Monounsaturated fatty acyl-coenzyme A is predictive of atherosclerosis in human apoB-100 transgenic, LDLr$^{-/-}$ mice

Thomas A. Bell, III, Martha D. Wilson, Kathryn Kelley, Janet K. Sawyer, and Lawrence L. Rudel[1]

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

Abstract **ACAT2, the enzyme responsible for the formation of cholesteryl esters incorporated into apolipoprotein B-containing lipoproteins by the small intestine and liver, forms predominantly cholesteryl oleate from acyl-CoA and free cholesterol. The accumulation of cholesteryl oleate in plasma lipoproteins has been found to be predictive of atherosclerosis. Accordingly, a method was developed in which fatty acyl-CoA subspecies could be extracted from mouse liver and quantified. Analyses were performed on liver tissue from mice fed one of four diets enriched with one particular type of dietary fatty acid: saturated, monounsaturated, n-3 polyunsaturated, or n-6 polyunsaturated. We found that the hepatic fatty acyl-CoA pools reflected the fatty acid composition of the diet fed. The highest percentage of fatty acyl-CoAs across all diet groups was in monoacyl-CoAs, and values were 36% and 46% for the n-3 and n-6 polyunsaturated diet groups and 55% and 62% in the saturated and monounsaturated diet groups, respectively. The percentage of hepatic acyl-CoA as oleoyl-CoA was also highly correlated to liver cholesteryl ester, plasma cholesterol, LDL molecular weight, and atherosclerosis extent. These data suggest that replacing monounsaturated with polyunsaturated fat can benefit coronary heart disease by reducing the availability of oleoyl-CoA in the substrate pool of hepatic ACAT2, thereby reducing cholesteryl oleate secretion and accumulation in plasma lipoproteins.**—Bell, T. A., III, M. D. Wilson, K. Kelley, J. K. Sawyer, and L. L. Rudel. **Monounsaturated fatty acyl-coenzyme A is predictive of atherosclerosis in human apoB-100 transgenic, LDLr$^{-/-}$ mice.** ***J. Lipid Res.*** **2007.** 48: **1122–1131.**

Supplementary key words acyl-coenzyme A:cholesterol acyltransferase 2 • stearoyl-coenzyme A desaturase 1 • saturated fat • polyunsaturated fat • dietary fat • cholesterol • cholesteryl ester • low density lipoprotein receptor-deficient • apolipoprotein B-100

Today, coronary heart disease (CHD) remains a leading cause of death of men and women in the United States, despite advances in the treatment and management of the disease (1). The seminal studies of heart disease in Framingham, Massachusetts, firmly established the relationship between cholesterol circulating on LDLs and HDLs and overall CHD risk (2). The consumption of a diet high in cholesterol has long been associated with an increase in LDL cholesterol (3), thus increasing the chance of a coronary event. The relationships between the different types of dietary fat and CHD have been less clear. Earlier studies indicated that a diet high in saturated fat increased plasma cholesterol, a polyunsaturated fat diet appeared to decrease total plasma cholesterol (TPC), and a monounsaturated fat-enriched diet appeared to be neutral with regard to the effects on plasma cholesterol (4). A study was conducted in humans by Mattson and Grundy (5) to examine the effects these different types of dietary fats had on lipoprotein cholesterol distribution. Their study found that a diet with saturated fat increased TPC, primarily through the increase of LDL cholesterol levels. Lower TPC concentrations attributable to a reduction in both LDL and HDL cholesterol occurred with polyunsaturated fat in the diet. The diet enriched with monounsaturated fat also decreased LDL cholesterol, but without any HDL cholesterol lowering, leading the authors to conclude that replacement of saturated fat with monounsaturated fat in the diet would lead to more favorable effects on lipoprotein cholesterol distribution and CHD risk than replacement with polyunsaturated fat.

A similar diet study was conducted in nonhuman primates to directly assess the effects of the different dietary fatty acids on atherosclerosis (6). The effects of the different fatty acid-enriched diets on TPC and lipoprotein cholesterol distribution in nonhuman primates were remarkably similar to the findings in Mattson and Grundy's study (5). However, when coronary artery atherosclerosis was quantified, monkeys fed monounsaturated fat had developed as much atherosclerosis as those fed saturated fat and more than in the monkeys fed polyunsaturated fat.

Manuscript received 11 December 2006 and in revised form 18 January 2007.

Published, JLR Papers in Press, February 4, 2007.
DOI 10.1194/jlr.M600526-JLR200

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

The monkeys fed monounsaturated fat were found to have enlarged, cholesteryl oleate-enriched LDLs compared with monkeys fed polyunsaturated fat, which may account for the discrepancy between HDL and LDL cholesterol concentrations and atherosclerosis. The relationship between cholesteryl ester (CE) secretion from the liver, LDL size, and the cholesteryl oleate content of LDLs was significantly correlated to the atherosclerosis measurements made in these animals (7).

The accumulation of cholesteryl oleate in plasma is attributed to the activity of the enzyme ACAT2. ACAT2 is the second of two isoforms of ACAT that are responsible for the production in tissues of CE from free cholesterol (FC) and fatty acyl-CoA (8–11). The CE formed in the hepatocyte can then be available for incorporation into the neutral lipid core of nascent lipoproteins secreted from the liver.

A study similar to the one conducted in nonhuman primates was also conducted in mice (12). In that study, human apolipoprotein B-100 (apoB-100) transgenic, low density lipoprotein receptor-deficient ($LDLr^{-/-}$) mice were fed one of five diets containing fats enriched in ω-3 polyunsaturated, ω-6 polyunsaturated, saturated, *cis*-monounsaturated, or *trans*-monounsaturated fatty acids. A chow-based diet was also used as a control. The results of this study paralleled the findings of the monkey studies (6), in that the monounsaturated fat diet promoted as much atherosclerosis as the saturated fat diet. By comparison, mice fed both types of polyunsaturated fat were protected against atherosclerosis. The increase in atherosclerosis was accompanied by an increase in monounsaturated CE in circulating apoB-containing lipoproteins, further implicating ACAT2 as playing a central role in diet-induced atherosclerosis.

One of the potential methods of regulating CE secretion in hepatocytes is by the availability of the substrate for ACAT2, fatty acyl-CoA. However, little is known about how dietary fat type alters tissue acyl-CoA composition and subsequent ACAT2 activity, so we developed a reproducible method for the quantitation of mouse liver fatty acyl-CoA concentrations using high-performance liquid chromatography. We hypothesized that alterations of dietary fatty acids would shift the hepatic fatty acyl-CoA composition toward the composition of the diet, so that mice fed diets enriched in saturated and monounsaturated fat would have more monoacyl-CoA available for esterification by ACAT2. Such changes are anticipated to promote the development of atherosclerosis.

MATERIALS AND METHODS

Mice and diets

The mice used in these studies were human apoB-100 transgenic, $LDLr^{-/-}$ animals provided by Dr. Helen Hobbs at the University of Texas Southwestern Medical Center in Dallas, and these mice have been described in a previous publication (13). In brief, this mouse strain is a hybrid cross between the $LDLr^{-/-}$ mouse described originally by Ishibashi et al. (14), which is a hybrid of the 129sv and C57BL/6 strains, and the human apoB-100 transgenic mouse (15), which is a hybrid of the SJL and C57BL/6B strains. Breeding indicated that the $LDLr^{-/-}$ and apoB overexpression traits were homozygous, and the mice exhibited a massive increase in apoB-100-containing LDL. At 8–9 weeks of age, mice were assigned to one of four diet groups consisting of 25 to 38 mice per group with at least 10 members of each gender in each group. All animals were housed in our American Association for the Accreditation of Laboratory Animal Care-approved animal facility, and their care was supervised by a veterinarian. All animal procedures were preapproved by the institutional animal care and use committee.

The diets have been described in detail in a previous publication (12). Briefly, the diets consisted of 10% of calories from fat and 0.02% cholesterol by weight. The diets were enriched in saturated, *cis*-monounsaturated, or ω-6 or ω-3 polyunsaturated fatty acids. After 16 weeks on the diet, the animals were anesthetized with ketamine and xylazine. A terminal blood sample was taken in EDTA by heart puncture, and the liver and aorta were removed. The blood samples were promptly spun at 12,500 rpm in a refrigerated ultracentrifuge for 15 min to separate the plasma from the packed red blood cells. The plasma was stored at −20°C in a 10% sucrose solution. After the liver samples were removed, they were snap-frozen in liquid nitrogen and stored at −80°C. The aortas were stored in 10% neutral buffered formalin.

Long-chain fatty acyl-CoA standards

The following standards were purchased from Sigma (St. Louis, MO): arachidonoyl-CoA (20:4 CoA), heptadecanoyl-CoA (17:0 CoA), linoleoyl-CoA (18:2 CoA), myristoyl-CoA (14:0 CoA), oleoyl-CoA (18:1 CoA), palmitoleoyl-CoA (16:1 CoA), palmitoyl-CoA (16:0 CoA), and stearoyl-CoA (18:0 CoA). The docosahexaenoyl-CoA (22:6 CoA) standard was purchased from Avanti Polar Lipids (Alabaster, AL). The standards were solubilized and stored at −20°C in 60% isopropanol and 40% 25 mM potassium phosphate buffer (pH 4.9); combining equimolar amounts of each standard created a standard mix. Aliquots of this standard mix were used to generate a standard curve consisting of 25, 12.5, 6.25, 3.125, and 1.56 nmol of fatty acyl-CoA. The standards were run on our HPLC system according to the protocol described below. The heptadecanoyl-CoA was used as an internal standard to measure the percentage recovery of all fatty acyl-CoAs.

Fatty acyl-CoA extraction from mouse liver

Many of the published methods for the extraction of fatty acyl-CoA from tissue involve the use of solid-phase extraction cartridges (16–18), an approach that we found resulted in a low and highly variable recovery of fatty acyl-CoA. Our method involves a series of extractions to remove neutral lipid, which upon completion results in a sample consisting primarily of fatty acyl-CoA and residual phospholipid. In our hands, this method provided a consistent recovery of internal standard and chromatograms that were comparable within the diet groups.

The fatty acyl-CoA extraction protocol occurred as follows: 1.25 ml of 50 mM potassium phosphate (pH 7.2), 1.25 ml of isopropanol, and 50 μl of glacial acetic acid were added to a 30 ml screw-top tube and vortexed. Then, 500 mg of frozen liver and 6.25 nmol of the internal standard (17:0 CoA) was added to the tube, and the contents were homogenized with a Polytron until the tissue was homogeneously pulverized. Next, 2.5 ml of petroleum ether was added, and the solution was rehomogenized. The tube was then placed in a TH-4 swinging-bucket rotor of a Beckman model TJ-6 centrifuge and spun at 1,500 *g* for 15 min. The upper petroleum ether phase containing neutral lipid was removed and discarded, and the petroleum ether ex-

traction was repeated. Subsequently, 100 μl of saturated ammonium sulfate was added, and the tube contents were rehomogenized. Next, 2.5 ml of methanol-chloroform (2:1) was added, and the solution was vortexed and allowed to sit at room temperature for 30 min. The tube was subsequently spun for 30 min at 1,500 *g*, and the aqueous supernatant was separated from the pellet and saved in a separate screw-top glass tube. Then, 2.5 ml of methanol-chloroform-water (5:2.5:1) was added to the 30 ml screw-top tube containing the pellet, and the contents were vortexed. The tube was allowed to sit at room temperature for 30 min and was spun again for 30 min at 1,500 *g*. The supernatant was once again recovered and added to the rest of the saved supernatant. The pellet in the 30 ml screw-top tube was discarded. Afterward, 2.5 ml of water was added to the tube containing the pooled supernatant to promote phase separation, and the tube was then rigorously vortexed and spun for 20 min at 1,500 *g*. The supernatant consisting primarily of methanol was recovered and dried down in a rotary evaporator at 33°C until all of the liquid had been removed. The sample was then collected for HPLC analysis by resuspension in 500 μl of 10 mM potassium phosphate buffer containing 1 mM β-mercaptoethanol (pH 5.3). This procedure consistently resulted in the recovery of >65% of added [^{14}C]oleoyl-CoA.

High-performance liquid chromatography procedures

The Beckman Coulter System Gold HPLC setup used for these analyses consisted of a 508 Autosampler, a 126 Solvent Module, and a 168 Detector. The equipment was controlled and results were analyzed by System Gold's 32 Karat software. Volumes of 50 μl of sample or standard in phosphate buffer were injected onto a Waters Symmetry reverse-phase C_{18} 4.6 × 250 mm column. The gradient in which the samples and standards were run at a constant flow rate of 1 ml/min consisted of two components: 70% 25 mM potassium phosphate buffer and 30% acetonitrile (pH 4.9) (A) and 100% acetonitrile (B). At time zero, when the sample was injected, the gradient was 100% A; after 5 min, the gradient was changed to 15% B over 5 min. Then, the gradient was gradually changed to 45% B over 35 min, held at 45% B for 5 min, and changed again to 100% B over 2 min. The gradient was held at 100% B for 15 min and was returned to 100% A over 5 min; it was held there for an additional 15 min before the run was concluded. The fatty acyl-CoA elution times from the column varied according to the chain length, and the degree of unsaturation of the acyl carbon chain and mass were monitored by the absorbance of the CoA group measured at 254 nm. The total amount of each type of fatty acyl-CoA subtype for each sample tested was determined by comparing the peak area of the sample with that of the standard curve. Representative chromatograms of the standard curve (**Fig. 1A**), an ω-3 polyunsaturated fat-fed mouse (Fig. 1B), and a monounsaturated fat-fed mouse (Fig. 1C) are shown in Fig. 1.

Standards for eicosapentaenoyl-CoA (20:5 CoA) are currently unavailable. The location and concentration of eicosapentaenoyl-CoA in the chromatograms of ω-3 polyunsaturated fat-fed mice was identified by the following methods. The elution time of eicosapentaenoyl-CoA was crudely extrapolated by comparing its structure with the structures and elution times of the other polyunsaturated CoAs (18:2, 20:4, and 22:6 CoAs). The eicosapentaenoyl-CoA was predicted to coelute with myristoyl-CoA at ~31 min into the gradient run. The peaks in this region were collected and injected onto another reverse-phase C_{18} column on a Hewlett-Packard 1100 HPLC system connected to a Micromass Quattro II mass spectrometer. The samples were washed with deionized water to remove the phosphate buffer and then eluted from the column with 100% acetonitrile. The ratio of eicosapentaenoyl-CoA to myristoyl-CoA was determined by scanning negative ion mass spectrometry of mass/charge ratios from −400 to −600 *m/z*. Mice fed diets other than the ω-3 polyunsaturated fat diet had only trace amounts of eicosapentaenoyl-CoA.

Plasma lipid and lipoprotein measurements

TPC, FC, and triglyceride (TG) concentrations were measured using enzymatic assays as described previously (19, 20). The lipoprotein subclasses were isolated from fresh aliquots of plasma from individual mice according to methods described previously (21). Whole plasma was injected onto a Superose 6 chromatography column, which was subsequently run at 0.5 ml/min with 0.9% NaCl containing 0.05% EDTA (pH 7.4) and 0.05% NaN_3. The average LDL particle size, estimated as LDL molecular weight in g/μmol, was measured from a standard curve constructed from elution times of LDL of premeasured size plotted against particle size expressed in g/μmol (21). Fractions containing VLDL, LDL, and HDL were collected and pooled according to elution times. Aliquots of isolated lipoproteins were then used for enzymatic measurement of TPC, FC, and TG.

Liver and aorta lipid measurements

Liver CE concentration was estimated after performing chloroform-methanol extraction on ~100 mg of liver tissue according to the Bligh and Dyer method (22). An aliquot of liver lipid extract was then solubilized in 1% Triton X-100 solution, and total cholesterol and FC contents were determined by enzymatic assays as described previously (20). The amount of esterified cholesterol was found by subtracting FC from total cholesterol, and the difference was then multiplied by 1.67 to convert it to CE mass. The extent of aortic atherosclerosis was measured by quantifying the accumulation of CE in the entire aorta, including the entire vessel from the heart to the iliac bifurcation, by methods that have already been published (12). Briefly, the formalin-preserved aortas were cleaned by removing all adherent adipose and connective tissues. The lipids were extracted in 2:1 chloroform-methanol with 5α-cholestane added as an internal standard, and FC and total cholesterol (after saponification) were measured by gas-liquid chromatography. Some of the characteristics of atherosclerosis in this model have been described (13); lesions include both intracellular and extracellular lipid, including a significant amount of FC (12) that presumably accumulates after lysosomal CE hydrolysis. Aortic atherosclerosis as quantified by CE concentration (12) represents a comparator of lesion extent that can be used for individual animals as well as diet groups; thus, this was the end point chosen for this study.

Quantification of mRNA levels

Real-time PCR analyses of liver tissue were performed according to methods described previously (23). Primers used for these measurements were as follows: ACS1 forward, 5′-GCTGCCGACGGGATCAG-3′; ACS1 reverse, 5′-TCCAGACACATTGAGCATGTCAT-3′; ACC forward, 5′-TGACAGACTGATCGCAGAGAAAG-3′; ACC reverse, 5′-TGGAGAGCCCCACACACA-3′; SCD1 forward, 5′-GAGAGAAGGTGAAGACAGTGC-3′; SCD1 reverse, 5′-GACCATGAGAATGATGTTCC-3′; ACAT2 forward, 5′-GACTTGGTGCAATGGACTCG-3′; ACAT2 reverse, 5′-GGTCTTGCTTGTAGAATCTGG-3′; cyclophilin forward, 5′-TGGAGAGCACCAAGACAGACA-3′; cyclophilin reverse, 5′-TGCCGGAGTCGACAATGAT-3′.

Statistical analyses

All statistical analyses were performed using the SAS Institute's JMP program, version 5.0.1.2.

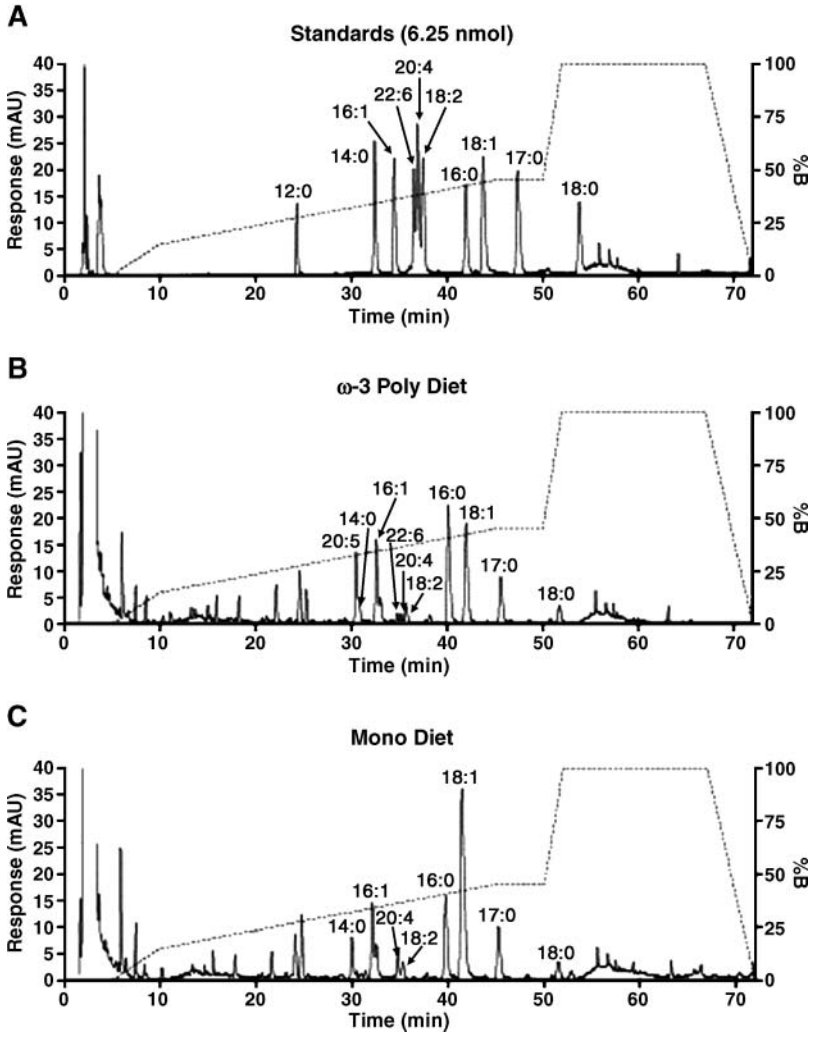

Fig. 1. Representative chromatograms of standards run at a 6.25 nmol concentration (A), a sample from an n-3 polyunsaturated fat diet (Poly)-fed mouse (B), and a sample from a monounsaturated fat diet (Mono)-fed mouse (C). The left y axis represents the signal response at an optical density of 254, and the right y axis represents the percentage of component B (100% acetonitrile). mAU, milliabsorbance units.

RESULTS

The ability of the different fatty acid-enriched diets to cause shifts in the hepatic fatty acyl-CoA composition is shown in **Table 1**. No significant differences among diet groups in the percentage of myristoyl-CoA, palmitoyl-CoA, stearoyl-CoA, and palmitoleoyl-CoA were observed, and together, these fatty acyl-CoA species accounted for between 45% and 55% of hepatic acyl-CoA species. The highest percentage of any one hepatic fatty acyl-CoA observed across the different diet groups was oleoyl-CoA. Percentages of oleoyl-CoA were lowest in animals fed the ω-3 polyunsaturated diet, at 21.4%, a value not significantly different from the 30.6% in the livers of the mice fed the

TABLE 1. Effects of diet on liver fatty acyl-CoA distribution

Diet Group	C14:0	C16:0	C18:0	C16:1	C18:1	C18:2	C20:4	C20:5	C22:6
ω-3 polyunsaturated fat	4.0 ± 0.0 a	24.9 ± 2.9 a	11.2 ± 1.8 a	14.9 ± 2.5 a	21.4 ± 1.6 a	5.0 ± 0.7 a	2.7 ± 0.6 a	10.9 ± 1.8 a	5.0 ± 1.7 a
ω-6 polyunsaturated fat	5.3 ± 1.1 a	22.1 ± 1.5 a	9.3 ± 2.7 a	15.1 ± 1.3 a	30.6 ± 2.7 a,b	15.3 ± 2.1 b	2.3 ± 0.5 a	0.0 ± 0.0 b	0.0 ± 0.0 b
Saturated fat	4.0 ± 1.4 a	20.6 ± 2.3 a	11.5 ± 2.4 a	15.8 ± 2.1 a	38.8 ± 2.8 b,c	4.8 ± 1.1 a	3.7 ± 1.3 a	0.0 ± 0.0 b	0.0 ± 0.0 b
Monounsaturated fat	2.7 ± 1.3 a	17.8 ± 0.7 a	10.6 ± 2.7 a	15.1 ± 2.6 a	46.9 ± 3.7 c	3.4 ± 0.8 a	3.6 ± 1.1 a	0.0 ± 0.0 b	0.9 ± 0.5 b

Values represent percentage means ± SEM of liver fatty acyl-CoA for each acyl-CoA subtype in each diet group. The livers of four mice were sampled in each diet group, and different letters reflect significant differences ($P < 0.05$ by Tukey's honestly significant difference test).

ω-6 polyunsaturated diet. The livers of mice fed the saturated fat diet had significantly higher percentages of oleoyl-CoA (38.8%). Mice fed the monounsaturated fat-enriched diet had the highest percentage of hepatic oleoyl-CoA at 46.9%, a higher percentage than in livers of either of the polyunsaturated fat diet groups but similar to that of the saturated fat diet group. The percentage of hepatic acyl-CoA as linoleoyl-CoA in livers of mice fed the ω-6 polyunsaturated diet was significantly higher compared with livers of mice in the other diet groups. Interestingly, this observation did not hold true for the other ω-6 polyunsaturated acyl-CoA that was measured, arachidonyl-CoA, which was only a minor component that did not vary significantly across the diet groups. The only animals that exhibited significant amounts of ω-3 polyunsaturated acyl-CoAs were those fed the ω-3 polyunsaturated diet.

The liver CE fatty acid percentage distribution (**Table 2**) reflected the hepatic acyl-CoA percentage distribution in many cases. The largest difference among diet groups was in the percentage of monounsaturated CE, with the percentage of hepatic cholesteryl oleate as high as 64.8% in the livers of the monounsaturated fat group and only 33.3% in the liver of the ω-3 polyunsaturated fat group. Interestingly, the mice fed the ω-3 polyunsaturated diet exhibited significantly higher percentages of both hepatic cholesteryl palmitate and stearate compared with livers from mice fed the saturated and monounsaturated diets. Livers from mice in the ω-6 polyunsaturated diet group had significantly lower percentages of cholesteryl palmitate than the livers from mice fed ω-3 polyunsaturated fat, whereas the percentage of cholesteryl stearate was intermediate between the mice fed ω-3 polyunsaturated fat and those fed saturated or monounsaturated fat. The percentages of hepatic cholesteryl myristoleate and palmitoleate did not vary significantly across the different diet groups. The only diet groups that displayed significantly higher percentages of polyunsaturated CE were those fed polyunsaturated fat, a trend also observed in the percentage distribution of fatty acyl-CoA shown in Table 1. The livers from the ω-6 polyunsaturated diet group had significantly higher percentages of both cholesteryl linoleate and arachidonate compared with livers in the other diet groups. Curiously, the higher percentage of cholesteryl arachidonate did not follow from an increase in the arachidonyl-CoA percentage. The livers of the ω-3 polyunsaturated diet group had significantly higher percentages of both cholesteryl eicosanoate (20:5) and docosahexaenoate (22:6) than the livers of the other diet groups.

To compare the overall lipid balance in the livers of the different dietary fatty acid groups, hepatic total cholesterol, FC, CE, TG, phospholipid, and total acyl-CoA concentrations were measured as summarized in **Table 3**. The mice fed monounsaturated or saturated fat had higher hepatic total cholesterol, FC, and CE concentrations compared with the mice fed either type of polyunsaturated fat, and these lipids were similar in concentration in the ω-3 and ω-6 polyunsaturated fat groups. The monounsaturated and saturated fat diet groups also had almost twice

TABLE 2. Effects of diet on liver CE fatty acid composition

Diet Group	Saturated Fat		Monounsaturated Fat			ω-6 Polyunsaturated Fat		ω-3 Polyunsaturated Fat		Other
	16:0	18:0	14:1	16:1	18:1	18:2	20:4	20:5	22:6	
ω-3 polyunsaturated fat	16.2 ± 1.4 a	9.5 ± 1.2 a	12.3 ± 1.6 a	4.8 ± 1.5 a	33.3 ± 3.1 a	7.6 ± 0.6 a	3.3 ± 0.3 a	7.3 ± 2.8	5.0 ± 0.3 a	0.8 ± 0.4 a
ω-6 polyunsaturated fat	7.6 ± 1.4 b	7.2 ± 1.0 a,b	9.1 ± 2.2 a	2.5 ± 0.8 a	38.4 ± 2.7 a	20.1 ± 1.7 b	12.5 ± 2.6 b	—	0.9 ± 0.1 b	1.9 ± 0.9 a
Saturated fat	9.4 ± 0.3 b	5.2 ± 0.5 b	4.8 ± 2.6 a	4.2 ± 0.7 a	60.4 ± 5.7 b	4.5 ± 0.9 a	4.1 ± 0.6 a	—	0.3 ± 0.2 b,c	7.1 ± 3.5 a
Monounsaturated fat	7.3 ± 0.9 b	4.0 ± 0.8 b	3.3 ± 1.8 a	4.4 ± 0.9 a	64.8 ± 5.7 b	4.6 ± 1.0 a	4.6 ± 1.7 a	—	0.2 ± 0.1 c	6.8 ± 2.2 a

CE, cholesteryl ester. Values represent percentage means ± SEM for each CE subtype in each diet group. The livers of four mice were sampled in each diet group, and different letters reflect significant differences ($P < 0.05$ by Tukey's honestly significant difference test).

TABLE 3. Effects of diet on liver lipids

Diet Group	TC	FC	CE	TG	PL	Fatty Acyl-CoA
	mg/g wet weight					*nmol/g wet weight*
ω-3 polyunsaturated fat	≅3.2 (0.1) a	2.2 (0.1) a	1.6 (0.1) a	39.1 (8.0) a	11.2 (0.3) a	62.4 (24) a
ω-6 polyunsaturated fat	4.6 (0.3) a	2.4 (0.1) a	3.7 (0.5) a	63.0 (7.3) a,b	11.0 (0.2) a	96.3 (41) a
Saturated fat	7.5 (0.6) b	3.3 (0.2) b	7.1 (1.0) b	74.0 (10) b	11.0 (0.2) a	51.3 (15) a
Monounsaturated fat	8.5 (1.0) b	3.0 (0.1) b	9.2 (1.5) b	76.1 (11) b	11.0 (0.3) a	75.0 (30) a

FC, free cholesterol; PL, phospholipid; TC, hepatic total cholesterol; TG, triglyceride. Values represent means and (SEM) for 27–34 mice in each diet group for liver TC, FC, CE, TG, and PL values. Total fatty acyl-CoA measurements were sampled in four mice from each diet group. Different letters indicate significant differences ($P < 0.05$ by Tukey's honestly significant difference test).

the concentration of liver TG compared with the ω-3 polyunsaturated fat diet group, whereas the liver TG values of the ω-6 polyunsaturated diet group fell between them. There were no significant differences in liver phospholipid and total acyl-CoA concentrations among the diet groups.

Table 4 presents data comparing the strength of association between the summed percentage of the two primary forms of monoacyl-CoA, palmitoleoyl-CoA and oleoyl-CoA, and plasma lipid and lipoprotein parameters, liver CE, liver TG, and aortic CE. Similar comparisons were made with the sum of the percentages for palmitoyl-CoA and stearoyl-CoA. The significance of the regression coefficient, R^2, for each comparison was evaluated with an *F*-test, and the results are shown. Statistically significant linear relationships were found between the percentage of monoacyl-CoA and TPC, LDL molecular weight, VLDL-cholesterol, LDL-cholesterol, liver CE, and aortic CE. The relationships between monoacyl-CoA percentage and LDL molecular weight, liver CE, and aortic CE were particularly strong ($P < 0.005$). No significant associations were found between the hepatic monoacyl-CoA and plasma TG, HDL-cholesterol, and liver TG. **Figure 2** shows the regression lines comparing the percentage of liver monoacyl-CoA with TPC (Fig. 2A) and plasma TG (Fig. 2B) of the diet-fed mice. By contrast, no statistically significant associations were found between the summed percentage of saturated acyl-CoA and any of the lipid, lipoprotein, or atherosclerosis measurements tested.

Figure 3 demonstrates the relationships between the percentage of oleoyl-CoA and the percentages of cholesteryl oleate and stearate. The scatterplot shown in Fig. 3A clearly illustrates the strong positive relationship ($R^2 = 0.80$, $P < 0.0001$) between the percentage of hepatic oleoyl-CoA and the percentage of cholesteryl oleate in the liver. For hepatic cholesteryl stearate, the inverse was found to be true: the percentage of oleoyl-CoA increased as the percentage of cholesteryl stearate decreased, as summarized in Fig. 3B, and this negative relationship was

TABLE 4. Responses of monounsaturated and saturated acyl-CoA to proatherogenic markers and atherosclerosis

	Percentage 16:1 + 18:1 CoA		Percentage 16:0 + 18:0 CoA	
Response	R^2	ANOVA (*P* by *F*-test)	R^2	ANOVA (*P* by *F*-test)
Total plasma cholesterol	0.43	0.01	0.06	0.40
Plasma TG	0.01	0.69	0.07	0.37
LDL molecular weight	0.52	0.004	0.09	0.31
VLDL-cholesterol	0.49	0.008	0.17	0.16
LDL-cholesterol	0.39	0.03	0.0000	0.98
HDL-cholesterol	0.08	0.37	0.02	0.61
Liver CE	0.59	0.001	0.15	0.16
Liver TG	0.19	0.117	0.12	0.22
Aortic CE	0.53	0.003	0.20	0.11

Values reflect the strength of the relationship (R^2 and *F*-test) between the percentage of liver monounsaturated and saturated fatty acyl-CoA and the listed plasma lipid, lipoprotein, LDL size (LDL molecular weight), liver lipid, and atherosclerosis measurements. The regression analyses were conducted using data collected from 16 mice.

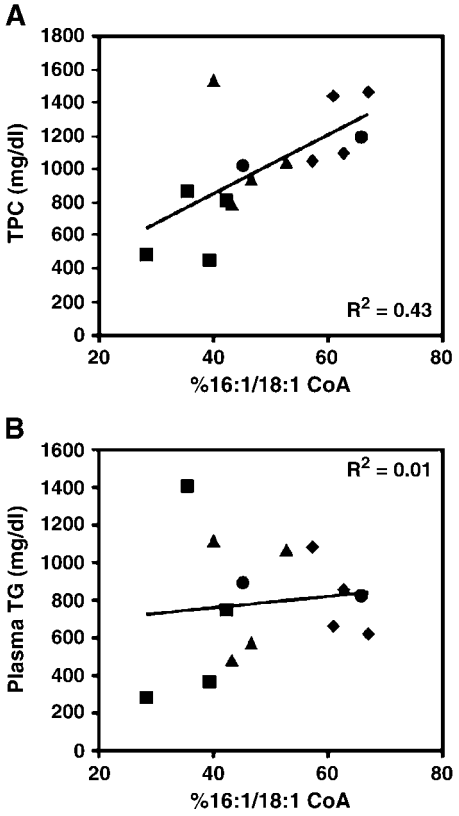

Fig. 2. Relationships between the percentage of hepatic monoacyl-CoA and total plasma cholesterol (TPC; A) and plasma triglyceride (TG; B) in mice fed diets enriched in different types of dietary fat. The data points represent individual mice, and the different symbols represent the diet groups: squares, n-3 polyunsaturated fat; triangles, n-6 polyunsaturated fat; circles, saturated fat; diamonds, monounsaturated fat.

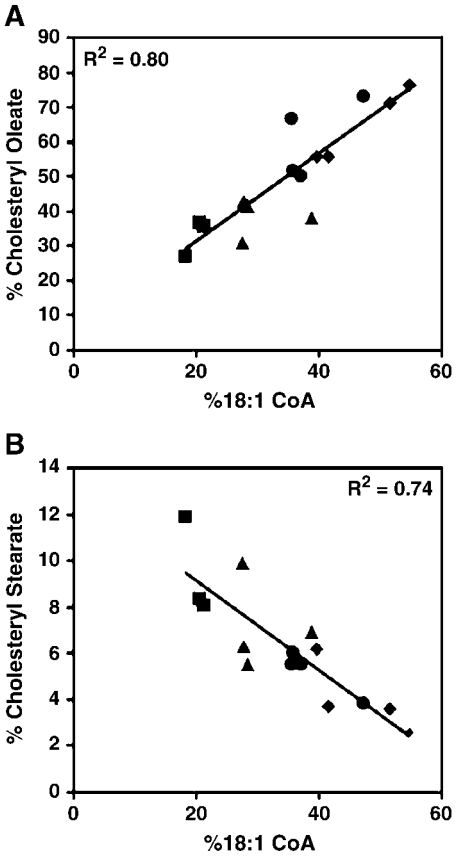

Fig. 3. Relationships between the percentage of hepatic oleoyl-CoA and the percentage of hepatic cholesteryl oleate (A) and cholesteryl stearate (B) in mice fed diets enriched in different types of dietary fat. The data points represent individual mice, and the different symbols represent the diet groups: squares, n-3 polyunsaturated fat; triangles, n-6 polyunsaturated fat; circles, saturated fat; diamonds, monounsaturated fat.

highly significant ($R^2 = 0.74$, $P < 0.0001$). Similar relationships were not found when the other acyl-CoA and CE subtypes were compared.

Figure 4 shows the analyses of the expression of key hepatic genes involved in the synthesis of fatty acyl-CoA and CE. Figure 4A, B compare the mRNA levels of the acyl-coenzyme A synthetase 1 (ACS1) and acyl-coenzyme A carboxylase (ACC) genes. Significant differences were not found among the different diet groups, but the averages were higher in the livers of mice fed saturated and monounsaturated fat compared with those fed polyunsaturated fat. Figure 4C summarizes the effects of the different diets on the expression of stearoyl-coenzyme A desaturase 1 (SCD1). The lowest levels of expression of SCD1 were found in the mice fed either type of polyunsaturated fat. In the mice fed saturated fat, a 6.5-fold increase in SCD1 expression occurred that was significantly higher than that in the livers of polyunsaturated fat-fed mice. The expression of SCD1 in the monounsaturated fat-fed mice was intermediate between the saturated and polyunsaturated groups. No significant differences in the expression of ACAT2 were found among the four diet groups (Fig. 4D).

DISCUSSION

By developing a method for the quantification of hepatic fatty acyl-CoA, we were able to examine the effects that different types of dietary fats had on mouse liver fatty acyl-CoA concentration and percentage fatty acyl-CoA distribution. It was then possible to correlate these changes to the liver CE compositions and to plasma lipoprotein end points. Although the different fatty acid-enriched diets did not have a major effect on overall fatty acyl-CoA concentration (Table 3), the dietary fatty acid composition did have a significant effect on the proportions of individual fatty acyl-CoAs in liver (Table 1). The acyl-CoA subtype most affected was oleoyl-CoA, the major acyl-CoA in liver of most diet groups. Livers from mice fed the diets high in saturated and monounsaturated fat exhibited the highest percentage of oleoyl-CoA. This percentage of oleoyl-CoA in the hepatic fatty acyl-CoA substrate pool was highly correlated with the percentage of liver cholesteryl oleate (Fig. 3), suggesting that it estimates the ACAT2 substrate acyl-CoA pool. Mice fed saturated and monounsaturated fat had higher oleoyl-CoA percentages than mice fed polyunsaturated fat (Table 2) as well as higher liver CE concentrations (Table 3). When the effects of dietary fat on liver CE concentration and liver CE fatty acid composition were considered together, the net result was an increase in the amount of cholesteryl oleate in the livers of mice fed saturated and monounsaturated fat. The dietary fatty acid effect on liver cholesteryl oleate content is strikingly similar and likely related to shifts in the percentage of LDL cholesteryl oleate across the diet groups that were observed in a previous atherosclerosis study conducted in these mice (12). The mice fed saturated and *cis*-monounsaturated fat had more than double the percentage of LDL cholesteryl oleate compared with mice fed polyunsaturated fat. Thus, the clear indication of our data is that the availability of oleoyl-CoA in the liver is responsible in a major way for the accumulation and secretion of ACAT2-derived cholesteryl oleate.

The presumed effects of monoacyl-CoA on ACAT2 CE production and secretion from the liver appeared to have downstream consequences on plasma lipoprotein cholesterol, such that the percentage of monoacyl-CoA was positively correlated to TPC, LDL size (LDL molecular weight), and LDL-cholesterol. The percentage of monoacyl-CoA was also positively correlated to atherosclerosis, measured by aortic CE concentration, presumably as a result of the associated plasma lipoprotein cholesterol changes. Relationships between monoacyl-CoA and the liver lipid, plasma lipid, and atherosclerosis measurements were not duplicated when similar analyses were performed with saturated acyl-CoA (Table 4). Because ACAT2 synthesizes saturated CE as well as monounsaturated CE (24), the atherogenic potential of these two types of CE may not be equivalent, although the greater abundance of cholesteryl oleate may be the reason for any apparent paradox. In summary, these analyses highlight the proatherogenic potential of monounsaturated fat in the diet. The accumulation of monounsaturated CE in LDL in plasma appears

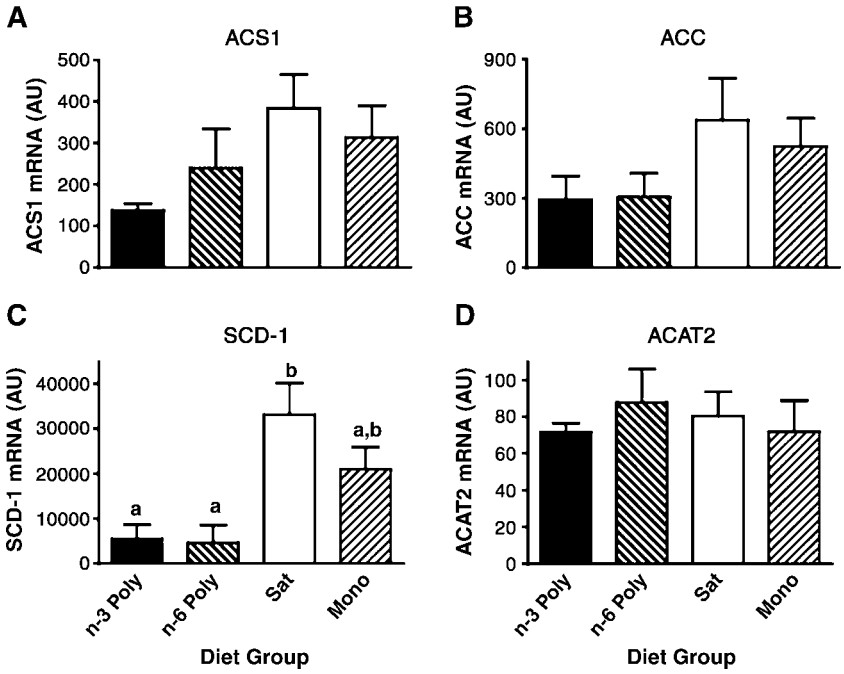

Fig. 4. Quantification of mRNA of liver acyl-coenzyme A synthetase 1 (ACS1; A), acyl-coenzyme A carboxylase (ACC; B), stearoyl-coenzyme A desaturase 1 (SCD-1; C), and ACAT2 (D) by real-time PCR in diet-fed mice. Columns represent averages (±SEM) for four mice per diet group, and different letters represent significant differences between diet groups ($P < 0.05$ by Tukey's honestly significant difference test). AU, arbitrary units.

to drive atherosclerosis. These data also illustrate the accumulation of neutral lipids in the liver driven by monounsaturated fatty acids. The oleoyl-CoA that accumulates appears to be readily available for cholesterol esterification and for TG formation. This effect of dietary monounsaturated fat on the liver is often missed when people consider the Mediterranean diet, rich in olive oil, as the healthiest diet (25). Perhaps the many other differences in this diet compared with the Western diet, and not the olive oil, provide the benefits (26).

The effect of LDL size on CHD risk has been examined in clinical studies (27, 28). Both large and small LDL particle sizes have been associated with increased CHD risk. The contradiction regarding the role of LDL size in CHD limits the utility of LDL size as a predictor of CHD. In our monkey studies (6, 7, 29), increases in LDL size and atherosclerosis were associated with an enrichment of LDL with ACAT2-derived monounsaturated CE, as the type of dietary fat fed was shifted from polyunsaturated to saturated and monounsaturated. Similar dietary fat-induced effects on ACAT2 activity, LDL size, and atherosclerosis have also been documented in mice (our unpublished data). Furthermore, a recent study in humans found a positive relationship between monounsaturated CE in plasma and carotid artery intimal thickness (30). These findings indicate that beyond LDL size, the CE fatty acid composition, and specifically the cholesteryl oleate enrichment of LDL, may be an important indicator of CHD risk.

Figure 3 gives further insight into the relationships between monoacyl-CoA, as oleoyl-CoA, and saturated and monounsaturated CE. Across the different diet groups, the increase in the percentage of oleoyl-CoA was associated with an increase in the percentage of cholesteryl oleate. Conversely, as the percentage of oleoyl-CoA increased, a decrease in the percentage of cholesteryl stearate was observed. These results indicate a conversion of stearoyl-CoA to oleoyl-CoA, and the enzyme commonly associated with this activity is SCD1 (31). We measured the expression of genes that are central in the metabolic pathways responsible for governing acyl-CoA pool size and species distribution, as well as ACAT2. Figure 4C shows the significant shifts in SCD1 expression across the different diet groups. Compared with mice fed saturated fat, SCD1 expression is significantly reduced in both of the diet groups fed polyunsaturated fat. The mRNA levels of mice fed monounsaturated fat were intermediary between those of polyunsaturated and saturated fat-fed mice. This effect of dietary fat on SCD1 expression would likely have consequences on the amount of monoacyl-CoA available in the liver as substrate for CE production by ACAT2 and is consistent with the differences observed in liver fatty acyl-CoA and CE fatty acid percentage composition across the different diet groups.

The effect of polyunsaturated fat in reducing SCD1 expression is likely related to the increase in the ratio of saturated to monounsaturated CE in mice fed ω-3 polyunsaturated fat compared with mice fed saturated or monounsaturated fat (Table 2). The observation that SCD1 activity is reduced by polyunsaturated fat has been known for some time, from the studies of Jeffcoat and James (32). The reduction in SCD1 message and activity by

polyunsaturated fat feeding is attributed to polyunsaturated fat downregulating SCD1 expression by interfering with liver X receptor and sterol-regulatory element binding protein 1c transcription factor activity (33–35). The effects that dietary polyunsaturated fat had on ACS1 and ACC expression (Fig. 4A, B), other known targets of liver X receptor and sterol-regulatory element binding protein 1c (33), were similar but less marked. By contrast, there was no effect of dietary fat type on ACAT2 expression (Fig. 4D), a finding consistent with the observation made in HepG2 cells (36) and with in vivo studies carried out in nonhuman primates (L. L. Rudel, unpublished observations).

In conclusion, we developed a reliable method for the quantification of liver fatty acyl-CoA with which we were able to examine the effects that feeding different types of dietary fats had on fatty acyl-CoA distribution. We found that mice fed diets high in saturated and monounsaturated fat exhibited an increase in oleoyl-CoA that was accompanied by an increase in liver ACAT2-derived CE, primarily in the form of cholesteryl oleate. These data further suggested that diet-induced ACAT2-derived cholesteryl oleate was responsible for increased atherosclerosis and was attributable more to an increase in oleoyl-CoA than to altered ACAT2 expression. A major component in the shift in liver lipids caused by dietary saturated and monounsaturated fat may have been the increase in SCD1 expression. The increased hepatic lipid accumulation in the animals fed saturated and monounsaturated fat appears related to the oleoyl-CoA accumulation. These data suggest that a probable mechanism by which saturated fat exerts its proatherogenic nature is via its conversion to monounsaturated acyl-CoA in the liver, with an additional consequence being hepatic neutral lipid accumulation. These findings suggest that polyunsaturated fat is a more suitable replacement than monounsaturated fat for dietary saturated fat.

This research was supported by National Institutes of Health Grants HL-49373, HL-24736, and AT-0027820.

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