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Effect of epigallocatechin gallate on bile production

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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
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BADF	Bile acid-dependent fraction
BAIF	Bile acid-independent fraction
BE	Biliary excretion
BSEP	Bile salt export pump
C4	7α-Hydroxy-4-cholesten-3-one
CAR	Constitutive androstane receptor
CDCA	Chenodeoxycholic acid
CYP7A1	Cholesterol 7α-hydroxylase
DCA	Deoxycholic acid
DMEM	Dulbecco's modified Eagle's medium
EE	Ethinylestradiol
EGCG	Epigallocatechin gallate
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
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LRH-1	Liver receptor homologue-1
LXR	Liver X receptor
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
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. Souhrn

Vliv epigallokatechingalátu na tvorbu žluče

Epigallokatechingalát (EGCG), významný katechin zeleného čaje, působil příznivě u řady experimentálních modelů poškození jater. Jeho efekt na tvorbu žluče a jaterní metabolismus cholesterolu však nebyl dosud zcela objasněn. V předkládané disertační práci byl studován účinek EGCG na tvorbu žluče a homeostázu žlučových kyselin a cholesterolu jak u zdravých potkanů tak u potkanů s intrahepatální cholestázou navozenou podáváním ethinylestradiolu. Podávání EGCG potkanům vedlo ke snížení toku žluče o 23 %. Paracelulární permeabilita a biliární exkrece žlučových kyselin nebyla ovlivněna, zatímco biliární exkrece glutathionu po podávání EGCG byla snížena významně (o 70 %). Stejně tak významné bylo snížení exprese proteinu Mrp2, hlavního transportéru pro glutathion. EGCG výrazně zvýšil biliární exkreci cholesterolu a fosfolipidů. Tyto změny těsně korelovaly se zvýšenou expresí Abcg5/8 (ATP-binding cassette transporter G5/8) a Sr-B1 (scavenger receptor class B type 1) a se sníženou expresí acyl-CoA:cholesterol acyltransferázy (Acat2). EGCG u potkanů rovněž zvýšil plazmatické koncentrace žlučových kyselin. Zatímco exprese hlavních jaterních transportérů žlučových kyselin se nezměnila, exprese Cyp7a1, klíčového enzymu v syntéze žlučových kyselin, byla po podávání EGCG výrazně zvýšena. Zvýšená syntéza žlučových kyselin u těchto zvířat byla také potvrzena zvýšenými plazmatickými hladinami 7α-hydroxy-4-cholesten-3-onu. EGCG naopak výrazně downreguloval hlavní transportéry žlučových kyselin (Asbt a Ostα) a regulační molekuly (Shp a Fgfl15) v ileu.

Při současném podávání EGCG s ethinylestradiolem, nevykazoval katechin žádný dodatečný účinek na navozenou cholestázu, ale vedl ke snížení plazmatické hladiny celkového cholesterolu a VLDL cholesterolu. Dále podávání EGCG zabránilo zvýšení hmotnosti jater a kumulaci cholesterolu v játrech, navozené podáváním ethinylestradiolu. Tento účinek byl spojen s odpovídající redukcí exprese Acat2 v játrech.

Tato studie prokázala schopnost EGCG zvýšit plazmatické hladiny žlučových kyselin, zejména prostřednictvím Cyp7a1 upregulace, a snížit produkci žluče prostřednictvím snížení MRP2-závislého toku žluče. EGCG také zvýšilo biliární exkreci cholesterolu prostřednictvím upregulace Abcg5/8 a zabránilo u cholestatických potkanů hromadění cholesterolu v játrech prostřednictvím snížené exprese Acat2. Tato data významně přispívají k současným poznatkům o vlivu EGCG na homeostázu žlučových kyselin a cholesterolu.

2. Summary

Effect of epigallocatechin gallate

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 Fgfl15) 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.

3. Introduction

3.1. 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.

Bile is an isotonic aqueous solution (~97% water) containing bile acids, phospholipids (predominantly phosphatidylcholine), cholesterol, bilirubin, glutathione, proteins, and inorganic ions. 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 BAIF, although this varies with the species and responses to enteric hormones.[1]

3.1.1. Biliary bile acid excretion

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. 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.[2] 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.[3]

3.1.1.1. 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. Primary bile acids, cholic acid and chenodeoxycholic acid, are synthesized from cholesterol exclusively in the liver. At least 14 enzymes have been shown to take part in the bile acid synthesis. 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.[4] Bile acid synthesis within the liver is completed when primary bile acids are conjugated to taurine or glycine.

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. FXR functions as a sensor for intracellular levels of bile acids, which are endogenous ligands of this receptor. To suppress CYP7A1 gene expression, activated FRX induces expression of small heterodimeric partner (SHP), which in turn inhibits activity of hepatocyte nuclear factor-4 α (HNF4α) and liver receptor homologue-1 (LRH-1), which mediate CYP7A1 transcription.[4]

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. Released Fgf15 binds to the liver-specific receptor Fgfr4 (fibroblast growth factor receptor 4) and suppresses Cyp7a1 transcription.[5]

In rodents, but not in humans, Cyp7a1 expression can be induced by activation of liver X receptor α (Lxrα).[6] 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.

3.1.1.2. 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.

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. All of the above-mentioned bile acid transporters are

mainly regulated by the class II sub-family of the nuclear receptor superfamily. 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 α (PPRAα), constitutive androstane receptor (CAR), LXRα, and the common partner RXR.[7] Other nuclear receptors involved in bile acid transporter gene regulation are SHP, HNF4α, and LRH-1.[8]

From all these nuclear receptors, FXR plays the key role in regulation of bile acid transporters. In rats, Fxr activation indirectly suppresses Ntcp transcription via Shp, which interferes with basal Ntcp expression regulated by Rar/Rxr.[8] 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.

3.1.2. 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. 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.[9] 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.[10]

3.1.3. Biliary cholesterol excretion

The whole body cholesterol homeostasis is regulated at several levels: cholesterol *de novo* synthesis, hepatic catabolism, 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[11], 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.[12] Further, expression and function of ATP-binding cassette transporters sub-family G (ABCG), multidrug resistance protein (MDR) 3 and scavenger receptor class B type 1 (SR-B1) are essential for biliary cholesterol excretion.[13]

3.1.4. 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. Mdr2 knockout mice not only lack biliary phosphatidylcholine excretion[14], but also present significantly decreased biliary excretion of cholesterol[12]. It has been shown that at least minimal amount of phospholipids is needed for normal cholesterol excretion into bile.[15]

3.1.5. Biliary paracellular transport

Bile components can enter bile canaliculi not only by hepatocyte excretion but also by paracellular pathway. Specialized structures between adjacent hepatocytes (desmosomes, tight-junctions) seal the bile canaliculi 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.[16] This, in turn, can decrease bile flow, as exemplified in estrogen-treated rats.[17-18] Hence, the proper function of blood-biliary barrier is considered to play an important role in bile production.

3.2. 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.[19-20]

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.[21] 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.[21-22] A cup of green tea may contain 100-200 mg of EGCG.[23] 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.

3.2.1. 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, liver injury induced by carbon tetrachloride or D-galactosamine or prevented ischemia/reperfusion-induced liver apoptosis.[24-28] 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.[29-30]

To date, little is known about the effect of EGCG on biliary physiology or bile acid homeostasis. Li et al.[31] 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.[32] The opposite effect of EGCG on human CYP7A1 expression was described in human hepatoma (HepG2) cells, where EGCG induced CYP7A1mRNA levels.[33] 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.[34] 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.[34] Cases of green tea-related hepatotoxicity have been comprehensively reviewed elsewhere.[35-36]

3.2.2. 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.[37-40] 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.[41] Several animal studies reported that green tea or its extract reduced plasma cholesterol and liver cholesterol accumulation during high fat or high cholesterol diet.[42-49]

Pure EGCG also proved its ability to attenuate a diet-induced hypercholesterolemia and cholesterol accumulation in the liver.[50] EGCG has been shown to interfere with micellar solubilization of cholesterol in the intestine and thus decrease cholesterol intestinal absorption.[51] Later studies confirmed increased cholesterol fecal excretion in EGCG-treated animals.[50,52] Therefore, it is generally accepted that EGCG exerts its hypocholesterolemic activity through reduced intestinal absorption of cholesterol and its enhanced fecal excretion.[53]

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.[54] 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.[55] EGCG was also shown as a potent *in vitro* inhibitor of squalene epoxidase and 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, two important enzymes in the cholesterol biosynthetic pathway.[56-57] Several reports showed that hepatocytes incubated in the presence of EGCG secreted less apolipoprotein B-100 into the medium.[54,58-59]

4. Aims of the study

The overall objective of the present postdoctoral research 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

5. Materials and methods

5.1. Reagents, animals and experimental design

EGCG and ethinylestradiol were purchased from Cayman Chemical and Sigma-Aldrich, respectively. 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.

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 Fig. 6. 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.

If we extrapolate data from Kao et al.,[60] 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.[19]

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 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 were taken at designated time intervals after injection of the sugars. 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 and small intestines were harvested. Bile flow was determined gravimetrically without correction for relative density, assuming a bile density of 1.0 g/mL. Feces were collected from two cages in each group during the last three days of the treatment.

5.2. 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.[61] Plasma concentrations of 7α-hydroxy-4-cholesten-3-one (C4), a marker of Cyp7a1 activity, were measured by high-performance liquid chromatography (HPLC) as previously described.[62]

5.3. 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 at the beginning of bile collection and a 4-h pharmacokinetic study was performed. Plasma and biliary concentrations of melibiose and rhamnose were determined by HPLC method.[63] 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.

5.4. 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).

5.5. 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.[64]

5.6. Glutathione measurements

Concentrations of reduced (GSH) and oxidized (GSSG) glutathione in bile and liver tissue were analyzed separately using HPLC method on a Shimadzu system.[65] Total glutathione was calculated as the sum of reduced glutathione and oxidized glutathione (GSH + 2GSSG).

5.7. 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).

5.8. 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). RNA was 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).[66] The relative changes in gene expression were analyzed using ΔΔCt method. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase expression.

5.9. 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 $100\ 000 \times g$ for 1 h. Isolated canalicular membrane fractions were prepared according to Ito et al.[67] Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblot detection were performed as previously described.[66]

5.10. Gene reporter assay for human FXR

HepG2 cell line was purchased from the European Collection of Cell Cultures (Salisbury, UK). 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, expression plasmid pCMX-GAL4-FXR containing FXR ligand binding domain and pRL-TK using Lipofectamine 2000 (Life Technologies). Following 24 h of stabilization, HepG2 cells were treated with EGCG, chenodeoxycholic acid, and vehicle (DMSO) for 24 h. Cells were analyzed for firefly luciferase activity normalized to *Renilla* luciferase activity (Dual-Luciferase Reporter Assay, Promega).

5.11. 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[65] 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. In both sets of experiments, hepatocytes were cultered in collagen-sandwich configuration. 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 for 24 h incubation.

5.12. 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). 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.

5.13. HepG2 cell culture and treatment

HepG2 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM containing 10% fetal bovine serum (Gibco) and penicillin/streptomycin (100 U/mL, 100 μg/mL). At 50% to 70% confluence, cells were treated with DMSO or EGCG for 24 h in serum-free medium.

5.14. Rat Cyp7a1 promoter activity

Rat Cyp7a1 promoter reporter construct (pGL2-Cyp7a1) was provided from J. Y. L. Chiang (Northeastern Ohio University). 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, pCMV-LXRα and pCMV-RXRα expression plasmids and phRL-CMV. Transfection was performed in triplicates in 24-well plates using Lipofectamin 2000 (Invitrogen). After 24 h, cells were treated with DMSO, EGCG and 22-(R)-hydroxycholesterol 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.

5.15. Human CYP7A1 promoter activity

Human CYP7A1 promoter reporter construct (pGL3-CYP7A1) was a gift from J. Y. L. Chiang (Northeastern Ohio University). 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). For transfection experiments, medium was changed to OPTI-MEM medium and cells were cotransfected with pGL3-CYP7A1, pGL3 and phRL-CMV using X-Treme HP (Roche). Twenty-four hours later, cells were treated with DMSO, EGCG and CDCA 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 5.14.

5.16. 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 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).

6. Results

6.1. 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. 1).
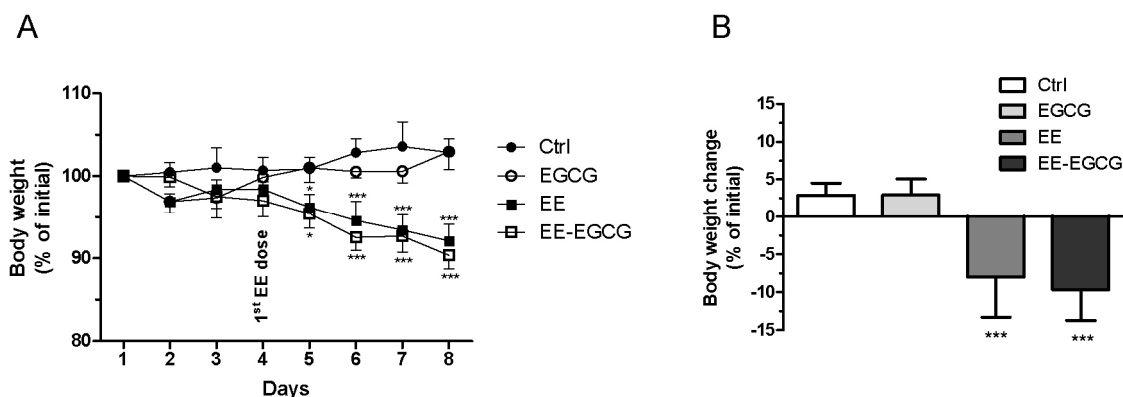
Figure 1. Time course of body weight change (A) and body weight change at the end of experiment (B) as a percentage of initial body weight. Data are expressed as means ± SD for 6-7 animals per group. Significantly different from the control group: * P < 0.05, *** P < 0.001.

6.2. 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. 2). On the other side, ethinylestradiol caused a significant liver weight increase, which was blunted by EGCG coadministration. 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.
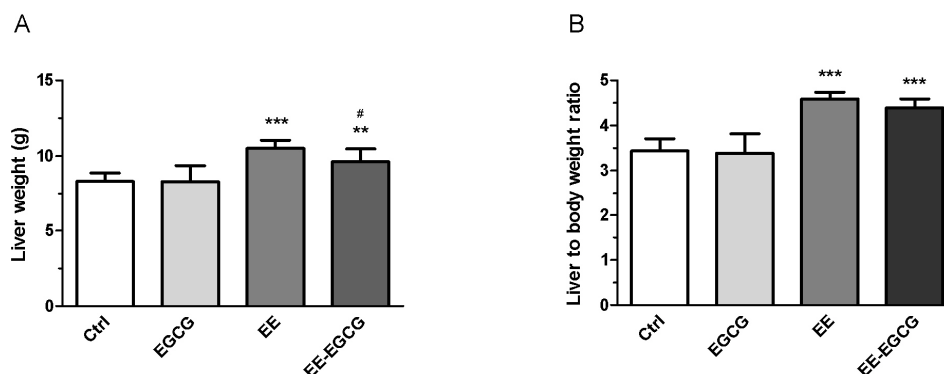
Figure 2. Liver weight (A) and liver weight to body weight ratio (B). Data represent means ± SD for 6-7 animals per group. Significantly different from the control group: ** P < 0.01, *** P < 0.001. Significantly different from EE group: # P < 0.05.

6.3. 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 1), but it did not further raise ethinylestradiol-elevated plasma bile acids in EE-EGCG group. 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 1. 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 (detection limit 0.01 mM).

6.4. 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. 3). Interestingly, the average bile flow rate in EGCG-treated rats was reduced by 23% compared to the control group ($P < 0.05$). Ethinylestradiol administration to rats diminished bile flow by $\sim 60\%$ ($P < 0.001$) regardless of EGCG treatment as the effect of ethinylestradiol prevailed over the effect of EGCG.
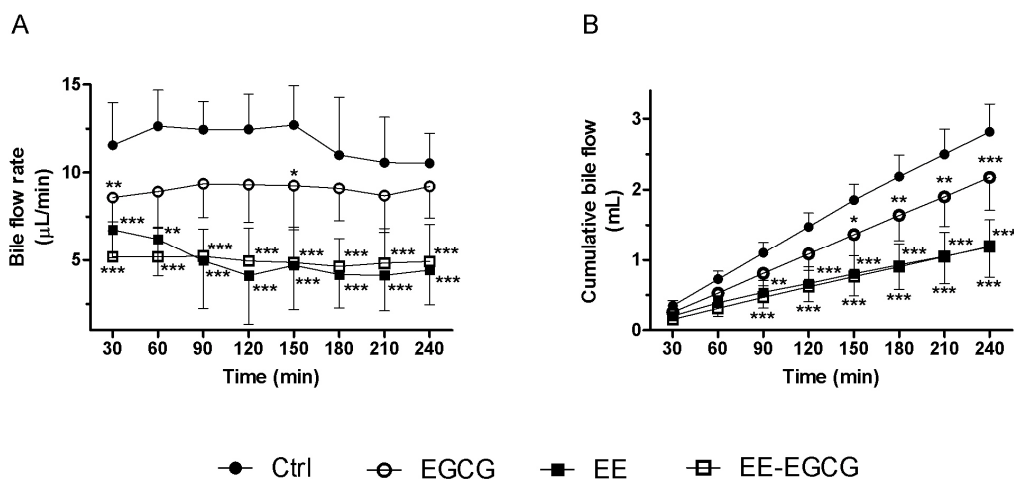
Figure 3. Bile flow expressed as a time course of the bile flow rate (A) and cumulative bile flow (B). Values are means ± SD for 6-7 animals per group. Significantly different from the control group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

6.5. Function of blood-biliary barrier

The ratio of biliary excretion of melibiose to rhamnose is a marker of blood-biliary barrier impairment. This ratio was markedly increased in the groups receiving ethinylestradiol and ethinylestradiol plus EGCG (Fig. 4), 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,[68] this particular permeability test was utilized in ethinylestradiol-treated rats for the first time.
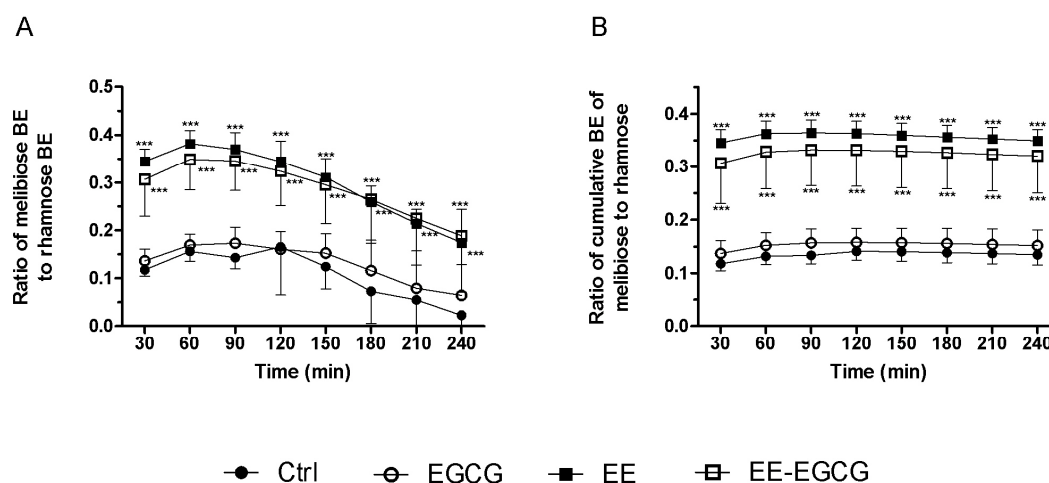
Figure 4. Ratio of melibiose biliary excretion (BE) to rhamnose biliary excretion (A) and ratio of melibiose cumulative biliary excretion to rhamnose cumulative biliary excretion (B). Values are means ± SD for 6-7 animals per group. Significantly different from the control group: *** $P < 0.001$.

6.6. Biliary excretion of bile acids, glutathione and lipids

EGCG treatment did not affect biliary output of bile acids compared to controls (Fig. 5), but reduced biliary glutathione excretion by $\sim 70\%$ ($P < 0.001$). 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, $P < 0.001$).

A

B

C

D
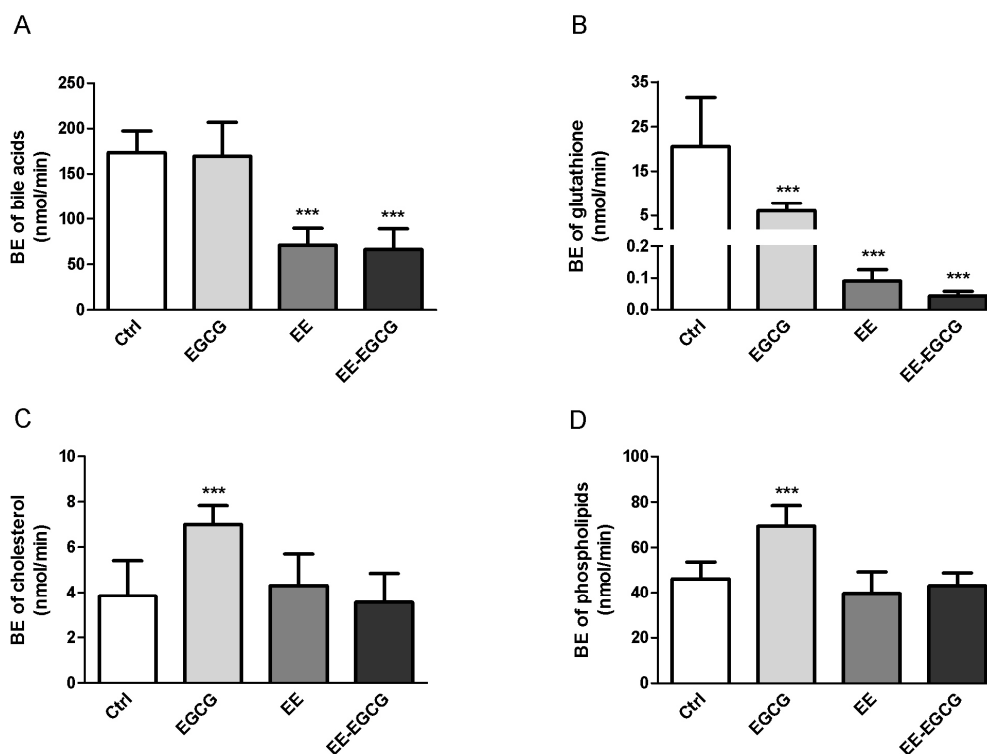
Figure 5. Biliary excretion of bile acids (A), glutathione (B), cholesterol (C), and phospholipids (D). Values are means ± SD for 6-7 animals per group. Significantly different from the control group: *** $P < 0.001$.

6.7. Lithogenic index

Both EGCG and ethinylestradiol significantly increased formation of lithogenic bile (Fig. 6).
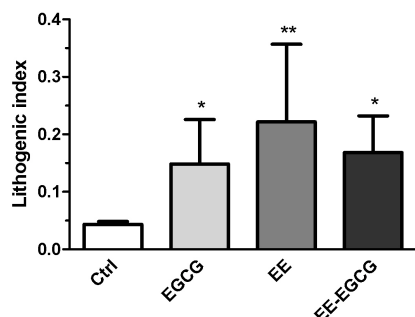
Figure 6. Lithogenic index. Values are means ± SD for 6-7 animals per group. Significantly different from the control group: * $P < 0.05$, ** $P < 0.01$.

6.8. Fecal bile acid excretion

A

B
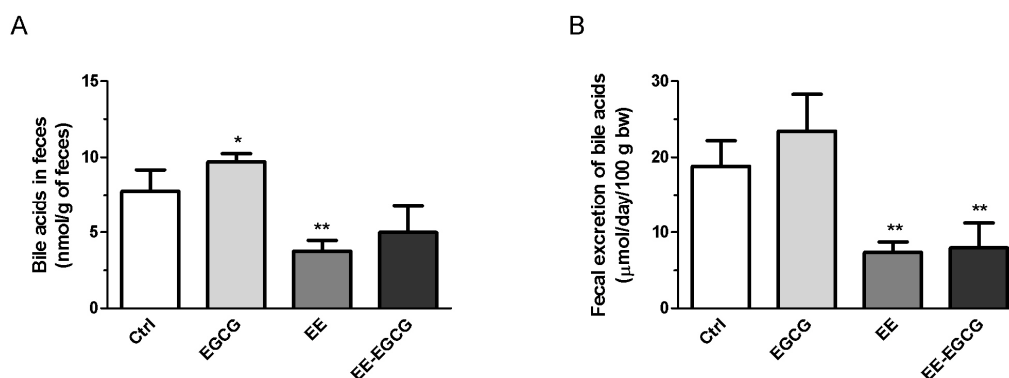
Figure 7. Concentration of total bile acids in the feces (A) and fecal bile acid excretion (B). Values are means ± SD for 4 samples per group. Significantly different from the control group: * $P < 0.05$, ** $P < 0.01$.

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EGCG-treated rats had higher concentrations of bile acids in feces than the control rats (Fig. 7A). The fecal bile acid excretion tended to be increased after EGCG treatment, although this was not statistically significant (Fig. 7B).

6.9.   Liver glutathione concentration

EGCG-treated rats showed increased concentrations of oxidized glutathione, but unchanged levels of the reduced form and total glutathione compared to controls (Fig. 8A-C). This, however, made the GSH/GSSG ratio decrease significantly (Fig. 8D). 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 8. Liver concentrations of reduced glutathione (A), oxidized glutathione (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: ## $P < 0.01$, ### $P < 0.001$.
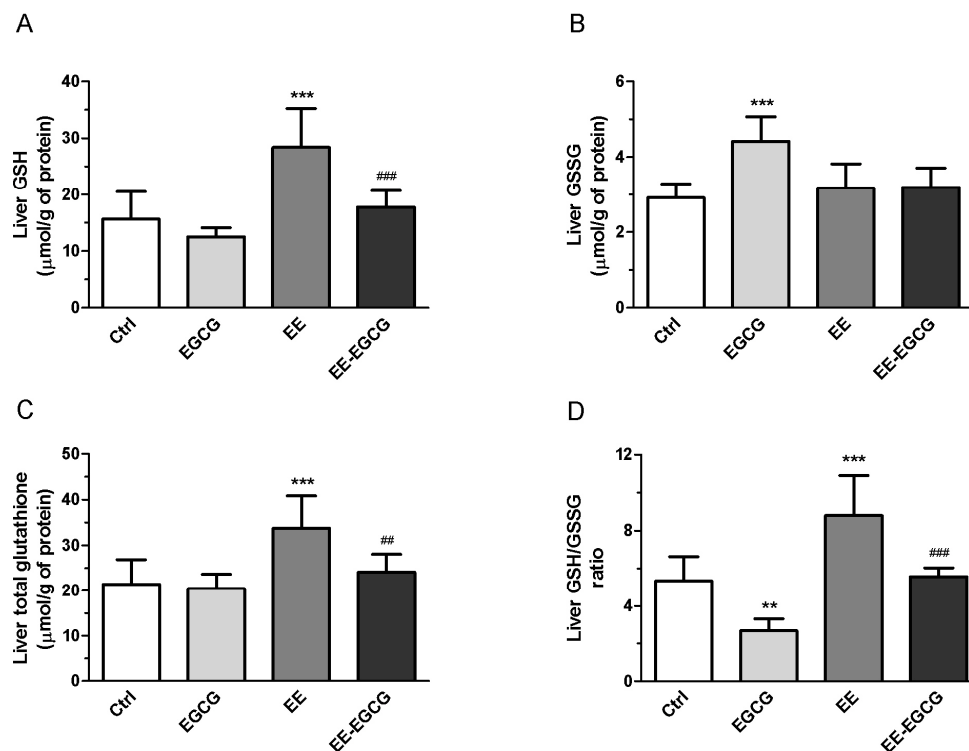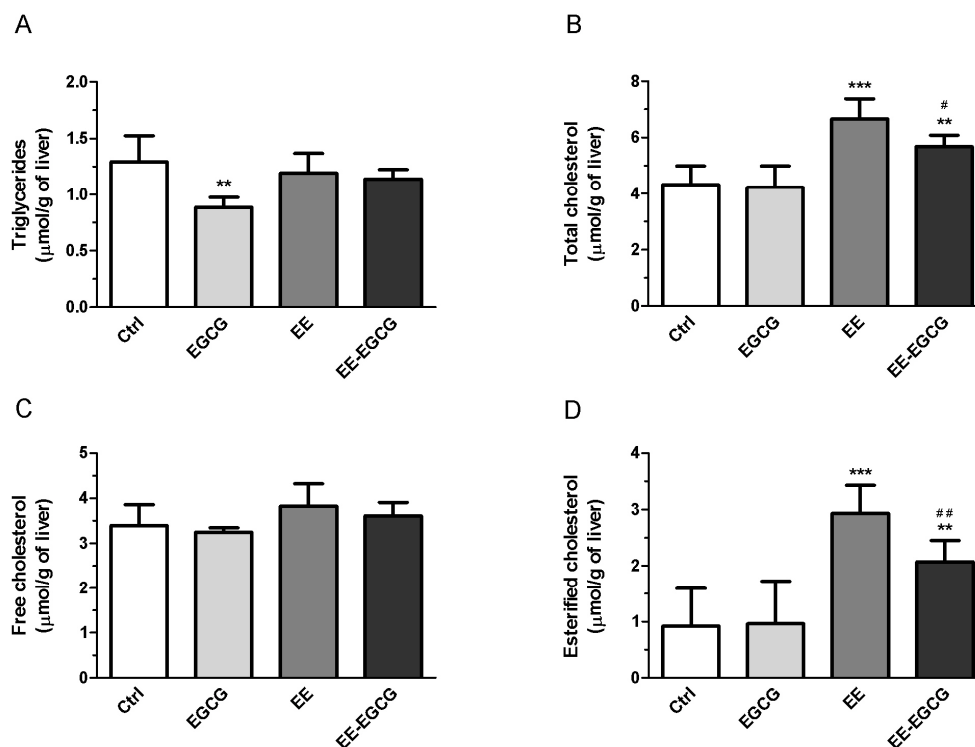
6.10.   Liver concentrations of lipids

A moderate decline in liver triglycerides was observed in EGCG-treated rats, whereas no such change occurred when EGCG was coadministered with ethinylestradiol (Fig. 9A). 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. 9B, 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 9. 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: [#] $P < 0.05$, [##] $P < 0.01$.

6.11. 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. 10A). Nevertheless, their protein expression was not changed compared to controls (Fig. 10B). On the other hand, EGCG nearly doubled protein expression of Mrp4 ($P < 0.001$).

Similarly to previous reports, ethinylestradiol significantly reduced gene expression of Ntcp and Oatp1a4 and strongly upregulated Mrp3 (Fig. 10A). In ethinylestradiol-treated group, protein expression of Bsep, Ntcp, Oatp1a1, Oatp1a4, Mrp2 was reduced, whereas Mrp4 protein mass was increased (Fig. 10B). EGCG coadministration to ethinylestradiol did not markedly alter gene or protein expression, except for the increase in Oatp1a4 mRNA and Mrp4 protein.

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. 11). On the contrary, EGCG administered to ethinylestradiol-treated rats increased mRNA levels of Asbt, Ostα and Ostβ compared to rats administered with ethinylestradiol only.

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. 12). 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.

A

B

C
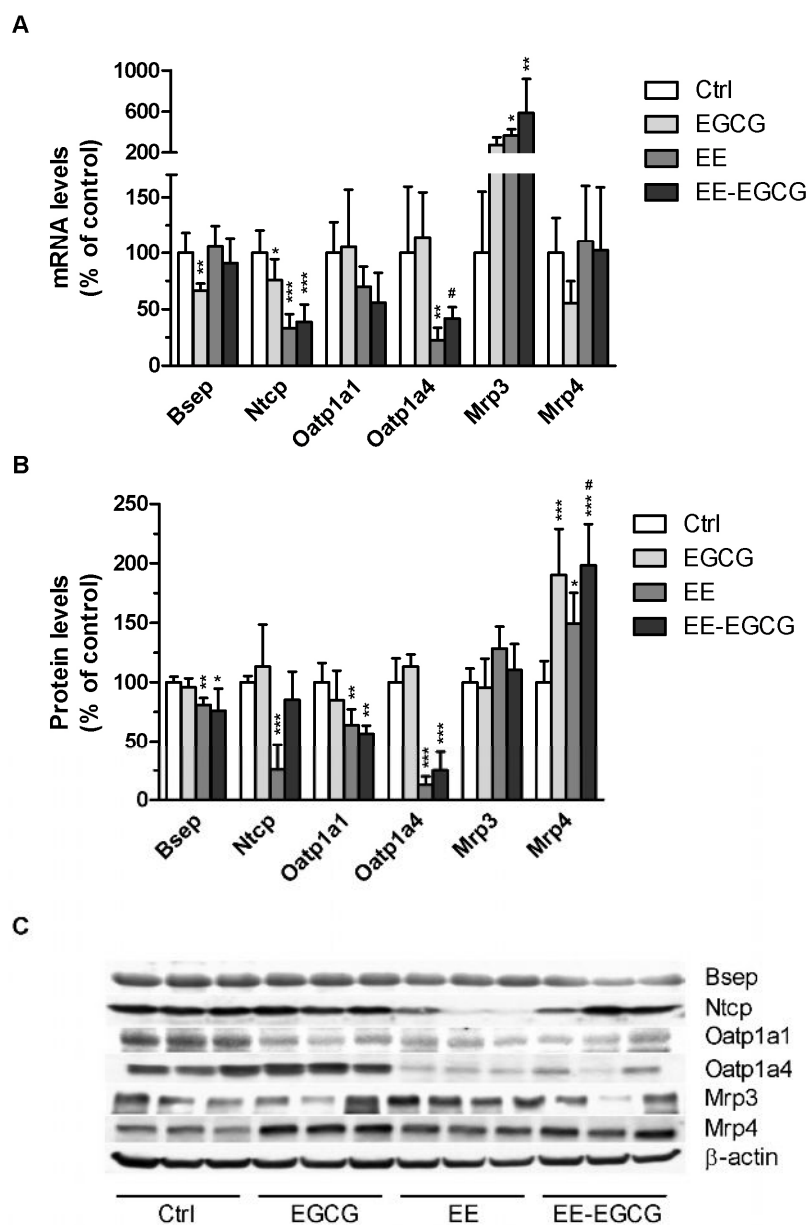
Figure 10. Hepatic expression of bile acid transporters at the mRNA (A) and protein levels (B). Representative immunoblots are shown (C). Values are means ± SD for 6-7 animals per group. Significantly different from the control group: * P < 0.05, ** P < 0.01, *** P < 0.001. Significantly different from EE-treated group: # P < 0.05.
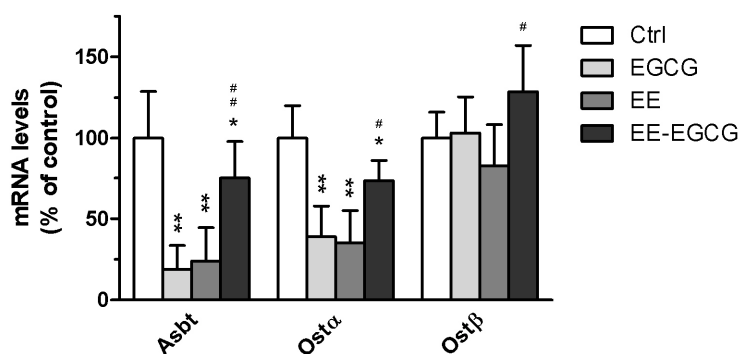
Figure 11. Gene expression of the major bile acid transporters in the ileum. Values are means ± SD for 6-7 rats per group. Significantly different from the control group: * P < 0.05, ** P < 0.01. Significantly different from EE group: # P < 0.05, ## P < 0.01.

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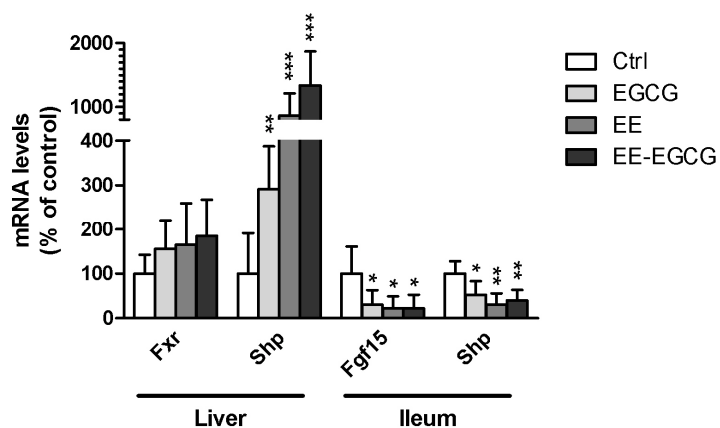
Figure 12. Gene expression of the major regulators of bile acid homeostasis in the liver and ileum. Values are means ± SD for 6-7 rats per group. Significantly different from the control group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

6.12. 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. 13A).

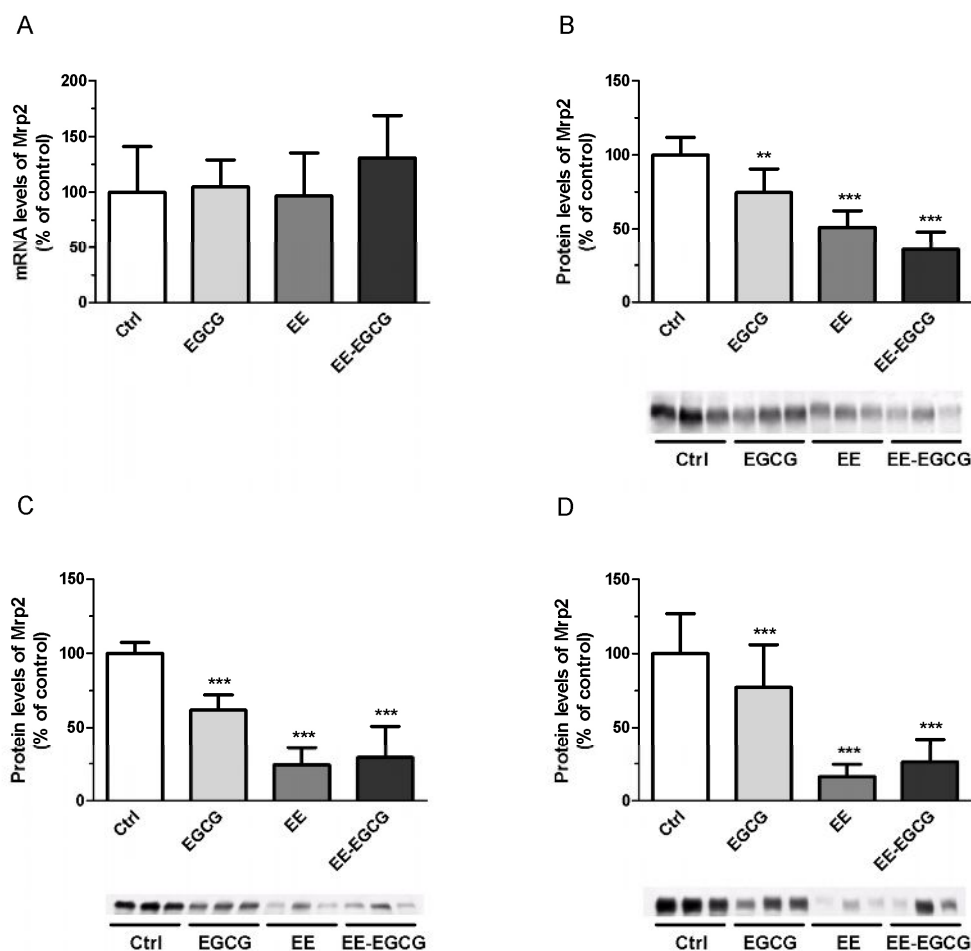
Figure 13. Hepatic expression of Mrp2 at the mRNA (A) and protein levels (B-D). Mrp2 protein was detected in the whole cell lysate (B), membrane-enriched fraction (C) and isolated canalicular membrane fraction (D). Values are means ± SD for 6-7 animals per group. Significantly different from the control 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. 13B) 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. 13C, 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 ($r = 0.86$, $P < 0.0001$).

6.13. 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. 14A). 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. 14A). Gene expression of the main biliary phospholipid transporter, Mdr2, did not differ between the groups.
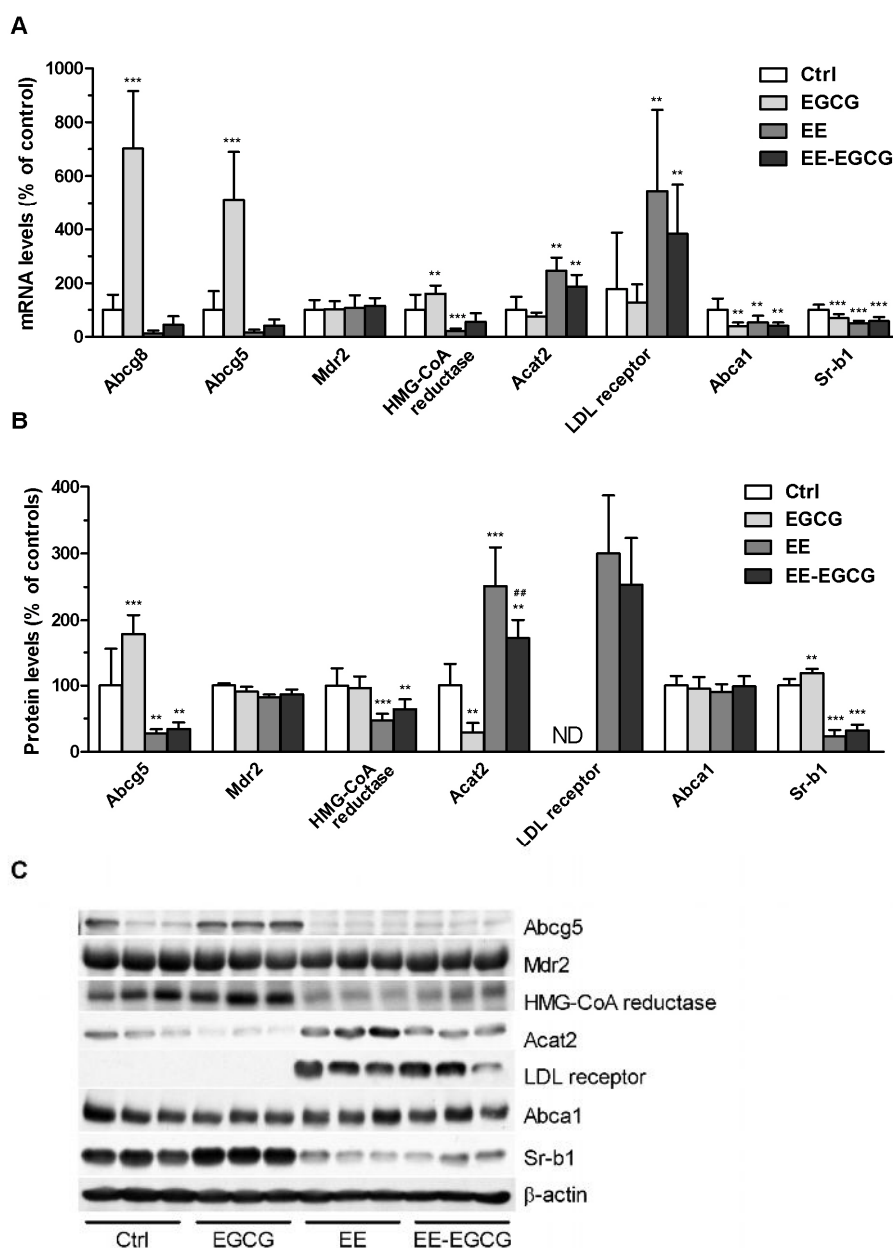
Figure 14. Hepatic expression of key regulators of cholesterol metabolism at the mRNA (A) and protein levels (B). Representative immunoblots are shown (C). Values are means ± SD for 6-7 animals per group. Significantly different from the control group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Significantly different from EE-treated group: # $P < 0.05$.

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. 14B). 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.

Expression of selected nuclear receptors and transcription factors (and their downstream target genes) involved in cholesterol metabolism regulation was also assessed (Table 2). 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 2. 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. 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.

6.14. 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. 15). As shown previously,[69] chenodeoxycholic acid was a potent FXR agonist in activating gene reporter construct via the pCMX-GAL4-FXR.
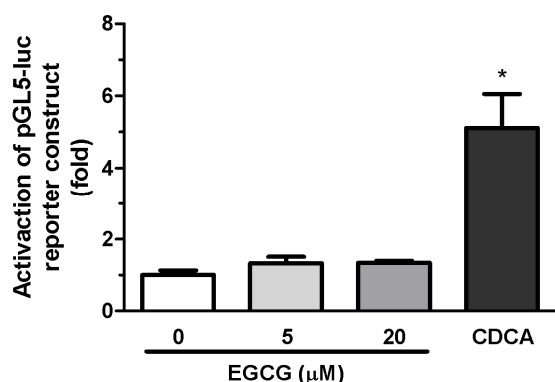
Figure 15. Interaction of EGCG and human FXR. Chenodeoxycholic acid (CDCA, 50 μM) was used as a positive control in activating pGL5-luc reporter construct via the pCMX-GAL4-FXR. Data are shown as the mean fold activation (normalized to the *Renilla* luciferase activity) relative to the vehicle treatment (DMSO). Significantly different from vehicle treatment: * P < 0.05.

6.15. 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.[4] In EGCG-treated rats, hepatic expression of

Cyp7a1 was increased by 5 and 1.5-fold at mRNA and protein levels, respectively (Fig. 16A, 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. 16C).
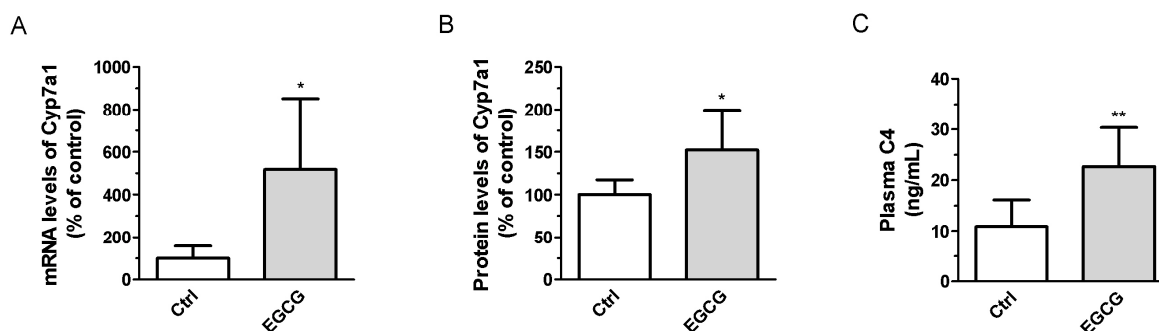
Figure 16. Effect of EGCG on bile acid synthesis *in vivo*. EGCG treatment in rats increased expression of Cyp7a1 at both mRNA (A) and protein (B) levels and doubled plasma levels of C4 (C). Values are means ± SD for 7 rats per group. Significantly different from the control group: * P < 0.05.

6.16.   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. 17)

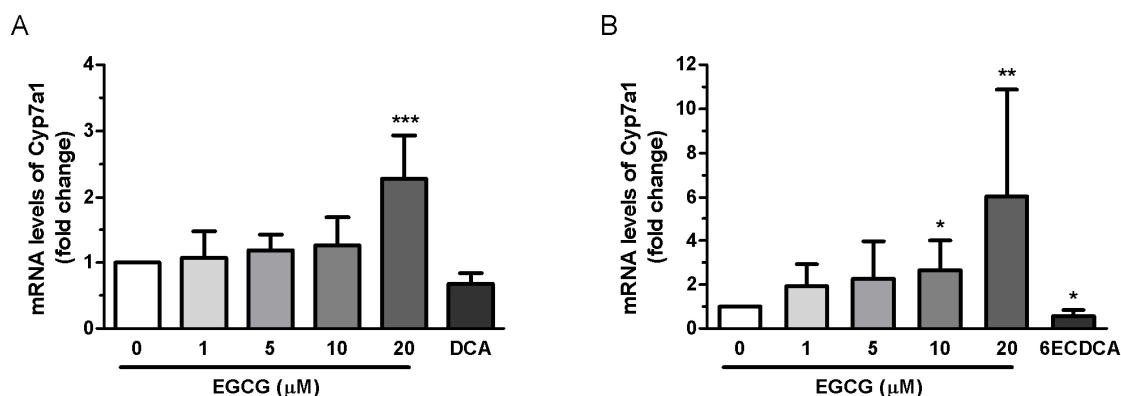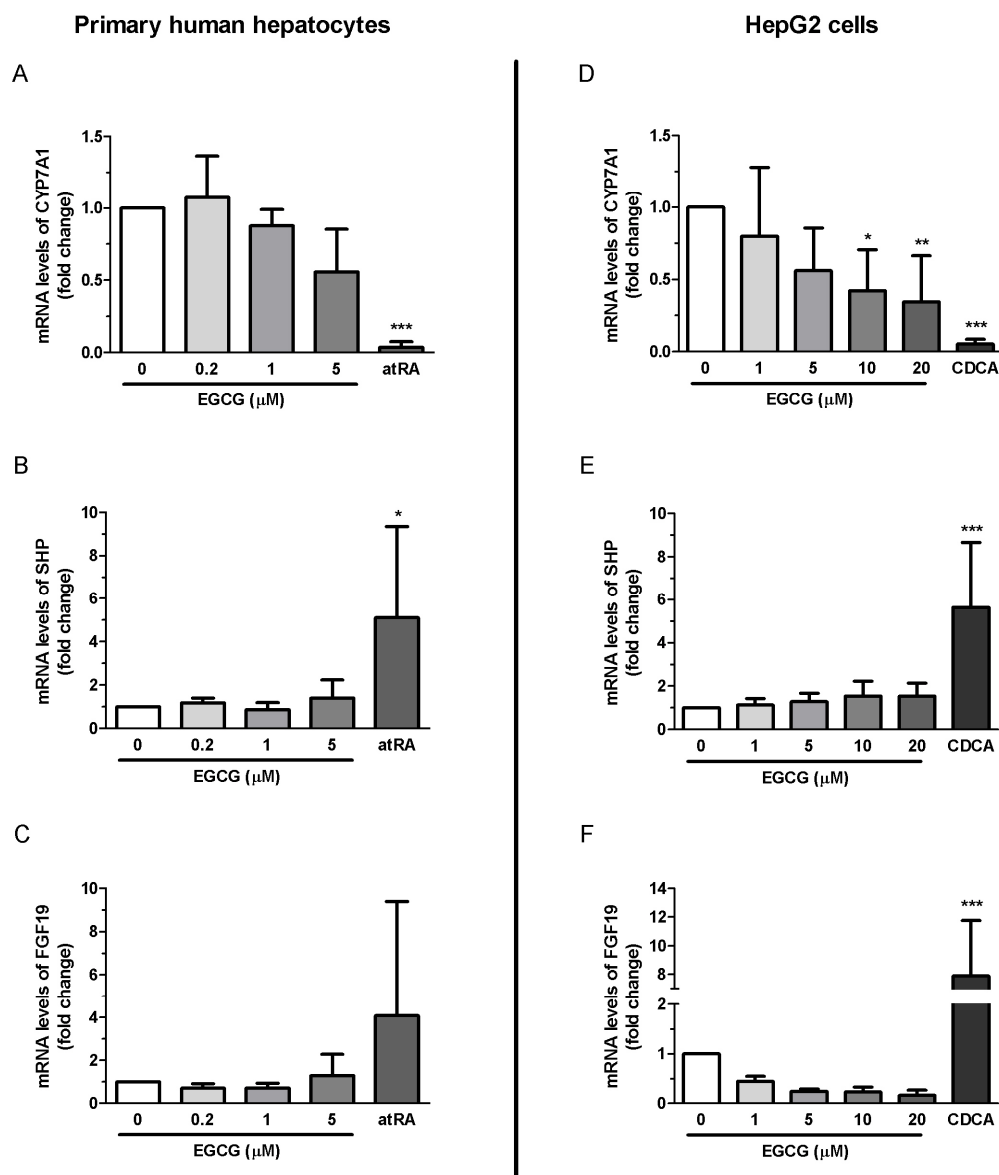**Figure 17.** Gene expression of Cyp7a1 in primary hepatocytes isolated from either Wistar rats (A) or Sprague Dawley rats (B), cultured in a collagen-sandwich configuration and treated with EGCG (1–20 μM) for 24 h. DCA and 6ECDCA were used as negative controls that downregulate Cyp7a1 mRNA expression. 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.

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. 18A). 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. 18D-F).

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. 19A). 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. 19B). CDCA (50μM) treatment was used as a negative control.

Primary human hepatocytes   **HepG2 cells**

Figure 18. 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. Significantly different from the vehicle-treated cells: * P < 0.05, ** P < 0.01, *** P < 0.001.
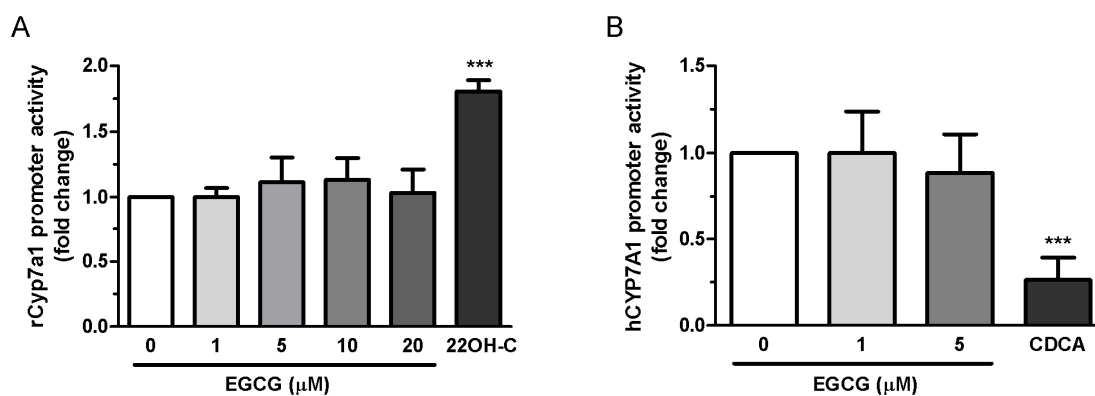
Figure 19. Effect of EGCG on rat Cyp7a1 (A) and human CYP7A1 promoter activity (B) in gene reporter assay. CDCA (50 µM) and 22OH-C (5 µM) were used as a negative and positive control for human and rat promoter activity, respectively. Values are means ± SD for 4 independent experiments. Significantly different from the vehicle-treated cells: *** P < 0.001.

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7.   Discussion

7.1.   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. 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.[10] 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.[9] Since EGCG did not affect biliary bile acid excretion, 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.[70] Nevertheless, EGCG and its methyl metabolites were shown to be substrates for MRP2.[71]

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.[72] 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.[31] 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. 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, 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[31], 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).

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.[73] 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.[74] 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.[4] 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 (Fig. 20).
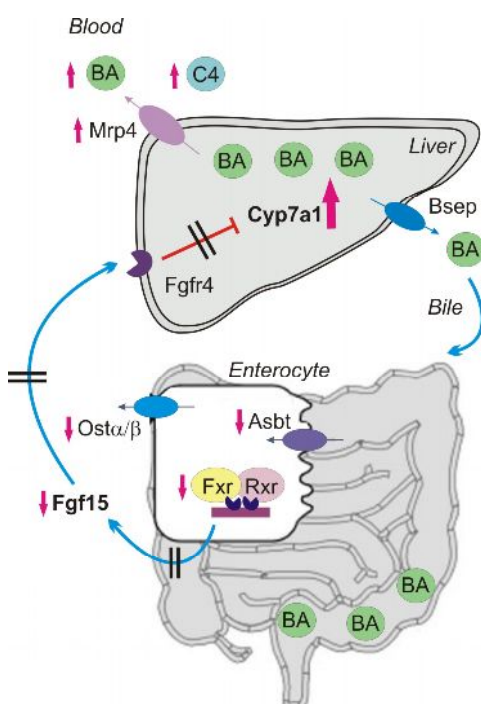
Figure 20. Schematic representation of the proposed mechanism of the EGCG effect on bile acid homeostasis in rats. EGCG-treated rats showed downregulation of the main bile acid transporters in the intestine, Asbt and Ostα. Consequently decreased reabsorption of bile acids in the ileum was documented 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.

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.[33] 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.[33] or with various modifications.

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.[72] BADF is likely diminished by trans-inhibition of Bsep after secretion of estrogen metabolites into bile via Mrp2.[75] Further, estrogens alter hepatocyte bile acid uptake by decreasing the expression of basolateral transporters such as Ntcp and Oatps.[76] Molecular changes detected in ethinylestradiol-treated animals in this study fully complied with the current concept of this form of cholestasis.[76-78] 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.

7.2.   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.[13] 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.[79] On the other hand, overexpression of human ABCG5/8 in mice promoted biliary cholesterol secretion.[80] In addition, it has been

shown that expression of MDR2 is required for ABCG5/8-mediated biliary cholesterol secretion.[81] This is consistent with the former finding that biliary cholesterol excretion is dramatically decreased in Mdr2-deficient mice.[82] Finally, biliary cholesterol excretion can be regulated by Sr-b1, which is a selective HDL cholesterol receptor.[83] Since HDL cholesterol is the preferred source of biliary cholesterol, Sr-b1 knockout mice have biliary cholesterol excretion decreased nearly to 50%.[84]

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[85-86] or inhibition of Acat2, i.e., decreased cholesterol esterification.[87-88] 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.[87-88] In accordance, EGCG treatment in rats decreased plasma levels of VLDL cholesterol, the production of which is also mediated by Acat2. Since biliary cholesterol and VLDL cholesterol seem to have a common hepatic pool,[89] 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.[54,58-59]

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.[80]

EGCG has exerted hypocholesterolemic effect in both preclinical and clinical settings.[41] 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.[90-92]

Ethinylestradiol at the given dose is well known to cause liver cholesterol accumulation in rats.[68] This effect is primary mediated by substantially induced expression of LDL receptor and thus increased uptake of plasma LDL by hepatocyte.[93] In addition, in ethinylestradiol-treated rats plasma total cholesterol and triglycerides decline while newly secreted VLDL particles are enriched with esterified cholesterol.[88] Green tea has been shown to prevent cholesterol accumulation in the liver of rats on high-cholesterol diet.[47-49] 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 in this parameter have been reported.[94-96] 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.[97] However, information on EGCG interaction with nuclear receptors is scarce. The ability of EGCG to modulate FXR activity is discussed above. EGCG has also been shown to modulate activity of transcription factor Nrf2.[98] In the present study, an inverse pattern in the expression of key liver lipid regulators was observed compared to Nrf2 knockout mice,[99] 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.[100] 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.[101] 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.[102-103] 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 21.
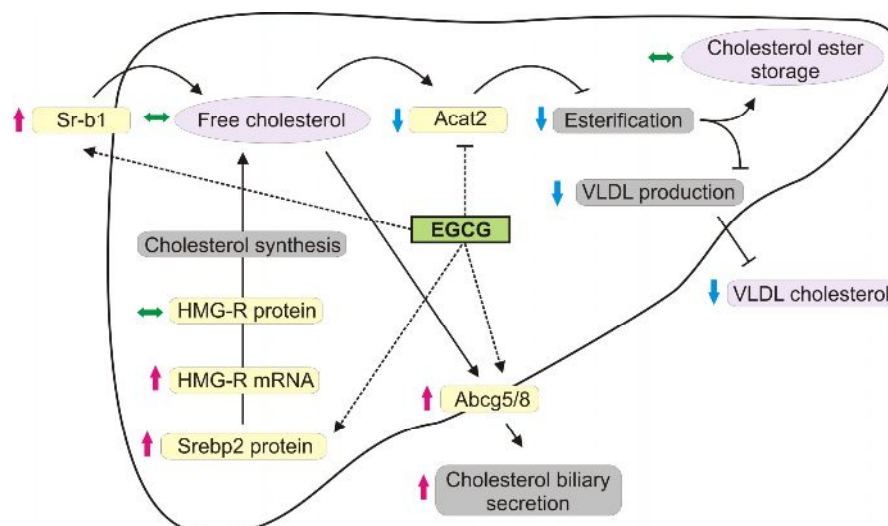
Figure 21. Schematic representation of the proposed mechanism of EGCG action on liver cholesterol metabolism. EGCG induced Sr-b1, which mediates uptake of HDL cholesterol, the main source of biliary cholesterol. It is proposed that the EGCG-decreased Acat2 expression and consequently reduced cholesterol 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.

8.   Conclusions

8.1.   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.

8.2.   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.

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10. List of publications and lectures

Publications

1. <u>Hirsova P</u>**, 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 and Micuda S. Cholestatic effect of epigallocatechin gallate in rats is mediated via decreased expression of Mrp2.** *Toxicology* **2012, accepted for publication. IF: 3.681**

2. <u>Hirsova P</u>**, Kolouchova G, Dolezelova E, Cermanova J, Hyspler R, Kadova Z, Micuda S. Epigallocatechin gallate enhances biliary cholesterol secretion in healthy rats and lowers plasma and liver cholesterol in ethinylestradiol-treated rats.** *Eur J Pharmacol* **2012, 691(1-3):38-45. IF: 2.516**

3. Kolouchova G, Brcakova E, <u>Hirsova P</u>, Sispera L, Tomsik P, Cermanova J, Hyspler R, Slanarova M, Fuksa L, Lotkova H *et al*: Pravastatin modulates liver bile acid and cholesterol homeostasis in rats with chronic cholestasis. *J Gastroenterol Hepatol* 2011, 26(10):1544-1551. IF: 2.410

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Poster presentation

1. <u>Hirsova P</u>, Kolouchova G, Brcakova E, Cermanova J, Micuda S: Pravastatin modulates hepatic iron metabolism during obstructive cholestasis in rats. Liver Meeting 2011, 2-8.11.2011, San Francisco, CA, USA

2. <u>Hirsova P</u>, Kolouchova G, Brcakova E, Cermanova J, Fuksa L, Sispera L, Tomsik P, Hyspler R, Safka V, Micuda S: Green tea flavonoid epigallocatechin gallate changes hepatic cholesterol and bile acid processing in normal and cholestatic rats. Liver Meeting 2010, 29.10-2.11.2010, Boston, MA, USA

3. <u>Hirsova P</u>, Kolouchova G, Brcakova E, Fuksa L, Cermanova J, Mokry J, Sispera L, Tomsik P, Micuda S. Effect of epigallocatechin gallate on liver injury during intrahepatic cholestasis in rats. 38. Májové hepatologické dny, 12.-14.5.2010, Karlovy Vary

4. <u>Hirsova P</u>, Muchova L, Kolouchova G, Brcakova E, Fuksa L, Cermanova J, Vitek L, Micuda S: The model for evaluation of the bile formation mechanisms during ethinylestradiol-induced cholestasis – the influence of heme oxygenase 1. 59. Farmakologické dny, 2.-4.9.2009, Bratislava

Lectures

1. <u>Hirsova P</u>, Kolouchova G, Brcakova E, Lastuvkova H, Fuksa L, Cermanova J, Sispera L, Tomsik P, Hyspler R, Safka V, Micuda S: Effect of epigallocatechin gallate on liver injury during intrahepatic cholestasis in rats. Květinův den – nultý ročník mezioborového semináře mladých farmakologů a toxikologů, 3.6.2010, Brno.

2. <u>Hirsova P</u>, Kolouchova G, Brcakova E, Micuda S: The study of potential importance of epigallocatechin gallate in the prevention and treatment of the liver injury caused by intrahepatic and extrahepatic cholestasis. XVI. Vědecká konference, 25.1.2012, Hradec Králové.