Nuclear receptors in control of cholesterol transport
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CHAPTER 5

Abcg5/Abcg8-independent pathways contribute to hepatobiliary cholesterol secretion in mice

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ABSTRACT

The ABC half-transporters ABCG5 and ABCG8 heterodimerize into a functional complex that mediates secretion of plant sterols and cholesterol by hepatocytes into bile and their apical efflux from enterocytes. We have addressed the putative rate-controlling role of Abcg5/Abcg8 in hepatobiliary cholesterol excretion in mice during (maximal) stimulation of this process. Despite similar bile salt (BS) excretion rates, basal total sterol and phospholipid (PL) output rates were reduced by 82 % and 35 %, respectively, in chow-fed Abcg5^-/- mice compared to wild-type mice. Upon infusion with the hydrophilic bile salt TUDCA, similar relative increases in bile flow, BS output, PL output and total sterol output were observed in wild-type, Abcg5^+/+ and Abcg5^-/- mice. Maximal cholesterol and PL output rates in Abcg5^-/- mice were only 15 % and 69 %, respectively, of wild-type values. Infusion of increasing amounts of the hydrophobic bile salt TDCA increased cholesterol excretion 3.0- and 2.4-fold in wild-type and Abcg5^-/- mice, but rapidly induced cholestasis in Abcg5^-/- mice. Treatment with the LXR agonist T0901317 increased the maximal sterol excretion capacity in wild-type mice (4-fold), concomitant with induction of Abcg5/Abcg8 expression, but not in Abcg5^-/- mice. In a separate study, mice were fed chow containing 1 % w/w cholesterol. As expected, hepatic expression of Abcg5 and Abcg8 was strongly induced (5-fold and 4-fold) in wild-type but not in liver x-receptor α-deficient (Lxra^-/-) mice. Surprisingly, hepatobiliary cholesterol excretion was increased to the same extent, i.e., 2.2-fold in wild-type mice and 2.0-fold in Lxra^-/- mice, upon cholesterol-feeding. Our data confirm that Abcg5, as part of the Abcg5/Abcg8 heterodimer, strongly controls hepatobiliary cholesterol secretion in mice. However, our data demonstrate that Abcg5/Abcg8 heterodimer-independent, inducible routes exist that can significantly contribute to total hepatobiliary cholesterol output.
INTRODUCTION

Mutations in the ATP-binding-cassette (ABC) half-transporters ABCG5 and ABCG8 cause sitosterolemia\(^1,2\) which is characterized by the accumulation of plant sterols in the body\(^3\). Data indicate that ABCG5 and ABCG8, which are highly expressed in liver and small intestine, heterodimerize into a functional complex\(^1,4\). Mutations in either one of the genes cause the biochemical hallmarks of the disease in humans\(^1,2\) as well as in mouse models\(^5,6\).

The daily intake of plant sterols, i.e., sitosterol and campesterol, from a "Western-type" diet is in the same order of magnitude as that of cholesterol. However, only trace amount of plant sterols are absorbed in healthy subjects\(^7,8\). ABCG5/ABCG8 mediates efflux of plant sterols from enterocytes back into the intestinal lumen and their excretion into bile, thus limiting their accumulation in the body\(^1,9\). Expression of the Abcg5 and Abcg8 genes is controlled by the liver X-receptor (LXR\(\alpha/NR1H3\)) and possibly by the liver-receptor-homologue 1 (LRH-1)\(^10,11\). LXR is activated by oxysterols and hence is considered a cellular "cholesterol sensor"\(^12\). Kosters et al.\(^13\) have demonstrated that, across various mouse models, a strong correlation exists between biliary cholesterol excretion (normalized for bile salt and phospholipid excretion) and hepatic Abcg5/g8 expression\(^13\). Overexpression of Abcg5/Abcg8 in transgenic mice\(^14\) or induction of their expression via activation of LXR with synthetic ligands\(^6,11\) or dietary cholesterol feeding\(^15,16\) in wild-type mice is associated with a strongly increased hepatobiliary cholesterol excretion. Deletion of the genes, either Abcg5\(^5\) or Abcg8\(^5\) or both\(^17\), in general has the opposite effects. Importantly, biliary cholesterol content is also reduced in heterozygote Abcg5\(^{+/+}\), Abcg8\(^{+/+}\) and Abcg5\(^{+/+}\)/Abcg8\(^{+/+}\) mice\(^5,6,17\), indicating a high degree of control of the functional heterodimer in the secretion process. However, it is of importance to note that residual cholesterol secretion is still observed in the complete knock-out mice (10-20% of wild-type\(^16,17\)). In addition, it appeared that diosgenin-induced hypersecretion of cholesterol in mice occurs in the absence of Abcg5/Abcg8 induction\(^18\) although the presence of functional Abcg5/Abcg8 is required for the effect to occur\(^16\). Of note, in most of the studies mentioned, cholesterol content of gallbladder bile was determined rather than biliary output rates. In contrast to the mouse\(^13\), recent data demonstrate that in human liver transplantation patients no relationship exists between normalized biliary cholesterol excretion and hepatic ABCG5 and ABCG8 gene expression\(^19\).

Thus it appears likely to assume that hepatobiliary cholesterol secretion can occur by both Abcg5/g8-dependent and Abcg5/g8 independent routes, the former being (quantitatively) the most important one in mice under basal conditions. In this study, we have used two strategies to address the quantitative contribution of both (putative) pathways under stressed conditions, i.e., during infusion of hydrophilic and hydrophobic bile salts in control and LXR-agonist-treated wild-type, Abcg5\(^{+/+}\) and Abcg5\(^{-/-}\) mice and upon feeding a high-cholesterol diet in wild-type and Lxra\(^-/-\) mice. The data from these studies provide evidence to suggest that, under certain metabolic conditions, Abcg5/Abcg8-independent routes significantly contribute to total hepatobiliary cholesterol output in mice.
MATERIALS AND METHODS

Animals and diets.
Mice homozygous (Abcg5\(^{-/-}\)) and heterozygous (Abcg5\(^{+/-}\)) for the disruption of the Abcg5 gene and their wild-type littermates were used\(^6\). Animals were housed in temperature-controlled rooms (21°C) with 12 hours light cycling and received standard mouse chow (Arie Blok, Woerden, The Netherlands) and water \textit{ad libitum}. The diet contained 0.017 % (w/w) cholesterol and 0.045 % (w/w) plant sterols.

\textit{Lxra}\(^{-/-}\) mice, generated by Deltagen, Inc. (Redwood City, USA) using standard gene-targeting methods, were kindly provided by Tularik Inc. (South San Francisco, USA). In short, a 42 bp fragment corresponding to a segment of exon 2 was replaced by a beta-galactosidase cDNA and a phosphoglycerate kinase promoter-driven neomycin resistance cassette. The remaining procedure followed that described for the Abcg5\(^{-/-}\) mice\(^6\). Mice were genotyped via PCR using allele-specific primers (wild-type: GTTTCTCTCCCCCTATCTCTCTAGGGAGAC; CACCCATTCTCCCCGTGTCTCTCTTG; knockout: GGGCCAGCTCTTCTCCCACTCAT). Mice homozygous (\textit{Lxra}\(^{-/-}\)) and heterozygous (\textit{Lxra}\(^{+/-}\)) for the disruption of the Lxra gene and their wild-type littermates received either standard mouse chow or chow diet containing 1 % cholesterol (wt/wt; Arie Blok, Woerden, The Netherlands) for two weeks. Male mice of 2-4 months were used. All experimental procedures were approved by the local Ethical Committee for Animal Experiments.

\textit{Abcg5}\(^{-/-}\) mice. \textit{Abcg5}\(^{-/-}\) and wild-type mice were fed either standard laboratory chow or chow supplemented with the synthetic LXR-agonist T0901317 (0.015 %, w/w; Cayman Chemicals, Ann Arbor, MI, USA) for 7 days. \textit{Abcg5}\(^{+/-}\) mice received standard chow only.

Female mice of 3-6 months were used. Mice were anaesthetized by intraperitoneal injection with Hypnorm (fentanyl/fluanisone, 1 ml/kg) and diazepam (10 mg/kg). Bile was collected by cannulation of the gallbladder. After two basal bile samples of 15 minutes, mice were continuously infused with tauroursodeoxycholate (TUDCA; Calbiochem/Merck Biosciences, Darmstadt, Germany) or taurodeoxycholate (TDCA; Calbiochem/Merck Biosciences) in PBS via the jugular vein. Infusion rates were increased in a stepwise manner: 150, 300, 450, 600 nmol/min and 25, 50, 75, 100 nmol/min for TUDCA and TDCA, respectively. During bile collection, body temperature was stabilized using an humidified incubator. At the end of the collection period, animals were killed by cardiac puncture. Livers were excised and weighed.

Gene expression, plasma lipid composition and hepatic lipid contents was studied in an independent experiment. 5-7 months old male \textit{Abcg5}\(^{-/-}\), \textit{Abcg5}\(^{+/-}\) and wild-type mice were fed standard laboratory chow. Mice were anesthetized with isoflurane and killed by cardiac puncture. Blood was collected in EDTA-containing tubes. Livers were excised and weighed. Aliquots were snap frozen in liquid nitrogen and stored at -80°C for biochemical analyses and RNA isolation.

\textit{Lxra}\(^{-/-}\) mice. \textit{Lxra}\(^{-/-}\), \textit{Lxra}\(^{+/-}\) and \textit{Lxra}\(^{+/+}\) mice were anaesthetized by intraperitoneal injection with Hypnorm and diazepam as described above. Bile was collected by cannulation of the gallbladder for 30 minutes. Body temperature was stabilized using an humidified incubator. Subsequently, animals were killed by cardiac puncture and livers were excised and weighed. Aliquots were snap frozen in liquid nitrogen and stored at -80°C for biochemical analyses and RNA isolation. TUDCA infusion in \textit{Lxra}\(^{-/-}\) mice and wild-type littermates fed a high-cholesterol diet was performed as described above.
Analytical procedures.
Biliary bile salt concentrations were measured enzymatically\textsuperscript{20}. Biliary phospholipid and sterol concentrations in the Abcg5 experiments were determined as described previously\textsuperscript{21}. No distinction was made between cholesterol and plant sterols, as enzymatic cholesterol assays have been found to measure both\textsuperscript{22}. In Lxra mice, phospholipids and cholesterol in bile were determined as described by Böttcher \textit{et al.}\textsuperscript{23} and Gamble \textit{et al.}\textsuperscript{24}, respectively, after extraction according to Bligh and Dyer\textsuperscript{25}. The same extraction method was applied for hepatic lipids, after which commercially available kits were used for the determination of unesterified and total cholesterol (Wako, Neuss, Germany), and for triglycerides (Roche, Mannheim, Germany). Pooled plasma samples from all animals of one group were used for lipoprotein separation by fast protein liquid chromatography (FPLC) as described previously\textsuperscript{26}.

RNA isolation and PCR procedures.
Total RNA was extracted from frozen tissues with TriReagent (Sigma, St. Louis, MO, USA) and quantified photometrically. cDNA synthesis was performed using recombinant M-MLV reverse transcriptase (10 U/µl), the appropriate buffer, dNTPs (500 µM), random nonamers (1 µM), RNase inhibitor (2 U/µl; all from Sigma) and total RNA (50 ng/µl). The reaction mix was incubated for 10 minutes at 25°C for primer annealing, 60 minutes at 37°C for synthesis and 5 minutes at 94°C to denature the RT enzyme. Real-time quantitative PCR was performed using an Applied Biosystems 7700 sequence detector as previously described\textsuperscript{27}. Primers were obtained from Invitrogen (Carlsbad, USA). Fluorogenic probes, labeled with 6-carboxy-fluorescein (FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA), were made by Eurogentec (Seraing, Belgium). Primers and probes used in these studies have been described elsewhere (Sterol regulatory element binding protein (Srebp)\textsubscript{1a}, Srebp1c, Srebp2, Lxra, scavenger receptor (Srb)\textsubscript{1}, acyl-coenzymeA: cholesterol acyltransferase (Acat)\textsubscript{1}, Acat2, 3-hydroxy-3-methylglutaryl-CoA reductase (Hmgr), cytochrome P-450 (Cyp)\textsubscript{7a1}, Cyp27, Abca1, Abcg5, Abcg8 multidrug resistance protein, (Mdr)\textsubscript{2}, BS export protein (Bsep), and 18S rRNA\textsuperscript{27}; \textit{beta actin} and LDL-receptor (Ldlr)\textsuperscript{28}; Abcg1, Abcg2\textsuperscript{29}; Niemann-Pick disease-1 (Npc1)-like 1 (Npc\textsubscript{1}l1) and 36b4\textsuperscript{30}; and Mrp2 (Abcc2)\textsuperscript{31}, with the exception of Cyclophilin (XM\textsubscript{356256}; forward CAGATCGAGGGATCGATTCAG, reverse TCAC-CACTTGACACCCTCATTC, probe CTCCTCCACCTGAGACAGAAGATGCA). All data of the Abcg5 experiment were subsequently normalized to the median of \textit{beta-actin}, 36b4, 18S rRNA, and cyclophilin as described by Vandesompele \textit{et al.}\textsuperscript{32}. In the Lxra experiments, \textit{beta-actin} alone was used for normalization.

Statistics
Statistical analyses were performed using SPSS 10.1 for Windows (SPSS Inc., Chicago, USA). Differences between genotypes were evaluated using the Mann-Whitney-U-test. A p-value smaller than 0.05 was considered statistically significant.


**RESULTS**

*Plasma and hepatic lipid composition in Abcg5−/− mice.*

It has been previously reported\(^6\) that Abcg5−/− mice have elevated plasma triglyceride levels compared to wild-type mice, whereas plasma cholesterol concentrations (measured by gas chromatography) are decreased. To establish the distribution of plasma sterols across the various lipoprotein classes, plasma samples were subjected to FPLC separation (Fig. 1). Total sterol (cholesterol + plant sterols) distribution was virtually identical in the two genotypes and almost exclusively present in the HDL-sized fractions.

![Figure 1: Distribution of sterols in plasma lipoprotein fractions of wild-type and Abcg5−/− mice. Blood was collected via cardiac puncture and pooled before FPLC analysis. Analysis was performed as described in MATERIALS AND METHODS.](image)

### TABLE 1. Liver parameters of male Abcg5+/+, Abcg5+/− and Abcg5−/− mice on chow diet

<table>
<thead>
<tr>
<th></th>
<th>Abcg5+/+</th>
<th>Abcg5+/−</th>
<th>Abcg5−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio liver weight/body weight</td>
<td>0.049 ± 0.002</td>
<td>0.049 ± 0.004</td>
<td>0.058 ± 0.002(^a)</td>
</tr>
<tr>
<td>Total sterols (nmol/mg)</td>
<td>5.14 ± 0.64</td>
<td>5.52 ± 0.72</td>
<td>5.24 ± 0.43</td>
</tr>
<tr>
<td>Sterol ester (nmol/mg)</td>
<td>0.77 ± 0.29</td>
<td>0.79 ± 0.50</td>
<td>0.43 ± 0.19(^a)</td>
</tr>
<tr>
<td>Unesterified sterols (nmol/mg)</td>
<td>4.37 ± 0.53</td>
<td>4.73 ± 0.26</td>
<td>4.80 ± 0.44</td>
</tr>
<tr>
<td>Triglycerides (nmol/mg)</td>
<td>19.2 ± 15.4</td>
<td>11.7 ± 9.8</td>
<td>8.9 ± 3.5</td>
</tr>
<tr>
<td>Phospholipids (nmol/mg)</td>
<td>34.8 ± 1.4</td>
<td>36.0 ± 0.4</td>
<td>36.8 ± 2.1</td>
</tr>
</tbody>
</table>

Male Abcg5+/+, Abcg5+/− and littermate control mice, 5-7 months old, were fed standard laboratory chow. Mice were anesthetized with isoflurane and killed by cardiac puncture. Livers were excised and weighed. Aliquots were snap frozen in liquid nitrogen and stored at -80°C for biochemical analyses. Lipids were extracted and analysed as described in Materials and Methods. No distinction was made between cholesterol and other sterols. \(^a\) indicates significant difference from wild-type group (Mann-Whitney-U-test, p<0.05). n=5-7 per group.

As previously reported\(^6\), liver weight was slightly increased in Abcg5−/− mice compared to wild-type littermates (Table 1). This was not due to steatosis, because triglyceride concentrations were not increased in the knock-out mice. The concentrations of phospholipids as well as that of total sterols and unesterified sterols were almost identical between all groups. Only sterol ester concentrations in Abcg5−/− mice were reduced by 45%.
compared to wild-type and heterozygotes. It should be noted that sterol concentrations were measured enzymatically and consisted of both cholesterol and plant sterols; it has been previously shown that plant sterols comprise up to 42% of total sterols in livers of Abcg5−/− mice while plant sterols are present in low amounts in livers of wild-type mice.

Hepatic gene expression.

In male mice, hepatic gene expression was determined by real-time RT-PCR (Table 2). The mRNA levels of regulatory genes (Srebp1a, 1c, 2; Lxra) were identical in all groups. Expression of hepatic cholesterol uptake systems, i.e., Ldlr and Srb1, also did not differ between groups. Western blot analysis of isolated plasma membranes confirmed that also Sr-b1 protein levels were not different between genotypes (data not shown). On the other hand, expression of cholesterol metabolism genes in liver was different in Abcg5−/− mice compared to wild-type and Abcg5+/+ mice: the expression of Hmgcr, rate-controlling for cholesterol synthesis was reduced by 50% in Abcg5−/− mice. Expression of Cyp7a1,
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encoding the enzyme responsible for the majority of bile salt synthesis, was upregulated by 82% in Abcg5\(^{-/-}\) mice, whereas that of Cyp27 was unchanged. Expression levels of Acat1 and Acat2, involved in cholesteryl ester formation, were not impaired in Abcg5\(^{-/-}\) and Abcg5\(^{-/-}\) mice compared to wild-type controls. Gene expression of a wide spectrum of transporters potentially involved in cholesterol transport was screened: only the expression of Abca1 (+24%) and Npc1/11 (+41%) did show significant increases in Abcg5\(^{-/-}\) mice compared to their wild-type littermates. Expression of Abcg1, Abcg2, Abcg8, Npc1, Mrp2 and Bsep was identical in all three genotypes. As anticipated, Abcg5 mRNA was virtually absent (5% mRNA remaining compared to wild-type) in Abcg5\(^{-/-}\) mice (Figure 2A). Surprisingly, Abcg5 expression in heterozygous mice was 76% of wild-type values. No effect on the expression of the phospholipid flippase Mdr2 (Abcb4) was noticed (Figure 2B).

**Figure 2:** Hepatic Abcg5 and Mdr2 mRNA expression levels (above) and hepatobiliary sterol and phospholipid output rates (below) of Abcg5\(^{-/-}\), Abcg5\(^{-/-}\) and Abcg5\(^{-/-}\) mice. A and B: Liver pieces were snap frozen in liquid nitrogen. mRNA extraction, cDNA synthesis and TaqMan PCR analysis was done as described in Materials and Methods. All data were normalized to the median of the expression levels of beta-actin, 36b4, 18S rRNA, and cyclophilin as described by Vandesompele et al.\(^{13}\). * indicates significant difference from wild-type group (Mann-Whitney-U-test, \(p<0.05\)). C and D: Mice were anaesthetized by intraperitoneal injection with Hypnorm (fentanyl/fluanisone, 1 ml/kg) and diazepam (10 mg/kg). Bile was collected by cannulation of the gallbladder for 15 minutes. Biliary phospholipid and sterol concentrations were determined enzymatically as described previously;** no distinction was made between cholesterol and plant sterols. * indicates significant difference from wild-type group (Mann-Whitney-U-test, \(p<0.05\)).

**Biliary sterol and phospholipid secretion rates are decreased in Abcg5\(^{-/-}\) mice.** Abcg5\(^{-/-}\) mice have been reported to have decreased hepatobiliary cholesterol and phospholipid secretion rates, while for Abcg5\(^{-/-}\) mice only gallbladder concentration data are available. Therefore, hepatic bile was collected from Abcg5\(^{+/+}\), Abcg5\(^{-/-}\) and Abcg5\(^{-/-}\) mice for 15 minutes immediately after creation of a gallbladder fistula to determine basal biliary lipid output rates. Abcg5 knockout mice presented with a significantly increased bile flow compared to heterozygotes and wild-type littermates (8.9 ± 2.3 in Abcg5\(^{-/-}\) mice vs. 6.6 ± 1.5

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and 5.0 ± 2.9 μl/min/100 g body weight in Abcg5+/+ and Abcg5+/- mice, respectively. Hepatobiliary sterol (Fig. 2C) and phospholipid excretion rates (Fig. 2D) were significantly decreased in both heterozygous and homozygous knockout mice compared to wild-type controls. Bile salt output rates were unaffected, i.e., 325 ± 121, 309 ± 198 and 332 ± 164 nmol/min/100 g body weight in Abcg5+/+, Abcg5+/- and Abcg5-/- mice, respectively. Moreover, gas-chromatographic analysis revealed that biliary bile salt composition was not significantly different between Abcg5+/+, Abcg5+/- and Abcg5-/- mice (data not shown).

**Biliary lipid excretion increases upon infusion with TUDCA in Abcg5-/-, Abcg5+/- and wild-type mice.**

Systemic infusion of hydrophilic bile salts increases bile flow and facilitates hepatobiliary lipid secretion in wild-type mice33. To investigate whether this forced flow could restore impaired phospholipid- and sterol secretion in Abcg5-/- mice, we infused Abcg5+/+, Abcg5+/- and Abcg5-/- mice with increasing concentrations of tauroursodeoxycholate (TUDCA). Bile flow was increased in Abcg5-/- mice compared to wild-type and heterozygote mice already under basal conditions. This effect was even more pronounced at higher infusion rates (data not shown).

As depicted in Figure 3, phospholipid excretion increased upon TUDCA infusion in all genotypes (+140 %, +280 % and +150 % in Abcg5+/+, Abcg5+/- and Abcg5-/- mice, respectively, during infusion of the highest dose of TUDCA). In parallel, hepatobiliary sterol excretion also increased in all strains (+230 %, +700 %, and +120 % Abcg5+/+, Abcg5+/- and Abcg5-/- mice, respectively). Noteworthy, the initially low sterol excretion rate in Abcg5-/- mice recovered upon infusion of TUDCA to reach wild-type levels. As a consequence, the

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**Figure 3:** Hepatobiliary phospholipid and sterol output rates and the sterol:phospholipid ratio under basal conditions and during infusion with TUDCA in Abcg5+/+, Abcg5+/- and Abcg5-/- mice. TUDCA was infused via the jugular vein in a stepwise manner as described in Materials and Methods: 150, 300, 450, 600 nmol/min. Phospholipids and sterols were measured enzymatically as described previously21; no distinction was made between cholesterol and plant sterols. * indicates significant difference between basal level and maximal level during TUDCA infusion (Mann-Whitney-U-test, p<0.05).
sterol:phospholipid-ratios in Abcg5+/− mice and in Abcg5−/− mice were significantly lower than in wild-type mice on the basal level, but upon infusion with TUDCA the ratios normalized in Abcg5+/− mice but not in Abcg5−/− mice.

**Biliary lipid excretion increases upon infusion with TDCA in Abcg5−/− and wild-type mice.**

To determine whether a more hydrophobic bile salt would restore hepatobiliary cholesterol excretion more effectively than TUDCA in Abcg5−/− mice, Abcg5−/− mice and littermate controls were infused with increasing amounts of the hydrophobic bile salt taurodeoxycholate (TDCA, Figure 4). At infusion rates of up to 75 nmol/min, bile flow and hepatobiliary bile salt output were indistinguishable between the two groups. At 100 nmol TDCA/min, the highest infusion rate tested, bile of the Abcg5 knockout mice turned red and bile flow dramatically decreased.

Both phospholipid excretion curves and sterol excretion curves were lower in the Abcg5−/− mice compared to wild-type controls. However, both phospholipid (3.5-times) and sterol (2.4-times) excretion increased in Abcg5−/− mice upon infusion with TDCA (at the maximal capacity of hepatobiliary lipid secretion upon an infusion rate of 75 nmol/min). This increase was not statistically different from that in wild-type mice (2.1-times for phospholipids and 3.5-times for sterols).

![Figure 4: Bile flow and hepatobiliary bile salt, phospholipid and sterol output rates during infusion with increasing amounts of TDCA in Abcg5−/− and wild-type mice. TDCA was infused via the jugular vein at rates of 25, 50, 75 and 100 nmol/min. Measurements were done as described in MATERIALS AND METHODS. The arrow points at the appearance of red-colored bile in Abcg5−/− mice](image-url)
The Abcg5-independent sterol secretion is not induced upon LXR activation.

LXR is involved in the regulation of cholesterol homeostasis at various levels. To unravel if the remaining sterol excretion capacity in Abcg5\(^{-/-}\) mice is LXR-dependent, wild-type and Abcg5\(^{-/-}\) mice were fed the LXR agonist T0901317 for two weeks. Similar to results obtained previously\(^{28}\), LXR activation led to the presence of large, triglyceride-rich HDL particles in Abcg5\(^{-/-}\) mice (data not shown). As also reported previously\(^{27}\), treatment with T0901317 yielded an increased hepatobiliary sterol secretion (+210 %) and a reduced phospholipid secretion (-35 %), resulting in a relative sterol hypersecretion in wild-type mice. In Abcg5\(^{-/-}\) mice, however, no increase in sterol secretion was observed. In contrast, a slight drop of the already low sterol secretion rate occurred (from 1.1 to 0.7 nmol/min/100 g BW).

Upon infusion with TUDCA, phospholipid secretion rates more than doubled in all groups. The maximal excretory rate for sterols went up 4 times in wild-type mice treated with the LXR agonist compared to non-treated wild-type mice. However, in Abcg5\(^{-/-}\) mice no difference was observed between LXR-treated and non-treated mice. Figure 5 visualizes the effect of LXR activation in Abcg5\(^{-/-}\) mice and wild-type littermates. The sterol:bile salt ratio was clearly increased in wild-type mice upon treatment with T0901317, whereas the relationships between bile salt and cholesterol output in untreated or T0901317-treated Abcg5\(^{-/-}\) mice were similar.

Cholesterol feeding increases hepatobiliary cholesterol excretion in wild-type and Lxra\(^{-/-}\) mice.

To test whether the reported increase in hepatobiliary cholesterol excretion upon cholesterol feeding is LXR- (and Abcg5/Abcg8) dependent, Lxra\(^{-/-}\), Lxra\(^{-/-}\) and wild-type control mice were fed a diet containing 1 % cholesterol to increase delivery of cholesterol to the liver. Hepatic sterol(ester) contents increased to similar levels in wild-type and heterozygote mice, but much stronger in the Lxra\(^{-/-}\) mice. As anticipated\(^{15}\), hepatic expression of Abcg5 and Abcg8 was increased in control mice and heterozygotes on high-cholesterol diet (Figure 6).
In Lxra\(^{-/-}\) mice on high cholesterol diet, Abcg5 and Abcg8 expression did not differ from that in chow-fed Lxra\(^{+/+}\) mice. Bile flow, biliary bile salt and phospholipid excretion rates did not differ between the groups. Surprisingly, hepatobiliary cholesterol excretion was increased in all mice on high cholesterol diet, regardless of genotype (Figure 6).

**Hepatobiliary cholesterol excretion in Lxra\(^{-/-}\) mice is correlated with increased phospholipid secretion upon infusion of TUDCA.**

In principle, it could be speculated that basal expression levels of Abcg5 and Abcg8 are sufficiently high to allow for the increased hepatobiliary cholesterol transport in the absence of LXR. To test this hypothesis, we infused Lxra\(^{-/-}\) and wild-type mice fed high-cholesterol diet with increasing amounts of TUDCA to determine the maximal secretory rates for biliary lipids. As depicted in Figure 7, bile flow was slightly higher in Lxra\(^{-/-}\) mice than in littermate controls, whereas bile salt output was identical. Unexpectedly, hepatobiliary cholesterol secretion rates were identical in Lxra\(^{-/-}\) and wild-type mice even at the highest TUDCA infusion rate applied. Moreover, the hepatobiliary excretion of phospholipids was up to 200% increased in Lxra\(^{-/-}\) mice compared to wild-type littermates. As a result, the cholesterol/phospholipid ratio indicated a relative hypersecretion in wild-type (ratio 0.176) compared to Lxra\(^{-/-}\) mice (ratio 0.088).
DISCUSSION

The mechanism by which cholesterol molecules are excreted from the hepatocyte into the bile still represents an unsolved problem in lipid biochemistry. The discovery of the ABC-half transporters Abcg5 and Abcg8 as important players herein \(^1,2\) has established the involvement of these transporter proteins, but mechanistic issues concerning the actual excretion process have remained in the dark. Knocking out the genes encoding Abcg5, Abcg8, or both in mice dramatically reduces biliary cholesterol concentrations \(^5,6,17\), which was taken to indicate that Abcg5 and Abcg8 function as a heterodimer in the excretion process. The fact that heterozygous knock-out mice and rodents in which Abcg5/Abcg8 expression is modulated \(^6,11,14-16\) show a clear phenotype for biliary cholesterol excretion underscores the important role of the Abcg5/Abcg8 heterodimer in control of the secretion process. Currently, two major models have been proposed concerning the mode of action of the Abcg5/Abcg8 heterodimer in hepatobiliary cholesterol excretion. The first model, basically proposed by Wittenburg and Carey \(^3\), postulates that the Abcg5/Abcg8 heterodimer translocates (“flops”) cholesterol from the inner leaflet of the canalicular membrane to the...
outer one and thereby provides a continuous supply of cholesterol for (bile salt-facilitated) excretion. An alternative model by Small\textsuperscript{35} proposes that cholesterol reaches the outer leaflet by diffusion. There, the Abcg5/Abcg8 heterodimer would "lift" cholesterol from its membraneous environment to be more easily available for micellization by bile salts present in the lumen of the canalculus. In this study we wanted to address the extent of rate-control exerted by the Abcg5/Abcg8 heterodimer in hepatobiliary (chole)sterol secretion under conditions of (maximal) induction of this process.

First, it was demonstrated that sterols present in plasma of $\text{Abcg5}^{-/-}$ mice, like in wild-type mice, are almost exclusively present in HDL-sized fractions. Furthermore, hepatic mRNA and protein expression of the HDL-receptor Sr-b1 was not affected by $\text{Abcg5}$-deficiency. This excludes differences in "delivery" of plasma cholesterol to the liver as a cause for the observed differences in biliary cholesterol secretion. However, it cannot be ruled out that enlargement of HDL particles upon LXR activation\textsuperscript{28,36} leads to a differential processing by livers from wild-type and knock-out mice.

In a very recent study\textsuperscript{16}, Yu and colleagues have elegantly demonstrated that gallbladder cholesterol concentrations correlate with $\text{Abcg5}/\text{Abcg8}$ expression levels in transgenic mice containing 1, 10, or 16 copies of $\text{Abcg5}/\text{Abcg8}$. Likewise, Kosters et al.\textsuperscript{13} have demonstrated a high correlation between "normalized" biliary choleseterol secretion and hepatic $\text{Abcg5}/\text{Abcg8}$ mRNA expression when various mouse models were compared. In this study, we quantified hepatobiliary cholesterol excretion rates, rather than gallbladder concentrations, in wild-type, $\text{Abcg5}^{+/-}$ and $\text{Abcg5}^{-/-}$ mice. We have recently reported that cholesterol concentrations in the gallbladder of $\text{Abcg5}^{-/-}$ mice are reduced by 50\% compared to wild-type mice\textsuperscript{6}. In this study, we found a much stronger reduction of biliary cholesterol output rates, as measured after cannulation of the gallbladder. The discrepancy between the low hepatic cholesterol output rates and relatively higher downstream concentrations in the gallbladder may be caused by processes in the gallbladder itself. It can be speculated that gallbladder epithelial cells are able to deliver cholesterol to the bile, particularly when detergent biliary bile salts are not shielded sufficiently by phospholipids and cholesterol.

Both hepatobiliary phospholipid and sterol secretion were impaired under basal, non-stimulated conditions in $\text{Abcg5}^{+/-}$ and $\text{Abcg5}^{-/-}$ mice. Hepatic expression of $\text{Abcg5}$ was reduced by only 24\% in the heterozygotes, indicative for compensatory up-regulation of transcription of the remaining allele, whereas that of $\text{Mdr2}$ ($\text{Abcb4}$) was not affected. The relatively small reduction of $\text{Abcg5}$ expression in heterozygotes was associated with a reduction by approximately 60\% of biliary sterol secretion. Therefore, it is reasonable to conclude that, under these conditions, $\text{Abcg5}$ expression controls a large fraction of hepatobiliary sterol secretion and partly, either directly or indirectly, also influences phospholipid secretion. It is tempting to speculate that reduced phospholipid secretion associated with $\text{Abcg5}$-deficiency is a secondary phenomenon, most likely related to altered canalicular membrane structure or biliary micelle composition. It is well-established that, under physiological circumstances, biliary phospholipid and cholesterol concentrations are coupled to that of bile salts\textsuperscript{37}. To exclude the possibility that changes in biliary bile salt composition contributed to the reduced phospholipid and cholesterol excretion rates, bile salt composition was determined. It is noteworthy that biliary bile salt composition was indistinguishable between the three genotypes, as was expression of the canalicular bile salt transporters $\text{Bsep}$ ($\text{Abcb11}$) and $\text{Mrp2}$ ($\text{Abcc2}$).
The high degree of control on biliary excretion was overcome when biliary bile salt secretion was stimulated by infusion of the hydrophilic bile salt TUDCA. Under these conditions, both sterol and phospholipid secretion rates approximated wild-type levels in the heterozygotes, resulting in a normalization of the sterol:phospholipid ratio in bile. This indicates that a high bile salt flux creates a situation in which biliary sterol and phospholipid excretion becomes less dependent of Abcg5 expression levels. Under the same conditions, biliary sterol- and phospholipid secretion in Abcg5\(^{-/-}\) mice did increase substantially, indicating that sterol secretion is inducible in the absence of Abcg5, but remained low when compared to Abcg5\(^{+/+}\) and Abcg5\(^{-/-}\) mice. It is tempting to speculate that the observed increase reflects an Abcg5/Abcg8-independent, but bile salt-dependent part of biliary cholesterol secretion that contributes approximately 20 % of maximal output under chow-fed conditions.

Infusion of the hydrophobic bile salt TDCA restored cholesterol secretion, but not phospholipid secretion, in mice lacking Mdr2 (Abcb4)\(^{33}\). TDCA infusions at low concentrations increased phospholipid and sterol secretion in Abcg5\(^{-/-}\) mice at the same relative rate as observed in wild-type mice, however, at a much lower absolute level. At higher infusion rates of TDCA, bile of Abcg5\(^{-/-}\) mice only turned red and the mice became cholestatic. It is likely that induction of cholestasis reflects deterrent effects of intraluminal TDCA exerted on canalicular membranes in the absence of sufficient amounts of cholesterol in the outer leaflet of the canalicular membrane. This rapid induction of cholestasis by TDCA may be considered as support, but definitively not as proof, for a floppase mode of action of the Abcg5/Abcg8 heterodimer, since the liftase model would predict cholesterol to be present in similar or even higher amounts in the outer leaflet of the canalicular membrane in Abcg5\(^{-/-}\) mice compared to wild-type mice. It should be realized, however, that the exact mechanisms of TDCA-induced cholestasis is not yet known.

In a series of classical papers\(^{38-41}\), Yousef and colleagues studied the effects of different bile salts on bile flow and biliary lipid composition in rats. Upon infusion of hydrophobic bile salts, typically phospholipid secretion declined first, followed by decreases in bile flow, bile salt output and cholesterol output. Concomitantly with the decline in phospholipid output, phospholipid composition changed from mainly phosphatidylcholine to more phosphatidylethanolamine and sphingomyelins, which was attributed to partial solubilization of the canalicular membrane\(^{41}\). Based on these results, these authors concluded that insufficient supply of phosphatidylcholine to the canalicular plasma membrane was the cause of bile salt-induced cholestasis. Our data from TDCA-infused Abcg5\(^{-/-}\) mice, however, differ in the kinetics of the process reported by Yousef et al.: in Abcg5\(^{-/-}\) mice, the maximal secretory rate for phospholipids and bile salts as well as the maximal bile flow rate were reached earlier than that of sterols. This would indicate that in the outer leaflet of the canalicular membrane, sterols were present - even in the absence of Abcg5 - which could be "dissolved" by hydrophobic micelles. However, the appearance of red bile suggests that the late increase in sterol excretion in the Abcg5\(^{-/-}\) mice may also be caused by hepatic micro-bleedings which could theoretically provide erythrocyte membranes as a source for the sterols measured in bile. In any case, our data demonstrate that the absence of a functional Abcg5/Abcg8 heterodimer renders the mouse susceptible to bile salt-induced cholestasis.

Treatment with the LXR agonist T0901317 has been demonstrated to increase expression of Abcg5, Abcg8, Abca1 and other genes involved in cholesterol transport\(^{11,15}\).
LXR activation dramatically increased sterol excretion in wild-type mice under basal conditions and upon infusion with TUDCA. However, no additional effect of LXR activation was observed in Abcg5-/- mice. Thus, the remaining sterol secretion in Abcg5-deficient mice is independent from LXR-activated systems. Interestingly, Sehayek and colleagues reported that Abcg5/Abcg8-independent loci regulate plasma plant sterol levels in mice. This further supports the hypothesis that other mechanisms than Abcg5/Abcg8-mediated transport exist to regulate sterol homeostasis.

To further substantiate the existence of Abcg5-independent cholesterol secretion, we examined a model in which the expression of this transporter remains unchanged upon loading of the liver with dietary cholesterol, i.e., the Lxra-/- mouse on a high cholesterol diet. Wild-type mice show a strong up-regulation of hepatic Abcg5/Abcg8 expression when challenged with a high cholesterol diet. This response is mediated via LXR and, consequently, abolished in Lxra-/- mice. Nevertheless, both wild-type and Lxra-/- mice showed a significant increase in hepatobiliary cholesterol excretion, independent of the Abcg5/Abcg8 expression level. This could either mean that the Abcg5/Abcg8 heterodimer is not rate-controlling under these conditions or that other routes compensate for this system under this particular stress. Increased hepatobiliary phospholipid excretion in Lxra-/- mice without induction of Mdr2/Abcb4 expression might indicate that enhanced micelle formation at the outer leaflet of the canalicular membrane could play a role under this particular circumstances.

Across various mouse models, a strong correlation exists between biliary cholesterol excretion and hepatic Abcg5/Abcg8 expression, with the notable exception of the diosgenin-treated mouse. Diosgenin is a plant sterol-like compound known to induce hypersecretion of cholesterol into bile, possibly dependent on PXR activation, but independent of the expression of Abcg5/Abcg8. In contrast to the mouse data published by Kosters et al., in human liver transplantation patients no relationship between (normalized) biliary cholesterol excretion and ABCG5 and ABCG8 expression was found, indicating at least relatively large contributions of Abcg5/Abcg8-independent cholesterol excretion in this specific patient population. In mice, absence of LXRs in combination with a high dietary cholesterol intake seems to add another model in which Abcg5/Abcg8 expression does not correlate with cholesterol excretion rates. We, therefore, favor the hypothesis that (an) LXR- and Abcg5/Abcg8-independent route(s) of biliary cholesterol secretion might come into play in specific situations.

Taken together, our studies support the notion that Abcg5/Abcg8 has rate-controlling function for the majority of hepatobiliary cholesterol transport in mice under "basal" conditions and that it may function as a floppase. However, a considerable fraction of cholesterol may reach the bile via an Abcg5/Abcg8-independent route, particularly when the secretory process is stimulated by "classical" approaches, i.e., bile salt infusion or cholesterol feeding. Thus, in other words, changes in hepatic Abcg5/Abcg8 expression alone do not always predict changes in the actual metabolic flux of interest, i.e., the hepatobiliary cholesterol secretion rate.
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