Plasma Cholesteryl Esters Provided by Lecithin:Cholesterol Acyltransferase and Acyl-Coenzyme A:Cholesterol Acyltransferase 2 Have Opposite Atherosclerotic Potential

Richard G. Lee, Kathryn L. Kelley, Janet K. Sawyer, Robert V. Farese, Jr, John S. Parks, Lawrence L. Rudel

Abstract—Evidence suggests that ACAT2 is a proatherogenic enzyme that contributes cholesteryl esters (CEs) to apoB-containing lipoproteins, whereas LCAT is an antiatherogenic enzyme that facilitates reverse cholesterol transport by esterifying free cholesterol on HDL particles. We hypothesized that deletion of LCAT and ACAT2 would lead to absence of plasma CEs and reduced atherosclerosis. To test this hypothesis, ACAT2−/− LCAT−/− LDLr−/−, ACAT2−/− LDLr−/−, and LCAT−/− LDLr−/− mice were fed a 0.15% cholesterol diet for 20 weeks. In comparison to LDLr−/− mice, the total plasma cholesterol (TPC) of ACAT2−/− LCAT−/− LDLr−/− mice was 67% lower because of the complete absence of plasma CEs, leading to 94% less CE accumulation in the aorta. In the LCAT−/− LDLr−/− mice, TPC and atherosclerosis were significantly higher because of increased accumulations of ACAT2-derived CE. In ACAT2−/− LDLr−/− mice, again compared with LDLr−/− mice, TPC was 19% lower, whereas atherosclerosis was 88% lower. Therefore, the absence of ACAT2 led to a significant reduction in TPC although benefits in reduction of atherosclerosis were much more pronounced. Overall, the data suggest that ACAT2-derived CE is the predominant atherogenic lipid in blood, and that an important goal for prevention of atherosclerosis is to limit ACAT2-derived CE accumulation in lipoproteins. (Circ Res. 2004;95:998-1004.)

Key Words: LCAT ■ ACAT2 ■ atherosclerosis ■ cholesterol ■ cholesteryl esters ■ lipoproteins

Atherosclerotic degeneration is a complex process that underlies coronary heart disease, the number one cause of premature death in the United States.1 Since the early studies of atherosclerosis, the accumulation of plasma lipoprotein cholesteryl esters (CEs) in the intima of arteries has been thought to be central to the development of the disease.2 This accumulation occurs when the arterial influx of CEs within the core of apoB lipoproteins originating from the liver is outweighed by the efflux of free cholesterol (FC) on HDL particles. Efflux of cholesterol onto HDL is driven by a diffusion gradient of FC, itself maintained by the esterification of cholesterol within the HDL particle. Two enzymes are thought to be responsible for the synthesis of plasma CE, ie, acyl-CoA:cholesterol acyltransferase 2 (ACAT2)3−5 and lecithin:cholesterol acyltransferase (LCAT).6

ACAT is an integral membrane protein localized to the rough ER that catalyzes a reaction in which the fatty acid of an acyl-CoA molecule, typically oleoyl-CoA, is esterified to cholesterol, generating CE.7 The initial evidence for existence of an ACAT enzyme specific to the liver and small intestine came from characterization of ACAT1 KO mice in which esterification activity was absent in the adrenal, but was not significantly reduced in the liver and small intestine.8 This evidence led to the discovery of a second ACAT isoform, designated ACAT2, specific to the liver and small intestine of monkeys, mice, and humans.9−11 When immunofluorescent analysis was performed on nonhuman primate liver and jejunum using antibodies specific for ACAT1 and ACAT2, ACAT2 was the only isoform found in hepatocytes and enterocytes, the cells that secrete and assemble apoB lipoproteins.12 A similar finding has recently been made for human liver.13 In addition, several types of evidence suggest that ACAT2, and not ACAT1, may be proatherogenic because of its ability to synthesize CEs for incorporation into apoB-containing lipoproteins that are secreted into the plasma.14,15 The recent observation of reduced atherosclerosis in the apoE−/− ACAT2−/− double knockout mouse supports this suggestion.16

The other enzyme important in synthesis of plasma lipoprotein CE is LCAT, a glycoprotein that is secreted by the liver into the blood. The most potent activator of LCAT is apoA-I, and most, but not all, of the LCAT mass is associated with HDL.17 By esterifying HDL free cholesterol, LCAT is thought to promote reverse cholesterol transport by maintain-
ing a FC gradient between HDL and peripheral tissues and by generating spherical HDL that can accumulate more CE mass in the core of the particle. Deletion of LCAT would be expected to enhance atherosclerosis by interfering with this process, and this has been observed in LCAT−/− mice. However the antiatherogenic properties of LCAT have been called into question because of independent studies showing that deletion of LCAT had opposite effects on the development of atherosclerosis in mice. Whereas the focus of most investigations of LCAT has been on its role in HDL metabolism, there is growing interest in the participation of LCAT in the potentially proatherogenic process of apoB particle CE synthesis because of findings that it may also synthesize many of the long chain (>18 carbons) CE species in apoB containing lipoproteins.

An objective of the present study was to establish whether LCAT and ACAT2 are the only two genes responsible for synthesis of plasma CE. Additionally, we hypothesized that the two enzymes may have opposing roles, ie, synthesis of CE by LCAT is antiatherogenic because of its role in reverse cholesterol transport and synthesis of polyunsaturated CE, whereas ACAT2 promotes atherosclerosis by promoting accumulation of saturated and monounsaturated CE in apoB-containing lipoprotein particles. In this scenario, gene deletion of LCAT alone should have a proatherogenic effect, whereas gene deletion of ACAT2 should have an antiatherogenic effect. Deletion of both enzymes together should prevent atherosclerosis through a major reduction in plasma CE levels. To test these hypotheses, we developed LDLr−/− mice, a genetically engineered mouse model that facilitates atherosclerosis evaluation, with deletions of LCAT, ACAT2, or both enzymes together.

Materials and Methods

Mice and Diets

Single LDLr−/−, double ACAT2−/−/LDLr−/− and LCAT−/−/LDLr−/−, and triple ACAT2−/−/LCAT−/−/LDLr−/− KO male mice were created as described in the expanded Materials and Methods (see online data supplement available at http://circres.ahajournals.org). A diet with trans monounsaturated fatty acid-enriched fat (10% of energy as fat) supplemented with cholesterol (0.18% w/w) was then fed daily for 20 weeks (online Table 1). Lipids and lipoproteins were analyzed as described in the online data supplement.

Cholesterol Absorption Analysis by Dual Fecal Radioisotope Method

After 8 weeks on diet, cholesterol absorption was performed using the dual fecal isotope method as described previously. The protocol is described in detail in the expanded Materials and Methods section (see online data supplement).

Analysis of Liver Lipid Composition

At the time of analysis, ~100 mg of liver was thawed and minced, and lipids were extracted in 2:1 chloroform: methanol at room temperature overnight. The protein was quantitatively separated from the lipid extract which was then dried down under N2 and redissolved in a measured volume of CHCl3:MeOH, 2:1 v/v. Dilute H2SO4 was added, 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 CHCl3 was then added, and the solvent was evaporated. Deionized H2O was then added to each tube and vortexed until the solution was clear. Lipids were then quantified using enzymatic assays described for plasma lipid analysis (see online data supplement).

Quantification of Atherosclerosis

Atherosclerosis was evaluated by both morphometric and biochemical methods as described previously. A detailed description is provided in the expanded Materials and Methods section.

Statistical Analyses

Data were evaluated using 1-way ANOVA for genotype with post hoc analyses by Fisher protected least significant difference test. Statistical significance was considered at P<0.05. The outcomes for post hoc analyses are as indicated.

Results

Our first objective was to develop an atherosclerosis-susceptible mouse that lacked the LCAT and ACAT2 genes, which was accomplished by breeding mice with the desired traits. At 6 to 7 weeks of age, males of each genotype including LDLr−/−, ACAT2−/−/LDLr−/−, LCAT−/−/LDLr−/−, and ACAT2−/−/LCAT−/−/LDLr−/−, began eating an atherogenic diet that was continued for 20 weeks. Body weights were monitored periodically throughout the study. It was found that the ACAT2−/−/LDLr−/− mice gained significantly more weight in comparison to each of the other groups, which were not significantly different with respect to weight gain (online Figure 1).

Genotype-specific differences in total plasma cholesterol levels were already apparent two weeks into the diet period (Figure 1), with LCAT−/−/LDLr−/− and ACAT2−/−/LDLr−/− mice having significantly higher TPC levels than ACAT2−/−/LDLr−/− and ACAT2−/−/LCAT−/−/LDLr−/− mice (averages of 541 and 620 mg/dL versus 252 and 164 mg/dL, respectively). As time on diet progression to week 20, the TPC of LCAT−/−/LDLr−/− and ACAT2−/−/LDLr−/− mice steadily increased, whereas the

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TPC (mg/dL)</th>
<th>FC (mg/dL)</th>
<th>CE (mg/dL)</th>
<th>TG (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDLr−/−</td>
<td>833 (45)†</td>
<td>286 (13)‡</td>
<td>199 (22)‡</td>
<td></td>
</tr>
<tr>
<td>ACAT2−/−/LDLr−/−</td>
<td>637 (57)†</td>
<td>261 (19)*</td>
<td>628 (73)†</td>
<td>705 (105)†</td>
</tr>
<tr>
<td>LCAT−/−/LDLr−/−</td>
<td>1245 (86)‡</td>
<td>529 (34)‡</td>
<td>1177 (103)‡</td>
<td>660 (114)†</td>
</tr>
<tr>
<td>ACAT2−/−/LCAT−/−/LDLr−/−</td>
<td>259 (26)§</td>
<td>302 (21)*</td>
<td>0 (19)§</td>
<td>255 (37)*</td>
</tr>
</tbody>
</table>

Plasma isolated from the blood of mice fed diet for 20 weeks were analyzed for plasma concentrations of total cholesterol (TPC), free cholesterol (FC), cholesteryl ester (CE), and triglyceride (TG) as described in Materials and Methods. Values are expressed as means with SEM in parenthesis. Different symbols denote statistically significant differences (P<0.05) between values in the same column, whereas values with the same symbols are not significantly different (P>0.05).
Figure 1. Total plasma cholesterol concentrations of KO mice during diet feeding. LDLr⁻/⁻ (○), ACAT2⁺/⁻ LDLr⁻/⁻ (●), LCAT⁻/⁻ LDLr⁻/⁻ (■), and ACAT2⁻/⁻ LCAT⁻/⁻ LDLr⁻/⁻ (□) were fed a 0.18% diet for 20 weeks with time points taken at 2, 8, 12, and 16 weeks and TPC of each time point was determined as described in Material and Methods. The number of animals analyzed in each genotype is as indicated for Table 1. Each point represents the average with error bars representing SEM.

Table 2. Distribution of Cholesterol in VLDL, LDL, and HDL Size Fractions

<table>
<thead>
<tr>
<th>Genotype</th>
<th>VLDL-C</th>
<th>LDL-C</th>
<th>HDL-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDLr⁻/⁻ (n=10)</td>
<td>308 (41)*</td>
<td>431 (16)*</td>
<td>66 (5)*</td>
</tr>
<tr>
<td>ACAT2⁺/⁻ LDLr⁻/⁻ (n=9)</td>
<td>84 (18)†</td>
<td>454 (45)*</td>
<td>99 (7)†</td>
</tr>
<tr>
<td>LCAT⁻/⁻ LDLr⁻/⁻ (n=13)</td>
<td>1025 (89)*</td>
<td>257 (13)†</td>
<td>0 (0)‡</td>
</tr>
<tr>
<td>ACAT2⁻/⁻ LCAT⁻/⁻ LDLr⁻/⁻ (n=9)</td>
<td>250 (28)*</td>
<td>9 (4)‡</td>
<td>9 (2)‡</td>
</tr>
</tbody>
</table>

Total plasma cholesterol concentration was multiplied by the percentage of cholesterol found under each peak to determine VLDL-C, LDL-C, and HDL-C. Statistical analysis was denoted as described in Table 1.
This was supported by the fact that both the percent of palmitate (17.1 ± 0.6% versus 7.0 ± 0.4%) and oleate (50.3 ± 0.8% versus 31.2 ± 0.8%) in CEFA of apoB lipoproteins were higher in the LCAT−/− LDLr−/− mice. Conversely, the ratio was significantly lower in ACAT2−/− LDLr−/− when compared with LDLr−/− mice, indicating an increased contribution of LCAT-derived CE. This was evident by the enrichment of polyunsaturated fatty acids such as linoleate (30.2 ± 0.7% versus 22.5 ± 0.7) and arachidonate (28.8 ± 1.4% versus 10.8 ± 0.6%) in apoB lipoprotein CE of ACAT2−/− LDLr−/− versus LDLr−/− mice, respectively. There were no significant differences in the ratio of saturated + monounsaturated to polyunsaturated fatty acids of the PL among the three genotypes, indicating that there were no substantial differences in the fatty acid substrate pools for LCAT among the groups of mice.

The liver lipid compositions of the mice were also measured (Table 3). Hepatic FC concentrations of the LCAT−/− LDLr−/− mice were significantly higher than FC concentrations in the other groups of mice where FC concentrations were comparable. Hepatic CE concentrations of the ACAT2−/− LDLr−/− and ACAT2−/− LCAT−/− LDLr−/− mice were lower by 84% and 98%, respectively, when compared with LDLr−/− controls and CE concentrations were significantly higher in the LCAT−/− LDLr−/− mice. Regression analysis demonstrated that a positive correlation existed between hepatic CE concentrations and week 20 TPC (r = 0.81) (online Figure 3). A significant difference in liver TG concentration was the 200% to 300% higher value in the ACAT2−/− LDLr−/− mice. A significantly lower hepatic TG concentration was found in the triple KO mice. There were only small differences in hepatic PL concentrations among the genotypes with the LCAT deficient strains having slightly higher values. Real-time PCR analysis of hepatic ACAT2 and LCAT mRNA expression showed that absence of hepatic LCAT mRNA did not have an effect on the expression of hepatic ACAT2 mRNA, and vice versa (online Figure 4).

In an effort to explain the higher TG levels in livers of ACAT2−/− LDLr−/− mice, we looked at hepatic sterol regulatory binding protein-1c (SREBP-1c) mRNA levels by real-time PCR. SREBP-1c is a sterol-sensitive transcription factor important in the synthesis of FA and TG.26 We found no significant differences in SREBP-1c mRNA levels among the four genotypes (data not shown).

To determine whether the rather large differences in lipid levels led to an overall change in the size of the liver, the liver weight as a percentage of total body weight was calculated (data not shown). Significant differences were observed including an increase in the percent liver weight in LCAT−/− LDLr−/− mice when compared with LDLr−/− control mice (6.16 ± 0.24% versus 5.56 ± 0.12%, respectively) and a decrease in ACAT2−/− LCAT−/− LDLr−/− mice compared with controls (4.59 ± 0.11%). No differences in percent liver weight were found in ACAT2−/− LDLr−/− mice (5.64 ± 0.11%) when compared with controls.

Two methods were used to quantify the amount of atherosclerosis that developed in the animals. Initially, the percent of aortic surface covered with atherosclerotic plaque was determined (Figure 4A). The aortae of LCAT−/− LDLr−/− mice had significantly (~3-fold) more of the surface area covered with lesion when compared with those of LDLr−/− control mice (15.3 ± 1.0% versus 4.9 ± 0.8%), whereas the aortae of both the ACAT2−/− LDLr−/− mice (1.1 ± 0.8%) and ACAT2−/− LCAT−/− LDLr−/− mice (0 ± 0.0%) had significantly

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**TABLE 3. Hepatic Lipid Compositions of KO Mice**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Lipid Concentration, μg/mg protein</th>
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<tbody>
<tr>
<td></td>
<td>FC</td>
</tr>
<tr>
<td>LDLr−/− (n=18)</td>
<td>16.9 (1.8)*</td>
</tr>
<tr>
<td>ACAT2−/− LDLr−/−</td>
<td>19.3 (1.4)*</td>
</tr>
<tr>
<td>LCAT−/− LDLr−/−</td>
<td>30.7 (3.2)†</td>
</tr>
<tr>
<td>ACAT2−/− LCAT−/−</td>
<td>16.6 (1.4)*</td>
</tr>
</tbody>
</table>

One hundred to 200 mg of liver from diet fed mice was analyzed for protein and lipid content. Results are expressed as microgram lipid divided by milligram of hepatic protein. Statistical analysis is denoted as described in Table 1.
less surface area with lesion. The greatest extent of lesion involvement typically occurred within the proximal third of the aorta (including the arch).

Lipids were then extracted from the whole aorta and the aortic CE accumulation was quantified by GLC (Figure 4B). Results were similar to the morphometric analysis, with aortic CE synthesis of plasma CE and the atherogenic25,28,29 presumably because of the limited ability of macrophages to mobilize cholesterol from these CE.30 When even more of these CE accumulated in plasma lipoproteins, as in the LCAT−/− LDLr−/− mice (Figure 3A), the extent of atherosclerosis generally extended beyond decreases in plasma cholesterol concentration. Earlier data have suggested that the cholesteryl oleate and cholesteryl palmitate synthesized by ACAT2 are particularly atherogenic25,28,29 presumably because of the limited ability of macrophages to mobilize cholesterol from these CE.30 When even more of these CE accumulated in plasma lipoproteins, as in the LCAT−/− LDLr−/− mice (Figure 3A), the extent of atherosclerosis was even higher (Figure 4). Higher plasma cholesterol concentrations as well as modified CE composition characterized the LCAT−/− mice and likely contributed to the higher extent of atherosclerosis, as would the decreased ability to transport cholesterol from the periphery to the liver via the hypothesized reverse cholesterol transport pathway.18

The effects of the gene deletion of ACAT2 or LCAT had contrasting effects on plasma lipoproteins. Deletion of ACAT2 lowered TPC because of a decrease in plasma CEs, and the plasma lipoprotein profile showed a lower VLDL cholesterol and a higher HDL cholesterol (Table 2) as well as a smaller-sized LDL particle. All of these changes are consistent with the decreased atherogenicity observed in these animals. It is interesting to note that in the mouse strains that synthesize plasma CEs (all genotypes but the ACAT2−/− mutant), the extent of atherosclerosis generally followed the concentrations of VLDL-C. Because VLDL contain primarily ACAT2-derived CEs, this may be another indication that ACAT2-derived CEs promote atherosclerosis. It also was noteworthy that LDL-C levels in the ACAT2−/− LDLr−/− mice were not significantly different than in

**Figure 4.** Measurements of atherosclerosis end points in mice. A, Morphometric analysis of aortas isolated from LDLr−/− (rr, n=10 and n=17 for A and B, respectively), ACAT2−/− LDLr−/− (aarr, n=9 and n=12 for A and B, respectively), LCAT−/− LDLr−/− (lirr n=9 for both panels), and ACAT2−/− LCAT−/− LDLr−/− (aallrr, n=9 for both panels) mice. Values are expressed as percent surface area of aorta covered in lesion. B, GLC analysis of CE/H11005 for both panels) mice. Values are expressed as percent

**Figure 5.** Regression analysis of (A) total plasma cholesterol and aortic cholesteryl ester content, (B) plasma cholesteryl and aortic cholesteryl ester content, and (C) hepatic CE content and aortic CE content. Shown are least squares best fit regression lines representing data from all LDLr−/− (△), ACAT2−/− LDLr−/− (♦), LCAT−/− LDLr−/− (■), and ACAT2−/− LCAT−/− LDLr−/− (○) mice.

Discussion

This study allowed us to determine the importance of LCAT and ACAT2 in both the synthesis of plasma CE and the development of atherosclerosis. We found that deletion of both LCAT and ACAT2 genes led to the complete absence of plasma CE, indicating that there are no compensatory mechanisms, such as ACAT1, to synthesize plasma CE. A striking observation was that in ACAT2−/− LDLr−/− mice, a 25% lower plasma CE concentration was associated with an 88% decrease in aortic CE accumulation. This observation suggests that ACAT2−/− mice have 25% less CE in the plasma, which is consistent with a decreased ability to transport cholesterol from the peripheral tissues. This decrease might be due to the decreased production of CE in ACAT2−/− mice, which in turn results in a lower concentration of CE in the plasma.

Consistent with this observation, the aortic CE accumulation was quantified by GLC (Figure 4B). Results were similar to the morphometric analysis, with aortic CE accumulation of 2.9±11.2 versus 23.8±1.9 μg CE/mg aortic protein in ACAT2−/− LDLr−/− mice and ACAT2−/− LCAT−/− LDLr−/− mice had significantly less aortic CE accumulation (2.9±0.8 and 1.4±0.8 μg CE/mg protein, respectively). Across all animals, there was a significant correlation between percent surface area involvement and μg CE/mg protein of r=0.79, a result similar to that found in an earlier study using the same experimental protocols.22 Regression analysis showed that the positive correlation between TPC and aortic CE accumulation (r=0.75) and the positive correlation between hepatic CE concentration and aortic CE concentration (r=0.78) were of a similar magnitude, whereas the correlation between plasma CE and aortic CE concentrations was also statistically significant (r=0.65) (Figure 5).
LDLr−/− mice even though the LDL particles were smaller in ACAT2−/− LDLr−/− mice. Analysis of apoB lipoprotein CEs in ACAT2-deficient mice showed enrichment in polyunsaturated fatty acids. These findings suggest that LCAT is able to compensate for the loss of ACAT2-derived CEs in the LDL fraction although it was only able to partially compensate in the VLDL fraction. The resulting LDL particles are smaller with less cholesteryl ester per particle presumably resulting from the lower CEs in VLDL. It has been hypothesized that the compensatory increase in LCAT activity in ACAT2−/− mice may also contribute to the increase in HDL cholesteryl.15

Another factor contributing to the reduced atherosclerosis extent in ACAT2-deficient mice may be the replacement of cholesteryl ester with triglyceride in the core of apoB-containing lipoproteins. However, hypertriglyceridemia has also been considered as a risk factor for atherosclerosis, leading to speculation that enrichment of apoB-containing lipoprotein particles with triglyceride could be proatherogenic. In ACAT2 gene deletion experiments in the apoE−/− mice, triglyceride levels were not elevated, making it difficult to ascertain the effects of elevated plasma triglycerides and triglyceride-rich lipoproteins.16 However in the LDLr−/− background of these studies, deletion of ACAT2 led to mild hypertriglyceridemia when compared with controls, yet atherosclerosis decreased dramatically in these mice. These data provide a clear indication that, when compared with plasma CEs, triglyceride is less effective in promoting atherosclerosis development. The elevation of plasma apoB-containing lipoprotein triglyceride in the ACAT2−/− LDLr−/− mice may be associated with the increase in body mass, and weight gain may be a possible negative side effect of ACAT2 inhibition. The mechanism resulting in the increase in triglyceride accumulation in the ACAT2−/− mice is unclear at the present time and needs further study. Further, it is unclear why deletion of LCAT together with the deletion of ACAT led to lower plasma TG levels.

Loss of LCAT led to an increase in TPC because of increases in both FC and CEs (Table 1). Furthermore, the atherogenicity of the lipoprotein profile was greater apparent-ly because of the increased VLDL cholesterol and the complete absence of HDL. The relative TPC concentrations observed in the LCAT−/− LDLr−/− mice differ from the results of Furbee et al,19 who saw no difference in TPC when compared with LDLr−/− controls, and Lambert et al20 who saw decreases in TPC when compared with controls. The use of trans fatty acid enriched fat in the diet of this study may partly explain the contradictory results. In the LCAT−/− LDLr−/− mice, our atherosclerosis results agree with those generated by Furbee et al19 in that the absence of LCAT led to increased atherosclerosis in comparison to LDLr−/− control mice. It has been argued that loss of LCAT would be antiatherosclerotic because of the decreases in LDL cholesterol concentrations.6 We speculate that the proatherogenic effects of the loss of LCAT, through limiting the ability to maintain the FC gradient between peripheral tissue and HDL necessary for reverse cholesterol transport, may outweigh the antiatherogenic effects of the decrease in LDL. Further, in LCAT deficiency in our study, VLDL cholesterol concentrations were greatly increased with ACAT2-derived cholesteryl esters even though LDL cholesterol concentrations were decreased (Table 2). This shift, together with the cholesteryl ester composition changes and HDL depletion, were sufficient to counterbalance any apparently anti-atherogenic effects of reduced LDL cholesterol concentrations.

Analysis of cholesterol absorption resulted in all three KO animals having significantly decreased cholesterol absorption (≈20%) when compared with LDLr−/− controls when they were fed the 0.18% cholesterol, trans fat diet (Figure 2). In ACAT2−/− LDLr−/− mice fed a chow diet (0.02% cholesterol), cholesterol absorption was not significantly different from that in wild-type mice but when ACAT2−/− mice were fed an 1.25% cholesterol diet, cholesterol absorption was 85% less than in wild-type mice, leading the authors to speculate that a compensatory mechanism occurs with increased amounts of cholesterol in the absence of ACAT2.15 A possible compensatory mechanism is related to results generated by Iqbal et al13 in CaCO2 cells. An apoB-independent secretion pathway apparently involving ABCA1, suggests that HDL may be a cholesterol acceptor in the absorption pathway. In the absence of ACAT2, it is possible that cholesteryl that would normally be secreted in chylomicrons is transported to the site within the enteroocyte to be effluxed to apoA-I via ABCA1. This pool of cholesterol may be expanded when cholesterol-enriched diets are fed. This also could provide a possible explanation for the decrease of cholesterol absorption in the LCAT−/− LDLr−/− mice as the absence of HDL in these mice may inhibit the apoB-independent secretion pathway thereby inhibiting cholesterol absorption. Further investigation is necessary to determine whether the cell culture results will carry over to in vivo models.

Deletion of ACAT2 led to depletion of CE in the liver, whereas deletion of LCAT led to CE enrichment in the liver when compared with controls. Hepatic CE enrichment taken together with the increased VLDL-C in the plasma of the LCAT−/− LDLr−/− mice suggests that ACAT2 expression is upregulated in the liver of the LCAT−/− LDLr−/− mice. Recently, it has been shown that ACAT2 is upregulated, primarily through posttranscriptional mechanisms, by the presence of increased levels of dietary cholesterol.32 It is not clear to us at this time why the absence of LCAT resulted in increased cholesterol concentrations in the liver of these mice. The positive correlation between hepatic CE content and TPC, as well as between hepatic CE and aortic CE is another indication that hepatic ACAT2 activity contributes importantly in the development of atherosclerosis.

In conclusion, these studies in mouse models suggest that there are only two enzymes that possess the ability to synthesize CE, ACAT2 and LCAT. Loss of the ability to synthesize plasma cholesteryl esters did not cause obvious detrimental effects but did eliminate the appearance of atherosclerotic lesions in the aorta. Our findings support the long-standing hypothesis that accumulation of CE from blood plasma in the intima of arteries is central to the development of atherosclerosis. Our data further support the importance of ACAT2 in the development of atherosclerosis. This enzyme facilitates cholesterol absorption in the intestine and incorpo-
ration of atherogenic CEs into apoB-containing plasma lipoproteins, which then appear to accumulate in the aortic intima promoting atherosclerotic lesion development. The fact that the decrease in TPC was disproportionately less than the decrease in atherosclerosis in ACAT2 KO mice suggests that other factors, such as increased HDL-C cholesterol, increased polyunsaturated CE, and smaller LDL particles with fewer CE per particle, contribute important antiatherosclerotic effects in the presence of ACAT2 deficiency.

Acknowledgments

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References

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**Expanded Materials and Methods**

All mice used in this study were housed in the medical center animal resources facility (AAALAC approved) and the institutional animal care and use committee approved all animal protocols. LDLr<sup>−/−</sup> mice on the C57Bl/6 genetic background were purchased from Jackson Laboratories. The ACAT2<sup>−/−</sup> LDLr<sup>−/−</sup> mice (75% C57Bl/6, 25% 129Sv/Jae) were crossed with LCAT<sup>−/−</sup> LDLr<sup>−/−</sup> mice (90% C57Bl/6, 10% 129Sv/Jae) to generate ACAT2<sup>+/−</sup> LCAT<sup>+/−</sup> LDLr<sup>−/−</sup> mice. Progeny were then backcrossed to LCAT<sup>−/−</sup> LDLr<sup>−/−</sup> and litters were screened for the ACAT2<sup>+/−</sup> LCAT<sup>+/−</sup> LDLr<sup>−/−</sup> genotype by PCR, using primers and protocols described previously<sup>1,2</sup>. ACAT2<sup>+/−</sup> LCAT<sup>+/−</sup> LDLr<sup>−/−</sup> mice were then bred to develop the desired ACAT2<sup>−/−</sup> LCAT<sup>−/−</sup> LDLr<sup>−/−</sup> triple KO genotype (80% C57Bl/6, 20% 129Sv/Jae). For sedation during retro-orbital blood collection, mice were administered 1 µl/gm body weight of a 50 mg/ml ketamine:10 mg/ml xylazine solution. For sedation prior to termination mice were administered 2 µl/gm body weight of the 50 mg/ml ketamine:10 mg/ml xylazine solution.

**Diet Composition**

Diet was prepared by thoroughly mixing 10 kg of the dry ingredients listed in Supplement Table 1 with 3.5 liters of water. The resulting dough was then divided into 400 gm portions and compressed into a cake and wrapped in wax paper. The cake was frozen at -20°C for storage. For feeding, diet was thawed and 10 gm/day was given to each mouse for the duration of the study. The trans
monounsaturated enriched fat was used because it led to more extensive atherosclerosis development than diets containing other fatty acid enrichments³.

**Blood and Tissue collection**

At 2, 8, 12, and 16 week time points, mice were sedated, body weights were measured and blood was collected from the retro-orbital sinus. At 20 weeks after mice were sedated, a terminal blood sample was taken via heart puncture, and after exsanguination, the liver and aorta were harvested ³. Livers were immediately minced and frozen in liquid nitrogen, while the aortas were pinned flat then placed in phosphate buffered saline in 1% formalin for morphometric analysis and subsequent chemical analysis.

**Lipid and Lipoprotein measurements**

After blood collection, EDTA-plasma was immediately isolated by centrifugation at 5000 x g for 15 min. at 4°C. Total cholesterol (TPC), FC, and triglyceride (TG) concentrations in plasma were determined by enzymatic analysis (FC using kit from Wako Chemicals USA, TC and TG with kit from Roche Diagnostics). The amount of esterified cholesterol was calculated by subtracting the free cholesterol from the total cholesterol and the difference was multiplied by 1.67 to convert it to CE mass. Cholesterol distribution among lipoprotein classes was determined after separation by gel filtration chromatography similarly to the method described previously ⁴. A 30 µl aliquot of plasma was diluted 1:1 in phosphate buffered saline (PBS) and centrifuged at
5000 x g to remove any particulate debris. The samples were injected onto a Superose 6 HR 10/30 (Amersham Pharmacia) chromatography column, which was subsequently run at 0.4 ml/min. with eluant containing 0.9% NaCl, 0.05% EDTA, pH 7.4, and 0.05% NaN₃. The column effluent was mixed with total cholesterol enzymatic reagent (Infinite) and passed through a knitted reaction coil in a 37°C H₂O jacket with absorbance read at 546 nm. The signal was subsequently integrated using Chrom Perfect Spirit software (Justice Laboratory Software). VLDL-C, LDL-C, and HDL-C concentrations were determined by multiplying the TPC concentration by the cholesterol percentage within the elution region for each lipoprotein class.

**Fatty acid Methyl Ester (FAME) Analysis of Cholesteryl Esters from Lipoproteins with d<1.063 g/ml**

Samples of plasma were adjusted to a density of 1.063 g/ml with KBr and were then centrifuged at 100K for 4.5 hrs in a TLA 100.2 rotor. The top fraction containing the VLDL, IDL, and LDL lipoproteins were collected after slicing the tube. FAME analysis was then carried out as described previously. Briefly, the lipids were extracted with the Bligh-Dyer procedure and the CE were isolated by thin layer chromatography (Silica Gel 60, EM Sciences). The CE spot from the silica gel was scraped. Methanol containing 0.5 N NaOH was added to each sample and they were heated to 100°C. After cooling, 14% BF₃ in methanol was added, and the samples were again heated to 100°C. Next, isooctane and saturated NaCl were added and the samples were centrifuged to ensure clean
separation of phases. The isooctane phase was collected and the FAME species were quantified by gas-liquid chromatography (CP-Sil 88, 0.25mm ID X 100m, Varian) using the temperature program and detection system described previously 7.

**Cholesterol absorption analysis by dual fecal radioisotope method.**

An isotopic dose of 0.1 μCi of $^{14}$C cholesterol (Amersham Life Sciences) and 0.2 μCi of $^3$H-β-sitosterol (NEN Life Sciences), repurified by high performance liquid chromatography, was added to 200 μl of a soybean oil mixture. An aliquot of the dose was counted in triplicate to determine the $^{14}$C/$^3$H ratio of the initial dose 7. The mixture was taken into a plastic 1cc syringe that was fitted with a 20 gauge, 1 1/2” curved animal feeding needle (Popper & Sons, Inc., New Hyde Park, NY). Animals were then gavaged with the dose and placed individually in a wire bottom cage with free access to food and water for 3 days. All feces was collected over the 3 days and stored at 4°C until analysis.

The combined fecal sample was then homogenized in ethanol using a polytron homogenizer in a final volume of 10 ml. An aliquot of the slurry was saponified by adding 50% KOH and heating at 80°C for 1 hour. Neutral lipids were isolated by adding dH$_2$O and hexane and, after thorough mixing, harvesting the upper phase into a scintillation vial. The hexane was evaporated and scintillation cocktail (Biosafe II, Research Products International Corp.) was added. Radioactivity was counted on $^3$H/$^{14}$C dual isotope program with quench
correction (Beckman Coulter LS 6500) and percent cholesterol absorption was calculated using the following equation:

\[
\left( \frac{^{14}C/^{3}H \text{ dosing mixture} - ^{14}C/^{3}H \text{ feces}}{^{14}C/^{3}H \text{ dosing mixture}} \right) \times 100 = \text{Percent cholesterol absorption}
\]

Quantification of Atherosclerosis

At the time of necropsy, aortas were removed from the mouse beginning at the aortic sinus and ending at the iliac bifurcation. After cleaning all adventitia from the aortas they were opened longitudinally along the ventral midline, pinned flat on a black wax surface, and submersion fixed in 10% neutral buffered formalin for a minimum of 24 hours. Images of the aortas were captured using a Sony digital camera (Model DXC-S500) and evaluated for atherosclerosis development using Scion Image software (Version 1.62). Percent of surface involved with atherosclerosis was calculated after measuring total aortic surface area and surface area with visible atherosclerosis involvement.

For biochemical quantification, aortic lipids were then extracted from the fixed aortas in 2:1 CHCl₃:MeOH with 5α-cholestane added as an internal standard. Extracts were dried down, brought up in hexane, and free and total cholesterol were measured by gas-liquid chromatography as described previously ⁸. The delipidated tissue was then digested and dissolved in 1N NaOH, and total protein was determined by the method of Lowry et al ⁹.
Real-Time PCR Analysis of ACAT2 and LCAT mRNA expression

Total mRNA was extracted from 250-350 mg of liver tissue with Trizol (Invitrogen Life Technologies) using the protocol provided by the manufacturer. The mRNA was resuspended in 300 µl of DEPC H₂O, and 1 µg of mRNA was reverse transcribed to cDNA using Omniscript reverse transcriptase under the following conditions: 37°C for 1 hr., 93°C for 5 min. The cDNA was diluted 1:10 using DEPC H₂O and equal amounts of cDNA from four animals of the same genotype were pooled and two pooled samples of each genotype were analyzed by real time PCR. The PCR reaction was done in triplicate with 5 µl of pooled sample, 12.5 µl of cyber green master mix (includes DNA polymerase and dNTPs), 5.5 µl of DEPC H₂O, and 1 µl of each primer (20 picomoles) for a final reaction volume of 25 µl. The ACAT2 forward primer was 5’-GGGCTGCTGAATTTCACCAT -3’ and the ACAT2 reverse primer was 5’-GAAGAGAAAGGTCCACATCAGGAT -3’. The LCAT forward primer was 5’-GCTGGCCTGGTAGAGGAGATG –3’ and the LCAT reverse primer was 5’-CCAAGGCTATGCCCAATGA -3’. The PCR reaction 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 seconds and 60°C for 1 min. The fluorescence measurement used to calculate Ct is made at the 60°C point. A dissociation curve was run at the end of the reaction to ensure a single amplification product. At the same time that the samples were being run, a positive control using a constant mouse liver cDNA was run using 10 µl of either undiluted, a 1:10 dilution, or 1:100 dilution of liver
RNA. Ct values were entered into the following equation to determine the arbitrary unit value: $1 \times 10^9 \times e^{(-0.6931 \times \text{Ct})}$. All values were then normalized to the cyclophilin mRNA expression of the sample in order to take total mRNA concentration into account. The cyclophilin forward primer was 5’-TGGAGAGCACCAAGACAGACA -3’ and the cyclophilin reverse primer was 5’-TGCCGGAGTCGACAATGAT -3’.
Reference List


**Additional Figures and Supporting Information**

**Figure 1. Body weights of KO mice during diet feeding.** Body weights of LDLr\(^{-/-}\) (Δ), ACAT2\(^{-/-}\) LDLr\(^{-/-}\) (杢), LCAT\(^{-/-}\) LDLr\(^{-/-}\) (ν), and ACAT2\(^{-/-}\) LCAT\(^{-/-}\) LDLr\(^{-/-}\) (O) were measured at the 0, 2, 8, 12, 16, and 20 week time points. The number of animals in each genotype are the same as Table 1. * denotes a statistically significant difference of \(p<0.05\) when compared to LDLr\(^{-/-}\) mice.

**Figure 2. Cholesterol absorption analysis of KO mice.** After 8 weeks on diet, cholesterol absorption was quantified in the LDLr\(^{-/-}\) (rr, n=5), ACAT2\(^{-/-}\) LDLr\(^{-/-}\) (aarr, n=4), LCAT\(^{-/-}\) LDLr\(^{-/-}\) (llrr, n=4), and ACAT2\(^{-/-}\) LCAT\(^{-/-}\) LDLr\(^{-/-}\) (aallrr, n=4) mice via the dual fecal radioisotope method as described in the Materials and Methods. Different letters denote statistically significant differences of \(p<0.05\).

**Figure 3. Regression analysis of total plasma cholesterol and hepatic cholesteryl ester concentrations.** Regression lines represent data from all LDLr\(^{-/-}\) (Δ), ACAT2\(^{-/-}\) LDLr\(^{-/-}\) (杢), LCAT\(^{-/-}\) LDLr\(^{-/-}\) (ν), and ACAT2\(^{-/-}\) LCAT\(^{-/-}\) LDLr\(^{-/-}\) (O) in which TPC and hepatic CE concentrations were measured.

**Figure 4. Real-time quantification of ACAT2 and LCAT mRNA in KO mouse liver.** Real time PCR analysis was carried out as described in Materials and Methods on pooled samples from livers of LDLr\(^{-/-}\) (rr), ACAT2\(^{-/-}\) LDLr\(^{-/-}\) (aarr), LCAT\(^{-/-}\) LDLr\(^{-/-}\) (llrr), and ACAT2\(^{-/-}\) LCAT\(^{-/-}\) LDLr\(^{-/-}\) (aallrr) mice using primers specific to either ACAT2 (A) or LCAT (B). Results are normalized to cyclophilin expression and are expressed as arbitrary units. The results for each genotype are an average of two pooled samples.
### Supplemental Table 1

<table>
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<tr>
<th>Ingredients</th>
<th>g/100 g dry weight</th>
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<tr>
<td>Trans enriched fat*</td>
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<tr>
<td>Fish oil</td>
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* The fatty acid composition of the *trans* enriched fat included 17.5% 16:0, 19.7% *trans* 18:1 (trans double bond at C10 and C11 are the major isomers), 19.1% *cis* 18:1, and 17.7% 18:2. The fat was purchased as a blend from AC Humko, Memphis, TN. The energy breakdown was 11% of energy as fat, 71% of energy as carbohydrates, and 18% of energy as protein.

†Vitamin mix included 2 mg/100 g diet for each of alpha-tocopherol, gamma tocopherol, and tenox 20A as additional antioxidants.
Figure 1

Body Weight (gm) vs. Week
Figure 2

Cholesterol Absorption (%)
Figure 3

The graph shows a scatter plot of TPC (mg/dl) versus \( \mu \text{g Liver CE/mg prot} \). The correlation coefficient is indicated as \( r = 0.81 \).
Figure 4

A

ACAT2/Cyclophilin (A.U.)

rr  aarr  llrr  aallrr

B

LCAT/Cyclophilin (A.U.)

rr  aarr  llrr  aallrr