Effect of Fish Oil on Lipoproteins, Lecithin:Cholesterol Acyltransferase, and Lipid Transfer Protein Activity in Humans

Mavis Abbey, Peter Clifton, Mark Kestin, Bryan Belling, and Paul Nestel

A group of 33 mildly hypercholesterolemic men were stratified into three groups on diets closely matched except for the polyunsaturated fatty acid supplement. The first group received 14 g/day of linoleic acid (safflower oil); the second group, 9 g of α-linolenic acid (linseed oil); and the third group, 3.8 g of n-3 fatty acids (fish oil). Only fish oil lowered plasma triglycerides (by 24% at 6 weeks, \( p < 0.05 \)) compared to safflower oil. Very low density lipoprotein (VLDL) apoprotein (apo) B, triglyceride, and cholesterol fell significantly with the fish-oil diet (\( p < 0.05 \)). There was a strong correlation between the changes in VLDL triglyceride and LDL cholesterol with the fish-oil diet (\( r = -0.84, p < 0.002 \)). High density lipoprotein (HDL) cholesterol fell slightly in all three groups (\( p < 0.02 \) with the linseed-oil diet only). However, the apo A-I/A-II ratio rose by 5% (\( p < 0.05 \)), and the HDLα/HDLβ protein ratio increased by 28% with the fish-oil diet (\( p < 0.05 \)). Fish oil reduced the capacity for transfer of cholesteryl ester between LDL and HDL by 23% (\( p < 0.02 \) compared to baseline), reduced plasma lecithin:cholesterol acyltransferase activity by 21% (\( p < 0.05 \)), and reduced maximal stimulated thromboxane production by 9% (\( p < 0.05 \)). Thus fish oil produced three potentially beneficial changes: significant decreases in VLDL concentration and in thromboxane production and an increase in the HDLα/HDLβ ratio. The increase in the average HDL particle size probably reflected reduced cholesteryl ester acceptor capacity within the smaller pool of VLDL, as well as the decline in lipid transfer activity in plasma involving transfer protein itself, LDL, and HDL. (Arteriosclerosis 10:85–94, January/February 1990)

It is well established that fish oils containing high concentrations of the n-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are very potent at lowering plasma triglycerides.1–8 The effect of fish oils on plasma cholesterol in patients with hypercholesterolemia appears to depend on the source of the oil. Fish oils have been reported both to raise low density lipoprotein (LDL) cholesterol9,10 and to lower it.1 The effect on high density lipoprotein (HDL) appears to be dose-dependent, with reductions in total HDL cholesterol with 12 g or more of n-3 fatty acids per day.6,8 The n-3 fatty acids of plant origin (α-linolenic acid) have been little studied in relation to lipoprotein metabolism. It is known that limited conversion of α-linolenic acid to EPA occurs, but the biological effects of this degree of EPA enrichment are not known.11

Lipid transfer protein (LTP), which facilitates transfers of cholesteryl ester, triglyceride, and phospholipid between plasma lipoproteins12 and lecithin:cholesterol acyltransferase (LCAT), the enzyme which promotes esterification of free cholesterol in plasma,13 are key mediators of normal lipid metabolism in man. Little is known about the effect of fish oil and other polyunsaturated oils on these two systems. LTP may be quite central to atherogenesis, since species without LTP activity, such as the rat and pig,14 are relatively resistant to atherosclerosis. The effect of fish oil in inhibiting the development of atherosclerosis in experimental animals may, therefore, be partly mediated through changes in LTP activity in addition to fish oil's well characterized effects on thrombogenic and inflammatory responses.7,15–18

This experiment was designed to compare the effects of n-3 fatty acids of plant and marine origin with n-6 fatty acids in mildly hypercholesterolemic, normotriglyceridemic adult men, with an emphasis on changes in lipoprotein metabolism. We have demonstrated that fish oil reduces plasma triglyceride, plasma LCAT, plasma lipid transfer activities, and thromboxane generation, but increases LDL cholesterol levels. The blood pressure results from this study have been reported elsewhere.17

Methods

Materials

1α,2α(β)-3H cholesterol, 40 to 60 Ci/mmol, was obtained from The Radiochemical Centre, Amersham, UK. Bovine serum albumin (Fraction V) was obtained from Sigma Fine Chemical, St. Louis, MO. Ready Value
Subjects
A total of 33 men were recruited from among the volunteers who were screened for hyperlipidemia. The men were mildly hypercholesterolemic on average (mean, 6.38 mmol/l; range, 5.3 to 7.9 mmol/l). Their mean age (±SEM) was 47.4±2.5 years old, and their mean body mass index (BMI), which did not change significantly during the dietary intervention period, was 26.1±0.9 kg/m². Their alcohol intake was <40 g/day, and the mean intake was not significantly different between the three groups. The men were advised not to change their exercise patterns throughout the trial. The exclusion criteria were: alcohol consumption >40 g/day, treatment likely to affect either blood pressure or lipoprotein metabolism, or any metabolic disorder other than mild hypercholesterolemia. The study was approved by the Human Ethics Committee of the CSIRO Division of Human Nutrition, Adelaide, Australia, and informed consent was obtained from the men.

Experimental Design
All subjects commenced a diet containing 30% energy from fat (less than 5% energy from polyunsaturated fatty acids) plus a safflower-oil supplement (14 g/day) for 3 weeks. Blood pressures and blood samples for lipoprotein levels were taken on 3 consecutive days at the end of this period (Figure 1). The 33 men were then randomly allocated to either continue on the safflower-oil supplement or change to the fish-oil or to the linseed-oil supplement for a further 6 weeks. The study was performed double-blind. The safflower-oil group was thus designed to be neutral in its effect on cholesterol, while the other two groups were to highlight significant differences between n-6 fatty acids and n-3 fatty acids of marine and vegetable origin. There was no significant difference between the three groups in mean plasma cholesterol and triglyceride at the end of the baseline period.

Diet
The men were instructed carefully, were provided with sample menus, and were taught to identify and quantify their fat intake using simplified food tables. Compliance with the background diet was monitored and found to be highly satisfactory from a total of 8 days’ weighed inventory food records over the 6-week experimental period.

The major sources of fatty acids tested were a commercial fish-oil concentrate (Max-EPA), linseed oil (α-linolenic acid), and safflower oil (linoleic acid). The characteristic tastes of the supplements (provided as a beverage) were masked with natural and artificial flavors, and the men were advised to consume each supplement at the same time each day. The composition of the supplements is shown in Table 1. The amounts of fatty acids were chosen so that linoleic acid provided about 6% of energy; the amount of marine n-3 fatty acids resembled the commonly used dose of Max-EPA; for α-linolenic acid, we provided about two and a half times as much as marine n-3 fatty acids on the basis that the plant source is not as potent in animal models. The supplements were balanced for C12:0 to C16:0 fatty acids, total monounsaturated fatty acids, total polyunsaturated fatty acids, and cholesterol by using a mixture of vegetable oils and pure cholesterol.

Measurements
The men fasted for 12 hours and avoided alcohol for 24 hours before each visit. Venous blood (20 ml) was then taken with minimal occlusion into tubes containing ethylenediaminetetraacetic acid (EDTA, 1 mg/ml final concentration) as anticoagulant, and the plasma was separated by low-speed centrifugation (3000 rpm for 10 minutes).

Plasma total cholesterol and triglyceride concentrations were determined by enzymatic methods on an automated centrifugal analyzer (Cobas Bio, Roche, NJ). HDL cholesterol was determined similarly after precipitating LDL and very low density lipoprotein (VLDL) with heparin/MnCl₂, and VLDL and intermediate density lipoprotein (IDL) cholesterol and triglyceride were determined after ultracentrifugal isolation from plasma of the density <1.006 g/ml and 1.006 to 1.019 g/ml fractions. LDL cholesterol was calculated as the total cholesterol minus (HDL cholesterol + VLDL cholesterol + IDL cholesterol).
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Table 1. Composition of Supplement per 188 g (Average Daily Portion)

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Safflower oil</th>
<th>Linseed oil</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (MJ)*</td>
<td>1.29</td>
<td>1.29</td>
<td>1.29</td>
</tr>
<tr>
<td>Protein (g)*</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Carbohydrate (g)*</td>
<td>8.2</td>
<td>8.2</td>
<td>8.2</td>
</tr>
<tr>
<td>Total lipid (g)*</td>
<td>28.3</td>
<td>28.3</td>
<td>28.3</td>
</tr>
<tr>
<td>Cholesterol (mg)t</td>
<td>62</td>
<td>60</td>
<td>62</td>
</tr>
<tr>
<td>Saturated fatty acids (g)t</td>
<td>6.2</td>
<td>6.4</td>
<td>6.8</td>
</tr>
<tr>
<td>C12:0-C16:0 saturated fatty acids (g)t</td>
<td>4.5</td>
<td>4.9</td>
<td>5.1</td>
</tr>
<tr>
<td>Monounsaturated fatty acids (g)t</td>
<td>6.2</td>
<td>7.0</td>
<td>6.2</td>
</tr>
<tr>
<td>Polyunsaturated fatty acids (g)t</td>
<td>14.5</td>
<td>13.5</td>
<td>13.9</td>
</tr>
<tr>
<td>N-6 polyunsaturated fatty acids (g)t</td>
<td>14.3</td>
<td>4.3</td>
<td>10.2</td>
</tr>
<tr>
<td>N-3 polyunsaturated fatty acids (g)t</td>
<td>0.2</td>
<td>9.2</td>
<td>3.8</td>
</tr>
<tr>
<td>N-6/N-3†</td>
<td>76</td>
<td>0.5</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*Calculated from food tables. †Chemically analyzed.

Apolipoproteins were measured by using immunoturbidimetry on the Cobas-Bio centrifugal analyzer. Boehringer antisera (Boehringer-Mannheim) and Hyland Omega (Hyland Diagnostics, Malvern, PA) standards were used. The coefficient of variation of the assays was <5%.

Analysis

Paired t tests were used to compare the 6-week experimental values and the baseline (after the 3-week safflower-oil period). One-way analysis of variance (ANOVA) was used to compare the 6-week values for all three experimental groups, although they were not entirely comparable, since the safflower-oil group had taken a single supplement for 9 weeks.

Preparation of Low Density Lipoprotein and High Density Lipoprotein

Plasma density was adjusted with KBr, and each subject's LDL (d=1.019 to 1.055 g/ml) and HDL (d=1.07 to 1.21 g/ml) were isolated by sequential ultracentrifugation. The HDL for substrate in LTP assay Method A was prepared from plasma of one individual and stored at 4°C. All lipoprotein samples were dialyzed extensively against LTP assay buffer (100 mM sodium phosphate/0.01% [wt/vol] EDTA/0.02% [wt/vol] NaN₃, pH 7.4).

Preparation of Lipoprotein-deficient Plasma

After isolation of lipoproteins from the plasma of each subject, the d>1.21 g/ml lipoprotein-deficient fraction was extensively dialyzed against an LTP assay buffer. Lipoprotein-deficient plasma prepared from the plasma of one man was stored in small aliquots at -20°C to be used as a standard in the LTP assay.

Preparation of Radiolabeled Low Density Lipoprotein

LDL (d=1.019 to 1.055 g/ml) labeled with ³H in the esterified cholesterol moiety was prepared from the plasma of each subject as previously described. The radioactivity (mean±SD) in these samples was 1.7×10⁶±9×10² cpm/aliquot used in the LTP assay (n=30). Radiolabeled LDL to be used as the substrate in LTP assay Method A was prepared from plasma of one man and was stored at 4°C. The radioactivity in this sample was 4×10⁶ cpm/aliquot for each LTP assay. The stability of the radiolabeled LDL was monitored by assaying a standard preparation of lipoprotein-deficient plasma with each batch of samples. There was no significant difference between the batches.

Lipid Transfer Protein Activity Assay

Two methods were used. Method A measured the amount of lipid transfer protein activity in the lipoprotein-deficient fraction of plasma, while Method B measured the ability of the endogenous plasma lipoproteins to donate and accept cholesteryl ester.

Method A

Plasma LTP activity was measured as previously described. Lipoprotein-deficient plasma from each subject was dialyzed extensively against LTP assay buffer (ultracentrifugation resulted in a 1:5 dilution) and was tested for its ability to promote transfer of ³H-esterified cholesterol from LDL to HDL during 3-hour incubations at 37°C. Several aliquots of lipoprotein-deficient plasma (diluted 1:5) from each man were assayed to establish the linearity of the assay. Standard lipoprotein-deficient plasma (diluted 1:5) was assayed with each batch of samples to monitor repeatability. The LTP activity was calculated as previously described, with the number of units being the rate constant, k, for the transfer of radiolabeled tracer between lipoproteins:

\[-\ln[1-(\text{HDL}_t)/(\text{LDL}_0)]=Kt\]

where (HDL)₀ and (LDL)₀ are the number of dpm in HDL and LDL at times (t3h) and zero, respectively.

Method B

LTP activity was determined as for Method A, except that HDL and radiolabeled LDL isolated from each man's plasma was incubated with a standard lipoprotein-deficient plasma diluted 1:5. Determination of the linearity of transfer and calculation of the activity were as given for Method A.
\textbf{Lecithin:Cholesterol Acyltransferase Activity}

LCAT activity was measured as the difference in free cholesterol concentration in plasma before and after incubation for 40 minutes and 3 hours at 37°C. Free cholesterol concentration was measured enzymatically by using an automated centrifugal analyzer (Cobas Bio, Roche, Diagnostica, Nutley, NJ).

\textbf{Fatty Acid Composition of Total Plasma Lipids and High Density Lipoprotein Esterified Cholesterol}

Lipids were extracted from plasma (200 μl) by using chloroform/methanol/0.1M HCl (4:2:1) containing 0.005% butylated hydroxytoluene (BHT) as antioxidant. Lipids were methylated\textsuperscript{22} and extracted with petroleum spirit. Contaminating free fatty acids and cholesterol were removed by chromatography on Biosil A. The eluate was dried under N\textsubscript{2}, samples were taken up in isocitrate, and an aliquot was injected onto a vitreous silica (30 M x0.53 mm ID) cross-linked free fatty acid phase (FFAP) gas-liquid chromatography column for separation of fatty acids by using a Hewlett-Packard 5711A gas chromatograph (Hewlett-Packard, Avondale, PA).

Lipids were extracted from HDL samples containing approximately 200 μg esterified cholesterol by using the method of Dole\textsuperscript{22} with 0.005% BHT in the extraction solution. HDL esterified cholesterol was separated from the other lipids by thin-layer chromatography\textsuperscript{24} on silica gel G by using petroleum spirit, diethyl ether, acetic acid (90:15:1), containing 0.005% BHT as the developing solvent. The lipids were stained by exposure to iodine vapor. HDL esterified cholesterol was methylated\textsuperscript{22} and extracted with petroleum spirit. Contaminating free fatty acids and cholesterol were removed from the fatty acid methyl esters by chromatography on acid-washed Florisil. The columns were prepared by adding 0.125 g Florisil to glass Pasteur pipettes blocked with glass wool. The columns were washed once with chloroform and twice with petroleum spirit (40° to 60°C boiling point), and the sample was applied immediately and eluted with 3 x 0.2 ml petroleum spirit/1% diethyl ether at a flow rate of approximately 5 ml/min. The combined eluate was dried under N\textsubscript{2}, and subjected to gas-liquid chromatography as explained for plasma fatty acids.

\textbf{High and Low Density Lipoprotein Particle Size}

Lipoproteins were separated from 1 ml of plasma by a single centrifugation at a background density of 1.21 g/ml in a 50.3 rotor (Beckman) for 17 hours at 50 000 rpm. Polyacrylamide gradient gel electrophoresis of HDL was performed by using slab gradient gels (2.5% to 27% acrylamide, Gradient Laboratories, Sydney, Australia). An aliquot of 30 μl of lipoprotein sample mixed with 10 μl of a solution containing 40% sucrose and 0.01% bromphenol blue was applied directly to the gel. Electrophoresis was performed in a nondenaturing buffer of Tris borate, pH 8.35, for 17 hours at 160 V. The LDL Stokes' radii were calculated by reference to co-electrophoresed standards of thyroglobulin (8.50 nM), ferritin (6.10 nM), lactate dehydrogenase (4.08 nM), and bovine serum albumin (3.55 nM) from a high molecular weight electrophoresis calibration kit (Pharmacia Fine Chemicals, Uppsala, Sweden). The gels were fixed in 10% sulfosalicylic acid for 1 hour, stained for 4 hours in 0.04% Coomassie G-250 in 3.5% perchloric acid, and destained in 5% acetic acid. The gels were scanned with a laser densitometer (2202 ultrascan, LKB, Bromma, Sweden) and were quantified with a Hewlett-Packard 3390a integrator.

\textbf{Maximal Stimulated Thromboxane Production}

Whole blood (1 ml) was incubated at 37°C for 1 hour. The clot was allowed to retract fully at 4°C overnight, and the serum was collected and immediately frozen at -20°C.\textsuperscript{25} All samples were assayed in one batch by radioimmunoassay. The antibody recognized both thromboxane B\textsubscript{2} and thromboxane B\textsubscript{3}.

\textbf{Results}

\textbf{Plasma Lipid Levels}

As expected, n-3 fatty acids of marine origin lowered plasma triglycerides by 24% at 6 weeks (p<0.05 compared to the safflower-oil group, p<0.01 compared to baseline) (Table 2). In 9 of the 11 subjects taking fish oil, triglycerides were lowered. There was no significant relationship between the initial triglyceride value and the decrease in triglycerides with fish-oil supplementation (r=-0.42, p>0.1). A triglyceride-lowering effect was seen within 2 weeks, with the maximum change (32% decrease) seen after 4 weeks. The n-3 fatty acid of vegetable origin (linseed oil) had no significant effect on plasma triglycerides, while the safflower-oil group did not change. Plasma cholesterol concentration did not change in the fish-oil group, but fell modestly in the other two groups. The experiment was designed with the expectation that the plasma cholesterol would not change in the safflower-oil group after the 3-week baseline period. The further fall was due either to a continued lowering of cholesterol by safflower oil or to an unrelated temporal effect. The net effect of n-3 fatty acids, therefore, was a higher plasma cholesterol when compared to the effect of n-6 fatty acids (safflower oil).

\textbf{Plasma Lipoprotein Levels}

Both VLDL cholesterol and VLDL triglyceride were significantly lowered by the fish-oil supplement (p<0.02 compared to the baseline period and p<0.01 compared to the safflower-oil group at the end of the study). Linseed oil and safflower oil had no effect (Table 2). Although IDL cholesterol and IDL triglyceride were both lowered by fish oil (25% and 28%, respectively, in 7 of 11 subjects), this effect was not significant. HDL cholesterol fell in all three diet groups, with a significant fall of 8% in the linseed-oil group (p<0.02 compared to baseline). LDL cholesterol fell marginally in the safflower-oil group (4%) and in the linseed-oil group (2%), while in the fish-oil group the LDL rose by 5%. This rise was significant (p<0.05) when compared to the change in both the safflower-oil and linseed-oil groups. There was a strong correlation (r=-0.84, p<0.002) between the fall in VLDL triglyceride and the rise in LDL cholesterol.
Plasma Apolipoprotein Levels

Plasma apo B levels fell in all three groups (p<0.05 in fish-oil group) with no significant difference between the dietary supplements (Table 3). With fish oil, VLDL apolipoprotein (apo) B fell by nearly 50% (p<0.01), while IDL apo B fell by 25% (p>0.05). LDL apo B fell in both safflower-oil and linseed-oil groups and rose marginally in the fish-oil group (p>0.05). Apo A-I fell significantly in all three groups (p<0.05, safflower oil; p<0.001, linseed oil and fish oil), but there was no difference between groups. There was a significant decrease in apo A-II after 6 weeks of linseed-oil and fish-oil supplementation (p<0.05, p<0.005, respectively), but there was no significant difference between these two groups. The apo A-I/apo A-II ratio rose by 5% with fish oil, while it fell with both vegetable oils (p<0.01).

Plasma Fatty Acids

Plasma fatty acids reflected major specific fatty acids in the dietary supplements, indicating satisfactory compliance. Fish-oil supplementation with this relatively modest dose of Max-Epa produced a sevenfold increase in the levels of plasma EPA and a threefold increase in plasma DHA, primarily at the expense of linoleic and oleic acids (Table 4). In the linseed-oil group, α-linolenic acid was partly elongated to EPA in vivo, with a twofold increase in plasma EPA levels accompanying a nearly fourfold increase in plasma linolenic acid levels. However, there was virtually no further elongation to DHA in 6 weeks and, as noted previously, no fall in plasma triglyceride. There were highly significant correlations between the fall in plasma triglyceride levels and the increase in both EPA and DHA in the fish-oil group (r=-0.76 and -0.84, respectively, p<0.01), but no correlation was noted between plasma triglyceride and EPA levels in the linseed-oil group.

High Density Lipoprotein Cholesteryl Ester Fatty Acids

The changes in HDL cholesteryl ester fatty acids were similar to those observed in plasma fatty acids. The major change in HDL cholesteryl ester fatty acids after 6 weeks of fish-oil supplementation was a fourfold increase in EPA (p<0.01) mainly at the expense of linoleic acid. DHA was

Table 2. Effects of Various Dietary Lipids on Plasma Lipids and Lipoproteins

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Supplement</th>
<th>Safflower oil</th>
<th>Linseed oil</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 weeks</td>
<td>6 weeks</td>
<td>0 weeks</td>
<td>6 weeks</td>
</tr>
<tr>
<td>Plasma lipid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglyceride</td>
<td>1.43±0.66</td>
<td>1.42±0.56</td>
<td>1.90±0.91</td>
<td>1.85±0.85</td>
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<tr>
<td>Cholesterol</td>
<td>6.34±1.06</td>
<td>6.14±1.00</td>
<td>6.55±0.96</td>
<td>6.35±0.98</td>
</tr>
<tr>
<td>Lipoprotein lipid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.32±0.17</td>
<td>1.28±0.17</td>
<td>1.29±0.40</td>
<td>1.18±0.37§</td>
</tr>
<tr>
<td>VLDL cholesterol</td>
<td>0.37±0.27</td>
<td>0.40±0.23</td>
<td>0.62±0.40</td>
<td>0.63±0.39</td>
</tr>
<tr>
<td>IDL cholesterol</td>
<td>0.16±0.11</td>
<td>0.15±0.08</td>
<td>0.17±0.11</td>
<td>0.17±0.12</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>4.49±1.00</td>
<td>4.31±0.93</td>
<td>4.47±0.96</td>
<td>4.37±0.86</td>
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<tr>
<td>VLDL triglyceride</td>
<td>0.68±0.46</td>
<td>0.70±0.37</td>
<td>1.10±0.70</td>
<td>1.09±0.57</td>
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<tr>
<td>IDL triglyceride</td>
<td>0.09±0.06</td>
<td>0.07±0.04</td>
<td>0.10±0.06</td>
<td>0.09±0.06</td>
</tr>
</tbody>
</table>

Values are given in g/l and are the mean±SD.

* p<0.05 compared to safflower oil, † p<0.01 compared to safflower-oil group, § p<0.01 compared to baseline.

HDL = high density lipoprotein, VLDL = very low density lipoprotein, IDL = intermediate density lipoprotein, LDL = low density lipoprotein.

Table 3. Effects of Various Dietary Lipids on Apolipoproteins

<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>Supplement</th>
<th>Safflower oil</th>
<th>Linseed oil</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 weeks</td>
<td>6 weeks</td>
<td>0 weeks</td>
<td>6 weeks</td>
</tr>
<tr>
<td>Apo B</td>
<td>1.35±0.40</td>
<td>1.30±0.37</td>
<td>1.66±0.66</td>
<td>1.60±0.66</td>
</tr>
<tr>
<td>Apo A-I</td>
<td>1.49±0.17</td>
<td>1.39±0.20‡</td>
<td>1.52±0.20</td>
<td>1.40±0.23†</td>
</tr>
<tr>
<td>Apo A-II</td>
<td>56.5±7.9</td>
<td>55.9±9.3</td>
<td>60.3±8.8</td>
<td>55.4±11.0‡</td>
</tr>
<tr>
<td>Apo A-I/apo A-II</td>
<td>2.65±0.30</td>
<td>2.57±0.37‡</td>
<td>2.55±0.20</td>
<td>2.52±0.23</td>
</tr>
<tr>
<td>VLDL apo B</td>
<td>0.14±0.10</td>
<td>0.16±0.11</td>
<td>0.26±0.17</td>
<td>0.23±0.14</td>
</tr>
<tr>
<td>IDL apo B</td>
<td>0.04±0.03</td>
<td>0.03±0.02</td>
<td>0.05±0.02</td>
<td>0.05±0.02</td>
</tr>
<tr>
<td>LDL apo B</td>
<td>1.17±0.42</td>
<td>1.11±0.40</td>
<td>1.36±0.68</td>
<td>1.28±0.68</td>
</tr>
</tbody>
</table>

Values are given in g/l and are the mean±SD.

* p<0.05 compared to safflower oil, † p<0.01 compared to safflower oil, § p<0.01 compared to baseline, ‡ p<0.01 compared to baseline.

Apo = apolipoprotein. See the legend for Table 2 for an explanation of the other abbreviations.

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not detected in HDL cholesteryl esters before or after fish-oil supplementation.

Supplementation with linseed oil for 6 weeks resulted in a fivefold increase ($p<0.001$) in HDL cholesteryl ester linolenic acid, with elongation and desaturation to EPA, as observed in plasma fatty acids (Table 4), resulting in a threefold increase in EPA ($p<0.001$).

There were no significant changes in HDL cholesteryl ester fatty acids after 6 weeks of safflower-oil supplementation.

**Lipid Transfer Protein Activity**

Baseline data for 30 individuals in the study gave a linear response using Method A, in which 50, 100, and 200 μL aliquots of a 1:5 dilution of each subject’s lipoprotein-deficient plasma were assayed. Method B, in which each subject’s HDL and radiolabeled LDL were incubated with 50, 100, and 200 μL aliquots of standard lipoprotein-deficient plasma (diluted 1:5), gave a curvilinear response. Accordingly, only values obtained in the linear region of the curve were used to calculate LTP activity units. Baseline values (means±SEM) for LTP activity with Method A were 2.73±0.15, 2.88±0.18, and 2.96±0.23 units/ml/3 hours for safflower-oil, linseed-oil, and fish-oil groups, respectively. Method B gave baseline values of 2.65±0.14, 2.55±0.15, and 2.69±0.17 units/ml/3 hours for safflower-oil, linseed-oil, and fish-oil groups, respectively.

Using Method A to determine LTP activity (assaying each subject’s lipoprotein-deficient plasma) resulted in a significant reduction in activity ($p<0.05$) of 12% in the safflower-oil group and 13% in the fish-oil group after 6 weeks of dietary intervention. In the linseed-oil group, LTP activity was marginally increased by 2% after 6 weeks and was not significantly different from baseline. There were no significant differences between the three dietary groups (Figure 2A). Method B (assaying each subject’s HDL and radiolabeled LDL) gave a similar trend in the reductions in LTP activity for all groups compared to baseline after 6 weeks of dietary intervention. Activity was reduced 7%, 16%, and 23% in the safflower-oil, linseed-oil, and fish-oil groups, respectively, but this was only significant ($p<0.02$) for the fish-oil group (Figure 2B).

Figure 2. The change in lipid transfer protein (LTP) activity after 6 weeks of dietary intervention. The change in LTP activity was measured by Method A (A) in which each man’s lipoprotein-deficient plasma was used in the assay, and by Method B (B) in which each man’s high density lipoprotein and 3H-esterified cholesteryl-low density lipoprotein were used in the assay. Values represent mean±SD for 10 persons in each group.

**Lecithin:Cholesterol Acyltransferase Activity**

LCAT activity, measured as the change in mass of free cholesterol, gave a curvilinear response in the 3-hour time period examined (Figure 3). Baseline values (means±SEM) for 3-hour incubations were not significantly different between the three dietary groups, 147±22, 155±9, and 172±18 μmol/l free cholesterol esterified for safflower-oil, linseed-oil, and fish-oil groups, respectively. After 6 weeks of dietary intervention, LCAT activity was reduced relative to baseline in all three groups: 5%, 9%, and 21%, respectively, for the safflower-oil, linseed-oil, and fish-oil groups. The reduction from baseline with fish oil was significant ($p<0.05$) with individual changes of 35, 20, 3, -6, -22, -46, -62, -92, -94, and -99 μmol/l/3 hours. The difference between the three groups at 6 weeks was not significant (Figure 4).
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Figure 3. Lecithin-cholesterol acyltransferase (LCAT) activity in plasma before and after 6 weeks of dietary intervention. The LCAT activity was measured as the difference in mass of free cholesterol in plasma before incubation and after incubation at 37°C for 40 minutes and 3 hours. Plasma samples were obtained before (a) and after (c) 6 weeks of dietary intervention. The values represent means±SD for 10 persons.

There were significant negative correlations between the decrease in LCAT activity and the increase in EPA in both plasma and HDL cholesteryl esters (r=-0.62, p<0.05, and r=-0.80, p<0.02, respectively) in the group fed linseed oil. The same trend was apparent between the decrease in LCAT activity and the increase in EPA in HDL cholesteryl esters in the group fed fish oil, although it did not reach significance (r=-0.46, 0.1>p>0.05).

Figure 4. Change in lecithin-cholesterol acyltransferase (LCAT) activity after 6 weeks of dietary intervention. LCAT activity was measured as the difference in mass of free cholesterol before incubation and after a 3-hour incubation at 37°C. The difference in LCAT activity between baseline values and values obtained after 6 weeks of dietary intervention are expressed as means±SD for 10 persons.

Low and High Density Lipoprotein Particle Size Distribution

LDL particle size as assessed by gradient gel electrophoresis was unchanged by dietary supplementation. HDL particle size distribution was significantly altered by fish-oil supplementation. The proportion of particles of HDL$_2$ size (radius >4.4 nm) increased by 27.5%±9.1% (mean±1SD) in the fish-oil group, while in the safflower-oil group the increase was 6.6%±12.8% (p<0.005). No change occurred in the linseed-oil group. A representative HDL profile from a fish-oil-treated man is shown in Figure 5. The increase in the HDL$_2$/HDL$_3$ ratio in the fish-oil group is consistent with the 5% increase in the apo A-I/apo A-II ratio (Table 3), and with the small decrease in HDL cholesterol compared to the large 10% reduction in apo A-I. The apo A-I/HDL cholesterol ratio changed from 1.19 to 1.08 after fish-oil supplementation, indicating the presence of protein-depleted, cholesterol-enriched particles, i.e., a shift to particles in which the protein/lipid ratio was closer to that seen in HDL$_2$ than in HDL$_3$. In the linseed-oil group, the apo A-I and HDL cholesterol both fell by 8%, while in the safflower-oil group, HDL cholesterol fell by 3%, and the apo A-I fell by 7%, consistent with a small increase in the HDL$_2$/HDL$_3$ ratio.

Thromboxane Production

Fish-oil feeding produced an 8.6% decrease in maximal stimulated thromboxane production in whole blood, from 228±58 ng/ml to 208±61 ng/ml, while safflower oil produced a 10% increase (p<0.05) and linseed oil had no effect.

Discussion

The most consistent effect noted with fish-oil feeding is a reduction in plasma and VLDL triglyceride levels in both normolipidemic and hyperlipidemic subjects. In these mildly hypercholesterolemic subjects, we have demonstrated that the triglyceride lowering was directly corre-
Fish oil produced a small, but significant, 5% increase in LDL cholesterol, despite the balancing of cholesterol and saturated fat content in the three dietary supplements. No relationship was noted between the initial LDL cholesterol and the effect of fish oil. This finding has been reported in other studies of normal and hypercholesterolemic persons. Lowering of LDL cholesterol in normal and hypercholesterolemic persons has been found when large doses of salmon oil, which contains relatively more DHA than does Max-EPA, were used. It is also possible that polyunsaturated acids other than DHA or EPA may affect LDL cholesterol levels. Furthermore, in most studies IDL and LDL have been isolated together, so that a dramatic fall in IDL cholesterol may mask a small rise in LDL cholesterol, especially in hyperlipidemic persons. In this study, we noted a 25% fall in IDL cholesterol, but a small rise in LDL cholesterol when the men switched from a safflower-oil supplement during the baseline period to a fish-oil supplement. One of the reasons for a rise in LDL cholesterol despite a fall in VLDL and IDL cholesterol may be in the response of the LDL receptor. Wong and Nestel27 demonstrated that EPA completely suppressed specific binding of LDL to HepG2 cells in culture. Roach et al.28 recently noted down-regulation of LDL receptor activity in rat liver with fish-oil feeding. We found no change in HDL cholesterol levels, while others have reported increases,3,6,10 no change,2 or reductions8,8 in HDL cholesterol with fish-oil feeding.

Fish-oil supplementation resulted in a fourfold increase in EPA in HDL cholesteryl esters, mainly at the expense of linoleic acid. DHA did not reach detectable levels in HDL cholesteryl esters, even though DHA in plasma increased twofold. This result is evidence of the preferential incorporation of EPA into cholesteryl esters and DHA into triglycerides,29 reflecting the increased participation of EPA in the LCAT reaction,41 the reduced transfer of other species of cholesteryl ester from VLDL, or a combination of both.

There was a 21% reduction (p<0.05) in LCAT activity after fish-oil supplementation. The increase in EPA in HDL cholesteryl esters after supplementation with linseed oil (the result of elongation and desaturation of α-linolenic acid) was apparently not sufficient to lower LCAT activity significantly, although there was a significant negative correlation between EPA concentration and the decrease in activity of the enzyme.

A significant reduction in LCAT activity has previously been observed with dietary mackerel.29 The reduction in LCAT activity in the fish-oil group in our studies is probably the result of the n-3 fatty acid enrichment of phospholipids. Parks et al.32 have recently reported a reduced rate of LCAT-catalyzed cholesteryl ester formation when n-3-enriched phospholipids from fish-oil-fed monkeys were used in an esterification assay compared with phospholipids from lard-fed monkeys. This group also reports that there was no effect of fish-oil feeding on LCAT mass or reactivity of the enzyme with recombinant particles containing egg yolk lecithin.

Morton23 has shown that there is substrate competition for transfer of cholesteryl ester species by LTP, which is dependent on substrate specificity rather than availability. The order of specificity was shown to be 18:1>18:2>20:4>16:0=18:0 with the transfer rate for 18:1 being about 50% higher than for 16:0. Specificity appears to be dependent on acyl group composition in the order of monounsaturated, polyunsaturated, and saturated fatty acids. In support of this, our results show that the rate of LTP activity (measured using each person’s LDL and HDL and a standard source of LTP activity) decreased with the degree of cholesteryl ester fatty acid unsaturation, with LTP activity being lowest in the men after fish-oil supplementation, in which EPA increased fourfold in HDL cholesteryl esters. Studies in diabetic persons suggest that there may be another factor contributing to the reduction in LTP activity. Fielding et al.34 have reported a marked reduction in LTP activity in persons with diabetes, due to an increase in the free cholesterol-phospholipid ratio in VLDL and LDL. In these current studies, the reduction in LCAT activity would perhaps have been
sufficient to cause an increase in lipoprotein free cholesterol since less was utilized in the LCAT reaction. This may, in turn, have contributed to the reduction observed in LTP activity. An increase in a specific LTP inhibitor may also contribute to a reduction in LTP activity; however, this was not measured in these studies.

An effect not previously noted with fish-oil supplementation was the increase in the apo A-I/apo A-II ratio. As particles contain apo A-I and apo A-II in a fixed ratio of 2:1, an increase in the apo A-I/apo A-II ratio means an increase in the number of particles containing apo A-I only, a decrease in the number of particles containing apo A-I and apo A-II, or both. The HDL₂ fraction contains predominantly apo A-I only particles and HDL₃ predominantly mixed particles. Thus an increase in the relative proportions of apo A-I only particles to mixed particles is likely to lead to an increase in the HDL₂/HDL₃ ratio, as was observed on gradient gels. The decreased protein/lipid ratio in HDL after fish-oil supplementation is also consistent with the observed changes in the ratio of HDL₂ to HDL₃. Vandongen et al. observed a significant increase in HDL₃ cholesterol in diabetic persons treated with a similar dose of fish oil, while in nonhuman primates, fish-oil feeding produced a selective decrease in intermediate-size, HDL₃ subpopulations. In nonhuman primates, LDL particle size and molecular weight were also decreased by fish-oil supplementation, but in this study LDL particle size did not change. Reduced LTP activity with fish-oil treatment may underlie the increase in the HDL₂/HDL₃ ratio, since individuals with absent or markedly reduced LTP activity have increased HDL cholesterol, mainly associated with HDL₂. This particular form of familial hyperalphalipoproteinemia (FHALP) is associated with longevity. Heterozygous FHALP patients have LTP activity levels between those of normal persons and homozygous FHALP patients and also have an increased apo A-I/apo A-II ratio, indicating that even a partial reduction in LTP activity may be associated with important beneficial changes in lipoprotein distribution. The fall in VLDL triglyceride concentrations with fish-oil treatment would also increase the HDL₂/HDL₃ ratio, due to reduced acceptor lipoprotein mass. The magnitude of the reduction in LTP activity observed after fish-oil supplementation may be physiologically important. Any reduction in transfer of cholesterol from HDL to the atherogenic apo B-containing lipoproteins must be considered beneficial, and, in addition, an increase in the proportion of HDL₂ particles and an increase in apo A-I/apo A-II ratio are also factors associated with reduced risk of coronary artery disease.

Heterozygous LCAT-deficient subjects whose plasma LCAT activity is only about half the normal level have reduced apo A-I levels, implying a reduction in HDL₂, possibly due to the fact that LCAT contributes to the conversion of HDL₃ to HDL₂. In these current studies, it appears that the decrease in LTP activity that might have shifted the HDL distribution toward HDL₂ and consequently increased the apo A-I/apo A-II ratio was sufficient to mask any opposing effect through the reduction in LCAT activity. The physiological significance of the reduction in LCAT activity observed in these experiments is not clear. It is possible that a reduction in LCAT activity may have an adverse effect by reducing reverse cholesterol transport. Alternatively, it may be beneficial in reducing the amount of cholesteryl ester available for transfer to atherogenic apo B-containing lipoproteins.

Fish-oil supplementation at this modest dose produced a small, but significant, reduction in maximal thromboxane generation, and 10 g/day of DHA/EPA has been found to produce a 50% fall in thromboxane, while 1 g/day apparently is without effect. Our results are consistent with previous findings since the dose used was about half of the larger dose. The lowering of plasma thromboxane, the changes in VLDL and LDL lipids, and in HDL particle size, plus our previously reported reduction in blood pressure would be expected to more than counteract the adverse effects of a 5% rise in LDL cholesterol on cardiovascular risk. However, if fish oil is used to lower plasma triglycerides or reduce stenosis after angioplasty, LDL cholesterol levels should be monitored closely. Six weeks of fish-oil supplementation had significant effects on LTP and LCAT activities and resulted in changes in HDL distribution. The physiological effects of such changes may be important in changing risk factors for coronary artery disease.

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