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ACAT2 Deficiency Limits Cholesterol Absorption in the Cholesterol-Fed Mouse: Impact on Hepatic Cholesterol Homeostasis

Joyce J. Repa,[1,2] Kimberly K. Buhman,[3] Robert V. Farese, Jr.,[3] John M. Dietschy,[1] and Stephen D. Turley[1]

Acyl CoA:cholesterol acyltransferase (ACAT) 2 is the major cholesterol-esterifying enzyme in mouse enterocytes and hepatocytes. Male ACAT2$^{+/+}$ and ACAT2$^{-/-}$ mice were fed chow containing added cholesterol (0%-0.500% w/w) for 24 days. Over this range, fractional cholesterol absorption in the ACAT2$^{+/+}$ mice fell from 41.4% ± 6.6% to 21.0% ± 5.2%, and in their ACAT2$^{-/-}$ counterparts it fell from 35.1% ± 4.5% to 7.9% ± 0.8%. The mass of dietary cholesterol absorbed (mg/d per 100 g body weight) increased from 1.2 ± 0.2 to 14.7 ± 4.4 in the ACAT2$^{+/+}$ mice and from 1.0 ± 0.2 to 5.5 ± 0.6 in those without ACAT2. In the ACAT2$^{+/+}$ mice, hepatic cholesterol concentrations increased as a function of intake despite compensatory changes in cholesterol and bile acid synthesis and in the expression of adenosine triphosphate–binding cassette transporter G5 (ABCG5) and ABC transporter G8 (ABCG8). In contrast, in ACAT2$^{-/-}$ mice in which the amount of cholesterol absorbed at the highest intake was only 37% of that in the ACAT2$^{+/+}$ mice, suppression of synthesis was a sufficient adaptive response; there was no change in bile acid synthesis, ABCG5/G8 expression, or hepatic cholesterol concentration. The expression of adenosine triphosphate–binding cassette transporter A1 (ABCA1) in the jejunum was markedly elevated in the ACAT2$^{-/-}$ mice, irrespective of dietary cholesterol level. In conclusion, although ACAT2 deficiency limits cholesterol absorption, the extent to which it impacts hepatic cholesterol homeostasis depends on cholesterol intake. Loss of ACAT2 activity may result in unesterified cholesterol being absorbed via an ABCA1-mediated basolateral efflux pathway. (HEPATOLOGY 2004;40:1088–1097.)

The central role of the liver in regulating the production and clearance of low density lipoprotein (LDL) cholesterol is well defined.[1–3] Although the balance of cholesterol across the liver is articulated through the interplay of many complex pathways,[4] it is largely the amount of cholesterol reaching the liver from the small intestine that determines not only how much cholesterol the liver itself synthesizes, but also the amount of cholesterol that is esterified in the hepatocyte and either stored in lipid droplets or incorporated into nascent very low density lipoproteins, the precursors of most of the LDL cholesterol in the circulation.[1]

In humans, the absorption of cholesterol leads to the net delivery of hundreds of milligrams of cholesterol to the liver each day.[5,6] The majority of this cholesterol is carried in esterified form in the core of chylomicrons which, after removal of much of their triacylglycerol content, become remnant particles that are rapidly cleared by the liver.[7] The main steps in the absorption process have been defined previously.[6] Most recently, Niemann-Pick C1-like 1 (NPC1L1) was found to play a major role in facilitating the uptake of cholesterol and other noncholesterol sterols by the enterocyte.[8] Ezetimibe—a potent inhibitor of cholesterol absorption—and its analogs inhibit the activity of NPC1L1 and diminish the cholesterol content of chylomicrons reaching the circulation.[8,9] The enterohepatic movement of cholesterol can be regulated by

Abbreviations: ACAT, acyl CoA:cholesterol acyltransferase; ABCG5, adenosine triphosphate–binding cassette transporter G5; ABCG8, adenosine triphosphate–binding cassette transporter G8; ABCA1, adenosine triphosphate–binding cassette transporter A1; LDL, low density lipoprotein; NPC1L1, Niemann-Pick C1-like 1; mRNA, messenger RNA; SREBP-1, sterol regulatory element-binding protein 1; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

From the Departments of [1]Internal Medicine and [2]Physiology, University of Texas Southwestern Medical Center, Dallas, TX; and the [3]Gladstone Institute of Cardiovascular Disease, San Francisco, CA.

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K.K.B. is currently affiliated with Washington University Medical School, St. Louis, MO.

Address reprint requests to: Stephen D. Turley, Ph.D. Department of Internal Medicine, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd, Dallas, TX 75390-8887. E-mail: stephen.turley@utsouthwestern.edu; fax: 214-648-9761.

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manipulating other steps in the absorption process, including the hydrolysis of lipids or micellar solubilization of cholesterol within the lumen, or the esterification of cholesterol within the enterocyte by acylCoA:cholesterol acyltransferase (ACAT).[10–14] Numerous ACAT inhibitors have been found to be effective in preventing hepatic cholesterol accumulation and hypercholesterolemia in animal models.[11] In contrast, three separate ACAT inhibitors evaluated in humans showed little or no LDL cholesterol–lowering action.[15–17]

The identity and pathophysiology of cholesterol esterifying enzymes in the major organs of several species have been well defined.[18–21] In the mouse, ACAT1 is distributed in many tissues, but ACAT2 is the dominant esterifying enzyme in the liver and small intestine.[19] Mice bearing selective deletion of the genes for either ACAT1 or ACAT2 have been characterized in detail.[19,22] In ACAT2 knockout mice fed a basal low-cholesterol rodent diet, fractional cholesterol absorption was not different than in wild-type controls.[19] However, when a diet enriched with cholesterol, triacylglycerol, and cholic acid was given, fractional cholesterol absorption was significantly lower in the ACAT2-deficient mice compared with $ACAT^{+/+}$ control mice. Furthermore, unlike their controls, the $ACAT2^{-/-}$ mice fed this diet maintained a near normal hepatic cholesterol concentration and exhibited rates of cholesterol synthesis in the liver similar to those of chow-fed mice. Together, these findings suggested that the importance of ACAT2 activity in regulating cholesterol absorption and hepatic cholesterol homeostasis might vary depending on dietary cholesterol intake. To further investigate this possibility, we measured cholesterol absorption and various parameters of hepatic cholesterol metabolism in $ACAT2^{+/+}$ and $ACAT2^{-/-}$ mice fed chow enriched with graded amounts of cholesterol only. The data show that ACAT2 deficiency impeded but did not totally prevent the absorption of additional cholesterol as the dietary cholesterol intake was raised. The adaptive changes in hepatic cholesterol metabolism that ensued were qualitatively the same in mice of both genotypes, although less pronounced in the case of the ACAT2-deficient animals.

Materials and Methods

Animals and Diets. ACAT2-deficient mice were generated as described previously[19] and maintained on a mixed strain background (C57BL/6:129/SvJae), as were matching $ACAT2^{+/+}$ controls. All experiments were performed in male mice 4 to 6 months of age that were housed individually and initially maintained on a cereal-based rodent diet (Wayne Lab Blox, No. 8604) (Harlan Teklad, Madison, WI). This basal diet had an inherent cholesterol and total lipid content of approximately 0.02% w/w and 4% w/w, respectively.[23] The mice were subsequently fed the meal form of 8604 containing added cholesterol at levels of 0%, 0.125%, 0.250%, 0.375%, and 0.500% w/w for 24 days. All mice were fed their respective diets *ad libitum* and were studied in the fed state toward the end of the 12-hour dark phase of the lighting cycle. The liver was removed, and the gallbladder, with contents, was placed in 1 mL of methanol. Jejunum was also removed in one study. All experiments were approved by the Institutional Animal Care and Research Advisory Committee.

Fecal Bile Acid Excretion. After 18 days on their respective diets, stools were collected over 3 days and their total bile acid content was measured.[23] Fecal bile acid excretion was expressed as μmol/d per 100 g body weight.

Intestinal Cholesterol Absorption. Cholesterol absorption was measured by a fecal dual-isotope method.[23] After 21 days on their respective diets, the mice were dosed intragastrically with a mixture of 2 μCi [5,6-^{3}H]sitostanol and 1 μCi [4-^{14}C]cholesterol. They were then housed in fresh cages and stools were collected over the following 3 days. Aliquots of stool and the dosing mixture were extracted, and the ratio of ^{14}C to ^{3}H in each was determined.[23] These values were used to calculate fractional (percent) absorption values. These data, together with the daily food intake of the mice and cholesterol content of their respective diets, were used to calculate the approximate milligrams of dietary cholesterol absorbed per day per 100 g body weight. Food intake averaged approximately 14 g/d per 100 g body weight irrespective of genotype or dietary cholesterol level.

Liver and Plasma Cholesterol Levels and Biliary Bile Acid Composition. Liver and plasma total cholesterol concentrations were determined as described.[23] The cholesterol concentration in the jejunum was not measured because the mucosa was used for messenger RNA (mRNA) analysis. Biliary bile acid composition was determined by high-performance liquid chromatography.[23]

RNA Analysis. Five micrograms of poly (A+) RNA prepared from pooled liver samples were subjected to electrophoresis and Northern blot analysis using the ^{32}P-labeled probes indicated. The amount of radioactivity in each band was quantified using a PhosphoImager (Molecular Dynamics, Sunnyvale, CA), normalized to the signal generated by β-actin, and mathematically adjusted to establish a unit of 1 for the $ACAT2^{+/+}$ mice fed the basal diet alone.[24] In one study in which the mice were fed chow with either 0% or 0.5% added cholesterol, mRNA levels for various proteins in jejunal mucosa were determined by quantitative real-time polymerase chain reac-

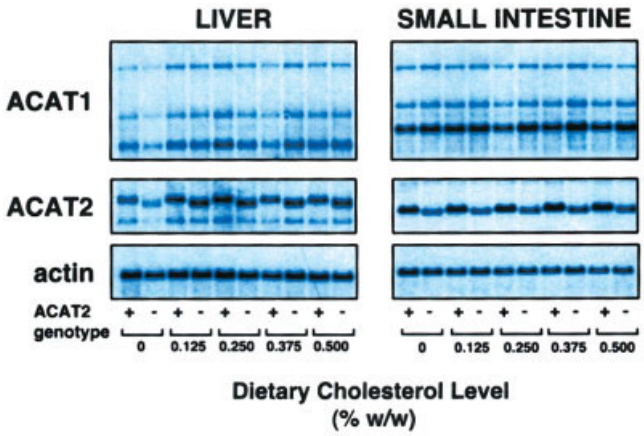

Fig. 1. Relative level of expression of mRNA for ACAT1 and ACAT2 in the liver and small intestine of ACAT2$^{+/+}$ and ACAT2$^{-/-}$ mice fed cholesterol-enriched diets. Matching groups of ACAT2$^{+/+}$ and ACAT2$^{-/-}$ mice were fed a diet containing added cholesterol at levels of 0%, 0.125%, 0.250%, 0.375%, and 0.500% w/w for 24 days. The relative level of expression of mRNA for both ACAT1 and ACAT2 in the small intestine and liver was determined using Northern blot analysis. For the ACAT2 genotype, plus and minus signs refer to ACAT2$^{+/+}$ and ACAT2$^{-/-}$ animals, respectively. Each band represents the relative mRNA level determined in a sample composed of equal amounts of RNA from 6 animals in each group. As documented previously, ACAT1 mRNA appears as 3 transcript sizes.[44] The ACAT2 transcript in the mutant animals is truncated and does not produce a functional protein. ACAT1, acyl CoA:cholesterol acyltransferase 1; ACAT2, acyl CoA:cholesterol acyltransferase 2.

tion assay.[24–26] These proteins included NPC1L1, the primer sequence for which is as follows: forward, 5′TGGACTGGAAGGACCATTTCC; reverse, 5′GACAGGTGCCCCGTAGTCA.

Analysis of Data. Where appropriate, data are reported as the mean ± 1 SEM. GraphPad Prism software (GraphPad, San Diego, CA) was used to perform all statistical analyses. Differences between means were tested for statistical significance ($P < .05$) using either one-way or two-way ANOVA with ACAT2 genotype and dietary cholesterol level as factors, using transformed data if unequal variances were evident among the groups.

Results

Although ACAT2 is the dominant esterifying enzyme in the small intestine and liver, ACAT1 is also present in both organs.[19,21] Therefore, we evaluated whether or not the relative expression of mRNA for ACAT1 in these organs varied as a function of either ACAT2 genotype or dietary cholesterol level. The data in Fig. 1 show that the mRNA levels for ACAT1 were about the same in both ACAT2$^{-/-}$ and ACAT2$^{+/+}$ mice at all dietary cholesterol levels. This figure also shows that, in the case of the ACAT2$^{+/+}$ mice, the relative mRNA levels for ACAT2 in the small intestine and liver did not change consistently as a function of dietary cholesterol content. It should be noted here that the determination of the mRNA levels for ACAT2 via Northern blot analysis results in the detection of a truncated transcript, which does not produce a functional protein.[19]

The body weights of the mice of both genotypes averaged approximately 30 to 33 g at all dietary cholesterol levels (Fig. 2A). On the higher cholesterol diets, modest hepatomegaly was seen in the ACAT2$^{+/+}$ mice but not their ACAT2$^{-/-}$ counterparts (Fig. 2B). The data in Fig. 3 illustrate the effect of varying dietary cholesterol content on both fractional cholesterol absorption (Fig. 3A) and the mass of dietary cholesterol absorbed (Fig. 3B). In mice fed the diet with no added cholesterol, there was no genotypic difference in either the fractional or absolute levels of absorption. However, as the dietary cholesterol content was raised, especially to levels above 0.250% w/w, both the fractional and absolute levels of absorption were

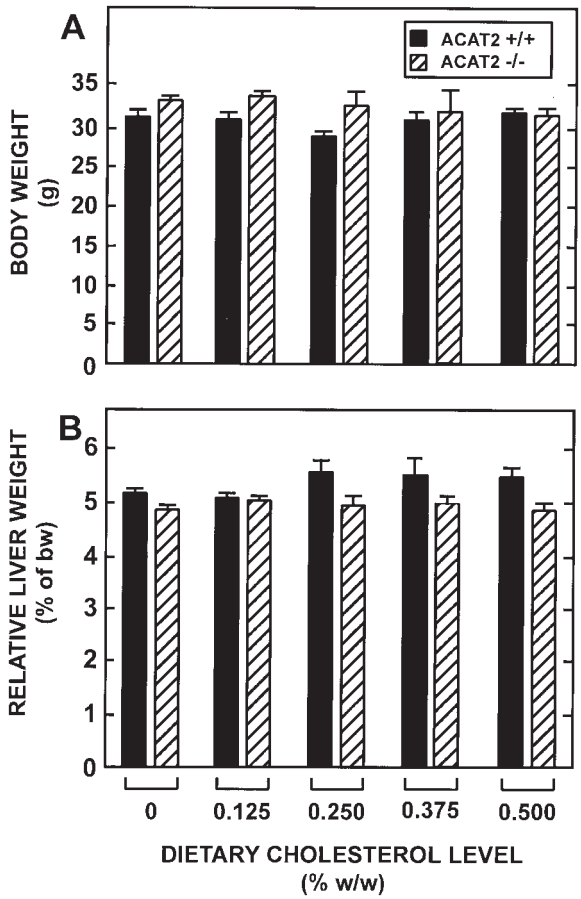

Fig. 2. Body and relative liver weights in ACAT2$^{+/+}$ and ACAT2$^{-/-}$ mice fed cholesterol-enriched diets. These data pertain to the same groups of animals discussed in the legend to Fig. 1. Values are the mean ± 1 SEM of data from 6 animals in each group. Two-way ANOVA revealed a significant effect ($P < .05$) of ACAT2 genotype on (A) body weight and (B) relative liver weight. ACAT2, acyl CoA:cholesterol acyltransferase 2; bw, body weight.

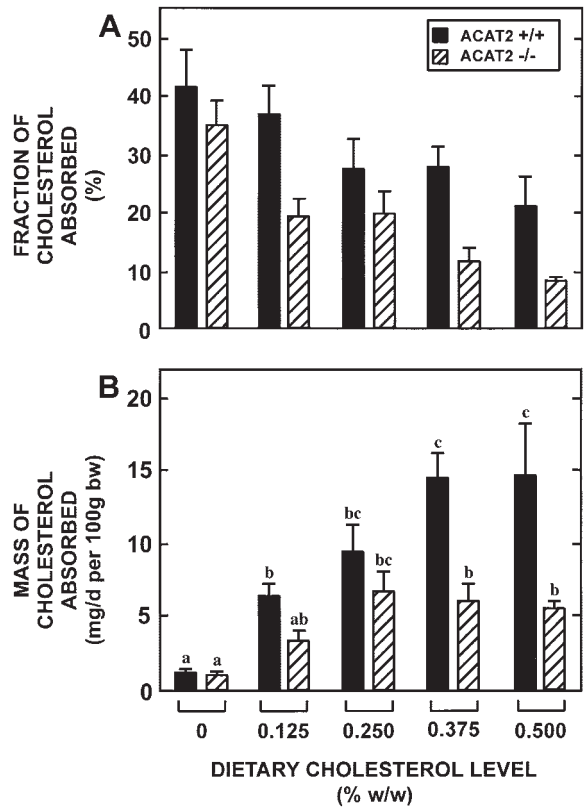

Fig. 3. Fractional and absolute levels of intestinal cholesterol absorption in ACAT2$^{+/+}$ and ACAT2$^{-/-}$ mice fed cholesterol-enriched diets. After 21 days on their respective diets, the mice were dosed intragastrically with labeled sterols. Stools were collected over the following 3 days for the measurement of (A) fractional cholesterol absorption. (B) The mass of dietary cholesterol absorbed was calculated from the fractional absorption value, the dietary cholesterol content, and the daily food intake, which averaged 14 g/d per 100 g body weight. Values are the mean ± 1 SEM of data from 6 animals in each group. Two-way ANOVA revealed a significant effect ($P < .05$) of both ACAT2 genotype and dietary cholesterol level on the fractional and absolute levels of cholesterol absorption. A significant interaction was observed only for the mass of cholesterol absorbed, thus allowing for a *post hoc* Newman-Keuls test of statistical significance among all groups. Bars designated with the same letter are not statistically different ($P \geq .05$). ACAT2, acyl CoA:cholesterol acyltransferase 2; bw, body weight.

decisively lower in the ACAT2$^{-/-}$ mice than in their ACAT2$^{+/+}$ controls. At the two highest dietary cholesterol levels, the mass of cholesterol absorbed reached a plateau of 5 to 6 mg/d per 100 g body weight in the ACAT2$^{-/-}$ mice compared with 14 to 15 mg/d per 100 g body weight in their corresponding controls. Although ACAT2 deficiency clearly reduced the amount of dietary cholesterol absorbed as dietary cholesterol intake increased, the amount of cholesterol absorbed by the mice lacking ACAT2 at the highest levels of intake was nevertheless approximately fourfold more than the amount absorbed by mice of either genotype fed the diet with no added cholesterol.

The genotypic and diet-related differences in cholesterol absorption were reflected in the total cholesterol concentration in the liver (Fig. 4A) and plasma (Fig. 4B). Although in absolute terms the changes in concentration were modest, the data show that, unlike their matching ACAT2$^{+/+}$ controls, the ACAT2$^{-/-}$ mice maintained normal hepatic cholesterol concentrations at all levels of cholesterol intake. This was particularly noteworthy for the ACAT2$^{-/-}$ mice given that at the higher levels of cholesterol intake these mice absorbed approximately four times more dietary cholesterol compared with their counterparts given the basal diet alone. The hierarchy of adaptive changes in intrahepatic cholesterol handling that occurred in both types of mice as a function of their dietary cholesterol intake was delineated by measuring the relative mRNA

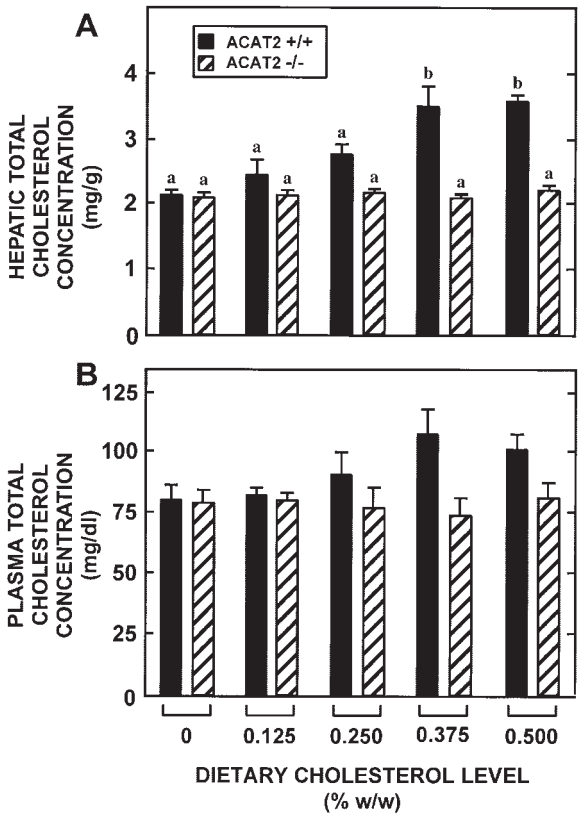

Fig. 4. Hepatic and plasma cholesterol concentrations in ACAT2$^{+/+}$ and ACAT2$^{-/-}$ mice fed cholesterol-enriched diets. These data were obtained from the groups of mice described in the legends for Figs. 1 and 2. Values are the mean ± 1 SEM of data from 6 animals in each group. (A) Two-way ANOVA revealed a significant effect of both genotype and diet—as well as a significant interaction between these two factors—on hepatic cholesterol concentrations. This allowed us to evaluate the 10 treatment groups independently using a *post hoc* Newman-Keuls test of significance. The bars designated with the same letter are not significantly different ($P \geq .05$). (B) No significant interaction of genotype and diet was found for plasma cholesterol concentrations, but these did vary significantly ($P < .05$) as a function of genotype. ACAT2, acyl CoA: cholesterol acyltransferase 2.

expression of multiple proteins that regulate cholesterol homeostasis in the liver, as well as two parameters of bile acid metabolism. The data in Fig. 5 show the relative mRNA levels for 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) synthase (Fig. 5A), HMG CoA reductase (Fig. 5B), and the LDL receptor (Fig. 5C). These values were determined by Northern blot analysis on pooled liver samples. Clearly, there was an adaptive downregulation of cholesterol synthesis in the liver of both types of mice as their dietary cholesterol intake was raised. However, without exception, the degree of downregulation of both HMG CoA synthase and HMG CoA reductase was less in the ACAT2$^{-/-}$ mice than it was in their matching ACAT2$^{+/+}$ controls. This was also generally true for the mRNA levels for the LDL receptor (Fig. 5C).

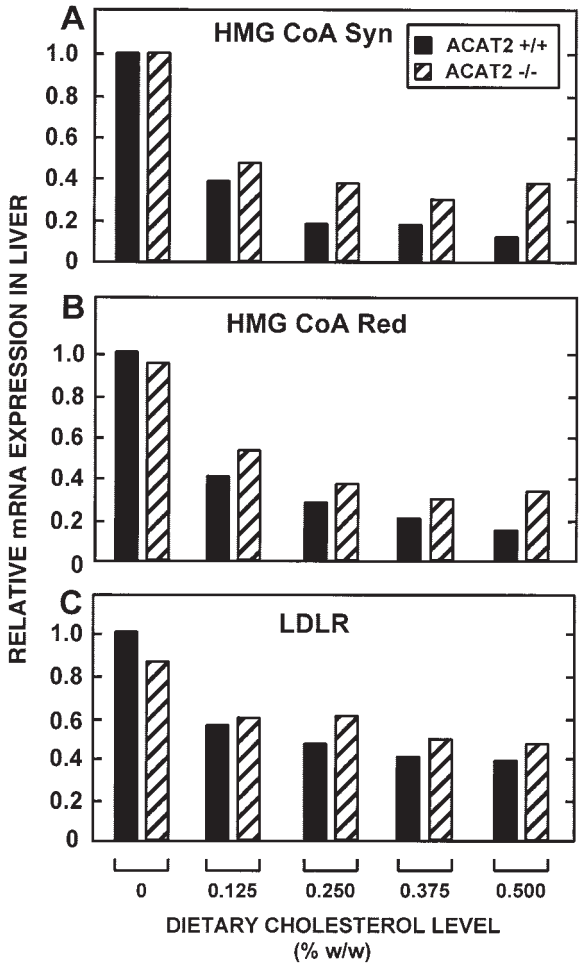

Fig. 5. Relative level of expression of mRNA for (A) HMG CoA synthase, (B) HMG CoA reductase, and (C) the LDL receptor in the livers of ACAT2$^{+/+}$ and ACAT2$^{-/-}$ mice fed cholesterol-enriched diets. Liver samples from the mice that were used in the experiments described in the legends for Figs. 1 and 2 were pooled and used for mRNA analysis. Each histogram indicates the relative amount of mRNA established by Northern blot analysis and represents measurements in pooled livers from 6 animals in each group. HMG CoA Syn, 3-hydroxy-3-methylglutaryl coenzyme A synthase; HMG CoA Red, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDLR, low density lipoprotein receptor.

A major focus of the hepatic mRNA expression analyses was the relative levels of adenosine triphosphate–binding cassette transporters A1 (ABCA1), G5 (ABCG5), and G8 (ABCG8). In contrast to the level of expression of ABCA1 (Fig. 6A), which remained about the same in all groups of mice, the expression of ABCG5 (Fig. 6B) and ABCG8 (Fig. 6C) showed marked genotypic and diet-related differences. The mRNA levels for these two proteins increased progressively with dietary cholesterol intake in the ACAT2$^{+/+}$ mice, but not in their ACAT2$^{-/-}$ counterparts. At the two highest dietary cholesterol levels tested, the mRNA levels for both proteins in the ACAT2$^{+/+}$ mice was two- to threefold greater than it was in matching ACAT2$^{-/-}$ mice. The pattern of expression of sterol regulatory element-binding protein 1 (SREBP-1) (Fig. 6D) closely reflected that of ABCG5 and ABCG8 in mice of both genotypes. Together, the data in Fig. 6 correlate well not only with the other mRNA data (see Fig. 5), but also with the differences between the two types of mice in the net amount of cholesterol that was delivered to the liver as their intake was raised. Thus, in the ACAT2$^{+/+}$ mice, but not in the ACAT2$^{-/-}$ mice, the increase in cholesterol reaching the liver was sufficient to elicit an increase in mRNA levels for both ABCG5 and ABCG8.

Although the mRNA data for HMG CoA synthase and reductase showed a compensatory downregulation of hepatic sterol synthesis in ACAT2$^{+/+}$ mice (and to a lesser extent in mice lacking ACAT2), as their cholesterol intake was raised it is possible that other adaptive mechanisms such as induction of bile acid synthesis also occurred. Two parameters were examined: biliary bile acid composition (Fig. 7A) and fecal bile acid excretion (Fig. 7B). In the mouse, cholic and muricholic acid are the dominant bile acids synthesized, and the ratio of these two bile acids in bile shifts dramatically with genetic or dietary induced changes in the rate of total bile acid synthesis.[13,27] As shown in Fig. 7A, the ratio of cholic to muricholic acid in the bile of the ACAT2$^{+/+}$ mice fell progressively as the dietary cholesterol level was raised. This change was not seen in the bile of the ACAT2$^{-/-}$ mice. Hence, at dietary levels of 0.250% and above, the bile of the ACAT2$^{+/+}$ mice had a noticeably lower enrichment with cholic acid compared to the bile of their matching ACAT2$^{-/-}$ controls. These genotypic differences in biliary bile acid composition correlated well with the differences between both types of mice in their rate of fecal bile acid

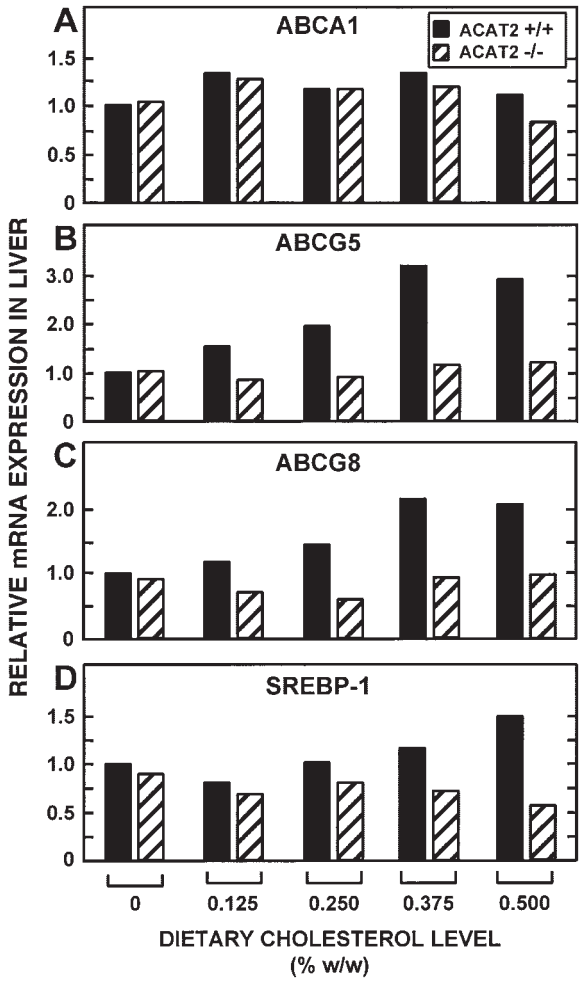

Fig. 6. Relative level of expression of mRNA for (A) ABCA1, (B) ABCG5, (C) ABCG8, and (D) SREBP-1 in the livers of ACAT2$^{+/+}$ and ACAT2$^{-/-}$ mice fed cholesterol-enriched diets. Liver samples from the mice that were used in the experiments described in the legends for Figs. 1 and 2 were pooled and used for mRNA analysis. Each histogram indicates the relative amount of mRNA found for ABCA1, ABCG5, ABCG8, and SREBP-1 using Northern blot analysis and represents measurements in pooled livers from 6 animals in each group. SREBP-1, sterol regulatory element-binding protein 1.

excretion (Fig. 7B). While mice of both genotypes showed higher rates of bile acid excretion as their cholesterol intake increased, the change was clearly more decisive in the ACAT2$^{+/+}$ mice than in their ACAT2$^{-/-}$ counterparts.

The final set of data shows the changes in the relative mRNA levels for five proteins in the jejunal mucosa as a function of genotype and dietary cholesterol intake (Fig. 8). For this study, mice of both genotypes were fed chow with either 0% or 0.5% added cholesterol. As shown in Fig. 8A, the mRNA levels for NPC1L1 were lower in the mice fed the high-cholesterol diet—particularly in the ACAT2$^{-/-}$ group—where they fell by approximately 50% compared with the levels of expression in the mice fed chow alone. A similar result was found for HMG CoA synthase (Fig. 8B). Irrespective of dietary cholesterol intake, the relative mRNA levels for both ABCG5 (Fig. 8C) and ABCG8 (Fig. 8D) were consistently higher in the ACAT2$^{-/-}$ mice compared with their matching ACAT2$^{+/+}$ controls. However, these changes were modest compared with those for ABCA1 (Fig. 8E). In the ACAT2$^{-/-}$ mice fed chow with no added cholesterol, the mRNA levels for ABCA1 were 4-fold greater than in ACAT2$^{+/+}$ mice fed the same diet. Cholesterol feeding raised the mRNA levels for ABCA1 in mice of both genotypes,

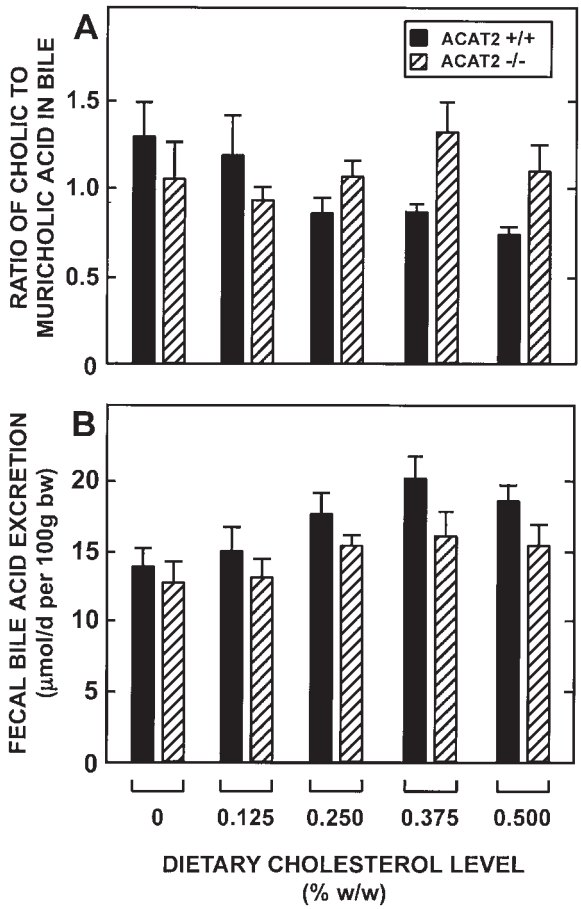

Fig. 7. Biliary bile acid composition and rate of fecal bile acid excretion in ACAT2$^{+/+}$ and ACAT2$^{-/-}$ mice fed cholesterol-enriched diets. (A) Bile acid composition of gallbladder bile and (B) rate of fecal bile acid excretion were determined in the same groups of mice that were used for the experiments described in the legends for Figs. 1 and 2. Values are the mean ± 1 SEM of data from 6 animals in each group. Two-way ANOVA revealed a significant effect ($P < .05$) of both ACAT2 genotype and dietary cholesterol level on the rate of fecal bile acid excretion, but not on biliary bile acid composition. No significant interaction between these variables was observed. ACAT2, acyl CoA:cholesterol acyltransferase 2.

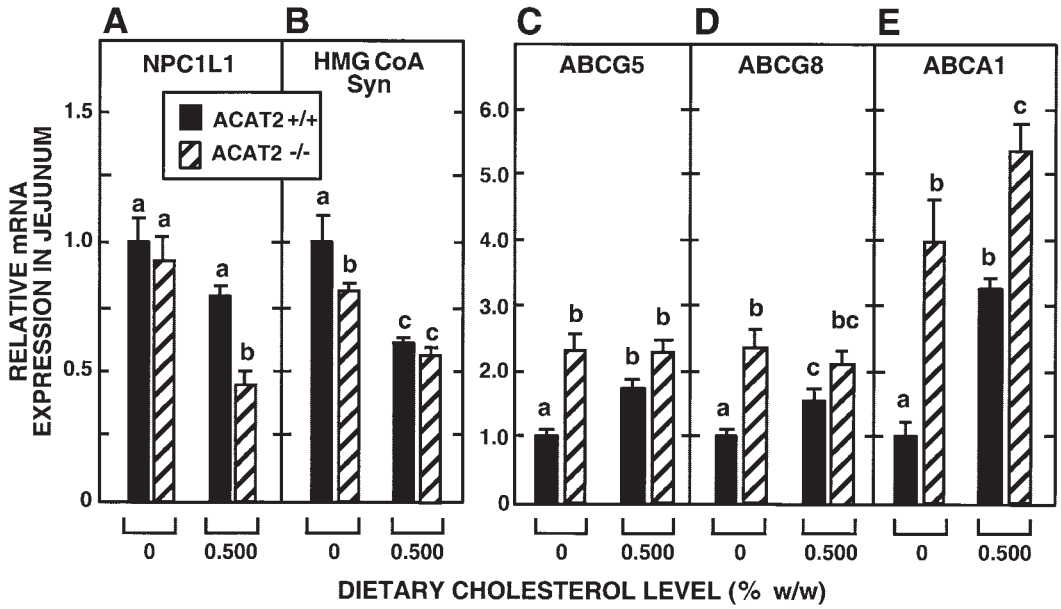

Fig. 8. Relative expression of mRNA for various proteins in the jejunum of ACAT2$^{+/+}$ and ACAT2$^{-/-}$ mice fed cholesterol-enriched diets. The jejunal mucosa was removed from mice that had been fed chow containing added cholesterol at levels of either 0% or 0.5% w/w. The relative mRNA level for each of the proteins (A-E) was determined in mucosa from individual mice using quantitative real-time polymerase chain reaction assay. Values are the mean ± 1 SEM of data from 4 to 6 mice in each group. These data were subjected to one-way ANOVA. Bars designated with the same letter are not statistically significant ($P \geq .05$). mRNA, messenger RNA; Niemann-Pick C1-like 1; HMG CoA Syn, 3-hydroxy-3-methylglutaryl coenzyme A synthase.

but the level was decisively greater in mice lacking ACAT2 activity.

Discussion

The finding that ACAT2 deficiency prevents atherosclerosis in the apolipoprotein E-deficient mouse[28] underscores the importance of fully defining the role of ACAT2, the dominant cholesterol esterifying enzyme in the human intestine,[20] in regulating the enterohepatic flux of cholesterol. While this goal will be greatly aided by the development of specific ACAT2 inhibitors,[29] other approaches like the one used in the current study are also needed.

In evaluating the present data, two points concerning the animal model and the cholesterol absorption measurements warrant emphasis. The first relates to the extent of loss of cholesterol-esterifying activity in the enterocytes and hepatocytes of mice lacking ACAT2. When this knockout was first described, it was shown that cholesterol ester formation was essentially fully abolished in both the intestine and liver, irrespective of whether the mice were fed a plain chow diet or a semisynthetic atherogenic regimen.[19] The finding in those studies that the loss of ACAT2 did not result in upregulation of ACAT1 in either the intestine or liver was confirmed in the present study, in which the relative mRNA levels for ACAT1 were found to remain low and constant in both tissues regardless of the genotype of the mice or the level of cholesterol in their diet.

The second point relates to the challenge of quantifying the mass of cholesterol that reaches the liver from the lumen of the small bowel. Although lymphatic diversion theoretically yields a direct measure of the quantity of cholesterol that is absorbed,[30,31] cholesterol absorption in mice is most routinely measured as a fractional (percentage) value using either a dual isotope plasma or fecal ratio method, as was the case in the present study.[31] The fractional absorption value yielded by these techniques can be used to calculate an approximate figure for the mass of cholesterol absorbed, providing the amount of cholesterol entering the intraluminal pool each day is known. This latter value can be difficult to determine with precision because, in addition to dietary and biliary cholesterol, other cholesterol comes from sloughed mucosal cells, the ingestion of stools, and fur licking. Although the input of cholesterol from these latter three sources has not been quantified for the mouse, it can be calculated from other published data that in adult mice consuming plain rodent chow, approximately 3 to 4 mg of cholesterol from the diet and a similar amount from biliary secretion enter the lumen per day per 100 g body weight.[32] When mice are fed a cholesterol-rich diet, the quantity of cholesterol entering the intestinal lumen daily is easier to estimate because the contribution from this exogenous source

swamps the cholesterol derived from the other sources. Thus, although cholesterol feeding usually raises the biliary cholesterol concentration in normal mice, often this increase is no more than 2- to 3-fold when the dietary intake is raised 50-fold or more.[24] Hence, as the dietary cholesterol content increases, the calculated absolute rate of dietary cholesterol absorption more closely approximates the total amount of cholesterol that is absorbed. In the present study, the approximate dietary cholesterol intake of the mice that were fed chow plus 0%, 0.125%, 0.250%, 0.375%, and 0.500% cholesterol was calculated to be 2.8, 17.5, 35.0, 52.5, and 70.0 mg/d per 100 g body weight, respectively. In the case of the mice given the basal diet alone, the calculated absorption rate of dietary cholesterol was probably, at most, only about half of the total amount absorbed given the approximate equal contributions of biliary and dietary cholesterol when mice are fed chow only. However, in the mice fed the diet with 0.500% added cholesterol, the rate of total cholesterol absorption was likely essentially equal to that calculated for dietary cholesterol.

With these points taken into consideration, two conclusions regarding the importance of ACAT2 as a regulator of cholesterol absorption can be drawn. One is that this regulatory role becomes greater as dietary cholesterol intake increases. Thus, while there was little difference in the mass of cholesterol absorbed between mice of both genotypes when they were fed the basal diet alone, in the group fed chow containing 0.500% w/w cholesterol, its absorption in the ACAT2-deficient mice was only a third of that in matching $ACAT2^{+/+}$ controls. Even at the lowest level of dietary cholesterol enrichment (0.125% w/w), the mass of cholesterol absorbed by the $ACAT2^{-/-}$ mice was only about half of that absorbed by their matching $ACAT2^{+/+}$ controls. Clearly, then, the extent to which intestinal ACAT2 regulates the movement of cholesterol into the lymph varies with dietary cholesterol intake.

The second conclusion is that, in the face of a complete absence of ACAT2 in enterocytes, there is still an increase in net cholesterol movement from the lumen to the lymph when more cholesterol is consumed. This follows from the finding that the mass of cholesterol absorbed by $ACAT2^{-/-}$ mice fed diets containing cholesterol at levels of 0.250% w/w or more was, on average, at least four times the amount absorbed by $ACAT2^{+/+}$ or $ACAT2^{-/-}$ mice fed the basal diet alone. Although some of this cholesterol absorption might be facilitated through the activity of ACAT1 in the enterocyte, these data also imply that other mechanisms may exist that facilitate the passage of unesterified cholesterol from the intestine into the circulation. Because the synthesis of apolipoprotein AI, the principal apolipoprotein of high density lipoproteins, occurs partly in the intestinal mucosa,[33] it is conceivable that nascent high density lipoprotein particles might carry unesterified cholesterol out of enterocytes. Studies in Caco-2 cells point to the existence of an apolipoprotein B–independent pathway for the transport of cholesterol out of enterocytes.[34] ABCA1, which is located on the basolateral surface of enterocytes and other cell types,[35–37] facilitates cholesterol efflux[38,39] and so could potentially be involved in delivering unesterified cholesterol from enterocytes into the circulation. This contention is firmly supported by our finding that the loss of ACAT2 from enterocytes leads to a decisive increase in the level of expression of mRNA for ABCA1.

The conclusions drawn from the absorption data (Fig. 3) are supported by the changes in relative hepatic mRNA expression for genes involved in the regulation of cholesterol homeostasis in the liver. Here several findings are noteworthy. One is that the mRNA levels for HMG CoA synthase and reductase, both established indices of the rate of cholesterol synthesis, fell markedly in mice of both genotypes. However, the extent of downregulation in both of these enzymes was considerably less in the $ACAT2^{-/-}$ mice than in matching $ACAT2^{+/+}$ controls. This is fully consistent with the finding that while the ACAT2-deficient mice absorbed more cholesterol than mice of either genotype fed only the basal diet, the amount of cholesterol absorbed by the mutants was always less than it was in matching $ACAT2^{+/+}$ mice fed the same amount of cholesterol. The second finding relates to the mRNA levels for the LDL receptor, which was clearly down-regulated with cholesterol feeding. Again, however, the fall in mRNA for the LDL receptor in the $ACAT2^{-/-}$ mice was overall less than it was in their $ACAT2^{+/+}$ controls. The third observation concerns the changes in the expression of ABCG5, ABCG8, and SREBP-1 and their relationship to the genotypic and diet-related differences in hepatic cholesterol concentrations. Several studies in different types of mice have shown that the expression of target genes of the nuclear receptor liver X receptor α such as ABCG5, ABCG8, and SREBP-1 rises when the concentration of cholesterol in liver increases.[24,40,41] The present data are consistent with those findings. Thus, in the $ACAT2^{+/+}$ mice that absorbed more cholesterol than their $ACAT2^{-/-}$ counterparts, the downregulation of *de novo* synthesis, together with an induction of bile acid synthesis, were insufficient to fully compensate for the increased delivery of cholesterol from the intestine. Hence, there was a net accumulation of cholesterol in the liver, and this was accompanied by an increase in the expression of ABCG5, ABCG8, and SREBP-1. In the case of the cholesterol-fed $ACAT2^{-/-}$ mice, there was no consistent change in hepatic cholesterol concentration or

mRNA expression for ABCG5, ABCG8, and SREBP-1. Although downregulation of cholesterol synthesis in hepatocytes was clearly a major adaptive response to the increase in cholesterol delivery from the intestine, the fecal bile acid excretion data suggest that there might also have been an increase in bile acid synthesis. Such an effect of ACAT2 deficiency would be consistent with the documented effects of the ACAT inhibitor avasimibe on bile acid synthesis in the rat.[42]

In summary, although this study raises several new questions, perhaps the most important one involves the mechanisms whereby cholesterol gets absorbed with no ACAT2 activity, and little ACAT1 activity, in enterocytes. Ordinarily, the bulk of the cholesterol in chylomicrons is esterified.[7,9,30] The data in Fig. 8 suggest that when cholesterol intake increases in the face of ACAT2 deficiency, there is a greater buildup of unesterified cholesterol in enterocytes than when ACAT2 is present, and that this is compensated for by a greater degree of downregulation of sterol synthesis and a more marked induction of expression of ABCG5, ABCG8, and particularly ABCA1. Given the location of ABCA1 on the basolateral surface[35–37] and its role in facilitating cellular cholesterol efflux,[38,39] one might speculate that some of the excess unesterified cholesterol is packaged with locally synthesized apolipoprotien AI into nascent high density lipoprotein particles that enter the circulation. This thesis, which is supported by the finding that loss of ABCA1 in the mouse results in a decrease in fractional cholesterol absorption and accelerated fecal sterol loss,[43] could be tested by repeating the absorption measurements described in Fig. 3 in mice that are deficient in both ACAT2 and ABCA1, or in ACAT2 knockout mice that are concurrently treated with ezetimibe. In the case of the double knockout, the genotypic difference seen in the mRNA level for HMG CoA synthase (Fig. 8B) should be further magnified. However, in mice lacking ACAT2 and given ezetimibe, the genotypic differences seen in the expression of several proteins, particularly HMG CoA synthase and ABCA1, should no longer appear given that ezetimibe inhibits the NPC1L1-mediated uptake of sterols by enterocytes.[8]

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