ACAT2 stimulates cholesteryl ester secretion in apoB-containing lipoproteins

Ryan E. Temel, Li Hou, Lawrence L. Rudel, and Gregory S. Shelness[1]

Department of Pathology, Section on Lipid Sciences, Wake Forest University School of Medicine, Winston-Salem, NC 27157

Abstract **Previous studies in nonhuman primates revealed a striking positive correlation between liver cholesteryl ester (CE) secretion rate and the development of coronary artery atherosclerosis. CE incorporated into hepatic VLDL is necessarily synthesized by ACAT2, the cholesterol-esterifying enzyme in hepatocytes. We tested the hypothesis that the level of ACAT2 expression, in concert with cellular cholesterol availability, affects the CE content of apolipoprotein B (apoB)-containing lipoproteins. In a model system of lipoprotein secretion using COS cells cotransfected with microsomal triglyceride transfer protein and truncated forms of apoB, ACAT2 expression resulted in a 3-fold increase in microsomal ACAT activity and a 4-fold increase in the radiolabeled CE content of apoB-lipoproteins. After cholesterol-cyclodextrin (Chol-CD) treatment, CE secretion was increased by 27-fold in ACAT2-transfected cells but by only 7-fold in control cells. Chol-CD treatment also caused the percentage of CE in the apoB-lipoproteins to increase from 3% to 33% in control cells and from 16% to 54% in ACAT2-transfected cells. In addition, ACAT2-transfected cells secreted 3-fold more apoB than control cells. These results indicate that under all conditions of cellular cholesterol availability tested, the relative level of ACAT2 expression affects the CE content and, hence, the potential atherogenicity, of nascent apoB-containing lipoproteins.—** Temel, R. E., L. Hou, L. L. Rudel, and G. S. Shelness. **ACAT2 stimulates cholesteryl ester secretion in apoB-containing lipoproteins. *J. Lipid Res.* 2007.** 48: **1618–1627.**

Supplementary key words atherosclerosis • lipid mobilization • lipoprotein assembly • very low density lipoprotein • apolipoprotein B • triglycerides • acyl-coenzyme A:cholesterol acyltransferase 2

It is well established that increased plasma concentrations of LDL and other apolipoprotein B (apoB)-containing lipoproteins are risk factors for the development of coronary artery disease. In humans and other mammalian species, the cholesteryl esters (CEs) in LDL particles are synthesized by two different enzymes, LCAT and ACAT2. LCAT, a liver-derived plasma protein, acts primarily on plasma HDL to produce cholesteryl linoleate and arachidonate (1). In many mammalian species, LCAT-derived HDL-CE can subsequently be transferred to apoB-containing lipoproteins by cholesteryl ester transfer protein (2).

ACAT2 also synthesizes CE found in LDL, yet the characteristics of LCAT and ACAT2 are dramatically different. Similar to the related enzyme, ACAT1 (3), ACAT2 is an endoplasmic reticulum-localized transmembrane protein that transfers the fatty acyl moiety of acyl-CoA to free cholesterol, producing mainly cholesteryl oleate and palmitate. As ACAT2 expression is limited to hepatocytes and enterocytes, ACAT2-derived CE can be packaged directly into nascent apoB-containing lipoproteins or stored as neutral lipid droplets in the cytosol (4–9).

Although CEs synthesized by both LCAT and ACAT2 are found within plasma apoB-containing lipoproteins, ACAT2-derived CE appears to be more atherogenic. Liver perfusion studies in African green monkeys fed high-fat, cholesterol-enriched diets demonstrated that hepatic ACAT activity was positively correlated with CE secretion rates, plasma LDL-CE content, and the degree of coronary artery atherosclerosis (10). Studies in several mouse models have also shown a relationship between ACAT2-derived CE and atherosclerosis. When ACAT2-deficient ($ACAT2^{-/-}$) mice were crossed with atherosclerosis-susceptible apoE-deficient ($ApoE^{-/-}$) or low density lipoprotein receptor-deficient ($LDLr^{-/-}$) mice, almost no atherosclerosis was observed (11, 12). Because the $ACAT2^{-/-}$ $ApoE^{-/-}$ and $ACAT2^{-/-}$ $LDLr^{-/-}$ mice displayed significantly lower plasma concentrations of cholesteryl oleate and cholesteryl palmitate in apoB-containing lipoproteins, it was concluded that plasma accumulation of ACAT2-derived CE promotes atherogenesis in mice (11, 12). The specific contribution of liver ACAT2 to atherogenesis was further established by Bell et al. (13), who demonstrated that antisense oligonucleotide-mediated inhibition of liver ACAT2 limited atherosclerosis development

Manuscript received 5 September 2006 and in revised form 1 March 2007.

Published, JLR Papers in Press, April 16, 2007.
DOI 10.1194/jlr.M700109-JLR200

Abbreviations: AP, alkaline phosphatase; apoB, apolipoprotein B; CD, methyl-β-cyclodextrin; CE, cholesteryl ester; Chol-CD, free cholesterol solubilized in methyl-β-cyclodextrin; LDLr, low density lipoprotein receptor; MTP, microsomal triglyceride transfer protein; PL, phospholipid; TG, triglyceride.

[1] To whom correspondence should be addressed.
e-mail: gshelnes@wfubmc.edu

in apoB-100-only LDLr$^{-/-}$ mice. In contrast to the reduction of atherosclerosis in ACA2$^{-/-}$ mice, genetic inactivation of LCAT in either ApoE$^{-/-}$ or LDLr$^{-/-}$ mice resulted in an enrichment of apoB-containing lipoproteins with ACAT2-derived CE and significantly increased atherosclerosis (14).

Although the ACAT2-derived CE content of apoB-containing lipoproteins is an important mediator of atherosclerosis development, little is known regarding the quantitative relationships among ACAT2 expression, cellular cholesterol availability, and the composition of nascent apoB-containing lipoproteins (15). Therefore, we explored the consequences of ACAT2 expression on the lipid composition of newly secreted apoB-containing lipoproteins in a transfected cell-based system, in which ACAT2 expression and exogenous cholesterol can be readily manipulated. Although ACAT2 is regulated allosterically by substrate availability (16), we observed that under all conditions of cellular cholesterol content, ACAT2 protein mass and activity dramatically affected the CE content of nascent apoB-containing lipoproteins. These results suggest that even under conditions of cholesterol excess, partial therapeutic inhibition of ACAT2 may reduce the CE content and, hence, the potential atherogenicity, of newly secreted apoB-containing lipoproteins.

EXPERIMENTAL PROCEDURES

Transient transfection and metabolic radiolabeling of COS cells

COS-1 cells were cultured in 150 mm dishes containing DMEM and 10% FBS (complete medium), as described previously (17). Transfections were performed using FuGENE 6 (Roche Applied Science) and a total of 17.5 μg of DNA per dish at a mass ratio of 1:1.5:1 [human microsomal triglyceride transfer protein (MTP) large subunit/human apoB-34 or apoB-50/African green monkey ACAT2 or truncated human placental alkaline phosphatase (AP)]. Where indicated, the mass of ACAT2 plasmid was titrated from 0 to 5 μg, with a corresponding decrease in AP plasmid. In all other experiments, the maximum dose of ACAT2 plasmid was used (5 μg). The apoB constructs contained a His_6 tag at their C termini for affinity purification (17, 18) (see below). The assembly and secretion of both apoB-34 and apoB-50 are fully dependent upon coexpression with MTP (18, 19). Twenty-four hours after transfection, cells were washed with PBS and metabolically radiolabeled for 24 h in serum-free DMEM containing 10 μCi/ml [^{3}H]oleate, 0.5% fatty acid-free BSA, and 20 μCi/ml [^{35}S]methionine/cysteine, as described previously (17, 18).

For the experiment involving the ACAT inhibitor CP-113,818 (20), 24 h after transfection, cells were incubated for 2 h in complete medium containing either 1.25 μg/ml CP-113,818 or vehicle [0.025% (v/v) DMSO]. The cells were then metabolically radiolabeled with [^{3}H]oleate, as described above, also in the presence of either 1.25 μg/ml CP-113,818 or vehicle.

Radiolabeled lipid composition of COS cells and affinity-purified lipoproteins

Affinity purification of apoB-34- and apoB-50-containing lipoproteins and analysis of radiolabeled lipids were performed as described (17, 18). Briefly, the medium and cells from two 150 mm dishes were combined and the His_6-tagged apoB-containing lipoproteins were purified by nickel-nitrilotriacetic-agarose affinity chromatography (Qiagen). Lipids were extracted from purified lipoproteins (17, 18) or from COS-1 cells lysed in 0.1 N NaOH, as described (21). The extracted lipids were separated by TLC and quantitated by liquid scintillation counting.

Preparation of cholesterol-cyclodextrin solution

Methyl-β-cyclodextrin (CD; Sigma-Aldrich) was dissolved in water at a concentration of 344 mM. Four hundred milligrams of purified cholesterol (Nu-Chek Prep, Inc.) was added to 20 ml of CD solution in a 50 ml glass tube and rotated overnight at room temperature, followed by incubation at 60°C with periodic vortexing for 2 h. After cooling to room temperature, the solution was passed through a 0.45 μm HT Tuffryn membrane syringe filter (Gelman) and stored at 4°C in a glass tube. The cholesterol concentration of the final solution was 19 mg/ml, as determined by Cholesterol/HP assay kit (Roche Applied Bioscience); the CD/cholesterol molar ratio was ~7.

Loading of cells with free cholesterol and analysis of cholesterol mass

Free cholesterol solubilized in methyl-β-cyclodextrin (Chol-CD) was added to preheated (37°C) complete DMEM-10% FBS such that the final concentration of the CD was 5.0 mM and the exogenous free cholesterol concentration was ~311 μg/ml. After incubation at 37°C for 1 h, the medium was filter-sterilized through a 0.22 μm membrane and 12 ml was added to each dish of COS cells. Control dishes received an equivalent volume of complete medium alone (DMEM-10% FBS). The addition of Chol-CD increased the total cholesterol content of the medium, as determined by gas-liquid chromatography (see below), from 31 to 66 μg/ml. After incubation at 37°C for 2 h, the cells were washed with PBS and metabolically radiolabeled with [^{3}H]oleate for 24 h at 37°C. After harvesting medium, cells were washed with PBS and cell pellets were recovered by scraping. Cells were lysed with 0.1 N NaOH and homogenized by shearing through a 28 gauge needle. The internal standard, 5α-cholestane (20.6 μg), was added and lipids were extracted from cell lysates and medium by the method of Bligh and Dyer (22). Cholesterol mass was measured by gas-liquid chromatography as described previously (21). The difference in mass between total and free cholesterol was multiplied by 1.7 to calculate the CE mass. The protein concentrations of the cell lysates were determined using a modified Lowry assay. Cell viability was determined by a colorimetric MTT assay (23).

Quantitation of apoB-50 secretion and cellular accumulation

COS-1 cells cultured in 100 mm dishes were transfected with a total of 8 μg of DNA at a mass ratio of 1:1.5:1 (MTP/apoB-50/ACAT2 or AP). Twenty-four hours after transfection, cells were incubated for 2 h at 37°C with complete medium or complete medium supplemented with Chol-CD, as described above. The cells were washed with PBS and metabolically radiolabeled with 100 μCi/ml [^{35}S]methionine/cysteine for 3 h at 37°C. After harvesting medium, cells were washed with PBS and lysed, as described (24). ApoB-50 was immunoprecipitated from the medium and cell lysates using anti-human apoB polyclonal antibody (Academy Bio-Medical) and separated by 6% SDS-PAGE. After fixation and drying, gels were exposed to Fujifilm BAS-MS imaging plates. The plates were scanned and the images acquired using a Fujifilm BAS-5000 imager and Image Reader software (version 1.8; Fujifilm). The apoB-50 band intensity was quantified using the Fujifilm Multi Gauge software and normalized to cellular protein mass.

Microsomal ACAT activity and immunoblot analyses

An aliquot of washed cells was resuspended in ACAT homogenization buffer (0.25 M sucrose, 1 mM EDTA, and 0.1 M K_2HPO_4, pH 7.4) supplemented with 10 μl/ml protease inhibitor cocktail, containing 4-(2-Aminoethyl)-bezenesulfonylfluoride, aprotinin, leupeptin, bestatin, pepstatin, and E-64 (Sigma; catalog No. P 8340), and microsomes were isolated as described previously (21). Microsomes (100 μg of protein) and 1 mg of fatty acid-free human serum albumin (Miles) dissolved in ACAT reaction buffer were placed into a 16 × 100 mm glass tube. After bringing the final volume of the reaction to 300 μl with ACAT reaction buffer, cholesterol dissolved in hydroxypropyl-β-cyclodextrin was added to the tube such that the final concentration of exogenous cholesterol was 0.5 nmol/μg microsomal protein. The tube containing the ACAT reaction mixture was incubated for 30 min in a shaking 37°C water bath. After adding 30 nmol of [1-^{14}C]oleoyl-CoA (7,885 dpm/nmol) (Amersham Pharmacia Biotech), the tube was vortexed and placed back into the water bath for 10 min. The ACAT reaction was terminated and the lipids were extracted as described (21). The extracted lipids were resuspended in $CHCl_3$ containing 1 mg/ml cholesteryl oleate, applied to a polyester-backed silica gel TLC plate (PE SIL G; Whatman), and separated using 70:30:1 hexane-ethyl ether-acetic acid as the running solvent. The separated lipids were visualized using I_2 vapor, and the CE band was cut from the plate and subjected to scintillation counting. Quantitation of relative ACAT mass was achieved by immunoblot analysis, as described previously (25, 26). The immunoreactive proteins were visualized by exposing the blots to Biomax MS scientific imaging film (Kodak). Relative ACAT2 protein mass was quantified by densitometry of the exposed film using the ChemiImager 5500 (Alpha Innotech).

RESULTS

Impact of ACAT2 expression on CE incorporation into apoB-containing lipoproteins

To determine whether ACAT2 expression affects CE incorporation into apoB-containing lipoproteins, COS cells were transiently transfected with plasmids encoding a His_6-tagged form of apoB-34 or apoB-50 (17, 18), human MTP, and either a control plasmid (AP) or African green monkey ACAT2 (27). The cells were metabolically radiolabeled for 24 h with [^{3}H]oleate and [^{35}S]methionine/cysteine in serum-free medium containing 0.5% fatty acid-free BSA.

AP- and ACAT2-transfected cells displayed similar levels of endogenous ACAT1 protein (**Fig. 1A**, upper blot). However, ACAT2 protein was detected only in ACAT2-transfected cells (Fig. 1A, lower blot). Although the expression of endogenous COS cell ACAT1 produced a basal level of microsomal ACAT activity in AP-transfected cells, transfection with ACAT2 increased microsomal ACAT activity by 3- to 4-fold (Fig. 1B). Cells transfected with apoB-34 or apoB-50 displayed similar amounts of ACAT protein and ACAT activity (Fig. 1A, B).

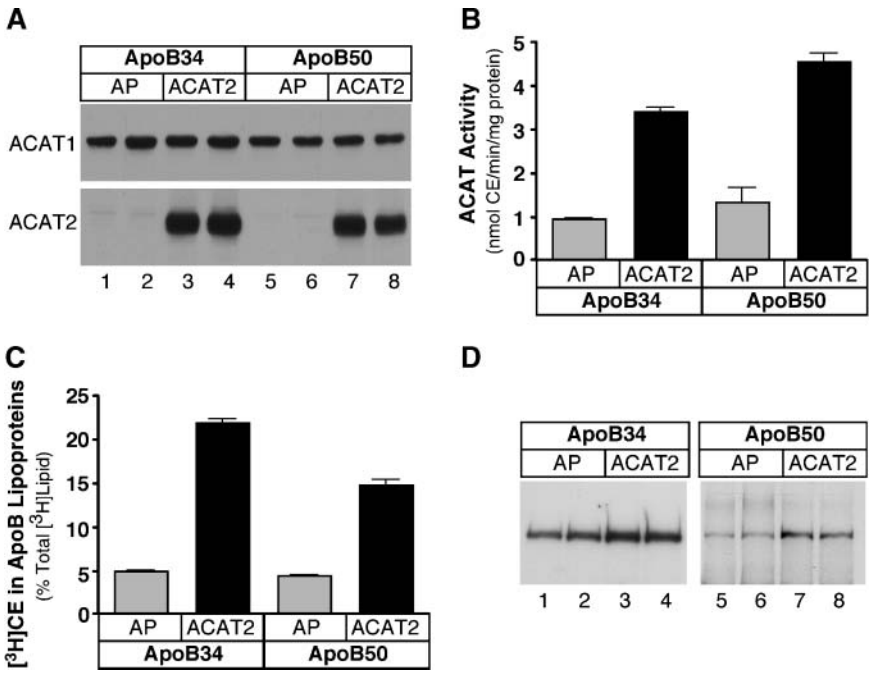

Fig. 1. Impact of ACAT2 expression on cholesteryl ester (CE) enrichment of apolipoprotein B (apoB)-containing lipoproteins. Duplicate dishes of COS cells were transfected with microsomal triglyceride transfer protein (MTP), apoB-34 or apoB-50, and either alkaline phosphatase (AP) or ACAT2, as indicated. Cells were metabolically radiolabeled with [^{3}H]oleate and [^{35}S]methionine/cysteine for 24 h. A: Immunoblot analysis of ACAT1 and ACAT2. B: Microsomal ACAT activity (means ± range). C: Percentage radiolabeled CE content of apoB-containing lipoproteins {apoB-associated [^{3}H]CE/apoB-associated [^{3}H](phospholipid + triglyceride + CE) × 100} (means ± range). D: Immunoprecipitation, SDS-PAGE, and fluorography of radiolabeled apoB recovered from medium aliquots. The experiment was repeated with similar outcomes (data not shown).

The impact of increased ACAT2 protein expression and activity on the core lipid composition of the secreted apoB-containing lipoproteins was dramatic. Compared with particles secreted by AP-transfected cells, apoB-34- and apoB-50-containing lipoproteins secreted from ACAT2-transfected cells displayed 4.5- and 3.3-fold increases, respectively, in the percentage of CE (Fig. 1C). The expression of ACAT2 also increased the number of apoB-containing particles secreted from the cells. Secretion of apoB-34 and apoB-50 was increased 22% and 32%, respectively, by ACAT2- versus AP-transfected cells (Fig. 1D). These results demonstrate that the expression of ACAT2 by COS cells increased microsomal ACAT activity and caused both an increase in apoB-34- and apoB-50-containing lipoprotein secretion and an increase in particle CE content.

To determine whether ACAT2 activity was necessary for the increased CE content of apoB-containing lipoproteins, COS cells expressing apoB-34, MTP, and either AP or ACAT2 were metabolically radiolabeled with [^{3}H]oleate for 24 h in the presence or absence of the ACAT inhibitor CP-113,818 (20). Similar to the results in Fig. 1C, apoB-containing lipoproteins from ACAT2-transfected cells contained 6-fold more CE relative to AP controls (**Fig. 2**). The increased incorporation of ACAT2-derived CE into the apoB-containing lipoproteins caused the triglyceride (TG) content of the particles to decrease from 74% to 60% but had no effect on phospholipid (PL) content. CP-113,818 treatment of ACAT2-transfected cells decreased the CE and increased the TG content of the apoB-containing lipoproteins to levels virtually identical to those observed in AP cells. Immunoblot analysis revealed that CP-113,818 did not decrease the level of endogenous ACAT1 or exogenous ACAT2 (data not shown). These results indicate that ACAT2 must be enzymatically active to enrich apoB-containing lipoproteins with CE.

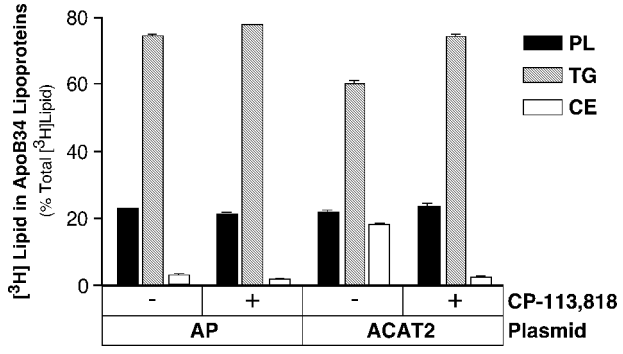

Fig. 2. Effect of ACAT inhibition on the CE content of apoB-containing lipoproteins. Duplicate dishes of COS cells were transfected with MTP, apoB-34, and either AP or ACAT2, as indicated. Twenty-four hours after transfection, cells were incubated for 2 h with either 1.25 μg/ml CP-113,818 or vehicle [0.025% (v/v) DMSO]. The cells were then metabolically radiolabeled with [^{3}H]oleate for 24 h in the presence of either 1.25 μg/ml CP-113,818 or vehicle, and the percentage [^{3}H]lipid content of the affinity-purified apoB-containing lipoproteins (means ± range) was determined. PL, phospholipid; TG, triglyceride.

Quantitative relationships among ACAT2 mass, activity, and the CE content of apoB-containing lipoproteins

We explored whether there is a quantitative relationship between the expression of ACAT2 and the extent of CE enrichment in apoB-containing lipoproteins. COS cells were transiently transfected with fixed amounts of apoB-34 and MTP and varying amounts of ACAT2 and labeled with [^{3}H]oleate, as described above. A linear relationship was observed between ACAT2 plasmid mass and both ACAT2 protein expression (**Fig. 3A**; $r^2 = 0.98$) and ACAT activity (Fig. 3B; $r^2 = 0.99$). Although increasing ACAT2 expression caused an increase in the CE content of apoB-34-containing lipoproteins (Fig. 3C, **Table 1**), a significant hyperbolic relationship was observed ($r^2 = 0.92$). This result suggested that at higher levels of ACAT2 protein expression and activity, depletion of the free cholesterol substrate pool was not fully compensated for by endogenous synthesis, thereby limiting further CE incorporation

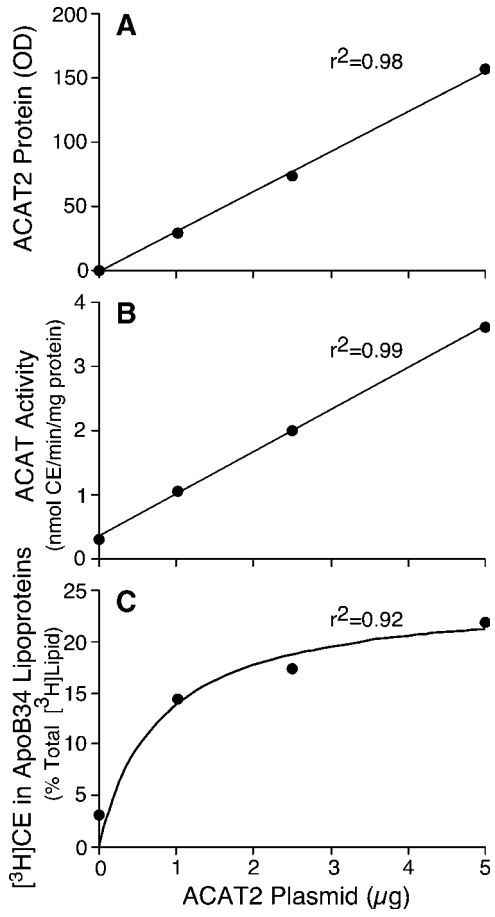

Fig. 3. Relationship among ACAT2 protein mass, activity, and apoB-containing lipoprotein CE content. Duplicate dishes of COS cells were transiently transfected with MTP, apoB-34, and 0, 1, 2.5, or 5 μg of ACAT2, as indicated. Cells were radiolabeled with [^{3}H]oleate for 24 h. Relationships are shown between ACAT2 plasmid mass and relative ACAT2 protein mass (A), microsomal ACAT activity (OD, optical density) (B), and the percentage [^{3}H]CE in apoB-34-containing lipoproteins (C). Each data point represents the mean of duplicate samples. The data in A and B were fit to a linear regression. The data in C were fit to a hyperbolic curve.

TABLE 1. Effect of ACAT2 on the radiolabeled lipid composition of apoB-34-containing lipoproteins

ACAT2 Plasmid (μg)	Percentage Total [^{3}H]Lipid PL	TG	CE	CE/TG	PL/CE+TG
0	19.1	77.7	3.3	0.042	0.24
1	16.9	68.7	14.5	0.211	0.20
2.5	19.0	63.5	17.4	0.274	0.24
5	20.3	57.8	22.0	0.380	0.25

ApoB, apolipoprotein B; CE, cholesteryl ester; PL, phospholipid; TG, triglyceride. Values shown are means of duplicate samples.

into apoB-containing lipoproteins. Although ACAT2 expression increased the CE content of apoB-34-containing particles, the surface-to-core lipid ratio (PL/TG+CE) did not change appreciably (Table 1). Although the use of [^{3}H]oleate did not allow us to measure the free cholesterol content of the lipoprotein surface, it appears that ACAT2-mediated incorporation of CE into apoB-containing lipoproteins caused a corresponding decrease in core TG.

Effects of cholesterol loading and ACAT2 expression on cellular CE content

As the previous experiment indicated that the free cholesterol content of the COS cells may become limiting at high ACAT2 levels, we explored the impact of exogenous cholesterol loading on cellular lipid and apoB particle composition in ACAT2- and AP-transfected cells. COS cells were transiently transfected with plasmids encoding apoB-34, MTP, and either AP or ACAT2. The cells were then incubated for 2 h in either complete medium (DMEM with 10% FBS) or complete medium supplemented with Chol-CD. After the cholesterol-loading period, cells were switched to serum-free medium and incubated for 24 h with [^{3}H]oleate and 0.5% BSA, as described above.

Immunoblot analysis revealed that preincubation with Chol-CD-supplemented medium did not change the amount of ACAT1 or ACAT2 protein present in either AP- or ACAT2-transfected cells (**Fig. 4A**). Likewise, microsomal ACAT activity was unaffected by cholesterol loading, although, as observed previously, ACAT2-transfected cells displayed a significant increase in ACAT activity relative to AP-transfected controls (Fig. 4B). Cholesterol loading with Chol-CD increased the cellular free cholesterol mass by ~2-fold and the esterified cholesterol mass by ~5-fold in both AP- and ACAT2-transfected cells (Fig. 4C). Surprisingly, the mass of cellular free and esterified cholesterol was not significantly affected by ACAT2 expression, despite the dramatic increase in microsomal ACAT activity (Fig. 4B). Compared with exposure to complete medium alone, incubation with Chol-CD-supplemented medium caused cell viability to decrease by 32% for the AP-transfected cells but by only 15% in ACAT2-transfected cells (Fig. 4D). Although not reflected by the cellular levels

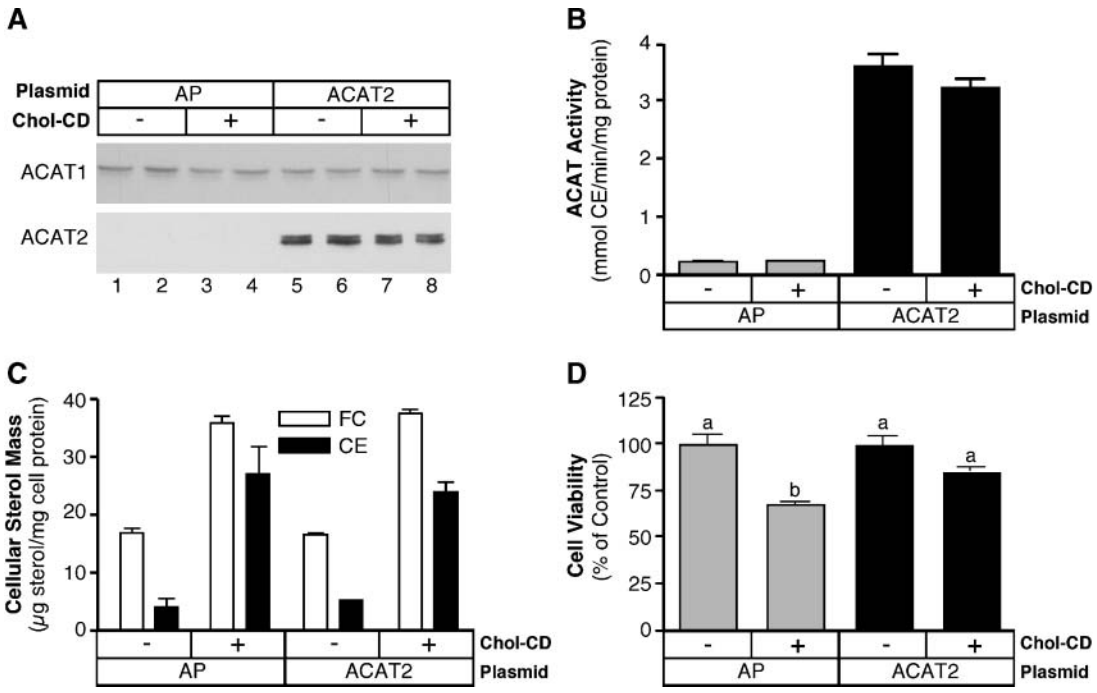

Fig. 4. Effect of cholesterol loading on cellular ACAT mass and activity, cholesterol content, and cell viability. Duplicate dishes of COS cells were transiently transfected with MTP, apoB-34, and either AP or ACAT2, as indicated. Cells were incubated without (−) or with (+) free cholesterol solubilized in methyl-β-cyclodextrin (Chol-CD), as indicated, and then radiolabeled with [^{3}H]oleate and processed, as described for Fig. 1. A: Immunoblot analysis of ACAT1 and ACAT2. B: Microsomal ACAT activity. C: Cellular free cholesterol (FC) and CE mass. D: Cell viability. For B and C, values shown represent means ± range. The experiment was repeated with similar outcomes (data not shown). For D, AP-transfected, non-cholesterol-loaded cells were used as controls, and values shown represent means ± SEM (n = 4). Statistically significant differences were determined by ANOVA (Tukey-Kramer honestly significant difference) and are indicated by different letters ($P < 0.05$).

of free cholesterol and CE, the increased cell viability of the ACAT2- versus AP-transfected cells may be attributable to enhanced esterification of a cytotoxic pool of free cholesterol that can accumulate in the endoplasmic reticulum after cholesterol loading (28).

The effects of ACAT2 and cholesterol loading on cellular lipids were further explored by metabolic radiolabeling of cells with [^{3}H]oleate. In both AP- and ACAT2-transfected cells, the cellular [^{3}H]CE content increased by ~10-fold in response to Chol-CD (**Fig. 5A**), a somewhat higher value than the ~5-fold increase observed when CE mass was measured by GC (Fig. 4C). In addition, unlike CE mass, [^{3}H]CE content increased by 1.8-fold in Chol-CD-treated ACAT2- versus AP-transfected cells. Cholesterol loading with Chol-CD reduced the [^{3}H]TG content in both AP- and ACAT2-transfected cells by ~40%; however, the [^{3}H]PL content of cells remained constant, irrespective of transfection or cholesterol-loading conditions (Fig. 5A).

Impact of cellular CE enrichment and ACAT2 expression on the lipid composition of apoB-containing lipoproteins

The lipid composition of apoB-34-containing lipoproteins was dramatically affected by both manipulation of ACAT activity and Chol-CD loading of cells. After incubation with complete medium supplemented with Chol-CD, AP-transfected cells secreted 6-fold more [^{3}H]CE compared with AP-transfected cells treated with complete medium alone (Fig. 5B). Secretion of [^{3}H]TG in apoB-34 particles was reduced by 88%, creating an overall 65% reduction in total core [^{3}H]lipid secretion (Fig. 5B). Hence, cholesterol loading in the absence of ACAT2 overexpression created aberrant, apparently smaller particles with a surface-to-core lipid ratio of 0.98 (**Table 2**).

In the absence of Chol-CD treatment, ACAT2 expression increased [^{3}H]CE secretion by 8.6-fold, primarily by increasing the proportion of [^{3}H]CE in apoB-34-containing lipoproteins from 3% to 16% of total [^{3}H]lipid (Fig. 5B, Table 2). Relative to non-cholesterol-loaded, AP-transfected cells, cholesterol-loaded ACAT2-transfected cells displayed a 27-fold increase in [^{3}H]CE secretion and an increase from 3% to 54% in the proportion of [^{3}H]CE contributing to total radiolabeled lipid, well above the 22% maximum value observed when cells were cultured in the absence of exogenous cholesterol (Fig. 3C, Table 1). The enhanced secretion of [^{3}H]CE in Chol-CD-treated, ACAT2-expressing cells was accompanied by a 75% reduction in [^{3}H]TG (Fig. 5B), resulting in maintenance of the surface-core lipid ratio of apoB-34 particles (Table 2). These results suggest that the levels of cellular free cholesterol and ACAT expression contribute significantly to the CE content of apoB-containing lipoproteins.

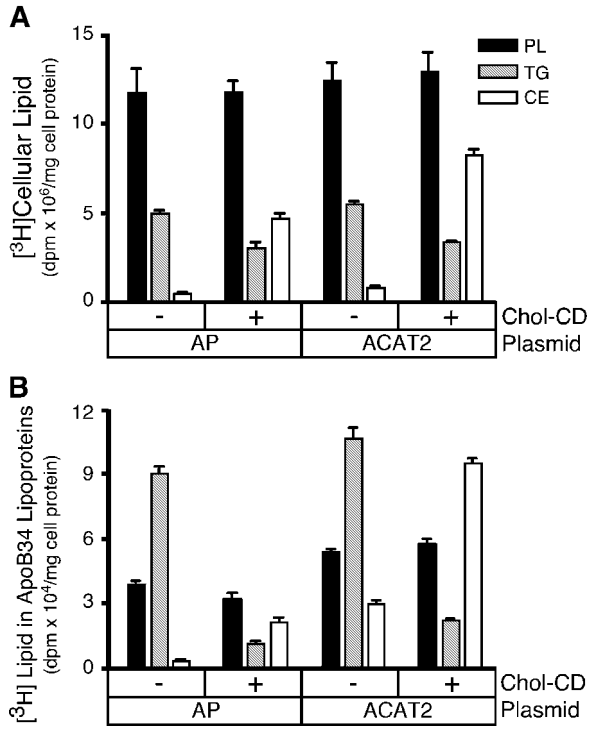

Fig. 5. Effect of ACAT2 overexpression and cholesterol loading on the lipid composition of cells and apoB-34-containing lipoproteins. Duplicate dishes of COS cells were transfected with MTP, apoB-34, and either AP or ACAT2, as indicated, followed by radiolabeling with [^{3}H]oleate. A: Radiolabeled lipid content of cells. B: Radiolabeled lipid content of apoB-34-containing lipoproteins. All values are means of duplicate samples ± range. The experiment was repeated with similar outcomes (data not shown).

Effect of cellular cholesterol content and ACAT2 expression on apoB secretion

The results in Fig. 1D indicated that ACAT2 expression increased the secretion of apoB. To more thoroughly define the impact of ACAT2 on apoB secretion, COS cells were transiently transfected with plasmids encoding apoB-50, MTP, and either AP or ACAT2. After incubation with either complete medium or complete medium supplemented with Chol-CD, the cells were metabolically radiolabeled with [^{35}S]methionine/cysteine for 3 h. ApoB-50 was then quantitated by immunoprecipitation, SDS-PAGE, and phosphorimager analysis.

Similar to the results observed in Fig. 1D, ACAT2 caused a 3.2-fold increase in apoB-50 secretion relative to the AP controls ($P < 0.05$) (**Fig. 6A**, compare lanes 1–3 with 7–9). Likewise, under conditions of cholesterol loading, ACAT2 resulted in a 3.6-fold increase in apoB-50 secretion ($P < 0.05$) (Fig. 6A, compare lanes 4–6 with 10–12). However, Chol-CD reduced apoB-50 secretion by 36% in ACAT2-transfected cells ($P < 0.05$) (Fig. 6A, compare lanes 7–9 with 10–12).

TABLE 2. Effect of ACAT2 and cholesterol loading on the radiolabeled lipid composition of apoB-34-containing lipoproteins

		Percentage Total [^{3}H]Lipid				
Plasmid	Medium	PL	TG	CE	CE/TG	PL/CE+TG
AP	−Chol-CD	29	68	3	0.04	0.41
AP	+Chol-CD	49	17	33	1.90	0.98
ACAT2	−Chol-CD	28	56	16	0.28	0.39
ACAT2	+Chol-CD	33	13	54	4.20	0.49

AP, alkaline phosphatase; Chol-CD, free cholesterol solubilized in methyl-β-cyclodextrin. Values shown are means of duplicate samples.

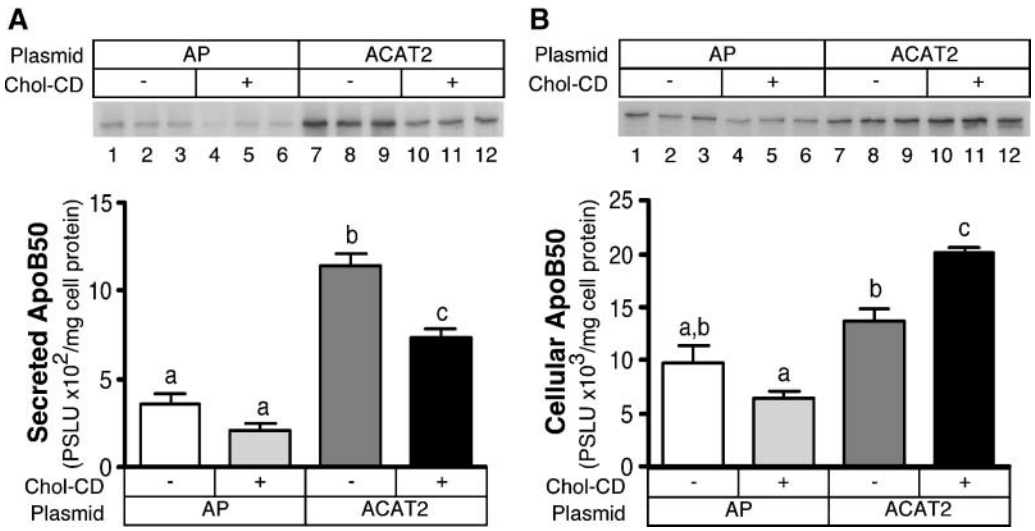

Fig. 6. Effect of ACAT2 expression and cholesterol loading on apoB-50 secretion. Triplicate dishes of COS cells were transiently transfected with apoB-50, MTP, and either AP or ACAT2, as indicated. Cells were incubated without (−) or with (+) Chol-CD, as indicated, and then radiolabeled with [^{35}S]methionine/cysteine for 3 h. The apoB-50 that was secreted into the medium (A) or accumulated within the cell (B) was immunoprecipitated, separated by SDS-PAGE, and quantitated by phosphorimager analysis. PSLU, photostimulated luminescence units. Statistically significant differences as determined by ANOVA (Tukey-Kramer honestly significant difference) are indicated by different letters ($P < 0.05$).

The reduction in apoB secretion observed in Chol-CD-loaded ACAT2 cells was accompanied by in increase in its intracellular accumulation (Fig. 6B, compare lanes 1–9 with 10–12). Although not reaching statistical significance, apoB-50 secretion also appeared to decrease in Chol-CD-treated AP cells (Fig. 6A, compare lanes 1–3 with 4–6). As observed previously (Fig. 4A), the cholesterol content of the ACAT2-transfected cells did not alter the expression of ACAT2 protein (data not shown). Overall, these results indicate that in addition to its effects on particle CE content, ACAT2 expression also significantly stimulates apoB-50 secretion, irrespective of cellular cholesterol content.

DISCUSSION

One of the strongest predictors of coronary artery atherosclerosis in nonhuman primates is the rate of hepatic CE secretion (10). As hepatic cholesteryl oleate is produced intracellularly by the enzyme ACAT2 (7, 8, 25, 29), we used a simplified and well-characterized cell-based system to establish quantitative relationships among ACAT protein mass and activity, cellular CE content, and the composition of nascent apoB-containing lipoproteins. These experiments revealed that under conditions of limiting endogenous cholesterol availability, ACAT2 overexpression increased the radiolabeled CE content of apoB-34- and apoB-50-containing lipoproteins from ~5% to 15% and 22%, respectively (Fig. 1C). However, as the supply of free cellular cholesterol became limiting, the CE content of apoB-containing particles became refractory to further increases in ACAT2 expression (Fig. 3C). When exogenous cholesterol was provided to cells via Chol-CD complexes, ACAT2 expression exerted a further dramatic impact on CE secretion. In cholesterol-loaded control (AP) cells, CE secretion increased by ~6-fold relative to nonloaded control cells (Fig. 5B). In ACAT2-overexpressing cells, a similar extent of cholesterol loading caused a disproportionate 27-fold increase in CE secretion (Fig. 5B). These results demonstrate that under a disparate range of conditions of cellular cholesterol content, the extent of ACAT2 expression regulated the mobilization of CE for apoB-containing lipoprotein assembly.

Although both AP- and ACAT2-expressing COS cells accumulated similar levels of cellular CE after cholesterol loading with Chol-CD (Figs. 4C, 5A), the ACAT2 cells were better able to secrete CE on apoB-containing particles (Fig. 5B, Table 2). Although this could be attributable simply to overexpression of ACAT2, it is also possible that ACAT2, by virtue of its distinct structure and membrane topology (30–32), is better adapted to interact with components of the apoB-lipoprotein secretory pathway. This prediction is consistent with the finding that ACAT2 expression is confined to hepatocytes and enterocytes and is responsible for the synthesis of all CE present in nascent apoB-containing lipoproteins (5, 9, 25, 33). Furthermore, in studies by Liang et al. (15), stable transfection of ACAT1 or ACAT2 in rat hepatoma cells caused similar increases in cellular CE content, yet ACAT2 cells secreted 2-fold more apoB than ACAT1 cells, suggesting a specialized role of ACAT2 in apoB secretion. However, in these studies, apoB and CE secretion were not normalized to ACAT mass or activity (15), and our attempts to directly compare ACAT1 with ACAT2 were compromised by the inability to obtain transfected ACAT1 activity levels comparable to those observed in ACAT2 cells (27). Hence, the relative potential of

ACAT1 versus ACAT2 to provide CE for apoB-containing lipoprotein assembly is still not fully defined, although it is clear from these and other studies that both enzymes are capable of producing CE for apoB assembly and secretion (15, 34).

It is generally assumed that during the translation of apoB, a nascent first-step precursor particle is formed, which then acquires additional lipid after trafficking to a distal site within the secretory pathway (35–39). An issue relevant to the current studies is the relationship between the composition of lipids added to apoB during the first versus the second (posttranslational) step of lipoprotein assembly (40). The production of truncated apoB-containing lipoproteins in MTP-cotransfected nonhepatic cells is generally thought to recapitulate only the first step in lipoprotein assembly (18). Nonetheless, MTP is required for both steps of lipoprotein assembly and the lipid transfer rate for MTP-mediated CE transfer is ~70% of that observed for TG (41). The studies here confirm that the relative lack of discrimination between TG and CE displayed by MTP in vitro also exists in vivo, as there appears to be little selectivity with respect to the incorporation of CE versus TG into nascent lipoprotein particles, particularly when accompanied by ACAT2 expression. Therefore, we postulate that the facile enrichment of nascent apoB-containing lipoproteins with CE observed in this study may also be operational under conditions that support second-step assembly.

In this study, we found that ACAT2 expression in COS cells increased apoB-50 secretion by ~3-fold (Fig. 6A). This outcome is in agreement with previous studies demonstrating that ACAT activity and/or CE mass can have a stimulatory effect on the secretion of apoB (42–46). Most notably, Spady, Willard, and Meidell (34) demonstrated that adenovirus-mediated overexpression of ACAT1 in $LDLr^{-/-}$ mice resulted in increased hepatic CE accumulation and enhanced hepatic apoB and CE secretion. More recently, Liang et al. (15) demonstrated that overexpression of either ACAT1 or ACAT2 in rat hepatoma cells increased the secretion and cellular accumulation of radiolabeled CE to about the same extent, but ACAT2 expression caused a greater increase in apoB secretion than ACAT1. Although ACAT2 expression in our studies consistently increased apoB secretion, we unexpectedly found that Chol-CD treatment of ACAT2-expressing cells, which increases cellular CE content (Figs. 4C, 5A), caused a reduction in apoB-50 secretion (Fig. 6). Because cholesterol-loaded, ACAT2-transfected cells displayed not only a dramatic increase in CE but also a substantial reduction in TG (Fig. 5A), it is possible that CE is not used as efficiently as TG in the cotranslational lipidation of apoB. It is also possible, however, that the increase in cellular free cholesterol adversely affects other aspects of the lipoprotein assembly process.

Although our in vitro data and those of many others suggest a specialized role for ACAT in apoB lipoprotein assembly, ACAT2 expression and CE synthesis are not essential for apoB-containing particle secretion in vivo (5, 11, 12). In addition, we have reported that during isolated liver perfusion, the perfusate accumulation rate of apoB was similar for ACAT2-sufficient and -deficient mice (33). Thus, we believe that the primary role of ACAT2 expression, relevant to atherogenesis, is to enrich apoB-containing lipoproteins with cholesteryl oleate and palmitate, as opposed to increasing the number of secreted apoB-containing particles.

The studies described here have important implications for the use of ACAT2 inhibitors as a means of limiting CE secretion and, hence, atherosclerosis progression. It has long been known that ACAT is stimulated allosterically by cholesterol, a finding that explains the dramatic increase in cellular CE content produced by cholesterol loading (16). Indeed, exogenous cholesterol loading had a greater impact on cellular CE than did the overexpression of ACAT2 (Figs. 4C, 5A). Therefore, the question arises whether excess cholesterol availability would counteract the partial therapeutic inhibition of ACAT2. In the cell-based system described here, it is clear that although cholesterol availability is an important regulator of ACAT2, the relative level of ACAT2 expression had a dramatic impact on the CE content and, therefore, the potential atherogenicity, of newly secreted apoB-containing lipoproteins (Figs. 1C, 3C, 5B). These results suggest that even partial therapeutic inhibition or silencing of ACAT2, particularly in the liver, will both reduce the CE content of apoB-containing lipoproteins and confer atheroprotection. Indeed, recent studies by Bell et al. (13) indicate that antisense oligonucleotide-mediated silencing of liver ACAT2 limited atherogenesis in apoB-100-only $LDLr^{-/-}$ mice fed an atherogenic diet. It will be of interest in future studies to determine quantitative relationships between ACAT2 and CE content of newly secreted lipoproteins in vivo.

In conclusion, the current studies demonstrate that the level of ACAT2 expression in concert with free cholesterol availability determines the CE content of apoB-containing lipoproteins. These findings validate previous correlative studies linking liver ACAT activity with the rate of cholesteryl oleate secretion and further support ACAT2 as an attractive target for achieving plasma cholesterol decreases and inhibition of atherosclerosis progression.

This work was supported by National Institutes of Health Grants HL-49373 (G.S.S. and L.L.R.) and HL-024736 (L.L.R.). R.E.T. was supported by National Institutes of Health Training Grant HL-07115 and by a postdoctoral fellowship from the Mid-Atlantic Affiliate of the American Heart Association.

REFERENCES

1. Glomset, J. A. 1968. The plasma lecithins:cholesterol acyltransferase reaction. *J. Lipid Res.* **9:** 155–167.
2. Morton, R. E., and D. B. Zilversmit. 1982. Purification and characterization of lipid transfer protein(s) from human lipoprotein-deficient plasma. *J. Lipid Res.* **23:** 1058–1067.
3. Chang, C. C., H. Y. Huh, K. M. Cadigan, and T. Y. Chang. 1993. Molecular cloning and functional expression of human acyl-coenzyme A:cholesterol acyltransferase cDNA in mutant Chinese hamster ovary cells. *J. Biol. Chem.* **268:** 20747–20755.
4. Avramoglu, R. K., K. Cianflone, and A. D. Sniderman. 1995. Role of the neutral lipid accessible pool in the regulation of secretion

of apoB-100 lipoprotein particles by HepG2 cells. *J. Lipid Res.* **36:** 2513–2528.
5. Buhman, K. K., M. Accad, S. Novak, R. S. Choi, J. S. Wong, R. L. Hamilton, S. Turley, and R. V. Farese, Jr. 2000. Resistance to diet-induced hypercholesterolemia and gallstone formation in ACAT2-deficient mice. *Nat. Med.* **6:** 1341–1347.
6. Meiner, V. L., S. Cases, H. M. Myers, E. R. Sande, S. Bellosta, M. Schambelan, R. E. Pitas, J. McGuire, J. Herz, and R. V. Farese, Jr. 1996. Disruption of the acyl-CoA:cholesterol acyltransferase gene in mice: evidence suggesting multiple cholesterol esterification enzymes in mammals. *Proc. Natl. Acad. Sci. USA.* **93:** 14041–14046.
7. Chang, T. Y., C. C. Chang, S. Lin, C. Yu, B. L. Li, and A. Miyazaki. 2001. Roles of acyl-coenzyme A:cholesterol acyltransferase-1 and -2. *Curr. Opin. Lipidol.* **12:** 289–296.
8. Rudel, L. L., R. G. Lee, and T. L. Cockman. 2001. Acyl coenzyme A:cholesterol acyltransferase types 1 and 2: structure and function in atherosclerosis. *Curr. Opin. Lipidol.* **12:** 121–127.
9. Parini, P., M. Davis, A. T. Lada, S. K. Erickson, T. L. Wright, U. Gustafson, S. Sahlin, C. Einarsson, M. Eriksson, B. Angelin, et al. 2004. ACAT2 is localized to hepatocytes and is the major cholesterol-esterifying enzyme in human liver. *Circulation.* **110:** 2017–2023.
10. Rudel, L. L., J. Haines, J. K. Sawyer, R. Shah, M. S. Wilson, and T. P. Carr. 1997. Hepatic origin of cholesteryl oleate in coronary artery atherosclerosis in African green monkeys. Enrichment by dietary monounsaturated fat. *J. Clin. Invest.* **100:** 74–83.
11. Willner, E. L., B. Tow, K. K. Buhman, M. Wilson, D. A. Sanan, L. L. Rudel, and R. V. Farese, Jr. 2003. Deficiency of acyl CoA:cholesterol acyltransferase 2 prevents atherosclerosis in apolipoprotein E-deficient mice. *Proc. Natl. Acad. Sci. USA.* **100:** 1262–1267.
12. Lee, R. G., K. K. Kelly, J. K. Sawyer, R. V. Farese, Jr., J. S. Parks, and L. L. Rudel. 2004. Plasma cholesteryl esters provided by lecithin: cholesterol acyltransferase and acyl-coenzyme A:cholesterol acyltransferase 2 have opposite atherosclerotic potential. *Circ. Res.* **95:** 998–1004.
13. Bell, T. A., 3rd, J. M. Brown, M. J. Graham, K. M. Lemonidis, R. M. Crooke, and L. L. Rudel. 2006. Liver-specific inhibition of acyl-coenzyme A:cholesterol acyltransferase 2 with antisense oligonucleotides limits atherosclerosis development in apolipoprotein B100-only low-density lipoprotein receptor$^{-/-}$ mice. *Arterioscler. Thromb. Vasc. Biol.* **26:** 1814–1820.
14. Furbee, J. W., Jr., J. K. Sawyer, and J. S. Parks. 2002. Lecithin: cholesterol acyltransferase deficiency increases atherosclerosis in the low density lipoprotein receptor and apolipoprotein E knockout mice. *J. Biol. Chem.* **277:** 3511–3519.
15. Liang, J. J., P. Oelkers, C. Guo, P. C. Chu, J. L. Dixon, H. N. Ginsberg, and S. L. Sturley. 2004. Overexpression of human diacylglycerol acyltransferase 1, acyl-CoA:cholesterol acyltransferase 1 or acyl-CoA:cholesterol acyltransferase 2 stimulates secretion of apolipoprotein B-containing lipoproteins in McA-RH7777 cells. *J. Biol. Chem.* **279:** 44938–44944.
16. Liu, J., C. C. Chang, E. J. Westover, D. F. Covey, and T. Y. Chang. 2005. Investigating the allosterism of acyl-CoA:cholesterol acyltransferase (ACAT) by using various sterols: in vitro and intact cell studies. *Biochem. J.* **391:** 389–397.
17. Sellers, J. A., L. Hou, H. Athar, M. M. Hussain, and G. S. Shelness. 2003. A Drosophila microsomal triglyceride transfer protein homolog promotes the assembly and secretion of human apolipoprotein B—implications for human and insect lipid transport and metabolism. *J. Biol. Chem.* **278:** 20367–20373.
18. Shelness, G. S., L. Hou, A. S. Ledford, J. S. Parks, and R. B. Weinberg. 2003. Identification of the lipoprotein initiating domain of apolipoprotein B. *J. Biol. Chem.* **278:** 44702–44707.
19. Gordon, D. A., H. Jamil, D. Sharp, D. Mullaney, Z. Yao, R. E. Gregg, and J. Wetterau. 1994. Secretion of apolipoprotein B-containing lipoproteins from HeLa cells is dependent on expression of the microsomal triglyceride transfer protein and is regulated by lipid availability. *Proc. Natl. Acad. Sci. USA.* **91:** 7628–7632.
20. Marzetta, C. A., Y. E. Savoy, A. M. Freeman, C. A. Long, J. L. Pettini, R. E. Hagar, P. B. Inskeep, K. Davis, A. F. Stucchi, and R. J. Nicolosi. 1994. Pharmacological properties of a novel ACAT inhibitor (CP-113,818) in cholesterol-fed rats, hamsters, rabbits, and monkeys. *J. Lipid Res.* **35:** 1829–1838.
21. Temel, R. E., A. K. Gebre, J. S. Parks, and L. L. Rudel. 2003. Compared with acyl-CoA:cholesterol O-acyltransferase (ACAT) 1 and lecithin:cholesterol acyltransferase, ACAT2 displays the greatest capacity to differentiate cholesterol from sitosterol. *J. Biol. Chem.* **278:** 47594–47601.
22. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
23. Denizot, F., and R. Lang. 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Methods.* **89:** 271–277.
24. Shelness, G. S., K. C. Morris-Rogers, and M. F. Ingram. 1994. Apolipoprotein B48-membrane interactions. Absence of transmembrane localization in nonhepatic cells. *J. Biol. Chem.* **269:** 9310–9318.
25. Lee, R. G., M. C. Willingham, M. A. Davis, K. A. Skinner, and L. L. Rudel. 2000. Differential expression of acyl-CoA:cholesterol acyltransferase 1 and acyl-CoA:cholesterol acyltransferase 2 among cells within liver, intestine, kidney, and adrenal of nonhuman primates. *J. Lipid Res.* **41:** 1991–2001.
26. Rudel, L. L., M. Davis, J. Sawyer, R. Shah, and J. Wallace. 2002. Primates highly responsive to dietary cholesterol up-regulate hepatic ACAT2, and less responsive primates do not. *J. Biol. Chem.* **277:** 31401–31406.
27. Anderson, R. A., C. Joyce, M. Davis, J. W. Reagan, M. Clark, G. S. Shelness, and L. L. Rudel. 1998. Identification of a form of acyl-CoA:cholesterol acyltransferase specific to liver and intestine in nonhuman primates. *J. Biol. Chem.* **273:** 26747–26754.
28. Maxfield, F. R., and I. Tabas. 2005. Role of cholesterol and lipid organization in disease. *Nature.* **438:** 612–621.
29. Chang, C. C. Y., N. Sakashita, K. Ornvold, O. Lee, E. T. Chang, R. Dong, S. Lin, C-Y. G. Lee, S. C. Strom, R. Kashyap, et al. 2000. Immunological quantitation and localization of ACAT-1 and ACAT-2 in human liver and small intestine. *J. Biol. Chem.* **275:** 28083–28092.
30. Lin, S., D. Cheng, M. S. Liu, J. Chen, and T. Y. Chang. 1999. Human acyl-CoA:cholesterol acyltransferase-1 in the endoplasmic reticulum contains seven transmembrane domains. *J. Biol. Chem.* **274:** 23276–23285.
31. Joyce, C. W., G. S. Shelness, M. A. Davis, R. G. Lee, K. Skinner, R. A. Anderson, and L. L. Rudel. 2000. ACAT1 and ACAT2 membrane topology segregates a serine residue essential for activity to opposite sides of the endoplasmic reticulum. *Mol. Biol. Cell.* **11:** 3675–3687.
32. Lin, S., X. H. Lu, C. C. Y. Chang, and T. Y. Chang. 2003. Human acyl-coenzyme A:cholesterol acyltransferase expressed in Chinese hamster ovary cells: membrane topology and active site location. *Mol. Biol. Cell.* **14:** 2447–2460.
33. Lee, R. G., R. Shah, J. K. Sawyer, R. L. Hamilton, J. S. Parks, and L. L. Rudel. 2005. ACAT2 contributes cholesteryl esters to newly secreted VLDL, whereas LCAT adds cholesteryl ester to LDL in mice. *J. Lipid Res.* **46:** 1205–1212.
34. Spady, D. K., M. N. Willard, and R. S. Meidell. 2000. Role of acyl-coenzyme A:cholesterol acyltransferase-1 in the control of hepatic very low density lipoprotein secretion and low density lipoprotein receptor expression in the mouse and hamster. *J. Biol. Chem.* **275:** 27005–27012.
35. Alexander, C. A., R. L. Hamilton, and R. J. Havel. 1976. Subcellular localization of B apoprotein of plasma lipoproteins in rat liver. *J. Cell Biol.* **69:** 241–263.
36. Kilinski, A., S. Rustaeus, and J. E. Vance. 2002. Microsomal triglyceride transfer protein is required for lumenal accretion of triacylglycerol not associated with apoB, as well as for apoB lipidation. *J. Biol. Chem.* **277:** 31516–31525.
37. Gusarova, V., J. L. Brodsky, and E. A. Fisher. 2003. Apolipoprotein B100 exit from the endoplasmic reticulum (ER) is COPII-dependent, and its lipidation to very low density lipoprotein occurs post-ER. *J. Biol. Chem.* **278:** 48051–48058.
38. Wang, Y., K. Tran, and Z. Yao. 1999. The activity of microsomal triglyceride transfer protein is essential for accumulation of triglyceride within the microsomes in McA-RH7777 cells. *J. Biol. Chem.* **274:** 27793–27800.
39. Fisher, E. A., and H. N. Ginsberg. 2002. Complexity in the secretory pathway: the assembly and secretion of apolipoprotein B-containing lipoproteins. *J. Biol. Chem.* **277:** 17377–17380.
40. Rustaeus, S., K. Lindberg, P. Stillemark, C. Claesson, L. Asp, T. Larsson, J. Borén, and S. O. Olofsson. 1999. Assembly of very low density lipoprotein: a two-step process of apolipoprotein B core lipidation. *J. Nutr.* **129 (Suppl. 2S):** 463–466.

41. Jamil, H., J. K. Dickson, Jr., C-H. Chu, M. W. Lago, J. K. Rinehart, S. A. Biller, R. E. Gregg, and J. R. Wetterau. 1995. Microsomal triglyceride transfer protein. Specificity of lipid binding and transport. *J. Biol. Chem.* **270:** 6549–6554.
42. Carr, T. P., R. L. Hamilton, Jr., and L. L. Rudel. 1995. ACAT inhibitors decrease secretion of cholesteryl esters and apolipoprotein B by perfused livers of African green monkeys. *J. Lipid Res.* **36:** 25–36.
43. Musanti, R., L. Giorgini, P. Lovisolo, A. Pirillo, A. Chiari, and G. Ghiselli. 1996. Inhibition of acyl-CoA:cholesterol acyltransferase decreases apolipoprotein B-100-containing lipoprotein secretion from HepG2 cells. *J. Lipid Res.* **37:** 1–14.
44. Zhang, Z. J., K. Cianflone, and A. D. Sniderman. 1999. Role of cholesterol ester mass in regulation of secretion of apoB100 lipoprotein particles by hamster hepatocytes and effects of statins on that relationship. *Arterioscler. Thromb. Vasc. Biol.* **19:** 743–752.
45. Wilcox, L. J., P. H. R. Barrett, R. S. Newton, and M. W. Huff. 1999. ApoB100 secretion from HepG2 cells is decreased by the ACAT inhibitor CI-1011—an effect associated with enhanced intracellular degradation of apoB. *Arterioscler. Thromb. Vasc. Biol.* **19:** 939–949.
46. Burnett, J. R., L. J. Wilcox, D. E. Telford, S. J. Kleinstiver, P. H. R. Barrett, R. S. Newton, and M. W. Huff. 1999. Inhibition of ACAT by avasimibe decreases both VLDL and LDL apolipoprotein B production in miniature pigs. *J. Lipid Res.* **40:** 1317–1327.