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The effect of a dietary supplement of *n*-3 polyunsaturated fat on platelet lipid composition, platelet function and platelet plasma membrane fluidity in healthy volunteers

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1. Eight healthy male volunteers (aged 22–39 years) supplemented their normal daily diet with 15 g encapsulated fish oil (MaxEPA) for a 6 week period. Fasting blood samples were taken before, at the completion of and 3 months after the period of supplementation.

2. Evaluation of nutrient intakes showed that the intake of *n*-3 polyunsaturated fatty acids rose significantly ($P < 0.01$) during supplementation. This was reflected in changes in the fatty acid composition of platelet phosphatidyl choline (PC) and phosphatidyl ethanolamine (PE) without any changes in phosphatidyl serine, phosphatidyl inositol or sphingomyelin.

3. In both PC and PE there were significant ($P < 0.05$) increases in the levels of 18:1*n*-9 and 20:5*n*-3 fatty acids and a significant ($P < 0.05$) decrease in 20:4*n*-6 during supplementation. 16:0 rose significantly in PC ($P < 0.05$) while in PE, 18:0 fell and both 22:5*n*-3 and 22:6*n*-3 rose significantly ($P < 0.05$).

4. There were no significant effects of fish-oil supplementation on serum lipids, platelet cholesterol: phospholipid, collagen-induced platelet aggregation or collagen-induced platelet thromboxane B_2 production. However, there was a significant correlation ($P < 0.001$; $r + 0.63$) between total phospholipid arachidonic acid and platelet thromboxane production.

5. The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene was used to determine whether fish-oil supplementation altered fluorescence polarization of isolated platelet plasma membrane and, by inference, platelet plasma membrane fluidity. No significant effect of fish-oil supplementation on fluorescence polarization was seen.

In recent years, there has been a rapid expansion in studies into the effects of marine oils, rich in *n*-3 polyunsaturated fatty acids (PUFA), on various physiological variables (Herold & Kinsella, 1986). These have included studies of the effects of *n*-3 PUFA on serum lipids (Seynor *et al.* 1984) and the eicosanoid regulation of both haemostasis (Sanders, 1985) and inflammation (Salmon & Terano, 1985). All these variables may play a role in atherogenesis and thrombogenesis (Ross, 1986). Most studies have focused on the potential of eicosapentaenoic acid (EPA; 20:5*n*-3) to influence the synthesis of prostaglandins and leukotrienes, and on the effect of EPA on the biological potency of these compounds. However, while fish-oil supplements increase the concentration of EPA in membrane phospholipids, they also lead to variable increases in the longer chain *n*-3 PUFA, e.g. docosapentaenoic acid (DPA; 22:5*n*-3) and docosahexanoic acid (DHA; 22:6*n*-3), as well as to changes in the levels of some saturated, monounsaturated and *n*-6 PUFA, i.e. linoleic acid (18:2*n*-6) and arachidonic acid (20:4*n*-6). These extensive changes in membrane phospholipid fatty acid composition have led many authors to speculate as to the possibility that fish-oil feeding might also alter membrane fluidity (Sanders, 1985). Such changes in membrane fluidity might alter the expression of cell receptors or the activity of key membrane-located enzymes (Clandinin *et al.* 1985). Were this to occur, the sensitivity of platelets to stimuli such as collagen or fibrinogen might change, as might the activity of key enzymes in arachidonic acid metabolism such as adenylate cyclase (*EC* 4.6.1.1) or

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phospholipase (*EC* 3 . 1 . 4 . 1). Thus, fish-oil feeding could exert an effect on platelet function independent of its effect on platelet thromboxane A_2 synthesis. In the present study, variation in polarization values of the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) were used as an inverse indicator of platelet plasma membrane (PPM) fluidity in healthy volunteers, before, during and after a fish-oil supplement. Other variables which might influence membrane fluidity or which might be altered by fish-oil feeding were also studied.

MATERIALS AND METHODS

Subjects

Eight healthy male volunteers (aged 22–39 years) participated in the study which was approved by the Ethics Committee of the Hampshire Area Health Authority. Each volunteer supplemented his diet with 15 g fish oil (MaxEPA; Seven Seas, Hull)/d in 1-g gelatin capsules. This provided 2·25 g EPA and 1·35 g DHA, plus 69 mg cholesterol/d. Weighed food intake surveys (3 or 7 d) were carried out before and during the period of 6 weeks of fish-oil supplementation. It was a requirement of the Ethics Committee that 3 months should elapse between each fasting blood sample of 200 ml, the minimum volume needed for isolating sufficient platelet plasma membrane for fluidity studies. Thus, blood samples were taken 6 weeks before the period of supplementation, at the end of this period and 3 months after its cessation. All subjects abstained from medication for at least 7 d before donating blood.

Biochemical methods

PPM was prepared by the glycerol lysis techniques (Barber *et al.* 1971) and characterized by the activities of key marker enzymes: adenylate cyclase (Houslay *et al.* 1976), 5′-nucleotidase (*EC* 3 . 1 . 3 . 5) (Arnich & Wallach, 1971), ubiquinol–cytochrome-*c* reductase (*EC* 1 . 10 . 2 . 2) (Tisdale, 1976), phosphodiesterase 1 (*EC* 3 . 1 . 4 . 1) (Taylor *et al.* 1977). Lactate dehydrogenase (*EC* 1 . 1 . 1 . 27) was assayed by incubation with lactic acid followed by spectrophotometric analysis of NADH production at 340 nm. Lactate dehydrogenase activity indicated almost 75% lysis of platelets. Adenylate cyclase, 5′-nucleotidase and phosphodiesterase were present in the PPM in quantities of 4·9, 12·2 and 3·8 (respectively) times higher than in the platelet homogenate. The higher concentration of these membrane-bound enzymes in the PPM contrasting with the lower level of the cytoplasmic enzyme (0·39 times) ubiquinol–cytochrome-*c* oxidoreductase, indicated adequate purity of the isolated PPM.

Fluorescence polarization was measured using the fluorescent probe as previously described (Shinitzky & Barenholz, 1978). Platelet aggregation in response to ADP and collagen was determined using a Payton Dual Channel aggregometer at 37° and 900 rev/min. Saline (9 g sodium chloride/1; 200 μl) was added to 200 μl platelet-poor plasma or 200 μl platelet-rich plasma for 100% and zero light transmissions respectively. PPM levels of cholesterol and phospholipid were detemined by the methods of Gamble *et al.* (1978) and Raheja *et al.* (1973) respectively. Platelet and plasma lipids were extracted in chloroform–methanol (2:1 v/v). Phospholipids were isolated by thin-layer chromatography in a solvent system of light petroleum (b.p. 40–60)–diethyl ether–formic acid (80:20:2, by vol., with 10 mg butylated hydroxytoluene). Individual phospholipids were separated using two-dimensional thin-layer chromatography: chloroform–methanol–alkali (0·88 ammonia)–water (90:54:8·8:2·2, by vol.) followed by chloroform–acetone–methanol–acetic acid–water (48:63:16:16:8, by vol.). The stationary phase was Kieselgel 60 H plus 30 g magnesium carbonate/kg. The fatty acid composition of lipid fractions was

Table 1. *Dietary composition, serum lipids, platelet lipid composition and platelet function studies in eight healthy male volunteers, before, during and after 42 d of supplementation with 15 g fish oil (MaxEPA)/d*

(Mean values with their standard errors)

	Pre-supplementary		Supplementary		Post-supplementary	
	Mean	SE	Mean	SE	Mean	SE
Dietary composition:						
Energy (MJ)	11·0	0·9	11·7	0·7	10·4	0·7
Fat (% energy)	38·3	1·5	40·6	1·3	34·4	2·4
Fibre (g)	25·2	1·9	27·6	2·5	29·9	4·5
Cholesterol (mg)	150	30	293	36	162	33
n-3 PUFA (g)	2·5	0·5	6·0*	0·2	2·1*	0·4
n-6 PUFA (g)	22·6	3·3	21·1	2·5	14·4	5·1
Serum lipids (mmol/l):						
Cholesterol	3·0	1·2	4·0	0·3	3·6	0·3
Triglyceride	0·9	0·2	0·8	0·2	1·0	0·3
Platelet lipids (nmol/mg protein):						
Cholesterol	190	17	170	5	167	14
Phospholipid	647	61	670	68	498	37
Cholesterol:phospholipid	0·32	0·05	0·27	0·03	0·34	0·02
PPM fluorescence polarization	0·221	0·003	0·230	0·005	0·240*	0·007
Platelet aggregation (% maximum):						
ADP (final concentration μM):						
2·5	44	8	66	6	58	5
5	49	12	75*	3	66	4
10	73	2	86*	3	72	8
Collagen (final concentration; $\mu g/10^8$ platelets):						
2·5	36	11	37	7	42	9
5·0	54	8	66	6	67	7
Platelet TxB_2 ($ng/10^8$ platelets) with 5 μg collagen	11·9	1·2	9·9	0·9	10·9	0·6

PUFA, polyunsaturated fatty acids; PPM, platelet plasma membrane; TxB_2, thromboxane B_2.
Mean value was significantly different from the pre-supplementary value: $^*P < 0{\cdot}05$.

Table 2. *Fatty acid compositions (% w/w) of platelet phospholipids in eight healthy male volunteers, before, during and after 42 d of supplementation with 15 g fish oil (MaxEPA)/d*

(Mean values with their standard errors)

Fatty acid...	16:0		18:0		18:1		18:2*n*-6		20:4*n*-6		20:5*n*-3		22:5*n*-3		22:6*n*-3	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Phosphatidyl serine																
Pre-supplement	7·5	3·2	44·8	4·7	24·6	2·4	3·1	1·6	18·5	2·4	tr		tr		tr	
Supplement	5·2	1·5	49·8	3·3	22·6	1·7	1·5	0·4	17·5	1·9	tr		tr		tr	
Post-supplement	11·3	2·9	43·4	3·0	22·3	1·3	0·8	0·5	23·6	2·4	tr		tr		tr	
Phosphatidyl inositol																
Pre-supplement	24·6	6·4	32·9	2·7	6·3	0·8	1·5	0·3	32·3	4·4	tr		tr		tr	
Supplement	26·7	6·2	29·2	4·5	10·1	2·0	3·5	3·3	27·1	5·5	tr		tr		tr	
Post-supplement	16·0	3·5	35·7	3·7	11·6	4·1	1·2	0·7	37·1	5·3	tr		tr		tr	
Phosphatidyl choline																
Pre-supplement	34·1	2·3	15·4	0·5	21·1	1·1	9·1	0·8	17·3	1·5	tr		tr		tr	
Supplement	42·6*	2·0	14·6	1·5	25·1*	0·6	7·4	0·9	7·9*	1·9	1·2*	0·2	tr		tr	
Post-supplement	41·1*	1·4	14·5	0·6	23·5	0·8	9·1	0·7	12·0*	0·8	tr		tr		tr	
Phosphatidyle ethanolamine																
Pre-supplement	17·7	6·0	23·1	1·4	8·3	1·6	4·5	1·0	45·2	6·3	tr		1·7	0·1	0·6	0·2
Supplement	18·6	3·9	16·8*	1·8	17·6*	4·4	6·1	1·1	30·3*	5·4	3·7*	1·1	2·8*	0·6	3·4*	0·7
Post-supplement	7·9	1·7	22·2	1·1	5·3	0·9	3·3	0·7	58·7	3·1	tr		1·2	0·3	1·2	0·4

Fatty acid...	16:0		18:0		18:1		18:2*n*-6		20:0		22:0		24:0		24:1	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Sphingomyelin																
Pre-supplement	43·2	5·3	11·5	2·0	5·9	2·3	6·7	4·4	3·1	0·5	18·8	4·0	4·1	1·3	5·5	1·6
Supplement	48·4	6·2	9·1	1·2	5·3	1·3	1·8	0·6	3·1	0·6	14·7	3·0	2·7	0·8	4·3	1·0
Post-supplement	49·2	5·0	13·3	1·4	3·7	1·4	tr		2·8	0·8	17·4	3·3	2·0	0·5	3·0	0·8

tr, trace, i.e. < 1 % (w/w).

Mean value was significantly different from the pre-supplementary value: * $P < 0·05$.

analysed by gas–liquid capillary-column chromatography following transmethylation in sodium methoxide. The conditions of this chromatographic separation were as follows: wall coated, open tubular fused silica column, 50 m × 0·22 m (internal diameter) and film thickness 0·18 μm, liquid phase CP 5·188, column temperature 170°, injection temperature 260°, detector temperature 250°, oven programme temperature increase from 170 to 180° over 3 min, 3 min at 3°/min, 1 min at 180° followed by 6°/min to 210° for 10 min; carrier gas hydrogen, delivery of 400 mm linear velocity/s. Platelet thromboxane B_2 was determined by radioimmunoassay (New England Nuclear) following aggregation of platelet-rich plasma in saline (1:1, v/v) with 5 μg collagen.

Dietary analysis

Dietary information was analysed using conventional tables of food composition (Paul & Southgate, 1978).

Statistical methods

Statistical analysis was carried out using the analysis of variance. Mean values were compared using Duncan's multiple range test.

RESULTS

There was a high degree of supplement compliance with no side effects or weight changes noted. The only dietary variable to change during the period of supplementation was the intake of *n*-3 PUFA which rose by 250% ($P < 0·05$). No significant effects on plasma lipids or on platelet levels of cholesterol or phospholipid were observed. There was no significant change in fluorescence polarization values of isolated PPM during the supplementary period. However, at the final blood sample, the fluorescence polarization value had increased significantly ($P < 0·05$) over that obtained with the initial blood sample. Platelet aggregation in response to collagen or to low levels of ADP was not altered during the period of fish-oil supplementation. At 5 and 10 μM-ADP (final concentration) there was a significant increase in platelet aggregation during the period of fish-oil supplementation ($P < 0·05$) which persisted at the 10 μM level into the post-supplementary period. There were no significant changes in platelet thromboxane B_2 production. These results are given in Table 1. The fatty acid compositions of platelet individual phospholipids are given in Table 2. The period of fish-oil supplementation did not produce any significant changes in the fatty acid compositions of platelet phosphatidyl serine, phosphatidyl inositol or sphingomyelin. In phosphatidyl choline, the levels of oleic acid (18:1*n*-9) and EPA (20:5*n*-3) increased significantly during the period of fish-oil supplementation while that of arachidonic acid (20:4*n*-6) fell significantly during that period ($P < 0·05$). Palmitic acid (16:0) was increased significantly both during and after the period of supplementation ($P < 0·05$). There were significant increases ($P < 0·05$) during the period of supplementation in the phosphatidyl ethanolamine levels of oleic acid, EPA, DPA (22:5*n*-3) and DHA (22:6*n*-3). The levels of stearic acid (18:0) and arachidonic acid fell significantly during this period ($P < 0·05$).

DISCUSSION

As would be expected, the period of fish-oil supplementation led to a substantial increase in the daily intake of *n*-3 PUFA although the total intake of *n*-3 and *n*-6 PUFA remained a relatively constant proportion of energy intake over the three study periods. The dietary intakes are in general agreement with previously recorded information (Sanders &

Roshani, 1983; Church *et al.* 1984; Reeves *et al.* 1984). The low levels of serum cholesterol recorded in this study may reflect the nature of the subjects' diets which were characterized by a high ratio of PUFA:saturated fatty acids for each of the three periods (0·62 (SE 0·06), 0·60 (SE 0·05) and 0·55 (SE 0·11) respectively). (This contrasts with the UK average of 0·23.) The diets were also characterized by high intakes of fibre and low intakes of cholesterol.

The reported effects of *n*-3 PUFA supplements on blood lipids have been variable (Herold & Kinsella, 1986). Some studies agree with the findings of the present study that fish-oil supplements do not influence blood lipids in man (Seiss *et al.* 1980; Terano *et al.* 1983). Others have reported a hypolipidaemic effect of fish-oil supplements in hyperlipidaemic patients (Hirai *et al.* 1983; Phillipson *et al.* 1985) and in normolipidaemic individuals (Goodnight *et al.* 1981; Sanders & Roshani, 1983; Seynor et al. 1984; Harris *et al.* 1984; Illingworth *et al.* 1984). In general, the reported hypotriglyceridaemic effects of fish-oil supplements are more consistent than the reported hypocholesterolaemic effect (Sanders, 1985). These widely different results may reflect variation in the degree of exposure, source of *n*-3 PUFA and initial blood lipid values as well as dietary changes other than *n*-3 PUFA intake.

In the present study, the period of fish-oil supplementation led to an increase in the platelet total phospholipid level of EPA (0·5–3·8 % w/w) and to a decrease in the level of arachidonic acid (31·5–23·1 % w/w). These changes are in agreement with the many studies of the effect of *n*-3 PUFA supplements on platelet total phospholipid fatty acids in man (Sanders, 1985). In the present study, the effects were confined to phosphyatidyl choline and phosphatidyl ethanolamine which agrees with the findings of previous studies (Brox *et al.* 1981; Fisher & Weber, 1983; Ahmed & Holub, 1984; Galloway *et al.* 1985).

The effects of *n*-3 PUFA supplements on platelet aggregation in man have been extensively reported and reveal widely different results: increased ADP-induced platelet aggregation (Sanders *et al.* 1981) which agrees with the findings of the present study; no effect of ADP on platelet aggregation (Hirai *et al.* 1983); increased ADP-induced platelet aggregation (Seiss *et al.* 1980; Terano *et al.* 1983). In the present study, collagen-induced platelet aggregation and thromboxane B_2 release was not influenced by dietary supplements of fish oil in spite of extensive incorporation of EPA into platelet phospholipids. Sanders & Hochland (1983) found that platelet aggregation in response to collagen in healthy volunteers receiving fish-oil supplements was significantly reduced at low levels of stimulation (0·5 μg collagen) but not at high levels (1·0 and 10·0 μg). In contrast, thromboxane B_2 production was significantly reduced only at the higher levels of stimulation (10 μg). However, the intake of EPA in these studies was 3·6 g/d contrasting with the intake of 2·25 g/d in the present study. Galloway *et al.* (1985) also found that lower intakes of EPA (1·8 g/d) did not alter collagen-induced platelet thromboxane A_2 release. While the present study failed to record an effect of *n*-3 PUFA supplements on either collagen-induced aggregation or thromboxane A_2 release, a highly significant ($P < 0{\cdot}001$) correlation ($r + 0{\cdot}63$, n 22) was observed between the production of platelet thromboxane A_2 (measured as thromboxane B_2) and platelet phospholipid arachidonic acid levels. Clearly, while the reduction in the mean levels of arachidonic acid, noted during the period of fish-oil supplementation, was not sufficiently consistent to suppress platelet thromboxane B_2 release, this correlation indicates the capacity of *n*-3 supplements to inhibit thromboxane A_2 synthesis when the supplementation is successful in displacing platelet arachidonic acid.

Membrane fluidity can be influenced by a variety of factors, both physical and chemical. Of the physical effectors, temperature, pressure, pH and calcium ions are the most important, their effect being virtually instantaneous. Of the chemical effectors, the most important are: protein:lipid, sphingomyelin:phosphatidyl choline, acyl-chain length and

degree of unsaturation, cholesterol:phospholipid and the sum of the molar proportions of linoleic and oleic acids (Stubbs, 1983; Berlin *et al.* 1987). In the present study, the only variable of PPM lipid composition which altered during the period of fish-oil supplementation was the composition of phospholipid acyl chains. No significant effects of dietary supplementation on phospholipid:cholesterol, sphingomyelin:phosphatidyl choline or protein:lipid in platelet-rich plasma were noted. Furthermore, the distribution of platelet individual phospholipids was not altered by dietary supplementation. It would therefore appear that modification of the fatty acid composition of human platelet phospholipids alone will not influence PPM fluidity. Berlin *et al.* (1987), working with rabbit PPM, found no effect of dietary fat source (maize oil, cocoa butter and milk-fat) on membrane fluidity although these authors did find that the addition of 2 g cholesterol/kg to the cocoa-butter diet did significantly lower membrane fluidity. In contrast, Rand *et al.* (1986) showed a significant increase in platelet membrane fluidity in whole platelets of rats fed on high-fat diets (50% energy) compared with low-fat diets (5% energy). However, these authors did not find a significant difference between oils rich in *n*-3 PUFA (marine oil) and *n*-6 PUFA (sunflower oil).

Some of these differences may relate to the tendency of DPH to report localized changes in membrane fluidity given that lipid domains of varying composition are likely to occur (Kannagi *et al.* 1981).

Thus, it is unlikely that the effects of *n*-3 PUFA on platelet function, reported both for animals and man, is related to changes in membrane fluidity.

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