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# The effects of dietary ($n-3$) fatty acid supplementation on lipid dynamics and composition in rat lymphocytes and liver microsomes

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**Rats were fed diets devoid of ($n-3$) fatty acids (olive oil supplementation) or high in ($n-3$) fatty acids (fish oil supplementation) for a period of 10 days. In spleen lymphocytes and liver microsomes derived from animals fed fish oil diets, relatively high levels of ($n-3$) eicosapentaenoic (20:5), docosapentaenoic (22:5) and docosahexaenoic acids (22:6) were obtained compared to minimal levels when fed the olive oil diet. When the average lipid motional properties were examined by measuring the fluorescence anisotropy of diphenylhexatriene, no significant different was found between intact liver microsomes from animals fed the two diets. However, when lipid motion was examined in vesicles of phosphatidylcholine, isolated from the microsomes from fish oil fed animals (21.4% ($n-3$) fatty acids), the fluorescence anisotropy was significantly less than the corresponding phosphatidylcholine from olive oil fed animals (5.6% ($n-3$) fatty acids), indicating a more disordered or fluid bilayer in the presence of higher levels of ($n-3$) fatty acids. Phosphatidylethanolamine ($n-3$) fatty acids were also elevated after fish oil supplementation (41.3% of total fatty acids), compared to the level after olive oil supplementation (21.4%). The major effect of the fish oil supplementation was a replacement of ($n-6$) arachidonic acid by the ($n-3$) fatty acids and when this was 'modeled', using liposomes of synthetic lipids, 1-palmitoyl-2-arachidonyl($n-6$) or docosahexaenoyl($n-3$)-phosphatidylcholine, significant differences in lipid motional properties were found, with the docosahexaenoate conferring a more disordered or fluid lipid environment. Thus it appears that although lipid order/fluidity can be significantly decreased by increases in the highly unsaturated ($n-3$) fatty acid levels, alterations in membrane domain organization and/or phospholipid molecular species composition effectively compensated for the changes, at least as far as average lipid motional properties in the intact membranes was concerned.**

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Abbreviations: Fatty acids are abbreviated as follows: Number of carbons : number of *cis*-double bonds; for example, 20:5 is a 20 carbon fatty acid with five *cis*-double bonds. Phosphatidylcholine molecular species are shown in the following manner: 16:0/18:1-PC: 1-palmitoyl-2-oleoyl-phosphatidylcholine.

## Introduction

The effect of changing fatty acyl unsaturation on membrane lipid dynamics has been widely studied, as recently reviewed [1]. However, the fatty acids that are most unsaturated, namely ($n-3$) 20:5, 22:5 and 22:6 have received relatively little attention. These fatty acids are nevertheless

found at high levels in many membranes such as those from brain and retinal rod outer segments. Recently, interest has been intensified in the functional role of $(n-3)$ fatty acids as modulators of prostaglandin biosynthesis [2]. Other studies have connected high dietary levels of $(n-3)$ fatty acids (from marine sources) with low incidence of heart disease in humans [2–4], and recent work has connected specific dietary fatty acid intake with heart lesions in the rat [5]. Specific effects of $(n-3)$ fatty acyl supplementation in in vitro cell culture systems, particularly membrane transport, have been described [6,7].

In view of the above studies, it is of importance to discover what effects elevation of $(n-3)$ fatty acids might have on membrane lipid dynamics and organization. Previous studies in this area have indicated that a membrane with elevated 22:6 will be more disordered [8], as shown in an ESR study of brain phosphatidylserines. In $^2$H-NMR studies the gel to liquid-crystalline phase transition of 16:0/22:6-PC was found to lie between $-3$ to $-10$°C which is similar to that 16:0/16:1-PC [9]. However, it has also been shown by $^2$H-NMR that compared to 16:1 the 22:6 chain considerably disorders the adjacent 16:0, *sn*-1 chain [10]. Thus it is to be expected that introduction of highly unsaturated $(n-3)$ fatty acyl moieties into the lipid bilayer would disturb the packing and reduce lipid order.

Previously, we used in vitro supplementation to alter the fatty acyl group composition of lymphocytes [11]. In the present investigation, however, we have used dietary rather than in vitro means to change the degree of unsaturation. The effects on the fatty acid composition of $(n-3)$ fatty acyl supplementation brought about by fish oil supplementation compared to controls fed olive oil were examined in lymphocytes and liver microsomes, and lipid motional properties were assessed using fluorescence anisotropy measurements of diphenylhexatriene. For comparison, synthetic phosphatidylcholine molecular species with differing unsaturation were also examined.

## Methods

Wistar rats (150–200 g) were fed diets supplemented with fish or olive oil (20% by weight), consisting of a standard laboratory rat chow supplemented with either olive oil or with Maxepa or Menhaden fish oil (Table I) both fish oil supplemented diets giving essentially similar results. The data presented here for the lymphocytes was with Maxepa fish oil and for the liver microsomes was with Menhaden fish oil. After 10 days, spleen lymphocytes [11] and liver microsomes [12] were prepared as previously described. The purity of the microsomal fraction was assessed by measuring glucose-6-phosphatase and 5′-nucleotidase as previously described [13,14].

Total lipid extractions were performed according to Bligh and Dyer [15]. Cholesterol was determined as described elsewhere [16] and phospholipids by determination of inorganic phosphorus [17]. The phospholipid class composition was determined by separating lipid extracts by TLC [18] visualizing the lipids by iodine vapor and scrapping from the plate, followed by determination of inorganic phosphorous.

Phospholipids were isolated by silicic acid chromatography and classes separated. After visualizing with 0.05% dichlorofluorescein in 50% methanol, the lipids were scraped from the plate and the silicic acid washed with 4 M $NH_4OH$. Finally the lipids were eluted with methanol/chloroform/water (87 : 10 : 3, v/v). In later experiments, an HPLC separation method was used to purify phospholipid classes [19]. Lipids

TABLE I

MAJOR FATTY ACIDS OF THE DIETARY SUPPLEMENTATIONS

| Fatty acid | Olive oil | Maxepa fish oil | Menhaden fish oil |
|---|---|---|---|
| 14:0 | * | 7.8 | 6.4 |
| 16:0 | 11.7 | 14.7 | 18.6 |
| 16:1 | 1.0 | 9.1 | 10.8 |
| 18:0 | 2.4 | 2.8 | 14.6 |
| 18:1 | 73.3 | 17.0 | 3.2 |
| 18:2($n-6$) | 10.2 | 1.7 | 0.4 |
| 20:4($n-6$) | 0.9 | 1.5 | 4.7 |
| 20:5($n-3$) | * | 16.4 | 15.8 |
| 22:5($n-3$) | * | 4.2 | 2.0 |
| 22:6($n-3$) | * | 16.1 | 12.0 |

* Not detectable, trace amounts.

were quantified by determination of lipid phosphorus and protein by the method of Lowry et al. [20]. The synthetic lipids, 16:0/18:1-PC, 16:0/20:4-PC and 16:0/22:6-PC were from Avanti Lipids, Birmingham, AL.

Labelling of cells, microsomes and multilamellar liposomes of lipid extracts with diphenylhexatriene (Aldrich) or 3-, 9- and 12-anthroylstearates (Molecular Probes Eugene, OR) was performed as previously described [11,21]. Steady-state fluorescence anisotropy measurements were made using an SLM 4800 spectrofluorimeter. Excitation was at 360 nm (diphenylhexatriene) or 380 nm (anthroylstearates) and emission was monitored after passing through a narrow band pass interference filter (430 or 450 nm) or suitable low fluorescence red pass filters. The anisotropy was calculated as described previously [21]. Fluorescence lifetimes were measured as described elsewhere [22] using non-linear least-squares analysis software. The (steady state) fluorescence anisotropy of diphenylhexatriene contains contributions from both the lipid order and rate of motion (see review in Ref. 23). Generally the lipid order term predominates, more especially in membranes below the gel-liquid crystalline phase transition temperature or in the presence of cholesterol or proteins. The term 'membrane fluidity' should be more properly reserved with reference to lipid motion. In contrast to the situation with diphenylhexatriene, the anthroylstearate probes mainly reflect the rate of lipid motion [24,25], since the residual anisotropy (the order component) is relatively low.

## Results and Discussion

The effects of supplementing diets with olive or fish oils on rat spleen lymphocyte and liver microsome phosphatidylcholine and ethanolamine fatty acid composition is shown in Table II. The dietary olive oil ($n-3$) fatty acid content is negligible while that of the fish oil diet is around 30%, and this difference is reflected in the composition of the composition of the major phospholipid classes. In phosphatidylcholine, supplementation with fish oil resulted in decreased 18:1 and 20:4 and an elevated 16:0, 20:5 and 22:6, as compared to the effect of olive oil. In spite of these differences, there was no significant difference in the unsaturation index. The phosphatidylethanolamine of fish oil fed animals also showed elevated 20:5, 22:5 and 22:6 as with phosphatidylcholine but to a greater extent, the unsaturation index being greater in the phosphatidylethanolamine from animals fed the fish oil supplemented diet. Analy-

TABLE II

FATTY ACID COMPOSITION OF RAT SPLEEN LYMPHOCYTE AND LIVER MICROSOME PHOSPHOGLYCERIDES

Figures presented are means (±S.D. ($n=3$)). U.I., unsaturation index (percent of each fatty acid ×(double bonds/fatty acid)×1/100.

| | Spleen lymphocytes | | | | Liver microsomes | | | |
|---|---|---|---|---|---|---|---|---|
| | phosphatidylcholine | | phosphatidylethanolamine | | phosphatidylcholine | | phosphatidylethanolamine | |
| | olive oil | fish oil | olive oil | fish oil | olive oil | fish oil | olive oil | fish oil |
| 16:0 | 36.8 (1.0) | 43.8 (1.6) | 10.6 (0.2) | 10.4 (0.7) | 20.2 (0.9) | 32.7 (0.3) | 16.8 (1.2) | 17.9 (0.2) |
| 16:1 | 2.0 (0.7) | 1.5 (0.1) | 1.9 (0.6) | 2.5 (1.0) | 0.6 (0.1) | 3.4 (0.1) | 1.7 (0.6) | 1.7 (0.1) |
| 18:0 | 8.9 (0.5) | 8.8 (0.3) | 23.6 (2.1) | 19.6 (1.2) | 28.4 (0.9) | 19.5 (0.2) | 22.8 (1.6) | 21.7 (0.5) |
| 18:1 | 22.6 (0.2) | 16.7 (0.6) | 12.5 (0.9) | 9.3 (1.3) | 13.6 (0.2) | 9.8 (0.1) | 10.1 (1.1) | 5.9 (0.7) |
| 18:2($n-6$) | 10.3 (0.4) | 12.2 (0.9) | 3.7 (0.4) | 5.3 (1.0) | 8.1 (0.3) | 3.7 (0.3) | 5.2 (0.2) | 3.1 (0.1) |
| 20:4($n-6$) | 15.8 (0.6) | 5.8 (0.4) | 38.0 (1.0) | 15.4 (0.6) | 23.6 (0.8) | 9.4 (0.1) | 27.3 (1.3) | 8.1 (0.7) |
| 20:5($n-3$) | 0.4 (0.2) | 4.0 (0.4) | 0.5 (0.1) | 10.4 (0.4) | 0.2 (0.1) | 10.6 (0.1) | 0.5 (0.1) | 8.5 (0.2) |
| 22:5($n-3$) | 0.8 (0.1) | 2.9 (0.3) | 3.4 (0.3) | 10.2 (0.3) | 0.2 (0.1) | 1.4 (0.1) | 2.1 (0.3) | 4.0 (0.1) |
| 22:6($n-3$) | 1.3 (0.2) | 4.1 (0.4) | 5.4 (0.3) | 17.0 (0.4) | 5.2 (0.5) | 9.4 (0.4) | 14.0 (1.2) | 22.8 (0.8) |
| %sat. FA | 46.7 | 42.6 | 34.2 | 30.0 | 48.6 | 52.2 | 39.6 | 39.6 |
| U.I. | 1.22 | 1.26 | 2.26 | 2.89 | 1.58 | 1.75 | 2.28 | 2.82 |

sis of the molar ratios of phosphatidylcholine/phosphatidylethanolamine (olive: $1.7 \pm 0.1$; fish: $1.8 \pm 0.1$, $n = 5$) and cholesterol/phospholipid (olive: $0.37 \pm 0.02$; fish: $0.33 \pm 0.02$, $n = 5$) showed no differences between olive and fish oil feeding.

The differences in the level of $(n - 3)$ fatty acids achieved by the two diets seemed sufficient to warrant examination for possible differences in the motional properties of the lymphocyte lipids. In previous studies, we also examined whole spleen lymphocytes [11], but the in vitro culture system used for fatty acyl supplementation introduced triacylglycerols into the cytoplasm which took up the fluorophore probe diphenylhexatriene, used to monitor lipid order, so that the signal from the membrane was partially masked. In the current study, since the supplementation was dietary, this problem was avoided. However, another problem was that the diphenylhexatriene distributed throughout the subcellular organelles. This makes any effects difficult to interpret unless the membranes are isolated. Attempts to use thymocytes (which are obtainable in greater yields than spleen lymphocytes) for purified plasma membrane preparations were frustrated by the finding that incorporation of the $(n - 3)$ fatty acids were rather modest, and we therefore looked for an alternative system. Liver microsomes were finally chosen since they provided a membrane enriched in these fatty acids and a large amount of material was available for separation of phospholipid classes from the isolated membranes.

Liver microsomes were found to incorporate high levels of the $(n - 3)$ fatty acids as shown in Table II. However, no differences were found in the levels of glucose-6-phosphatase (microsomal marker), 5′-nucleotidase (indicator of plasma membrane contamination), cholesterol/phospholipid ratios or phospholipid classes as shown in Table III. The nature of the difference in fatty acid composition between fish-oil and olive-oil derived phosphatidylcholine and phosphatidylethanolamine was, in fact, rather similar for the lymphocytes and microsomes, although the phospholipid molecular species distribution may differ in the two tissues. Again, the major difference was in the elevated $(n - 3)$ 20:5, 22:5 and 22:6 at the expense of 20:4 in the fish oil derived lipids as compared to the olive oil. The fluorescence anisotropy measurements on intact microsomes, and liposomes of extracted total lipids or phospholipids were virtually identical in both olive and fish oil derived samples (Table IV). Fluorescence lifetime measurements were also performed, since large differences would complicate interpretation of differences in the fluorescence anisotropy values; however, the values were found to be similar for intact membranes from animals on either diet. We also used the anthroylstearate probes since the anisotropy is more a reflection of the rate of lipid motion (see Methods). The anisotropy values for 3-, 9- and 12-anthroylstearates in intact membranes (25°C) were 0.116, 0.089 and 0.061 (olive diet) and 0.117, 0.091 and 0.059 (fish diet), showing no differences could be detected.

When liposomes made from phosphatidylcholine isolated from microsomes obtained form fish oil fed animals (21.4% $(n - 3)$ fatty acids) were examined, the lipids were significantly more disordered (lower fluorescence anisotropy) compared to phosphatidylcholine from olive oil fed animals (5.6% $(n - 3)$ fatty acids), the anisotropy values at 37°C being 0.069 and 0.082, respectively (Table IV). Phosphatidylethanolamine also showed significant supplementation with $(n - 3)$ fatty acids after fish oil dietary supplementation (41.3%)

TABLE III

ENZYME ACTIVITIES AND LIPID COMPOSITION OF LIVER MICROSOMES FROM ANIMALS FED OLIVE AND FISH OIL DIETS

| | | Olive oil diet | Fish oil diet |
|---|---|---|---|
| Glucose-6-phosphatase [a] | Expt. 1 | 14.9 | 11.3 |
| | Expt. 2 | 11.4 | 13.8 |
| 5′-Nucleotidase [a] | Expt. 1 | 2.90 | 2.17 |
| | Expt. 2 | 1.87 | 2.02 |
| Cholesterol/phospholipid (molar ratio) | Expt. 1 | 0.112 | 0.101 |
| | Expt. 2 | 0.100 | 0.113 |
| Phosphatidylcholine [b] | | 61.0 ± 6.0 | 61.9 ± 2.4 |
| Phosphatidylethanolamine [b] | | 20.5 ± 2.3 | 21.6 ± 2.4 |
| Phosphatidylserine + phosphatidylinositol [b] | | 13.5 ± 0.4 | 13.6 ± 2.4 |
| Lysophosphatidylcholine [b] | | 1.4 ± 0.4 | 1.3 ± 0.7 |
| Sphingomyelin [b] | | 3.5 ± 0.1 | 1.7 ± 0.7 |

[a] $\mu$M $P_i$ released/min per mg protein.

[b] mole% of total phospholipids (mean of three determinations ± S.D.)

TABLE IV

FLUORESCENCE ANISOTROPY AND LIFETIMES OF DIPHENYLHEXATRIENE INCORPORATED INTO LIVER MICROSOMES

| Diet | Temp (°C) | Anisotropy ($r$) [a] | Difference ($r_{olive} - r_{fish}$) | $A_1$ | $\tau_1$ (ns) | $A_2$ | $\tau_2$ (ns) |
|---|---|---|---|---|---|---|---|
| Intact microsomes | | | | | | | |
| Olive | 37 | $0.111 \pm 0.007$ | $-0.002 \pm 0.003$ [b] ($n = 5$) | 0.09 | 2.02 | 0.91 | 9.08 |
| Fish | 37 | $0.109 \pm 0.006$ | | 0.11 | 1.94 | 0.89 | 9.06 |
| Olive | 25 | 0.143 | $-0.001$ | – | – | – | – |
| Fish | 25 | 0.142 | | – | – | – | – |
| Liposomes of extracted phosphatidylcholine | | | | | | | |
| Olive | 37 | $0.082 \pm 0.008$ | $-0.013 \pm 0.007$ [c] ($n = 5$) | 0.12 | 2.76 | 0.88 | 8.74 |
| Fish | 37 | $0.069 \pm 0.007$ | | 0.15 | 3.18 | 0.85 | 9.34 |
| Olive | 25 | 0.094 | $-0.010$ | – | – | – | – |
| Fish | 25 | 0.084 | | – | – | – | – |
| Liposomes of total lipids | | | | | | | |
| Olive | 25 | 0.116 | $-0.001$ | – | – | – | – |
| Fish | 25 | 0.115 | | – | – | – | – |
| Liposomes of phospholipids | | | | | | | |
| Olive | 25 | 0.090 | 0.001 | – | – | – | – |
| Fish | 25 | 0.091 | | – | – | – | – |
| Synthetic lipids | | | | | | | |
| 16:0/18:1-PC | 25 | $0.102 \pm 0.001$ ($n = 3$) | | 0.13 | 2.46 | 0.87 | 8.65 |
| 16:0/20:4-PC | 25 | $0.084 \pm 0.001$ | | 0.16 | 2.77 | 0.84 | 8.83 |
| 16:0/22:6-PC | 25 | $0.062 \pm 0.001$ | | 0.13 | 1.82 | 0.87 | 7.73 |

[a] Steady-state fluorescence anisotropy.
[b] Difference not significant ($P < 0.2$, paired $t$-test).
[c] Difference significant ($P < 0.01$).
[d] $A_1$ and $A_2$ pre-exponents of fluorescence lifetimes.

compared to the olive oil supplementation (16.6%), and similar effects on lipid order might be expected. However, since stable bilayer cannot be made from unsaturated phosphatidylethanolamines, this could not be confirmed.

The $(n-3)$ fatty acyl supplementation was found to result in a considerable decrease in $(n-6)$ 20:4 in both lymphocytes and microsomes. We therefore addressed the question of the significance of replacing 20:4 by the more unsaturated $(n-3)$ fatty acids by examining liposomes of synthetic phospholipid molecular species. In a previous study, the disordering effect of increasing unsaturation in liposomes of phosphatidylcholines was examined for the series 16:0/18:1-PC, 16:0/18:2-PC and 16:0/20:4-PC, and a trend towards lipid disordering with increasing polyunsaturation noted [21]. Here we extended the study of 16:0/22:6-PC. Table IV shows the difference in fluorescence anisotropy when the *sn*-2 position in phosphatidylcholine is taken by either 18:1, 20:4 $(n-6)$ or 22:6$(n-3)$. It can be seen that the increased unsaturation leads to considerable lipid disordering (lower fluorescence anisotropy). On the basis of these model membrane measurements and those on phosphatidylcholine from olive and fish oil dietary animals showing the effects of unsaturation, differences in average lipid motional properties in intact membranes from the two diets might be expected. However, such differences were not forthcoming as presented above, and we conclude that as far as average or 'bulk' lipid order is

concerned, as detected by diphenylhexatriene, compensation may have occurred at the level of individual phospholipid molecular species.

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