JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2005, 53, 7295−7301 7295

Antiatherogenic Effects of Structured Lipid Containing Conjugated Linoleic Acid in C57BL/6J Mice

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Conjugated linoleic acids (CLA) were enzymatically acidolyzed with olive oil to produce structured lipids (SL), and their antiatherosclerotic properties were investigated in C57BL/6J mice. Twenty-eight mice were divided into four groups and fed control diet or atherogenic diets supplemented with high cholesterol and high fat (HCHF) containing 5% of lard, olive oil, or SL based on control diet for 4 weeks. The supplementation of SL diet (0.6% CLA) significantly reduced the levels of serum total cholesterol and total triglyceride and increased high-density lipoprotein cholesterol level as compared to lard and olive oil diet groups ($p < 0.05$). The activity of liver acyl CoA:cholesterol acyltransferase (ACAT) of mice fed the SL diet was significantly lower than that of mice fed the lard or olive oil diet. A reduced formation of aortic fatty streak was observed in SL group. The extent of CLA incorporation depended on tissues or types of phospholipids. More CLA was incorporated in adipose tissue (1.85 mol %) than in the liver (0.33 mol %). Besides, more CLA was found in phosphatidylethanolamine (PE) (0.47 mol %) than in phosphatidylcholine (PC) (0.05 mol %) of hepatic phospholipids. Hepatic phospholipids (PC and PE) of mice fed the SL diet contained reduced contents of arachidonic and linoleic acid compared with mice fed the olive oil or lard diet. The present study suggests that SL could be considered as a functional oil for preventing risks of atheroscelerosis.

KEYWORDS: Structured lipids; conjugated linoleic acid; atherosclerosis; C57BL/6J mice; cholesterol

INTRODUCTION

Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of linoleic acid (C18:2n−6) containing conjugated double bonds at *c*10 and *c*12 or at *c*9 and *c*11 with possible cis and trans combination. CLA occurs predominantly in meat and dairy products from ruminants, because it is formed by rumen microorganisms involved in linoleic acid metabolism (*1*). An important biological active isomer is *c*9,*t*11 CLA (rumenic acid), representing ∼80% of the total CLA in ruminant fat, whereas commercially produced CLA mixtures available in a diet supplementation contained *c*9,*t*11 CLA and *t*10,*c*12 CLA as the main isomers in equal amounts, which have been the focus of most of the research for a biological activity of CLA. The effects of CLA on animal metabolism are currently under investigation, and the recent research discovered that CLA has been shown to have beneficial physiological effects including inhibiting cancer risk, enhancing immune response, and reducing atherosclerosis and fat gain (*1*−*5*).

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Effects of dietary CLA in protecting against atherosclerosis in animals have been reported. However, it remains unclear whether dietary CLA prevents atherosclerotic disease. Supplementation with CLA on atherogenic diet reduced the concentration of total serum cholesterol and triacylglyerol and increased the high-denisty lipoprotein (HDL) cholesterol/total cholesterol ratio in most animal models including mice and hamsters (*6*−*8*). For example, diets containing 0.1 or 0.5% CLA lowered the severity of aortic arch lesions by 40 and 60% in rabbits, respectively (*9*). Another study showed that hamsters fed 1% CLA as free fatty acids decreased aortic fatty streaks formation and low-denisty lipoprotein (LDL) oxidation relative to control. Thus, CLA seemed to reduce the development of early aortic atherosclerosis, possibly through decreased LDL susceptibility to oxidation (*8*). Besides, in hamsters, CLA (2 g/100 g of diet) reduced the activity of acyl CoA:cholesterol acyltransferase (ACAT), which plays a critical role in the development of atherosclerosis via foam cell transformation of macrophages and/or smooth muscle (*10*). In contrast, the C57BL/6 mouse, fed on atherogenic diet with either 2.5 or 5 g of CLA/kg for 15 weeks, developed significantly greater aortic fatty streak area than mouse fed control diet (*6*). Therefore, further research is required to identify antiatherogenic effects of CLA in animals as well as humans.

10.1021/jf050626e CCC: $30.25 © 2005 American Chemical Society
Published on Web 08/04/2005

CLA is typically present in food from animal sources, but vegetable oils contain little CLA. Moreover, CLA itself is a free fatty acid that is not a desirable form for diets. Thus, an incorporation of CLA into triacylglycerol (TAG) molecules of vegetable oil is desirable, and lipase-mediated acidolysis is one of the developed reactions (*11*). Modified lipids are so-called structured lipids (SLs), in which any lipid can change its fatty acids composition and/or positional distribution in TAG molecules by enzymatic or chemical process (*12*).

In our study, SL was produced with CLA mixture and olive oil by lipase-catalyzed acidolysis, and the effects of dietary CLA in TAG molecules were investigated on early atherosclerosis in hyperlipidemic C57BL/6J mice. The atherosclerotic parameters including serum lipid profiles and in vivo enzyme activities of acyl CoA:cholesterol acyltransferase (ACAT) in liver and lipoprotein-associated phospholipase A_2 (Lp-PLA_2) in serum were studied. Additionally, the fatty acid composition was analyzed to determine whether SL affected the fatty acid profile of hepatic phospholipids.

MATERIALS AND METHODS

Synthesis of SL. Extra virgin olive oil was supplied by CJ Food Co. (Seoul, Korea), and CLA mixture (70 CLA) was kindly donated by Livemax Co. (Sungnam, Korea). The CLA mixture consisted of 32.8% *c*9,*t*11 CLA, 32.2% *t*10,*c*12 CLA, 3.8% other CLA isomers, 17.1% oleic acid, 11.1% palmitic acid, and 2.2% stearic acid. Immobilized lipases from *Rhizomucor miehei* (Lypozyme, RM IM) were purchased from Novo Nordisk Biochem North America, Inc. (Franklinton, NC). All solvents used were obtained from Fisher Scientific (Seoul, Korea). Lypozyme RM IM (3 g, 5 wt % of total substrates) and 9 mL of hexane were added to a mixture of olive oil (30 g) and CLA (28.9 g, 1:3 molar ratio) in a 250-mL Erlenmeyer flask with a screw cap. The reaction mixture was incubated for 24 h in a shaking water bath (200 rpm) at 55 °C. The reactant was filtered, under vacuum, to remove the lipases from the reaction mixture and was poured through an anhydrous sodium sulfate column. The reaction mixture was mixed with 3−4 drops of phenolphthalein solution and titrated with 0.5 N KOH solution in 20% ethanol (pH end point 7.2). After titration, saturated NaCl (60 mL) solution was vigorously added, and the hexane phase was then isolated. After passing through an anhydrous sulfate column, hexane was evaporated by a rotary evaporator under vacuum, and SL was collected. The typical reaction described above was conducted several times to get enough SL for feeding.

Animals and Diets. The male C57BL/6J mice, 5 weeks old, were obtained from the laboratory of the Experimental Animal in Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejon, Korea). The mice were fed normal mice chow diet (CRF-1, Oriental Yeast Inc., Tokyo, Japan) for 1 week prior to the start of the experimental diet (**Table 1**). After the acclimation, the mice were then randomly distributed into four groups ($n = 7$) and kept at 22 ± 1 °C and 55 ± 5% relative humidity in a room under a 12-h light−dark cycle. They were given ad libitum access to water and assigned diets for 4 weeks. The experimental diets and fatty acid compositions are described in **Tables 1** and **2**. The lard, olive oil, and SL groups were fed a high-cholesterol−high-fat (HCHF) mixture diet consisting of 1.25% cholesterol, 0.5% sodium cholate, and an additional 20% fat that contains 7.5% cacao butter, 7.5% lard, and 5% lard, olive oil, or SL. Body weights were measured weekly throughout experimental periods. This study was maintained in accordance with guidelines of animal experiments by the committee of the KRIBB.

Serum Analysis. Blood was collected from the retro-orbital sinus, using heparinized microhematocrit capillary tubes (Sigma 02-668-66) after 12 h of food deprivation. The serum triglyceride, total cholesterol, HDL-cholesterol, glucose, glutamic oxaloacetic transaminase (GOT), and γ-glutamic pyruvic transaminase (γ-GPT) concentrations were determined using a Hitachi 7020 automatic blood analyzer.

Acyl-CoA:Cholesterol Acyltransferase Assay. Liver microsomal fractions of the mice were prepared by ultracentrifugation (*13*), using a Beckman L8-M (Palo Alto, CA) and SW55.1 rotor. The in vivo ACAT activity in the mouse liver was measured according to the method described by Cho et al. (*14*). The hepatic microsome was mixed with potassium phosphate buffer, bovine serum albumin, cholesterol, and distilled water and then preincubated at 37 °C for 15 min. The reaction was proceeded by adding [1-^{14}C]oleoyl-CoA solution (0.05 μCi, final concentration = 10 μM) at 37 °C for 15 min and was stopped by adding 2-propanol/heptane (4:1, v/v) solution. After termination of the reaction by adding heptane and assay buffer, the radioactivity of an aliquot of the supernatant was measured in a liquid scintillation counter (1450 Microbeta Trilux, Wallac Oy, Turku, Filand). ACAT activity was expressed as synthesized cholesteryl oleate picomoles per minute per milligram of microsomal protein.

Lipoprotein-Associated Phospholipase A_2 Assay. The in vivo activity of the Lp-PLA_2 in the mouse serum was measured according to the method described by Stafforini et al. (*15*) with minor modification (*16*). The activities of Lp-PLA_2 were expressed as picomoles per hour per milligram.

Liver Cholesterol. The lipid extract was saponified by adding 2 mL of 3% KOH in methanol and heating for 20 min at 70 °C. After cooling, 1 mL of distilled water was added, and the unsaponfiable fraction including cholesterol was extracted by 2 mL of hexane twice. The hexane phase was evaporated and dissolved in 5 mL of acetone. The cholesterol separation was made on a reversed phase column (Nova-Pak C18, 60 Å 4 μm, 3.9 × 150 mm) using high-performance liquid chromatography (HPLC, Younglin, Anyang, Korea) with acetonitrile/methanol (50:50, v/v) containing 3% water (v/v) as a mobile phase. The flow rate was 0.8 mL/min, and a Sedex 75 evaporative light scattering detector (ELSD) was used. Standard cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO).

Table 1. Composition of Experimental Diets

		high-cholesterol–high-fat (HCHF) diet[a]		
diet	control	lard	olive oil	SL (g/100 g)
CRF-1[b]	100	82.14	82.14	82.14
cholesterol		1.03	1.03	1.03
sodium cholate		0.41	0.41	0.41
cacao butter		6.16	6.16	6.16
lard		10.3	6.16	6.16
olive oil			4.1	
SL				4.1

[a] HCHF diet was supplemented with 1.25% cholesterol, 15% fat (7.5% cacao butter and 7.5% lard), 0.5% sodium cholate, and 5% lard, olive oil, or SL–olive oil–CLA oil based on CRF-1. [b] CRF-1 (Tokyo, Japan) consisted of protein, 22.6 g; fat, 5.6 g; carbohydrate, 53.8 g; fiber, 3.3 g; ash, 6.6 g; water, 8.1 g; vitamin A, 3783 IU; vitamin D_3, 503 IU; vitamin E, 21.2 mg; vitamin K_3, 0.16 mg; vitamin B_1, 4.44 mg; vitamin B_2, 3.06 mg; vitamin C, 14 mg; vitamin B_6, 1.26 mg; vitamin B_{12}, 12.2 μg; inositol, 431 mg; biotin, 27.8 μg; pantothenic acid, 7.07 mg; niacin, 14.6 mg; choline, 0.31 g; and folic acid, 0.25 mg/100 g.

Table 2. Fatty Acid Composition (Mole Percent) of Experimental Diets[a]

		HCHF diet		
fatty acid	control	lard	olive oil	SL
myristic (14:0)	0.98 ± 0.0	1.05 ± 0.10	0.81 ± 0.01	0.87 ± 0.0
palmitic (16:0)	25.35 ± 0.25	25.30 ± 0.04	22.85 ± 0.01	23.47 ± 0.03
palmitoleic (16:1)	1.08 ± 0.01	1.17 ± 0.10	0.99 ± 0.02	0.99 ± 0.03
stearic (18:0)	22.30 ± 0.33	21.61 ± 1.56	19.58 ± 0.24	20.60 ± 0.02
oleic (18:1)	34.79 ± 0.1	34.49 ± 0.32	42.07 ± 0.02	37.19 ± 0.03
linoleic (18:2)	14.44 ± 0.0	15.20 ± 0.87	12.79 ± 0.14	12.94 ± 0.03
linolenic (18:3)	1.05 ± 0.0	1.18 ± 0.14	0.91 ± 0.03	0.93 ± 0.03
*c*9,*t*11 CLA[b]	–[c]	–	–	1.64 ± 0.01
*t*10,*c*12 CLA	–	–	–	1.45 ± 0.01
∑ SFA	48.63 ± 2.5	46.91 ± 1.3	43.24 ± 3.6	44.94 ± 2.5
∑ MUFA	35.87 ± 1.9	36.71 ± 0.5	43.06 ± 1.6	38.18 ± 1.1
∑ PUFA	15.49 ± 0.3	16.38 ± 0.6	13.70 ± 0.2	16.96 ± 0.9

[a] Values are expressed as a mean ± standard deviations. [b] *t*, trans; *c*, cis. [c] –, not detected.

Liver Phospholipids. Liver (100 mg) in a vial was disrupted by sonication for 2 min and then extracted with 5 mL of chloroform/methanol (2:1, v/v) in a shaking incubator for 2 h at 175 rpm and 40 °C (*17*). The aliquot of the lipid extract was filtered, and 1 mL of 10% NaCl solution was added and vortexed. After centrifugation (1500 rpm, 10 min, 4 °C), the lower chloroform phase was collected and dried under nitrogen. For separation of phospholipids from lipid extract, 2 mL of acetone was added, mixed by vortexing for 1 min, and left on ice for 1 h. After centrifugation (1500 rpm, 10 min), the supernatant was discarded. The same procedure was repeated. The pellet, rich in phospholipids, was dried under nitrogen and redissolved in chloroform/methanol for further analysis. The concentrations of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were measured by HPLC equipped with a Yonglin SP930D dual pump and a Sedex 75 ELSD. The analytical column was a Spherisorb silica (5 μm, 250 × 4.6 mm i.d., Waters, Milford, MA), and the operation temperature of the ELSD was set at 40 °C. Nitrogen was used as a nebulizing gas at a pressure of 2.2 bar. The mobile phase reservoirs contained solvent A of chloroform/methanol/ammonium hydroxide (80:19.5:0.5, v/v/v) and solvent B of methanol/water/ammonium hydroxide (80:19.5:0.5, v/v/v). The following linear gradient condition was used: 0−20% B from 0 to 14 min, 20−25% from 14 to 30 min, and returning to 0% B from 30 to 35 min at a flow rate of 1 mL/min. The PC and PE standards were purchased from Sigma Chemical Co.

Atherosclerotic Lesion Analysis. Evaluation of aortic lesion formation was performed according to the method described by Paigen et al. (*18*) with slight modification (*19*). After removal of the heart, it was perfused through the left ventricle and fixed in 30 mL of 4% paraformaldehyde for 24 h. The isolated upper heart/aortic sinus was embedded in Tissue-Tek OCT compound (Fisher Scientific, Pittsburgh, PA) and was frozen. The frozen tissue blocks were placed on a cryotome (model AS620, Shandon, Pittsburgh, PA), and 10-μm serial sections of the ascending aorta were saved on 3-(aminopropyl)triethoxysilane-coated slides, until the aortic sinus appeared. The sectioned slides were stained with Oil Red-O and hematoxylin for visualization of atherosclerotic lesion.

Fatty Acid Compositions of Liver and Adipose Tissue. Lipids were extracted from liver and adipose tissue (500 mg each) according to the method of Ametaj et al. (*17*) with a modification. Using phospholipid fractions from the liver lipid, the PC and PE were separated by thin-layer chromatography (TLC) on silica gel 60 F plates (20 × 20 cm, Merck, Darmstadt, Germany) in a tank containing chloroform/methanol/acetic acid/water (100:75:7:4, v/v/v/v) and visualized under 340 nm by staining with primuline solution (5 mg/100 mL of acetone/water, 80:20, v/v). The band corresponding to PC and PE was scraped into a vial for analysis of fatty acid composition. The lipid extract, PC, or PE from the liver was methylated with 3 mL of BF_3 in methanol in a 90 °C oven for 150 min. After cooling to room temperature, 1 mL of distilled water and 2 mL of pentane were added and vortexed for 1 min. The upper pentane layer was collected and evaporated, and the dry residue was dissolved in hexane. The lipid from the adipose tissue was methylated with 3 mL of 6% H_2SO_4 in methanol at 70 °C for 1 h and extracted with 2 mL of hexane. The hexane phases of total lipid, PC, and PE from liver and total lipid from adipose tissue were passed through an anhydrous sodium sulfate column and concentrated under nitrogen. The fatty acids of the liver and adipose tissues were analyzed on a fused-silica capillary column (SP-Wax, 60 m × 0.25 mm i.d., Supelco, Bellefonte, PA) using a Hewlett-Packard 6890 gas chromatograph with an autoinjector and flame ionization detector (Agilent Technologies, Little Falls, DE). The column was held at 100 °C for 1 min and then increased to 220 °C, at the rate of 10 °C/min and held for 65 min. Nitrogen was used as the carrier gas with a total gas flow rate of 1 mL/min (constant flow mode) and split mode 50:1. The temperatures of the injector and detector were 250 and 260 °C, respectively. Heptadecanoic acid (C17:0) was used as an internal standard, and triplicate analyses performed as previously (*11*).

Statistical Analysis. Data are expressed as the mean ± standard deviation (SD). Analysis of variance with Duncan's multiple-range test was performed to evaluate the difference between the groups, using the Statistical Analysis System version 6.0 (SAS Institute, Cary, NC). Statistical significance was defined as $p < 0.05$.

Table 3. Dietary Effects on Body Weight and Liver Function[a]

	control	HCHF diet		
		lard	olive oil	SL
initial body wt (g)	24.2 ± 1.6	24.7 ± 1.5	23.3 ± 1.3	22.1 ± 1.1
final body wt (g)	28.9 ± 1.8	28.4 ± 1.6	28.4 ± 2.0	27.0 ± 5.7
liver wt (g)	1.39 ± 0.21b	2.10 ± 0.19a	1.95 ± 0.19a	1.91 ± 0.15a
liver wt in body wt (%)	4.8 ± 0.5b	7.3 ± 0.6a	6.8 ± 0.3a	7.1 ± 0.3a
adipose tissue wt in body wt (%)	0.30 ± 0.02b	0.36 ± 0.01b	0.5 ± 0.03a	0.14 ± 0.01c
GOT[b] (units/L)	79.8 ± 28.9	116.5 ± 50.5	84.3 ± 16.3	115.0 ± 27.2
γ-GPT[c] (units/L)	19.2 ± 4.9	40.7 ± 16.7	47.3 ± 41.1	58.0 ± 32.2

[a] Means in the same row not sharing a common letter are significantly different ($p < 0.05$) among groups. [b] GOT, glutamic oxaloacetic transaminase. [c] γ-GPT, glutamic pyruvic transaminase.

Table 4. Dietary Effects on Serum Lipid[a]

	control	HCHF diet		
		lard	olive oil	SL
total cholesterol (mg/dL)	98.4 ± 7.2d	274.4 ± 40.3a	244.2 ± 3.8b	205.6 ± 13.6c
HDL-cholesterol (mg/dL)	59.0 ± 10.7a	48.29 ± 11.5b	45.14 ± 6.4b	62.0 ± 4.3a
HDL/total cholesterol, ratio (%)	65.26 ± 16.7a	21.2 ± 2.9c	19.8 ± 2.9d	30.4 ± 2.7b
total triglyceride (mg/dL)	66.3 ± 17.0c	104.6 ± 26.8a	90 ± 11.2ab	70.2 ± 12.6bc
glucose (mg/dL)	147.0 ± 11.3	152.0 ± 15.9	174.4 ± 15.5	167.8 ± 27.2

[a] Values with different letters in the same row are significantly different among groups at $p < 0.05$.

RESULTS

Body-Weight Gain and Liver Function. The body-weight gains during the 4 weeks of the experimental period were not significantly different among groups ($p < 0.05$) (**Table 3**). The liver weights of mice consuming theHCHF diet were higher than those from mice receiving control diet (normal mice chow). Besides, the percentage of adipose tissue weight in body weight was significantly lower in the SL group compared with the other three groups. Because previous studies demonstrated that dietary CLA induces a decrease in body fat accumulation in animals including pigs, rats, and mice (*20*−*22*), this observation is directed at confirming those studies. The serum GOT) and GPT values of the SL group were 115 and 58 units/L, respectively, and those values were not significantly different among dietary groups ($p < 0.05$).

Serum Lipid Profiles. Serum total cholesterol and triglyceride concentration of mice fed the HCHF diet were higher than those of mice fed the control diet (normal chow) (**Table 4**). Serum total cholesterol and triglyceride concentration of mice fed the lard diet were increased up to 2.8- and 1.6-fold, respectively, in comparison to those of mice fed the control diet. However, the serum total cholesterol and total triglyceride concentration of mice fed the SL diet were 25 and 32% lower than those of mice fed the lard diet and 16 and 22% lower than those of mice fed the olive oil diet ($p < 0.05$). In contrast, the HDL-cholesterol concentration of the SL group was the highest among the HCHF diet groups. Also, the ratio of HDL-cholesterol to total cholesterol in the SL group was the highest among the groups, showing 43 and 54% increases compared with the lard and olive oil groups, respectively ($p < 0.05$). No significant difference in serum glucose concentration was observed among dietary groups (**Table 4**).

Liver Lipids and Cholesterol. The effects of different dietary fats on the liver lipid contents are shown in **Table 5**. The lipid content was significantly lower in mice fed the SL diet than in

Table 5. Dietary Effects on Liver Lipid[a]

	control	HCHF diet		
		lard	olive oil	SL
total lipids (mg/g)	54.7 ± 1.25c	83.6 ± 0.11a	88.6 ± 0.0a	67.8 ± 0.03b
cholesterol (μmol/g)	2.69 ± 0.004c	21.84 ± 3.31a	20.48 ± 0.02a	15.46 ± 0.16b
phospholipids (mg/g)				
phosphatidylcholine	4.62 ± 0.005b	6.92 ± 0.018a	7.10 ± 0.012a	4.05 ± 0.013b
phosphatidylethanolamine	2.10 ± 0.001a	1.78 ± 0.001a	2.57 ± 0.004a	0.80 ± 0.001b

[a] Values with different letters in the same row are significantly different between groups at $p < 0.05$.

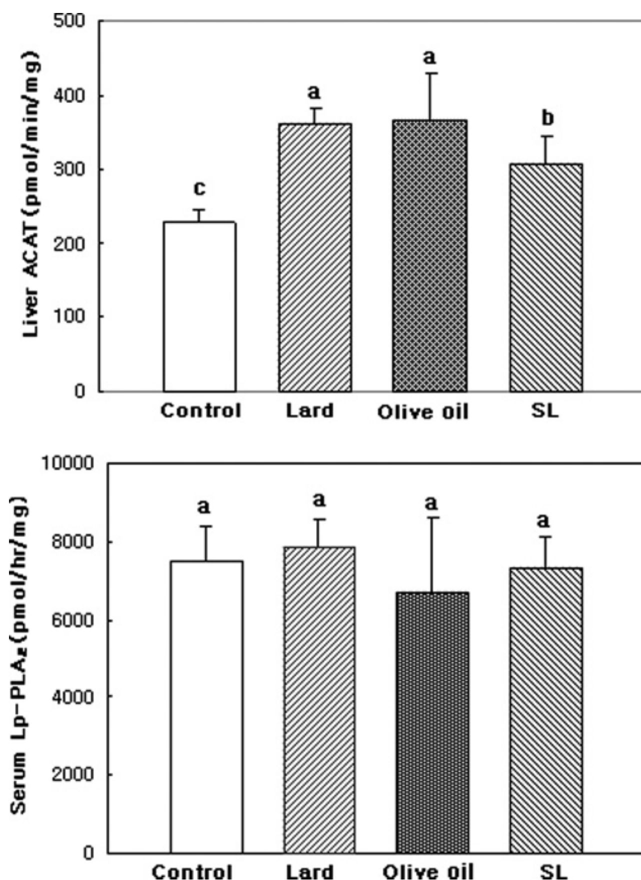

Figure 1. Different activities of liver acyl-CoA:cholesterol acyltransferase (ACAT) and serum lipoprotein-associated phospholipase A_2 (Lp-PLA_2) depending on diets in C57BL/6J mice. Values with different letters are significantly different at $p < 0.05$.

mice fed the lard or olive oil diets ($p < 0.05$). In the livers of mice fed the HCHF diet, 6−8-fold accumulation of cholesterol was observed in comparison to control diet. However, the concentration of liver cholesterol in the SL group was significantly lower than that in the lard and olive oil groups ($p < 0.05$). PC and PE are the major phospholipids of cell membranes, and their concentrations in liver were determined with HPLC. As shown in **Table 5**, mice fed the SL diet had significantly lower levels of PC and PE than mice fed the other diets ($p < 0.05$), whereas no significant difference in the level of liver phospholipids was observed between the lard and olive oil groups ($p < 0.05$).

Effect on in Vivo Enzyme Activities. As shown in **Figure 1**, the liver ACAT activities in HCHF groups (lard, olive oil, and SL) were considerably elevated up to 1.6-fold compared to the control group. Among them, the SL group showed 16 and 15% decreased liver ACAT activity (308 pmol/min/mg) compared to the olive oil (367 pmol/min/mg) and lard groups (361 pmol/min/mg), respectively ($p < 0.05$), whereas no difference in activities between the olive oil and lard groups was observed. The in vivo serum Lp-PLA_2 activity was analyzed, and the HCHF diet during 4 weeks did not cause a significant difference in Lp-PLA_2 activity among dietary groups.

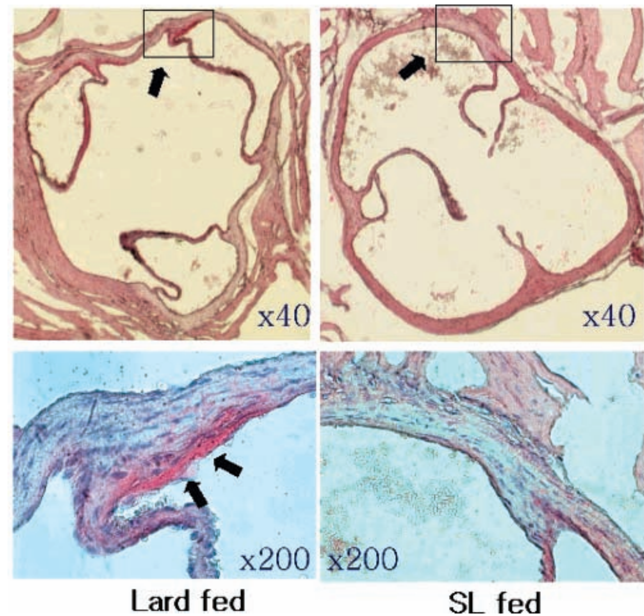

Figure 2. Cross sections of mouse aortic sinus and lesion histology from representative mice. Lesions were visualized after staining with Oil Red-O and hematoxylin.

Atherosclerotic Lesion. Most mice fed the lard diet for 4 weeks developed an atherosclerotic lesion in sectioned aortic sinus, as indicated in the left panel of **Figure 2**. However, the lesion was not observed in mice fed the SL diet, which suggests that the SL diet induces a resistance to the early development of atherosclerosis and fatty streak formation even though the HCHF diet was provided.

Fatty Acid Composition of Liver and Adipose Tissue. Fatty acid compositions of the livers and adipose tissues in mice fed the different diets were determined (**Table 6**). The liver fatty acid compositions between control and HCHF diet groups (lard, olive oil, and SL) were significantly different ($p < 0.05$), but the composition among HCHF diet groups was quite similar except for the content of CLA isomers. In HCHF diet groups, ∼44−52 mol % was monounsaturated fatty acids (MUFA) (mainly in the form of oleic acid, C18:1) in HCHF diet groups, 30−34 mol % was saturated fatty acids (SFA) (mainly palmitic acid, C16:0, and stearic acid, C18:0), and 19−22 mol % was polyunsaturated fatty acid (PUFA) (mainly linoleic acid, C18:2). After 4 weeks of experiment, the content of CLA isomers in the liver of mice fed the SL diet was 0.33 mol %, in which *c*9,*t*11 CLA and *t*10,*c*12 CLA composed 0.18 and 0.15 mol %, respectively. The fatty acid composition of adipose tissue was significantly different among groups ($p < 0.05$). In adipose tissue of HCHF diet groups, MUFA ranged from 51 to 59% among groups, and the content of oleic acid was the highest in the SL diet fed group ($p < 0.05$). It is known that the composition of CLA isomers depended on different types of tissues (*23*). In the present study, more CLA isomers were found in adipose tissue than in liver. The relative content of CLA to total fatty acids in liver tissue was not similar to the content of dietary CLA in the SL diet, suggesting that CLA in the diet was transported into the liver or assimilated in tissues.

Table 6. Fatty Acids Composition (Mole Percent) of Livers and Adipose Tissues from Mice Fed Different Diets[a]

fatty acid	control	HCHF diet lard	HCHF diet olive oil	HCHF diet SL
		Liver		
14:0	0.56 ± 0.16	0.24 ± 0.04	0.3 ± 0.16	0.28 ± 0.03
16:0	31.71 ± 0.56a	21.7 ± 0.45b	19.84 ± 3.14b	22.68 ± 0.17b
16:1	2.81 ± 0.13	2.66 ± 0.02	2.96 ± 0.06	2.36 ± 0.42
18:0	12.76 ± 1.14	9.22 ± 0.51	9.84 ± 1.32	10.64 ± 0.94
18:1	20.16 ± 0.3b	45.08 ± 0.41a	48.53 ± 7.10a	41.66 ± 1.39a
18:2	22.73 ± 2.19a	15.99 ± 0.21b	13.8 ± 2.25b	16.41 ± 0.92b
20:4	9.16 ± 1.86a	5.07 ± 0.21b	4.65 ± 0.16b	5.64 ± 0.14b
CLA isomers	0.02 ± 0.0b	0.04 ± 0.01b	0.08 ± 0.0b	0.33 ± 0.02a
Σ SFA	45.03 ± 0.42a	31.16 ± 0.37b	29.98 ± 2.24b	33.6 ± 0.45b
Σ MUFA	22.97 ± 0.27b	47.74 ± 0.29a	51.49 ± 6.21a	44.02 ± 1.08a
Σ PUFA	32.01 ± 2.02a	21.1 ± 0.16b	18.53 ± 0.13b	22.38 ± 0.51b
		Adipose Tissue		
14:0	0.93 ± 0.42a	1.14 ± 0.12a	1.1 ± 0.04a	0.68 ± 0.02b
16:0	22.86 ± 3.91a	21.1 ± 0.86a	20.85 ± 0.42a	18.33 ± 0.27b
16:1	4.71 ± 1.2a	4.18 ± 0.12a	4.83 ± 0.02a	2.98 ± 0.18b
18:0	2.52 ± 0.08d	7.14 ± 0.08a	5.99 ± 0.28b	5.05 ± 0.23c
18:1	33.79 ± 3.84d	47.0 ± 0.84c	49.81 ± 0.51b	56.51 ± 0.17a
18:2	34.59 ± 3.84a	19.13 ± 0.18b	16.83 ± 0.33c	14.43 ± 0.64d
20:4	0.54 ± 0.36a	0.26 ± 0.03b	0.52 ± 0.07a	0.19 ± 0.03b
CLA isomers	0.06 ± 0.01b	0.06 ± 0.02b	0.09 ± 0.02b	1.85 ± 0.07a
Σ SFA	26.31 ± 1.92	29.38 ± 0.41	27.94 ± 0.29	24.06 ± 0.12
Σ MUFA	38.5 ± 0.15d	51.18 ± 0.72c	54.64 ± 0.52b	59.49 ± 0.35a
Σ PUFA	35.19 ± 3.01a	19.45 ± 0.07b	17.44 ± 0.15b	16.47 ± 0.29b

[a] Values with different letters in the same row are significantly different at $p < 0.05$.

Table 7. Fatty Acid Compositions (Mole Percent) of Phosphatidylethanolamine and Phosphatidylcholine from Livers of Mice Fed Different Diets[a]

fatty acid	control	HCHF diet lard	HCHF diet olive oil	HCHF diet SL
		Phosphatidylethanolamine		
14:0	1.19 ± 0.23	1.17 ± 0.42	0.93 ± 0.38	1.25 ± 0.67
16:0	16.82 ± 1.54b	20.44 ± 8.37a	20.52 ± 0.15a	21.15 ± 0.88a
16:1	1.84 ± 0.09	1.41 ± 0.59	1.08 ± 0.59	1.34 ± 0.97
18:0	20.89 ± 0.34	23.10 ± 7.49	25.42 ± 4.26	34.66 ± 11.02
18:1	30.87 ± 3.05	37.91 ± 7.18	26.48 ± 6.78	29.06 ± 10.08
18:2	14.38 ± 1.13a	10.39 ± 4.28ab	14.27 ± 0.43a	6.80 ± 1.05b
20:4	13.85 ± 0.11a	5.58 ± 2.53b	11.20 ± 4.3a	5.26 ± 3.35b
CLA isomers	0.17 ± 0.1	–[b]	0.14 ± 0.0	0.47 ± 0.1
Σ SFA	38.9 ± 0.84b	44.71 ± 0.92ab	46.87 ± 8.72ab	57.06 ± 14.53a
Σ MUFA	32.5 ± 3.47a	39.32 ± 5.21a	27.56 ± 6.53a	30.4 ± 16.58a
Σ PUFA	28.61 ± 1.19a	15.97 ± 4.87b	25.61 ± 5.26a	12.53 ± 2.21b
		Phosphatidylcholine		
14:0	0.36 ± 0.04	0.51 ± 0.22	0.42 ± 0.04	0.4 ± 0.05
16:0	37.35 ± 1.93	38.07 ± 1.46	36.83 ± 2.33	40.24 ± 1.57
16:1	1.29 ± 0.23	1.35 ± 0.42	1.0 ± 0.15	0.96 ± 0.15
18:0	14.65 ± 1.29a	11.50 ± 0.36b	11.83 ± 0.67b	10.98 ± 0.94b
18:1	17.29 ± 0.75b	29.62 ± 2.73a	28.68 ± 1.21a	32.17 ± 3.53a
18:2	22.90 ± 0.73a	16.76 ± 0.9b	17.36 ± 0.74b	13.58 ± 3.46b
20:4	6.16 ± 0.62a	2.19 ± 0.16c	3.88 ± 0.91b	1.62 ± 0.59c
CLA isomers	–	–	–	0.05 ± 0.0
Σ SFA	52.36 ± 3.01a	50.08 ± 1.56a	49.08 ± 2.13a	51.62 ± 1.23a
Σ MUFA	18.58 ± 0.64b	30.97 ± 2.51a	29.68 ± 0.96a	33.13 ± 3.12a
Σ PUFA	29.06 ± 2.37a	18.95 ± 1.62c	21.24 ± 1.1b	15.25 ± 4.26c

[a] Values with different letters are significantly different at $p < 0.05$. [b] –, not detected.

Fatty Acids Composition of Liver Phospholipids. In the fatty acid compositions of liver PC and PE (**Table 7**), PE contained more CLA isomers than PC, and such different incorporation rates of CLA isomers may suggest the specific role of CLA isomers in individual phospholipids, which have different metabolic functions (*23*). The different fatty acid composition was found between PC and PE of mice liver. Except for the SL group, oleic acid was the major fatty acid in PE of control, lard, and olive oil groups, whereas palmitic acid was the most abundant in PC among the four groups. Liver phospholipids (PC and PE) of mice fed the SL diet contained significantly reduced contents of arachidonic and linoleic acid compared with mice fed the olive oil diet ($p < 0.05$).

DISCUSSION

Serum lipid and lipoprotein levels are mainly used to predict the atherogenic status of animals and humans because atherogenesis is positively correlated with elevated levels of serum total cholesterol, LDL-cholesterol, and triglyceride and with reduced level of HDL-cholesterol. Thus, the result of the present study suggested an early antiathergenic effect of the SL diet on mice and makes a good agreement with other studies using dietary CLA fed rabbits (*24*), hamsters (*25*, *26*), and mice (*6*).

Olive oil has beneficial effects on a number of risk factors for atherosclerosis. Oleic acid (MUFA), the major fatty acid in olive oil, lowered both total and LDL-cholesterol compared with SFA and reduced oxidized LDL compared with PUFA. However, their effects on HDL-cholesterol metabolism remained unclear (*27*). In the present study, the consumption of the olive oil diet reduced the level of total cholesterol and triglyceride by 11 and 13% compared to the lard diet, respectively. However, these levels were higher than those in SL fed mice, whereas the HDL-cholesterol level was lower than that in the SL group. Therefore, SL might have a more protective role against atherosclerosis than olive oil. In experimental animal models under MUFA and SFA diets, SFA are known as poor substrates for ACAT. These fatty acids allow more unesterified cholesterol concentration in endoplasmic reticulum (ER) to down-regulate the LDL receptor through the sterol-regulatory element binding protein (SREBP) pathway and then resulted in an elevated LDL-cholesterol concentration in the plasma and subsequently increased the synthesis of cholesteryl ester by activating ACAT (*28*, *29*). In contrast, MUFA (i.e., oleic acid), a preferred substrate for ACAT, shows a decreasing effect on the LDL-cholesterol level, and a MUFA-rich diet causes a higher concentration of cholesteryl ester (75% of cholesteryl oleate) in the livers of monkeys, indicating that oleic acid stimulates ACAT activity (*30*).

An atherosclerotic lesion was not observed in the aortic sinus of the SL fed mice, whereas early atherosclerotic lesions including fatty streaks were detected in lard fed mice. This result indicated that SL induced a resistance of fatty streak formation. Consistent with our observation, other data in previous studies showed that the reduction of lesion formation by CLA was correlated with changes of serum lipid profiles such as lowered serum total cholesterol concentration or elevated HDL-cholesterol to total cholesterol ratio (*9*, *24*, *25*). However, other reports showed that no significant correlations between the lesion development and serum lipid profile were observed in mice fed 0.25 or 0.5 g of CLA/100 g of diet, suggesting that fatty streak formation by CLA was independent of serum cholesterol and lipoprotein profile (*6*).

The mechanisms for the potential antiatherogenic effect of dietary CLA remain poorly understood because the atherosclerotic mechanism is very complex and many factors are involved. However, one of the possible mechanisms for decreasing serum cholesterol by dietary CLA may involve a down-regulation of ACAT activity (*26*). The earliest detectable lesion of atherosclerosis is the fatty streak, which is macrophage-driven foam cells and later evolves into the fibrous plaque. The inhibition of ACAT activity might contribute to reducing foam cell formation. We examined whether SL supplementation inhibits

the activity of ACAT in hyperlipidemic mice. The in vivo liver ACAT activity in the SL group was significantly decreased compared with the lard or olive oil group ($p < 0.05$). Liver ACAT plays a critical role in the production and secretion of very low density lipoprotein (VLDL) cholesterol via formation of cholesteryl esters. Therefore, this observation suggests that the antiatherogenic effect of CLA might be originated in the down-regulation of liver ACAT activity, leading to reduction of both serum total cholesterol and triglyceride. In the intestine, ACAT is involved in cholesterol absorption because the majority of dietary free cholesterol is converted to cholesteryl esters by ACAT in the enterocyte and subsequently assembled in the chylomicron and secreted into the lymphatic system. According to the study of Yeung et al. (*26*), the inhibition of intestinal ACAT activity by CLA supplementation was also found in hamsters, showing the decreased activity of intestinal ACAT by 58% when compared with control. Furthermore, the levels of hepatic cholesterol and serum total cholesterol were significantly lower in CLA supplemented hamsters. The present study showed the significantly reduced concentration of hepatic cholesterol in mice fed the SL diet among HCHF diets ($p < 0.05$), and this observation might be associated with decreased cholesterol absorption due to the inhibition of intestinal ACAT by SL supplementation. Therefore, further investigation for the effect of SL on intestinal ACAT would be needed.

We also examined in vivo serum Lp-PLA_2 in C57BL/6J mice. The Lp-PLA_2 is an independent atherogenic factor (*31*), which is attached to LDL as an inactive form. When activated by oxidized LDL, Lp-PLA_2 hydrolyzes phosphatidylcholine, the main phospholipid component in LDL, into oxidized fatty acid (mainly arachidonic acid) and lyso-phosphatidylcholine, which are known as inflammatory mediators for attracting monocytes and exacerbating atherogenic process (*10*). The study of Leach et al. (*32*) supported the view that Lp-PLA_2 plays a significant role in the development of atherosclerotic lesions because its activity was 6-fold higher in the arteries of rabbits fed an atherogenic diet for 8 weeks than in those of rabbits fed a control diet. In the present study, the activity of Lp-PLA_2 was not significantly altered by the experimental diets. Because the effect of dietary CLA on the Lp-PLA_2 activity has not been well reported and the in vivo effect on short-term feeding was carried out in this study, a study having a longer feeding period should be performed to find out whether CLA affects the activity of Lp-PLA_2.

Another possible hypothesis on the contribution to antiatherogenic effect of dietary CLA might be related to an interference of its metabolites with eicosanoid biosynthesis. Hepatic phospholipids (PC and PE) of mice fed the SL diet contained lower contents of arachidonic and linoleic acid than mice fed the olive oil or lard diet. This study showed that the dietary CLA in SL might be associated with the reduced concentration of linoleic acid in hepatic phospholipids, suggesting a modulation of fatty acid composition and subsequent eicosanoid metabolism. CLA is readily incorporated into phospholipids in cell membranes, and the isomers are converted into conjugated C18:3 (*c*6,*c*9,*t*11 or *c*6,*t*10,*c*12 18:3) and further subsequently metabolized by desaturation and elongation to form conjugated eicosatrienoic acid (C20:3) or conjugated arachidonic acid (C20:4) (*33*, *34*). On the basis of this finding, membrane-bound CLA is likely to compete with linoleic acid for Δ^6-desaturase and/or elongase that convert linoleic acid into arachidonic acid and that subsequently can lead to decreasing the formation of phospholipids-associated arachidonic acid, resulting in reduction of arachidonic acid-derived eicosanoids such as prostaglandin E_2 (*35*). Because such eicosanoids are associated with a stimulation of cardiovascular disease including atherosclerosis, a reduction of arachidonic acid by CLA might be beneficial against the diseases.

In conclusion, this short period of study suggests that SL appears to be antiatherogenic in hyperlipidemic C57BL/6J mice as shown by improved serum lipid profile and reduced hepatic ACAT activity.

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Received for review March 20, 2005. Revised manuscript received June 30, 2005. Accepted July 1, 2005. This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health and Welfare, Republic of Korea (02-PJ1-PG1-CH15-0001).

JF050626E