Effects of n–3 fatty acids on the NMR profile of plasma lipoproteins


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Abstract The effects of fish oil supplementation (14.5 g n–3 fatty acids/day) on plasma lipoprotein particles in healthy volunteers were assessed by high resolution 13C and 1H nuclear magnetic resonance (NMR) spectroscopy. Resonances not previously observed in the 13C and 1H spectra of plasma and isolated lipoproteins were detected after fish oil ingestion. The 13C resonances, centered at 14.3, 127.1, and 131.6 ppm, have been assigned to specific carbon groups (CH2–CH=CH–, CH3–CH2–CH = CH–CH=CH–, CH3–CH2–C=CH–CH2–, respectively) in eicosapentaenoic acid (C20:5n–3) and docosahexaenoic (C22:6n–3) DHA. The new lipid resonance observed in the 1H spectra of plasma (0.941 ppm) is consistent with the incorporation of these n–3 fatty acids into lipoprotein particles. The presence of increased EPA and DHA in plasma lipids was confirmed by gas-liquid chromatography. A marked reduction in the intensity of the methylene signal from very low density lipoproteins (VLDL) was also observed with fish oil. This reduction arises from a decrease in plasma triglyceride concentration (ca. 18%) and a reduction in the number of VLDL particles. Transverse relaxation studies of isolated VLDL and low density lipoprotein (LDL) showed significant elevation in the T2 of the –(CH2)3– and CH2= signals from non-n–3 fatty acids. The relaxation characteristics and signal intensity of the novel 1H peak (0.941 ppm) point to the existence of n–3 enriched microenvironments within lipoprotein particles. These findings suggest that incorporation of EPA and DHA into VLDL and LDL, after fish oil ingestion, leads to significant alteration in the molecular architecture of lipoprotein particles. These findings suggest that incorporation of EPA and DHA into VLDL and LDL, after fish oil ingestion, leads to significant alteration in the molecular architecture of lipoprotein particles.

Supplementary key words transverse relaxation • lipid mobility • microenvironments

In recent years, there has been increasing interest in the effects of dietary fish oil on plasma lipid metabolism (1–3). The main effect of dietary fish oil appears to be a lowering of total plasma triglyceride and VLDL concentrations (1). This is due to a combination of factors, including inhibition of triglyceride secretion and an apparent enhancement of triglyceride removal (1, 4, 5). Several reports have shown a significant rise in the cholesterol/triglyceride ratio contained in VLDL and LDL, suggesting smaller lipoprotein particles (4). These studies have concentrated mainly on determining the concentration of eicosapentaenoic acid (EPA: C20:5n–3) and docosahexaenoic (DHA: C22:6n–3) in different lipoprotein fractions and/or their effects on relative levels of plasma lipids and lipoprotein fractions. The effects that n–3 fatty acids may have on the structure and function of plasma lipoproteins have not been fully investigated. Parks and Rudel (5) and Nenseter et al. (6) have shown that dietary fish oil can induce changes in the thermal transition of LDL particles. However, due to the methodology used in their studies, little could be ascertained as to the general organization of the lipid components within the intact lipoprotein particles. To address this question we have in this study used nuclear magnetic resonance spectroscopy.

High resolution nuclear magnetic resonance (NMR) spectroscopy is a non-destructive technique which is finding increasing favor in metabolic studies. NMR spectroscopy has been applied to the study of a range of metabolic questions in a variety of biological systems, including body fluids (7, 8) and tissue extracts (9). NMR spectroscopy has also been applied to the study of isolated lipoproteins, yielding valuable information regarding their composition and structure (10). Recent improvements in NMR techniques and data processing have opened the possibility of studying different lipoprotein fractions in intact plasma (11–13). These meth-
ods enable complete qualitative and quantitative measurements of different lipoprotein fractions directly from the \(^1\)H NMR spectra of intact plasma.

The purpose of the present study was to examine the effects of dietary fish oil on plasma lipoproteins, principally VLDL and LDL, by \(^1\)H and \(^13\)C NMR spectroscopy. A combination of chemical shift and spin-echo measurements suggests that fish oil induces marked changes in the physical properties, as well as the composition, of lipoprotein particles. The use of NMR spectroscopy to assess the effects of dietary supplementation on the physicochemical status of plasma lipoprotein particles is discussed.

SUBJECTS AND METHODS

Subjects

Nine healthy volunteers (29–40 years old) took part in this study. All subjects had normal liver function and normal fasting plasma triglyceride concentrations (0.62 ± 0.17 mmol/l). From 7 days before and throughout the study, subjects excluded alcohol and fish from their normal diet.

Protocol and blood samples

All volunteers consumed 50 ml/day MaxEPA fish oil (EPA: 17.0%, DHA: 11.9% and DPA (docosapentaenoic acid): 2.7%, expressed as g/100 g of fish oil) for 7 days. The fish oil was kindly supplied by Seven Seas Ltd, Hull UK. This amount of fish oil corresponds to 15.8 g of n-3 fatty acids per day. All blood samples were drawn after a 12-h fast, immediately prior to starting fish oil (baseline samples) and again after 7 days of fish oil supplementation. Venous blood samples for lipid and NMR analyses were collected in tubes containing sodium EDTA and phenylmethylsulfonylfluoride (PMSF). PMSF was added to prevent enzymatic lipoprotein degradation (14). Plasma (ca. 20 ml/subject) was immediately separated by centrifugation at 700 g for 20 min at 4°C.

Lipoprotein fractionation

Lipoprotein fractions were isolated by sequential flotation ultracentrifugation as described by Lindgren et al., with small modifications (15). Basically, the density of whole plasma was adjusted to 1.25 g/ml with KBr and the solution was spun at 150,000 g (Beckman 70.1 Ti rotor, 45,000 rpm) at 15°C for 20 h. The top of each tube was sliced off and the lipoproteins were collected using a pasteur pipette and the density was increased with solid KBr. The lipoproteins were then loaded into Sorvall 03988 polyallomer ultracentrifuge tubes and the samples were carefully overlaid with a solution of NaCl 0.15 M, EDTA 1 mM. Tubes were spun at 162,000 g (Sorvall TV8 65B rotor, 45,000 rpm) for 1 h at 15°C. Tubes were then allowed to stand at room temperature for 30 min to allow the VLDL fraction to move up the walls of each tube. Each lipoprotein fraction could be seen as a distinct band within the gradient. The top of each tube was sliced off and the various fractions were collected with a pasteur pipette. The density of each fraction was then sequentially adjusted to isolate VLDL (<1.006 g/ml), LDL (1.006–1.063 g/ml) and high density lipoprotein particles (HDL: 1.063–1.21 g/ml). Density was adjusted by adding solid KBr and all centrifugation was at 150,000 g (Beckman 70.1 Ti rotor) for 20 h at 15°C. Each tube was sliced and the top 3 ml was collected. These volumes of each lipoprotein fractions were more than sufficient for all subsequent NMR and GC measurements. After separation, lipoprotein fractions (1–4 ml) were dialyzed for 24 h against NaCl 0.15 M, EDTA 1 mM, CaCl2 0.2 mM, NaN3 15 mM. Fractions were stored at 4°C, for a period of less than 12 h, until NMR analysis. Minimal cross contamination and effective separation of the lipoprotein fractions was confirmed by determining apolipoprotein content of each fraction, after delipidation, by polyacrylamide gel electrophoresis. Briefly, the Laemmli SDS polyacrylamide gel electrophoresis method was used (Bio-Rad Laboratories, Richmond, CA), with a 3–18% resolving gel (16).

Plasma lipids and glucose

Total plasma triglyceride concentration was measured using standard enzymatic test kits (Boehringer, Mannheim, Germany) (17). Total plasma cholesterol, plasma LDL, and plasma HDL cholesterol concentrations were determined enzymatically (18–20). HDL cholesterol was determined using the method described by Lopes-Virella et al. (20) where VLDL and LDL are precipitated by adding phosphotungstic acid and magnesium ions to the sample. LDL cholesterol concentration was measured using polyvinyl sulfate precipitation (19). Total plasma eicosapentaenoic acid (EPA; C20:5n–3) and docosahexaenoic acid (DHA; C22:6n–3) concentrations were determined by capillary gas–liquid chromatography (GLC), as described by Sanders,
TABLE 2. Plasma and LDL EPA and DHA concentrations determined by GLC

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Total Plasma</th>
<th></th>
<th>Total LDL</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EPA</td>
<td>DHA</td>
<td>EPA</td>
<td>DHA</td>
</tr>
<tr>
<td>Control</td>
<td>1.06 ± 0.46</td>
<td>3.63 ± 1.18</td>
<td>0.73 ± 0.37</td>
<td>2.64 ± 0.34</td>
</tr>
<tr>
<td>Fish oil, 7 days</td>
<td>13.02 ± 2.49</td>
<td>6.52 ± 1.44</td>
<td>18.15 ± 2.44</td>
<td>5.93 ± 0.64</td>
</tr>
<tr>
<td>Significance of change</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Values are given as means ± SEM. EPA and DHA (n = 9) are expressed as mass % of total fatty acids. *Ultracentrifugally isolated LDL.

Hinds, and Pereira (21) and Chen, Menard, and Cun- nane (22) and expressed as mass % of total plasma fatty acids. Plasma glucose levels were measured using standard enzymatic methods (23). All analyses were performed in duplicate.

NMR measurements

$^{13}$C and $^1$H NMR measurements were carried out on a JEOL GSX500 spectrometer operating in quadrature detection mode at 125.7 and 500 MHz, respectively. All spectra were recorded at constant temperature (37°C).

$^{13}$C NMR

Proton-decoupled $^{13}$C NMR of plasma and lipoprotein fractions were obtained essentially as described by Peeling et al. (24), with small modifications. A 30° pulse angle with a 1 sec relaxation delay was used to acquire fully relaxed spectra. Due to the low sensitivity of $^{13}$C, all spectra required more than 10,000 scans for good signal to noise ratio, leading to a total scan time of 12 h. This long acquisition time meant that determination of absolute $T_2$ and $T_1$ values for the $^{13}$C resonances were not feasible. Free-induction-decays (FID) were zero-filled to 64K and an exponential line broadening of 2 Hz was applied before Fourier transformation.

Fig. 1. $^{13}$C proton-decoupled spectra of plasma: (A) before; (B) after fish oil supplementation. Peak assignments: a-f, lipids (see Table 3); G, glucose (96.2 ppm); P, protein related peak (40.3 ppm).
\(^1\text{H NMR}\)

A sample (0.5 ml) of intact plasma or isolated lipoprotein fractions, VLDL, LDL and HDL, was placed in a 5-mm diameter NMR tube (Fluorochem Limited, UK) and 0.05 ml of D\(_2\)O was added as a field-frequency lock. Samples were allowed to equilibrate at 37°C in the spectrometer for 10 min prior to acquisition. The intense water peak and the broad protein resonances were suppressed by the application of a gated secondary irradiation at the water resonance. The Hahn spin-echo (90°-180°-collect) spectra of plasma were obtained with a \(\tau = 60\) ms and a repetition time of 10 sec (25, 26). Single pulse spectra were obtained with a pulse angle of 35° and a total relaxation delay of 5 sec to allow full relaxation.

Transverse (spin-spin) relaxation times \((T_2)\) of the methylene \((-\text{CH}_2-)\) and methyl \((-\text{CH}_3-)\) signals of VLDL and LDL were obtained by the use of CPMG spin-echo sequence with a 1 ms delay and increasing from 2 to 600 ms (7). The FIDs were zero-filled to 32K prior to Fourier transform. Plasma spectra were cross-referenced to the methyl group resonances of lactate at 1.33 ppm and N-acetyl resonances at 2.02 ppm for chemical shift.

**Data analysis**

\(^1\text{H NMR}\) spectra of whole plasma and isolated lipoproteins were analyzed using standard JEOL spectrometer software, allowing direct measurements of chemical...
shift, peak areas, and $T_2$ measurements. $^{13}$C resonances were measured after computer-based baseline correction of the areas of interest. Spectra were internally scaled relative to the glucose resonance at 96.2 ppm and the protein-related peak at 40.3 ppm (24), both of which were unaffected by fish oil supplementation. Peak areas of the resonances in the baseline plasma spectrum were compared to those obtained from the plasma spectra after 7 days of fish oil supplementation to give the peak height ratios for each subject. Each subject served as his own control.

Carbon and proton NMR signals were assigned on the bases of chemical shifts from previously published data and chemical shifts obtained from spectra of isolated lipoproteins and pure lipids (7, 8, 10, 24–27).

**Statistical analysis**

All values presented here are expressed as mean ± SEM. Statistical analysis was performed by Student's paired t-test and changes were considered significant when $P < 0.05$.

**RESULTS**

Changes in plasma lipid concentrations after 7 days fish oil supplementation are shown in Table 1. Plasma triglycerides concentrations decreased by 18% ($P < 0.03$), while total plasma cholesterol concentration and the LDL/HDL cholesterol ratio showed no significant changes. Furthermore, the proportions of EPA and DHA in total plasma were raised ca. 10-fold ($P < 0.001$) and 2-fold ($P < 0.001$) respectively (Table 2). Similarly, isolated LDL particles showed marked increases in relative levels of EPA (20-fold; $P < 0.001$) and DHA (2-fold; $P < 0.01$). No changes in plasma glucose concentration were observed (pre: $4.92 ± 0.60$ vs. post: $5.21 ± 0.45$ mmol/l).

Typical $^{13}$C NMR spectra of intact plasma and isolated LDL before and after fish oil supplementation are shown in Fig. 1 and Fig. 2. All the spectra were acquired at constant temperature (37°C). The spectrum from control plasma consists of resonances from low molecular weight metabolites, including glucose, glutamine, lactate, alanine, and lipids from lipoprotein particles superimposed on a broad protein envelope (24). Using $^{13}$C NMR, distinctive chemical groups can be distinguished by their chemical shift, including specific sites in the fatty acid chains and cholesterol (Table 3) (10, 24). In particular, as shown in Fig. 1A, resonance f (54.1 ppm) has been assigned to the choline groups in phospholipids (–N+(CH3)3) from HDL and LDL particles; resonance e (29.5 ppm) to the fatty acyl methylene (–(CH2)n–) carbons; resonance b (14.1 ppm) to the terminal fatty acid methyl (CH3–) carbon; resonance a (11.9 ppm) to the C-18 carbon of cholesterol. Two resonances dominate the olefinic region of the spectrum (Fig. 2C) and can be assigned to monounsaturated fatty acids (i, 129.7 ppm: –CH2=CH–CH2–) and polyunsaturated fatty acids (i, 129.7 ppm:–CH=CH–CH=CH2– and h, 127.4 ppm:–CH=CH–CH=CH–) in the plasma spectra from all volunteers: at 14.3 ppm (b'), 121.1 ppm (g), and 131.6 ppm (j). These resonances have been assigned, based on

### Table 3. Assignment, chemical shift, and peak height ratios of the $^{13}$C spectra of intact plasma before and after 7 days fish oil supplementation

<table>
<thead>
<tr>
<th>Resonances</th>
<th>Chemical Shift ($\delta$ ppm)</th>
<th>Peak Ratio Fish oil/Control</th>
<th>Significance of Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Cholesterol C18</td>
<td>11.9</td>
<td>1.20 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>(b) CH3-CH=CH2</td>
<td>14.1</td>
<td>1.16</td>
<td></td>
</tr>
<tr>
<td>(b') CH3-CH2=CH = CH– (n–5)</td>
<td>14.3</td>
<td>a</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>(c) CH3-CH2=CH2</td>
<td>22.6</td>
<td>0.91 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>(d) CH = CH–CH=CH–CH–CH</td>
<td>25.6</td>
<td>1.67 ± 0.31</td>
<td>$P &lt; 0.02$</td>
</tr>
<tr>
<td>(e) Fatty acyl.–(CH2)n–</td>
<td>29.5</td>
<td>0.72 ± 0.17</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>(f) Choline.–N+(CH3)3</td>
<td>54.1</td>
<td>1.14</td>
<td></td>
</tr>
<tr>
<td>(g) CH3–CH2–CH = CH–CH2 (n–3)</td>
<td>127.1</td>
<td>a</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>(h) Polyunsaturation Index</td>
<td>127.7/129.9</td>
<td>1.74 ± 0.46</td>
<td>$P &lt; 0.01$</td>
</tr>
<tr>
<td>(i)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(j) CH3–CH–CH = CH– (n–3)</td>
<td>131.6</td>
<td>a</td>
<td>$P &lt; 0.01$</td>
</tr>
</tbody>
</table>

Values are given as means ± SEM.

*Unique resonances from n-3 fatty acids (b', g, and i) were present only in $^{13}$C spectra of plasma samples taken after fish oil supplementation. Therefore, no fish oil/control ratios are available.
Fig. 3. $^{13}$C-proton-decoupled spectra of isolated LDL: (A) before and (B) after fish oil supplementation. Resonance assignment is shown in Fig. 1 and Table 3.
chemical shift and literature values, to \( \text{CH}_3\text{CH}_2\text{CH} = \text{CH}^- \) (b': 14.3 ppm), \( \text{CH}_3\text{CH}_2\text{CH} = \text{CH}^- \) (g: 127.1 ppm), and \( \text{CH}_3\text{CH}_2\text{CH} = \text{CH}^- \) (j: 131.6 ppm) of n-3 fatty acids, respectively (27). Fish oil also led to significant changes in the intensity of other lipid resonances (Table 3). Peak e (-(\text{CH}_2)_n-) at 29.5 ppm was consistently reduced (ca. 15%; \( P < 0.01 \)), while resonance d (25.6 ppm), arising from -CH = CH-CH\text{=CH}-CH- carbons, was markedly increased (ca. 72%, \( P < 0.01 \)). Furthermore, the polyunsaturation index, defined as the peak height ratio of signals h/i (127.7/129.7 ppm), was significantly increased (ca. 83%; \( P < 0.01 \)) in all plasma samples after fish oil supplementation. No significant changes in the relative intensities of the resonances arising from cholesterol \( \text{C}-18 \) (a: 11.9 ppm), fatty acid terminal methyls (b: 14.1 ppm), and choline -N\textsuperscript{3} (CH\textsubscript{3})\textsubscript{3} (f: 54.2 ppm) were observed. The \( ^{13} \text{C} \) NMR spectra of isolated LDL is shown in Fig. 3. The spectra consist principally of signals from lipids, including triglycerides, phospholipids, cholesterol, and cholesteryl esters (10). As with intact plasma, supplementation of fish oil led to an increase in the polyunsaturation index and the appearance of resonances (b', g, and j) not previously observed. These peaks were assigned, based on their chemical shift and lipid extract analysis, to specific chemical groups of n-3 fatty acids. Similar resonances were observed in the spectra of isolated VLDL and HDL particles (results not shown).

The resonances at 14.3 and 14.1 ppm can be used to determine, by \( ^{13} \text{C} \) NMR, the relative plasma levels of EPA + DHA after fish oil ingestion. The results were compared to those obtained by GLC from the same samples. Good agreement between the two methods was observed (GLC: 19.8 ± 3.5% vs. NMR: 17.6 ± 1.77%). Similar agreement was observed for the LDL levels of EPA + DHA as measured by GLC and NMR (GLC: 24.1 ± 4.2% vs. NMR: 20.0 ± 1.6%).

\(^1\text{H} \) spectra of blood plasma have been extensively reported in the literature (7, 8). Resonances from lipoproteins arise mainly from their lipid components (fatty acids from triglycerides, cholesteryl esters, and phospholipids) as contributions from apolipoproteins are too broad to be observed under standard \( ^1\text{H} \) NMR conditions (10). The signals arising from different lipoprotein fractions in the \( ^1\text{H} \) NMR spectra of plasma are assigned in Fig. 4A: peaks 1 (0.858 ppm) and 2 (0.863 ppm) which overlap, assigned to the CH\textsubscript{3} groups of HDL and LDL respectively; peak 3 (0.886 ppm) to the CH\textsubscript{3} groups of VLDL; peaks 5 (1.25 ppm), and peak 6 (1.279 ppm) to -(\text{CH}_2)_n- groups of LDL and VLDL, respectively (11, 25). After fish oil supplementation, the plasma spectra were significantly altered, Fig. 4B. Resonances from VLDL (peaks 3 and 6) are completely absent (>99% reduction) and a new lipid resonance, centred at 0.941 ppm (*) and of similar intensity as peak 2, can be observed. The spin-echo \( ^1\text{H} \) spectra of isolated
LDL and VLDL, before fish oil, are shown in Fig. 5A and Fig. 6B, respectively. The methyl and methylene resonances shown in these spectra arise from non-n-3 fatty acids (27, 28). After fish oil supplementation, the spectra from both lipoprotein fractions show the presence of a new lipid resonance centre at 0.941 ppm. A similar resonance was detected in the $^1H$ NMR spectra of isolated HDL (results not shown). The new peak (*) has been assigned, on basis of its chemical shift and the $^{13}C$ NMR findings, to the methyl group of EPA and DHA within the lipoprotein particles.

The effects of fish oil supplementation on the transverse relaxation ($T_2$) of the LDL and VLDL methyl, methylene and (*) lipid signals are shown in Table 4. Spin-echo spectra were recorded at constant temperature (37°C) to minimize $T_2$ changes known to arise from temperature variation. The $T_2$ of the methyl ($\text{CH}_3$) signals from LDL (112 ± 28 ms vs. 162 ± 37 ms; $P < 0.05$) and VLDL (182 ± 24 ms vs. 268 ± 19 ms; $P < 0.01$) were significantly elevated after fish oil supplementation. Similar changes, although of lesser magnitude, were observed for the $T_2$ of the methylene ($-\text{(CH}_2)_n-$) signals from LDL (52 ± 12 ms vs. 78 ± 21 ms; $P < 0.02$) and VLDL (88 ± 17 ms vs. 106 ± 24 ms; $P < 0.01$). The $T_2$ of the lipid resonance (*) arising from EPA and DHA were relatively longer and for both VLDL (326 ± 42 ms) and LDL (227 ± 33 ms) compared to the other methyl lipid signals within the same particles.

![Fig. 5. 500 MHZ spin-echo $^1H$ NMR spectra of isolated VLDL (A) before, (B) after fish oil supplementation. The new lipid resonance at 0.941 ppm (*) has been assigned to methyl proton ($\text{CH}_3$-$\text{CH}_2$-$\text{CH} = \text{CH}$-) from EPA and DHA. The resonance at 1.284 ppm has been identified as a contaminant arising from the lipoprotein isolation procedure.](image)

**DISCUSSION**

In the present study, we have used high resolution NMR to examine the effects of fish oil supplementation on the plasma lipoprotein molecular architecture. The NMR properties of lipoprotein particles in isolation and in intact plasma have been extensively analyzed (10–13, 29). Each lipoprotein fraction (chylomicrons, VLDL, LDL, HDL) has its own spectral characteristics including chemical shift, lipid signal intensities, longitudinal ($T_1$) and transverse ($T_2$) relaxation times (7, 8, 10–13, 29). These features allow the identification of different lipoprotein fractions in the $^1H$ NMR spectra of intact plasma or serum (11–13, 29). Furthermore, the $^1H$ and $^{13}C$ NMR properties of the lipoprotein lipid signals can shed light on the physico-chemical state of lipoprotein particles, including chemical composition, structural organization and molecular motion (7, 10, 11, 24–26, 29).

The fish oil supplement used in this study (MaxEPA) consists mainly of triglycerides, in which 28.9% (mass) are represented by EPA and DHA. These n-3 fatty acids are thought to be the active components of fish oil and are consistently incorporated into plasma lipids (1, 29–31). In this study, and as previously shown by others, fish oil supplementation led to a significant decrease in plasma triglyceride concentration and an increase in the concentration of EPA and DHA. No changes in choles-
terol levels were observed, even at this relatively high concentration of fish oil.

The lipid changes induced by fish oil had noticeable effects on the NMR profile of intact plasma and isolated lipoproteins. In the absence of fish oil supplementation, the $^1$H and $^{13}$C lipid resonances arise principally from the fatty acids of triglycerides and phospholipids including linoleic, oleic, and palmitic acids (10, 11, 24). After fish oil supplementation, new $^{13}$C (14.3, 127.1, and 131.6 ppm) and $^1$H (0.941 ppm) resonances were observed in the spectra of intact plasma and isolated lipoproteins. Gunstone (27) has shown that a number of chemical groups from EPA and DHA have very distinctive chemical shifts and can be differentiated from those of other fatty acids by NMR. The new $^{13}$C and $^1$H resonances have been assigned, based on their chemical shift and NMR characteristics, to specific carbon groups of the EPA and DHA moiety in the lipoprotein particles. Furthermore, we have shown that these specific resonances can be utilized to determine the relative levels of these fatty acids in intact plasma and isolated lipoprotein fractions.

The disappearance of the VLDL resonances from the $^1$H NMR spectra of intact plasma after fish oil supplementation is consistent with the decrease in triglyceride
concentration (1, 30-32). Moreover, the fact that the methylene (-(CH\_2\_n)-) resonance from VLDL particles is absent in the 1H NMR spectra of intact plasma after fish supplementation suggests that the n-3 resonance observed in the spectra of intact plasma samples arises predominantly from LDL particles.

The transverse relaxation, chemical shift, and intensity of the signals of plasma lipoproteins are determined by a number of factors including composition and packing. Furthermore, these NMR parameters can shed light on the structural organization of lipoprotein particles (10). In this study, the T2 relaxation times of the VLDL and LDL lipoprotein methyl and methylene resonances from non-n-3 fatty acids were significantly increased after fish oil supplementation. This implies an increase in relative mobility of the acyl chain within the lipoprotein particles. The incorporation of n-3 fatty acids, therefore, appears to disrupt the hydrophobic domains of VLDL and LDL, resulting in alteration in the packing and increased isotropic mobility of the lipid components within lipoprotein particles (10, 11). This supports previous findings suggesting alteration in core fluidity being induced by increased amounts of n-3 polyunsaturated fatty acids in LDL (5, 6).

Interestingly, the intensity of the new peak at 0.941 ppm, in the 1H spin-echo spectra of plasma and isolated lipoproteins, was similar to those from non-n-3 fatty acids, although the relative level of EPA and DHA was <20% of the total lipid concentration. Furthermore, its T2 was notably longer than those from non-n-3 lipids. These findings are surprising as one would expect the T2 of all lipid components within a given type of lipoprotein to be similar. There are several possible explanations for these findings, including the existence of a fraction of LDL and VLDL particles that contains most of the n-3 fatty acid components detected by NMR and GLC. This would give rise to lipoprotein particles with NMR characteristics distinct from normal LDL and VLDL. No such lipoprotein fraction has been observed during separation by ultracentrifugation techniques. Another more plausible explanation is the existence of "microenvironments" rich in EPA and DHA within the lipoprotein particles. The lipid constituents within these microenvironments will thus give rise to 1H signals with NMR relaxation characteristics very different from those of other lipids within the same lipoprotein particle. Furthermore, as there appears to be an overall alteration in the architecture of the lipoproteins, reflected in this study as changes in lipid mobility, the n-3 microenvironments must be distributed throughout the lipoprotein particles.

The existence of lipid microenvironments has been previously postulated from work carried out with surfactant vesicles and intact cells (33-35). Gordon et al. (33) used electron spin resonance (ESR) spectroscopy to measure membrane fluidity in platelet and hepatocytes and suggested the existence of cholesterol-rich and cholesterol-poor lipid domains in the membranes. Similarly, the use of the fluorescent probe merocyanine (MC540) suggests that the incorporation of DHA into cell membranes may produce DHA-rich, cholesterol-poor domains (34, 35). This would, in turn, lead to a variable distribution of plasma membrane proteins and possible local variations in biochemical activity. Our results appear to suggest that similar n-3-rich domains may be present in plasma lipoprotein particles. This is the first time that such a phenomenon has been observed in plasma lipoproteins. The possible effects of these microenvironments on the function and uptake of plasma lipoproteins are unclear and warrant further investigation.

In conclusion, our results show for the first time that fish oil supplementation leads to significant alterations in plasma lipoprotein architecture. These alterations arise from changes in the lipid composition and physical characteristics of the lipoprotein particles, particularly the possible existence of n-3-rich micro-domains. Our findings suggest that multinuclear NMR may be a powerful tool in the study of the effects of n-3 fatty acids on intact lipoprotein particles.

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