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FISH OIL PREPARATIONS RICH IN DOCOSAHEXAENOIC ACID MODIFY PLATELET RESPONSIVENESS TO PROSTAGLANDIN-ENDOPEROXIDE/THROMBOXANE A_2 RECEPTOR AGONISTS

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Abstract—The effects of daily dietary supplementation for 6 weeks with either 4.5 g eicosapentaenoic acid (EPA) and 3.35 g docosahexaenoic acid (DHA) (group I, EPA/DHA = 1.33) or 3.5 g EPA and 6.4 g DHA (group II, EPA/DHA = 0.54) on platelet responsiveness to the stable prostaglandin (PG)-endoperoxide analogue 9,11-dideoxy,9α-11α-methanoepoxy-$PGF_{2\alpha}$ (U 46619) were studied in healthy volunteers. Dose–response curves (DRC) of U 46619-induced platelet aggregation were analysed by computerized non-linear curve fitting. In group I, the concentration of U 46619 required for half-maximum platelet aggregation (EC_{50}) remained unchanged, whereas the Hill coefficient decreased from 6.2 to 3.3 ($P < 0.02$). In group II, characterized by a high intake of DHA, a considerable increase of EC_{50} from 0.3 to 1.4 μM was found ($P < 0.02$). These results suggest different effects of EPA and DHA on the platelet thromboxane/endoperoxide-amplifying system. The considerable shift of the DRC in group II suggests a direct effect of DHA on the presentation of the endoperoxide receptor and/or post-receptoral events.

Epidemiological studies suggest a lower occurrence of thrombotic vascular disease in populations consuming large amounts of marine fish oil. This beneficial effect has been predominantly attributed to inhibition of platelet function by eicosapentaenoic acid (EPA‡) and docosahexaenoic acid (DHA). These fatty acids are readily incorporated into the membrane lipid core and cause a reduced synthesis of thromboxane (Tx)A_2 by displacing the precursor arachidonate from the phospholipid pool. In addition, the biologically less active thromboxane species TxA_3 is generated from membrane-incorporated EPA (for review, see Ref. 1). More recent data provide evidence that additional and more efficient mechanisms may be involved in the inhibition of platelet function by cis-unsaturated fatty acids. These include inhibition of platelet cyclooxygenase [2], modifications of post-receptor signal transduction (phosphoinositole cycle) [3] and alterations in membrane microviscosity causing changes in receptor surface expression [4]. *In vitro*, EPA (C20:5) and DHA (C22:6) were shown to interfere specifically with the binding and the pro-aggregatory effect of the stable endoperoxide analogue 9,11-dideoxy,9α-11α-methanoepoxy-prostaglandin (PG)$F_{2\alpha}$ (U 46619) [5]. Since the inhibitory potentials of the two compounds seem to be different, the present study was designed to evaluate whether dietary supplementation with different amounts of EPA and DHA (EPA/DHA ratios 1.33 and 0.54, respectively) modifies differentially platelet responsiveness to the stable endoperoxide analogue U 46619.

MATERIALS AND METHODS

Study design. Twelve healthy male volunteers (age 25–38 years), recruited from the hospital staff, were included in the study. In addition to their normal diets, the volunteers of group I were supplemented with 4.5 g EPA and 3.35 g DHA daily, whereas group II received a daily supplementation with 3.5 g EPA and 6.5 g DHA. The preparations were taken for 6 weeks. No further medication was allowed. DHA- and EPA-containing capsules were provided by Dr Lisa Carr-Fischer, Fresenius, Bad Homburg, Germany. Blood samples were obtained before, and 3 and 6 weeks after starting dietary supplementation. During the study, bleeding time was monitored using the Simplate I device (Organon Teknika, Eppelheim, Germany).

Platelet preparation and aggregation studies. Venous blood samples were drawn into one ninth volume of anticoagulant (sodium citrate, 4.8%). Platelet aggregation studies were done turbidometrically with platelet-rich plasma (PRP), adjusted with autologous platelet-poor plasma to a final count of 3×10^{11}/L. Platelet aggregation was determined by monitoring changes in light

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‡ Abbreviations: DHA, docosahexaenoic acid; DRC, dose–response curve; EC_{50}, concentration required for half-maximal effect; EPA, eicosapentaenoic acid; PG, prostaglandin; PGH_2, prostaglandin-endoperoxide; PRP, platelet-rich plasma; Tx, thromboxane; U 46619, 9,11-dideoxy,9α-11α-methanoepoxy-$PGF_{2\alpha}$.

transmission using a Lab-aggregometer (Fresenius) as described previously [6].

Dose–response curves (DRCs) of U 46619-induced platelet aggregation were analysed by a four parameter logistic equation according to DeLean *et al.* [7]. The concentration of U 46619 required for half-maximum aggregation (EC_{50}) and the slope of the DRC (Hill coefficient) were calculated by computerized non-linear curve fitting (MATHLAB^R, Math Works Inc., South Natick, MA, U.S.A.).

The anti-aggregatory effectiveness of the stable prostacyclin analogue iloprost (13-methyl-18,19-didehydrocarbacyclin; Schering AG, Berlin, Germany) was determined in PRP by measurements of the half-maximum concentration of the compound required to inhibit primary platelet aggregation induced by adenosine diphosphate (0.6 μM).

Incorporation of EPA and DHA into membranes. To monitor compliance during the study and to assess resorption of the fatty acids, incorporation of EPA and DHA into membrane phospholipids was determined. Lipid composition of red blood cell ghosts was monitored after methylation by gas chromatography according to Clemens *et al.* [8].

TxB_2 and TxB_3 determination by HPLC. PRP was stimulated with 5 U/mL thrombin (5 min, 37°). The reaction was stopped by the addition of methanol, and samples were extracted on C18-RP columns using 16,16-dimethyl-PGE_2 as internal standard. Afterwards, prostanoids were derivatized into 2,4-dimethoxyanilides, separated by HPLC (Waters Novapack; Methanol/H_2O 65:35) and quantified by electrochemical detection according to Knospe *et al.* [9].

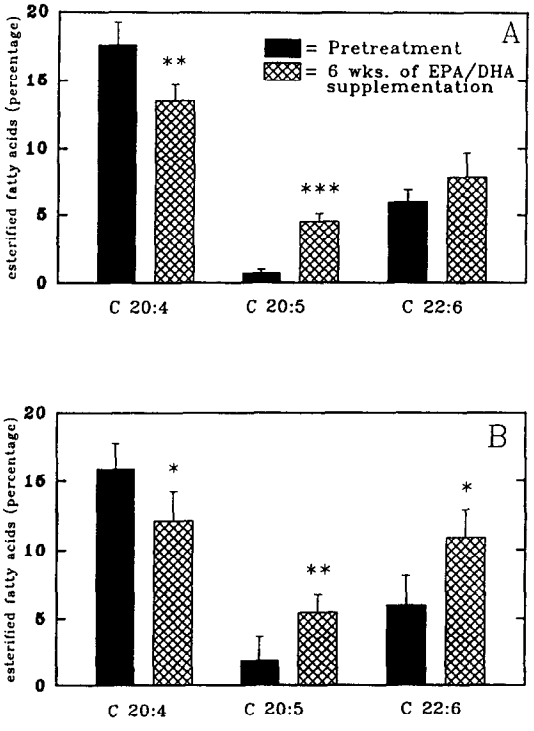

Fig. 1. Incorporation of EPA and DHA into red blood cell membranes. Membrane lipid composition was determined in red blood cell ghosts by gas chromatography. (A) Group I (high dose EPA); (B) group II (high dose DHA). Symbols represent means ± SD. *P < 0.05; **P < 0.01; ***P < 0.001.

RESULTS

Incorporation of EPA and DHA into cell membranes

The partitioning of DHA and EPA into membranes was determined in red blood cell ghosts (Fig. 1). In both groups, a significant reduction of membrane arachidonic acid was observed. As to be expected, the DHA content of membranes from the high dose DHA group was significantly increased as compared with group I (7.8 ± 1.9 vs 10.8 ± 2% of total fatty acids; $P < 0.05$). Nevertheless, the increase in DHA in plasma did not differ between the two groups (6.0 ± 1.0 vs 5.6 ± 1.1% of total fatty acids) after 6 weeks dietary supplementation.

Bleeding time and platelet aggregation studies

In both groups, only a modest and statistically non-significant increase in Simplate I bleeding times could be observed (Table 1). Dietary supplementation with both regimens did not significantly affect collagen (2.5 μg/mL) and ADP (0.6 μM)-induced platelet aggregation. The application of high doses of DHA (group II) resulted in increased sensitivity of the platelets to iloprost. In this group, responsiveness to the stable endoperoxide analogue U 46619 was considerably impaired.

U 46619-induced platelet aggregation

In further experiments, platelet responsiveness to U 46619 was determined over a concentration range of 30–3000 nM. DRCs obtained for the two groups are given in Figs 2 and 3. The DRCs were analysed by non-linear computerized curve fitting. In group I, the concentration of U 46619 required for half-maximum platelet aggregation (EC_{50}) remained unchanged after dietary supplementation (412 ± 51 vs 416 ± 183 nM), whereas the Hill coefficient decreased from 6.2 ± 3.0 to 3.3 ± 0.6 ($P < 0.02$). In group II, a considerable increase in EC_{50} from 364 ± 190 to 1442 ± 1146 nM was found ($P < 0.02$). The computerized analysis of the DRCs did not reveal significant changes in the Hill coefficients in this group (6.5 ± 3.6 vs 6.5 ± 4.0).

Synthesis of TxB_2 and TxB_3 by thrombin-stimulated platelets

In group II, the synthesis of TxB_2 and TxB_3 was monitored after stimulation of the platelets with 5 U/mL thrombin (Fig. 4). The application of EPA and DHA resulted in only a modest decrease in TxB_2 formation. Displacement of arachidonic acid by EPA within the membrane phospholipid pool lead to a continuous increase in TxB_3 formation by the platelets throughout the observation period.

DISCUSSION

Supplementation with high doses of DHA and EPA causes only a modest inhibition of platelet capacity to synthesize TxA_2 and results in the

Table 1. Bleeding time and platelet aggregation studies before and 6 weeks after dietary supplementation with fish oils containing different amounts of EPA and DHA

	Group I (4.5 g EPA/3.35 g DHA)		Group II (3.5 g EPA/6.4 g DHA)	
	Before	After	Before	After
Bleeding time (min)	7.5 ± 1.5	8.2 ± 1.6	7.0 ± 1.5	8.0 ± 1.7
Collagen (2.5 μg/mL)-stimulated platelet aggregation (reaction time; sec)	52 ± 6	56 ± 5	42 ± 19	54 ± 17
IC_{50} of iloprost on ADP-stimulated platelet aggregation (pg/mL)	237 ± 71	227 ± 149	254 ± 71	162 ± 46*
Platelet aggregation induced by 500 nM U 46619 (% transmission)	23 ± 10	22 ± 8	29 ± 12	3 ± 3†
ADP (0.6 μM)-induced aggregation (% transmission)	14 ± 5	16 ± 3	13 ± 4	17 ± 11

Group I: EPA/DHA ratio 1.33; group II: EPA/DHA ratio 0.54.
* $P < 0.05$; † $P < 0.01$ as compared to pretreatment value.
Data are given as means ± SD.

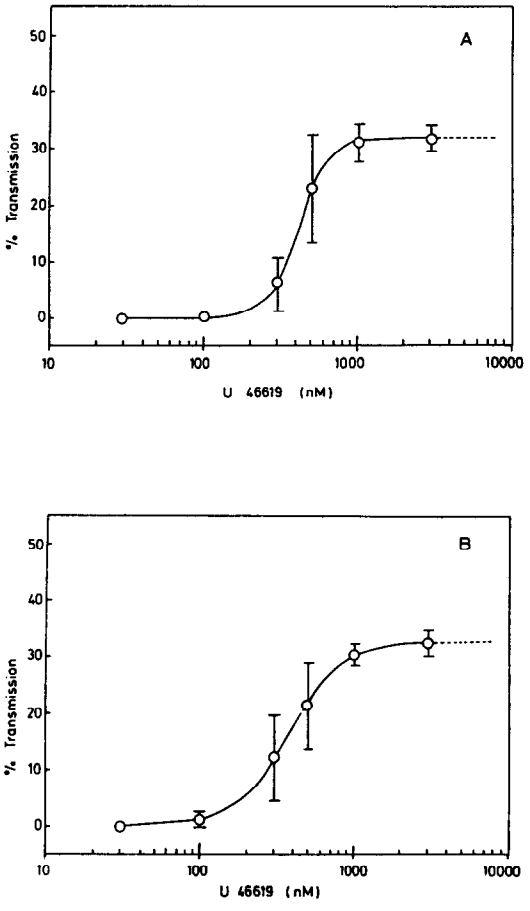

Fig. 2. DRCs of U 46619-induced platelet aggregation of group I before (A) and after (B) a 6 week diet containing 4.5 g EPA and 3.35 g DHA daily. The slopes of the DRCs were significantly different (6.2 ± 3.0 vs 3.3 ± 0.6; $P < 0.02$). Symbols represent means ± SEM.

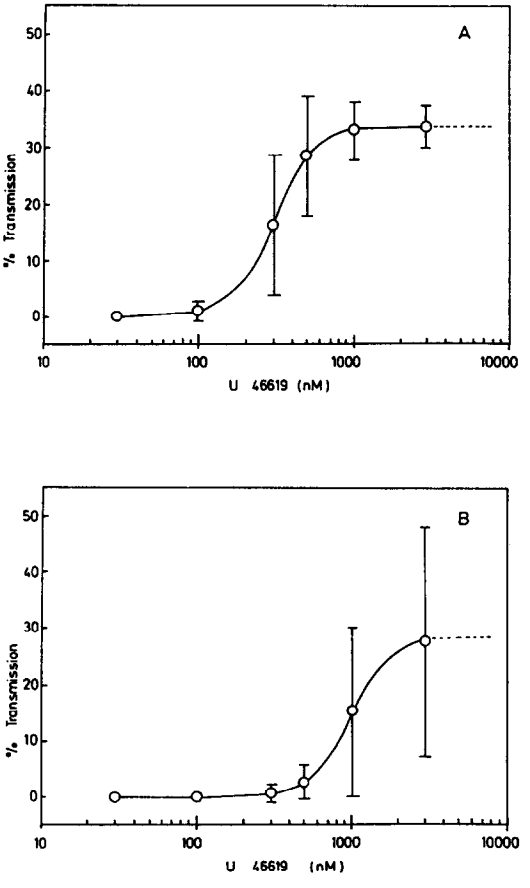

Fig. 3. DRCs of U 46619-induced platelet aggregation of group II before (A) and after (B) a 6 week diet containing 3.5 g EPA and 6.4 g DHA daily. The EC_{50} was significantly different after dietary supplementation (364 ± 190 nM vs 1442 ± 1146 nM; $P < 0.02$). Symbols represent means ± SEM.

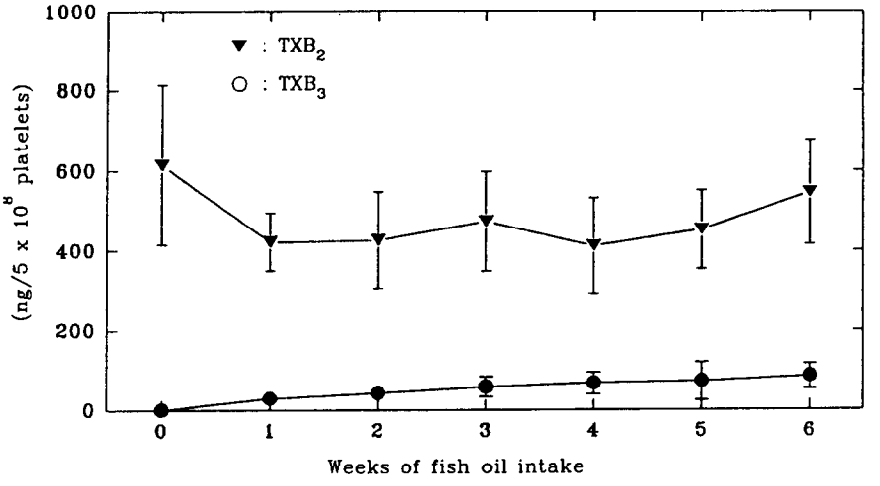

Fig. 4. Synthesis of TxB_2 and TxB_3 by thrombin (5 U/mL)-stimulated platelets during the application of fish oil in group II. Symbols represent means ± SD.

formation of EPA-derived TxA_3. Usually, more than 90% inhibition of the TxA_2 generating system is required to obtain significant inhibition of *in vivo* TxA_2 synthesis [10]. Thus, the modest reductions in platelet capacity to form thromboxane found by us and others [11] hardly explain the antithrombotic potential of marine fish oils. In this study we have shown that platelet responsiveness to the endoperoxide analogue U 46619 can be differentially modified by dietary supplementation with fish oil preparations depending on their EPA and DHA contents. A reduction of the platelet responsiveness to U 46619, as reflected by a considerable shift of the DRCs to the right, was only observed in the "high dose DHA" group (EPA/DHA ratio: 0.54). On the other hand, in the "high dose EPA" group (EPA/DHA ratio: 1.33), only a reduced slope of the DRCs could be observed. Because our investigations were done in platelets not pretreated with a cyclooxygenase inhibitor, we cannot exclude the possibility that the reduced cooperative effect, as reflected by a decrease in the slope of the DRCs in group I, results from a moderate decrease in platelet TxA_2 synthesis. However, these alterations in platelet TxA_2 generation cannot explain the considerable shift of the DRCs of U 46619-induced platelet aggregation during high dose DHA. This was confirmed by us in a further series of experiments, demonstrating that pretreatment of PRP (N = 4) with indomethacin (10 μM) does not significantly affect the EC_{50} of U 46619-induced platelet aggregation (EC_{50} 450 ± 110 vs 440 ± 175 nM).

The reason for the reduced platelet responsiveness to the endoperoxide analogue and thromboxane mimetic found in the "high dose DHA group" remains open to question. Plasma DHA and EPA did not differ in the two groups. This largely precludes the possibility that these fatty acids might have specifically competed with different potencies at the prostaglandin-endoperoxide (PGH_2)/TxA_2 receptor level [5]. Our analyses of the lipid composition of erythrocyte ghosts clearly demonstrate that EPA and DHA are readily partitioned into the membrane lipid core. During long term application of fish oils EPA and DHA increase approximately in parallel in phospholipids of red cells and platelets [11]. The resulting changes in the membrane lipid composition have been shown to alter membrane microviscosity and to modify the accessability of ligands to their corresponding binding sites [12, 13]. This has been demonstrated previously by us for the platelet prostacyclin (PGI_2) receptor, which behaves as a syndromic protein: an increase in membrane microviscosity by the incorporation of cholesterol induced a decrease, whereas cis-unsaturated fatty acids or membrane-fluidizing compounds (e.g. dibucaine, pentoxifylline) caused enhanced receptor surface expression [14, 15]. Platelet PGH_2/TxA_2 receptors are apparently regulated in an opposite fashion: *in vitro* studies with cholesterol-enriched platelet membranes demonstrated a significant increase in specific binding of the TxA_2/PGH_2 receptor antagonist [^{3}H]SQ 29548 [16]. Our findings would therefore favourably converge with the concept that PGH_2/TxA_2 receptors behave as antidromic proteins. In this case, one would expect that fluidizing agents like DHA decrease the accessibility of U 46619 to its corresponding binding sites. This hypothesis has been corroborated by direct [^{3}H]U 46619 binding studies. The enrichment of platelet membranes with DHA *in vitro* caused a considerable decrease in the binding affinity (4.8-fold decrease) whereas EPA caused only a minor effect [17]. Since these experiments were done in platelet preparations free of non-esterified fatty acids, direct competition at the receptor level can largely be excluded. Therefore, intercalation of highly unsaturated acyl chains (six double bounds) into the membrane lipid core and the resulting changes in the physical membrane properties appear to be a reasonable explanation. Moreover, recent studies on the effects of EPA and

DHA on the binding of [^{3}H]U 46619 and the PGH_2/TxA_2 receptor antagonist [^{3}H]SQ 29548 to CHAPS-solubilized PGH_2/TxA_2 receptors point towards the possibility that these fatty acids may also modify receptor function by interacting directly with the functional site of the receptor or by altering its conformation by associating with its hydrophobic domains [18].

The biological significance of TxA_2 in the pathogenesis of thromboembolic cardiovascular complications has been proven by a series of investigations on *in vivo* TxA_2 formation (e.g. measurements of urinary dinor-TXB_2 excretion) and by recent studies on the efficacy of low dose aspirin [19]. When platelets are stimulated by weak agonists, PGH_2/TxA_2 serves as a potent signal amplifier. Over the past several years, modulation of the responsiveness of the target tissues to PGH_2/TxA_2 (e.g. by thromboxane receptor antagonists) has attracted much attention [20]. Our present study suggests that similar effects can be achieved by modulation of membrane TxA_2 receptor expression. Thus, the application of marine fish oils with a high DHA/EPA ratio may evolve as a promising adjunct for the prophylaxis of thromboembolic vascular disease.

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