Life Sciences 232 (2019) 116643

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

High soluble endoglin levels regulate cholesterol homeostasis and bile acids turnover in the liver of transgenic mice

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ARTICLE INFO

Keywords:
Endoglin
Liver
Bile production
Bile acids
Cholesterol

ABSTRACT

Aims: Increased plasma soluble endoglin concentrations (sEng) are frequently detected in metabolic disorders accompanied with hypercholesterolemia in serum, but effect of sEng on the cholesterol biochemistry is unknown. Cholesterol and bile acids (BA) are important products of liver metabolism with numerous functions within the organism. Turnover of these substances requires precise regulation due to potential toxicities during their cumulation. In this study, we hypothesized that high sEng levels affect cholesterol homeostasis and BA turnover in mice liver.

Main methods: Nine-month-old transgenic male mice overexpressing human sEng and wild-type mice underwent plasma, bile, stool, and organ samples analysis by analytical, qRT-PCT and Western blot methods.

Key findings: sEng mice demonstrated decreased plasma total and LDL cholesterol concentrations due to upregulation of hepatic Sr-b1 and Ldlr receptors, increased liver cholesterol content, and increased Abcg8-mediated cholesterol efflux into bile. sEng also increased conversion of cholesterol into bile acids (BA) via upregulation of Cyp7a1 and increased Mdr1 expression. Plasma concentrations of BA were increased in sEng mice due to their enhanced reabsorption via ileum. Increased hepatic disposition of BA led to their increased biliary excretion coupled with choleretic activity.

Significance: For the first time, we have shown that high sEng plasma levels affect cholesterol and BA homeostasis on the basis of complex liver and intestinal effects. The significance of these findings for pathophysiology of diseases associated with increased sEng concentrations remains to be elucidated in prospective studies.

1. Introduction

Soluble endoglin (sEng) is free form of membrane bound Endoglin (CD 105) that is a transmembrane glycoprotein acting as coreceptor of the transforming growth factor-β (TGF-β) superfamily, which is expressed in the plasma membrane of diverse cells (e.g in endothelial cells, macrophages, fibroblasts or hepatic stellate cells) [1,2]. sEng can be released to the extracellular fluid and plasma by MMP14 (matrix metalloproteinase 14)-mediated shedding of membrane-bounded endoglin [3,4]. Plasma levels of sEng are increased in various cardiovascular and metabolic diseases including hypercholesterolemia, type II diabetes mellitus, preeclampsia and arterial hypertension [5], but as well in liver diseases [6,7]. It is of interest to mention that sEng is not only a biomarker increased during these pathological conditions, but it may also participate in their pathogenesis as demonstrated by abrogation of endothelial/vascular dysfunction in mice with high sEng plasma concentrations [8]. However, its potential effect on cholesterol metabolism has never been studied so far.

Regulation of cholesterol turnover and its metabolism to bile acids belongs to the essential functions of the liver. Despite numerous

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https://doi.org/10.1016/j.lfs.2019.116643
Received 26 April 2019; Received in revised form 1 July 2019; Accepted 9 July 2019
Available online 09 July 2019
0024-3205/

positive effects of cholesterol, once cumulated, it is involved in pathogenesis of numerous diseases such as atherosclerosis and ischemic heart disease [9,10]. Similarly, BA are not only necessary for lipid digestion in intestine, but also mediate significant regulatory function within liver glucose and lipid metabolisms, and upon cumulation during cholestasis, they are major harmful mediators imposing tissue damage in the liver and extrahepatic tissues. The major route for the elimination of cumulated cholesterol from organism is via its biliary excretion, and by its conversion to bile acids (BA), the substances responsible for the bile formation, and for intestine and lipid homeostasis, but also acting as hormones in regulating glucose and triglyceride homeostasis [11,12]. Synthesis and further processing of cholesterol and BA are regulated by the modulation of crucial steps, which includes Hmg-CoA reductase (Hmgcr) for cholesterol synthesis, and Cyp7a1 for BA synthesis [13,14]. The rate-limiting transporters for the secretion of these substances into bile are Abcg5/8 and Mdr2 for cholesterol/phosphatidylcholine and Bsep for BA, respectively. Regulation of these pathways is complex, and their modulation or alteration may prevent or promote liver disease, respectively [11,15].

Taken together with the fact that sEng levels are increased in pathological conditions associated with increased cholesterol concentrations in plasma and metabolic liver impairment, generates question whether high sEng levels may affect liver functions. Thus, we hypothesized that high sEng levels will modulate cholesterol homeostasis and bile acid turnover in liver. We found that sEng modulates plasma concentrations of cholesterol and increases plasma concentrations of BA thanks to complex changes in the expression of responsible enzymes and transporters in liver. We also identified the inducing effect of sEng on essential drug metabolizing enzyme, and biliary transporter. We thus propose that high plasma levels of sEng modulate liver cholesterol- and drug-processing pathways and could modify pathophysiology and drug effects during respective cardiometabolic disorders.

2. Materials and methods

2.1. Chemicals

Bile acids reference standards of tauromuricholic acid (TMCA), tauroursodeoxycholic acid (TUDCA), taurodeoxycholic acid (TDCA), glycoursodeoxycholic acid (GUDCA) and muricholic acid (MCA) were purchased from Steraloids (Newport, RI, USA). Taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), cholic acid (CA), ursodeoxycholic acid (UDCA), chenodeoxycholic acid (CDCA), litocholic acid (LCA) and deoxycholic acid (DCA) were obtained from Sigma-Aldrich (Prague, Czech Republic). Obeticholic acid (OCA) used as internal standard was from MedChemExpress (Monmouth Junction, NJ, USA) and hyodeoxycholic acid (HDCA) from Santa Cruz Biotechnology (Dallas, TX, USA). Ammonium formate, formic acid, acetonitrile and methanol (all in LC-MS grade) were purchased from Sigma-Aldrich (Prague, Czech Republic). The water was purified with a MilliQ system (Schwalbach, Germany).

2.2. Animals and experimental design

Transgenic mice overexpressing human sEng (*Sol-Eng*$^+$) on the CBAxC57BL/6 J background were generated at the Genetically Modified Organisms Generation Unit (University of Salamanca, Spain), as previously described [4]. Three-month-old Sol-Eng$^+$ male mice overexpressing human soluble endoglin (sEng group) and CBAxC57BL/6 J wild-type mice (WT) with undetectable plasma levels of human sEng (n = 6 in both groups) fed with a chow diet for next 6 months were used. The animals were housed under a 12-h light cycle and constant temperature (22 ± 1 °C) and humidity with free access to tap water and a standard laboratory pellet diet. All experiments were performed in accordance to the directive of the EEC (86/609/EEC) and the use of animals was approved by the Ethical Committee for the protection of animals against cruelty at Faculty of Pharmacy in Hradec Kralove, Charles University (Reg. Number: 21558/2013–2), and the Bioethics Committee of the University of Salamanca (Permit Number: 006–201400038812). All 9 months old mice were placed into metabolic cages where the stool was collected for 24 h. Collected stools were dried for 72 h at the room temperature and BA were isolated as described previously [16] with slight modifications. To calculate rate of fractional cholesterol synthesis, 24 h before in vivo clearance study, mice received i.p. bolus of deuterium oxide to enrich total body water pool to 1% and drunk water enriched by 1.5% deuterium oxide for 24 h as described previously [17]. Mice were fasted overnight before the beginning of *in vivo* clearance study.

2.3. In vivo clearance study

Under the anesthesia induced by pentobarbital (50 mg/kg), the gallbladder was cannulated under the stereomicroscope for bile collection in pre-weighed tubes for 45 min. The body temperature of the animals was maintained at 37 °C by keeping the animals on a heated platform. At the end of the experiment, all mice were sacrificed by exsanguination from vena cava, livers and ileum (5 cm proximal to caecum) were immediately frozen in liquid nitrogen. Plasma samples were obtained from the whole blood by centrifugation at 2000 × *g* for 5 min at 4 °C. Samples were stored at −80 °C until analysis.

2.4. Analytical methods

Plasma lipoprotein fractions were prepared as described previously [18]. Plasma concentration of total cholesterol was analyzed by commercial kit Cholesterol 250 (Erba Lachema s.r.o., Brno, Czech Republic). Biliary concentrations of phospholipids were determined by Phosphotidylcholine Assay kit (Sigma-Aldrich, St.Louis, USA). Concentrations of oxidized glutathione (GSSG) in bile were analyzed using validated HPLC method with fluorescence detection as described previously [19]. Liver concentration of cholesterol was assayed by commercial kit Cholesterol Assay Kit (Cayman Chemical, Michigan, USA). BA concentrations in plasma were measured using LC-MS method as described previously [20].

2.5. Analysis of bile acids in bile and feces

2.5.1. Liquid chromatography

Quantitative analyses were performed on a 1200 series Agilent liquid chromatograph (Palo Alto, CA, USA) composed of a degasser, quaternary pump, cooled autosampler and thermostated column compartment. Chromatographic separation of 16 endogenous bile acids and OCA (internal standard) was performed using a Kinetex XB-C18 core shell column 150 × 3 mm, 2.6 um (Phenomenex, Torrance, USA) with CrudCatcher disposable in-line filter. Mobile phase flowing at rate 0.3 mL/min consisted of solvent A (MPA) with 0.5 mM ammonium formate and formic acid (0.01%, v/v) in mixture of water:-methanol:propan-2-ol (600:400:40, v/v) and solvent B (MPB) with 0.5 mM ammonium formate and 0.01% formic acid in mixture of methanol:propan-2-ol:water (950:100:50:, v/v). Gradient program was as follows: 0–2 min, 100% MPA; 2–5 min, 18% MPB; 5–25 min, 18–91% MPB; 25–30 min, 100% MPB. The column was then re-equilibrated for 10 min with 100% MPA. Separation was carried out at temperature 40 °C and the sample injection volume was 2 uL. Samples were held at 10 °C during the analysis. Data processing was performed with Thermo Fisher Xcalibur software.

2.5.2. Mass spectrometry

Separated bile acids were detected using an ion trap LCQ Fleet mass spectrometer (Thermo Fisher Scientific, San Jose, USA) operated in ESI negative mode. Optimized settings of mass spectrometer were as

follows: Spray voltage −4.5 kV, Capillary voltage −75 V, Capillary temperature 275 °C, Tube lens −125 V, Sheath gas 40 arbitrary units (AU), Auxiliary gas 30 AU, and Sweep gas 1 AU. Full scan mode in the range of *m/z* 200–600 was used for detection of bile acids. Scanning was performed with maximum injection time 150 ms and 3 micro-scans. The mobile phase flow was diverted into the mass spectrometer only between 8 and 32 min to minimize contamination of the ion source. Chromatograms were constructed from extracted ion currents at m/z 514.4 (TMCA, TCA), 498.3 (TUDCA, TCDCA, TDCA), 464.3 (GCA), 448.3 (GUDCA, GCDCA, GDCA), 417.3 (OCA), 407.3 (MCA, CA), 391.3 (UDCA, HDCA, CDCA, DCA) and 375.3 (LCA) with isolation width 1 Da.

2.5.3. Sample preparation

50μL of feces ethanolic extract was 4× diluted with methanol:water (1:1, v/v) solution containing internal standard (OCA, c = 12.5 μM). Mixture was thoroughly vortexed and centrifuged (5 min, 10 °C, 14.000 ×*g*). 50 μL of the solution were then transferred to an auto-sampler vial with a 250 μL glass insert and subjected to analysis.

2.6. Quantitative real time RT-PCR

Gene expression by mRNA quantification was performed by reverse transcription-polymerase chain reaction (qRT-PCR) on 7500 HT Fast Real-Time PCR System (Applied Biosystems, Foster City, USA) as previously described [19]. Primers used for analysis are specified in Table 1. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) gene was used as reference for normalizing data in livers and because Gapdh expression differed between experimental groups in ileum, we have used β-actin for this purpose. (Applied Biosystems, Foster City, USA).

Table 1
Pre-designed TaqMan® Gene Expression Assay kits (Life Technologies) used for quantitative real-time RT-PCR.

Gene symbol	Transporter/receptor	Life Technologies cat. number
Slc10a1	Ntcp	Mm00441421_m1
Slco1a4	Oatp1a4	Mm00453136_m1
Abca1		Mm00442646_m1
Abcc3	Mrp3	Mm00551550_m1
Abcc4	Mrp4	Mm1226380_m1
Abcb11	Bsep	Mm00445168_m1
Abcc2	Mrp2	Mm00496899_m1
Abcb1a	Mdr1a	Mm00440761_m1
Abcb1b	Mdr1b	Mm00440736_m1
Abcb4	Mdr2	Mm00435630_m1
Abcg5		Mm00446241_m1
Abcg8		Mm00445980_m1
Acat2		Mm00782408_s1
Hmgcr		Mm01282499_m1
Ldlr		Mm01177349_m1
Cyp7a1		Mm00484150_m1
Cyp8b1		Mm00501637_s1
Cyp27a1		Mm00470430_m1
Cyp2b10		Mm00456588_mh
Cyp3a11		Mm00731567_m1
Scrab1	Sr-b1	Mm00450234_m1
Srebf2		Mm0130692_m1
Nr1h3	Lxr-α	Mm00443451_m1
mice Eng		Mm00468256_m1
human Eng		Hs00923996_m1
IL6		Mm00446190_m1
Acta2	α-SMA	Mm01546133_m1
Tgf-β1		Mm01178820_m1
Col1a1		Mm00801666
Pdgfb		Mm00440677_m1
Nqo1		Mm01253561_m1
Hmox		Mm00516005_m1
Slc51a	Ost-α	Mm00521530_m1
Slc51b	Ost-β	Mm00619242_m1
Slc10a2	Asbt	Mm00488258_m1
Fgf15		Mm00433278_m1
Nr1h4	Fxr	Mm00436425_m1
Actb	β-Actin	Mm01205647_m1
Gapdh		Mm99999915_g1

Table 2
Primary and secondary antibodies used in Western blot.

Protein	Source	Dilution	Secondary antibody dilution
Lxr-α	Abcam (ab45279)	1:500	1:1000
IL6	Abcam (ab6672)	1:1000	1:2000
Eng	Santa Cruz (sc-19,793)	1:500	1:3000
Abcg8	Thermo Fisher (PA1–16798)	1:1000	1:2000
Cyp7a1	Thermo Fisher (PA5–15216)	1:1000	1:2000
Cyp8b1	Thermo Fisher (PA5–37088)	1:1000	1:2000
Cyp27a1	Thermo Fisher (PA5–27946)	1:1000	1:2000
Hmgcr	Thermo Fisher (PA5–37367)	1:1000	1:2000
Ldlr	Cayman (10007665)	1:1000	1:2000
Sr-b1	Novus (NB400–104)	1:2000	1:4000
Srebp2	Abcam (ab30682)	1:1000	1:2000
Ntcp	Santa Cruz (sc98485)	1:3000	1:6000
Oatp1a1	Thermo Fisher (PA5–42445)	1:500	1:1000
Abca1	Thermo Fisher (PA1–16789)	1:500	1:2000
Mrp4	Abcam (ab77184)	1:2000	1:4000
Mrp2	Thermo Fisher (PA5–49997)	1:500	1:1000
Bsep	Thermo Fisher (PA5–13105)	1:1000	1:2000
Mdr1	Cell Signaling (13,978	1:1000	1:2000
Mdr2	Abcam (ab71792)	1:1000	1:2000
Oatp1a4	Abcam (ab224610)	1:1000	1:2500
Fgf15	Abcam (ab225942)	1:1000	1:2000
Acat2	Cayman (100027)	1:1000	1:2000
Ostα	Biorbyt (orb185685)	1:250	1:2000
Ostβ	Bioss (bs2128R)	1:250	1:2000
Asbt	Thermo Fisher (PA-18990)	1:500	1:2000
Gapdh	GE-HealthCare (NA9340)	1:8000	1:10000
β-actin	Sigma (AC-74)	1:2000	1:4000

2.7. Western blot analysis

A crude plasma membrane was prepared from mice liver homogenates as described previously [20]. Proteins (50 mg of liver and ileum) were separated by SDS-PAGE, transferred to PVDF membrane (Millipore, NY, USA) and incubated with appropriate antibodies (Table 2). The immunoreactive bands on the autoradiography films were quantified using QuantityOne imaging software (Bio-Rad Laboratories, Hercules, CA). The equal loading of proteins onto the gel was confirmed by the immunodetection of Gapdh in liver and because Gapdh expression differed between experimental groups in ileum, we have used β-actin for this purpose.

2.8. Histology

Livers were collected immediately after death and fixed in 10% neutral buffered formalin, embedded in paraffin, and 10% cut to 4–5 μm thick sections. Light microscopy of hematoxylin-eosin stained liver sections was performed as previously described [21].

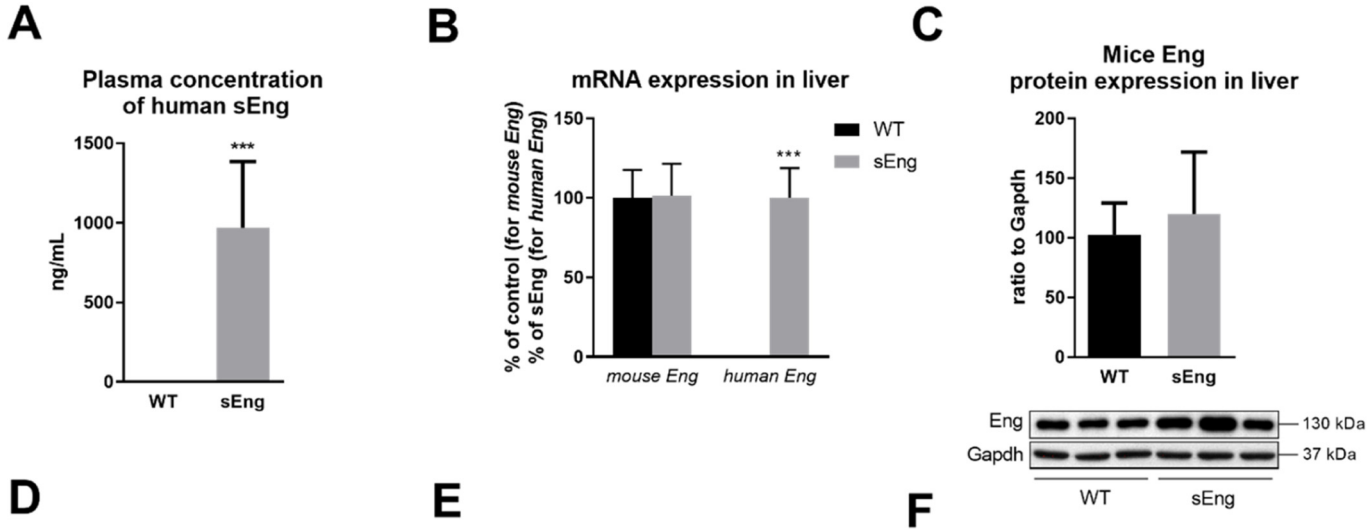
2.9. Statistical analysis

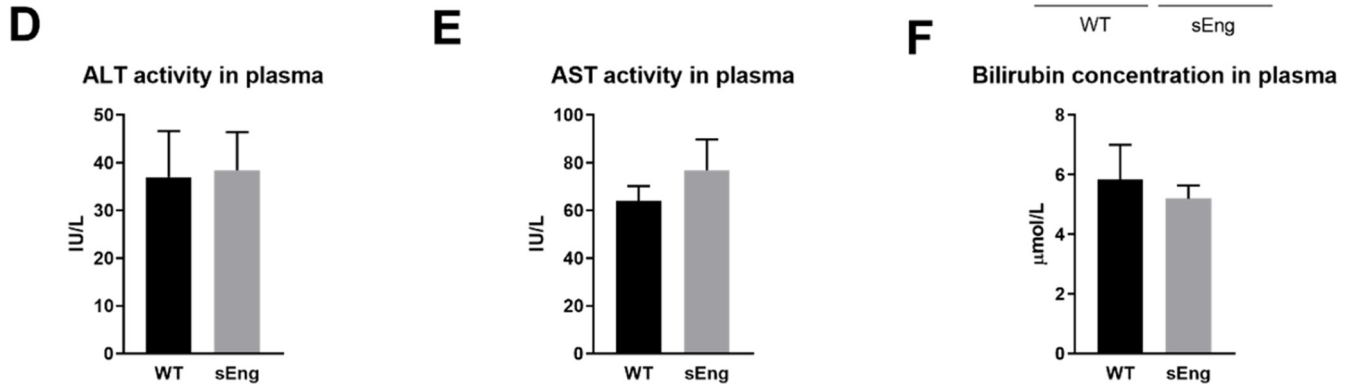
All experimental data are expressed as mean ± SD. Direct group-group comparisons were carried out using one-way ANOVA, Mann-Whitney test. All analyses were performed using Graphpad Prism 7.0 software (San Diego, California, USA). A difference of $P < 0.05$ was considered statistically significant.

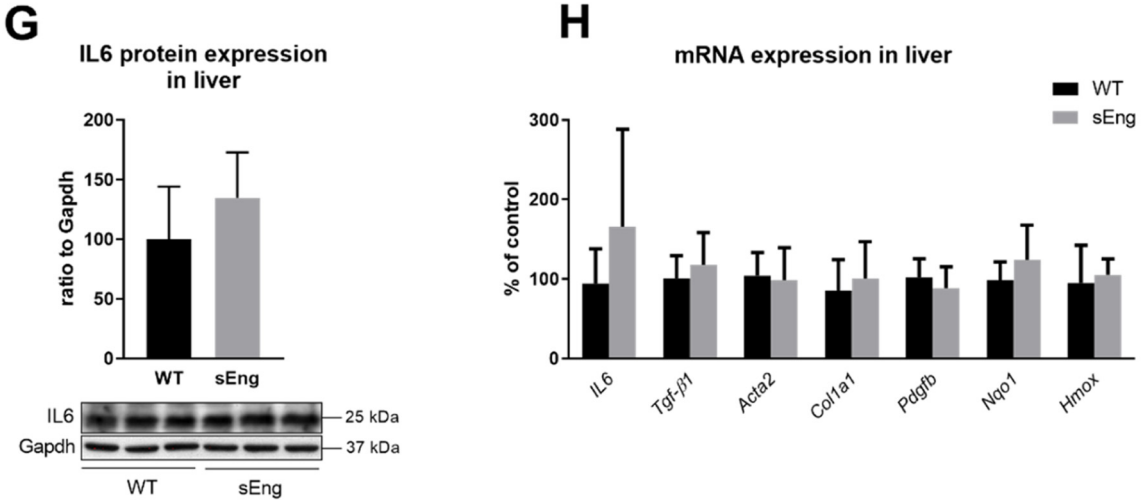
3. Results

3.1. Transgenic sEng mice showed marked plasma sEng levels without modulation of endogenous mice plasma sEng, and mice liver Eng expression

We performed ELISA to analyze human sEng concentrations in mice plasma, and qRT-PCR and Western blot to assess liver expression of mice and human Eng. Only transgenic mice demonstrated significant







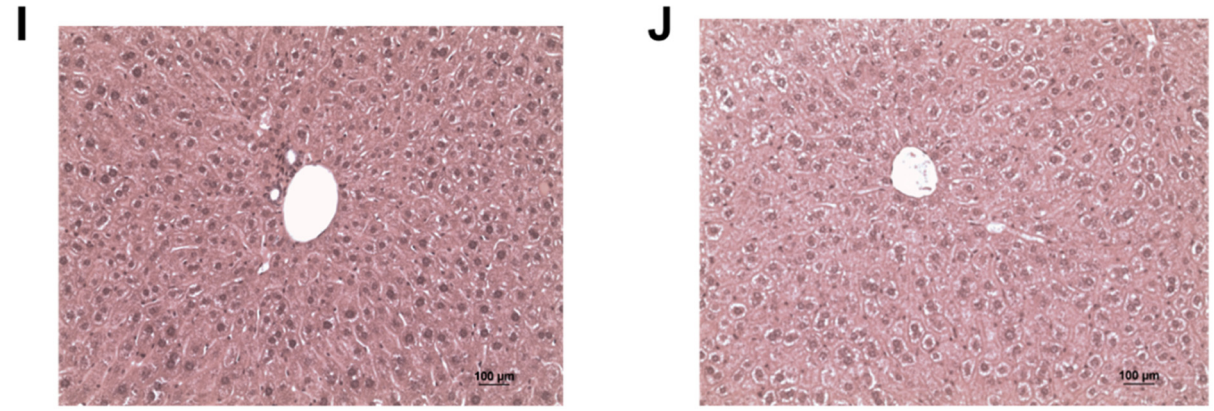


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Fig. 1. High plasma levels of sEng levels without modulation of endogenous mice plasma sEng, and mice liver in transgenic mice without any effect on inflammation, fibrosis and oxidative stress in liver. Plasma concentration of human sEng (A), mRNA expression of mice and human endoglin in liver (B), protein expression of mice endoglin in liver (C), activity of ALT and AST in plasma (D, E), plasma concentration of bilirubin (F), protein expression IL-6 in liver (G), gene expression of *IL6*, fibrogenic (*Tgf-β1*, Acta2, *Col1a1*, *Pdgfb*), oxidative stress markers (*Nqo1*, *Hmox*) (H) and hematoxylin and eosin-stained liver sections (I, J) of wild-type (WT) and sEng mice. Bar 100 μm. For Western blot analysis (C, G): top: results are presented as ratio to Gapdh; bottom: representative immunoblots. Human sEng levels in control mice were below the detection limit. Data are presented as the mean ± SD (n = 6). ***$P < 0.001$, Mann-Whitney *U* test.

sEng concentrations in plasma, and it was undetectable in wild-type (WT) animals (Fig. 1A). Similarly, only livers of sEng mice showed expression of human endoglin mRNA in comparison to WT animals (Fig. 1B). The liver expressions of mice endoglin mRNA and protein were not different between WT and sEng mice (Fig. 1B, C).

3.2. sEng does not affect inflammation, fibrosis and oxidative stress in liver of healthy animals

Ability of human sEng to modulate mice endoglin pathways in the present mice model was recently demonstrated by our group in aortas of high fat diet fed sEng animals [8]. To screen for potential liver effects of sEng, we analyzed liver histological sections, plasma biochemistry, and the expression of inflammation-related genes in otherwise healthy transgenic sEng animals. High human sEng in plasma did not induce changes in plasma transaminases activity (ALT, AST) or concentration of total bilirubin (Fig. 1D, E, F). Similarly, sEng did not modify liver IL6 protein or gene expression of major proinflammatory (*IL6*), fibrogenic (*Tgf-β1, Acta2, Col1a1, Pdgfb*) or oxidative stress markers (*Nqo1, Hmox1*) in comparison with wild-type animals (Fig. 1G, H). Moreover, no morphological changes in liver histology with respect to steatosis, inflammation and fibrosis were found in both control and sEng mice (Fig. 1I, J).

3.3. sEng reduces plasma cholesterol concentration and increases liver cholesterol concentration via up-regulation of LDL-receptor mediated by Srebp2

Increased release of sEng accompanies hypercholesterolemia [22], but the effect of sEng on cholesterol turnover is unknown. To investigate whether sEng induces changes in cholesterol biochemistry and its liver uptake and synthesis, we analyzed cholesterol plasma and liver concentrations and mRNA and protein expression of responsible transporters and enzymes. In comparison to WT mice, sEng significantly decreased plasma concentrations of total cholesterol to 88% and LDL cholesterol to 78% without any significant change of the VLDL and HDL profile (Fig. 2A). Moreover, sEng mice demonstrated significant increase in liver concentrations of cholesterol by 22% when compared to WT mice (Fig. 2B). These changes in plasma and liver cholesterol concentrations in sEng transgenic mice accompanied significant up-regulation of transporters responsible for cholesterol uptake into hepatocytes, Sr-b1 and Ldlr both at mRNA as well as protein levels (Fig. 2D, E).

Analysis of liver cholesterol fractional synthesis did not reveal any significant change in sEng mice in comparison with WT group (Fig. 2C). In agreement, we have not detected significant change between sEng and WT groups in mRNA and protein expression of Hmgcr, the rate limiting enzyme in cholesterol de novo synthesis (Fig. 2D, E). Moreover, we did not find any significant change in mRNA and protein expression of Acat2, which is responsible for the esterification of cholesterol and Abca1, the transporter mediating efflux of cholesterol to plasma (Fig. 2D, E). These findings further support the fact, that increased uptake of cholesterol from blood is the main mechanism of increased liver and reduced plasma cholesterol concentrations in sEng mice.

Two nuclear receptors have central role in the regulation of cholesterol synthesis, metabolism, and transport in hepatocytes, Srebf2 - Sterol regulatory element-binding transcription factor 2, and Lxrα (Liver X receptor). PCR analysis of these nuclear receptors did not reveal any significant change in the expression of their mRNA (Fig. 2D). In contrast, sEng group demonstrated significant increase of Srebp2 protein expression (Fig. 2F) which corresponds with upregulation of Ldlr via activation of Ldlr gene transcription (Fig. 2D).

3.4. sEng increased biliary excretion of cholesterol and its conversion to bile acids

In order to further study the mechanisms of sEng-mediated increase in liver and reduction in plasma cholesterol concentrations, we studied two principal hepatic pathways responsible for its elimination – biliary secretion, and conversion to bile acids. First, collection of bile with consequent cholesterol quantification revealed that sEng significantly increased biliary secretion of cholesterol to 145% compared with WT (Fig. 3A). It corresponded with significantly increased mRNA (to 134%) and protein (to 149%) expression of Abcg8 (Fig. 3B, C), the protein representing the Abcg5/Abcg8 heterodimeric transporter complex responsible for cholesterol biliary efflux at the canalicular membrane of hepatocytes (Fig. 3). Second, sEng led to a significant up-regulation of Cyp7a1 protein (Fig. 3E) the rate limiting enzyme for the BA synthesis from cholesterol. Neither the protein expression of other crucial enzymes of BA synthesis such as Cyp8b1 (an enzyme important in the classic pathway for BA synthesis), and Cyp27a1 (an enzyme important in the alternative pathway of BA synthesis), nor the gene expression of these enzymes, including Cyp7a1, were significantly changed in sEng mice in comparison with WT group (Fig. 3D, E).

3.5. sEng induces plasma concentration of BA and BA-dependent choleretic effect

Upregulation of Cyp7a1 suggested influence of sEng on BA turnover. To investigate this effect, we analyzed BA concentrations in plasma and bile. Concentrations of total BA and their spectra in plasma were significantly increased same as biliary secretion of BA in sEng mice when compared with WT (Fig. 4A, B). The total BA concentration in bile was calculated as a sum of concentrations of individual BA measured by LC-MS method and we found significant induction of biliary secretion of total BA (Fig. 4B). Most of 16 measured BA were found at the concentrations below the quantification limit of the method (0.13 mM). Therefore, we finally quantified 5 BA in bile. We have found significantly increased biliary excretion of tauromuricholic acid (TMCA) and taurocholic acid (TCA) to 328% and 386%, respectively in sEng mice (Fig. 4C). It corresponds with previous findings that TMCA and TCA are the predominant BA in the bile acid pool in mice [12]. Increased biliary secretion of BA was associated with corresponding significant increase in bile flow rate (Fig. 4D). Biliary secretion of glutathione (Fig. 4E), which represents BA-independent component of bile formation, was not modified in sEng mice. It indicates, that choleretic effect of sEng is BA-dependent.

The detergent properties of BA concentrated in bile are compensated by biliary secretion of phospholipids [23] to form micelle. In sEng mice, we found significantly increased biliary secretion of phospholipids (Fig. 4F) despite the absence of change in the expression of their Mdr2 transporter both at mRNA and protein level (Fig. 4G, H).

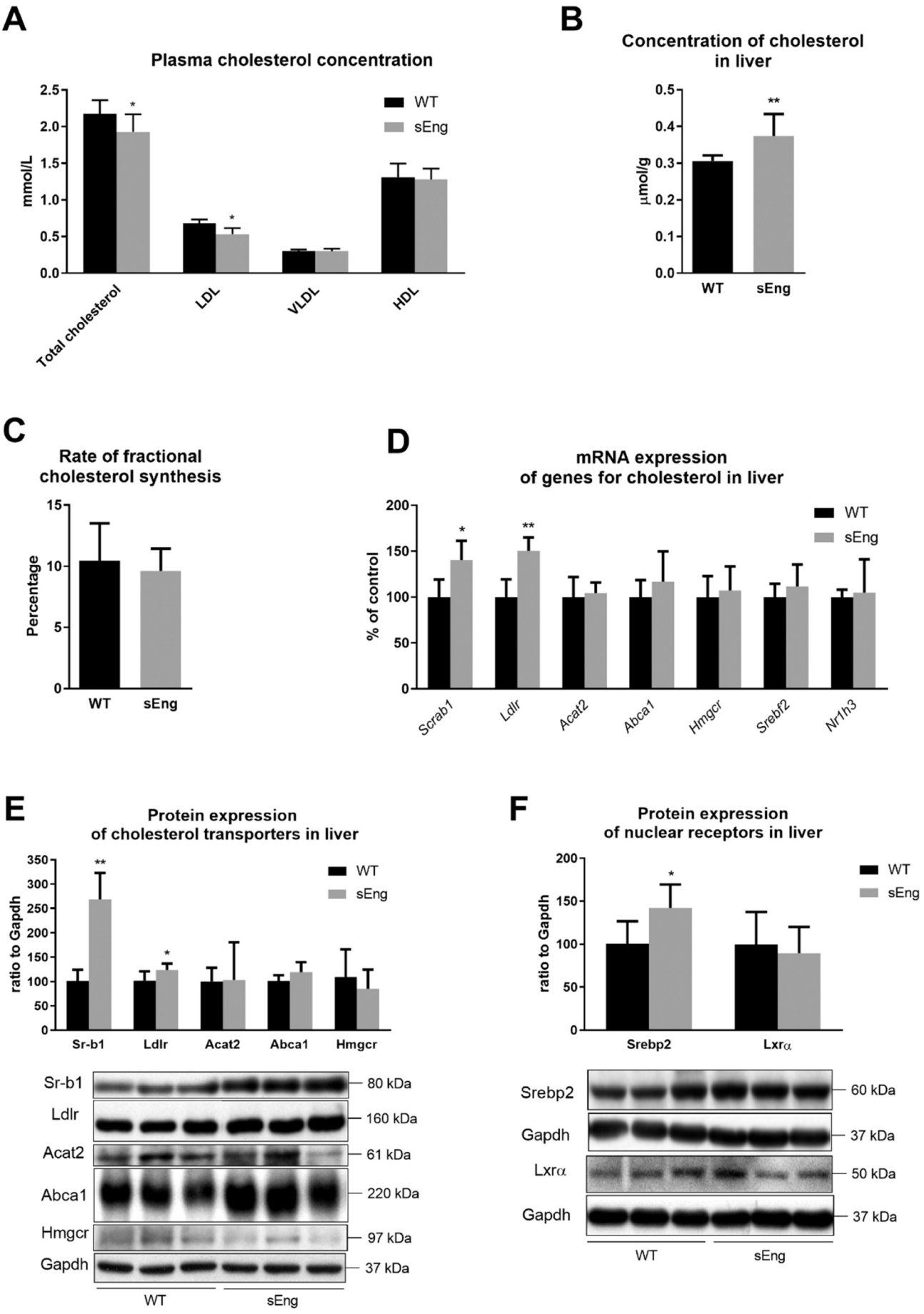

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Fig. 2. sEng reduces plasma cholesterol concentration and increases liver cholesterol concentration via up-regulation of LDL-receptor mediated by Srebp2. Plasma (A) and liver (B) concentration of cholesterol, fractional cholesterol synthesis rate (C), mRNA expression of genes for cholesterol transporter in liver (D), protein expression of cholesterol transporters (E) and nuclear receptors (F) in liver wild-type (WT) and sEng mice. Data are presented as the mean ± SD (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$, Mann-Whitney *U* test. For Western blot analysis (E, F): top: results are presented as ratio to Gapdh; bottom: representative immunoblots.

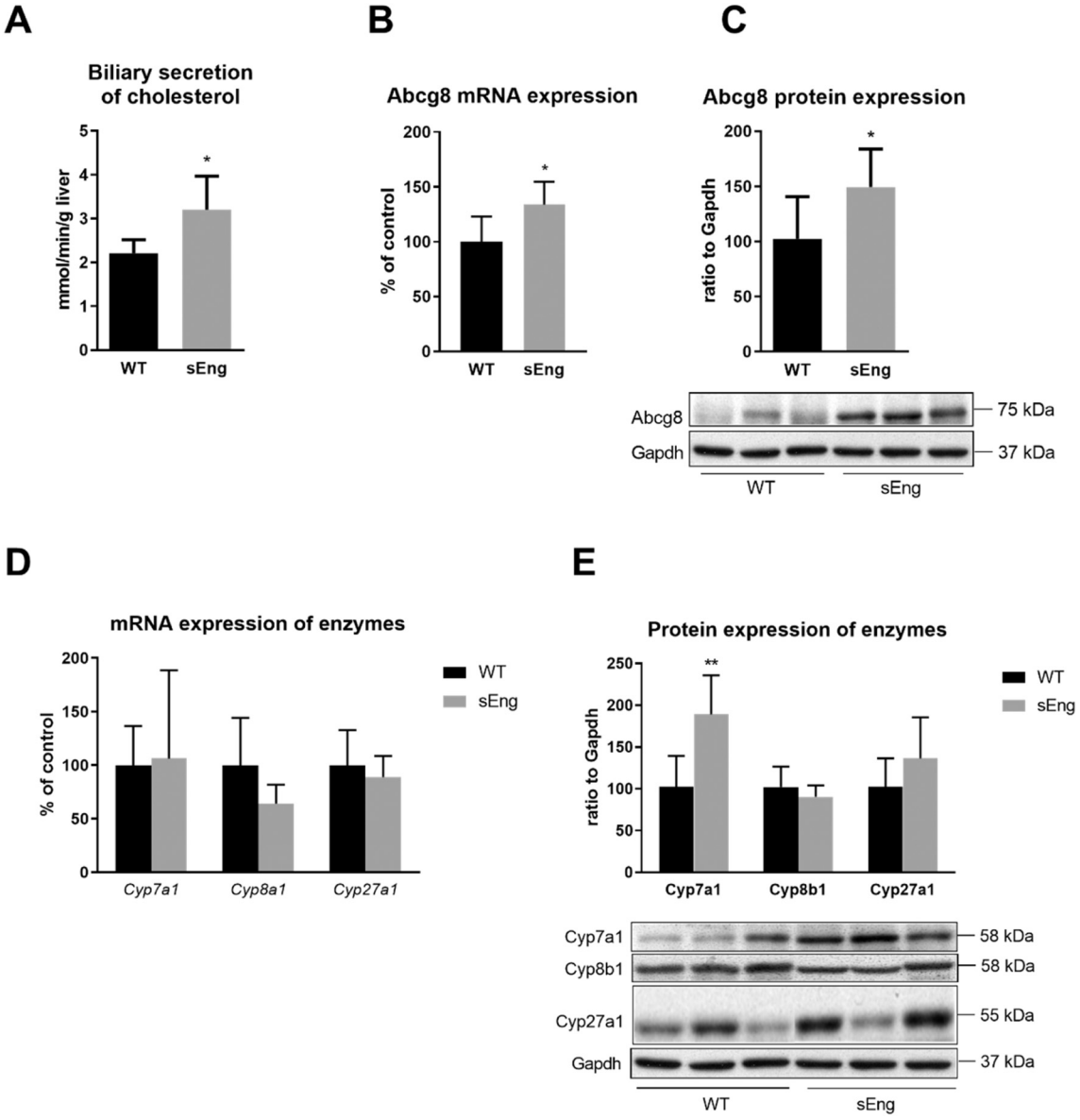

Fig. 3. sEng increased biliary excretion of cholesterol and its conversion to bile acids. Biliary secretion of cholesterol (A), mRNA (B) and protein (C) expression of Abcg8 transporter in liver, mRNA (D) and protein (E) expression of enzymes involved in BA metabolism in liver of wild-type (WT) and sEng mice. Data are presented as the mean ± SD (n = 7). $^{*}P < 0.05$, $^{**}P < 0.01$, Mann-Whitney *U* test. For Western blot analysis (C, E): top: results are presented as ratio to Gapdh; bottom: representative immunoblots.

3.6. sEng activates liver drug metabolizing and transporting pathways and induces hepatocyte Ntcp uptake transporter for BA

To describe the mechanisms responsible for the sEng-mediated induction of BA biliary secretion and bile formation, we performed analysis of gene and protein expression of transporters involved in these processes (Fig. 5). In comparison to WT animals, high plasma levels of sEng led to a significant increase in the mRNA level of the Mrp2 (*Abcc2* gene) transporter (Fig. 5A), which contributes to BA-independent bile flow via biliary secretion of glutathione. This change was not reflected by Mrp2 protein (Fig. 5B). Importantly, we detected induction of *Abcb1* gene encoding major canalicular transporter for biliary secretion of drugs, Mdr1 (Fig. 5A). Absence of changes in mRNA of other transporters excludes regulation of BA biliary secretion by transcriptional modulation. On the other hand, *Abcc2* and *Abcb1b* are typically regulated by stimulation of xenosensor nuclear receptors, CAR (Constitutive androstane receptor), and PXR (Pregnane X receptor) [24]. We therefore analyzed mRNA expression of *Cyp2b10*, the target gene for CAR, and *Cyp3a11* (Fig. 5A), the target gene of PXR. Significant induction of *Cyp3a11*, together with induction of *Abcc2* and *Abcb1b*, indicates activation of PXR receptor in sEng mice. Subsequent Western blot analysis confirmed induction of Mdr1 protein and indicates potential of sEng to increase biliary efflux of drugs, which are substrates of this transporter (Fig. 5B).

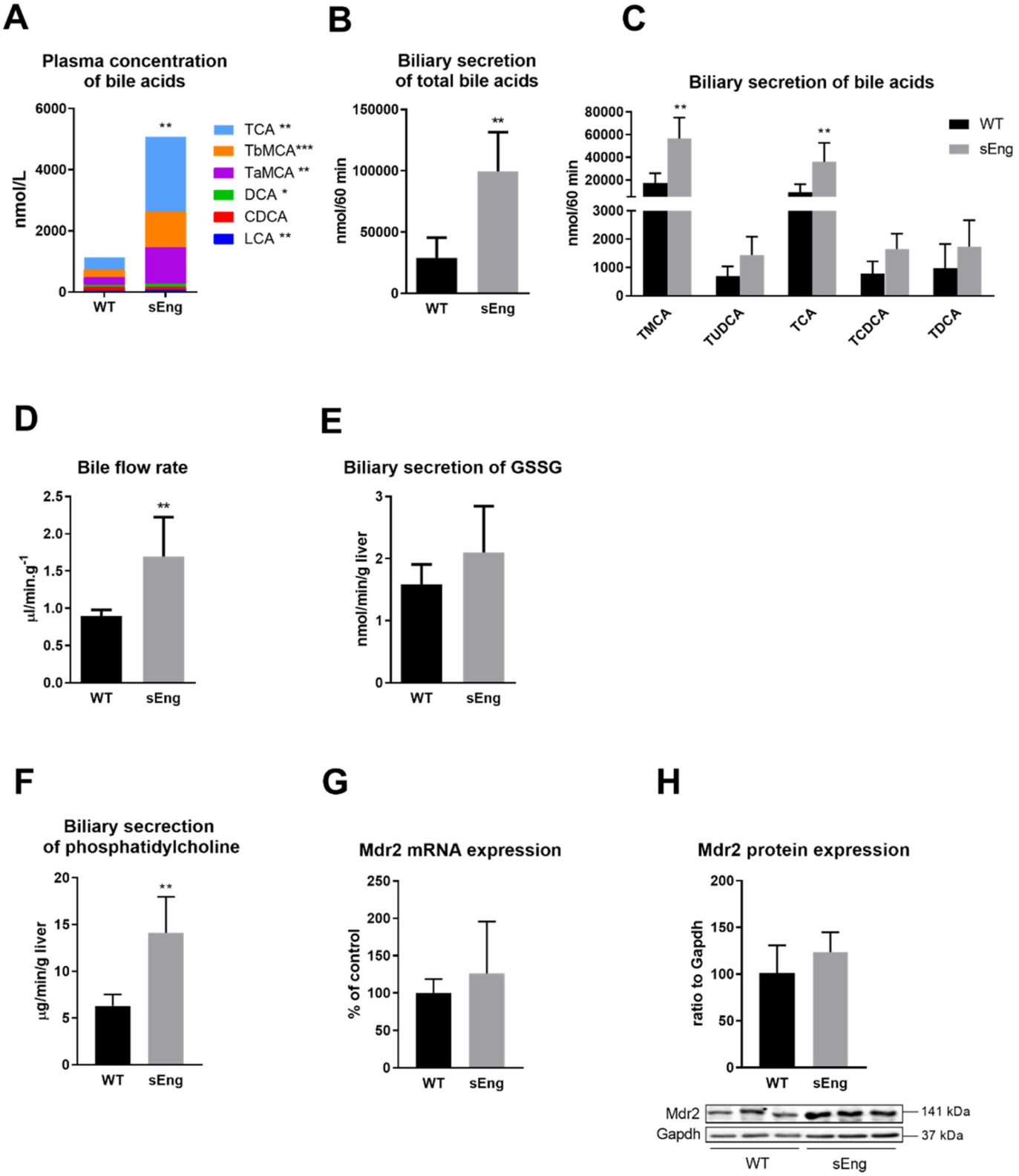

Fig. 4. sEng induces plasma concentration of BA and BA-dependent choleretic effect. Plasma concentration of total and individual BA (A), biliary secretion of total BA (B) and BA - spectra (C), bile flow rate (D), biliary secretion of glutathione (E) and phosphatidylcholine (F), mRNA (G) and protein (H) expression of Mdr2 transporter in liver of wild-type (WT) and sEng mice. Total BA concentration in plasma and bile was calculated as a sum of individual BA quantified in the sample. LC-MS measurements were performed in bile collected within 45 min. Data are presented as the mean ± SD (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$, Mann-Whitney *U* test. For Western blot analysis (H): top: results are presented as ratio to Gapdh; bottom: representative immunoblots.

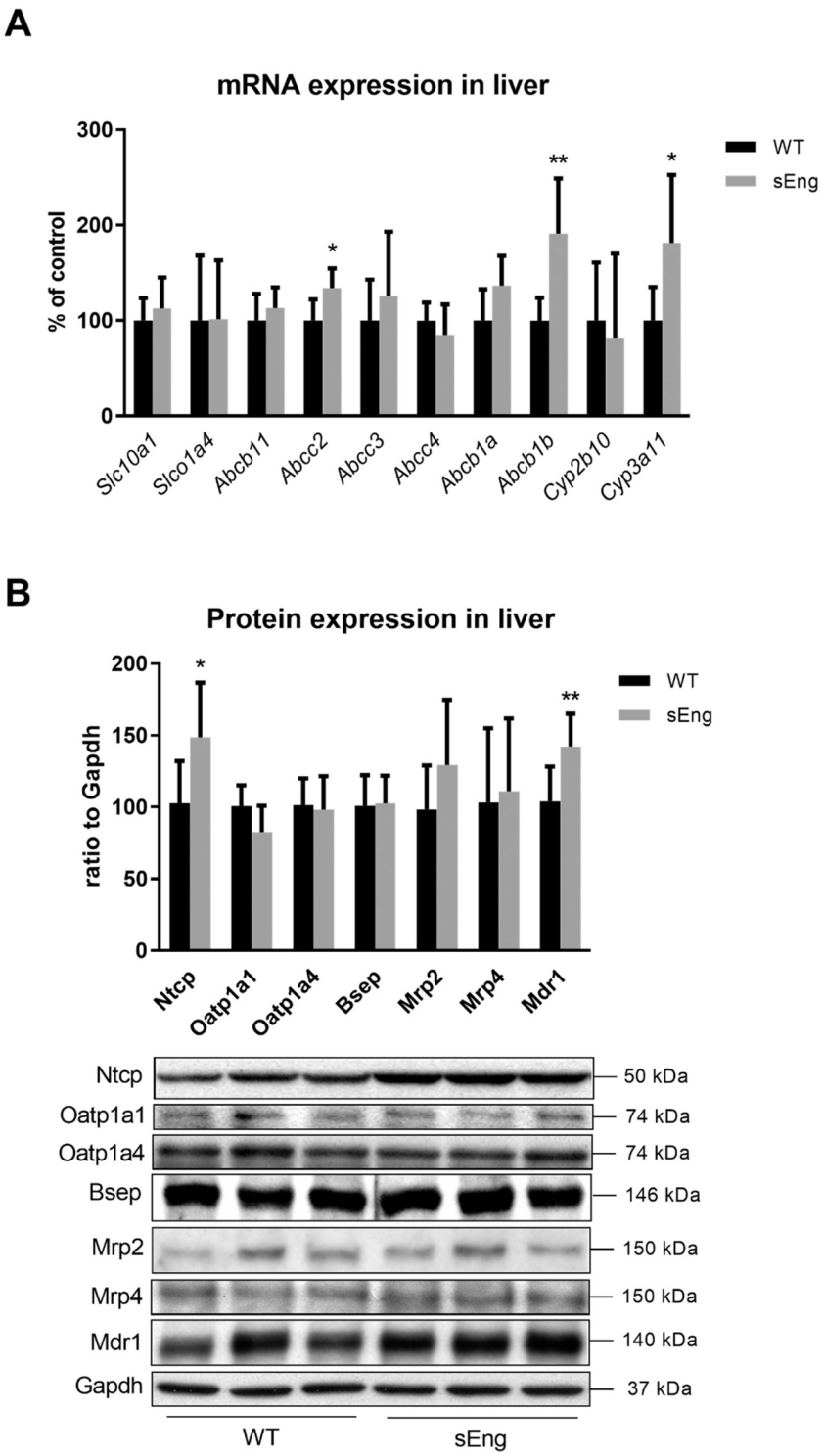

Fig. 5. sEng activates liver drug metabolizing and transporting pathways and induces hepatocyte Ntcp uptake transporter for BA. Gene (A) and protein (B) expression of transporters and enzymes involved in metabolism and transport of BA in wild-type (WT) and sEng mice. Data are presented as the mean ± SD (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$, Mann-Whitney *U* test. For Western blot analysis (B): top: results are presented as ratio to Gapdh; bottom: representative immunoblots.

In agreement with mRNA analysis, majority of BA liver transporters such as Bsep, the rate limiting protein for BA biliary secretion, were not changed by sEng at protein level. The exception was only upregulation of Ntcp, the major transporter responsible for uptake of BA into hepatocytes from portal circulation (Fig. 5B). This effect corresponds with increased plasma concentration of total BA due to increased reabsorption in ileum in sEng mice.

3.7. sEng increases BA reabsorption from stool

BA undergo significant enterohepatic recycling with > 90% of BA secreted to bile being reabsorbed back to portal circulation in ileum [25]. To elucidate this process, we quantified BA loss via feces using LC-MS method (Fig. 6). Out of 16 measured BA, only 8 were contained in stool in sufficient amount to reach quantification limit of the method, 1.25 μM (Fig. 6A). BA present in stool mainly in unconjugated form,

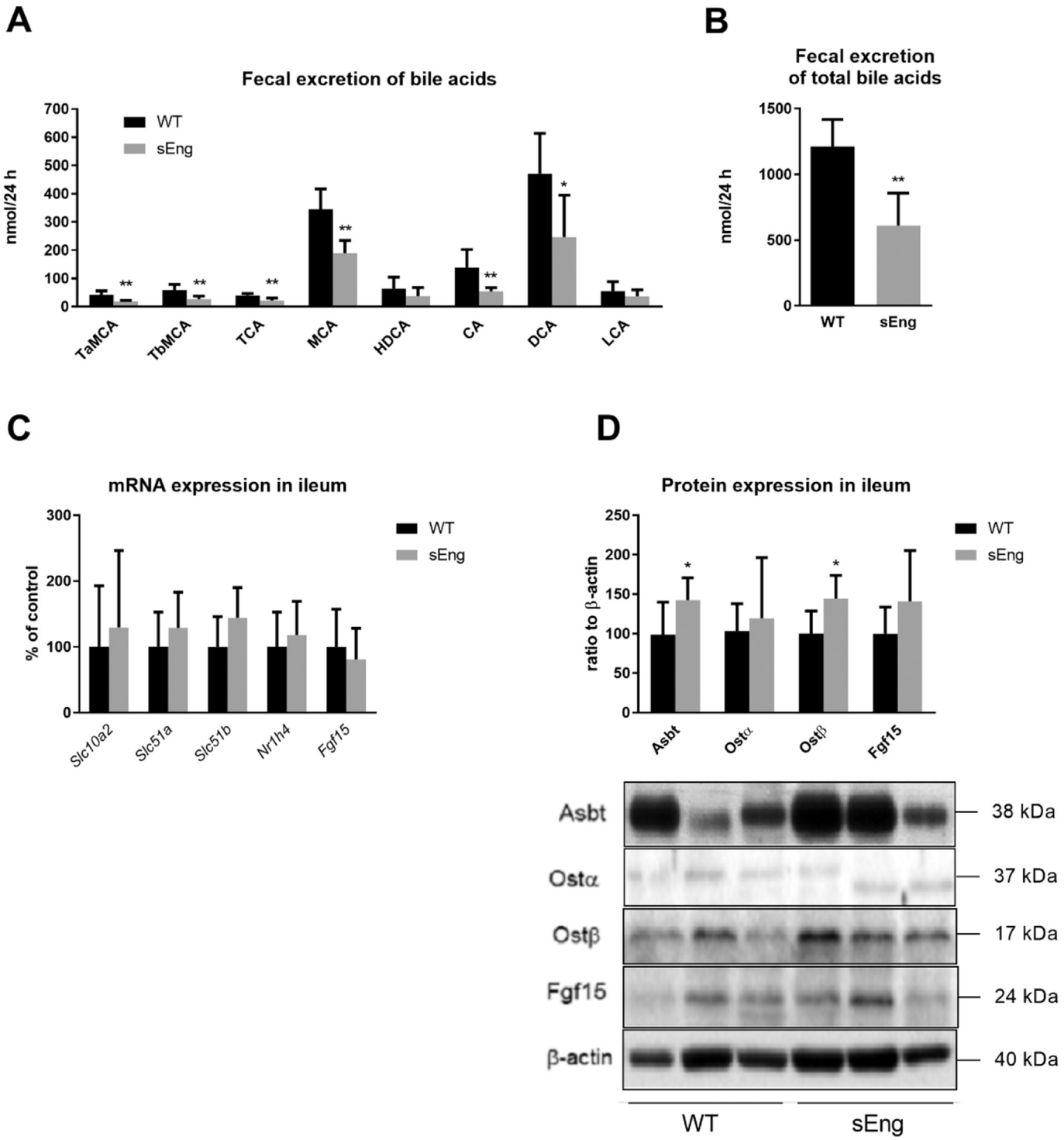

Fig. 6. sEng increases BA reabsorption from stool. The fecal excretion of individual (A) and total (B) BA in stool, mRNA (C) and protein (D) expression of major transporters for BA reabsorption in ileum. Total BA concentration in stool was calculated as a sum of individual BA quantified in the sample. Data are presented as the mean ± SD (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$, Mann-Whitney *U* test. For Western blot analysis (D): top: results are presented as ratio to β-actin; bottom: representative immunoblots.

which reflect extensive deconjugation of BA by bacterial flora. The most abundant BA were muricholic (MCA) and deoxycholic acid (DCA) (Fig. 6A). Stool content of majority of detected BA declined in sEng mice compared with WT animals. The total BA excretion via the stool was subsequently calculated as a sum of individual BA. High sEng mice showed significant reduction in fecal excretion of total BA. (Fig. 6B). Coupled with increased BA delivery to intestine via increased biliary secretion, reduced BA fecal excretion indicates increased BA reabsorption in ilea of sEng mice.

To determine mechanism of increased BA reabsorption in intestine of sEng mice, we analyzed mRNA and protein of major BA transporters in ileum (Fig. 6C, D). Although gene expressions of these molecules showed tendencies for induction, they were insignificant due to marked interindividual variability (Fig. 6C). In contrast, Western blot analysis confirmed significantly increased expression of Asbt, an uptake transporter at apical membrane of enterocytes, as well as Ostβ, an excretory

transporter at basolateral membrane of enterocyte in sEng mice (Fig. 6D).

4. Discussion

sEng plasma levels are increase during hypecholesterolemia but the effect of sEng on the liver functions, especially those related to cholesterol and bile acid metabolism has not been elucidated so far. By using specific transgenic sEng mice, herein we show, for the first time, that increased plasma sEng activates liver mechanisms targeted at reducing cholesterol plasma concentrations by its redistribution from plasma to hepatocytes and consequently to bile. Uptake of cholesterol into hepatocytes was stimulated by sEng via induction of both major transporters at basolateral membrane, Sr-b1, mediating uptake of HDL cholesterol from plasma, and LDL receptor, responsible for uptake of LDL cholesterol [26,27]. In agreement, mice models with absence of either of these transporters demonstrate increased plasma cholesterol concentrations and development of atherosclerosis [28,29], while their overexpression are protective against diet-induced hypercholesterolemia [30]. Our data show that sEng reduced plasma cholesterol mainly via reduction of LDL cholesterol. The reason why HDL cholesterol was not modified in sEng mice in spite of Sr-b1 induction is unknown. We therefore studied Srebp-2, the major Ldlr transcriptional regulator [31] and we indeed confirmed Srebp-2 upregulation by sEng. Given that cholesterol synthesis was unchanged, and its metabolism and biliary excretion was even increased, our observations suggests that hepatic Srebp-2-Ldlr-mediated uptake is the main mechanism responsible for the increased hepatic cholesterol content induced by high sEng levels and resulting in decreased plasma concentration of cholesterol.

In addition to increased cholesterol uptake into liver, sEng mice showed also an increase in biliary cholesterol secretion. This principal excretory route for unmetabolized cholesterol depends mainly on the activities of Abcg5/Abcg8 and Mdr2 canalicular transporters. Abcg5/Abcg8 heterodimer complex transport directly cholesterol. Its absence in Abcg5/Abcg8-deficient mice markedly lowers biliary cholesterol concentration [32], while increased biliary cholesterol secretion is detected after Abcg5/Abcg8 overexpression [33]. However, proper biliary cholesterol secretion requires also intact Mdr2 transporter. Mdr2 is primary responsible for the biliary secretion of phospholipids [34,35] to form micelle with BA. Micelles enable transport of lipophilic agents in bile including cholesterol. Thus, absence of Mdr2 transporter leads to reduced cholesterol biliary excretion. We detected induction of Abcg5/Abcg8 complex without modulation of Mdr2, which indicates that increased biliary secretion of cholesterol is related to induction Abcg5/Abcg8 protein, and the transport may be further promoted by increased hepatic cholesterol disposition mediated by its increased uptake from plasma.

The second crucial pathway for cholesterol elimination from organism is its liver conversion into BA with their consequent biliary secretion. The rate limiting enzyme initiating BA synthesis from cholesterol is Cyp7a1. The rate limiting transporter mediating biliary secretion of BA is Bsep [11]. Our results uncovered significant upregulation of Cyp7a1 at protein level in sEng mice, but enzymes following in the cascade of BA metabolic conversions and controlling the synthesis of neutral (Cyp8b1), and acidic (Cyp27a1) BA were not modulated. Reason for discrepancy between mRNA and protein expression of Cyp7a1 is unknown, because this enzyme is considered as mainly regulated at transcriptional step [25]. Posttranslational modification of Cyp7a1 opens new field of research and will be analyzed in the following studies. However, isolated Cyp7a1 induction was in agreement with a proportional increase in biliary secretion of major BA from neutral and acidic pathway, TCA and TMCA, respectively. Simultaneous absence of change in the expression of Bsep, and upregulation of Ntcp, the major transporter for uptake of BA from blood to hepatocytes indicates that increased biliary secretion of BA was the consequence of their increased hepatocyte disposition in response to their increased synthesis, and uptake from blood. Secreted BA are major osmotic driving force to generate bile, thus we propose that choleretic effect detected in sEng mice was BA-dependent. BA-independent bile flow was not involved as suggested by unchanged biliary secretion of its main component, glutathione and its major canalicular transporter, Mrp2 [11].

Majority of BA entering the intestine via biliary tract undergo enterohepatic recycling, i.e. they are reabsorbed in ileum and thereafter avidly cleared from portal blood by hepatocytes [25]. Our results show reduced content of BA in the stool of sEng animals despite increased BA biliary secretion. It indicates increased BA reabsorption in ileum. This finding is supported by up-regulation of Asbt and Ostβ, the major transporters for reuptake of BA from intestine at apical and basolateral membranes of enterocytes, respectively. The spectra of BA in the stool showed the highest contribution of unconjugated secondary BA, MCA and DCA onto the decreased fecal BA excretion, which complies with the extensive deconjugation and conversion of BA into secondary BA by intestinal flora [36]. Following reabsorption, BA were transported by portal blood to the liver, where they are taken up into hepatocytes mainly by Ntcp transporter [25]. Mice with sEng showed significant increase of BA systemic concentrations due to the increased BA reabsorption in ileum, and their increased synthesis in the liver. Together with induced Ntcp transporter our results indicate that increased reabsorption of BA in ileum is the major mechanism responsible for increased BA systemic concentrations. This finding is important for cardiometabolic disorders because BA acts as hormones and regulates energy homeostasis through the modulation of glucose and lipid metabolism via multiple intracellular receptors in various organs and tissues [37]. In agreement, a positive association was demonstrated between BA and diabetes, when BA pool and fecal BA were increased in diabetic patients with uncontrolled hyperglycemia. Moreover, higher plasma BA levels were found in patients with diabetes mellitus type 2 [38]. In addition, there is also a positive relationship between hypertension and BA levels in diabetic patients and in patients with NASH (non-alcoholic steatohepatitis), where BA affected hepatic lipid metabolism [39,40].

Mdr1/MDR1 is apical transporting protein expressed on cells with excretory or barrier function, and within the liver it mediates biliary secretion of numerous clinically available drugs or their metabolites [41]. Cyp3a11 is mice orthologue of human CYP3A4, the enzyme responsible for metabolism of > 30% of drugs. Cyp3a11 and Mdr1 share substrate specificity and co-operate in the elimination of many therapeutic agents. They share also transcriptional regulation by PXR receptor, a “master” xenobiotic sensor regulating the expression of a wide variety of genes involved in the elimination of xenobiotics. Enzyme inducers producing serious drug/food-drug interactions are commonly agonists of this nuclear receptor [42]. Significant induction of Mdr1, and Cyp3a11 in sEng mice strongly suggest stimulation of PXR. Because sEng mice were on standard chow diet, PXR might be stimulated only by endogenous compound cumulating within the liver. CA (cholic acid) was recently described as a potent agonist of PXR [43] and its taurine conjugate TCA was also markedly increased in bile of sEng animals. It indicates increased CA intrahepatic disposition, because Bsep transporter was not modified. Therefore, we suggest that sEng may increase elimination of Cyp3a11/Mdr1 substrates by CA-mediated PXR stimulation. It is of interest to mention that choleretic effect of sEng shown in this study can together with the parallel induction of Mdr1 further affect pharmacotherapy of above-mentioned drugs as demonstrated in other studies, where these both effects occurred simultaneously [44,45]. Therefore, the exact role of sEng in modulation of drugs pharmacokinetics must be elucidated in future studies.

Previous studies demonstrated sEng as a plasma biomarker of atherosclerosis [46], arterial hypertension [47], hypercholesterolemia [48], and type II diabetes mellitus [47,49]. In addition, the presence of sEng aggravated endothelial/vascular function in aorta [8] and induced

preeclampsia phenotype in mice [4]. In this study, the presence of high sEng in plasma of otherwise healthy animals significantly reduced cholesterol and increased BA plasma concentrations due to a complex effect on their synthesis and excretion. The beneficial reduction of plasma cholesterol by sEng was mild and within physiological values, but the BA raised marked over this range which threatens organism with organ toxicities. Taken together, our data indicate mechanisms how the increased plasma sEng may modify pathophysiology of associated diseases. Further studies are therefore required to uncover regulatory role of sEng on liver cholesterol and BA metabolism in various disorders.

5. Conclusions

In conclusion, our data provides new evidence that high plasma levels of sEng increases systemic plasma concentrations of BA due to their increased reabsorption in ileum, what leads to the increased uptake of BA into hepatocytes, and biliary secretion coupled with BA-dependent choleretic activity. Moreover, we have demonstrated that increased sEng levels reduced plasma concentrations of total and LDL cholesterol by increasing its uptake to hepatocytes, conversion to BA, and secretion into bile. These data provide important insight into effects of high sEng levels on cholesterol and bile acids metabolism. Furthermore, our result also implies significant potential of sEng to increase liver elimination of numerous drugs through Mdr1 transporter. These findings indicate significant potential of sEng to modify lipid metabolism and drug pharmacokinetics, and supports further studies uncovering role of high sEng levels in disorders associated with hypercholesterolemia.

Acknowledgement

We gratefully acknowledge the skillful technical assistance of Jitka Hajkova and the care of animals of Katerina Sildbergerova.

Grants

This study was supported by grants from the Ministry of Health of the Czech Republic grant [AZV; 150/52/75201], Efficiency and safety improvement of current drugs and nutraceuticals: advanced methods – new challenge grant [EFSA-CDN; CZ.02.1.01/0.0/0.0/16_019/0000841] co-funded by European Regional Development Fund (ERDF), and Specific University Research Projects [SVV-260414 and Progres Q40/05] and Grant Agency of Charles University [GAUK No. 1166119]. It was also funded by Instituto de Salud Carlos III from Spain thorough the project (PI16/00460) co-funded by European Regional Development Fund/European Social Fund “Investing in your future” and the by Kidney Research Network [REDINREN; RD016/0009/0025] co-funded by European Regional Development Fund/European Social Fund “Investing in your future”.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

E. D., S. M. designed research. E. D., A. P., I. C. I. S., J. C., H. L., S. M. performed research. M. H., R. H., A. T. contributed analytic tools. E. D., I. C. I. S., analyzed data. E. D., S. M., I. C. I. S., wrote the paper. E. D., P. N., S. M. participated in research discussion. All authors participated in the revision and approved final manuscript. P. N. and S. M. are the guarantors of this work and had full access to all the data in the study.

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