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

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Submitted 9 December 2005; accepted in final form 9 April 2006

MUTATIONS in the ATP binding-cassette (ABC) half-transporters ABCG5 and ABCG8 cause sitosterolemia (3, 23), which is characterized by the accumulation of plant sterols in the body (4). Data indicate that ABCG5 and ABCG8, which are highly expressed in the liver and small intestine, heterodimerize into a functional complex (3, 12). Mutations in either one of the genes cause the biochemical hallmarks of the disease in humans (3, 23) as well as in mouse models (18, 27). 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 amounts of plant sterols are absorbed in healthy subjects (14, 30). ABCG5/ABCG8 mediates the efflux of plant sterols from enterocytes back into the intestinal lumen and their excretion into bile, thus limiting their accumulation in the body (3, 15). The expression of the Abcg5 and Abcg8 genes is controlled by the liver X receptor (LXRα/NR1H3) and possibly by liver receptor homolog (LRH)-1 (8, 43). LXR is activated by oxysterols and hence is considered as a cellular “cholesterol sensor” (16).

Kosters et al. (21) have demonstrated that, across various mouse models, a strong correlation exists between biliary cholesterol excretion [normalized for bile salt (BS) and phospholipid (PL) excretion] and hepatic cholesterol secretion (21). Overexpression of Abcg5/Abcg8 in transgenic mice (42) or the induction of their expression via activation of LXR with synthetic ligands (27, 43) or dietary cholesterol feeding (29, 40) in wild-type mice is associated with strongly increased hepatobiliary cholesterol excretion. The deletion of these genes, either Abcg5 (27), Abcg8 (18), or both (41), in general has the opposite effects. Importantly, biliary cholesterol content is also reduced in heterozygote Abcg5+/−, Abcg8+/−, and Abcg5+/−/Abcg8+/− mice (18, 27, 41), 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 knockout mice [10–20% of wild-type values (40, 41)]. In addition, it appears that the diosgenin-induced hypersecretion of cholesterol in mice occurs in the absence of Abcg5/Abcg8 induction (20), although the presence of functional Abcg5/Abcg8 is required for the effect to occur (40). Of note, in most of the studies mentioned, the cholesterol content of gallbladder bile was determined rather than biliary output rates. In contrast to the mouse (21), recent data demonstrate that in human liver transplantation patients, no relationship exists between normalized biliary cholesterol excretion and hepatic ABCG5 and ABCG8 gene expression (11).

Thus it appears likely to assume that hepatobiliary cholesterol secretion can occur by both Abcg5/Abcg8-dependent and independent routes, with the former being (quantitatively) the most important one in mice under basal conditions. In this study, we used two strategies to address the quantitative contribution of both (putative) pathways under stressed condi-
tions, i.e., during the infusion of hydrophilic and hydrophobic BSs in control and LXR agonist-treated wild-type, Abcg5\(^{-/-}\), and Abcg5\(^{+/+}\) mice and upon feeding a high-cholesterol diet in wild-type and LXR-α-deficient (Lxra\(^{-/-}\)) mice. The data from these experiments provide evidence to suggest that, under certain metabolic conditions, Abcg5/Abcg8-independent routes significantly contribute to the 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 (Abcg5\(^{+/-}\)) littermates were used (27). Animals were housed in temperature-controlled rooms (21°C) with 12:12-h light-dark cycling and received standard mouse chow (Arie Blok; Woerden, The Netherlands) and water ad libitum. The diet contained 0.017% (wt/wt) cholesterol and 0.045% (wt/wt) plant sterols.

Lxra\(^{-/-}\) mice, generated by Deltagen using standard gene-targeting methods, were kindly provided by Tularik (San Francisco, CA). In short, a 42-bp fragment corresponding to a segment of exon 2 was replaced by β-galactosidase cDNA and a phosphoglycerase kinase promoter-driven neomycin resistance cassette. The remaining procedure followed that described for the Abcg5\(^{-/-}\) mice (27). Mice were genotyped via PCR using allele-specific primers (wild type: 5’-GGTCTTCCTCCCTATCTATAGGGAGAC-3’ and 5’-CACCTC-ATTCTCATGTCCTTCTT-3’; knockout: 5’-GGGCCAGCTCA-TTCCTCCCCACTCAT-3’). Mice homozygous (Lxra\(^{-/-}\)) and heterozygous (Lxra\(^{+/+}\)) for the disruption of the Lxra gene and their wild-type (Lxra\(^{+/-}\)) littermates received either standard mouse chow or chow diet containing 1% (wt/wt) cholesterol (Arie Blok) for 2 wk. Male mice of 2–4 mo were used.

All experimental procedures were approved by the local Ethical Committee for Animal Experiments. Abcg5\(^{-/-}\) mice. Abcg5\(^{+/+}\) and wild-type mice were fed either standard laboratory chow or chow supplemented with the synthetic LXR agonist T0901317 [0.015% (wt/wt), Cayman Chemicals; Ann Arbor, MI] for 7 days. Livers were excised and weighed. Aliquots were snap-frozen in liquid nitrogen and stored at –80°C for biochemical analyses and RNA isolation.

**Biliary Procedures**

Biliary BS concentrations were measured enzymatically (24). Biliary PL and sterol concentrations in the Abcg5 experiments were determined as described previously (9). No distinction was made between cholesterol and plant sterols, because enzymatic cholesterol assays have been found to measure both (25). In Lxra mice, PLs and cholesterol in bile were determined as described by Böttcher et al. (6) and Gamble et al. (10), respectively, after the extraction according the method described by Bligh and Dyer (5). 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 (36).

**RNA Isolation and PCR Procedures**

Total RNA was extracted from frozen tissues with TriReagent (Sigma; St. Louis, MO) and quantified photometrically. cDNA synthesis was performed using recombinant Moloney murine leukemia virus reverse transcriptase (10 U/µl), the appropriate buffer, dNTPs (500 µM), random nonamers (1 µM), RNAsin inhibitor (2 U/µl; all from Sigma), and total RNA (10 ng/µl). The reaction mix was incubated for 10 min at 25°C for primer annealing, 60 min at 37°C for synthesis, and 5 min at 94°C to denature the reverse transcriptase enzyme. Real-time quantitative PCR was performed using an Applied Biosystems 7700 sequence detector as previously described (28). Primers were obtained from Invitrogen. Fluorogenic probes, labeled with 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine ( TAMRA), were made by Eurogentec (Seraing, Belgium). Primers and probes used in these experiments have been described elsewhere [sterol regulatory element binding protein (Srebp)1a, Srebp1c, Srebp2, Lxra, scavenger receptor B (Srb1), acyl-coenzyme A: cholesterol acyltransferase (Acat)1, Acat2, 3-hydroxy-3-methylglutaryl-CoA reductase (Hmgcr), cytochrome P-450 (Cyp7a1, Cyp27, Abca1, Abcg5, Abcg8, multidrug resistance protein (Mdr)2, BS export protein (Bsep), and 18S RNA (28); β-actin and LDL receptor (Ldlr) (13); Abcg1 and Abcg2 (22); Niemann-Pick disease-1 (Npc1)-like 1 (Npc1l1) and 36b4 (33); and Mrp2 (Abcc2) (19); with the exception of cyclophilin (XM_356256; forward 5’-CAGATCGAGGGATCGAT-3’; reverse 5’-TCACACCTTGACACCCCTTACC-3’; and probe 5’-CTCCTCCACATTGGAGACAAGATGCA-3’)]. All data of the Abcg5 experiments were subsequently normalized to the median of β-actin, 36b4, 18S RNA, and cyclophilin as described by Vandesompele et al. (34). In the Lxra experiments, β-actin alone was used for normalization.

**Statistics**

Statistical analyses were performed using SPSS 10.1 for Windows (SPSS; Chicago, IL). Differences between genotypes were evaluated using the Mann-Whitney U-test. A P value of <0.05 was considered as statistically significant.

**RESULTS**

**Plasma and Hepatic Lipid Composition in Abcg5\(^{-/-}\) Mice**

It has been previously reported (27) that Abcg5\(^{-/-}\) mice have elevated plasma triglyceride levels compared with 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). The total sterol (cholesterol + plant) distribution was virtually identical in the two genotypes and almost exclusively present in the HDL-sized fractions.

As previously reported (27), liver weights were slightly increased in Abcg5<sup>−/−</sup> mice compared with wild-type littermates (Table 1). This was not due to steatosis, because triglyceride concentrations were not increased in the knockout mice. The concentrations of PLs as well as those of total sterols and unesterified sterols were almost identical between all groups. Only sterol ester concentrations in Abcg5<sup>−/−</sup> mice were reduced by 45% compared with wild-type and heterozygous mice. 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 the livers of Abcg5<sup>−/−</sup> mice, whereas plant sterols are present in low amounts in the livers of wild-type mice.

Table 1. Liver parameters of male Abcg5<sup>+/+</sup>, Abcg5<sup>−/−</sup>, and Abcg5<sup>+/−</sup> mice on the chow diet

<table>
<thead>
<tr>
<th></th>
<th>Abcg5&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Abcg5&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Abcg5&lt;sup&gt;+/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio of liver weight to body weight</td>
<td>0.049±0.002</td>
<td>0.049±0.004</td>
<td>0.058±0.002*</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*</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>

Values are means ± SD; n = 5–7 mice/group. Male Abcg5<sup>−/−</sup>, Abcg5<sup>+/−</sup>, and littermate control Abcg5<sup>+/+</sup> mice, (5–7 mo old) were fed standard chow diet measured (WT) and ATP-binding cassette half-transporter Abcg5 knockout (KO; Abcg5<sup>−/−</sup>) mice. Blood was collected via cardiac puncture and pooled before fast protein liquid chromatography (FPLC) analysis. Analysis was performed as described in MATERIALS AND METHODS. VLDL, very-low-density lipoprotein; IDL/LDL, intermediate-density lipoprotein; LDL, high-density lipoprotein; ctrl, control.

Table 2. Hepatic mRNA expression levels in male Abcg5<sup>+/+</sup>, Abcg5<sup>−/−</sup>, and Abcg5<sup>+/−</sup> mice on the chow diet measured by real-time RT-PCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Abcg5&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Abcg5&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Abcg5&lt;sup&gt;+/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Srebp1a</td>
<td>1.00±0.11</td>
<td>1.11±0.11</td>
<td>1.06±0.12</td>
</tr>
<tr>
<td>Srebp1c</td>
<td>1.00±0.41</td>
<td>1.08±0.29</td>
<td>0.95±0.42</td>
</tr>
<tr>
<td>Srebp2</td>
<td>1.00±0.14</td>
<td>1.08±0.35</td>
<td>0.78±0.17</td>
</tr>
<tr>
<td>Lxra</td>
<td>1.00±0.08</td>
<td>0.99±0.12</td>
<td>1.05±0.08</td>
</tr>
<tr>
<td>Ldlr</td>
<td>1.00±0.11</td>
<td>1.18±0.24</td>
<td>0.78±0.28</td>
</tr>
<tr>
<td>Srb1</td>
<td>1.00±0.20</td>
<td>1.06±0.19</td>
<td>0.98±0.10</td>
</tr>
<tr>
<td>Hmgcr</td>
<td>1.00±0.35</td>
<td>1.36±0.56</td>
<td>0.50±0.23&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acatl</td>
<td>1.00±0.11</td>
<td>0.94±0.25</td>
<td>1.19±0.20</td>
</tr>
<tr>
<td>Acat2</td>
<td>1.00±0.18</td>
<td>1.24±0.28</td>
<td>0.89±0.32</td>
</tr>
<tr>
<td>Cyp7a1</td>
<td>1.00±0.59</td>
<td>0.85±0.66</td>
<td>1.82±0.59</td>
</tr>
<tr>
<td>Cyp27</td>
<td>1.00±0.13</td>
<td>1.24±0.14</td>
<td>1.22±0.22</td>
</tr>
<tr>
<td>Abcg8</td>
<td>1.00±0.19</td>
<td>1.41±0.44</td>
<td>1.26±0.37</td>
</tr>
<tr>
<td>Abca1</td>
<td>1.00±0.12</td>
<td>1.01±0.09</td>
<td>1.24±0.11&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Abcg1</td>
<td>1.00±0.36</td>
<td>0.98±0.21</td>
<td>1.04±0.13</td>
</tr>
<tr>
<td>Abcg2</td>
<td>1.00±0.29</td>
<td>0.96±0.19</td>
<td>0.98±0.20</td>
</tr>
<tr>
<td>Npc1</td>
<td>1.00±0.33</td>
<td>0.91±0.43</td>
<td>1.19±0.20</td>
</tr>
<tr>
<td>Npc1l1</td>
<td>1.00±0.13</td>
<td>0.97±0.60</td>
<td>1.21±0.26&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bsep</td>
<td>1.00±0.18</td>
<td>1.00±0.13</td>
<td>0.99±0.05</td>
</tr>
<tr>
<td>Mrp2</td>
<td>1.00±0.32</td>
<td>1.37±0.43</td>
<td>1.26±0.36</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 5–8 mice/group. All data were normalized to the median of β-actin, 36b4, 18S rRNA, and cyclophilin as described by Vandesompele et al. (34). Male Abcg5<sup>+/+</sup>, Abcg5<sup>−/−</sup>, and littermate control Abcg5<sup>+/−</sup> mice, (5–7 mo 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 analyzed as described in MATERIALS AND METHODS. No distinction was made between cholesterol and other sterols. *Significant difference from the wild-type group (Mann-Whitney U-test, P < 0.05).

Hepatic Gene Expression

In male mice, hepatic gene expression was determined by real-time RT-PCR (Table 2). mRNA levels of regulatory genes (Srebp1a, Srebp1c, Srebp2, and Lxra) were identical in all groups. The 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 Srb1 protein levels were not different between genotypes (data not shown). On the other hand, the expression of cholesterol metabolism genes in the liver was different in Abcg5<sup>−/−</sup> mice compared with wild-type and Abcg5<sup>+/−</sup> mice: the expression of Hmgcr, which is a rate-controlling enzyme for cholesterol synthesis, was reduced by 50% in Abcg5<sup>−/−</sup> mice. The expression of Cyp7a1, encoding the enzyme responsible for the majority of BS synthesis, was upregulated by 82% in Abcg5<sup>−/−</sup> mice, whereas that of Cyp27 was unchanged. The expression levels of Acatl and Acat2, which are involved in cholesteryl ester formation, were not impaired in Abcg5<sup>−/−</sup> and Abcg5<sup>+/−</sup> mice compared with wild-type controls.

The gene expression of a wide spectrum of transporters potentially involved in cholesterol transport was screened: only the expression of Acatl (+ 24%) and Npc1l1 (+ 41%) showed significant increases in Abcg5<sup>−/−</sup> mice compared with their wild-type littermates. The expression of Ablg1, Ablg2, Ablg5, Npc1, Mrp2, and Bsep were identical in all three genotypes. As anticipated, Abcg5 mRNA was virtually absent (5% mRNA remaining compared with wild-type mice) in Abcg5<sup>−/−</sup> mice.
Surprisingly, Abcg5 expression in heterozygous mice was 76% of wild-type values. No effect on the expression of the PL flippase Mdr2 (Abcb4) was noticed (Fig. 2B).

**Biliary Sterol and PL Secretion Rates Are Decreased in Abcg5<sup>−/−</sup> Mice**

Abcg8<sup>−/−</sup> mice have been reported to have decreased hepatobiliary cholesterol and PL secretion rates (18), whereas for Abcg5<sup>−/−</sup> mice only gallbladder concentration data are available (27). Therefore, hepatic bile was collected from Abcg5<sup>+/+</sup>, Abcg5<sup>−/−</sup>, and Abcg5<sup>−/−</sup> mice for 15 min immediately after the creation of a gallbladder fistula to determine basal biliary lipid output rates. Abcg5 knockout mice presented with a significantly increased bile flow compared with heterozygotes and wild-type littermates (8.9 ± 2.3 µl·min<sup>−1</sup>·100 g body wt<sup>−1</sup> in Abcg5<sup>−/−</sup> mice vs. 6.6 ± 1.5 and 5.0 ± 2.9 µl·min<sup>−1</sup>·100 g body wt<sup>−1</sup> in Abcg5<sup>+/+</sup> and Abcg5<sup>−/−</sup> mice, respectively). Hepatobiliary sterol (Fig. 2C) and PL excretion rates (Fig. 2D) were significantly decreased in both heterozygous and homozygous knockout mice compared with wild-type controls. BS output rates were unaffected, i.e., 325 ± 121, 309 ± 198, and 332 ± 164 nmol·min<sup>−1</sup>·100 g body wt<sup>−1</sup> in Abcg5<sup>+/+</sup>, Abcg5<sup>−/−</sup>, and Abcg5<sup>−/−</sup> mice, respectively. Moreover, gas-chromatographic analysis revealed that the biliary BS composition was not significantly different among Abcg5<sup>+/+</sup>, Abcg5<sup>−/−</sup>, and Abcg5<sup>−/−</sup> mice (data not shown).

**Biliary Lipid Excretion Increases Upon Infusion With TUDCA in Abcg5<sup>−/−</sup> and Abcg5<sup>+/−</sup> Mice**

The systemic infusion of hydrophilic BSs increases bile flow and facilitates hepatobiliary lipid secretion in wild-type mice (26). To investigate whether this forced flow could restore impaired PL and sterol secretion in Abcg5<sup>−/−</sup> mice, we infused Abcg5<sup>+/+</sup>, Abcg5<sup>−/−</sup>, and Abcg5<sup>−/−</sup> mice with increasing concentrations of TUDCA. Bile flow was increased in Abcg5<sup>−/−</sup> mice compared with wild-type and heterozygous mice already under basal conditions. This effect was even more pronounced at higher infusion rates (data not shown).

As depicted in Fig. 3, PL excretion increased upon TUDCA infusion in all genotypes (+140%, +280%, and +150% in Abcg5<sup>+/+</sup>, Abcg5<sup>−/−</sup>, and Abcg5<sup>−/−</sup> mice, respectively, during the infusion of the highest dose of TUDCA). In parallel, hepatobiliary sterol excretion also increased in all strains (+230%, +700%, and +120% in Abcg5<sup>+/+</sup>, Abcg5<sup>−/−</sup>, and Abcg5<sup>−/−</sup> mice, respectively). Of note, the initially low sterol excretion rate in Abcg5<sup>−/−</sup> mice recovered upon the infusion of TUDCA to reach wild-type levels. As a consequence, the sterol-to-PL ratios in Abcg5<sup>−/−</sup> mice and Abcg5<sup>−/−</sup> mice were significantly lower than in wild-type mice at the basal level, but, upon the infusion with TUDCA, the ratios normalized in Abcg5<sup>−/−</sup> mice but not in Abcg5<sup>−/−</sup> mice.

**Biliary Lipid Excretion Increases Upon Infusion With TDCA in Abcg5<sup>−/−</sup> and Abcg5<sup>+/−</sup> Mice**

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

Both PL excretion curves and sterol excretion curves were lower in Abcg5<sup>−/−</sup> mice compared with wild-type controls. However, both PL (3.5 times) and sterol (2.4 times) excretion increased in Abcg5<sup>−/−</sup> mice upon the 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 PLs and 3.5 times for sterols).
Upon the infusion with TUDCA, PL secretion rates more than doubled in all groups. The maximal excretory rate for sterols went up four times in wild-type mice treated with the LXR agonist compared with nontreated wild-type mice. However, in Abcg5<sup>−/−</sup> mice, no differences were observed between LXR-treated and nontreated mice. Figure 5 shows the effect of LXR activation in Abcg5<sup>−/−</sup> mice and wild-type littermates. The sterol-to-BS ratio was clearly increased in wild-type mice upon treatment with T0901317, whereas the relationships between BS and cholesterol output in untreated or T0901317-treated Abcg5<sup>−/−</sup> mice were similar.

**Cholesterol Feeding Increases Hepatobiliary Cholesterol Excretion in Lxra<sup>+/+</sup> and Lxra<sup>−/−</sup> Mice**

To test whether the reported increase in hepatobiliary cholesterol excretion upon cholesterol feeding is LXR (and Abcg5/Abcg8) dependent, Lxra<sup>−/−</sup>, Lxra<sup>−/+</sup>, and wild-type control mice were fed a diet containing 1% cholesterol to increase the delivery of cholesterol to the liver. Hepatic sterol(ester) contents increased to similar levels in wild-type and heterozygous mice but were much stronger in Lxra<sup>−/−</sup> mice. As anticipated (29), the hepatic expressions of Abcg5 and Abcg8 were increased in control and heterozygous mice fed a high-cholesterol diet (Fig. 6). In Lxra<sup>−/−</sup> mice fed a high-cholesterol diet, Abcg5 and Abcg8 expressions did not differ from those in chow-fed Lxra<sup>−/−</sup> mice. Bile flow and biliary BS and PL excretion rates did not differ between the groups. Surprisingly, hepatobiliary cholesterol excretion was increased in all mice fed a high-cholesterol diet regardless of genotype (Fig. 6).

**Hepatobiliary Cholesterol Excretion in Lxra<sup>−/−</sup> Mice Is Correlated With Increased PL 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<sup>−/−</sup> and wild-type mice fed a high-cholesterol diet with increasing amounts of TUDCA to determine the maximal secretory rates for biliary lipids. As shown in Fig. 7, bile flows were slightly higher in Lxra<sup>−/−</sup> mice than in littermate controls, whereas the BS output was identical. Unexpectedly, hepatobiliary cholesterol secretion rates were identical in Lxra<sup>−/−</sup> and wild-type mice even at the highest TUDCA infusion rate applied. Moreover, the hepatobiliary excretion of PLs was up to 200% increased in Lxra<sup>−/−</sup> mice compared with wild-type littermates. As a result, the cholesterol-to-PL ratio indicated a relative hypersecretion in wild-type mice (ratio, 0.176) compared with Lxra<sup>−/−</sup> mice (ratio, 0.088).

The mRNA expression of Mdr2/[Abcb4], the gene coding for the canalicular PL flippase Mdr2 P-glycoprotein, was measured in liver tissues of Lxra<sup>−/−</sup>, Lxra<sup>−/+</sup>, and wild-type mice fed chow and high-cholesterol diets. All groups but that of Lxra<sup>−/−</sup> mice fed a high-cholesterol diet showed similar expression levels; in Lxra<sup>−/−</sup> mice, however, Mdr2 expression was reduced to 60% of the wild-type level (data not shown).

**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 (3, 23) 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 (18, 27, 41), which was taken to indicate that Abcg5 and Abcg8 function as a heterodimer in the excretion process. The fact that heterozygous knockout mice and rodents in which Abcg5/Abcg8 expression is modulated (27, 29, 40, 42, 43) show a clear phenotype for biliary cholesterol excretion underscores the important role of the Abcg5/Abcg8 heterodimer in the 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 (37), 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 (BS-facilitated) excretion. An alternative model by Small (32) 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 BSs 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 (cholesterol) secretion under conditions of (maximal) induction of this process.

First, it was demonstrated that sterols present in plasma of Abcg5/Abcg8 mice, like in wild-type mice, are almost exclusively present in HDL-sized fractions. Furthermore, hepatic mRNA and protein expression of the HDL receptor Srb1 was not affected by Abcg5 deficiency. This excludes differences in the “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 (13, 17) leads to a differential processing by livers from wild-type and knockout mice.

In a very recent study (40), Yu and colleagues have elegantly demonstrated that gallbladder cholesterol concentrations correlate with Abcg5/Abcg8 expression levels in transgenic mice containing 1, 10, or 16 copies of Abcg5/Abcg8. Likewise, Kosters et al. (21) have demonstrated a high correlation between “normalized” biliary cholesterol secretion and hepatic Abcg5/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, Abcg5+/−, and Abcg5−/− mice. We recently reported that cholesterol concentrations in the gall-
bladder of Abcg5\(^{-/-}\) mice are reduced by 50% compared with wild-type mice (27). 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 BSs are not shielded sufficiently by PLs and cholesterol.

Both hepatobiliary PL and sterol secretion were impaired under basal, nonstimulated conditions in Abcg5\(^{+/+}\) and Abcg5\(^{-/-}\) mice. The hepatic expression of Abcg5 was reduced by only 24% in the heterozygotes, indicative for compensatory upregulation of transcription of the remaining allele, whereas that of Mdr2 (Abcb4) was not affected. The relatively small reduction of Abcg5 expression in heterozygotes was associated with a reduction by \(-60\%\) of biliary sterol secretion. Therefore, it is reasonable to conclude that, under these conditions, Abcg5 expression controls a large fraction of hepatobiliary sterol secretion and partly, either directly or indirectly, also influences PL secretion. It is tempting to speculate that the reduced PL secretion associated with Abcg5 deficiency is a secondary phenomenon, most likely related to an altered canalicular membrane structure or biliary micelle composition. It is well established that, under physiological circumstances, biliary PL and cholesterol concentrations are coupled to those of BSs (35). To exclude the possibility that changes in biliary BS composition contributed to the reduced PL and cholesterol excretion rates, the BS composition was determined. It is noteworthy that the biliary BS compositions were indistinguishable between the three genotypes as were the expressions of the canalicular BS transporters Bsep (Abcb11) and Mrp2 (Abcc2).

The high degree of control on biliary excretion was overcome when biliary BS secretion was stimulated by an infusion of the hydrophilic BS TUDCA. Under these conditions, both sterol and PL secretion rates approximated wild-type levels in
the heterozygotes, resulting in a normalization of the sterol-to-PL ratio in bile. This indicates that a high BS flux creates a situation in which biliary sterol and PL excretion becomes less dependent of Abcg5 expression levels. Under the same conditions, biliary sterol and PL secretion in Abcg5<sup>−/−</sup> mice did increase substantially, indicating that sterol secretion is inducible in the absence of Abcg5, but remained low compared with Abcg5<sup>+/+</sup> and Abcg5<sup>+/−</sup> mice. It is tempting to speculate that the observed increase reflects an Abcg5/Abcg8-independent, but BS-dependent, part of biliary cholesterol secretion that contributes ~20% of maximal output under chow-fed conditions.

Infusion of the hydrophobic BS TDCAs restored cholesterol secretion, but not PL secretion, in mice lacking Mdr2 (Abcb4) (26). TDCAs infusions at low concentrations increased PL and sterol secretion in Abcg5<sup>−/−</sup> mice at the same relative rates as observed in wild-type mice, however, at a much lower absolute level. At higher infusion rates of TDCAs, the bile of Abcg5<sup>−/−</sup> mice only turned red and the mice became cholestatic. It is likely that the induction of cholestasis reflects detergent effects of intraluminal TDCAs 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 TDCAs may be considered as support, but definitively not as proof, for a floppase mode of action of the Abcg5/Abcg8 heterodimer, because the flipase model would predict cholesterol to be present in similar or even higher amounts in the outer leaflet of the canalicular membrane in Abcg5<sup>−/−</sup> mice compared with wild-type mice. It should be realized, however, that the exact mechanisms of TDCA-induced cholestasis are not yet known.

In a series of classical papers (1, 2, 38, 39), Yousef and colleagues studied the effects of different BSs on bile flow and biliary lipid composition in rats. Upon the infusion of hydrophobic BSs, typically PL secretion declined first, followed by decreases in bile flow, BS output, and cholesterol output. Concomitantly with the decline in PL output, the PL composition changed from mainly phosphatidylcholine to more phosphatidylethanolamine and sphingomyelins, which was attributed to partial solubilization of the canalicular membrane (38). On the basis of these results, these authors concluded that an insufficient supply of phosphatidylcholine to the canalicular plasma membrane was the cause of BS-induced cholestasis. Our data from TDCA-infused Abcg5<sup>−/−</sup> mice, however, differ in the kinetics of the process reported by Yousef et al.; in Abcg5<sup>−/−</sup> mice, the maximal secretory rate for PLs and BSs 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 Abcg5<sup>−/−</sup> mice may also be caused by hepatic microbleedings, 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 BS-induced cholestasis.

Treatment with the LXR agonist T0901317 has been demonstrated to increase the expressions of Abcg5, Abcg8, Abca1, and other genes involved in cholesterol transport (29, 43). 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<sup>−/−</sup> mice. Thus the remaining sterol secretion in Abcg5-deficient mice is independent from LXR-activated systems. Interestingly, Sehayek and colleagues (31) 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 remained unchanged upon loading of the liver with dietary choleseterol, i.e., the Lxra<sup>−/−</sup>-mouse fed a high-cholesterol diet. Wild-type mice show a strong upregulation of hepatic Abcg5/Abcg8 expression when challenged with a high-cholesterol diet (29). This response is mediated via LXR and, consequently, abolished in Lxra<sup>−/−</sup>-mice. Nevertheless, both wild-type and Lxra<sup>−/−</sup>-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 PL excretion in Lxra<sup>−/−</sup>-mice without the 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 circumstance.

Across various mouse models, a strong correlation exists between biliary cholesterol excretion and hepatic Abcg5/Abcg8 expression (21), with the notable exception of the dioxigenin-treated mouse. Diosgenin is a plant sterol-like compound known to induce hypersecretion of cholesterol into bile (7), possibly dependent on pregnane X receptor activation, but independent of the expression of Abcg5/Abcg8 (20). In contrast to the mouse data published by Kosters et al. (21), in human liver transplantation patients, no relationship between (normalized) biliary cholesterol excretion and ABCG5 and ABCG8 expression was found (11), indicating at least relatively large contributions of Abcg5/Abcg8-independent cholesterol excretion in this specific patient population. In mice, the absence of LXR-α 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 LXR- and Abcg5/Abcg8-independent route(s) of biliary cholesterol secretion might come into play in specific situations.

Taken together, our results support the notion that Abcg5/Abcg8 has a 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., BS 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.

**ACKNOWLEDGMENTS**

We thank Juul F. W. Baller and Renze Boverhof for excellent technical assistance and Dr. A. K. Groen for helpful discussions.
Parts of this study have been presented at the annual meeting of the American Association for the Study of Liver Diseases in Boston, MA, in 2004 and published in abstract form.

GRANTS

This study was supported by The Netherlands Organization for Scientific Research Grant 912-02-063. T. Plösch is the recipient of a Dr. Dekker fellowship from the Dutch Heart Foundation (2004T048).

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