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Effects of bile salts on hepatic lipoprotein production

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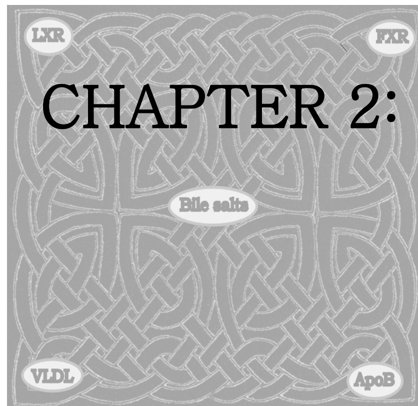

CHAPTER 2: The role of transhepatic bile salt flux in the control of hepatic secretion of triacylglycerol-rich lipoproteins in vivo in rodents

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ABSTRACT

Bile salts (BS) have been shown to suppress the secretion of Very-Low-Density Lipoprotein-Triglyceride (VLDL-TG) in rat and human hepatocytes *in vitro*. In the present study we investigated whether the transhepatic BS flux affects VLDL-TG concentration and hepatic VLDL-TG secretion *in vivo.* In rats, the transhepatic BS flux was quantitatively manipulated by 1-wk chronic bile diversion (BD), followed by intraduodenal infusion with taurocholate (TC) or saline for 6h. In mice, the transhepatic BS flux was manipulated by a 3-wk dietary supplementation with TC (0.5wt%) or cholestyramine (2wt%). In rats, BD followed by saline or TC infusion did not affect plasma TG concentration, hepatic TG production rate or VLDL lipid composition compared to control rats. In mice supplemented for 3 weeks with TC or cholestyramine, the transhepatic BS flux was increased by 335% and decreased by 48%, respectively, compared to controls. Among the three experimental groups of mice, an inverse relationship between transhepatic BS flux and either plasma TG concentration (R^2=0.89) or VLDL-TG production rate (R^2=0.87) was observed, but differences were relatively small. Present data support the concept that BS can reduce VLDL-TG concentration and inhibit hepatic TG secretion *in vivo*, however, this occurs only at supraphysiological transhepatic BS fluxes in mice.

INTRODUCTION

The parenchymal cells of the liver secrete significant amounts of lipids into bile and blood. Biliary lipid secretion mainly involves secretion of unesterified cholesterol and phospholipids (PL), mostly phosphatidylcholines. Hepatic lipid secretion into the blood occurs mainly in the form of Very-Low-Density Lipoproteins (VLDL), which are composed of triacylglycerol (TG), cholesteryl ester (CE), cholesterol, PL and apolipoproteins (apoB, apoE, apoC's). Apolipoprotein B is the key structural protein [1]. Each VLDL particle contains one apoB molecule and, therefore, apoB concentration is related to the amount of VLDL particles. BS have been reported to affect both hepatic lipid secretion into bile and into blood [2, 28, 17, 35].

For a long time it is known that BS stimulate biliary cholesterol and PL secretion in a dose-dependent fashion [2]. More recently, it was shown that BS dose-dependently inhibit the secretion of VLDL by primary human and rat hepatocytes *in vitro* [3,4]. BS-mediated regulation of hepatic VLDL secretion may thus represent a novel physiological function of BS. Indirect indications that the transhepatic BS flux affects hepatic VLDL secretion can be derived from clinical observations. Patients with familial hypertriglyceridemia (FHT) were shown to experience intestinal BS malabsorption [5,6], which is supposed to decrease the transhepatic BS flux. The patients developed a further increase in plasma TG concentrations after interruption of the enterohepatic circulation by 18h-withdrawal of bile [7]. Interruption of the enterohepatic circulation in healthy humans with cholestyramine or after ileal exclusion is associated with elevated plasma TG concentrations [8,9]. Finally, treatment of hypertriglyceridemic patients with chenodeoxycholate for gallstone dissolution has been reported to coincide with a decrease in plasma TG concentrations [10].

BS can affect expression of multiple genes via direct and indirect actions. BS biosynthesis is directly regulated via a negative feedback mechanism; BS act as natural ligands for the nuclear farnesoid X receptor (FXR) [11] which leads to the transcription of small heterodimer partner (SHP) [12]. Via FXR and elevated SHP, cholesterol-7a-hydroxylase gene expression is repressed [13], which is the rate-limiting enzyme in BS biosynthesis. Indirectly, BS are involved in regulation of cholesterol metabolism. During BS biosynthesis cholesterol is converted to oxysterols, which are known ligands for the liver X receptor (LXR) that is shown to stimulate expression of cholesterol 7α-hydroxylase [14]. Cholesterol depletion caused by increased BS synthesis activates sterol

regulatory element-binding proteins (SREBP's), a family of transcription factors, to stimulate expression of genes involved in cholesterol synthesis (HMG-CoA synthase and HMG-CoA reductase) and fatty acid metabolism [15,16]. BS have been speculated to regulate VLDL secretion via SREBP-mediated expression of lipogenic enzymes [17].

In the present study we investigated the effects of acute and chronic manipulation of the transhepatic BS flux on hepatic TG production rate in rats and mice *in vivo*. Biliary BS secretion rates were manipulated between ~8% and ~400% of the physiological values. Our results indicate that only supraphysiological transhepatic BS fluxes lower plasma TG concentrations and inhibit VLDL-TG production *in vivo* in mice. An important physiological effect of the transhepatic BS flux in control of hepatic VLDL-TG production in rodents seems therefore unlikely.

EXPERIMENTAL PROCEDURES

Materials

Triton WR-1339, taurocholate, fatty acid-free bovine serum albumin, oleic acid and cholestyramine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Triglyceride and (total) cholesterol assay were from Roche (Mannheim, Germany), free cholesterol and phospholipids assay were from WAKO Chemicals GmbH (Neuss, Germany). ^{3}H-glycerol was purchased from New England Nuclear (Boston, MA, USA), SDS gels were from Biorad (Herculus, CA, USA) and cell culture materials were from Costar (Cambridge, MA, USA).

Animals and diets

Rats. Male Wistar rats (Harlan Laboratories, Zeist, The Netherlands), weighing 300 - 370 g were kept in a light- and temperature-controlled facility (lights on: 06.00 - 18.00 h). Animals had free access to chow (RMH-B, Hope Farms BV, Woerden, The Netherlands) and tap water.

Mice. Male wild-type C57BL/6J mice (obtained from IFFACredo, Rijswijk, The Netherlands), 2 - 3 months old, were used. The animals were kept in a light- and temperature-controlled facility (lights on: 06.00 - 18.00 h) and had free access to tap water

and standard chow (RMH-B, Hope Farms BV, Woerden, The Netherlands) until the start of the experiment.

Experimental protocols were approved by the Ethical Committee for Animal Experiments, Faculty of Medical Sciences, University of Groningen.

In vivo experiments

Rats

Rats were equipped with permanent catheters in bile duct, duodenum and jugular vein, as described by Kuipers *et al.*[18]. This experimental model allows for physiological studies in unanaesthetized rats with long-term bile diversion without interference of stress or restraint. After surgery, catheters in bile duct and duodenum were chronically interrupted for 1 week. Animals were allowed to recover from surgery for 6 days. On day 7, after an overnight fast, bile diverted rats were intraduodenally infused with 0.9% (w/v) NaCl solution (control, 1.5 ml/h, n=4) or with taurocholate solution (dose 9.3 µmol/h/100 g BW, n=5) for 6 h. Taurocholate was dissolved in 0.9% (w/v) NaCl in a concentration of 22.3 mM (pH 7.4), immediately before use. Bile was collected at 1 h intervals during infusion. At time point 2 h after starting the infusion, a 1 ml blood sample was obtained via the jugular vein catheter and was immediately transferred to an EDTA-containing tube. The Triton WR-1339 procedure [19] was used to measure the hepatic VLDL-triglyceride production rate. After obtaining the 2 h blood sample, Triton WR-1339 (12% in PBS, 0.5 ml/100 g BW) was administered intravenously. Rats with intact enterohepatic circulation served as controls and were also administered Triton WR-1339. Blood samples (0.2 ml) were taken at regular intervals (t = 0 h, t = 0.5 h, t = 1 h, t = 2 h, t = 3 h and t = 4 h after Triton administration) via the catheter in the jugular vein and a final sample (2 ml) at 6 h after start of the infusion. Linear regression analysis was performed on plasma TG concentration at time points 1, 2, 3 and 4 h in individual animals. The thus obtained slopes, reflecting individual TG production rates, were normalized to body weight and used to determine mean TG production rates per experimental group. Plasma VLDL/LDL fraction (d < 1.019 g/ml) was isolated by density gradient ultracentrifugation in an Optima TM TLX (Beckman Instruments, Inc., Palo Alto, CA, USA) table top ultracentrifuge (120 000 rpm, 100 min., 4°C).

Mice

Mice were fed the standard diet (RMH-B, Hope Farms B.V., Woerden, The Netherlands) or the standard diet supplemented with either 0.5% (w/w) taurocholate or 2% (w/w) cholestyramine for three weeks (each group n=6). At time point three weeks, after an overnight fast, mice (n=6 per diet group) were anaesthetized with halothane and a large blood sample was collected by cardiac puncture. Blood was immediately transferred to tubes containing EDTA. Subsequently, the liver was perfused via de portal vein with ice-cold saline to remove remaining blood, quickly excised and weighted. Small pieces of liver tissue were obtained to be used for RNA isolation and, as the remaining liver tissue, frozen in liquid nitrogen and stored at -80°C until analysis. Separate groups of animals were used for measurement of hepatic VLDL-triglyceride production rates by the Triton WR-1339 method (n=6 per group). After an overnight fast, blood samples (75 µl) were obtained before and at regular intervals after i.v. administration of Triton WR-1339 (12.5 mg/100 µl phosphate-buffered saline) by tail bleeding. Linear regression analysis was performed on plasma TG concentration at time points 0.5, 1, 2 and 3 h in individual animals. The thus obtained slopes, reflecting individual TG production rates, were normalized to body weight and used to determine mean TG production rates per experimental group. The plasma VLDL/IDL fraction was isolated by density gradient ultracentrifugation from a large final blood sample collected by hart puncture as described. Separate groups of mice were used to analyze the biliary secretion rates of bile salts under control, taurocholate- or cholestyramine-supplemented conditions (n=6 per diet group). After three weeks on either diet the gallbladder of mice was canulated under Hypnorm (fentanyl/ fluanisone, 1 ml/kg) and Diazepam (10 mg/kg) anesthesia and bile was collected for 30 minutes. Subsequently, a large blood sample was collected by cardiac puncture and transferred into an EDTA-containing tube for VLDL isolation by density gradient ultracentrifugation as described.

In vitro experiments

In vitro measurement of VLDL-TG secretion

Isolation and culture of mouse hepatocytes was performed as described previously [20,21] using wild type C57BL6/J mice. Hepatocytes were plated in 35 mm 6-well plastic dishes (Costar Corp., Cambridge, MA, USA), precoated with collagen (Serva Feinbiochemica, Heidelberg, Germany) at a density of 1.0 x 10^6 cells/well in 2 ml of William's E medium

containing insulin, fetal calf serum (FCS), dexamethasone and penicillin/streptomycin. After overnight incubation the medium was removed and hepatocytes were washed with hormone-free and FCS-free (HF-SF) William's E medium and subsequently incubated with HF-SF William's E medium for 4 hours. After four hours the medium was removed and replaced by 1 ml HF-SF William's E medium per well containing 25 µM [^{3}H]-glycerol (4.4 µCi/well), 0.75 mM oleate (C18:1) complexed with bovine serum albumin and, in the experimental group only, 200 µM taurocholate. After 24 hours incubation the medium was collected and centrifuged to remove debris. Hepatocytes were washed with ice-cold Hank's balanced salt solution (HBSS) and scraped into 2 ml of HBSS for lipid extraction.

Analytical techniques

Fast Protein Liquid Chromatography (FPLC)

For lipoprotein size fractionation 0.2 ml plasma samples from individual rats or pooled plasma samples from mice (totaling 0.2 ml, n=6 per group) were separated by Fast Protein Liquid Chromatography (FPLC) on a Superose 6 HR10/30 column (Amersham Pharmacia Biotech, Upsalla, Sweden). Triglyceride and total cholesterol concentrations in the obtained fractions (0.5 ml) were measured as described below.

Hepatic mRNA expression levels

Total RNA from mouse liver samples was isolated by using the Trizol method (Gibco BRL, Grand Island, NY). Isolated total RNA was converted to single stranded cDNA by a reverse transcription procedure with M-Mulv-RT (Boehringer Mannheim/Roche, Mannheim, Germany) according to manufacturer's protocol. cDNA levels were measured by real-time PCR using the ABI prism 770 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). For the PCR amplification studies, an amount of cDNA corresponding to 10 ng of total RNA was amplified using the qPCR core kit (Eurogentec, Seraing, Belgium) essentially according to the manufacturer's protocol and optimized for amplification of the particular gene using the appropriate forward and reverse primers (GIBCO, Paisly, UK) and a template specific 3'-TAMRA, 5'-6-FAM labeled Double Dye Oligonucleotide probe (Eurogentec, Seraing, Belgium). In the same experiments, calibration curves were run on serial dilutions of an 8x concentrated cDNA solution as used in the assay, resulting in a series containing 8x, 4x, 2x, 1x, 0.5x, 0.25x,

0.125x and 0.063x of the cDNA present in the assay incubation. Both assay and calibration incubations were done simultaneously. During amplification, breakdown of the probe released the fluorescent 6-FAM-dye, resulting in increase in fluorescence. The fluorescence data were processed using the software program ABI Sequence Detector v1.6.3 (System Applied Biosystems, Foster City, CA, USA). All quantified expression levels were within the linear part of the calibration curves and were calculated using these curves. The primer sequences used are listed in **Table 1**.

Table 1. Real-time PCR primers

Gene name	Type	Sequence 5'-3'	Gen-Bank Accession No.
ß-Actin	Forward	AGC CAT GTA CGT AGC CAT CCA	NM_007393
	Reverse	TCT CCG GAG TCC ATC ACA ATG	
	Probe	TGT CCC TGT ATG CCT CTG GTC GTA CCA C	
Apob	Forward	GCC CAT TGT GGA CAA GTT GAT C	AW012827
	Reverse	CCA GGA CTT GGA GGT CTT GGA	
	Probe	AAG CCA GGG CCT ATC TCC GCA TCC	
Mttp	Forward	CAA GCT CAC GTA CTC CAC TGA AG	NM_008642
	Reverse	TCA TCA TCA CCA TCA GGA TTC CT	
	Probe	ACC GCA AGA CAG CGT GGG CTA CA	
Fas	Forward	GGC ATC ATT GGG CAC TCC TT	AF127033
	Reverse	GCT GCA AGC ACA GCC TCT CT	
	Probe	CCA TCT GCA TAG CCA CAG GCA ACC TC	
Dgat1	Forward	GGT GCC CTG ACA GAG CAG AT	NM_010046
	Reverse	CAG TAA GGC CAC AGC TGC TG	
	Probe	CTG CTG CTA CAT GTG GTT AAC CTG GCC A	
Apo-a1	Forward	CCC AGT CCC AAT GGG ACA	NM_009692
	Reverse	CAG GAG ATT CAG GTT CAG CTG TT	
	Probe	CAA ACT GGG ACA CAT AGT CTC TGC CGC T	
Hmgcoar	Forward	CCG GCA ACA ACA AGA TCT GTG	BB664708
	Reverse	ATG TAC AGG ATG GCG ATG CA	
	Probe	TGT CGC TGC TCA GCA CGT CCT CTT C	
Acat2	Forward	GGT GGA ACT ATG TGG CCA AGA	NM_011433
	Reverse	CCA GGA TGA AGC AGG CAT AGA	
	Probe	CAA ACA GCC CAG GAC CTG GGC AAA G	
Fxr	Forward	CGC TGA GAT GCT GAT GTC TTG	U09416
	Reverse	CCA TCA CTG CAC ATC CCA GAT	
	Probe	ATG ATC ACA AGT TCA CCC CGC TCC TCT	

Apob: apolipoprotein B; *Mttp*: microsomal triglyceride transfer protein; *Fas*: fatty acid synthase; *Dgat1*: diacylglycerol acyltransferase 1; *Apo-a1*: apolipoprotein A-1; *Hmgcoar*: 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; *Acat2*: acyl-coenzyme A: cholesterol acyltransferase-2; *Fxr*: farnesoid x receptor.

Lipid analyses

Plasma lipids (triglycerides, total cholesterol, phospholipids and unesterified cholesterol) were measured using commercially available assay kits (triglyceride and (total) cholesterol assay kit from Roche (Mannheim, Germany), free cholesterol and phospholipids assay kit from WAKO Chemicals GmbH (Neuss, Germany)). Hepatic lipid concentrations and biliary lipid concentrations were determined after lipid extraction as described previously [22].

Medium lipids secreted by mouse hepatocytes were extracted as mentioned above. TG was separated form other lipids by thin-layer chromatography (TLC) with hexane/diethyl ether/acetic acid (80/20/1) as developing solvent. After iodine staining, the spots containing ^{3}H-TG (as a measure for VLDL-TG secretion) were scraped into vials and assayed for radioactivity by scintillation counting.

Western Blotting of apoB

Secreted apoB in medium of mouse hepatocytes in the absence or presence of 200 μM TC was concentrated with fumed silica according to the methods described by Vance *et al.* [23]. ApoB was separated from other proteins by SDS-PAGE using 4-15% gradient gels (Ready Gels, BioRad, Hercules CA, USA), transferred to nitrocellulose membranes (Hyperbound, Amersham Pharmacia Biotech, Roosendaal, The Netherlands), incubated with sheep-anti-human apoB (Roche, Mannheim, Germany) and donkey-anti-sheep IgG HRP and visualized by ECL detection (Both Amersham Pharmacia Biotech, Upsalla, Sweden) according to the manufacturer's instructions. The relative intensity of the apoB100 and apoB48 bands was determined using a CCD camera of Image Masters VDS system (Amersham Pharmacia Biotech, Upsalla, Sweden).

MTP triacylglycerol transport activity

The activity of the microsomal triglyceride transport protein was measured in isolated mouse liver microsomes in the absence or presence of different amount of taurocholate; i.e., 0.5, 1.0, 5.0 and 50 μM. Livers were removed from wild type (C57BL6/J) mice (n=5) and homogenized in buffer containing 50 mM tris (pH 7.4), 250 mM sucrose, 1 mM EDTA, 5 μg/ml leupeptin (Sigma Chemical Co., St. Louis, MO, USA), 1 mM benzamidine (Sigma Chemical Co., St. Louis, MO, USA) and 1 mM PMSF (Sigma Chemical Co., St. Louis, MO, USA). Microsomes were isolated by centrifugation: 30 minutes at 10000 g to remove plasma membranes, 30 minutes at 18000 rpm in a Kontron TST41.14 rotor to remove the nuclei and finally 60 minutes at 28000 rpm in the same rotor to pellet the microsomes. The pellet thus obtained was resuspended for at least 17 h in a hypotonic buffer (1 mM tris (pH 7.4), 0.02% saponin (Sigma)) to release microsomal lumenal content. MTP was further purified by applying the samples onto a DEAE cellulose (Sigma) column in 10 mM phosphate buffer (pH 6.8), washing the column with 30 mM NaCl-containing phosphate buffer and eluting the protein with the same buffer in the presence of

220 mM NaCl. Samples were concentrated to ~300 μl with Biomax-30 (Millipore, Ettenleur, The Netherlands). The assay was done essentially as described before [24] as adjusted by Ritchie *et al.* [25].

Miscellaneous methods

Protein concentrations in liver and in isolated mouse hepatocytes were determined according to Lowry *et al.* [26] using bovine serum albumin as standard (Pierce). Bile salt concentration in bile was determined by an enzymatic fluorimetric assay [27].

Statistics

All values represent mean ± standard deviation for the number of animals or experiments indicated. Statistical analysis of three groups was assessed by Kruskal Wallis Test. Mann Whitney U analysis was performed to determine differences between control groups and either one of the experimental groups. Level of significance was set at $p<0.05$. Analysis was performed using SPSS for Windows software (SPSS, Chicago, IL, USA).

RESULTS

Effects of low BS flux on plasma TG concentration and TG production rate in rats

Bile-diverted rats were intraduodenally infused with either saline or TC (dose 9.3 μmol/h/100 g BW) for 6 h. The biliary BS secretion rate during TC infusion plateaued after 3 h of infusion at a value of 17 $\mu mol.h^{-1}.100g\ BW^{-1}$, which is ~60% of that reported in intact rats (28-33 $\mu mol.h^{-1}.100g\ BW^{-1}$ [18,28]). BS secretion rates in the saline-infused rats remained constant at a rate of 2 $\mu mol.h^{-1}.100g\ BW^{-1}$. After 1 wk of bile diversion plasma TG concentrations (**Figure 1**, **panel A**) were similar in BD rats (prior to infusion) and control rats with intact enterohepatic circulation. The rate of increase in plasma TG, reflecting the hepatic production of VLDL-TG (**Figure 1**, **panel B)** was studied by the Triton WR-1339 method. Between 1 h and 4 h after injection of Triton WR-1339, the TG production rates were similar in all three animal groups.

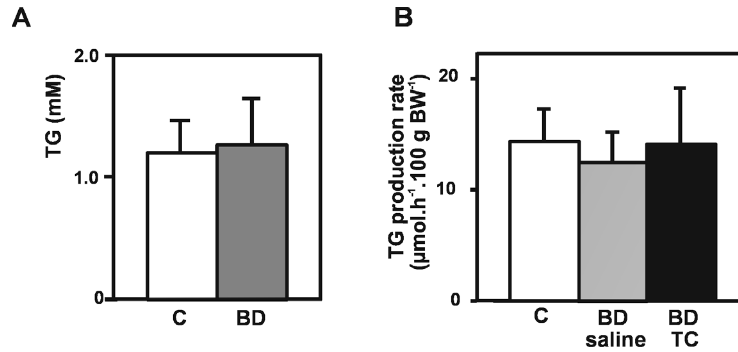

Figure 1. Plasma TG concentration and TG production rate after manipulation of the BS secretion rate in rats. A. Plasma TG concentrations in control (intact) rats (□) (n=4) and bile-diverted (BD) rats (■) (n=9) after 7 days of chronic bile diversion. **B**. 2 hours after the start of infusion (PBS or TC) Triton WR-1339 was administered intravenously to all rats and blood samples were taken at fixed time points. For each animal, TG production rates was calculated from plasma TG accumulation vs. time curve and corrected for bodyweight. Values represent mean ± S.D.. Per group n=4 (control, saline) or n=5 (TC), (control: □; saline: ■; TC: ■).

Effects of low BS flux on lipoprotein lipids

Table 2 shows the lipid composition in the VLDL fraction (d < 1.019 g/ml) of plasma from control rats and BD rats infused with TC or saline obtained 4 h after Triton WR-1339 injection. The lipoprotein fraction with density d < 1.019 g/ml was isolated by ultracentrifugation as described in materials and method section. The data indicate that neither the TG concentration nor the total cholesterol concentration in the VLDL/LDL fraction was affected by BD or by TC infusion after BD.

Table 2. Plasma lipid parameters in control rats and in BD rats (saline- or TC-infused) 4 h after Triton WR-1339 administration.

		Control (n=4)	BD Saline-infused (n=4)	BD TC-infused (n=5)
Total in plasma:	**TG (mM):**	7.7 ± 1.8	7.1 ± 0.4	8.8 ± 1.9
	Cholesterol (mM):	4.2 ± 0.7	3.0 ± 0.4*	3.1 ± 0.4*
Plasma VLDL/IDL fraction:	**TG (mM):**	6.9 ± 1.9 (71%)	5.6 ± 1.4 (68%)	7.5 ± 4.0 (72%)
(d < 1.019 g/ml)	**Cholesterol (mM):**	1.4 ± 0.1 (14%)	1.2 ± 0.3 (15%)	1.5 ± 0.4 (14%)
	PL (mM):	1.4 ± 0.2 (14%)	1.4 ± 0.3 (17%)	1.4 ± 0.8 (13%)

Lipids in plasma and lipoprotein fraction (d < 1.019 g/ml) were determined in control rats or bile-diverted rats (saline- or TC-infused for 6 h) 4h after Triton WR-1339 injection. Lipoprotein fraction with density d < 1.019 g/ml was isolated from plasma by density gradient ultracentrifugation as described in material and method section. Data represent mean values ± SD. * P < 0.05, significantly different from control.

In vitro suppression of VLDL-TG secretion and apoB secretion in mouse hepatocytes

The inhibitory action of BS on TG secretion *in vitro* has been reported in human [3] and rat hepatocytes [4], but not in mouse hepatocytes. To exclude species specificity we confirmed that VLDL-TG secretion by primary mouse hepatocytes is also inhibited by incubation with bile salts (**Figure 2**). Mouse hepatocytes were labeled with [^{3}H]-glycerol for 24 h in medium with or without taurocholate as described in the method section. In four independent experiments (each experimental condition at least in triple) we observed a marked reduction in the secretion of VLDL-^{3}H-TG (**Panel A**; - 72% ± 9 compared to controls, $p < 0.01$) and of apoB (**Panel B**; apoB100: -61% ± 13 compared to controls, apoB48: -36% ± 9 compared to controls, $p < 0.05$), when mouse hepatocytes were incubated with 200 µM TC during 24 h. These data confirm that BS suppress VLDL-TG and apoB secretion *in vitro* in mouse hepatocytes to the same extent as in human and rat hepatocytes.

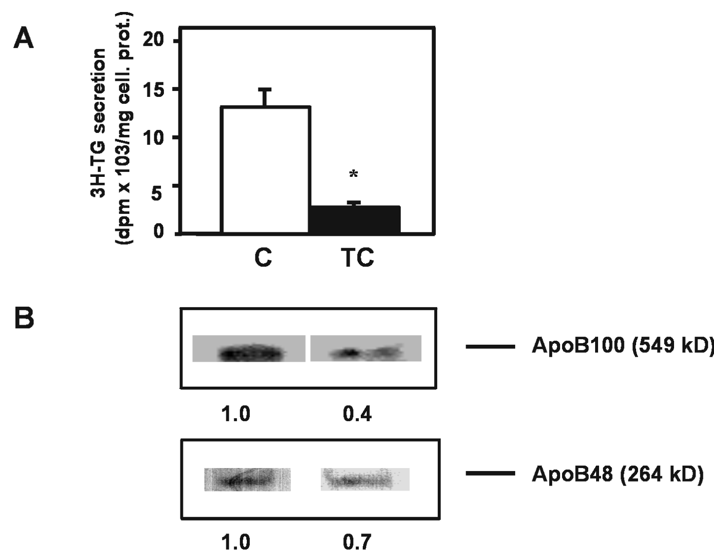

Figure 2. VLDL-TG and apoB secretion in cultured mouse hepatocytes after 24 h incubation with or without 200 µM TC. A. ^{3}H-VLDL-TG secretion by primary mouse hepatocytes in the presence or absence of 200 µM TC after [^{3}H]-glycerol (25 µM) labeling for 24 h. ^{3}H-VLDL-TG secretion is presented as mean dpm per mg cellular protein ± SD from 4 independent experiments. (control:□; TC: ■).* p<0.05, significantly different from control.

B. Secreted apoB was extracted from combined medium and separated by SDS PAGE. Secreted apoB100 and apoB48 from TC-containing medium were compared with secreted apoB100 and apoB48 from control medium.

Effects of low and high BS flux on plasma TG concentration and TG production rate in mice

Figure 3A shows biliary BS secretion rates in mice after feeding the control diet or the same diet supplemented with either TC or cholestyramine. The BS secretion rate was 335% higher in TC-supplemented mice and 48% lower in cholestyramine-supplemented mice, compared to controls.

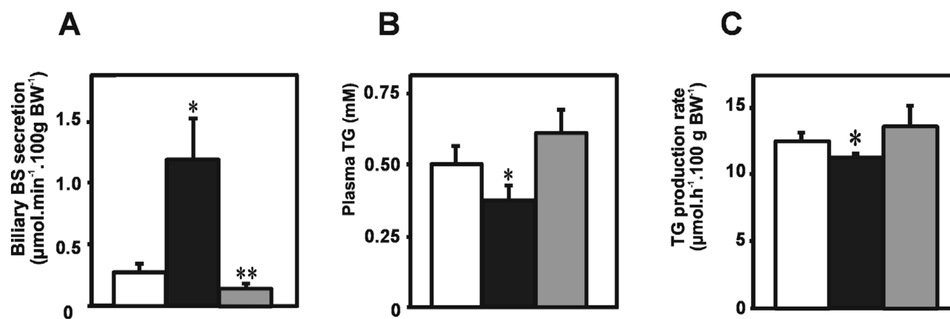

Figure 3. Plasma lipid parameters in control mice and mice supplemented with TC or cholestyramine for 3 weeks. Control diet: □; TC diet: ■; cholestyramine diet: ■. **A.** After a 3-wk control diet or TC-diet (0.5%) or cholestyramine-diet (2%) (n=6 mice per group) biliary bile salt secretion rates were determined in µmol per minute per 100 g bodyweight. **B.** Basal plasma TG concentrations in control mice (n=5), TC-fed mice (n=6) and cholestyramine-fed mice (n=6). **C.** Mice in each diet group (n=6 per group) were intravenously injected with Triton WR-1339 and the hepatic TG production rate was calculated from TG accumulation vs. time curve for each animal and corrected for bodyweight.* $p<0.05$, significantly different from control group; ** Cholestyramine-fed group significantly different from control group. Values are represented as mean ± S.D..

Both plasma TG concentration (**Figure 3, panel B**) and the rate of increase in plasma TG, reflecting VLDL-TG production rate after Triton injection (**Figure 3, panel C**), were decreased in TC-fed mice compared to controls (-26% and -10% respectively). Cholestyramine feeding tended to evoke opposite effects, but differences were not statistically significant.

In the previous *in vitro* experiments, an inverse relationship between BS concentration and hepatocyte VLDL-TG secretion had been found [3,4]. We investigated in the *in vivo* condition in mice whether an analogous dose-response relationship exists between transhepatic BS flux (biliary BS secretion rate) and either plasma TG concentration or plasma TG production after Triton injection. The mean biliary BS secretion rate appeared inversely related to either the mean plasma TG concentration or the mean TG production rates (**Figure 4**). Ideally, data on both bile secretion and plasma TG concentration or hepatic TG production are obtained from individual mice. However, reliable information on each of these parameters in individual animals cannot be obtained, since Triton WR-1339 treatment affects bile secretion [46]. It is therefore only possible to

relate the mean biliary BS secretion rates obtained from one set of animals with data on plasma TG concentration and plasma TG production obtained from another set of mice. Naturally both groups of animals had otherwise undergone the same dietary and experimental treatment.

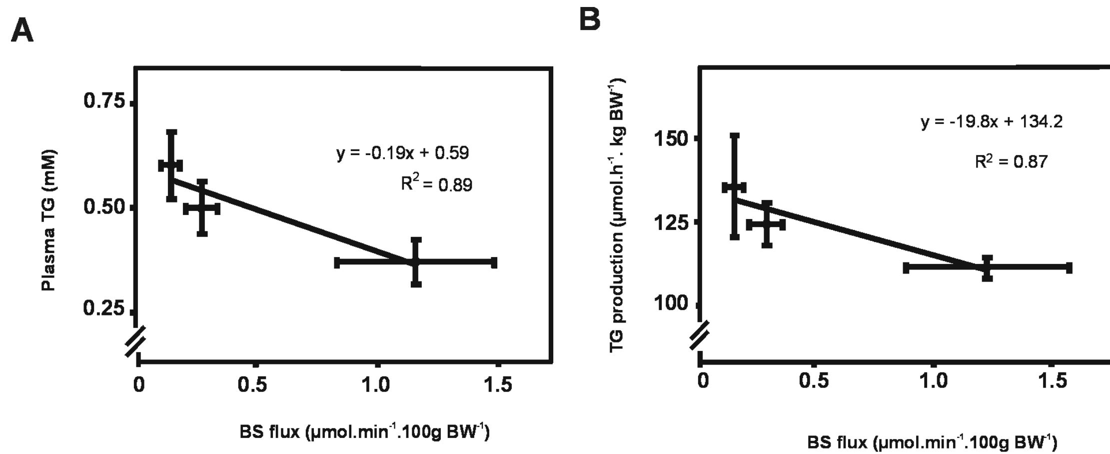

Figure 4. Relationship between BS secretion rate and plasma TG concentration or TG production rate in mice. In separate mice within one diet group biliary BS secretion rate and plasma TG concentration and TG production rate were determined. The mean biliary BS secretion rate per diet group was plotted against the mean plasma TG concentration per diet group (**panel A**) or plotted against the mean TG production rate per diet group (**panel B**) n=6 per group. **Panel A** and **panel B** show a direct inverse relationship between mean biliary BS secretion rate and either mean plasma TG concentration or mean TG production rate.

Lipoprotein lipid distribution and VLDL composition

Pooled plasma samples (n=6 per diet group) were fractionated by FPLC (**Figure 5**). The decrease in plasma TG concentration in the TC-supplemented group could mainly be attributed to a decrease in VLDL-TG (fraction 13-17) and IDL/LDL-TG (**Figure 5**, **panel A**).

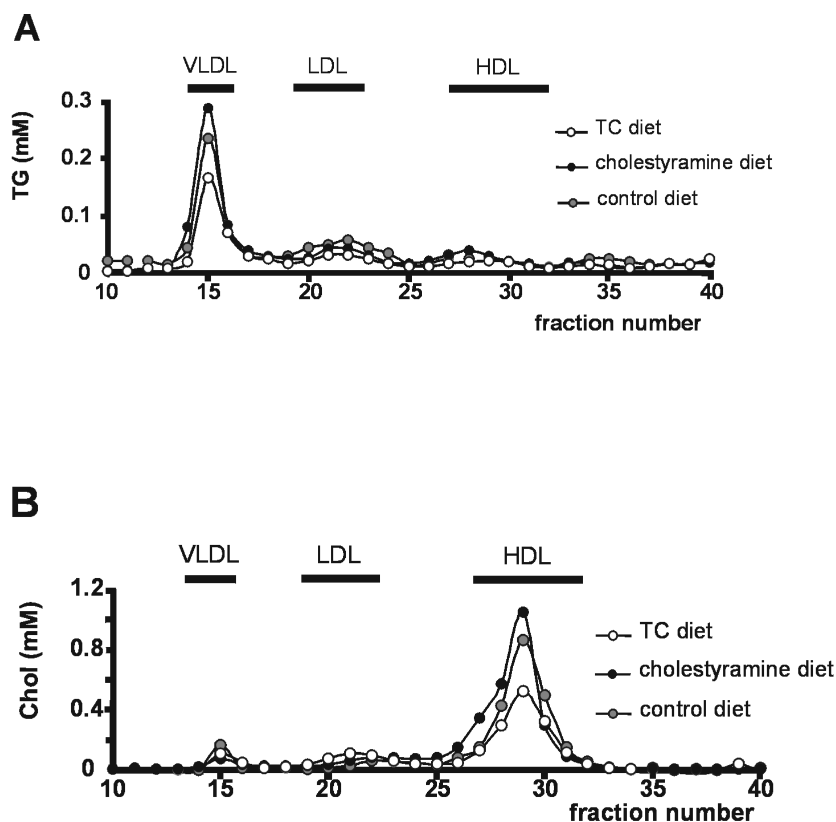

Figure 5. TG and cholesterol profiles after FPLC size chromatography of pooled plasma from control mice, TC-fed mice and cholestyramine-fed mice. For lipoprotein size fractionation 0.2 ml of pooled plasma obtained after 3 weeks on the diet) from 6 individual mice per group (control diet, TC-diet or cholestyramine-diet), was separated by FPLC. Plasma triglyceride (panel A) and plasma cholesterol (panel B) profiles were determined in each fraction (0.5 ml) as described in materials and method section. Data represent values of lipid concentrations (mM) in pooled plasma from 6 individual mice per group.

Plasma of cholestyramine-fed mice shows a slight increase in VLDL-TG. VLDL-cholesterol concentrations in TC-fed mice were similar compared to those of controls, but HDL-cholesterol was decreased by ~50%. Cholestyramine feeding resulted in opposite results compared with the TC-supplementation: higher HDL-cholesterol and lower VLDL-cholesterol (**Figure 5**, **panel B**).

Analysis of the lipid contents of the isolated VLDL/LDL particles by density gradient ultracentrifugation (density d < 1.019 g/ml) revealed a decrease by 36% in TG content in VLDL/LDL of TC-fed animals (**Table 3**) and a profound increase in cholesteryl ester (CE) content. No significant changes were observed in phospholipid and free cholesterol content. Hepatic lipid contents in the three diet groups show the same pattern: the livers of TC-fed show decreased TG content and a profoundly increased CE content.

Table 3. Animal characteristics of control mice and mice on either TC or cholestyramine diet.

		Control (n=6)	TC-diet (n=5)	Cholestyramine diet (n=6)
Weight (g):	**Body weight:**	24 ± 2	22 ± 2	25 ± 2
	Liver weight:	1.1 ± 0.0	1.1 ± 0.1	1.1 ± 0.1
Total in plasma (mM):	**TG:**	0.50 ± 0.06	0.37 ± 0.05*	0.60 ± 0.08
	Cholesterol:	2.12 ± 0.29	1.96 ± 0.13	2.21 ± 0.26
Plasma lipoprotein fraction	**TG:**	0.45 ± 0.06 (67%)	0.29 ± 0.09* (41%)	0.56 ± 0.13 (70%)
(d < 1.019 g/ml), (mM):	**F-Chol:**	0.06 ± 0.01 (9%)	0.08 ± 0.02 (11%)	0.06 ± 0.01 (8%)
	CE:	0.03 ± 0.01 (4%)	0.18 ± 0.03* (27%)	0.04 ± 0.01 (5%)
	PL:	0.13 ± 0.08 (19%)	0.15 ± 0.02 (21%)	0.14 ± 0.02 (18%)
Liver (nmol/ mg prot.):	**TG:**	65 ± 16	51 ± 10	62 ± 11
	F-Chol:	58 ± 10	60 ± 7	56 ± 4
	CE:	N.D.	7.0 ± 1.2*	N.D.
	PL:	292 ± 33	189 ± 15*	282 ± 19

Lipid concentrations were determined in plasma lipoprotein fractions (d<1.019 g/ml) isolated by density gradient ultracentrifugation and in liver homogenates as described in 'Experimental procedures'.N.D.: Not detectable. * $P < 0.05$, significantly different from control.

Effects of low and high BS flux on expression of genes involved in lipoprotein metabolism and on MTP TG transfer activity

To study whether the decreased plasma TG concentration and the decreased hepatic TG production in TC-fed mice was due to decreased expression of apoB or MTP, we determined steady state mRNA levels in livers of control mice, TC-fed mice and cholestyramine-fed mice. No major changes were observed in steady state mRNA levels of *ApoB* or *Mttp* (**Figure 6**).

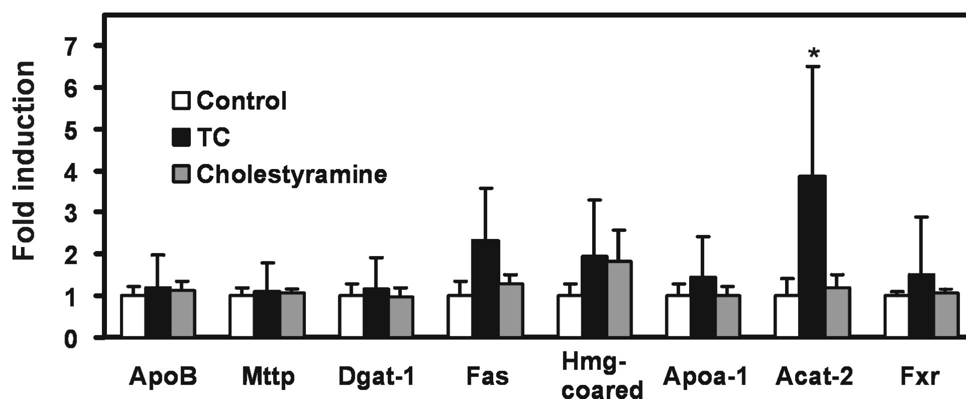

Figure 6. Effects of low and high BS flux on expression of genes involved in lipoprotein metabolism. Basal mRNA expression levels of hepatic genes involved in lipoprotein metabolism determined by Real-time PCR Expression levels of the above mentioned genes in control mice, TC- and cholestyramine-fed mice are represented as relative induction compared to ß-actin expression level (set to 1).

We also determined hepatic steady state mRNA expression levels of other genes (possibly) involved in lipoprotein metabolism: fatty acid synthase (*Fas*), diacylglycerol acyltransferase 1 (*Dgat1*), apolipoprotein A-1 (*ApoAI)*, HMG-CoA reductase (*Hmgcoar*), acyl-coenzyme A: cholesterol acyltransferase-2 (*Acat-2*) and farnesoid x activated receptor (*Fxr*) (**Figure 6**). A 3-fold induction in Acat-2 expression was observed in livers of TC-supplemented mice. No major changes were detected in mRNA levels of *Fas*, *Dgat-1*, *apoA1*, *Hmgcoar* and *Fxr*. In the livers of TC-fed mice we observed a severe downregulation of *Cyp7*α expression at mRNA level (25 fold) and a moderate decrease in *Cyp27* (sterol 27 hydroxylase) mRNA (1.3 fold). Hepatic mRNA expression of *Cyp7*α and *Cyp27* in cholestyramine-fed mice was moderately increased (1.9 fold and 1.5 fold, respectively) (data not shown).

To examine whether BS directly interfere with MTP transfer activity, the MTP triglyceride transfer activity was measured in the presence of different amounts of TC. MTP protein was isolated from livers from wild type C57BL/6J mice who had been given normal chow diet. Increasing amounts of BS did not alter the MTP transfer activity (represented as transferred ^{3}H-TG per hour per µg MTP protein). Only in the presence of an excessive concentration of 50 µM TC did the MTP transfer activity show a slight decrease (**Figure 7**).

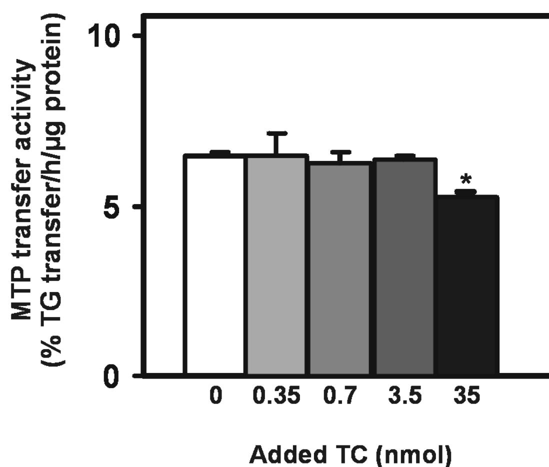

Figure 7. MTP triglyceride transport activity in the presence of different concentrations TC. Livers from control C57BL/6J mice (n=5) were removed and microsomes were isolated. MTP was further purified, concentrated and used in MTP activity assay as described in 'Experimental procedures'. TC was added to the reaction mixture (0.5, 1, 5 and 50 µM, end concentration) before the start of assay. The assay was performed as described.

DISCUSSION

Previously we demonstrated in human and rat hepatocytes *in vitro* that BS inhibit VLDL-TG secretion in a dose-dependent fashion [3,4]. In the present study we investigated whether the magnitude of the transhepatic BS flux affects the VLDL secretion *in vivo*. Our results indicate that a high transhepatic BS flux is associated with relatively minor decreases in plasma VLDL-TG concentration (-26%) and hepatic VLDL-TG production (-11%) in mice. Cholestyramine treatment inhibited the transhepatic BS flux, but did not significantly alter VLDL-TG production and VLDL composition in mice. In rats, no significant effects on hepatic TG production rate were observed upon chronic bile diversion, which decreases biliary bile salt secretion rate by 92%. Based on these data we conclude that the transhepatic bile salt flux does not exert a major regulatory role on hepatic VLDL-TG secretion in rats and mice under physiological conditions.

For manipulation of the transhepatic BS flux in rats we used a well-characterised model [18] with permanent catheters in heart, duodenum and bile duct. During chronic bile diversion, only BS originating from *de novo* synthesis are secreted into the bile and hepatic BS synthesis and cholesterol synthesis are markedly upregulated [29]. VLDL-TG production rate, plasma TG concentration and VLDL lipid composition were similar in rats with a physiological transhepatic BS flux (control rats with intact enterohepatic circulation), with completely absent transhepatic BS flux (chronically bile-diverted rats) or with a partially restored transhepatic BS flux (53% of normal, bile-diverted rats intraduodenally infused with taurocholate). Komai *et al.* [30] also reported unchanged TG production rates in 48-h-bile-diverted rats, consistent with our present observations in 7-

day-bile-diverted rats. Other authors reported that feeding rats cholestyramine increased plasma TG concentrations by 10% [31], which is inconsistent to our data in chronically bile-diverted rats. From other studies [32,33], including from ourselves [34], cholestyramine has been demonstrated to reduce biliary BS secretion and bile flow. No data, however, have been provided on plasma VLDL production rate or plasma VLDL lipid composition during cholestyramine treatment. The present data indicate that a reduced transhepatic BS flux does not necessarily affect VLDL-TG secretion in rats *in vivo*.

BS had previously been demonstrated to inhibit VLDL secretion by rat hepatocytes *in vitro* [4]. The discrepancy between the *in vivo* results and the previously obtained *in vitro* data could be related to several factors. In contrast to the *in vitro* conditions, the *in vivo* model involves polarized hepatocytes with an efficient hepatobiliary BS transport. Rat hepatocytes *in vitro* that are incubated with BS concentrations similar to postprandial concentrations in portal blood may reach higher intracellular BS concentrations than those reached during their flux through the hepatocyte *in vivo*. It may be that the intracellular concentrations reached in hepatocytes are of more importance for inhibition of VLDL secretion than the actual flux of bile salts through the hepatocyte. *In vivo*, postprandial, hormonal and possibly neuronal factors influence hepatocyte function, in contrast to the conditions *in vitro*.

The BS flux in mice *in vivo* was manipulated by feeding the mice control diet or the same diet containing either TC, or cholestyramine for three weeks. The TC-containing diet resulted in a 4-fold increase in BS flux, which was associated with a 26% decreased plasma TG concentration. On the other hand, cholestyramine treatment decreased the biliary BS secretion rate by 50%, which was associated with slightly elevated plasma TG concentrations. This TG elevation was, however, not statistically significant, which is consistent with the results obtained in bile-diverted rats (this study) and with Repa *et al.* [35]. When the data from the three different experimental set-ups were combined, an inverse relationship between BS flux rates and either plasma TG concentration or TG production rate was found in mice (**Figure 4**).

These data support the hypothesis that the transhepatic BS flux can affect VLDL secretion. However, an important regulatory role of BS on hepatic VLDL production is unlikely under physiological conditions.

Although rat and mouse hepatocytes react similarly to BS *in vitro*, *in vivo* rats and mice show different reactions upon manipulation of the BS flux. The presence of the gallbladder in mice could account for this difference. Specific characteristics of the C57BL/6J mouse strain, which are vulnerable to diet-induced changes in hepatic and plasma lipid metabolism, might also contribute.

In mouse hepatocytes *in vitro* VLDL triglyceride and apoB secretion were inhibited to a similar extent (**Figure 2**), suggesting that BS suppress the number of VLDL particles secreted. Similar results had been obtained in rat and human hepatocytes *in vitro* [3,4]. The potential mechanism(s) underlying the inhibition of VLDL secretion, however, is (are) not known. It is until now unclear whether the inhibition is primarily mediated by inhibition of the lipid availability for lipoprotein assembly, or, alternatively, by interfering with the routing of apoB through the secretory pathway. No changes in *ApoB* mRNA expression were observed in the three diet groups (**Figure 6**), as expected [36]. BS could stimulate post-translational apoB degradation, preventing the assembly of lipoprotein particles. However, intracellular (hepatic) concentrations of triglycerides were not accumulated in mouse hepatocytes or in livers of taurocholate-treated mice. An alternative explanation could involve bile salt-induced inhibition of the activity of the microsomal triglyceride transfer protein (MTP). MTP facilitates the initial lipidation of apoB and is involved in the transfer of lipids to the primordial VLDL particle [37]. *Mttp* mRNA expression was not affected by either an increased or decreased BS flux in mice (**Figure 6**), nor was the TG transfer activity of the semi-purified MTP protein affected in the presence of increasing amounts of TC (**Figure 7**). These data suggest that BS suppress VLDL-TG secretion via a mechanism not involving MTP. Dietary TC supplementation caused a profound change in VLDL composition with a 6-fold increase in CE concentration (**Table 3**). The amount of intracellular CE has been suggested to play a role in the VLDL secretion rate [38,39]. In parallel to the increased CE contents, the TG concentrations in the VLDL core were decreased. It cannot be excluded that the CE merely replaced TG as VLDL core component, in the light of the reduced TG content in liver of TC-fed mice (**Table 3**). The enzyme ACAT-2 is suggested to be responsible for CE secretion into apoB-containing lipoproteins [40,41]. Real-time PCR analysis showed a 3-fold induction in mRNA expression of *ACAT-2* in livers of TC-supplemented mice (**Figure 6**). Thus, the presence of increased levels of CE in liver and VLDL of TC-fed mice coincides with increased ACAT-2 expression. However, from these data we cannot conclude whether the amount of

secreted VLDL particles is decreased or merely that TG is replaced by CE in the VLDL core. Administration of a radio-labeled amino acid prior to Triton WR-1339 injection could provide more information on apoB secretion *in vivo* and therefore, more information about the amount of secreted apoB-containing lipoproteins.

It is known that BS stimulate biliary cholesterol and PL secretion in a dose-dependent fashion [2]. In TC-fed mice, the hepatic PL content was significantly decreased (**Table 3**), while biliary PL secretion was 2-fold increased (data not shown). PL drainage from the liver via increased secretion into bile might suppress VLDL assembly in the liver and therefore inhibit VLDL secretion. Yao and Vance [42] showed that selective inhibition of phosphatidylcholine (PC) biosynthesis in rats by feeding a choline-deficient diet, led to a 60% reduction of VLDL in plasma and an increased TG content in liver. Yet, in the present study, we observed a tendency towards decreased TG content in liver of TC-fed mice, which suggests another mechanism than PC-deficiency underlying the suppression of VLDL secretion.

Depletion of BS by bile diversion or cholestyramine administration is reported to upregulate cholesterol-7α-hydroxylase, the key enzyme in BS synthesis [29,43]. Kang *et al*. [44] proposed that an upregulation of cholesterol-7α-hydroxylase activity causes an increased VLDL TG secretion via upregulation of SREBP's. Our present data do not support this hypothesis. In the chronic bile diversion rat model, cholesterol-7α-hydroxylase expression and activity are upregulated several fold [29]. In addition, HMG-CoA reductase activity is strongly induced, in accord with SREBP activation [15,16]. However, in our hands, VLDL-TG production rate in rats was not significantly affected.

The knock-out mouse models with disrupted farnesoid X receptor gene (*FXR*(-/-)) [45] or with disruption of the sterol 27-hydroxylase gene (*Cyp27*(-/-)) [35] are both reported to have severely decreased BS pool sizes (-50% and -75%, respectively). Interestingly, plasma TG levels are increased in both models (2.4-fold in *FXR*(-/-), 2-fold in *Cyp27*(-/-), respectively). However, the biliary BS secretion rates and the VLDL-TG production rates have not been reported in either model. It is therefore not possible to address the possible contribution of the transhepatic BS flux on the hepatic VLDL-TG secretion in these models.

In summary, our data indicate that under physiological conditions the transhepatic BS flux does not exert major control on the hepatic VLDL-TG production rates in rats *in vivo*. In mice, an inverse relationship between the transhepatic BS flux rates and either

plasma TG concentration or VLDL-TG production rate was observed, but only at supraphysiological BS rates.

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