Diosgenin stimulation of fecal cholesterol excretion in mice is not NPC1L1 dependent

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Abstract Diosgenin exists in some food supplements and herbal medicines and lowers plasma cholesterol by increasing fecal cholesterol excretion. It is believed that diosgenin promotes fecal cholesterol excretion by stimulating biliary cholesterol secretion and decreasing intestinal cholesterol absorption. Niemann-Pick C1-like 1 (NPC1L1) was recently identified as an essential protein for intestinal cholesterol absorption. To determine the relative contribution of biliary secretion and intestinal absorption of cholesterol in diosgenin-stimulated fecal cholesterol excretion, wild-type (WT) and NPC1L1-knockout (L1KO) mice were fed a diet with or without 1% diosgenin. Fecal cholesterol excretion (µmol/day/100 g body weight) increased in diosgenin-fed WT and L1KO mice from 4.2 to 52 and from 63 to 140, respectively. Surprisingly, this increase in diosgenin-treated versus untreated L1KO mice (77) was even greater than that seen in diosgenin-treated versus untreated WT mice (47.8). Additionally, WT and L1KO mice fed the diosgenin diet had similar increases in biliary cholesterol concentration, despite unaltered hepatic expression of the hepatobiliary cholesterol transporter, ATP binding cassette transporters G5 and G8. Facilitated cholesterol excretion in diosgenin-treated WT and L1KO mice was associated with decreased hepatic and plasma cholesterol and increased liver expression of cholesterol synthetic genes. In contrast, diosgenin had no effect on the intestinal expression of NPC1L1 and cholesterol synthetic genes. In an in vitro assay, diosgenin was unable to block NPC1L1-dependent cholesterol uptake. In conclusion, diosgenin stimulation of fecal cholesterol excretion is independent of NPC1L1-mediated cholesterol absorption.—Temel, R. E., J. M. Brown, Y. Ma, W. Tang, L. L. Rudel, Y. A. Ioannou, J. P. Davies, and L. Yu. Diosgenin stimulation of fecal cholesterol excretion in mice is not NPC1L1 dependent. J. Lipid Res. 2009, 50: 915−923.

Supplementary key words bile • intestine • feces

The tubers of Dioscorea, commonly known as yams, have been used for centuries as herbal medicine for the treatment of various conditions, including infections, cancer, diabetes, and rheumatism (1). One pharmacologically active compound found in many Dioscorea species is diosgenin, a steroidal sapogenin. When fed to rats, diosgenin is absorbed, although at a level that is 5- to 10-fold less than that of cholesterol, and accumulates at the highest concentrations in the adrenals, liver, and gastrointestinal tract (2). Through a poorly defined mechanism, diosgenin can dramatically and selectively increase the secretion of biliary cholesterol but not phospholipids or bile salts (3–5). The heterodimer of two ATP binding cassette half-transporters G5 and G8 (ABCG5/G8), which mediates the normal hepatobiliary secretion of cholesterol (6–8), is essential for diosgenin-stimulated biliary cholesterol secretion. However, we and others (9, 10) have shown that diosgenin has no effect on hepatic ABCG5/G8 expression at both the mRNA and protein level. The nuclear receptor pregnane X receptor (PXR) also appears to be important because its genetic disruption moderately attenuates diosgenin-stimulated biliary cholesterol secretion (9).

In addition to biliary cholesterol secretion, many other aspects of hepatic cholesterol metabolism are altered by diosgenin. In rats fed diosgenin, cholesterol synthesis is significantly increased in liver homogenates, isolated hepatocytes, and whole liver in vivo (3, 4, 11). Diosgenin treatment of rats fed a high-cholesterol diet blocks the accumulation of cholesterol in the liver (3, 12). Moreover, ACAT activity is reduced by 30% in isolated hepatocytes and 40% in liver microsomes isolated from rats consuming diosgenin (4, 13). These findings indicate that diosgenin profoundly impacts hepatic cholesterol metabolism.

Abbreviations: ABC, ATP binding cassette transporter; EGFP, enhanced green fluorescent protein; HMGCR, HMG-CoA reductase; HMGCS, HMG-CoA synthase; HPβCD, hydroxypropyl-β-cyclodextrin; L1KO, Niemann-Pick C1-Like 1-knockout; MβCD, methyl-β-cyclodextrin; NPC1L1, Niemann-Pick C1-Like 1; PXR, pregnane X receptor; qPCR, quantitative PCR; SREBP2, sterol regulatory element binding protein 2; WT, wild-type.

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Many of the effects of diosgenin on hepatic cholesterol metabolism, such as increased cholesterol synthesis and decreased hepatic cholesterol content, are believed to stem from its impact on cholesterol absorption. In mice and rats treated with diosgenin, fractional cholesterol absorption is significantly decreased and fecal cholesterol excretion is significantly increased (3, 14). One protein that has a major role in cholesterol absorption is Niemann-Pick C1-Like 1 (NPC1L1). NPC1L1 is a sterol-sensing domain-containing polytopic transmembrane protein (15) that is highly expressed on the apical surface of absorptive enterocytes (16–18). Genetic inactivation of NPC1L1 in mice causes a 70–80% reduction in cholesterol absorption, a phenotype similar to that seen in mice treated with a potent cholesterol absorption inhibitor ezetimibe (16, 17). A biochemical study showed that NPC1L1 is the target of ezetimibe (19). In addition, cells transfected with NPC1L1 display increased ezetimibe-sensitive uptake of free cholesterol from the culture media (20–23). Thus, it is believed that NPC1L1 facilitates the movement of free cholesterol from the gut lumen into enterocytes.

To determine the relative contribution of biliary secretion and intestinal absorption of cholesterol in diosgenin-stimulated fecal cholesterol excretion, wild-type (WT) and NPC1L1-knockout (L1KO) mice were fed a synthetic diet with or without 1% diosgenin. Unexpectedly, we found that diosgenin promotes fecal cholesterol excretion independently of NPC1L1-mediated cholesterol absorption.

MATERIALS AND METHODS

Animals and diets

L1KO mice have been described previously (24). WT and L1KO mice with a pure C57BL/6 background were housed in a specific pathogen-free animal facility in plastic cages in a temperature-controlled room (22°C) with a daylight cycle from 6AM to 6PM. The mice were fed ad libitum a standard rodent chow diet (5P00Prolab; LabDiet) prior to diosgenin feeding and had free access to water. At 12–13 weeks of age, female WT and L1KO mice were fed a synthetic low-fat (10% of energy as palm enriched fat), low-cholesterol (0.015% w/w) diet supplemented without or with 1% diosgenin. Unexpectedly, we found that diosgenin promotes fecal cholesterol excretion independently of NPC1L1-mediated cholesterol absorption.

TABLE 1. Composition of experimental diets based on dry weight

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Control Diet</th>
<th>Diosgenin Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palm oil</td>
<td>4.0000</td>
<td>4.0000</td>
</tr>
<tr>
<td>Fish oil</td>
<td>0.2000</td>
<td>0.2000</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>35.0000</td>
<td>35.0000</td>
</tr>
<tr>
<td>Dextrin</td>
<td>17.0000</td>
<td>17.0000</td>
</tr>
<tr>
<td>Sucrose</td>
<td>17.0000</td>
<td>17.0000</td>
</tr>
<tr>
<td>Casein</td>
<td>8.0000</td>
<td>8.0000</td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>4.0000</td>
<td>4.0000</td>
</tr>
<tr>
<td>Alphacel</td>
<td>7.2687</td>
<td>6.2688</td>
</tr>
<tr>
<td>β-Sitosterol</td>
<td>0.0103</td>
<td>0.0103</td>
</tr>
<tr>
<td>Hegsted salts</td>
<td>5.0000</td>
<td>5.0000</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>2.5000</td>
<td>2.5000</td>
</tr>
<tr>
<td>MTS-50 (vitamin E)</td>
<td>0.0191</td>
<td>0.0191</td>
</tr>
<tr>
<td>Tenox 20A</td>
<td>0.0018</td>
<td>0.0018</td>
</tr>
<tr>
<td>Diosgenin</td>
<td>0.0000</td>
<td>1.0000</td>
</tr>
<tr>
<td>Totals</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Measurement of hepatic, plasma, and biliary lipid concentrations

After a 4 h fast, gallbladder bile and blood were collected from mice that had been fed the synthetic diets for 14 d. The liver was removed and snap-frozen in liquid nitrogen. Plasma total cholesterol concentration was determined using the Cholesterol/HP enzymatic assay kit (Roche). To determine plasma lipoprotein cholesterol distribution, plasma lipoprotein classes were separated by gel filtration chromatography as described previously (26).

For analysis of liver cholesterol concentration, ~100 mg of liver was placed into a glass tube containing 104 μg 5α-cholestan. Lipids were extracted in 2:1 CHCl₃:methanol at room temperature overnight. Total and free cholesterol mass was measured by gas-liquid chromatography as described previously (27). The extracted piece of liver was solubilized in 1 N NaOH, and the protein content of the lysate was determined using a modified Lowry assay (28).

For analysis of biliary lipid concentrations, a measured volume (5–10 μl) of bile was extracted by sequential addition of 2:1 CHCl₃:MeOH, CHCl₃, and water. A measured aliquot of the organic phase was dried down and then analyzed for cholesterol and phospholipid content by solubilizing the lipids in Triton X-100/water (25) and employing the Cholesterol/HP (Roche) and Phospholipids B (Wako) enzymatic assay kits. The aqueous phase was analyzed for bile acid content using an enzymatic assay employing hydroxycholesterol dehydrogenase (25).

Determination of diosgenin effects on ACAT activity

Microsomes were isolated from snap-frozen liver and resuspended in ACAT buffer (0.1 M K₂PO₄, pH 7.4) as described previously (29). In a final volume of 300 μl ACAT buffer, microsomes (100 μg protein) were mixed with fatty acid free BSA (1 mg) and either water or cholesterol solubilized in hydroxypropyl-β-cyclodextrin (Chol-HPβCD), such that the exogenous cholesterol concentration was 0.5 nmol cholesterol/μg microsomal protein. The mixture was incubated for 30 min in a 37°C shaking water bath, followed by the addition of 30 nmol [1-14C]oleoyl CoA (Amersham Biosciences). After a 20 min incubation in a 37°C shaking water bath, the reaction was terminated by adding 6 ml of CHCl₃:MeOH (2:1), and microsomal ACAT activity was determined as described previously (27).

For experiments in which diosgenin was added to the microsomes in vitro, microsomes were isolated and pooled from five WT mice fed the control diet. In a final volume of 2,700 μl ACAT buffer, microsomes (900 μg protein) were mixed with fatty acid free BSA (9 mg) and either water or Chol-HPβCD, such that the exogenous cholesterol concentration was 0.125, 0.25, or 0.5 nmol.
cholesterol/μg microsomal protein. The mixture was incubated at 37°C for 30 min, and the microsomes were resuspended by centrifugation at 100,000 rpm in a TLA 100.3 rotor for 15 min at 4°C. After washing with ACAT buffer, the microsomal pellet was suspended in 900 μl ACAT buffer, and aliquots were taken to determine protein concentration and cholesterol content (27). In a final volume of 300 μl ACAT buffer, the cholesterol-loaded microsomes (~100 μg) were mixed with fatty acid free BSA (1 mg) and either ethanol (vehicle) or 1 mM diosgenin, such that final diosgenin concentration was 5 or 10 μM. The mixture was incubated for 30 min at 37°C. After adding 30 nmol [1-14C]oleoyl CoA, microsomal ACAT activity was determined as described above.

**RESULTS**

Diosgenin increases fecal cholesterol excretion in both WT and L1KO mice

The efficiency of intestinal cholesterol absorption dramatically affects the output of cholesterol in the feces. To determine the importance of this pathway in diosgenin-stimulated fecal cholesterol excretion, WT mice and L1KO mice were fed a low-cholesterol diet (0.015% w/w) either lacking or containing 1% diosgenin. As expected, fecal excretion of neutral sterols (cholesterol and its bacterial metabolites) increased in diosgenin-fed WT mice from 4.2 to 52 μmol/day/100 g body weight (BW) (Fig. 1A). As a result of disrupted cholesterol absorption, fecal neutral sterol excretion was also significantly elevated in L1KO versus WT mice on the control diet (63 μmol/day/100 g BW). Surprisingly, fecal neutral sterol excretion was more than doubled when L1KO mice were treated with diosgenin (140 μmol/day/100 g BW) (Fig. 1A). The increase of fecal neutral sterol excretion in diosgenin-treated versus untreated L1KO mice (77 μmol/day/100 g BW) was even greater than that seen in diosgenin-treated versus untreated WT mice (47.8 μmol/day/100 g BW). In line with the increased fecal neutral sterol excretion, fractional cholesterol absorption decreased from 83% to 17% in WT mice fed the diosgenin versus control diet (Fig. 1B). However, fractional cholesterol absorption was undetectable in the L1KO mice fed the control diet and thus could not be further suppressed by diosgenin (Fig. 1B). The additional fecal neutral sterol excretion in diosgenin-treated L1KO mice must be derived from excretion of endogenous cholesterol. These data indicate that diosgenin stimulation of fecal neutral sterol excretion was largely independent of intestinal cholesterol absorption.

Diosgenin does not block NPC1L1-dependent cholesterol uptake

To directly determine whether diosgenin inhibits NPC1L1 function, McArdle RH7777 rat hepatoma cells stably expressing EGFP or human NPC1L1-EGFP (L1-EGFP) were used. Using these cells, we have previously established a functional assay for NPC1L1 (20). In the absence of depletion of cellular cholesterol by MβCD, cholesterol uptake was similar for the EGFP and L1-EGFP cells treated

![Fig. 1](https://example.com/fig1.png)  
**Fig. 1.** Effects of diosgenin on fecal neutral sterol excretion (A) and fractional cholesterol absorption (B) in WT and L1KO mice. Each column represents the mean (±SEM) from six WT mice and eight L1KO mice. Statistically significant differences are indicated by different superscript letters (P < 0.05).
with vehicle, 30 μM ezetimibe, or 10 μM diosgenin (Fig. 2). Following 1 h pretreatment of cells with MβCD that induces NPC1L1 translocation from intracellular compartments to the cell surface (20), cholesterol uptake was increased by 2.3-fold and 3.6-fold in EGFP and L1-EGFP cells treated with vehicle, respectively. Thus, expression of NPC1L1-EGFP was able to stimulate cholesterol uptake by 35% in these cells. Ezetimibe, a potent inhibitor of NPC1L1 (19), was able to completely abolish the NPC1L1-mediated increase in cholesterol uptake observed in the MβCD-pretreated L1-EGFP cells. In contrast, diosgenin concentrations of 10 μM (Fig. 2) or 20 μM (data not shown) had no effect on cholesterol uptake by either the MβCD-pretreated EGFP or L1-EGFP cells.

Diosgenin does not alter intestinal expression of sterol transporters and cholesterol synthetic genes

Even though diosgenin did not appear to directly block NPC1L1 function in vitro, diosgenin could have been disrupting cholesterol absorption in vivo by altering the gene expression of NPC1L1 or other proteins involved in cholesterol trafficking through enterocytes. Based on intestinal mRNA levels, the expression of NPC1L1 was similar in WT mice fed the control and diosgenin-containing diets (Fig. 3A). Additionally, diosgenin treatment of WT and L1KO mice did not change the mRNA expression of ABCG5, ABCG8, and ABCA1. In contrast, the expression of Cyp3a11 (cytochrome P450, family 3, subfamily a, polypeptide 11), a PXR target gene, was increased 8.8- and 6.8-fold in WT and L1KO mice treated with diosgenin (Fig. 3B), a finding consistent with previous studies (9, 10). These results indicate that diosgenin did not block cholesterol absorption by altering the expression of genes involved in cholesterol movement through intestinal epithelial cells.

Because of the near absence of cholesterol uptake by NPC1L1-deficient enterocytes, intestinal mRNA expression of HMG-CoA synthase (HMGCS), a key gene in the cholesterol synthetic pathway, is significantly upregulated in L1KO mice (17). If diosgenin was significantly inhibiting the NPC1L1-dependent movement of cholesterol into WT enterocytes, we predicted that mRNA expression of genes in the cholesterol synthetic pathway would be increased to an extent similar to that in L1KO enterocytes. Similar to previously published results, the mRNA levels for HMGCS, HMG-CoA reductase (HMGCR), and sterol regulatory element binding protein 2 (SREBP2) were increased in L1KO versus WT mice fed the control diet (Fig. 3C). However, compared with their respective genotype controls, WT and L1KO mice treated with diosgenin displayed no change in mRNA expression of genes regulating cholesterol synthesis. These results suggest that diosgenin does not alter cholesterol movement from the gut lumen into enterocytes.

Diosgenin treatment of L1KO and WT mice causes a similar increase in biliary cholesterol without affecting hepatic expression of ABCG5/G8

Since fecal neutral sterol excretion is dictated by not only the efficiency of cholesterol absorption but also the
level of biliary cholesterol secretion, the lipid composition of gallbladder bile was analyzed. Biliary cholesterol concentration was similar in WT and L1KO mice fed the control diet (Table 2). However, compared with their respective genotype controls, the diosgenin-treated WT and L1KO mice displayed a similar and significant increase (~3.5-fold) in biliary cholesterol concentration. In contrast, no significant difference in biliary phospholipid concentration was observed for the four treatment groups, and the only significant difference in bile acid concentration was a 26% decrease in the bile of WT mice fed the diosgenin diet compared with that of the L1KO mice fed the control diet (Table 2). Because bile can be concentrated in the gallbladder potentially altering lipid concentrations, molar percentages of biliary lipids were calculated. The molar percentages of biliary cholesterol were similar in WT and L1KO mice fed the control diet and increased ~4-fold in the WT and L1KO mice fed the diosgenin diet (Table 2). Likely as a result of the increased cholesterol content, the molar ratio of bile acids in the diosgenin-treated mice decreased from ~80% to ~70%. In contrast, the molar percentages of phospholipids were similar among the four treatment groups. These results indicate that diosgenin treatment of both WT and L1KO mice drastically increased biliary cholesterol content, which in turn explains the increased fecal neutral sterol excretion in these animals.

The heterodimer of ABCG5 and ABCG8 is a major determinant of biliary cholesterol secretion (6, 7). However, we and others (9, 10) have previously shown that diosgenin treatment does not alter hepatic ABCG5/G8 mRNA and protein levels. Consistent with the previous studies, hepatic mRNA levels of ABCG5 and ABCG8 did not change in diosgenin-treated WT and L1KO mice (Fig. 4), despite a dramatic upregulation of mRNAs for HMGCS, HMGCR, and SREBP2, genes related to cholesterol synthesis. The hepatic mRNAs for ABCB4 and ABCB11, the proteins responsible for hepatobiliary transport of phospholipids and bile acids, respectively, were largely unaffected by diosgenin. Hepatic NPC1L1 mRNA levels were very low and also not affected by diosgenin treatment in WT mice (data not shown). These results indicate that increased hepatic expression of biliary lipid transporters was not responsible for the elevated level of biliary cholesterol secretion observed in diosgenin-treated mice. In addition, diosgenin had no effect

Table 2. Effects of diosgenin on the lipid composition of gallbladder bile

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>Chol</th>
<th>PL</th>
<th>BS</th>
<th>Chol</th>
<th>PL</th>
<th>BS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/ml</td>
<td>% molar</td>
<td></td>
<td></td>
<td>µmol/ml</td>
<td>% molar</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>Control</td>
<td>5.1 (0.4)a</td>
<td>29 (2)a</td>
<td>138 (6)a,b</td>
<td>3.0 (0.3)a</td>
<td>17 (0.8)a</td>
<td>80 (0.9)a</td>
</tr>
<tr>
<td>WT</td>
<td>Diosgenin</td>
<td>18 (1.3)b</td>
<td>27 (2)a</td>
<td>111 (12)a</td>
<td>12 (0.5)b</td>
<td>17 (0.8)a</td>
<td>71 (1)b</td>
</tr>
<tr>
<td>L1KO</td>
<td>Control</td>
<td>7.2 (0.2)a</td>
<td>35 (2)a</td>
<td>149 (7)b</td>
<td>3.8 (0.2)a</td>
<td>18 (0.5)a</td>
<td>78 (0.5)a</td>
</tr>
<tr>
<td>L1KO</td>
<td>Diosgenin</td>
<td>26 (2.5)c</td>
<td>33 (1)a</td>
<td>124 (8)a,b</td>
<td>14 (1.9)b</td>
<td>18 (0.7)a</td>
<td>68 (2)b</td>
</tr>
</tbody>
</table>

After being fed for 14 d the diet supplemented without (control) or with diosgenin (1% w/w), mice were fasted for 4 h and bile was collected from the gallbladder for the determination of biliary concentrations of cholesterol (Chol), phospholipids (PL), and bile salts (BS). Each column represents mean (± SEM) of six samples. Statistically significant differences for each column of values are indicated by different superscript letters (P < 0.05).
on the hepatic mRNA levels of cholesterol 7α-hydroxylase, the rate-limiting enzyme in the classical pathway of bile acid synthesis (Fig. 4).

**Diosgenin treatment of WT and L1KO mice decreases liver and plasma cholesterol and increases hepatic cholesterol synthesis**

By promoting biliary cholesterol secretion, diosgenin has the potential to reduce hepatic cholesterol stores. Alternatively, increases in hepatic cholesterol stores may raise biliary cholesterol secretion. To determine the relationship between biliary cholesterol secretion and hepatic cholesterol stores in diosgenin-treated mice, liver cholesterol was analyzed. There was a trend toward a decrease in hepatic esterified cholesterol in WT mice fed the diosgenin versus the control diet (6.0 versus 9.8 mg/g protein) (Fig. 5A). L1KO mice consuming either the control or the diosgenin diet also tended to have less hepatic esterified cholesterol (4.7 and 4.9 mg/g protein) than WT controls. Hepatic content of free cholesterol, which was 12.6 and 12.1 mg/g protein in WT and L1KO controls, respectively, was reduced significantly to 10.5 and 10.8 mg/g protein in WT and L1KO mice treated with diosgenin (Fig. 5B).

Reductions in hepatic cholesterol content often lead to the feedback upregulation of cholesterol synthesis. As shown in Fig. 4, HMGCS, HMGCR, and SREBP2 mRNA were increased 3.1-, 2.1-, and 1.5-fold in WT mice fed the diosgenin versus control diet. Although already increased by 2-fold in control L1KO mice, HMGCS and HMGCR mRNA levels were elevated 6.5- and 5.1-fold in L1KO mice treated with diosgenin compared with WT mice on the control diet. These results indicate that diosgenin-treated WT and, to a greater extent, L1KO mice had a compensatory increase in hepatic cholesterol synthesis.

To define how the diosgenin-induced biliary and hepatic cholesterol changes influence whole-body cholesterol homeostasis, the plasma concentration and lipoprotein distribution of cholesterol was measured. Consumption of diosgenin caused plasma total cholesterol concentrations to decrease from 103 to 73 mg/dl in WT mice and from 93 to 70 mg/dl in L1KO mice (Fig. 6A). This decrease was reflected by significant reductions in the plasma concentrations of both LDL- and HDL-cholesterol. Plasma LDL-cholesterol decreased from 18 to 6.7 mg/dl in WT mice and from 10 to 5.6 mg/dl in L1KO mice (Fig. 6B). Plasma HDL-cholesterol decreased from ~80 to ~60 mg/dl in both WT and L1KO mice (Fig. 6C). Thus, diosgenin reduced total, HDL-, and LDL-cholesterol, which could have reflected either decreased production or increased clearance of lipoproteins by the liver.

**Diosgenin does not directly decrease hepatic ACAT activity**

One possible explanation for the decreased LDL-cholesterol in diosgenin-treated mice could be a reduction in cholesteryl ester incorporation into VLDL. Hepatic ACAT2 is responsible for the synthesis of cholesteryl es-

![Fig. 5.](image-url) Diosgenin treatment significantly reduces hepatic-free cholesterol content. Total lipids were extracted from livers of mice fed the control or diosgenin diet for 14 d, and hepatic content of esterified cholesterol (A) and free cholesterol (B) was determined as described in Materials and Methods. All values were normalized to the protein content of the extracted piece of liver. Each column represents the mean (± SEM) of six samples. Statistically significant differences are indicated by different letters (P < 0.05).

![Fig. 6.](image-url) Diosgenin significantly decreases plasma LDL- and HDL-cholesterol. A: Total plasma cholesterol (TPC) concentrations. B: Plasma LDL-cholesterol. C: Plasma HDL-cholesterol. Each column represents the mean (± SEM) of six samples. Statistically significant differences are indicated by different letters (P < 0.05).
ters that are packaged into VLDL (32). Previous studies have shown that hepatic ACAT activity can be decreased by diosgenin (4, 13). Therefore, hepatic microsomes were isolated from control and diosgenin-treated WT and L1KO mice, and ACAT activity was assayed. Compared with WT mice fed the control diet, WT mice fed the diosgenin diet and L1KO mice fed either diet displayed a significant 30% decrease in microsomal ACAT activity (Fig. 7A). Because cholesterol availability within the microsomes can significantly impact ACAT activity, the microsomes were loaded with free cholesterol using HPβCD as a carrier (Chol-HPβCD), and the ACAT activity was measured. ACAT activity was increased in the cholesterol-loaded (Fig. 7B) versus nonloaded microsomes (Fig. 7A), but with addition of exogenous cholesterol, ACAT activity was similar regardless of genotype or treatment.

To determine whether diosgenin could directly inhibit ACAT, hepatic microsomes were isolated from WT mice fed the control diet and were incubated with either water or increasing concentrations of Chol-HPβCD. Subsequently, microsomal cholesterol content was measured and ACAT activity was assayed in the presence of either vehicle or diosgenin (5 and 10 μM). Regardless of the cholesterol content of the microsomes, ACAT activity was decreased on average by 7% and 13% in the presence of 5 and 10 μM diosgenin versus vehicle (Fig. 7C, D). This result indicates that diosgenin is not a specific inhibitor of ACAT and that in vivo diosgenin likely decreases hepatic ACAT activity by limiting cholesterol availability to the enzyme.

**DISCUSSION**

It was believed that diosgenin promotes fecal cholesterol excretion by inhibiting intestinal cholesterol absorption and enhancing biliary cholesterol secretion. In this study, we found that diosgenin significantly increases biliary cholesterol and hepatic expression of cholesterol synthetic genes in both WT and L1KO mice. However, diosgenin does not require NPC1L1 to promote fecal cholesterol excretion. In an in vitro assay, diosgenin has no effects on NPC1L1-dependent cholesterol uptake. Additionally, it does not alter intestinal expression of sterol transporters and cholesterol synthetic genes. Given that the primary defect in L1KO mice is the blockade of intestinal cholesterol absorption (16, 17), these findings indicate that diosgenin stimulation of fecal cholesterol excretion is primarily attributable to its impact on hepatic cholesterol metabolism rather than NPC1L1-dependent intestinal cholesterol absorption. Alternatively, diosgenin may increase intestinal cell sloughing, which may partially explain the increased fecal cholesterol excretion observed in the diosgenin-treated mice. In the absence of NPC1L1, diosgenin could possibly facilitate this process to a greater extent, thereby resulting in a further increase in fecal cholesterol loss in L1KO mice.

**Fig. 7.** Diosgenin does not directly inhibit hepatic ACAT activity. ACAT activities were measured as described in Materials and Methods in liver microsomes (A) containing endogenous free cholesterol or (B) loaded exogenously with free cholesterol using Chol-CD. Each column represents the mean ± SEM of five samples. Statistically significant differences are indicated by different letters ($P < 0.05$). C and D: Microsomes from control diet-fed WT mice were loaded with different amounts of free cholesterol using Chol-CD. ACAT activity was measured in the presence of 5 and 10 μM diosgenin or vehicle (ethanol). The columns represent the means of duplicate samples ± range. The experiment was repeated with similar outcomes (data not shown).
Because of the structural similarities between diosgenin and cholesterol, it was possible that NPC1L1 would be necessary for the absorption of diosgenin. Compared with WT mice, L1KO mice displayed a significant decrease in the concentration of diosgenin in the plasma (0.34 versus 0.5 mg/dl) and liver (0.34 versus 0.51 mg/g protein). Nevertheless, similar to WT mice, L1KO mice treated with diosgenin had increases in fecal neutral sterol excretion (Fig. 1A), biliary cholesterol concentration (Table 2), and mRNA expression of hepatic genes in the cholesterol biosynthetic pathway (Fig. 4). This data shows that 1) although reduced, the concentration of diosgenin in the body of L1KO mice was sufficient to significantly alter cholesterol metabolism; and 2) the absorption of diosgenin may be influenced by but does not require NPC1L1 expression.

WT mice treated with diosgenin or ezetimibe display significantly decreased fractional cholesterol absorption and increased fecal neutral sterol excretion. However, based on the following evidence, diosgenin, unlike ezetimibe (19, 20, 33), does not appear to directly target NPC1L1: 1) ezetimibe but not diosgenin was able to block NPC1L1-dependent cholesterol uptake in vitro (Fig. 2); 2) presumably due to less cholesterol uptake by enterocytes, feedback upregulation of cholesterol synthetic gene expression in the small intestine occurred in ezetimibe-treated mice and L1KO mice (17, 34) but not in diosgenin-treated mice (Fig. 3C); 3) probably because of less cholesterol available to activate the nuclear receptor liver X receptor (34), the intestinal expression of ABCA1 was dramatically reduced in ezetimibe-treated mice and L1KO mice (17, 34, 35) but not in diosgenin-treated mice (Fig. 3B); and 4) diosgenin treatment neither affected NPC1L1 expression in the intestine (Fig. 3B) nor altered subcellular localization of NPC1L1-EGFP fusion protein in cultured cells (data not shown).

One possible explanation for reduced fractional cholesterol absorption in diosgenin-treated WT mice is that diosgenin promotes biliary cholesterol secretion (Table 2), resulting in more cholesterol delivered to the gut lumen and dilution of the isotopic cholesterol tracer used to measure fractional cholesterol absorption. Consistent with this scenario are the findings that transgenic mice overexpressing ABCG5/G8 predominantly in the liver had increased biliary cholesterol secretion and reduced fractional cholesterol absorption (7) and that biliary cholesterol secretion was inversely correlated with the percentage of intestinal cholesterol absorption in C57BL/6 mice fed different amounts of dietary cholesterol and in transgenic mice over-expressing scavenger receptor class B type 1 in the liver (36). Whatever the mechanism is, diosgenin does not seem to directly inhibit NPC1L1, an essential protein in the intestinal cholesterol absorption pathway.

A dramatic and consistent finding in diosgenin-treated animals is the elevation in biliary cholesterol secretion (3–5, 9, 10, 13, 14) (Table 2). However, the molecular mechanism by which diosgenin stimulates biliary cholesterol excretion is unknown. Although the presence of the hepatobiliary sterol transporter ABCG5/G8 is required for diosgenin to drive cholesterol from hepatocytes into bile (9, 10), diosgenin treatment has no effects on the hepatic mRNA and protein levels of ABCG5/G8 (9, 10) (Fig. 4). Thus, alterations in ABCG5/G8 expression are not responsible for the diosgenin-mediated induction of biliary cholesterol secretion. Diosgenin may act on steps before ABCG5/G8. In two studies, diosgenin was shown to induce expression of nuclear receptor PXR target genes in the intestine and liver (9, 10), suggesting that diosgenin may be a PXR agonist and PXR may regulate diosgenin-stimulated secretion of cholesterol. Indeed, mice deficient in PXR have a modestly attenuated response to diosgenin (9). It was previously reported that liver microsomes and hepatocytes isolated from diosgenin-treated rats displayed decreased ACAT activity (4, 13). We also observed a significant 30% reduction in ACAT activity in hepatic microsomes isolated from WT mice treated with diosgenin (Fig. 7A). It was possible that diosgenin acts as an inhibitor of ACAT in the liver, thereby increasing the availability of free cholesterol for ABCG5/G8-dependent biliary secretion. However, when loaded exogenously with cholesterol, microsomes from control and diosgenin-treated WT mice displayed a similar level of ACAT activity. Moreover, minimal effects on ACAT activity were observed when diosgenin was added to control, WT mouse microsomes. Thus, diosgenin most likely decreases hepatic ACAT activity by limiting the amount of cholesterol available to the enzyme through promoting biliary cholesterol secretion and not by directly inhibiting ACAT. Although rather unlikely, it is also conceivable that an ACAT inhibitor is formed endogenously from a metabolite of diosgenin. Further studies are required to identify factors (proteins or lipids) responsible for diosgenin-induced biliary cholesterol secretion.

Another consistent finding in diosgenin-treated rats and mice is the increase in hepatic cholesterol synthesis (3, 4, 10, 11) (Fig. 4), which was believed to be the result of decreased intestinal cholesterol absorption. However, in L1KO mice lacking intestinal cholesterol absorption and already having a compensatory increase in hepatic cholesterol synthesis, diosgenin was able to drive further upregulation of cholesterol synthetic gene expression in the liver (Fig. 4). This finding clearly indicates that diosgenin has a direct effect on hepatic cholesterol synthesis. Further studies to define how diosgenin directly alters hepatic cholesterol synthesis are warranted.

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