Hepatic ACAT Activity in African Green Monkeys Is Highly Correlated to Plasma LDL Cholesteryl Ester Enrichment and Coronary Artery Atherosclerosis

Timothy P. Carr, John S. Parks, and Lawrence L. Rudel

Previous studies and this study of African green monkeys show a strong positive correlation between plasma low density lipoprotein (LDL) size and the extent of coronary artery atherosclerosis (CAA). Increased LDL size was principally due to the accumulation of cholesteryl oleate molecules within the particle core, suggesting that many of these cholesteryl esters were of tissue origin, i.e., from the acyl-coenzyme A: cholesterol acyltransferase (ACAT) reaction instead of the lecithin: cholesterol acyltransferase (LCAT) reaction. The current study was conducted to test the hypothesis that ACAT in the liver is the source of the increased numbers of cholesteryl oleate molecules in plasma LDL particles that appear to increase the atherogenic potential of LDL. Monkeys were fed diets rich in fat (lard, safflower oil, or fish oil) and cholesterol for 3–6 years before liver perfusion, ACAT assay, and evaluation of CAA. Hepatic ACAT activity was positively correlated with hepatic cholesteryl ester secretion (r=0.61, p<0.001), plasma LDL cholesteryl ester content (r=0.60, p<0.0001), and the extent of CAA (r=0.62, p<0.0001). The number of cholesteryl oleate molecules within LDL increased proportionally with LDL size in each of the diet groups. Hepatic cholesteryl oleate concentration was correlated with the accumulation of cholesteryl oleate in liver perfusate (r=0.72, p<0.01) and with plasma LDL cholesteryl oleate content (r=0.73, p<0.0001). Our interpretation is that these data, obtained in a relevant primate model of CAA, suggest that hepatic ACAT increases the atherogenicity of LDL by augmenting both the secretion by the liver and accumulation in plasma LDL of cholesteryl oleate.

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KEY WORDS • liver • acyl-coenzyme A: cholesterol acyltransferase • cholesteryl ester • hepatic secretion • low density lipoproteins • atherosclerosis • primates

Nonhuman primates consuming atherogenic diets develop hypercholesterolemia characterized by an increased low density lipoprotein (LDL) concentration and enlargement of LDL particle size. Increased LDL particle size has been highly correlated with the extent and severity of coronary artery atherosclerosis (CAA). Increased LDL size is characterized by an increase in the number of cholesteryl ester molecules in the core of the particle, with smaller increases in the surface constituents of LDL. Since the majority of plasma LDL is believed to arise from the intravascular catabolism of hepatic apoprotein (apo) B100-containing lipoproteins, increased LDL size and cholesteryl ester concentration are the likely result of increased cholesteryl ester synthesis and secretion in apoB100 lipoproteins by the liver. Indeed, liver perfusion studies in African green monkeys fed high-fat, high-cholesterol diets have demonstrated significant positive correlations between the rate of hepatic cholesterol secretion and both plasma cholesterol concentration and LDL particle size. This relation exists even when the type of fat and amount of cholesterol in the diet are altered. These data suggest that mechanisms regulating lipoprotein synthesis and secretion by the liver play a critical role in determining the size and concentration of plasma LDL and, consequently, the development of CAA.

Acyl-coenzyme A: cholesterol acyltransferase (ACAT) catalyzes the intracellular formation of cholesteryl ester from fatty acyl-coenzyme A (CoA) and free cholesterol. In the liver, this reaction is undoubtedly responsible for the majority of cholesteryl ester secreted with apoB100-containing lipoproteins. The fatty acid specificity of ACAT has been systematically studied in rats, with oleoyl-CoA being the preferred substrate and other acyl-CoAs being somewhat less efficiently used. This contrasts with the plasma lecithin: cholesterol acyltransferase (LCAT) reaction, for which cholesteryl linoleate is the primary product. Studies of isolated hepatocytes...
Plasma Lipid and Lipoprotein Characterization

Animals were restrained with ketamine (10 mg/kg), and from 10-ml blood samples taken after an overnight fast. While much information is lacking concerning hepatic ACAT activity and lipoprotein assembly and secretion, these observations appear to implicate ACAT as a potential regulatory enzyme in the production of lipoproteins by the liver.

In this study we document a relation between hepatic ACAT activity and plasma LDL cholesteryl ester enrichment in African green monkeys consuming high-fat, cholesterol-containing diets. Three types of dietary fat (representing saturated, n-6 polyunsaturated, and n-3 polyunsaturated) were studied because each fat type is known to affect hepatic cholesterol secretion, plasma LDL size, and liver cholesteryl ester content, possibly through effects on hepatic ACAT activity. Animals consuming monkey chow were included in the study for comparison, since liver cholesteryl ester content is minimal in such animals. High correlations were found between hepatic ACAT activity, liver cholesteryl ester secretion, plasma LDL cholesteryl ester content, and CAA, suggesting that the enrichment of plasma LDL with cholesteryl olate via hepatic ACAT represents an atherogenic modification of plasma LDL.

Methods

Animals and Diets

Adult male African green monkeys of the grivet subspecies (Cercopithecus aethiops subsp aethiops) were purchased from Primate Imports (Port Washington, N.Y.). Three diet groups were established, each containing 42% of calories as fat and 0.8 mg cholesterol/ kcal. Approximately half of the fat calories were derived from lard, safflower oil, or fish oil and half from egg yolk and/or egg yolk replacement. Detailed diet compositions have been previously published. A fourth diet group was fed monkey chow for comparison. All animals were fed 90 kcal/kg body wt per day.

The animals used in this study were part of two ongoing studies of atherosclerosis, which accounts for the unequal group sizes reported herein. In one study, monkeys were fed diets containing lard and safflower oil for 5–6 years. In the second study, monkeys were fed the same lard-containing diet and a fish oil–containing diet for approximately 3 years. Since the composition of the diets differed only in the type of fat, the data from both studies have been combined. To justify this approach, data from the lard-fed group of both studies were compared by Student’s t test analysis. The only parameter that was different between studies was the extent of atherosclerosis, since the animals in one study had more time to develop the disease. Accordingly, the atherosclerosis data have been presented on a per-study basis.

Plasma Lipid and Lipoprotein Characterization

Plasma lipid levels were determined every 2 months from 10-ml blood samples taken after an overnight fast. Animals were restrained with ketamine (10 mg/kg), and blood was drawn from the femoral vein into Vacutainer tubes containing 1 mg/ml EDTA as an anticoagulant. Plasma was isolated after removal of red blood cells by low-speed centrifugation. Total plasma cholesterol and triglyceride concentrations were determined using automated enzymatic procedures with the Technicon RA-5000 (Technicon Instruments Corp., Tarrytown, N.Y.). Plasma high density lipoprotein (HDL) cholesterol concentration was determined after heparin–manganese precipitation of apoB-containing lipoproteins using the methods described in the Manual of Laboratory Operations of the Lipid Research Clinics Program.

Plasma lipoproteins were further characterized from 30-ml blood samples taken at least once from each animal during the course of the study. Plasma was isolated and adjusted to contain 0.1% EDTA, 0.1% NaNO₃, 80 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin. The density of the plasma was adjusted to 1.225 g/ml, and the lipoproteins were isolated by ultracentrifugation at 50,000 rpm for 24 hours (15°C) in a Beckman Ti 70.1 rotor. The lipoprotein classes were then separated by gel filtration chromatography, and the LDL molecular weights were determined as described previously. Plasma LDL percentage compositions were directly determined using enzymatic assays for free cholesterol (Biochemical Diagnostics, Inc., Edge-wood, N.Y.), total cholesterol, and triglyceride (Boehringer Mannheim Diagnostics, Indianapolis, Ind.). LDL phospholipid was determined by measuring inorganic phosphorus, and protein was estimated according to the method of Lowry et al with bovine serum albumin (fraction V) as the reference standard.

Lipid Perfusion

Perfusion of isolated livers was performed as described previously. The perfusion medium consisted of Krebs-Henseleit original Ringer’s bicarbonate buffer (pH 7.4) containing glucose, amino acids, insulin, hydrocortisone, penicillin, streptomycin, and washed human erythrocytes at 22% hematocrit. Recirculating perfusion was initially performed for 90 minutes to allow the release of plasma lipoproteins that remained in the liver before perfusion. The perfusate was then replaced with fresh medium and allowed to recirculate for 2 or 3 hours. Perfusate aliquots (10 ml) were taken every 20 minutes (for the 2-hour perfusion) or 30 minutes (for the 3-hour perfusion) for measurement of lipids and apoB. Red blood cells were removed from the perfusate by centrifugation, and the perfusate “plasma” was adjusted to 0.1% EDTA, 0.1% NaNO₃, 80 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin. Liver viability was monitored by color, rate of bile production, rate of oxygen consumption, and appearance of transaminases in the perfusate. Livers with data showing evidence of failure during perfusion were excluded.

Perfusate time-point aliquots were analyzed for triglyceride concentration by enzymatic assay. For the
The standard ACAT assay started with a 30-minute exogenous cholesterol. The reaction was initiated by the preincubation at 37°C containing 200 μg microsomal per assay in detergent 33 was intended to eliminate the effect of substrate availability on enzyme activity. The reaction was stopped by the addition of 6 ml chloroform/methanol (2:1, vol/vol) containing 15 μg cholesteryl oleate as a carrier. The phases were separated by the addition of 1.2 ml of 0.88% KCl, and an aliquot of the lower phase was removed. Cholesteryl esters were then isolated by TLC for determination of total 14C radioactivity by scintillation spectrometry.

Measurement of Coronary Artery Atherosclerosis

The extent of CAA was quantified in the hearts of the monkeys consuming the high-fat, cholesterol-containing diets (no CAA was present in the monkey chow group). The extent of intimal thickening and the percent lumen stenosis were determined by computer-assisted morphometric measurement of five cross sections from each of the three main branches of the coronary tree (left anterior descending, left circumflex, and right coronary artery). Intimal area (i.e., lesion area) was determined by subtracting lumen area from the area within the internal elastic lamina. Percent lumen stenosis was calculated as the intimal area divided by the area within the internal elastic lamina multiplied by 100. The data are expressed as the average value of the 15 arterial cross sections from each animal. Maximum intimal thickness was measured at the thickest portion of each cross section and represents the single largest measurement of the three coronary arteries.

Statistical Analyses

All values in the tables are presented as the mean±SEM. Statistical comparison among diet groups was determined by one-way analysis of variance (ANOVA) with Fisher’s post hoc least significant difference test using the STATVIEW software (Abacus Concepts, Inc., Berkeley, Calif.). Repeated-measures ANOVA was used to determine significant differences in perfusate accumulation of lipoprotein lipids and apoproteins using the SUPERANOVA software (Abacus Concepts, Inc.). Measurements of CAA, i.e., artery intimal area, maximum intimal thickness, and percent lumen stenosis, were logarithmically transformed before statistical analysis when distribution of the data was not normal.

Results

Characteristic aspects of the plasma lipoproteins of African green monkeys in each of the treatment groups are presented in Table 1. Total plasma cholesterol concentrations were highest in the lard-containing diet group and lowest in the group fed monkey chow. Plasma cholesterol concentrations were significantly lower in both polyunsaturated fat groups compared with the saturated fat group. HDL cholesterol concentrations were lowest in the fish oil and chow groups and were significantly higher in the safflower oil– and lard-containing diet groups. Animals consuming lard had the highest LDL cholesterol concentrations and the largest average size of LDL particles, as measured by LDL molecular weight. LDL cholesterol concentrations were significantly lower in safflower oil–fed monkeys, and although the average LDL particle size tended to be smaller, it was not significantly different compared with

Hepatic Lipids

Lipids were extracted from the liver by the method of Folch et al. 28 Hepatic phospholipid content was determined by measuring inorganic phosphorus in an aliquot of the lipid extract. 26 Triton X-100 was added to a separate aliquot of the extract, and the solvent was evaporated under nitrogen. The lipids were solubilized in water, and commercially available enzymatic assays were used for the determination of triglyceride, total cholesterol, and free cholesterol. 32 The final concentration of Triton X-100 in the enzymatic assays was 0.5%. Liver cholesteryl esters were isolated by TLC, and the fatty acid composition was determined by GLC as described above. 29

ACAT Assay

ACAT activity was measured in microsomes isolated from liver samples taken at the beginning and end of perfusion. Liver biopsies were taken from the same lobe of each liver, although we found no regional differences in ACAT activity in control livers. Triton X-100 was added to a separate aliquot of the extract, and the solvent was evaporated under nitrogen. The lipids were solubilized in water, and commercially available enzymatic assays were used for the determination of triglyceride, total cholesterol, and free cholesterol. 32 The final concentration of Triton X-100 in the enzymatic assays was 0.5%. Liver cholesteryl esters were isolated by TLC, and the fatty acid composition was determined by GLC as described above. 29

Addition of 30 nmol [14C]oleoyl-CoA (5,000–10,000 dpm/ nmol) in a final volume of 300 μl. After a 2-minute incubation period, the reaction was stopped by the addition of 6 ml chloroform/methanol (2:1, vol/vol) containing 15 μg cholesteryl oleate as a carrier. The phases were separated by the addition of 1.2 ml of 0.88% KCl, and an aliquot of the lower phase was removed. Cholesteryl esters were then isolated by TLC for determination of total 14C radioactivity by scintillation spectrometry.
that of the lard group. Monkeys consuming fish oil had significantly lower LDL cholesterol concentrations and LDL particles of significantly smaller average size compared with the lard group. ApoB concentrations were highest in the fish oil and lard groups, lower in the safflower oil group, and lowest in the group fed monkey chow.

Lipid concentration and ACAT activity in the livers of most of the study animals were determined for each diet group, and the results are shown in Table 2. Monkeys consuming dietary fat enriched with lard tended to have the highest concentrations of cholesteryl esters in the liver, although the difference failed to reach statistical significance when compared with the average cholesteryl ester concentrations of the safflower oil or fish oil groups. ACAT activities in the livers of animals fed lard and safflower oil were similar and were significantly greater than the average activities in the fish oil group or the monkey chow group.

The rates of accumulation of free cholesterol, cholesteryl ester, triglyceride, and apoB during liver perfusion were measured and expressed as milligrams per hour per 100 grams of liver (Table 3). The average accumulation rate of free cholesterol in the lard group was significantly higher compared with the monkey chow group and tended to be higher than the polyunsaturated fat groups (although not significantly so). Cholesteryl ester secretion in livers from lard-fed monkeys was significantly greater compared with the fish oil and monkey chow groups and tended to be higher compared with the safflower oil group (p=0.11). The average rate of apoB secretion by the liver was highest in the safflower oil group and lowest in the monkey chow group. Triglyceride accumulation rates appeared to be lowest in the fish oil group compared with other diet groups, and this difference is likely to be biologically important.24

As apparent in the SEMs of the aforementioned tables, individual animal differences in the various end points were large. These differences were used to help establish relations between hepatic ACAT activity and cholesteryl ester and apoB accumulations during perfusion among individual animals of all diet groups as shown in Figure 1. A statistically significant correlation (r=0.61, p<0.001, panel A) between ACAT activity and cholesteryl ester accumulation by the liver was found for all diet groups. In contrast, ACAT activity was not correlated with hepatic apoB accumulation (panel B).

Figure 2 shows a direct correlation between hepatic ACAT activity and LDL cholesteryl ester content, expressed as the number of molecules per LDL particle. A positive and statistically significant correlation (r=0.60, p<0.001) was demonstrated when all data were plotted. Significant relations were not demonstrated within the safflower oil, the fish oil, or the chow groups when analyzed separately, but the data from each group fit the overall regression line well. The presence of correlations within individual diet groups was made difficult by the limited range of values in ACAT activity. More animals fed the lard-containing diet were studied and more variability was apparent compared with the other groups. Regression analysis of ACAT activity and LDL cholesteryl ester content within the lard group (r=0.66, p<0.001) or among the animals of the remaining three groups (r=0.48, p<0.01) produced similar regression equations, suggesting that a similar relation existed.

The fatty acid composition of hepatic cholesteryl esters is presented in Table 4. Livers from monkeys fed safflower oil exhibited a significantly lower percentage of cholesteryl palmitate, stearate, and oleate and an increased percentage of cholesteryl linoleate compared with other diet groups. The lard (and chow) group had the highest percentage of cholesteryl oleate. The fish oil

### Table 1. Plasma Lipoprotein Characteristics of African Green Monkeys Fed Monkey Chow or High-Fat, High-Cholesterol Diets

<table>
<thead>
<tr>
<th>Diet group</th>
<th>n</th>
<th>Total plasma cholesterol</th>
<th>HDL cholesterol</th>
<th>LDL cholesterol</th>
<th>Plasma apoB</th>
<th>LDL molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>7</td>
<td>113±13*</td>
<td>64±7*</td>
<td>29±6*</td>
<td>27±4*</td>
<td>2.58±0.03*</td>
</tr>
<tr>
<td>Lard</td>
<td>22</td>
<td>372±28†</td>
<td>86±6†</td>
<td>233±24†</td>
<td>104±6†</td>
<td>3.28±0.09†</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>13</td>
<td>276±30†</td>
<td>89±8†</td>
<td>142±26†</td>
<td>74±7†</td>
<td>3.08±0.08†</td>
</tr>
<tr>
<td>Fish oil</td>
<td>9</td>
<td>231±37†</td>
<td>57±5*</td>
<td>166±42†</td>
<td>112±13†</td>
<td>2.68±0.10†</td>
</tr>
</tbody>
</table>

HDL, high density lipoprotein; LDL, low density lipoprotein; apo, apoprotein.

All values represent the mean±SEM and are in milligrams per deciliter except for LDL molecular weight, which is in grams per micromole. Means within the same column bearing unlike symbols are significantly different (p<0.05) as determined by one-way analysis of variance and Fisher's post hoc least significant difference test.

### Table 2. Hepatic Lipid Levels and ACAT Activity of African Green Monkeys Fed Monkey Chow or High-Fat, High-Cholesterol Diets

<table>
<thead>
<tr>
<th>Diet group</th>
<th>n</th>
<th>Phospholipid</th>
<th>Triglyceride</th>
<th>Free cholesterol</th>
<th>Cholesteryl ester</th>
<th>ACAT activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>6</td>
<td>32.4±1.2*</td>
<td>8.20±1.68†</td>
<td>2.58±0.11†</td>
<td>1.63±0.12*</td>
<td>528±124*</td>
</tr>
<tr>
<td>Lard</td>
<td>17</td>
<td>33.2±1.2*</td>
<td>8.75±1.11*</td>
<td>3.22±0.25*</td>
<td>4.70±0.82†</td>
<td>905±82†</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>12</td>
<td>35.9±0.9*</td>
<td>4.98±0.29†</td>
<td>2.81±0.10†</td>
<td>2.95±0.68†</td>
<td>1,016±86†</td>
</tr>
<tr>
<td>Fish oil</td>
<td>9</td>
<td>26.1±2.7†</td>
<td>12.45±2.59*</td>
<td>2.42±0.08†</td>
<td>2.81±0.70†</td>
<td>578±61*</td>
</tr>
</tbody>
</table>

ACAT, acyl-coenzyme A:cholesterol acyltransferase.

All values represent the mean±SEM and are in milligrams per gram wet weight except for ACAT activity, which is in picomoles per minute per milligram. Means within the same column bearing unlike symbols are significantly different (p<0.05) as determined by one-way analysis of variance and Fisher's post hoc least significant difference test.
### Table 3. Accumulation of Lipoprotein Constituents in Recirculating Liver Perfusate of African Green Monkeys Fed High-Fat, High-Cholesterol Diets

<table>
<thead>
<tr>
<th>Diet group</th>
<th>Triglyceride</th>
<th>Free cholesterol</th>
<th>Cholesteryl ester</th>
<th>Apoprotein B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>6</td>
<td>1.18±0.24†</td>
<td>6</td>
<td>0.90±0.27†</td>
</tr>
<tr>
<td>Lard</td>
<td>16</td>
<td>2.32±0.44‡</td>
<td>9</td>
<td>1.37±0.14‡</td>
</tr>
<tr>
<td>Safflower oil</td>
<td>11</td>
<td>1.81±0.13‡</td>
<td>11</td>
<td>1.79±0.43‡</td>
</tr>
<tr>
<td>Fish oil</td>
<td>6</td>
<td>1.64±0.29‡†</td>
<td>5</td>
<td>1.08±0.16‡†</td>
</tr>
</tbody>
</table>

*Liver perfusion was performed for 2 hours, and aliquots of perfusate were removed every 20 minutes for determination of lipoprotein constituents. Livers of fish oil-fed and some lard-fed animals were perfused for 3 hours, and aliquots of perfusate were removed every 30 minutes for analysis. Accumulation rate was calculated from the slope of linear regression analysis for lipoprotein lipids and apoprotein B. Values represent the mean±SEM and are in milligrams per hour per 100 grams of liver. Means within the same column bearing unlike symbols are significantly different (p<0.05) as determined by repeated-measures analysis of variance and Fisher’s post hoc least significant difference test.

Group exhibited the highest percentage of hepatic cholesteryl palmitate and cholesteryl esters containing n-3 polyunsaturated fatty acids; the other diet groups had no detectable n-3 polyunsaturated cholesteryl esters.

To determine the contribution of each molecular species of cholesteryl ester to LDL particle enlargement (Figure 3), cholesteryl ester compositions were measured in the LDL of individual animals. The content of cholesteryl palmitate in LDL was relatively constant among individuals in all diet groups (200–500 molecules per particle) and was not proportional to particle size. The content of cholesteryl oleate was positively correlated to LDL particle size for all diet groups, and the data for each diet group fit the same regression line; no diet-related difference was found by analysis of covariance. The magnitude and extent of the increase in cholesteryl ester content in relation to LDL particle size were greatest for cholesteryl oleate, suggesting that this cholesteryl ester was of primary importance in determining LDL size. The content of cholesteryl linoleate was diet specific; LDL from safflower oil-fed monkeys contained the highest amount of cholesteryl linoleate (1,000–1,300 molecules per particle), and the amount per particle increased with particle size. The lard group LDL contained about 700 molecules of cholesteryl linoleate per LDL particle, and the fish oil group LDL contained about 300 molecules per particle, but in neither case did the content of cholesteryl linoleate increase with particle size.

Because of the direct relation between LDL particle size and cholesteryl oleate content shown in Figure 3,

**Figure 1.** Scatterplots showing the relation between hepatic ACAT activity and liver perfusate accumulation of cholesteryl ester (panel A) and apoprotein (apo) B100 (panel B). The correlation coefficient in panel A was r=0.61 (p<0.001), whereas hepatic ACAT activity was not correlated with perfusate apoB accumulation. ACAT activity and perfusate accumulation of cholesteryl ester and apoB were determined as described in the “Methods” section. ACAT, acyl-coenzyme A:cholesterol acyltransferase.

**Figure 2.** Scatterplot showing the relation between hepatic ACAT activity and plasma LDL cholesteryl ester content. The correlation coefficient was r=0.60 (p<0.001). ACAT activity was determined as described in the “Methods” section. Plasma LDL cholesteryl ester content was calculated based on LDL percentage composition and LDL molecular weight. ACAT, acyl-coenzyme A:cholesterol acyltransferase; LDL, low density lipoprotein.
TABLE 4. Hepatic Cholesterol Ester Fatty Acid Percentage Composition

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Chow (n=7)</th>
<th>Lard (n=18)</th>
<th>Safflower oil (n=12)</th>
<th>Fish oil (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>1.3±0.2*</td>
<td>0.8±0.04†</td>
<td>0.8±0.1†</td>
<td>2.0±0.1‡</td>
</tr>
<tr>
<td>16:0</td>
<td>8.4±0.5*</td>
<td>8.4±0.4*</td>
<td>6.2±0.4†</td>
<td>11.1±0.8§</td>
</tr>
<tr>
<td>16:1</td>
<td>1.1±0.1*</td>
<td>1.5±0.2*</td>
<td>0.3±0.1†</td>
<td>3.5±0.4‡</td>
</tr>
<tr>
<td>18:0</td>
<td>7.3±0.4*</td>
<td>7.7±0.3*</td>
<td>4.5±0.3†</td>
<td>7.0±0.5*</td>
</tr>
<tr>
<td>18:1</td>
<td>57.6±2.6*</td>
<td>57.8±0.9*</td>
<td>39.7±1.0†</td>
<td>46.0±2.2‡</td>
</tr>
<tr>
<td>18:2</td>
<td>10.6±0.5*</td>
<td>7.8±0.4†</td>
<td>26.0±0.7‡</td>
<td>7.2±0.4†</td>
</tr>
<tr>
<td>20:3</td>
<td>4.3±0.5*</td>
<td>3.0±0.3†</td>
<td>5.7±0.3‡</td>
<td>1.9±0.1§</td>
</tr>
<tr>
<td>20:4</td>
<td>2.1±0.3*†</td>
<td>2.5±0.3*</td>
<td>3.0±0.3*</td>
<td>1.2±0.2†</td>
</tr>
<tr>
<td>22:4</td>
<td>1.8±0.1*†</td>
<td>2.2±0.3*</td>
<td>2.7±0.3*</td>
<td>0.8±0.2†</td>
</tr>
<tr>
<td>20:4 (n-3)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1.5±0.3</td>
</tr>
<tr>
<td>20:5 (n-3)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2.4±0.9</td>
</tr>
<tr>
<td>22:5 (n-3)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>5.4±1.3</td>
</tr>
<tr>
<td>22:6 (n-3)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1.4±0.6</td>
</tr>
<tr>
<td>Others</td>
<td>8.4±1.5</td>
<td>8.3±0.8</td>
<td>11.0±0.7</td>
<td>9.6±0.5</td>
</tr>
</tbody>
</table>

ND, not detected.

Values represent the mean±SEM. Means within the same row bearing unlike symbols are significantly different (p<0.05) as determined by one-way analysis of variance and Fisher's post hoc least significant difference test.

correlation statistics were used to examine the relations between LDL cholesteryl oleate content and liver cholesteryl oleate accumulation and secretion. The size-related increase in LDL cholesteryl oleate content was correlated to hepatic cholesteryl oleate concentration (r=0.73, p<0.0001) and to perfusate cholesteryl oleate accumulation rate (r=0.67, p<0.01). The correlation coefficient for cholesteryl oleate concentration in liver and perfusate cholesteryl oleate accumulation was r=0.72 (p<0.01). Finally, the extent of CAA was quantified morphometrically, and the results are presented in Table 5. Because the animals in this report were part of two ongoing studies of atherosclerosis in which monkeys in Study I were fed atherogenic diets twice as long as monkeys in Study II, the data have been presented according to study. The animals fed the lard-enriched diet in either study had the most atherosclerosis, and this was true when intimal area or percent stenosis was examined. Lard-fed monkeys in Study I had more atherosclerosis than lard-fed monkeys of Study II. The average amount of CAA in both the fish oil and safflower oil groups was low compared with their lard-fed counterparts. The major correlates of CAA are presented in Figure 4. The CAA data have been logarithmically transformed to fit a normal distribution. Nearly identical results were obtained when CAA was expressed as intimal area or percent stenosis; therefore, only data pertaining to intimal area are presented. Data from each study of atherosclerosis are identified separately in Figure 4. Plasma LDL cholesteryl ester content (Figure 4A), LDL cholesterol concentration (Figure 4B), and hepatic ACAT activity (Figure 4C) were all positively correlated with coronary artery intimal area. Although LDL cholesteryl ester content and LDL cholesterol concentration were correlated with CAA, multiple regression analysis demonstrated that hepatic ACAT activity was the only independent variable contributing significantly to the variance of CAA. These data imply that the relations between LDL cholesteryl...
TABLE 5. Effects of Dietary Fat on Coronary Artery Atherosclerosis in African Green Monkeys

<table>
<thead>
<tr>
<th>Study</th>
<th>Fat</th>
<th>n</th>
<th>Intimal area (mm²)</th>
<th>Maximum intimal thickness (mm)</th>
<th>Lumen stenosis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lard</td>
<td>14</td>
<td>0.432±0.147</td>
<td>0.320±0.072</td>
<td>19.1±5.8</td>
</tr>
<tr>
<td></td>
<td>Safflower oil</td>
<td>14</td>
<td>0.112±0.060</td>
<td>0.194±0.063</td>
<td>6.8±3.4</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td></td>
<td>0.054†</td>
<td>0.20†</td>
<td>0.078†</td>
</tr>
<tr>
<td></td>
<td>Fish oil</td>
<td>12</td>
<td>0.034±0.009</td>
<td>0.103±0.019</td>
<td>3.7±0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>0.010±0.002</td>
<td>0.059±0.006</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td></td>
<td>0.026†</td>
<td>0.077†</td>
<td>0.010‡</td>
</tr>
</tbody>
</table>

*Monkeys in this report were part of two ongoing studies of atherosclerosis. Animals in Study I were fed their high-fat diets for 5–6 years. Animals in Study II (see Reference 35) were fed their diets for approximately 3 years. Values represent the mean±SEM.

†Student’s t test was performed after logarithmic transformation of the data because of nonnormal distribution.

‡Student’s t test.

Ester content, LDL cholesterol concentration, and CAA may reflect the contribution of hepatic ACAT activity to LDL cholesteryl ester enrichment.

Discussion

The data of this manuscript directly implicate hepatic ACAT in promoting atherogenesis in nonhuman primates by modifying the cholesteryl ester content and composition of plasma LDL. The observation was facilitated by studying individual animal differences among groups of African green monkeys fed diets containing cholesterol and three different dietary fats. Associations were found between liver cholesteryl oleate synthetic activity via ACAT, cholesteryl oleate accumulation in liver, and cholesteryl oleate secretion measured as accumulation during liver perfusion. Furthermore, liver ACAT activity was correlated with plasma LDL particle size as determined by cholesteryl ester accumulation in the core of the LDL particle. Larger LDL were enriched primarily with cholesteryl oleate, the primary product of the reaction catalyzed by ACAT. The enrichment of LDL particles with cholesteryl oleate leads to an increased phase-transition temperature of the LDL core. This property and/or other properties of modified, cholesteryl oleate–enriched LDL particles appear to promote CAA, as evidenced by the high correlation with coronary artery intimal area (Figure 4).

Figure 5 shows a schematic diagram depicting the hypothesis we have developed based on the data. In monkeys fed diets enriched in cholesterol and saturated or n-6 polyunsaturated fat, an increased amount of cholesterol is available from intestinal absorption for esterification in the liver and, in an as-yet-undefined way, signals for increased activity of ACAT. The increased synthesis of cholesteryl esters (primarily cholesteryl oleate) by ACAT results in increased secretion into plasma of cholesteryl esters in apoB100-containing lipoproteins by the liver. Through the processes of intravascular metabolism, these precursor lipoproteins become LDLs that are enriched in ACAT-derived cholesteryl esters. Cholesteryl ester–enriched LDL accumulate in plasma, presumably in part because hepatic LDL receptors are downregulated by the increased influx of cholesterol into the liver, and result in accumulation of cholesteryl ester in the wall of the coronary artery. The increased deposition of cholesteryl ester in the artery is most likely due to an increased propensity for deposition of the cholesteryl oleate–enriched LDL. We presume that modification of the cholesteryl ester composition of LDL must affect the ability of the

![Figure 4](http://atvb.ahajournals.org/)
We have noted that the number of cholesteryl oleate molecules in plasma LDL increased proportionally to particle size in all of the diet groups across the entire range of LDL molecular weights observed in this study (Figure 3). Several observations suggest that the cholesteryl oleate molecules that accumulate in plasma LDL are largely derived from hepatic ACAT. First, cholesteryl oleate was shown to be the major product of ACAT activity in rat liver.\textsuperscript{10,11} Although ACAT is able to use other fatty acyl substrates, oleoyl-CoA is clearly the preferred substrate. Second, we found that cholesteryl oleate was the most abundant cholesteryl ester in the monkey liver (Table 4), which reflects the preference of oleoyl-CoA as a substrate. Third, the distribution of cholesteryl ester species in the liver closely parallels the distribution of cholesteryl esters in VLDL secreted by the liver.\textsuperscript{8,9} Plasma LDL are derived primarily from the intravascular catabolism of VLDL,\textsuperscript{2} suggesting that VLDL cholesteryl esters become the cholesteryl esters of LDL. Fourth, ACAT activity was directly correlated with cholesteryl ester mass secretion by the liver (Figure 1). Finally, hepatic ACAT activity was also directly correlated with plasma LDL cholesteryl oleate content ($r=0.47$, $p<0.001$; data not shown) when data from all diet groups were combined.

It might be argued that LDL cholesteryl ester enrichment was also due to increased activity of LCAT in the plasma. Cholesteryl linoleate, the major product of LCAT,\textsuperscript{12,13} was the most abundant LDL cholesteryl ester in normal-sized LDL of both the lard and safflower oil diet groups and increased proportionally to particle size in the safflower oil group (Figure 3). However, we have recently found that in plasma LDL, the proportion of cholesteryl linoleate molecules derived from LCAT was significantly less in monkeys fed safflower oil compared with lard, fish oil, or oleate-rich safflower oil (J.T. Thornburg and L.L. Rudel, unpublished observations). We would argue that the size-related increase in LDL cholesteryl linoleate content observed in the safflower oil group is likely due to an increased hepatic synthesis and secretion of cholesteryl linoleate by hepatic ACAT. From the data in Tables 3 and 4, one can calculate that the amount of hepatic cholesteryl linoleate was greatest in the safflower oil group, which presumably reflects the ability of ACAT to use linoleoyl-CoA as a substrate when the intracellular pools of linoleoyl-CoA are increased relative to those of oleoyl-CoA. Furthermore, we have shown that the livers from animals fed high-fat diets secrete cholesteryl esters with a composition similar to the hepatic cho-

**Figure 5.** Schematic summary of cholesteryl ester (CE) metabolism in African green monkeys fed diets rich in fat and cholesterol. This diagram integrates data obtained from measurements of liver CE metabolism and secretion, plasma CE metabolism, and the extent and severity of coronary artery atherosclerosis. An increased amount of dietary cholesterol is available from intestinal absorption for esterification in the liver, increasing the activity of acyl-coenzyme A: cholesterol acyltransferase (ACAT). Increased CE synthesis results in increased CE secretion into plasma via hepatic apoprotein (apo) B100-containing lipoproteins (apoB-Lp). ACAT activity and CE secretion are lower in monkeys fed fish oil compared with animals fed lard or safflower oil. The apoB-Lp secreted by the liver are metabolized intravascularly, resulting in low density lipoproteins (LDL) that are enriched in ACAT-derived CE. Safflower oil-fed monkeys exhibit fewer plasma LDL particles, assuming one molecule of apoB per particle (represented by the letter B). Monkeys fed fish oil exhibit LDL particles of smaller size. LDL from lard-fed animals are specifically enriched in cholesteryl oleate (dark-shaded areas), whereas LDL from animals fed safflower oil are enriched in cholesteryl linoleate (light-shaded areas). An accumulation of cholesteryl oleate–enriched LDL in plasma facilitates deposition of CE in the coronary artery wall.
cholesteryl esters and that the livers from monkeys fed safflower oil secrete cholesteryl esters relatively enriched in cholesteryl linoleate. This observation was confirmed among the diet groups of the present study. Compared with animals consuming the lard diet, which is relatively rich in oleate (about 44% of total fatty acids), the safflower oil group showed similar mean rates of hepatic cholesteryl ester secretion (Table 3), similar ACAT activities (Table 2), and similar LDL molecular weights (Table 1), even though the cholesteryl oleate to linoleate ratio in LDL was much lower in the safflower oil group. Taken together, the data provide evidence that plasma LDL enlargement in monkeys consuming diets rich in fat and cholesterol may be attributed primarily to the accumulation of cholesteryl esters derived from hepatic ACAT more than from LCAT and that the accumulation of cholesteryl linoleate and cholesteryl oleate in the safflower oil group actually results from hepatic secretion of ACAT-derived esters.

Hepatic ACAT activity was not correlated with apoB secretion by the liver (measured as accumulation during perfusion) but was correlated with cholesteryl ester secretion (Figure 1). Assuming that apoB-containing particles secreted by the liver contain only one apoB molecule per particle, these results indicate the lack of a relation between ACAT activity and the number of secreted particles but do suggest that ACAT determines the amount of cholesteryl ester that becomes associated with apoB before particle secretion. In studies with cultured rat hepatocytes, Drevon et al demonstrated that stimulation of ACAT with 25-hydroxycholesterol or mevalonolactone increased the amount of cholesteryl ester secreted with VLDL, although apoB was not measured. Erickson and Fielding reported that in the human hepatoma cell line HepG2, stimulation with mevalonolactone increased ACAT activity and cholesteryl ester secretion but did not affect the formation or secretion of apoB. Alternatively, Sniderman and coworkers (Cianflone et al) recently reported that in HepG2 cells, stimulation of intracellular cholesteryl ester formation with oleate or the inhibition of ACAT with a variety of compounds, including Sandoz 58-035, a specific ACAT inhibitor, produced parallel changes in apoB secretion. We have found that the addition of the Parke-Davis ACAT inhibitor CI-976 to recirculating liver perfusion medium decreased the secretion of both cholesteryl ester and apoB. One explanation for the apparent discrepancy among these findings may be the degree to which ACAT contributes to hepatic lipoprotein secretion, i.e., a minimal ACAT activity may be necessary for apoB secretion, but excess cholesteryl availability, as indicated by cholesteryl ester accumulation in the liver, would result in an increased amount of cholesteryl ester synthesized by ACAT and incorporated into secreted apoB-containing lipoprotein particles. In this scenario, partial inhibition of ACAT during recirculating liver perfusion and in HepG2 cells was apparently adequate to inhibit enough ACAT activity to decrease it below the minimum requirement for apoB secretion.

Finally, it is important to discuss how these findings in primates may be related to the situation in humans, in whom a correlation between LDL of smaller size and premature atherosclerosis has been described. We have hypothesized that the reason human beings do not accumulate enlarged LDL in the circulation, as do their nonhuman primate counterparts, is because they maintain higher plasma triglyceride concentrations, which facilitate triglyceride for cholesteryl ester exchange into LDL. After clearance of the transferred triglyceride, LDL particle size is reduced. If this hypothesis is correct, then secretion of cholesteryl oleate–enriched apoB100-containing lipoproteins by the human liver may occur analogously to that in nonhuman primates, and the predisposition of these particles to promote atherosclerosis may well be similar to that in nonhuman primates. Recent evidence in cholesteryl ester transfer protein–deficient patients strongly suggests that cholesteryl esters in VLDL and LDL are largely derived from the liver. Therefore, it may be important to consider the role of hepatic ACAT activity in humans when analyzing for potential antiatherogenic effects of ACAT inhibitors. While this effect may be apparent from analysis of plasma LDL cholesterol concentrations, analysis of LDL cholesteryl ester compositions would also be considered important.

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Hepatic ACAT activity in African green monkeys is highly correlated to plasma LDL cholesteryl ester enrichment and coronary artery atherosclerosis.

T P Carr, J S Parks and L L Rudel

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