Effect of fish oil versus lard diets on the chemical and physical properties of low density lipoproteins of nonhuman primates

John S. Parks* and Bill C. Bullock

Department of Comparative Medicine, Bowman Gray School of Medicine, Wake Forest University Medical Center, 300 South Hawthorne Road, Winston-Salem, NC 27103

Abstract Twenty-four adult male African green grivet monkeys were fed diets containing 42% of calories as lard or menaden oil and 0.76 mg of cholesterol/kcal for a period of 8 months. Plasma samples from fasting animals were then taken and low density lipoproteins (LDL) were isolated by ultracentrifugation and agarose column chromatography. The LDL were analyzed chemically, and physical properties of the particles were studied by differential scanning calorimetry. The fish oil group had significantly smaller LDL (2.91 vs. 3.43 g/μmol), which contained fewer molecules per particle of all lipid constituents, except triglyceride, compared to the lard-fed animals. The fish oil-fed group had 15% of the total cholesteryl esters as n-3 fatty acyl species and the number of n-5, but not n-6, cholesteryl esters per LDL particle was proportional to LDL size. The numbers of saturated and monounsaturated cholesteryl ester species per LDL particle were highly correlated with LDL size for both diet groups. The LDL of the fish oil group had broad reversible thermotropic transitions that were 12-13°C lower than those of the lard group. These transitions were indicative of order-disorder transitions of the LDL core cholesteryl esters. The peak transition temperature of LDL of the lard group was proportional to the ratio of saturated and monounsaturated cholesteryl ester species (CEFA ratio). However, the much lower peak transition temperature of the LDL of the fish oil group was not related to the CEFA ratio nor to the triglyceride content of the particles, but rather, to the n-3 cholesteryl ester content of the particles. Studies of cholesteryl ester model systems demonstrated that relatively small amounts of n-3 cholesteryl esters (<15% of total cholesteryl ester) could result in a lowering of the peak transition temperature of cholesteryl linoleate similar to that seen for intact LDL. We conclude that n-3 cholesteryl esters in small quantities have a marked disordering effect on the core cholesteryl esters of LDL, resulting in a striking depression of LDL transition temperature. In addition, we conclude that n-3 cholesteryl esters are preferentially utilized relative to n-6 cholesteryl esters to increase the number of cholesteryl esters per LDL particle with LDL enlargement in fish oil-fed animals. — Parks, J. S., and B. C. Bullock. Effect of fish oil versus lard diets on the chemical and physical properties of low density lipoproteins of nonhuman primates. J. Lipid Res. 1987. 28: 175-182.

Supplementary key words n-3 fatty acids • cholesteryl esters • differential scanning calorimetry

Low density lipoproteins (LDL) are spherical emulsion particles that contain a core of nonpolar lipid (cholesteryl ester and triglyceride) stabilized by a surface of phospholipid, free cholesterol, and protein (1), and are the major carriers of cholesteryl esters in plasma. Although numerous studies have shown that high concentrations of plasma LDL in human beings and experimental animals fed dietary cholesterol are associated with premature atherosclerosis (2-5), the atherogenic properties of LDL are not well understood.

Studies of dietary cholesterol-induced atherosclerosis in several species of nonhuman primates have shown that the size of the LDL particle (termed LDL molecular weight) is the single most important predictor of coronary atherosclerosis (3-5, 13). The atherogenic nature of the large LDL may be related to the chemical and physical properties of the particles. Large LDL contain proportionately more cholesteryl esters per particle than other chemical constituents (6, 7) and have been shown to deliver more cholesterol to cells in culture compared to small LDL (8-10). Thus, the large LDL may be more atherogenic because of the increased number of cholesteryl ester molecules they can deliver to cells.

Another potential atherogenic feature of LDL may relate to the physical properties of the particles. Core cholesteryl esters of LDL exhibit thermotropic transitions indicative of liquid crystalline to liquid transitions (1, 7). The transition temperature of LDL is dependent on the fatty acyl composition of the cholesteryl esters as well as the triglyceride to cholesteryl ester ratio (1). In studies of

**Abbreviations:** LDL, low density lipoproteins; DSC, differential scanning calorimetry; TLC, thin-layer chromatography; GLC, gas-liquid chromatography; CEFA, cholesteryl ester fatty acid; TG, triglyceride; CE, cholesteryl ester; C before the abbreviation of the fatty acid (e.g., 20:5) refers to cholesteryl.

*To whom reprint requests should be addressed.
nonhuman primates fed saturated fat diets, the number of saturated and monounsaturated cholesteryl ester species increased out of proportion to the number of polyunsaturated species as LDL molecular weight increased (7). This resulted in a transition temperature for the cholesteryl esters of large LDL that was above the body temperature of the animal (7). Thus, large LDL from saturated fat-fed animals had liquid crystalline cores at body temperature. This property may contribute to the atherogenic nature of LDL, since it has been shown that cells in culture loaded with liquid crystalline cholesteryl esters have lower cholesterol efflux rates than cells loaded with cholesteryl esters in the liquid (melted) state (11).

Recently, there has been increasing interest in the effect of diets rich in n-3 fatty acids on plasma lipoprotein concentrations. This interest has been stimulated by the observation that such diets are associated with a lower incidence of coronary heart disease in Eskimo populations (12). Although there are numerous studies on the effect of n-3 fatty acid-rich diets on plasma lipoprotein concentrations, few have investigated the effect of such diets on lipoprotein composition or on the physical properties of lipoproteins. This study was undertaken to compare the effect of saturated versus fish oil diets on LDL molecular weight enlargement and on the physical properties of LDL cholesteryl esters in the African green monkey, a nonhuman primate species that has been used extensively for atherosclerosis research (4, 5).

METHODS

Animals

Twenty-four adult male African green grivets (Cercopithecus aethiops aethiops) were purchased from Primate Imports (Port Washington, NY). Animals were fed diets containing 42% of the calories as fat with 11 g of lard or menhaden oil/100 g of diet. The diets contained 0.76 mg of cholesterol/kcal provided as dried egg yolk. All other aspects of the diets were similar and detailed compositions of the diets are published elsewhere (14). The animals were trained to eat 30-min meals twice daily to minimize oxidation of the dietary fatty acids.

Lipoprotein isolation

Blood samples for lipoprotein analyses were taken after the animals had consumed the experimental diets for 8 months. Details of blood sampling and lipoprotein isolation are given elsewhere (14). Briefly, lipoproteins were isolated from plasma by the combined techniques of ultracentrifugation and agarose column chromatography (15). LDL molecular weight was quantitated using this method as detailed previously (6).

Differential scanning calorimetry (DSC)

LDL cholesteryl ester transition temperatures and enthalpies were determined using a Du Pont 1090 DSC (Du Pont Instruments, Wilmington, DE) as previously described (1, 16). LDL samples were concentrated, using a combination of Amicon ultrafiltration cones and Centriflo membranes (Amicon Corp., Danvers, MA), to a final concentration of 25 to 150 mg of cholesterol/ml. Seventy-five μl of concentrated LDL was hermetically sealed in stainless-steel pans for analysis. Samples were heated from −10°C to 60°C and then cooled back to −10°C at 5°C/min. After a 10-min equilibration at −10°C, the samples were reheated to 120°C to effect thermal de-naturation and cooled back to 0°C at 5°C/min. After DSC analysis the pans were opened and the contents were extracted with 10 ml of chloroform–methanol 2:1. Aliquots of the extract were taken for total cholesterol, free and esterified cholesterol, and fatty acid quantitation as described below. Calorimetric enthalpies were quantitated from the area of expanded DSC plots, using a Hipad digitizer (Houston Instruments, Austin, TX) interfaced with a microcomputer. The DSC was standardized using cyclohexane and gallium.

DSC experiments of purified cholesteryl linoleate and cholesteryl docosahexaenoate (Nu-Chek-Prep, Elysian, MN) were also performed. Chloroform solutions were made for each of the two cholesteryl esters (10 mg/ml) and the purity of each was determined to be > 97% by thin-layer chromatography (6) and high performance liquid chromatography (17). Aliquots of the two solutions were taken and dried to a small volume before transfer to DSC pans. The aliquots taken resulted in a total of 5 mg of cholesteryl ester per pan with varying ratios of cholesteryl linoleate to cholesteryl docosahexaenoate as indicated (see Fig. 6). The remaining chloroform was evaporated from the DSC pan under a stream of N2 and the pans were vacuum-desiccated overnight at 4°C to remove trace quantities of CHCl3. The DSC pans were then hermetically sealed and were subjected to DSC analysis. After equilibration at −20°C for 10 min, the samples were heated at 5°C/min to 60°C and then cooled back to −20°C (first run). After another 10 min equilibration at −20°C, the heating and cooling cycle was repeated (second run).

Chemical analyses

Protein concentrations were determined by the method of Lowry et al. (18) using bovine serum albumin as the standard, after extraction of samples with hexane to remove turbidity. Total cholesterol was determined on intact LDL by an enzymatic procedure (19). Phospholipids were quantitated by the method of Fiske and SubbaRow (20). Extracts of LDL samples from DSC pans were quantitated
for total cholesterol using the o-phthalaldehyde method (21). Aliquots of samples extracted from DSC pans were subjected to thin-layer chromatography to separate free and esterified cholesterol and triglyceride (6). Free and esterified cholesterol extracted from the TLC plate were quantitated by the o-phthalaldehyde assay (21) and triglyceride extracts were quantitated by the method of Sardesai and Manning (22). After cholesterol quantitation, the hexane phase was discarded from the saponification tubes originally containing the esterified cholesterol and the lower phase, which contained the fatty acid soaps, was acidified using eight or nine drops of concentrated sulfuric acid. The fatty acids were extracted into 10 ml of hexane; 10 μg of heptadecanoate was added to the tubes as an internal standard and the fatty acids were methylated for GLC analysis (23).

**Gas-liquid chromatography (GLC)**

Cholesteryl ester fatty acids were quantitated using a Varian 3700 gas-liquid chromatograph (Palo Alto, CA) equipped with a 30 m x 0.25 mm fused silica column containing a liquid phase of OV351-30 N. Methylated fatty acid samples containing heptadecanoate as internal standard were dissolved in undecane and 0.5 μl containing ~2 μg of total fatty acid was injected onto the column. The gas flow rate of the column was 1 ml/min. The column temperature was held at 165°C for 1 min after sample injection, followed by a programmed 3°C/min increase to 226°C and was then maintained at 226°C for an additional 15 min. Retention times of individual fatty acids were determined using a standard mixture obtained from Nu-Chek-Prep (Elysian, MN). Recovery of individual fatty acids was quantitated using the heptadecanoate internal standard.

**Statistical analyses**

All values are reported as mean ± standard error of the mean. Data were analyzed for statistical significance by the Student's t-test (24). Graphical data were subjected to the linear regression analysis to determine the line of best fit (24).

**RESULTS**

The effects of fish oil diets on plasma lipids and lipoproteins of these animals have been given in detail elsewhere (14). Briefly, animals fed the fish oil diet had significantly lower total plasma cholesterol (133%), HDL cholesterol (33%), and apoA-I (30%) concentrations when compared to the lard-fed group. Although the fish oil diets resulted in a similar percentage reduction of LDL cholesterol concentration on average (34%), the decrease was not statistically significant. Plasma triglyceride concentrations were low (20–30 mg/dl) for animals in both diet groups but were significantly higher for the fish oil group. The lipid and apoprotein compositions of plasma LDL and HDL were similar for both diet groups. However, the fish oil group had a significantly lower mass of HDL subfractions of intermediate size (80–88 Å) and intermediate density (d 1.10–1.13 g/ml) relative to lard-fed animals.

LDL were isolated from the plasma of animals fed lard or fish oil diets by a combination of ultracentrifugation and agarose column chromatography, which allows quantitation of LDL molecular weight. These LDL were analyzed chemically and the within-particle chemical constituents were calculated (Table 1). LDL molecular weight was significantly lower in the fish oil group compared to the lard-fed group but LDL particle concentration was similar for both diet groups. There were significant differences in all LDL lipid constituents between the two groups with the number of molecules of phospholipid, cholesteryl ester, and free cholesterol per LDL particle being less in the fish oil group. In contrast, there were approximately twice as many triglyceride molecules per LDL particle in fish oil- versus lard-fed animals.

The LDL cholesteryl ester fatty acid composition for each animal was determined by gas-liquid chromatography and the results are shown in Table 2. Cholesteryl palmitate (C 16:0), oleate (C 18:1), and linoleate (C 18:2) were the predominant LDL cholesteryl esters in both diet groups. The fish oil-fed animals had 55% fewer molecules of cholesteryl oleate and cholesteryl linoleate and a significantly greater number of cholesteryl myristate (C 14:0) and cholesteryl palmitate (C 16:0) molecules per LDL particle compared to the lard-fed group. Approximately 15% of the cholesteryl esters derived from the LDL of fish oil-fed animals contained n-3 fatty acids (C 20:5, C 22:5, and C 22:6).

Since it is known that the number and fatty acyl composition of cholesteryl esters change as a function of LDL molecular weight in saturated fat-fed nonhuman primates (6, 7), we were interested in the effect of fish oil diets on the relationship between LDL size and cholesteryl ester composition. Fig. 1 shows a plot of LDL molecular weight versus molecules of cholesteryl ester species per LDL particle. The fatty acyl species of cholesteryl esters are grouped as saturated, monounsaturated, polyunsaturated (excluding n-3 fatty acids), and n-3. In both diet groups saturated and monounsaturated cholesteryl esters increased in number as LDL molecular weight increased. However, for the lard-fed monkeys, monounsaturated cholesteryl esters showed the sharpest increase with LDL molecular weight enlargement. As LDL size increased, saturated and monounsaturated cholesteryl esters increased proportionally in the fish oil group. Polyunsaturated cholesteryl esters (C 18:2 and C 20:4) showed little variation in number with LDL size, but the n-3 cholesteryl ester species (C 20:5, C 22:5, and C 22:6) did...
show a significant increase in number as LDL molecular weight increased.

**Fig. 2** shows a plot of LDL molecular weight versus the cholesteryl ester fatty acid (CEFA) ratio, that is, the ratio of saturated (Δ0) and monounsaturated (Δ1) to polyunsaturated (Δ2+) cholesteryl ester species. In this plot the n-3 cholesteryl esters are included with the polyunsaturated species. The CEFA ratio was significantly correlated with LDL molecular weight for both diet groups, although the slope of the line was less for the fish oil group compared to the lard group.

To determine the effect of fish oil diets on the interrelationships of LDL size, cholesteryl ester composition, and physical properties, we performed a series of DSC experiments. **Fig. 3** shows DSC scans for LDL from representative lard- and fish oil-fed monkeys. Upon heating the LDL samples from -10°C to 60°C, one broad endotherm was seen (Fig. 3A and E) and a single exotherm was observed when the samples were cooled from 60°C to -10°C (Fig. 3B and F). The exotherm had a peak temperature that was 10°C lower than that of the corresponding endotherm. A second heating and cooling of the samples was conducted from -10°C to 120°C to -10°C to effect LDL particle denaturation. The second heating of the samples resulted in two endotherms (Fig. 3C and G); one at low temperature (25°C–45°C), which was nearly identical to that of the first heating run, and a broad high temperature endotherm (70°C–90°C) indicative of particle denaturation (1, 7). In most cases a bimodal exotherm was seen upon cooling the denatured LDL (Fig. 3D) from 120°C to -10°C; however, only a single exotherm was seen with some samples (Fig. 3H). Note that the lower temperature endotherms and exotherms shown in Fig. 3 occurred at -10°C lower for the fish oil-fed animal compared to the lard-fed animal.

DSC analyses were performed on all LDL samples; however, two of the LDL samples from the fish oil group contained too little mass to give an adequate DSC scan. The peak transition temperature was determined for the first heating and cooling scan of each LDL sample, and these data are summarized in **Table 3**. The peak transition temperature is indicative of the transition of the LDL cholesteryl ester core from an ordered to disordered state, in the case of the heating scan, and from a disordered to ordered state, in the case of the cooling scan. The mean peak transition temperatures for heating and cooling scans were 12–13°C lower for the LDL of the fish oil-fed group compared to the lard-fed group. The enthalpy of the transition of the first heating run is given in Table 3 and is in the range of values typical of that for cholesteryl esters undergoing an order-to-disorder transition. The transition enthalpy was typical of that for cholesteryl esters (0.8 cal/g; ref. 25) and was not significantly different between the two diet groups.

To ascertain the relationship between the peak melting transition of the LDL and the size and cholesteryl ester composition of the LDL, plots were made of LDL molecular weight for both diet groups, although the slope of the line was less for the fish oil group compared to the lard group.

**Table 1.** Particle characteristics of LDL isolated from African green monkeys fed lard or fish oil diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>LDL Molecular Weight (μmol)</th>
<th>LDL Particle Concentration (μM)</th>
<th>Molecules/LDL Particle</th>
<th>AA</th>
<th>PL</th>
<th>TG</th>
<th>CE</th>
<th>FC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lard (n = 12)</td>
<td>3.43 ± 0.12*</td>
<td>1.79 ± 0.34</td>
<td>69.14 ± 173</td>
<td>1006 ± 31</td>
<td>26 ± 3</td>
<td>2431 ± 138</td>
<td>801 ± 47</td>
<td></td>
</tr>
<tr>
<td>Fish oil (n = 12)</td>
<td>2.91 ± 0.12</td>
<td>1.53 ± 0.23</td>
<td>63.07 ± 234</td>
<td>871 ± 54</td>
<td>63 ± 6</td>
<td>1957 ± 128</td>
<td>680 ± 36</td>
<td></td>
</tr>
<tr>
<td>P Value</td>
<td>0.007</td>
<td>NS</td>
<td>NS</td>
<td>0.04</td>
<td>0.001</td>
<td>0.002</td>
<td>0.009</td>
<td></td>
</tr>
</tbody>
</table>

*Values calculated using the following molecular weights: AA, amino acid (100); PL, phospholipid (775); TG, triacylglyceride (900); CE, cholesteryl ester (667); and FC, free cholesterol (386).

` Mean ± SEM.

All values are means ± SEM.

**Table 2.** LDL within-particle cholesteryl ester (CE) compositions

<table>
<thead>
<tr>
<th>Cholesterol Ester Fatty Acid</th>
<th>CE Molecules/LDL Particle</th>
<th>Lard</th>
<th>Fish Oil</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>13 ± 5*</td>
<td>35 ± 4</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>340 ± 18</td>
<td>450 ± 29</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>16:1</td>
<td>68 ± 9</td>
<td>93 ± 11</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>175 ± 22</td>
<td>123 ± 20</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>18:1</td>
<td>915 ± 83</td>
<td>408 ± 44</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>18:2</td>
<td>682 ± 21</td>
<td>303 ± 16</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>20:4</td>
<td>172 ± 10</td>
<td>197 ± 14</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>20:5</td>
<td>ND</td>
<td>222 ± 20</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>22:5</td>
<td>23 ± 15</td>
<td>36 ± 19</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>22:6</td>
<td>3 ± 2</td>
<td>34 ± 4</td>
<td>0.0001</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SEM.

1 Not significant at P = 0.05 by Student’s t-test.

1 Not detectable.
Fig. 1. Plot of number of cholesteryl ester molecules per LDL particle as a function of LDL molecular weight (i.e., LDL size). LDL molecular weights were quantitated by agarose column chromatography and the number of cholesteryl ester species was calculated from the cholesteryl ester distribution of individual LDL, determined by capillary gas-liquid chromatography (see Methods). Cholesteryl fatty acyl species were grouped as: saturated (C 16:0 + C 18:0), monounsaturated (C 16:1 + C 18:1), polyunsaturated (C 18:2 + 20:4), and n-3 (C 20:5 + C 22:5 + C 22:6). Note that n-3 cholesteryl ester species were not included with the polyunsaturated species. Each point represents data from an individual animal consuming the lard (○) or fish oil (■) diet and the line of best fit is shown for each data set. All lines of best fit had a significant slope and regression coefficient (P < 0.05) except those for the polyunsaturated cholesteryl esters (upper right hand panel).

the particles. However, no such relationship was apparent for the fish oil group (Figs. 4 and 5); that is, there was little effect of the LDL particle size or cholesteryl ester composition on the melting behavior of the particles.

Previous studies of human (1) and nonhuman primate (7) LDL have shown that the cholesteryl ester fatty acid ratio and the triglyceride to cholesteryl ester ratio are the two most important determinants of the transition temperature of LDL cholesteryl esters. The mean values of these ratios for the two diet groups are given in Table 3. The CEFA ratio was not significantly different between the two groups. However, there was a small but statistically significant difference of the TG/CE ratio.

To determine the effect of small amounts of an n-3 cholesteryl ester on the melting behavior of cholesteryl linoleate, model systems of varying ratios of cholesteryl linoleate (C 18:2) and cholesteryl docosahexaenoate (C 22:6) were made and were analyzed by DSC. Fig. 6 shows the results of the second heating run, which is indicative of the transition of the cholesteryl esters from a liquid crystalline to liquid state. The first heating run (not shown) resulted in the melting of the crystalline form of the cholesteryl linoleate and upon cooling a liquid to liquid crystalline transition occurred. The second heating scan of the cholesteryl linoleate (Fig. 6, top scan) showed the typical liquid crystalline to liquid transition that has been described previously (25). Cholesteryl docosahexaenoate had no detectable transitions between -20°C to 60°C (Fig. 6, bottom scan), which is in keeping with the reported

2The first heating run of the cholesteryl ester mixtures resulted in an endothermic transition typical of a crystalline to liquid transition. Since crystalline to liquid transitions do not occur in intact LDL, we have shown only the DSC scans of the liquid crystalline to liquid transitions, which do occur in intact LDL.
Fig. 2. Ratio of the saturated (Δ0) + monounsaturated (Δ1) to poly-
unsaturated (Δ2+) cholesteryl ester species versus LDL molecular
weight of animals fed lard (■) or fish oil (○) diets. Each point represents
data from an individual animal. Note that n-3 cholesteryl esters are in-
cluded as polyunsaturated species (Δ2+) in this graph. The line of best
fit is shown for each data set and both lines had a significant slope and
correlation coefficient; (lard; r = 0.87, P = 0.001 and fish oil; r = 0.60,
P = 0.038).

DISCUSSION

The LDL from nonhuman primates fed lard and fish oil diets exhibited broad, reversible thermotropic transitions in the range of body temperature indicative of core cholesteryl esters undergoing an order–disorder transition (Fig. 3). The ordered phase of cholesteryl esters in intact LDL particles resembles that of isolated cholesteryl esters in a smectic liquid crystalline phase by X-ray diffraction analysis (1, 26). Pure cholesteryl esters have relatively narrow DSC liquid crystalline transitions (Fig. 6 and ref. 25). However, the cholesteryl ester transitions of LDL are crystalline to liquid transition temperature of −72°C for this ester (25). With increasing percentages of cholesteryl docosahexaenoate (2.6 to 15%) relative to cholesteryl linoleate, the liquid crystalline to liquid transition temperature of the cholesteryl linoleate decreased from 33°C to 20°C. In addition, the transition became broader and bimodal in appearance.

Fig. 3. Differential scanning calorimetry (DSC) of LDL from a lard-fed (left) and a fish oil-fed (right) animal. DSC scans were made on 75-μl aliquots of LDL (5–7 mg of LDL cholesterol) hermetically sealed in stainless-steel pans; all scans were conducted at a rate of 5°C/min. Panels A and E contain the first heating scan from −10°C to 60°C; panels B and F contain the first cooling scan from 60°C to −10°C; panels C and G show the second heating scan from −10°C to 120°C; and panels D and H show the second cooling scan from 120°C to −10°C. Melting transitions (endotherms) result in a downward deflection from baseline while crystallization transitions (exotherms) result in an upward deflection from baseline.
broad because of the heterogeneity of cholesteryl ester species, the presence of core triglyceride, and the small domain size of LDL cholesteryl esters (1). Studies of human LDL have shown that there is an inverse relationship between the peak melting temperature of LDL cholesteryl esters and the triglyceride to cholesteryl ester (TG/CE) ratio (1). Nonhuman primates fed saturated fat have <1% LDL mass as triglyceride and, in this situation, the transition temperature of the LDL cholesteryl esters is determined by the ratio of saturated and monounsaturated to polyunsaturated cholesteryl ester species (i.e., CEFA ratio; ref. 7).

LDL from fish oil-fed animals had DSC transition temperatures that were 12-13°C lower than those of the lard-fed group (Table 3 and Fig. 3). To explain this difference we first examined the CEFA ratio of the LDL of these animals since the TG/CE ratio usually plays a minor role in determining monkey LDL transition temperatures. The lard-fed monkeys had a CEFA ratio of 1.73 ± 0.15 (Table 3) and this ratio was significantly correlated with LDL peak melting temperature (Fig. 5) as well as LDL molecular weight (Fig. 4). Similar results for saturated fat-fed cynomolgus monkeys have been reported previously (7). The CEFA ratio of the fish oil group (1.40 ± 0.07) was not significantly different from the lard group (Table 3).

As seen in Table 2, this was because the ratio of the two most predominant esters of LDL, cholesteryl oleate and cholesteryl linoleate, was identical for both diet groups (i.e., CO/CL = 1.34), despite a decrease in number of molecules per particle of these esters in the fish oil group compared with the lard group. Even though there was a significant correlation between LDL molecular weight and the CEFA ratio for the fish oil-fed animals (Fig. 2), there was no significant relationship between the peak melting temperature and either the CEFA ratio (Fig. 5) or LDL molecular weight (Fig. 4) for this group.

We next sought to explain the marked difference in transition temperatures between the two groups of animals on other factors such as the TG or n-3 cholesteryl ester content of the LDL particles. The TG/CE ratio was slightly but significantly higher for the fish oil group (Table 3) and, based on the earlier studies of Deckelbaum, Shipley, and Small (1), this difference in TG/CE ratio could explain only a difference of 1-2°C in the transition temperature of the LDL between the two groups. However, one apparent difference in the LDL of the two groups was the enrichment of n-3 cholesteryl esters of the fish oil group (Table 2). We hypothesized that n-3 cholesteryl esters, because of their extremely low transition temperatures (−72°C), might disrupt the liquid crystalline phase of other cholesteryl esters in a manner similar to that of triglycerides. To test this hypothesis, we studied the DSC melting behavior of cholesteryl linoleate with increasing amounts of an n-3 cholesteryl ester (C 22:6). With increasing percentages of C 22:6, there was a de-

### Table 3. Physiochemical properties of grivet monkey LDL

<table>
<thead>
<tr>
<th>Diet</th>
<th>Temperature (°C)</th>
<th>ΔH (cal/g)</th>
<th>CEFA Ratio</th>
<th>TG/CE Ratio (wt/wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heating</td>
<td>Cooling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lard</td>
<td>37.9 ± 0.8</td>
<td>29.9 ± 0.8</td>
<td>1.08 ± 0.13</td>
<td>1.73 ± 0.15</td>
</tr>
<tr>
<td>Fish oil</td>
<td>26.0 ± 1.0</td>
<td>16.7 ± 1.1</td>
<td>0.88 ± 0.13</td>
<td>1.40 ± 0.07</td>
</tr>
<tr>
<td>P Value</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

*All values are mean ± SEM; n = 10 to 12 for all values.

*Peak melting temperature of the first heating or cooling runs (−10°C → 60°C → −10°C) measured by differential scanning calorimetry.

CEFA = cholesteryl ester fatty acid ratio; saturated (Δ0) + monounsaturated (Δ1) to polyunsaturated (Δ2 ± ) species ratio.

NS = not significant at P = 0.05 by Student's t-test.

![Fig. 4. A plot of LDL peak melting temperature versus LDL molecular weight. Peak melting temperatures and molecular weights of individual animal LDL were determined by differential scanning calorimetry and agarose column chromatography, respectively (see Methods). Each point represents data from an individual animal consuming the lard (●) or fish oil (○) diet. The line of best fit is shown for the lard group (r = 0.82; P = 0.001). There was not a significant relationship between peak transition temperature and LDL molecular weight for the fish oil group. Two of the 12 fish oil-fed animals had amounts of LDL that were too low to give an adequate DSC transition.](image-url)
increase in the peak transition temperature and a broadening of the endothermic transition of cholesteryl linoleate (Fig. 6). At 15% C 22:6, a percentage of n-3 cholesteryl ester similar to that of fish oil-derived LDL (Table 2), there was a 13°C lowering of the transition temperature of cholesteryl linoleate, which was almost identical to the difference in LDL transition temperatures between the lard and fish oil groups (Table 3). Thus, we conclude that a small amount of n-3 cholesteryl esters, because of their very low transition temperatures, can markedly lower the transition temperature of LDL without significantly altering the CEFA ratio.

Livers of monkeys fed n-6 polyunsaturated fatty acids have more cholesteryl ester and less triglyceride and secrete hepatic VLDL, a presumed precursor of plasma LDL, that contain more cholesteryl esters per particle than do the livers of saturated fat-fed animals. Since plasma LDL of polyunsaturated (n-6) fat-fed animals are smaller and contain fewer cholesteryl esters per particle than the plasma LDL of saturated fat-fed animals, extensive intravascular metabolism of hepatic VLDL particles, with net removal of cholesteryl esters, must occur during LDL formation (27). This removal mechanism is likely mediated by lipid transfer protein and triglyceride lipases in plasma (28). A similar metabolic scheme may operate with n-3 polyunsaturated fat feeding, such that smaller LDL with fewer cholesteryl esters result (Table 1), although there are no data as yet to support such a mechanism. It has been shown, though, that both n-3 and n-6 polyunsaturated fat diets stimulated liver acyl CoA:cholesterol acyltransferase (ACAT) activity relative to saturated fat diets (29, 30). These observations would be consistent with the accumulation of cholesteryl esters by the liver and the secretion of cholesteryl ester-enriched VLDL with both n-3 and n-6 fatty acid feeding.

Feeding fish oil to monkeys resulted in significantly smaller LDL, compared to the lard group, with fewer molecules per LDL particle of all lipid constituents except triglyceride (Table 1). However, the number of LDL particles in plasma (µM concentration) was not different between the two groups (Table 1). LDL enlargement in saturated fat-fed animals is accompanied by a preferential increase in saturated and monounsaturated cholesteryl esters relative to other chemical constituents (6, 7). A similar result was found for both the lard- and fish oil-fed animals of this study (Fig. 1); in both groups the monounsaturated cholesteryl esters showed the greatest increase in number with increasing LDL size, while the n-6 polyunsaturated cholesteryl esters (C 18:2 and C 20:4) showed little change. In other studies we have found that when animals are fed diets rich in n-6 polyunsaturated fatty acids, the number of cholesteryl linoleate

---

**Fig. 5.** A plot of LDL peak melting temperature versus the ratio of saturated (Δ0) + monounsaturated (Δ1) to polyunsaturated (Δ2+) cholesteryl ester species of LDL. Peak melting temperature and cholesteryl ester fatty acid compositions were determined by DSC and gas-liquid chromatography, respectively. Each point represents an observation for an individual animal consuming the lard (■) or fish oil diet (○). The line of best fit is shown for the lard group (r = 0.89; P = 0.001). There was no significant relationship between the peak melting temperature and the cholesteryl ester fatty acid ratio for the fish oil group.

---

**Fig. 6.** DSC scans of mixtures of cholesteryl linoleate (C 18:2) and cholesteryl docosahexaenoate (C 22:6). Five-mg samples of total cholesteryl ester of the indicated percentages were placed in DSC pans for analysis. Samples were heated and cooled once (-20°C → 60°C → -20°C) to melt the crystalline phase and allow the cholesteryl ester to recrystallize in a liquid crystalline phase (data not shown). After a 10-min equilibration at -20°C, the samples were heated from -20°C to 60°C at 5°C/min. The sensitivity of the DSC was 0.2 mW/cm and was the same for all scans. Downward deflections from baseline are melting (endothermic) transitions indicative of the melting of cholesteryl esters from a liquid crystalline to liquid phase.
molecules per particle increases with increasing LDL size (4, 31). However, with fish oil feeding, cholesteryl linoleate and cholesteryl arachidonate did not increase in number as LDL enlargement occurred and the n-3 cholesteryl esters were the only group of polyunsaturated cholesteryl esters which were significantly correlated with LDL size (Fig. 1). This occurred even though there were more n-6 polyunsaturated esters (C 18:2 and C 20:4) per particle than n-3 esters (Fig. 1 and Table 2). Although the exact mechanisms of LDL enlargement are unknown, these results suggest that n-3 fatty acids are effective in competing for the pathways normally responsible for putting more cholesteryl linoleate molecules into larger LDL when polyunsaturated fat is fed.

Our findings may have important implications concerning the development of atherosclerosis in these animals. If cholesteryl esters from fish oil-derived LDL are deposited in atherosclerotic plaques, the increased concentration of n-3 cholesteryl esters could act as an impurity and lower the transition temperature of the plaque cholesteryl esters. If this resulted in a greater percentage of plaque cholesteryl esters that are in a liquid rather than liquid–crystalline state at body temperature, the potential for greater cholesteryl efflux from arterial plaques might exist (11).

The authors gratefully acknowledge the technical expertise of Ms. Martha Kennedy and Joy Martin and we thank Mrs. Linda Odham for her excellent assistance in manuscript preparation. The authors also wish to thank Drs. John Babiak and Lawrence R. Rudel for their helpful comments and constructive criticism of the work. This research was supported by National Heart, Lung, and Blood Institute grants HL-14164 (Specialized Center of Research in Arteriosclerosis), HL-24736 and HL-30342.

Manuscript received 26 March 1986 and in revised form 17 October 1986.

REFERENCES

23. Rogozinski, M. 1964. The methanol–sulfuric acid es-
terification methods. II. An improved extraction procedure. 
J. Gas Chromatogr. 2: 328–329.
Methods. 6th ed. Iowa State University Press, Ames, IA.
importance: cholesteryl esters, cholesterol, triglyceride. In 
Surface Chemistry of Biological Systems. M. Blank, editor. 
Shipley. 1977. Structure of human plasma low-density 
lipoproteins: molecular organization of the central core. 
Effects of the degree of saturation of dietary fat on the 
hepatic production of lipoproteins in the African green 
28. Deckelbaum, R. J., S. Eisenberg, Y. Oschry, E. Bethbul, I. 
Sharon, and T. Olivecrona. 1982. Reversible modification 
of human plasma low density lipoproteins toward triglyceride-rich precursors. A mechanism for losing excess 
of dietary fat saturation on acylcoenzyme A:cholesterol 
acyltransferase activity of rat liver microsomes. J. Lipid Res. 
Spector. 1983. Dietary fat saturation and hepatic acylcoen-
zyme A:cholesterol acyltransferase activity. Effect on n-3 
polyunsaturated and long-chain saturated fat. Atheroscle-
rus. 3: 242–248.
1986. Low density lipoproteins in atherosclerosis. J. Lipid 
Res. 27: 465–474.