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High dose eicosapentaenoic acid ethyl ester: effects on lipids and neutrophil leukotriene production in normal volunteers

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1 A 93% pure ethyl ester of eicosapentaenoic acid was investigated for tolerability and biochemical effects on neutrophil leukotriene synthesis and plasma lipoproteins when given in high dose. Six healthy volunteers received 6 g eicosapentaenoic acid ethyl ester daily for 6 weeks, followed by a 4 week wash-out and then 18 g daily for 6 weeks.
2 There was inhibition of neutrophil leukotriene B_4 and 5-hydroxyeicosatetraenoic acid synthesis, with no significant differences between low and high dose.
3 There was a dose dependent increase in leukotriene B_5 and 5-hydroxyeicosapentaenoic acid synthesis.
4 Plasma triglycerides were reduced maximally on 6 g daily, with no greater suppression at 18 g daily.
5 Plasma cholesterol was only suppressed significantly at 18 g daily.
6 The 6 g daily dose was well tolerated but the 18 g daily dose produced diarrhoea and steatorrhoea.

Keywords 5,8,11,14,17-eicosapentaenoic acid leukotrienes B lipoproteins

Introduction

Interest in the possible health benefits of omega-3 fatty acids was stimulated by the epidemiological observations of Bang and Dyerberg in Greenland Eskimos (Bang *et al.*, 1976), and these polyunsaturated marine lipids have been shown to have beneficial effects on the cardiovascular system (Leaf & Weber, 1988), and anti-inflammatory properties (Lee *et al.*, 1985). Eicosapentaenoic acid (EPA) is thought to be the most active omega-3 fatty acid (Terano *et al.*, 1987). Traditional Eskimo diets contain as much as 14 g EPA (Sanders, 1987), but a more modest intake of 2–5 g in fish-eating communities such as in Japan also seems to be of benefit.

Consumption of 0.5 kg of mackerel daily is needed even to achieve EPA intakes comparable with those in Japan, and therefore therapeutic intervention has often depended on the use of fish oil concentrates containing the triglycerides of EPA and docosahexaenoic acid (DHA). Since EPA is bound to the second limb of glycerol, triglycerides can only provide a maximum of 30% EPA. In practice, commercially available oils deliver 18–25% EPA, and trials have used daily doses of 3–4 g. A few studies have used high doses of 8–30 g EPA (Bjorneboe *et al.*, 1987; Illingworth *et al.*, 1984; Phillipson *et al.*, 1985; Prescott *et al.*, 1985; Ziboh *et al.*, 1986), but this causes problems because of the large volumes that need to be consumed. EPA is available in a highly purified form (93%) as the ethyl ester and has been used in 3–4 g doses in some studies (Payan *et al.*, 1986; Terano *et al.*, 1987). Use of this ester has been proposed as

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a means of achieving higher levels of supplementation (Knapp & Fitzgerald, 1989). We have investigated the acceptability and biochemical effects of the ethyl ester of EPA when used in high doses.

Methods

Six healthy male research workers (age range 30–41 years) gave informed consent to supplementation of their diet with a 93% pure eicosapentaenoic acid ethyl ester (EPA-EE) containing 0.2% α-tocopherol as antioxidant (Nippon Suisan Kaisho, Tokyo, Japan). The oil also contained 2.6% 18 : 4 ethyl ester, 1% 20 : 4 (n3) ethyl ester, and 2.6% 20 : 4(n6) ethyl ester. The peroxide value was 3.95 μg kg^{-1}. The oil was stored at 4° C, under an atmosphere of nitrogen. During the trial volunteers continued with their normal diet. For the first 6 weeks they received 6 g EPA-EE day^{-1} (7.2 ml). There followed a wash-out period of 4 weeks, followed by supplementation with 18 g day^{-1} (21.5 ml) for 6 weeks. For safety reasons the order of treatment was not randomized, because higher doses of this preparation had not been used widely. The response to 6 g could thus be monitored in all volunteers before giving them the 18 g dosage. Blood samples were taken at 09.00 h, in the supine position, after a 12 h fast.

Red cell membrane fatty acids

Red cells were separated by dextran sedimentation from EDTA-anticoagulated blood and lysed in distilled water. After washing in distilled water, lipids were extracted into chloroform : methanol (1 : 2) by the method of Bligh & Dyer (1959). All solutions contained added 0.01% butylated hydroxytoluene (BHT) weight/volume. Samples were methylated by alkaline methanolysis and heating with 14% boron trifluoride in methanol. Esters were extracted into hexane and analysed by gas chromatography (Sanders & Younger, 1981).

Neutrophil stimulation

After separation by gradient centrifugation (Boyum, 1968), neutrophils were suspended in phosphate buffered saline (PBS) at 2×10^7 cells ml^{-1}. Aliquots of 0.5 ml were incubated with calcium chloride (final concentration 1 mM), at 37° C for 5 min, and then calcium ionophore A23187 (Sigma) added. After 5 min, 0.5 ml ice-cold methanol was added. Supernatants were frozen (−40° C) until analysed by reverse-phase h.p.l.c. using a 150 × 4.8 mm Resolve C18 column with 5μ spherical particles (Waters-Millipore). Solvents were 0.05 M ammonium acetate in water, and methanol (Wilkinson *et al.*, 1985), flow rate 1.2 ml min^{-1}. Peaks were monitored by u.v. absorption at 270 nm (leukotrienes) and 236 nm (hydroxyeicosatetraenoic acids, HETEs). Peaks were identified by retention time in comparison with mixtures of known composition [retention times 2.65 min (20-hydroxyleukotriene B_4, LTB_4), 9.8 min (LTB_5), 11.6 and 12.3 min (6-trans isomers of LTB_4), 14.1 min (LTB_4), 20.9 min (5-hydroxyeicosapentaenoic acid, 5-HEPE), 25.2 min (5-HETE)], and were quantified by integrated optical density, calibrated against three concentrations of standards, run with each assay. Intra-assay variation for duplicate injections gave a coefficient of variation of 2.9%. Duplicate stimulation of neutrophils with an interval of 2 weeks, prior to dietary intervention gave a coefficient of variation of 18.8%.

Lipid analysis

EDTA-anticoagulated blood containing sodium azide was kept at 4° C and analysed within 5 days for cholesterol and triglyceride by standard enzymatic procedures, after separation of lipid subfractions by ultracentrifugation and precipitation (Fehily *et al.*, 1988).

Statistics

The data were analysed by non-parametric methods. Median values with range are quoted in the text, and statistical significance assessed by Friedman's two-way analysis of variance. A *P*-value of 0.05 was deemed significant, and paired comparisons were made by the Wilcoxon test against the initial (week 0) baseline.

Results

There were no abnormalities of blood count during low or high dose, in particular no fall in platelet count, median $256 \times 10^9 l^{-1}$ (range 138–281) pretreatment, $268 \times 10^9 l^{-1}$ (244–279) after 6 weeks, and $249 \times 10^9 l^{-1}$ (233–271) after 16 weeks. During the 6 g day^{-1} EPA-EE dosage period all volunteers tolerated the oil well. However, during the 18 g day^{-1} dosage period all volunteers developed diarrhoea and passed obvious unchanged oil in the stool. Two volunteers dropped out after 1 and 3 weeks on this high dose as a result of the steatorrhoea, and one reduced the dose to 10 g for the final 2 weeks. No

Table 1 Red cell membrane fatty acid levels during supplementation with EPA-EE. Values before the study (week 0), after 6 g daily (week 6), after 4 week wash-out (week 10), and after 18 g daily (week 16). Values expressed as percent total lipids, median and (range). $n = 6$ (4 only at week 16).

Fatty acid		*Low dose* *Start*	*Low dose* *End*	*High dose* *Start*	*High dose* *End*
18:0	Stearic acid	13.5 (12.1–14.1)	13.2 (9.8–15.6)	11.7 (8.7–15.2)	12.6 (10.9–15.8)
18:1(n6)	Oleic acid	11.5 (10.6–11.9)	12.3 (9.1–14.9)	11.8 (8.7–14.3)	11.2 (8.2–13.4)
18:2(n6)	Linoleic acid	9.5 (6.9–10.0)	8.7 (7.6–9.0)	9.1 (7.0–10.5)	7.2*† (6.8–8.1)
18:3(n6)	Linolenic acid	0.3 (0.3–0.4)	0.2 (0.04–0.3)	0.2 (0.06–0.2)	nd
20:3(n6)	Gamma-linolenic acid	1.7 (1.1–2.3)	1.2 (1.1–1.6)	1.5 (1.2–2.1)	1.1 (0.7–1.6)
20:4(n6)	Arachidonic acid	13.2 (11.4–15.3)	11.2* (10.4–12.2)	11.2 (6.4–14.9)	8.3* (7.0–11.6)
20:5(n3)	Eicosapentaenoic acid	0.8 (0.6–2.7)	3.0* (2.5–4.6)	1.6 (1.0–2.1)	6.0*† (4.1–7.3)
22:6(n3)	Docosahexaenoic acid	5.3 (4.7–5.8)	4.0* (3.1–4.6)	4.5 (1.4–8.0)	3.2* (2.8–5.0)

* $P < 0.05$ compared with baseline, † $P < 0.05$ compared with 6 week value.
Fatty acids shown as chain length : number of double bonds. Abbreviations: nd = nil detected.

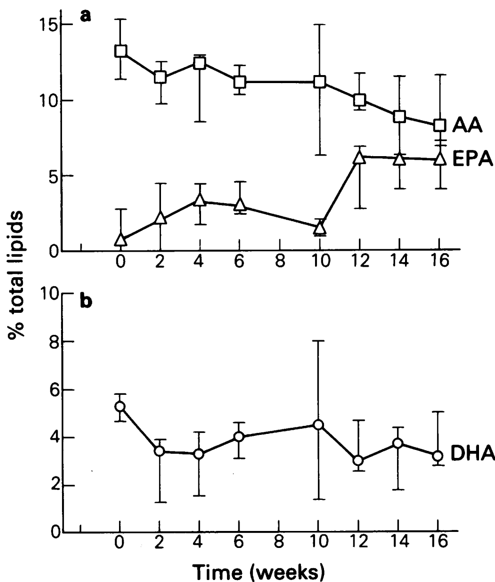

Figure 1 Changes in red cell membrane fatty acids in response to EPA-EE supplements 6 g daily weeks 0–6, 18 g daily weeks 10–16. Expressed as % of total lipids, median and range. $n = 6$ ($n = 4$ for week 14 and 16).

other side-effects were noted. There was no significant change in body weight during the trial (mean weight 76.2 kg at entry, 75.9 kg at 16 weeks).

Fatty acids

Changes in red cell membrane EPA, DHA and arachidonic acid (AA) occurred within 2 weeks of starting the low dose (Figure 1). EPA rose and reached a plateau of 3.3% of total lipids at 4 weeks. During the wash-out phase (week 6–10) the level returned to basal. On high dose, levels rose more rapidly to 6.2% after 2 weeks, and 6.0% after 6 weeks, significantly higher than levels on the low dose ($P = 0.043$). AA fell during both periods, from 13.2% before dosing, reaching a nadir of 8.3% at the end of the high dose period. Differences between high and low doses were not significant. DHA fell very rapidly from a basal value of 5.3% to 3.4% after 2 weeks, with similar changes during the high dose period. Other fatty acids are shown in Table 1: there was a significant fall in linoleic acid after the 18 g dose.

Neutrophil leukotriene production

There was a dose dependent stimulation of eicosanoid synthesis by ionophore (Figure 2), with 5 μM A23187 producing near maximal synthesis. After 6 weeks of supplementation with EPA-EE, there was significant suppression of LTB_4 (Figure 3a). As shown in Table 2 this was most marked at submaximal ionophore stimulation, with 51% inhibition at 1.25 μM A23187 (P = 0.014). During the 4-week wash-out period neutrophil LTB_4 synthesis returned to baseline, and fell again after 6 weeks at 18 g EPA-EE daily. The reduction was not significantly greater than after the 6 g dose. LTB_5 synthesis, derived from EPA, rose slightly after the 6 g dose, returned to undetectable levels after the wash-out period, and in comparison to the 6 g dose, reached significantly higher levels after 18 g daily, $P = 0.025$ (Figure 3b). Parallel changes were observed in the monohydroxy products with a fall in the AA-derived 5-HETE, that was not dose-dependent, and a dose-dependent rise in EPA-derived 5-HEPE. In parallel to LTB_4 there was also suppression of 6-trans-LTB_4 isomer synthesis and 20-hydroxy-LTB_4, the latter implying that reduction in LTB_4 was not due to increased LTB_4 metabolism.

Plasma lipids (Table 3)

Total triglyceride fell during the 6 g dose, mainly due to a fall in VLDL triglyceride. Levels did not return to basal values during the wash-out period, and there was no further fall during the 18 g dose. There was no significant change in total cholesterol during the 6 g dose, but levels fell significantly during the 18 g dose, due mainly to a fall in low density lipoprotein (LDL) cholesterol.

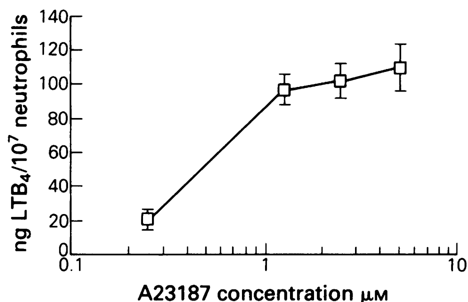

Figure 2 LTB_4 concentrations in supernatants of peripheral blood neutrophils stimulated with calcium ionophore A23187 for 5 min, measured by h.p.l.c. ($n = 6$). Results expressed as mean ± s.e. mean.

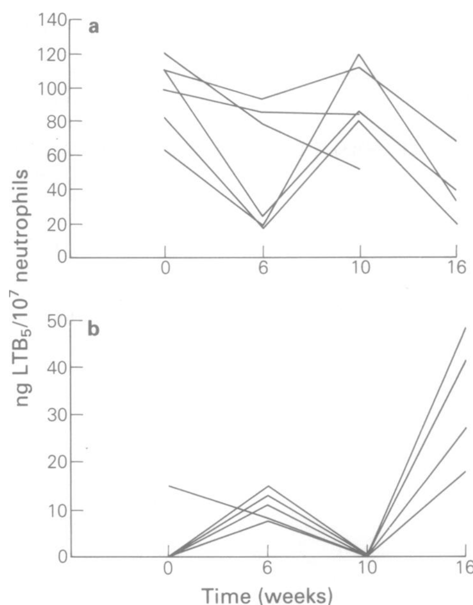

Figure 3 a) LTB_4 levels in supernatants of peripheral blood neutrophils, stimulated with calcium ionophore A23187, 1.25 μM, for 5 min. b) LTB_5 levels in response to 5 μM ionophore. Week 0–6 6 g EPA-EE daily, weeks 6–10 washout, weeks 10–16 18 g EPA-EE daily.

Discussion

No previous studies have reported diarrhoea as a side-effect of omega-3 fatty acid ingestion, although several studies have used high doses. However, these have used triglycerides of EPA, and it is possible that the ethyl ester of EPA is poorly absorbed in comparison with the triglyceride. Lipase activity is not normally rate limiting in fat digestion. In the case of triglycerides of long-chain polyunsaturated fatty acids, proximity of double bonds to the carboxy terminal seems to reduce lipase activity, even when the fatty acids are present at the triglyceride 1 and 3 positions (the preferential sites for lipase activity) (Entressangels *et al.*, 1966). Recent work has also shown that ethyl esters of EPA are very poorly absorbed in comparison with the 2-substituted triglyceride, free acid or salt (El Boustani *et al.*, 1987; Lawson & Hughes, 1988). There is some evidence that ethyl esters are poor substrates for hydrolysis by lipase. The extent of EPA incorporation into red cell membrane lipids is considerably lower at both doses than might be expected if absorption were complete. Other studies do however show wide variation in

Table 2 Eicosanoid concentrations (measured by h.p.l.c.) in supernatants of peripheral blood neutrophils stimulated with calcium ionophore at concentrations shown, for 5 min. Results expressed as ng/10^7 neutrophils, median (range). Values before the study (week 0), after 6 g daily (week 6), after 4 week wash-out (week 10), and after 18 g daily (week 16)

Eicosanoid		*Low dose* *Start*	*Low dose* *End*	*High dose* *Start*	*High dose* *End*
LTB_4	1.25 μM	104 (63–120)	51* (16–92)	84 (50–118)	34* (18–66)
	5 μM	105 (68–150)	90 (65–120)	100 (82–132)	42 (33–150)
LTB_5	1.25 μM	nd (nd)	nd (nd–14)	nd (nd)	17 (nd–27)
	5 μM	nd (nd–15)	9.7 (nd–15)	nd (nd)	27*† (18–48)
LTB_4 + LTB_5	1.25 μM	112 (71–128)	62* (24–102)	92 (58–126)	51 (26–93)
	5 μM	113 (76–165)	101 (76–128)	108 (90–140)	67 (51–191)
5-HETE + 5-HEPE	1.25 μM	95 (70–168)	58 (16–110)	63 (18–197)	83 (52–96)
	5 μM	138 (90–238)	129 (83–202)	114 (78–218)	167 (105–290)
5-HETE	1.25 μM	87 (62–160)	44 (nd–78)	50 (10–189)	41 (27–54)
	5 μM	130 (82–230)	89 (75–150)	99 (70–210)	67* (nd–190)
5-HEPE	1.25 μM	nd (nd)	13 (nd–35)	nd (nd–13)	39 (25–49)
	5 μM	nd (nd)	36 (nd–92)	nd (nd–18)	100 (78–110)
6-trans LTB_4	1.25 μM	52 (25–64)	16* (nd–40)	37 (6.6–90)	13* (nd–33)
	5 μM	63 (41–74)	54 (20–81)	41 (25–110)	27 (nd–55)
20–OH LTB_4	1.25 μM	115 (48–160)	53 (34–140)	130 (36–150)	30 (23–52)
	5 μM	120 (86–160)	104 (76–160)	140 (52–140)	43 (34–133)

* $P < 0.05$ compared with baseline, † $P < 0.05$ compared with 6 week value.
nd = Nil detected.

levels of incorporation, varying from 3.7% total red cell lipids after 6 weeks of cod liver oil containing 1.8 g EPA daily (Sanders *et al.*, 1981) and 5% red cell phospholipids after 4 weeks of cod liver oil containing 0.85 g EPA daily (Von Schacky *et al.*, 1985), up to 8% total red cell lipids after 3 weeks of 'Maxepa' containing 1.9 g EPA (Bruckner *et al.*, 1987). A 75% pure ethyl ester, giving 3.3 g EPA daily for 4 weeks gave 4% of red cell phospholipids. On the 18 g daily dose, the peak EPA incorporation of 6.2% was significantly higher than incorporation at the 6g daily dose, and represents an EPA/AA ratio of 0.70 (compared with 0.06 at baseline and 0.27 on 6 g day^{-1}). Although a number of factors including volunteer compliance, preparation of oil, and perhaps autoregulatory processes maintaining cell membrane composition may account

Table 3 Plasma lipids during supplementation with EPA-EE. Values before the study (week 0), after 6 g daily (week 6), after 4 week wash-out (week 10), and after 18 g daily (week 16). Values expressed in mM, as median (range). $n = 6$ (4 only at week 16)

Lipid	*Low dose* Start	*Low dose* End	*High dose* Start	*High dose* End
Total cholesterol	4.9 (4.3–6.6)	4.9 (3.8–6.2)	4.5* (4.2–5.7)	4.3*† (3.7–5.6)
VLDL cholesterol	0.27 (0.15–0.79)	0.30 (0.04–0.60)	0.23 (0.10–0.64)	0.31 (0.03–0.44)
LDL cholesterol**	3.7 (3.1–5.2)	3.8 (2.0–4.8)	3.3 (3.1–4.1)	3.2 (2.8–4.5)
HDL cholesterol	0.94 (0.72–1.1)	0.97 (0.74–1.4)	0.87 (0.67–1.4)	0.87 (0.85–1.1)
Total triglycerides	1.0 (0.51–1.5)	0.66* (0.36–1.5)	0.68 (0.40–1.5)	0.69* (0.36–1.3)
VLDL triglycerides	0.60 (0.17–1.8)	0.28 (0.05–0.6)	0.20 (0.08–0.90)	0.51 (0.16–0.52)
LDL triglycerides	0.25 (0.0–0.54)	0.31 (0.10–0.65)	0.25 (0.18–0.78)	0.56 (0.20–0.56)

** Overall significant differences by Friedman's 2-way analysis of variance, $P < 0.05$
* $P < 0.05$ compared with baseline, † $P < 0.05$ compared with 6 week value.

for these variations, the failure to produce high levels in this study is presumably mostly due to malabsorption.

Increased synthesis of LTB_5 and 5-HEPE by neutrophils in response to calcium ionophore after EPA-EE supplementation reflects the neutrophil membrane incorporation of EPA. Red cell membrane incorporation was measured rather than neutrophil incorporation, but in other studies the levels in different cell types has been shown to rise similarly with EPA supplementation (Von Schacky *et al.*, 1985). There was greater synthesis of LTB_5 and 5-HEPE following the higher dose. In contrast the suppression of ionophore-stimulated LTB_4 and 5-HETE synthesis in response to EPA-EE supplements appeared to be maximal at the 6 g day^{-1} dose, and was not greater at the 18 g day^{-1} dose. It has been suggested that the main effect of EPA-derived products in reducing LTB_4 synthesis is via competitive inhibition by LTA_5 at the level of LTA_4 hydrolase (Nathaniel *et al.*, 1985). Our data support this with significant suppression of total B leukotrienes (LTB_4 + LTB_5), at sub-maximal ionophore stimulation, but not of total monohydroxy products (5-HETE + 5-HEPE), which are derived non-enzymatically from 5-hydroperoxyeicosatetraenoic acid or 5-hydroperoxyeicosapentaenoic acid at an earlier step in the lipoxygenase pathway. It is interesting that with higher levels of LTB_5 synthesis there is not more profound inhibition of LTB_4 synthesis, and presumably implies that LTA_4 is still a preferred substrate for LTA_4 hydrolase. Other studies have failed to show greater than 50% inhibition of LTB_4 synthesis, using lower doses of EPA, and high dose therapy does not give significantly greater inhibition.

The plasma lipoprotein results showed that the effect on triglycerides was the most marked. Suppression was near maximal at 6 g EPA-EE day^{-1} (66% of basal) with no further reduction at 18 g day^{-1}, in keeping with other studies, using fish oil triglyceride concentrates (Prescott *et al.*, 1985). The effect on cholesterol was not significant at the 6 g dose, and only fell significantly with 18 g day^{-1}, to 87% of basal values. It is thought that EPA exerts its action by reduction in very low density lipoprotein (VLDL) synthesis, and the most consistent effect on cholesterol levels is a reduction in VLDL cholesterol. In standard dose of 2–4 g day^{-1} EPA can increase LDL cholesterol (Demke *et al.*, 1988). In our study the effect on VLDL cholesterol was

equivocal, and at 6 g day^{-1} LDL cholesterol did not change, whilst at 18 g day^{-1} there was a decrease. This is in keeping with other studies showing that high dose EPA lowers LDL cholesterol (Illingworth *et al.*, 1984). The study was not large enough to investigate this effect further, but does suggest that high dose EPA may inhibit cholesterol as well as triglyceride synthesis.

In conclusion this study has shown that dietary supplementation with high doses of EPA in the form of the ethyl ester is likely to be limited by malabsorption. Despite this problem high dose therapy did achieve greater suppression of plasma cholesterol. Neutrophil LTB_4 synthesis was not inhibited more at high dose, in spite of greater LTB_5 synthesis, and therefore high dose therapy is unlikely to give any greater anti-inflammatory effect. Alternative ways of administering high dose EPA should be explored, such as the use of the free acid, or the salt of EPA, where absorption would be complete, and palatability improved, and would enable further investigation of these actions of EPA.

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