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

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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.

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 at the apical plasma membrane of hepatocytes. Disruption of either one or both 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. In a previous study, 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, however, and there are indications that other pathways, independent of ABCG5/G8, promote cholesterol transfer into bile. Furthermore, it is unclear whether the stimulation of biliary cholesterol secretion is a direct effect of TH. It may be mediated by nuclear liver x receptor-alpha (LXRα), the expression of which has recently been reported to be positively regulated by TH receptor-beta (TRβ) in the mouse. LXRs regulate the transcription of several genes involved in cholesterol metabolism and the administration of the LXR agonist T0901317 to mice increases hepatic ABCG5/G8 gene expression and biliary cholesterol concentration and secretion. Thus, experimental evidence indicates that LXRs may mediate effects of TH on cholesterol metabolism.

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 LXRs 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 exerted by ABCG5/G8; (2) this TH-induced biliary cholesterol secretion is independent of LXRs; 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 LXRα 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.

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, 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 Ct method was used to quantify the results. The following primer sequences were used: Gapdh forward 5'-ttgctcgttgagctga-3'; Gapdh reverse 5'-cctgctcttctgtg-3'; Abcg5 forward 5'-aatgctttagctcctc-3'; Abcg5 reverse 5'-ccactatgatacaggctctc-3'; Abcg8 forward 5'-tccatcctcggagacagat-3'; Abcg8 reverse 5'-tgtgtccgtcgtggatctga-3'; Lxra forward 5'-tgattggagttggcaccat-3'; Lxra reverse 5'-gtgaaaaggacctctcgaagtg-3'; Hmgcr forward 5'-aatgctgtgaatctgtttccc-3'; Hmgcr reverse 5'-tgttgcagcctctctacttgga-3'; Cyp7a1 forward 5'-aatgctgtgaatctgtttccc-3'; Cyp7a1 reverse 5'-tgttgcagcctctctacttgga-3'; Ldlr forward 5'-gtccggatattcgctatctcctgcttcacgatgacaatga-3'; Ldlr reverse 5'-cctgcttcacgatgacaatgagcaccggatgtgagaatctgtttccca-3'; Gapdh forward 5'-ttgctcgttgagctga-3'; Gapdh reverse 5'-cctgctcttctgtg-3'; Abcg5 forward 5'-aatgctttagctcctc-3'; Abcg5 reverse 5'-ccactatgatacaggctctc-3'; Abcg8 forward 5'-tccatcctcggagacagat-3'; Abcg8 reverse 5'-tgtgtccgtcgtggatctga-3'; Lxra forward 5'-tgattggagttggcaccat-3'; Lxra reverse 5'-gtgaaaaggacctctcgaagtg-3'; Hmgcr forward 5'-aatgctgtgaatctgtttccc-3'; Hmgcr reverse 5'-tgttgcagcctctctacttgga-3'; Cyp7a1 forward 5'-aatgctgtgaatctgtttccc-3'; Cyp7a1 reverse 5'-tgttgcagcctctctacttgga-3'; Ldlr forward 5'-gtccggatattcgctatctcctgcttcacgatgacaatga-3'; Ldlr reverse 5'-cctgcttcacgatgacaatgagcaccggatgtgagaatctgtttccca-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 H2O 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/hexamethyl disilazane/chlorotrimethylsilane (3:2:1, v/v/v) at 60°C for 10 min by adding 400 μL of toluene, 100 μL of MeOH, and 25 μL of trimethylsilyl diazomethane, and samples were then dried under nitrogen at 60°C. Samples were silylated with pyridine/hexamethyl disilazane/chlorotrimethyl silane (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. D4-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 LXRα gene expression was unaltered in Abcg5−/− mice, while reduced in T3-treated 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+/+
mice, biliary cholesterol and phospholipids were increased 1.8- and 1.3-fold, respectively. Compared to untreated Abcg5<sup>−/−</sup> mice, cholesterol and phospholipids were unaltered in T3-treated Abcg5<sup>−/−</sup> mice.

The concentration of total BAs was unaltered in Abcg5<sup>−/−</sup> mice. T3 treatment of Abcg5<sup>+/+</sup> and Abcg5<sup>−/−</sup> 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<sup>+/+</sup> mice, whereas the PL/BA ratio was unaltered. In Abcg5<sup>−/−</sup> mice, the C/PL ratio was decreased by 40%, and the C/BA and PL/BA ratios were unaltered. T3 treatment of Abcg5<sup>−/−</sup> mice did not alter the ratios.

Under basal conditions, bile flow was the same in Abcg5<sup>+/+</sup> and Abcg5<sup>−/−</sup> mice. T3 treatment increased bile flow to similar extents in Abcg5<sup>+/+</sup> (1.9-fold) and in Abcg5<sup>−/−</sup> (1.8-fold) mice.

**Effects of T3 on Biliary Composition of BAs in Abcg5<sup>+/+</sup> and Abcg5<sup>−/−</sup> mice.** T3 treatment decreased the biliary proportion of deoxycholic acid (DCA) in Abcg5<sup>+/+</sup> and Abcg5<sup>−/−</sup> 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<sup>−/−</sup> mice, and there was a trend to an increased proportion of CDCA in the Abcg5<sup>−/−</sup> mice (P = 0.05). Alpha-muricholic acid (α-MCA) was increased by T3 treatment in both Abcg5<sup>+/+</sup> and Abcg5<sup>−/−</sup> mice 2.1- and 3.4-fold, respectively. Biliary proportions of β-muricholic acid (β-MCA), urso-deoxycholic acid (UDCA), and lithocholic 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

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**Table 1. Effects of T3 Treatment on Body Weight, Biliary Lipids, and Bile Flow in Abcg5<sup>−/−</sup> Mice and in Their WT Counterparts (Abcg5<sup>+/+</sup>).**

| No. of Animals | Abcg5<sup>−/−</sup> (n = 6) | Abcg5<sup>−/−</sup> T3 (n = 5) | Abcg5<sup>+/+</sup> (n = 5) | Abcg5<sup>+/+</sup> 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/BAs | 0.004 ± 0.001 | 0.008 ± 0.001† | 0.002 ± 0.000§ | 0.004 ± 0.001*  |
| Ratio phospholipid/BAs | 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<sup>+/+</sup>.

§P < 0.001 and †P < 0.01 versus Abcg5<sup>−/−</sup> T3.

¶P < 0.001 versus Abcg5<sup>−/−</sup>.
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 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.** LXRα 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.

### 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 ± 1±         | 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⁻/⁻.
T3-treated Lxra\(^{+/+}\) and Lxra\(^{-/-}\) mice, whereas the biliary proportions of \(\beta\)-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 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
lipid-lowering effects in humans by selectively stimulating TRβ in the liver has revitalized the interest for understanding the molecular effects of TH.\textsuperscript{4,6} We here analyzed TRβ in the liver has revitalized the interest for lipid-lowering effects in humans by selectively stimulating TRβ in the liver has revitalized the interest for understanding the molecular effects of TH.\textsuperscript{4,6} We here analyzing the role of the ABCG5/G8 half-transporter 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\textsuperscript{12,13} and double\textsuperscript{11,14-16} ABCG5/G8 knockout models. Second, hepatic overexpression of scavenger receptor class B, member 1 (SR-BI) in Abcg5\textsuperscript{-/-} mice can restore their initially decreased biliary cholesterol secretion to WT levels.\textsuperscript{23} And third, since transintestinal cholesterol efflux occurs in Abcg5\textsuperscript{-/-} and Abcg8\textsuperscript{-/-} 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\textsuperscript{-/-} and WT mice of the same genetic background (Abcg5\textsuperscript{+/+}) with T3. In line with previous results,\textsuperscript{18} TH treatment increased hepatic gene expression of

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

| No. of Animals | Lxra\textsuperscript{-/-} (n = 7) | Lxra\textsuperscript{-/-} T3 (n = 7) | Lxra\textsuperscript{-/-} (n = 7) | Lxra\textsuperscript{-/-} 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                   |
| BSs, 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†                  |
| Cholesterol, nmol/mL | 190 ± 30                      | 21,900 ± 1,320                | 21,400 ± 1,120                | 2,930 ± 1,490                 |
| 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\textsuperscript{+/+}.

§P < 0.001 versus Lxra\textsuperscript{-/-} T3.

P < 0.001 and ¶P < 0.05 versus Lxra\textsuperscript{-/-}.

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

| No. of Animals | Lxra\textsuperscript{-/-} (n = 7) | Lxra\textsuperscript{-/-} T3 (n = 7) | Lxra\textsuperscript{-/-} (n = 7) | Lxra\textsuperscript{-/-} 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\textsuperscript{+/+}.

§P < 0.001, †P < 0.01, and ¶P < 0.05 versus Lxra\textsuperscript{-/-} T3.

P < 0.001, **P < 0.01, and ††P < 0.05 versus Lxra\textsuperscript{-/-}.
ABCG5/G8 in Abcg5+/+ mice, but failed to increase Abcg5 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 12α-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 LXRα 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, 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 eprotiromide, 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.

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