AUTOIMMUNE, CHOLESTATIC AND BILIARY DISEASE

Stimulation of Murine Biliary Cholesterol Secretion by Thyroid Hormone is Dependent on a Functional ABCG5/G8 Complex

Ylva Bonde,[1,2] Torsten Plösch,[3] Folkert Kuipers,[3] Bo Angelin,[1,2] and Mats Rudling[1,2]

Secretion of cholesterol into bile is important for the elimination of cholesterol from the body. Thyroid hormone (TH) increases biliary cholesterol secretion and hepatic gene expression of adenosine triphosphate (ATP)-binding cassette, subfamily G (WHITE), member 5 (ABCG5) and ATP-binding cassette, subfamily G (WHITE), member 8 (ABCG8), two half-transporters that act as a heterodimeric complex promoting sterol secretion. In addition, nuclear liver x receptor-alpha (LXRa), also regulated by TH, induces gene expression of ABCG5/G8. We here investigated if the TH-induced stimulation of biliary cholesterol secretion is mediated by the ABCG5/G8 complex *in vivo*, and if so, whether LXRa is involved. Mice homozygous for disruption of *Abcg5* (*Abcg5*$^{-/-}$) or *Lxra* (*Lxra*$^{-/-}$) and their wild-type counterparts were treated with triiodothyronine (T3) for 14 days and compared to untreated mice of corresponding genetic backgrounds. Bile was collected by gallbladder cannulation, and liver samples were analyzed for gene expression levels. Basal biliary cholesterol secretion in *Abcg5*$^{-/-}$ mice was 72% lower than in *Abcg5*$^{+/+}$ mice. T3 treatment increased cholesterol secretion 3.1-fold in *Abcg5*$^{+/+}$ mice, whereas this response was severely blunted in *Abcg5*$^{-/-}$ mice. In contrast, biliary cholesterol secretion in T3-treated *Lxra*$^{+/+}$ and *Lxra*$^{-/-}$ mice was increased 3.5- and 2.6-fold, respectively, and did not differ significantly. *Conclusions*: TH-induced secretion of cholesterol into bile is largely dependent on an intact ABCG5/G8 transporter complex, whereas LXRa is not critical for this effect. (HEPATOLOGY 2012;56:1828-1837)

High plasma total and low-density lipoprotein (LDL) cholesterol levels are linked to an enhanced risk of developing premature atherosclerosis. Thyroid hormone (TH) is an important regulator of cholesterol metabolism and hyperthyroidism is commonly associated with decreased—and hypothyroidism with increased—plasma cholesterol concentrations.[1-3] TH is known to exert a number of beneficial effects on cholesterol and lipoprotein metabolism,[4] and promising results have recently been reported from the clinical development of liver-selective TH analogs, such as eprotirome.[5,6] One of the mechanisms by which TH may lower plasma cholesterol is by an increased secretion of biliary cholesterol,[7] a main route for elimination of cholesterol from the body.[8]

An important and presumably rate-limiting step in the process of biliary secretion of cholesterol is mediated by the half-transporters ATP-binding cassette, subfamily G (WHITE), member 5 (ABCG5) and

ATP-binding cassette, subfamily G (WHITE), member 8 (ABCG8). By heterodimerization with each other, these structures form a functional complex that promotes the transport of cholesterol and plant sterols from liver cells into bile[9-11] at the apical plasma membrane of hepatocytes.[10,11] Disruption of either one[12,13] or both[11,14-16] genes reduces biliary cholesterol concentration and secretion in mice. In contrast, induction of hepatic ABCG5/G8 gene expression is associated with increased biliary cholesterol concentration and secretion.[15-17] In a previous study,[18] biliary cholesterol secretion was strongly reduced in hypophysectomized rats as compared with intact animals, a finding associated with markedly reduced hepatic ABCG5/G8 gene expression. The administration of TH increased biliary cholesterol secretion and, concomitantly, hepatic ABCG5/G8 gene expression levels were increased. This suggests that TH-induced stimulation of biliary cholesterol secretion may be mediated by ABCG5/G8.

Hepatic gene expression of ABCG5/G8 is not always concurrent with biliary cholesterol secretion,[16,19-21] however, and there are indications that other pathways, independent of ABCG5/G8, promote cholesterol transfer into bile.[11,22,23] Furthermore, it is unclear whether the stimulation of biliary cholesterol secretion is a direct effect of TH. It may well be mediated by nuclear liver x receptor-alpha (LXRa), the expression of which has recently been reported to be positively regulated by TH receptor-beta (TRb) in the mouse.[24] LXRa regulates the transcription of several genes involved in cholesterol metabolism[25] and the administration of the LXR agonist T0901317 to mice increases hepatic ABCG5/G8 gene expression and biliary cholesterol concentration and secretion.[15,17,26] Thus, experimental evidence indicates that LXRa may mediate effects of TH on cholesterol metabolism.[27]

Here, we investigated whether the induction of biliary cholesterol secretion by TH is dependent on the ABCG5/G8 complex or if other mechanisms are involved. Furthermore, the question of if LXRa is important for the effect of TH on biliary cholesterol secretion was explored.

We present three novel findings: (1) Biliary cholesterol secretion induced by TH is predominantly excerted by ABCG5/G8; (2) this TH-induced biliary cholesterol secretion is independent of LXRa; and (3) a minor part of the TH-induced stimulation of biliary cholesterol secretion occurs independently of the ABCG5/G8 complex.

Materials and Methods

Animals and Treatments. Male mice (3-5 months of age) were used in the experiments. In the first experiment, mice homozygous for the disruption of the ABCG5 gene ($Abcg5^{-/-}$) and their wild-type (WT) counterparts ($Abcg5^{+/+}$) were divided into the following groups: $Abcg5^{+/+}$ (n = 6); $Abcg5^{+/+}$ T3 (n = 5); $Abcg5^{-/-}$ (n = 5); and $Abcg5^{-/-}$ T3 (n = 6). In the second experiment, mice homozygous for the disruption of the LXRa gene ($Lxra^{-/-}$) and their WT counterparts ($Lxra^{+/+}$) were divided into the following groups: $Lxra^{+/+}$; $Lxra^{+/+}$ T3; $Lxra^{-/-}$; and $Lxra^{-/-}$ T3 (all groups: n = 7). For detailed descriptions of how knockout mice were generated, see previous reports.[13,21]

Animals were housed in a temperature-controlled environment, with lights on from 6 a.m. to 6 p.m. They had free access to drinking water and mouse chow. Groups treated with T3 ($Abcg5^{+/+}$ T3, $Abcg5^{-/-}$ T3, $Lxra^{+/+}$ T3, and $Lxra^{-/-}$ T3) received drinking water supplemented with 0.5 μg of T3/mL (3,3′,5-triiodo-L-thyronine; Sigma-Aldrich, St Louis, MO) and 0.01% albumin (bovine serum albumin; Sigma-Aldrich). After 14 days of treatment, mice were anesthetized by an intraperitoneal injection of Hypnorm (fentanyl/fluanisone, 1 mL/kg) and diazepam (10 mg/kg). Bile was collected for 30 minutes from cannulated gallbladders, as previously described,[21] and blood was collected by heart puncture at the end of the bile-collection period. After animals had been killed by cervical dislocation, livers were removed and immediately frozen in liquid nitrogen and stored at −80°C. All experimental procedures were approved by the Local Ethical Committee for Animal Experiments of the University of Groningen.

RNA Isolation and Real-Time PCR Measurements. Total RNA was extracted from individual samples of liver and proximal small intestine using TRIzol Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. cDNA synthesis was

performed using Omniscript reverse transcriptase (Qiagen, Hilden, Germany). Quantitative real-time PCR was performed with SYBRGreen PCR MasterMix on a 7500 Fast Real-Time PCR System, and primers were designed using Primer Express Software 2.0 (Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and hypoxanthine guanine phosphoribosyl transferase (*Hprt*) were used as endogenous controls, and the comparative C_t method was used to quantify the results. The following primer sequences were used: *Gapdh* forward 5′-tgtgtccgtcgtggatctga-3′; *Gapdh* reverse 5′-cctgcttcaccaccttcttgat-3′; *Abcg5* forward 5′-aatgctgtg aatctgtttccca-3′; *Abcg5* reverse 5′-ccacttatgatacaggcca tcct-3′; *Abcg8* forward 5′-tccatcctcggagacacgat-3′; *Abcg8* reverse 5′-gctgatgccgatgacaatga-3′; *Lxra* forward 5′-gctct gctcattgccatcag-3′; *Lxra* reverse 5′-tgttgcagcctctctactt gga-3′; *Hprt* forward 5′-ggtgaaaaggacctctcgaagtg-3′; *Hprt* reverse 5′-atagtcaagggcatatccaacaaca-3′; *Cyp7a1* forward 5′-agcacctaaacaacctgccagtacta-3′; *Cyp7a1* reverse 5′-gtccggatattcaaggatgca-3′; *Hmgcr* forward 5′-tgattggagttggcaccat-3′; *Hmgcr* reverse 5′- tggccaacactga catgc-3′; *Ldlr* forward 5′-ggatggctatacctaccccctcaa-3′; and *Ldlr* reverse 5′-cacatcgtcctccaggctg-3′.

Assay of Biliary Cholesterol Concentration and Secretion. 25 μL of bile was used for this assay. After Folch extraction, dried samples were hydrolyzed with 1 mL of 0.5 M KOH at 70°C for 90 min. Samples were extracted by the addition of 1 mL of H_2O and 5 mL of hexane. After centrifugation at 3,000 rpm for 5 min, the upper phase was evaporated under nitrogen and silylated with pyridine/hexametyldisilazane/chlorotrimetylsilane (3:2:1, v/v/v) at 60°C for 30 min. After evaporation, the product was redissolved in hexane and analyzed using gas chromatography/mass spectrometry (GC/MS). D_7-cholesterol was used as internal standard. Biliary cholesterol secretion was calculated for each individual by multiplying the cholesterol concentration by the volume of bile secreted per minute and per 100 g of body weight.

Assay of Biliary Phospholipid Concentration and Secretion. Phospholipids were extracted from individual bile samples, as previously described.[28] The concentration was subsequently determined as in Böttcher et al.[29] Secretion of phospholipids was calculated for each individual by multiplying the concentration of phospholipids by the volume of bile secreted per minute and per 100 g of body weight.

Assay of Biliary Bile Acid Concentration and Secretion. 2 μL of bile were hydrolyzed with 0.5 mL of 5 M NaOH in 90% EtOH at 67°C for 90 min. Then, 0.5 mL of H_2O and 3 mL of cyklohexane were added and samples were centrifuged at 2,000 rpm for 10 min before upper phase was removed. This was repeated once before acidification of samples with 200 μL of 6 M HCl. Ether was added to extract bile acids (BAs) and H_2O was added to collected ether extracts, which were centrifuged at 2,000 rpm for 10 min before the upper phase was collected and evaporated under nitrogen at 60°C. Methylation was carried out at room temperature for 10 min by adding 400 μL of toluene, 100 μL of MeOH, and 25 μL of trimethylsilyldiazomethane, and samples were then dried under nitrogen at 60°C. Samples were silylated with pyridine/hexametyldisilazane/chlorotrimetylsilane (3:2:1, v/v/v) at 60°C for 30 min and thereafter dried under nitrogen, redissolved in hexane, and analyzed using GC/MS. D_4-labeled BAs were used as internal standards. BA secretion was calculated for each individual by multiplying the sum of concentrations of specific BAs by the volume of bile secreted per minute and per 100 g of body weight.

Statistical Analyses. Data show means ± standard error of the mean (SEM). The significance of differences between groups was tested by 1-way ANOVA, followed by post-hoc comparisons according to Tukey's test, using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

Results

Effects of T3 on Hepatic Gene Expression in Abcg5$^{+/+}$ and Abcg5$^{-/-}$ Mice. Hepatic ABCG5 and ABCG8 gene expressions were both increased 1.5-fold in T3-treated *Abcg5*$^{+/+}$ mice (Fig. 1). ABCG8 gene expression was unaltered in *Abcg5*$^{-/-}$ control and in T3-treated *Abcg5*$^{-/-}$ mice. Hepatic LXRa gene expression was unaltered in *Abcg5*$^{-/-}$ mice, while reduced in T3-treated *Abcg5*$^{+/+}$ and *Abcg5*$^{-/-}$ mice (by 23% and 10%, respectively), as compared to respective controls. Compared to untreated *Abcg5*$^{+/+}$ mice, CYP7A1, hydroxymethylglutaryl coenzyme A reductase (HMG CoA red), and LDLr gene expressions were increased 4.6-, 3.7-, and 1.6-fold, respectively, in T3-treated *Abcg5*$^{+/+}$ mice, whereas they were unaltered in untreated *Abcg5*$^{-/-}$ mice. In T3-treated *Abcg5*$^{-/-}$ mice, gene expressions of HMG CoA red and LDLr were unaltered, whereas CYP7A1 gene expression was 2.9-fold increased.

Biliary Cholesterol, Phospholipids, BAs, and Bile Flow in T3-Treated Abcg5$^{+/+}$ and Abcg5$^{-/-}$ Mice. In *Abcg5*$^{-/-}$ mice, biliary cholesterol and phospholipid concentrations were reduced by 75% and 46%, respectively (Table 1). In T3-treated *Abcg5*$^{+/+}$
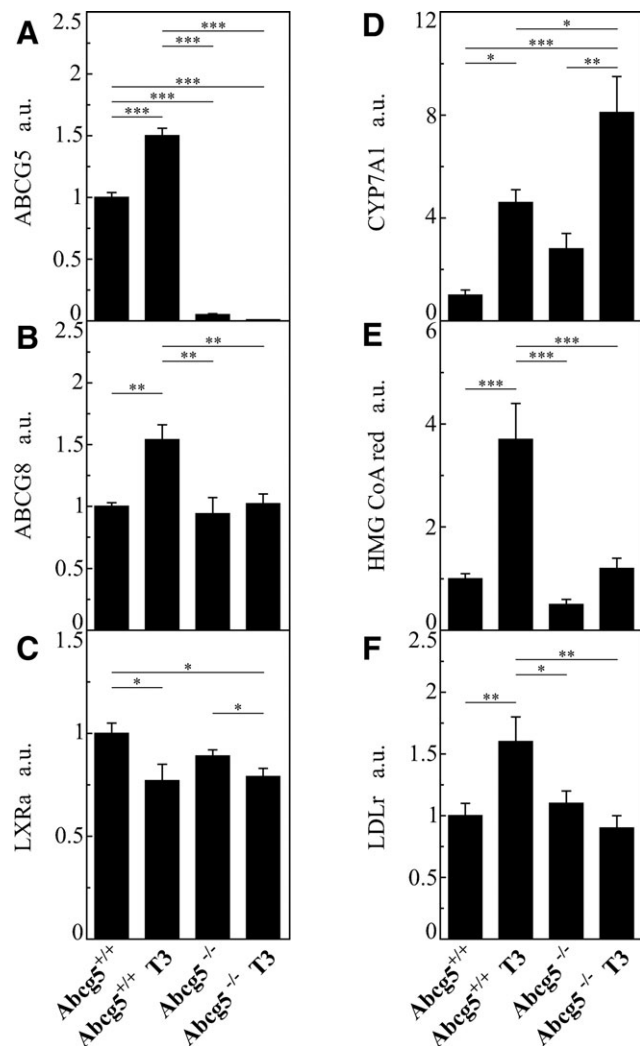
Fig. 1. Effects of T3 treatment on hepatic gene expression of ABCG5 (A), ABCG8 (B), LXRa (C), CYP7A1 (D), HMG CoA red (E), and LDLr (F) in $Abcg5^{-/-}$ mice and in their WT counterparts ($Abcg5^{+/+}$). Number of animals (n) per group: $Abcg5^{+/+}$ n = 6; $Abcg5^{+/+}$ T3 n = 5; $Abcg5^{-/-}$ n = 5; and $Abcg5^{-/-}$ T3 n = 6. Data are presented as mean ± SEM. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

mice, biliary cholesterol and phospholipids were increased 1.8- and 1.3-fold, respectively. Compared to untreated $Abcg5^{-/-}$ mice, cholesterol and phospholipids were unaltered in T3-treated $Abcg5^{-/-}$ mice.

The concentration of total BAs was unaltered in $Abcg5^{-/-}$ mice. T3 treatment of $Abcg5^{+/+}$ and $Abcg5^{-/-}$ mice did not significantly change biliary BA concentration compared to respective controls. Both the C/PL ratio and C/BA ratio were increased (1.4- and 1.9- fold, respectively) in T3-treated $Abcg5^{+/+}$ mice, whereas the PL/BA ratio was unaltered. In $Abcg5^{-/-}$ mice, the C/PL ratio was decreased by 40%, and the C/BA and PL/BA ratios were unaltered. T3 treatment of $Abcg5^{-/-}$ mice did not alter the ratios. Under basal conditions, bile flow was the same in $Abcg5^{+/+}$ and $Abcg5^{-/-}$ mice. T3 treatment increased bile flow to similar extents in $Abcg5^{+/+}$ (1.9-fold) and in $Abcg5^{-/-}$ (1.8-fold) mice.

Effects of T3 on Biliary Composition of BAs in Abcg5$^{+/+}$ and Abcg5$^{-/-}$ mice. T3 treatment decreased the biliary proportion of deoxycholic acid (DCA) in $Abcg5^{+/+}$ and $Abcg5^{-/-}$ mice by 56% and 55%, respectively (Table 2). The proportion of cholic acid (CA) tended to be reduced in T3-treated animals. Chenodeoxycholic acid (CDCA) was increased 1.8-fold by T3 treatment in $Abcg5^{-/-}$ mice, and there was a trend to an increased proportion of CDCA in the $Abcg5^{+/+}$ mice ($P = 0.05$). Alpha-muricholic acid (α-MCA) was increased by T3 treatment in both $Abcg5^{+/+}$ and $Abcg5^{-/-}$ mice 2.1- and 3.4-fold, respectively. Biliary proportions of β-muricholic acid (β-MCA), ursodeoxycholic acid (UDCA), and litocholic acid (LCA) were unaltered.

Importance of a Functional ABCG5/ABCG8 Complex for the Stimulation of Biliary Cholesterol Secretion by T3. Biliary cholesterol secretion was increased

Table 1. Effects of T3 Treatment on Body Weight, Biliary Lipids, and Bile Flow in $Abcg5^{-/-}$ Mice and in Their WT Counterparts ($Abcg5^{+/+}$)

No. of Animals	$Abcg5^{+/+}$ (n = 6)	$Abcg5^{++}$ T3 (n = 5)	$Abcg5^{-/-}$ (n = 5)	$Abcg5^{-/-}$ T3 (n = 6)
Body weight, g	28 ± 1	31 ± 1	28 ± 1	32 ± 1
Cholesterol, nmol/mL	180 ± 10	320 ± 10*	50 ± 5*·§	70 ± 10*·§
Phospholipids, nmol/mL	6,060 ± 320	7,890 ± 420‡	3,300 ± 180*·§	4,460 ± 400‡,§
BAs, nmol/mL	48,000 ± 9,770	44,800 ± 8,530	22,100 ± 3,290	18,700 ± 1,110‡
Ratio cholesterol/phospholipid	0.03 ± 0.003	0.04 ± 0.002†	0.01 ± 0.000*·§	0.02 ± 0.002*·§
Ratio cholesterol/BA	0.004 ± 0.001	0.008 ± 0.001†	0.002 ± 0.000§	0.004 ± 0.001$^{\parallel}$
Ratio phospholipid/BA	0.14 ± 0.02	0.20 ± 0.03	0.16 ± 0.03	0.24 ± 0.02‡
Bile flow, μL/min	2.0 ± 0.1	3.9 ± 0.3*	2.1 ± 0.2§	3.7 ± 0.3*·¶

Data are presented as mean ± SEM.

*$P < 0.001$, †$P < 0.01$, and ‡$P < 0.05$ versus $Abcg5^{+/+}$.

§$P < 0.001$ and $^{\parallel}P < 0.01$ versus $Abcg5^{+/+}$ T3.

¶$P < 0.001$ versus $Abcg5^{-/-}$.

Table 2. Effects of T3 Treatment on Biliary BA Composition in Abcg5$^{-/-}$ Mice and in Their WT Counterparts (Abcg5$^{+/+}$)

No. of Animals	Abcg5$^{+/+}$ (n = 6)	Abcg5^{++} T3 (n = 5)	Abcg5$^{-/-}$ (n = 5)	Abcg5$^{-/-}$ T3 (n = 6)
CA				
nmol/mL	23,500 ± 3,700	20,100 ± 3,330	11,500 ± 2,150‡	7,540 ± 840†,¶
% of total	51 ± 3	46 ± 2	51 ± 3	40 ± 3
CDCA				
nmol/mL	170 ± 10	260 ± 30‡	100 ± 10§	160 ± 20$^{|}$
% of total	0.4 ± 0.05	0.7 ± 0.09	0.5 ± 0.06	0.9 ± 0.08*,**
α-MCA				
nmol/mL	1,990 ± 410	4,220 ± 1,210	430 ± 70$^{|}$	1,190 ± 70¶
% of total	4.0 ± 0.4	9.0 ± 1.1*	2.0 ± 0.1§	6.0 ± 0.4‡,¶,$^{#}$
β-MCA				
nmol/mL	21,300 ± 5,720	19,700 ± 4,400	9,420 ± 1,200	9,490 ± 800
% of total	42 ± 3	44 ± 3	44 ± 3	51 ± 3
DCA				
nmol/mL	510 ± 80	200 ± 10†	470 ± 70¶	180 ± 30†,††
% of total	1.0 ± 0.1	0.5 ± 0.1‡	2.0 ± 0.1*,§	1.0 ± 0.2$^{#}$
UDCA				
nmol/mL	570 ± 100	350 ± 20	200 ± 30†	170 ± 20*
% of total	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
LCA				
nmol/mL	20 ± 0	20 ± 1	20 ± 0	20 ± 1
% of total	0.02 ± 0.02	0.02 ± 0.02	0.1 ± 0.00†,$^{|}$	0.1 ± 0.00*,$^{|}$

Data are presented as mean ± SEM.
*$P < 0.001$, †$P < 0.01$, and ‡$P < 0.05$ versus Abcg5$^{+/+}$.
§$P < 0.001$, $^{||}P < 0.01$, and ¶$P < 0.05$ versus Abcg5$^{+/+}$ T3.
$^{#}P < 0.001$, **$P < 0.01$, and $^{††}P < 0.05$ versus Abcg5$^{-/-}$.

3.1-fold in T3-treated Abcg5$^{+/+}$ mice (Fig. 2). Basal secretion of biliary cholesterol in Abcg5$^{-/-}$ mice was only 28% of that observed in untreated Abcg5$^{+/+}$ mice. In T3-treated Abcg5$^{-/-}$ mice, biliary cholesterol secretion was unaltered compared to Abcg5$^{-/-}$ mice, and did not differ from that of untreated Abcg5$^{+/+}$ mice. Biliary cholesterol secretion in T3-treated Abcg5$^{-/-}$ mice was 79% lower than in T3-treated Abcg5$^{+/+}$ mice. Biliary phospholipid secretion was unaltered in Abcg5$^{-/-}$ mice. T3 treatment increased phospholipid secretion 2.3-fold in Abcg5$^{+/+}$ mice and 2.1-fold in Abcg5$^{-/-}$ mice, compared to respective controls. Total BA secretion was unaltered in Abcg5$^{-/-}$ mice. T3 treatment of Abcg5$^{+/+}$ and Abcg5$^{-/-}$ mice tended to increase BA secretion, but the differences did not reach statistical significance.

Effects of T3 on Hepatic Gene Expression in Lxra$^{+/+}$ and Lxra$^{-/-}$ Mice. LXRa gene expression was unaltered in T3-treated Lxra$^{+/+}$ mice, whereas ABCG5 and ABCG8 gene expression levels were both increased 2.1- and 1.5-fold, respectively (Fig. 3). Gene expressions of ABCG5 and ABCG8 were unaltered in Lxra$^{-/-}$ mice, whereas they were increased in T3-treated Lxra$^{-/-}$ mice (1.8- and 1.7-fold, respectively), as compared to Lxra$^{-/-}$ mice. Gene expressions of ABCG5/G8 in proximal small intestine were unaltered (data not shown). Hepatic CYP7A1, HMG CoA red, and LDL receptor (LDLr) gene expressions were unaltered in Lxra$^{-/-}$ mice and in T3-treated Lxra$^{+/+}$

mice. In T3-treated Lxra$^{-/-}$ mice, HMG CoA red and LDLr gene expressions were unaltered, whereas CYP7A1 gene expression was increased 4.1-fold, compared with untreated Lxra$^{-/-}$ mice.

Effects of T3 Treatment on Biliary Cholesterol, Phospholipids, BAs, and Bile Flow in Lxra$^{+/+}$ and Lxra$^{-/-}$ Mice. Biliary cholesterol and phospholipids were unaltered in Lxra$^{-/-}$ mice, and T3 treatment of Lxra$^{+/+}$ or Lxra$^{-/-}$ mice did not alter biliary cholesterol or phospholipid concentrations, as compared to respective controls (Table 3). Further, the total concentration of biliary BAs did not differ between groups. C/PL and C/BA ratios were unaltered in T3-treated Lxra$^{+/+}$ mice, whereas the PL/BA ratio was 1.4-fold increased. None of the ratios were altered in the Lxra$^{-/-}$ mice. T3 treatment of Lxra$^{-/-}$ mice increased both the C/BA and PL/BA ratio (1.6- and 1.4-fold, respectively), whereas the C/PL ratio was unaltered, as compared to untreated Lxra$^{-/-}$ mice. Bile flow was unaltered in Lxra$^{-/-}$ mice. However, T3 treatment increased bile flow by 2.4-fold in Lxra$^{+/+}$ and 2.1-fold in Lxra$^{-/-}$ mice.

Effects of T3 on Biliary Composition of BAs in Lxra$^{+/+}$ and Lxra$^{-/-}$ Mice. CDCA was increased by T3 treatment in Lxra$^{+/+}$ and Lxra$^{-/-}$ mice (2.0- and 1.7-fold, respectively) (Table 4). α-MCA was increased by T3 treatment in Lxra$^{+/+}$ and Lxra$^{-/-}$ mice (2.2- and 1.8-fold, respectively). There was a tendency toward decreased biliary proportions of DCA and CA in
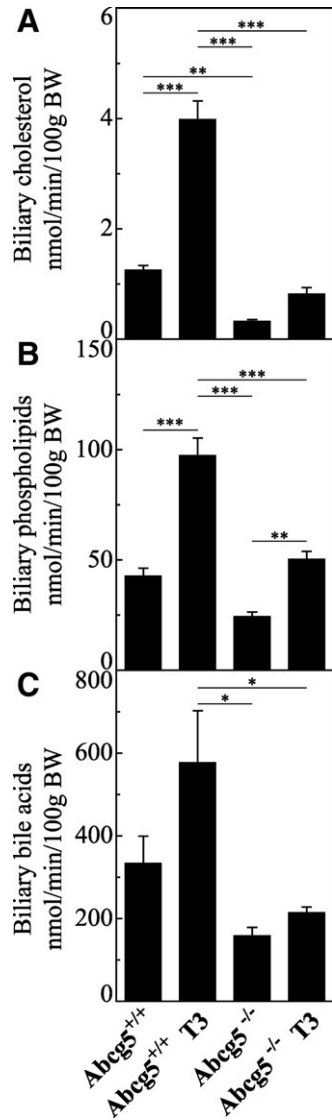
Fig. 2. Effects of T3 treatment on secretion of biliary cholesterol (A), phospholipids (B), and BAs (C) in $Abcg5^{-/-}$ mice and in their WT counterparts ($Abcg5^{+/+}$). Number of animals (n) per group: $Abcg5^{+/+}$ n = 6; $Abcg5^{+/+}$ T3 n = 5; $Abcg5^{-/-}$ n = 5; $Abcg5^{-/-}$ T3 n = 6. Data are presented as mean ± SEM. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

was unchanged in the groups, although there was a trend to an increased secretion in T3-treated mice.

Discussion

TH exerts a number of important regulatory effects on cholesterol, lipid, and lipoprotein metabolism.[4] These include stimulation of hepatic lipase activity, induction of hepatic LDL receptors, promotion of cholesterol breakdown to BAs, and cholesterol excretion into bile. Furthermore, there is evidence that TH may promote reverse cholesterol transport through stimulation of high-density lipoprotein (HDL) clearance.[4,30] Many of the positive actions of TH in lipid metabolism are constrained to the liver, and the recent demonstration of the possibility to achieve pronounced
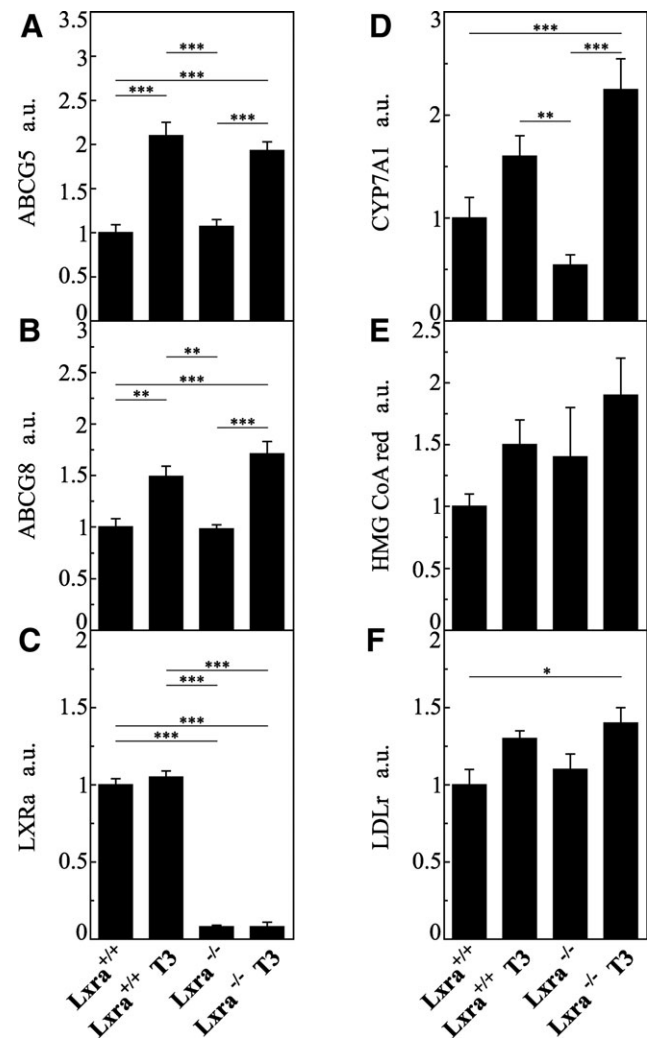
Fig. 3. Effects of T3 treatment on hepatic gene expression of ABCG5 (A), ABCG8 (B), LXRa (C), CYP7A1 (D), HMG CoA red (E), and LDLr (F) in in $Lxra^{-/-}$ mice and in their WT counterparts ($Lxra^{+/+}$). Number of animals per group: n = 7. Data are presented as mean ± SEM. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

T3-treated $Lxra^{+/+}$ and $Lxra^{-/-}$ mice, whereas the biliary proportions of β-MCA, UDCA, and LCA were unaltered.

Biliary Cholesterol Secretion Is Induced by T3 independent of **Lxra.** Biliary cholesterol secretion was similar in untreated $Lxra^{+/+}$ and $Lxra^{-/-}$ mice (Fig. 4). In response to T3 treatment, it increased 3.5-fold in $Lxra^{+/+}$ and 2.6-fold in $Lxra^{-/-}$ mice, to similar levels. Phospholipid secretion was unchanged in $Lxra^{-/-}$ mice. In T3-treated $Lxra^{+/+}$ mice and $Lxra^{-/-}$ mice, phospholipid secretion increased 2.3- and 2.2-fold, compared to respective controls. Secretion of total BAs

Table 3. Effects of T3 Treatment on Body Weight, Biliary Lipids, and Bile Flow in Lxra$^{-/-}$ Mice and in Their WT Counterparts (Lxra$^{+/+}$)

No. of Animals	Lxra$^{+/+}$ (n = 7)	Lxra$^{+/+}$ T3 (n = 7)	Lxra$^{-/-}$ (n = 7)	Lxra$^{-/-}$ T3 (n = 7)
Body weight, g	34 ± 1	40 ± 1†	36 ± 1	38 ± 2
Cholesterol, nmol/mL	190 ± 30	320 ± 60	340 ± 80	470 ± 50‡
Phospholipids, nmol/mL	5,870 ± 610	6,710 ± 930	6,670 ± 710	7,610 ± 320
BAs, nmol/mL	49,200 ± 5,390	43,900 ± 9,560	53,400 ± 7,330	41,900 ± 3,480
Ratio cholesterol/phospholipid	0.03 ± 0.00	0.05 ± 0.00	0.05 ± 0.01	0.06 ± 0.05‡
Ratio cholesterol/BA	0.004 ± 0.001	0.008 ± 0.001	0.007 ± 0.001	0.011 ± 0.001*,¶
Ratio phospholipid/BA	0.12 ± 0.01	0.17 ± 0.01‡	0.13 ± 0.01	0.19 ± 0.01†,¶
Bile flow, μL/min	1.7 ± 0.1	4.1 ± 0.5*	1.7 ± 0.2§	3.5 ± 0.2*,‖

Data are presented as mean ± SEM.
*$P < 0.001$, †$P < 0.01$, and ‡$P < 0.05$ versus Lxra$^{+/+}$.
§$P < 0.001$ versus Lxra$^{+/+}$ T3.
‖$P < 0.001$ and ¶$P < 0.05$ versus Lxra$^{-/-}$.

lipid-lowering effects in humans by selectively stimulating TRb in the liver has revitalized the interest for understanding the molecular effects of TH.[4-6] We here explored by which mechanisms TH exerts its powerful effects on biliary cholesterol secretion by specifically analyzing the role of the ABCG5/G8 half-transporter complex in mice. This complex has been shown to be of major importance for sterol excretion into bile, but there are also data indicating that ABCG5/G8-independent mechanisms may promote cholesterol secretion. First, biliary cholesterol secretion/concentration is not completely abolished in single[12,13] and double[11,14-16] ABCG5/G8 knockout models. Second, hepatic overexpression of scavenger receptor class B, member 1 (SR-BI) in Abcg5$^{-/-}$ mice can restore their initially decreased biliary cholesterol secretion to WT levels.[23] And third, since transintestinal cholesterol efflux occurs in Abcg5$^{-/-}$ [22] and Abcg8$^{-/-}$ [31] mice via additional pathways not yet defined, such mechanisms may operate also in the liver.

To determine to which extent the strong stimulation of biliary cholesterol secretion induced by TH is mediated by the ABCG5/G8 complex, we treated Abcg5$^{-/-}$ and WT mice of the same genetic background (Abcg5$^{+/+}$) with T3. In line with previous results,[18] TH treatment increased hepatic gene expression of

Table 4. Effects of T3 Treatment on Biliary BA Composition in Lxra$^{-/-}$ Mice and in Their WT Counterparts (Lxra$^{+/+}$)

No. of Animals	Lxra$^{+/+}$ (n =7)	Lxra$^{+/+}$ T3 (n =7)	Lxra$^{-/-}$ (n =7)	Lxra$^{-/-}$ T3 (n =7)
CA				
nmol/mL	21,900 ± 3,320	18,900 ± 4,170	28,300 ± 4,700	19,300 ± 2,200
% of total	44 ± 4	43 ± 3	51 ± 3	45 ± 2
CDCA				
nmol/mL	670 ± 50	1,120 ± 180‡	730 ± 80	1.050 ± 80
% of total	1.0 ± 0.1	3.0 ± 0.4†	2.0 ± 0.2‖	3.0 ± 0.3‡,††
α-MCA				
nmol/mL	2,860 ± 390	5,810 ± 1,560	3,970 ± 790	5,200 ± 370
% of total	6.0 ± 0.5	13.0 ± 0.9*	7.0 ± 0.6§	13.0 ± 0.9*,#
β-MCA				
nmol/mL	21,400 ± 2,610	16,300 ± 3,610	18,100 ± 2,930	14,900 ± 1,210
% of total	43 ± 3	37 ± 2	34 ± 4	36 ± 2
DCA				
nmol/mL	1,320 ± 130	710 ± 110‡	1,370 ± 200¶	630 ± 70†,**
% of total	3.0 ± 0.4	2.0 ± 0.2	3.0 ± 0.7	2.0 ± 0.2
UDCA				
nmol/mL	1,000 ± 90	1,060 ± 220	910 ± 110	830 ± 30
% of total	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1
LCA				
nmol/mL	60 ± 2	60 ± 3	60 ± 2	50 ± 1
% of total	0.1 ± 0.01	0.2 ± 0.02	0.1 ± 0.03	0.1 ± 0.02

Data are presented as mean ± SEM.
*$P < 0.001$, †$P < 0.01$, and ‡$P < 0.05$ versus Lxra$^{+/+}$.
§$P < 0.001$, ‖$P < 0.01$, and ¶$P < 0.05$ versus Lxra$^{+/+}$ T3.
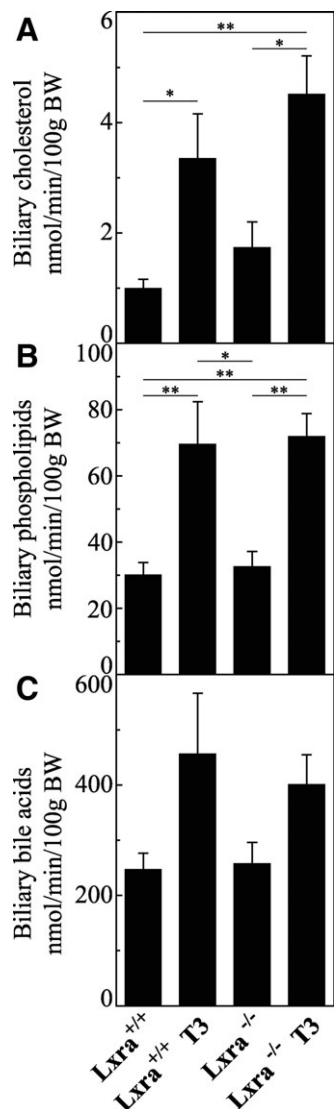#$P < 0.001$, **$P < 0.01$, and ††$P < 0.05$ versus Lxra$^{-/-}$.

Fig. 4. Effects of T3 treatment on secretion of biliary cholesterol (A), phospholipids (B), and BAs (C) in $Lxra^{-/-}$ mice and in their WT counterparts ($Lxra^{+/+}$). Number of animals per group: n = 7. Data are presented as mean ± SEM. **P < 0.01; *P < 0.05.

ABCG5/G8 in $Abcg5^{+/+}$ mice, but failed to increase ABCG8 gene expression in $Abcg5^{-/-}$ mice. This lack of response may be the result of a disruption in a regulatory region of $Abcg8$ caused in the procedure of disrupting $Abcg5$. The ABCG5 and ABCG8 genes are orientated in a head-to-head manner in the genome within 400 base pairs of each other. This implies that putative binding sites for transcription factors for one gene may be positioned within the opposite gene. Therefore, the insertion of the LacZ/Neo cassette used to disrupt the ABCG5 gene has been shown to also indirectly influence the expression of the other gene (ABCG8).[13]

Biliary cholesterol secretion was strongly reduced in $Abcg5^{-/-}$ mice, to only 28% of that in $Abcg5^{+/+}$

mice. T3 treatment increased biliary cholesterol secretion 3.1-fold in $Abcg5^{+/+}$ mice, whereas in $Abcg5^{-/-}$ mice, this response was blunted. These results demonstrate that stimulation of biliary secretion of cholesterol by T3 treatment of mice is largely dependent on an intact ABCG5/G8 complex. However, T3 treatment restored the low biliary secretion of cholesterol in $Abcg5^{-/-}$ mice up to the basal rate observed in $Abcg5^{+/+}$ mice. This suggests that, although a functional ABCG5/G8 complex is required for the major stimulation of biliary cholesterol secretion by T3, there is also an additional, ABCG5/G8-independent, mechanism.

The increased secretion in $Abcg5^{-/-}$ mice occurred simultaneously with a T3-induced doubled flow rate of bile, regardless of the genetic background of the animals. Thus, one explanation for the non-ABCG5/G8 driven cholesterol secretion could be that it reflects the combined results of simple diffusion of cholesterol and the biliary capacity to bind cholesterol. The T3-induced flow rate of bile would then modulate the total output of diffusible lipophilic compounds such as cholesterol and phospholipids, as observed, and may in turn be related to circulatory effects exerted by the hormone. In addition to the markedly (3-fold) increased secretion of cholesterol, gene expression of the rate-limiting enzyme in BA synthesis, cholesterol 7α-hydroxylase (cytochrome P450 [CYP]7A1), was 4.6-fold increased by T3 treatment in $Abcg5^{+/+}$ mice. These changes were accompanied with increased gene expression levels of the LDLr and HMG CoA red, the rate-limiting enzyme in cholesterol synthesis (2- and 4-fold, respectively), suggesting that the increased hepatic turnover of cholesterol is balanced by an increased *de novo* synthesis of cholesterol and by an increased uptake of cholesterol from the circulation. Consistent with previous results,[13] the concentration of total BAs in bile was unchanged in $Abcg5^{-/-}$ mice. In spite of an increased bile flow rate, and in contrast to the effect on the secretion of cholesterol, the secretion of total BAs was unaltered by T3 treatment. T3 treatment decreased the proportion of DCA and CA, whereas the proportions of CDCA and α-MCA increased. These results are in line with the concept that TH suppresses the BA synthetic enzyme, sterol 12-α-hydroxylase (CYP8B1), as has previously been shown.[32-34]

Activation of LXR by selective agonists has similar effects on hepatic ABCG5/G8 gene expression levels and biliary cholesterol secretion as TH.[15,17] It has been reported that LXRa is positively regulated at the transcriptional level by TRb.[24] We therefore

investigated the role of LXRa in the TH-induced stimulation of biliary cholesterol secretion. For this purpose, $Lxra^{-/-}$ and $Lxra^{+/+}$ mice with the same genetic background were treated with T3. Biliary cholesterol secretion rates did not differ between T3-treated $Lxra^{+/+}$ and $Lxra^{-/-}$ mice. These results clearly indicate that the stimulation of biliary cholesterol secretion in response to T3 is independent of LXRa. Mean levels of CYP7A1, HMG CoA red, and LDLr gene expressions were higher in T3-treated $Lxra^{+/+}$ mice, compared to the controls. However, as opposed to the response in T3-treated $Abcg5^{+/+}$ mice, mean levels were not statistically significantly different.

Because LXRa agonists have been shown to possess adverse side effects,[35-37] the apparent absence of LXRa involvement in TH-induced responses on biliary cholesterol is promising from a therapeutic point of view, since available novel thyromimetics, such as eprotirome, should thus not be expected to present such side effects.

In conclusion, we have demonstrated that the ability of TH to stimulate the secretion of cholesterol into bile is largely mediated by the ABCG5/G8 complex, whereas LXRa does not seem to be of importance for this effect.

References

1. Peters JP, Man EB. The interrelations of serum lipids in patients with thyroid disease. J Clin Invest 1943;22:715-720.

2. Peters JP, Man EB. The significance of serum cholesterol in thyroid disease. J Clin Invest 1950;29:1-11.

3. Scottolini AG, Bhagavan NV, Oshiro TH, Abe SY. Serum high-density lipoprotein cholesterol concentrations in hypo- and hyperthyroidism. Clin Chem 1980;26:584-587.

4. Angelin B, Rudling M. Lipid lowering with thyroid hormone and thyromimetics. Curr Opin Lipidol 2010;21:499-506.

5. Berkenstam A, Kristensen J, Mellstrom K, Carlsson B, Malm J, Rehnmark S, et al. The thyroid hormone mimetic compound KB2115 lowers plasma LDL cholesterol and stimulates bile acid synthesis without cardiac effects in humans. Proc Natl Acad Sci U S A 2008;105: 663-667.

6. Ladenson PW, Kristensen JD, Ridgway EC, Olsson AG, Carlsson B, Klein I, et al. Use of the thyroid hormone analogue eprotirome in statin-treated dyslipidemia. New Engl J Med 2010;362:906-916.

7. Gebhard RL, Stone BG, Andreini JP, Duane WC, Evans CD, Prigge W. Thyroid hormone differentially augments biliary sterol secretion in the rat. I. The isolated-perfused liver model. J Lipid Res 1992;33: 1459-1466.

8. Ferezou J, Coste T, Chevallier F. Origins of neutral sterols in human feces studied by stable isotope labeling (D and 13C). Existence of an external secretion of cholesterol. Digestion 1981;21:232-243.

9. Berge KE, Tian H, Graf GA, Yu L, Grishin NV, Schultz J, et al. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. Science 2000;290:1771-1775.

10. Graf GA, Li WP, Gerard RD, Gelissen I, White A, Cohen JC, Hobbs HH. Coexpression of ATP-binding cassette proteins ABCG5 and ABCG8 permits their transport to the apical surface. J Clin Invest 2002;110:659-669.

11. Graf GA, Yu L, Li WP, Gerard R, Tuma PL, Cohen JC, Hobbs HH. ABCG5 and ABCG8 are obligate heterodimers for protein trafficking and biliary cholesterol excretion. J Biol Chem 2003;278:48275-48282.

12. Klett EL, Lu K, Kosters A, Vink E, Lee MH, Altenburg M, et al. A mouse model of sitosterolemia: absence of Abcg8/sterolin-2 results in failure to secrete biliary cholesterol. BMC Med 2004;2:5.

13. Plosch T, Bloks VW, Terasawa Y, Berdy S, Siegler K, Van Der Sluijs F, et al. Sitosterolemia in ABC-transporter G5-deficient mice is aggravated on activation of the liver-X receptor. Gastroenterology 2004;126: 290-300.

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

15. Yu L, York J, von Bergmann K, Lutjohann D, Cohen JC, Hobbs HH. Stimulation of cholesterol excretion by the liver X receptor agonist requires ATP-binding cassette transporters G5 and G8. J Biol Chem 2003;278:15565-15570.

16. Yu L, Gupta S, Xu F, Liverman AD, Moschetta A, Mangelsdorf DJ, et al. Expression of ABCG5 and ABCG8 is required for regulation of biliary cholesterol secretion. J Biol Chem 2005;280:8742-8747.

17. Plosch T, Kok T, Bloks VW, Smit MJ, Havinga R, Chimini G, et al. Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X receptor is independent of ABCA1. J Biol Chem 2002; 277:33870-33877.

18. Galman C, Bonde Y, Matasconi M, Angelin B, Rudling M. Dramatically increased intestinal absorption of cholesterol following hypophysectomy is normalized by thyroid hormone. Gastroenterology 2008; 134:1127-1136.

19. Kosters A, Frijters RJ, Schaap FG, Vink E, Plosch T, Ottenhoff R, et al. Relation between hepatic expression of ATP-binding cassette transporters G5 and G8 and biliary cholesterol secretion in mice. J Hepatol 2003;38:710-716.

20. Geuken E, Visser DS, Leuvenink HG, de Jong KP, Peeters PM, Slooff MJ, et al. Hepatic expression of ABC transporters G5 and G8 does not correlate with biliary cholesterol secretion in liver transplant patients. HEPATOLOGY 2005;42:1166-1174.

21. Plosch T, van der Veen JN, Havinga R, Huijkman NC, Bloks VW, Kuipers F. Abcg5/Abcg8-independent pathways contribute to hepatobiliary cholesterol secretion in mice. Am J Physiol Gastrointest Liver Physiol 2006;291:G414-G423.

22. van der Veen JN, van Dijk TH, Vrins CL, van Meer H, Havinga R, Bijsterveld K, et al. Activation of the liver X receptor stimulates transintestinal excretion of plasma cholesterol. J Biol Chem 2009;284: 19211-19219.

23. Wiersma H, Gatti A, Nijstad N, Oude Elferink RP, Kuipers F, Tietge UJ. Scavenger receptor class B type I mediates biliary cholesterol secretion independent of ATP-binding cassette transporter g5/g8 in mice. HEPATOLOGY 2009;50:1263-1272.

24. Hashimoto K, Matsumoto S, Yamada M, Satoh T, Mori M. Liver X receptor-alpha gene expression is positively regulated by thyroid hormone. Endocrinology 2007;148:4667-4675.

25. Zhao C, Dahlman-Wright K. Liver X receptor in cholesterol metabolism. J Endocrinol 2010;204:233-240.

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

27. Gullberg H, Rudling M, Salto C, Forrest D, Angelin B, Vennstrom B. Requirement for thyroid hormone receptor beta in T3 regulation of cholesterol metabolism in mice. Mol Endocrinol 2002;16:1767-1777.

28. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911-917.

29. Böttcher CFJ van Gent CM, Pries C. A rapid and sensitive sub-micro phosphorus determination. Anal Chim Acta 1961:203-204.

30. Johansson L, Rudling M, Scanlan TS, Lundasen T, Webb P, Baxter J, et al. Selective thyroid receptor modulation by GC-1 reduces serum lipids and stimulates steps of reverse cholesterol transport in euthyroid mice. Proc Natl Acad Sci U S A 2005;102:10297-10302.

31. van der Velde AE, Vrins CL, van den Oever K, Kunne C, Oude Elfer-ink RP, Kuipers F, Groen AK. Direct intestinal cholesterol secretion contributes significantly to total fecal neutral sterol excretion in mice. Gastroenterology 2007;133:967-975.

32. Ellis EC. Suppression of bile acid synthesis by thyroid hormone in primary human hepatocytes. World J Gastroenterol 2006;12: 4640-4645.

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

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

35. Grefhorst A, Elzinga BM, Voshol PJ, Plosch T, Kok T, Bloks VW, et al. Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. J Biol Chem 2002;277:34182-34190.

36. Groot PH, Pearce NJ, Yates JW, Stocker C, Sauermelch C, Doe CP, et al. Synthetic LXR agonists increase LDL in CETP species. J Lipid Res 2005;46:2182-2191.

37. Katz A, Udata C, Ott E, Hickey L, Burczynski ME, Burghart P, et al. Safety, pharmacokinetics, and pharmacodynamics of single doses of LXR-623, a novel liver X-receptor agonist, in healthy participants. J Clin Pharmacol 2009;49:643-649.