

Studies on the effect of dietary fish oil on the physical and chemical properties of low density lipoproteins in cynomolgus monkeys

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Abstract To determine the effect of isocaloric substitution of dietary fish oil for lard on the physical and chemical properties of plasma low density lipoproteins (LDL), ten adult male cynomolgus monkeys were fed diets containing 11% (by weight) fish oil or lard in a crossover study consisting of two 15-week periods with a 6-week washout period in between. The atherogenic diets contained 40% of calories as fat with 0.26 mg cholesterol/kcal. Periodic measurements of plasma lipids were made throughout the study and a large blood sample was taken near the end of each 15-week period for LDL isolation and characterization, and for quantification of plasma apolipoproteins. Values for both studies were combined (mean \pm SE; n = 10) by diet. Significantly lower high density lipoprotein (HDL) cholesterol (28 ± 2 vs. 57 ± 8 mg/dl), apoA-I (53 ± 11 vs. 88 ± 7 mg/dl), and apoE (4.2 ± 0.9 vs. 8.2 ± 1.5 mg/dl) concentrations were found when the animals were consuming the fish oil versus the lard diet, respectively, but total plasma cholesterol (408 ± 35 vs. 416 ± 14 mg/dl), LDL cholesterol (356 ± 34 vs. 331 ± 17 mg/dl), and apoB (227 ± 35 vs. 205 ± 23 mg/dl) levels were not affected. LDL size was smaller during fish oil feeding (4.2 ± 0.1 vs. 4.9 ± 0.1 g/ μ mol) and LDL particle concentration was greater (2.3 ± 0.2 vs. 1.8 ± 0.1 μ M). During fish oil feeding LDL cholesteryl esters (CE) and phospholipids (PL) were enriched in n-3 fatty acids and were relatively poor in 18:1 and 18:2. LDL CE transition temperature was about 11°C lower during fish oil feeding (32 ± 1 vs. $44 \pm 0.5^\circ\text{C}$) and was positively correlated with the number of saturated, monounsaturated, and n-6 polyunsaturated CE molecules per LDL. The results suggested that the range of transition temperatures among individual animal LDL was primarily determined by the number of monounsaturated CE, and the accumulation of n-3 polyunsaturated CE in LDL during fish oil feeding uniformly lowered the transition temperature of the LDL particle. There was a significant decrease in the percentage of LDL phosphatidylcholine (59 ± 1 vs. $72 \pm 1\%$) and an increase in lysophosphatidylcholine (13 ± 1 vs. $5 \pm 1\%$) and sphingomyelin (22 ± 1 vs. $17 \pm 1\%$) during fish oil feeding relative to that of lard. **Conclusion** We conclude that striking changes in the physical and chemical properties of plasma LDL can occur when fish oil is isocalorically substituted for lard in the diet with no apparent effect on total plasma or LDL cholesterol concentrations. These diet-induced changes may affect the atherogenic potential of plasma LDL. — Parks, J. S., and A. K. Gebre. Studies on the effect of dietary fish oil on the physical and chemical properties

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Supplementary key words lipoproteins • n-3 fatty acids • nonhuman primates • phospholipids • transition temperature • cholesteryl esters

Previous studies of nonhuman primates have shown that low density lipoprotein (LDL) size was one of the most powerful predictors of coronary artery atherosclerosis (1-3). Many nonhuman primates respond to atherogenic diets containing saturated fat and cholesterol with a large increase in LDL cholesterol concentration and in LDL size (1-3). As LDL size increases so does the liquid crystalline to liquid transition temperature of the cholesteryl ester (CE) core of the particle because the size increase is mainly due to an increase in the number of molecules of saturated and monounsaturated CE per particle, which have transition temperatures above body temperature (4, 5). It has been hypothesized that the LDL with liquid crystalline CE cores at body temperature are more atherogenic than are LDL particles with liquid CE cores (3, 5). Recently, it was shown that cynomolgus monkeys fed an atherogenic diet containing n-6 polyunsaturated fat (safflower oil) had larger LDL particles, lower HDL concentrations, and less coronary artery atherosclerosis compared to a group fed a diet in which lard was isocalorically substituted for safflower oil (6). These results suggested that properties of LDL other than

Abbreviations: LDL, low density lipoproteins; HDL, high density lipoproteins; CE, cholesteryl esters; PL, phospholipids; TPC, total plasma cholesterol; TG, triglycerides; DSC, differential scanning calorimetry; TLC, thin-layer chromatography; VLDL, very low density lipoproteins; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; ILDL, intermediate-sized low density lipoproteins; SM, sphingomyelin; PI, phosphatidylinositol; PE, phosphatidylethanolamine; FC, free cholesterol; HPLC, high performance liquid chromatography.

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size might be important in determining the atherogenic potential of LDL. One such property could be the physical state of the core CE of LDL.

Nonhuman primates, like their human counterparts, have variable plasma lipid and lipoprotein responses to diets enriched in saturated fat and cholesterol (7). Cynomolgus monkeys are among the most responsive to atherogenic diets while African green monkeys are among the least responsive (1, 8). We have investigated the effect of type of dietary fat on the physical and chemical properties of LDL and on atherosclerosis development in previous studies of African green monkeys, a species that is relatively resistant to diet-induced atherosclerosis (5, 9, 10). In those studies we compared the effects of fish oil isocalorically substituted for lard because it seemed likely that these two dietary fats would give us the maximum opportunity to detect differences in the measured endpoints. The purpose of the present study was to determine the effect of isocaloric substitution of fish oil for lard on the physical and chemical properties of LDL using the cynomolgus monkey, a species that is relatively susceptible to diet-induced atherosclerosis compared to the African green monkey (1). The study had a crossover dietary design so that each animal served as its own control. We found that major changes in the chemical and physical properties of LDL were induced by dietary fish oil compared to lard even though total plasma and LDL cholesterol were not affected.

METHODS

Experimental design

Ten adult male cynomolgus monkeys (*Macaca fascicularis*) were provided for the study through an NHLBI-sponsored nonhuman primate models program at the Bowman Gray School of Medicine. Prior to the start of the study the colony animals had been maintained on a monkey chow diet supplemented with egg yolk (colony diet) for at least 2 years. The colony diet contained 40% of calories as fat and 0.4 mg cholesterol/kcal. Two groups of five animals each were selected so that total plasma cholesterol (TPC), HDL cholesterol (HDL-C), and triglyceride (TG) concentrations were not significantly different between the two groups while consuming the colony diet. After group assignments were made the animals were subjected to a monkey chow "wash-out" phase for 8 weeks to bring plasma lipids back to baseline before initiation of the experimental diets. A crossover study consisting of two phases of approximately 15 weeks each with a 5-week monkey chow "wash out" phase in between the experimental diet phases was carried out. During Phase 1 of the study, Group 1 received the lard diet and Group 2 received the fish oil diet; during Phase 2, Groups 1 and

2 consumed the fish oil and lard diets, respectively. Periodic blood samples were taken throughout the study for measurement of TPC, HDL-C, and TG concentrations (11). A large blood sample was taken from each animal for apolipoprotein quantification and detailed lipoprotein analyses at ~15 weeks into each dietary phase. The body weights of the animals at the beginning and the end of the study were 6.3 ± 0.3 and 6.9 ± 0.4 kg, respectively. The protocol for this study was approved by the Institutional Animal Care and Use Committee.

Diets

The experimental diets contained 40% of calories as fat with 0.26 mg cholesterol/kcal. Half of the fat calories were derived from lard or menhaden oil; the other half were from egg yolk or egg yolk replacement, a low cholesterol mixture that resembles egg yolk in composition. Diets similar to these have been used in studies of African green monkeys and detailed diet compositions have been published (12). In the present study the amount of egg yolk was reduced to give 0.26 mg cholesterol/kcal of diet because cynomolgus monkeys are more responsive to dietary cholesterol than are African green monkeys (1). Processed menhaden oil was obtained from the Southeast Fisheries Center (Charleston, SC) through the Nutrition Committee Fish Oil Test Material Program. Alpha-tocopherol, Tenox GT-1 (mixture of tocopherols; Eastman Chemical Products, Kingsport, TN) and Tenox 20A (tertiary butyl-hydroquinone, Eastman Chemicals) were added to the lard diet to a final concentration of 16.4 mg, 13.2 mg, and 11 mg/100 g diet, respectively, to balance the amounts contained in the fish oil diet (antioxidants were added to processed fish oil). All other dietary constituents were similar to those given in our previous publication (12). Diets were made in 10-kg batches and were stored frozen until needed. Prior to feeding, the diets were allowed to thaw overnight at 4°C and the animals were fed 30-min meals (15 g diet/kg body wt per meal) twice daily. The fish oil group consumed, on average, 7.4 g n-3 fatty acids/day (7.9 g n-3 fatty acids/1000 calories).

Lipoprotein analyses

Blood samples were taken from animals after an overnight (18 h) fast. Ketamine hydrochloride (10 mg/kg) was used to restrain each animal while blood was taken from the femoral vein into chilled tubes (4°C) containing 0.1% EDTA and 0.02% NaN₃ (final concentrations) at pH 7.4. Lipoproteins ($d < 1.225$ g/ml) were isolated from plasma by ultracentrifugation and fractionated by size using a 1.6×50 cm Superose 6B HPLC column (13). A Rainin HPLC system equipped with an ISCO ultraviolet detector and interfaced with a Macintosh computer were used for the lipoprotein separations. Lipoproteins were eluted

from the Superose column at a rate of 1 ml/min with 0.9% NaCl, 0.01% EDTA, and 0.01% NaN₃, pH 7.4. The column was calibrated with LDL of known molecular weight (14) and a standard curve that related elution volume to LDL molecular weight (i.e., particle size) was used for the samples of this study.² Cholesterol distribution of the isolated lipoproteins and chemical compositions of LDL were performed as described previously (5). LDL cholesteryl ester (CE) and phospholipid (PL) fatty acid compositions were determined by gas-liquid chromatography. Briefly, the LDL were extracted with chloroform-methanol 2:1, the CE and PL were separated by thin-layer chromatography (TLC; ref. 5), and the separated lipids were methylated for fatty acid analysis by the procedure of Metcalfe and Schmitz (15). The fatty acid methyl esters were separated on a 30 m × 0.25 mm (I.D.) DB-225 column (J & W Scientific, Folsom, CA) using a Hewlett-Packard model 5890A gas-liquid chromatograph equipped with a cool on-column capillary injector, automatic sampler, flame ionization detector, and Hewlett Packard 3396 integrator. The column had a 0.25- μ m film thickness and a 1 m × 0.53 mm I.D. retention gap. During fatty acid separation the column temperature was increased at a rate of 1°C/min from 165° to 220°.

LDL PL headgroup distribution was determined by TLC. Aliquots of chloroform-methanol-extracted LDL (~400 μ g PL) were applied to TLC plates (Silica Gel 60) and the PL species were separated using a chloroform-methanol-acetic acid-water 65:45:12:6 solvent system. The individual PL classes were visualized with I₂ vapor, scraped separately, and eluted from the silica gel using 2 × 2 ml of chloroform-methanol-water 1:2.5:0.5. Inorganic phosphorus was then assayed (16) and the percentage distribution of PL classes was calculated.

Plasma apolipoproteins A-I, B, and E were assayed using an enzyme-linked immunosorbent (ELISA) assay as described previously (17, 18). Transition temperatures of LDL were determined by differential scanning calorimetry (DSC) using a Dupont 1090 Thermal analyzer (Dupont Instruments, Wilmington, DE) or a Microcal ultrasensitive DSC (MicroCal, Inc., Northampton, MA). Details of the DSC analysis have been given previously (5). Samples analyzed with the Microcal DSC did not need to be concentrated before analysis because of the increased sensitivity of the instrument compared to the Dupont DSC. Identical transition temperatures were obtained for a subset of LDL samples analyzed with both instruments.

²LDL is not a molecule but an aggregate of different molecules of lipid and protein that form discrete particles. The term "LDL molecular weight" is used to refer to the mass (grams) of LDL lipid and protein constituents per μ mole of LDL particles.

Data analysis

Values are presented as mean \pm standard error of the mean. In most cases the data from both phases were averaged by diet group. A paired *t*-test was used for statistical analysis.

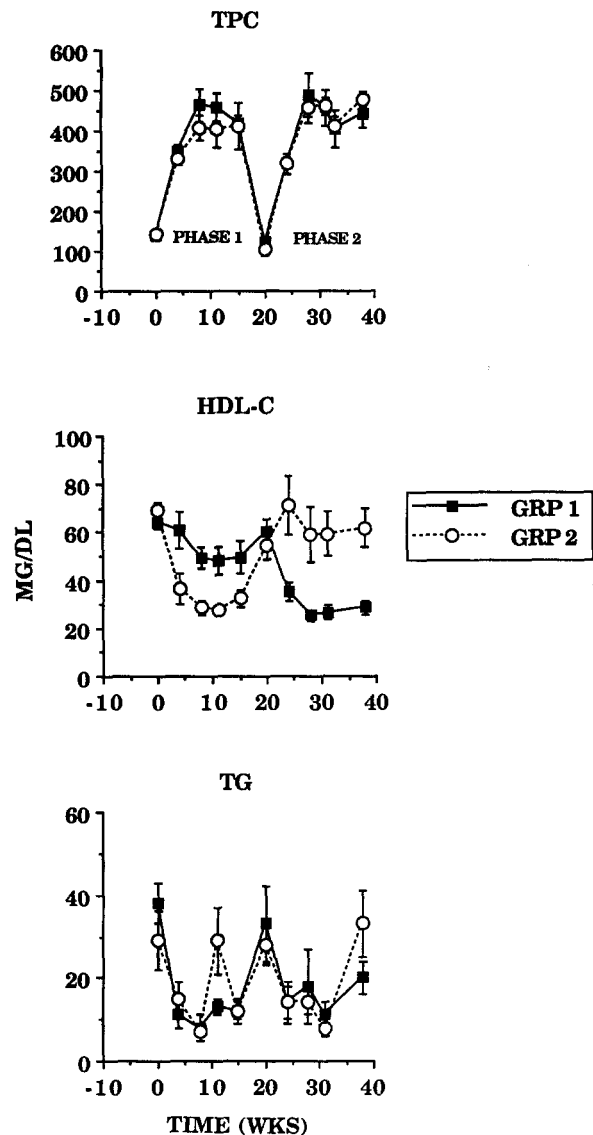


Fig. 1. Total plasma cholesterol (TPC), HDL cholesterol (HDL-C), and plasma triglyceride (TG) concentrations of cynomolgus monkeys consuming lard and fish oil diets. Two groups of five animals each were chosen so that TPC, HDL-C, and plasma TG concentrations were not significantly different between groups while they were consuming a monkey chow diet supplemented with egg yolk (0.4 mg/cholesterol/kcal; 40% of calories as fat). After an 8-wk monkey chow washout period, the animals were started on the experimental diets at time 0. Group 1 received the lard diet during phase 1 and the fish oil diet during phase 2; Group 2 received the same diets in opposite order. There was a monkey chow washout phase between phases 1 and 2 (wk 16–21) to bring plasma lipids back to baseline values. Values are mean \pm SEM (n = 5).

RESULTS

TPC, HDL-C, and TG concentrations determined periodically during the study are shown in Fig. 1. While consuming monkey chow (time 0) the animals of groups 1 and 2 had TPC concentrations of 139 ± 10 and 141 ± 9 mg/dl, respectively. TPC values increased rapidly after initiation of the experimental diets for both groups and had stabilized at 400–460 mg/dl by week 8. During the monkey chow wash-out phase (weeks 16–21) TPC values returned to baseline. Results similar to those of Phase 1 were obtained for TPC concentrations when the animals were “crossed over” to the opposite diet during Phase 2 of the study. At no time during the study were the TPC concentrations different between the two groups.

HDL-C concentrations decreased when the animals were switched from monkey chow at time 0 to the experimental diets and they were significantly lower for the animals fed fish oil (Group 2 in Phase 1); at week 11 the values were 48 ± 6 (lard) versus 28 ± 2 mg/dl (fish oil). The animals fed the lard diet during Phase 1 (Group 1) showed a decrease in HDL-C levels when switched to the fish oil diet in Phase 2; the opposite trend was shown by the animals in Group 2; i.e., an increase in HDL-C when switched from the fish oil to the lard diet.

Plasma TG concentrations were variable throughout the study for both groups and were low compared to those of humans. There was no apparent difference in plasma TG concentrations between the two groups during Phase 1 or Phase 2 of the study.

Plasma lipoprotein cholesterol distribution for animals consuming the two diets is shown in Table 1. Large blood samples were taken from each animal at 15 weeks (Phase 1) and 33 weeks (Phase 2) for lipoprotein isolation and characterization. The data in Table 1 are combined by diet for both phases of the study. Total plasma, VLDL + ILDL, and LDL cholesterol concentrations were not affected by the substitution of fish oil for lard in the diet. However, HDL cholesterol concentrations were significantly lower when the animals were consuming the fish oil diet compared to the lard diet.

TABLE 1. Plasma lipoprotein cholesterol distribution for cynomolgus monkeys consuming diets containing lard or fish oil

Diet	Plasma Cholesterol Concentration			
	Total	VLDL + ILDL	LDL	HDL
	<i>mg/dl</i>			
Lard (n = 10)	416 ± 14^a	29 ± 5	331 ± 17	57 ± 8
Fish oil (n = 10)	408 ± 35	24 ± 4	356 ± 34	28 ± 2
P Value ^b	NS	NS	NS	0.009

^aValues are mean \pm SEM.

^bPaired *t*-test; NS, not significant at $P = 0.05$.

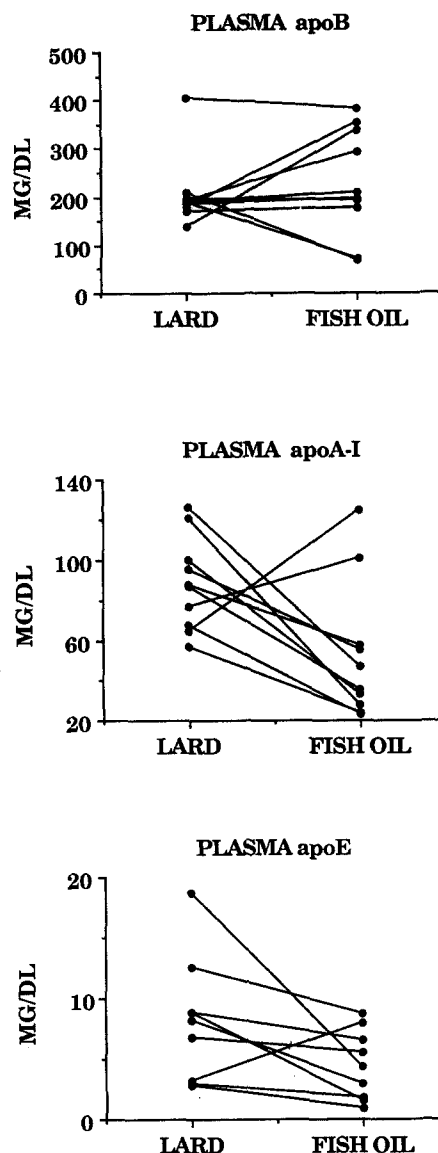


Figure 2. Plasma apolipoprotein concentrations of cynomolgus monkeys consuming a lard or fish oil diet. Plasma samples for enzyme-linked immunosorbent assay of the apolipoproteins were collected at weeks 15 and 33 (see Fig. 1). Values from both phases of the study for each animal are shown. The mean \pm SEM for the lard and fish oil diet, respectively, are as follows: apoB, 205 ± 23 vs. 227 ± 35 ; 88 ± 7 vs. 53 ± 11 , $P=0.04$ and apoE, 8.2 ± 1.5 vs. 4.2 ± 0.9 , $P=0.03$.

Plasma concentrations for apolipoproteins B, A-I, and E are shown in Fig. 2 for individual animals during the study. Apolipoprotein concentrations were determined on the plasma samples taken for lipoprotein analyses during each phase of the study (i.e., weeks 15 and 33). Plasma apoB concentrations were not significantly affected by the type of dietary fat (205 ± 23 [lard] vs. 227 ± 35 mg/dl [fish oil]). However, 8 of 10 animals had lower plasma apoA-I concentrations when consuming the fish oil compared to the lard diet (53 ± 11 vs. 88 ± 7 mg/dl; respectively; $P = 0.04$). In addition, 9 of 10 animals consuming

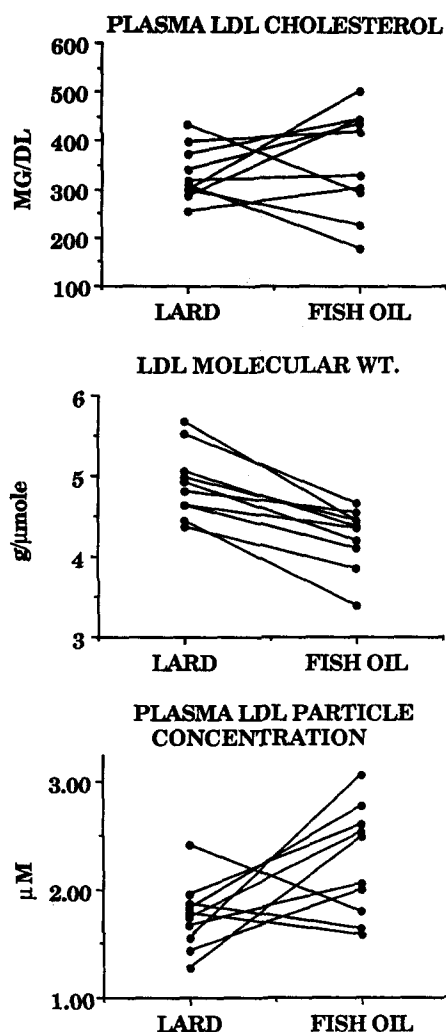


Fig. 3. Plasma LDL cholesterol concentration, LDL molecular weight, and LDL particle concentration of cynomolgus monkeys fed a lard or fish oil diet. Analyses were done at weeks 15 and 33 of the study; values from both phases of the study for each animal are shown. The mean \pm SEM for the lard vs. fish oil diet, respectively, are as follows: LDL cholesterol, 331 ± 17 vs. 356 ± 34 ; LDL molecular weight, 4.90 ± 0.13 vs. 4.23 ± 0.12 , $P < 0.0001$ and LDL particle concentration, 1.8 ± 0.1 vs. 2.3 ± 0.2 , $P < 0.0004$.

the fish oil diet had lower concentrations of plasma apoE (4.2 ± 0.9 [fish oil] vs. 8.2 ± 1.5 mg/dl [lard]; $P = 0.03$).

The effect of the fish oil diet on LDL particle size and concentration is shown in **Fig. 3**; plasma LDL cholesterol concentration is also shown for reference. Although plasma LDL cholesterol concentration was not affected by the type of dietary fat (**Table 1** and **Fig. 3**), LDL particle size, measured as LDL molecular weight,² was smaller for all animals during consumption of the fish oil diet (4.23 ± 0.12 vs. 4.90 ± 0.13 g/ μ mol; $P < 0.0001$). LDL particle concentration was significantly higher when the animals were consuming the fish oil versus lard diet (2.3 ± 0.2 vs. 1.8 ± 0.1 μ M; $P < 0.0004$) as 7 of 10 animals showed an increase in the number of LDL particles in plasma.

Detailed compositions of plasma LDL were performed to determine the effect of dietary lard versus fish oil on LDL chemical composition. The data are shown in **Table 2** in terms of molecules of each chemical constituent per LDL particle. There was no diet-induced difference in the content of protein or TG of LDL. However, while consuming the fish oil diet, the monkeys had significantly fewer PL, FC, and CE molecules per LDL particle compared to the results obtained during lard diet feeding. The LDL weight percentage chemical composition is given in parentheses for reference. The only statistically significant differences detected were a higher percentage protein ($P = 0.0096$) and triglyceride ($P = 0.048$) when the animals were consuming the fish oil diet.

The effect of diet on LDL CE fatty acid composition was determined by gas-liquid chromatography (**Table 3**). Cholesteryl oleate and cholesteryl linoleate were the major CE species of LDL regardless of the type of dietary fat. During fish oil feeding, cholesteryl eicosapentaenoate became a major CE species in LDL and there were significantly more CE molecules containing 16:0, 20:5-(n-3), 22:5 (n-3), and 22:6 (n-3) compared to when the animals were consuming the lard diet. However, fewer CE mole-

TABLE 2. Chemical composition of plasma LDL^a

Dietary Fat	Amino Acids	molecules/LDL particle				LDL Molecular Weight ^b	
		PL	TG	FC	CE	g/ μ mol	
Lard (n = 10)	8600 ± 130 (19.4 \pm 0.3) ^c	1275 ± 30 (20.2 \pm 0.2)	45 ± 4 (0.8 \pm 0.1)	1136 ± 74 (8.9 \pm 0.5)	3732 ± 142 (50.7 \pm 0.7)	4.90 ± 0.13	
Fish oil (n = 10)	8300 ± 120 (21.6 \pm 0.8)	1074 ± 42 (19.6 \pm 0.4)	66 ± 10 (1.5 \pm 0.3)	790 ± 67 (7.2 \pm 0.6)	3191 ± 156 (50.1 \pm 1.4)	4.23 ± 0.12	
<i>P</i> Value ^d	NS	0.0001	NS	0.015	0.019	0.0001	

^aMean \pm SEM. To calculate molecules per particle the following molecular weight values were used: amino acids, 110; PL (phospholipid), 775; TG (triglyceride), 900; FC (free cholesterol), 386; and CE (cholesteryl ester), 667.

^bLDL is not a molecule but an aggregate of different molecules of lipid and protein that form discrete particles. The term "LDL molecular weight" is used to refer to the mass (grams) of LDL lipid and protein constituents per μ mole of LDL particles.

^c% Composition (wt) given in parentheses.

^dPaired *t*-test for molecules/LDL particle composition and LDL molecular weight; NS, not significant at $P = 0.05$.

TABLE 3. Cholesteryl ester fatty acid composition of cynomolgus monkey plasma LDL

Cholesteryl Ester Fatty Acid	Cholesteryl Ester Molecules/LDL		P Value ^c
	Lard (n = 10)	Fish Oil (n = 10)	
16:0	295 ± 22 ^a	(7.7) ^b	360 ± 27 (11.0) 0.04
16:1	53 ± 6	(1.4)	68 ± 11 (2.1) NS
18:0	345 ± 28	(9.2)	178 ± 15 (5.5) 0.001
18:1	1589 ± 102	(42.4)	969 ± 61 (30.3) 0.0001
18:2 (n-6)	1133 ± 52	(30.4)	709 ± 22 (22.4) 0.0001
20:4 (n-6)	107 ± 6	(3.0)	119 ± 8 (4.0) NS
20:5 (n-3)	10 ± 6	(0.3)	499 ± 49 (16.0) 0.0001
22:5 (n-3)	2 ± 1	(0.1)	5 ± 1 (0.2) 0.03
22:6 (n-3)	10 ± 2	(0.3)	67 ± 7 (2.2) 0.0001
Other	251 ± 49	(5.2)	208 ± 49 (6.3) NS

^aMean ± SEM.^bValue in parentheses is mean weight percentage of individual CE fatty acids.^cPaired *t*-test; NS, not significant at *P* = 0.05.

cules containing 18:0, 18:1, and 18:2 were present in LDL during fish oil feeding. Cholesteryl arachidonate content of LDL was unaffected by diet.

The effect of the fish oil diet on LDL PL headgroup and fatty acid distribution was also determined. **Table 4** contains the results of the LDL PL fatty acid analysis. The substitution of fish oil for lard in the diet had no effect on the content of 16:0, 16:1, and 18:0 in LDL PL. Dietary fish oil resulted in a significant increase in the percentage of n-3 fatty acids (20:5, 22:5, and 22:6) compared to lard and a significant reduction in the percentage of 18:1, 18:2, 20:4, and 22:5 (n-6) in plasma LDL PL. The largest changes in LDL PL composition due to consumption of the lard versus fish oil diet occurred for 18:2 (26% vs. 10%) and 20:5 (1% vs. 17%).

The results of the LDL PL headgroup distribution as a function of diet are shown in **Table 5**. LDL analyzed during fish oil feeding had significantly more LPC and SM and less PC than did LDL isolated during feeding of the lard diet. The percentage of LDL PS + PI and PE was not affected by the substitution of fish oil for lard in the diet.

We next investigated the effect of the experimental diets on the transition temperature of LDL CE, which has been shown in previous studies to depend on the CE fatty acid composition and the CE/TG ratio of LDL (4, 5). The transition temperature is indicative of a liquid crystalline to liquid transition of the CE of LDL (19). **Table 6** lists the CE peak transition temperature of LDL, determined by differential scanning calorimetry, the ratio of saturated and monounsaturated to polyunsaturated CE species (CE fatty acid ratio), and the CE/TG weight ratio. The average peak melting temperature of LDL CE was reduced by 11°C with the substitution of fish oil for lard in the diet (43.5° vs. 32.3°C). The LDL CE fatty acid ratio and the CE/TG ratio also were significantly reduced when the animals were consuming the fish oil diet.

To better understand the effect of the experimental diets on LDL size and CE composition, we categorized the LDL CE species as saturated (18:0, 16:0), monounsaturated (16:1, 18:1), n-6 polyunsaturated (18:2, 20:4), and n-3 polyunsaturated (20:5, 22:5, 22:6) and plotted the number of CE in each category against LDL size (i.e., LDL molecular weight; **Fig. 4**). Because the number of n-3 CE species was <1% of the total CE in LDL when animals were consuming the lard diet (Table 3), these data were omitted from the graph. Each point represents data from an individual animal and data from each of the 10 animal are presented twice, so that values during both the lard and fish oil feeding phase are shown. There was a statistically significant relationship between LDL size and the number of saturated CE (top panel; *r* = 0.77) as well as the number of monounsaturated CE species (middle panel; *r* = 0.95) and n-6 polyunsaturated CE species (bottom panel; *r* = 0.69). However, by comparing the slopes of the graphs it was evident that for a unit increase

TABLE 4. Phospholipid fatty acid composition of cynomolgus monkey plasma LDL

Fatty Acid	Lard (n = 10)	Fish Oil (n = 10)	P Value ^b
	<i>wt %</i>		
16:0	15.7 ± 1.2 ^a	15.7 ± 1.2	NS
16:1	0.7 ± 0.1	0.5 ± 0.1	NS
18:0	21.7 ± 0.6	23.2 ± 0.8	NS
18:1	12.0 ± 0.4	8.6 ± 0.2	0.0001
18:2 (n-6)	25.6 ± 0.8	9.6 ± 0.4	0.0001
20:4 (n-6)	10.3 ± 0.4	6.5 ± 0.2	0.0001
22:5 (n-6)	0.6 ± 0.1	0.1 ± 0.03	0.0005
20:5 (n-3)	1.0 ± 0.4	16.8 ± 1.3	0.0001
22:5 (n-3)	0.9 ± 0.1	2.2 ± 0.2	0.002
22:6 (n-3)	3.0 ± 0.4	9.2 ± 0.8	0.0005
Other	8.2 ± 0.6	7.5 ± 0.8	NS

^aMean ± SEM.^bPaired *t*-test. NS, not significant at *P* = 0.05.

TABLE 5. Phospholipid class percentage distribution of cynomolgus monkey plasma LDL

Diet	LPC	SM	PC	PS + PI	PE
Lard (n = 10)	5.3 ± 1.0 ^a	16.6 ± 0.6	71.9 ± 1.1	4.1 ± 0.6	2.1 ± 0.2
Fish Oil (n = 10)	12.9 ± 0.9	22.2 ± 0.7	58.9 ± 0.8	4.2 ± 0.3	1.7 ± 0.2
<i>P</i> Value ^b	0.0016	0.0003	0.0001	NS	NS

^aMean ± SEM. LPC, lysophosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PS + PI, phosphatidylserine + phosphatidylinositol; and PE, phosphatidylethanolamine.

^bPaired *t*-test. NS, not significant at *P* = 0.05.

in LDL molecular weight, there was a 3- to 4-fold greater increase in the number of monounsaturated CE molecules per LDL compared to saturated CE. There was no relationship between LDL molecular weight and the number of n-3 polyunsaturated CE species in LDL when animals consumed the fish oil diet (bottom panel). Furthermore, the total number of polyunsaturated CE species (n-3 and n-6) in LDL was not influenced by substitution of fish oil for lard in the diet (1400 ± 77 [fish oil] vs. 1263 ± 56 [lard] molecules/LDL).

The relationship between the number of CE species per LDL particle and the LDL CE transition temperature is shown in Fig. 5. In general, there was an increase in the number of saturated, monounsaturated, and n-6 polyunsaturated CE species with increasing LDL transition temperature (*T*_m). There was no apparent change in the number of n-3 CE molecules with increasing LDL transition temperature when animals were fed the fish oil diet; a similar comparison could not be made when the animals were consuming the lard diet because few n-3 CE were present in the plasma LDL (Fig. 5, Table 3). Two interesting points can be made from the data in Fig. 5. First, the transition temperatures were consistently lower when the animals were consuming the fish oil diet, and second, the monounsaturated CE species likely made the greatest contribution to the range of transition temperatures observed for the LDL of individual animals since the number of monounsaturated CE ranged from 660 to 2270 among LDL while the range of values for saturated CE was much smaller (420–820).

DISCUSSION

In a study designed to use each animal as its own control, we investigated the effect of isocaloric substitution of dietary fish oil for lard on plasma lipids, apolipoproteins and on the physical and chemical properties of LDL. Dietary fish oil significantly lowered plasma HDL cholesterol, apoA-I, and apoE relative to lard but had no effect on plasma total or LDL cholesterol, apoB, or triglyceride concentrations. Although LDL cholesterol concentrations were unaffected by diet, there were more LDL particles of smaller size when the fish oil diet was

fed; these LDL had fewer PL, FC, and CE molecules per particle, were enriched in n-3 fatty acids and relatively poor in 18:1 and 18:2 in both the CE and PL fractions, had less PC and relatively more LPC and sphingomyelin, and had lower CE transition temperatures. Thus, both surface and core constituents of LDL were altered by dietary fish oil. These data suggest that the physical, chemical, and biological properties (see below) can be markedly altered by the substitution of dietary fish oil for lard without affecting the concentration of plasma LDL cholesterol and emphasize the importance of investigating more than just the plasma LDL cholesterol-lowering effect of dietary constituents that potentially may be used to reduce atherosclerotic risk.

We hypothesize that modifications in particle composition induced by the lard diet result in LDL that are more atherogenic compared to the fish oil diet for several reasons. Results from previous studies have shown that LDL size is one of the most powerful predictors of coronary artery atherosclerosis in nonhuman primates (1–3). The larger LDL that result when the lard diet is fed presumably can deliver more cholesteryl ester per particle to cells or connective tissue in the arterial wall compared to the smaller LDL particles isolated from animals fed the fish oil diet. This point is supported by cell culture studies where larger LDL resulted in more cellular CE accumulation compared to smaller LDL when equivalent concentrations of LDL cholesterol were incubated with cells (20). The LDL from animals fed lard have more saturated CE, which exist in a liquid crystalline state at body tempera-

TABLE 6. Physical and chemical characteristics of cynomolgus monkey LDL

Diet	<i>T</i> _m (°C)	CE Fatty Acid Ratio	CE/TG Ratio (wt)
Lard (n = 10)	43.5 ± 0.5 ^a	1.84 ± 0.12	67.8 ± 8.3
Fish Oil (n = 10)	32.3 ± 0.9	1.15 ± 0.09	44.5 ± 7.8
<i>P</i> Value ^b	0.0001	0.0002	0.0001

^aMean ± SEM. *T*_m, peak transition temperature of LDL CE obtained upon heating the LDL samples from 5° to 65°C at a rate of 5°/min in a differential scanning calorimeter. CE fatty acid ratio, ratio of (saturated + monounsaturated)/polyunsaturated CE species.

^bPaired *t*-test.

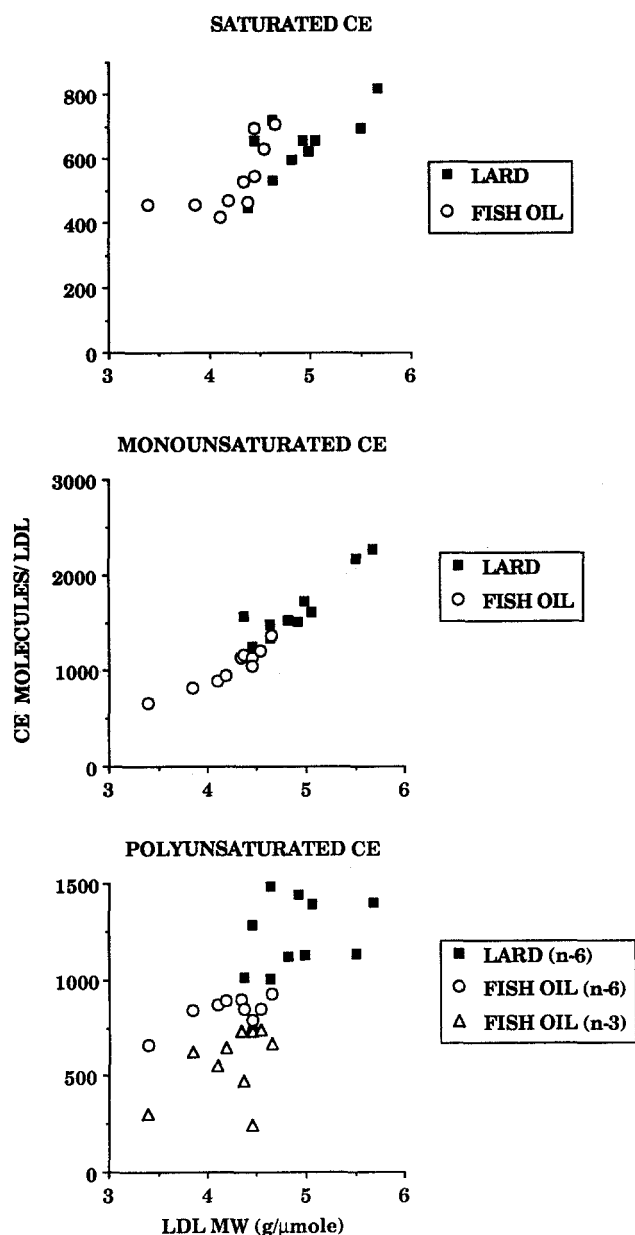


Fig. 4. Relationships between LDL molecular weight and the number of saturated CE (18:0, 16:0), monounsaturated CE (18:1, 16:1), n-6 polyunsaturated (18:2, 20:4), and n-3 polyunsaturated CE (20:5, 22:5, 22:6) molecules per LDL particle. Values from both phases of the study for each animal are shown. Because the number of n-3 CE species was <1% of the total CE in LDL when animals were consuming the lard diet, these data were omitted from the graph. Correlation coefficients for each graph are as follows: saturated CE, $r = 0.77$, monounsaturated CE, $r = 0.95$, and n-6 polyunsaturated CE, $r = 0.69$. There was no significant correlation between LDL molecular weight and the number of n-3 CE when the animals were fed the fish oil diet.

ture (5). We have shown a direct correlation between the saturation of LDL CE (i.e., CE fatty acid ratio) and the amount of free cholesterol ($r = 0.63$) and esterified ($r = 0.59$) cholesterol measured in the abdominal aortas of African green monkeys at necropsy (10). In cell culture studies, liquid crystalline CE was found to have a slower

rate of efflux compared to CE in the liquid state (21). Both of these studies suggest that LDL with liquid crystalline CE cores are more atherogenic. In preliminary studies we have found that LDL derived from cynomolgus monkeys fed the lard diet bind isolated arterial proteoglycans 3-5 times more avidly than do LDL isolated from the same animals fed the fish oil diet (22). All of these data are consistent with the hypothesis that the isocaloric substitution of fish oil for lard will reduce the atherogenic potential of LDL.

LDL exhibit a transition temperature that can be monitored by DSC and is indicative of a liquid crystalline to liquid transition of the core CE of the particle (4, 5, 19). Results from past studies have shown that the transition temperature of LDL CE is proportional to the ratio of saturated and monounsaturated to polyunsaturated CE species (CE fatty acid ratio) and to the CE to TG ratio (4, 5, 19). LDL CE transition temperatures were significantly higher when the animals in this study were consuming the lard versus the fish oil diet and there was a significant association between the transition temperature and the CE fatty acid ratio as well as the CE to TG ratio. However, the variation in the CE to TG ratio could explain <1°C of the 11°C difference in CE transition temperature between LDL isolated during lard versus fish oil feeding, based on results from model systems (19). Therefore, relationships among the different species of CE and transition temperatures were analyzed (Fig. 5). Although there was a significant positive trend between LDL CE transition temperature and the number of saturated, monounsaturated, and n-6 polyunsaturated CE species, the increase in the number of n-6 polyunsaturated and saturated CE species probably played a minor role in determining the range of transition temperatures observed for individual animals. Cholesteryl linoleate, the major n-6 polyunsaturated CE of these LDL (Table 3), has a liquid crystalline to liquid transition temperature that is 11-12°C below that of the LDL obtained after feeding the lard diet and, as such, could only contribute to a lowering of the mean transition temperature of the LDL isolated when the lard diet was fed to the animals. The number of saturated CE species was one-third to one-half that of the monounsaturated CE species and did not demonstrate as much variability in number with increasing LDL size (Fig. 4). Taken together, these data suggest that the primary determinants of LDL CE transition temperature for the animals of this study were the number of monounsaturated and n-3 polyunsaturated CE species. The range of transition temperatures appeared to be determined, in large part, by the number of monounsaturated CE, which showed the greatest increase in number with increasing LDL size (Fig. 4) and the n-3 polyunsaturated CE, which did not vary in number with LDL size (Fig. 4), but uniformly lowered the transition temperature of the particle. We

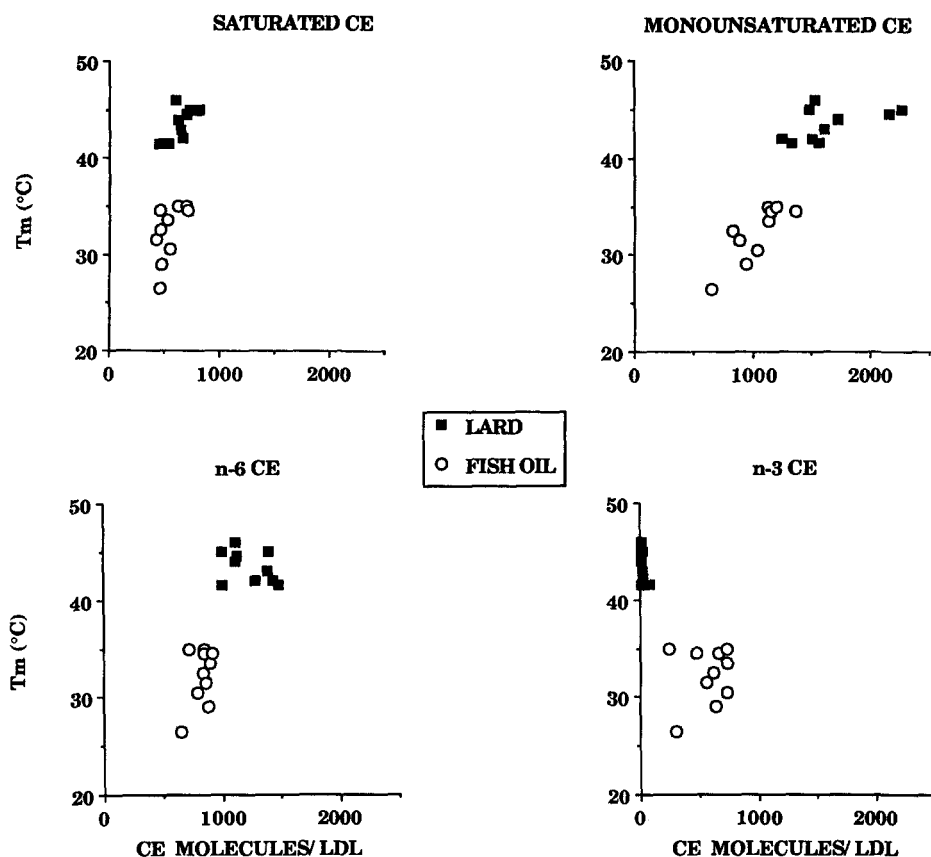


Fig. 5. Relationships between the number of CE species per LDL particle and the CE transition temperature (T_m) of LDL. Values from both phases of the study for each animal are shown.

have previously shown in a binary phase model system that increasing amounts of cholesteryl docosahexaenoic acid will lower the transition temperature of cholesteryl linoleate in a manner similar to that described for TG (5, 19). This effect is due to the ability of a low melting constituent (i.e., n-3 CE, triolein) to disrupt the crystalline or liquid crystalline state of a higher melting constituent (i.e., cholesteryl oleate, cholesteryl linoleate) (5, 19). Therefore, we conclude that factors that determine the number of monounsaturated and n-3 polyunsaturated CE molecules in plasma LDL are the most important in determining the range and absolute value of the LDL transition temperatures.

We also noted that the transition temperature of LDL CE was $\sim 6^\circ\text{C}$ higher for cynomolgus monkeys compared to that observed in a previous study of African green monkeys regardless of the type of dietary fat (5). This difference appears to reflect the increased number of cholesteryl oleate molecules in LDL of cynomolgus monkeys (~ 600 more) compared to that of the African green monkey since the number of saturated CE was similar for the two species within each dietary fat group and the number of n-3 and n-6 polyunsaturated CE was

actually higher in LDL of cynomolgus monkeys. These data again suggest an important role of cholesteryl oleate as a major determinant of LDL CE transition temperature.

Our data suggest a role for the liver in determining the atherogenic potential of plasma LDL. There is a strong correlation between hepatic content of CE ($r = 0.9$, ref. 11) and plasma LDL size, which is correlated with the CE fatty acid ratio and the transition temperature of LDL (5). If transition temperature is an important atherogenic feature of plasma LDL, as we have hypothesized, then the liver may also play an important role in lowering the transition temperature of LDL when fish oil is fed by the increased secretion of n-3 polyunsaturated CE in hepatic VLDL, which can be catabolized to plasma LDL (11). Although the lecithin:cholesterol acyltransferase (LCAT) reaction may be responsible for some increase in the number of LDL n-3 polyunsaturated CE in plasma, we speculate that this will be a small contribution compared to that of the liver inasmuch as we have shown, using an in vitro assay system, that plasma phospholipids derived from monkeys eating diets containing fish oil were poor substrates for the LCAT reaction (23). These studies sug-

gest that a thorough knowledge of hepatic CE metabolism is necessary to understand the role of LDL in atherosclerosis development.

The plasma lipid and lipoprotein response to isocaloric substitution of dietary fish oil for lard was different for African green monkeys in a previous study (5) compared to the cynomolgus monkeys of this study. As indicated in the Methods section the diets for these two studies were very similar except for the reduction in dietary cholesterol in the present study so that the range of TPC values was 200–400 mg/dl for both studies. In the previous study it took 5 months of diet consumption before the group fed fish oil had significantly lower TPC and LDL-cholesterol concentrations compared to the group fed the lard diet (10). However, in the present study there was no apparent difference in TPC or LDL cholesterol throughout the entire study and we have continued to monitor the animals consuming their Phase 2 diets for up to 7 months and have found no change in these measurements. Among the nonhuman primate species investigated, cynomolgus monkeys tend to be the most responsive to dietary-induced hyperlipidemia while African green monkeys are among the least responsive. Results from preliminary studies have suggested that cholesterol absorption is decreased in African green monkeys consuming fish oil versus lard in the diet while no difference was found between diet groups for the cynomolgus monkeys of this study during Phase 1 (M. Anthony, J. R. Crouse, J. S. Parks, unpublished data). In addition, hepatic cholesterol content, which is highly correlated with VLDL cholesterol accumulation rate during liver perfusion ($r = 0.85$; ref. 11) and is lower in African green monkeys fed fish oil compared to lard, may not be affected by substitution of dietary fish oil for lard in cynomolgus monkeys. Contrary to the cholesterol response, the effects of dietary fish oil on plasma apolipoproteins concentrations were similar for both species of nonhuman primates (i.e., lower apoA-I and apoE with fish oil feeding but no change in apoB).

Plasma TG concentrations for the cynomolgus monkeys of this study were low compared to those of human beings and were not affected by dietary fish oil (Fig. 1). In a previous study African green monkeys fed a fish oil-containing diet had plasma TG concentrations that were slightly but significantly higher than those of animals fed the lard diet (10). Nonhuman primates fed high fat diets apparently have low plasma TG concentrations due to a higher amount of post-heparin plasma lipolytic activity and a higher ratio of lipoprotein lipase to hepatic lipase compared to human beings (24). Human beings respond to fish oil with a lowering of plasma TG which is different from our findings in nonhuman primates (10, 25). However, we have shown that secretion of TG from perfused livers is approximately 50% lower in African green monkeys fed fish oil compared to those fed lard (11,

26). Therefore, the influence of dietary fish oil on plasma TG concentrations in nonhuman primates apparently reflects the efficient clearance of plasma TG more than the decrease in hepatic production.

At equivalent TPC concentrations cynomolgus monkeys have larger LDL particles than do African green monkeys (8). However, both species respond to dietary fish oil with a reduction in LDL size (Table 2) (5, 10). In cynomolgus monkeys, but not African green monkeys, plasma LDL particle concentration was increased 28% with fish oil versus lard diet consumption and plasma apoB concentrations were 11% higher on average (Fig. 2). Since the protein content per LDL particle was not affected by the substitution of dietary fish oil for lard, these results seem contradictory. However, there may be two possible explanations for this outcome. First, there may be more of the total plasma apoB distributed in the LDL fraction during the feeding of the fish oil versus lard diet. Second, we observed a high correlation between whole plasma apoE and LDL molecular weight ($r = 0.84$) in the cynomolgus monkeys (data not shown). Since the LDL molecular weight values were higher when the lard diet was fed, it is likely that a substantial amount of apoE in whole plasma was associated with the LDL fraction. In fact, a study has shown that LDL preferentially acquire the increased amount of apoE in the plasma of nonhuman primates that are hyperresponsive to dietary cholesterol (27) and that the apoE is mainly associated with the larger, lighter LDL subfractions (8). Therefore, a greater proportion of the LDL protein was likely to be apoE rather than apoB when the animals were consuming the lard diet. If this speculation is correct, then the monkeys fed the fish oil diet would have a greater number of smaller LDL particles that are relatively poor in apoE compared to lard-fed counterparts. Studies are now underway to better define the effect of dietary fish oil on the apoE and apoB content of LDL subfractions.

Dietary fish oil was found to alter not only the fatty acid composition of LDL PL but also the PL headgroup distribution in the cynomolgus monkeys of our study. To our knowledge this is the first report of an effect of dietary fish oil on LDL PL headgroup distribution. The metabolic significance of this change and its effect on LDL particle structure is unknown. Presumably, the change in PL headgroup of LDL with diet reflects a change in hepatic phospholipid production; however, since there was an increase in LPC and a decrease in PC content of LDL during fish oil consumption, the possibility of increased hydrolysis of PC cannot be eliminated. Further studies are needed to understand how dietary fish oil affects hepatic and LDL PL metabolism and how the changes induced in LDL PL affect particle structure. ■

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