Effect of Dietary Supplementation With n-3 Polyunsaturated Fatty Acids on Physical Properties and Metabolism of Low Density Lipoprotein in Humans


The effects of marine n-3 polyunsaturated fatty acids were investigated in relation to the chemical and physical properties of low density lipoprotein (LDL) and how these changes affected LDL metabolism in humans. The subjects received supplements of six capsules daily, each capsule containing 1 g of either highly concentrated ethyl esters of n-3 fatty acids (85% eicosapentaenoic acid and docosahexaenoic acid) (n = 12) or corn oil (56% linoleic and 26% oleic acid) (n = 11). After 4 months of oil supplementation, the following changes were observed in the lipid moiety of the n-3–enriched LDL particles compared with LDL from the corn oil group: LDL cholesteryl ester, as well as the amount of total lipids of LDL, was significantly lower (0.97 ± 0.12 versus 1.19 ± 0.23 mg/mg protein and 1.88 ± 0.40 versus 2.45 ± 0.31 mg/mg, respectively; mean ± SD, n = 6, p < 0.05); the amount of eicosapentaenoic and docosahexaenoic acids and the unsaturation index increased (104.0 versus 29.4 µg/mg protein and 6.64 versus 5.49, respectively); and differential scanning calorimetry showed that LDL cholesteryl ester melting temperature was lowered by 2°C (27.6 ± 0.8°C versus 29.5 ± 0.2°C). The only effect observed on the protein moiety was an increase in the ratio of apolipoprotein (apo) B to cholesterol (0.66 ± 0.17 versus 0.82 ± 0.14 mg/mg cholesterol; p < 0.05). Circular dichroism spectra of LDL indicated an α-helix content of 46.5±% in apo B from both groups. No difference was observed by 13C nuclear magnetic resonance spectroscopy in the ratio of "active" to "normal" lysine residues of apo B. No detectable differences in the size of n-3 fatty acid–enriched LDL particles versus control LDL could be measured by either electron microscopy of negatively stained LDL (24.5 ± 2.0 versus 25.0 ± 1.5 nm) or dynamic light scattering (24.9 ± 0.9 versus 24.9 ± 0.4 nm). LDL from the fish oil and corn oil groups showed similar susceptibility to Cu2+-catalyzed lipid peroxidation, as indicated by the amount of lipid peroxides formed during the oxidation time, and degradation of oxidatively modified LDL in J774 macrophages as a function of Cu2+ oxidation time. No effect of n-3 fatty acids was observed on LDL metabolism. Specific uptake and degradation of n-3 fatty acid–enriched LDL were similar to those for control LDL in HepG2 cells as well as in human skin fibroblasts, and they showed the same ability to stimulate cholesteryl ester synthesis. Peripheral blood mononuclear cells from the two supplementation groups were able to take up a similar amount of LDL regardless of the source of LDL. No difference was observed with respect to the amount of mRNA specific for the LDL receptor, LDL receptor–related protein, and 3-hydroxy-3-methylglutaryl coenzyme A reductase in the peripheral blood mononuclear cells. We conclude that marine n-3 polyunsaturated fatty acids induced some changes in the lipid moiety of LDL; however, these changes had no measurable effect on the cellular metabolism of LDL.

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Numerous experimental and clinical studies have suggested that the marine n-3 polyunsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have beneficial effects on the development of atherosclerosis and lipoprotein metabolism. However, the mechanism by which dietary n-3 fatty acids may exert their effects is not fully understood. The n-3 fatty acids are reported to have a wide range of biologic effects that may be related to atherogenesis. Some of the effects occur through...
plasma lipids.\textsuperscript{4} It is well established that n-3 fatty acids reduce the level of plasma triacylglycerol and very low density lipoprotein. Variable effects have been demonstrated on low density lipoprotein (LDL) and high density lipoprotein cholesterol levels.\textsuperscript{3} Parks and Rudel\textsuperscript{4} have shown, in nonhuman primates, that dietary fish oil reduced the particle size of LDL and lowered cholesteryl ester transition temperature, probably by forming a less atherogenic LDL particle. It is important to know if the fatty acids found in fish oil have similar effects on the physical properties of human LDL. Moreover, Yla-Herttuala et al\textsuperscript{7} recently reported evidence for the presence of oxidatively modified LDL in atherosclerotic lesions of rabbits and humans. Parthasarathy et al\textsuperscript{8} showed that LDL rich in oleic acid was remarkably resistant to oxidative modification compared with n-6 fatty acid–enriched LDL. Thus, it is important to investigate whether dietary supplementation with marine n-3 polyunsaturated fatty acids renders human LDL particles more susceptible to oxidative modification.

The present study was undertaken to establish the effect of marine n-3 fatty acids on the chemical and physical properties of LDL in humans and how the changes affect the metabolism of the LDL particles. All the methods used to highlight these questions showed that the changes observed in the lipid moiety of LDL had no measurable effect on the cellular metabolism of the LDL particles.

**Methods**

**Materials**

Gelatin capsules of corn oil or highly concentrated ethyl esters of n-3 fatty acids (K85) were kindly provided by Norsk Hydro (Porsgrunn, Norway). \textsuperscript{[\textsuperscript{\textsuperscript{125}}I]}NaI, [\textsuperscript{\textsuperscript{1-\textsuperscript{14}}}C]oleate, and [\textsuperscript{\textsuperscript{13}}C]formaldehyde were purchased from Du Pont–New England Nuclear (Boston, Mass.), and [\textsuperscript{\textsuperscript{13}}C]formaldehyde (99% isotope enrichment) as a 20% solution in water was from ICN (Costa Mesa, Calif.). Sodium cyanoborohydride from Aldrich (Milwaukee, Wis.) was recrystallized from methylene chloride before use. Bovine serum albumin (fraction V) and fatty acid–free albumin were obtained from Sigma Chemical Co. (St. Louis, Mo.). The apolipoprotein (apo) B radioimmunoassay kit was from Pharmacia Diagnostics AB (Uppsala, Sweden), and the color reagent for determination of lipid peroxides was from Merck (cholesterol kit, No. 14106, Rahway, N.J.). Dolecco's modified Eagle's medium, RPMI-1640, penicillin, streptomycin, amphotericin B, and heat-inactivated fetal calf serum were purchased from Flow Laboratories (McLean, Va.), and Lymphoprep was from Nycomed A/S (Oslo, Norway). Tissue-culture dishes were supplied by CoStar (Cambridge, Mass.).

**Subjects**

The study was carried out in connection with a larger dietary intervention study that assessed the effect of marine n-3 fatty acids on psoriasis and atopic dermatitis. In the present study, the treatment group (n=12) consisted of six women and six men ranging in age from 27 to 63 years, whereas the control group (n=11) included nine women and two men from 23 to 70 years old. The subjects were randomly selected to provide lipoproteins and peripheral blood mononuclear cells for metabolic studies. All the subjects were normolipidemic except that one subject in the fish oil group showed an elevated plasma cholesterol concentration (8.4 mmol/l), and one subject in the control group showed elevated plasma concentrations of both cholesterol and triacylglycerol (11.3 and 4.1 mmol/l, respectively). Therefore, the results apply to normolipidemic states. None of the patients had used systemic drug treatment during the 4 weeks before the start of the study. LDL was isolated from six individuals in each supplementation group, and from four persons in each group LDL was radiolabeled for uptake and degradation studies (see below).

**Study Design**

The patients were randomly allocated to receive supplementation with either corn oil or highly concentrated ethyl esters of n-3 fatty acids (K85) (Table 1). The subjects received six gelatin capsules daily for 4 months, each capsule containing 1 g of oil. Evaluation of dietary intake of relevant nutrients was performed by a self-administered questionnaire, and calculations were done on the basis of data in the national food composition data base (Table 2). Before the experiment started, there were no significant differences in dietary intakes of energy-providing nutrients between the two groups of subjects receiving either corn oil or fish oil. Fatty acid composition of plasma phospholipids was determined before and after treatment. All other data presented were obtained from plasma, LDL, and peripheral blood mononuclear cells isolated after 4 months of dietary supplementation.

**Lipoproteins**

LDL was prepared by vertical-rotor gradient ultracentrifugation according to Chung et al\textsuperscript{9} and modified as

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Corn oil</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>9.3</td>
<td>ND</td>
</tr>
<tr>
<td>16:1 n-7</td>
<td>0.1</td>
<td>ND</td>
</tr>
<tr>
<td>18:0</td>
<td>2.0</td>
<td>ND</td>
</tr>
<tr>
<td>18:1 n-9</td>
<td>26.2</td>
<td>ND</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>56.3</td>
<td>0.1</td>
</tr>
<tr>
<td>18:3 n-3</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>20:1</td>
<td>ND</td>
<td>0.1</td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>ND</td>
<td>3.8</td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>ND</td>
<td>47.1</td>
</tr>
<tr>
<td>22:5 n-3</td>
<td>ND</td>
<td>2.7</td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>ND</td>
<td>29.6</td>
</tr>
<tr>
<td>Other n-3</td>
<td>ND</td>
<td>5.4</td>
</tr>
<tr>
<td>Other n-6</td>
<td>ND</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Six percent to 10% of the total fatty acids either were not identified or were present in a concentration less than 0.1%. ND, not detectable.
**Table 2. Daily Dietary Intake as Evaluated by Food Frequency Questionnaire**

<table>
<thead>
<tr>
<th>Intake</th>
<th>Corn oil</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (MJ)</td>
<td>8.9±3.1</td>
<td>7.2±1.9</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>15.8±2.6</td>
<td>18.4±2.8</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>49.2±3.3</td>
<td>47.3±7.4</td>
</tr>
<tr>
<td>Alcohol (%)</td>
<td>0.5±0.7</td>
<td>2.3±2.2</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>34.5±4.5</td>
<td>31.9±6.3</td>
</tr>
<tr>
<td>Saturates (g)</td>
<td>32.9±17.7</td>
<td>23.3±7.4</td>
</tr>
<tr>
<td>Monoenes (g)</td>
<td>28.8±14.1</td>
<td>19.3±7.0</td>
</tr>
<tr>
<td>Polyenes (g)</td>
<td>14.6±5.5</td>
<td>13.6±4.1</td>
</tr>
<tr>
<td>Total n-3</td>
<td>1.7±1.1</td>
<td>6.1±0.7</td>
</tr>
<tr>
<td>20:5 n-3</td>
<td>0.1±0.2</td>
<td>2.8±0.2</td>
</tr>
<tr>
<td>22:6 n-3</td>
<td>0.3±0.3</td>
<td>1.9±0.3</td>
</tr>
<tr>
<td>Total n-6</td>
<td>11.9±4.5</td>
<td>6.5±3.4</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>11.7±4.5</td>
<td>6.1±3.4</td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>0.2±0.1</td>
<td>0.3±0.1</td>
</tr>
</tbody>
</table>

Results are mean±SD; n=11 (corn oil) and n=12 (fish oil). *p<0.01 vs. corn oil; **p<0.001 vs. corn oil.

The LDL density profile for patients receiving fish oil concentrate showed insignificantly higher densities, ranging from 1.036±0.011 to 1.075±0.004 g/ml compared with 1.034±0.007 to 1.070±0.003 g/ml for the corn oil group (Figure 1). Protein concentration was determined using bovine serum albumin as the standard. The LDL concentration of apo B was measured by radioimmunoassay.

**Figure 1. Distribution of protein (optical density at 280 nm [O.D.280]) and density (g/ml) in the fractions of a discontinuous gradient of 0.9% NaCl and a concentrated lipoprotein fraction with density of 1.30 g/ml.** Plasma was adjusted to a density of 1.25 g/ml with solid KBr and centrifuged in a Beckman VTI 50 vertical rotor for 16 hours at 10°C at 50,000 rpm. Ten milliliters of this concentrated lipoprotein fraction, adjusted to a density of 1.30 g/ml, was layered under 30 ml of 0.9% NaCl by means of a peristaltic pump at a speed of 1 ml/min and centrifuged as described above for 180 minutes. Two-milliliter fractions were collected, and the O.D.280 and refractive indexes measured. Peaks correspond to high density lipoprotein, low density lipoprotein, and very low density lipoprotein (from left to right). Fractions 9–13, corresponding to low density lipoprotein, were pooled.

**Nesveter et al Effect of n-3 Fatty Acids on LDL in Humans**

**13C and 14C Labeling of Low Density Lipoprotein**

The reductive methylation procedure of Jentoft and Dearborn was employed to introduce 13C CH3 groups into the amino groups of lysine (Lys) residues of apo B-100 on the LDL particles. Conditions described previously were used to convert about 10% of the Lys residues to the [13C]dimethyl derivative. The HCHO was doped with a trace of H3CHO to give a known specific activity; the 14C counts were used to monitor the incorporation of H3CHO into Lys residues to form dimethyl-Lys, and thereby to compute the degree of reductive methylation of the apo B-100. The degree of methylation is an average of the values computed from the incorporation of 13C/formaldehyde (by scintillation counting) and of [14C]formaldehyde (by nuclear magnetic resonance [NMR]); for a given sample the values agreed to within 0.1%. When the degree of labeling of Lys residues was <15%, there was no detectable change in the electrophoretic mobility of the LDL on agarose gels or any conformational change of apo B-100. There was no degradation of apo B-100 as monitored by electrophoresis on sodium dodecyl sulfate–3% polyacrylamide 1% agarose gels.

**125I Labeling of Low Density Lipoprotein**

Isolated LDL was labeled with 125I-tyramine cellulose (125I-TLC-LDL), and final preparations were digested extensively against phosphate-buffered saline (PBS: 0.15 M NaCl, 20 mM NaH2PO4, and 1 mM EDTA; pH 7.4). More than 96% of the radioactivity was precipitated by 10% (wt/vol) trichloroacetic acid. The final specific activity of 125I-labeled LDL was 406±160 and 396±128 cpm/ng protein for LDL from corn oil and fish oil groups, respectively (n=4 for each group).

**Oxidative Modification of Low Density Lipoprotein**

LDL and 125I-TLC-LDL were oxidatively modified in a cell-free system, and lipid peroxides were determined as described by El-Saadani et al. Freshly isolated LDL with butylated hydroxytoluene (BHT) added (final concentration, 10 μM) was stored at 4°C and used within 1 week. Before use, LDL was dialyzed extensively against PBS (pH 7.4) to remove EDTA and BHT. LDL was incubated for 1, 6, or 24 hours at 37°C in the presence of 5 μM CuCl2 in PBS containing 2.5 μM ascorbic acid. Lipid peroxidation was assessed by an isodometric method using the color reagent of a kit for enzymatic determination of cholesterol.

**Low Density Lipoprotein Particle Size**

LDL particle size was determined by negative-staining electron microscopy as described by Collins and Phillips or by dynamic light scattering (photometer correlation spectroscopy). LDL preparations measured by photon correlation spectroscopy were diluted in PBS and filtered through a 0.45-μm filter just before measurement (Sterivex, Millipore, Bedford, Mass.). A Spectra Physics Model 2020 argon ion laser was used as the light source. The detected scattering intensity was monitored. A reference specimen was used to adjust the
optical alignment for each experiment. Data were analyzed by the method of cumulants.

**Cell Cultures**

The human hepatoma cell line HepG2, human skin fibroblasts, and the murine macrophage-like cell line J774 were maintained in Dulbecco's modified Eagle's medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin B (4 μg/ml), and 10% heat-inactivated fetal calf serum at 37°C in a 5% CO₂ atmosphere. Before the experiments were performed, the cells were grown in media containing 5% lipoprotein-deficient serum for 24 hours. Peripheral blood mononuclear cells from the patients were separated from defibrinated blood by means of flotation on Lymphoprep.  

The cells were suspended in RPMI-1640 medium and supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal calf serum.

**Metabolism of Low Density Lipoprotein by Cells**

Uptake and degradation of LDL by HepG2 cells, human skin fibroblasts, J774 cells, and peripheral blood mononuclear cells were measured as described previously.  

Specific uptake of LDL was calculated as the difference between uptake of labeled LDL in the absence or presence of excess amounts of unlabeled LDL (500 μg/ml). Cholesterol esterification in HepG2 cells and human skin fibroblasts was measured by incorporation of [1,14]C oleate into cholesteryl ester.  

The cells were extracted with chloroform/methanol (2:1, vol/vol), and radiolabeled cholesteryl esters were isolated by thin-layer chromatography on silica gel plates using hexane/diethyl ether/acetic acid (80:20:1, vol/vol/vol).

**NMR Measurements**

The LDL samples contained 15–25 mg protein in 0.15 M NaCl, 1 mM EDTA, and 0.02% NaN₃, pH 7.6, with deuterium oxide added as the NMR lock compound to increase the volume by 20% to 1.5 ml.  

13C NMR spectra at 126 MHz were obtained on a Bruker AM 500 spectrometer. The conditions employed to determine chemical shifts and resonance intensities have been described previously.  

**Circular Dichroism Spectroscopy**

The conformation of LDL apo B-100 protein was monitored by circular dichroism spectroscopy using a Jasco 41A spectropolarimeter employing procedures described previously.  

The molar ellipticity at 222 nm was employed to compute α-helix content because of the small circular dichroism contribution from LDL lipids at this wavelength.  

**Differential Scanning Calorimetry**

Differential scanning calorimetry with LDL particles was conducted with a MicroCal MC-2 scanning microcalorimeter (Northampton, Mass.) equipped with a downscanning accessory and matched tantalum sample and reference cells. Samples were exhaustively dialyzed at 4°C against saline (0.15 M NaCl, 1 mM EDTA, 0.02% NaN₃, [pH 7.4]), and LDL and saline solution were degassed under vacuum. LDL (7–10 mg esterified cholesterol per cell) was scanned from 3°C to 60°C against saline solution in the reference cell at heating rates of 90°C/hr. Saline baselines were subtracted from the LDL sample scans. The peak melting temperature (Tₘ) for a given transition was taken to be the temperature of maximum excess heat capacity.

**Triacylglycerol and Phospholipid**

Triacylglycerol and phospholipid were determined by gas-liquid chromatography as fatty acid methyl esters.  

Triheptadecanoin and diheptadecanoyl phosphatidylcholine were used as internal standards for triacylglycerols and phospholipids, respectively. A Shimadzu gas-liquid chromatograph (GC-14A) equipped with an apolar capillary column (FS-SE 30; 50 m×0.32 mm i.d.; Macherey-Nagel, FRG) was used, with helium as the carrier gas (flow rate, 0.5 ml/min). The oven temperature was programmed to rise from 180°C to 215°C at 0.2–0.5°C/min.

**Total and Free Cholesterol**

Free and esterified cholesterol contents were determined by gas-liquid chromatography using stigmasteryl, coprostanol as the internal standard. The fatty acids in cholesteryl esters were determined as fatty acid methyl esters, as described previously for triacylglycerol and phospholipid.

**RNA Analysis**

After centrifugation on Lymphoprep, the suspension of peripheral blood mononuclear cells was washed twice in RPMI-1640 and resuspended in Ringer's acetate. RNA from the cells was isolated by a lithium chloride/urea procedure, essentially as described before. The 2.5-kb Bgl II fragment of the pHRed-102 cDNA clone (No. 57042, American Type Culture Collection, Rockville, Md.) was used as a probe for hybridization to a human liver cDNA library.  

LDL receptor-related protein mRNA was detected by using a 5.6-kb cDNA probe. This probe was constructed from several overlapping clones (LRP 1, 2, and 4) originally isolated from a human liver cDNA library.  

The plasmid was modified by double digestion with Xho I and Sma I and religation to eliminate the poly-A tail. The 2.6-kb Kpn I HindIII fragment of this modified clone was used as the probe. All probes were prepared by randomly primed extension. Standardization of RNA amounts was done by hybridization of the filters to a kinase-labeled oligonucleotide (19 bases) specific for 18S rRNA. For quantification of signals on the blots, the autoradiogram was scanned densitometrically on a Molecular Dynamics computing densitometer.

**Statistical Analysis**

All results are presented as mean±SD. The Mann-Whitney two-sample test was used for calculation of statistical significance of differences between groups. The level of significance was set at p<0.05.

**Results**

**Plasma Lipoths**

The fatty acid composition of plasma phospholipids reflected the dietary fatty acid content, including that in
The diet-induced increase of n-3 fatty acids appeared to be highest in the cholesteryl ester component of LDL (not phospholipids) in the LDL particles after 4 months of fish oil supplementation were an increase in EPA and DHA concentrations of cholesteryl esters, triacylglycerols, and phospholipids in the LDL particles after 4 months of fish oil supplementation were an increase in EPA and DHA (Table 5). The unsaturation index of the total LDL particle composition and fatty acid pattern of the LDL particles was similar in the two dietary groups before start of the experiment (Table 3). After 4 months of fish oil supplementation, phospholipids were significantly enriched in n-3 fatty acids (p<0.01 versus before treatment; p<0.001 versus the corn oil group after treatment), and the amount of n-6 fatty acids was significantly reduced (p<0.05 versus before treatment; p<0.01 compared with the corn oil group).

Low Density Lipoprotein Composition

Dietary supplementation with fish oil changed the lipid composition and fatty acid pattern of the LDL particles (Tables 4 and 5). The concentration of LDL cholesteryl esters was significantly lower in the fish oil group compared with the corn oil group (0.97 versus 1.19 mg/mg protein; n=6, p<0.05). The amount of total lipids per milligram LDL protein was significantly lower in the fish oil group (0.82±0.14 versus 0.66±0.17 mg/mg cholesterol in the corn oil group; p<0.05). However, the ratio (wt/wt) between surface-to-core components of the LDL particles was similar in the two dietary groups (1.53±0.10 and 1.53±0.51 in the fish oil and corn oil groups, respectively). The major changes in the fatty acid compositions of cholesteryl esters, triacylglycerols, and phospholipids in the LDL particles after 4 months of fish oil supplementation were an increase in EPA and DHA (Table 5). Polyunsaturated n-3 fatty acids of cholesteryl esters were significantly increased in LDL cholesteryl esters, triacylglycerols, and phospholipids after fish oil supplementation (p<0.01, p<0.05, and p<0.01, respectively; Table 5). The unsaturation index of the total LDL particle was 6.64 in the fish oil group compared with 5.48 in the corn oil group.

Low Density Lipoprotein Particle Size

No detectable difference in the size or shape of n-3 and n-6 fatty acid-enriched LDL particles could be detected by electron microscopy of negatively stained LDL (24.5±2.0 and 25.0±1.5 nm in the fish oil and corn oil LDL, respectively; n=4 in each group; 50 particles counted) or by the dynamic light-scattering method (24.9±0.9 and 24.9±0.4 nm in the fish oil and corn oil LDL, respectively; n=4 and n=5). The loss of core lipids in fish oil LDL did not appear to affect the particle size in any significant way. This observation is supported by volumetric calculations. The electrophoretic mobilities on agarose gels of corn oil and marine oil LDL were identical (data not shown).

Physical-Chemical Properties of Low Density Lipoprotein

Circular dichroism measurements were carried out between 184 and 260 nm to obtain the average secondary structure of apo B-100 of corn oil and marine oil LDL. Not surprisingly, the spectra were identical and indicate that the fish oil and corn oil capsules, indicating satisfactory compliance (Tables 1, 2, and 3). The fatty acid composition of plasma phospholipids was similar in the two dietary groups before start of the experiment (Table 3). After 4 months of fish oil supplementation, phospholipids were significantly enriched in n-3 fatty acids (p<0.01 versus before treatment; p<0.001 versus the corn oil group after treatment), and the amount of n-6 fatty acids was significantly reduced (p<0.05 versus before treatment; p<0.01 compared with the corn oil group).

Table 4. Effect of Corn Oil or Fish Oil Supplementation on Low Density Lipoprotein Particle Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Corn oil</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>FC</td>
<td>0.29±0.04</td>
<td>0.25±0.07</td>
</tr>
<tr>
<td>CE</td>
<td>1.19±0.23</td>
<td>0.97±0.12*</td>
</tr>
<tr>
<td>TG</td>
<td>0.20±0.08</td>
<td>0.17±0.07</td>
</tr>
<tr>
<td>PL</td>
<td>0.77±0.39</td>
<td>0.49±0.16</td>
</tr>
<tr>
<td>Protein</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Results are mean±SD (n=6 in each group).
FC, free cholesterol; CE, cholesteryl ester; TG, triacylglycerol; PL, phospholipid. *p<0.05 vs. corn oil.

Table 3. Effect of Corn Oil or Fish Oil Supplementation on Fatty Acid Composition of Plasma Phospholipids

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Corn oil</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturates</td>
<td>52±1±121</td>
<td>505±88</td>
</tr>
<tr>
<td>Monounsaturates</td>
<td>150±42</td>
<td>141±44</td>
</tr>
<tr>
<td>Polyenes (n-3)</td>
<td>98±31</td>
<td>101±42</td>
</tr>
<tr>
<td>Polyenes (n-6)</td>
<td>415±84</td>
<td>391±71</td>
</tr>
</tbody>
</table>

Results are in milligrams per liter and are mean±SD; n=11 (corn oil) and n=12 (fish oil).

n-3: tp<0.001 vs. corn oil after; *p<0.01 vs. fish oil before.
n-6: *p<0.01 vs. corn oil after; p<0.01 vs. fish oil before.
The a-helix content of the apo B-100 is 46±5% in both cases (Figure 2). To explore the protein conformation in more detail, the microenvironments of the Lys residues were examined by NMR, as previous studies have indicated that the triacylglycerol content of the LDL particles affects the conformation of apo B-100, with an abnormal apo B/E receptor recognition, due to a change in the ratio of "active" to "normal" Lys residues. The number of active and normal Lys residues resonating at chemical shifts of 43.2 and 42.8 ppm, respectively, were obtained from NMR spectra of the type shown in Figure 3. No difference was observed in the ratio of active to normal Lys between normal and marine oil LDL. The pH dependences of the Lys chemical shift indicate that the two types of Lys titrate with different pK values; active Lys has a pK of 8.9, while normal Lys has a pK of 10.5. Both types of LDL contained 17 pK 8.9 dimethyl Lys and 18 pK 10.5 dimethyl Lys. This is in agreement with previously published data, indicating that Lys exposed on the surface of LDL exists in two distinct chemical microenvironments.

The two types of LDL particles were examined by high-resolution differential scanning calorimetry (Figure 4). The calorimetric transition associated with reversible order–disorder transition of the core-located cholesteryl esters was examined. On heating intact LDL from 3°C to 60°C, an endothermic transition occurred between 20°C and 35°C; this transition was reversible on cooling (data not shown). Identical transitions were obtained with repeated runs between 3°C and 60°C. The mean onset temperature for LDL was 27.6±0.8°C versus 29.3–29.7°C in fish oil and corn oil LDL, respectively, n=4 and n=2). The completion temperature of the transition was increased by 2°C for

### Table 5. Effect of Corn Oil or Fish Oil Supplementation on Fatty Acid Composition of Low Density Lipoprotein Particles

<table>
<thead>
<tr>
<th>Component</th>
<th>CE</th>
<th>Fish oil</th>
<th>TG</th>
<th>Fish oil</th>
<th>PL</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/mg protein</td>
<td>µg/mg protein</td>
<td>µg/mg protein</td>
<td><strong>Results</strong> are mean±SD, n=6 in each group; CE, cholesteryl ester; TG, triacylglycerol; PL, phospholipid; ND, not detectable. The unsaturation index is the sum of (mole % unsaturated fatty acid x No. of double bonds)/100. *p&lt;0.05 vs. corn oil; tp&lt;0.01 vs. corn oil.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 2. Circular dichroism spectra at room temperature of human low density lipoprotein from subjects supplied with corn oil (-----) or fish oil (———). Spectra were recorded in a continuous scanning mode from 260 to 184 nm on a Jasco 41A spectropolarimeter calibrated with \( n \)-10-camphorsulfonic acid. A 0.1-cm quartz cuvette was used with a protein concentration of 33 \( \mu \)g/ml in 10 mMNaCl solution at pH 7.6. Both spectra are averages of eight acquisitions. Four samples from each dietary group were examined.

the marine oil LDL (37°C versus 35°C for fish oil versus corn oil LDL, respectively). Overall, the transition width spanned 20°C in fish oil LDL compared with 15°C in corn oil LDL, reflecting a 5°C broadening of the transition. No significant differences were observed between the two groups. In common with the other serum lipoproteins, LDL can be viewed in terms of an "oil-drop" model, where a core of cholesteryl ester and triacylglycerol molecules is encapsulated by a monolayer comprising phospholipids and apo B-100. The molecular organization of the core below the thermal transition temperature involves cholesteryl ester molecules that are thought to be extended and radially oriented. 39-41

Discussion

The present investigation examined the effects of dietary marine fatty acids on the structure-function relation of LDL particles. Our data demonstrate that it is possible to introduce increased amounts of C-20 and C-22 n-3 fatty acids in plasma LDL for triacylglycerol, cholesteryl ester, and phospholipids by dietary supplementation of 5 g EPA and DHA for 4 months (Table 5). There were small differences between the groups fed corn or fish oil for gross lipid composition (Table 4) and size of LDL particles. In common with the other serum lipoproteins, LDL can be viewed in terms of an "oil-drop" model, where a core of cholesteryl ester and triacylglycerol molecules is encapsulated by a monolayer comprising phospholipids and apo B-100. The molecular organization of the core below the thermal transition temperature involves cholesteryl ester molecules that are thought to be extended and radially oriented. 39-41

The reversible transition in intact LDL, which occurs close to body temperature of 37°C, is associated with an ordered-to-disordered phase change of cholesteryl esters within the LDL particle. The peak broadening and the corn oil and fish oil groups showed a similar ability to stimulate cholesteryl ester synthesis (Figure 6).
Figure 3. Proton-decoupled $^{13}$C nuclear magnetic resonance spectra (126 MHz) of human low density lipoprotein (LDL) from subjects supplied with corn oil (A) or fish oil (B). Lysine (Lys) residues in LDL have been converted to (\(^{13}\text{CH}_3\))Lys by reductive methylation. Spectra were obtained at 37°C on a Bruker AM500 spectrometer and were accumulated using 90° pulses of 14-usec duration and a recycling time of 1.37 seconds. Chemical shifts were measured relative to external, aqueous 1,4-dioxane (66.55 ppm). Spectra were processed with 2.0-Hz exponential filtering. Panel A shows the upfield region of the spectrum (37,820 acquisitions) of human LDL from a corn oil-fed subject (14 mg protein in 1.5 ml 0.15 M NaCl, 1 mM EDTA, 0.02% w/vol NaNO₃; pH 7.6), in which 9% of the Lys residues were methylated. Panel B shows (\(^{13}\text{CH}_3\))Lys resonances of human LDL from a fish oil-fed subject, in which 10% of the Lys residues were labeled (19 mg protein in 1.5 ml saline solution; 35,340 acquisitions). Two samples from each dietary group were examined. FA, Fatty acid.

A 2°C decrease in Tₑ observed for the marine oil LDL samples are consistent with increased disordering of the cholesteryl ester core of the LDL particle due to the increased amount of n-3 polyunsaturated fatty acids. Increasing triacylglycerol content lowers the transition temperature in intact LDL. However, because no major difference was observed in triacylglycerol content in the two types of LDL particles (Table 4), we attribute the
changes in the thermal transitions to the increased n-3 polyunsaturated fatty acid content of the cholesteryl esters.

Previous work has shown that the ratio of active to normal Lys in apo B-100 affects the relative affinity of LDL for the LDL receptor. NMR spectroscopic analysis of LDL from subjects supplied with corn or marine oil indicates that marine oil LDL has a similar ratio of active to normal Lys as LDL derived from subjects given corn oil supplementation. These data are in agreement with our finding that the receptor-mediated endocytosis of LDL by cells is similar for both dietary groups. It is possible that the physical state of the core of the LDL particle may affect the surface structure, thereby modulating its interaction with the LDL receptor. However, an increase in the n-3 polyunsaturated fatty acid content of the cholesteryl esters in marine oil LDL leads only to minor changes in the thermotropic behavior of the cholesteryl ester core, and there is no significant change in the average secondary structure of the apo B-100 molecule (Figure 2). Also, the net negative surface charge densities of corn oil and marine oil LDL particles are similar, as indicated by agarose gel electrophoresis. Thus, the relatively small change in core fluidity induced by increased amounts of n-3 polyunsaturated fatty acids in cholesteryl ester is
not significant for the function of the LDL particles, as evaluated by the uptake, degradation, and cholesterol esterification in fibroblasts and HepG2 cells (Figures 5 and 6). These findings are further supported by the finding that the amounts of mRNA for the LDL receptor, LDL receptor-related protein, and HMG-CoA reductase in peripheral blood mononuclear cells are unaffected by the type of fatty acid supplementation.

Oxidative modification was performed with LDL from two subjects in each dietary group. Thus, a final conclusion cannot be drawn from these experiments, and further study is in progress to extend this part of our work. Nevertheless, our results in the present study suggest that the susceptibility of LDL to copper oxidation is similar, irrespective of fatty acid supplement and despite the fact that the unsaturation index of LDL from subjects supplied with fish oil was increased (Figures 7 and 8). In contrast to these observations, Parthasarathy et al.44 found that in rabbits, LDL enriched with oleic acid was protected against oxidative modification compared with LDL rich in linoleic acid. The reason for their finding could be that an extensive dietary manipulation can be done with rabbit LDL, whereas we obtained relatively small alterations in human LDL. This could be explained by the fact that humans have a very high "background" of fatty acid intake, whereas the rabbit, in principle, is a vegetarian species with a very low basal intake of fatty acids. Another point of importance is that it is possible that n-3 fatty acids are peroxidized to a smaller extent than n-6 fatty acids. Fisher et al.43 have shown that feeding fish oil to humans causes a reduction in oxygen radical formation by their neutrophils and monocytes when the cells are activated in vitro. Recently, Saito et al.44 actually observed that LDL from rabbits fed a highly purified ethyl ester of EPA was less susceptible to Cu2+-catalyzed oxidative modification than control LDL. It is possible that the tight packing of n-3 fatty acids in complex membrane lipids makes the double bonds less available for oxygen interaction.45

From a dietary point of view, it is unrealistic to expect an intake of EPA and DHA greater than that ingested by the subjects of the present study (approximately 5 g/day). Thus, it is possible that our findings, showing a decreased Tm of LDL probably due to an increased amount of n-3 fatty acids with 20 and 22 carbon atoms, represent a physical condition that is not atherogenic via the oxidized LDL pathway in humans. On the other hand, it is likely that the suggested antiatherogenic effects of fish oils operate via other mechanisms than the oxidized LDL pathway.

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