Intestinal cholesterol absorption is substantially reduced in mice deficient in both ABCA1 and ACAT2

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Abstract **The process of cholesterol absorption has yet to be completely defined at the molecular level. Because of its ability to esterify cholesterol for packaging into nascent chylomicrons, ACAT2 plays an important role in cholesterol absorption. However, it has been found that cholesterol absorption is not completely inhibited in ACAT2-deficient (ACAT2 KO) mice. Because ABCA1 mRNA expression was increased 3-fold in the small intestine of ACAT2 KO mice, we hypothesized that ABCA1-dependent cholesterol efflux sustains cholesterol absorption in the absence of ACAT2. To test this hypothesis, cholesterol absorption was measured in mice deficient in both ABCA1 and ACAT2 (DKO). Compared with wild-type, ABCA1 KO, or ACAT2 KO mice, DKO mice displayed the lowest level of cholesterol absorption. The concentrations of hepatic free and esterified cholesterol and gallbladder bile cholesterol were significantly reduced in DKO compared with wild-type and ABCA1 KO mice, although these measures of hepatic cholesterol metabolism were very similar in DKO and ACAT2 KO mice. We conclude that ABCA1, especially in the absence of ACAT2, can have a significant effect on cholesterol absorption, although ACAT2 has a more substantial role in this process than ABCA1.**—Temel, R. E., R. G. Lee, K. L. Kelley, M. A. Davis, R. Shah, J. K. Sawyer, M. D. Wilson, and L. L. Rudel. **Intestinal cholesterol absorption is substantially reduced in mice deficient in both ABCA1 and ACAT2.** ***J. Lipid Res.*** **2005.** 46: **2423–2431.**

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Because the level of cholesterol absorption can influence plasma cholesterol concentrations (1), disruption of intestinal cholesterol transport by pharmacological means has been proven to reduce the concentration of plasma LDL cholesterol, the latter a risk factor that is tightly linked to the development of coronary heart disease. For example, administration of ezetimibe, a cholesterol absorption inhibitor, caused LDL cholesterol concentrations to decrease by 20%, whereas a reduction of almost 60% was achieved when ezetimibe and a statin were given together (2). Thus, inhibition of intestinal cholesterol absorption may provide an effective means of preventing coronary heart disease, the single leading cause of death in the United States (3).

Although it is recognized that cholesterol absorption can have a significant impact on plasma cholesterol levels, many of the molecular mechanisms controlling cholesterol absorption remain to be elucidated. One cellular component of cholesterol absorption appears to be ACAT2. ACAT2 is a transmembrane protein that is expressed in the rough endoplasmic reticulum of enterocytes (4, 5) and esterifies free cholesterol that has been either synthesized de novo or internalized from the intestinal lumen (6, 7). Because a limited amount of free cholesterol can associate with the phospholipid monolayer of chylomicrons, the production of cholesteryl ester by ACAT2 allows cholesterol to be more efficiently packaged into apolipoprotein B (apoB)-containing lipoproteins formed during lipid absorption. For example, after a meal, cholesteryl ester can represent up to 78% of the total cholesterol in chylomicrons (8–12), which carry >85% of the total cholesterol secreted by the small intestine into lymph (7, 9). Thus, under normal circumstances, ACAT2 esterifies the majority of cholesterol absorbed by the body.

Direct evidence for the involvement of ACAT2 in cholesterol absorption comes from studies of ACAT2-deficient (ACAT2 KO) mice. In these studies, it was shown that as dietary cholesterol content was incrementally increased, ACAT2 KO mice absorbed proportionally less cholesterol than wild-type mice (13, 14). Although this finding implicated ACAT2 as a significant factor in cholesterol absorption, the incomplete inhibition of this process in ACAT2

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Abbreviations: apoB, apolipoprotein B; DKO, deficient in both ABCA1 and ACAT2; KO, -deficient; LXR, liver X receptor; NPC1L1, Niemann-Pick C1-like 1; SR-BI, scavenger receptor class B type I; TPC, plasma total cholesterol.

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KO mice indicated that cholesterol absorption may be mediated by additional mechanisms. One of these pathways may be via ABCA1. Expressed in enterocytes and many other cell types in the body (15), ABCA1 is a transmembrane protein that mediates the efflux of phospholipids and free cholesterol to lipid-poor apolipoproteins, such as apoA-I and apoE (16, 17).

Based on the current literature, the importance of ABCA1 in cholesterol absorption is unclear. Using CaCo-2 cells, an immortalized cell line with properties similar to those of enterocytes, it has been shown that free cholesterol can be effluxed, presumably via ABCA1, from the basolateral membrane of the cells (18–20). In animal models, cholesterol absorption has been shown to decrease by 13% in ABCA1 KO mice (21) and by 79% in ABCA1 KO chickens (22). In contrast, other studies have reported that ABCA1 KO mice displayed either similar (23) or significantly increased (24) cholesterol absorption compared with wild-type mice. A patient with Tangier disease had a cholesterol absorption percentage similar to those of control subjects (25). Therefore, ABCA1 appears to mediate only a limited amount of cholesterol absorption. However, when other components in the process are not functioning normally, such as when ACAT2 is missing, ABCA1 might assume a more significant role.

The main line of evidence supporting the hypothesis that cholesterol absorption is sustained by ABCA1 in the absence of ACAT2 comes from a study showing that ABCA1 mRNA expression was significantly greater in ACAT2 KO compared with wild-type small intestine (14). Because the regulatory pool of free cholesterol may be increased in enterocytes of ACAT2 KO mice, the increase in ABCA1 expression is likely mediated by liver X receptor (LXR) activation (26). In turn, an increase in ABCA1-mediated cholesterol efflux to lipid-poor apolipoproteins may serve as a means of cholesterol absorption in ACAT2 KO mice. To test the hypothesis that cholesterol absorption can be partially maintained by ABCA1 in the absence of ACAT2, the present study of intestinal cholesterol transport and hepatic cholesterol metabolism was conducted using mice deficient in both ABCA1 and ACAT2 (DKO mice).

MATERIALS AND METHODS

Mice

All mice were maintained in an American Association for Accreditation of Laboratory Animal Care-approved animal facility under protocols approved by the institutional animal care and use committee at Wake Forest University School of Medicine. Male wild-type mice (50% C57Bl/6, 50% 129Sv/Jae) were purchased from Jackson Laboratories, and male ACAT2 KO mice (50% C57Bl/6, 50% 129Sv/Jae) were provided by Dr. Robert J. Farese, Jr. (13). To generate DKO mice, ACAT2 KO mice were crossed with ABCA1 KO mice (50% FVB, 50% 129Sv/Jae) provided by CV Therapeutics. ABCA1 KO mice were created by Dr. Eddy Rubin at Berkeley National Laboratory and lack exons 17–22, which encode nucleotide binding domain 1. $ABCA1^{+/-}$ $ACAT2^{+/-}$ siblings were then mated to establish the following lines of mice: wild-type ($ABCA1^{+/+}$ $ACAT2^{+/+}$), ABCA1 KO ($ABCA1^{-/-}$ $ACAT2^{+/+}$), ACAT2 KO ($ABCA1^{+/+}$ $ACAT2^{-/-}$), and DKO ($ABCA1^{-/-}$ $ACAT2^{-/-}$).

Fractional cholesterol absorption

Male wild-type and ACAT2 KO mice at least 6 weeks of age and 6–7 week old female wild-type, ABCA1 KO, ACAT2 KO, and DKO mice were offered 10 g/day of a low-fat (20% of energy as palm-enriched fat), moderate-cholesterol (0.17%, w/w) diet. After feeding the diet to the males for 10 days and to the females for 4 weeks, the mice were gavaged with 0.1 μCi of [4-^{14}C]cholesterol (Amersham Pharmacia Biotech) and 0.2 μCi of [22,23-^{3}H]sitosterol (New England Nuclear) dissolved in 100 μl of soybean oil. Each mouse was individually housed in a cage with a wire bottom and was allowed free access to diet and water for 3 days. The feces were collected and homogenized in 95% ethanol using a Polytron PT 1200 homogenizer (Kinematica). An aliquot of the fecal slurry was saponified by adding 50% KOH to a final concentration of 5% (w/v) and heating at 65°C for 2 h. Neutral lipids were extracted by adding hexane and deionized water, vortexing, and centrifuging at 2,000 *g* for 10 min. The hexane phase was transferred to a scintillation vial and dried at 65°C under N_2. In addition, aliquots of the initial [^{14}C]cholesterol/[^{3}H]sitosterol dose were placed into scintillation vials. Bio-Safe II scintillation fluid (Research Products International) was added to the vials, and the [^{14}C]cholesterol and [^{3}H]sitosterol counts were measured in a scintillation spectrometer. Percentage cholesterol absorption was calculated using the following equation:

$$\frac{(^{14}\mathrm{C}/^{3}\mathrm{H}\ \text{dose ratio} - {}^{14}\mathrm{C}/^{3}\mathrm{H}\ \text{feces ratio})}{^{14}\mathrm{C}/^{3}\mathrm{H}\ \text{dose ratio}} \times 100$$

Fecal neutral sterol excretion

After being fed the low-fat, moderate-cholesterol diet for 5 weeks, female mice were singly housed as described above. After a 3 day fecal collection, the mice were weighed, and the feces were collected, dried in a 70°C vacuum oven, weighed, and crushed into a fine powder. A measured mass (50–100 mg) of feces was placed into a glass tube containing 103 μg of 5α-cholestane as an internal standard. The feces were saponified and the neutral lipids were extracted into hexane as described above. Mass analysis of the extracted neutral sterols was conducted by gas-liquid chromatography as described previously (27). Fecal neutral sterol mass represents the sum of cholesterol, coprostanol, and coprostanone in each sample. Fecal neutral sterol excretion was expressed as mg sterol/day/100 g body weight.

Plasma, hepatic, and biliary lipid concentrations

After being fed the low-fat, moderate-cholesterol diet for 6 weeks, mice were fasted for 4 h and euthanized. Blood was collected by heart puncture and was placed into a tube containing protease inhibitor cocktail (Sigma) dissolved in 5% EDTA, 5% NaN_3. Gallbladder bile was collected, and then the liver was removed, weighed, and snap-frozen in liquid N_2. The small intestine from the pyloric valve to the cecum was removed, cleaned of fat and luminal contents while on ice, and cut into either three sections of equal length for the male ACAT2 KO experiments or five pieces of equal length for the female DKO experiments. The intestinal sections were snap-frozen in liquid N_2 and stored along with the liver and bile samples at −80°C. The blood was centrifuged at 12,000 *g* for 10 min at 4°C, and the plasma was analyzed for total and free cholesterol concentrations using the Cholesterol/HP (Roche) and the Free Cholesterol C (Wako) enzymatic assay kits, respectively. Plasma lipoprotein cholesterol distribution was determined after separation of lipoprotein classes from whole plasma by gel filtration chromatography as described previously (28).

For analysis of the liver lipid composition, ~100 mg of liver was thawed, minced, and weighed in a glass tube. Lipids were extracted in 2:1 $CHCl_3$/methanol at room temperature overnight. The protein was quantitatively separated from the lipid extract, which was then dried down under N_2 and redissolved in a measured volume of 2:1 $CHCl_3$/methanol. Dilute H_2SO_4 was added to the sample, which was then vortexed and centrifuged to split the phases. The aqueous upper phase was aspirated and discarded, and an aliquot of the bottom phase was removed and dried down; 1% Triton X-100 in $CHCl_3$ was then added, and the solvent was evaporated (29). Deionized water was then added to each tube and vortexed until the solution was clear. Lipids were then quantified using the Triglycerides/GB kit (Roche) plus the enzymatic cholesterol assays described above.

For analysis of biliary lipid concentrations, a measured volume (~10 μl) of bile was placed into a glass tube and the neutral lipids were extracted and analyzed as described for liver. Aliquots of the aqueous phase of the extraction were analyzed for bile acid content using an enzymatic assay using hydroxysteroid dehydrogenase (30).

Real-time PCR analysis of intestinal and hepatic mRNA levels

Total mRNA was extracted from ~100 mg of liver and proximal small intestine with Trizol (Invitrogen Life Technologies) using the protocol provided by the manufacturer. The mRNA was resuspended in 300 μl of diethyl pyrocarbonate water, and 1 μg of mRNA was reverse transcribed to cDNA using Omniscript reverse transcriptase (Qiagen) under the following conditions: 37°C for 1 h and 93°C for 5 min. The cDNA was diluted 1:10 using diethyl pyrocarbonate water, and real-time PCR was done in triplicate with 5 μl of cDNA, 12.5 μl of SYBR GREEN PCR master mix (Applied Biosystems), 5.5 μl of diethyl pyrocarbonate water, and 1 μl of forward and reverse primer (20 pmol) for a final reaction volume of 25 μl. The primer sequences are presented in **Table 1**. PCR was then run on the Sequence Detection System 7000 (Applied Biosystems) using the following conditions: 50°C for 2 min, 94°C for 10 min, and 40 cycles of 94°C for 10 s and 60°C for 1 min. The fluorescence measurement used to calculate threshold cycle (Ct) was made at the 60°C point. A dissociation curve was run at the end of the reaction to ensure a single amplification product. Ct values were entered into the following equation to determine the arbitrary unit value: $1 \times 10^9 \times e(-0.6931 \times Ct)$. All values were then normalized to either GAPDH or cyclophilin mRNA concentration of the sample to take total mRNA concentration into account.

RESULTS

Intestinal cholesterol absorption in ABCA1 KO and ACAT2 KO mice

To verify that cholesterol absorption was reduced in ACAT2 KO mice fed a semisynthetic diet without the fiber-rich properties of chow, male wild-type and ACAT2 KO mice were fed a low-fat (20% of energy as palm oil), moderate-cholesterol (0.17%, w/w) diet and fractional cholesterol absorption was measured using the fecal dual-isotope method. Similar to other studies (13, 14), fractional cholesterol absorption was decreased from 53% in wild-type mice to 35% in ACAT2 KO mice (**Fig. 1A**). However, much of the cholesterol was still absorbed by the ACAT2 KO mice, suggesting that, in the absence of ACAT2, redundant pathways were allowing cholesterol absorption to be sustained. Because previous evidence indicated that ABCA1 could play a role in cholesterol absorption (14, 21), real-time PCR analysis of ABCA1 mRNA expression in the small intestine was conducted. Compared with wild-type mice, ACAT2 KO mice had an almost 3-fold increase in the expression of ABCA1 (Fig. 1B). This result supported the hypothesis that increased expression of ABCA1 in the absence of ACAT2 allowed cholesterol absorption to be partially maintained.

To test this hypothesis, ABCA1 KO mice were mated with ACAT2 KO mice and the offspring were crossed to create DKO mice. Cholesterol absorption was then measured in female DKO mice along with wild-type, ABCA1 KO, and ACAT2 KO mice fed the low-fat, moderate-cholesterol diet. Using the fecal dual-isotope method, cholesterol absorption was decreased from 63% in wild-type mice to 25% in DKO mice (**Fig. 2A**). Fractional cholesterol absorption was also reduced to 55% in ABCA1 KO mice and to 35% in ACAT2 KO mice, and the sum of these decreases was similar to the decline displayed by the DKO mice. The extent of cholesterol absorption was also estimated by analyzing the excretion of neutral sterols in the feces. In agreement with the fractional cholesterol absorption data, sterol excretion was significantly increased from 11 mg/day/100 g body weight in the wild-type mice to 26 mg/day/100 g body weight in the DKO mice (Fig. 2B). The ACAT2 KO mice also showed a significant increase in neutral sterol excretion (18 mg/day/100 g body weight), whereas the mean value was not significantly higher in the ABCA1 KO mice (14 mg/day/100 g body weight). The data show that cholesterol absorption can be mediated by both ABCA1 and ACAT2 and that this process was greatly decreased when both of these proteins were absent.

Real-time PCR analysis was used to determine whether any differences occurred in the expression of genes involved in the trafficking of cholesterol through the enterocytes. For this experiment, RNA was isolated from the duode-

TABLE 1. Primer sequences used for real-time PCR analysis

Gene	Primer Sequence (5′→3′)
ATP binding cassette transporter A1	CGTTTCCGGGAAGTGTCCTAGCTAGAGATGACAAGGAGGATGGA
ATP binding cassette transporter G5	TGGATCCAACACCTCTATGCTAAAGGCAGGTTTTCTCGATGAACTG
Cholesterol 7-α hydroxylase	AGCAACTAAACAACCTGCCAGTACTAGTCCGGATATTCAAGGATGCA
Cyclophilin	TGGAGAGCACCAAGACAGACATGCCGGAGTCGACAATGAT
Glyceraldehyde-3-phosphate dehydrogenase	TGTGTCCGTCGTGGATCTGACCTGCTTCACCACCTTCTTGAT
3-Hydroxy-3-methylglutaryl-CoA synthase 1	GCCGTGAACTGGGTCGAAGCATATATAGCAATGTCTCCTGCAA
Niemann-Pick C1-like protein 1	ATCCTATCCCTGGACTTGCTGCTCAGTGAGGCTGGTGTTATGCG
Sterol-regulatory element binding protein 1c	GGAGCCATGGATTGCACATTGGCCCGGGAAGTCACTGT

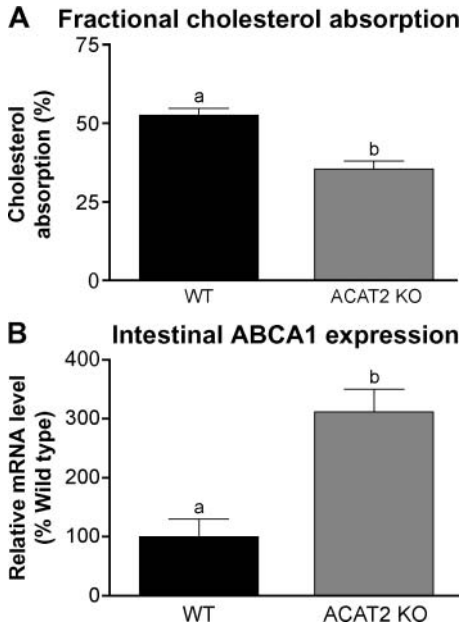

Fig. 1. Intestinal cholesterol absorption and ABCA1 expression in ACAT2-deficient (ACAT2 KO) mice. A: Male mice were fed a low-fat (20% energy as fat), moderate-cholesterol (0.17%, w/w) diet for 10 days, and fractional cholesterol absorption was measured using the fecal dual-isotope method. The columns represent means ± SEM of 16 measurements for wild-type (WT) mice and 14 measurements for ACAT2 KO mice. B: Total RNA was isolated from the proximal third of the small intestine, and real-time PCR analysis was conducted to determine the expression of ABCA1. All values were normalized to the GAPDH mRNA concentration of the samples, and the mean of the wild-type samples was arbitrarily designated as 100%. The columns represent means ± SEM of four samples. Statistically significant differences were determined by unpaired Student's *t*-test and are indicated by different letters ($P < 0.05$).

num and proximal jejunum. The mRNA of Niemann-Pick C1-like 1 (NPC1L1), which is the target of the cholesterol absorption inhibitor ezetimibe (31), was decreased to ~60% of that of the wild type in the small intestine of DKO mice (**Fig. 3A**). A similar decrease in NPC1L1 mRNA was also observed for the small intestine of ACAT2 KO mice, although no significant change was seen in the intestine of ABCA1 KO mice. Because ABCG5, in concert with ABCG8, is believed to efflux excess cholesterol from enterocytes into the lumen of the intestine (32, 33), the mRNA level of this LXR-sensitive gene was also determined. Although a trend toward increased expression was evident for the ACAT2 KO mice, the amounts of ABCG5 mRNA were not significantly different in the small intestines of the four different genotypes of mice (Fig. 3B).

Hepatic cholesterol metabolism in ABCA1 KO and ACAT2 KO mice

It was hypothesized that because of significant decreases in cholesterol absorption, the livers of DKO mice would reflect the changes in intestinal cholesterol metabolism. Accordingly, hepatic lipid compositions were determined. Although no statistically significant differences were found between wild-type and ABCA1 KO mouse livers, the livers of DKO mice contained 30% less free cholesterol and 99% less cholesteryl ester (**Fig. 4A**, **B**). In addition, a reduction of 64% was observed for hepatic triglyceride content of DKO versus wild-type and ABCA1 KO mice (Fig. 4C). ACAT2 KO mice had similar reductions in hepatic concentrations of these three lipids as DKO mice. Thus, even though the DKO mice displayed lower fractional cholesterol absorption than the ACAT2 KO mice (Fig. 2), the livers from these two genotypes showed similarly decreased concentrations of free cholesterol, cholesteryl ester, and triglyceride.

The lipid composition of gallbladder bile collected from fasted mice was also analyzed. No significant differences in cholesterol, bile salt, and phospholipid concentrations were found upon comparison of DKO and ACAT2 KO gallbladder bile (**Table 2**). However, the concentrations of cholesterol and phospholipids were significantly decreased in the DKO and ACAT2 KO mice compared with the wild-type and ABCA1 KO mice (Table 2). Because the bile can

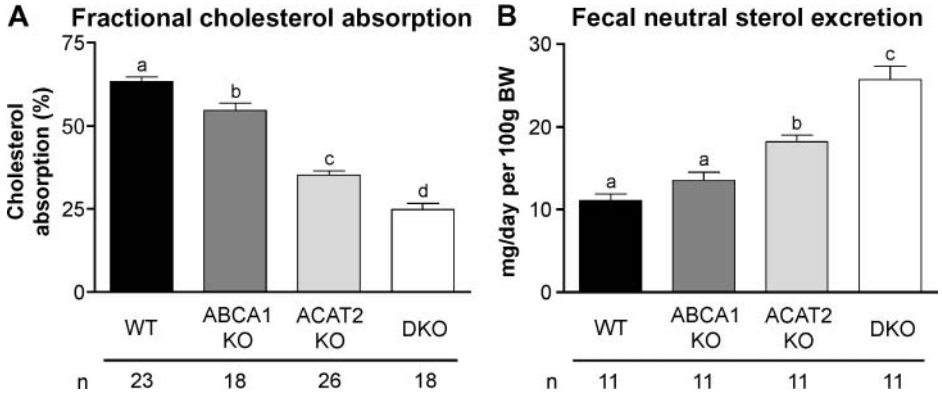

Fig. 2. Intestinal cholesterol absorption in mice deficient in both ABCA1 and ACAT2 (DKO mice). A: Female mice were fed a low-fat, moderate-cholesterol diet for 4 weeks, and fractional cholesterol absorption was measured using the fecal dual-isotope method. B: After feeding female mice a low-fat, moderate-cholesterol diet for 5 weeks, feces were collected for 3 days and the amount of neutral sterol excreted into the feces was determined using gas-liquid chromatography. Each column represents the mean ± SEM of n samples. Statistically significant differences were determined for each data set by ANOVA (Tukey-Kramer honestly significant difference) and are indicated by different letters ($P < 0.05$). BW, body weight; WT, wild type.

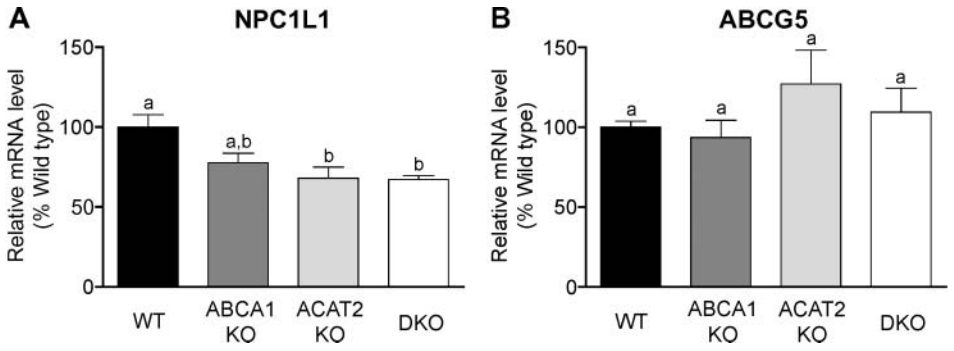

Fig. 3. Small intestinal expression of genes involved in cholesterol absorption. The small intestine was removed from female mice fed a low-fat, moderate-cholesterol diet for 6 weeks. Total RNA was isolated from the proximal two-fifths of the small intestine, and real-time PCR analysis was then conducted to determine the mRNA level of Niemann-Pick C1-like 1 (NPC1L1; A) and ABCG5 (B). All values were normalized to the cyclophilin mRNA concentration of the samples, and the mean of the wild-type (WT) samples was arbitrarily designated as 100%. Each column represents the mean ± SEM of five samples. Statistically significant differences were determined for each data set by ANOVA (Tukey-Kramer honestly significant difference) and are indicated by different letters ($P < 0.05$).

be concentrated while being collected in the gallbladder, the percentage molar composition of the bile was calculated. The bile from DKO and ACAT2 KO mice contained a lower molar percentage of cholesterol than that from wild-type mice, whereas ABCA1 KO mouse bile contained a significantly higher molar percentage of cholesterol. ACAT2 KO and DKO mice also had bile with lower molar percentages of phospholipids and higher molar percentages of bile acids than ABCA1 mice. These differences could be indications of an increased tendency for bile acid secretion in mice that cannot esterify cholesterol in the hepatocyte. The results also showed that in spite of significant differences in cholesterol absorption, cholesterol excretion into the bile was similar in DKO and ACAT2 KO mice.

The expression of some of the genes involved in hepatic cholesterol homeostasis was also examined. Using real-time PCR, it was found that DKO mice compared with ABCA1 KO mice displayed significantly higher expression of mRNA for HMG-CoA synthase (**Fig. 5A**), which is the rate-limiting enzyme in cholesterol synthesis. In addition, the mRNA level of the LXR-responsive gene ABCG5 (Fig. 5B) was significantly reduced from wild-type levels in the DKO liver. Significant differences in the mRNA of 7α-hydroxylase were not found in the DKO liver (Fig. 5C). Thus, the data are consistent with an increase in cholesterol synthesis and a decrease in the availability of cholesterol for secretion into bile in DKO mice. However, the differences in regulation of the mRNA expression of these genes were relatively small, and the compensatory mechanisms of the reduced intestinal cholesterol absorption in the livers of the DKO mice were apparently relatively subtle and not clearly evident in mRNA measurements.

Effects of ABCA1 and ACAT2 deficiency on the concentration and lipoprotein distribution of plasma cholesterol

Based on the decreased availability of hepatic cholesterol in DKO mice and previous reports showing that the ab-

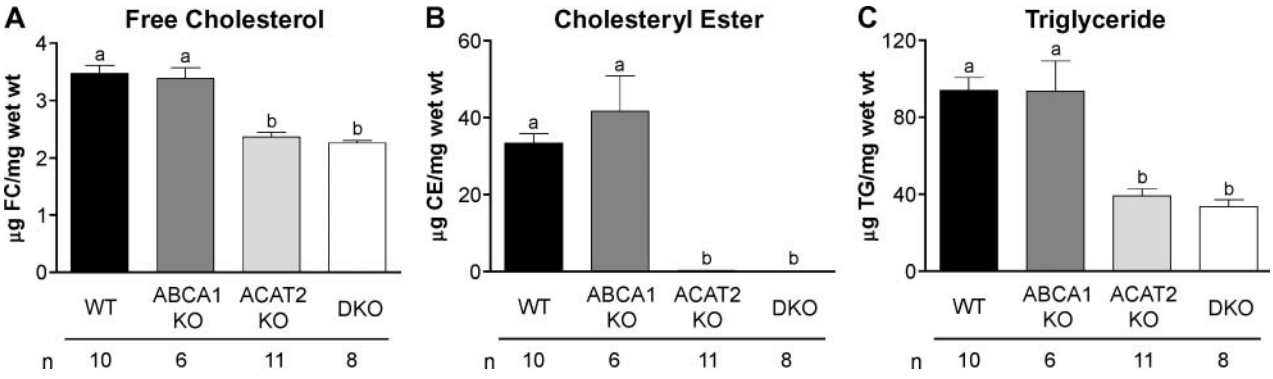

Fig. 4. Hepatic lipid concentrations of DKO mice. Using 2:1 $CHCl_3$/methanol, total lipids were extracted from weighed pieces of liver that had been removed from female mice fed a low-fat, moderate-cholesterol diet for 6 weeks. Enzymatic kits were used to determine the total cholesterol, free cholesterol (FC), and triglyceride (TG) contents of the lipid extracts. Cholesteryl ester (CE) content was calculated by multiplying the difference between total and free cholesterol mass by 1.67. All values were normalized to the wet weight of the extracted pieces of liver. Each column represents the mean ± SEM of n samples. Statistically significant differences were determined for each data set by ANOVA (Tukey-Kramer honestly significant difference) and are indicated by different letters ($P < 0.05$). WT, wild type.

TABLE 2. Lipid composition of gallbladder bile

Genotype	Cholesterol	Phospholipid	Bile Salt	Cholesterol	Phospholipid	Bile Salt
		μmol/ml			*% molar*	
Wild type (n = 13)	11 ± 1.7 a	41 ± 5.5 a	121 ± 17 a	6.0 ± 0.4 a	24 ± 1.1 a, b	70 ± 1.4 a, b
ABCA1 KO (n = 11)	11 ± 1.0 a	41 ± 4.0 a	100 ± 10 a	7.4 ± 0.5 b	27 ± 1.2 a	65 ± 1.7 b
ACAT2 KO (n = 15)	3.6 ± 0.4 b	26 ± 2.3 b	87 ± 6.0 a	3.0 ± 0.2 c	22 ± 1.0 b	75 ± 1.0 a
DKO (n = 10)	3.0 ± 0.6 b	23 ± 2.9 b	79 ± 6.6 a	2.8 ± 0.3 c	22 ± 1.0 b	76 ± 1.3 a

DKO, deficient in both ABCA1 and ACAT2; KO, -deficient. After being fed a moderate-cholesterol diet for 6 weeks, mice were fasted for 4 h and bile was removed from the gallbladder. A measured volume of bile was extracted with 2:1 $CHCl_3$/methanol, and the organic phase was analyzed using enzymatic kits to determine the cholesterol and phospholipid concentrations. The aqueous phase was analyzed using an enzymatic assay to determine the bile salt concentration. Each column represents means ± SEM of n samples. Statistically significant differences were determined for each column of values by ANOVA (Tukey-Kramer honestly significant difference) and are indicated by different letters ($P < 0.05$).

sence of either ABCA1 or ACAT2 causes a significant reduction in plasma cholesterol (13, 24), it was predicted that DKO mice would have the lowest plasma cholesterol concentrations of the four genotypes. Compared with wild-type and ACAT2 KO mice, the plasma total cholesterol (TPC) concentration of DKO mice was significantly lower (**Table 3**). However, unexpectedly, 2.5-fold more TPC was present in the plasma of DKO versus ABCA1 KO mice. This was not the result of an increase in HDL cholesterol but instead was attributable to a higher concentration of cholesterol in VLDL- and LDL-sized particles. Interestingly, almost all of the cholesterol found in the plasma of DKO mice was free cholesterol, which was not the case for any of the other genotypes. Thus, the higher than expected TPC concentration of the DKO mice resulted from the presence of free cholesterol-enriched VLDL- and LDL-sized particles.

DISCUSSION

In the current study, DKO mice were used to test the hypothesis that, in the absence of ACAT2, intestinal cholesterol absorption can be partially maintained by ABCA1. Fractional cholesterol absorption was decreased significantly in ACAT2 KO versus wild-type mice (35% vs. 63%),

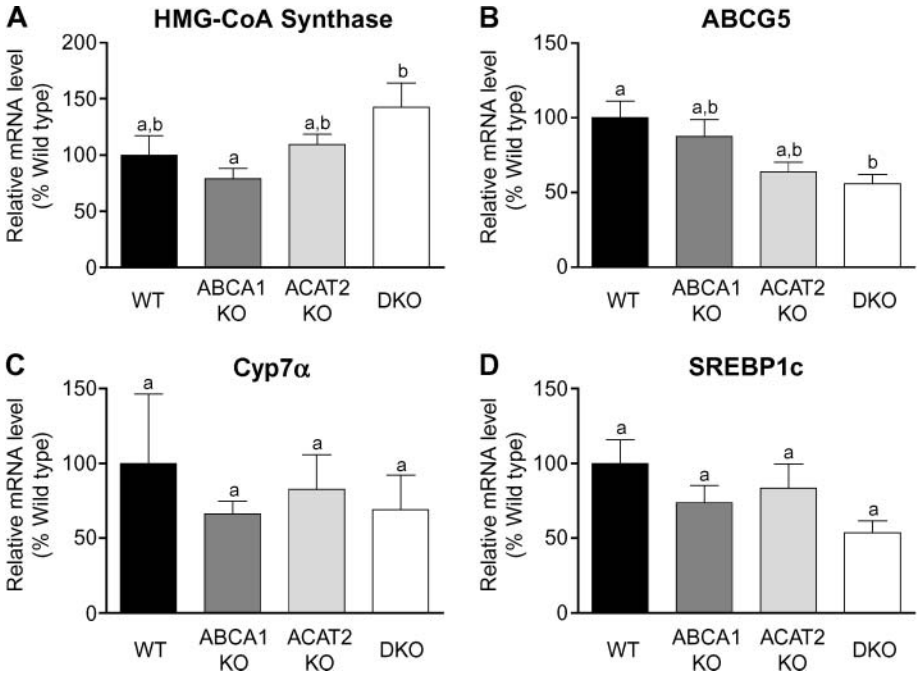

Fig. 5. Expression of genes involved in hepatic lipid metabolism. Total RNA was isolated from liver removed from female mice fed a low-fat, moderate-cholesterol diet for 6 weeks. After being reverse transcribed, the mRNA levels of HMG-CoA synthase (A), ABCG5 (B), 7α-hydroxylase (Cyp7α; C), and sterol-regulatory element binding protein 1c (SREBP1c; D) were determined using real-time PCR analysis. All values were normalized to the cyclophilin mRNA concentration of the samples, and the mean of the wild-type (WT) samples was arbitrarily designated as 100%. Each column represents the mean ± SEM of five samples. Statistically significant differences were determined for each data set by ANOVA (Tukey-Kramer honestly significant difference) and are indicated by different letters ($P < 0.05$).

TABLE 3. Concentration and lipoprotein distribution of plasma cholesterol

Genotype	Total Cholesterol	Free Cholesterol	VLDL + LDL Cholesterol	HDL Cholesterol
		mg/dl		
Wild type (n = 7)	111 ± 6.1 a	23 ± 1.5 a	34 ± 1.9 a, b	77 ± 4.7 a
ABCA1 KO (n = 8)	18 ± 2.1 b	2.7 ± 0.6 b	16 ± 1.9 b	1.2 ± 0.3 b
ACAT2 KO (n = 7)	81 ± 3.4 c	26 ± 1.4 a	18 ± 3.2 b	63 ± 1.6 c
DKO (n = 7)	50 ± 9.8 d	47 ± 9.0 c	49 ± 9.8 a	0.8 ± 0.3 b

Plasma was isolated from fasted mice and analyzed for total and free cholesterol concentrations using enzymatic kits. Lipoprotein cholesterol distribution was determined by separating plasma using gel-exclusion chromatography linked with an inline total cholesterol assay. The values represent means ± SEM of n measurements. Statistically significant differences were determined for each column of values by ANOVA (Tukey-Kramer honestly significant difference) and are indicated by different letters ($P < 0.05$).

but an even greater reduction was observed for DKO mice (25%) (Fig. 2A). In addition, fecal neutral sterol excretion was increased by 130% in DKO mice but by only 64% in ACAT2 KO mice (Fig. 2B). Although a statistically significant decrease in fractional cholesterol absorption was seen in ABCA1 KO mice (down to 55% from 63%) (Fig. 2A), this effect was small and did not result in a significant increase in neutral sterol excretion (Fig. 2B). These results show that ABCA1 contributes minimally to cholesterol absorption when ACAT2 is functional. In agreement with this conclusion, previous studies have shown that fecal neutral sterol excretion is similar in ABCA1 KO and wild-type mice (23) and that fractional cholesterol absorption is decreased by only 13% in ABCA1 KO mice (21) compared with 60–85% in ACAT2 KO mice (13, 14). However, in the absence of ACAT2, the increased expression of ABCA1 (Fig. 1B) (14), presumably in response to an accumulation of intracellular free cholesterol, oxysterol formation, and subsequent transcriptional upregulation via LXR, may partially compensate for the lack of cholesterol esterification and provide for some movement of cholesterol into the body.

The mechanism by which ABCA1 mediates cholesterol absorption is currently unknown. Most likely, it involves ABCA1-dependent efflux of free cholesterol from the basolateral surface of the enterocytes to lipid-poor apolipoproteins such as apoA-I. In agreement with this hypothesis, studies in CaCo-2 cells have shown that free cholesterol can be trafficked from the apical to the basolateral surface of the cell and subsequently effluxed, presumably via ABCA1, on HDL particles (18–20). Alternatively, ABCA1 may have an indirect role in cholesterol absorption as a result of its requirement in the formation of mature HDL particles. In this scenario, cholesterol from the enterocytes would be effluxed to mature, spherical HDLs via a receptor other than ABCA1. Two possible candidate receptors are ABCG1 and scavenger receptor class B type I (SR-BI), both of which efflux cholesterol to spherical HDLs (34, 35). Although SR-BI KO mice do not display a defect in cholesterol absorption (36), cholesterol efflux mediated by SR-BI, which is expressed on the basolateral membrane of enterocytes (37, 38), could become important in ACAT2 KO mice. ABCG1 could also aid ACAT2 KO mice in cholesterol absorption. LXR agonist treatment of CaCo-2 cells increased ABCG1 expression and cholesterol efflux to exogenously added HDL (19). However, in the presence or absence of LXR agonist, ABCG1 expression in mice was detected only in resident macrophages of the small intestine and not in enterocytes (39). Thus, it is also possible that another currently unknown receptor could mediate the efflux of cholesterol to mature HDL. Further experiments will be necessary to define the exact role that ABCA1 plays in cholesterol absorption.

In the absence of both ABCA1 and ACAT2, the absorption of cholesterol was presumably limited to free cholesterol incorporation into the chylomicrons. Because the capacity of this mechanism is likely much more limited than esterification via the ACAT2-dependent pathway, unchecked movement of cholesterol into the DKO enterocytes could have had deleterious effects. Possibly to avoid the potential toxic effects of excess intracellular cholesterol, the expression of NPC1L1 was decreased significantly (Fig. 3A), an effect similar to that shown for wild-type and ACAT2 KO mice fed a high-cholesterol diet (14, 40). This downregulation of NPC1L1 in the enterocytes presumably led to a reduction in the movement of cholesterol from the lumen of the small intestine into the cells. The enterocytes also could have eliminated excess free cholesterol by upregulating the expression of ABCG5 and ABCG8, which presumably efflux free cholesterol back into the lumen of the small intestine (32). However, compared with wild-type mice, DKO mice did not display a significant increase in ABCG5 mRNA expression in the small intestine (Fig. 3B). This result, which is in accordance with data on wild-type and ACAT2 KO mice fed a high-cholesterol diet (14), indicates that regardless of genotype, ABCG5 mRNA may have been maximally expressed as a result of the consumption of a cholesterol-rich diet. Nevertheless, DKO enterocytes could have had either increased expression of ABCG5 and ABCG8 protein or improved functional activity from the existing ABCG5 and ABCG8 protein complexes. Thus, a combination of decreased cholesterol uptake caused by the downregulation of NPC1L1 and increased cholesterol efflux via ABCG5 and ABCG8 may serve to maintain free cholesterol levels in DKO enterocytes.

The liver is the clearinghouse for cholesterol associated with chylomicrons and HDL that originate from the small intestine. Therefore, it was predicted that hepatic cholesterol metabolism would mirror the amount of cholesterol absorption displayed by the four genotypes of mice. This

assumption was correct for the wild-type and ABCA1 KO mice because these two genotypes had similar levels of cholesterol absorption (Fig. 2) and nearly identical hepatic lipid concentrations (Fig. 4), gallbladder bile composition (Table 2), and hepatic mRNA expression of genes involved in cholesterol homeostasis (Fig. 5). These results are similar to those reported previously for wild-type and ABCA1 KO mice (23) and support the conclusion that in the presence of ACAT2, ABCA1 has an auxiliary role in cholesterol absorption. The idea that cholesterol absorption directly affects hepatic cholesterol homeostasis was also confirmed by findings in ACAT2 KO mice. Compared with wild-type and ABCA1 KO mice, ACAT2 KO mice absorbed significantly less cholesterol, which may contribute to an ~2-fold reduction in cholesterol concentration in gallbladder bile (Table 2). In addition, ACAT2 KO mice displayed decreases of 30% and 99% in hepatic free cholesterol and cholesteryl ester concentration, respectively (Fig. 4), the latter reduction being compounded by the hepatocytes lacking their sole cholesterol-esterifying enzyme, ACAT2 (13, 41, 42). Because DKO mice exhibited the largest decrease in cholesterol absorption (Fig. 2), an even greater alteration in hepatic cholesterol metabolism was expected for these mice. However, compared with ACAT2 KO mice, DKO mice displayed similar concentrations of biliary cholesterol, bile salt, and hepatic free cholesterol (Table 2, Fig. 4A). In addition, only slight differences between these two genotypes were seen for the hepatic expression of cholesterol-sensitive genes such as HMG-CoA synthase, ABCG5, and sterol-regulatory element binding protein 1c (Fig. 5). One explanation for the minimal variations in hepatic cholesterol metabolism in ACAT2 KO and DKO mice could relate to the differences in hepatic HDL formation. In liver-specific ABCA1 KO mice, the hepatocyte was found to be the most important cell in the formation of nascent HDL (43). Therefore, the greater amount of cholesterol absorbed by ACAT2 KO versus DKO mice could have been directed toward ABCA1-dependent HDL formation in the liver and away from excretion into bile and the synthesis of bile acids. Consequently, the amount of cholesterol available for excretion into bile could have been similar in ACAT2 KO and DKO mice.

Previous studies have shown that deficiencies in either ABCA1 or ACAT2 cause significant decreases in total plasma cholesterol. Because ABCA1 is essential in the formation of nascent HDL particles, ABCA1 deficiency results in nearly undetectable levels of HDL cholesterol (24, 44, 45). On the other hand, because ACAT2 creates cholesteryl ester that is packaged into chylomicrons and VLDL, ACAT2 KO mice display a significant decrease in cholesterol carried in apoB-containing lipoproteins (13, 28, 46). Because of these major effects on lipoprotein cholesterol, it was assumed that DKO mice would have lower TPC concentrations than mice lacking either ABCA1 or ACAT2. As expected, the TPC of DKO mice was significantly less than in both wild-type and ACAT2 KO mice (Table 3). However, because of an increase in cholesterol carried in VLDL- and LDL-sized particles, DKO mice had ~3-fold more TPC than ABCA1 KO mice. In fact, compared with the other three groups, DKO mice had the highest concentration of VLDL plus LDL cholesterol. Also unexpected was the finding that >90% of the cholesterol in the plasma of DKO mice was free cholesterol, compared with 30% or less for the other genotypes. Preliminary experiments to determine the cause of the increased concentrations of free cholesterol and VLDL/LDL cholesterol have shown that plasma LCAT activity is drastically reduced in DKO mice. This LCAT insufficiency in combination with the absence of ACAT2 results in the accumulation of particles in the LDL density range that lack cholesteryl ester and are enriched in free cholesterol and phospholipids (R. E. Temel, H. M. Alger, and L. L. Rudel, unpublished data). LDL particles with characteristics similar to those of DKO mice were also found in mice with null mutations in both LCAT and ACAT2 (47). The reasons for the buildup of these abnormal LDLs in plasma are not completely clear; however, cholesteryl esters derived from either ACAT2 or LCAT may be essential for maintaining the structural integrity of LDL. Accordingly, LDL particles lacking a core of cholesteryl ester could assume an irregular shape, thus changing the conformation of apoB or apoE and limiting the hepatic clearance of the LDL. To test these conclusions, experiments analyzing plasma lipoprotein metabolism in the DKO mice are currently under way.

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