Dietary Fish Oils Modify the Assembly of VLDL and Expression of the LDL Receptor in Rabbit Liver

Jane Wilkinson, Joan A. Higgins, Colin Fitzsimmons, David E. Bowyer

Abstract—Supplementation of the diet of rabbits with fish oil or sunflower oil resulted in significant changes in the lipoproteins and lipids in serum. Compared with chow-fed rabbits, dietary fish oils decreased very low density lipoprotein (VLDL), increased low density lipoprotein (LDL), and shifted the peak of the LDL to denser fractions, whereas sunflower oil increased high density lipoprotein and shifted LDL to the lighter fractions. The amount of LDL receptors in fish oil–fed rabbit liver decreased by >70% while there was only a small fall in these levels in sunflower oil–fed rabbit liver. The concentrations of apolipoprotein (apo) B in the subcellular organelles of the secretory compartment (rough and smooth endoplasmic reticulum and Golgi fractions) were also changed by dietary lipids. In both sunflower oil– and fish oil–fed liver, apo B was increased in the lumen of the rough endoplasmic reticulum compared with fractions from chow-fed rabbit liver. The apo B in the trans-Golgi lumen from fish oil–fed livers was reduced and occurred in particles of $d=1.21 \text{ g/mL}$. In contrast, apo B in the trans-Golgi lumen from livers of sunflower oil–fed rabbits was increased and occurred in particles of $d<1.21 \text{ g/mL}$. These results suggest that feeding of fish oils causes an interruption in the intracellular transfer of apo B and hence assembly of VLDL. This leads to an enrichment of the rough endoplasmic reticulum membranes with cholesterol, thus downregulating the expression of the LDL receptor.

Key Words: apolipoprotein B ■ rough endoplasmic reticulum ■ smooth endoplasmic reticulum ■ Golgi ■ cholesterol

Endogenous triacylglycerols synthesized by the liver are transported in the plasma as components of very low density lipoprotein (VLDL), which are precursors of LDL. An elevated level of LDL in the plasma is a well-established risk factor for atherosclerosis. Reduction of the rate at which VLDL is secreted is therefore an appropriate target for lowering LDL levels and atherosclerotic risk. Increased intake of dietary fish oils results in a fall in VLDL triacylglycerol, which may partly account for the observation that increased consumption of fish oils, enriched in the n-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), protects against cardiovascular disease.1,2 Experiments on hepatic cells and by inference, the intact liver, have shown that n-3 polyunsaturated fatty acids added to the incubation medium do not inhibit synthesis of triacylglycerol but do perturb intracellular events in the assembly of VLDL.3–5 It has also been demonstrated recently that prefeeding fish oils to rats inhibits secretion of apo B (particularly apo B-48) and triacylglycerol in VLDL by cultured hepatocytes.6,7 This reduction in secretion is accompanied by decreased lipolysis of intracellular triacylglycerol.7

VLDL consists of droplets of nonpolar lipids, triacylglycerol, and cholesterol ester stabilized by an outer “shell” of phospholipid, cholesterol, and apolipoproteins. Apo B is the major apolipoprotein of VLDL and is essential for their assembly and secretion. Both the human and rabbit liver secrete apo B-100 in contrast to the rat liver, which secretes both apo B-48 and apo B-100. Many investigations have suggested that apo B is synthesized in excess of that secreted and that the excess protein is degraded intracellularly.8–13 Newly synthesized apo B is either translocated to the lumen of the rough endoplasmic reticulum (RER) or remains membrane bound and is degraded.8–12 Kinetic studies on isolated hepatocytes have shown that the ER is the main site of degradation of membrane-bound apo B14,15 However, in hepatocytes, significant amounts of apo B are found in all membranes of the secretory pathway.16–20 When degradation of the membrane-bound form of apo B is inhibited, the protein accumulates in the trans-Golgi membrane.14,15 In the RER lumen, apo B is incorporated into lipid-poor VLDL-precursor particles of $d=1.21 \text{ g/mL}$.14,21,22 A large fraction of this pool of apo B is degraded in the RER lumen,14,15 presumably because it has not acquired the correct complement of lipids, is not properly folded, or is prevented in some way from moving on through the secretory pathway. In hepatocytes, most of the VLDL lipids are transferred to the lumen of the smooth endoplasmic reticulum (SER) and are assembled with the apo B—containing precursors in the lumen of the secretory compartments between the SER and the cis-Golgi.21,23 This 2-step model for VLDL assembly was

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proposed from early electron microscopic studies and is consistent with cell fractionation studies. Investigations of McArdle hepatoma cells have also concluded that apo B-48 is incorporated into lipoproteins in a 2-step pathway, although in these cells, the 2-step pathway for assembly of apo B-100–containing particles is less clear. The 2-step model has been disputed by results from studies on rat hepatocytes, which have suggested that assembly of VLDL occurs in a single cotranslational step in the lumen of the RER.

Addition of EPA and DHA to the incubation medium of cultured rat hepatocytes lowers LDL secretion and stimulates intracellular degradation of apo B, suggesting that fish oil fatty acids divert apo B from the secretory to the degradative pathway. In contrast, recent studies showed that secretion of apo B-48, but not of apo B-100, was inhibited by feeding fish oils to rats before the isolation and culture of hepatocytes, but that degradation of intracellular apo B was not affected. In the present study, we have investigated the effect of supplementing the diets of rabbits with fish oil or sunflower oil as a control on the intracellular pools of apo B-100 in rabbit liver. As a basis for these studies, we first determined the effect of the dietary oils on plasma lipoproteins. One of the major effects of a diet supplemented with fish oils was an increase in plasma LDL levels. We therefore also determined the expression of the LDL receptor (LDLR) in the liver, which is the main site of removal of LDL from the circulation. The results show that dietary oils have profound effects on plasma lipoproteins and alter the intracellular pools of apo B and lipids and that feeding of fish oils causes a fall of 70% in the expression of the LDLR in liver.

Methods

Materials

Iodixanol, commercially available as a 60% solution (Optiprep), was a gift from Bjorn Henriksen, Nycomed-Pharm AS, Oslo, Norway. Nunc 96-well microtiter plates were from Gibco-Life Technologies. Cholesterol standard and assay reagents were from Boehringer-Mannheim, and triacylglycerol standards and assay reagents were from Alpha Laboratories. Hydrogel lip-LO-p(a) agarose gel kits were from YSI Clandon. High-performance thin-layer chromatography plates were from Camlab. Reagents and materials were as described previously.

Animals and Diets

Fish oil (MaxEpa) was a gift from Dr Willum vas Dias, Seven Seas Ltd. This was stored in sealed drums at 4°C. After the drums had been opened and part of the contents removed, they were flushed with N₂ and resealed. The cholesterol content of the fish oil was determined as 1.5% (wt/wt) by quantitative high-performance thin-layer chromatography, and the cholesterol content of sunflower oil was adjusted to that of the fish oil by dissolving cholesterol in the warmed oil. The fatty acid compositions of fish oil, sunflower oil, and the chow diet (2.5% fat) are shown in the Table. The major differences were that chow and sunflower oil had a high concentration of the n-6 fatty acid linoleic and that fish oil had a high concentration of the n-3 fatty acids DHA and EPA.

Dwarf lop rabbits (6 months old, 2.56 ± 0.12 kg body weight) bred in the University of Sheffield Field laboratories were used for these studies. They were allowed free access to chow (2.5% fat, equivalent to 7% of the dietary calorie content; average intake, 95 g/d) and water and were maintained on a 12-hour light/dark cycle. In preliminary studies, animals were presented with diets supplemented with fish oil or sunflower oil (5% to 20%, wt/wt) as pastes or pellets.

<table>
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<th>Fatty Acid Composition of Dietary Lipids</th>
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<td>18:5, Arachidonic acid</td>
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<td>20:5, EPA</td>
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<td>22:6, DHA</td>
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Samples of each diet were analyzed by gas chromatography after production of the methyl esters. Results are mean of 2 determinations performed in duplicate and are consistent with the manufacturer's information. We are grateful to Drs A. Bennett and D. White, Department of Biochemistry, University of Nottingham, for performing these analyses.

However, the rabbits would not eat the diets containing either sunflower oil or fish oil. These oils (4.0 mL/kg, equivalent to an additional ~10% and 28% wt/wt, respectively, of the calorie content of the chow diet) were therefore administered by daily gavage (between 9 and 11 AM). To prevent oxidative damage, oils were taken into 10-mL syringes without air and stored in the freezer until use, when they were warmed to room temperature. Gavage was carried out by trained personnel in the University Field Laboratories, who also monitored food intake and the weight and health of the rabbits, to ensure that the diet had no adverse effect. Oils were administered daily for 2 weeks, and the last dose was given 24 hours before the animals were killed by an overdose of pentobarbitone administered intravenously.

Analysis of Plasma Lipoproteins

To monitor changes in serum lipids and lipoproteins during the course of the diets, 1 mL of blood was taken from the ear vein after 0, 7, and 14 days. The blood was allowed to clot and then centrifuged for 20 minutes at 2000 rpm. Serum was removed and the total cholesterol and triacylglycerol contents were determined. The lipoproteins were separated on Hydrogel agarose gels and stained with Sudan black, which is provided with the gels, according to the manufacturer's instructions. At the end of the diet period, the rabbits were killed, blood was taken by cardiac puncture, and the livers removed. Total triacylglycerol and cholesterol were determined in aliquots of serum. The lipoprotein classes (VLDL, LDL, and HDL) were separated by centrifugation on gradients of iodixanol. The lipoproteins in each gradient fraction were identified by electrophoresis on Hydrogel agarose gels followed by staining with Sudan black, and aliquots of each fraction were taken to determine the triacylglycerol and cholesterol contents.

Preparation of Subcellular Fractions From Rabbit Liver

Rabbit livers were homogenized in 0.25 mol/L sucrose, and total microsomes, rough microsomes (RER), smooth microsomes (SER), cis-enriched Golgi, and trans-enriched Golgi were prepared as described previously. The vesicular fractions were separated into membrane and luminal content fractions by treatment with sodium carbonate. A “cocktail” of protease inhibitors was added to the initial homogenate and all subcellular fractions prepared, which were stored frozen at −20°C and analyzed within 4 weeks.

Separation and Lipid Analysis of Microsomal Fractions in Gradients of Iodixanol

In some experiments, total microsomes from the livers of chow-fed, sunflower oil–fed, and fish oil–fed rabbits were separated into RER...
and SER in self-generating gradients of ioxanol, and membrane and luminal content fractions were prepared.\textsuperscript{21} Lipids were extracted from the fractions and analyzed by high-performance thin-layer chromatography.\textsuperscript{21,23}

**Determination of Apo B**

The apo B content of subcellular fractions, luminal content fractions, and plasma samples was determined by competition ELISA using a monoclonal antibody (MAC 31).\textsuperscript{31} A competition curve, using a range of concentrations of the LDL standard that contained only apo B, was prepared for each microtiter plate and was used to calculate the amount of apo B in the unknown samples. In some experiments, the proportion of apo B in the luminal content fractions that floated from a density of 1.21 was determined by adjusting the density of the content fraction to 1.21 with the addition of solid KBr, by layering from a density of 1.21 was determined by adjusting the density of the content fraction to 1.21 with the addition of solid KBr, by layering this beneath a KBr solution of $d=1.009$ g/mL and centrifugation at 400 000 rpm (105 000g) in an SW25.2 rotor for 20 hours. The layers were removed, dialyzed, and concentrated, and the apo B content was determined as previously described.\textsuperscript{14}

**Determination of the LDLR**

Detergent extracts of liver were prepared, separated by SDS–polyacrylamide gel electrophoresis, and probed with the monoclonal antibody MAC 188 raised against purified LDLR protein.\textsuperscript{31} The amount of bound MAC 188 was determined by quantitative Western blotting,\textsuperscript{32,33} and the amount of LDL protein was expressed in arbitrary units measured under standard densitometry conditions.

**Results**

**Effect of Dietary Oils on Serum Lipids and Lipoproteins**

The prediet serum levels of triacylglycerol, cholesterol, and apo B varied considerably between individual rabbits. In 15 rabbits the triacylglycerol ranged from 0.94 to 3.73 nmol/L (mean $\pm$ SEM, 1.80 $\pm$ 0.20); the cholesterol ranged from 1.38 to 4.62 nmol/L (mean $\pm$ SEM, 2.42 $\pm$ 0.20); and apo B ranged from 6.90 to 21.14 (mean $\pm$ SEM, 11.03 $\pm$ 1.54). Therefore, the percent change in triacylglycerol, cholesterol, and apo B relative to the starting levels was calculated for each individual animal (Figure 1). In fish oil–fed animals, there was no significant overall change in total triacylglycerol, although levels fell by as much as 40% in some animals. The total cholesterol levels rose $\approx 60\%$, and there was a striking increase (close to 400%) in plasma apo B (Figure 1). In rabbits fed sunflower oil, there was a small increase in total triacylglycerol and cholesterol after 1 week of the diet, and these returned to the starting levels after the second week of the diet, whereas apo B levels rose by $\approx 60\%$ after 1 week and this increase was sustained at 2 weeks (Figure 1).

Interpretation of measurements of components of total serum is difficult because all of the lipoprotein classes are pooled. Increases in triacylglycerol or cholesterol in 1 class of lipoprotein may be balanced by decreases in the lipids of another class of lipoprotein, resulting in no overall change. Therefore, we examined the effects of diets on VLDL, LDL, and HDL by separating the lipoproteins in self-generating ioxanol gradients followed by determination of the lipids (Figure 2). This method separates the classes of lipoproteins on the basis of density in a 3-hour centrifugation step, and the recovery of lipids from the gradient is $>90\%$.\textsuperscript{30} Dietary oils altered the profile of the serum lipoproteins compared with chow-fed rabbits. In fractions from fish oil–fed rabbits, the relative amount of VLDL seen in agarose gels was reduced, the amount of LDL increased and the peak of their distribution shifted to denser fractions, and HDLs were apparently reduced. In sera from sunflower oil–fed rabbits, there was a shift in the peak density of LDL to lighter fractions, overlapping distribution of the VLDL, and there was a large apparent increase in HDL.

In sera from fish oil–fed rabbits, triacylglycerol in the VLDL fraction fell compared with that in serum from chow-fed rabbits (Figure 3). However, the triacylglycerol content of LDL increased $\approx 5$-fold compared with the chow-fed controls. The cholesterol content of the LDL fractions also rose $>5$-fold compared with that of the chow-fed controls. In sera from sunflower oil–fed rabbits, there was no significant change in the VLDL lipid compared with chow-fed samples. However, there was an increase in both the triacylglycerol and cholesterol content of the HDL fractions. Dietary oils therefore produce complex changes in the plasma lipoproteins. Overall, fish oils decrease VLDL, increase LDL, and shift the LDL to denser fractions, whereas sunflower oils increase HDL and shift the LDL to lighter fractions.

**Effect of Dietary Oils on Expression of the LDLR in the Liver**

The liver is the main site of removal of LDL from the circulation. To investigate whether the increase in LDL found on feeding of fish oils was a result of decreased removal of LDL from the circulation, we determined the amount of LDLR protein in the livers of rabbits fed chow, sunflower oil, or fish oil diets for 2 weeks. Dietary fish oils lowered LDLR levels protein by $>70\%$ ($P>0.002$) compared with chow-fed controls, whereas in sunflower oil–fed animals, the level fell by 18% ($P>0.01$) (Figure 4).

**Effect of Dietary Oils on the Concentration of Apo B in Subcellular Fractions**

The major effect of feeding oils for 2 weeks on the concentration of apo B was a 60% reduction in the apo B (micro-
grams per milligram of fraction protein) of trans-Golgi prepared from livers of fish oil–fed rabbits compared with chow-fed controls (Figure 5). There was also a small increase in the apo B content of the RER from livers of fish oil–fed and sunflower oil–fed animals compared with that from chow-fed controls (Figure 5). Apart from these changes, the apo B content was similar in fractions prepared from livers of sunflower oil–fed rabbits to those in fractions prepared from chow-fed liver (Figure 5). For direct comparison of fractions from livers of animals fed different diets, the concentration of apo B (micrograms per milligram fraction protein) is given. However, when one takes into account the recoveries of marker enzymes from the total liver homogenate, it can be calculated as described previously \(^{17}\) that in livers from chow-fed or sunflower oil–fed rabbits, approximately two thirds of the apo B is in the ER, with the remainder in the Golgi. In the livers of fish oil–fed rabbits, the amount of apo B in the ER was unchanged compared with that in livers of chow-fed or sunflower oil–fed animals, but the apo B content in the Golgi fell by 60%.

When the subcellular fractions were separated into membrane and luminal contents, further differences between chow-fed and oil-fed rabbits were observed (Figure 6). In chow-fed animals, \(\approx 60\%\) of the apo B in the ER fractions was membrane bound; however, in both sunflower oil–fed and fish oil–fed animals, 5% to 15% of the apo B was in the membrane and the concentration of apo B in the lumen was \(>3\times\) that in the fractions from chow-fed fractions. The concentration of apo B in the lumen of the trans-Golgi from fish oil–fed liver was reduced by 50% compared with that in the chow-fed controls, with no significant effect on the membrane apo B, whereas in the trans-Golgi prepared from livers of sunflower oil–fed animals, the apo B concentration increased in the luminal contents by 50% and decreased in the membrane by 50%.

These observations suggest that increased dietary fat shifts apo B from the membrane to the lumen of the RER, ie, into the secretory pathway, and that fish oils, but not sunflower oils, interfere with the transit of apo B from the ER lumen to the Golgi lumen. In subcellular fractions from chow-fed rabbit liver, the proportion of luminal apo B that floated from a density of 1.21 increased in the luminal contents by 50% and decreased in the membrane by 50%.

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Effect of Dietary Lipids on the Lipid Composition of Microsomal Fractions

Dietary fish oils apparently reduce the movement of apo B from the lumen of the ER to the Golgi lumen and inhibit assembly of apo B with lipid. VLDL assembly involves transfer of cholesterol, cholesterol ester, and triacylglycerol from the sites of their synthesis in the ER membrane to the lumen. Transcription of the LDLR gene is controlled by a putative cholesterol pool in the ER membrane. The effect of dietary fish oil on expression of the LDLR therefore may be a consequence of changes in the membrane pools of cholesterol in the ER. To test this hypothesis, we determined the effect of dietary fish oils on the lipid composition of RER and SER separated on self-generating iodixanol gradients.

Figure 3. Effect of dietary oils on lipids of plasma lipoproteins separated on iodixanol gradients. Plasma lipoproteins were separated as in the legend to Figure 2. Aliquots of fractions were used to assay cholesterol and triacylglycerol levels. Results are mean of 3 separate experiments ±SD. Gradient fractions 1 to 12 correspond to lanes 3 to 14 in Figure 2. Lipoprotein classes were identified by comparison with Figure 1.

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The recoveries of protein, phospholipid, and NADPH cytochrome c reductase of the RER and SER were similar in chow-fed, fish oil–fed, and sunflower oil–fed animals, indicating that the different diets did not alter the relative amounts of these 2 subcellular organelles. In the ER fractions from chow-fed livers, the distribution of lipids was as previously described: triacylglycerol, cholesterol ester, and cholesterol each exhibited a peak coincident with the SER, and a large fraction of each of these lipids was in the lumen (Figure 8). In contrast, there was less cholesterol, cholesterol ester, and triacylglycerol in the SER from fish oil–fed livers compared with fractions from chow-fed animals, and these lipids were almost completely recovered in the membrane fractions. There was also a large increase in the cholesterol...
content of the RER from the fish oil–fed liver compared with chow-fed liver, and >90% of this was in the membrane fraction. In the fractions from sunflower oil–fed liver, there was an increase in the triacylglycerol of the RER membrane, and the cholesterol, cholesterol ester, and triacylglycerol contents of the SER were slightly lower than those in fractions from the chow-fed liver.

Discussion

A variety of approaches have been used in investigations of the effect of varying dietary lipid compositions on plasma lipoproteins. These include supplementation of the diet or isocaloric replacement of carbohydrate with fats of known composition, specific fatty acids, or esters of fatty acids. In the current study, to minimize the variables, we opted to supplement a low-fat chow diet with oil. This approach has also been used by other laboratories in studies of VLDL assembly.6,7 The variables between the dietary regimens are the amount of fat in the diet (2.5% to 12.5% wt/wt; 7% to 35% of the total calorie intake) and the composition of fatty acids (high n-6 and high n-3). The changes in apo B distribution and LDLR levels in the livers from rabbits fed different diets are clear-cut. However, it must be emphasized that the experimental diets are extreme in fatty acid composition compared with the normal human diet. Smaller changes...
Dietary Fish Oils and Lipoprotein Uptake and Assembly

may result when the fatty acid composition of the diet is varied in a more subtle way.

Increased consumption of fish oil by normal or hypertria-
cylglyceridemic individuals results in a fall in total plasma
triacylglycerol.1,2 The change in plasma cholesterol is vari-
able, and a reduction, increases, and no change have been
reported.1,2 Variations between individuals, differences in
baseline diets, or preexisting dyslipidemias have been implicated
as accounting for these variable effects. Animal exper-
iments have also shown varied and inconsistent effects of
increased consumption of fish oils on plasma lipids between
different species. One confounding factor is the amount of
cholesterol in the diet. In the rat, increased fish oil as part of a
low-cholesterol diet results in a fall in both plasma
cholesterol and triacylglycerol, whereas in the hamster, there
is little effect on plasma triacylglycerol and only a small fall
in plasma cholesterol.3,6 When cholesterol and saturated fat
are added to the diet together with fish oils, VLDL and LDL
cholesterol levels increase in the hamster but fall in the rat.4,6
In the present studies, we found that dietary fish oils increase
plasma cholesterol and apo B in rabbits and show no consistent change in plasma triacylglycerol. However, the
changes in lipoproteins are complex and involve all classes of
lipoprotein. Thus, dietary fish oils reduce VLDL and increase
LDL, whereas dietary sunflower oils increase HDL, with little effect on VLDL. The increased LDL in the fish oil–fed
rabbit is probably a consequence of the large fall in the expression of the LDLR that we found in the livers of these
animals.

The present investigation used cell fractionation to deter-
mine the intracellular pool sizes of apo B within the secretory
compartment of whole liver. The results reported for chow-
fed rabbit livers are similar to those reported previously.1,4
The validity of the cell fractionation procedures has been
described in detail elsewhere1,4,17,19; however, it must be
emphasized that the secretory compartment is a physical and
functional continuum. Assay of marker enzymes and use of
immunoblotting8,14,17,19,23 have shown that the trans-Golgi has
no detectable ER marker and is the only fraction with significant levels of the trans-Golgi network marker TGN38,
whereas the RER has very low contamination with UDP-
galactosyltransferase. The cis-Golgi network marker is pres-
ent at very low levels in the RER but at relatively high levels
in both the SER and cis-Golgi.23 The major site of apo B
degradation is the ER in hepatocytes.15,23 However, we have
consistently found that apo B is present at high concentrations in trans-Golgi membranes.16–19 This has also been observed in
rat hepatocytes by other laboratories.20 With respect to this
finding, we cannot exclude the possibility that the trans-Golgi
fraction is contaminated with elements derived from the ER.
However, if this is the case, then the contaminating elements
must have extremely high concentrations of apo B compared
with the total ER, lack ER markers, and respond specifically
to dietary fish oils.

Our observations indicate that dietary lipids significantly
affect the concentrations of apo B in subcellular fractions,
which reflect the size of intracellular pools.16,17,19 Increased
dietary lipid, either fish oil or sunflower oil, increases the
amount of apo B in the lumen of the RER, suggesting that

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