Effect of n-3 Fatty Acid Ethyl Ester Supplementation on Fatty Acid Composition of the Single Platelet Phospholipids and on Platelet Functions

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Twenty healthy male volunteers were randomly assigned to receive either four 1-g capsules of n-3 polyunsaturated fatty acids (PUFA) ethyl esters or four 1-g capsules of olive oil (as placebo) for a period of 4 months, followed by a 3-month wash-out period. Fatty acids of platelet phospholipid fractions, platelet aggregation, and thromboxane B_2 (TXB_2) formation were analyzed at 0, 2, and 4 months of treatment and at 1, 2, and 3 months of wash-out. During n-3 PUFA supplementation, accumulations of eicosapentaenoic (EPA), docosapentaenoic (DPA), and docosahexaenoic (DHA) acids were markedly increased after 2 months, with slight differences in further accumulation up to 4 months among the various phospholipid fractions. Significant decreases in platelet sensitivity to collagen, serum TXB_2 levels, and urinary TXB_2 metabolites were also observed following n-3 PUFA treatment. During the first and second month of wash-out, slight differences were observed in changes of various fatty acids among different phospholipid fractions, but after 3 months of wash-out, alterations were no longer detectable with respect to pretreatment values. After 3 months of wash-out, platelet function parameters also were returned to baseline. Thus, both platelet lipids and function are influenced by n-3 PUFA ethyl ester supplementation, and significant alterations are still detectable after 2 months of wash-out.

EPIDEMIOLOGIC STUDIES on Greenland Eskimos have suggested that diets rich in n-3 polyunsaturated fatty acids (PUFA) may be protective against cardiovascular disease, possibly affecting the so-called thromboxane/prostacyclin balance.[1,2] Thus, over the last two decades many studies have been performed on the effects of fish oil supplementation on platelet lipid composition, platelet function, and eicosanoid formation (for a review, see Kristensen et al[3]). Most trials have involved the use of diets supplemented by either large amounts of cod liver oil and other fish oils or large numbers of capsules containing fish oil extracts.[3] However, fish oils may increase dietary n-3 PUFA, but they also may contain a number of other fatty acids and different components.[4] Thus, from these studies, an evaluation of the specific effects of n-3 PUFA is not possible.

Recently, preparations of purified n-3 PUFA ethyl esters became widely available,[5,6] thus providing a selective supplementation with eicosapentaenoic (EPA) and/or docosahexaenoic acid (DHA). To date, only a few studies have examined the effects of purified n-3 PUFA preparations, usually EPA, on platelet composition and function.[7-12] In addition, platelet aggregation and thromboxane formation have been studied only after treatment periods of a few weeks. Thus, the time course of changes in platelet lipid composition and function either during or after treatment is not completely known. Therefore, the aim of this study was to investigate (1) the effects of purified EPA + DHA ethyl esters on fatty acid composition of the single platelet phospholipid fractions and their functional implications after a medium-term supplementation and (2) the time course of platelet changes after discontinuing treatment.

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SUBJECTS AND METHODS

Subjects

Twenty healthy male volunteers (aged 32 ± 4 years; range, 27 to 41) were recruited to participate in the study. They were considered eligible if total plasma cholesterol was less than 5.5 mmol/L, triglycerides (TG) were less than 2 mmol/L, and blood pressure was normal. All subjects had normal findings on physical examination and routine hematology analyses. A preliminary interview was performed to exclude subjects with extreme dietary habits. Participants were advised to avoid taking all drugs known to interfere with eicosanoid formation or lipid metabolism during the study period of 7 months. All subjects provided informed consent.

Study Design

The subjects were randomly assigned to receive either EPA and DHA ethyl esters (4 g/d) or olive oil as a placebo (4 g/d) for 4 months in a double-blind study. All subjects continued their usual diets during the study. They were prescribed four 1-g capsules of EPA + DHA (Esapent; Farmitalia-Carlo Erba, Milan, Italy) or placebo, taken with meals in three doses of one, one, and two capsules each. Customary diets were not otherwise altered. No specific monitoring of dietary intake was performed during the study. Each capsule of Esapent contained EPA 0.51 g and DHA 0.35 g (plus 0.14 g different n-3 and n-6 PUFA). Thus, the daily total dose was EPA 2.04 g and DHA 1.4 g. A parallel rather than a crossover protocol was used because of previous demonstration that a long wash-out period is required after discontinuation of fish oil for platelet membrane n-3 fatty acid levels to return to baseline values.[13]

It should be noted that all subjects were following a Mediterranean diet rich in olive oil, so that a 4-g/d olive oil supplementation has to be considered a true placebo unable to affect significantly the global dietary intake.

Participants in the study were instructed to keep in contact with the organizing center in case of any intercurrent health problem.

Blood Sampling

Venous blood samples were taken after an overnight fast immediately before the study, after 2 months of treatment, at the end of the 4-month treatment, and 1, 2, and 3 months after stopping n-3 PUFA supplementation.

Table 1. Characteristics of Subjects

	Active Treatment	Placebo Treatment
No.	10	10
Age (yr)	32 ± 4 (27-39)	33 ± 4 (27-41)
Smokers (no.)	3	2
Body weight (kg)	73 ± 7 (65-88)	72 ± 5 (65-78)
Body mass index	24 ± 2 (22-27)	23 ± 2 (21-26)
Systolic blood pressure (mm Hg)	121 ± 10 (105-130)	120 ± 12 (105-130)
Diastolic blood pressure (mm Hg)	81 ± 4 (70-85)	84 ± 3 (80-90)

Plasma Lipid Analysis

Plasma total cholesterol, high-density lipoprotein cholesterol (HDL-C), and TG were determined by commercial kits (Boehringer Mannheim Italia, Milan, Italy).

Platelet Lipid Analysis

Isolation of platelets. Platelet-rich plasma was obtained from citrated blood (0.13 mol/L trisodium citrate, 1/10 vol/vol) by centrifugation at 150 × *g* for 5 minutes at room temperature. The pH of platelet-rich plasma was adjusted to 6.5 with additional acid citrate dextrose (ACD). The platelet pellet, obtained by centrifugation for 20 minutes at 1,400 × *g*, was resuspended in HEPES buffer (pH 6.6), and 1 vol acid citrate dextrose was added to 14 vol of the platelet suspension. Platelets were washed twice and finally resuspended in 5 mL of the same HEPES buffer (pH 7.5) without addition of acid citrate dextrose.[14] Platelet pellets thus obtained were stored at −70°C until lipid extraction. Platelets were analyzed for protein content by the method of Lowry et al.[15]

Platelet lipid extraction and separation of phospholipid fractions. Platelet lipids were extracted by the method of Folch et al.[16] The different phospholipid fractions were separated by reverse-phase high-performance liquid chromatography (HPLC) according to the method reported by Schlager and Jordi.[17] A linear gradient obtained with two Waters 501 pumps (Millipore, Milford, MA) and a Waters model 660 solvent programmer (Millipore) was used for the separation of hexane:2-propanol:water from 6:8:0.75 to 6:8:14 (vol/vol). The gradient program was generated at a flow rate of 2 mL/min over a 30-minute period. Phospholipids were identified by monitoring absorbance at 205 nm with a Waters 486 spectrophotometric detector (Millipore). All solvents used in this study were HPLC-grade (Carlo Erba, Milan, Italy) and were degassed by sonication. The water used was HPLC-grade (BDH, Poole, UK). Samples were resuspended in 100 μL HPLC-grade chloroform (Carlo Erba) and injected with a rheodyne injection valve, equipped with a 100-μL loop, onto a Lichrospher Si 100 (10-μm) column (Merck, Darmstadt, Germany). Phospholipid content was determined colorimetrically (using dipalmitoyl lecithin as standard, Sigma Chemical, St Louis, MO) using the method reported by Stewart.[18]

Fatty acid analysis. Fatty acid methyl esters were analyzed using a Sigma 3 gas chromatograph (Perkin-Elmer, Norwalk, CT) with a split-splitless inlet. A fused silica gel capillary column (Supelco, Bellefonte, PA) of 30-m × 0.32-mm ID with a 0.25-μm layer and a splitless mode of injection was used. Dried helium was used as the carrier gas (linear velocity, 25 cm/s; split ratio, 100:1). The temperature at the injection port and at the hydrogen flame-ionization detector was 240°C. The initial column temperature was 185°C, and the rate of increase was 3°C/min until a final temperature of 200°C was reached. Identification of individual components was made by comparison of their retention time with standards (PUFA II, Matreya, Pleasant Gap, PA). Peaks were calculated by a computing integrator (model 1020, Perkin-Elmer) and expressed as percent of total fatty acid pool.

Platelet Aggregation

The minimum concentration of collagen (Semmelweiss, Mascia Brunelli, Milan, Italy) sufficient to induce 50% of the maximum aggregation response was determined in whole blood by the electrical impedance method[19] using a Whole-Blood Aggregometer (model 540, Chrono-Log, Havertown, PA).

Serum Thromboxane B_2

Thromboxane A_2 production in clotting blood, after incubation of 2 mL blood at 37°C for 30 minutes, was studied by measuring levels of its stable metabolite thromboxane B_2 (TXB_2) in serum[20] by enzyme immunoassay[21] using a commercial kit (Thromboxane B_2 EIA kit, Cayman Chemical, Ann Arbor, MI).

Urinary TXB_2 Derivatives

Aliquots of urine collected for the 24 hours preceding blood sampling were frozen and kept at −20°C until assayed. After adjusting the urine pH to between 4 and 4.5 with formic acid (99%, Carlo Erba), TXB_2, 11-dehydro-TXB_2, and 2,3-dinor-TXB_2 were extracted on SEP-PAK C18 cartridges (Millipore) and eluted with ethyl acetate according to the method reported by Siess and Dray.[22] Eluted metabolites were purified on reverse-phase HPLC (Millipore) according to the method reported by Patrignani et al.[23] Fractions corresponding to 11-dehydro-TXB_2 and 2,3-dinor-TXB_2 were measured as TXB_2 by enzyme-linked immunosorbent assay (Cayman Chemical) according to the method reported by Castagnoli et al.[21]

Statistical Analysis

Results are expressed as the mean ± SD. ANOVA and Student's *t* test for paired data were used to compare results obtained throughout the treatment. Student's *t* test for unpaired data was

Table 2. Effects of EPA + DHA (4 g/d) or Olive Oil (4 g/d) on Plasma Lipids

Plasma Lipid	Treatment			Recovery		
	Baseline	2 Months	4 Months	1 Month	2 Months	3 Months
EPA + DHA						
TC (mmol/L)	4.4 ± 0.4	4.3 ± 0.4	4.3 ± 0.5	4.2 ± 0.5	4.2 ± 0.5	4.2 ± 0.4
HDL-C (mmol/L)	1.2 ± 0.2	1.3 ± 0.2	1.3 ± 0.3	1.3 ± 0.2	1.3 ± 0.2	1.2 ± 0.2
TG (mmol/L)	1.2 ± 0.3	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.2	1.0 ± 0.1	1.0 ± 0.2
Olive oil						
TC (mmol/L)	4.2 ± 0.6	4.2 ± 0.5	4.2 ± 0.8	4.2 ± 0.4	4.1 ± 0.7	4.3 ± 0.5
HDL-C (mmol/L)	1.2 ± 0.4	1.3 ± 0.2	1.3 ± 0.3	1.2 ± 0.4	1.3 ± 0.2	1.3 ± 0.2
TG (mmol/L)	1.3 ± 0.3	1.1 ± 0.2	1.2 ± 0.3	1.1 ± 0.3	1.0 ± 0.2	1.1 ± 0.2

Abbreviation: TC, plasma total cholesterol.

Table 3. Effect of EPA + DHA Supplementation on Fatty Acids of Platelet PC (mol% of total)

Platelet PC Fatty Acid	Treatment			Recovery		
	Baseline	2 Months	4 Months	1 Month	2 Months	3 Months
18:2 n-6	6.40 ± 0.69	6.63 ± 0.85	7.08 ± 1.03*	6.59 ± 0.98	6.39 ± 0.92	6.32 ± 0.76
20:3 n-6	1.34 ± 0.26	1.14 ± 0.26*	1.04 ± 0.29†§	1.26 ± 0.32	1.29 ± 0.31	1.34 ± 0.23
20:4 n-6	10.11 ± 2.46	9.10 ± 2.23	9.13 ± 2.38	9.71 ± 2.34	9.45 ± 2.65	9.63 ± 2.40
20:5 n-3	0.21 ± 0.18	1.19 ± 0.44‡	1.40 ± 0.59‡	0.40 ± 0.12*	0.28 ± 0.07	0.26 ± 0.08
22:4 n-4	0.46 ± 0.09	0.48 ± 0.15	0.47 ± 0.15	0.56 ± 0.23	0.45 ± 0.12	0.53 ± 0.09
22:5 n-3	0.35 ± 0.12	0.56 ± 0.25†	0.75 ± 0.24‡	0.59 ± 0.20*	0.46 ± 0.17	0.40 ± 0.14
22:6 n-6	0.58 ± 0.24	1.02 ± 0.30‡	1.26 ± 0.36‡	0.99 ± 0.26‡	0.77 ± 0.23	0.74 ± 0.21

*$P < .05$ *v* baseline.
†$P < .01$ *v* baseline.
‡$P < .001$ *v* baseline.
§$P < .05$ *v* 2 months' treatment.

used for comparisons between the two treatment groups. P less than .05 was used as the criterion for statistical significance.

RESULTS

The two groups were well balanced for age, blood pressure, smoking habits, and body mass index (Table 1). They were also similar for baseline plasma cholesterol and TG levels (Table 2). Compliance as assessed by capsule count was more than 90%, and all subjects completed the protocol. Two subjects consuming olive oil reported abdominal discomfort and diarrhea after 7 and 14 days of treatment, respectively. Symptoms subsided after a transient reduction of daily olive oil supplementation from four to two capsules. Five of 10 subjects taking EPA + DHA reported occasional fish aftertaste. No other side effects were experienced.

The subjects' diet throughout the study was not directly assessed by specific methods. However, all subjects stated that their diet did not change, and in no subjects were body mass index changes greater than ±2% versus baseline observed.

Plasma Lipids

No significant changes were observed in plasma lipids after n-3 PUFA supplementation, even if a trend to decreased levels of TG was detectable after 2 and 4 months of treatment. No changes were seen in the group treated with olive oil as placebo (Table 2).

Changes in Platelet Phospholipid Fatty Acids

No changes in the fatty acid composition of different phosphoglyceride fractions were observed in the group of 10 subjects treated with olive oil (data not shown).

The amounts of n-3 and n-6 PUFA esterified in different phosphoglyceride fractions during n-3 PUFA supplementation are listed in Tables 3 through 6.

In phosphatidylcholine (PC), n-3 PUFA were significantly increased after 2 months of supplementation. Both absolute and relative increases were greater for EPA than for docosapentaenoic acid (DPA) and DHA. After 4 months, a further slight increase (nonsignificant) was observed for all three n-3 PUFA. Among n-6 PUFA, a significant 10% increase in 18:2 n-6 and a significant decrease in 20:3 n-6 were observed, whereas 22:4 n-6 was unchanged and a mean 10% decrease in arachidonic acid (AA) did not reach statistical significance. One month after stopping treatment, EPA, DPA, and DHA levels were still significantly higher with respect to baseline values, whereas after 2 and 3 months of recovery the fatty acid pattern was returned to the pretreatment state (Table 3).

In phosphatidylethanolamine (PE), n-3 PUFA were already increased after 2 months of supplementation. From 2 to 4 months of treatment, EPA, DPA, and DHA remained at a steady level. At completion of the treatment period, 18:2 n-6 was also increased, but no changes were seen in the other n-6 PUFA. One month after stopping treatment, not only n-3 PUFA, as in PC, but also 18:2 n-6

Table 4. Effect of EPA + DHA Supplementation on Fatty Acids of Platelet PE (mol% of total)

Platelet PE Fatty Acid	Treatment			Recovery		
	Baseline	2 Months	4 Months	1 Month	2 Months	3 Months
18:2 n-6	2.53 ± 0.53	2.89 ± 0.45	3.48 ± 0.66†	3.41 ± 0.64†	2.83 ± 0.63	2.67 ± 0.79
20:3 n-6	0.59 ± 0.15	0.66 ± 0.12	0.59 ± 0.11	0.65 ± 0.11	0.60 ± 0.12	0.51 ± 0.28
20:4 n-6	27.26 ± 5.17	26.66 ± 4.85	26.82 ± 4.34	27.88 ± 4.10	28.75 ± 5.64	27.25 ± 4.61
20:5 n-3	0.28 ± 0.15	2.06 ± 0.87‡	2.24 ± 0.96‡	0.81 ± 0.38*	0.40 ± 0.18	0.33 ± 0.16
22:4 n-4	2.25 ± 0.56	1.87 ± 0.51	1.89 ± 0.72	2.24 ± 0.48	2.21 ± 0.48	1.96 ± 0.54
22:5 n-3	1.26 ± 0.56	2.43 ± 0.82*	2.56 ± 0.66‡	2.05 ± 0.50*	1.84 ± 0.64	1.38 ± 0.56
22:6 n-6	1.60 ± 0.55	2.55 ± 0.78*	2.56 ± 0.64‡	2.39 ± 0.42*	1.89 ± 0.59	1.81 ± 0.49

*$P < .05$ *v* baseline.
†$P < .01$ *v* baseline.
‡$P < .001$ *v* baseline.

Table 5. Effect of EPA + DHA Supplementation on Fatty Acids of Platelet PI (mol% of total)

Platelet PI Fatty Acid	Treatment			Recovery		
	Baseline	2 Months	4 Months	1 Month	2 Months	3 Months
18:2 n-6	1.29 ± 0.63	1.45 ± 0.61	1.86 ± 0.58†	1.65 ± 0.66	1.22 ± 0.63	1.16 ± 0.63
20:3 n-6	1.20 ± 0.27	1.30 ± 0.43	1.45 ± 0.43	1.26 ± 0.39	1.20 ± 0.35	1.16 ± 0.26
20:4 n-6	14.93 ± 2.42	14.99 ± 2.21	14.71 ± 2.33	14.94 ± 2.77	15.11 ± 2.37	15.11 ± 2.35
20:5 n-3	0.13 ± 0.04	0.74 ± 0.44†	0.84 ± 0.54†	0.15 ± 0.06	0.16 ± 0.01	0.18 ± 0.11
22:4 n-4	0.73 ± 0.25	0.88 ± 0.38	0.88 ± 0.45	0.80 ± 0.32	0.68 ± 0.18	0.64 ± 0.18
22:5 n-3	0.38 ± 0.24	0.81 ± 0.17‡	1.04 ± 0.28‡	0.71 ± 0.27†	0.48 ± 0.23	0.38 ± 0.26
22:6 n-6	0.56 ± 0.34	1.22 ± 0.45*	1.61 ± 0.53‡	1.02 ± 0.22*	0.97 ± 0.26*	0.49 ± 0.41

*$P < .05$ *v* baseline.
†$P < .01$ *v* baseline.
‡$P < .001$ *v* baseline.
§$P < .05$ *v* 2 months' treatment.

content was still higher than before the study. However, the decrease of EPA (both in percent and in absolute terms) was more marked than those of DPA and DHA. After 2 months of recovery, no differences were seen with respect to baseline levels (Table 4).

In phosphatidylinositol (PI), a significant incorporation of n-3 PUFA after 2 and 4 months of supplementation was detectable. After 4 months, a significant increase in 18:2 n-6 levels was also found. In comparison to PC and PE, in PI the EPA increase was less marked than DPA and DHA increases, and EPA levels rapidly returned to baseline after 1 month of recovery, whereas DPA and DHA levels were still elevated. The rate of decrease in DHA levels was slower than that in DPA, and after 2 months of recovery, DHA levels were still higher than in the baseline sample (Table 5).

In phosphatidylserine (PS), only trace amounts of n-3 PUFA were detectable in baseline samples. Changes induced by n-3 PUFA supplementation were qualitatively similar to those observed in the other fractions. n-3 PUFA incorporation was scanty, such as in PI, even if detectable in all subjects (Table 6).

In no fraction were significant changes in AA levels seen throughout the study. Neither were significant differences observed between the baseline fatty acid pattern and that obtained 3 months after stopping the study.

Platelet Aggregation Studies

For each subject, the minimum concentration of collagen sufficient to induce 50% of the maximum aggregation response (threshold) was determined in whole blood. Supplementation with n-3 PUFA induced an increase in the threshold concentration for aggregation to collagen in whole blood, whereas placebo had no effect (Table 7). This effect was already significant after 2 months and persisted at the end of treatment. The threshold for aggregation to collagen was still increased, but less markedly, 1 month after the end of supplementation, whereas changes were no longer detectable after 2 and 3 months of recovery.

Serum TXB_2

A significant decrease in serum TXB_2 was seen after 2 months of n-3 PUFA supplementation (Fig 1). A further inhibition was also detectable at the end of treatment (4 months). Serum TXB_2 levels after 1 and 2 months of recovery were still significantly lower than before the study, but after 3 months they had returned to baseline values. No changes were seen after placebo treatment.

Urinary TXA_2 Derivatives

The pattern of urinary 11-dehydro-TXB_2 and 2,3-dinor-TXB_2 is shown in Figs 2 and 3, respectively. In three subjects treated with n-3 PUFA, baseline levels of 11-dehydro-TXB_2 were particularly high (Fig 2). In one of the three subjects, baseline levels of 2,3-dinor-TXB_2 were also markedly high (Fig 3). A decrease in the two metabolites was found in all but one subject after 2 months of EPA + DHA supplementation, with a nadir at the end of treatment. The decrease was marked in subjects with high

Table 6. Effect of EPA + DHA Supplementation on Fatty Acids of Platelet PS (mol% of total)

Platelet PS Fatty Acid	Treatment			Recovery		
	Baseline	2 Months	4 Months	1 Month	2 Months	3 Months
18:2 n-6	2.41 ± 0.83	2.59 ± 1.15	2.80 ± 0.99	2.56 ± 0.88	2.85 ± 1.29	2.45 ± 0.79
20:3 n-6	0.59 ± 0.35	0.70 ± 0.26	0.83 ± 0.33	0.73 ± 0.39	0.74 ± 0.35	0.58 ± 0.33
20:4 n-6	25.97 ± 4.84	23.50 ± 5.94	24.56 ± 5.09	25.85 ± 4.40	25.65 ± 4.94	26.07 ± 4.52
20:5 n-3	0.15 ± 0.13	0.91 ± 0.73†	1.05 ± 0.82†	0.27 ± 0.24*	0.16 ± 0.18	0.13 ± 0.10
22:4 n-4	1.05 ± 0.56	1.19 ± 0.58	1.15 ± 0.49	1.03 ± 0.41	0.97 ± 0.46	1.11 ± 0.48
22:5 n-3	0.28 ± 0.24	0.83 ± 0.52†	0.95 ± 0.24‡	0.34 ± 0.21	0.28 ± 0.30	0.22 ± 0.15
22:6 n-6	0.36 ± 0.37	0.75 ± 0.46†	0.87 ± 0.49*	0.57 ± 0.42*	0.37 ± 0.38	0.35 ± 0.38

*$P < .05$ *v* baseline.
†$P < .01$ *v* baseline.
‡$P < .001$ *v* baseline.

Table 7. Collagen Aggregation Threshold (μg/mL) in Whole Blood

	Treatment			Recovery		
	Baseline	2 Months	4 Months	1 Month	2 Months	3 Months
EPA + DHA	0.56 ± 0.15	0.72 ± 0.19*	0.73 ± 0.16†	0.64 ± 0.15*	0.58 ± 0.15	0.54 ± 0.17
Olive oil	0.57 ± 0.15	0.52 ± 0.13	0.53 ± 0.13	0.59 ± 0.12	0.54 ± 0.15	0.55 ± 0.14

*$P < .01$ *v* baseline.
†$P < .001$ *v* baseline.

baseline levels, whereas it was scanty in subjects with normal levels. After stopping supplementation, the levels of TXA_2 urinary metabolites progressively returned to baseline levels. Olive oil treatment was not followed by changes in urinary TXA_2 derivatives.

DISCUSSION

The results of this study indicate that (1) supplementation with EPA + DHA ethyl esters induces important modifications in platelet membrane lipid composition; (2) these modifications are still detectable for several weeks after stopping supplementation, with different patterns in different phospholipid fractions; and (3) changes in platelet lipid composition are associated with a marked decrease in platelet TXA_2 formation.

Plasma Lipids

Only a trend for decreased levels of plasma TG was found during treatment, in contrast to other studies.[24] This is likely due to the low-dose n-3 PUFA administered. Moreover, it should be noted that TG levels were particularly low in the healthy subjects investigated in the present study.

Changes in Platelet Lipid Composition

Only a few studies have evaluated changes in single platelet phospholipid fractions after n-3 PUFA supplementation. This is of crucial importance, since different platelet phospholipids may play different functional roles. In particular, PI, the major source of AA, plays an important role in platelet functions related to hemostasis. Over the past few years, n-3 PUFA supplementation has been reported to increase the incorporation of EPA, DPA, and DHA in platelet PC and PE but not in PI and PS.[25] However, these results were derived from a study performed with supplementation for a few weeks (< 4) with fish oil at a dosage of less than 2 g n-3 PUFA per day.[25] In contrast, results of the present study indicate that EPA + DHA ethyl esters, when administered at a dosage of approximately 4 g/d for 4 months, also induce an incorporation of n-3 PUFA in PI and PS. Recent observations confirm the importance of both treatment duration and dosage for the incorporation of n-3 PUFA in platelet phospholipids.[11,12] In PI and PS, EPA rapidly decreased after stopping treatment, whereas DHA levels decreased more slowly. In contrast, AA changes in different phospholipids were variable in different subjects, and we did not observe a consistent decrease in AA

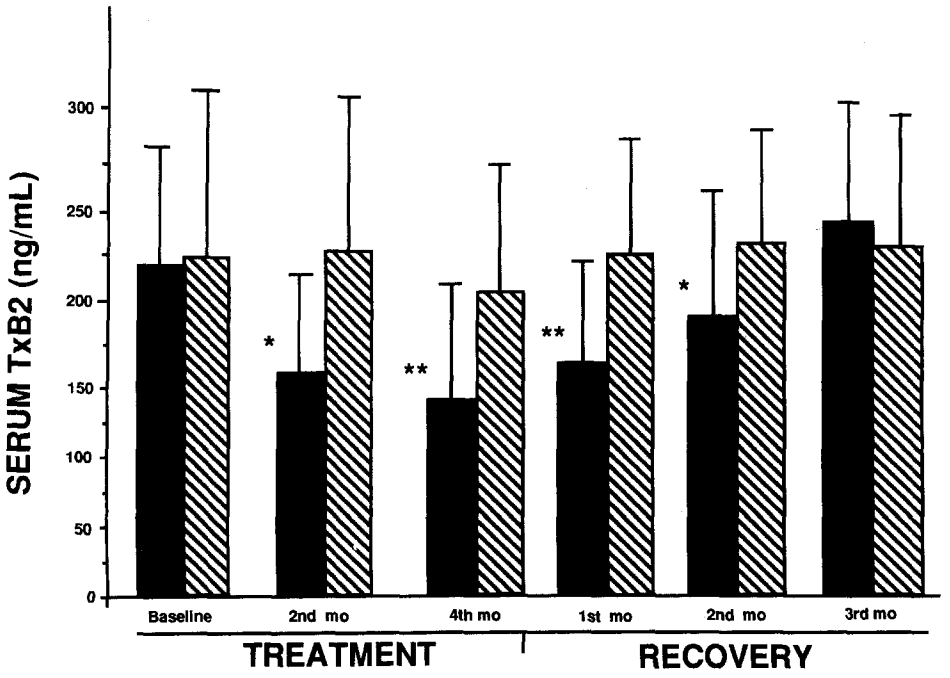

Fig 1. Serum TXB_2 levels during EPA + DHA (■) and olive oil (▨) supplementation. *$P < .01$ *v* baseline. **$P < .001$ *v* baseline.

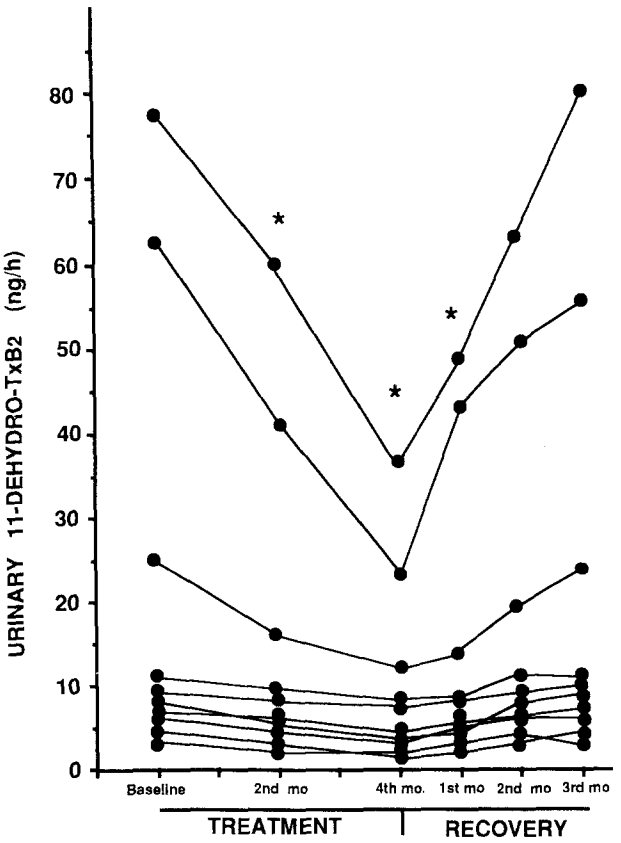

Fig 2. Urinary 11-dehydro-TXB_2 output during EPA + DHA supplementation. *$P < .05$ *v* baseline.

levels after n-3 PUFA supplementation. These results are at variance with previous studies in which fish oils had been given,[25] but are in agreement with more recent investigations.[10,11] On the other hand, these results obtained with a EPA + DHA ethyl ester preparation are not comparable to results of studies using fish oils or a selective supplementation with only EPA[7,10] due to possible interferences between EPA and DHA.[8,24,26]

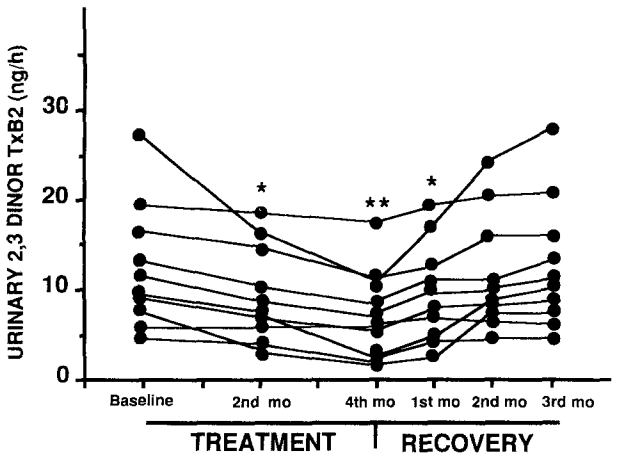

Fig 3. Urinary 2,3-dinor-TXB_2 output during EPA + DHA supplementation. *$P < .01$ *v* baseline. **$P < .001$ *v* baseline.

In the present study, a significant increase in linoleic acid (18:2 n-6) levels was found in different platelet phospholipid fractions after n-3 PUFA supplementation. This result was unexpected and is not in agreement with results of other studies.[10,11,25] Actually, linoleic acid is a precursor of AA, and n-3 PUFA inhibit the synthesis of AA from linoleic acid,[27] possibly leading to its accumulation. However, in this study, no clear decrease was observed in AA levels even if we consider that the dietary fatty acid intake was not modified but was only supplemented with n-3 PUFA, leaving unmodified the nutritional sources for AA independently of in vivo synthesis from linoleic acid.

In a recent investigation in which EPA + DHA ethyl esters 3 g/d were administered to healthy subjects, increased levels of n-3 PUFA in platelet phospholipids were reported up to 24 weeks after stopping supplementation.[11] Some differences in the subjects investigated (50% females), the dosage of n-3 PUFA, and the methodology could explain, at least in part, the different results with respect to our study. However, a comparison of the results of the two studies is difficult because only the fatty acid composition of total phospholipids (and not of the different fractions) was examined by Marangoni et al.[11]

Platelet Functional Changes

The EPA + DHA supplementation used in this study (4 g/d for 4 months) induced a significant decrease in both collagen-induced platelet aggregation and TXA_2 production. However, the time course of these functional variations during n-3 PUFA supplementation does not seem to correspond exactly to that of the membrane lipid modifications. Actually, inhibition of aggregation was already maximal after 2 months and persisted for only 1 month after the end of dietary supplementation, whereas n-3 PUFA accumulation in platelets and serum TXB_2 reached maximal levels at the end of treatment and were still detectable after 2 months of wash-out. However, the conclusion that platelet sensitivity to collagen returns to normal before TXA_2 platelet production must be stated cautiously because of the low sensitivity of platelet function assays in vivo. In any case, on the whole, these data suggest that functional changes are not simply a direct effect of membrane fatty acid changes.

It is difficult to comment on the relevance of the observed changes in platelet sensitivity to collagen. Over the last few years, platelet aggregation in vitro has been reported to be a useful biologic marker for the prediction of coronary events and mortality both in apparently healthy men and in particular subsets of patients.[28,29] On the other hand, several clinical studies have demonstrated that inhibition of platelet function by drugs exerts beneficial effects via secondary prevention of vascular events in different groups of vascular patients.[30] Thus, a reduction of platelet activity could be of importance. However, any specific comment on the possible relevance of the changes we observed in

platelet aggregation after n-3 PUFA therapy could only be speculative.

Although TXB_2 serum levels decreased in all subjects after n-3 PUFA therapy, in terms of urinary TXA_2 derivatives, marked decreases were only evident in subjects with high baseline values, as reported by others.[31] The decrease in thromboxane-generating capacity ($<50\%$) cannot by itself explain the decrease in platelet response to collagen, because only 5% of normal TXA_2 production is necessary to support normal platelet aggregation responses.[32] These data suggest that the effects of n-3 PUFA on TXA_2 synthesis may be greater when it is above the normal range. However, further specifically designed studies are necessary to clarify this point.

On the other hand, a discrepancy among changes in membrane n-3 PUFA, inhibition of platelet aggregation, and decreases in TXA_2 platelet production had already been reported.[33-37] Actually, even if a substrate substitution (n-3 PUFA in the place of AA) plays some role in platelet functional changes, other possible mechanisms should be considered, such as the formation of n-3 PUFA–derived epoxides, which are endowed with potent antiaggregatory effects[38] and may act independently of the TXA_2 pathway, or an impaired coupling of collagen to its receptors.[31] Finally, n-3 PUFA not only inhibit enzymatic steps in TXA_2 synthesis,[26] but also exert inhibitory effects on TXA_2/prostaglandin-H_2 receptors.[39]

In summary, although the effects of n-3 PUFA supplementation on platelet aggregation and TXA_2 formation are indeed in the direction of decreased platelet activation, the magnitude of the effects is modest. This suggests that mechanisms other than changes in platelet function may contribute to the reported beneficial effects of n-3 PUFA on cardiovascular function.[3,24]

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