesting that CA is a major activator of FXR in mice (Murphy et al., 2005).

Cholestyramine (resin), a non-absorbable ionic exchange resin, is a cholesterol-lowering drug that binds BAs in the intestine and increases their fecal excretion (Thompson, 1971). The decreased enterohepatic circulation of BAs results in increased catabolism of cholesterol into BAs. The ultimate mechanism for decreasing cholesterol after resin treatment is the increase in mRNA expression and enzyme activity of Cyp7a1, which is the rate-limiting enzyme in BA biosynthesis (Thompson, 1971; Nilsson et al., 2007). Although the resin is a non-absorbable drug, it has been found to regulate glucose homeostasis and to improve atherosclerotic coronary heart disease, which are thought to have little to do with its lipid lowering effects (Bays and Goldberg, 2007). The binding efficiency of various BAs to resin varies (Thompson, 1971). The relatively hydrophobic BAs (DCA and LCA) bind more strongly to the resin than do the hydrophilic BAs (CA and MCA) at the pH of the intestine (about 6.0) (Whiteside et al., 1966; Thompson, 1971; Story and Kritchevsky, 1976). Thus, feeding mice resin is likely to not only decrease absolute BA concentrations in liver, but also alter BA composition, as well as expression of genes involved in BA biosynthesis and transport. Therefore, the

purpose of this study was to determine the effects of resin on BA concentrations and BA composition in the livers of mice, as well as the subsequent alteration in the expression of genes encoding BA biosynthesis enzymes, transporters, and transcription factors in both liver and ileum.

5.3 Results

Total BA Concentrations in Serum and Liver. As shown in Fig 5-1, feeding mice 2% resin in the diet tended to increase the total BA concentration in serum and significantly decreased total BA concentration in liver 80%.

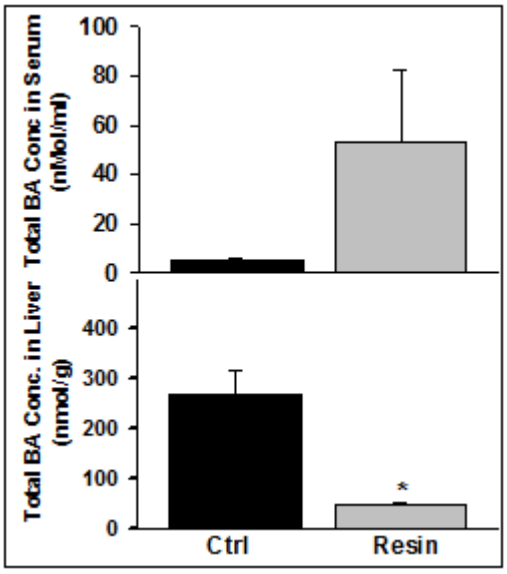


Fig 5-1. Total BA concentrations in sera and livers of mice fed a control diet and a 2% resin-supplemented diet for one week. Data are presented as Mean ± S.E.M (n=5). *P<0.05.

Individual BA Concentrations in Serum. As shown in Fig 5-2, feeding mice the resin-supplemented diet surprisingly increased T-CA as well as the total CA (CA plus T-CA). In contrast, feeding mice the resin supplemented diet trended to

increase the concentrations of other BAs (DCA, LCA, UDCA, and their taurine conjugates), but significantly decreased β-MCA in sera.

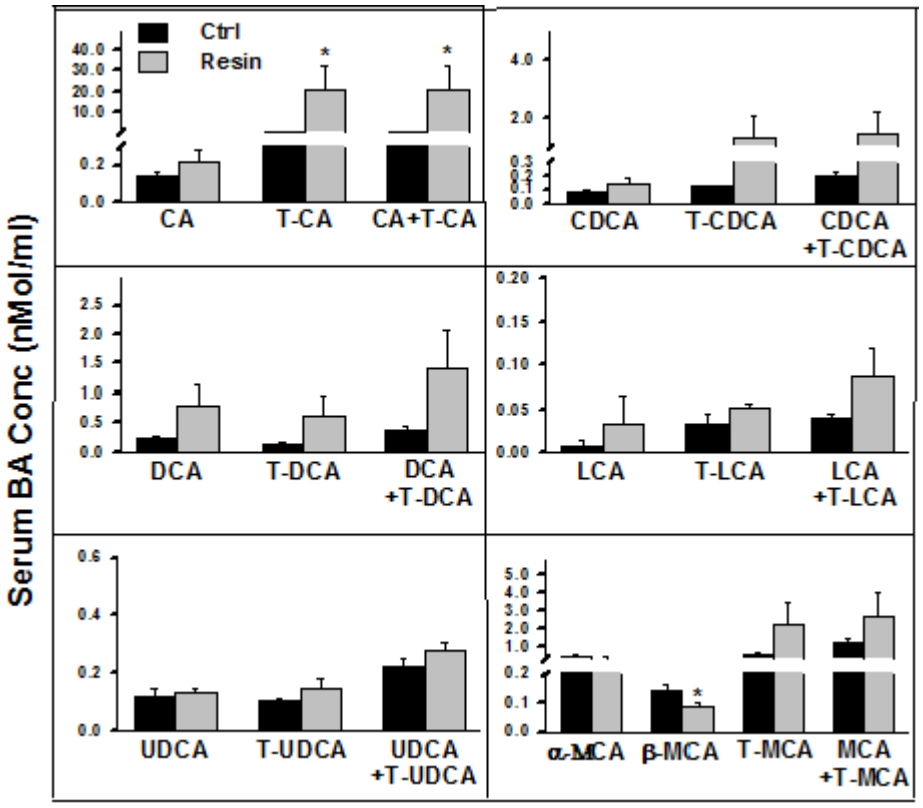


Fig 5-2. BA concentrations in sera of mice fed a control diet and a 2% resin-supplemented diet for one week. Data are presented as Mean ± S.E.M (n=5). *P<0.05.

Individual BA Concentrations in Liver. As shown in Fig 5-3, feeding mice 2% resin decreased the concentrations of various BAs in liver. For example, CA, T-CA, and total CA decreased 70-80%, and CDCA decreased 37% by feeding the resin, whereas T-CDCA and total CDCA were not significantly altered by feeding the resin. DCA, T-DCA, and total DCA decreased 35-60%, and LCA, T-LCA, total LCA,

UDCA, T-UDCA, and total UDCA all decreased about 85%. In addition, feeding mice the resin decreased MCAs and T-MCAs (α-, β-, and ω-MCA) 80-99%. Feeding mice the resin also decreased HDCA, MDCA and their taurine conjugates 65-94%.

BA composition in Liver. Feeding mice the resin-containing diet not only decreased total BA concentrations in liver, but also altered the percentage of each BA relative to total BAs in liver. As shown in Fig 5-4, the percentage of the total CA (CA+T-CA) in the livers of mice fed the resin increased from 39.4 to 63.8%, whereas, the percentage of the total MCA (MCAs+T-MCAs) decreased from 52.7 to 14.4%. The percentage of the total CDCA (CDCA+T-CDCA), that of the total DCA (DCA+T-DCA), and that of the total LCA (LCA+T-LCA) in the livers of mice fed the resin also increased from 1.5 to 7.6%, 2.7 to 9.8%, and 0.2 to 1.0% respectively. The percentage of other BAs relative to the total BA concentration in the livers of mice fed the resin, including the total UDCA and the total 6-OH BAs (HDCA and MDCA plus their taurine conjugates), did not change.

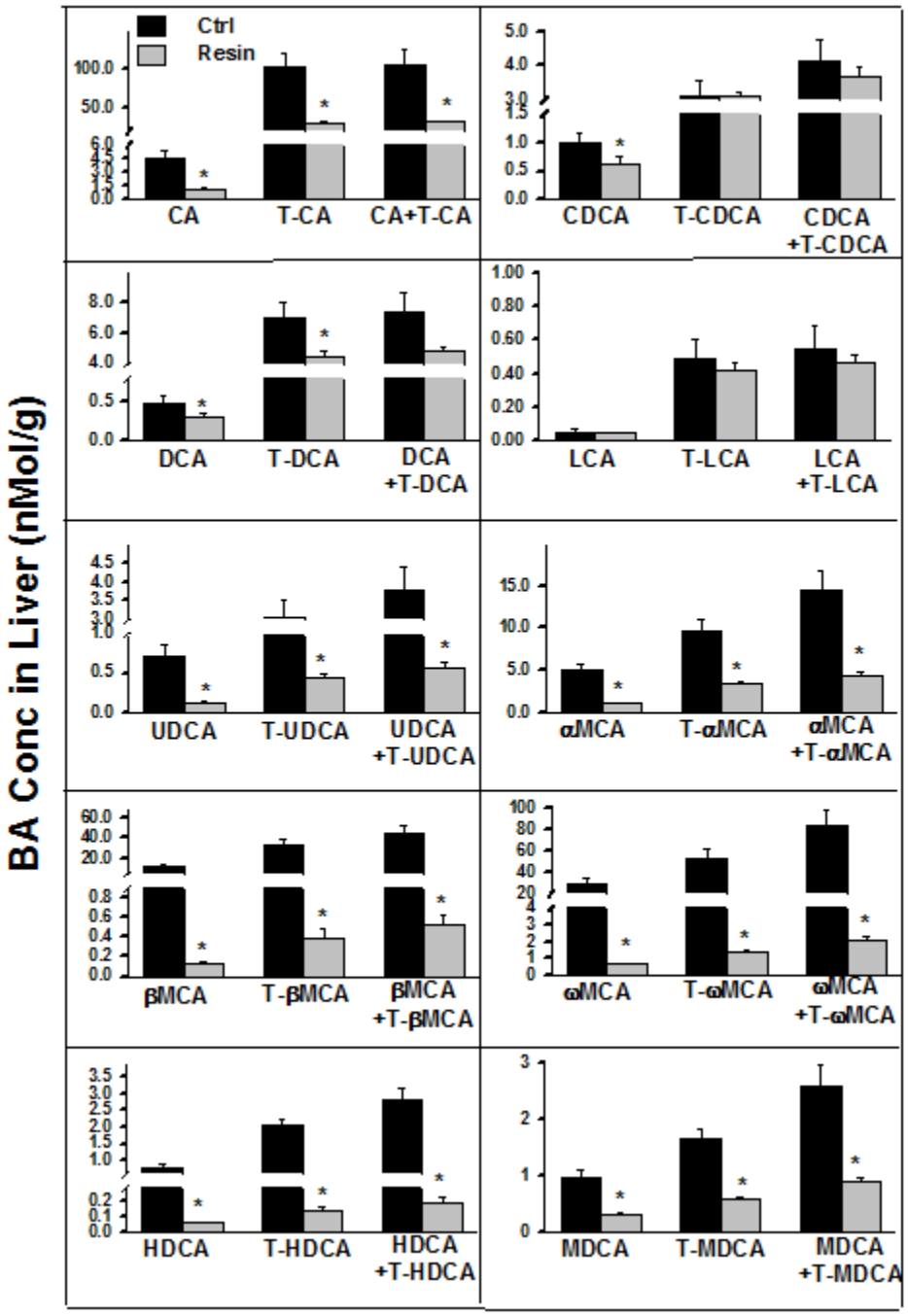


Fig 5-3. BA concentrations in livers of mice fed a control diet and a 2% resin-supplemented diet for one week. Data are presented as Mean ± S.E.M (n=5). *P<0.05.

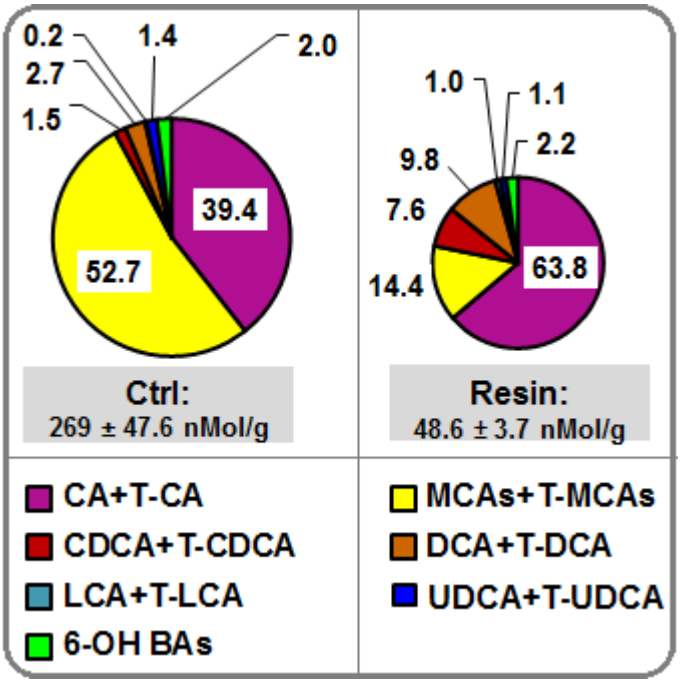


Fig 5-4. Liver BA composition (% of total BAs) in mice fed a control diet and a 2% resin supplemented diet for one week.

Effect of feeding the resin-containing diet on messenger RNA expression of BA-biosynthesis enzymes in liver. As shown in 5-5A, after feeding the resin-containing diet for one week, Cyp7a1 mRNA expression was increased about 180%. Feeding the resin-supplemented diet increased Cyp8b1 about 100%.

Feeding mice the resin-containing diet had no effect on Cyp27a1 mRNA expression (Fig 5-5A). Whereas the resin-containing diet almost tripled Cyp7a1 mRNA expression, it did not alter Cyp7b1 mRNA expression.

Effect of feeding the resin-containing diet on messenger RNA expression of uptake transporters in liver. As shown in Fig 5-5B, after feeding the resin-containing diet for one week, Ntcp mRNA expression was not altered.

Members of the organic anion transporter family (Oatp/Slco) function as uptake transporters for a wide variety of xenobiotics and endogenous chemicals, including BAs. In addition to Ntcp, Oatp1a1, 1a4, and 1b2 are also thought to be involved in BA uptake into liver (Jacquemin et al., 1994; Kullak-Ublick et al., 1994; Meier and Stieger, 2002; Hagenbuch and Meier, 2003). As shown in Fig 5-5B, feeding mice the resin-containing diet increased Oatp1a4 mRNA about 70%, but it did not alter Oatp1a1 or 1b2 mRNA expression.

Effect of feeding the resin-containing diet on messenger RNA expression of efflux transporters in liver. The effect of feeding the resin-supplemented diet on hepatic efflux transporter mRNA expression is shown in Fig 5-5C. After feeding the resin-containing diet for one week, the mRNA of the BA-efflux transporter Bsep was decreased about 18.6%.

In addition to the efflux of BAs by Bsep into canaliculi, Mrps are involved in BA efflux from hepatocytes to either bile (Mrp2) or blood (Mrp3 and 4) (Keppler and Konig, 1997; Meier and Stieger, 2002). The resin-containing diet had no effect on Mrp2 mRNA expression, but increased the mRNA expression of Mrp3 and 4 (22% and 150%, respectively) (Fig 5-5C). Ostα and β are localized on the sinusoidal membranes of hepatocytes, mediating the efflux of BAs back into blood. As shown in Fig 5-5C, after feeding the resin, Ostα/β mRNA was not significantly altered.

Effect of feeding the resin-containing diet on the messenger RNA expression of Abcg5 and Abcg8 in liver. Abcg5 and g8 are half-transporters for cholesterol elimination from hepatocytes into bile. The effect of the resin-containing diet on Abcg5 and g8 mRNA expression is shown in Fig 5-5D. After feeding the resin-containing diet for one week, the mRNA of Abcg5 and g8 did not change.

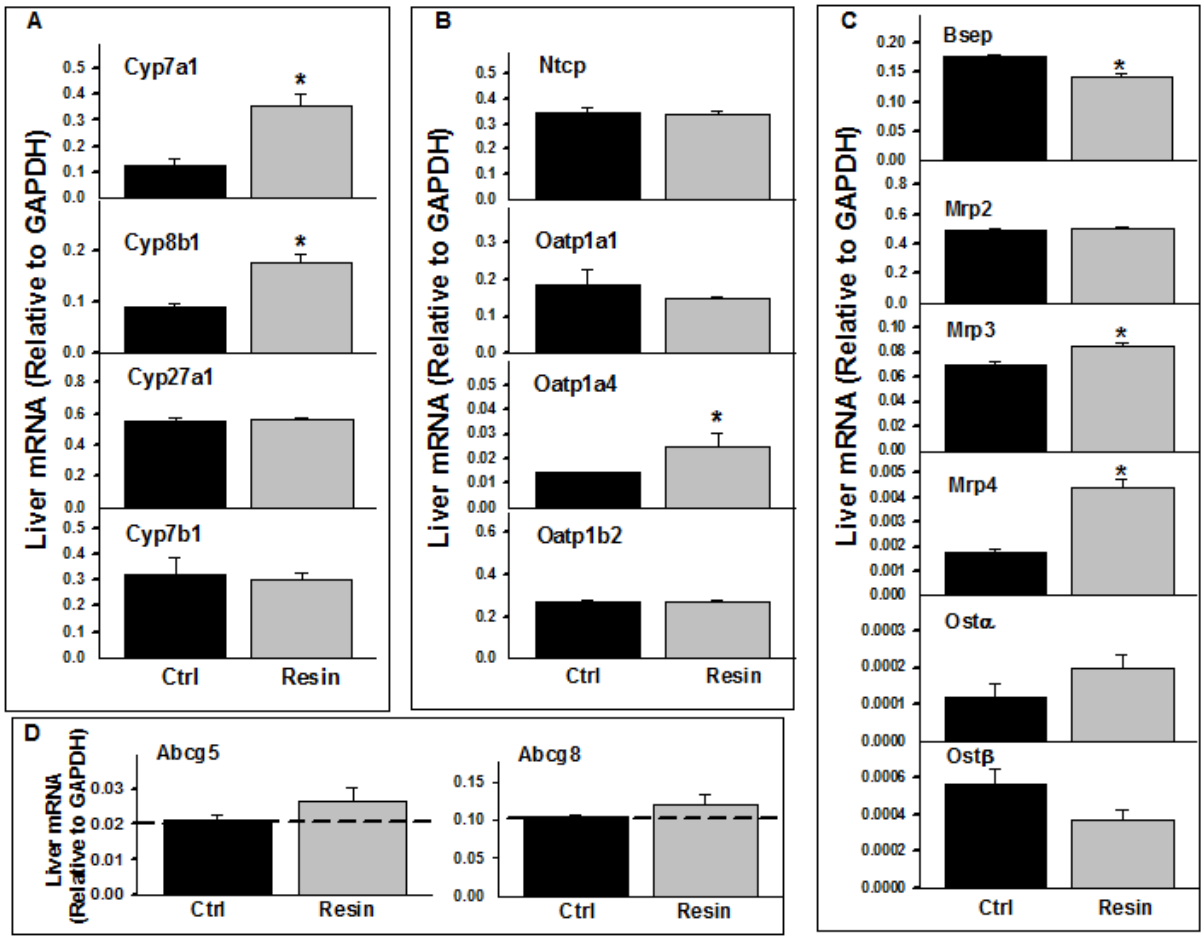


Fig 5-5. Messenger RNA expression of genes involved in BA biosynthesis (A), BA uptake (B), BA efflux (C), as well as cholesterol efflux transporters Abcg5 and Abcg8 (D) in livers of mice fed a 2% resin-supplemented diet for one week. Data are presented as Mean ± S.E.M (n=5). *P<0.05.

Effect of feeding the resin-containing diet on the messenger RNA expression of nuclear receptors and their target genes in liver. As shown in Fig 5-6, feeding mice the resin-containing diet does not appear to affect the mRNA expression of the nuclear receptor FXR, or its activation, because its target gene SHP did not increase in liver. Fig 5-6 also shows that feeding the resin-containing diet had no effect on the expression of PXR mRNA expression, but it decreased the expression of the PXR-target gene Cyp3a11 about 10%. The resin-containing diet did not increase mRNA of Nrf2, but increased the expression of its target gene Nqo1 80%, indicating that feeding the resin-containing diet activated Nrf2.

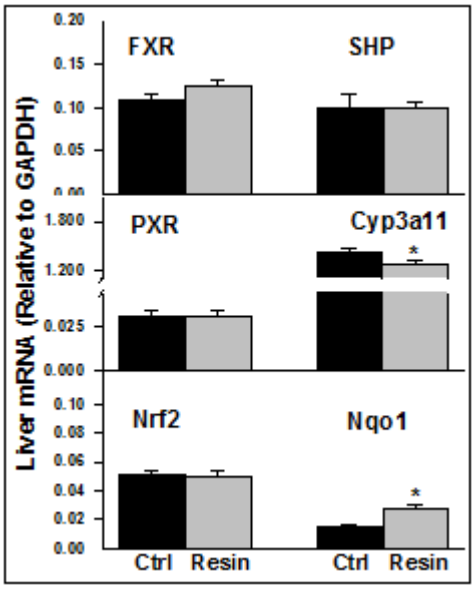


Fig 5-6. Messenger RNA expression of nuclear receptors and their target genes in livers of mice fed a 2% resin-supplemented diet for one week. Data are presented as Mean ± S.E.M (n=5). *P<0.05.

Effect of feeding the resin-containing diet on the messenger RNA expression of BA transporters in ileum. The effects of the resin-containing diet on BA transporters in the ileum are shown in Fig 5-7. In the ileum, the BA uptake transporter Asbt is localized on the apical membrane of enterocytes (Kramer et al., 1982; Wong et al., 1994; Shneider et al., 1995). Asbt is responsible for the uptake of BAs from the intestinal lumen into enterocytes. After feeding the resin-containing diet for one week, Asbt mRNA expression was increased 66%.

The cytosolic intestinal BA-binding protein (Ibabp) is responsible for the intracellular transport of BAs from the apical side to the basolateral side of enterocytes (Meier and Stieger, 2002). After feeding mice the resin-containing diet for one week, the Ibabp mRNA expression did not change (Fig 5-7).

The organic solute transporter (Ostα/Ostβ) is the main BA transporter that effluxes BAs from enterocytes into blood. The resin did not alter Ostα/Ostβ mRNA expression (Fig 5-7). In addition to Ostα/Ostβ, the multiple drug resistant protein Mrp3 is located on the basal membrane of enterocytes and might have a role in BA efflux from inside the enterocytes to the portal blood. As shown in Fig 5-7, the resin-containing diet did not alter the mRNA expression of Mrp3.

Effect of feeding the resin-containing diet on the messenger RNA expression of nuclear receptors and their target genes in ileum. As shown in Fig 5.8, FXR mRNA in the ileum was not altered by feeding the resin-containing diet. Although feeding mice the resin-containing diet did not alter the FXR-target gene SHP, it markedly decreased the FXR-target gene Fgf15 in the ileum.

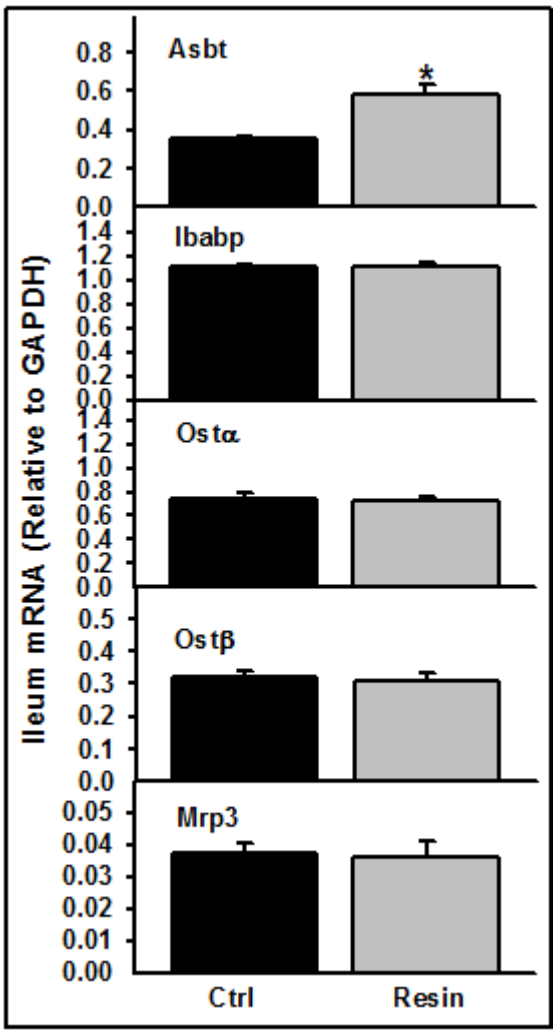


Fig 5-7. Messenger RNA expression of BA transporters in ileum of mice treated with 2% Resin in the diets for one week. Data are presented as Mean ± S.E.M (n=5). *P<0.05.

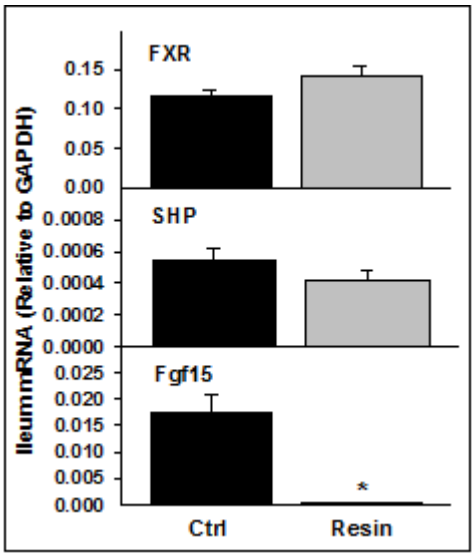


Fig 5-8. Messenger RNA expression of nuclear receptors and their target genes in ileum of mice fed a 2% resin-supplemented diet for one week. Data are presented as Mean ± S.E.M (n=5). *P<0.05.

5.4 Discussion

The present study characterizes the pharmacological effects of the resin (2% in the diet) on BA concentrations in sera and liver, as well as the expression of genes involved in BA homeostasis. The present data indicate that the resin-containing diet not only decreased the total liver BA concentration as expected, but also altered the liver BA composition. The alterations in BA concentration and composition in the enterohepatic circulation after feeding mice the resin-containing diet resulted in a diminished ileum FXR-Fgf15 signaling, but it did not affect the hepatic FXR-SHP signaling.

An unexpected finding in this study was that the serum total BA concentration tended to increase and the T-CA in serum increased significantly. It has been noticed for decades that the cholestyramine resin is able to improve the glucose homeostasis in diabetes patients and various potential mechanisms for glucose-lowering effects of resin have been proposed (Staels, 2009). In this study, we report that the serum BA composition is altered after resin-feeding. The total CA concentration was increased whereas the βMCA concentration decreased. Accordingly, we propose an original hypothesis in regarding to the glucose lowering effects by cholestyramine: The increased CA in the systemic circulation may cause activation of the TGR5 receptor in the skeletal muscles and the lipid tissues, resulting in increased glucose metabolism.

Even though the total liver BA concentration decreased 80% after feeding mice the 2% resin-containing diet, the percentage of the total CA (CA+T-CA) relative to the total BAs in the liver was increased from 39.4 to 63.8%. The concentration of the total CA in the livers of the resin-fed mice (30 nmol/g) is 29.1% of that of the control mice (106 nmol/g) (Fig 5-4). The increase in the percentage of the total CA after the 2% resin-containing diet is at least partially due to the increased expression of Cyp8b1 (Fig 5-5) and thus the *de novo* biosynthesis of CA.

BAs are known activators of FXR (Grober et al., 1999; Makishima et al., 1999). BAs not only activate the FXR-SHP pathway in liver, but also activate the FXR-Fgf15 signaling pathway in the ileum of mice (Inagaki et al., 2005). The fibroblast growth factor 15 (Fgf15) in mouse intestine has been shown to be induced

by CA feeding (Inagaki et al., 2005), and Fgf15 is thought to travel via the portal vein to the liver where it activates the cell surface fibroblast growth factor receptor (FGFR4), causing repression of hepatic Cyp7a1. Both the FXR-SHP signaling pathway in liver (Goodwin et al., 2000) and the FXR-Fgf15 signaling pathway in ileum are involved in inhibition of Cyp7a1 expression (Inagaki et al., 2005; Kim et al., 2007).

It might be expected that the decreased concentration of BAs in mouse liver after feeding the resin-containing diet would result in diminished activation of FXR and thus reduced expression of SHP in liver, resulting in an increase in Cyp7a1 (Fig 5-5). Consistent with previous reports (Thompson, 1971; Nilsson et al., 2007), feeding mice the resin-containing diet increased the rate-limiting BA-biosynthesis enzyme in the classic pathway, Cyp7a1. However, the present data demonstrate that neither SHP nor FXR mRNA expression in liver is altered by feeding mice the resin-containing diet (Fig 5-6). In marked contrast, Fgf15 mRNA was decreased about 90% by feeding mice the resin (Fig 5-.8). In addition, consistent with previous reports (Repa et al., 2002; Shibata et al., 2007), the positive regulator of Cyp7a1, the liver X receptor (LXR), was not activated after the resin-containing diet, indicated by unchanged expression of its target gene Abcg5/g8 (Fig 5-5D). Thus, the increased expression of Cyp7a1 in mice fed the resin-containing diet is more likely due to decreased expression of Fgf15 in ileum, rather than altered FXR-SHP signaling and/or activation of LXR in liver.

The resin containing diet also induced the mRNA of Cyp8b1, an enzyme necessary for the biosynthesis of CA, but did not increase the mRNA of enzymes in the alternative pathway, such as Cyp27a1 or Cyp7b1. The increased expression of Cyp8b1 is likely the reason for the increased percentage of CA relative to total BAs in liver after feeding the resin-containing diet (Fig 5-5). Our previous BA-feeding study showed that feeding mice non-hepatotoxic doses of CA (0.03% in the diet) or its secondary BA, DCA, activated FXR-Fgf15 signaling in ileum and reduced Cyp8b1 in liver (Chapter 4). Consistent with this previous study, the current studies suggest that the FXR-Fgf15 signaling pathway from the intestine appears to play an especially important role in regulating Cyp8b1 in liver.

The present data also demonstrate that feeding the resin-containing diet results in a small decrease (10%) in the putative PXR-target gene Cyp3a11 and an increase (80%) in the Nrf2-target gene Nqo1 (Fig 5-6). The effects of feeding the resin-containing diet on the expression of these two genes (Cyp3a11 and Nqo1) are similar to those observed after feeding of BAs at hepatotoxic doses (Chapter 4). Although the two studies may appear contradictory in that one increased BA concentrations in liver, whereas the other decreased BA concentrations in liver, both studies resulted in increased percentages of the secondary BA, DCA (from 2.7 to 9.8%), which is known to activate Nrf2 (Tan et al., 2007). Thus the increased secondary BA concentrations in liver after either BA- or resin-feeding are likely the reason for Nrf2 activation.

The present study indicates that feeding the resin-containing diet did not alter the expression of uptake transporters, Ntcp, Oatp1a1, and Oatp1b2 in mouse livers. It has been hypothesized that high concentrations of BAs in liver activates FXR, which then induces the production of the repressor SHP, causing a decrease in the mRNA expression of the BA uptake transporter Ntcp (Gartung et al., 1996; Sauer et al., 2000). However, an *in vitro* study indicates that inhibition of the Ntcp promoter activity by SHP is species-dependent; that is, SHP inhibits rat Ntcp, but not mouse or human Ntcp (Jung et al., 2004). Our previous study also demonstrated that feeding mice low-non-hepatotoxic doses of BAs did not affect the mRNA expression of Ntcp, Oatp1a1, and 1b2 (Chapter 4). Thus, BAs do not appear to be involved in basal regulation of these BA transporter genes via the hepatic FXR-mediated pathway.

Similar to the effects of feeding BAs on Oatp1a4 expression (Chapter 4), feeding mice the resin-containing diet also increased Oatp1a4 mRNA expression. Oatp1a4 expression is regulated by Nrf2 (Cheng et al., 2005). As mentioned previously, Nrf2 was activated by feeding both the resin-containing diet and BA-supplemented diets. Thus, both the BA-feeding study and the present resin-feeding study suggest that the increased Oatp1a4 may be due to Nrf2 activation.

Bsep and Mrp2 are located on the canalicular membrane of hepatocytes and mediate the efflux of BAs into bile canaliculi. BAs are thought to regulate Bsep expression primarily via their nuclear receptor FXR (Ananthanarayanan et al., 2001; Plass et al., 2002). The present data show that feeding mice the resin-containing

diet resulted in a small decrease (10%) in the expression of Bsep, but had no effect on the mRNA expression of Mrp2. Our previous study with feeding non-hepatotoxic doses of BAs to mice also indicates that BAs do not have major effects on Bsep regulation (Chapter 4). In addition, feeding the resin-containing diet did not affect Mrp2 mRNA, another BA efflux transporter on the canalicular membrane of hepatocytes.

An unexpected finding in the present study is that hepatic Mrp3 and Mrp4 mRNA was increased after resin feeding. Increased Mrp3 and Mrp4 expression during cholestasis is considered to be a compensatory mechanism for the removal of toxic BAs from hepatocytes (Slitt et al., 2007). Thus, the increased expression of Mrp3 and 4 in the present study may also contribute to increased BA concentrations in serum (Fig 5-2). Activation of Nrf2 is known to induce Mrp3 and Mrp4 in mice (Maher et al., 2008). Both the resin-feeding study and the BA-feeding study showed that the Nrf2 receptor was activated. Thus, the increases in Mrp3 and 4 mRNA expressions after feeding the resin-containing diet might be due to Nrf2 activation.

The organic solute transporter (a heterodimer of Ostα and β) is located on the basolateral membrane of both hepatocytes and cholangiocytes (Ballatori et al., 2005). Both Ostα and β are thought to be regulated by FXR (Boyer et al., 2006). The present data demonstrate that the resin did not affect Ostα and β mRNA expression in liver. This might be because the hepatic FXR was not activated after feeding mice the resin-containing diet.

Feeding the resin-containing diet almost completely abolished the expression of the ileum FXR target gene Fgf15 mRNA (Fig 5-8). The decrease in Fgf15 mRNA may be due to a reduction of the major FXR agonist in mice fed resin-containing diet, namely CA. However, the decreased activation of FXR after feeding the resin-containing diet did not affect the mRNA expression of other FXR target genes SHP, Ostα/β and Ibabp (Grober et al., 1999; Goodwin et al., 2000; Lu et al., 2000) . Surprisingly, the mRNA of Asbt in ileum, another FXR target gene, was increased 66% after feeding mice the resin-containing diet (Fig 5-7), suggesting that a FXR-independent signaling pathway may exist in regulating the Asbt.

In summary, feeding mice the resin-containing diet decreased the total BA concentrations in liver 80% and altered BA composition in liver. The ileum FXR-Fgf15 signaling pathway was inhibited 97%, whereas, the hepatic FXR-SHP signaling pathway was not affected by feeding the resin-containing diet. The diminished FXR-Fgf15 signaling in the ileum is likely responsible for the increased mRNA expression of Cyp7a1 and Cyp8b1. In addition, the mRNA expression of the sinusoidal BA efflux transporters Mrp3 and Mrp4 were increased by feeding the resin-containing diet, which may be due to activation of Nrf2, a known regulator of Mrp transporters. However, no marked alterations in either the BA uptake transporter Ntcp or the efflux transporter, Bsep, were observed in mice fed the resin-containing diet.

CHAPTER 6

SUMMARY AND CONCLUSION

6.1 Altered BA Composition in Liver Contributes to the Hepatotoxicity of Individual BAs.

The major function of BAs was thought to facilitate lipid absorption in the intestine for a long time after the Nobel Prize laureate Heinrich Wieland determined the molecular structures of BAs in 1927 (Witkop, 1992). The discovery of FXR in 1999 as a BA receptor ushered in a new era of BA research (Parks et al., 1999; Wang et al., 1999). BAs are now recognized as endocrine molecules that regulate the homeostasis of BAs, cholesterol, glucose, and energy. Given all these important physiological functions of BAs, BAs and their receptors as potential pharmaceutical targets for the treatment of various metabolic diseases have been proposed (Lefebvre et al., 2009; Zollner and Trauner, 2009).

However, in the long history of BA research, a simple but fundamental question has been overlooked, that is, what is the proper dose for each individual BA to study their physiological functions or toxicity *in vivo*? This question was addressed in the first study (Chapter three) of my dissertation, using a strictly designed experimental system. The data showed: 1) LCA, DCA, and CDCA at 1.0% in the diets were lethal, whereas CA at 1% and UDCA at 3.0% were not lethal. 2) Using both serum ALT activity and BA concentration as indices of liver function, the doses of each BA that produce hepatotoxicity are as follows: LCA 0.03% or above, DCA at 0.1% or above, and CDCA and CA at 0.3% in the diet or above, whereas, UDCA at 0.3% in the diet or above might be hepatotoxic because it only caused increases in serum BA concentration, but not serum ALT. 3) Feeding BAs (CA,

CDCA, DCA, or LCA) at hepatotoxic doses did not alter the total liver BA concentration; however it altered the liver BA composition, featured by increases in the percentage of the fed-BAs, and decreases in the percentage of MCAs.

In conclusion, the imbalance in the BA composition in liver after feeding mice hepatotoxic doses of BAs appears to contribute to their hepatotoxicity. Furthermore, to study the physiological or pharmacological functions of each BA, non-hepatotoxic doses of BA should be used, namely, CA and CDCA at 0.1% or lower, DCA at 0.03% or lower, and LCA at 0.01% in the diet. Given the fact that high doses of UDCA produced increased serum BA concentration, UDCA at 0.1% or lower in the diets are recommended to use for the study of its pharmacological functions.

6.2 Adaptive Gene Expression in Response to Altered Liver BA Composition in Mice Fed Non-hepatotoxic Doses or Hepatotoxic Doses of BAs

FXR plays important roles in the regulation of BA biosynthesis and transport. It has been established that both the hepatic FXR and the ileum FXR are involved in regulation of the BA biosynthesis enzyme Cyp7a1 (Goodwin et al., 2000; Inagaki et al., 2005). In 2007, it was reported that the hepatic FXR-SHP signaling pathway plays a more important role in regulation of Cyp8b1, which determines the ratio between CA and CDCA, whereas, the ileum FXR-Fgf15 signaling pathway plays a major roles in the regulation of Cyp7a1 (Kim et al., 2007). The first study in this dissertation determined the low-non-hepatotoxic doses and high-hepatotoxic doses of each BA. The data obtained from the first study (chapter three) also showed that

feeding mice BAs at either low-non-hepatotoxic or high hepatotoxic doses did not increase the total liver concentration of BAs, suggesting possible adaptive responses in the expression of genes involved in BA biosynthesis and transport. Thus, the purpose of the study described in chapter four of this dissertation was to investigate the adaptive responses in the expression of genes involved in BA homeostasis, and the possible roles of the hepatic and the ileum FXR signaling pathways in regulating these genes in mice fed low-non-hepatotoxic doses and high-hepatotoxic doses of BAs.

The data indicate that only the target gene of FXR, SHP, was induced by all five BAs at both low-non-hepatotoxic and high-hepatotoxic doses, but not the target genes of other nuclear receptors, suggesting that the five BAs specifically activated FXR. Corresponding to the increased SHP mRNA in liver, the Cyp7a1 mRNA, but not the Cyp8b1 mRNA in liver, was decreased in mice fed all doses of BAs, suggesting that the FXR-SHP signaling pathway was involved in suppression of Cyp7a1, but not Cyp8b1. In contrast, the ileum FXR-target gene Fgf15 was induced by CA and DCA at low-non-hepatotoxic doses, but not by these doses of CDCA or LCA, suggesting that activation of FXR in the ileum is BA species dependent. Corresponding to the BA species specific activation of the ileum FXR-Fgf15 signaling, the hepatic Cyp8b1 mRNA was inhibited only by CA and DCA, but not CDCA or LCA at non-hepatotoxic doses, indicating that the ileum FXR-Fgf15 mRNA plays major role in suppression of Cyp8b1. In contrast to the effects of the five BAs on the BA biosynthesis enzymes, low-non-hepatotoxic doses of the five BAs did not

have obvious effects on mRNA expression of the BA transporters, but high-hepatotoxic doses of the five BAs decreased the mRNA expression of the major BA uptake transporter Ntcp. Interestingly, the cholesterol transporter Abcg5/g8 was induced by all five BAs at both low-non-hepatotoxic and high-hepatotoxic doses, whereas, the mRNA of the phospholipid transporter Mdr2 was induced by all five BAs only at high-hepatotoxic doses.

In conclusion, feeding the five BAs at non-hepatotoxic doses and hepatotoxic doses triggers different, but partially overlapping, adaptations in the gene expression involved in BA and lipid homeostasis. At low-non-hepatotoxic doses, all BA species inhibited Cyp7a1 mRNA expression, apparently via activation of the hepatic FXR-SHP signaling pathway, resulting in reduced *de novo* biosynthesis of BAs. The BA-species specific inhibition of Cyp8b1 by CA and DCA, is likely mediated by activating the ileum FXR-Fgf15 signaling pathway. The decreased expression of Cyp8b1 results in decreased biosynthesis of CA and DCA, but not CDCA and LCA, accordingly, the balance of BA composition in liver is maintained and probably aids in preventing hepatotoxicity of the fed-BAs. At high-hepatotoxic doses, all BAs inhibited both BA biosynthesis enzymes (Cyp7a1 and Cyp8b1) and the major BA-uptake transporter, Ntcp, which probably contributes to maintaining the total liver BA concentration. The increased expression of lipid transporters Abcg5/g8 and Mdr2 may result in increased secretion of lipids into bile, which may contribute to reduction of the hepatobilliary damage by BAs.

6.3 BA Sequestration by Resin-feeding increased the mRNA expression Cyp7a1 and Cyp8b1 due to diminished ileum FXR-Fgf15 signaling, but not reduced hepatic FXR-SHP signaling

An increased mRNA expression of Cyp7a1 was found after resin administration. Even though the mechanism for up-regulation of Cyp7a1 mRNA expression by resin has been investigated or proposed, such as the roles of nuclear receptors in liver (FXR, LXR, HNF4α), no conclusive mechanism has been found (Shibata et al., 2007). Our previous BA-feeding study demonstrated that the primary murine BA, CA, at very low-non-hepatotoxic dose (0.03% in the diet) suppressed Cyp7a1 mRNA expression, probably via activation of both hepatic FXR-SHP signaling pathway and the ileum FXR-Fgf15 signaling pathway (Chapter 4). The resin removes BAs from the normal enterohepatic circulation by sequestration of BAs in the intestine. Thus, the increased mRNA expression of Cyp7a1 by resin-feeding is likely because the loss of BAs diminished the suppressive effect of ileum FXR-Fgf15 signaling, which is activated by CA under normal condition.

Mice were fed 2% resin in their diets for one week. The BAs in both the serum and the liver, as well as the mRNA involved in BA homeostasis were quantified. As expected, feeding mice the resin decreased the total liver BA concentration 80%, however, contrary to expectation, resin-feeding did not decrease the total serum BA concentration, but rather it increased the CA and T-CA

concentrations in sera. Only the β-MCA in sera was decreased, but not the other BAs. Increased serum total CA concentration may be responsible for the serum glucose-lowering effect that the resin was found to have in type 2 diabetes patient (Staels, 2009). In addition, the resin also altered the liver BA composition, in which there is an increase in the percentage of the total CA and that of the secondary BA DCA. The mRNA of the BA-biosynthesis enzymes, Cyp7a1 and Cyp8b1, was increased 180 and 100% respectively. However, the FXR-target gene SHP in liver was not decreased. In contrast, the ileum Fgf15 mRNA expression was significantly decreased. Feeding the resin did not alter the BA uptake transporters on the sinusoidal membrane nor did it altered the efflux transporters on the canalicular membrane, however, the efflux transporters on the sinusoidal membrane of hepatocytes, Mrp3 and 4, were increased by the resin 22 and 150% respectively. The increased Mrp3 and 4 might be responsible for the increased serum BA concentration.

In conclusion, the diminished FXR-Fgf15 signaling after resin-feeding is responsible for the decreased mRNA expression of Cyp7a1 and Cyp8b1. The altered liver BA composition is likely responsible for the increased expression of Mrp3 and 4, which may contribute to the increased serum BA concentration.

6.4 Significance of the studies

Knowing the non-hepatotoxic dose of each BA is critical in studying BA physiological or pharmacological functions. Data generated by feeding mice a non-hepatotoxic, rather than a hepatotoxic dose of individual BA, will provide more certainty in clarifying the signaling function of that BA, because there is no toxic effects which might obscure the BA functions.

The novel concept brought out in my dissertation that the altered liver BA composition without an increase in the total liver BA concentration causes hepatotoxicity and can trigger BA receptor FXR mediated signaling pathway will lead to a new direction in BA research field and also will provide a new theory for drug discovery.

Another exciting finding in my dissertation is that the resin can increase serum BA (total CA) concentration. This finding will provide a new perspective for the understanding of the mechanism by which the resin lowers the serum glucose level in type 2 diabetes patients. In addition, the altered BA composition after resin feeding resulted in an increased expression of the BA efflux transporter Mrp3 and 4 which might be the reason why the serum BA concentration is increased.

BIBLIOGRAPHY

Alnouti Y, Csanaky IL and Klaassen CD (2008) Quantitative-profiling of bile acids and their conjugates in mouse liver, bile, plasma, and urine using LC-MS/MS. *J Chromatogr B Analyt Technol Biomed Life Sci* **873**:209-217.

Alrefai WA and Gill RK (2007) Bile acid transporters: structure, function, regulation and pathophysiological implications. *Pharm Res* **24**:1803-1823.

Ambros-Rudolph CM, Glatz M, Trauner M, Kerl H and Mullegger RR (2007) The importance of serum bile acid level analysis and treatment with ursodeoxycholic acid in intrahepatic cholestasis of pregnancy: a case series from central Europe. *Arch Dermatol* **143**:757-762.

Ananthanarayanan M, Balasubramanian N, Makishima M, Mangelsdorf DJ and Suchy FJ (2001) Human bile salt export pump promoter is transactivated by the farnesoid X receptor/bile acid receptor. *J Biol Chem* **276**:28857-28865.

Andersson U, Yang YZ, Bjorkhem I, Einarsson C, Eggertsen G and Gafvels M (1999) Thyroid hormone suppresses hepatic sterol 12alpha-hydroxylase (CYP8B1) activity and messenger ribonucleic acid in rat liver: failure to define known thyroid hormone response elements in the gene. *Biochim Biophys Acta* **1438**:167-174.

Ando H, Tsuruoka S, Yamamoto H, Takamura T, Kaneko S and Fujimura A (2005) Regulation of cholesterol 7alpha-hydroxylase mRNA expression in C57BL/6 mice fed an atherogenic diet. *Atherosclerosis* **178**:265-269.

Araya Z, Tang W and Wikvall K (2003) Hormonal regulation of the human sterol 27-hydroxylase gene CYP27A1. *Biochem J* **372**:529-534.

Attili AF, Angelico M, Cantafora A, Alvaro D and Capocaccia L (1986) Bile acid-induced liver toxicity: relation to the hydrophobic-hydrophilic balance of bile acids. *Med Hypotheses* **19**:57-69.

Azer SA, Klaassen CD and Stacey NH (1997) Biochemical assay of serum bile acids: methods and applications. *Br J Biomed Sci* **54**:118-132.

Ballatori N, Christian WV, Lee JY, Dawson PA, Soroka CJ, Boyer JL, Madejczyk MS and Li N (2005) OSTalpha-OSTbeta: a major basolateral bile acid and steroid

transporter in human intestinal, renal, and biliary epithelia. *Hepatology* **42**:1270-1279.

Ballatori N and Truong AT (1992) Glutathione as a primary osmotic driving force in hepatic bile formation. *Am J Physiol* **263**:G617-624.

Bays HE and Goldberg RB (2007) The 'forgotten' bile acid sequestrants: is now a good time to remember? *Am J Ther* **14**:567-580.

Bazzoli F, Roda A, Fromm H, Sarva RP, Roda E and Barbara L (1982) Relationship between serum and biliary bile acids as an indicator of chenodeoxycholic and ursodeoxycholic acid-induced hepatotoxicity in the rhesus monkey. *Dig Dis Sci* **27**:417-424.

Becker S, Reinehr R, Graf D, vom Dahl S and Haussinger D (2007) Hydrophobic bile salts induce hepatocyte shrinkage via NADPH oxidase activation. *Cell Physiol Biochem* **19**:89-98.

Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P and Berkenstam A (1998) Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc Natl Acad Sci U S A* **95**:12208-12213.

Biava C (1964a) STUDIES ON CHOLESTASIS. THE FINE STRUCTURE AND MORPHOGENESIS OF HEPATOCELLULAR AND CANALICULAR BILE PIGMENT. *Lab Invest* **13**:1099-1123.

Biava CG (1964b) STUDIES ON CHOLESTASIS. A RE-EVALUATION OF THE FINE STRUCTURE OF NORMAL HUMAN BILE CANALICULI. *Lab Invest* **13**:840-864.

Bjorkhem I (1992) Mechanism of degradation of the steroid side chain in the formation of bile acids. *J Lipid Res* **33**:455-471.

Bjorkhem I, Diczfalusy U and Lutjohann D (1999) Removal of cholesterol from extrahepatic sources by oxidative mechanisms. *Curr Opin Lipidol* **10**:161-165.

Blumberg B, Sabbagh W, Jr., Juguilon H, Bolado J, Jr., van Meter CM, Ong ES and Evans RM (1998) SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev* **12**:3195-3205.

Bodin K, Lindbom U and Diczfalusy U (2005) Novel pathways of bile acid metabolism involving CYP3A4. *Biochim Biophys Acta* **1687**:84-93.

Bogan AA, Cohen FE and Scanlan TS (1998) Natural ligands of nuclear receptors have conserved volumes. *Nat Struct Biol* **5**:679-681.

Botham KM and Boyd GS (1983) The metabolism of chenodeoxycholic acid to beta-muricholic acid in rat liver. *Eur J Biochem* **134**:191-196.

Boulias K, Katrakili N, Bamberg K, Underhill P, Greenfield A and Talianidis I (2005) Regulation of hepatic metabolic pathways by the orphan nuclear receptor SHP. *EMBO J* **24**:2624-2633.

Bourguet W, Germain P and Gronemeyer H (2000) Nuclear receptor ligand-binding domains: three-dimensional structures, molecular interactions and pharmacological implications. *Trends Pharmacol Sci* **21**:381-388.

Bouscarel B, Fromm H and Nussbaum R (1993) Ursodeoxycholate mobilizes intracellular Ca2+ and activates phosphorylase a in isolated hepatocytes. *Am J Physiol* **264**:G243-251.

Boyer JL, Trauner M, Mennone A, Soroka CJ, Cai SY, Moustafa T, Zollner G, Lee JY and Ballatori N (2006) Upregulation of a basolateral FXR-dependent bile acid efflux transporter OSTalpha-OSTbeta in cholestasis in humans and rodents. *Am J Physiol Gastrointest Liver Physiol* **290**:G1124-1130.

Bremmelgaard A and Alme B (1980) Analysis of plasma bile acid profiles in patients with liver diseases associated with cholestasis. *Scand J Gastroenterol* **15**:593-600.

Brendel C, Schoonjans K, Botrugno OA, Treuter E and Auwerx J (2002) The small heterodimer partner interacts with the liver X receptor alpha and represses its transcriptional activity. *Mol Endocrinol* **16**:2065-2076.

Burwen SJ, Schmucker DL and Jones AL (1992) Subcellular and molecular mechanisms of bile secretion. *Int Rev Cytol* **135**:269-313.

Cattori V, van Montfoort JE, Stieger B, Landmann L, Meijer DK, Winterhalter KH, Meier PJ and Hagenbuch B (2001) Localization of organic anion transporting polypeptide 4 (Oatp4) in rat liver and comparison of its substrate specificity with Oatp1, Oatp2 and Oatp3. *Pflugers Arch* **443**:188-195.

Chawla A, Saez E and Evans RM (2000) "Don't know much bile-ology". *Cell* **103**:1-4.

Chen LJ, Imperato TJ and Bolt RJ (1978) Enzymatic sulfation of bile salts. II. Studies on bile salt sulfotranferase from rat kidney. *Biochim Biophys Acta* **522**:443-451.

Chen W and Chiang JY (2003) Regulation of human sterol 27-hydroxylase gene (CYP27A1) by bile acids and hepatocyte nuclear factor 4alpha (HNF4alpha). *Gene* **313**:71-82.

Chen W, Owsley E, Yang Y, Stroup D and Chiang JY (2001) Nuclear receptor-mediated repression of human cholesterol 7alpha-hydroxylase gene transcription by bile acids. *J Lipid Res* **42**:1402-1412.

Cheng X, Maher J, Dieter MZ and Klaassen CD (2005) Regulation of mouse organic anion-transporting polypeptides (Oatps) in liver by prototypical microsomal enzyme inducers that activate distinct transcription factor pathways. *Drug Metab Dispos* **33**:1276-1282.

Chiang JY (2002) Bile acid regulation of gene expression: roles of nuclear hormone receptors. *Endocr Rev* **23**:443-463.

Chiang JY (2009) Bile acids: regulation of synthesis. *J Lipid Res*.

Chiang JY, Kimmel R and Stroup D (2001) Regulation of cholesterol 7alpha-hydroxylase gene (CYP7A1) transcription by the liver orphan receptor (LXRalpha). *Gene* **262**:257-265.

Chiang JY and Stroup D (1994) Identification and characterization of a putative bile acid-responsive element in cholesterol 7 alpha-hydroxylase gene promoter. *J Biol Chem* **269**:17502-17507.

Cohen BI and Raicht RF (1981) Effects of bile acids on colon carcinogenesis in rats treated with carcinogens. *Cancer Res* **41**:3759-3760.

Copaci I, Micu L, Iliescu L and Voiculescu M (2005) New therapeutical indications of ursodeoxycholic acid. *Rom J Gastroenterol* **14**:259-266.

Crestani M, Sadeghpour A, Stroup D, Galli G and Chiang JY (1998) Transcriptional activation of the cholesterol 7alpha-hydroxylase gene (CYP7A) by nuclear hormone receptors. *J Lipid Res* **39**:2192-2200.

Crosignani A, Podda M, Battezzati PM, Bertolini E, Zuin M, Watson D and Setchell KD (1991) Changes in bile acid composition in patients with primary biliary cirrhosis induced by ursodeoxycholic acid administration. *Hepatology* **14**:1000-1007.

Cui Y, Konig J, Buchholz JK, Spring H, Leier I and Keppler D (1999) Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. *Mol Pharmacol* **55**:929-937.

Dahm LJ, Schultze AE and Roth RA (1991) An antibody to neutrophils attenuates alpha-naphthylisothiocyanate-induced liver injury. *J Pharmacol Exp Ther* **256**:412-420.

Davies G and Henrissat B (1995) Structures and mechanisms of glycosyl hydrolases. *Structure* **3**:853-859.

De Fabiani E, Mitro N, Anzulovich AC, Pinelli A, Galli G and Crestani M (2001) The negative effects of bile acids and tumor necrosis factor-alpha on the transcription of cholesterol 7alpha-hydroxylase gene (CYP7A1) converge to hepatic nuclear factor-4: a novel mechanism of feedback regulation of bile acid synthesis mediated by nuclear receptors. *J Biol Chem* **276**:30708-30716.

De Fabiani E, Mitro N, Gilardi F, Caruso D, Galli G and Crestani M (2003) Coordinated control of cholesterol catabolism to bile acids and of gluconeogenesis via a novel mechanism of transcription regulation linked to the fasted-to-fed cycle. *J Biol Chem* **278**:39124-39132.

Delzenne NM, Calderon PB, Taper HS and Roberfroid MB (1992) Comparative hepatotoxicity of cholic acid, deoxycholic acid and lithocholic acid in the rat: in vivo and in vitro studies. *Toxicol Lett* **61**:291-304.

Denson LA, Sturm E, Echevarria W, Zimmerman TL, Makishima M, Mangelsdorf DJ and Karpen SJ (2001) The orphan nuclear receptor, shp, mediates bile acid-induced inhibition of the rat bile acid transporter, ntcp. *Gastroenterology* **121**:140-147.

Desmet VJ (1995) Histopathology of cholestasis. *Verh Dtsch Ges Pathol* **79**:233-240.

Desmet VJ, Krstulovic B and Van Damme B (1968) Histochemical study of rat liver in alpha-naphthyl isothiocyanate (ANIT) induced cholestasis. *Am J Pathol* **52**:401-421.

Ding X, Lichti K, Kim I, Gonzalez FJ and Staudinger JL (2006) Regulation of constitutive androstane receptor and its target genes by fasting, cAMP, hepatocyte nuclear factor alpha, and the coactivator peroxisome proliferator-activated receptor gamma coactivator-1alpha. *J Biol Chem* **281**:26540-26551.

Doerner KC, Gurley EC, Vlahcevic ZR and Hylemon PB (1995) Regulation of cholesterol 7 alpha-hydroxylase expression by sterols in primary rat hepatocyte cultures. *J Lipid Res* **36**:1168-1177.

Downes M, Verdecia MA, Roecker AJ, Hughes R, Hogenesch JB, Kast-Woelbern HR, Bowman ME, Ferrer JL, Anisfeld AM, Edwards PA, Rosenfeld JM, Alvarez JG, Noel JP, Nicolaou KC and Evans RM (2003) A chemical, genetic, and structural analysis of the nuclear bile acid receptor FXR. *Mol Cell* **11**:1079-1092.

Dubuisson C, Cresteil D, Desrochers M, Decimo D, Hadchouel M and Jacquemin E (1996) Ontogenic expression of the Na(+)-independent organic anion transporting polypeptide (oatp) in rat liver and kidney. *J Hepatol* **25**:932-940.

Dyrszka H, Chen T, Salen G and Mosbach EH (1975) Toxicity of chenodeoxycholic acid in the rhesus monkey. *Gastroenterology* **69**:333-337.

Elferink RP, Tytgat GN and Groen AK (1997) Hepatic canalicular membrane 1: The role of mdr2 P-glycoprotein in hepatobiliary lipid transport. *FASEB J* **11**:19-28.

Elias E (1999) URSO in obstetric cholestasis: not a bear market. *Gut* **45**:331-332.

Eloranta JJ and Kullak-Ublick GA (2005) Coordinate transcriptional regulation of bile acid homeostasis and drug metabolism. *Arch Biochem Biophys* **433**:397-412.

Eppens EF, van Mil SW, de Vree JM, Mok KS, Juijn JA, Oude Elferink RP, Berger R, Houwen RH and Klomp LW (2001) FIC1, the protein affected in two forms of hereditary cholestasis, is localized in the cholangiocyte and the canalicular membrane of the hepatocyte. *J Hepatol* **35**:436-443.

Eyssen HJ, De Pauw G and Van Eldere J (1999) Formation of hyodeoxycholic acid from muricholic acid and hyocholic acid by an unidentified gram-positive rod

termed HDCA-1 isolated from rat intestinal microflora. *Appl Environ Microbiol* **65**:3158-3163.

Falany CN, Fortinberry H, Leiter EH and Barnes S (1997) Cloning, expression, and chromosomal localization of mouse liver bile acid CoA:amino acid N-acyltransferase. *J Lipid Res* **38**:1139-1148.

Falany CN, Johnson MR, Barnes S and Diasio RB (1994) Glycine and taurine conjugation of bile acids by a single enzyme. Molecular cloning and expression of human liver bile acid CoA:amino acid N-acyltransferase. *J Biol Chem* **269**:19375-19379.

Fang Y, Studer E, Mitchell C, Grant S, Pandak WM, Hylemon PB and Dent P (2007) Conjugated bile acids regulate hepatocyte glycogen synthase activity in vitro and in vivo via Galphai signaling. *Mol Pharmacol* **71**:1122-1128.

Faubion WA, Guicciardi ME, Miyoshi H, Bronk SF, Roberts PJ, Svingen PA, Kaufmann SH and Gores GJ (1999) Toxic bile salts induce rodent hepatocyte apoptosis via direct activation of Fas. *J Clin Invest* **103**:137-145.

Fickert P, Fuchsbichler A, Marschall HU, Wagner M, Zollner G, Krause R, Zatloukal K, Jaeschke H, Denk H and Trauner M (2006) Lithocholic acid feeding induces segmental bile duct obstruction and destructive cholangitis in mice. *Am J Pathol* **168**:410-422.

Fickert P, Trauner M, Fuchsbichler A, Stumptner C, Zatloukal K and Denk H (2002) Cytokeratins as targets for bile acid-induced toxicity. *Am J Pathol* **160**:491-499.

Fickert P, Zollner G, Fuchsbichler A, Stumptner C, Pojer C, Zenz R, Lammert F, Stieger B, Meier PJ, Zatloukal K, Denk H and Trauner M (2001) Effects of ursodeoxycholic and cholic acid feeding on hepatocellular transporter expression in mouse liver. *Gastroenterology* **121**:170-183.

Fischer S, Beuers U, Spengler U, Zwiebel FM and Koebe HG (1996) Hepatic levels of bile acids in end-stage chronic cholestatic liver disease. *Clin Chim Acta* **251**:173-186.

Fuchs M (2003) Bile acid regulation of hepatic physiology: III. Regulation of bile acid synthesis: past progress and future challenges. *Am J Physiol Gastrointest Liver Physiol* **284**:G551-557.

Fujino T, Une M, Imanaka T, Inoue K and Nishimaki-Mogami T (2004) Structure-activity relationship of bile acids and bile acid analogs in regard to FXR activation. *J Lipid Res* **45**:132-138.

Funk C (2008) The role of hepatic transporters in drug elimination. *Expert Opin Drug Metab Toxicol* **4**:363-379.

Furutani M, Arii S, Higashitsuji H, Mise M, Fukumoto M, Takano S, Nakayama H, Imamura M and Fujita J (1995) Reduced expression of kan-1 (encoding putative bile acid-CoA-amino acid N-acyltransferase) mRNA in livers of rats after partial hepatectomy and during sepsis. *Biochem J* **311 (Pt 1)**:203-208.

Garg A and Grundy SM (1994) Cholestyramine therapy for dyslipidemia in non-insulin-dependent diabetes mellitus. A short-term, double-blind, crossover trial. *Ann Intern Med* **121**:416-422.

Gartung C, Ananthanarayanan M, Rahman MA, Schuele S, Nundy S, Soroka CJ, Stolz A, Suchy FJ and Boyer JL (1996) Down-regulation of expression and function of the rat liver Na+/bile acid cotransporter in extrahepatic cholestasis. *Gastroenterology* **110**:199-209.

Gerloff T, Stieger B, Hagenbuch B, Madon J, Landmann L, Roth J, Hofmann AF and Meier PJ (1998) The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. *J Biol Chem* **273**:10046-10050.

Goodwin B, Jones SA, Price RR, Watson MA, McKee DD, Moore LB, Galardi C, Wilson JG, Lewis MC, Roth ME, Maloney PR, Willson TM and Kliewer SA (2000) A regulatory cascade of the nuclear receptors FXR, SHP-1, and LRH-1 represses bile acid biosynthesis. *Mol Cell* **6**:517-526.

Gores GJ, Miyoshi H, Botla R, Aguilar HI and Bronk SF (1998) Induction of the mitochondrial permeability transition as a mechanism of liver injury during cholestasis: a potential role for mitochondrial proteases. *Biochim Biophys Acta* **1366**:167-175.

Greim H, Czygan P, Schaffner F and Popper H (1973a) Determination of bile acids in needle biopsies of human liver. *Biochem Med* **8**:280-286.

Greim H, Trulzsch D, Czygan P, Hutterer F, Schaffner F, Popper H, Cooper DY and Rosenthal O (1973b) Bile acid formation by liver microsomal systems. *Ann N Y Acad Sci* **212**:139-147.

Greim H, Trulzsch D, Roboz J, Dressler K, Czygan P, Hutterer F, Schaffner F and Popper H (1972) Mechanism of cholestasis. 5. Bile acids in normal rat livers and in those after bile duct ligation. *Gastroenterology* **63**:837-845.

Grober J, Zaghini I, Fujii H, Jones SA, Kliewer SA, Willson TM, Ono T and Besnard P (1999) Identification of a bile acid-responsive element in the human ileal bile acid-binding protein gene. Involvement of the farnesoid X receptor/9-cis-retinoic acid receptor heterodimer. *J Biol Chem* **274**:29749-29754.

Grundy SM, Ahrens EH, Jr. and Salen G (1971) Interruption of the enterohepatic circulation of bile acids in man: comparative effects of cholestyramine and ileal exclusion on cholesterol metabolism. *J Lab Clin Med* **78**:94-121.

Gujral JS, Farhood A, Bajt ML and Jaeschke H (2003) Neutrophils aggravate acute liver injury during obstructive cholestasis in bile duct-ligated mice. *Hepatology* **38**:355-363.

Gujral JS, Hinson JA, Farhood A and Jaeschke H (2004a) NADPH oxidase-derived oxidant stress is critical for neutrophil cytotoxicity during endotoxemia. *Am J Physiol Gastrointest Liver Physiol* **287**:G243-252.

Gujral JS, Liu J, Farhood A and Jaeschke H (2004b) Reduced oncotic necrosis in Fas receptor-deficient C57BL/6J-lpr mice after bile duct ligation. *Hepatology* **40**:998-1007.

Guo GL, Lambert G, Negishi M, Ward JM, Brewer HB, Jr., Kliewer SA, Gonzalez FJ and Sinal CJ (2003) Complementary roles of farnesoid X receptor, pregnane X receptor, and constitutive androstane receptor in protection against bile acid toxicity. *J Biol Chem* **278**:45062-45071.

Gupta S, Stravitz RT, Dent P and Hylemon PB (2001) Down-regulation of cholesterol 7alpha-hydroxylase (CYP7A1) gene expression by bile acids in primary rat hepatocytes is mediated by the c-Jun N-terminal kinase pathway. *J Biol Chem* **276**:15816-15822.

Hagenbuch B and Meier PJ (1994) Molecular cloning, chromosomal localization, and functional characterization of a human liver Na+/bile acid cotransporter. *J Clin Invest* **93**:1326-1331.

Hagenbuch B and Meier PJ (2003) The superfamily of organic anion transporting polypeptides. *Biochim Biophys Acta* **1609**:1-18.

Halestrap AP and Brennerb C (2003) The adenine nucleotide translocase: a central component of the mitochondrial permeability transition pore and key player in cell death. *Curr Med Chem* **10**:1507-1525.

Hatano E (2007) Tumor necrosis factor signaling in hepatocyte apoptosis. *J Gastroenterol Hepatol* **22 Suppl 1**:S43-44.

Heuman DM (1989) Quantitative estimation of the hydrophilic-hydrophobic balance of mixed bile salt solutions. *J Lipid Res* **30**:719-730.

Heuman DM, Hylemon PB and Vlahcevic ZR (1989) Regulation of bile acid synthesis. III. Correlation between biliary bile salt hydrophobicity index and the activities of enzymes regulating cholesterol and bile acid synthesis in the rat. *J Lipid Res* **30**:1161-1171.

Heuman DM, Mills AS, McCall J, Hylemon PB, Pandak WM and Vlahcevic ZR (1991) Conjugates of ursodeoxycholate protect against cholestasis and hepatocellular necrosis caused by more hydrophobic bile salts. In vivo studies in the rat. *Gastroenterology* **100**:203-211.

Hirohashi T, Suzuki H, Takikawa H and Sugiyama Y (2000) ATP-dependent transport of bile salts by rat multidrug resistance-associated protein 3 (Mrp3). *J Biol Chem* **275**:2905-2910.

Hofmann AF (1994) Pharmacology of ursodeoxycholic acid, an enterohepatic drug. *Scand J Gastroenterol Suppl* **204**:1-15.

Hofmann AF and Hagey LR (2008) Bile acids: chemistry, pathochemistry, biology, pathobiology, and therapeutics. *Cell Mol Life Sci* **65**:2461-2483.

Hofmann AF and Small DM (1967) Detergent properties of bile salts: correlation with physiological function. *Annu Rev Med* **18**:333-376.

Holt JA, Luo G, Billin AN, Bisi J, McNeill YY, Kozarsky KF, Donahee M, Wang da Y, Mansfield TA, Kliewer SA, Goodwin B and Jones SA (2003) Definition of a novel growth factor-dependent signal cascade for the suppression of bile acid biosynthesis. *Genes Dev* **17**:1581-1591.

Houten SM, Watanabe M and Auwerx J (2006) Endocrine functions of bile acids. *EMBO J* **25**:1419-1425.

Huang L, Zhao A, Lew JL, Zhang T, Hrywna Y, Thompson JR, de Pedro N, Royo I, Blevins RA, Pelaez F, Wright SD and Cui J (2003) Farnesoid X receptor activates transcription of the phospholipid pump MDR3. *J Biol Chem* **278**:51085-51090.

Inagaki T, Choi M, Moschetta A, Peng L, Cummins CL, McDonald JG, Luo G, Jones SA, Goodwin B, Richardson JA, Gerard RD, Repa JJ, Mangelsdorf DJ and Kliewer SA (2005) Fibroblast growth factor 15 functions as an enterohepatic signal to regulate bile acid homeostasis. *Cell Metab* **2**:217-225.

Ito S, Fujimori T, Furuya A, Satoh J, Nabeshima Y and Nabeshima YI (2005) Impaired negative feedback suppression of bile acid synthesis in mice lacking betaKlotho. *J Clin Invest* **115**:2202-2208.

Ito S, Kinoshita S, Shiraishi N, Nakagawa S, Sekine S, Fujimori T and Nabeshima YI (2000) Molecular cloning and expression analyses of mouse betaklotho, which encodes a novel Klotho family protein. *Mech Dev* **98**:115-119.

Iwamura K (1982) Clinical aspects of bile acid metabolism in liver diseases. *Tokai J Exp Clin Med* **7**:7-29.

Jacquemin E, Hagenbuch B, Stieger B, Wolkoff AW and Meier PJ (1994) Expression cloning of a rat liver Na(+)-independent organic anion transporter. *Proc Natl Acad Sci U S A* **91**:133-137.

Jaeschke H, Gores GJ, Cederbaum AI, Hinson JA, Pessayre D and Lemasters JJ (2002) Mechanisms of hepatotoxicity. *Toxicol Sci* **65**:166-176.

Jahan A and Chiang JY (2005) Cytokine regulation of human sterol 12alpha-hydroxylase (CYP8B1) gene. *Am J Physiol Gastrointest Liver Physiol* **288**:G685-695.

James O, Cullen J and Bouchier IA (1975) Chenodeoxycholic acid therapy for gallstones: effectiveness, toxicity and influence on bile acid metabolism. *Q J Med* **44**:349-367.

Jung D, Hagenbuch B, Fried M, Meier PJ and Kullak-Ublick GA (2004) Role of liver-enriched transcription factors and nuclear receptors in regulating the human, mouse, and rat NTCP gene. *Am J Physiol Gastrointest Liver Physiol* **286**:G752-761.

Kantrow SP, Tatro LG and Piantadosi CA (2000) Oxidative stress and adenine nucleotide control of mitochondrial permeability transition. *Free Radic Biol Med* **28**:251-260.

Katsuma S, Hirasawa A and Tsujimoto G (2005) Bile acids promote glucagon-like peptide-1 secretion through TGR5 in a murine enteroendocrine cell line STC-1. *Biochem Biophys Res Commun* **329**:386-390.

Kawamata Y, Fujii R, Hosoya M, Harada M, Yoshida H, Miwa M, Fukusumi S, Habata Y, Itoh T, Shintani Y, Hinuma S, Fujisawa Y and Fujino M (2003) A G protein-coupled receptor responsive to bile acids. *J Biol Chem* **278**:9435-9440.

Keppler D and Konig J (1997) Hepatic canalicular membrane 5: Expression and localization of the conjugate export pump encoded by the MRP2 (cMRP/cMOAT) gene in liver. *FASEB J* **11**:509-516.

Keppler D and Konig J (2000) Hepatic secretion of conjugated drugs and endogenous substances. *Semin Liver Dis* **20**:265-272.

Kerr TA, Saeki S, Schneider M, Schaefer K, Berdy S, Redder T, Shan B, Russell DW and Schwarz M (2002) Loss of nuclear receptor SHP impairs but does not eliminate negative feedback regulation of bile acid synthesis. *Dev Cell* **2**:713-720.

Kim I, Ahn SH, Inagaki T, Choi M, Ito S, Guo GL, Kliewer SA and Gonzalez FJ (2007) Differential regulation of bile acid homeostasis by the farnesoid X receptor in liver and intestine. *J Lipid Res* **48**:2664-2672.

Kliewer SA (2003) The nuclear pregnane X receptor regulates xenobiotic detoxification. *J Nutr* **133**:2444S-2447S.

Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, Perlmann T and Lehmann JM (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* **92**:73-82.

Kliewer SA and Willson TM (2002) Regulation of xenobiotic and bile acid metabolism by the nuclear pregnane X receptor. *J Lipid Res* **43**:359-364.

Kneuer C, Honscha W, Gabel G and Honscha KU (2007) Adaptive response to increased bile acids: induction of MDR1 gene expression and P-glycoprotein activity in renal epithelial cells. *Pflugers Arch* **454**:587-594.

Kodali P, Wu P, Lahiji PA, Brown EJ and Maher JJ (2006) ANIT toxicity toward mouse hepatocytes in vivo is mediated primarily by neutrophils via CD18. *Am J Physiol Gastrointest Liver Physiol* **291**:G355-363.

Kouzuki H, Suzuki H, Stieger B, Meier PJ and Sugiyama Y (2000) Characterization of the transport properties of organic anion transporting polypeptide 1 (oatp1) and Na(+)/taurocholate cotransporting polypeptide (Ntcp): comparative studies on the inhibitory effect of their possible substrates in hepatocytes and cDNA-transfected COS-7 cells. *J Pharmacol Exp Ther* **292**:505-511.

Kramer W, Bickel U, Buscher HP, Gerok W and Kurz G (1982) Bile-salt-binding polypeptides in plasma membranes of hepatocytes revealed by photoaffinity labelling. *Eur J Biochem* **129**:13-24.

Krell H and Enderle GJ (1993) Cholestasis: pathophysiology and pathobiochemistry. *Z Gastroenterol* **31 Suppl 2**:11-15.

Kubitz R, Helmer A and Haussinger D (2005) Biliary transport systems: short-term regulation. *Methods Enzymol* **400**:542-557.

Kullak-Ublick GA, Hagenbuch B, Stieger B, Wolkoff AW and Meier PJ (1994) Functional characterization of the basolateral rat liver organic anion transporting polypeptide. *Hepatology* **20**:411-416.

Kullak-Ublick GA, Stieger B and Meier PJ (2004) Enterohepatic bile salt transporters in normal physiology and liver disease. *Gastroenterology* **126**:322-342.

Kwo P, Patel T, Bronk SF and Gores GJ (1995) Nuclear serine protease activity contributes to bile acid-induced apoptosis in hepatocytes. *Am J Physiol* **268**:G613-621.

Lee JW, Cheong JH, Lee YC, Na SY and Lee SK (2000) Dissecting the molecular mechanism of nuclear receptor action: transcription coactivators and corepressors. *Exp Mol Med* **32**:53-60.

Lefebvre P, Cariou B, Lien F, Kuipers F and Staels B (2009) Role of bile acids and bile acid receptors in metabolic regulation. *Physiol Rev* **89**:147-191.

Lehmann JM, Kliewer SA, Moore LB, Smith-Oliver TA, Oliver BB, Su JL, Sundseth SS, Winegar DA, Blanchard DE, Spencer TA and Willson TM (1997) Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *J Biol Chem* **272**:3137-3140.

Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT and Kliewer SA (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* **102**:1016-1023.

Leppa S and Bohmann D (1999) Diverse functions of JNK signaling and c-Jun in stress response and apoptosis. *Oncogene* **18**:6158-6162.

Lew JL, Zhao A, Yu J, Huang L, De Pedro N, Pelaez F, Wright SD and Cui J (2004) The farnesoid X receptor controls gene expression in a ligand- and promoter-selective fashion. *J Biol Chem* **279**:8856-8861.

Leyland H, Gentry J, Arthur MJ and Benyon RC (1996) The plasminogen-activating system in hepatic stellate cells. *Hepatology* **24**:1172-1178.

Li-Hawkins J, Gafvels M, Olin M, Lund EG, Andersson U, Schuster G, Bjorkhem I, Russell DW and Eggertsen G (2002) Cholic acid mediates negative feedback regulation of bile acid synthesis in mice. *J Clin Invest* **110**:1191-1200.

Li MK and Crawford JM (2004) The pathology of cholestasis. *Semin Liver Dis* **24**:21-42.

Liblau RS, Singer SM and McDevitt HO (1995) Th1 and Th2 CD4+ T cells in the pathogenesis of organ-specific autoimmune diseases. *Immunol Today* **16**:34-38.

Lida T, Momose T, Tamura T, Matsumoto T, Chang FC, Goto J and Nambara T (1989) Potential bile acid metabolites. 14. Hyocholic and muricholic acid stereoisomers. *J Lipid Res* **30**:1267-1279.

Lin BC, Wang M, Blackmore C and Desnoyers LR (2007) Liver-specific activities of FGF19 require Klotho beta. *J Biol Chem* **282**:27277-27284.

Louis H, Van Laethem JL, Wu W, Quertinmont E, Degraef C, Van den Berg K, Demols A, Goldman M, Le Moine O, Geerts A and Deviere J (1998) Interleukin-10 controls neutrophilic infiltration, hepatocyte proliferation, and

liver fibrosis induced by carbon tetrachloride in mice. *Hepatology* **28**:1607-1615.

Lu TT, Makishima M, Repa JJ, Schoonjans K, Kerr TA, Auwerx J and Mangelsdorf DJ (2000) Molecular basis for feedback regulation of bile acid synthesis by nuclear receptors. *Mol Cell* **6**:507-515.

Ma K, Saha PK, Chan L and Moore DD (2006) Farnesoid X receptor is essential for normal glucose homeostasis. *J Clin Invest* **116**:1102-1109.

Macrides TA, Faktor DA, Kalafatis N and Amiet RG (1994) Enzymic sulfation of bile salts. Partial purification and characterization of an enzyme from the liver of the shark Heterodontus portusjacksoni that catalyses the sulfation of the shark bile steroid 5 beta-scymnol. *Comp Biochem Physiol Biochem Mol Biol* **107**:461-469.

Maher JM, Aleksunes LM, Dieter MZ, Tanaka Y, Peters JM, Manautou JE and Klaassen CD (2008) Nrf2- and PPAR alpha-mediated regulation of hepatic Mrp transporters after exposure to perfluorooctanoic acid and perfluorodecanoic acid. *Toxicol Sci* **106**:319-328.

Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR and Mangelsdorf DJ (2002) Vitamin D receptor as an intestinal bile acid sensor. *Science* **296**:1313-1316.

Makishima M, Okamoto AY, Repa JJ, Tu H, Learned RM, Luk A, Hull MV, Lustig KD, Mangelsdorf DJ and Shan B (1999) Identification of a nuclear receptor for bile acids. *Science* **284**:1362-1365.

Mangelsdorf DJ and Evans RM (1995) The RXR heterodimers and orphan receptors. *Cell* **83**:841-850.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P and Evans RM (1995) The nuclear receptor superfamily: the second decade. *Cell* **83**:835-839.

Mannes GA, Stellaard F and Paumgartner G (1982) Increased serum bile acids in cirrhosis with normal transaminases. *Digestion* **25**:217-221.

Martinez-Diez MC, Serrano MA, Monte MJ and Marin JJ (2000) Comparison of the effects of bile acids on cell viability and DNA synthesis by rat hepatocytes in primary culture. *Biochim Biophys Acta* **1500**:153-160.

Maruyama T, Miyamoto Y, Nakamura T, Tamai Y, Okada H, Sugiyama E, Itadani H and Tanaka K (2002) Identification of membrane-type receptor for bile acids (M-BAR). *Biochem Biophys Res Commun* **298**:714-719.

Mason JI and Boyd GS (1978) The suppressive effect of the catatoxic steroid, pregnenolone-16alpha-carbonitrile, on liver microsomal cholesterol-7alpha-hydroxlyase. *Steroids* **31**:849-854.

Matsuzaki Y, Bouscarel B, Le M, Ceryak S, Gettys TW, Shoda J and Fromm H (1997) Effect of cholestasis on regulation of cAMP synthesis by glucagon and bile acids in isolated hepatocytes. *Am J Physiol* **273**:G164-174.

McCarter JD and Withers SG (1994) Mechanisms of enzymatic glycoside hydrolysis. *Curr Opin Struct Biol* **4**:885-892.

Meier PJ and Stieger B (2002) Bile salt transporters. *Annu Rev Physiol* **64**:635-661.

Menke JG, Macnaul KL, Hayes NS, Baffic J, Chao YS, Elbrecht A, Kelly LJ, Lam MH, Schmidt A, Sahoo S, Wang J, Wright SD, Xin P, Zhou G, Moller DE and Sparrow CP (2002) A novel liver X receptor agonist establishes species differences in the regulation of cholesterol 7alpha-hydroxylase (CYP7a). *Endocrinology* **143**:2548-2558.

Miki S, Mosbach EH, Cohen BI, Mikami T, Infante R, Ayyad N and McSherry CK (1993) Metabolism of beta-muricholic acid in the hamster and prairie dog. *J Lipid Res* **34**:1709-1716.

Milkiewicz P, Mills CO, Roma MG, Ahmed-Choudhury J, Elias E and Coleman R (1999) Tauroursodeoxycholate and S-adenosyl-L-methionine exert an additive ameliorating effect on taurolithocholate-induced cholestasis: a study in isolated rat hepatocyte couplets. *Hepatology* **29**:471-476.

Miyai K, Richardson AL, Mayr W and Javitt NB (1977) Subcellular pathology of rat liver in cholestasis and choleresis induced by bile salts. 1. Effects of lithocholic, 3beta-hydroxy-5-cholenoic, cholic, and dehydrocholic acids. *Lab Invest* **36**:249-258.

Miyake JH, Wang SL and Davis RA (2000) Bile acid induction of cytokine expression by macrophages correlates with repression of hepatic cholesterol 7alpha-hydroxylase. *J Biol Chem* **275**:21805-21808.

Miyata M, Matsuda Y, Tsuchiya H, Kitada H, Akase T, Shimada M, Nagata K, Gonzalez FJ and Yamazoe Y (2006) Chenodeoxycholic acid-mediated activation of the farnesoid X receptor negatively regulates hydroxysteroid sulfotransferase. *Drug Metab Pharmacokinet* **21**:315-323.

Miyata M, Tozawa A, Otsuka H, Nakamura T, Nagata K, Gonzalez FJ and Yamazoe Y (2005) Role of farnesoid X receptor in the enhancement of canalicular bile acid output and excretion of unconjugated bile acids: a mechanism for protection against cholic acid-induced liver toxicity. *J Pharmacol Exp Ther* **312**:759-766.

Miyazaki K, Nakayama F and Koga A (1984) Effect of chenodeoxycholic and ursodeoxycholic acids on isolated adult human hepatocytes. *Dig Dis Sci* **29**:1123-1130.

Montet JC, Lindheimer M, Reynier MO, Crotte C, Bontemps R and Gerolami A (1982) Mixed micelle properties and intestinal cholesterol uptake. *Biochimie* **64**:255-261.

Mosbach EH (1972) Hepatic synthesis of bile acids. Biochemical steps and mechanisms of rate control. *Arch Intern Med* **130**:478-487.

Moschetta A and Kliewer SA (2005) Weaving betaKlotho into bile acid metabolism. *J Clin Invest* **115**:2075-2077.

Muller M and Jansen PL (1997) Molecular aspects of hepatobiliary transport. *Am J Physiol* **272**:G1285-1303.

Murphy C, Parini P, Wang J, Bjorkhem I, Eggertsen G and Gafvels M (2005) Cholic acid as key regulator of cholesterol synthesis, intestinal absorption and hepatic storage in mice. *Biochim Biophys Acta* **1735**:167-175.

Mutoh S, Osabe M, Inoue K, Moore R, Pedersen L, Perera L, Rebolloso Y, Sueyoshi T and Negishi M (2009) Dephosphorylation of threonine 38 is required for nuclear translocation and activation of human xenobiotic receptor CAR (NR1I3). *J Biol Chem* **284**:34785-34792.

Nestel PJ and Grundy SM (1976) Changes in plasma triglyceride metabolism during withdrawal of bile. *Metabolism* **25**:1259-1268.

Nilsson LM, Abrahamsson A, Sahlin S, Gustafsson U, Angelin B, Parini P and Einarsson C (2007) Bile acids and lipoprotein metabolism: effects of cholestyramine and chenodeoxycholic acid on human hepatic mRNA expression. *Biochem Biophys Res Commun* **357**:707-711.

Norlin M and Wikvall K (2007) Enzymes in the conversion of cholesterol into bile acids. *Curr Mol Med* **7**:199-218.

Oda H, Kuroki S, Yamashita H and Nakayama F (1990) Effects of bile acid feeding on hepatic deoxycholate 7 alpha-hydroxylase activity in the hamster. *Lipids* **25**:706-710.

Ourlin JC, Lasserre F, Pineau T, Fabre JM, Sa-Cunha A, Maurel P, Vilarem MJ and Pascussi JM (2003) The small heterodimer partner interacts with the pregnane X receptor and represses its transcriptional activity. *Mol Endocrinol* **17**:1693-1703.

Palmer RH and Ruban Z (1966) Production of bile duct hyperplasia and gallstones by lithocholic acid. *J Clin Invest* **45**:1255-1267.

Pandak WM, Bohdan P, Franklund C, Mallonee DH, Eggertsen G, Bjorkhem I, Gil G, Vlahcevic ZR and Hylemon PB (2001) Expression of sterol 12alpha-hydroxylase alters bile acid pool composition in primary rat hepatocytes and in vivo. *Gastroenterology* **120**:1801-1809.

Pandak WM, Vlahcevic ZR, Heuman DM, Redford KS, Chiang JY and Hylemon PB (1994) Effects of different bile salts on steady-state mRNA levels and transcriptional activity of cholesterol 7 alpha-hydroxylase. *Hepatology* **19**:941-947.

Parks DJ, Blanchard SG, Bledsoe RK, Chandra G, Consler TG, Kliewer SA, Stimmel JB, Willson TM, Zavacki AM, Moore DD and Lehmann JM (1999) Bile acids: natural ligands for an orphan nuclear receptor. *Science* **284**:1365-1368.

Patel T, Bronk SF and Gores GJ (1994) Increases of intracellular magnesium promote glycodeoxycholate-induced apoptosis in rat hepatocytes. *J Clin Invest* **94**:2183-2192.

Plaa GL and Priestly BG (1976) Intrahepatic cholestasis induced by drugs and chemicals. *Pharmacol Rev* **28**:207-273.

Plass JR, Mol O, Heegsma J, Geuken M, Faber KN, Jansen PL and Muller M (2002) Farnesoid X receptor and bile salts are involved in transcriptional regulation of the gene encoding the human bile salt export pump. *Hepatology* **35**:589-596.

Popper H (1968) Cholestasis. *Annu Rev Med* **19**:39-56.

Popper H and Schaffner F (1963) FINE STRUCTURAL CHANGES OF THE LIVER. *Ann Intern Med* **59**:674-691.

Qatanani M and Moore DD (2005) CAR, the continuously advancing receptor, in drug metabolism and disease. *Curr Drug Metab* **6**:329-339.

Quinn CM, Jessup W, Wong J, Kritharides L and Brown AJ (2005) Expression and regulation of sterol 27-hydroxylase (CYP27A1) in human macrophages: a role for RXR and PPARgamma ligands. *Biochem J* **385**:823-830.

Radominska A, Treat S and Little J (1993) Bile acid metabolism and the pathophysiology of cholestasis. *Semin Liver Dis* **13**:219-234.

Ramaiah SK and Jaeschke H (2007) Role of neutrophils in the pathogenesis of acute inflammatory liver injury. *Toxicol Pathol* **35**:757-766.

Rao A, Haywood J, Craddock AL, Belinsky MG, Kruh GD and Dawson PA (2008) The organic solute transporter alpha-beta, Ostalpha-Ostbeta, is essential for intestinal bile acid transport and homeostasis. *Proc Natl Acad Sci U S A* **105**:3891-3896.

Redinger RN (2003) The role of the enterohepatic circulation of bile salts and nuclear hormone receptors in the regulation of cholesterol homeostasis: Bile salts as ligands for nuclear hormone receptors. *Can J Gastroenterol* **17**:265-271.

Reinehr R, Becker S, Keitel V, Eberle A, Grether-Beck S and Haussinger D (2005) Bile salt-induced apoptosis involves NADPH oxidase isoform activation. *Gastroenterology* **129**:2009-2031.

Reinehr R, Graf D and Haussinger D (2003) Bile salt-induced hepatocyte apoptosis involves epidermal growth factor receptor-dependent CD95 tyrosine phosphorylation. *Gastroenterology* **125**:839-853.

Reinehr R and Haussinger D (2007) CD95 activation in the liver: ion fluxes and oxidative signaling. *Arch Biochem Biophys* **462**:124-131.

Repa JJ, Berge KE, Pomajzl C, Richardson JA, Hobbs H and Mangelsdorf DJ (2002) Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta. *J Biol Chem* **277**:18793-18800.

Rodriguez-Garay EA (2003) Cholestasis: human disease and experimental animal models. *Ann Hepatol* **2**:150-158.

Rolo AP, Oliveira PJ, Moreno AJ and Palmeira CM (2001) Chenodeoxycholate is a potent inducer of the permeability transition pore in rat liver mitochondria. *Biosci Rep* **21**:73-80.

Rolo AP, Oliveira PJ, Moreno AJ and Palmeira CM (2003a) Chenodeoxycholate induction of mitochondrial permeability transition pore is associated with increased membrane fluidity and cytochrome c release: protective role of carvedilol. *Mitochondrion* **2**:305-311.

Rolo AP, Palmeira CM, Holy JM and Wallace KB (2004) Role of mitochondrial dysfunction in combined bile acid-induced cytotoxicity: the switch between apoptosis and necrosis. *Toxicol Sci* **79**:196-204.

Rolo AP, Palmeira CM and Wallace KB (2003b) Mitochondrially mediated synergistic cell killing by bile acids. *Biochim Biophys Acta* **1637**:127-132.

Russell DW (2003) The enzymes, regulation, and genetics of bile acid synthesis. *Annu Rev Biochem* **72**:137-174.

Russell DW and Setchell KD (1992) Bile acid biosynthesis. *Biochemistry* **31**:4737-4749.

Saile B and Ramadori G (2007) Inflammation, damage repair and liver fibrosis--role of cytokines and different cell types. *Z Gastroenterol* **45**:77-86.

Salvioli G, Salati R, Bondi M, Fratalocchi A, Sala BM and Gibertini A (1982) Bile acid transformation by the intestinal flora and cholesterol saturation in bile. Effects of Streptococcus faecium administration. *Digestion* **23**:80-88.

Sauer P, Stiehl A, Fitscher BA, Riedel HD, Benz C, Kloters-Plachky P, Stengelin S, Stremmel W and Kramer W (2000) Downregulation of ileal bile acid absorption in bile-duct-ligated rats. *J Hepatol* **33**:2-8.

Savas U, Wester MR, Griffin KJ and Johnson EF (2000) Rabbit pregnane X receptor is activated by rifampicin. *Drug Metab Dispos* **28**:529-537.

Schaffner F and Popper H (1959) Electron microscopic study of human cholestasis. *Proc Soc Exp Biol Med* **101**:777-779.

Schmiedlin-Ren P, Thummel KE, Fisher JM, Paine MF, Lown KS and Watkins PB (1997) Expression of enzymatically active CYP3A4 by Caco-2 cells grown on extracellular matrix-coated permeable supports in the presence of 1alpha,25-dihydroxyvitamin D3. *Mol Pharmacol* **51**:741-754.

Schmucker DL, Ohta M, Kanai S, Sato Y and Kitani K (1990) Hepatic injury induced by bile salts: correlation between biochemical and morphological events. *Hepatology* **12**:1216-1221.

Scholmerich J, Becher MS, Schmidt K, Schubert R, Kremer B, Feldhaus S and Gerok W (1984) Influence of hydroxylation and conjugation of bile salts on their membrane-damaging properties--studies on isolated hepatocytes and lipid membrane vesicles. *Hepatology* **4**:661-666.

Searle J, Kerr JF and Bishop CJ (1982) Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. *Pathol Annu* **17 Pt 2**:229-259.

Seol W, Choi HS and Moore DD (1995) Isolation of proteins that interact specifically with the retinoid X receptor: two novel orphan receptors. *Mol Endocrinol* **9**:72-85.

Setchell KD, Rodrigues CM, Clerici C, Solinas A, Morelli A, Gartung C and Boyer J (1997) Bile acid concentrations in human and rat liver tissue and in hepatocyte nuclei. *Gastroenterology* **112**:226-235.

Shibata S, Hayakawa K, Egashira Y and Sanada H (2007) Roles of nuclear receptors in the up-regulation of hepatic cholesterol 7alpha-hydroxylase by cholestyramine in rats. *Life Sci* **80**:546-553.

Shneider BL, Dawson PA, Christie DM, Hardikar W, Wong MH and Suchy FJ (1995) Cloning and molecular characterization of the ontogeny of a rat ileal sodium-dependent bile acid transporter. *J Clin Invest* **95**:745-754.

Sinakos E, Marschall HU, Kowdley KV, Befeler A, Keach J and Lindor K (2010) Bile acid changes after high-dose ursodeoxycholic acid treatment in primary sclerosing cholangitis: Relation to disease progression. *Hepatology* **52**:197-203.

Sinal CJ, Tohkin M, Miyata M, Ward JM, Lambert G and Gonzalez FJ (2000) Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell* **102**:731-744.

Siviero I, Ferrante SM, Meio IB, Madi K and Chagas VL (2008) Hepatobiliary effects of cholic and lithocholic acids: experimental study in hamsters. *Pediatr Surg Int* **24**:325-331.

Slitt AL, Allen K, Morrone J, Aleksunes LM, Chen C, Maher JM, Manautou JE, Cherrington NJ and Klaassen CD (2007) Regulation of transporter expression in mouse liver, kidney, and intestine during extrahepatic cholestasis. *Biochim Biophys Acta* **1768**:637-647.

Smit JJ, Schinkel AH, Oude Elferink RP, Groen AK, Wagenaar E, van Deemter L, Mol CA, Ottenhoff R, van der Lugt NM, van Roon MA and et al. (1993) Homozygous disruption of the murine mdr2 P-glycoprotein gene leads to a complete absence of phospholipid from bile and to liver disease. *Cell* **75**:451-462.

Sodeman T, Bronk SF, Roberts PJ, Miyoshi H and Gores GJ (2000) Bile salts mediate hepatocyte apoptosis by increasing cell surface trafficking of Fas. *Am J Physiol Gastrointest Liver Physiol* **278**:G992-999.

Sokol RJ, Winklhofer-Roob BM, Devereaux MW and McKim JM, Jr. (1995) Generation of hydroperoxides in isolated rat hepatocytes and hepatic mitochondria exposed to hydrophobic bile acids. *Gastroenterology* **109**:1249-1256.

Soroka CJ, Lee JM, Azzaroli F and Boyer JL (2001) Cellular localization and up-regulation of multidrug resistance-associated protein 3 in hepatocytes and cholangiocytes during obstructive cholestasis in rat liver. *Hepatology* **33**:783-791.

Spivey JR, Bronk SF and Gores GJ (1993) Glycochenodeoxycholate-induced lethal hepatocellular injury in rat hepatocytes. Role of ATP depletion and cytosolic free calcium. *J Clin Invest* **92**:17-24.

St-Pierre MV, Kullak-Ublick GA, Hagenbuch B and Meier PJ (2001) Transport of bile acids in hepatic and non-hepatic tissues. *J Exp Biol* **204**:1673-1686.

Staels B (2009) A review of bile acid sequestrants: potential mechanism(s) for glucose-lowering effects in type 2 diabetes mellitus. *Postgrad Med* **121**:25-30.

Staudinger J, Liu Y, Madan A, Habeebu S and Klaassen CD (2001a) Coordinate regulation of xenobiotic and bile acid homeostasis by pregnane X receptor. *Drug Metab Dispos* **29**:1467-1472.

Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH and Kliewer SA (2001b) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci U S A* **98**:3369-3374.

Story JA and Kritchevsky D (1976) Comparison of the binding of various bile acids and bile salts in vitro by several types of fiber. *J Nutr* **106**:1292-1294.

Stravitz RT, Rao YP, Vlahcevic ZR, Gurley EC, Jarvis WD and Hylemon PB (1996) Hepatocellular protein kinase C activation by bile acids: implications for regulation of cholesterol 7 alpha-hydroxylase. *Am J Physiol* **271**:G293-303.

Stravitz RT, Vlahcevic ZR, Gurley EC and Hylemon PB (1995) Repression of cholesterol 7 alpha-hydroxylase transcription by bile acids is mediated through protein kinase C in primary cultures of rat hepatocytes. *J Lipid Res* **36**:1359-1369.

Sueyoshi T, Moore R, Sugatani J, Matsumura Y and Negishi M (2008) PPP1R16A, the membrane subunit of protein phosphatase 1beta, signals nuclear translocation of the nuclear receptor constitutive active/androstane receptor. *Mol Pharmacol* **73**:1113-1121.

Svegliati-Baroni G, De Minicis S and Marzioni M (2008) Hepatic fibrogenesis in response to chronic liver injury: novel insights on the role of cell-to-cell interaction and transition. *Liver Int* **28**:1052-1064.

Takikawa H (2002) Hepatobiliary transport of bile acids and organic anions. *J Hepatobiliary Pancreat Surg* **9**:443-447.

Tan KP, Yang M and Ito S (2007) Activation of nuclear factor (erythroid-2 like) factor 2 by toxic bile acids provokes adaptive defense responses to enhance cell survival at the emergence of oxidative stress. *Mol Pharmacol* **72**:1380-1390.

Thomas C, Pellicciari R, Pruzanski M, Auwerx J and Schoonjans K (2008) Targeting bile-acid signalling for metabolic diseases. *Nat Rev Drug Discov* **7**:678-693.

Thompson K, Maltby J, Fallowfield J, McAulay M, Millward-Sadler H and Sheron N (1998) Interleukin-10 expression and function in experimental murine liver inflammation and fibrosis. *Hepatology* **28**:1597-1606.

Thompson WG (1971) Cholestyramine. *Can Med Assoc J* **104**:305-309.

Thummel KE, Brimer C, Yasuda K, Thottassery J, Senn T, Lin Y, Ishizuka H, Kharasch E, Schuetz J and Schuetz E (2001) Transcriptional control of intestinal cytochrome P-4503A by 1alpha,25-dihydroxy vitamin D3. *Mol Pharmacol* **60**:1399-1406.

Tietz PS, Marinelli RA, Chen XM, Huang B, Cohn J, Kole J, McNiven MA, Alper S and LaRusso NF (2003) Agonist-induced coordinated trafficking of functionally related transport proteins for water and ions in cholangiocytes. *J Biol Chem* **278**:20413-20419.

Trauner M, Fickert P, Halilbasic E and Moustafa T (2008) Lessons from the toxic bile concept for the pathogenesis and treatment of cholestatic liver diseases. *Wien Med Wochenschr* **158**:542-548.

Trauner M, Meier PJ and Boyer JL (1998) Molecular pathogenesis of cholestasis. *N Engl J Med* **339**:1217-1227.

Ujhazy P, Ortiz D, Misra S, Li S, Moseley J, Jones H and Arias IM (2001) Familial intrahepatic cholestasis 1: studies of localization and function. *Hepatology* **34**:768-775.

Uppal H, Toma D, Saini SP, Ren S, Jones TJ and Xie W (2005) Combined loss of orphan receptors PXR and CAR heightens sensitivity to toxic bile acids in mice. *Hepatology* **41**:168-176.

Vlahcevic ZR, Eggertsen G, Bjorkhem I, Hylemon PB, Redford K and Pandak WM (2000) Regulation of sterol 12alpha-hydroxylase and cholic acid biosynthesis in the rat. *Gastroenterology* **118**:599-607.

Vlahcevic ZR, Stravitz RT, Heuman DM, Hylemon PB and Pandak WM (1997) Quantitative estimations of the contribution of different bile acid pathways to total bile acid synthesis in the rat. *Gastroenterology* **113**:1949-1957.

Vyvoda OS, Coleman R and Holdsworth G (1977) Effects of different bile salts upon the composition and morphology of a liver plasma membrane preparation. Deoxycholate is more membrane damaging than cholate and its conjugates. *Biochim Biophys Acta* **465**:68-76.

Wagner M, Halilbasic E, Marschall HU, Zollner G, Fickert P, Langner C, Zatloukal K, Denk H and Trauner M (2005) CAR and PXR agonists stimulate hepatic bile acid and bilirubin detoxification and elimination pathways in mice. *Hepatology* **42**:420-430.

Wagner M and Trauner M (2005) Transcriptional regulation of hepatobiliary transport systems in health and disease: implications for a rationale approach to the treatment of intrahepatic cholestasis. *Ann Hepatol* **4**:77-99.

Walker IA, Nelson-Piercy C and Williamson C (2002) Role of bile acid measurement in pregnancy. *Ann Clin Biochem* **39**:105-113.

Wang DQ and Tazuma S (2002) Effect of beta-muricholic acid on the prevention and dissolution of cholesterol gallstones in C57L/J mice. *J Lipid Res* **43**:1960-1968.

Wang DQ, Tazuma S, Cohen DE and Carey MC (2003a) Feeding natural hydrophilic bile acids inhibits intestinal cholesterol absorption: studies in the gallstone-susceptible mouse. *Am J Physiol Gastrointest Liver Physiol* **285**:G494-502.

Wang H, Chen J, Hollister K, Sowers LC and Forman BM (1999) Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. *Mol Cell* **3**:543-553.

Wang J, Einarsson C, Murphy C, Parini P, Bjorkhem I, Gafvels M and Eggertsen G (2006) Studies on LXR- and FXR-mediated effects on cholesterol homeostasis in normal and cholic acid-depleted mice. *J Lipid Res* **47**:421-430.

Wang L, Han Y, Kim CS, Lee YK and Moore DD (2003b) Resistance of SHP-null mice to bile acid-induced liver damage. *J Biol Chem* **278**:44475-44481.

Wang R, Lam P, Liu L, Forrest D, Yousef IM, Mignault D, Phillips MJ and Ling V (2003c) Severe cholestasis induced by cholic acid feeding in knockout mice of sister of P-glycoprotein. *Hepatology* **38**:1489-1499.

Watanabe M, Houten SM, Mataki C, Christoffolete MA, Kim BW, Sato H, Messaddeq N, Harney JW, Ezaki O, Kodama T, Schoonjans K, Bianco AC and Auwerx J (2006) Bile acids induce energy expenditure by promoting intracellular thyroid hormone activation. *Nature* **439**:484-489.

Watanabe M, Houten SM, Wang L, Moschetta A, Mangelsdorf DJ, Heyman RA, Moore DD and Auwerx J (2004) Bile acids lower triglyceride levels via a pathway involving FXR, SHP, and SREBP-1c. *J Clin Invest* **113**:1408-1418.

Watkins RE, Wisely GB, Moore LB, Collins JL, Lambert MH, Williams SP, Willson TM, Kliewer SA and Redinbo MR (2001) The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. *Science* **292**:2329-2333.

Wheeler HO (1972) Secretion of bile acids by the liver and their role in the formation of hepatic bile. *Arch Intern Med* **130**:533-541.

Whiteside CH, Fluckiger HB and Sarett HP (1966) Comparison of in vitro bile acid binding capacity and in vivo hypocholesteremic activity of cholestyramine. *Proc Soc Exp Biol Med* **121**:153-156.

Witkop B (1992) Remembering Heinrich Wieland (1877-1957). Portrait of an organic chemist and founder of modern biochemistry. *Med Res Rev* **12**:195-274.

Wolf G (2002) Intestinal bile acids can bind to and activate the vitamin D receptor. *Nutr Rev* **60**:281-283.

Wong MH, Oelkers P, Craddock AL and Dawson PA (1994) Expression cloning and characterization of the hamster ileal sodium-dependent bile acid transporter. *J Biol Chem* **269**:1340-1347.

Wu T, Xie C, Bhaskarabhatla M, Yan M, Leone A, Chen SS, Zhou XJ, Putterman C and Mohan C (2007) Excreted urinary mediators in an animal model of experimental immune nephritis with potential pathogenic significance. *Arthritis Rheum* **56**:949-959.

Xie W, Radominska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES, Waxman DJ and Evans RM (2001) An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc Natl Acad Sci U S A* **98**:3375-3380.

Xu G, Li H, Pan LX, Shang Q, Honda A, Ananthanarayanan M, Erickson SK, Shneider BL, Shefer S, Bollineni J, Forman BM, Matsuzaki Y, Suchy FJ, Tint GS and Salen G (2003) FXR-mediated down-regulation of CYP7A1 dominates LXRalpha in long-term cholesterol-fed NZW rabbits. *J Lipid Res* **44**:1956-1962.

Xu G, Pan LX, Li H, Shang Q, Honda A, Shefer S, Bollineni J, Matsuzaki Y, Tint GS and Salen G (2004) Dietary cholesterol stimulates CYP7A1 in rats because farnesoid X receptor is not activated. *Am J Physiol Gastrointest Liver Physiol* **286**:G730-735.

Yamagata K, Daitoku H, Shimamoto Y, Matsuzaki H, Hirota K, Ishida J and Fukamizu A (2004) Bile acids regulate gluconeogenic gene expression via small heterodimer partner-mediated repression of hepatocyte nuclear factor 4 and Foxo1. *J Biol Chem* **279**:23158-23165.

Yang JI, Yoon JH, Myung SJ, Gwak GY, Kim W, Chung GE, Lee SH, Lee SM, Kim CY and Lee HS (2007) Bile acid-induced TGR5-dependent c-Jun-N terminal kinase activation leads to enhanced caspase 8 activation in hepatocytes. *Biochem Biophys Res Commun* **361**:156-161.

Yang Y, Zhang M, Eggertsen G and Chiang JY (2002) On the mechanism of bile acid inhibition of rat sterol 12alpha-hydroxylase gene (CYP8B1) transcription: roles of alpha-fetoprotein transcription factor and hepatocyte nuclear factor 4alpha. *Biochim Biophys Acta* **1583**:63-73.

Yerushalmi B, Dahl R, Devereaux MW, Gumpricht E and Sokol RJ (2001) Bile acid-induced rat hepatocyte apoptosis is inhibited by antioxidants and blockers of the mitochondrial permeability transition. *Hepatology* **33**:616-626.

Yu L, Li-Hawkins J, Hammer RE, Berge KE, Horton JD, Cohen JC and Hobbs HH (2002) Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. *J Clin Invest* **110**:671-680.

Zhang M and Chiang JY (2001) Transcriptional regulation of the human sterol 12alpha-hydroxylase gene (CYP8B1): roles of heaptocyte nuclear factor 4alpha in mediating bile acid repression. *J Biol Chem* **276**:41690-41699.

Zhang Y, Lee FY, Barrera G, Lee H, Vales C, Gonzalez FJ, Willson TM and Edwards PA (2006) Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice. *Proc Natl Acad Sci U S A* **103**:1006-1011.

Zollner G, Fickert P, Fuchsbichler A, Silbert D, Wagner M, Arbeiter S, Gonzalez FJ, Marschall HU, Zatloukal K, Denk H and Trauner M (2003a) Role of nuclear bile acid receptor, FXR, in adaptive ABC transporter regulation by cholic and ursodeoxycholic acid in mouse liver, kidney and intestine. *J Hepatol* **39**:480-488.

Zollner G, Fickert P, Silbert D, Fuchsbichler A, Marschall HU, Zatloukal K, Denk H and Trauner M (2003b) Adaptive changes in hepatobiliary transporter expression in primary biliary cirrhosis. *J Hepatol* **38**:717-727.

Zollner G and Trauner M (2009) Nuclear receptors as therapeutic targets in cholestatic liver diseases. *Br J Pharmacol* **156**:7-27.

APPENDIX

Suppliers

Chemicals

Sigma Aldrich Chemical Company, St, Louis, MO:

CA:

Brand Name: Fluka

Purity: ≥99.0%

CDCA:

Brand Name: Sigma

Purity: ≥95%

DCA:

Brand Name: Fluka,

Purity: ≥99.0%

LCA:

Brand Name: Sigma

Purity: ≥97%

UDCA:

Brand Name: Sigma

Purity: ≥99% (Sigma)