ACAT inhibitors decrease secretion of cholesteryl esters and apolipoprotein B by perfused livers of African green monkeys

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Abstract To test the hypothesis that newly synthesized cholesteryl esters are required for hepatic lipoprotein assembly and secretion, isolated livers of African green monkeys were perfused with medium containing an ACAT inhibitor. Three ACAT inhibitors with different structural and chemical properties were used: CI-976 (Parke-Davis), CP-113818 (Pfizer), or PD-138142-15 (Parke-Davis). Each compound produced variable effects on secretion of lipids and apolipoproteins. A significant decrease in the secretion rates of cholesteryl ester and apoB was common to all three inhibitors, indicating that fewer lipoprotein particles were secreted during ACAT inhibition. Triacylglycerol secretion was decreased in the presence of CP-113818 and PD-138142-15 but no decrease in triacylglycerol secretion was observed with CI-976. Particles secreted in the presence of CI-976 were enriched in triacylglycerol relative to apoB. Effects on secretion of other apolipoproteins (apoA-I, apoA-II, and apoE) were variable. When all data were combined, the percent inhibition of cholesteryl ester secretion and apoB secretion in the presence of ACAT inhibitors was positively correlated (r = 0.84), whereas a similar relationship was not observed between triacylglycerol and apoB. While the results demonstrate some lack of specificity for these ACAT inhibitors, the complimentary results using the three different ACAT inhibitors suggest that secretion of cholesteryl ester and apoB are coordinately regulated. The data suggest that newly synthesized cholesteryl esters may participate in and promote the assembly and secretion of hepatic apoB-containing lipoproteins. The availability of triacylglycerol during lipoprotein assembly, while also important, would appear to serve a role separate from that of cholesteryl ester.—**Carr, T. P., R. L. Hamilton, Jr., and L. L. Rudel.** ACAT inhibitors decrease secretion of cholesteryl esters and apolipoprotein B by perfused livers of African green monkeys. *J. Lipid Res.* 1995. **36:** 25–36.

Supplementary key words very low density lipoprotein • apolipoprotein • triacylglycerol • cholesterol

The primary physiologic function of hepatic very low density lipoproteins (VLDL) is to transport newly synthesized triacylglycerol from the liver to peripheral tissues for the maintenance of energy balance in the body. In this process, VLDL secreted by the liver are depleted of triacylglycerol by lipoprotein lipase largely in muscle and adipose tissue and the resulting remnant particles are either removed from the circulation or are remodeled and converted to cholesteryl ester-rich low density lipoproteins (LDL) (1). The concentration of LDL in the plasma is highly correlated with the incidence of coronary heart disease (2). Kinetic studies in humans suggest that an overproduction of VLDL by the liver could lead to atherogenic concentrations of LDL (3–5). The mechanisms which govern VLDL production, however, are incompletely understood.

Apolipoprotein B-100 (apoB) is the primary secretory protein with which triacylglycerol and other lipid components must associate in order for VLDL to be secreted by the liver. The requirement of apoB for lipid transport in VLDL is quite evident in the monogenic disease, abetalipoproteinemia, in which apoB is not present in plasma and affected individuals lack VLDL, LDL, and chylomicrons (6). ApoB is synthesized in the rough endoplasmic reticulum (ER) and contains several hydrophobic segments. The hydrophobic properties of the protein apparently allow some association with the membrane during VLDL particle assembly (7, 8). However, disagreement exists as to where and how particle maturation takes place prior to secretion into the plasma. The early studies of Stein and Stein (9) using electron microscopy suggested that VLDL particles were assembled largely in the ER. Alexander, Hamilton, and Havel (10) provided evidence using electron microscopy that triacylglycerol-rich particles form in the smooth ER and coalesce with apoB at the junction of the rough and smooth ER.

Abbreviations: HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins; apo, apolipoprotein; ER, endoplasmic reticulum; GLC, gas-liquid chromatography; TLC, thin-layer chromatography.

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Kinetic studies using cultured rat hepatocytes (11) and HepG2 cells (12, 13) indicated that most of the triacylglycerol became associated with apoB in the ER and that traversing the ER is the rate-limiting step in VLDL assembly. Conversely, kinetic studies by Bamberger and Lane (14, 15) using chick hepatocytes indicated that apoB associated tightly with the Golgi membrane and that transit through the Golgi was much slower than through the ER, implying that the immature VLDL particles are held up in the Golgi to allow acquisition of lipid prior to secretion. Additional studies using rat (16, 17) and chick (18) hepatocytes suggest that the majority of phospholipid secreted with VLDL is added to maturing particles in the Golgi. Most recently, studies by Rusinol, Verkade, and Vance (19) provide evidence that apoB-containing particles present in isolated ER vesicles have a similar amount of lipid associated as plasma VLDL. Despite the uncertainty concerning the rate-limiting step in VLDL particle assembly, a limited capacity of the Golgi membranes to synthesize triacylglycerol exists (20) and the picture that emerges is that apoB and triacylglycerol associate prior to arrival in the Golgi with further maturation of the nascent VLDL particle occurring during transit through the Golgi. Finally, the newly assembled VLDL are partitioned into secretory vesicles which eventually fuse with the cell surface releasing the particles into the space of Disse (21).

Regulation of VLDL assembly and secretion in the liver depends largely on the availability of newly synthesized triacylglycerol (reviewed in refs. 22, 23) increased amounts of which potentially can affect the number of secreted particles (i.e., copies of apoB) but, more likely, the triacylglycerol content of particles containing apoB. Hepatic VLDL also contain cholesteryl ester in the particle core, although a metabolic role of cholesteryl ester in the production of VLDL is not established. The enzyme that synthesizes cholesteryl esters, acyl-CoA:cholesterol acyltransferase (ACAT), appears to be located in the rough ER (24–26), whereas triacylglycerol appears to be synthesized largely in the smooth ER (20, 27). As cholesteryl esters are produced at the site of apoB synthesis and are secreted by the liver in VLDL in proportion to hepatic cholesteryl ester content and ACAT activity (28, 29), ACAT is a candidate for a role in the regulation of VLDL assembly early in the secretory pathway. Cianflone et al. (30) recently reported that inhibition of ACAT in HepG2 cells resulted in reduced cholesteryl ester and apoB secretion. The same authors (31) reported that treatment of HepG2 cells with glucose increased triacylglycerol secretion but did not alter the secretion of either cholesteryl ester or apoB. In turnover studies of apoB in VLDL and LDL in normocholesterolemic pigs, Huff et al. (32) reported that ACAT inhibitors decreased the production of VLDL apoB. Clearly, several types of data suggest an association between apoB secretion and cholesteryl ester synthesis by ACAT with subsequent secretion of the cholesteryl ester in the apoB-containing particle. In a recent paper by Tanaka et al. (33), a correlation between apoB secretion and cholesteryl ester content of rabbit hepatocytes was found, although no ACAT activity or cholesteryl ester secretion was measured. These authors hypothesized that a relationship exists whereby higher intracellular cholesteryl ester content prevents some apoB degradation (thereby permitting secretion) in the rabbit hepatocyte.

The current study was designed to examine the hypothesis that apoB secretion is dependent on ACAT-catalyzed cholesteryl ester synthesis using perfused livers of African green monkeys. Pharmacological doses of three different ACAT inhibitors were added separately to recirculating liver perfusate and the relative changes in the secretion of apoB and lipids was studied. The unique advantage of using these perfused primate livers to examine the relationship between ACAT activity and lipoprotein secretion is the ability to manipulate enzyme activity in the intact liver, thereby providing information pertinent to the in vivo situation in which an association between hepatic ACAT activity, plasma LDL cholesteryl ester content, and coronary artery atherosclerosis has been defined (29).

MATERIALS AND METHODS

Animals

Adult male African green monkeys (*Cercopithecus aethiops*) were purchased from Primate Imports, Port Washington, NY, and from the breeding colony at Hahnemann Medical School. The monkeys used in this study were part of two ongoing studies of atherosclerosis and were killed at the end of the atherosclerosis induction phase. Animals were fed diets containing 0.8 mg cholesterol/kcal and either 42% of calories as fat (34) or 35% of calories as fat (35). All monkeys consumed 90 kcal/kg body weight per day for 4–6 years prior to the liver perfusions.

ACAT inhibitor preparation

Three ACAT inhibitors were used in this study: CI-976 (36) and PD-138142-15 (37, 38) were kindly provided by Drs. Brian Krause and Roger Newton of Parke-Davis Pharmaceutical Research (Warner-Lambert Company, Ann Arbor, MI) and CP-113818 (39) was a generous gift from Dr. Carol Marzetta (Pfizer Central Research, Groton, CT). CI-976 and CP-113818 are water-insoluble and, therefore, were complexed with bovine serum albumin for administration to recirculating liver perfusate. This procedure allowed solubilization of the compounds in an aqueous medium and provided a vehicle by which the compounds could be taken up by hepatic parenchymal cells in the intact organ. To prepare the drug/albumin complex, CI-976 was dissolved in ethanol and CP-113818 was dissolved in dimethyl sulfoxide. A solution of bovine serum albumin (fatty acid-free, Sigma Chemical Co.) was

made with deionized water and the dissolved drugs were added dropwise to the warm albumin solution and then incubated overnight in a shaking water bath at 37°C. The molar ratio of drug:albumin in the final preparation was approximately 1 for both compounds. PD-138142-15 was dissolved in deionized water and added directly to the liver perfusate. **Figure 1** shows the relative potency of the ACAT inhibitors when added to in vitro enzyme assays. The concentrations of CI-976, CP-113818, and PD-138142-15 at which enzyme activity was inhibited 50% were approximately 1.4, 0.2, and 3.0 μM, respectively.

Liver perfusion

Animals were anesthetized with ketamine hydrochloride (10 mg/kg) and perfusion of isolated livers was performed as described previously (40, 41). Perfusate medium consisted of Krebs-Henseleit original Ringer bicarbonate buffer (pH 7.4) containing glucose, amino acids, insulin, hydrocortisone, penicillin, streptomycin, 1–3% bovine serum albumin, and washed human erythrocytes at 22% hematocrit. Recirculating perfusion was initially performed for 60 min ("flush period") to allow the release of plasma lipoproteins remaining in the liver prior to perfusion. Sodium taurocholate was infused during perfusion at a rate of 0.016 μmol/min per g liver. In some experiments where noted, an equimolar mixture of oleic and linoleic acid was infused at 0.050 μmol/min per g liver. This infusion of fatty acids did not significantly alter hepatic secretion of lipids or apolipoproteins; therefore, the data have been combined. After the flush period, the perfusate was replaced with fresh medium and recirculated through the liver for 120 min in the absence of ACAT inhibitors ("control period"). Perfusate was then replaced again with fresh medium containing an ACAT inhibitor and recirculated for an additional 120 min ("drug period"). To test the effect of time of perfusion, two consecutive 120-min periods were performed in five livers in the absence of ACAT inhibitors.

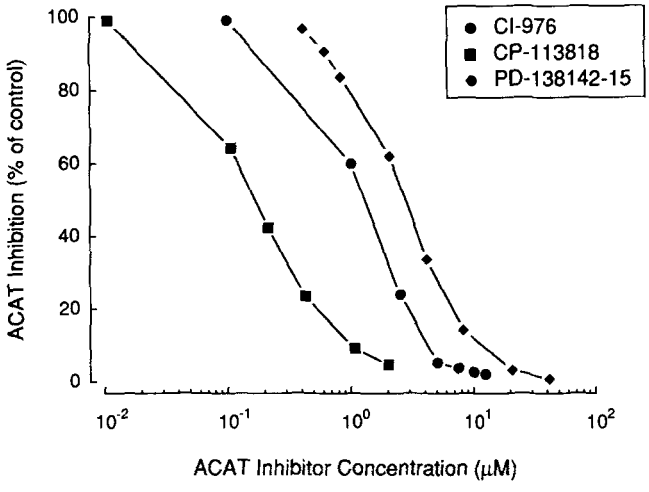

Fig. 1. Relative potency of ACAT inhibitors. CI-976 and CP-113818 were complexed with bovine serum albumin before addition to in vitro assays containing liver microsomes. PD-138142-15 was dissolved in phosphate buffer for addition to the enzyme assays. The assay was performed in 300 μl 0.1 M phosphate buffer containing 200 μg microsomal protein from monkey liver, 1.0 mg bovine serum albumin, and 50 nmol cholesterol in detergent (ref. 28). After incubation for 30 min at 37°C, the reaction was initiated by adding 30 nmol [^{14}C]oleoyl-CoA and incubated for 2 min. The reaction was stopped by adding 6 ml chloroform–methanol 2:1. Typical ACAT specific activities for ACAT in our experiments are between 200 and 1200 pmol/min per mg protein. The IC_{50} values for CI-976, CP-113818, and PD-138142-15 were approximately 1.4, 0.2, and 3.0 μM, respectively.

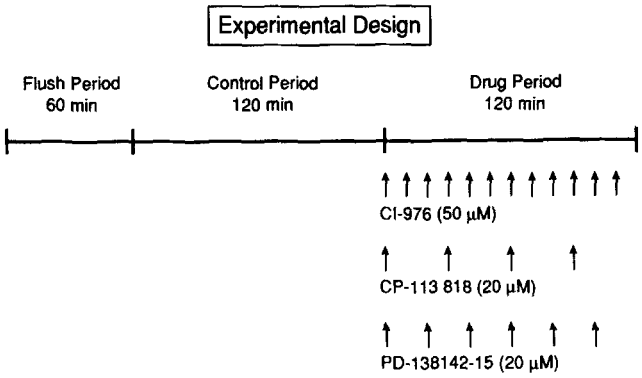

Fig. 2. Experimental design. Recirculating liver perfusion was performed for 60 min to "flush" the liver of plasma lipoproteins prior to the start of the experiment. The perfusate was replaced with fresh medium and recirculated for 120 min in the absence of ACAT inhibitors (control period). Perfusate was then replaced with fresh medium containing ACAT inhibitors and recirculated for an additional 120 min (drug period). CI-976 was added to attain an initial concentration of 50 μM, followed by equivalent doses every 10 min, as indicated by the arrows. The initial concentration of CP-113818 was 20 μM, followed by equivalent doses every 30 min. PD-138142-15 was added to attain an initial concentration of 20 μM, followed by equivalent doses every 20 min.

Perfusate aliquots (10 ml) were taken every 20 min and assayed for free cholesterol, cholesteryl ester, triacylglycerol, phospholipid, and apolipoproteins (apo) B, E, A-I, and A-II (see Analytical procedures). Perfusate accumulation rates of lipids and apolipoproteins were calculated as the slope of linear regression analysis of the six time-point samples taken during the control and drug periods. Bile was collected via a bile duct cannula at 30-min intervals throughout each perfusion experiment. Liver viability was monitored by color, rate of bile production, rate of oxygen consumption, and appearance of transaminases in the perfusate. Data from livers that showed evidence of failure were excluded from the study.

Figure 2 shows the experimental design, with the "flush," "control," and "drug" periods. In the drug period, CI-976 was added to perfusate for an initial concentration of 50 μM. African green monkey liver exhibited the ability to quickly metabolize CI-976 as evidenced by the rapid appearance of drug metabolites in the perfusate (42). Therefore, equivalent doses of CI-976 were added to

the perfusate every 10 min (represented by arrows) to approximate a level near the initial concentration. It was impossible to know how much drug actually reached the enzyme at its site of action. Because CI-976 was complexed with albumin, each dose increased the albumin concentration in this period by 0.25%. The effects of increasing albumin concentration were tested in three control livers and only the secretion of apoA-II was significantly affected (secretion was reduced 20%). Increasing albumin concentration did not affect secretion of the other apolipoproteins or any of the lipids. To control for the addition of increasing albumin concentration with CP-113818, albumin devoid of drug was added to the control period to mimic the addition of the albumin/drug complex. CP-113818 was added to perfusate at an initial concentration of 20 μM with equivalent doses added every 30 min. PD-138142-15 was added to perfusate dissolved in deionized water (no additional albumin) at an initial concentration of 20 μM, followed by equivalent doses every 20 min.

Analytical procedures

Apolipoproteins B, E, A-I, and A-II were measured by enzyme-linked immunosorbent assays (43, 44). Perfusate triacylglycerol, total cholesterol, and free cholesterol were determined enzymatically (45) and phospholipid was quantified by measuring inorganic phosphorus (46). The results of the enzymatic assays used for determining perfusate total cholesterol were verified in perfusate from ten livers by the *o*-phthalaldehyde procedure of Rudel and Morris (47). Perfusate free and esterified cholesterol concentrations were verified by first separating the lipids by thin-layer chromatography using Empore TLC sheets (Analytichem International, Harbor City, CA) and quantifying by gas–liquid chromatography (GLC). The free cholesterol was quantified by GLC using a 15 m DB17 column (J & W Scientific, Folsom, CA) with a film thickness of 1.0 μm and stigmasterol as the internal standard; esterified cholesterol was first saponified before quantifying by GLC. Biliary lipids were extracted according to Bligh and Dyer (48). Biliary cholesterol was measured enzymatically (45) and phospholipid was quantified by measuring inorganic phosphorus (46). The enzymatic steroid dehydrogenase procedure was used to measure total bile acids (49).

Perfusate apoB-containing lipoproteins were isolated by a combination of gel filtration chromatography and ultracentrifugation. Liver perfusate was first concentrated approximately 20-fold using Amicon ultrafiltration membranes (W. R. Grace & Co., Beverly, MA) and then applied to a 4% agarose gel filtration column to separate the apoB- and apoA-I-containing lipoproteins (50). The apoB-containing lipoproteins were centrifuged at density 1.006 g/ml and the floated lipoproteins were collected from the top of the tube. The density of the bottom fraction was adjusted to 1.063 g/ml and again centrifuged; the floated lipoproteins of density 1.006–1.063 g/ml were then collected. Lipoprotein fractions from experiments using PD-138142-15 and CP-113818 were shipped to Dr. Robert Hamilton Jr. at the University of California Medical Center in San Francisco for electron microscopic examination. Lipoprotein particle sizes were determined from electron micrographs of each lipoprotein preparation. Chemical composition of the lipoproteins was determined using enzymatic assays for triacylglycerol, free and total cholesterol (45); phospholipid was quantified by measuring inorganic phosphorus (46) and protein was estimated by the method of Lowry et al. (51). Lipoprotein composition was expressed as moles of lipid per mole of apoB and as the percent mass distribution.

ACAT activity was measured as described previously (29) from liver samples taken at the beginning of perfusion and at the end of the control and drug periods. Briefly, liver samples (200–400 mg) were homogenized in ice-cold phosphate buffer containing 0.25 M sucrose and centrifuged for 15 min at 12,000 *g* (4°C) to remove cell debris. The supernatant was centrifuged for 60 min at 100,000 *g* (4°C) and the resulting microsomal pellet was suspended in a small volume of phosphate buffer. The microsomal preparation was immediately frozen in liquid nitrogen for brief storage at −80°C. Freezing the microsomes did not affect ACAT activity, as has been reported in other species (26, 52). Triglyceride synthetase and acyl-CoA hydrolase activities were measured simultaneously with ACAT activity by scraping the TLC plates corresponding to triglyceride and free fatty acid and counting for total ^{14}C radioactivity.

Statistical analyses

Data in tables represent means ± standard errors of the mean (SEM). Perfusate accumulation rates of lipids and apolipoproteins were calculated from the slope of linear regression analysis of the six time-point samples taken during the control and drug periods. Differences in accumulation rates between the control and drug periods were determined by paired *t*-test.

RESULTS

The secretion of free cholesterol, cholesteryl ester, triacylglycerol, and phospholipid during recirculating liver perfusion is presented in **Fig. 3.** Likewise, the secretion of apoB, apoE, apoA-I, and apoA-II is shown in **Fig. 4.** Two identical, consecutive 120-min periods of perfusion were used test the effect of time on the secretion (i.e., accumulation in perfusate) of lipids and apolipoproteins. The rates of secretion were calculated from the slopes of the linear regression analyses using the six time points between 20

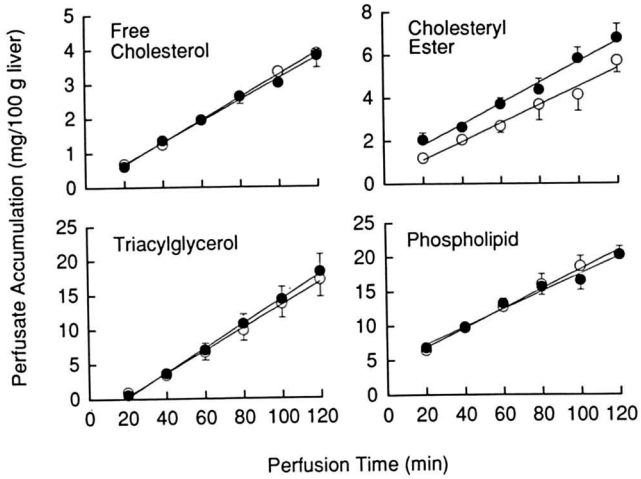

Fig. 3. Effect of time on the accumulation of free cholesterol, cholesteryl ester, triacylglycerol, and phospholipid in recirculating liver perfusate. Aliquots of perfusate were removed every 20 min for determination of lipid concentration. The filled circles represent the first of two consecutive 120-min periods of perfusion, and the open circles represent the second 120-min period of perfusion. Each point is the mean ± SEM of five experiments. The best fit line was determined by linear regression analysis.

and 120 min. Paired *t*-tests of the secretion rates between the two periods revealed no significant differences for any of the lipoprotein constituents, indicating that secretion was linear throughout the 5-h experimental period.

Figure 5 shows the effects of ACAT inhibitors added during recirculating liver perfusion. The ACAT inhibitors were added to the second of two consecutive 120-min periods of perfusion and the secretion rates of lipids and apolipoproteins were determined for both the control and drug periods. Differences in the secretion rates between periods were determined by paired *t*-test, and the effects of each ACAT inhibitor are presented as the percent of the control period.

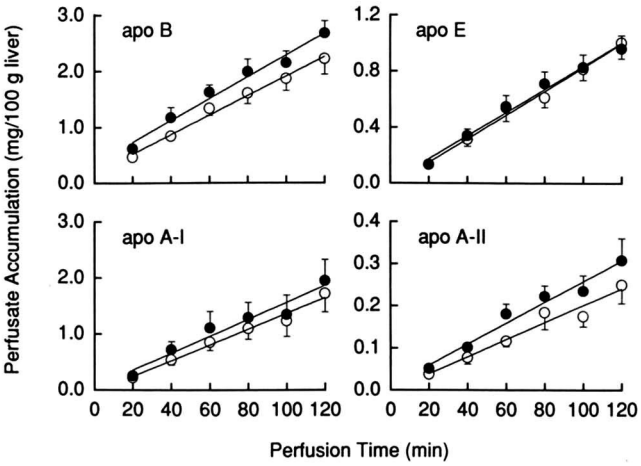

Fig. 4. Effect of time on the accumulation of apolipoproteins B, E, A-I, and A-II in recirculating liver perfusate. The remaining details are as in Fig. 3.

Addition of CI-976 to liver perfusate significantly decreased cholesteryl ester secretion by 35% (Fig. 5). Triacylglycerol, free cholesterol, and phospholipid secretion was not affected. Hepatic secretion of apoB, apoE, apoA-I, and apoA-II was decreased 43, 33, 30, and 47%, respectively. Because CI-976 was added to perfusate as an albumin/drug complex, control experiments were conducted in five livers in which only albumin, devoid of drug, was added to perfusate (data not shown). The secretion of apoA-II was decreased 20% in the presence of increased albumin concentration during the final perfusion period, whereas the secretion of the other constituents was not affected. Thus, the effect of CI-976 on apoA-II secretion was probably less than the apparent 47% decrease.

CP-113818 significantly decreased cholesteryl ester secretion 37% and triacylglycerol secretion 45% (Fig. 5). The secretion of free cholesterol and phospholipid was not altered. ApoB secretion was decreased 26% and apoA-II secretion was decreased 16%. The secretion of apoE was significantly increased 29% by CP-113818.

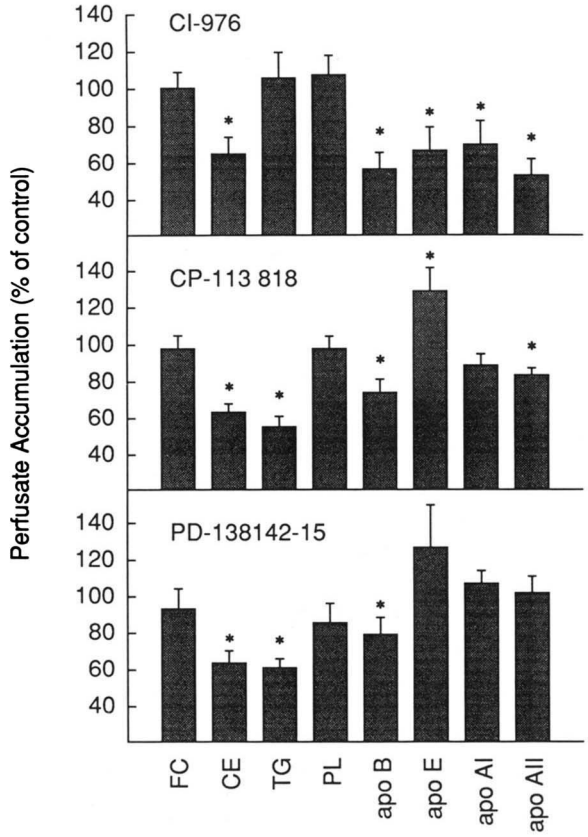

Fig. 5. Effect of ACAT inhibitors on the accumulation of lipids and apolipoproteins in recirculating liver perfusate. The ACAT inhibitors were added to the second of two consecutive 120-min periods of perfusion as described in Materials and Methods. The data are expressed as means ± SEM; n = 8 for CI-976, n = 12 for CP-113818, and n = 6 for PD-138142-15. FC, free cholesterol; CE, cholesteryl ester; TG, triacylglycerol; PL, phospholipid. Significant differences ($P < 0.05$) are indicated by an asterisk (*).

Addition of PD-138142-15 significantly decreased the secretion of cholesteryl ester (36%) and triacylglycerol (39%), but did not affect the secretion of free cholesterol or phospholipid (Fig. 5). The secretion of apoB was decreased 21%. ApoE secretion tended to be increased, although this difference was not statistically significant. The secretion of apoA-I and apoA-II was not affected by PD-138142-15.

Figure 6 shows the relationship between the change in secretion rates of apoB and cholesteryl ester as affected by the ACAT inhibitors. A highly significant correlation of $r = 0.84$ was observed for all three drugs. No correlation was detected between the absolute secretion rates of apoB and cholesteryl ester in the absence or presence of ACAT inhibitors (data not shown). In addition, no relationship was observed between the percentage change in apoB secretion rate and the percentage change in triacylglycerol secretion rate (data not shown).

The effects of the ACAT inhibitors on the hepatic secretion of apoB-containing lipoproteins are presented in **Table 1.** Assuming that each lipoprotein particle contains only one apoB molecule, these data represent the number of lipoprotein particles secreted in the absence and presence of the ACAT inhibitors. In all cases, the majority of apoB-containing lipoproteins secreted during the control period were isolated at density <1.006 g/ml. When CI-976 was added to recirculating perfusate, the secretion of lipoproteins of density 1.006–1.063 g/ml appeared to be affected to a greater extent, although this difference failed to reach statistical significance and represents only a small sample size. When CP-113818 and PD-138142-15 were added to perfusate, the greatest inhibitory effect ($P < 0.01$) occurred with lipoproteins of density <1.006 g/ml; the secretion of lipoproteins of density 1.006–1.063 g/ml was not significantly affected by either of these two ACAT inhibitors.

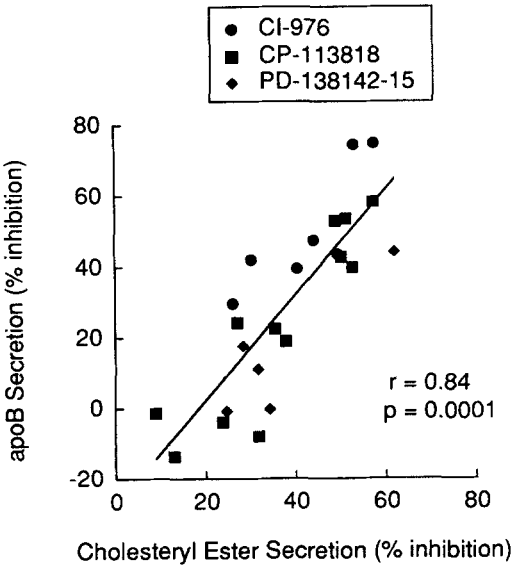

Fig. 6. Relationship between the percent inhibition of cholesteryl ester secretion and apoB secretion by the liver during ACAT inhibition. Each point represents the result from an individual liver perfusion experiment.

TABLE 1. Effect of ACAT inhibitors on hepatic secretion of apoB-containing lipoproteins

ACAT Inhibitor[a]	ApoB Secretion Rate[b]	
	d<1.006 g/ml	d 1.006–1.063 g/ml
	mg/h · 100 g liver	
CI-976		
Absent	0.82 ± 0.35	0.68 ± 0.36
Present	0.73 ± 0.16	0.31 ± 0.15
CP-113818		
Absent	0.96 ± 0.09	0.56 ± 0.09
Present	0.54 ± 0.09[c]	0.58 ± 0.07
PD-138142-15		
Absent	1.37 ± 0.29	0.69 ± 0.16
Present	0.91 ± 0.22[c]	0.84 ± 0.08

Values represent means ± SEM; n = 3 for CI-976, n = 12 for CP-113818, n = 5 for PD-138142-15.

[a]Livers were perfused for 2 h with no ACAT inhibitor present. Perfusate was replaced with fresh perfusate containing an ACAT inhibitor and livers were perfused for an additional 2 h.

[b]ApoB-containing lipoproteins were isolated by gel filtration chromatography, then centrifuged sequentially at densities 1.006 and 1.063 g/ml. The percent distribution of apoB in each fraction was used to determine the secretion rates of apoB in each fraction.

[c]Significantly different ($P < 0.01$) compared with the period of perfusion containing no ACAT inhibitor using two-tailed paired *t*-test.

The composition of apoB-containing lipoproteins of density <1.006 g/ml secreted by the liver are shown in **Table 2.** Data are expressed as mole of lipid per mole apoB; assuming one molecule of apoB per lipoprotein particle, the data can be viewed as the number of lipid molecules per particle. In the presence of CI-976, the particles became enriched in triacylglycerol, whereas the numbers of molecules per particle of the other lipids were not significantly affected. When CP-113818 or PD-138142-15 was added to perfusate, the lipoproteins of density <1.006 g/ml became significantly depleted of triacylglycerol and phospholipid. PD-138142-15 caused a specific reduction in the number of cholesteryl ester molecules per particle. In no case was the number of free cholesterol molecules per particle in this density range affected by ACAT inhibitors.

The composition of apoB-containing lipoproteins of density 1.006–1.063 g/ml secreted by the liver are shown in **Table 3.** The only ACAT inhibitor to significantly affect composition of lipoproteins in this density range was CP-113818, which reduced the number of cholesteryl ester molecules per particle. The number cholesteryl ester molecules per particle tended to be lower in the presence of PD-138142-15, but this reduction was not statistically significant ($P = 0.09$).

TABLE 2. Composition of liver perfusate lipoproteins of density < 1.006 g/ml

ACAT Inhibitor[a]	Lipoprotein Lipid Composition[b]			
	Free Cholesterol	Phospholipid	Cholesteryl Ester	Triacylglycerol
	mol/mol apoB			
CI-976				
Absent	1118 ± 67	2042 ± 87	1515 ± 111	3373 ± 448
	(6.7 ± 0.8)	(24.3 ± 0.7)	(15.5 ± 2.1)	(45.6 ± 3.8)
Present	918 ± 129	2303 ± 151	1346 ± 80	4816 ± 432'
	(4.5 ± 0.3)	(22.8 ± 0.6)	(11.4 ± 0.6)	(54.6 ± 1.2)
CP-113818				
Absent	880 ± 50	1738 ± 83	1647 ± 236	3593 ± 205
	(5.3 ± 0.3)	(20.9 ± 0.9)	(17.0 ± 2.0)	(48.9 ± 2.3)
Present	808 ± 94	1630 ± 74'	1607 ± 306	3172 ± 201'
	(5.3 ± 0.6)	(21.3 ± 0.8)	(17.1 ± 2.4)	(47.5 ± 2.3)
PD-138142-15				
Absent	877 ± 61	1945 ± 39	1864 ± 349	4048 ± 350
	(4.9 ± 0.6)	(21.3 ± 1.2)	(16.6 ± 1.9)	(50.0 ± 0.8)
Present	979 ± 67	1608 ± 75'	1207 ± 132'	3211 ± 307'
	(6.6 ± 0.4)	(21.8 ± 1.6)	(13.7 ± 1.0)	(49.0 ± 1.8)

Values represent means ± SEM; n = 3 for CI-976; n = 12 for CP-113818; n = 5 for PD-138142-15.
[a]Livers were perfused for 2 h with no ACAT inhibitor present. Perfusate was replaced with fresh perfusate containing an ACAT inhibitor and livers were perfused for an additional 2 h.
[b]Values in parentheses represent the percent mass composition.
'Significantly different ($P < 0.05$) compared with the period of perfusion containing no ACAT inhibitor using two-tailed paired *t*-test.

Table 4 shows the average particle diameter of perfusate lipoproteins from four individual experiments. Lipoproteins from experiments using CP-113818 and PD-138142-15 were examined by electron microscopy. In each case, the particle diameter became smaller or did not change when the ACAT inhibitors were added to the perfusate.

The production of bile and biliary lipids during liver perfusion in the presence of ACAT inhibitors was also examined (**Table 5**). Addition of CI-976 to recirculating

TABLE 3. Composition of liver perfusate lipoproteins of density 1.006–1063 g/ml

ACAT Inhibitor[a]	Lipoprotein Lipid Composition[b]			
	Free Cholesterol	Phospholipid	Cholesteryl Ester	Triacylglycerol
	mol/mol apoB			
CI-976				
Absent	1474 ± 586	2705 ± 609	984 ± 307	801 ± 146
	(11.8 ± 4.0)	(45.0 ± 6.1)	(15.6 ± 6.4)	(16.1 ± 3.2)
Present	2334 ± 423	4021 ± 383	783 ± 272	1212 ± 411
	(14.7 ± 2.7)	(50.9 ± 5.0)	(8.5 ± 3.0)	(17.5 ± 5.8)
CP-113818				
Absent	1417 ± 211	2222 ± 266	1402 ± 157	882 ± 94
	(11.6 ± 0.8)	(37.0 ± 1.8)	(21.4 ± 2.3)	(18.0 ± 2.0)
Present	1741 ± 147	2566 ± 263	1248 ± 162'	764 ± 77
	(14.3 ± 0.7)	(41.6 ± 1.7)	(17.7 ± 2.0)	(14.9 ± 1.4)
PD-138142-15				
Absent	1027 ± 100	2567 ± 755	1875 ± 471	1373 ± 423
	(8.6 ± 1.6)	(36.0 ± 3.0)	(22.8 ± 2.3)	(21.5 ± 3.8)
Present	1167 ± 143	2086 ± 154	1484 ± 307	1257 ± 388
	(10.6 ± 2.0)	(35.5 ± 2.5)	(20.4 ± 2.4)	(22.0 ± 4.5)

Values represent means ± SEM; n = 3 for CI-976, n = 12 for CP-113818, n = 5 for PD-138142-15.
[a]Livers were perfused for 2 h with no ACAT inhibitor present. Perfusate was replaced with fresh perfusate containing an ACAT inhibitor and livers were perfused for an additional 2 h.
[b]Values in parentheses represent the percent mass composition.
'Significantly different ($P < 0.05$) compared with the period of perfusion containing no ACAT inhibitor using two-tailed paired *t*-test.

TABLE 4. Effect of ACAT inhibitors on liver perfusate lipoprotein size

	Particle Diameter[b]			
	d < 1.006 g/ml		d 1.006–1.063 g/ml	
Animal[a]	Drug Absent	Drug Present	Drug Absent	Drug Present
	Å			
681	494	407	318	319
665	453	406	300	291
674	613	512	458	383
701	557	553	431	417
Mean	529	470	377	353

[a]Livers from animals 681 and 665 were perfused with ACAT inhibitor CP-113818. Livers from animals 674 and 701 were perfused with PD-138142-15.

[b]Lipoprotein particle diameters were determined from 4–6 electron micrographs of each lipoprotein preparation. Approximately 80 lipoprotein particles were measured from each photograph.

perfusate caused a significant reduction in the rate of secretion of biliary phospholipid and cholesterol; the secretion rate of bile acids was not affected. CP-113818 and PD-138142-15 did not affect the secretion rates of bile or biliary lipids.

DISCUSSION

This study was conducted to test the hypothesis that the assembly and secretion of apoB-containing lipoproteins by the liver is dependent on cholesteryl ester synthesis by ACAT. Isolated livers of African green monkeys were perfused with medium containing one of three ACAT inhibitors: CI-976, CP-113818, or PD-138142-15. Each compound produced variable effects on the hepatic secretion of other lipids and apolipoproteins. However, in common among all three ACAT inhibitors was a significant decrease in both cholesteryl ester and apoB secretion, indicating that fewer lipoprotein particles were secreted during enzyme inhibition. This finding occurred even when triacylglycerol secretion was not inhibited, as was seen with CI-976, in which case the apoB-containing VLDL were enriched in triacylglycerol (Table 2). Triacylglycerol secretion was inhibited together with cholesteryl ester and apoB secretion when CP-113818 and PD-138142-15 were used, indicating that the different ACAT inhibitors exerted independent and variable effects on lipoprotein secretion. In contrast, the percent change in cholesteryl ester and apoB secretion in the presence of all of the ACAT inhibitors was positively correlated ($r = 0.84$), whereas no similar relationship was detected between triacylglycerol and apoB. These data suggest that newly synthesized cholesteryl esters resulting from the activity of ACAT may participate in the assembly and secretion of apoB-containing lipoproteins, possibly serving early in the assembly and secretion process to prevent apoB from entering a degradation pathway (33).

The availability of triacylglycerol for apoB lipoprotein assembly and secretion appears to be a separate important factor in the perfused primate liver. CI-976 was the first compound tested and we observed that triacylglycerol secretion by the liver was maintained at a similar rate, even though fewer lipoprotein particles were being secreted. Field, Albright, and Mathur (53) have characterized the effects of CI-976 on fatty acyl-CoA hydrolase, triacylglycerol synthetase, HMG-CoA reductase, and ACAT in CaCo-2 cells and found the drug to be a specific, competitive inhibitor of ACAT. We also found no effect of CI-976 on fatty acyl-CoA hydrolase in microsomes isolated from the intact liver. At the 35% inhibition level (of hepatic ACAT) that we achieved, our observations support the suggestion that CI-976 may be relatively specific for ACAT inhibition leaving triacylglycerol synthesis and

TABLE 5. Bile and biliary lipid secretion during recirculating liver perfusion

ACAT Inhibitor[a]	Bile Secretion	Biliary Lipid Secretion		
		Bile Acids	Phospholipid	Cholesterol
	ml/h	*μmol/h · 100 g liver*		
CI-976				
Absent	2.93 ± 0.48	152 ± 29	11.65 ± 2.69	6.78 ± 1.33
Present	2.86 ± 0.67	147 ± 36	8.24 ± 2.21[b]	4.40 ± 1.06[b]
CP-113818				
Absent	1.99 ± 0.13	166 ± 15	8.60 ± 1.05	5.14 ± 0.57
Present	1.99 ± 0.16	181 ± 16	8.14 ± 1.16	4.92 ± 0.64
PD-138142-15				
Absent	2.09 ± 0.23	219 ± 22	9.73 ± 1.23	6.26 ± 1.13
Present	2.71 ± 0.53	225 ± 14	11.02 ± 1.63	6.90 ± 0.79

Values represent means ± SEM; n = 8 for CI-976, n = 10 for CP-113818, n = 6 for PD-138142-15.

[a]Livers were perfused for 2 h with no ACAT inhibitor present. Perfusate was replaced with fresh perfusate containing an ACAT inhibitor and livers were perfused for an additional 2 h.

[b]Significantly different ($P < 0.01$) compared with the period of perfusion containing no ACAT inhibitor using two-tailed paired *t*-test.

secretion unaffected (see Fig. 5). It is possible that a greater percentage inhibition of ACAT by CI-976 could result in reduced triacylglycerol synthesis and secretion, but we were unable to achieve further inhibition of enzyme activity with this compound. We found that CI-976 was rapidly converted to a metabolite by monkey livers so that the amounts of inhibitor that actually reach the intracellular site(s) of the enzyme must remain uncertain.

Addition of CP-113818 and PD-138142-15 to liver perfusate resulted in decreased cholesteryl ester and apoB, as well as triacylglycerol, secretion. Early studies using perfused rat livers (54, 55) demonstrated that the secretion of hepatic triacylglycerol was dependent on the availability of fatty acids for triacylglycerol synthesis, although we have observed that African green monkey livers do not increase triacylglycerol secretion more than 20% when fatty acids were infused into the perfusate (unpublished data, T. P. Carr, J. S. Parks, and L. L. Rudel). An important question, however, was whether the infusion of exogenous fatty acids would alter the inhibitory effect of the ACAT inhibitors on triacylglycerol secretion during liver perfusion. No differences were detected in the secretion of any of the lipids or apolipoproteins during perfusion with fatty acids in the absence or presence of CP-113818 (data not shown). It is possible that CP-113818 and PD-138142-15 may have directly inhibited triacylglycerol synthesis and secretion, and that this decrease in triacylglycerol secretion was related to the decrease in apoB secretion. In any case, these inhibitors did not appear to be as specific as CI-976. The fact that one compound could inhibit apoB and cholesteryl ester secretion without simultaneously affecting triacylglycerol secretion established the concept that apoB secretion can be separately regulated from triacylglycerol secretion. Conversely, as cholesteryl ester secretion and apoB were similarly affected in the presence of all ACAT inhibitors, the case for coordinate regulation of secretion of these two molecules is stronger, although it cannot be considered proven.

The secretion of free cholesterol and phospholipid was unaffected by any of the ACAT inhibitors. While we did not isolate and characterize the high density lipoproteins (HDL) secreted by the livers during perfusion, it is likely that much of the free cholesterol and phospholipid in perfusate would have been recovered in the HDL fraction (41, 56). In view of the decreased production of apoB-containing lipoproteins, it may be that free cholesterol and phospholipid secretion was maintained by the secretion of HDL particles. Another possibility is that the apoB-containing lipoproteins secreted in the presence of the ACAT inhibitors contained excess "surface" lipid that subsequently appeared in the HDL fractions in perfusate as a result of interaction of apoE and apoA-I with the surface lipids during recirculating perfusion.

The ACAT inhibitors exhibited variable effects on the hepatic secretion of apolipoproteins. The data of Fig. 5 show that addition of CI-976 appeared to decrease the secretion of all the apolipoproteins measured in this study. Because we had to consider the possibility that some of this decrease was due to the high concentrations of albumin achieved with the repeated additions of drug on albumin, control experiments using only albumin during the final perfusion period (without CI-976) were done. Albumin alone resulted in lower averages for secretion rates of many of the apolipoproteins, although apoA-II secretion was the only statistically significant decrease (data not shown). In all cases, the addition of CI-976 to perfusate resulted in a significantly greater inhibition of apolipoprotein secretion compared to albumin alone, suggesting that the inhibitory effect of the drug on the secretion of individual apolipoproteins was real and was not secondary to an albumin effect.

In the experiments with CP-113818, which was also complexed with albumin, the amount of albumin used was much less than with CI-976 and an equivalent dose of albumin without drug was added to the control period in each case. This ACAT inhibitor significantly increased the secretion of apoE, while apoA-I secretion remained unchanged. A similar pattern of effects on apolipoprotein secretion was seen for PD-139142-15. ApoE appeared to be increased (albeit not significantly) and apoA-I and apoA-II were not changed. The variable effects on apolipoprotein secretion make it unlikely that the increase in albumin concentration used to carry CI-976 and CP-113818 in perfusate directly inhibited apolipoprotein synthesis in general, as has been observed for apoB synthesis in HepG2 cells (57).

As with our previous report (29), no significant relationship was detected between the absolute secretion rates of cholesteryl ester and apoB. However, in the presence of ACAT inhibitors, a highly significant relationship was observed between the percent change in cholesteryl ester secretion and the percent change in apoB secretion (Fig. 6). The livers used in this study were obtained from African green monkeys fed a high level of cholesterol (0.8 mg/kcal) that results in hepatic cholesteryl ester accumulation and ACAT activities 2-fold higher than when animals are fed monkey chow (29). We have previously noted that hepatic cholesteryl ester concentration is directly correlated with hepatic cholesterol secretion (28). These observations are consistent with the possibility that, at some minimal level, newly synthesized cholesteryl ester (from ACAT) may be necessary for the secretion of apoB-containing lipoproteins. However, the bulk of cholesteryl ester secreted when liver contains excess cholesterol, as when cholesterol is fed, is not needed for apoB particle secretion (and thus the absolute rates of secretion of cholesteryl ester and apoB would not correlate). We as-

sume that the threshold requirement for newly synthesized cholesteryl ester was approached only in the presence of the ACAT inhibitors. Excess cholesteryl ester in the liver could result in the secretion of apoB-containing lipoproteins enriched in cholesteryl ester, but the amount of cholesteryl ester synthesis above a putative threshold would not further increase the number of apoB-containing lipoprotein particles, although it could increase cholesteryl ester content of the particles.

A concept that accounts for several aspects of nascent hepatic VLDL assembly and secretion was first proposed by Alexander, Hamilton, and Havel (10) and has received recent support from studies in transfected HepG2 cells (58). The hypothesis is termed the two-step hypothesis. In the first step, newly synthesized full-length apoB associates and binds to the rough ER membrane before it attains a neutral lipid core. Subsequently, the core may be assembled as a result of the activity of the microsomal triglyceride transfer protein (59). Lipidation of the core presumably releases apoB from the rough ER membrane into the lumen of the ER as small HDL- or LDL-sized particles. In the second step, a triacylglycerol-containing particle (presumably a lipid droplet surrounded by a monolayer of phospholipid), which is synthesized largely in the smooth ER, coalesces or fuses with the primary apoB-containing particle to form a nascent VLDL. Nascent lipoprotein particles are then transported to the Golgi apparatus where they are further remodeled and partitioned into secretory vesicles (15). In the context of this hypothesis, a role for ACAT-derived cholesteryl esters would appear in the first step by facilitating the association of apoB with the membrane or by participating in core lipidation. If apoB does not become associated with lipid in an appropriate way during particle assembly, the apolipoprotein may enter a degradation pathway. ACAT could function to assure appropriate assembly of apoB with a particle for secretion (33).

Taken together, the evidence from the present study suggests that ACAT inhibition primarily resulted in reduced numbers of apoB molecules destined for lipoprotein assembly and secretion. We presume this is due to a need for newly synthesized cholesteryl esters in the capture of apoB for lipoprotein particle assembly and secretion. The data further indicate that, in some cases, triacylglycerol availability was also affected. We were unable to determine whether this effect was a primary or secondary effect of ACAT inhibition.

Catharine J. Andresen, Jeffery L. Haines, Robert D. Runk, and Ramesh Shah provided excellent technical assistance. This work was supported by the National Heart, Lung, and Blood Institute grants HL-24736, HL-49373, and HL-14237, and by grants from Parke-Davis and Pfizer. TPC was supported by an Individual National Research Service Award DK-08558.

Manuscript received 29 November 1993 and in revised form 23 May 1994.

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