Charles University in Prague

Faculty of Medicine in Hradec Kralove

Ph.D. program

Medical Pharmacology

Effect of epigallocatechin gallate on bile production

Vliv epigalokatechingalátu na tvorbu žluče

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Hradec Kralove, 2012   Defense date:

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October 15, 2012
Rochester, MN, USA

Acknowledgments

I wish to give thanks to all the people who have helped me throughout my Ph.D. study and contributed to this work. I am especially grateful to my Ph.D. advisor, Dr. Stanislav Mičuda, for the excellent guidance throughout my postdoctoral study. I am very thankful for the professional and friendly manner of his advisement during my Ph.D. project and for his great support in my carrier development.

My special thanks goes to Gabriela Karlasová and Hana Laštůvková for their everyday scientific and non-scientific support. I also would like to thank Eva Doleželová, Jolana Cermanová, Zuzana Kadová, Marie Zagórová, Jitka Hájková for their research collaboration and help with this project. I thank Dagmar Ježková, Kateřina, Sildbergová and Lenka Kriesfalusyová for taking care of my beloved animals, and Hana Krupičková, Radomír Hyšpler, Luděk Šispera, Martin Leníček, Petr Pávek and Otto Kučera for their assistance with a analyses.

I give thanks to all other colleagues at the Department of Pharmacology of the Faculty of Medicine in Hradec Kralove for providing frienship and scientic support, by also creating a pleasant working environment and having important coffee breaks. I am also very thankful to my US advisors Prof. James L. Boyer and Prof. Gregory J. Gores and other numerous colleagues from Yale Liver Center and Mayo Clinic, who were always very supportive, encouraging and inspiring.

Last but not least, I wish to express gratitude towards my parents and my whole family for their immense support throughout my studies.

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Abbreviations

22-OH-C	22-(R)-hydroxycholesterol
ABC	ATP-binding cassette
ABCA	ATP-binding cassette transporter sub-family A
ABCB	ATP-binding cassette transporter sub-family B
ABCC	ATP-binding cassette transporter sub-family C
ABCG	ATP-binding cassette transporter sub-family G
ACAT2	Acyl-coenzyme A:cholesterol acyltransferase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BADF	Bile acid-dependent fraction
BAIF	Bile acid-independent fraction
BARE	Bile acid response element
BE	Biliary excretion
BSEP	Bile salt export pump
C4	7α-Hydroxy-4-cholesten-3-one
CAR	Constitutive androstane receptor
CDCA	Chenodeoxycholic acid
CYP27A1	Sterol 27-hydroxylase
CYP7A1	Cholesterol 7α-hydroxylase
CYP7B1	Oxysterol 7α-hydroxylase
CYP8B1	Sterol 12α-hydroxylase
DCA	Deoxycholic acid
DR4	Direct repeat spaced by four nucleotides
DMEM	Dulbecco's modified Eagle's medium
EE	Ethinylestradiol
EGCG	Epigallocatechin gallate
ERK	Extracellular signal-regulated kinase
FASN	Fatty acid synthase
FGF	Fibroblast growth factor

FGFR	Fibroblast growth factor receptor
FXR	Farnesoid X receptor
HDL	High density lipoprotein
HMG-CoA	3-Hydroxy-3-methyl-glutaryl-coenzyme A
HNF	Hepatocyte nuclear factor
HPLC	High performance liquid chromatography
IBABP	Ileal bile acid binding protein
IR1	Inverted repeat spaced by one nucleotide
JNK	Jun N-terminal kinase
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LRH-1	Liver receptor homologue-1
LXR	Liver X receptor
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistance protein
MRP	Multidrug resistance-associated protein
NRF2	Nuclear factor (erythroid-derived 2)-like 2
NTCP	Na^+-taurocholate cotransporting polypeptide
OATP	Organic anion transporting polypeptide
OST	Organic solute transporter
PPAR	Peroxisome proliferator-activated receptor
PXR	Pregnane X receptor
RAR	Retinoic acid receptor
RXR	Retinoid X receptor
SD	Standard deviation
SHP	Small heterodimeric partner
SLC	Solute carrier
SR-B1	Scavenger receptor class B type 1
SREBP	Sterol element-binding protein
VDR	Vitamin D receptor
VLDL	Very low density lipoprotein

Nomenclature note: in this thesis, human gene and protein symbols are all capitalized. Rodent gene and protein symbols are in lower case with an initial capital.

1. Introduction

1.1. Bile production

Bile production represents one of the essential functions of the liver. Firstly, bile plays a vital role in digestion and absorption of lipids in the intestine. Secondly, bile serves for elimination of endogenous compounds and their metabolites, such as bilirubin, cholesterol, and hormones. Moreover, bile also provides an important excretory route for exogenous substances, toxins, drugs and their metabolites (Kuntz and Kuntz, 2008).

Bile components are primarily secreted by hepatocytes into bile canaliculi that eventually merge and form bile ductules. Primary bile is further modified by cholangiocyte secretion and absorption as it passes through the bile ductules and ducts. Bile production is driven by osmotic gradient formed by a continuous secretion of organic solutes from the hepatocytes into the bile canaliculi, followed by passive movement of water. Bile flow has two major components: bile acid-dependent fraction (BADF) and bile acid-independent fraction (BAIF). BADF is mainly based on the vectorial transport of bile acids from the sinusoidal blood to bile. BAIF is mainly generated by biliary secretion of glutathione. Cholangiocyte secretion of bicarbonate also contributes to the bile acid-independent mechanism, although this varies with the species and responses to enteric hormones (Trauner and Boyer, 2003). In humans, approximately 450 mL of primary bile is formed every day by equal contribution of both BADF and BAIF. The volume of hepatic bile is further increased by approximately 150 mL by cholangiocyte active secretion, which results in formation of so-called ductular (hepatic) bile (Kuntz and Kuntz, 2008).

Bile is an isotonic aqueous solution (~97% water) containing bile acids, phospholipids (predominantly phosphatidylcholine), cholesterol, bilirubin, glutathione, proteins, and inorganic ions (Fig. 1). Bile also contains minute amounts of antibodies and hormones. Being an aqueous solution, bile is more suitable for excretion of hydrophilic compounds. However, the presence of micelle forming bile acids above their critical micellar concentration allows solubilization of lipids in bile. Thus, water-soluble as well as lipid-soluble compounds are excreted via bile.
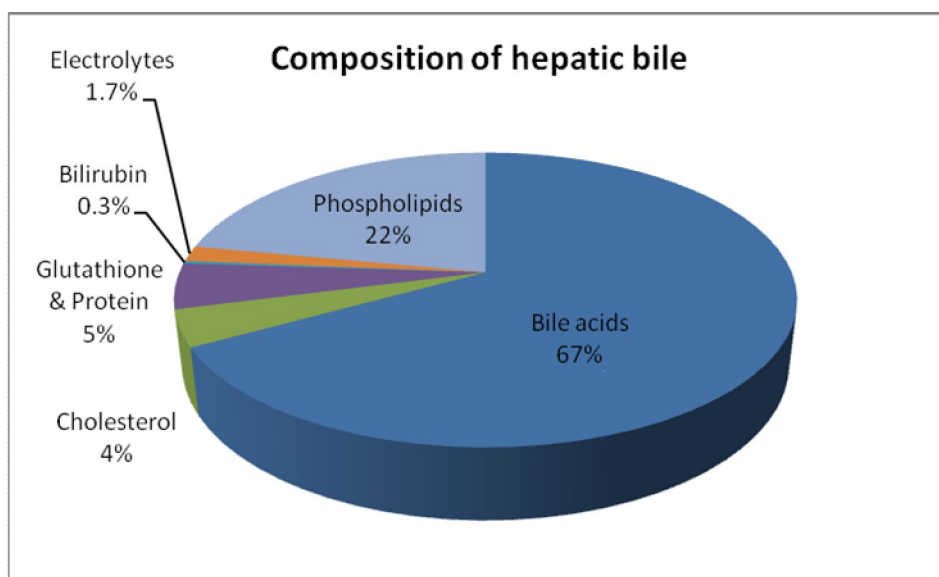
Figure 1. Schematic chart representing composition of human hepatic bile. Adapted from Kuntz and Kuntz (2008).

In human bile, the concentrations of individual constituents range from being slightly higher than in plasma (e.g., electrolytes except for chlorides, which are slightly lower) to being extremely concentrated such as bile acids (2-45 mM) and bilirubin (0.5-2 g/L) (Esteller, 2008). Human bile is also rich in lipids as the concentrations of cholesterol and phospholipids stretch between 0.6-3.2 g/L and 0.25-8.1 g/L, respectively. Bile acids together with cholesterol and phospholipids form mixed micelles in bile and the ratio between these three constituents is important for cholesterol solubility (Fig. 2). Imbalance in this ratio may raise propensity for gallstone formation.
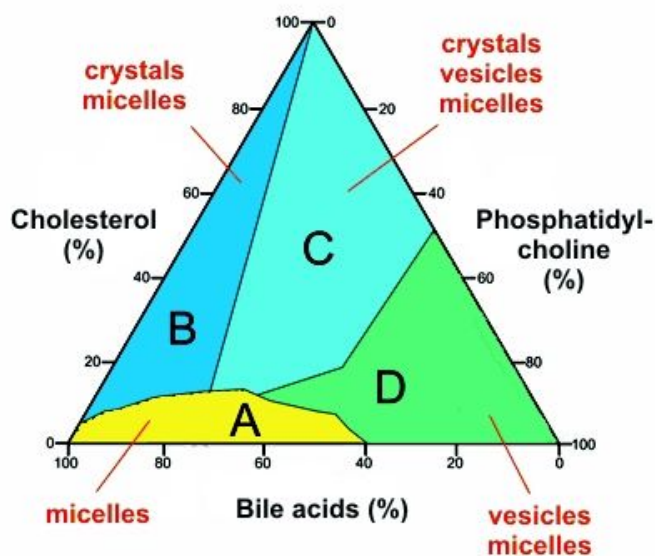
Figure 2. Equilibrium bile acid-phospholipid-cholesterol phase diagram. There are three types of solid particles that can be formed in the bile. These are crystals, micelles and vesicles. Area A is one-phase zone containing micelles only (cholesterol is completely soluble). Area B represents two-phase zone with micelles and crystals of cholesterol. Area C represents three-phase zone containing micelles, vesicles and crystal of cholesterol. Area D represents two-phase zone with micelles and vesicles. The bile components are expressed in mol percent. Adapted from a review article by Venneman and van Erpecum (2010).

1.1.1. Biliary bile acid excretion

Hepatocytes are polarized cells in which three different compartments of plasma membrane can be distinguished based on structural and functional characteristics. About 37% of plasma membrane (sinusoidal membrane) is in direct contact with Disse's space and mediates exchange of compounds between blood and hepatocytes. Bile secretory pole of the hepatocyte is represented by canalicular (apical) membrane, which accounts for 15% of the cell membrane. The remaining 50% of total cell surface forms lateral membrane with specialized structures allowing adhesion (desmosomes and tight-junctions) and communication (gap-junctions) between neighboring hepatocytes (Kuntz and Kuntz, 2008). Hepatocyte sinusoidal membrane together with lateral membrane is often designated as basolateral membrane.

Biliary excretion of bile acids is an active process involving a number of transport systems at both the basolateral and canalicular membranes of hepatocytes. Basolateral transporters are particularly important for bile formation since more than 95% of bile acids secreted into bile undergo enterohepatic recirculation. While unconjugated bile acids can penetrate through plasma membrane by passive diffusion (as weak acids they are

uncharged at physiological pH), bile acids conjugated with glycine or taurine require active transport systems for crossing plasma membrane. Basolateral hepatocyte uptake is directed against a concentration gradient between the portal blood and hepatocyte cytosol and is mediated by either sodium-dependent or sodium-independent mechanisms (Esteller, 2008). Following basolateral uptake, bile acids are transferred to the apical pole and excreted across the apical membrane into canalicular space. Canalicular excretion of bile acids is also an energy-dependent process as it takes place against a high concentration gradient. Because the capacity of bile acid basolateral uptake is 8-10 higher than the secretion rate at the canalicular membrane, the transport across the canalicular membrane is the rate-limiting step in biliary bile acid excretion (Kuntz and Kuntz, 2008).

Although the amount of bile acids secreted in bile correlates with the volume of osmotically secreted water, there is a significant variability in choleretic activity of individual bile acids. In general, monohydroxy bile acids function cholestatically, while dihydroxy and trihydroxy bile acids (see Fig. 3) have a choleretic effect. Some bile acids, especially ursodeoxycholic acid and its nor-derivatives, generate a volume of bile that is higher than it would be expected from their actual osmotic activity. It seems that such hypercholeresis is a result of their early reabsorption in cholangiocytes and immediate re-secretion by hepatocytes. This phenomenon has been termed the cholehepatic shunt pathway (Esteller, 2008).

1.1.1.1. Bile acid synthesis

Although bile acid pool undergoes extensive enterohepatic recirculation, a considerable amount of bile acids is loss through fecal excretion. Thus, *de novo* bile acid synthesis in the liver compensates for this bile acid loss. In humans, about 500 mg of bile acids are synthesized every day (Russell, 2003). Primary bile acids, cholic acid and chenodeoxycholic acid, are synthesized from cholesterol exclusively in the liver via two independent pathways, i.e., classic (neutral) and alternative (acidic) pathway (Fig. 3). The classic and alternative pathways are also designated as neutral and acidic, respectively, since the intermediates are either neutral sterols or carboxylic acids. At least 14 enzymes have been shown to take part in the bile acid synthesis (Chiang, 2009). Among these, cholesterol 7α-hydroxylase (CYP7A1) is a critical enzyme as it catalyzes the first and rate-limiting step of the classic pathway, which accounts for ~90% of bile acid synthesis in humans. CYP7A1 catalyzes hydroxylation of cholesterol into 7α-hydroxycholesterol,

which is then converted into 7α-hydroxy-4-cholesten-3-on (C4). Interestingly, plasma concentration of C4 has been identified as a reliable marker of CYP7A1/Cyp7a1 activity both in humans and laboratory animals (Axelson et al., 1991; Galman et al., 2003). In the liver, C4 is then metabolized into either cholic acid or chenodeoxycholic acid depending on the activity of sterol 12α-hydroxylase (CYP8B1). CYP8B1 activity, which favors cholic acid synthesis, thus determines the ratio between production of cholic acid and chenodeoxycholic acid. The alternative pathway is initiated by sterol 27-hydroxylase (CYP27A1) and leads selectively to the synthesis of chenodeoxycholic acid (Chiang, 2009).

Bile acid synthesis within the liver is completed when primary bile acids are conjugated to taurine or glycine. The conjugation is catalyzed by bile acid:CoA synthase and bile acid:amino acid transferase. Primary bile acids that escape absorption in the small intestine are then converted to secondary bile acids, deoxycholic acid and lithocholic acid. The conversion involves deconjugation and removal of 7α-hydroxyl from the primary bile acids by intestinal bacteria. A certain amount of secondary bile acids is also reabsorbed and returns to the liver, the rest is excreted in feces. Further metabolism of secondary bile acids in the liver or intestine yields tertiary bile acids, such as ursodeoxycholic acid and sulfolithocholic acid (Fig. 3).

Liver

Classic (neutral) pathway

Alternative (acidic) pathway

Intestine

Primary bile acids

Secondary bile acids

Liver + intestine

Tertiary bile acids
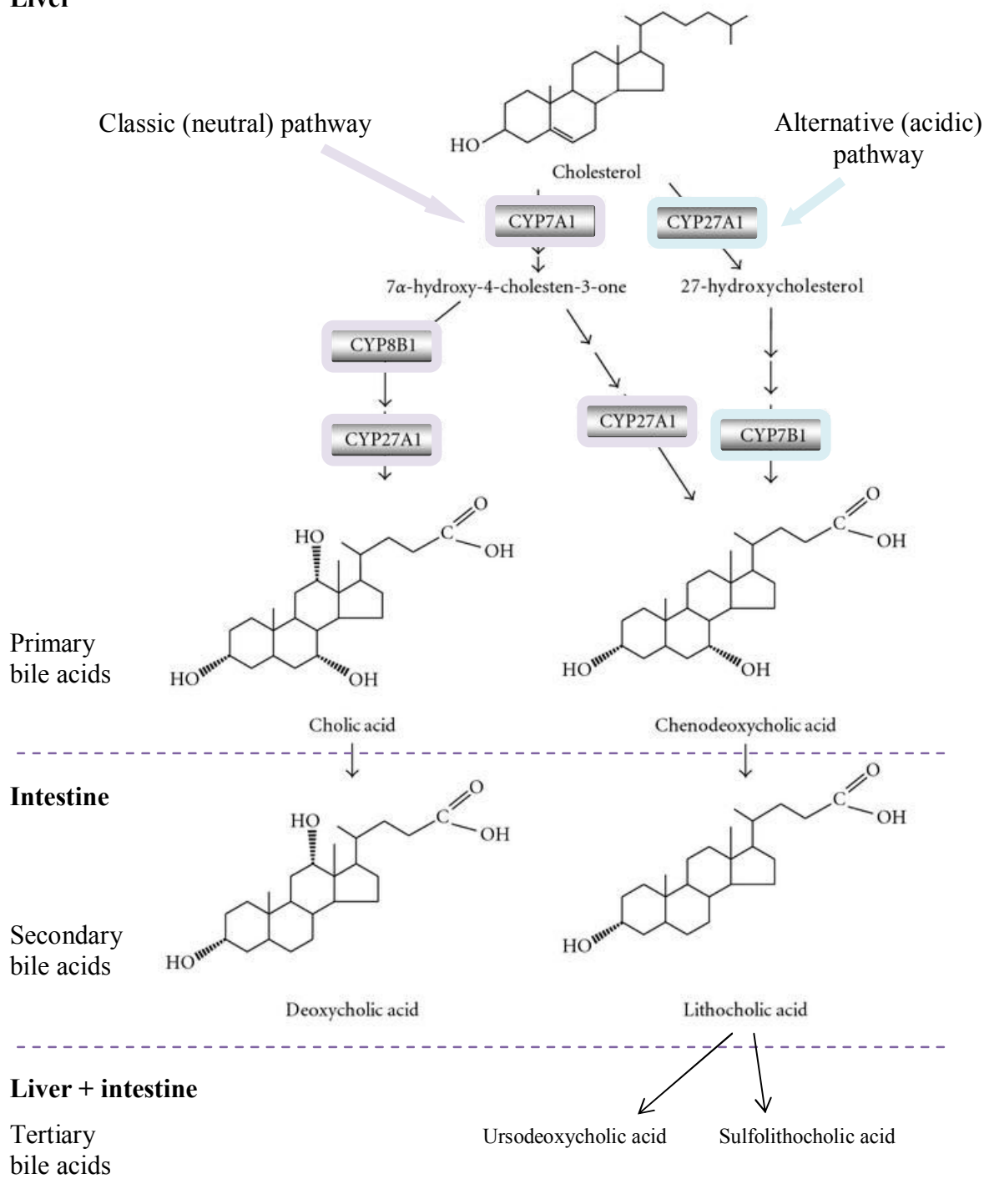
Figure 3. Schematic diagram showing bile acid synthesis. Bile acids are synthesized from cholesterol in the liver via two general pathways: the classic (neutral) pathway and the alternative (acidic) pathway. In humans, bile acid synthesis mainly produces two primary bile acids, cholic acid and chenodeoxycholic acid. Cholesterol 7α-hydroxylase (CYP7A1) catalyzes the first and rate-limiting step in the classic pathway while converting cholesterol into 7α-hydroxycholesterol. Sterol 27-hydroxylase (CYP27A1) initiates the alternative pathway by converting cholesterol into 27-hydroxycholesterol, which is then hydroxylated at 7α-position by oxysterol 7α-hydroxylase (CYP7B1). In the intestine, cholic acid and chenodeoxycholic acid are dehydroxylated at the 7α-position by bacterial enzymes to produce deoxycholic acid and lithocholic acid, respectively (secondary bile acids). Secondary bile acids can be transformed by further metabolism into tertiary bile acids, such as ursodeoxycholic acid and sulfolithocholic acid. Adapted from Chiang (2009) and Kuntz and Kuntz (2008).
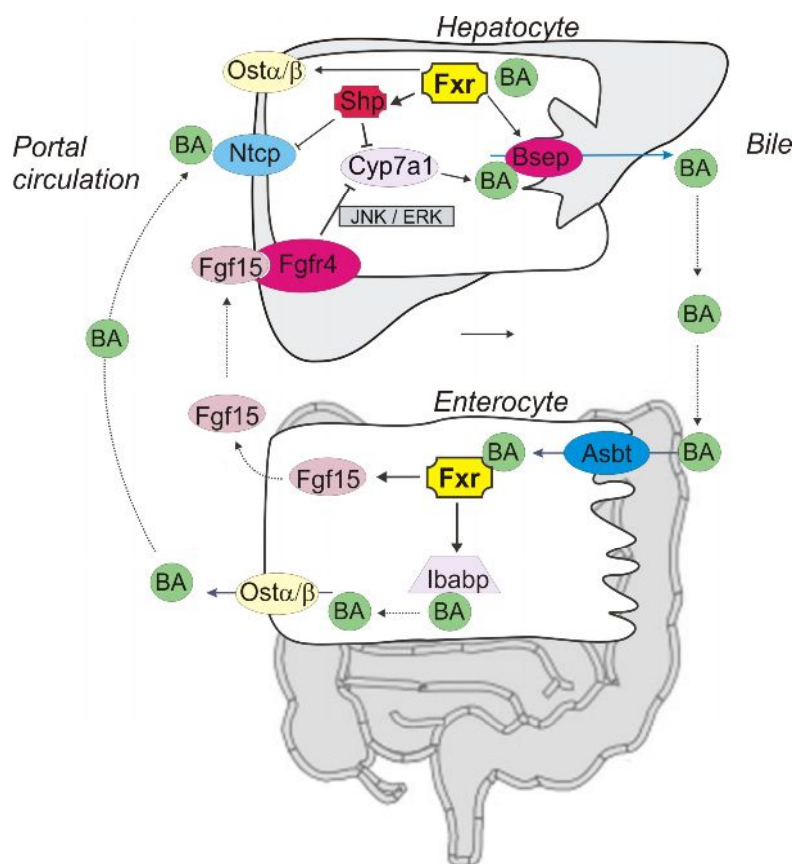
Regulation of bile acid synthesis

Bile acid synthesis in the liver is very tightly regulated since imbalance in bile acid homeostasis leads to a variety of hepatobiliary and intestinal disorders, such as cholestasis, gallstone formation, inflammatory bowel disease, etc. (Zhu et al., 2011). Bile acid synthesis is mainly regulated by feedback suppression of the key enzyme CYP7A1 by nuclear receptors and inflammatory cytokines.

Under normal conditions, activation of farnesoid X receptor (FXR) is the main mechanism of suppression of bile acid synthesis (Fig. 4). FXR functions as a sensor for intracellular levels of bile acids, which are endogenous ligands of this receptor. Especially hydrophobic bile acids have a potent FXR agonistic activity (Zhu et al., 2011). Both free and conjugated bile acids bind to the ligand binding domain of FXR, which in turn forms a heterodimer with retinoid X receptor (RXR). This FXR/RXR complex then binds to the inverted repeat of AGGTCA-like sequence with one nucleotide spacing (IR1) located in the promoter regions of FXR target genes to initiate gene transcription. To suppress CYP7A1 gene expression, activated FRX induces expression of small heterodimeric partner (SHP), which in turn inhibits activity of hepatocyte nuclear factor-4 alpha (HNF4α) and liver receptor homologue-1 (LRH-1), which mediate CYP7A1 transcription (Chiang, 2009).

Experiments with Shp knockout mice have pointed out existence of Shp-independent repression of Cyp7a1 by bile acids (Kerr et al., 2002). Bile acid-induced secretion of pro-inflammatory cytokines stimulates mitogen-activated protein kinase (MAPK) cascade leading to activation of JNK (Jun N-terminal kinase), which may inhibit HNF-4α activity. In addition, pregnane X receptor (PXR) and vitamin D receptor (VDR) may also suppress CYP7A1 transcription by Shp-independent mechanism (Chiang, 2009).

Gene expression of CYP7A1 is further modulated by fibroblast growth factor 19 (FGF19) or its orthologue Fgf15 in rodents. FGF19/Fgf15 is produced in the intestine following FXR activation by bile acids and is secreted into the portal blood to regulate bile acid synthesis in the liver (Inagaki et al., 2005). Released Fgf15 binds to the liver-specific receptor Fgfr4 (fibroblast growth factor receptor 4) and activate signaling pathways such as JNK or ERK (extracellular signal-regulated kinase). It has been shown that intestinal Fxr/Fgf15/Fgfr4 pathway plays a critical role in Cyp7a1 gene suppression in mice compared to hepatic Fxr/Shp pathway, which had minor effect on Cyp7a1 (Kong et al., 2012). In addition, human (but not mouse) hepatocytes are also able to express and secrete FGF19, which exerts its effect on CYP7A1 transcription by an autocrine or paracrine

mechanism (Song et al., 2009).

Figure 4. Regulation of bile acid homeostasis by farnesoid X receptor (Fxr). In the ileum, bile acids are reabsorbed by the activity of apical sodium-dependent bile acid transporter (Asbt) located to the brush border membrane. Under physiological conditions, bile acids within enterocytes activate Fxr that induces production of intestinal hormone fibroblast growth factor 15 (Fgf15). Fgf15 travels via the portal blood to the liver where it activates Fgfr4 (fibroblast growth factor receptor 4). Fgf15 binding to Fgfr4 activates signaling cascades such as Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), which then suppress the transcription of cholesterol 7α-hydroxylase (Cyp7a1) gene. Fxr also induces bile acid transport in both the liver and intestine. In hepatocytes, Fxr stimulates expression of bile salt export pump (Bsep), the main canalicular bile acid transporter. In enterocytes, Fxr induces expression of ileal bile acid binding protein (Ibabp) and organic solute transporter (Ost) α and β, which promotes enterohepatic recirculation of bile acids. Under cholestatic conditions, increased bile acids in the liver activate Fxr as well as other signaling pathways. Liver Fxr activation triggers transcription of small heterodimeric partner (Shp), which, in turn, represses transcription of Cyp7a1 and Na$^+$-taurocholate cotransporting polypeptide (Ntcp). At the same time, activated Fxr induces Ostα/β to promote bile acid efflux from the hepatocytes to the sinusoidal blood. Adapted from Zhu et al. (2011).

In rodents, but not in humans, Cyp7a1 expression can be induced by activation of liver X receptor α (Lxrα) (Goodwin et al., 2003). Once activated, Lxrα binds to a putative bile acid response element (BARE-I), which contains a DR4 (direct repeat spaced by four nucleotides) motif. DR4 motif Cyp7a1 promoter is conserved in many species but not in humans. Thus, in contrast to rodents, human CYP7A1 gene promoter is not affected by

LXRα activation (Chen et al., 2002). Increased bile acid synthesis via Lxrα-mediated Cyp7a1 induction is thought to be the reason for the relative resistance of rodents to develop hypercholesterolemia in models of high cholesterol diet. In contrast, Lxrα knockout mice fed with a cholesterol-enriched diet do not increase bile acid synthesis and have high liver cholesterol levels compared to wild-types (Peet et al., 1998).

Expression of CYP8B1 is also regulated by bile acid-activated FXR. Mouse Cyp8b1 can be downregulated by both liver Fxr/Shp and intestinal Fxr/Fgf15/Fgfr4 pathways (Kong et al., 2012). CYP8B1 promoter region also contains sterol response element and is thus regulated by sterol element-binding proteins (SREBP). SREBP1 increases CYP8B1 transcription and, on the contrary, CYP8B1 gene expression is suppressed by SREBP2 (Norlin and Wikvall, 2007).

1.1.1.2. Hepatic bile acid transporters

Function of hepatic bile acid transporters is crucial for biliary bile acid excretion. Na^+-taurocholate cotransporting polypeptide (NTCP) mediates sodium-dependent uptake of bile acids from the sinusoidal blood into hepatocytes. Sodium-independent basolateral uptake of bile acids is executed by transporters from organic anion transporting polypeptide (OATP) family. All NTCP and OATPs belong to solute carrier (SLC) family of transporting proteins. Alternative bile acid efflux from hepatocytes back to blood is mediated by organic solute transporter alpha and beta (OSTα/β) and transporters from multidrug resistance-associated protein (MRP) family. Canalicular bile salt export pump (BSEP) is the major transporter for bile acid excretion into bile (Fig. 5).
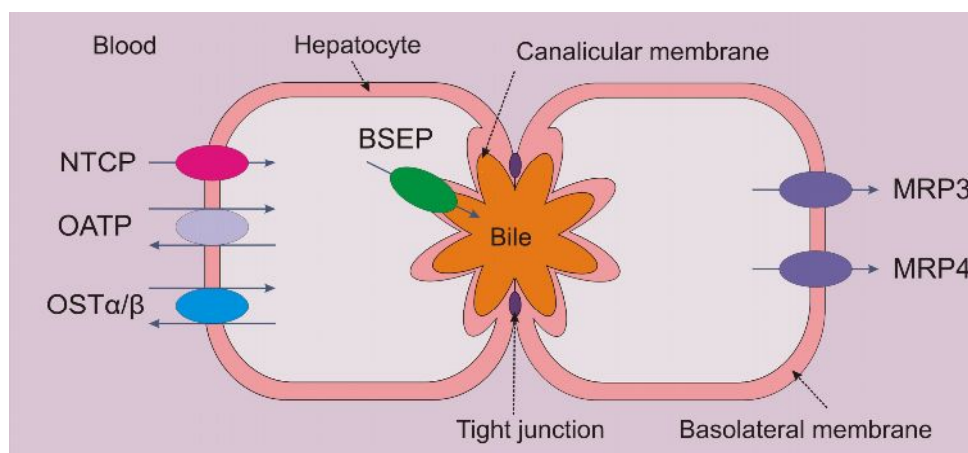
Figure 5. Schematic diagram showing localization of the main bile acid transporters in the hepatocytes. NTCP, Na^{+}-taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; OSTα/β, organic solute transporter α/β; BSEP, bile salt export pump; MRP, multidrug resistance-associated protein.

Na^{+}-taurocholate cotransporting polypeptide

First and foremost, transporter NTCP (encoded by SLC10A1 gene) mediates basolateral uptake of conjugated bile acids. With a lower affinity, it also transports unconjugated bile acids. For example, NTCP-mediated transport accounts for more than 80% of taurocholate uptake, but less than 50% of cholate transport (Trauner and Boyer, 2003). Similarly, NTCP has a higher affinity to dihydroxy bile acids than to trihydroxy ones (Stieger, 2011). NTCP utilizes a sodium gradient as a driving force for the transport of bile acids, which is basically a cotransport of two sodium ions with one bile acid molecule (Hagenbuch and Meier, 1996). The spectrum of NTCP substrates comprises both endogenous compounds and xenobiotics, e.g., bromosulfophtalein, dehydroepiandrosterone, estrone-3-sulfate, triiodo-thyronine, thyroxine, sulindac, and rosuvastatin (Stieger, 2011).

Organic anion transporting polypeptides

OATPs (encoded by SLC genes sub-family 21) represent a large group of transporters with an important role especially in drug transport. In contrast to NTCP, the transport activity of OATPs is sodium-independent. Specifically, the bidirectional transport of their substrates is driven by an antiport of bicarbonate, glutathione or glutathione conjugates (Konig, 2011). The substrate specificity of OATPs is rather broad and embraces a variety of organic anions and steroid hormones (Konig, 2011). Regarding bile acids, transporters OATP1A2, OATP1B1, OATP1B3 participate in basolateral uptake in humans; in rodents,

bile acid uptake is mediated by Oatp1a1, Oatp1a4, Oatp1b2 (Hagenbuch and Meier, 2004).

Multidrug resistance-associated proteins

These transporters belong to the family of ATP-binding cassette (ABC) transporters, specifically to ABCC sub-family. MRP1 (encoded by ABCC1 gene), MRP3 (ABCC3), MRP4 (ABCC4), MRP5 (ABCC5) and MRP6 (ABCC6) are localized to the hepatocyte basolateral membrane and their major role is to efflux endogenous compounds and xenobiotics from hepatocytes into the sinusoidal blood. With high affinity, MRP1 and MRP3 transport divalent bile acids, e.g., sulfated taurolithocholate and taurodeoxycholate. Mrp3 in rodents may also transport monovalent bile acids such as taurocholate and glycocholate (Keppler, 2011). Expression of MRP3 or MRP4 is low under normal circumstances but rises in cholestasis or MRP2-deficiency. Their increased expression and subsequently increased bile acid efflux into blood represent a compensatory mechanism for defective biliary excretion of bile acids and conjugated bilirubin, which otherwise would accumulate within the hepatocytes (Zollner and Trauner, 2008). From the whole ABCC sub-family, the only MRP2 is localized to the hepatocyte canalicular membrane. MRP2 transports compounds such as organic anions, glutathione, bile acids and conjugates with glutathione, glucuronide and sulfate, as well as unconjugated drugs (methotrexate, pravastatin, azithromycin) (Keppler, 2011). In the liver, MRP2 provides one of two basic mechanisms of bile production, i.e., BAIF of bile flow. The importance of its function is documented by significantly decreased bile production in Mrp2-deficient rats (Ito et al., 1997; Paulusma et al., 1999) or Mrp2-null mice (Chu et al., 2006). In humans, MRP2 deficiency manifests conjugated hyperbilirubinemia known as Dubin-Johnson syndrome (Konig et al., 1999). In these patients, MRP3 protein expression is induced to compensate for the low biliary excretion of organic anions (Zollner and Trauner, 2008).

Organic solute transporters

Heterodimeric complex of Ostα and Ostβ is localized to the basolateral membrane of hepatocytes. Both subunits are required for plasma membrane localization of the functional transporter (Soroka et al., 2008). Apart from bile acids, substrates of Ostα/β include estrone-3-sulfate, dehydroepiandrosteron-3-sulfate, dioxin or prostaglandin E2. Tissue expression of Ostα/β differs considerably among the species. In mice, these transporters are barely expressed in the liver, but are abundant in the intestine. Conversely, humans have higher expression of OSTα/β in the liver than in the intestine (Soroka et al., 2010a).

Ostα/β is normally expressed at rather low levels but its expression is strongly induced during cholestatic injury both in humans and rodents. Alternative bile acid efflux by Ostα/β, similarly to MRP3 and MRP4, helps divert bile acids towards renal excretion under cholestatic conditions (Zollner and Trauner, 2008). Interestingly, Ostα/β-deficient mice are protected against cholestasis since the lack of these transporters in the ileum and renal proximal tubules increases bile acid elimination into feces and urine, respectively (Soroka et al., 2010b; Soroka et al., 2011).

Bile salt export pump

ATP-dependent BSEP (encoded by ABCB11 gene) is the major canalicular bile acid transporter which exports bile acids from hepatocytes into bile canaliculi. BSEP transport activity is the rate limiting-step of biliary bile acid excretion and thus it is primary responsible for BADF of bile flow (Stieger, 2011). In addition, BSEP mediates biliary excretion of several drugs, e.g., pravastatin, sulindac, and vinblastine (Funk, 2008). Estrogen metabolites indirectly decrease BSEP activity by trans-inhibition, i.e., they block BSEP from the canalicular lumen side after their excretion into bile (Stieger, 2011). Mutation in the gene encoding BSEP causes progressive familial intrahepatic cholestasis type 2. Other clinically silent mutations can predispose women to develop intrahepatic cholestasis of pregnancy (Stieger, 2010).

Regulation of bile acid transporters

Bile acid transporters can be regulated at the level of expression (transcriptional and post-translational regulation) as well as at the level of transport activity. Regarding the gene expression, all of above mentioned bile acid transporters are mainly regulated by the class II sub-family of the nuclear receptor superfamily. For target gene regulation, these nuclear receptors form a heterodimer with the common binding partner RXR. Their complex then binds to corresponding nuclear receptor response elements in promoters of target genes. Until now, six nuclear receptors from class II have been shown to regulate gene expression of bile acid transporters: FXR, retinoic acid receptor (RAR), PXR, peroxisome proliferator-activated receptor alpha (PPRAα), constitutive androstane receptor (CAR), LXRα, and the common partner RXR (Trauner et al., 2011). Other nuclear receptors involved in bile acid transporter gene regulation are SHP, HNF4α, and LRH-1 (Kosters and Karpen, 2008).

From all these nuclear receptors, FXR plays the key role in regulation of bile acid transporters (Fig. 6). In rats, Fxr activation indirectly suppresses Ntcp transcription via Shp, which interferes with basal Ntcp expression regulated by Rar/Rxr (Kosters and Karpen, 2008). Shp can reduce Ntcp expression also via repression of Hnf4α and Hnf1α activity. In contrast, human NTCP promoter does not contain RAR/RXR and HNF4α response elements. In both humans and rodents, bile acid-activated FXR induces gene expression of BSEP and OSTα/β. MRP2 expression is also positively regulated by FXR.

Bile acids also activate PXR, CAR and VDR. These nuclear receptors play important roles in detoxification of bile acids, drugs, and xenobiotics. PXR and CAR have been shown to induce expression of MRP2/Mrp2 and MRP3/Mrp3. In addition, CAR also positively regulates expression of MRP4/Mrp4 and VDR induces Mrp3 (Wagner et al., 2010). Nuclear receptors do not exert their effects separately and the final effect on transporter gene expression is a result of a complex cross-talk between several nuclear receptors (Fig 6).
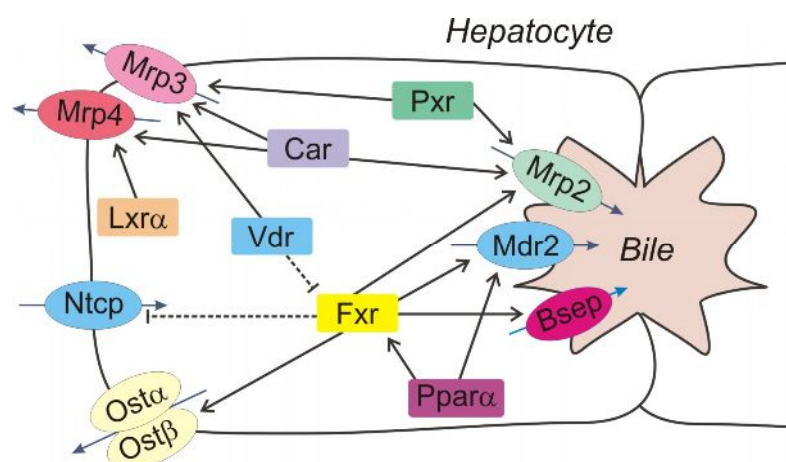
Figure 6. Gene regulation of hepatic bile acid transporters by nuclear receptors. Arrows represent stimulatory effect, while dotted lines indicate suppressive effects on the gene expression. Bsep, bile salt export pump; Car, constitutive androstane receptor; Fxr, farnesoid X receptor; Lxr, liver X receptor; Mdr, multidrug resistance protein; Mrp, multidrug resistance-associated protein; Ntcp, Na^{+}-taurocholate cotransporting polypeptide; OST, organic solute transporter; Ppar, peroxisome proliferator-activated receptor; Pxr, pregnane X receptor; Vdr, vitamin D receptor. Adapted from Zollner and Trauner (2009)

At the level of protein expression, bile acid transporters can be regulated by phosphorylation/dephosphorylation or altered plasma membrane insertion and endocytic retrieval. For example, increased cyclic adenosine monophosphate has been shown to stimulate Ntcp and Bsep insertion into the basolateral and canalicular membrane,

respectively (Kosters and Karpen, 2008).

Last but not least, a variety of compounds directly modulate the transport activity of bile acid transporters. Cyclosporine A, for instance, inhibits transport of both Ntcp and Bsep (Kosters and Karpen, 2008). Another classic example is trans-inhibition of Bsep activity by estrogens, as mentioned above (Stieger, 2011).

1.1.2. Biliary glutathione excretion

Biliary glutathione excretion represents an important mechanism of bile formation while it constitutes BAIF of bile flow. Glutathione, both in its reduced and oxidized form, is excreted into bile via MRP2/Mrp2 in humans and rodents (Fig. 7). The critical role of Mrp2 in biliary glutathione excretion was first shown in Mrp2-mutant rats (TR⁻ and EHBR rats), which presented dramatically decreased biliary glutathione output and bile flow compared to wild-type counterparts (Jemnitz et al., 2010). In Mrp2-deficient mice, biliary glutathione output is nearly abolished (~6% of wild-type littermates) and bile flow is ~25% lower than in wild-type mice (Chu et al., 2006).

1.1.2.1. Regulation of biliary glutathione excretion

Expression of MRP2 is known to be regulated at both transcriptional and post-transcriptional levels. In humans and rodents, it is mainly FXR that takes control over MRP2 transcription. Additionally, MRP2 transcription is positively regulated by activation of CAR and PXR. Response elements of all the above-mentioned nuclear receptors have been identified within the promoter region of MRP2 gene (Nies and Keppler, 2007). The post-translational regulation of MRP2 involves both increased insertion of the protein into the canalicular membrane as well as endocytic retrieval to intracellular pools (Nies and Keppler, 2007). For instance, clofibrate (Johnson and Klaassen, 2002) and ethinylestradiol (Trauner et al., 1997) treatments in rats decrease Mrp2 protein without affecting Mrp2 mRNA levels.
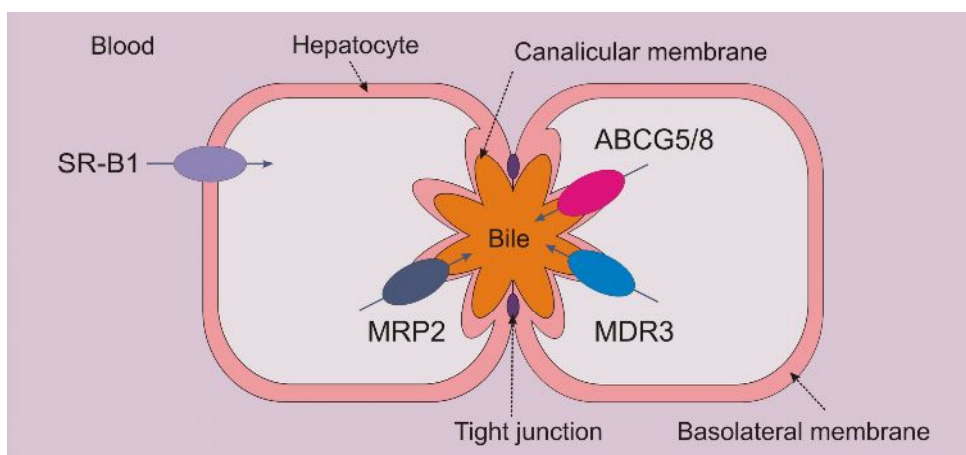
Figure 7. Schematic diagram showing localization of hepatic transporters for glutathione, cholesterol and phospholipids. MRP, multidrug resistance-associated protein; MDR, multidrug resistance protein; ABCG, ATP-binding cassette sub-family G, SR-B1, scavenger receptor class B type 1.

1.1.3. Biliary cholesterol excretion

The whole body cholesterol homeostasis is regulated at several levels: cholesterol *de novo* synthesis, hepatic catabolism (i.e., bile acid synthesis), intestinal absorption, and biliary excretion. Under normal circumstances, the amount of cholesterol excreted into bile is equal to the amount of cholesterol synthesized in the liver and absorbed from the intestine (Yamanashi et al., 2011), which points towards the importance of biliary cholesterol excretion.

Biliary cholesterol output is closely tied to biliary excretion of bile acids. Biliary cholesterol excretion is decreased in the absence of bile acids and, conversely, it is stimulated after bile acid administration (Oude Elferink et al., 1996). Further, expression and function of ATP-binding cassette transporters sub-family G (ABCG), multidrug resistance protein 3 (MDR3) and scavenger receptor class B type 1 (SR-B1) are essential for cholesterol excretion into bile (Fig. 7).

1.1.3.1. Hepatic cholesterol transporters

ATP-binding cassette transporters sub-family G member 5 and 8
At the hepatocyte canalicular domain, ABCG5 and ABCG8 form a heterodimeric complex that transports free cholesterol into bile canaliculi. ABCG5/8 complex is also expressed at the apical membrane of enterocytes and participates in intestinal cholesterol absorption

(Dikkers and Tietge, 2010). The critical role of ABCG5/8 in biliary cholesterol excretion has been illustrated by generation of Abcg5/8-deficient mice. Abcg5/8-null mice have biliary cholesterol output reduced by 80% compared to their littermates (Yu et al., 2002a). On the contrary, human ABCG5/8 transgenic mice have significantly enhanced cholesterol excretion into bile, which correlates with the inserted gene copy number and mRNA levels of ABCG5/8 in the liver of these mice (Yu et al., 2002b).

Scavenger receptor class B type 1

SR-B1 is a selective high density lipoprotein (HDL) receptor at the basolateral membrane of hepatocytes. It facilitates uptake of esterified cholesterol, which then becomes the main source of cholesterol excreted into bile (Dikkers and Tietge, 2010). Accordingly, biliary cholesterol excretion was decreased to nearly ~50% in mice lacking Sr-b1 (Wiersma et al., 2009). Conversely, hepatic Sr-b1 overexpression enhanced biliary cholesterol output in these transgenic mice (Kozarsky et al., 1997).

1.1.3.2. Regulation of biliary cholesterol excretion

Nuclear receptor LXRα plays a key role in regulation of biliary cholesterol excretion. Endogenous ligands of LXRα are oxidized derivatives of cholesterol (oxysterols). Ligand-activated LXRα forms a heterodimeric complex with RXR and then binds to LXR response elements within target gene promoters. In the liver, activation of LXRα leads to induction of Abcg5 and Abcg8 with a consequently increased biliary cholesterol excretion (Repa et al., 2002). Mice treated with LXRα agonist (T0901317) or fed with high cholesterol diet showed upregulation of Abcg5/8 and significantly increased biliary cholesterol output (Plosch et al., 2002). Abcg5/8 transcription is also positively regulated by Lrh-1 and Hnf4α (Freeman et al., 2004; Sumi et al., 2007).

1.1.4. Biliary phospholipid excretion

MDR3 (or its orthologue Mdr2 in rodents) functions as a flippase of phosphatidylcholine from the inner to the outer leaflet of hepatocyte canalicular membrane, where phosphatidylcholine is extracted by bile acids and excreted in bile (Fig. 7). Mdr2 knockout mice not only lack biliary phosphatidylcholine excretion (Smit et al., 1993), but also

present significantly decreased biliary excretion of cholesterol (Oude Elferink et al., 1996). It has been shown that at least minimal amount of phospholipids is needed for normal cholesterol excretion into bile (Smith et al., 1998).

In humans, mutation in MDR3 gene leads to progressive familial intrahepatic cholestasis type 1 (Oude Elferink et al., 2006). Heterozygous mutation in MDR3 can predispose for a variety of liver disorders, such as biliary cirrhosis, drug-induced cholestasis or cholestasis of pregnancy (Oude Elferink and Beuers, 2011).

1.1.4.1. Regulation of biliary phospholipid excretion

The notion that MDR3 is regulated by FXR arose from the observations in which hydrophobic, but not hydrophilic, bile acids induced gene expression of Mdr2/MDR3 both in rodents (Frijters et al., 1997; Gupta et al., 2000) and human hepatocytes (Jung et al., 2007). Indeed, FXR response element was identified in MDR3/Mdr2 gene promoter and expression of the transporter was inducible by treatment with a FXR agonist such as GW4064 (Huang et al., 2003).

1.1.5. Biliary paracellular transport

Bile components can enter bile canaliculi not only by hepatocyte excretion but also by paracellular pathway. As mentioned above, there are specialized structures between adjacent hepatocytes (desmosomes, tight-junctions) to seal the bile canaliculi and to prevent bile leakage into blood. In particular, tight junctions play a central role in regulating the movement of solutes, ions and water through the paracellular spaces between neighboring hepatocytes and forming thus so-called blood-biliary barrier. Impairment of the blood-biliary barrier function increases paracellular movement of solutes and causes regurgitation of fluid and electrolytes from bile canaliculi into the blood (Rahner et al., 1996). This, in turn, can decrease bile flow, as exemplified in estrogen-treated rats (Elias et al., 1983; Kan et al., 1989). Hence, the proper function of blood-biliary barrier is considered to play an important role in bile production.

1.1.6. Drugs and bile formation

A great variety of drugs have been shown to affect bile formation in the sense of both its decrease (cholestatic effect) and stimulation (choleretic effect). Drugs can trigger cholestasis by inhibiting hepatic transporter expression and function or, rarely, by inducing the vanishing bile duct syndrome. In addition, several drugs can induce cholestatic hepatitis through inflammation and hepatocyte injury. The spectrum of drugs with potential to induce cholestasis in humans is wide and comprises distinct drug classes such as oral contraceptives, penicillins, macrolide antibiotics, fluroquinolones, neuroleptics, nonsteroidal anti-inflammatory drugs and oral antidiabetics (Lindor, 2008). Regarding the underlying mechanisms of the cholestatic effect, many cases of drug-induced cholestasis are associated with a functional inhibition of bile acid transporters by the drug itself or its metabolites. For example, cyclosporine A, rifampicin, bosentan, troglitazone and glibenclamide inhibit transport activity of BSEP in dose-dependent manner. Estrogen and progesterone metabolites have been shown to trans-inhibit BSEP function after their secretion into bile via MRP2. Furthermore, bile production can be also reduced as a result of decreased expression of the major hepatobiliary transporters. This way estrogen metabolites decrease bile flow through diminished expression of Mrp2. In addition, genetic variations in transporter genes can also predispose to drug-induced cholestasis. BSEP and MDR3 gene polymorphisms and mutations have recently been described in drug-induced cholestasis (Padda et al., 2011).

Besides drugs, several natural compounds and toxins can also induce cholestasis. Alpha-naphtylisothiocyanate administration to rats causes chronic cholestatic injury characterized by bile duct injury and proliferation. Endotoxin administration to rodents decreases both BADF and BAIF of bile flow through reduced expression or redistribution of the main bile acid transporters such as Bsep, Ntcp, Oatp and Mrp2 (Rodriguez-Garay, 2003). Further, number of case reports described cholestasis induced by herbal supplements and infusions. These herbal preparations include *Rhamnus Purshiana* bark, Chinese traditional herb *Lycopodium Serrated*, chaparral leaf, germander extract, comfrey, grater celandine, and kava root (Lindor, 2008).

On the other side, several drugs and natural compounds have a choleretic effect and promote bile formation. First of all, dihydroxy (chenodeoxycholic, deoxycholic acid, ursodeoxycholic acid) and trihydroxy (cholic acid) bile acids exert choleretic effect due to enhanced biliary bile acid excretion. Some unconjugated dihydroxy acid actually induces

hypercholeresis, i.e., a choleresis that exceeds predicted values based on the osmotic action of the secreted bile acid. Hypercholeresis induced by ursodeoxycholic acid or nor-ursodeoxycholic acid has been attributed to the cholehepatic shunt mechanism, where the bile acids after their biliary excretion are being reabsorbed by cholangiocytes, returned to the hepatocyte, and resecreted into bile. By multiple mechanisms, ursodeoxycholic acid also stimulates expression and function of bile acid transporters and enzymes in bile acid synthesis and detoxification pathways. For instance, ursodeoxycholic acid promotes insertion of BSEP and MRP2 into canalicular membrane (Zollner and Trauner, 2008). Heretofore, ursodeoxycholic acid is the only approved drug for treatment of cholestatic liver diseases.

Spironolactone and benzylpenicillin have shown capacity to increase BAIF of bile flow. Spironolactone administration to rats increased biliary glutathione and bicarbonate secretion, which was associated with increased Mrp2 expression (Ruiz et al., 2005). In ethinylestradiol-treated rats, spironolactone restored ethinylestradiol-decreased biliary glutathione secretion and bile flow while increasing Mrp2 protein expression (Ruiz et al., 2007). Similarly, benzylpenicillin administration to rats also enhanced bile flow and biliary glutathione output in Mrp2-dependent manner (Ito et al., 2004; Fukami et al., 2011).

Several herbal choleretic drugs are known from traditional medicine. Cynarine isolated from artichoke (*Cynara scolymus*), curcumin (*Curcuma longa*) and liquiritigenin (*Glycyrrhiza glabra*) are known for their choleretic activity (Speroni et al., 2003). Curcumin has been shown to increase bile flow during cyclosporine-induced cholestasis in rats and to enhance both bile flow and biliary glutathione excretion in Mdr2 knockout mice (Deters et al., 1999; Baghdasaryan et al., 2010). Liquiritigenin, a flavonoid from liquorice, induced bile flow in rats through both increase in BADF and BAIF (Kim et al., 2009). Many Chinese traditional herbal medicines are known for their choleretic effect (Sasaki et al., 1990). Among these, genipin, the main active component of Inchin-ko-to, has been proved to significantly enhance Mrp2-mediated bile flow in rats (Shoda et al., 2004; Goto and Takikawa, 2010).

1.2. Epigallocatechin gallate (EGCG)

For centuries, green tea has been one of the most popular beverages worldwide. Regular consumption of green tea has been associated with a variety of health benefits. These purported preventive and therapeutic effects have recently attracted a significant attention from medical research. Currently, a great amount of research has focused on the role of green tea intake in prevention of cancer and cardiovascular disease (Yang et al., 2009; Hodgson and Croft, 2010).

Green tea is very rich in flavonoid compounds. Catechins, which represent the vast majority of these flavonoids, have been suggested to be responsible for the health promoting effects of the plant. These catechins make up 30% of the dry weight of green tea leaves and include EGCG, epigallocatechin, epicatechin gallate, and epicatechin (Wolfram et al., 2006). EGCG is the most abundant of these compounds (>50% of catechin content) and is generally considered to be the major active component of green tea (Nagle et al., 2006; Wolfram et al., 2006). A cup of green tea may contain 100–200 mg of EGCG (Zaveri, 2006). EGCG is currently a widely used dietary supplement available in numerous products either in purified form or as a part of tea or tea extract. In the weight reduction supplements, a daily dose of EGCG ranges between 115-270 mg.
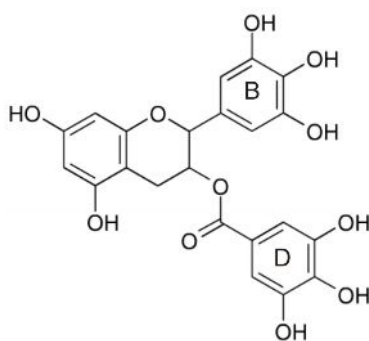
Figure 8. Chemical structure of EGCG.

EGCG has demonstrated a wide range of health benefits, which are often related to its potent antioxidant, anti-inflammatory and anticancer activities (Singh et al., 2011). EGCG has ability to chelate metal ions and scavenge free oxygen and nitrogen radicals. It has these properties due to the presence of phenolic groups in rings B and D of its structure

(see Fig. 8) (Zaveri, 2006). EGCG can exert antioxidant activity also indirectly via inhibition of redox-sensitive transcription factors such as nuclear factor- κB and activator protein-1 (Zaveri, 2006). Further, EGCG has been reported to inhibit pro-oxidative enzymes (lipoxygenase, cyclooxygenase, inducible nitric oxide synthase, xanthine oxidase) and, conversely, induce antioxidative enzymes and phase II biotransformation enzymes (glutathione S-transferase and superoxid dismutase) (Frei and Higdon, 2003). In this induction, transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2) seems to play a pivotal role (Na and Surh, 2008).

1.2.1. EGCG and liver injury

Many experimental studies have shown hepatoprotective action of EGCG in various models of liver injury. EGCG attenuated development of non-alcoholic steatohepatitis (Kuzu et al., 2008), liver injury induced by carbon tetrachloride (Yasuda et al., 2009; Tipoe et al., 2010) or D-galactosamine (Lin et al., 2009) or prevented ischemia/reperfusion-induced liver apoptosis (Giakoustidis et al., 2010). Most of these effects are ascribed to the anti-inflammatory activity of EGCG. Interestingly, two studies have shown protective effect of EGCG against cholestatic liver injury induced by bile duct ligation in mice and rats (Zhong et al., 2003; Kobayashi et al., 2010).

To date, little is known about the effect of EGCG on biliary physiology or bile acid homeostasis. Li et al. (2012) has recently reported a unique feature of EGCG to stimulate Fxr selectively in the mouse intestine and thus increase expression of its target gene Fgf15. Actually, this mechanism has been suggested to protect the liver against cholestasis as it may significantly reduce bile acid synthesis via Fgf15-mediated Cyp7a1 downregulation in the liver (Modica et al., 2012). The opposite effect of EGCG on human CYP7A1 expression was described in human hepatoma (HepG2) cells, where EGCG (5μM) induced CYP7A1mRNA levels by 4-fold (Lee et al., 2008). The authors also showed a direct stimulatory effect of EGCG on human CYP7A1 promoter activity.

In contrast to the hepatoprotective activity, liver toxicity of green tea or EGCG dietary supplements has been reported in humans. Liver injury in the affected individuals ranged from elevated liver transaminase levels to hepatitis and hepatocellular necrosis (Mazzanti et al., 2009). Also several cases of cholestasis have been described in humans after either administration of green tea/green tea extract with high doses of EGCG or

coadministration of green tea with estrogens, which are potentially cholestatic agents (Mazzanti et al., 2009). Cases of green tea-related hepatotoxicity have been comprehensively reviewed elsewhere (Sarma et al., 2008; Stickel et al., 2011).

1.2.2. EGCG and cholesterol metabolism

Positive effect of EGCG on cholesterol metabolism has recently attracted a considerable interest. Epidemiologic studies were the first to draw attention to the hypocholesterolemic activity of green tea when they showed a negative correlation between plasma cholesterol levels and green tea consumption (Kono et al., 1992; Stensvold et al., 1992; Kono et al., 1996; Tokunaga et al., 2002). Later, several clinical trials came to the same conclusion. A recent meta-analysis of 14 randomized controlled trials showed that green tea or its extract significantly decreases plasma total cholesterol and low density lipoprotein (LDL) cholesterol (Zheng et al., 2011). Several animal studies reported that green tea or its extract reduced plasma cholesterol (Murase et al., 2002; Hasegawa et al., 2003; Kobayashi et al., 2005; Bursill et al., 2007; Bursill and Roach, 2007) and liver cholesterol accumulation (Muramatsu et al., 1986; Yang and Koo, 1997; Alshatwi et al., 2011) during high fat or high cholesterol diet.

Pure EGCG also proved its ability to attenuate a diet-induced hypercholesterolemia and cholesterol accumulation in the liver (Raederstorff et al., 2003). EGCG has been shown to interfere with micellar solubilization of cholesterol in the intestine and thus to decrease cholesterol intestinal absorption (Ikeda et al., 1992). Later studies confirmed increased cholesterol fecal excretion in EGCG-treated animals (Raederstorff et al., 2003; Wang et al., 2006). Therefore, it is generally accepted that EGCG exerts its hypocholesterolemic activity through reduced intestinal absorption of cholesterol and its enhanced fecal excretion (Koo and Noh, 2007).

On the other hand, it is quite likely that EGCG exercises its effect on cholesterol metabolism also at another level distinct from intestinal absorption. A growing body of *in vitro* evidence suggests that EGCG may also affect cholesterol metabolism directly in the liver. DNA microarray analysis showed that EGCG treatment altered expression of many genes related to cholesterol metabolism in HepG2 cells (Goto et al., 2011). The most pronounce effect was the upregulation of LDL receptor. Another study demonstrated an increased LDL receptor binding activity and protein mass in HepG2 cells treated with

EGCG (Bursill and Roach, 2006). EGCG was also shown as a potent *in vitro* inhibitor of squalene epoxidase (Abe et al., 2000) and 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase (Cuccioloni et al., 2011), two important enzymes in the cholesterol biosynthetic pathway. Three reports showed that hepatocytes incubated in the presence of EGCG secreted less apolipoprotein B-100 into the medium (Yee et al., 2002; Li et al., 2006; Goto et al., 2011).

1.2.3. Pharmacokinetics of EGCG

Pharmacokinetic parameters in detail were studied in rats (Chen et al., 1997; Lin et al., 2007). After oral administration, EGCG is absorbed in jejunum and ileum (Moore et al., 2009), however, its bioavailability is rather low due to instability, low absorption and rapid metabolism and excretion. Bioavailability of EGCG significantly varies with the species. Only 2–5% of orally administered EGCG is absorbed systematically in rats, which is much lower than the 26% bioavailability in mice (Lambert et al., 2003). First-pass effect of EGCG (13% in rats) likely does not contribute significantly to the generally low bioavailability of the compound (Lee et al., 1995). After intravenous administration, distribution kinetics follows a two-compartment model. EGCG highly binds to plasma proteins (~93%). When EGCG (10 mg/kg) was administered intravenously to rats, biological half-life of the substance was 135 min, clearance and distribution volume were 72.5 mL/min/kg and 22.5 dL/kg, respectively. The highest tissue concentrations were detected in the intestine and kidneys, lower levels were found in the liver and lungs (Chen et al., 1997). EGCG is metabolized in the liver and intestine while undergoing methylation and conjugation with glucuronide and sulfate (Lambert et al., 2003). Besides bioavailability, metabolism of EGCG also differs among the species. Isolated rat liver cytosol has been shown to metabolize EGCG by methylation and sulfation more readily than human and mouse liver cytosol. The opposite is true for conjugation with glucuronide (Lambert et al., 2003). In addition, EGCG is further metabolized by intestinal microflora (Lambert et al., 2007). EGCG is probably excreted exclusively in bile as several studies have not detected its presence in urine (Lee et al., 1995; Chow et al., 2001; Van Amelsvoort et al., 2001; Lee et al., 2002).

Several studies evaluated EGCG kinetics also in humans. After drinking green tea infusion, plasma concentrations of EGCG remain generally below 0.5 μM. Intake of

EGCG-rich dietary supplements resulted in peak plasma levels of 5-7 µM (Yang et al., 2009). Bioavailability of EGCG in humans has not yet been studied due to the lack of an intravenous formulation.

It has been shown that peak plasma concentrations of EGCG increase after repeated administration in humans (Chow et al., 2003). Similarly, peak plasma concentrations of EGCG were approximately five times higher in fasting condition than when administered with food. Intake of EGCG supplements on empty stomach has been also associated with a higher incidence of adverse effects (Chow et al., 2005).

2. Aim of the study

The overall objective of the present Ph.D. thesis was to explore effect of EGCG on bile production in rats under both normal conditions and pathological conditions of ethinylestradiol-induced intrahepatic cholestasis.

The specific aims of the study were:

- To investigate effect of EGCG on bile formation and bile acid homeostasis
- To evaluate effect of EGCG on hepatic cholesterol excretion and metabolism

3. Materials and methods

EGCG and ethinylestradiol were purchased from Cayman Chemical and Sigma-Aldrich, respectively. Rhamnose and melibiose were purchased from Sigma. All other reagents and supplies were obtained as specified in the text.

3.1. Animals and experimental design

All animal experimental protocols were approved by the Animal Research Committee of the Faculty of Medicine in Hradec Kralove, Charles University in Prague. Female Wistar rats (230–250 g) were obtained from Anlab (Prague, Czech Republic). Animals were housed under controlled temperature and light conditions, on a natural 12h light–dark cycle and had free access to water and food throughout the experiments, but were fasted overnight before sacrifice.

Experiment 1
Rats were randomly divided into four groups, namely:

1. Control rats (Ctrl), receiving propylene glycol (vehicle of ethinylestradiol) s.c. for 5 consecutive days and saline (vehicle of EGCG) i.p. for 8 days;

2. EGCG-treated rats, receiving daily propylene glycol s.c. for 5 consecutive days and EGCG (50 mg/kg bw) i.p. for 8 days;

3. Ethinylestradiol-treated rats (EE), which were administered daily with ethinylestradiol (5 mg/kg bw) s.c. for 5 days and saline i.p. for 8 days;

4. EE-EGCG rats, which were coadministered with ethinylestradiol (5 mg/kg bw) s.c. for 5 days and EGCG (50 mg/kg bw) i.p. for 8 days.

Administration of ethinylestradiol (or its vehicle propylene glycol) was started on day 4 of EGCG (or saline) treatment, as illustrated in Figure 9. Bile collection, kinetic study of rhamnose and melibiose and sacrifice were carried out on day 9, i.e. a day after the last EGCG or ethinylestradiol dose.
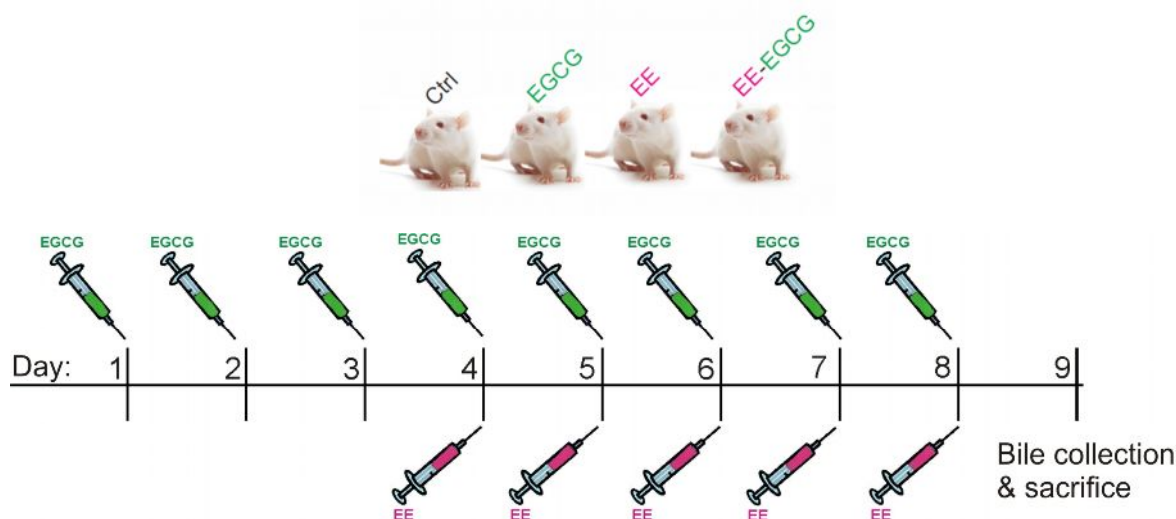
Figure 9. Schematic presentation of the study design. Control rats (Ctrl) received propylene glycol s.c. for 5 consecutive days and saline i.p. for 8 consecutive days. EGCG group received EGCG (50 mg/kg bw) i.p. for 8 days and propylene glycol s.c. injection for 5 consecutive days starting on day 4 of EGCG treatment. EE group received i.p. injections of saline daily for 8 days and EE s.c. injections for 5 consecutive days starting on day 4 of saline treatment. EE-EGCG group received EGCG (50 mg/kg bw) i.p. for 8 days and EE s.c. for 5 consecutive days starting on day 4 of EGCG administration.

If we extrapolate data from Kao et al. (2000), the dose of 50 mg/kg EGCG i.p. to rats may result in plasma concentrations of 1 μM after 1 hour and peak plasma concentrations of 2 μM after 2 hours. The plasma concentration of 1 μM is a similar concentration achievable in humans after either drinking ~8 cups of green tea or ingestion of EGCG-rich extracts (Yang et al., 2009). The intraperitoneal route of the administration may, by bypassing the intestine, secure a direct effect of EGCG on the liver. Moreover, it may compensate for the low and variable bioavailability of EGCG in rats (Lambert et al., 2003).

The bile collection and kinetic study of melibiose and rhamnose was performed on day 9 of the experiment. Rats were introduced to general anesthesia by a single i.p. injection of sodium pentobarbital (50mg/kg bw). The body temperature of rats was maintained at 37°C by placement of the animals on a heating platform. The abdominal cavity was opened and the common bile duct was exposed and cannulated. Thereafter, the right jugular vein was cannulated for melibiose/rhamnose administration and for the continuous infusion of normal saline (2 mL/h) to ensure hydration and to compensate for blood loss due to blood sampling. For blood sampling the left carotid artery was cannulated. After surgical preparations, rats received a single bolus dose of melibiose and rhamnose (100 mg/kg bw). Blood samples (~0.3 mL) were taken at designated time

intervals (–5, 4, 10, 30, 60, 90, 120, 180, and 240 min) after injection of the sugars. Plasma samples were obtained from the whole blood by centrifugation at $5000 \times g$ for 5 min at 4°C. Bile was collected in pre-weighed tubes at 30-min intervals. At the end of the kinetic study, animals were sacrificed by exsanguination and livers were harvested, weighed, snap frozen in liquid nitrogen and stored at –80°C until use. Bile flow was determined gravimetrically without correction for relative density, assuming a bile density of 1.0 g/mL.

Experiment 2

Experiment 2, which had the same design of the drug administration as Experiment 1, was performed to harvest tissue from the small intestine. Moreover, feces were collected from two cages in each group (2–3 animals per cage) during the last three days of the treatment.

3.2. Liver histology

Right lobe of the excised liver was fixed in 10% neutral buffered formalin. Paraffin-embedded liver tissue sections were prepared and stained with hematoxylin-eosin at the Department of Histology and Embryology, Faculty of Medicine in Hradec Kralove. Pictures were taken using Olympus BX51 microscope with Olympus DP71 camera.

3.3. Routine plasma biochemistry and 7α-hydroxy-4-cholesten-3-one measurements

Plasma bilirubin concentrations and liver enzyme activities were determined by routine laboratory methods on Cobas Integra 800 (Roche Diagnostics). Plasma triglycerides, total cholesterol, high density lipoprotein (HDL) cholesterol, and LDL cholesterol were determined by routine laboratory methods on a Hitachi automatic analyzer. Since HDL and LDL cholesterol were measured, we could calculate concentrations of very low density lipoprotein (VLDL) cholesterol by subtracting HDL and LDL cholesterol from total cholesterol (Sanchez-Muniz and Bastida, 2008). Plasma concentrations of C4 were measured by high-performance liquid chromatography (HPLC) at the Institute of Medical Biochemistry and Laboratory Diagnostics, 1st Faculty of Medicine of Charles University, as previously described (Lenicek et al., 2008). The chromatographic parameters were: Tessek SGX C18 column (4 × 250 mm, 4 μm), acetonitrile:water (95:5, vol/vol) mobile

phase, flow rate 1 mL/min, detection/reference wavelength 241/360 nm.

3.4. Evaluation of paracellular permeability

Hepatic tight junctional permeability (function of blood-biliary barrier) was assessed by a dual sugar permeability test. As mentioned above, on day 9 of the experiment rats under general anesthesia were injected with a single intravenous dose of melibiose and rhamnose (100 mg/kg) at the beginning of bile collection and 4-h pharmacokinetic study was performed. Plasma and biliary concentrations of melibiose and rhamnose were determined by HPLC method with a fluorescent detection as previously described (Tomsik et al., 2008). The ratio of biliary excretion of melibiose (disaccharide, which penetrates selectively across the paracellular junctions) to biliary excretion of rhamnose (monosaccharide, which permeates barriers by transcellular diffusion) was used as a marker of liver paracellular permeability.

Despite the fact that the biliary excretion of these sugars is minor compared to urinary excretion, the changes in biliary excretion of these sugars correlate very tightly with the function of blood-biliary barrier (Tomsik et al., 2008).

3.5. Bile acid measurements

Bile acids in plasma, bile, liver tissue and feces were assayed using a commercial kit (Total Bile Acids, Diazyme) and microplate reader Infinite 200 PRO (Tecan). While plasma samples were assayed directly, bile samples were diluted 1:300 before the measurement. Bile acids from liver tissue were extracted in 75% ethanol overnight and then heated up to 60°C for 2.5 h. After centrifugation (1500 × g, 10 min), the supernatant was assayed for bile acids. Fecal samples from the last day of drug administration were weighted and air-dried over 72-h period. Then 0.5 g of dried feces was minced and extracted in 10 mL of 75% ethanol at about 50°C for 2 h. The extract was centrifuged and total bile acids were measured in the supernatant. The daily feces output (g/day per 100 g of bw) and fecal bile acid content (μmol/g) were used to calculate the rate of bile acid excretion (μmol/day/100 g of bw).

3.6. Cholesterol, phospholipid and triglyceride measurements

Biliary concentrations of cholesterol and phospholipids were assessed by commercially available kits Cholesterol Liquid 500 (Pliva-Lachema Diagnostika) and Phosphatidylcholine Assay Kit (Cayman Chemical), respectively. Liver cholesterol and triglyceride concentrations were determined by commercial kits Cholesterol Assay Kit (Cayman Chemical) and Triglycerides 250 S (Pliva-Lachema Diagnostika), respectively, after extraction of hepatic lipids with chloroform-methanol 2:1 according to Lee et al. (2004). Hepatic esterified cholesterol concentrations were calculated by subtracting free cholesterol from total cholesterol.

3.7. Glutathione measurements

Concentrations of reduced (GSH) and oxidized (GSSG) glutathione in bile and liver tissue were analyzed separately using HPLC method (Kand'ar et al., 2007) on a Shimadzu system with fluorescence detection. The chromatographic conditions employed in the study were as follows: stationary phase SUPELCO Discovery C18 (4 mm × 150 mm, 5 μm), mobile phase methanol:phosphate buffer (15:85, vol/vol, 25 mM, pH 6.0), flow rate 0.5 mL/min, detector set at 350 nm and 450 nm (excitation and emission wavelengths, respectively). Total glutathione was calculated as the sum of reduced glutathione and oxidized glutathione (GSH + 2GSSG).

3.8. Lithogenic index

Lithogenic index was calculated according to the method of Thomas and Hofmann (1973) and represents cholesterol saturation in the sample. The lithogenic index is defined as a ratio of the actual molar percentage of cholesterol in the bile sample to the molar percentage of cholesterol that is theoretically soluble. The molar percentage of theoretically soluble cholesterol is calculated as the ratio of phospholipids : (phospholipids + bile acids).

3.9. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Liver tissue samples were homogenized using MagNA Lyser instrument (Roche) and total RNA was isolated with RNeasy Mini Kit (Qiagen). Total RNA from primary human hepatocytes, HepG2 cells and primary rat hepatocytes was isolated using TRI Reagent (Invitrogen or Sigma-Aldrich) and cleaned up with Qiagen kit (RNeasy MinElute Cleanup Kit). One to five micrograms of RNA were then converted into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was examined by qRT-PCR on 7500 HT Fast Real-time PCR system (Applied Biosystems). Each reaction was conducted with 30 ng of cDNA (total RNA equivalent) in duplicates or triplicates. Thermal cycling profile was 95°C for 20 sec, 40 cycles: 95°C for 3 sec, 60°C for 30 sec. TaqMan Fast Universal PCR Master Mix and pre-designed TaqMan Gene Expression Assay kits (Table 1) were purchased from Applied Biosystems. HMG-CoA reductase assay was purchased from Generi Biotech. The relative changes in gene expression were analyzed using ΔΔCt method. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase expression.

Table 1. Pre-designed TaqMan Gene Expression Assay kits used in the study.

Gene symbol	Synonym	Applied Biosystems cat. number
Abca1		Rn00710172_m1
Abcb4	Mdr2	Rn00562185_m1
Abcb11	Bsep	Rn00582179_m1
Abcc2	Mrp2	Rn00563231_m1
Abcc3	Mrp3	Rn01452854_m1
Abcc4	Mrp4	Rn01465702_m1
Abcg5		Rn00587092_m1
Abcg8		Rn00590367_m1
Acat2		Rn00596636_m1
Cyp7a1		Rn00564065_m1
Fasn		Rn00569117_m1
Fgf15		Rn00590708_m1
Gapdh		Rn01775763_g1
LDL receptor		Rn00598442_m1

Table 1. (continued)

Gene symbol	Synonym	Applied Biosystems cat. number
Lxrα		Rn00581185_m1
Nqo1		Rn00566528_m1
Nr0b2	Shp	Rn00589173_m1
Osta	Ostα	Rn01763289_m1
Ostb	Ostβ	Rn01767005_m1
Slc10a1	Ntcp	Rn00566894_m1
Slc10a2	Asbt	Rn00691576_m1
Slco1a1	Oatp1a1	Rn00755148_m1
Slco1a4	Oatp1a4	Rn00756233_m1
Sr-b1		Rn00580588_m1
Srebp1		Rn01495769_m1
Srebp2		Rn01502638_m1

3.10. Western blot

Liver samples were homogenized in RIPA buffer using MagNA Lyser instrument (Roche). Protein concentration was measured with the bicinchoninic acid assay (Thermo Pierce) using serum bovine albumin (Sigma) as a standard. Membrane-enriched fraction was prepared from cytosolic fraction after spinning at 100 000 × g for 1 h (Denk et al., 2004). Isolated canalicular membrane fractions were prepared according to Ito et al. (2001). Liver homogenates were incubated with sample buffer at room temperature for 30 min and separated on a polyacrylamide gel. After the proteins were transferred to a polyvinylidene fluoride membrane (Millipore), the membrane was blocked for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20. The membrane was then incubated with primary antibodies (Table 2) for 1 h or longer, washed, and incubated for 1 h with a peroxidase-conjugated secondary anti-mouse or anti-rabbit antibody (GE Healthcare). After washing three times, the membranes were developed using enhanced chemiluminescent reagent (Thermo Pierce) and subjected to autoluminography. The immunoreactive bands on the exposed films were scanned with a densitometer ScanMaker i900 (UMAX) and semiquantified using the QuantityOne imaging software (Bio-Rad). Beta-actin immunoblots were used as loading controls.

Table 2. Primary antibodies used in western blot analyses.

Protein	Source, catalog number	Dilution
Abca1	Novus, NB 100-2068	1:2000
Abcg5	Abcam, ab45279	1:1000
Acat2	Cayman, 100027	1:500
Bsep	Abcam, ab71793	1:1000
Cyp7a1	Abcam, ab 65596	1:1000
HMG-CoA reductase	Abcam, ab98018	1:1000
LDL receptor	Cayman, 10007665	1:1000
Mdr2	Abcam, ab71792	1:500
Mrp2	Alexis, ALX-801-037	1:500
Mrp3	Sigma, M0318	1:500
Mrp4	Abcam, ab32550	1:200
Ntcp	Santa Cruz, sc-98485	1:300
Oatp1a1	Millipore, AB3570P	1:5000
Oatp1a4	Millipore, AB3572P	1:3000
Sr-b1	Novus, NB 400-104	1:2000
Srebp2	Abcam, ab30682	1:1000
β-actin	Sigma, A5316	1:5000

3.11. Gene reporter assay for human FXR

HepG2 cell line was purchased from the European Collection of Cell Cultures (Salisbury, UK) and was passaged no more than 25 times. All cells were maintained at 37°C in a humidified atmosphere containing 5% carbon dioxide. For transient transfection gene reporter experiments, HepG2 cells were maintained in antibiotic-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% sodium pyruvate, and 1% nonessential amino acids (all from Sigma-Aldrich). HepG2 cells were cotransfected with the pGL5-luc luciferase reporter construct (150 ng/well in 48-well plate), 100 ng/well of the expression plasmid pCMX-GAL4-FXR containing FXR ligand binding domain (Adachi et al., 2005) and pRL-TK (30 ng/well) using Lipofectamine 2000 (Life Technologies). Following 24 h of stabilization, HepG2 cells were treated with EGCG (5 μM and 20 μM), chenodeoxycholic acid (CDCA; 50 μM), and vehicle (DMSO; 0.1% v/v) for 24 h. Cells were then lysed and analyzed for firefly luciferase activity normalized

to enzyme activity of the *Renilla* luciferase (Dual-Luciferase Reporter Assay, Promega).

3.12. Rat hepatocyte isolation, culture and treatment

Male Wistar rats (250–280 g) were used as liver donors and primary rat hepatocytes were isolated by collagenase perfusion (Kand'ar et al., 2007) at the Department of Physiology, Faculty of Medicine in Hradec Kralove. Sprague Dawley rats (170–200 g) as liver donors were obtained from Charles River (Wilmington, MA) and primary hepatocyte isolation was performed in the Liver Center of Yale University. All cells were maintained at 37°C in a humidified atmosphere containing 5% carbon dioxide. In both sets of experiments, hepatocyte suspensions were added to the precoated dishes at a density of 1×10^6 cells per 35-mm dish. Two hours after plating, the medium was aspirated and cells were overlaid with 70 µL of collagen solution in William's E medium (1.5 mg/mL, pH 7.4). One hour later, fresh supplemented William's E medium was added. William's E medium for Wistar rat hepatocytes contained fetal bovine serum (6%), glutamine (2 mM), penicillin (100 IU/mL), streptomycin (10 mg/mL), dexamethasone (0.1 µg/mL), insulin (0.08 IU/mL) and glucagon (8 ng/mL). William's E medium for Sprague Dawley rat hepatocytes contained fetal bovine serum (5%), glutamine (2 mM), penicillin (100 IU/mL), streptomycin (10 mg/mL), gentamicin (8 µg/mL), dexamethasone (0.1 µM) and insulin (4 mg/L). After 24 h, the medium was removed and replaced with a fresh serum-free William's E medium containing either DMSO or EGCG at concentration of 1-20 µM for 24 h incubation. Thereafter, cells were collected in TRI Reagent or Trizol Reagent (Sigma-Aldrich or Invitrogen) for gene expression analysis.

3.13. Primary human hepatocyte isolation, culture and treatment

Primary human hepatocytes were obtained from the Liver Tissue Procurement and Distribution System of the National Institutes of Health (Dr. Stephen Strom, University of Pittsburgh, Pittsburgh, PA). Human hepatocytes were maintained on collagen coated plates overlaid with Matrigel (BD Biosciences). All cells were maintained at 37°C in a humidified atmosphere containing 5% carbon dioxide. Culture medium (HMM medium, Lonza) contained dexamethasone (0.1 µM), insulin (0.1 µM), penicillin G (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (0.25 µg/mL). Twenty-four hours after Matrigel overlay, cells were treated with either DMSO or EGCG at concentration of

0.2-5 µM for 24 h. Cells were harvested in Trizol Reagent (Invitrogen) for gene expression analysis.

3.14. HepG2 cell culture and treatment

HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA) and were used within 20 passages. Cells were maintained in DMEM containing 10% fetal bovine serum (Gibco) and penicillin/streptomycin (100 U/mL, 100 µg/mL). All cells were maintained at 37°C in a humidified atmosphere containing 5% carbon dioxide. At 50% to 70% confluence, cells were treated with DMSO or EGCG at concentration of 1-20 µM for 24 h in serum-free medium. Afterwards, cells were harvested in Trizol reagent (Invitrogen) for gene expression analysis.

3.15. Rat Cyp7a1 promoter activity

The effect of EGCG on rat Cyp7a1 promoter activity was assessed in a dual-luciferase gene reporter assay from Promega. Rat Cyp7a1 promoter reporter construct (pGL2-Cyp7a1) was provided from J. Y. L. Chiang (Northeastern Ohio University) and is described in a previous report as p-416Luc (Crestani et al., 1995). HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in DMEM containing 10% fetal bovine serum (Gibco) and penicillin/streptomycin (100 U/mL, 100 µg/mL). When cells reached 80% confluence, the culture medium was changed to OPTI-MEM and cells were cotransfected with pGL2-Cyp7a1 (300 ng), pCMV-LXRα and pCMV-RXRα expression plasmids (both 75 ng) and 1.5 ng phRL-CMV per each well. Transfection was performed in triplicates in 24-well plates using Lipofectamin 2000 (Invitrogen). After 24 h, cells were treated with DMSO, EGCG (1-20 µM) and 22-(R)-hydroxycholesterol (5 µM) for an additional 24 h in 0.5% charcoal-stripped fetal bovine serum DMEM. Cell lysate was prepared with passive lysis buffer (Promega) and luminescence was measured by a Synergy2 Microplate Reader (BioTek). Firefly luciferase activity was normalized to *Renilla* luciferase activity to control for transfection efficiency and cell numbers. Data (firefly/*Renilla* luciferase activity) are expressed as fold increase over vehicle-treated cells.

3.16.   Human CYP7A1 promoter activity

A dual-luciferase gene reporter assay (Promega) was utilized to evaluate the effect of EGCG on human CYP7A1 promoter activity. HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in DMEM containing 10% fetal bovine serum (Gibco) and penicillin/streptomycin (100 U/mL, 100 µg/mL). Human CYP7A1 promoter reporter construct (pGL3-CYP7A1) was a gift from J. Y. L. Chiang (Northeastern Ohio University). For transfection experiments, medium was changed to OPTI-MEM medium and cells were cotransfected with pGL3-CYP7A1 (300 ng), pGL3 (200 ng) and 1.5 ng phRL-CMV in triplicates, using X-Treme HP (Roche) for each well in 24-well plates. Twenty-four hours later, cells were treated with DMSO, EGCG (1-5 µM) and CDCA (50 µM) for an additional 24 h in DMEM containing 0.5% charcoal-stripped fetal bovine serum. Passive lysis, luminescence reading and normalizing the activity were performed as described in method 3.16.

3.17.   Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Time-course experiments were analyzed using a two-way analysis of variance (ANOVA) followed by Bonferroni test for post-hoc analysis. For two-group comparison, Student's t-test was employed. For multiple-group comparison, data were analyzed by one-way ANOVA followed by Newman-Keuls test. Pearson correlation coefficients (r) were calculated. Differences were considered significant at P-value less than 0.05. All analyses were performed using GraphPad Prism 5.0 software (San Diego, USA).

4. Results

4.1. Body weight

The body weight of all animals was monitored on daily basis throughout the experiment. While EGCG treatment did not affect the body weight, administration of ethinylestradiol led to a moderate body weight loss (Fig. 10).
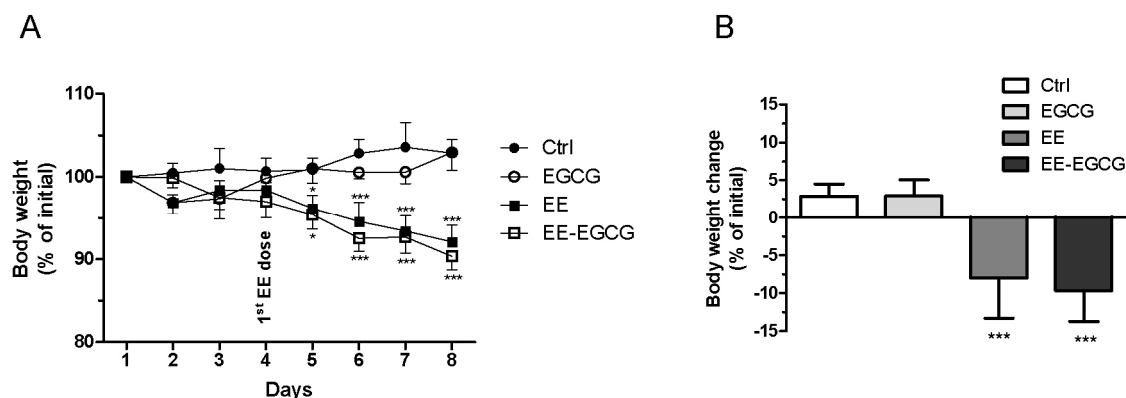
Figure 10. Time course of body weight change (A) and body weight change at the end of experiment (B) as a percentage of initial body weight. Data are expressed as means ± SD for 6-7 animals per group. Significantly different from the control group: * $P < 0.05$, *** $P < 0.001$.

4.2. Liver weight

At the end of the kinetic study, rat livers were harvested and weighted. EGCG administration alone had no effect on the liver weight (Fig. 11). On the other side, ethinylestradiol caused a significant liver weight increase, which was blunted by EGCG coadministration.
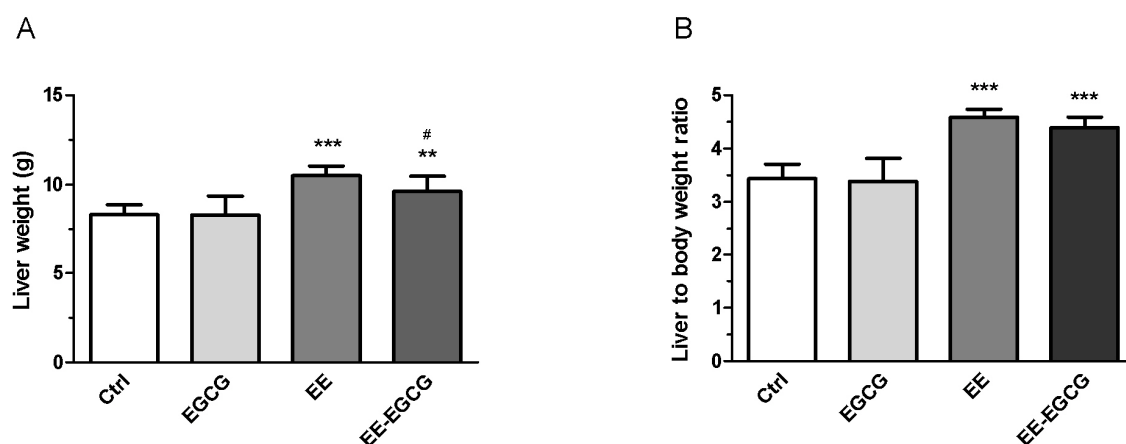

Figure 11. Liver weight (A) and liver weight to body weight ratio (B). Data represent means ± SD for 6-7 animals per group. Significantly different from the control group: ** $P < 0.01$, *** $P < 0.001$. Significantly different from EE group: [#] $P < 0.05$.

Figure 8B shows liver weight normalized to body weight. Since the body weight of EE-EGCG rats was slightly lower at the end of experiment compared to EE group, the difference in liver to body weight ratio between these two groups is no longer statistically significant.

4.3. Liver histology

Formalin-fixed paraffin-embedded liver sections were stained with hematoxylin-eosin and examined by light microscopy. Livers of all groups showed normal liver architecture and no hepatocyte injury (Fig. 12). Ethinylestradiol-induced cholestasis is a form of bland cholestasis that does not exhibit parenchymal damage (Lindor, 2008).
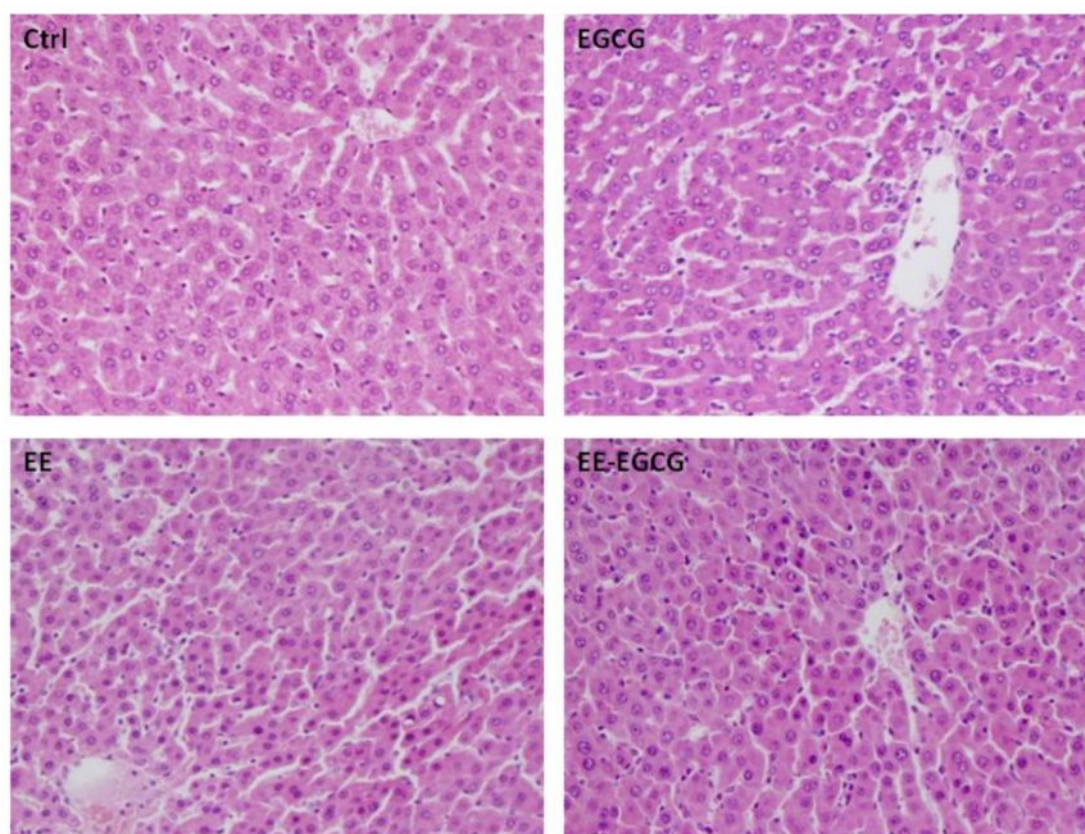
Figure 12. Photomicrographs of hematoxylin-eosin-stained sections of liver tissue from the control, EGCG, EE and EE-EGCG group (original magnification ×10).

4.4. Plasma biochemistry

The sole EGCG administration to rats did not significantly affect most of the plasma biochemistry markers except for the concentrations of bile acids and VLDL cholesterol. EGCG elevated plasma bile acids by 2-fold ($P < 0.05$) compared to controls (Table 3), but it

did not further raise ethinylestradiol-elevated plasma bile acids in EE-EGCG group.

Ethinylestradiol treatment in rats raised plasma bile acids and bilirubin compared to controls. Further, ethinylestradiol administration lowered plasma triglycerides, total cholesterol and HDL cholesterol while increased VLDL cholesterol fraction. Ethinylestradiol treatment also diminished levels of LDL cholesterol under the limit of detection. EGCG in combination with ethinylestradiol further reduced plasma total cholesterol (due to a decrease in VLDL cholesterol fraction), with no change in plasma HDL cholesterol compared with ethinylestradiol-only administration. EGCG also further lowered ethinylestradiol-reduced plasma levels of triglycerides.

Table 3. Plasma biochemistry

	Ctrl	EGCG	EE	EE-EGCG
Total bile acids (μM)	13.5 ± 5.5	27.2 ± 8.2[*]	39.3 ± 12.6[***]	41.4 ± 13.1[***]
Total bilirubin (μM)	2.9 ± 0.7	2.7 ± 0.8	6.4 ± 1.8[**]	8.6 ± 4.2[***]
ALT (μkat/L)	1.0 ± 0.5	1.0 ± 0.5	1.7 ± 0.4	0.7 ± 0.1
AST (μkat/L)	2.2 ± 1.2	2.1 ± 0.6	1.7 ± 0.4	1.4 ± 0.5
LDH (μkat/L)	3.2 ± 1.9	2.5 ± 0.5	2.6 ± 1.0	2.1 ± 0.7
Total protein (g/L)	40.2 ± 5.2	37.5 ± 2.2	39.4 ± 5.8	35.3 ± 1.7
Albumin (g/L)	28.6 ± 3.9	28.9 ± 1.4	27.0 ± 3.5	25.7 ± 1.9
Glucose (mM)	9.8 ± 1.2	8.8 ± 1.3	10.0 ± 3.0	8.0 ± 1.0
Plasma lipids				
Triglycerides (mM)	0.69 ± 0.18	0.87 ± 0.23	0.39 ± 0.23[*]	0.21 ± 0.04[***#]
Total cholesterol (mM)	1.25 ± 0.27	1.11 ± 0.17	0.41 ± 0.14[***]	0.16 ± 0.11[***##]
HDL cholesterol (mM)	0.87 ± 0.19	0.83 ± 0.18	0.04 ± 0.02[***]	0.03 ± 0.02[***]
LDL cholesterol (mM)	0.11 ± 0.06	0.08 ± 0.04	ND[a]	ND[a]
VLDL cholesterol (mM)	0.27 ± 0.08	0.20 ± 0.06[*]	0.40 ± 0.08[*]	0.15 ± 0.08[*###]

Values are means ± SD for 6-7 animals per group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase. Significantly different from the control group: [*] $P < 0.05$, [**] $P < 0.01$, [***] $P < 0.001$. Significantly different from EE group: [#] $P < 0.05$, [##] $P < 0.01$, [###] $P < 0.001$.

[a] ND, not detected in the majority of samples (detection limit 0.01 mM).

4.5. Bile flow

Following cannulation of the common bile duct, bile was collected in 30-minute intervals and bile flow rate and cumulative bile flow were calculated (Fig. 13). Interestingly, the average bile flow rate in EGCG-treated rats was reduced by 23% compared to the control group (9.1 ± 0.8 vs. 11.7 ± 0.7 µL/min, $P < 0.05$). Ethinylestradiol administration to rats diminished bile flow by ~ 60% regardless of EGCG treatment ($P < 0.001$) as the effect of ethinylestradiol prevailed over the effect of EGCG.
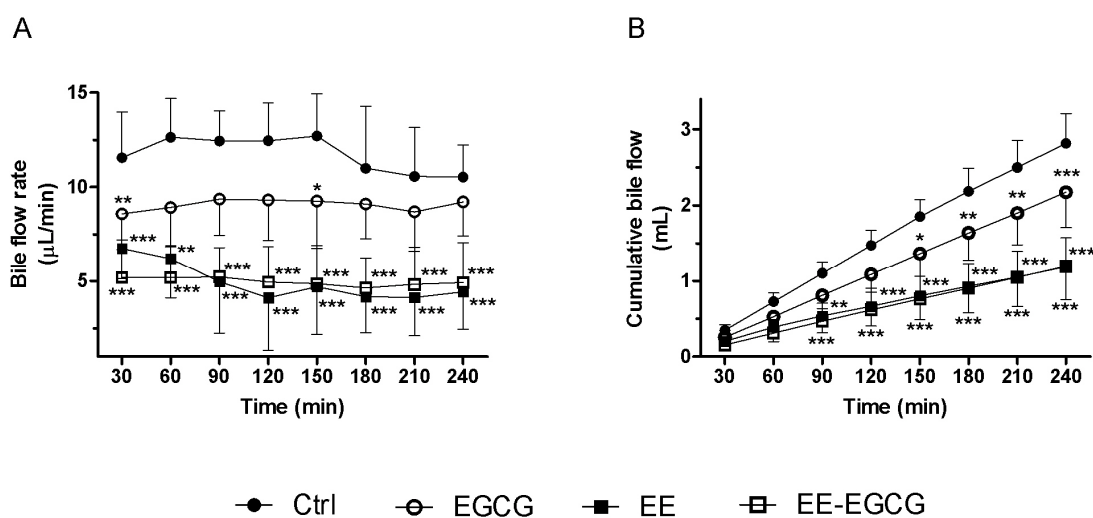
Figure 13. Bile flow expressed as a time course of the bile flow rate (A) and cumulative bile flow (B). Values are means ± SD for 6-7 animals per group. Significantly different from the control group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4.6. Function of blood-biliary barrier

Since the function of blood-biliary barrier significantly affects bile formation, this parameter was assessed by a previously described permeability test (Tomsik et al., 2008), which is based on intravenous administration of melibiose and rhamnose. Plasma and biliary concentrations of both sugars were measured and the ratio of biliary excretion of melibiose to rhamnose was calculated.

The mean plasma concentrations of melibiose and rhamnose did not differ significantly between groups throughout the kinetic study (Fig. 14).

A

B

C
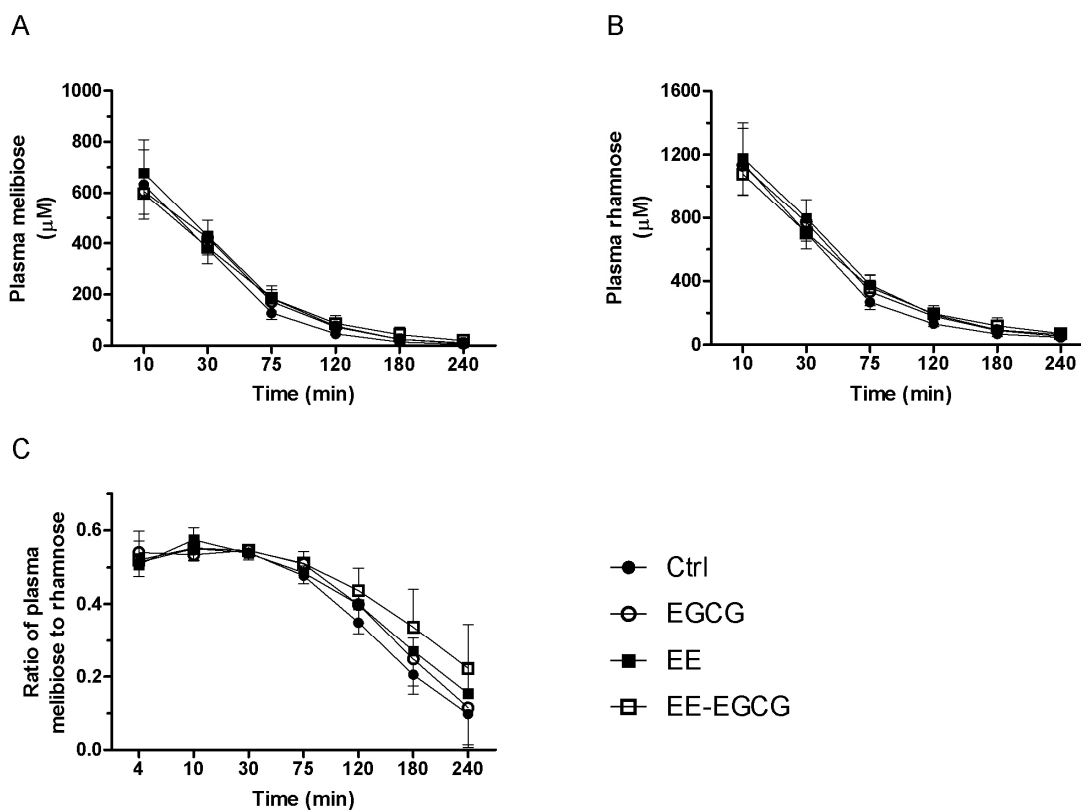
Figure 14. Plasma concentrations of melibiose (A) and rhamnose (B), and ratio of plasma concentration of melibiose to plasma concentration of rhamnose (C). Values are means ± SD for 6-7 animals per group.

Regarding the biliary excretion of melibiose and rhamnose, differences were observed in early phases of the bile collection (Fig. 15). Biliary excretion of melibiose was increased in the first two intervals in ethinylestradiol-treated rats. When expressed in cumulative fashion, ethinylestradiol-treated animals had a significantly higher biliary excretion of melibiose (Fig. 15C).
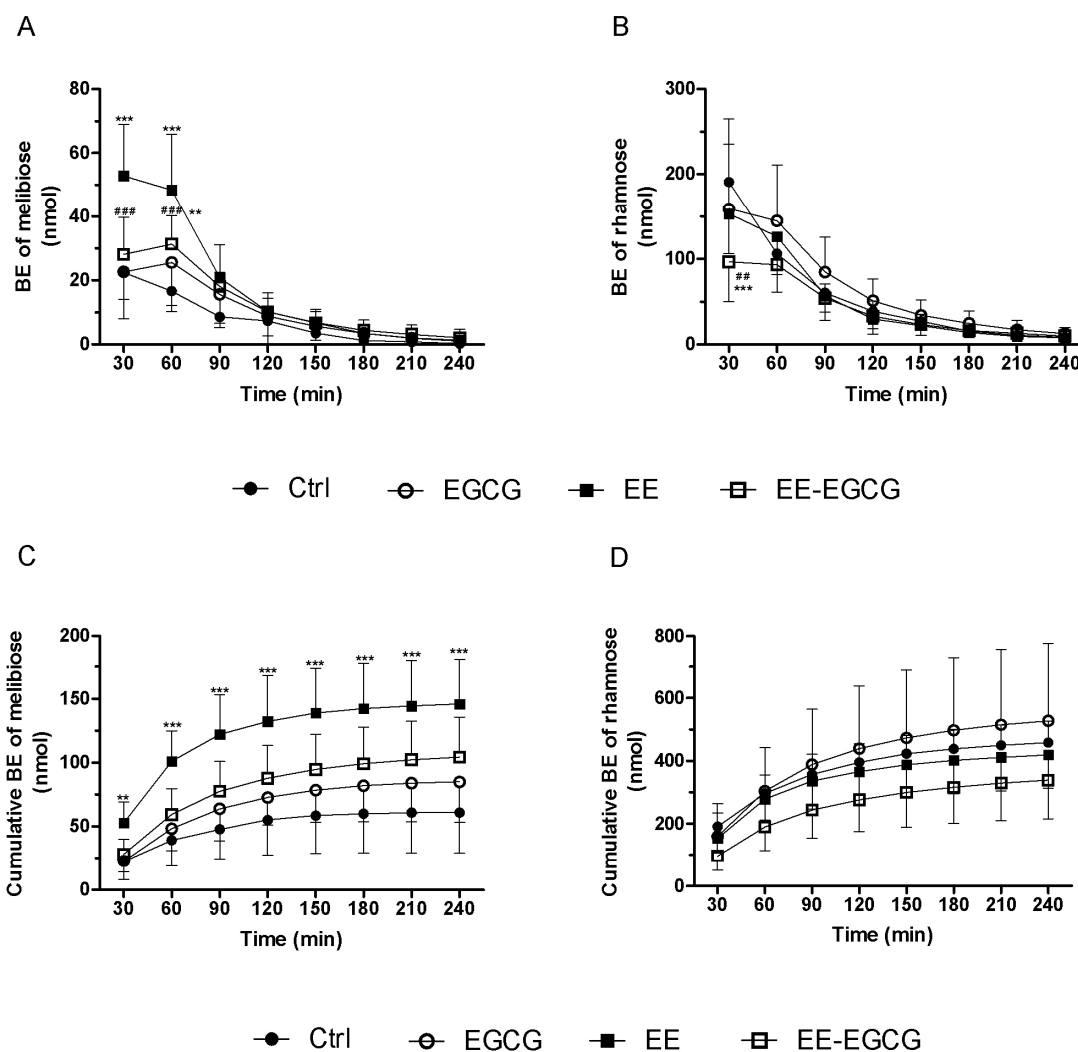
Figure 15. Biliary excretion of melibiose (A) and rhamnose (B) and cumulative biliary excretion of melibiose (C) and rhamnose (D). BE, biliary excretion. Values are means ± SD for 6-7 animals per group. Significantly different from the control group: ** $P < 0.01$, *** $P < 0.001$. Significantly different from EE-treated group: ## $P < 0.01$, ### $P < 0.001$.

The ratio of biliary excretion (or cumulative biliary excretion) of melibiose to rhamnose is a key marker of blood-biliary barrier impairment. The ratio was markedly increased in the groups receiving ethinylestradiol and ethinylestradiol plus EGCG (Fig. 16), which could allow for a paracellular regurgitation of bile constituents into the blood. Although ethinylestradiol administration is known to increase paracellular permeability in rat liver (Rodriguez-Garay, 2003), this particular permeability test was utilized in ethinylestradiol-treated rats for the first time.
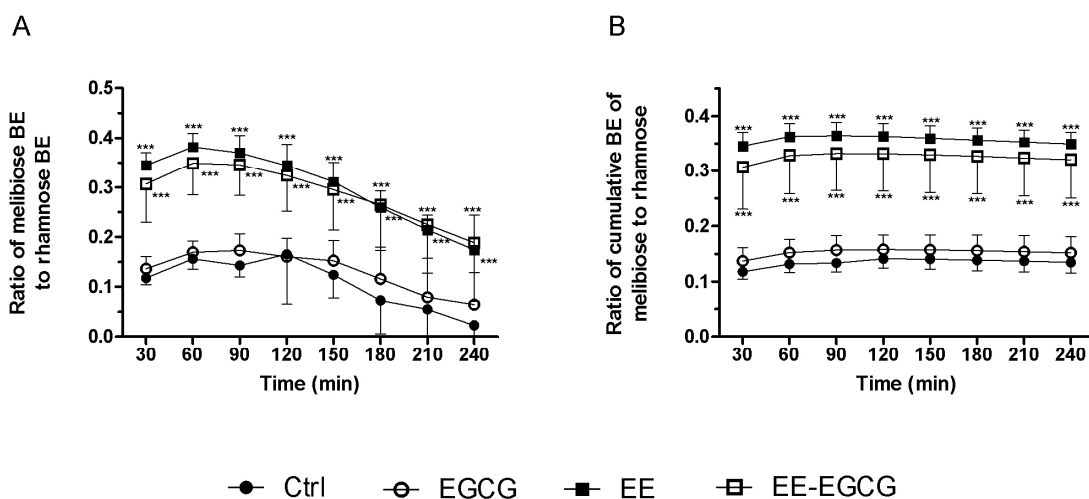
Figure 16. Ratio of melibiose biliary excretion to rhamnose biliary excretion (A) and ratio of melibiose cumulative biliary excretion to rhamnose cumulative biliary excretion (B). BE, biliary excretion. Values are means ± SD for 6-7 animals per group. Significantly different from the control group: *** $P < 0.001$.

4.7. Biliary excretion of bile acids, glutathione and lipids

Biliary concentrations of bile acids, glutathione, cholesterol and phospholipids were measured (Fig. 17) and their biliary excretion rates were calculated as a product of the concentration and bile flow rate.
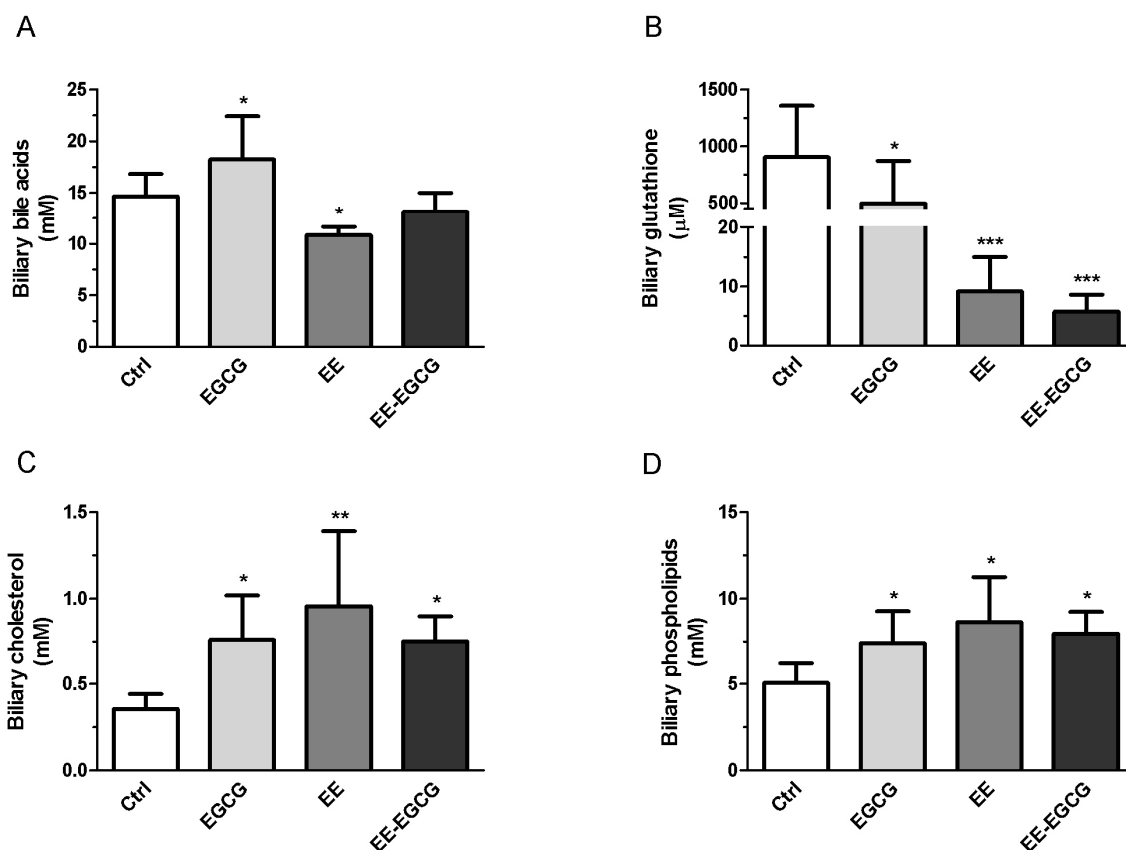
Figure 17. Biliary concentrations of bile acids (A), glutathione (B), cholesterol (C), and phospholipids (D). Values are means ± SD for 6-7 animals per group. Significantly different from the control group: ** $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

EGCG treatment did not affect biliary output of bile acids compared to controls (Fig. 18), but reduced biliary glutathione excretion by ~70% ($P < 0.001$). Interestingly, EGCG markedly enhanced biliary excretion of cholesterol and phospholipids by 84% and 50% ($P < 0.001$), respectively. Ethinylestradiol lowered bile acid excretion to 40% of the control value ($P < 0.001$) and nearly abolished glutathione biliary output (0.4% of controls, $P < 0.001$). EGCG coadministration to ethinylestradiol had no effect on these parameters compared to ethinylestradiol-only administration.

Figure 18. Biliary excretion of bile acids (A), glutathione (B), cholesterol (C), and phospholipids (D). BE, biliary excretion. Values are means ± SD for 6-7 animals per group. Significantly different from the control group: *** P < 0.001.

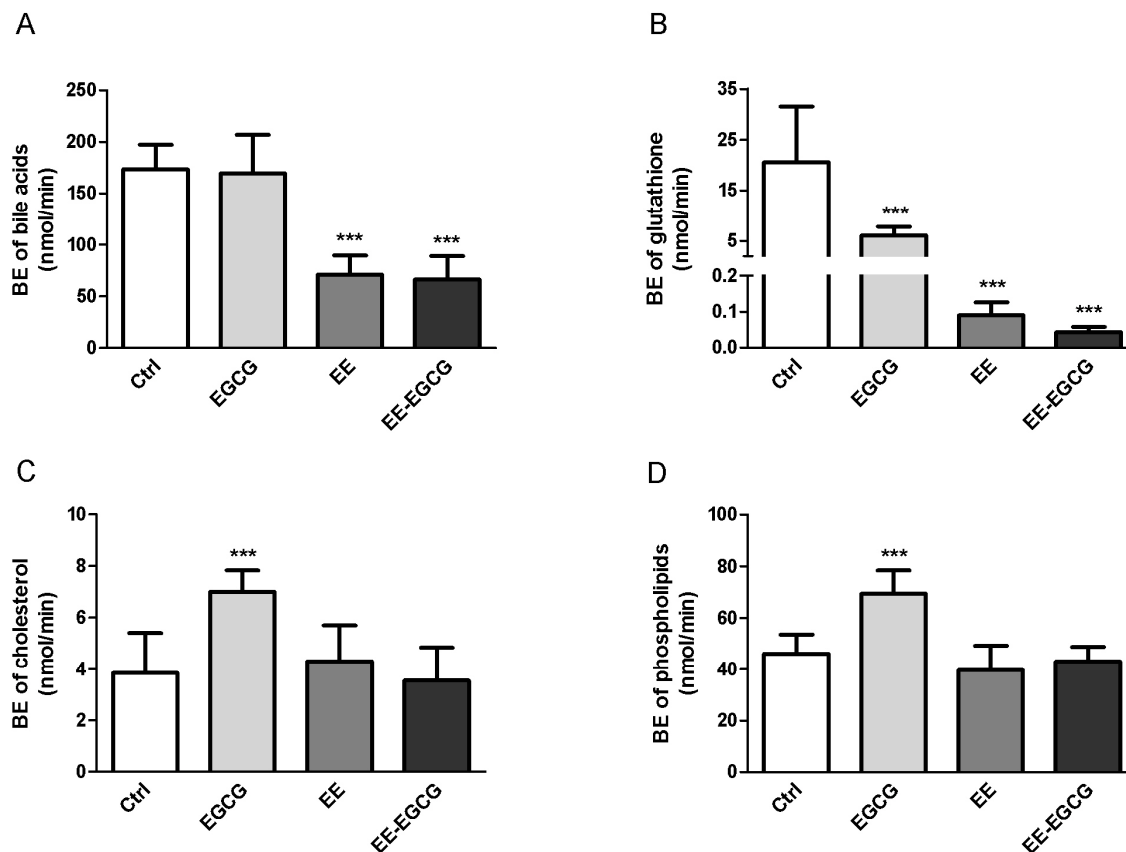Biliary bile acid excretion was evaluated in three different time points. Since the bile drainage due to bile duct cannulation interrupted enterohepatic circulation of bile acids, the early bile collection interval may best reflect the situation in the animals (Fig. 19).
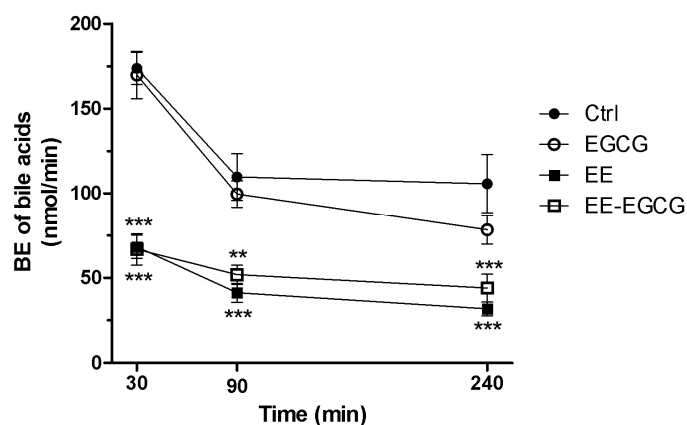
Figure 19. Biliary excretion of bile acids over the time periods. BE, biliary excretion. Values are means ± SD for 6-7 animals per group. Significantly different from the control group: ** P < 0.01, *** P < 0.001.

4.8. Lithogenic index

Lithogenic index for each animal was calculated. Both EGCG and ethinylestradiol (or their combination) significantly increased formation of lithogenic bile compared to controls (Fig. 20).
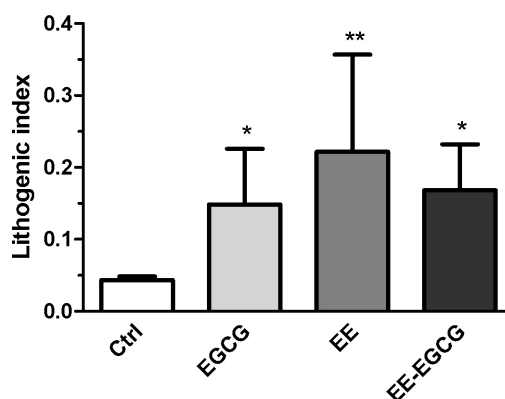
Figure 20. Lithogenic index. Values are means ± SD for 6-7 animals per group. Significantly different from the control group: * P < 0.05, ** P < 0.01.

4.9. Fecal bile acid excretion

EGCG-treated rats had a higher concentration of bile acids in feces (Fig. 21A) than the control rats. The fecal bile acid excretion tended to be increased after EGCG treatment, although this was not statistically significant (Fig. 21B). Bile acid content and fecal excretion were significantly lower in ethinylestradiol-treated rats compared to controls.

Figure 21. Concentration of total bile acids in the feces (A) and fecal bile acid excretion (B). Values are means ± SD for 4 samples per group. Significantly different from the control group: * P < 0.05, ** P < 0.01.

4.10. Liver bile acid concentration

Bile acid concentrations were measured in liver tissue. The mean hepatic bile acid concentrations remained unaffected by EGCG treatment (Fig. 22). Ethinylestradiol is known not to change hepatic bile acid levels (Davis and Kern, 1976).
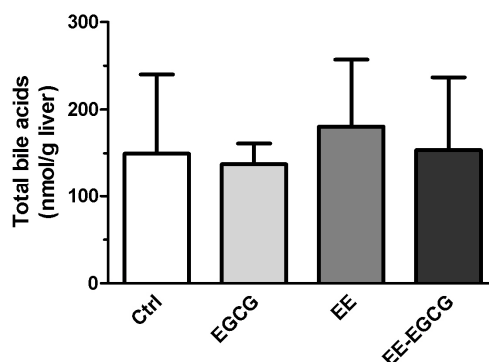
Figure 22. Hepatic concentrations of bile acids. Values are means ± SD for 6-7 animals per group.

4.11. Liver glutathione concentration

Both reduced and oxidized forms of glutathione were measured in liver tissue. EGCG-treated rats showed increased concentrations of oxidized glutathione, but unchanged levels of the reduced form and total glutathione compared to controls (Fig. 23A-C). This, however, made the GSH/GSSG ratio decrease significantly (Fig. 23D). Ethinylestradiol, on the other side, markedly raised levels of reduced glutathione and thus levels of the total glutathione since the reduced form is much more abundant than the oxidized form.
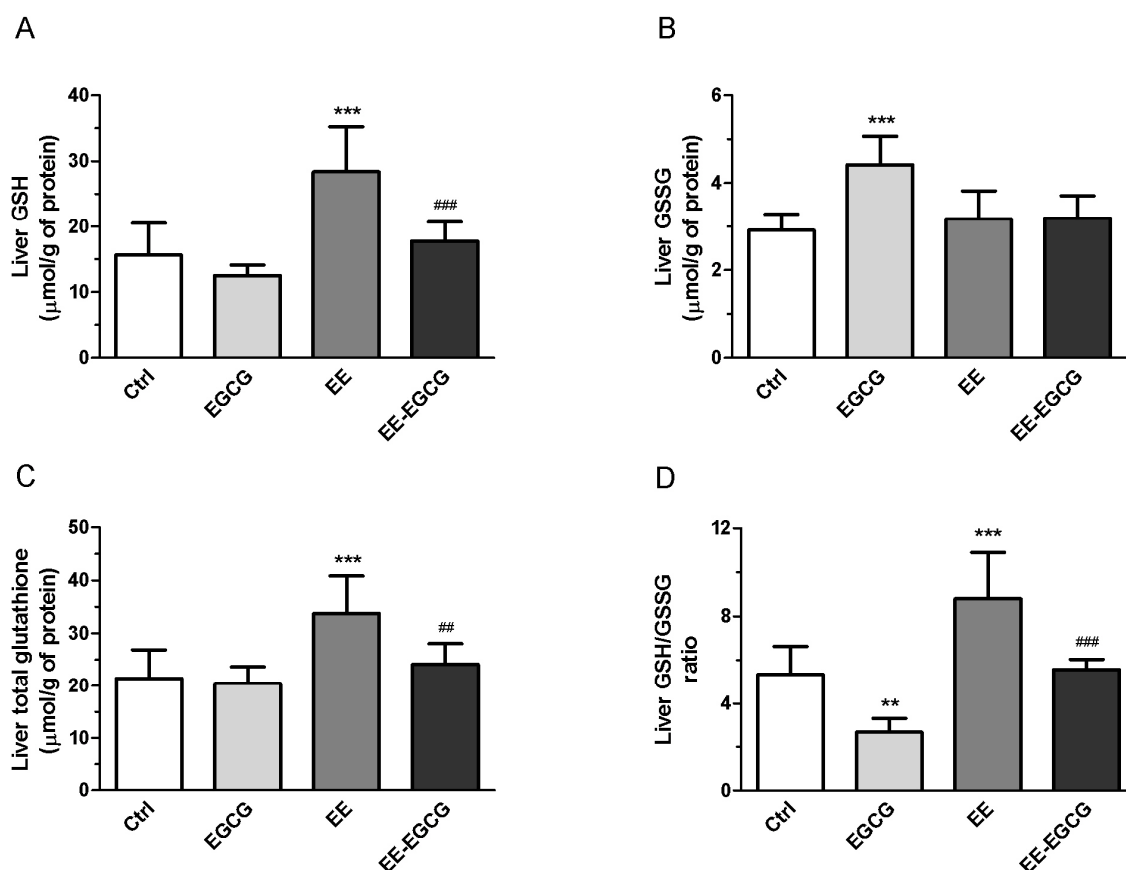
Figure 23. Liver concentrations of reduced glutathione (GSH; A), oxidized glutathione (GSSG; B), total glutathione (C) and ratio of reduced glutathione to oxidized glutathione (D). Values are means ± SD for 6-7 animals per group. Significantly different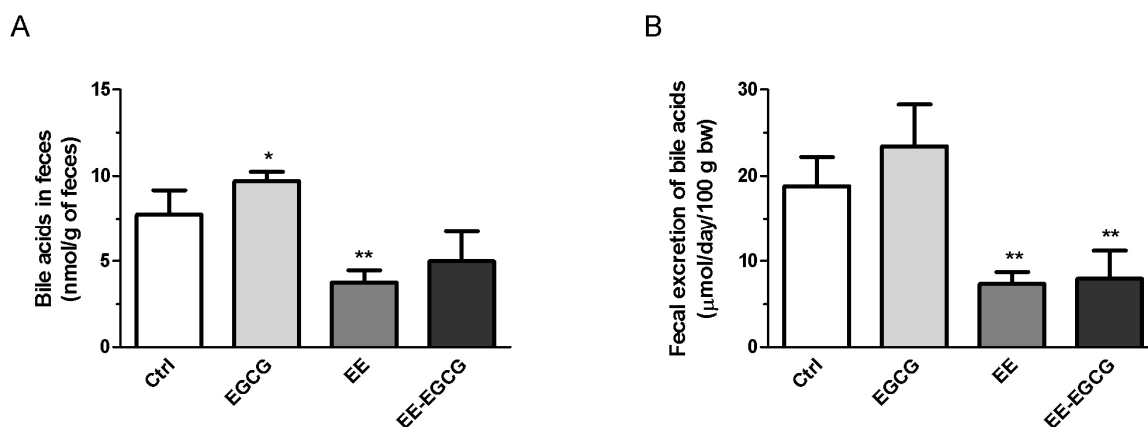 from the control group: ** P < 0.01, *** P < 0.001. Significantly different from EE-treated group: ## P < 0.01, ### P < 0.001.

4.12. Liver concentrations of lipids

A moderate decline in liver triglycerides was observed in EGCG-treated rats, whereas no such change occurred when EGCG was coadministered with ethinylestradiol (Fig. 24A). EGCG treatment alone affected neither free nor esterified cholesterol concentrations. On the contrary, ethinylestradiol treatment substantially increased liver cholesterol content, inducing hepatic cholesterol levels by ~50% compared to controls (P < 0.001) (Fig. 24B, D). This increase in hepatic total cholesterol is attributable to the increment in esterified cholesterol. When coadministered with ethinylestradiol, EGCG partially prevented ethinylestradiol-induced cholesterol accumulation and liver weight increase by lowering hepatic esterified cholesterol concentrations. In addition, cholesterol content per liver was tightly correlated with liver weight (r = 0.89, P < 0.0001) and with both gene and protein expression of acyl-coenzyme A:cholesterol acyltransferase (Acat2) (r = 0.82 and r = 0.71, respectively,

P < 0.001), the principal enzyme responsible for cholesterol esterification.
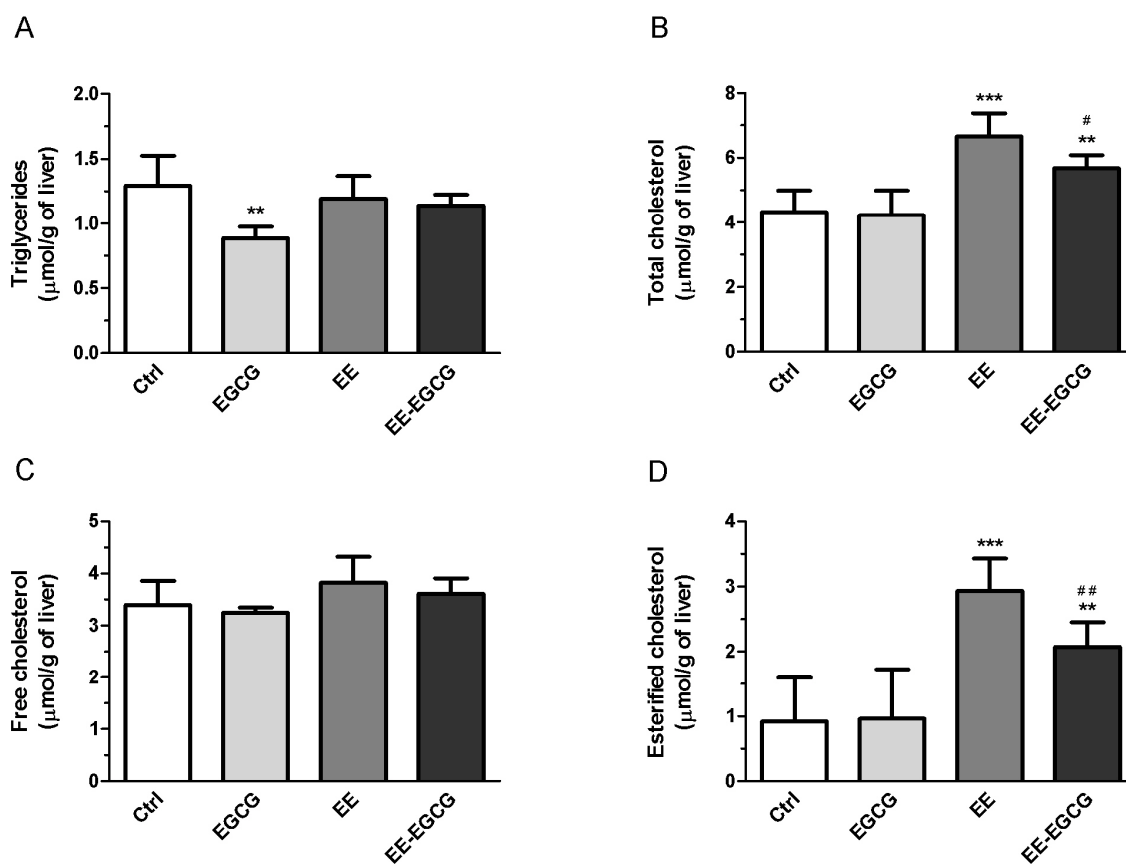
Figure 24. Liver concentrations of triglycerides (A), total cholesterol (B), free cholesterol (C) and esterified cholesterol (D). Values are means ± SD for 6-7 animals per group. Significantly different from the control group: ** P < 0.01, *** P < 0.001. Significantly different from EE-treated group: [#] P < 0.05, [##] P < 0.01.

4.13. Expression of molecules involved in bile acid homeostasis

To evaluate effect of EGCG at the molecular level, gene and protein expression profiles of the major hepatic bile acid transporters in the liver and intestine were examined by qRT-PCR and Western blotting. EGCG administration decreased mRNA levels of two major bile acid transporters at the canalicular (Bsep) and basolateral (Ntcp) membrane by 34% and 24% (P < 0.01 and P < 0.05), respectively (Fig. 25A). Nevertheless, their protein expression was not changed compared to controls (Fig. 25B). On the other hand, EGCG nearly doubled protein expression of Mrp4 (P < 0.001).

Similarly to previous reports (Geier et al., 2003; Fiorucci et al., 2005), ethinylestradiol significantly reduced gene expression of Ntcp and Oatp1a4 and strongly upregulated Mrp3 (Fig. 25A). In ethinylestradiol-treated group, protein expression of Bsep, Ntcp, Oatp1a1, Oatp1a4, Mrp2 was reduced, whereas Mrp4 protein mass was increased (Fig. 25B). EGCG

coadministration to ethinylestradiol did not markedly alter gene or protein expression, except for the increase in Oatp1a4 mRNA and Mrp4 protein.
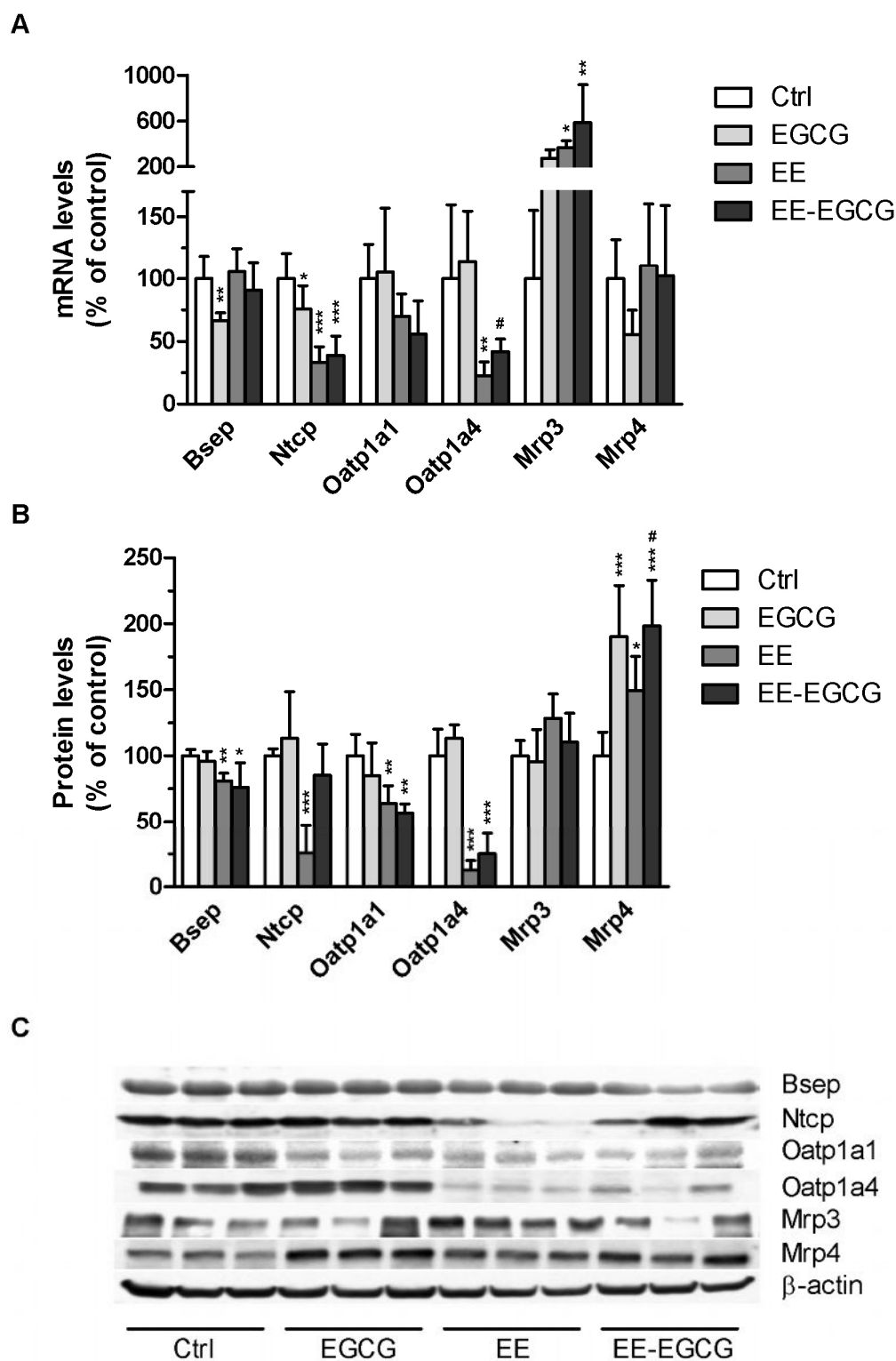
Figure 25. Hepatic expression of bile acid transporters at the mRNA (A) and protein levels (B). Representative immunoblots are shown (C). Bsep, bile salt export pump; Ntcp, Na^+-taurocholate co-transporting polypeptide; Oatp, organic anion transporting polypeptide; Mrp, multidrug resistance-associated protein. Values are means $\pm$ SD for 6-7 animals per group. Significantly different from the control group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significantly different from EE-treated group: # $P < 0.05$.

Gene expression of bile acid transporters was also evaluated in the ileum. All experimental groups showed reduced expression of the main ileal bile acid transporters, Asbt and Ostα, compared to controls (Fig. 26). On the contrary, EGCG administered to ethinylestradiol-treated rats increased mRNA levels of Asbt, Ostα and Ostβ compared to rats administered with ethinylestradiol only.
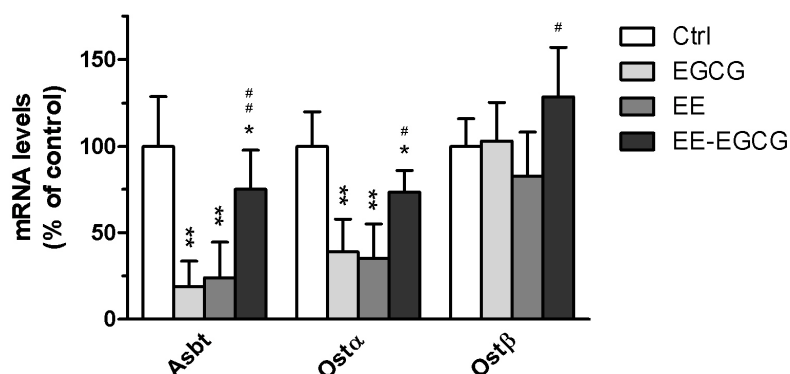
Figure 26. Gene expression of the major bile acid transporters in the ileum. Values are means ± SD for 6-7 rats per group. Asbt, apical sodium-dependent bile acid transporter; Ost, organic solute transporter. Significantly different from the control group: * P < 0.05, ** P < 0.01. Signifi-cantly different from EE group: [#] P < 0.05, [##] P < 0.01.

Next, the key regulators of bile acid homeostasis in the liver and intestine were evaluated at mRNA level. Fxr is the chief sensor of intracellular levels of bile acids, controlling their synthesis and transport. Its gene expression in the liver was not significantly altered by the treatment (Fig. 27). In all experimental groups, the expression of the orphan nuclear receptor Shp was markedly upregulated in the liver, but reduced in the ileum. Similarly, gene expression of Fgf15 in the ileum was significantly downregulated.
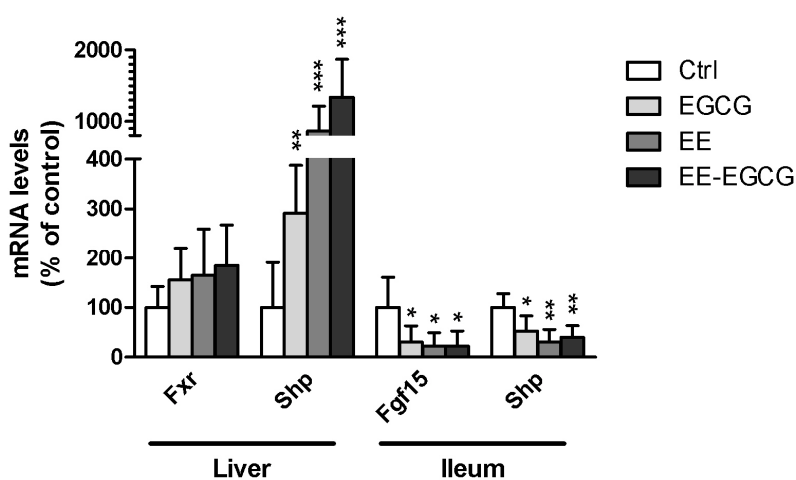
Figure 27. Gene expression of the major regulators of bile acid homeostasis in the liver and ileum. Values are means ± SD for 6-7 rats per group. Fxr, farnesoid X receptor; Shp, small heterodimer partner; Fgf15, fibroblast growth factor 15. Significantly different from the control group: * P < 0.05, ** P < 0.01, *** P < 0.001.

4.14. Expression of Mrp2

Since biliary glutathione excretion was significantly impaired in all treatment groups, hepatic expression of Mrp2, the key glutathione transporter, was explored. Surprisingly, none of the treatments affected mRNA levels of Mrp2 in the livers (Fig. 28A).
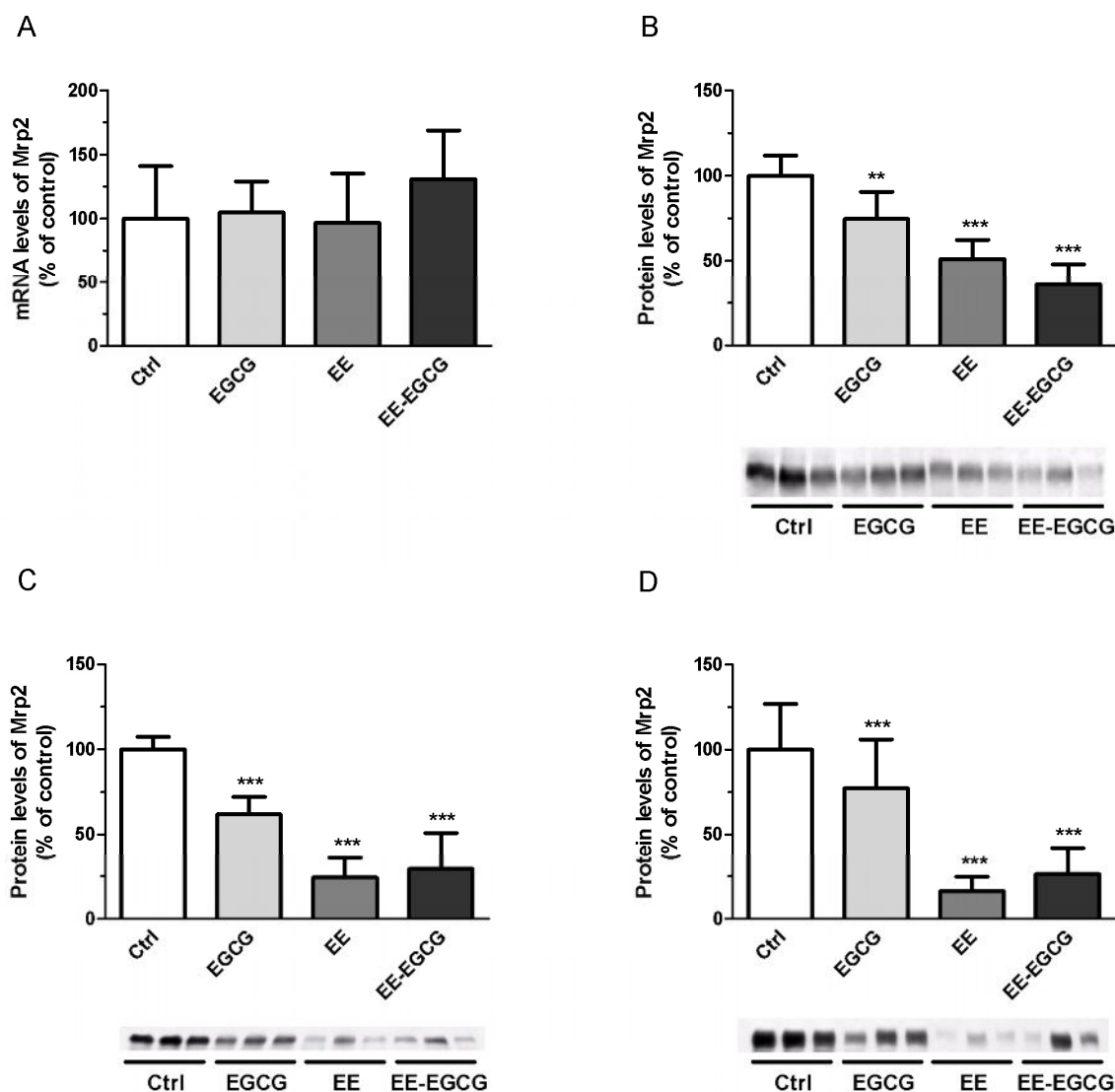
Figure 28. Hepatic expression of Mrp2 at the mRNA (A) and protein levels (B-D). Mrp2 protein was detected in the whole cell lysate (B), membrane-enriched fraction (C) and isolated canalicular membrane fraction (D). Mrp, multidrug resistance-associated protein. Values are means ± SD for 6-7 animals per group. Significantly different from the control group: ** $P < 0.01$, *** $P < 0.001$.

Mrp2 protein content was then examined in the whole cell lysate, which showed that EGCG treatment reduced protein mass of Mrp by ~25% ($p < 0.01$, Fig. 28B) compared to controls. Mrp2 protein content was then assessed in membrane-enriched fraction and isolated canalicular membrane fraction to see whether endocytic retrieval or redistribution from the canalicular membrane plays a role in Mrp2 decreased function. Mrp2 protein contents in both

membrane-enriched fraction and isolated canalicular membrane fraction were decreased to a similar extent (Fig. 28C, D) as the total amount of protein ruling out involvement of endocytic retrieval or redistribution. Protein expression of Mrp2 very tightly correlated with both biliary glutathione excretion ($r = 0.80$, P < 0.0001) and bile flow rate ($r = 0.86$, P < 0.0001).

4.15. Expression of molecules involved in cholesterol metabolism

Expression of a variety of enzymes, receptors and transporters involved in liver cholesterol synthesis, uptake, esterification and secretion was evaluated at both mRNA and protein levels. Compared to controls, EGCG treatment did not changed LDL receptor mRNA but downregulated gene expression of Sr-b1 (which mediates selective uptake of HDL cholesterol) and Abca1 (ATP-binding cassette transporter sub-family A member1, the main cholesterol basolateral efflux transporter) by 30% (P < 0.001) and 60% (P < 0.01), respectively (Fig. 29A). While EGCG did not alter Acat2 gene expression significantly, it increased HMG-CoA reductase mRNA by 60% (P < 0.01). Most strikingly, EGCG-treated rats exhibited a more than 5-fold increase in gene expression of Abcg5 and Abcg8 (P < 0.001), which function as a heterodimeric complex transporting cholesterol from the hepatocytes into bile. Ethinylestradiol significantly reduced mRNA abundance of Sr-b1, Abca1 and HMG-CoA reductase and markedly induced that of LDL receptor and Acat2 (Fig. 29A). Gene expression of the main biliary phospholipid transporter, Mdr2, did not differ between the groups.

To confirm changes found in gene expression, levels of respective proteins were assessed by Western blot. EGCG increased protein levels of Abcg5 and Sr-b1 by 77% (P < 0.001) and 20% (P < 0.01), respectively, and decreased Acat2 protein by 70% (P < 0.01) (Fig. 29B). Ethinylestradiol strongly induced LDL receptor protein mass, but no bands were detected in the control group, as reported previously by Bertolotti and Spady (1996), even if more protein from membrane-enriched fraction was used (data not shown). Ethinylestradiol-treated rats had lower protein levels of Abcg5 (P < 0.01), HMG-CoA reductase and Sr-b1 (P < 0.001) compared to controls. On the other side, ethinylestradiol caused a significant increase Acat2 protein (by ~2.5-fold, P < 0.001), which was blunted by EGCG coadministration (P < 0.01). Protein expression of Mdr2 and Abca1 was not change in all experimental groups.
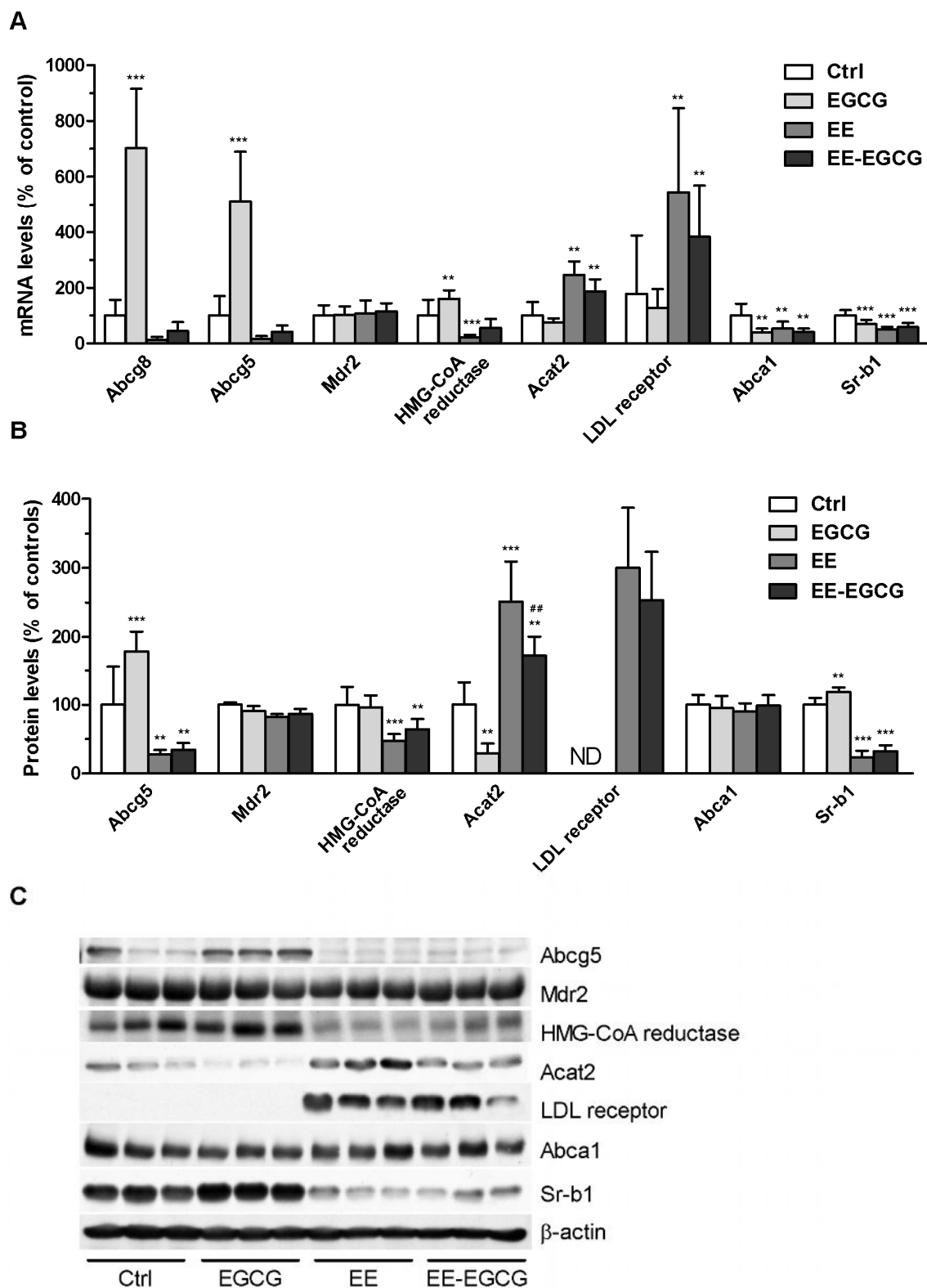
Figure 29. Hepatic expression of key regulators of cholesterol metabolism at the mRNA (A) and protein levels (B). Representative immunoblots are shown (C). Abcg, ATP-binding cassette transporter sub-family G; Mdr2, multidrug resistance protein2; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-coenzyme-A; Acat2, acyl-CoA:cholesterol acyltransferase 2; LDL, low density lipoprotein; Abca1, ATP-binding cassette transporter sub-family A member 1; Sr-b1, scavenger receptor class B type 1. Values are means ± SD for 6-7 animals per group. Significantly different from the control group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significantly different from ethinylestradiol-treated group: [#] $P < 0.05$.

Expression of selected nuclear receptors and transcription factors (and their downstream target genes) involved in cholesterol metabolism regulation was also assessed (Table 4). While EGCG had no significant effect on their expression, ethinylestradiol administration caused a marked induction of Shp and sterol regulatory element-binding protein (Srebp) 1 mRNA and decrease in mRNA levels of sterol Srebp2 (P < 0.001). EGCG supplementation in ethinylestradiol-treated rats normalized ethinylestradiol-induced gene expression of Srebp1 and further increased Shp. In addition, ethinylestradiol plus EGCG increased amount of the mature transcriptionally active form of Srebp2.

Table 4. Expression of selected genes and Srebp2 protein

	Ctrl	EGCG	EE	EE-EGCG
Relative mRNA abundance				
Lxrα	100 ± 31	65 ± 15	89 ± 19	117 ± 46
Srebp1	100 ± 38	77 ± 22	214 ± 53***	150 ± 45[##]
Srebp2	100 ± 19	102 ± 33	45 ± 9***	43 ± 5***
Fasn	100 ± 56	84 ± 37	55 ± 43	31 ± 12***
Nqo1	100 ± 39	105 ± 26	135 ± 79	134 ± 56
Shp	100 ± 91	291 ± 95	863 ± 350***	1340 ± 529***[#]
Relative protein abundance				
Srebp2	100 ± 26	186 ± 70	210 ± 69	284 ± 136[**]

Data are expressed as a percentage of the control value and represent means ± SD, n = 6–7. Protein levels of the active, i.e. cleaved, form of Srebp2 were evaluated. Lxrα, liver X receptor; Srebp, sterol regulatory element-binding protein; Fasn, fatty acid synthase; Nqo1, NAD(P)H dehydrogenase, quinone 1; Shp, small heterodimer partner. Significantly different: *** P < 0.001, ** P < 0.01, * P < 0.05 when compared to the control group; [##] P < 0.01, [#] P < 0.05 when compared to ethinylestradiol-treated group.

4.16. Gene reporter assay for human Fxr

Fusion expression construct with the ligand binding domains of human FXR fused to the DNA binding domain of the transcription factor GAL4 was used to examine interaction of EGCG and FXR. After 24-h treatment, no effect of EGCG on the activation of GAL4-FXR construct was actually seen in HepG2 cells (Fig. 30). As shown previously (Adachi et al., 2005), chenodeoxycholic acid was a potent FXR agonist in activating gene reporter construct

via the pCMX-GAL4-FXR.
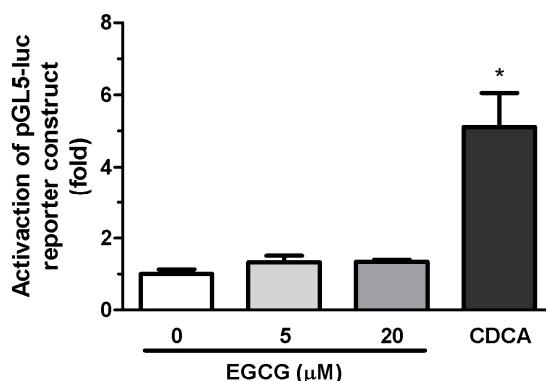
Figure 30. Interaction of EGCG and human FXR. Chenodeoxycholic acid (CDCA, 50 µM) was used as a positive control in activating pGL5-luc reporter construct via the pCMX-GAL4-FXR. Data are shown as the mean fold activation (normalized to the *Renilla* luciferase activity) relative to the vehicle treatment (DMSO). Significantly different from vehicle treatment: * P < 0.05.

4.17. Effect of EGCG on bile acid synthesis *in vivo*

Cholesterol 7α-hydroxylase plays a key role in regulation of bile acid synthesis since it catalyzes the first and rate-limiting step in the classic pathway of bile acid synthetic (Chiang, 2009). In EGCG-treated rats, hepatic expression of Cyp7a1 was increased by 5 and 1.5-fold at mRNA and protein levels, respectively (Fig. 31A, B). Bile acid synthesis in these rats was also assessed by plasma levels of C4, which is a well described marker of Cyp7a1 activity. The concentrations of C4 were actually doubled in EGCG-treated rats compared to controls (Fig. 31C).
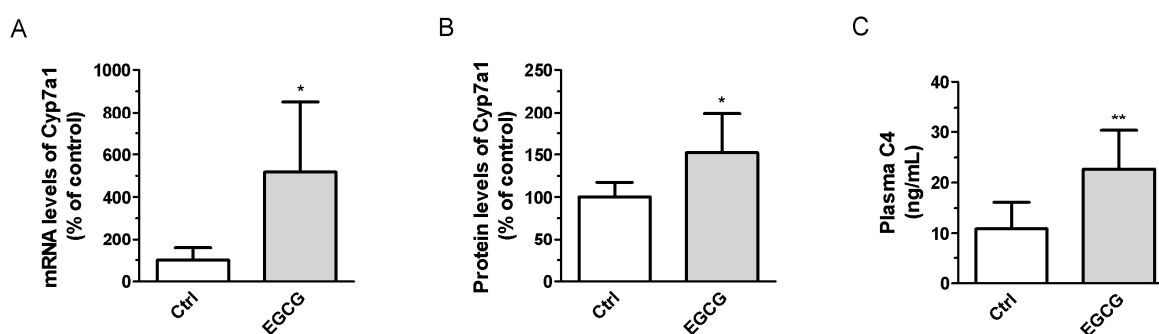
Figure 31. Effect of EGCG on bile acid synthesis *in vivo*. EGCG treatment in rats increased expression of Cyp7a1 at both mRNA (A) and protein (B) levels and doubled plasma levels of C4 (C). Values are means ± SD for 7 rats per group. C4, 7α-hydroxy-4-cholesten-3-one; Cyp7a1, cholesterol 7α-hydroxylase. Significantly different from the control group: * P < 0.05.

4.18. Effect of EGCG on bile acid synthesis *in vitro*

Since the EGCG-treated rats presented a 5-fold increase in Cyp7a1 mRNA levels, the direct effect of EGCG on Cyp7a1 gene expression was evaluated *in vitro*. Two series of experiments were conducted in primary rat hepatocytes cultured in a collagen-sandwich configuration and isolated from either Wistar or Sprague Dawley rats. These experiments showed that EGCG at 10 μM and 20 μM concentrations significantly induced rat Cyp7a1 mRNA *in vitro* (Fig. 32).
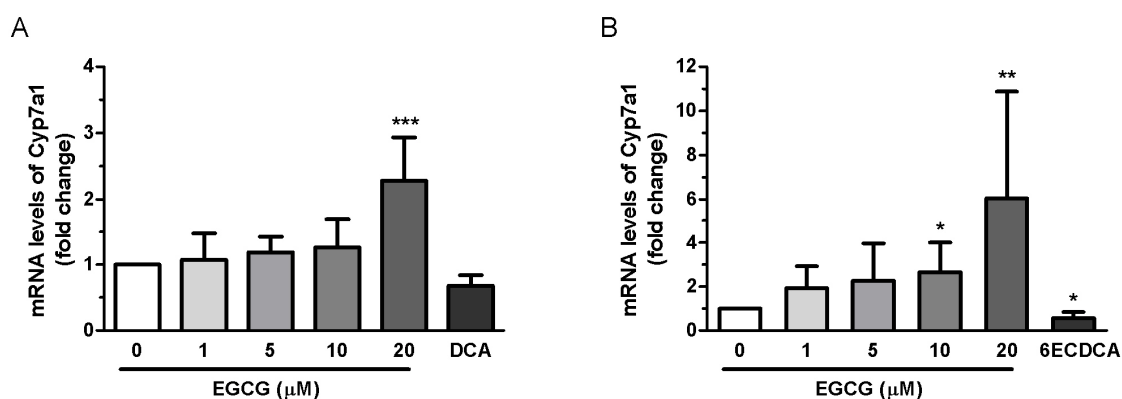
Figure 32. Gene expression of Cyp7a1 in primary hepatocytes isolated from either Wistar rats (A) or Sprague Dawley rats (B), cultured in a collagen-sandwich configuration and treated with EGCG (1–20 μM) for 24 h. DCA and 6ECDCA were used as negative controls that downregulate Cyp7a1 mRNA expression. Cyp7a1, cholesterol 7α-hydroxylase; DCA, deoxycholic acid; 6ECDCA, 6α-ethyl-chenodeoxycholic acid. Values are means ± SD for 3 (A) and 6 (B) independent experiments. Significantly different from the vehicle treatment: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
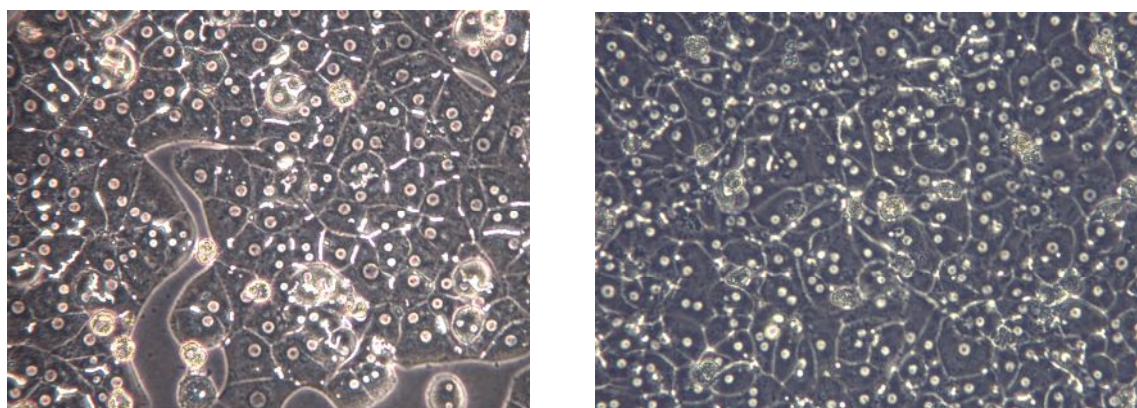
Figure 33. Illustrative microphotograph of primary rat hepatocytes cultured in a collagen-sandwich configuration (left) and primary human hepatocytes cultured in Matrigel matrix (taken with 40 × objective).

To address clinical relevance of the observed effect, EGCG effect on human CYP7A1 was tested in human primary hepatocytes and human hepatoma cell line. Surprisingly, the EGCG treatment in human hepatocytes showed a reverse effect to the one observed in the rat hepatocytes. In primary human hepatocytes, EGCG showed a tendency to decrease CYP7A1

expression, although this was not statistically significant (Fig. 34A). EGCG treatment (10-20 µM) in HepG2 cells resulted in a significant reduction in CYP7A1 mRNA, which was mediated by neither SHP nor FGF19 as it was the case for CYP7A1 downregulation induced by CDCA treatment (Fig. 34D-F).
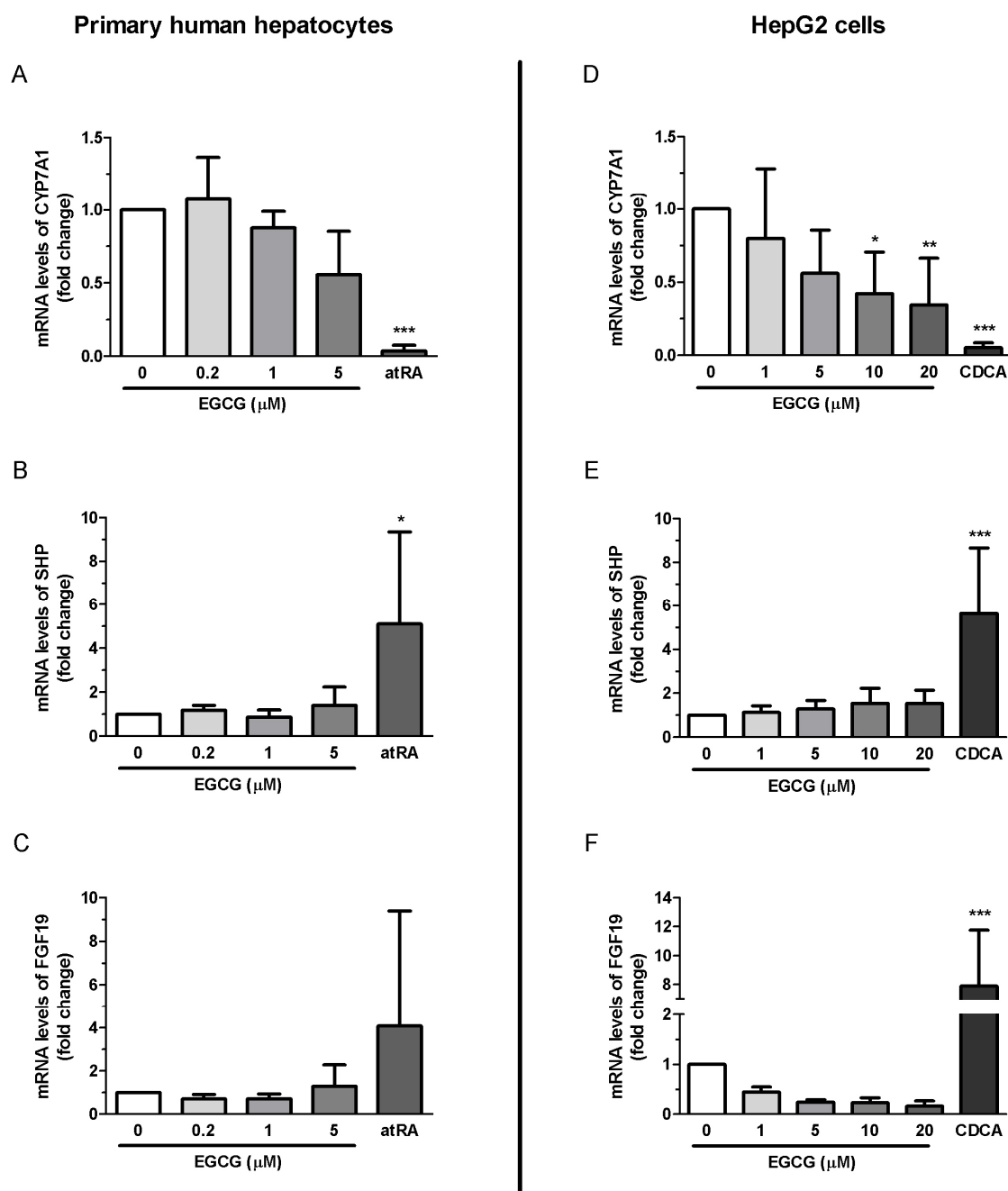
Figure 34. Effect of EGCG on CYP7A1 gene expression was studied in primary human hepatocytes (A-C) and HepG2 cells (D-F). atRA (1µM) and CDCA (50 µM) were used as negative controls for CYP7A1 mRNA expression. CYP7A1, cholesterol 7α-hydroxylase; SHP, small heterodimer partner; FGF19, fibroblast growth factor 19; atRA, all-trans retinoic acid; CDCA, chenodeoxycholic acid. Values are means ± SD for 3 (primary human hepatocyte) and 6 (HepG2 cells) independent experiments. Significantly different from the vehicle-treated cells: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

To assess whether EGCG affects Cyp7a1/CYP7A1 transcription directly, gene reporter assay was performed using both rat and human promoter of Cyp7a1/CYP7A1. EGCG had no effect on rat Cyp7a1 promoter activity, no matter if cells were transfected with reporter gene alone, cotransfected with the expression plasmid of HNF4α (not shown) or LXRα (Fig. 35A). As expected, the natural LXRα ligand, 22-(R)-hydroxycholesterol, increased activity of rat Cyp7a1 promoter. EGCG exerted no effect on human CYP7A1 promoter either (Fig. 35B). CDCA (50μM) treatment was used as a negative control.
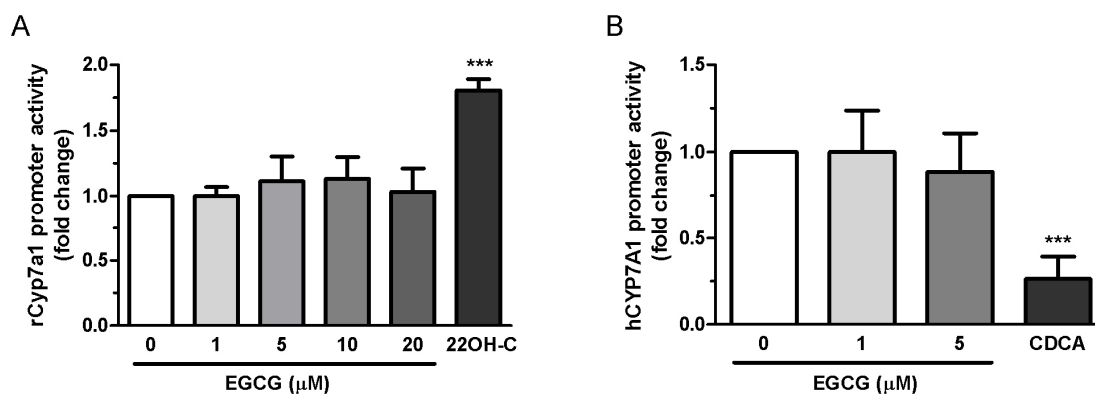
Figure 35. Effect of EGCG on rat Cyp7a1 promoter activity (A) and human CYP7A1 promoter activity (B) in gene reporter assay. CDCA (50 μM) and 22OH-C (5 μM) were used as a negative and positive control for human and rat promoter activity, respectively. hCyp7A1, human cholesterol 7α-hydroxylase; rCyp7a1, rat cholesterol 7α-hydroxylase; CDCD, chenodeoxycholic acid; 22OH-C, 22-(R)-hydroxycholesterol. Values are means ± SD for 4 independent experiments. Significantly different from the vehicle-treated cells: *** $P < 0.001$.

5. Discussion

5.1. Effect of EGCG on bile formation and bile acid homeostasis

EGCG has been reported to have beneficial effects in various experimental models of liver injury and diseases. On the other hand, a growing body of evidence has shown its toxic effect on the liver. Although there is a great interest in how EGCG affects liver pathophysiology, effect of EGCG on biliary physiology has not yet been studied. Thus, the present thesis investigated effect of EGCG on bile flow and bile acid homeostasis in rats.

One of the major findings of the study is that EGCG has a cholestatic activity in rats as its administration decreased both bile production and elevated plasma bile acids. Decreased bile flow after EGCG treatment was likely a consequence of reduced Mrp2 protein expression, which resulted in impaired biliary glutathione excretion (Fig. 18B). Hepatocyte canalicular transporter Mrp2 mediates biliary excretion of glutathione and various organic anions and their metabolites conjugated with glucuronate, glutathione or sulfate. Biliary excretion of these compounds creates osmotic driving force in hepatic bile formation (BAIF). In Mrp2 knockout mice, biliary glutathione excretion is nearly abolished (~6% of wild-type mice) and bile flow is ~25% lower than in wild-type mice (Chu et al., 2006). Similarly, compared to wild-type counterparts, Mrp2-mutant rats (TR$^-$ and EHBR rats) present dramatically decreased biliary glutathione excretion together with a 50% decrease in bile flow (Jemnitz et al., 2010). Since EGCG did not affect biliary bile acid excretion (BADF), the observed decrease in bile flow in EGCG-treated rats is very likely a result of Mrp2 protein reduction. The decreased biliary glutathione excretion confirmed decreased function of Mrp2. These findings were also indirectly supported by a tight correlation between bile production, glutathione excretion and Mrp2 protein expression. Direct inhibition of Mrp2 by EGCG may be excluded as EGCG has been shown to lack inhibitory effect on human MRP2 (Netsch et al., 2005). Nevertheless, EGCG and its methyl metabolites were shown to be substrates for MRP2 (Hong et al., 2003).

Since EGCG did not alter mRNA levels of Mrp2 in the liver, we can assume that the EGCG effect on Mrp2 is based on posttranscriptional mechanisms. Posttranslational regulation of plasma membrane transporters relates to the rate of protein synthesis, membrane positioning, endocytic retrieval and degradation. Especially endocytic retrieval and degradation seem to be relevant for Mrp2 regulation during cholestatic injury and drug treatment (Trauner et al., 1997; Johnson and Klaassen, 2002; Zinchuk et al., 2005; Micuda et

al., 2008). In the present study, EGCG likely did not alter Mrp2 membrane trafficking or endocytic retrieval as the Mrp2 content in the isolated canalicular membrane fraction reflected the overall content of the protein in whole cell lysate. Whether the decreased Mrp2 expression by EGCG is due to modified translation or impaired protein turnover requires further elucidation.

EGCG has recently been described as a unique modulator of mouse and human Fxr/FXR, the key regulator of whole body bile acid homeostasis (Li et al., 2012). In this report, EGCG moderately stimulated human FXR activity but, interestingly, inhibited FXR transactivation in the presence of bile acids or other FXR agonists. In addition, when administered to mice, EGCG induced transcription of Fxr target genes selectively only in the intestine but not in the liver (Li et al., 2012). In the present study, EGCG did not stimulate Fxr target genes in the rat liver (Bsep gene expression was rather reduced). In the intestine of EGCG-treated rats, contradictory to Li et al. (2012), Fxr target genes such as Shp and Ostα were significantly downregulated, which perhaps suggests alteration of Fxr at the given EGCG dose. Discrepancy between the described agonistic activity of EGCG on human FXR reporter and the expression of Fxr target genes in our animals was the reason for reproducing gene reporter experiments with human FXR. However, the activation of human FXR by EGCG was not confirmed in our settings. What caused these discrepancies is not known. The explanation might be a variable accumulation of endogenous coactivators (Li et al., 2012), gene reporter assay differences, species differences (mouse vs. rat vs. human) or dose and duration of the treatment (two days in mice vs. eight days in rats).

An intriguing question is whether human and rat FXR/Fxr are regulated differentially. Being aware of difficulties in interspecies interpretation, there is a significant concordance in the spectrum of FXR substrates and FXR-regulated target genes (including similarity in changes induced by cholestasis), which suggests a good probability that prediction the Fxr function is unbiased. Thus based on these data, it cannot be claimed that EGCG is an FXR agonist or that it acts *in vivo* rather like an antagonist. Relevant human data from clinical studies are needed to explain the discrepancy.

Another particular novel finding in the present study is that EGCG-treated rats presented elevated plasma bile acids, although the biliary bile acid excretion was not altered. Such a situation might be caused by either altered enterohepatic circulation of bile acids or increased bile acid biosynthesis (Li et al., 2010). At the level of the liver, enterohepatic recycling of bile acids was not apparently altered by EGCG because the biliary bile acid excretion was not changed. This was in agreement with unchanged protein expression of the

main bile acid transporters (Bsep, Ntcp, and Oatps). In contrast, the main bile acid transporters (Asbt, Ost) in the ileum were significantly downregulated. This may account for the increased bile acid content in feces of EGCG-treated rats, which was also observed after green tea administration (Yang and Koo, 2000). Decreased bile acid absorption is, however, in contradiction to the elevated plasma bile acid levels.

Regarding bile acid biosynthesis, EGCG administration to rats significantly increased mRNA and protein expression of Cyp7a1, the key and rate-limiting biosynthetic enzyme in the liver. The increased activity of this enzyme was further documented by increased concentrations of C4 in plasma of EGCG-treated rats. To confirm the EGCG-mediated induction of Cyp7a1 and obtain information on effective EGCG concentration, effect of EGCG was studied *in vitro* in primary rat hepatocyte. *In vitro* EGCG upregulated Cyp7a1 mRNA at concentrations above 10 µM. Gene reporter experiments, however, indicated that EGCG itself does not increase rat Cyp7a1 promoter activity. *In vivo*, a distinct mechanism which may cause the induction of Cyp7a1 is downregulation of intestinal Fgf15, which, in turn, is followed by disinhibition of Cyp7a1 expression in the liver (Chiang, 2009). Since the extent of plasma bile acid increase corresponded to the extent of plasma increase of C4 in EGCG-treated animals, the induction of Cyp7a1 is a likely cause of elevated plasma bile acids by EGCG. Based on our data, the mechanism of EGCG-mediated changes in bile acid turnover is summarized in Figure 36.

Effect of EGCG on CYP7A1 was also investigated in human hepatocytes. In contrast to the rat homologue, EGCG rather decreased expression of the human CYP7A1 gene and had no effect on CYP7A1 promoter activity. This observation was in a direct contradiction to a previously published report describing ability of EGCG to induce human CYP7A1 mRNA in HepG2 cells through increased CYP7A1 promoter activity (Lee et al., 2008). The reason for this discrepancy is unknown in spite of the fact that a number of experiments were performed either exactly according to Lee et al. (2008) or with various modifications.
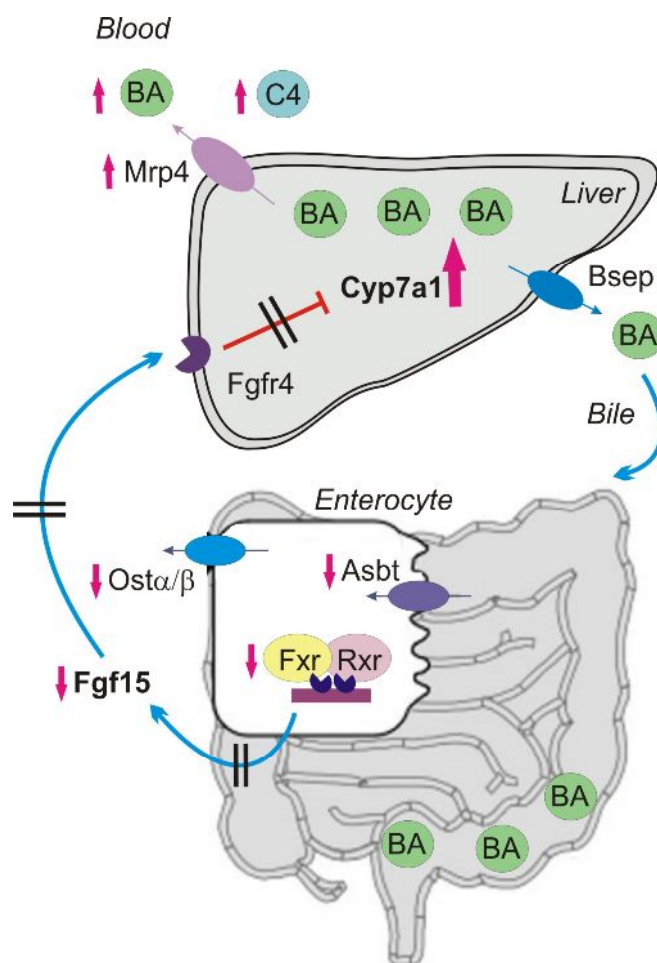
Figure 36. Schematic representation of the proposed mechanism of the EGCG effect on bile acid homeostasis in rats. EGCG-treated rats showed downregulation of the main bile acid transporters in the intestine, Asbt and Ostα. Consequently decreased reabsorption of bile acids in the ileum was documented by increased bile acid content in feces of EGCG-treated rats. On the other side, EGCG prevented transcription of Fgf15 within the enterocytes. Given that Fgf15 is to be transported to the liver to regulate basal Cyp7a1 gene expression by binding to its receptor Fgfr4, the lost of Fgf15 signaling caused disinhibition of Cyp7a1 transcription in the liver. Increased Cyp7a1 expression led to enhanced bile acid synthesis as evidenced by elevated plasma levels of C4. Since biliary bile acid excretion remained unchanged in EGCG-treated rats, the enhanced bile acid synthesis is a likely cause of raised plasma bile acids. Asbt, apical sodium-dependent transporter; BA, bile acid; Bsep, bile salt export pump; C4, 7α-hydroxy-4-cholesten-3-one; Cyp7a1, cholesterol 7α-hydroxylase; Fgf, fibroblast growth factor; Fgfr, fibroblast growth factor receptor; Fxr, farnesoid X receptor; Mrp, multidrug resistance-associated protein; Ost, organic solute transporter; Rxr, retinoid X receptor.

In parallel, EGCG effect was studied in ethinylestradiol-treated rats. Ethinylestradiol administration to rats is a well established model of intrahepatic cholestasis characteristic of decreased both BADF and BAIF of bile flow. In ethinylestradiol-treated rats, the lower BAIF has been attributed to a significant endocytic internalization of Mrp2 from the canalicular membrane into an intracellular compartment and further to reduced Mrp2 protein expression and reduced Mrp2 transport activity (Trauner et al., 1997; Mottino et al., 2002). BADF is likely diminished by trans-inhibition of Bsep after secretion of estrogen metabolites into bile

via Mrp2 (Stieger et al., 2000). Further, estrogens alter hepatocyte bile acid uptake by decreasing the expression of basolateral transporters such as Ntcp and Oatps (Simon et al., 1996; Geier et al., 2003). Molecular changes detected in ethinylestradiol-treated animals in this study fully complied with the current concept of this form of cholestasis (Geier et al., 2003; Fiorucci et al., 2005; Ruiz et al., 2007). Changes in the gene expression of bile acid transporters detected are summarized in Figure 37. It is of note that downregulation of ileal bile acid transporters in ethinylestradiol-treated rats has been described here for the first time and may signify a new adaptive mechanism in this type of cholestasis.

Under conditions of ethinylestradiol-induced cholestasis, EGCG showed only a tendency for further decrease in Mrp2 protein expression and biliary glutathione excretion, which was not reflected by a reduction in bile flow.
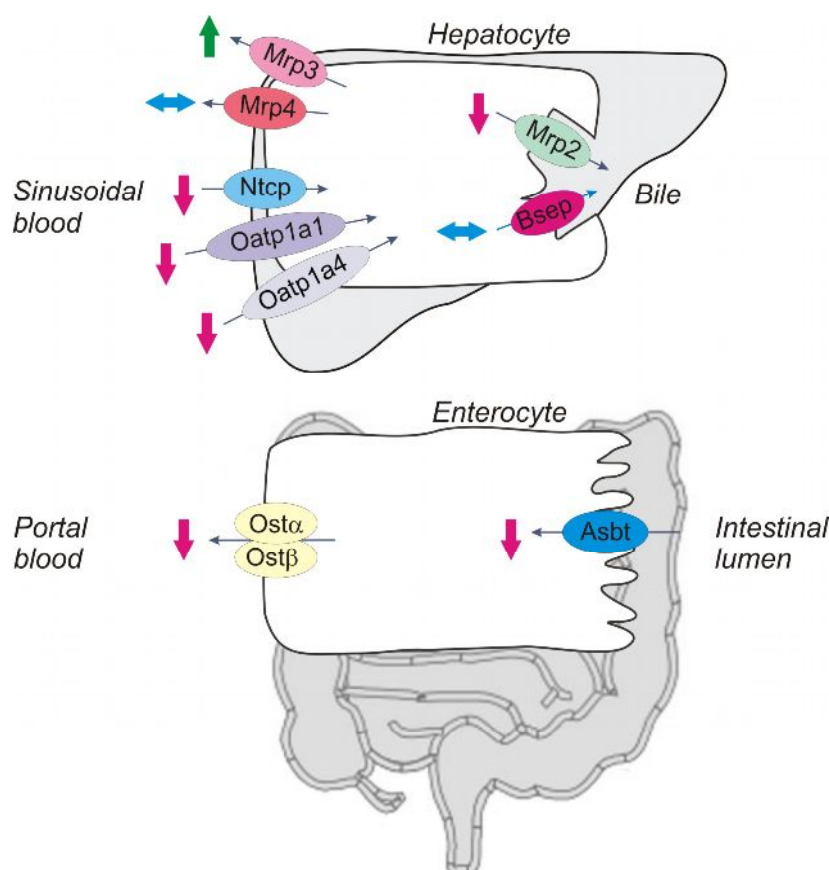
Figure 37. Schematic representation of changes in bile acid transporter gene expression in ethinylestradiol-treated rats. As an adaptive response to cholestasis, basolateral bile acid transporters are all downregulated to prevent accumulation of bile acids within the hepatocytes. Alternative basolateral efflux pumps may by upregulated to redirect bile acid elimination towards renal excretion. Expression of canalicular transporters Mrp2 and Bsep is not change at the mRNA level, but a variety of posttranscriptional mechanisms are involved in Mrp2 and Bsep decreased function. Downregulation of the main bile acid transporters in the ileum, Asbt and Ostα, was newly observed in ethinylestradiol-treated rats. Asbt, apical sodium-dependent transporter; Bsep, bile salt export pump; Mrp, multidrug resistance-associated protein; Ntcp, Na$^+$-taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; Ost, organic solute transporter.

5.2. Effect of EGCG on cholesterol metabolism

The present study investigated effect of EGCG on biliary cholesterol excretion and metabolism in the liver. The results showed that EGCG administration to rats significantly enhanced biliary cholesterol and phospholipid excretion. In addition, EGCG coadministration attenuated ethinylestradiol-induced liver cholesterol accumulation and liver weight increase.

Biliary excretion is a major route for eliminating cholesterol from the body (Dikkers and Tietge, 2010). The biliary cholesterol excretion rate is determined by both biliary bile acid secretion and activity of canalicular transporters ABCG5 and ABCG8. The importance of the ABCG5/8 complex in biliary cholesterol secretion was demonstrated by extremely low biliary cholesterol in Abcg5/8 knockout mice (Yu et al., 2002a). On the other hand, overexpression of human ABCG5/8 in mice promoted biliary cholesterol secretion (Yu et al., 2002b). In addition, it has been shown that expression of MDR2 is required for ABCG5/8-mediated biliary cholesterol secretion (Langheim et al., 2005). This is consistent with the former finding that biliary cholesterol excretion is dramatically decreased in Mdr2-deficient mice (Oude Elferink et al., 1995). Finally, biliary cholesterol excretion can be regulated by Sr-b1, which is a selective HDL cholesterol receptor (Wiersma et al., 2010). Since HDL cholesterol is the preferred source of biliary cholesterol, Sr-b1 knockout mice have biliary cholesterol excretion decreased nearly to 50% (Mardones et al., 2001).

In the present study, EGCG treatment doubled biliary cholesterol excretion in rats. In line with this finding, the expression of the crucial transporters Abcg5/8 was significantly increased by EGCG at both mRNA and protein levels. There was a modest increase in Sr-b1 protein too. Because biliary bile acid excretion remained unchanged in EGCG-treated rats, the data suggest that the upregulation of Abcg5/8 mediated the enhanced biliary cholesterol secretion in these rats.

Biliary cholesterol excretion may also be promoted by increased availability of free cholesterol within hepatocytes. This increased availability of free cholesterol can result from higher activity of HMG-CoA reductase, i.e., increase *de novo* cholesterol synthesis (Rigotti et al., 1989; Hooiveld et al., 1999) or inhibition of Acat2, i.e., decreased cholesterol esterification (Nervi et al., 1983; Nervi et al., 1984). In the present study, EGCG upregulated HMG-R mRNA but the protein levels remained unaltered. Given the unchanged HMG-CoA reductase protein, it can be speculated that increased cholesterol synthesis did not likely contribute to the enhanced biliary cholesterol secretion by EGCG. In contrast, EGCG-mediated induction of cholesterol biliary excretion strongly correlated with the decreased

expression of Acat2. The reduced expression of Acat2 and consequently reduced cholesterol esterification could transiently increase free cholesterol that is available for biliary secretion, as was previously demonstrated for progesterone and diosgenin (Nervi et al., 1983; Nervi et al., 1984). In accordance, EGCG treatment in rats decreased plasma levels of VLDL cholesterol, the production of which is also mediated by Acat2 (Shelness and Sellers, 2001) Since biliary cholesterol and VLDL cholesterol seem to have a common hepatic pool (Stone and Evans, 1992), the increased biliary loss of free cholesterol may compensate for the decreased VLDL production due to impaired Acat2 activity. As a result, the liver concentrations of free and esterified cholesterol were not finally affected by EGCG, but plasma VLDL cholesterol was significantly lowered in both groups receiving EGCG. In agreement, previous *in vitro* studies have reported that EGCG inhibited secretion of the essential VLDL protein, apolipoprotein B (Yee et al., 2002; Li et al., 2006; Goto et al., 2011).

EGCG-treated rats had a significantly higher biliary output of phospholipids compared to controls. Surprisingly, expression of Mdr2, the major phospholipid transporter, was not changed by EGCG. Thus, biliary phospholipid excretion may be coupled to the excretion of cholesterol in EGCG-treated rats, similarly as the overexpression of ABCG5/8 increased biliary phospholipids in mice (Yu et al., 2002b).

EGCG has exerted hypocholesterolemic effect in both preclinical and clinical settings (Zheng et al., 2011). In the present study, however, EGCG decreased plasma cholesterol significantly only in ethinylestradiol-treated animals, not in healthy rats. This is actually in accordance with several previous reports that detected cholesterol-lowering action of EGCG or green tea only when plasma cholesterol concentrations were altered by another treatment (Miura et al., 2001; Kaviarasan et al., 2008; Devika and Stanely Mainzen Prince, 2009).

Ethinylestradiol at the given dose is well known to cause liver cholesterol accumulation in rats (Rodriguez-Garay, 2003). This effect is primary mediated by substantially induced expression of LDL receptor and thus increased uptake of plasma LDL by hepatocyte (Bertolotti and Spady, 1996). In addition, in ethinylestradiol-treated rats plasma total cholesterol and triglycerides decline while newly secreted VLDL particles are enriched with esterified cholesterol (Nervi et al., 1984). Green tea has been shown to prevent cholesterol accumulation in the liver of rats on high-cholesterol diet (Muramatsu et al., 1986; Yang and Koo, 1997; Alshatwi et al., 2011). Similarly, EGCG in the present study attenuated liver cholesterol accumulation and liver weight increase induced by ethinylestradiol treatment. This preventive effect may be ascribed to the reduced Acat2 expression and to the consequent decrease in liver esterified cholesterol content and VLDL production. Reports on

biliary cholesterol excretion in ethinylestradiol-treated rats differ; both no change (Davis and Kern, 1976; Bonazzi et al., 1986) and decrease (Kamisako and Ogawa, 2003) in this parameter have been reported. In the present study, cholesterol excretion into bile was not altered by ethinylestradiol administration, although Abcg5 was significantly reduced. Elucidation of this discrepancy requires further investigation.

The mRNA data suggest that EGCG has ability to regulate expression of a variety of genes involved in cholesterol metabolism. However, what is the particular molecular mechanism of EGCG action remains an open question at this moment. Expression of the genes evaluated in the present study is regulated by several nuclear receptors and transcription factors. Among these, LXRα, SREBP1 and SREBP2 seem to play the key roles (Wagner et al., 2011). However, information on EGCG interaction with nuclear receptors is scarce. The ability of EGCG to modulate FXR activity is discussed above (Section 5.1.). EGCG has also been shown to modulate activity of transcription factor Nrf2 (Na and Surh, 2008). In the present study, an inverse pattern in the expression of key liver lipid regulators was observed compared to Nrf2 knockout mice (Huang et al., 2010), including 3-fold increase in Shp, a target gene of Nrf2. Nevertheless, the expression of Nqo1, another direct Nrf2 target, was not increased in EGCG-treated rats, suggesting that Nrf2 downstream target genes were not uniformly activated by EGCG. Kuhn et al. (2004) have shown that EGCG increases presence of the active form of SREBP2 via inhibition of its degradation by the ubiquitin-proteasome system in HepG2 cells. Similarly, in the present project, protein levels of Srebp2 active form were doubled in EGCG-treated rats compared to controls. However, from the other two known targets of Srebp2, only mRNA of HMG-CoA reductase was upregulated by EGCG, but not mRNA of LDL receptor. Further, EGCG strongly induced mRNA levels of Abcg5 and Abcg8, which are Lxrα target genes (Repa et al., 2002). Because other Lxrα targets, Abca1, Srebp1 and its downstream fatty acid synthase (Fasn), were not affected by EGCG in this study, it may be possible that Lrh-1 or Hnf4α contributed to Abcg5/8 upregulation (Freeman et al., 2004; Sumi et al., 2007). Taken together, the present findings indicate that the effect of EGCG on cholesterol metabolism is very complex and involves several regulatory pathways with a cross-talk between nuclear receptors and transcription factors. The proposed mechanism of EGCG action on cholesterol metabolism in the rat liver is depicted in Figure 38.
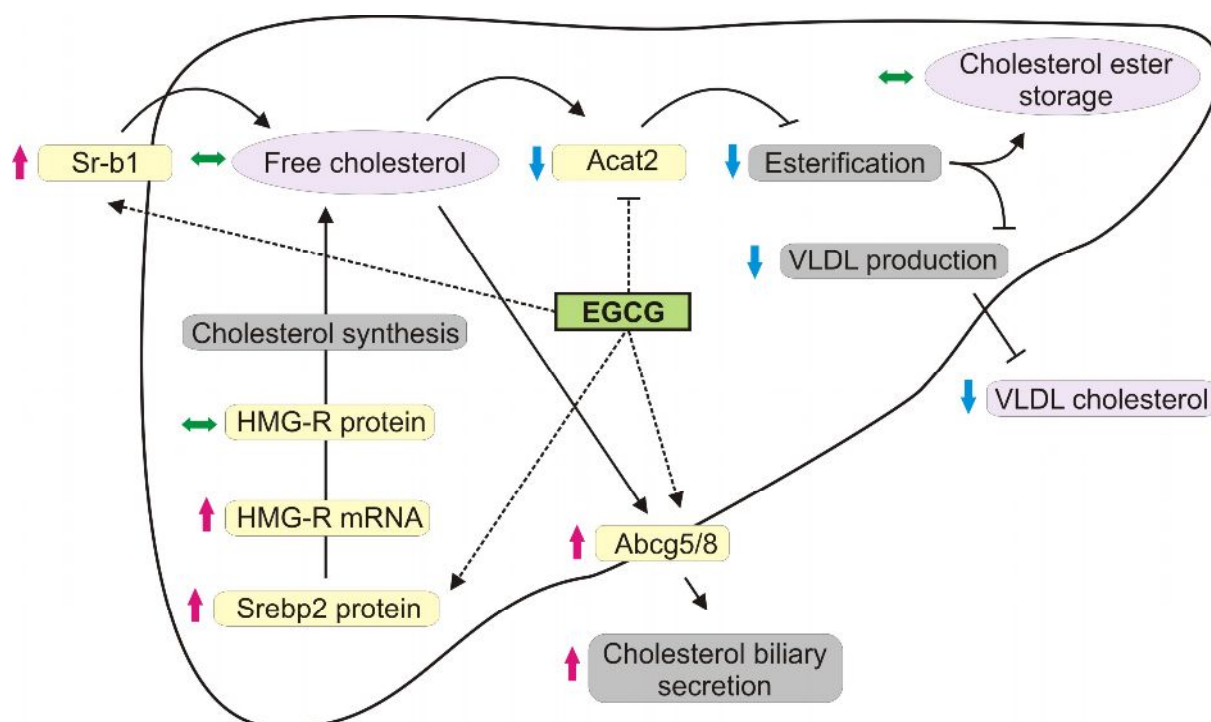
Figure 38. Schematic representation of the proposed mechanism of EGCG action on liver cholesterol metabolism in rats. EGCG induced protein expression of Sr-b1, which mediates selective uptake of HDL cholesterol, the main source of biliary cholesterol. On the contrary, EGCG decreased expression of Acat2, the enzyme responsible for hepatic cholesterol esterification. It is proposed that the EGCG-decreased Acat2 expression and consequently reduced cholesterol esterification diminished VLDL production and made excess free cholesterol available for biliary secretion. EGCG enhanced expression of the key biliary cholesterol transporters, Abcg5 and Abcg8, which together with higher free cholesterol availability promoted cholesterol excretion into bile. Although HMG-CoA reductase protein levels were not significantly changed by EGCG, both conversion of Srebp2 into its active form and expression of Srebp2 target gene were induced in EGCG-treated rats. Abcg, ATP-binding cassette transporter sub-family G; Acat2, acyl-coenzyme A:cholesterol acyltransferase 2; HMG-R, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase; Sr-b1, scavenger receptor class B type 1; Srebp, sterol element-binding protein; VLDL, very low density lipoprotein.

6. Conclusions

6.1. Effect of EGCG on bile formation and bile acid homeostasis

The present study has demonstrated that EGCG in rats raises plasma bile acid concentrations through Cyp7a1 upregulation and induces cholestasis by reduced Mrp2 protein expression and thus reduced biliary glutathione excretion.

While decreased intrabiliary pressure due to reduced bile formation may contribute to positive effects of EGCG in obstructive cholestasis, a potential for development of intrahepatic cholestasis by EGCG exists, which may caution against a long-term excessive use of EGCG-rich dietary supplements.

6.2. Effect of EGCG on cholesterol metabolism

This is the first study to show that EGCG supplementation significantly increases biliary cholesterol and phospholipid excretion. In addition, EGCG coadministration attenuates hepatic cholesterol accumulation and liver weight increase in ethinylestradiol-treated rats. These effects are attributable to upregulation of cholesterol transporters Abcg5/8 and Sr-b1 and to decreased expression of Acat2 by EGCG. The data provide new insights into mechanisms of EGCG action on cholesterol metabolism.

SUMMARY

Epigallocatechin gallate (EGCG), the major green tea catechin, has been shown to be protective in various experimental models of liver injury. Since its effect on biliary physiology and liver cholesterol homeostasis has not been thoroughly studied, the present study investigated effect of EGCG on bile flow, bile acid homeostasis and cholesterol metabolism in healthy and ethinylestradiol-treated rats. Compared to controls, EGCG treatment in rats decreased bile flow by 23%. Hepatic paracellular permeability and biliary bile acid excretion were not altered by EGCG administration, but biliary glutathione excretion was reduced by 70%. Accordingly, the main glutathione transporter at the hepatocyte canalicular membrane, multidrug resistance-associated protein 2 (Mrp2), was significantly decreased at the protein level. Interestingly, EGCG markedly enhanced biliary excretion of cholesterol and phospholipids. These changes tightly correlated with increased expression of ATP-binding cassette transporter G5 and G8 (Abcg5/8) and scavenger receptor class B type 1 and with decreased expression of acyl-CoA:cholesterol acyltransferase (Acat2). EGCG administration to rats also doubled plasma bile acid concentrations compared to controls. While protein expression of the main hepatic bile acid transporters was unchanged, the rate-limiting enzyme in the bile acid synthesis, Cyp7a1, was significantly increased by EGCG. Enhanced bile acid synthesis in these animals was also confirmed by a 2-fold increase in plasma marker of Cyp7a1 activity, 7α-hydroxy-4-cholesten-3-one. In contrast, EGCG markedly downregulated the major bile acid transporters (Asbt and Ostα) and regulatory molecules (Shp and Fgf15) in the ileum.

When EGCG was coadministered with ethinylestradiol, a potent cholestatic agent, it did not show any additional effect on the induced cholestasis but reduced plasma total cholesterol and VLDL cholesterol. Further, EGCG coadministration in ethinylestradiol-treated rats attenuated liver weight increase and liver cholesterol accumulation, which was linked with the corresponding reduction in Acat2 expression.

This study showed ability of EGCG to raise plasma bile acid concentrations, mainly through Cyp7a1 upregulation, and to decrease bile production through reduction in Mrp2-mediated bile flow. Despite the cholestatic effect, EGCG enhanced biliary cholesterol excretion and attenuated ethinylestradiol-induced liver cholesterol accumulation through Abcg5/8 upregulation and Acat2 reduction, respectively. Collectively, these data significantly contribute to the current knowledge of EGCG effect on bile acid and cholesterol homeostasis.

REFERENCES

Abe I, Seki T, Umehara K, Miyase T, Noguchi H, Sakakibara J and Ono T (2000) Green tea polyphenols: novel and potent inhibitors of squalene epoxidase. *Biochem Biophys Res Commun* 268:767-771.

Adachi R, Honma Y, Masuno H, Kawana K, Shimomura I, Yamada S and Makishima M (2005) Selective activation of vitamin D receptor by lithocholic acid acetate, a bile acid derivative. *J Lipid Res* 46:46-57.

Alshatwi AA, Al Obaaid MA, Al Sedairy SA, Ramesh E and Lei KY (2011) Black and green tea improves lipid profile and lipid peroxidation parameters in Wistar rats fed a high-cholesterol diet. *J Physiol Biochem* 67:95-104.

Axelson M, Bjorkhem I, Reihner E and Einarsson K (1991) The plasma level of 7 alpha-hydroxy-4-cholesten-3-one reflects the activity of hepatic cholesterol 7 alpha-hydroxylase in man. *FEBS Lett* 284:216-218.

Baghdasaryan A, Claudel T, Kosters A, Gumhold J, Silbert D, Thuringer A, Leski K, Fickert P, Karpen SJ and Trauner M (2010) Curcumin improves sclerosing cholangitis in Mdr2-/- mice by inhibition of cholangiocyte inflammatory response and portal myofibroblast proliferation. *Gut* 59:521-530.

Bertolotti M and Spady DK (1996) Effect of hypocholesterolemic doses of 17 alpha-ethinyl estradiol on cholesterol balance in liver and extrahepatic tissues. *J Lipid Res* 37:1812-1822.

Bonazzi P, Novelli G and Galeazzi R (1986) Effect of chenodeoxycholic acid on the alterations of biliary lipid secretion induced by ethynylestradiol in the rat. *Br J Exp Pathol* 67:341-348.

Bursill CA, Abbey M and Roach PD (2007) A green tea extract lowers plasma cholesterol by inhibiting cholesterol synthesis and upregulating the LDL receptor in the cholesterol-fed rabbit. *Atherosclerosis* 193:86-93.

Bursill CA and Roach PD (2006) Modulation of cholesterol metabolism by the green tea polyphenol (-)-epigallocatechin gallate in cultured human liver (HepG2) cells. *J Agric Food Chem* 54:1621-1626.

Bursill CA and Roach PD (2007) A green tea catechin extract upregulates the hepatic low-density lipoprotein receptor in rats. *Lipids* 42:621-627.

Chen JY, Levy-Wilson B, Goodart S and Cooper AD (2002) Mice expressing the human CYP7A1 gene in the mouse CYP7A1 knock-out background lack induction of CYP7A1 expression by cholesterol feeding and have increased hypercholesterolemia when fed a high fat diet. *J Biol Chem* 277:42588-42595.

Chen L, Lee MJ, Li H and Yang CS (1997) Absorption, distribution, elimination of tea polyphenols in rats. *Drug Metabolism and Disposition* 25:1045-1050.

Chiang JY (2009) Bile acids: regulation of synthesis. *J Lipid Res* 50:1955-1966.

Chow HH, Cai Y, Alberts DS, Hakim I, Dorr R, Shahi F, Crowell JA, Yang CS and Hara Y (2001) Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E. *Cancer Epidemiol Biomarkers Prev* 10:53-58.

Chow HH, Cai Y, Hakim IA, Crowell JA, Shahi F, Brooks CA, Dorr RT, Hara Y and Alberts DS (2003) Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals. *Clin Cancer Res* 9:3312-3319.

Chow HH, Hakim IA, Vining DR, Crowell JA, Ranger-Moore J, Chew WM, Celaya CA, Rodney SR, Hara Y and Alberts DS (2005) Effects of dosing condition on the oral bioavailability of green tea catechins after single-dose administration of Polyphenon E in healthy individuals. *Clin Cancer Res* 11:4627-4633.

Chu XY, Strauss JR, Mariano MA, Li J, Newton DJ, Cai X, Wang RW, Yabut J, Hartley DP, Evans DC and Evers R (2006) Characterization of mice lacking the multidrug resistance protein MRP2 (ABCC2). *Journal of Pharmacology and Experimental*

Therapeutics 317:579-589.

Crestani M, Stroup D and Chiang JY (1995) Hormonal regulation of the cholesterol 7 alpha-hydroxylase gene (CYP7). *J Lipid Res* 36:2419-2432.

Cuccioloni M, Mozzicafreddo M, Spina M, Tran CN, Falconi M, Eleuteri AM and Angeletti M (2011) Epigallocatechin-3-gallate potently inhibits the in vitro activity of hydroxy-3-methyl-glutaryl-CoA reductase. *J Lipid Res* 52:897-907.

Davis RA and Kern F, Jr. (1976) Effects of ethinyl estradiol and phenobarbital on bile acid synthesis and biliary bile acid and cholesterol excretion. *Gastroenterology* 70:1130-1135.

Denk GU, Soroka CJ, Takeyama Y, Chen WS, Schuetz JD and Boyer JL (2004) Multidrug resistance-associated protein 4 is up-regulated in liver but down-regulated in kidney in obstructive cholestasis in the rat. *J Hepatol* 40:585-591.

Deters M, Siegers C, Muhl P and Hansel W (1999) Choleretic effects of curcuminoids on an acute cyclosporin-induced cholestasis in the rat. *Planta Med* 65:610-613.

Devika PT and Stanely Mainzen Prince P (2009) Preventive effect of (-)epigallocatechin gallate on lipids, lipoproteins, and enzymes of lipid metabolism in isoproterenol-induced myocardial infarction in rats. *J Biochem Mol Toxicol* 23:387-393.

Dikkers A and Tietge UJ (2010) Biliary cholesterol secretion: more than a simple ABC. *World J Gastroenterol* 16:5936-5945.

Elias E, Iqbal S, Knutton S, Hickey A and Coleman R (1983) Increased tight junction permeability: a possible mechanism of oestrogen cholestasis. *Eur J Clin Invest* 13:383-390.

Esteller A (2008) Physiology of bile secretion. *World J Gastroenterol* 14:5641-5649.

Fiorucci S, Clerici C, Antonelli E, Orlandi S, Goodwin B, Sadeghpour BM, Sabatino G, Russo G, Castellani D, Willson TM, Pruzanski M, Pellicciari R and Morelli A (2005) Protective effects of 6-ethyl chenodeoxycholic acid, a farnesoid X receptor ligand, in estrogen-induced cholestasis. *Journal of Pharmacology and Experimental Therapeutics*

313:604-612.

Freeman LA, Kennedy A, Wu J, Bark S, Remaley AT, Santamarina-Fojo S and Brewer HB, Jr. (2004) The orphan nuclear receptor LRH-1 activates the ABCG5/ABCG8 intergenic promoter. *J Lipid Res* 45:1197-1206.

Frei B and Higdon JV (2003) Antioxidant activity of tea polyphenols in vivo: evidence from animal studies. *J Nutr* 133:3275S-3284S.

Frijters CM, Ottenhoff R, van Wijland MJ, van Nieuwkerk CM, Groen AK and Oude Elferink RP (1997) Regulation of mdr2 P-glycoprotein expression by bile salts. *Biochemical Journal* 321 (Pt 2):389-395.

Fukami M, Tanka A and Takikawa H (2011) Effect of penicillin G on the biliary excretion of cholephilic compounds in rats. *J Hepatobiliary Pancreat Sci* 18:684-688.

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

Galman C, Arvidsson I, Angelin B and Rudling M (2003) Monitoring hepatic cholesterol 7alpha-hydroxylase activity by assay of the stable bile acid intermediate 7alpha-hydroxy-4-cholesten-3-one in peripheral blood. *J Lipid Res* 44:859-866.

Geier A, Dietrich CG, Gerloff T, Haendly J, Kullak-Ublick GA, Stieger B, Meier PJ, Matern S and Gartung C (2003) Regulation of basolateral organic anion transporters in ethinylestradiol-induced cholestasis in the rat. *Biochim Biophys Acta* 1609:87-94.

Giakoustidis DE, Giakoustidis AE, Iliadis S, Koliakou K, Antoniadis N, Kontos N, Papanikolaou V, Papageorgiou G, Kaldrimidou E and Takoudas D (2010) Attenuation of liver ischemia/reperfusion induced apoptosis by epigallocatechin-3-gallate via down-regulation of NF-kappaB and c-Jun expression. *J Surg Res* 159:720-728.

Goodwin B, Watson MA, Kim H, Miao J, Kemper JK and Kliewer SA (2003) Differential regulation of rat and human CYP7A1 by the nuclear oxysterol receptor liver X receptor-alpha. *Mol Endocrinol* 17:386-394.

Goto H and Takikawa H (2010) Effect of genipin on cholestasis induced by estradiol-17beta-glucuronide and lithocholate-3-O-glucuornide in rats. *Hepatol Res* 40:524-529.

Goto T, Saito Y, Morikawa K, Kanamaru Y and Nagaoka S (2011) Epigallocatechin gallate changes mRNA expression level of genes involved in cholesterol metabolism in hepatocytes. *Br J Nutr*:1-5.

Gupta S, Todd Stravitz R, Pandak WM, Muller M, Reno Vlahcevic Z and Hylemon PB (2000) Regulation of multidrug resistance 2 P-glycoprotein expression by bile salts in rats and in primary cultures of rat hepatocytes. *Hepatology* 32:341-347.

Hagenbuch B and Meier PJ (1996) Sinusoidal (basolateral) bile salt uptake systems of hepatocytes. *Semin Liver Dis* 16:129-136.

Hagenbuch B and Meier PJ (2004) Organic anion transporting polypeptides of the OATP/ SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/functional properties. *Pflugers Arch* 447:653-665.

Hasegawa N, Yamda N and Mori M (2003) Powdered green tea has antilipogenic effect on Zucker rats fed a high-fat diet. *Phytotherapy Research* 17:477-480.

Hodgson JM and Croft KD (2010) Tea flavonoids and cardiovascular health. *Mol Aspects Med* 31:495-502.

Hong J, Lambert JD, Lee SH, Sinko PJ and Yang CS (2003) Involvement of multidrug resistance-associated proteins in regulating cellular levels of (-)-epigallocatechin-3-gallate and its methyl metabolites. *Biochem Biophys Res Commun* 310:222-227.

Hooiveld GJ, Vos TA, Scheffer GL, Van Goor H, Koning H, Bloks V, Loot AE, Meijer DK, Jansen PL, Kuipers F and Muller M (1999) 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) induce hepatic expression of the phospholipid translocase mdr2 in rats. *Gastroenterology* 117:678-687.

Huang J, Tabbi-Anneni I, Gunda V and Wang L (2010) Transcription factor Nrf2 regulates

SHP and lipogenic gene expression in hepatic lipid metabolism. *Am J Physiol Gastrointest Liver Physiol* 299:G1211-1221.

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.

Ikeda I, Imasato Y, Sasaki E, Nakayama M, Nagao H, Takeo T, Yayabe F and Sugano M (1992) Tea catechins decrease micellar solubility and intestinal absorption of cholesterol in rats. *Biochim Biophys Acta* 1127:141-146.

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 K, Koresawa T, Nakano K and Horie T (2004) Mrp2 is involved in benzylpenicillin-induced choleresis. *Am J Physiol Gastrointest Liver Physiol* 287:G42-49.

Ito K, Suzuki H, Hirohashi T, Kume K, Shimizu T and Sugiyama Y (1997) Molecular cloning of canalicular multispecific organic anion transporter defective in EHBR. *Am J Physiol* 272:G16-22.

Ito K, Suzuki H and Sugiyama Y (2001) Charged amino acids in the transmembrane domains are involved in the determination of the substrate specificity of rat Mrp2. *Mol Pharmacol* 59:1077-1085.

Jemnitz K, Heredi-Szabo K, Janossy J, Ioja E, Vereczkey L and Krajcsi P (2010) ABCC2/Abcc2: a multispecific transporter with dominant excretory functions. *Drug Metab Rev* 42:402-436.

Johnson DR and Klaassen CD (2002) Regulation of rat multidrug resistance protein 2 by classes of prototypical microsomal enzyme inducers that activate distinct transcription pathways. *Toxicol Sci* 67:182-189.

Jung D, Elferink MG, Stellaard F and Groothuis GM (2007) Analysis of bile acid-induced regulation of FXR target genes in human liver slices. *Liver International* 27:137-144.

Kamisako T and Ogawa H (2003) Regulation of biliary cholesterol secretion is associated with abcg5 and abcg8 expressions in the rats: effects of diosgenin and ethinyl estradiol. *Hepatol Res* 26:348-352.

Kan KS, Monte MJ, Parslow RA and Coleman R (1989) Oestradiol 17 beta-glucuronide increases tight-junctional permeability in rat liver. *Biochemical Journal* 261:297-300.

Kand'ar R, Zakova P, Lotkova H, Kucera O and Cervinkova Z (2007) Determination of reduced and oxidized glutathione in biological samples using liquid chromatography with fluorimetric detection. *J Pharm Biomed Anal* 43:1382-1387.

Kao YH, Hiipakka RA and Liao S (2000) Modulation of endocrine systems and food intake by green tea epigallocatechin gallate. *Endocrinology* 141:980-987.

Kaviarasan S, Viswanathan P, Ravichandran MK and Anuradha CV (2008) (-) Epigallocatechin Gallate (EGCG) Prevents Lipid Changes and Collagen Abnormalities in Chronic Ethanol-Fed Rats. *Toxicol Mech Methods* 18:425-432.

Keppler D (2011) Multidrug resistance proteins (MRPs, ABCCs): importance for pathophysiology and drug therapy. *Handb Exp Pharmacol*:299-323.

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 YW, Kang HE, Lee MG, Hwang SJ, Kim SC, Lee CH and Kim SG (2009) Liquiritigenin, a flavonoid aglycone from licorice, has a choleretic effect and the ability to induce hepatic transporters and phase-II enzymes. *Am J Physiol Gastrointest Liver Physiol* 296:G372-381.

Kobayashi H, Tanaka Y, Asagiri K, Asakawa T, Tanikawa K, Kage M and Yagi M (2010)

The antioxidant effect of green tea catechin ameliorates experimental liver injury. *Phytomedicine* 17:197-202.

Kobayashi M, Unno T, Suzuki Y, Nozawa A, Sagesaka Y, Kakuda T and Ikeda I (2005) Heat-epimerized tea catechins have the same cholesterol-lowering activity as green tea catechins in cholesterol-fed rats. *Biosci Biotechnol Biochem* 69:2455-2458.

Kong B, Wang L, Chiang JY, Zhang Y, Klaassen CD and Guo GL (2012) Mechanism of tissue-specific farnesoid X receptor in suppressing the expression of genes in bile-acid synthesis in mice. *Hepatology* 56:1034-1043.

Konig J (2011) Uptake transporters of the human OATP family: molecular characteristics, substrates, their role in drug-drug interactions, and functional consequences of polymorphisms. *Handb Exp Pharmacol*:1-28.

Konig J, Nies AT, Cui Y, Leier I and Keppler D (1999) Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. *Biochim Biophys Acta* 1461:377-394.

Kono S, Shinchi K, Ikeda N, Yanai F and Imanishi K (1992) Green tea consumption and serum lipid profiles: a cross-sectional study in northern Kyushu, Japan. *Prev Med* 21:526-531.

Kono S, Shinchi K, Wakabayashi K, Honjo S, Todoroki I, Sakurai Y, Imanishi K, Nishikawa H, Ogawa S and Katsurada M (1996) Relation of green tea consumption to serum lipids and lipoproteins in Japanese men. *J Epidemiol* 6:128-133.

Koo SI and Noh SK (2007) Green tea as inhibitor of the intestinal absorption of lipids: potential mechanism for its lipid-lowering effect. *J Nutr Biochem* 18:179-183.

Kosters A and Karpen SJ (2008) Bile acid transporters in health and disease. *Xenobiotica* 38:1043-1071.

Kozarsky KF, Donahee MH, Rigotti A, Iqbal SN, Edelman ER and Krieger M (1997)

Overexpression of the HDL receptor SR-BI alters plasma HDL and bile cholesterol levels. *Nature* 387:414-417.

Kuhn DJ, Burns AC, Kazi A and Dou QP (2004) Direct inhibition of the ubiquitin-proteasome pathway by ester bond-containing green tea polyphenols is associated with increased expression of sterol regulatory element-binding protein 2 and LDL receptor. *Biochim Biophys Acta* 1682:1-10.

Kuntz E and Kuntz HD: Hepatology (3rd edition), Springer Medizin Verlag Heidelberg 2008. ISBN 978-3-540-76838-8

Kuzu N, Bahcecioglu IH, Dagli AF, Ozercan IH, Ustundag B and Sahin K (2008) Epigallocatechin gallate attenuates experimental non-alcoholic steatohepatitis induced by high fat diet. *J Gastroenterol Hepatol* 23:e465-470.

Lambert JD, Lee MJ, Lu H, Meng X, Hong JJ, Seril DN, Sturgill MG and Yang CS (2003) Epigallocatechin-3-gallate is absorbed but extensively glucuronidated following oral administration to mice. *J Nutr* 133:4172-4177.

Lambert JD, Sang S and Yang CS (2007) Biotransformation of green tea polyphenols and the biological activities of those metabolites. *Mol Pharm* 4:819-825.

Langheim S, Yu L, von Bergmann K, Lutjohann D, Xu F, Hobbs HH and Cohen JC (2005) ABCG5 and ABCG8 require MDR2 for secretion of cholesterol into bile. *J Lipid Res* 46:1732-1738.

Lee MJ, Maliakal P, Chen L, Meng X, Bondoc FY, Prabhu S, Lambert G, Mohr S and Yang CS (2002) Pharmacokinetics of tea catechins after ingestion of green tea and (-)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability. *Cancer Epidemiol Biomarkers Prev* 11:1025-1032.

Lee MJ, Wang ZY, Li H, Chen L, Sun Y, Gobbo S, Balentine DA and Yang CS (1995) Analysis of plasma and urinary tea polyphenols in human subjects. *Cancer Epidemiol Biomarkers Prev* 4:393-399.

Lee MS, Park JY, Freake H, Kwun IS and Kim Y (2008) Green tea catechin enhances cholesterol 7alpha-hydroxylase gene expression in HepG2 cells. *Br J Nutr* 99:1182-1185.

Lee RG, Kelley KL, Sawyer JK, Farese RV, Jr., Parks JS and Rudel LL (2004) Plasma cholesteryl esters provided by lecithin:cholesterol acyltransferase and acyl-coenzyme a:cholesterol acyltransferase 2 have opposite atherosclerotic potential. *Circ Res* 95:998-1004.

Lenicek M, Juklova M, Zelenka J, Kovar J, Lukas M, Bortlik M and Vitek L (2008) Improved HPLC analysis of serum 7alpha-hydroxycholest-4-en-3-one, a marker of bile acid malabsorption. *Clin Chem* 54:1087-1088.

Li G, Lin W, Araya JJ, Chen T, Timmermann BN and Guo GL (2012) A tea catechin, epigallocatechin-3-gallate, is a unique modulator of the farnesoid X receptor. *Toxicol Appl Pharmacol* 258:268-274.

Li L, Stillemark-Billton P, Beck C, Bostrom P, Andersson L, Rutberg M, Ericsson J, Magnusson B, Marchesan D, Ljungberg A, Boren J and Olofsson SO (2006) Epigallocatechin gallate increases the formation of cytosolic lipid droplets and decreases the secretion of apoB-100 VLDL. *J Lipid Res* 47:67-77.

Li T, Owsley E, Matozel M, Hsu P, Novak CM and Chiang JY (2010) Transgenic expression of cholesterol 7alpha-hydroxylase in the liver prevents high-fat diet-induced obesity and insulin resistance in mice. *Hepatology* 52:678-690.

Lin BR, Yu CJ, Chen WC, Lee HS, Chang HM, Lee YC, Chien CT and Chen CF (2009) Green tea extract supplement reduces D-galactosamine-induced acute liver injury by inhibition of apoptotic and proinflammatory signaling. *J Biomed Sci* 16:35.

Lin LC, Wang MN, Tseng TY, Sung JS and Tsai TH (2007) Pharmacokinetics of (-)-epigallocatechin-3-gallate in conscious and freely moving rats and its brain regional distribution. *J Agric Food Chem* 55:1517-1524.

Lindor KD and Talwalkar JA: Cholestatic Liver Disease, Humana Press 2008.

ISBN 978-1-59745-118-5

Mardones P, Quinones V, Amigo L, Moreno M, Miquel JF, Schwarz M, Miettinen HE, Trigatti B, Krieger M, VanPatten S, Cohen DE and Rigotti A (2001) Hepatic cholesterol and bile acid metabolism and intestinal cholesterol absorption in scavenger receptor class B type I-deficient mice. *J Lipid Res* 42:170-180.

Mazzanti G, Menniti-Ippolito F, Moro PA, Cassetti F, Raschetti R, Santuccio C and Mastrangelo S (2009) Hepatotoxicity from green tea: a review of the literature and two unpublished cases. *Eur J Clin Pharmacol* 65:331-341.

Micuda S, Fuksa L, Brcakova E, Osterreicher J, Cermanova J, Cibicek N, Mokry J, Staud F and Martinkova J (2008) Zonation of multidrug resistance-associated protein 2 in rat liver after induction with dexamethasone. *J Gastroenterol Hepatol* 23:e225-230.

Miura Y, Chiba T, Tomita I, Koizumi H, Miura S, Umegaki K, Hara Y, Ikeda M and Tomita T (2001) Tea catechins prevent the development of atherosclerosis in apoprotein E-deficient mice. *J Nutr* 131:27-32.

Modica S, Petruzzelli M, Bellafante E, Murzilli S, Salvatore L, Celli N, Di Tullio G, Palasciano G, Moustafa T, Halilbasic E, Trauner M and Moschetta A (2012) Selective activation of nuclear bile acid receptor FXR in the intestine protects mice against cholestasis. *Gastroenterology* 142:355-365 e351-354.

Moore RJ, Jackson KG and Minihane AM (2009) Green tea (Camellia sinensis) catechins and vascular function. *Br J Nutr* 102:1790-1802.
Mottino AD, Cao J, Veggi LM, Crocenzi F, Roma MG and Vore M (2002) Altered localization and activity of canalicular Mrp2 in estradiol-17beta-D-glucuronide-induced cholestasis. *Hepatology* 35:1409-1419.

Muramatsu K, Fukuyo M and Hara Y (1986) Effect of green tea catechins on plasma cholesterol level in cholesterol-fed rats. *J Nutr Sci Vitaminol (Tokyo)* 32:613-622.

Murase T, Nagasawa A, Suzuki J, Hase T and Tokimitsu I (2002) Beneficial effects of tea

catechins on diet-induced obesity: stimulation of lipid catabolism in the liver. *Int J Obes Relat Metab Disord* 26:1459-1464.

Na HK and Surh YJ (2008) Modulation of Nrf2-mediated antioxidant and detoxifying enzyme induction by the green tea polyphenol EGCG. *Food Chem Toxicol* 46:1271-1278.

Nagle DG, Ferreira D and Zhou YD (2006) Epigallocatechin-3-gallate (EGCG): chemical and biomedical perspectives. *Phytochemistry* 67:1849-1855.

Nervi F, Bronfman M, Allalon W, Depiereux E and Del Pozo R (1984) Regulation of biliary cholesterol secretion in the rat. Role of hepatic cholesterol esterification. *Journal of Clinical Investigation* 74:2226-2237.

Nervi FO, Del Pozo R, Covarrubias CF and Ronco BO (1983) The effect of progesterone on the regulatory mechanisms of biliary cholesterol secretion in the rat. *Hepatology* 3:360-367.

Netsch MI, Gutmann H, Luescher S, Brill S, Schmidlin CB, Kreuter MH and Drewe J (2005) Inhibitory activity of a green tea extract and some of its constituents on multidrug resistance-associated protein 2 functionality. *Planta Med* 71:135-141.

Nies AT and Keppler D (2007) The apical conjugate efflux pump ABCC2 (MRP2). *Pflugers Arch* 453:643-659.

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

Oude Elferink RP and Beuers U (2011) Targeting the ABCB4 gene to control cholesterol homeostasis. *Expert Opin Ther Targets* 15:1173-1182.

Oude Elferink RP, Ottenhoff R, van Wijland M, Frijters CM, van Nieuwkerk C and Groen AK (1996) Uncoupling of biliary phospholipid and cholesterol secretion in mice with reduced expression of mdr2 P-glycoprotein. *J Lipid Res* 37:1065-1075.

Oude Elferink RP, Ottenhoff R, van Wijland M, Smit JJ, Schinkel AH and Groen AK (1995) Regulation of biliary lipid secretion by mdr2 P-glycoprotein in the mouse. *Journal*

of Clinical Investigation 95:31-38.

Oude Elferink RP, Paulusma CC and Groen AK (2006) Hepatocanalicular transport defects: pathophysiologic mechanisms of rare diseases. *Gastroenterology* 130:908-925.

Padda MS, Sanchez M, Akhtar AJ and Boyer JL (2011) Drug-induced cholestasis. *Hepatology* 53:1377-1387.

Paulusma CC, van Geer MA, Evers R, Heijn M, Ottenhoff R, Borst P and Oude Elferink RP (1999) Canalicular multispecific organic anion transporter/multidrug resistance protein 2 mediates low-affinity transport of reduced glutathione. *Biochemical Journal* 338 (Pt 2):393-401.

Peet DJ, Turley SD, Ma W, Janowski BA, Lobaccaro JM, Hammer RE and Mangelsdorf DJ (1998) Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. *Cell* 93:693-704.

Plosch T, Kok T, Bloks VW, Smit MJ, Havinga R, Chimini G, Groen AK and Kuipers F (2002) Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X receptor is independent of ABCA1. *J Biol Chem* 277:33870-33877.

Raederstorff DG, Schlachter MF, Elste V and Weber P (2003) Effect of EGCG on lipid absorption and plasma lipid levels in rats. *J Nutr Biochem* 14:326-332.

Rahner C, Stieger B and Landmann L (1996) Structure-function correlation of tight junctional impairment after intrahepatic and extrahepatic cholestasis in rat liver. *Gastroenterology* 110:1564-1578.

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.

Rigotti A, Marzolo MP, Ulloa N, Gonzalez O and Nervi F (1989) Effect of bean intake on biliary lipid secretion and on hepatic cholesterol metabolism in the rat. *J Lipid Res*

30:1041-1048.

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

Ruiz ML, Villanueva SS, Luquita MG, Ikushiro S, Mottino AD and Catania VA (2007) Beneficial effect of spironolactone administration on ethynylestradiol-induced cholestasis in the rat: involvement of up-regulation of multidrug resistance-associated protein 2. *Drug Metabolism and Disposition* 35:2060-2066.

Ruiz ML, Villanueva SS, Luquita MG, Sanchez-Pozzi EJ, Crocenzi FA, Pellegrino JM, Ochoa JE, Vore M, Mottino AD and Catania VA (2005) Mechanisms involved in spironolactone-induced choleresis in the rat. Role of multidrug resistance-associated protein 2. *Biochem Pharmacol* 69:531-539.

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

Sanchez-Muniz FJ and Bastida S (2008) Do not use the Friedewald formula to calculate LDL-cholesterol in hypercholesterolaemic rats. *European Journal of Lipid Science and Technology* 110:295-301.

Sarma DN, Barrett ML, Chavez ML, Gardiner P, Ko R, Mahady GB, Marles RJ, Pellicore LS, Giancaspro GI and Low Dog T (2008) Safety of green tea extracts : a systematic review by the US Pharmacopeia. *Drug Saf* 31:469-484.

Sasaki T, Ohta S, Kamogawa A and Shinoda M (1990) Protective effects of various Chinese traditional medicines against experimental cholestasis. *Chem Pharm Bull (Tokyo)* 38:513-516.

Shelness GS and Sellers JA (2001) Very-low-density lipoprotein assembly and secretion. *Curr Opin Lipidol* 12:151-157.

Shoda J, Miura T, Utsunomiya H, Oda K, Yamamoto M, Kano M, Ikegami T, Tanaka N, Akita H, Ito K, Suzuki H and Sugiyama Y (2004) Genipin enhances Mrp2 (Abcc2)-

mediated bile formation and organic anion transport in rat liver. *Hepatology* 39:167-178.

Simon FR, Fortune J, Iwahashi M, Gartung C, Wolkoff A and Sutherland E (1996) Ethinyl estradiol cholestasis involves alterations in expression of liver sinusoidal transporters. *Am J Physiol* 271:G1043-1052.

Singh BN, Shankar S and Srivastava RK (2011) Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. *Biochem Pharmacol* 82:1807-1821.

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.

Smith AJ, de Vree JM, Ottenhoff R, Oude Elferink RP, Schinkel AH and Borst P (1998) Hepatocyte-specific expression of the human MDR3 P-glycoprotein gene restores the biliary phosphatidylcholine excretion absent in Mdr2 (-/-) mice. *Hepatology* 28:530-536.

Song KH, Li T, Owsley E, Strom S and Chiang JY (2009) Bile acids activate fibroblast growth factor 19 signaling in human hepatocytes to inhibit cholesterol 7alpha-hydroxylase gene expression. *Hepatology* 49:297-305.

Soroka CJ, Ballatori N and Boyer JL (2010a) Organic solute transporter, OSTalpha-OSTbeta: its role in bile acid transport and cholestasis. *Semin Liver Dis* 30:178-185.

Soroka CJ, Mennone A, Hagey LR, Ballatori N and Boyer JL (2010b) Mouse organic solute transporter alpha deficiency enhances renal excretion of bile acids and attenuates cholestasis. *Hepatology* 51:181-190.

Soroka CJ, Velazquez H, Mennone A, Ballatori N and Boyer JL (2011) Ostalpha depletion protects liver from oral bile acid load. *Am J Physiol Gastrointest Liver Physiol* 301:G574-579.

Soroka CJ, Xu S, Mennone A, Lam P and Boyer JL (2008) N-Glycosylation of the alpha subunit does not influence trafficking or functional activity of the human organic solute transporter alpha/beta. *BMC Cell Biol* 9:57.

Speroni E, Cervellati R, Govoni P, Guizzardi S, Renzulli C and Guerra MC (2003) Efficacy of different Cynara scolymus preparations on liver complaints. *J Ethnopharmacol* 86:203-211.

Stensvold I, Tverdal A, Solvoll K and Foss OP (1992) Tea consumption. relationship to cholesterol, blood pressure, and coronary and total mortality. *Prev Med* 21:546-553.

Stickel F, Kessebohm K, Weimann R and Seitz HK (2011) Review of liver injury associated with dietary supplements. *Liver International* 31:595-605.

Stieger B (2010) Role of the bile salt export pump, BSEP, in acquired forms of cholestasis. *Drug Metab Rev* 42:437-445.

Stieger B (2011) The role of the sodium-taurocholate cotransporting polypeptide (NTCP) and of the bile salt export pump (BSEP) in physiology and pathophysiology of bile formation. *Handb Exp Pharmacol*:205-259.

Stieger B, Fattinger K, Madon J, Kullak-Ublick GA and Meier PJ (2000) Drug- and estrogen-induced cholestasis through inhibition of the hepatocellular bile salt export pump (Bsep) of rat liver. *Gastroenterology* 118:422-430.

Stone BG and Evans CD (1992) Evidence for a common biliary cholesterol and VLDL cholesterol precursor pool in rat liver. *J Lipid Res* 33:1665-1675.

Sumi K, Tanaka T, Uchida A, Magoori K, Urashima Y, Ohashi R, Ohguchi H, Okamura M, Kudo H, Daigo K, Maejima T, Kojima N, Sakakibara I, Jiang S, Hasegawa G, Kim I, Osborne TF, Naito M, Gonzalez FJ, Hamakubo T, Kodama T and Sakai J (2007) Cooperative interaction between hepatocyte nuclear factor 4 alpha and GATA transcription factors regulates ATP-binding cassette sterol transporters ABCG5 and ABCG8. *Mol Cell Biol* 27:4248-4260.

Thomas PJ and Hofmann AF (1973) Letter: A simple calculation of the lithogenic index of bile: expressing biliary lipid composition on rectangular coordinates. *Gastroenterology* 65:698-700.

Tipoe GL, Leung TM, Liong EC, Lau TY, Fung ML and Nanji AA (2010) Epigallocatechin-3-gallate (EGCG) reduces liver inflammation, oxidative stress and fibrosis in carbon tetrachloride (CCl4)-induced liver injury in mice. *Toxicology* 273:45-52.

Tokunaga S, White IR, Frost C, Tanaka K, Kono S, Tokudome S, Akamatsu T, Moriyama T and Zakouji H (2002) Green tea consumption and serum lipids and lipoproteins in a population of healthy workers in Japan. *Annals of Epidemiology* 12:157-165.

Tomsik P, Sispera L, Rezacova M, Niang M, Stoklasova A, Cerman J, Knizek J, Brcakova E, Cermanova J and Micuda S (2008) Increased melibiose/rhamnose ratio in bile of rats with acute cholestasis. *J Gastroenterol Hepatol* 23:1934-1940.

Trauner M, Arrese M, Soroka CJ, Ananthanarayanan M, Koeppel TA, Schlosser SF, Suchy FJ, Keppler D and Boyer JL (1997) The rat canalicular conjugate export pump (Mrp2) is down-regulated in intrahepatic and obstructive cholestasis. *Gastroenterology* 113:255-264.

Trauner M, Baghdasaryan A, Claudel T, Fickert P, Halilbasic E, Moustafa T and Zollner G (2011) Targeting nuclear bile acid receptors for liver disease. *Dig Dis* 29:98-102.

Trauner M and Boyer JL (2003) Bile salt transporters: molecular characterization, function, and regulation. *Physiol Rev* 83:633-671.

Van Amelsvoort JM, Van Hof KH, Mathot JN, Mulder TP, Wiersma A and Tijburg LB (2001) Plasma concentrations of individual tea catechins after a single oral dose in humans. *Xenobiotica* 31:891-901.

Venneman NG and van Erpecum KJ (2010) Pathogenesis of gallstones. *Gastroenterol Clin North Am* 39:171-183, vii.

Wagner M, Zollner G and Trauner M (2010) Nuclear receptor regulation of the adaptive

response of bile acid transporters in cholestasis. *Semin Liver Dis* 30:160-177.

Wagner M, Zollner G and Trauner M (2011) Nuclear receptors in liver disease. *Hepatology* 53:1023-1034.

Wang S, Noh SK and Koo SI (2006) Epigallocatechin gallate and caffeine differentially inhibit the intestinal absorption of cholesterol and fat in ovariectomized rats. *J Nutr* 136:2791-2796.

Wiersma H, Gatti A, Nijstad N, Kuipers F and Tietge UJ (2009) Hepatic SR-BI, not endothelial lipase, expression determines biliary cholesterol secretion in mice. *J Lipid Res* 50:1571-1580.

Wiersma H, Nijstad N, Gautier T, Iqbal J, Kuipers F, Hussain MM and Tietge UJ (2010) Scavenger receptor BI facilitates hepatic very low density lipoprotein production in mice. *J Lipid Res* 51:544-553.

Wolfram S, Raederstorff D, Preller M, Wang Y, Teixeira SR, Riegger C and Weber P (2006) Epigallocatechin gallate supplementation alleviates diabetes in rodents. *J Nutr* 136:2512-2518.

Yamanashi Y, Takada T, Yoshikado T, Shoda J and Suzuki H (2011) NPC2 regulates biliary cholesterol secretion via stimulation of ABCG5/G8-mediated cholesterol transport. *Gastroenterology* 140:1664-1674.

Yang CS, Wang X, Lu G and Picinich SC (2009) Cancer prevention by tea: animal studies, molecular mechanisms and human relevance. *Nat Rev Cancer* 9:429-439.

Yang TT and Koo MW (1997) Hypocholesterolemic effects of Chinese tea. *Pharmacol Res* 35:505-512.

Yang TT and Koo MW (2000) Chinese green tea lowers cholesterol level through an increase in fecal lipid excretion. *Life Sci* 66:411-423.

Yasuda Y, Shimizu M, Sakai H, Iwasa J, Kubota M, Adachi S, Osawa Y, Tsurumi H, Hara Y and Moriwaki H (2009) (-)-Epigallocatechin gallate prevents carbon tetrachloride-induced rat hepatic fibrosis by inhibiting the expression of the PDGFRbeta and IGF-1R. *Chem Biol Interact* 182:159-164.

Yee WL, Wang Q, Agdinaoay T, Dang K, Chang H, Grandinetti A, Franke AA and Theriault A (2002) Green tea catechins decrease apolipoprotein B-100 secretion from HepG2 cells. *Mol Cell Biochem* 229:85-92.

Yu L, Hammer RE, Li-Hawkins J, Von Bergmann K, Lutjohann D, Cohen JC and Hobbs HH (2002a) Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion. *Proc Natl Acad Sci U S A* 99:16237-16242.

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

Zaveri NT (2006) Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications. *Life Sci* 78:2073-2080.

Zheng XX, Xu YL, Li SH, Liu XX, Hui R and Huang XH (2011) Green tea intake lowers fasting serum total and LDL cholesterol in adults: a meta-analysis of 14 randomized controlled trials. *American Journal of Clinical Nutrition* 94:601-610.

Zhong Z, Froh M, Lehnert M, Schoonhoven R, Yang L, Lind H, Lemasters JJ and Thurman RG (2003) Polyphenols from Camellia sinenesis attenuate experimental cholestasis-induced liver fibrosis in rats. *Am J Physiol Gastrointest Liver Physiol* 285:G1004-1013.

Zhu Y, Li F and Guo GL (2011) Tissue-specific function of farnesoid X receptor in liver and intestine. *Pharmacol Res* 63:259-265.

Zinchuk V, Zinchuk O and Okada T (2005) Experimental LPS-induced cholestasis alters

subcellular distribution and affects colocalization of Mrp2 and Bsep proteins: a quantitative colocalization study. *Microsc Res Tech* 67:65-70.

Zollner G and Trauner M (2008) Mechanisms of cholestasis. *Clin Liver Dis* 12:1-26, vii.

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

APPENDIX I

Hirsova P, Kolouchova G, Dolezelova E, Cermanova J, Hyspler R, Kadova Z, Micuda S (2012) Epigallocatechin gallate enhances biliary cholesterol secretion in healthy rats and lowers plasma and liver cholesterol in ethinylestradiol-treated rats. *Eur J Pharmacol* 691(1-3):38-45.

Molecular and cellular pharmacology

Epigallocatechin gallate enhances biliary cholesterol secretion in healthy rats and lowers plasma and liver cholesterol in ethinylestradiol-treated rats

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ARTICLE INFO

ABSTRACT

The beneficial effect of the major green tea catechin, epigallocatechin gallate (EGCG), on cholesterol homeostasis has been studied mainly in relation to the intestinal absorption of cholesterol; however, how EGCG affects cholesterol metabolism in the liver is not entirely known. The present study investigated the effect of EGCG on liver cholesterol metabolism in healthy and ethinylestradiol-treated rats. EGCG treatment reduced plasma total cholesterol in ethinylestradiol-treated animals and very low density lipoprotein cholesterol in both groups receiving EGCG. In healthy rats, despite the decrease in bile flow, EGCG markedly enhanced biliary secretion of cholesterol and phospholipids. These changes were correlated with increased expression of ATP-binding cassette transporter G5 and G8 and scavenger receptor class B type 1, and decreased expression of acyl-CoA:cholesterol acyltransferase. Ethinylestradiol treatment caused marked hepatic cholesterol accumulation with a concomitant liver weight increase and plasma cholesterol reduction. In ethinylestradiol-treated rats, EGCG co-administration attenuated the increase in liver cholesterol and liver weight. Furthermore, EGCG blunted induction of acyl-CoA:cholesterol acyltransferase and raised reduced levels of ATP-binding cassette transporter G5 and G8 and 3-hydroxy-3-methyl-glutaryl-CoA reductase in ethinylestradiol-treated rats. In conclusion, this study has demonstrated for the first time the ability of EGCG to enhance biliary cholesterol secretion and to attenuate ethinylestradiol-induced liver cholesterol accumulation. Changes in the expression of relevant enzymes and transporters suggest evidence of another mechanism that may contribute to the overall effect of EGCG on cholesterol metabolism and imply new physiological consequences of this widely used compound.

1. Introduction

Green tea is one of the most popular beverages consumed worldwide. Drinking green tea has been associated with a variety of potential health benefits; among these, the positive effect on plasma lipoprotein cholesterol profile has attracted a considerable attention. Several animal studies reported that green tea or its extract reduced plasma cholesterol concentrations (Hasegawa et al., 2003; Chan et al., 1999; Murase et al., 2002) and liver cholesterol accumulation (Alshatwi et al., 2011; Muramatsu et al., 1986; Yang and Koo, 1997) during high fat or high cholesterol diet. Clinical trials and epidemiologic studies have confirmed the hypocholesterolemic effect of green tea also in humans (Kono et al., 1996; Tokunaga et al., 2002). A recent meta-analysis of 14 randomized controlled trials found that green tea or its extract significantly decreases plasma total cholesterol and low density lipoprotein (LDL) cholesterol (Zheng et al., 2011).

Polyphenolic compounds, known as catechins, have been suggested to be responsible for the health promoting effects of green tea. Epigallocatechin gallate (EGCG) is the most abundant of these catechins and is generally considered to be the major active component of the plant (Nagle et al., 2006; Wolfram et al., 2006). Regarding cholesterol-lowering effect, EGCG indeed proved its ability to attenuate a diet-induced hypercholesterolemia and cholesterol accumulation in the liver (Raederstorff et al., 2003). Ikeda et al. (1992) found that EGCG interferes with micellar solubilization of cholesterol in the intestine and thus decreases cholesterol absorption, as evidenced by later studies (Raederstorff et al., 2003; Wang et al., 2006). Hence, it is generally accepted that EGCG exerts its hypocholesterolemic effect through decreased intestinal absorption of cholesterol and its increased fecal excretion (Koo and Noh, 2007). On the other hand, several recent

in vitro studies suggested that EGCG may also affect cholesterol metabolism directly in the liver. Goto et al. (2011) showed that EGCG treatment altered expression of a number of genes related to cholesterol metabolism in human hepatoma cells; the strongest effect of EGCG was upregulation of LDL receptor. Another study demonstrated an increased LDL receptor binding activity and protein mass in hepatoma cells incubated with EGCG (Bursill and Roach, 2006). EGCG was also shown as a potent in vitro inhibitor of squalene epoxidase (Abe et al., 2000) and 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase (Cuccioloni et al., 2011), two important enzymes in the cholesterol biosynthetic pathway.

Taken together, the beneficial effect of EGCG on cholesterol metabolism may be mediated primarily by inhibition of intestinal cholesterol absorption, although a growing body of in vitro evidence suggests that EGCG may modulate cholesterol metabolism also in the liver. Nevertheless, a detailed in vivo study with focus on the liver is lacking. Therefore, the present study examines the effect of EGCG on liver cholesterol metabolism and biliary secretion, the major route of cholesterol excretion from the body. Additionally, the effect of EGCG was assessed in a pathophysiological condition in ethinylestradiol-treated rats, which are a well known animal model of both impaired bile secretion (Rodriguez-Garay, 2003) and hepatic cholesterol accumulation (Hayashi et al., 1986).

2. Material and methods

2.1. Reagents

EGCG and ethinylestradiol were purchased from Cayman Chemical and Sigma-Aldrich, respectively. All other reagents and supplies were obtained from Sigma-Aldrich and Bio-Rad Laboratories.

2.2. Animals and experimental design

All animal experimental protocols were approved by the Animal Research Committee of the Faculty of Medicine in Hradec Kralove, Charles University in Prague. Female Wistar rats (200–250 g) were obtained from Anlab (Prague, Czech Republic) and were housed under controlled temperature and light conditions, on a natural 12 h light–dark cycle. Animals had free access to food and water throughout the experiment, but were fasted overnight before sacrifice. Rats were randomly divided into four groups, namely:

1. Control rats (Ctrl), receiving propylene glycol (vehicle of ethinylestradiol) s.c. for five consecutive days, and saline (vehicle of EGCG) i.p. for eight consecutive days.
2. EGCG-treated rats, receiving daily propylene glycol s.c. for 5 consecutive days and EGCG (50 mg/kg bw) i.p. for 8 days.
3. Ethinylestradiol (EE)-treated rats, which were administered daily with ethinylestradiol (5 mg/kg bw) s.c. for 5 consecutive days and saline i.p. for 8 days.
4. EE–EGCG rats, which were co-administered with ethinylestradiol (5 mg/kg bw) s.c. for 5 days and EGCG (50 mg/kg bw, i.p.) for 8 days.

EGCG (or saline) administration to rats began three days before the start of ethinylestradiol (or propylene glycol) administration. If we extrapolate data from Kao et al. (2000), the dose of 50 mg/kg EGCG i.p. may result in plasma concentrations of 1 µmol/l after 1 h and peak plasma concentrations of 2 µmol/l after 2 h. The concentration of 1 µmol/l is a similar concentration achievable in human plasma either after drinking ∼8 cups of green tea or after ingestion of EGCG-rich extracts (Yang et al., 2009). The intraperitoneal route of administration may, by bypassing the intestine, secure a direct effect of EGCG on the liver. Moreover, it may compensate for the low and highly variable bioavailability of EGCG in rats (Lambert et al., 2003).

Bile collection was performed on the ninth day, i.e., one day after the last EGCG or ethinylestradiol dose, as previously described (Kolouchova et al., 2011b). At the end of bile collection, animals were sacrificed by exsanguination and the livers were harvested, weighed, snap frozen and stored at −80 °C until use. Bile flow was determined by gravimetry, assuming a bile density of 1.0 g/ml.

2.3. Plasma and biliary lipids and bile acids

Plasma triglycerides, total cholesterol, high density lipoprotein (HDL) cholesterol, and LDL cholesterol were determined by routine laboratory methods on a Hitachi automatic analyzer. Since HDL and LDL cholesterol were measured, we could calculate concentrations of VLDL cholesterol by subtracting HDL and LDL cholesterol from total cholesterol (Sanchez-Muniz and Bastida, 2008). Biliary concentrations of cholesterol and phospholipids were assessed by commercially available kits Cholesterol Liquid 500 (Pliva-Lachema Diagnostika) and Phosphatidylcholine Assay Kit (Cayman Chemical), respectively. Bile acids were assayed using a commercial kit from Diazyme.

2.4. Hepatic lipids

Liver cholesterol and triglyceride concentrations were determined by commercial kits Cholesterol Assay Kit (Cayman Chemical) and Triglycerides 250 S (Pliva-Lachema Diagnostika), respectively, after extraction of hepatic lipids with chloroform–methanol 2:1 according to Lee et al. (2004). Esterified cholesterol concentrations were calculated by subtracting free cholesterol from total cholesterol.

2.5. Real-time qRT-PCR

Gene expression was examined as previously described (Kolouchova et al., 2011b), with the exception that total RNA from liver tissue was isolated wit RNeasy Mini Kit (Qiagen). TaqMan Fast Universal PCR Master Mix and pre-designed TaqMan Gene Expression Assay kits (Supplemental Table 1) were from Applied Biosystems and HMG-CoA reductase primers and probe were purchased from Generi Biotech, Czech Republic. Glyceraldehyde 3-phosphate dehydrogenase was used as reference for normalizing the data.

2.6. Western blot

Protein expression was determined in whole-cell lysate prepared as previously described (Kolouchova et al., 2011a). Proteins (100 µg) were separated by SDS-PAGE, transferred to a PVDF membrane (Millipore) and incubated with appropriate antibodies (Supplemental Table 2). Horseradish peroxidase-conjugated secondary antibodies were from GE Healthcare and enhanced chemiluminescence reagents were from Thermo Pierce. Densitometry was performed using ScanMaker i900 (UMAX) and QuantityOne imaging software (BioRad). Equal protein loading was confirmed by immunodetection of beta-actin.

2.7. Statistical analysis

Experiments were carried out on 6–7 animals per group. Data are presented as means ± S.E.M. Data sets were analyzed by one-way analysis of variance, and individual group means were compared with Student's unpaired t test. Pearson correlation coefficients were calculated. Significance was assigned at $P < 0.05$. All statistical analyses were conducted using GraphPad Prism 5.0 software.

3. Results

3.1. Plasma lipids

EGCG treatment alone did not significantly affect plasma lipid levels, except for a reduction in VLDL cholesterol ($P < 0.05$) (Table 1). Ethinylestradiol administration lowered plasma triglycerides ($P < 0.05$), total cholesterol, HDL cholesterol ($P < 0.001$) and LDL cholesterol, while increased VLDL cholesterol fraction ($P < 0.05$). EGCG in combination with ethinylestradiol further reduced total cholesterol ($P < 0.01$) due to a decrease in plasma VLDL cholesterol ($P < 0.001$), with no change in plasma HDL cholesterol compared with ethinylestradiol-only administration. EGCG also further lowered ethinylestradiol-reduced plasma levels of triglycerides ($P < 0.05$).

3.2. Liver lipids

A moderate decline in liver triglycerides was observed in EGCG-treated rats ($P < 0.05$), whereas no such change occurred when EGCG was co-administered with ethinylestradiol (Table 2). EGCG treatment alone affected neither liver cholesterol concentration nor liver weight. On the contrary, ethinylestradiol

treatment substantially increased liver weight ($P < 0.001$) and liver cholesterol content, inducing hepatic cholesterol levels by ~50% compared to controls ($P < 0.001$). This increment in hepatic cholesterol is attributable to the elevated concentration of esterified cholesterol in the liver of ethinylestradiol-treated rats. When co-administered with ethinylestradiol, EGCG partially prevented the ethinylestradiol-induced cholesterol accumulation and liver weight increase by lowering hepatic esterified cholesterol concentrations. In addition, cholesterol content per liver was tightly correlated with liver weight ($r = 0.89$, $P < 0.0001$) and with both gene and protein expression of acyl-CoA:cholesterol acyl-transferase (ACAT2) ($r = 0.82$, $P < 0.001$, $r = 0.71$, $P < 0.001$, respectively), the principal enzyme responsible for cholesterol esterification in the liver (Supplemental Table 3).

3.3. Bile flow and biliary secretion of cholesterol and phospholipids

EGCG group had a substantially higher output of biliary cholesterol (by 84%) and phospholipids (by 50%) compared to controls ($P < 0.001$) (Table 3). In contrast, bile flow rate of EGCG-treated rats was lower than that of control animals ($P < 0.05$). Ethinylestradiol administration to rats diminished bile flow by ~60% regardless of EGCG treatment ($P < 0.001$) as the effect of EE prevailed over EGCG. Biliary secretion of cholesterol and phospholipids in groups receiving ethinylestradiol or ethinylestradiol plus EGCG did not differ from control values.

3.4. Expression of genes involved in liver cholesterol transport and metabolism

To gain greater insight into the molecular mechanisms underlying effects of EGCG on cholesterol homeostasis, we evaluated expression of a range of genes playing key roles in liver cholesterol synthesis, uptake, esterification, and secretion. While mRNA levels of LDL receptor were not altered by EGCG treatment, gene expression of scavenger receptor class B type 1 (SR-B1), which mediates selective uptake of HDL cholesterol, and ATP-binding cassette transporter A1 (ABCA1), the main cholesterol efflux transporter, were down-regulated by 30% and 60% ($P < 0.01$), respectively, compared to controls (Fig. 1A). EGCG did not significantly affect ACAT2 gene expression, but induced HMG-CoA reductase mRNA by 60% ($P < 0.05$) compared to controls. Most strikingly, EGCG-treated rats exhibited a > 5-fold induction of gene expression of the two ATP-binding cassette transporters of sub-family G, ABCG5 and ABCG8, that function as a heterodimeric complex transporting cholesterol from hepatocytes into the bile.

In livers of ethinylestradiol-treated rats, mRNA abundance of SR-B1, ABCA1, HMG-CoA reductase, ABCG5, and ABCG8 was significantly reduced while that of LDL receptor and ACAT2 was markedly induced (Fig. 1A).

Table 1
Plasma lipid concentrations.

	Ctrl	EGCG	EE	EE–EGCG
	mmol/l			
Triglycerides	0.69 ± 0.07	0.87 ± 0.09	0.39 ± 0.08[b]	0.21 ± 0.02[a,e]
Total cholesterol	1.25 ± 0.10	1.11 ± 0.06	0.41 ± 0.05[a]	0.16 ± 0.04[a,d]
HDL cholesterol	0.87 ± 0.07	0.83 ± 0.07	0.04 ± 0.01[a]	0.03 ± 0.0[a]
LDL cholesterol	0.11 ± 0.02	0.08 ± 0.02	ND	ND
VLDL cholesterol	0.27 ± 0.03	0.20 ± 0.02[b]	0.40 ± 0.03[b]	0.15 ± 0.04[b,c]

Data are presented as means ± S.E.M., n=6–7.

[a] $P < 0.001$ when compared to control group.
[b] $P < 0.05$ when compared to control group.
[c] $P < 0.001$ when compared to control group.
[d] $P < 0.01$ when compared to control group.
[e] $P < 0.05$ when compared to EE-treated group. ND—not detected in the majority of the samples.

Table 2
Liver weight and liver lipid concentrations.

	Ctrl	EGCG	EE	EE–EGCG
Liver weight (g)	8.3 ± 0.2	8.3 ± 0.4	10.4 ± 0.2[a]	9.6 ± 0.4[b,d]
Triglycerides (μmol/g liver)	1.29 ± 0.09	0.88 ± 0.04[b]	1.19 ± 0.06	1.14 ± 0.04
Total cholesterol (μmol/g liver)	4.3 ± 0.3	4.2 ± 0.3	6.5 ± 0.3[a]	5.7 ± 0.2[b,d]
Free cholesterol (μmol/g liver)	3.4 ± 0.2	3.3 ± 0.03	3.8 ± 0.2	3.6 ± 0.1
Esterified cholesterol (μmol/g liver)	0.9 ± 0.3	1.0 ± 0.3	2.7 ± 0.3[a]	2.1 ± 0.2[b,c]

Data are presented as means ± S.E.M., n=6–7.

[a] $P < 0.001$ when compared to control group.
[b] $P < 0.01$ when compared to control group.
[c] $P < 0.01$ when compared to control group.
[d] $P < 0.05$ when compared to EE-treated group.

Table 3
Bile flow and biliary output of cholesterol and phospholipids.

	Ctrl	EGCG	EE	EE–EGCG
Bile flow (μl/min)	11.7 ± 0.7	9.1 ± 0.8[b]	4.9 ± 0.6[a]	5.0 ± 0.8[a]
Cholesterol output (nmol/min)	3.8 ± 0.6	7.0 ± 0.3[a]	4.3 ± 0.6	3.6 ± 0.5
Phospholipid output (nmol/min)	46.2 ± 3.3	69.5 ± 3.4[a]	39.7 ± 3.4	43.2 ± 2.5

Data are presented as means ± S.E.M., $n = 6-7$.

[a] $P < 0.001$ when compared to control group.
[b] $P < 0.05$ when compared to control group.
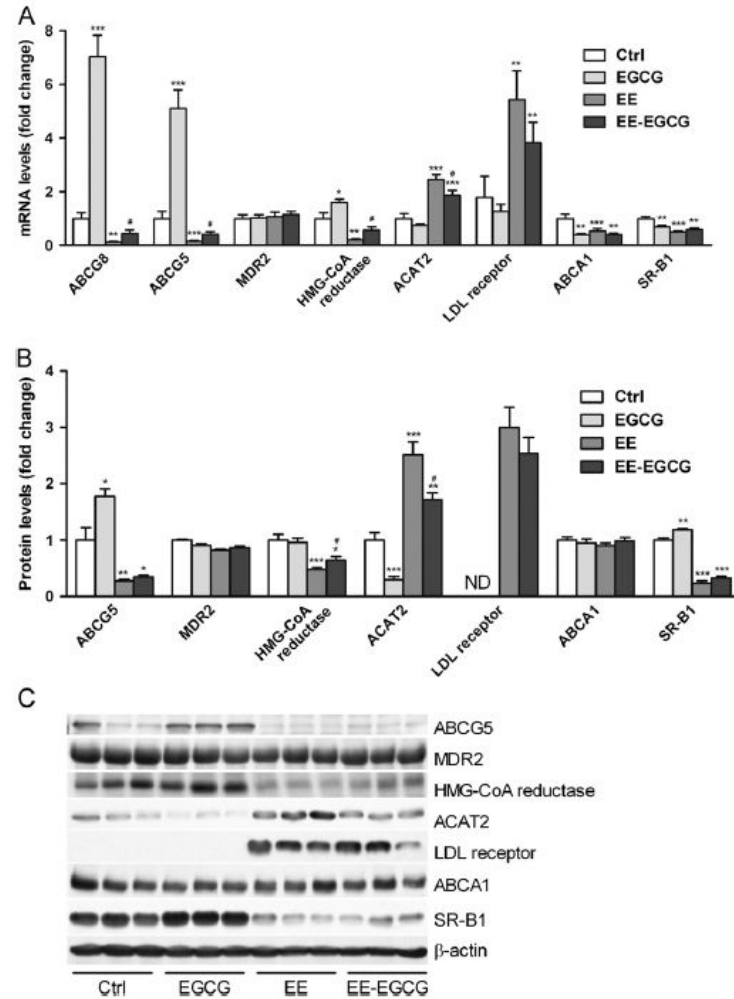
Fig. 1. Expression of genes (A) and proteins (B) related to liver cholesterol metabolism. The data from qRT-PCR and western blot are expressed relative to the level of the transcript and protein, respectively, in control rats. Bars are means ± S.E.M., $n = 6-7$. ND, not detected. LDL receptor protein levels (B) are expressed as relative units since the protein was not detected in the control group. Representative immunoblots are shown (C). Significantly different from control group: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Significantly different from EE group: #$P < 0.05$.

In EE-EGCG group, EGCG co-administration increased ethinyl-estradiol-reduced mRNA abundance of ABCG5, ABCG8 and HMG-CoA reductase by > 2-fold ($P < 0.05$) (Fig. 1A). Another positive effect of EGCG was a reduction in elevated ACAT2 mRNA levels ($P < 0.05$). EGCG treatment tended to lessen the strong upregulation of LDL receptor induced by ethinylestradiol ($P = 0.07$).

Gene expression of multidrug resistance protein 2 (MDR2), the main biliary phospholipid transporter, did not differ between all groups.

Table 4 shows gene expression of selected nuclear receptors, transcription factors and their downstream target genes. EGCG decreased gene expression of liver X receptor (LXR) by one third

Table 4
Expression of selected genes and SREBP2 protein.

	Ctrl	EGCG	EE	EE–EGCG
Relative mRNA abundance				
LXR	100 ± 11	65 ± 5[c]	89 ± 6	117 ± 18
SREBP1	100 ± 15	77 ± 8	214 ± 17[a]	150 ± 18[d]
SREBP2	100 ± 7	102 ± 12	45 ± 3[a]	43 ± 2[a]
FASN	100 ± 21	84 ± 14	55 ± 14	31 ± 4[c]
NQO1	100 ± 14	105 ± 9	135 ± 26	134 ± 23
SHP	100 ± 37	291 ± 36[b]	863 ± 123[a]	1340 ± 215[a]
Relative protein abundance				
SREBP2	100 ± 11	186 ± 29[c]	210 ± 28[b]	284 ± 56[b]

Data are expressed as a percentage of control and represent mean ± S.E.M, $n=6$–7.
Protein levels of the active, i.e., cleaved, form of SREBP2 were evaluated.

[a] $P < 0.001$ when compared to control group.
[b] $P < 0.01$ when compared to control group.
[c] $P < 0.05$ when compared to control group.
[d] $P < 0.05$ when compared to EE-treated group.

($P < 0.05$) and increased small heterodimer partner (SHP) mRNA levels by 3-fold compared to controls. Ethinylestradiol administration caused a marked induction of SHP and sterol regulatory-element binding protein 1 (SREBP1) mRNA, whereas mRNA levels of sterol regulatory-element binding protein 2 (SREBP2) decreased ($P < 0.001$). EGCG supplementation in ethinylestradiol-treated rats normalized ethinylestradiol-induced gene expression of SREBP1 to control values.

3.5. Expression of proteins involved in cholesterol transport and metabolism

To confirm changes found in gene expression, we assessed levels of respective proteins by Western blot. EGCG increased protein levels of ABCG5 and SR-B1 by 77% ($P < 0.05$) and 20% ($P < 0.01$), respectively, and decreased ACAT2 protein by 70% ($P < 0.001$) (Fig. 1B). On average, HMG-CoA reductase protein levels were not changed by EGCG. Ethinylestradiol strongly induced LDL receptor protein mass, but no bands were detected in the control group, as reported previously by Bertolotti and Spady (1996), even when more protein from membrane-enriched fraction was used (data not shown). Ethinylestradiol-treated rats had appreciably lower protein levels of ABCG5 ($P < 0.01$), HMG-CoA reductase and SR-B1 ($P < 0.001$) and higher levels of ACAT2 (by > 2-fold, $P < 0.001$) compared to controls. EGCG co-administration blunted ethinylestradiol-induced increase in ACAT2 protein levels and, conversely, increased ethinylestradiol-reduced protein mass of HMG-CoA reductase. Protein expression of MDR2 and ABCA1 was not change in all experimental groups. In addition, both EGCG and ethinylestradiol increased amount of the mature transcriptionally active form of SREBP2 (Table 4).

4. Discussion

To our knowledge, this is the first study to show that EGCG supplementation increases biliary cholesterol and phospholipid secretion. In addition, our data also demonstrate that EGCG co-administration attenuates liver cholesterol accumulation and liver weight increase during ethinylestradiol treatment in rats.

Biliary cholesterol secretion is an important route for cholesterol disposal from the body (Dikkers and Tietge, 2010). Biliary cholesterol output is not only driven by biliary bile acid secretion but also depends on the expression and activity of canalicular transporters such ABCG5/8 and MDR2. Particularly the obligate heterodimer ABCG5/8 is essential for biliary cholesterol secretion as evidenced by extremely low biliary cholesterol concentrations

in *Abcg5/8*-deficient mice (Yu et al., 2002a). Overexpression of ABCG5/8, on the other side, promotes biliary cholesterol output (Yu et al., 2002b). MDR2 functions as a key biliary phospholipid transporter; however, in *Mdr2* knockout mice biliary cholesterol is also dramatically decreased (Oude Elferink et al., 1995). It has been suggested that MDR2 is required for ABCG5/8-dependent biliary cholesterol secretion (Langheim et al., 2005). Finally, an important role of the HDL cholesterol receptor, SR-B1, in biliary cholesterol secretion has been shown recently (Mardones et al., 2001; Wiersma et al., 2010). Furthermore, HDL cholesterol is a preferred source of cholesterol secreted into the bile after the SR-B1-mediated uptake (Dikkers and Tietge, 2010).

In the present study, EGCG–treated rats presented an impressive, a nearly 2-fold, increment of biliary cholesterol output compared to controls. To explore the molecular mechanisms behind this phenomenon, key cholesterol transporters were evaluated. Indeed, expression of ABCG5 and ABCG8 was significantly increased by EGCG at both mRNA and protein levels together with a modest induction of SR-B1 protein. Since biliary bile acid output was not increased by EGCG (data not shown), our results indicate that especially upregulation of ABCG5/8 is primarily responsible for the enhanced biliary cholesterol secretion in EGCG-treated rats.

Previous studies have suggested that the biliary cholesterol secretion may be promoted by an increased availability of free cholesterol within hepatocytes resulting from either higher activity of HMG-CoA reductase (Hooiveld et al., 1999; Rigotti et al., 1989) or inhibition of the major enzyme for cholesterol esterification, ACAT2 (Nervi et al., 1984, 1983). EGCG has been shown to decrease cholesterol de novo synthesis in a concentration-dependent manner in human hepatoma cells (Bursill and Roach, 2006) and to inhibit in vitro activity of recombinant human HMG-CoA reductase (Cuccioloni et al., 2011). Similarly, in ovariectomized rats fed a high-fructose diet, dietary green tea down-regulated HMG-CoA reductase mRNA levels to control values (Shrestha et al., 2009). In our study, EGCG induced HMG-R mRNA, but the protein levels were not changed. Given the unchanged HMG-CoA reductase protein mass and the potential of EGCG to inhibit HMG-CoA reductase, we can speculate that increased synthesis of cholesterol does not likely contribute to the stimulating effect of EGCG on biliary cholesterol secretion. In contrast, the correlation analysis (Supplemental Table 3) revealed that EGCG-mediated induction of cholesterol biliary excretion may be related to the decreased expression of ACAT2. We therefore suggest that the decreased expression of ACAT2 and consequent reduction of cholesterol esterification could transiently increase free cholesterol that is available for biliary secretion, as was previously demonstrated for progesterone (Nervi et al., 1983) and diosgenin (Nervi et al., 1984). This is in line with our finding that EGCG decreased plasma VLDL cholesterol, the production of which is mediated by ACAT2 (Shelness and Sellers, 2001). Because biliary cholesterol and VLDL cholesterol seem to have a common hepatic pool (Stone and Evans, 1992), the increased biliary loss of free cholesterol may compensate for the decrease in VLDL synthesis due to ACAT2 downregulation. Therefore, the liver concentrations of free and esterified cholesterol were not finally affected by EGCG (Table 2), but plasma VLDL cholesterol was reduced in both groups receiving EGCG. The assumption that changes in VLDL production may be important for EGCG effect in the liver is supported by in vitro studies showing that EGCG inhibits apolipoprotein B secretion (Goto et al., 2011; Li et al., 2006; Yee et al., 2002). The complex effect of EGCG on hepatic cholesterol metabolism observed in our study is depicted in Fig. 2.

Despite the enhanced biliary phospholipid secretion by EGCG, no change in the expression of the main phospholipid transporter, MDR2, was detected in our study. What mechanism contributes
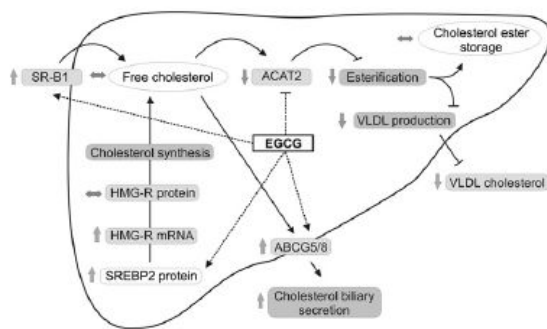
Fig. 2. Schematic diagram showing changes in liver cholesterol homeostasis in EGCG-treated rats. EGCG induced protein mass of SR-B1, which mediates selective uptake of HDL cholesterol by hepatocytes, and decreased expression of ACAT2, the major enzyme for cholesterol esterification. We propose that the decreased ACAT2 expression and consequently decreased cholesterol esterification diminished VLDL production and made free cholesterol available for biliary secretion. Moreover, expression of the main biliary cholesterol transporters, ABCG5 and ABCG8, was induced by EGCG at both gene and protein levels. EGCG increased both conversion of transcription factor SREBP2 to its active form and mRNA expression of SREBP2 target gene, HMG-CoA reductase. However, HMG-CoA reductase protein levels were not significantly changed by EGCG.

to the enhanced biliary phospholipid secretion by EGCG is unclear. Yu et al. (2002b) showed that ABCG5/8 overexpression in mice alone resulted in a modest increase in biliary phospholipid concentration. Thus biliary phospholipid output may be coupled to that one of cholesterol in EGCG-treated rats.

EGCG is well known for its plasma cholesterol-lowering effect (Zheng et al., 2011), although several animal studies reported that EGCG or green tea exerted its hypocholesterolemic action only when plasma cholesterol levels were altered with an additional treatment (Devika and Stanely Mainzen Prince, 2009; Kaviarasan et al., 2008; Miura et al., 2001). In agreement, we found that EGCG did not significantly decrease plasma total cholesterol in healthy but in ethinylestradiol-treated rats. Pharmacological doses of ethinylestradiol are also well known to markedly increase liver cholesterol content and liver weight in rats through induced expression of LDL receptor and thus increased liver uptake of LDL (Bertolotti and Spady, 1996). This is accompanied by a decrease in plasma cholesterol and triglycerides, and increase in cholesteryl ester content in newly secreted VLDL (Nervi et al., 1984). All these changes were confirmed by our data. In previous studies green tea prevented hepatic cholesterol accumulation in rats fed a cholesterol-enriched diet (Alshatwi et al., 2011; Muramatsu et al., 1986; Yang and Koo, 1997). In our study, EGCG demonstrated a similar effect during ethinylestradiol treatment. Although mechanisms of hepatic cholesterol accumulation differ greatly in these two conditions, our data may point to importance of the described pathways in EGCG effect on liver cholesterol homeostasis. As shown in Table 2, the reduction in liver cholesterol was accompanied by decreased liver weight and may be ascribed to the reduced expression of ACAT2 (Supplemental Table 3) and parallel decrease in liver esterified cholesterol and VLDL production. Similarly to previous reports (Bonazzi et al., 1986; Davis and Kern, 1976), and in contrast to another (Kamisako and Ogawa, 2003), biliary cholesterol secretion in ethinylestradiol-treated rats was not changed compared to controls, despite the profound reduction in ABCG5/8 expression. As to whether this effect is caused by an increased disposition of intracellular cholesterol in ethinylestradiol-treated animals requires further elucidation. EGCG co-administration to ethinylestradiol-treated rats raised ABCG5/8 gene expression, but the effect was not accompanied by increased protein levels of the

transporters, thus the biliary cholesterol secretion remained unchanged compared to ethinylestradiol-treated group.

The molecular mechanism underlying the effect of EGCG on the expression of genes and proteins related to cholesterol homeostasis remains an open question at the present time. However, from our mRNA data it is obvious that EGCG is capable of transcriptional regulation of a great variety of genes. The genes evaluated in our study can be regulated by various nuclear receptors and transcription factors, especially LXR, SREBP1 and SREBP2 (Wagner et al., 2011). However, data on modulation of nuclear receptor activity by EGCG are scarce. Up to date, EGCG has been shown to modulate activity of farnesoid X receptor (FXR) (Li et al., 2012) and nuclear factor (erythroid-derived 2)-like 2 (NRF2) (Na and Surh, 2008). EGCG was shown as a unique regulator of FXR as it moderately stimulated its activity, but inhibited FXR transactivation by other agonists (Li et al., 2012). Moreover, induction of FXR target genes after oral administration of EGCG was observed in the intestine but not in the liver (Li et al., 2012). In accordance, we did not detect stimulation of FXR target genes in the liver of EGCG-treated rats (data not shown). On the other hand, we observed an inverse pattern in the expression of key liver lipid regulators compared to *Nrf2* knockout mice (Huang et al., 2010) and found induction of SHP, a target gene of NRF2. However, the expression of another direct NRF2 target, NAD(P)H:-quinone oxidoreductase 1 (NQO1), was not increased in our study, suggesting that NRF2 downstream target genes were not uniformly activated by EGCG. Another study demonstrated that EGCG increased the active form of SREBP2 by inhibiting its degradation by the ubiquitin–proteasome pathway (Kuhn et al., 2004). We also found an increased protein mass of SREBP2 active form in EGCG-treated rats; however, from the two well known target genes of SREBP2 only HMG-CoA reductase mRNA was upregulated, in contrast to LDL receptor. EGCG strongly induced gene expression of ABCG5 and ABCG8, which are LXR target genes (Repa et al., 2002). Since other LXR target genes were not affected (ABCA1 or SREBP1 and its downstream FASN) by EGCG in our study, it may be possible that liver receptor homolog-1 (Freeman et al., 2004) or hepatocyte nuclear factor 4α (Sumi et al., 2007) contribute to the upregulation of ABCG5/8. Taken together, the results indicate that EGCG interferes with activation of LXR, NRF2 and SREBP2; however the effect of EGCG seems to be complex and involve many regulatory pathways with cross-talk between nuclear receptors and transcription factors.

5. Conclusions

In summary, the present study provides new evidence that EGCG significantly enhances biliary cholesterol and phospholipid secretion and decreases liver VLDL production in rats and attenuates the effect of ethinylestradiol on liver cholesterol accumulation and liver weight. These effects are attributable to upregulation of cholesterol transporters ABCG5/8 and SR-B1 and decreased expression of ACAT-2 by EGCG. Thus, our data offer new insights into mechanisms of EGCG effect on the cholesterol metabolism and may imply useful message for the use of this compound as potential intervention in the modulation of cholesterol liver homeostasis.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2012.06.034.

References

Abe, I., Seki, T., Umehara, K., Miyase, T., Noguchi, H., Sakakibara, J., Ono, T., 2000. Green tea polyphenols: novel and potent inhibitors of squalene epoxidase. Biochem. Biophys. Res. Commun. 268, 767–771.

Alshatwi, A.A., Al Obaaid, M.A., Al Sedairy, S.A., Ramesh, E., Lei, K.Y., 2011. Black and green tea improves lipid profile and lipid peroxidation parameters in Wistar rats fed a high-cholesterol diet. J. Physiol. Biochem. 67, 95–104.

Bertolotti, M., Spady, D.K., 1996. Effect of hypocholesterolemic doses of 17 alpha-ethinyl estradiol on cholesterol balance in liver and extrahepatic tissues. J. Lipid Res. 37, 1812–1822.

Bonazzi, P., Novelli, G., Galeazzi, R., 1986. Effect of chenodeoxycholic acid on the alterations of biliary lipid secretion induced by ethynylestradiol in the rat. Br. J. Exp. Pathol. 67, 341–348.

Bursill, C.A., Roach, P.D., 2006. Modulation of cholesterol metabolism by the green tea polyphenol (−)-epigallocatechin gallate in cultured human liver (HepG2) cells. J. Agric. Food. Chem. 54, 1621–1626.

Chan, P.T., Fong, W.P., Cheung, Y.L., Huang, Y., Ho, W.K., Chen, Z.Y., 1999. Jasmine green tea epicatechins are hypolipidemic in hamsters (Mesocricetus auratus) fed a high fat diet. J. Nutr. 129, 1094–1101.

Cuccioloni, M., Mozzicafreddo, M., Spina, M., Tran, C.N., Falconi, M., Eleuteri, A.M., Angeletti, M., 2011. Epigallocatechin-3-gallate potently inhibits the in vitro activity of hydroxy-3-methyl-glutaryl-CoA reductase. J. Lipid Res. 52, 897–907.

Davis, R.A., Kern Jr., F., 1976. Effects of ethinyl estradiol and phenobarbital on bile acid synthesis and biliary bile acid and cholesterol excretion. Gastroenterology 70, 1130–1135.

Devika, P.T., Stanely Mainzen Prince, P., 2009. Preventive effect of (−)epigallocatechin gallate on lipids, lipoproteins, and enzymes of lipid metabolism in isoproterenol-induced myocardial infarction in rats. J. Biochem. Mol. Toxicol. 23, 387–393.

Dikkers, A., Tietge, U.J., 2010. Biliary cholesterol secretion: more than a simple ABC. World J. Gastroenterol. 16, 5936–5945.

Freeman, L.A., Kennedy, A., Wu, J., Bark, S., Remaley, A.T., Santamarina-Fojo, S., Brewer Jr., H.B., 2004. The orphan nuclear receptor LRH-1 activates the ABCG5/ABCG8 intergenic promoter. J. Lipid Res. 45, 1197–1206.

Goto, T., Saito, Y., Morikawa, K., Kanamaru, Y., Nagaoka, S., 2011. Epigallocatechin gallate changes mRNA expression level of genes involved in cholesterol metabolism in hepatocytes. Br. J. Nutr., 1–5.

Hasegawa, N., Yamda, N., Mori, M., 2003. Powdered green tea has antilipogenic effect on Zucker rats fed a high-fat diet. Phytother. Res. 17, 477–480.

Hayashi, K., Koide, K., Hirata, Y., Ohtani, H., Yamada, K., Kajiyama, G., 1986. Metabolic changes in lipids of rat plasma and hepatocytes induced by 17 alpha-ethynylestradiol treatment. Biochim. Biophys. Acta 879, 140–148.

Hooiveld, G.J., Vos, T.A., Scheffer, G.L., Van Goor, H., Koning, H., Bloks, V., Loot, A.E., Meijer, D.K., Jansen, P.L., Kuipers, F., Muller, M., 1999. 3-hydroxy-3-methyl-glutaryl-coenzyme a reductase inhibitors (statins) induce hepatic expression of the phospholipid translocase mdr2 in rats. Gastroenterology 117, 678–687.

Huang, J., Tabbi-Anneni, I., Gunda, V., Wang, L., 2010. Transcription factor Nrf2 regulates SHP and lipogenic gene expression in hepatic lipid metabolism. Am. J. Physiol. Gastrointest. Liver Physiol. 299, G1211–G1221.

Ikeda, I., Imasato, Y., Sasaki, E., Nakayama, M., Nagao, H., Takeo, T., Yayabe, F., Sugano, M., 1992. Tea catechins decrease micellar solubility and intestinal absorption of cholesterol in rats. Biochim. Biophys. Acta 1127, 141–146.

Kamisako, T., Ogawa, H., 2003. Regulation of biliary cholesterol secretion is associated with abcg5 and abcg8 expressions in the rats: effects of diosgenin and ethinyl estradiol. Hepatol. Res. 26, 348–352.

Kao, Y.H., Hiipakka, R.A., Liao, S., 2000. Modulation of endocrine systems and food intake by green tea epigallocatechin gallate. Endocrinology 141, 980–987.

Kaviarasan, S., Viswanathan, P., Ravichandran, M.K., Anuradha, C.V., 2008. (−) Epigallocatechin gallate (EGCG) prevents lipid changes and collagen abnormalities in chronic ethanol-fed rats. Toxicol. Mech. Methods 18, 425–432.

Kolouchova, G., Brcakova, E., Hirsova, P., Cermanova, J., Fuksa, L., Mokry, J., Nachtigal, P., Lastuvkova, H., Micuda, S., 2011a. Modification of hepatic iron metabolism induced by pravastatin during obstructive cholestasis in rats. Life Sci. 89, 717–724.

Kolouchova, G., Brcakova, E., Hirsova, P., Sispera, L., Tomsik, P., Cermanova, J., Hyspler, R., Slanarova, M., Fuksa, L., Lotkova, H., Micuda, S., 2011b. Pravastatin modulates liver bile acid and cholesterol homeostasis in rats with chronic cholestasis. J. Gastroenterol. Hepatol. 26, 1544–1551.

Kono, S., Shinchi, K., Wakabayashi, K., Honjo, S., Todoroki, I., Sakurai, Y., Imanishi, K., Nishikawa, H., Ogawa, S., Katsurada, M., 1996. Relation of green tea consumption to serum lipids and lipoproteins in Japanese men. J. Epidemiol. 6, 128–133.

Koo, S.I., Noh, S.K., 2007. Green tea as inhibitor of the intestinal absorption of lipids: potential mechanism for its lipid-lowering effect. J. Nutr. Biochem. 18, 179–183.

Kuhn, D.J., Burns, A.C., Kazi, A., Dou, Q.P., 2004. Direct inhibition of the ubiquitin-proteasome pathway by ester bond-containing green tea polyphenols is associated with increased expression of sterol regulatory element-binding protein 2 and LDL receptor. Biochim. Biophys. Acta 1682, 1–10.

Lambert, J.D., Lee, M.J., Lu, H., Meng, X., Hong, J.J., Seril, D.N., Sturgill, M.G., Yang, C.S., 2003. Epigallocatechin-3-gallate is absorbed but extensively glucuronidated following oral administration to mice. J. Nutr. 133, 4172–4177.

Langheim, S., Yu, L., von Bergmann, K., Lutjohann, D., Xu, F., Hobbs, H.H., Cohen, J.C., 2005. ABCG5 and ABCG8 require MDR2 for secretion of cholesterol into bile. J. Lipid Res. 46, 1732–1738.

Lee, R.G., Kelley, K.L., Sawyer, J.K., Farese Jr., R.V., Parks, J.S., Rudel, L.L., 2004. Plasma cholesteryl esters provided by lecithin:cholesterol acyltransferase and acyl-coenzyme a:cholesterol acyltransferase 2 have opposite atherosclerotic potential. Circ. Res. 95, 998–1004.

Li, G., Lin, W., Araya, J.J., Chen, T., Timmermann, B.N., Guo, G.L., 2012. A tea catechin, epigallocatechin-3-gallate, is a unique modulator of the farnesoid X receptor. Toxicol. Appl. Pharmacol. 258, 268–274.

Li, L., Stillemark-Billton, P., Beck, C., Bostrom, P., Andersson, L., Rutberg, M., Ericsson, J., Magnusson, B., Marchesan, D., Ljungberg, A., Boren, J., Olofsson, S.O., 2006. Epigallocatechin gallate increases the formation of cytosolic lipid droplets and decreases the secretion of apoB-100 VLDL. J. Lipid Res. 47, 67–77.

Mardones, P., Quinones, V., Amigo, L., Moreno, M., Miquel, J.F., Schwarz, M., Miettinen, H.E., Trigatti, B., Krieger, M., VanPatten, S., Cohen, D.E., Rigotti, A., 2001. Hepatic cholesterol and bile acid metabolism and intestinal cholesterol absorption in scavenger receptor class B type I-deficient mice. J. Lipid Res. 42, 170–180.

Miura, Y., Chiba, T., Tomita, I., Koizumi, H., Miura, S., Umegaki, K., Hara, Y., Ikeda, M., Tomita, T., 2001. Tea catechins prevent the development of atherosclerosis in apoprotein E-deficient mice. J. Nutr. 131, 27–32.

Muramatsu, K., Fukuyo, M., Hara, Y., 1986. Effect of green tea catechins on plasma cholesterol level in cholesterol-fed rats. J. Nutr. Sci. Vitaminol. (Tokyo) 32, 613–622.

Murase, T., Nagasawa, A., Suzuki, J., Hase, T., Tokimitsu, I., 2002. Beneficial effects of tea catechins on diet-induced obesity: stimulation of lipid catabolism in the liver. Int. J. Obes. Relat. Metab. Disord. 26, 1459–1464.

Na, H.K., Surh, Y.J., 2008. Modulation of Nrf2-mediated antioxidant and detoxifying enzyme induction by the green tea polyphenol EGCG. Food Chem. Toxicol. 46, 1271–1278.

Nagle, D.G., Ferreira, D., Zhou, Y.D., 2006. Epigallocatechin-3-gallate (EGCG): chemical and biomedical perspectives. Phytochemistry 67, 1849–1855.

Nervi, F., Bronfman, M., Allalon, W., Depierreux, E., Del Pozo, R., 1984. Regulation of biliary cholesterol secretion in the rat. Role of hepatic cholesterol esterification. J. Clin. Invest. 74, 2226–2237.

Nervi, F.O., Del Pozo, R., Covarrubias, C.F., Ronco, B.O., 1983. The effect of progesterone on the regulatory mechanisms of biliary cholesterol secretion in the rat. Hepatology 3, 360–367.

Oude Elferink, R.P., Ottenhoff, R., van Wijland, M., Smit, J.J., Schinkel, A.H., Groen, A.K., 1995. Regulation of biliary lipid secretion by mdr2 P-glycoprotein in the mouse. J. Clin. Invest. 95, 31–38.

Raederstorff, D.G., Schlachter, M.F., Elste, V., Weber, P., 2003. Effect of EGCG on lipid absorption and plasma lipid levels in rats. J. Nutr. Biochem. 14, 326–332.

Repa, J.J., Berge, K.E., Pomajzl, C., Richardson, J.A., Hobbs, H., Mangelsdorf, D.J., 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.

Rigotti, A., Marzolo, M.P., Ulloa, N., Gonzalez, O., Nervi, F., 1989. Effect of bean intake on biliary lipid secretion and on hepatic cholesterol metabolism in the rat. J. Lipid Res. 30, 1041–1048.

Rodriguez-Garay, E.A., 2003. Cholestasis: human disease and experimental animal models. Ann. Hepatol. 2, 150–158.

Sanchez-Muniz, F.J., Bastida, S., 2008. Do not use the Friedewald formula to calculate LDL-cholesterol in hypercholesterolaemic rats. Eur. J. Lipid Sci. Technol. 110, 295–301.

Shelness, G.S., Sellers, J.A., 2001. Very-low-density lipoprotein assembly and secretion. Curr. Opin. Lipidol. 12, 151–157.

Shrestha, S., Ehlers, S.J., Lee, J.Y., Fernandez, M.L., Koo, S.I., 2009. Dietary green tea extract lowers plasma and hepatic triglycerides and decreases the expression of sterol regulatory element-binding protein-1c mRNA and its responsive genes in fructose-fed, ovariectomized rats. J. Nutr. 139, 640–645.

Stone, B.G., Evans, C.D., 1992. Evidence for a common biliary cholesterol and VLDL cholesterol precursor pool in rat liver. J. Lipid Res. 33, 1665–1675.

Sumi, K., Tanaka, T., Uchida, A., Magoori, K., Urashima, Y., Ohashi, R., Ohguchi, H., Okamura, M., Kudo, H., Daigo, K., Maejima, T., Kojima, N., Sakakibara, I., Jiang, S., Hasegawa, G., Kim, I., Osborne, T.F., Naito, M., Gonzalez, F.J., Hamakubo, T., Kodama, T., Sakai, J., 2007. Cooperative interaction between hepatocyte nuclear factor 4 alpha and GATA transcription factors regulates ATP-binding cassette sterol transporters ABCG5 and ABCG8. Mol. Cell Biol. 27, 4248–4260.

Tokunaga, S., White, I.R., Frost, C., Tanaka, K., Kono, S., Tokudome, S., Akamatsu, T., Moriyama, T., Zakouji, H., 2002. Green tea consumption and serum lipids and lipoproteins in a population of healthy workers in Japan. Ann. Epidemiol. 12, 157–165.

Wagner, M., Zollner, G., Trauner, M., 2011. Nuclear receptors in liver disease. Hepatology 53, 1023–1034.

Wang, S., Noh, S.K., Koo, S.I., 2006. Epigallocatechin gallate and caffeine differentially inhibit the intestinal absorption of cholesterol and fat in ovariectomized rats. J. Nutr. 136, 2791–2796.

Wiersma, H., Nijstad, N., Gautier, T., Iqbal, J., Kuipers, F., Hussain, M.M., Tietge, U.J., 2010. Scavenger receptor BI facilitates hepatic very low density lipoprotein production in mice. J. Lipid Res. 51, 544–553.

Wolfram, S., Raederstorff, D., Preller, M., Wang, Y., Teixeira, S.R., Riegger, C., Weber, P., 2006. Epigallocatechin gallate supplementation alleviates diabetes in rodents. J. Nutr. 136, 2512–2518.

Yang, C.S., Wang, X., Lu, G., Picinich, S.C., 2009. Cancer prevention by tea: animal studies, molecular mechanisms and human relevance. Nat. Rev. Cancer 9, 429–439.

Yang, T.T., Koo, M.W., 1997. Hypocholesterolemic effects of Chinese tea. Pharmacol. Res. 35, 505–512.

Yee, W.L., Wang, Q., Agdinaoay, T., Dang, K., Chang, H., Grandinetti, A., Franke, A.A., Theriault, A., 2002. Green tea catechins decrease apolipoprotein B-100 secretion from HepG2 cells. Mol. Cell Biochem. 229, 85–92.

Yu, L., Hammer, R.E., Li-Hawkins, J., Von Bergmann, K., Lutjohann, D., Cohen, J.C., Hobbs, H.H., 2002a. Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion. Proc. Nat. Acad. Sci. U.S.A. 99, 16237–16242.

Yu, L., Li-Hawkins, J., Hammer, R.E., Berge, K.E., Horton, J.D., Cohen, J.C., Hobbs, H.H., 2002b. Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. J. Clin. Invest. 110, 671–680.

Zheng, X.X., Xu, Y.L., Li, S.H., Liu, X.X., Hui, R., Huang, X.H., 2011. Green tea intake lowers fasting serum total and LDL cholesterol in adults: a meta-analysis of 14 randomized controlled trials. Am. J. Clin. Nutr. 94, 601–610.

Online Supporting Material

Supplemental Table 1	Pre-designed TaqMan® Gene Expression Assay kits (Applied Biosystems) used for quantitative real-time RT-PCR

Gene	Applied Biosystems cat. number
ABCA1	Rn00710172_m1
ABCG5	Rn00587092_m1
ABCG8	Rn00590367_m1
ACAT2	Rn00596636_m1
FASN	Rn00569117_m1
GAPDH	Rn01775763_g1
LDL receptor	Rn00598442_m1
LXR	Rn00581185_m1
MDR2	Rn00562185_m1
NQO1	Rn00566528_m1
SHP	Rn00589173_m1
SR-B1	Rn00580588_m1
SREBP1	Rn01495769_m1
SREBP2	Rn01502638_m1

Online Supporting Material

Supplemental Table 2   Primary antibodies used in Western blot

Protein	Source	Dilution
ABCA1	Novus, NB 100-2068	1:2000
ABCG5	Abcam, ab45279	1:1000
ACAT2	Cayman, 100027	1:500
HMG-CoA reductase	Abcam, ab98018	1:1000
LDL receptor	Cayman, 10007665	1:1000
MDR2	Abcam, ab71792	1:500
SR-B1	Novus, NB 400-104	1:2000
SREBP2	Abcam, ab30682	1:1000
beta-actin	Sigma, A5316	1:5000

APPENDIX II

Hirsova P, Karlasova G, Dolezelova E, Cermanova J, Zagorova M, Kadova Z, Hroch M, Sispera L, Tomsik P, Lenicek M, Vitek L, Pavek P, Kucera O, Cervinkova Z, Micuda S: Cholestatic effect of epigallocatechin gallate in rats is mediated via decreased expression of Mrp2. *Toxicology* (accepted for publication October 10, 2012)

TITLE PAGE

Cholestatic effect of epigallocatechin gallate in rats is mediated via decreased expression of Mrp2

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Abbreviations: Asbt, apical sodium-dependent bile salt transporter; ALT, alanine transaminase; AST, aspartate transaminase; Bsep, bile salt exporting pump; CDCA, chenodeoxycholic acid; Cyp7a1, cholesterol 7α-hydroxylase; C4, 7α-hydroxy-4-cholesten-3-one; EE, ethinylestradiol; EGCG, epigallocatechin gallate; Fgf, fibroblast growth factor;

Fxr, farnesoid X receptor; GSH, reduced glutathione; GSSG, oxidized glutathione; HPLC, high-performance liquid chromatography; Mrp, multidrug resistance-associated protein; Ntcp, Na^+-taurocholate cotransporting polypeptide; Oatp, organic anion transporting polypeptide; Ost, organic solute transporter; Shp, small heterodimer partner

Abstract

Epigallocatechin gallate (EGCG) has been shown to be protective in various experimental models of liver injury, although opposite effects have also been reported. Since its effect on biliary physiology has not been thoroughly investigated, the present study evaluated effect of EGCG on bile flow and bile acid homeostasis in rats. Compared to controls, EGCG treatment decreased bile flow by 23%. Hepatic paracellular permeability and biliary bile acid excretion were not altered by EGCG administration, but biliary glutathione excretion was reduced by 70%. Accordingly, the main glutathione transporter on the hepatocyte canalicular membrane, multidrug resistance-associated protein 2 (Mrp2), was significantly decreased at the protein level. EGCG administration also doubled plasma bile acid levels compared to controls. While protein levels of the main hepatic bile acid transporters were unchanged, the rate-limiting enzyme in the bile acid synthesis, Cyp7a1, was significantly increased by EGCG. Enhanced bile acid synthesis in these animals was also confirmed by a 2-fold increase in plasma marker 7α-hydroxy-4-cholesten-3-one. In contrast, EGCG markedly downregulated major bile acid transporters (Asbt and Ostα) and regulatory molecules (Shp and Fgf15) in the ileum. When EGCG was coadministered with ethinylestradiol, a potent cholestatic agent, it did not show any additional effect on the induced cholestasis. This study shows ability of EGCG to raise plasma bile acid concentrations, mainly through Cyp7a1 upregulation, and to decrease bile production through reduction in Mrp2-mediated bile acid-independent bile flow. In conclusion, our data demonstrate that under certain conditions EGCG may induce cholestasis.

Keywords: epigallocatechin gallate; bile formation; cholestasis; bile acids

1. Introduction

Bile production is one of the essential functions of the liver and is vital for both proper digestion of dietary lipids and elimination of lipid soluble endo- and xenobiotics, including drugs and toxins. The key step in the whole process is secretion of osmotically active compounds, especially bile acids and glutathione, across the hepatocyte canalicular membrane into the bile capillaries, followed by passive movement of water (Esteller, 2008). To generate the driving force for bile flow, solutes are transported into bile against a concentration gradient by energy-dependent transporters; bile salt exporting pump (Bsep) is essential for bile acid-dependent bile flow and multidrug resistance-associated protein (Mrp) 2 is crucial for bile acid-independent bile flow, which is based on the transport of glutathione and glutathione conjugates. Nevertheless, since bile acids are synthesized from cholesterol and the majority of their pool undergo excessive enterohepatic recirculation, several other transporters and enzymes in the liver and intestine are also crucial for proper functionality of the whole process (Esteller, 2008).

Epigallocatechin gallate (EGCG), the main active component of green tea, is currently a widely used natural compound available in numerous products either in purified form or as a part of tea or tea extracts. EGCG has demonstrated a great variety of health benefits, which are mainly related to its potent anti-oxidant, anti-inflammatory and anticancer activities (Singh et al., 2011). Among these, one of the prominent effects is its hepatoprotective action in various forms of liver injury (Ramesh et al., 2009; Ren et al., 2011; Tipoe et al., 2010), including cholestatic injury induced by bile duct ligation (Kobayashi et al., 2010; Zhong et al., 2003). Although most of these effects are ascribed to the anti-inflammatory activity of the compound, Li et al. (2012) has recently reported a unique feature of EGCG to stimulate farnesoid X receptor (Fxr), the master regulator of bile acid homeostasis, selectively in the intestine and thus increase expression of its target gene fibroblast growth factor 15 (Fgf15, a mouse homologue of human FGF19). This mechanism has been suggested to protect the liver against cholestasis as it may significantly reduce bile acid synthesis in the liver (Modica et al., 2012). However, the effect of EGCG on bile flow and corresponding mechanisms has not yet been thoroughly studied. In contrast, our recent data showed a bile flow decreased in EGCG-exposed rats (Hirsova et al., 2012). In fact, such information is of great interest since several cases of cholestasis have been reported in humans after either administration of high dose of EGCG/green tea or coadministration with other potentially cholestatic agents, such as ethinylestradiol (Mazzanti et al., 2009), a widely used contraceptive. The mechanism

involved may therefore account for possible toxic effects of EGCG reported recently (for review see Stickel et al., 2011).

The aim of the present study was to identify the effect of EGCG on overall bile production as well as to analyze separate effects on bile acid-dependent and bile acid-independent bile flow. In view of the current concept that FXR activators may be useful in treating intrahepatic cholestasis due to suppression of bile acid synthesis and promotion of bile acid efflux from hepatocytes (Zhu et al., 2011), the effect of EGCG in our study was further analyzed in a model of intrahepatic cholestasis induced by ethinylestradiol (EE).

2. Materials and Methods

2.1. Materials

Epigallocatechin gallate and ethinylestradiol were obtained from Cayman Chemical and Sigma-Aldrich, respectively. Rhamnose and melibiose were purchased from Sigma.

2.2. Animals and experimental design

All animal experimental protocols were approved by the Animal Research Committee of the Faculty of Medicine in Hradec Kralove, Charles University in Prague, Czech Republic. Female Wistar rats (230–250 g) were obtained from Anlab (Czech Republic) and were housed under controlled temperature and light conditions, on a natural 12 h light–dark cycle. Animals had free access to water and food throughout the experiment, but were fasted overnight before sacrifice. Because the decrease in bile flow by EGCG was first noticed in our previous study (Hirsova et al., 2012), we decided to use the same study design. Briefly, rats were randomly divided into four groups and were administered either EGCG 50 mg/kg body weight (EGCG group) or saline (control group, Ctrl) by i.p. injection once daily for 8 consecutive days. Groups EE and EE-EGCG received also ethinylestradiol (5 mg/kg body weight) s.c. once daily for 5 days, starting on day 4 of saline or EGCG treatment. This dose of EGCG (50 mg/kg) may result in a plasma concentration of 1 μM after 1 hour and peak plasma concentration of 2 μM after 2 hours. In humans, the plasma concentration of ~1 μM is achievable after either drinking ~8 cups of green tea or ingestion of EGCG-rich extracts (Hirsova et al., 2012).

Bile collection and sacrifice were performed on day 9, i.e. the next day after the last dose of EGCG. Bile collection was carried out as previously described (Hirsova et al., 2012). Plasma and bile samples from the initial collecting period (0–30 min) were used for further analyses of bile acids and glutathione as the early period best reflects the situation

under cholestasis.

2.3. Routine plasma biochemistry, bile acid and 7α-hydroxy-4-cholesten-3-one (C4) measurements

Plasma bilirubin levels and liver enzyme activities were determined by routine laboratory methods on Cobas Integra 800 (Roche Diagnostics). Bile acids in plasma and bile were assayed using a commercial kit (Diazyme). Plasma concentrations of C4 were measured by high-performance liquid chromatography (HPLC) as previously described by Lenicek et al. (2008). The chromatographic parameters were: Tessek SGX C18 column (4 × 250 mm, 4 μm), acetonitrile:water (95:5, vol/vol) mobile phase, flow rate 1 mL/min, detection/reference wavelength 241/360 nm. The detection limit was 1.2 μg/L.

2.4. Glutathione measurements

Concentrations of reduced (GSH) and oxidized (GSSG) glutathione were analyzed separately using an HPLC method (Kand'ar et al., 2007) on a Shimadzu system with fluorescence detection. The chromatographic conditions employed in the study were as follows: stationary phase SUPELCO Discovery C18 (4 mm × 150 mm, 5 μm), mobile phase methanol:phosphate buffer (15:85, vol/vol, 25 mM, pH 6.0), flow rate 0.5 mL/min, detector set at 350 nm and 450 nm (excitation and emission wavelengths, respectively). The limit of detection was 14 fmol and 5.6 fmol for GSH and GSSG, respectively. Total glutathione was calculated as the sum of reduced and oxidized forms of glutathione.

2.5. Evaluation of paracellular permeability in the liver

We assessed hepatic tight junctional permeability by a dual-sugar test based on quantifying biliary excretion of rhamnose and melibiose (Tomsik et al., 2008). Biliary concentrations of melibiose and rhamnose were determined by HPLC method with a fluorescent detection as previously described (Tomsik et al., 2008). The ratio of biliary excretion of melibiose (disaccharide, which penetrates selectively across the paracellular junctions) to biliary excretion of rhamnose (monosaccharide, which permeates barriers by transcellular diffusion) was used as a marker of the blood-biliary barrier function.

2.6. Rat hepatocyte isolation, culture and treatment

Rat hepatocytes were isolated by collagenase perfusion as previously described (Kand'ar et al., 2007) and cultured in William's E medium supplemented with fetal bovine serum

(10%), glutamine (2 mM), penicillin (100 IU/mL), streptomycin (10 mg/mL), dexamethasone (0.1 µg/mL), insulin (0.08 IU/mL) and glucagon (8 ng/mL) in a collagen sandwich configuration. After 24 h, medium was removed and replaced with a fresh serum-free medium containing either DMSO or EGCG at concentrations of 1–20 µmol/L. After 24 h incubation, cells were collected in TRI Reagent (Sigma-Aldrich) for gene expression analysis.

2.7. Quantitative real time RT-PCR

Total RNA from rat liver and ileum were isolated with RNeasy Mini Kit (Qiagen). Total RNA from primary rat hepatocytes was isolated using TRI Reagent (Sigma-Aldrich) and cleaned up with RNeasy MinElute Cleanup Kit (Qiagen). Gene expression was examined by quantitative real-time RT-PCR as previously described (Hirsova et al., 2012). TaqMan Fast Universal PCR Master Mix and pre-designed TaqMan Gene Expression Assay kits (Supplemental Table A) were purchased from Applied Biosystems. The glyceraldehyde 3-phosphate dehydrogenase gene was used as a reference for normalizing data.

2.8. Western blot

Protein expression in the liver was examined by Western blot as previously described (Hirsova et al., 2012). Briefly, proteins (100 µg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Millipore) and incubated with appropriate antibodies (Supplemental Table B). Horseradish peroxidase-conjugated secondary antibodies were from GE Healthcare and enhanced chemiluminescence reagents were from Thermo Pierce. Densitometry was performed using ScanMaker i900 (UMAX) and QuantityOne imaging software (BioRad). Protein levels were normalized to beta-actin levels.

2.9. Gene reporter assay

HepG2 cell line was purchased from the European Collection of Cell Cultures (Salisbury, UK) and was used within 25 passages after delivery. For transient transfection gene-reporter experiments, HepG2 cells were maintained in antibiotic-free Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% sodium pyruvate, and 1% nonessential amino acids. HepG2 cells were co-transfected with the pGL5-luc luciferase reporter construct, pCMX-GAL4-FXR expression plasmid containing farnesoid X receptor ligand binding domain (Adachi et al., 2005) and pRL-TK using Lipofectamine 2000 (Life

Technologies). Following 24 h of stabilization, HepG2 cells were treated with EGCG (5 µM and 20 µM), chenodeoxycholic acid (CDCA; 50 µM), and vehicle (DMSO; 0.1% v/v) for 24 h. Cells were then lysed and analyzed for firefly luciferase activity normalized to the Renilla luciferase activity (Dual-Luciferase Reporter Assay, Promega).

2.10. Statistical analysis

Data are expressed as the mean ± SD. Time-course experiments were analyzed using a two-way analysis of variance (ANOVA) followed by Bonferroni test for post-hoc analysis. For two-group comparison, Student's t-test was employed. Other data were analyzed using one-way ANOVA followed by Newman-Keuls post-hoc test. Pearson correlation coefficients were calculated. Differences were considered significant at p-value less than 0.05. All analyses were performed using GraphPad Prism 5.0 software (San Diego, USA).

3. Results

3.1. Plasmatic markers of liver injury

Compared to controls, the sole EGCG administration doubled plasma bile acid concentrations (Table 1). In contrast, EGCG did not further raise EE-elevated plasma bile acids in EE-EGCG group. Plasma levels of bilirubin and activities of alanine transaminase (ALT) and aspartate transaminase (AST) were not altered by EGCG treatment.

3.2. Bile flow and biliary excretion of bile acids and glutathione

To assess the effect of EGCG on bile flow, cumulative bile flow and biliary excretion of the main bile components were calculated. Compared to controls, rats treated with EGCG presented a significant decrease in cumulative bile flow and biliary excretion of glutathione by 23% and 70% (p < 0.001), respectively, with no change in biliary bile acid excretion (Fig. 1). As expected, EE decreased bile flow by both decreased biliary excretion of bile acids and glutathione. Combination of EE and EGCG decreased bile flow and biliary bile acid and glutathione excretion to the same extent as EE alone.

In addition, hepatic paracellular permeability (function of blood-biliary barrier) was assessed by a dual-sugar test based on biliary excretion of melibiose and rhamnose. Ethinylestradiol is well known to increase paracellular permeability in the liver, which allows for the regurgitation of bile constituents into the blood (Rahner et al., 1996). Higher ratio of biliary excretion of melibiose to rhamnose indicates increased paracellular permeability in both groups receiving EE (Fig. 1B). EGCG had no effect on blood-biliary

barrier function.

3.3. Expression of bile acid and glutathione transporters in the liver

To evaluate effect of EGCG at the molecular level, gene and protein expression profiles of the major hepatic bile acid and glutathione transporters were examined in the liver by qRT-PCR and Western blotting. EGCG administration decreased mRNA levels of Bsep, the major canalicular bile acid efflux transporter (Fig. 2A), and Na^+-taurocholate cotransporting polypeptide (Ntcp), the major basolateral bile acid uptake transporter, by 34% ($p < 0.01$) and 24% ($p < 0.05$), respectively. Nevertheless, their protein expression in the liver was not changed (Fig. 2B). At the protein level, EGCG induced Mrp4 by nearly 2-fold ($p < 0.001$) and reduced Mrp2 by 28% ($p < 0.01$). Protein expression of Mrp2 very tightly correlated with both biliary glutathione excretion ($r = 0.78$, $p < 0.0001$) and bile flow rate ($r = 0.84$, $p < 0.0001$) (Supplemental Table C).

Similarly to previous reports (Fiorucci et al., 2005; Geier et al., 2003), EE significantly reduced gene expression of Ntcp and organic anion transporting polypeptide (Oatp) 1a4, and strongly upregulated Mrp3. In EE-treated group, protein expression of Bsep, Ntcp, Oatp1a1, Oatp1a4, Mrp2 was reduced, whereas Mrp4 protein mass was increased. EGCG coadministration to EE did not markedly change gene and protein expression, except for the increase in Oatp1a4 mRNA and protein levels of Mrp4 and Ntcp.

3.4. Gene expression in the ileum

Expression of key genes involved in bile acid homeostasis was evaluated in the ileum. Individual treatment with EGCG or EE, as well as the combination of both compounds significantly lowered gene expression of Fgf15 and small heterodimer partner (Shp). Similarly, all these experimental groups showed reduced expression of the main ileal bile acid transporters, apical sodium-dependent bile salt transporter (Asbt) and organic solute transporter (Ost) α, compared to controls (Fig. 3). On the contrary, EGCG administered to EE-treated rats increased mRNA levels of Asbt, Ostα and Ostβ compared to rats administered with EE only.

3.5. EGCG effect on bile acid synthesis

In EGCG-treated rats, hepatic expression of the rate-limiting enzyme in bile acid synthesis, cholesterol 7α-hydroxylase (Cyp7a1), was increased by ~5 and 1.5-fold at mRNA and

protein levels, respectively (Fig. 4A, B). Bile acid synthesis was also assessed by plasma marker of Cyp7a1 activity, C4. The concentrations of C4 were actually doubled in EGCG-treated rats compared to controls (Fig. 4C). We tested whether upregulation of Cyp7a1 is reproducible in vitro in primary rat hepatocytes. EGCG at concentrations of 10 μM or lower had no effect on mRNA expression of Cyp7a1 (Fig. 4D), whereas concentration of 20 μM increased Cyp7a1 gene expression more than 2-fold (p < 0.001).

3.6. EGCG effect on human FXR

Fusion expression construct with the ligand binding domains of human FXR fused to the DNA binding domain of the transcription factor GAL4 was used to examine the effect of EGCG on FXR interaction. After 24 h treatment, we did not find any activation of GAL4-FXR construct in HepG2 treated with 5 or 20 μM EGCG (Fig. 5). CDCA, a potent FXR agonist, was used as a positive control in activating gene reporter construct via the pCMX-GAL4-FXR (Adachi et al., 2005). These data suggest that EGCG may not be an activator of human FXR in clinically relevant concentrations.

4. Discussion

The major finding of the study is that EGCG administration decreased bile production in rats as a consequence of reduced Mrp2 protein expression and thus decreased biliary glutathione excretion. Mrp2 is the major canalicular transporter for glutathione and various organic anions and their metabolites conjugated with glutathione, sulfate or glucuronate. Intracanalicular osmotic pressure generated by these compounds constitutes a bile acid-independent mechanism of bile formation. Data from Mrp2-mutant rats have demonstrated that absence of this protein leads to about 50% reduction in bile flow (Jemnitz et al., 2010) and significant accumulation of its substrates (Nies and Keppler, 2007). Similar effect is observed during blockade (e.g. cyclosporine) (Kobayashi et al., 2004) or downregulation of this transporter (Brcakova et al., 2009). Since EGCG had no effect on bile acid-dependent bile flow, the observed reduction in bile flow after EGCG treatment is very likely a consequence of decreased Mrp2 protein. Reduced function of the Mrp2 was confirmed by a corresponding decrease in biliary glutathione excretion. These results were also indirectly supported by a strong positive correlation detected between bile production, glutathione secretion and Mrp2 protein expression (Supplemental Table C). Since EGCG has been shown to lack inhibitory effect on Mrp2 (Netsch et al., 2005), we can assume that direct inhibition of the transporter by EGCG is likely not involved in the decreased

glutathione excretion despite the fact that EGCG and its methyl metabolites are substrates for human MRP2 (Hong et al., 2003) and the biliary route is the major pathway for EGCG excretion from the body (Chen et al., 1997).

Question arising from our data is how EGCG reduces Mrp2 protein content in the liver. Regulation of Mrp2 expression is complex and involves both transcriptional and posttranscriptional mechanisms. The major ligand-activated transcription factor for Mrp2 is constitutive androstane receptor with a contribution of pregnane X receptor and Fxr (Kast et al., 2002; Nies and Keppler, 2007). Posttranslational regulation is based on the balance between the rate of protein synthesis, canalicular membrane positioning, endocytic retrieval and degradation. Especially these later mechanisms seem to be relevant for Mrp2 regulation during various cholestatic conditions and after administration of several drugs, including ethinylestradiol, clofibrate or dexamethasone (Johnson and Klaassen, 2002; Micuda et al., 2008; Trauner et al., 1997; Zinchuk et al., 2005). Regarding EGCG, Li et al. (2012) recently demonstrated ability of EGCG to activate human FXR, but not constitutive androstane receptor, vitamin D receptor and retinoid X receptors, in the gene-reporter assay. However, in EGCG-fed mice an increased transcription of Fxr target genes was seen only in the intestine but not in the liver (Li et al., 2012). Our unchanged Mrp2 mRNA levels in the livers of EGCG-treated animals comply with this report. Moreover, the reduced Mrp2 protein mass (Fig. 2B) points towards posttranscriptional activity of the catechin. Whether the changes are due to modified translation or impaired Mrp2 protein turnover is not known at the present time. However, altered Mrp2 membrane trafficking or endocytic retrieval are not likely involved since Mrp2 content in our isolated canalicular membranes reflected the overall content of the protein (data not shown).

To verify the effect of EGCG on rat Fxr in the intestine, we also evaluated expression of Asbt and Fxr target genes in the ileum: Ostα/β, Fgf15 and Shp. In contradiction to Li et al. (2012), we have seen downregulation of these genes (Fig. 3), which suggests Fxr alteration by EGCG at the given dose. The reported significant direct agonistic effect of EGCG on the human FXR was also not reproduced in our settings (Fig.5). Whether the reasons for these discrepancies are a variable accumulation of endogenous coactivators (Li et al., 2012), gene-reporter assay differences, species differences (mouse vs. rat vs. human) or duration of treatment (two vs. eight days) requires further elucidation. It also raises the possibility of metabolic instability after EGCG administration. Nevertheless, downregulation of ileal transporters essential for bile acid absorption in EE-administered rats was described for the first time and may signify a new

adaptive mechanism for this type of cholestasis.

An interesting finding of our study was the increased plasma concentrations of bile acids in EGCG-treated rats. Interestingly, biliary excretion of bile acids, the major osmotic driving force for bile formation, was not changed in this group compared to controls. Such a situation indicates either modified enterohepatic recycling of bile acids or increased activity of synthetic enzymes. The major excretory route for bile acids from the body is their biliary excretion with the Bsep transporter being the rate-limiting step (Stieger, 2010). However, neither Bsep protein expression nor protein expression of basolateral bile acid uptake transporters (Ntcp, Oatps) were changed, which was in agreement with unchanged biliary excretion of bile acids after EGCG treatment (Fig. 1C). A direct inhibition of canalicular or basolateral transport of bile acids may be excluded since the clearance study was performed 24 hours after the last administration of EGCG. Considering that the reported half-life of EGCG in rats is 135 min (Chen et al., 1997) and the majority of the drug is eliminated from the body after five half-lives, the plasma concentration of EGCG at the time of bile collection would be too low to produce any significant inhibition of Bsep or other transporters. The second factor that modifies excretion of bile acids is their absorption in the ileum (Dawson et al., 2003). Annaba et al. (2010) recently indeed demonstrated in vitro direct inhibitory effect of EGCG on sodium-dependent transport of bile acids mediated by human ASBT, the key apical transporter for bile acid absorption in the ileum. Therefore, we looked at the expression of this transporter in the ileum and actually found its transcriptional downregulation, which may explain the observed increase in fecal bile acid loss after green tea administration (Yang and Koo, 2000). As this is in contradiction to elevated plasma bile acids after EGCG treatment, we focused on bile acid synthesis.

In EGCG-treated rats, we detected an increased expression of Cyp7a1, the rate-limiting enzyme in bile acid synthesis in the liver, which was further confirmed in vitro in rat hepatocytes and by increased plasma concentrations of C4, the marker of Cyp7a1 activity (Lenicek et al., 2008). This observation is in agreement with a formerly reported ability of EGCG to induce human CYP7A1 mRNA in HepG2 cells through increased promoter activity of CYP7A1 (Lee et al., 2008). Because we were unable to confirm increased activity of rat Cyp7a1 promoter by EGCG (unpublished observation), we can suggest also another mechanism which may contribute to the induction of this enzyme such as the downregulation of Fgf15 in intestine, which, in turn, may be followed by disinhibition of Cyp7a1 expression in the liver (Chiang, 2009). Although the mechanism of

EGCG action should be studied further, we may suggest that the induction of Cyp7a1 is the main cause of EGCG-elevated plasma bile acids since the extent of plasma bile acid increase complied with the extent of plasma C4 increase.

We tested the effect of the catechin in a model of intrahepatic cholestasis induced by ethinylestradiol. Nevertheless, at the given dose, EGCG showed only a tendency for further reduction in Mrp2 protein expression and biliary glutathione excretion (significant if evaluated by Student's t-test), which was not reflected by a reduction in bile flow. Molecular changes detected in EE-treated animals fully comply with the current concept of this form of cholestasis (Fiorucci et al., 2005; Geier et al., 2003; Ruiz et al., 2007) and were not modified by EGCG. Interestingly, melibiose-rhamnose permeability test revealed a permanent impairment of blood-biliary barrier, which was modulated by neither bile duct drainage during the bile collection nor EGCG administration. Collectively, these data suggest that whereas the reduced bile flow (and consequently decreased intrabiliary pressure) and decreased ileal bile acid absorption may contribute to the positive effects of EGCG in obstructive cholestasis, a potential exacerbation of intrahepatic cholestasis by EGCG may exist, which is warning information for long-term deliberate use of high doses of corresponding dietary supplements.

In conclusion, EGCG is known for its protective effects in various experimental models of liver injury. Our data, however, demonstrate that a higher dose or higher biological availability of the catechin may raise plasma bile acid concentrations and induce cholestasis. After exclusion of other possible mechanisms, we suggest that Cyp7a1 upregulation may stand behind or highly contribute to the increase in plasma bile acids. The cholestatic effect of EGCG in rats is mediated by reduced Mrp2 protein expression and thus decreased Mrp2-dependent fraction of bile flow.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

References

Adachi, R., Honma, Y., Masuno, H., Kawana, K., Shimomura, I., Yamada, S., Makishima, M., 2005. Selective activation of vitamin D receptor by lithocholic acid acetate, a bile acid derivative. J Lipid Res 46, 46-57.

Annaba, F., Kumar, P., Dudeja, A.K., Saksena, S., Gill, R.K., Alrefai, W.A., 2010. Green tea catechin EGCG inhibits ileal apical sodium bile acid transporter ASBT. Am J Physiol Gastrointest Liver Physiol 298, G467-473.

Brcakova, E., Fuksa, L., Cermanova, J., Kolouchova, G., Hroch, M., Hirsova, P., Martinkova, J., Staud, F., Micuda, S., 2009. Alteration of methotrexate biliary and renal elimination during extrahepatic and intrahepatic cholestasis in rats. Biol Pharm Bull 32, 1978-1985.

Chen, L., Lee, M.J., Li, H., Yang, C.S., 1997. Absorption, distribution, elimination of tea polyphenols in rats. Drug Metab Dispos 25, 1045-1050.

Chiang, J.Y., 2009. Bile acids: regulation of synthesis. J Lipid Res 50, 1955-1966.

Dawson, P.A., Haywood, J., Craddock, A.L., Wilson, M., Tietjen, M., Kluckman, K., Maeda, N., Parks, J.S., 2003. Targeted deletion of the ileal bile acid transporter eliminates enterohepatic cycling of bile acids in mice. J Biol Chem 278, 33920-33927.

Esteller, A., 2008. Physiology of bile secretion. World J Gastroenterol 14, 5641-5649.

Fiorucci, S., Clerici, C., Antonelli, E., Orlandi, S., Goodwin, B., Sadeghpour, B.M.,

Sabatino, G., Russo, G., Castellani, D., Willson, T.M., Pruzanski, M., Pellicciari, R., Morelli, A., 2005. Protective effects of 6-ethyl chenodeoxycholic acid, a farnesoid X receptor ligand, in estrogen-induced cholestasis. J Pharmacol Exp Ther 313, 604-612.

Geier, A., Dietrich, C.G., Gerloff, T., Haendly, J., Kullak-Ublick, G.A., Stieger, B., Meier, P.J., Matern, S., Gartung, C., 2003. Regulation of basolateral organic anion transporters in ethinylestradiol-induced cholestasis in the rat. Biochim Biophys Acta 1609, 87-94.

Hirsova, P., Kolouchova, G., Dolezelova, E., Cermanova, J., Hyspler, R., Kadova, Z., Micuda, S., 2012. Epigallocatechin gallate enhances biliary cholesterol secretion in healthy rats and lowers plasma and liver cholesterol in ethinylestradiol-treated rats. Eur J Pharmacol.

Hong, J., Lambert, J.D., Lee, S.H., Sinko, P.J., Yang, C.S., 2003. Involvement of multidrug resistance-associated proteins in regulating cellular levels of (-)-epigallocatechin-3-gallate and its methyl metabolites. Biochem Biophys Res Commun 310, 222-227.

Jemnitz, K., Heredi-Szabo, K., Janossy, J., Ioja, E., Vereczkey, L., Krajcsi, P., 2010. ABCC2/Abcc2: a multispecific transporter with dominant excretory functions. Drug Metab Rev 42, 402-436.

Johnson, D.R., Klaassen, C.D., 2002. Regulation of rat multidrug resistance protein 2 by classes of prototypical microsomal enzyme inducers that activate distinct transcription pathways. Toxicol Sci 67, 182-189.

Kand'ar, R., Zakova, P., Lotkova, H., Kucera, O., Cervinkova, Z., 2007. Determination of reduced and oxidized glutathione in biological samples using liquid chromatography with fluorimetric detection. J Pharm Biomed Anal 43, 1382-1387.

Kast, H.R., Goodwin, B., Tarr, P.T., Jones, S.A., Anisfeld, A.M., Stoltz, C.M., Tontonoz, P., Kliewer, S., Willson, T.M., Edwards, P.A., 2002. Regulation of multidrug resistance-associated protein 2 (ABCC2) by the nuclear receptors pregnane X receptor, farnesoid X-activated receptor, and constitutive androstane receptor. J Biol Chem 277, 2908-2915.

Kobayashi, H., Tanaka, Y., Asagiri, K., Asakawa, T., Tanikawa, K., Kage, M., Yagi, M., 2010. The antioxidant effect of green tea catechin ameliorates experimental liver injury. Phytomedicine 17, 197-202.

Kobayashi, M., Saitoh, H., Tadano, K., Takahashi, Y., Hirano, T., 2004. Cyclosporin A, but not tacrolimus, inhibits the biliary excretion of mycophenolic acid glucuronide possibly mediated by multidrug resistance-associated protein 2 in rats. J Pharmacol Exp Ther 309, 1029-1035.

Lee, M.S., Park, J.Y., Freake, H., Kwun, I.S., Kim, Y., 2008. Green tea catechin enhances cholesterol 7alpha-hydroxylase gene expression in HepG2 cells. Br J Nutr 99, 1182-1185.

Lenicek, M., Juklova, M., Zelenka, J., Kovar, J., Lukas, M., Bortlik, M., Vitek, L., 2008. Improved HPLC analysis of serum 7alpha-hydroxycholest-4-en-3-one, a marker of bile acid malabsorption. Clin Chem 54, 1087-1088.

Li, G., Lin, W., Araya, J.J., Chen, T., Timmermann, B.N., Guo, G.L., 2012. A tea catechin, epigallocatechin-3-gallate, is a unique modulator of the farnesoid X receptor. Toxicol Appl Pharmacol 258, 268-274.

Mazzanti, G., Menniti-Ippolito, F., Moro, P.A., Cassetti, F., Raschetti, R., Santuccio, C., Mastrangelo, S., 2009. Hepatotoxicity from green tea: a review of the literature and two unpublished cases. Eur J Clin Pharmacol 65, 331-341.

Micuda, S., Fuksa, L., Brcakova, E., Osterreicher, J., Cermanova, J., Cibicek, N., Mokry, J., Staud, F., Martinkova, J., 2008. Zonation of multidrug resistance-associated protein 2 in rat liver after induction with dexamethasone. J Gastroenterol Hepatol 23, e225-230.

Modica, S., Petruzzelli, M., Bellafante, E., Murzilli, S., Salvatore, L., Celli, N., Di Tullio, G., Palasciano, G., Moustafa, T., Halilbasic, E., Trauner, M., Moschetta, A., 2012. Selective activation of nuclear bile acid receptor FXR in the intestine protects mice against cholestasis. Gastroenterology 142, 355-365 e351-354.

Netsch, M.I., Gutmann, H., Luescher, S., Brill, S., Schmidlin, C.B., Kreuter, M.H., Drewe,

J., 2005. Inhibitory activity of a green tea extract and some of its constituents on multidrug resistance-associated protein 2 functionality. Planta Med 71, 135-141.

Nies, A.T., Keppler, D., 2007. The apical conjugate efflux pump ABCC2 (MRP2). Pflugers Arch 453, 643-659.

Rahner, C., Stieger, B., Landmann, L., 1996. Structure-function correlation of tight junctional impairment after intrahepatic and extrahepatic cholestasis in rat liver. Gastroenterology 110, 1564-1578.

Ramesh, E., Jayakumar, T., Elanchezhian, R., Sakthivel, M., Geraldine, P., Thomas, P.A., 2009. Green tea catechins, alleviate hepatic lipidemic-oxidative injury in Wistar rats fed an atherogenic diet. Chem Biol Interact 180, 10-19.

Ren, Y., Deng, F., Zhu, H., Wan, W., Ye, J., Luo, B., 2011. Effect of epigallocatechin-3-gallate on iron overload in mice with alcoholic liver disease. Mol Biol Rep 38, 879-886.

Ruiz, M.L., Villanueva, S.S., Luquita, M.G., Ikushiro, S., Mottino, A.D., Catania, V.A., 2007. Beneficial effect of spironolactone administration on ethynylestradiol-induced cholestasis in the rat: involvement of up-regulation of multidrug resistance-associated protein 2. Drug Metab Dispos 35, 2060-2066.

Singh, B.N., Shankar, S., Srivastava, R.K., 2011. Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. Biochem Pharmacol 82, 1807-1821.

Stickel, F., Kessebohm, K., Weimann, R., Seitz, H.K., 2011. Review of liver injury associated with dietary supplements. Liver Int 31, 595-605.

Stieger, B., 2010. Role of the bile salt export pump, BSEP, in acquired forms of cholestasis. Drug Metab Rev 42, 437-445.

Tipoe, G.L., Leung, T.M., Liong, E.C., Lau, T.Y., Fung, M.L., Nanji, A.A., 2010. Epigallocatechin-3-gallate (EGCG) reduces liver inflammation, oxidative stress and

fibrosis in carbon tetrachloride (CCl4)-induced liver injury in mice. Toxicology 273, 45-52.

Tomsik, P., Sispera, L., Rezacova, M., Niang, M., Stoklasova, A., Cerman, J., Knizek, J., Brcakova, E., Cermanova, J., Micuda, S., 2008. Increased melibiose/rhamnose ratio in bile of rats with acute cholestasis. J Gastroenterol Hepatol 23, 1934-1940.

Trauner, M., Arrese, M., Soroka, C.J., Ananthanarayanan, M., Koeppel, T.A., Schlosser, S.F., Suchy, F.J., Keppler, D., Boyer, J.L., 1997. The rat canalicular conjugate export pump (Mrp2) is down-regulated in intrahepatic and obstructive cholestasis. Gastroenterology 113, 255-264.

Yang, T.T., Koo, M.W., 2000. Chinese green tea lowers cholesterol level through an increase in fecal lipid excretion. Life Sci 66, 411-423.

Zhong, Z., Froh, M., Lehnert, M., Schoonhoven, R., Yang, L., Lind, H., Lemasters, J.J., Thurman, R.G., 2003. Polyphenols from Camellia sinenesis attenuate experimental cholestasis-induced liver fibrosis in rats. Am J Physiol Gastrointest Liver Physiol 285, G1004-1013.

Zhu, Y., Li, F., Guo, G.L., 2011. Tissue-specific function of farnesoid X receptor in liver and intestine. Pharmacol Res 63, 259-265.

Zinchuk, V., Zinchuk, O., Okada, T., 2005. Experimental LPS-induced cholestasis alters subcellular distribution and affects colocalization of Mrp2 and Bsep proteins: a quantitative colocalization study. Microsc Res Tech 67, 65-70.

Table 1

Plasma biochemistry

	Ctrl	EGCG	EE	EE-EGCG
Bile acids (μM)	13.3 ± 5.5	27.2 ± 8.2[c]	39.3 ± 12.6[a]	41.4 ± 13.1[a]
Bilirubin (μM)	2.9 ± 0.7	2.7 ± 0.8	6.4 ± 1.8[b]	8.6 ± 4.2[a]
ALT (μkat/L)	1.0 ± 0.5	1.0 ± 0.5	1.7 ± 0.4	0.7 ± 0.1
AST (μkat/L)	2.2 ± 1.2	2.1 ± 0.6	1.7 ± 0.4	1.4 ± 0.5

Data are expressed as means ± SD for 6-7 animals per group. Significantly different compared to controls: [a] $p < 0.001$, [b] $p < 0.01$, [c] $p < 0.05$.

Figure Legends

Fig. 1. Effect of EGCG on bile flow and biliary excretion. (A) EGCG and EE reduced cumulative bile flow by 23% and 58%, respectively. (B) Permeability of blood-biliary barrier was assessed by biliary excretion of melibiose and rhamnose. The higher ratio indicates an increased hepatic tight junctional permeability, which was seen in both groups receiving EE. (C) EGCG had no effect on biliary bile acid excretion. EE, either alone or in the combination with EGCG, decreased biliary bile acid excretion to ~40% of controls. (D) Biliary glutathione excretion declined to 30% after EGCG treatment while EE reduced this parameter below 1%. Data are expressed as means ± SD for 6-7 animals per group. Significantly different compared to the control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 2. Hepatic mRNA and protein levels. (A) EGCG decreased gene expression of bile acid transporters Bsep and Ntcp. (B) Apart from Mrp2 and Mrp4, protein expression of hepatic transporters was not altered by EGCG treatment. Data are expressed as percentage of the average expression in the control group. Bars are means ± SD for 6-7 animals per group. Significantly different compared to the control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Significantly different compared to EE group: [#] $p < 0.05$, [###] $p < 0.001$.

Fig. 3. mRNA expression of key genes in the ileum. Levels of Fgf15, Shp, Asbt and Ostα mRNA were downregulated in rats treated with EGCG. However, EGCG in the combination with EE increased gene expression of Asbt, Ostα and Ostβ compared to EE group. Data are expressed as percentage of the average expression in the control group. Bars are means ± SD for 6-7 animals per group. Significantly different compared to the control group: * $p < 0.05$, ** $p < 0.01$. Significantly different compared to EE group: [#] $p < 0.05$, [##] $p < 0.01$.

Fig. 4. Effect of EGCG on Cyp7a1 mRNA, protein, and activity. (A) EGCG upregulated gene expression of Cyp7a1 in rat liver. (B) Protein expression of Cyp7a1 was higher in rats treated with EGCG compared to controls. (C) Rats treated with EGCG showed a 2-fold increase in plasma C4, the marker of Cyp7a1 activity. (D) Primary rat hepatocytes cultured in a collagen-sandwich configuration were treated with 0, 1, 5, 10, 20 µM EGCG for 24 hours and the data presented are average from three independent experiments. EGCG at the

concentration of 20 µM doubled Cyp7a1 gene expression in rat hepatocytes in vitro. Significantly different compared to the control group/treatment: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig. 5. Interaction of EGCG with human FXR. The transient transfection gene reporter assay was performed in HepG2 cells co-transfected with the pGL5-luc luciferase reporter construct, expression plasmid pCMX-GAL4-FXR, and pRL-TK. After 24 h, HepG2 cells were treated with EGCG (5 µM and 20 µM), CDCA (50 µM) and vehicle of EGCG (DMSO) for 24 h. Firefly luciferase activity was normalized to the Renilla luciferase activity. Data are shown as mean fold activation ($\pm$ SD, $n = 3$) of normalized luciferase activity in vehicle-treated cells. Significantly different compared to the control treatment: * $p < 0.001$.
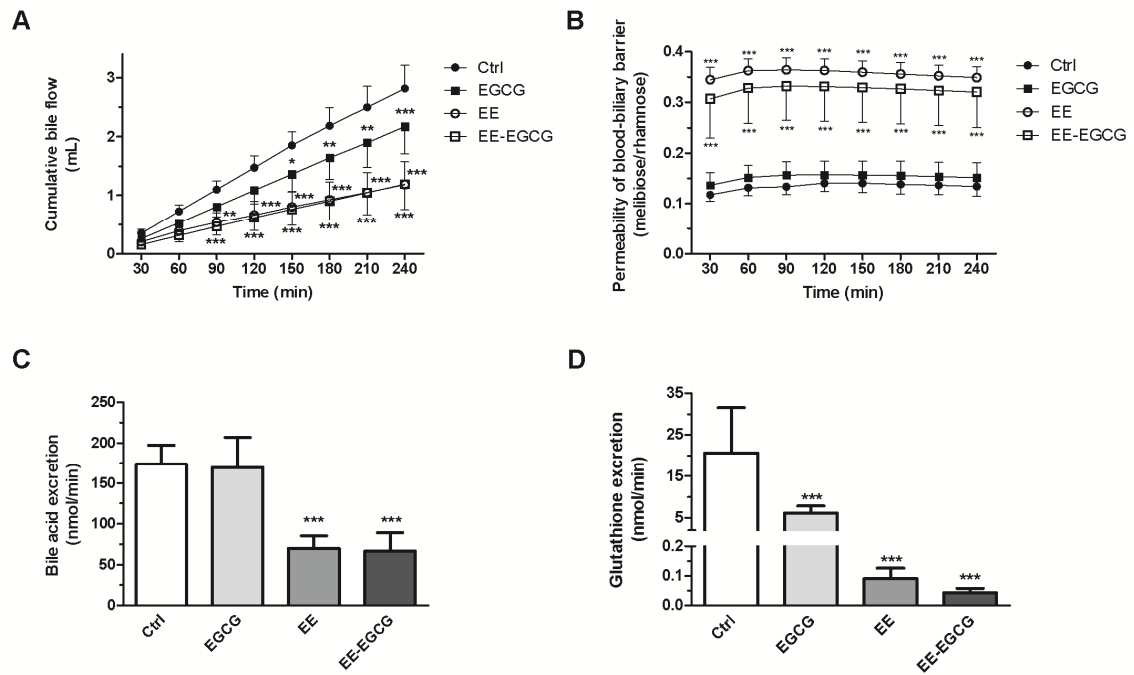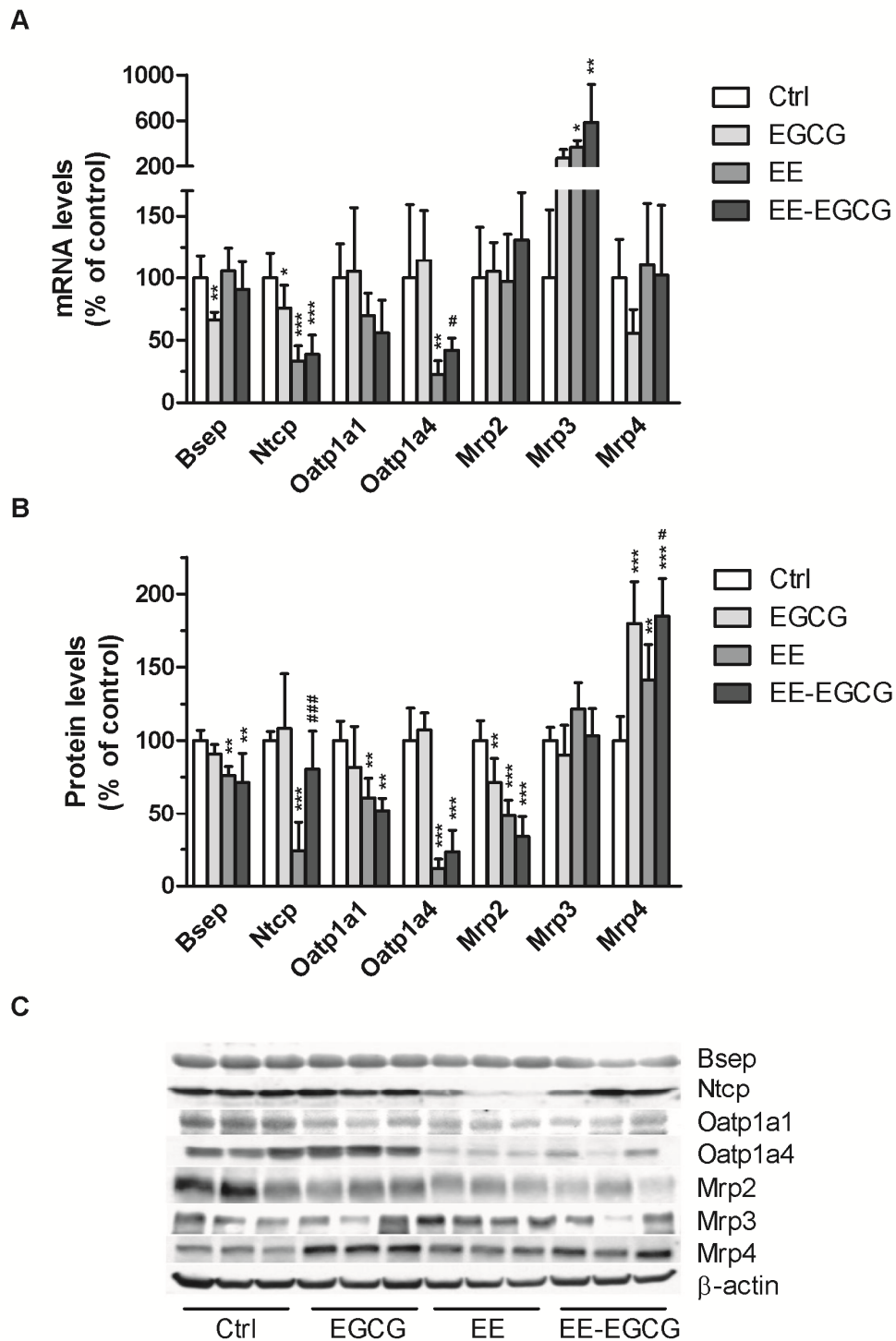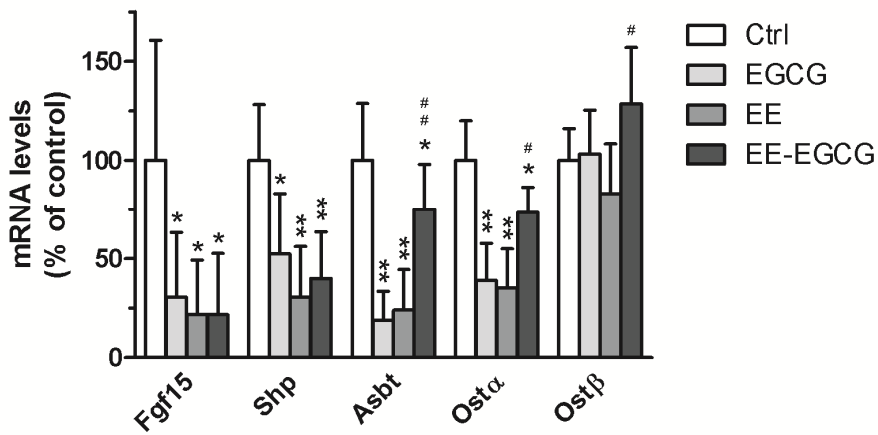
Figure 1

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