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Fish Oil Fatty Acids and Human Platelets: Dose-Dependent Decrease in Dienoic and Increase in Trienoic Thromboxane Generation

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ABSTRACT. Dietary enrichment of membrane phospholipids with n-3 (fish-oil-derived) fatty acids has attracted attention as a putative therapeutic regimen for suppression of inflammatory and coagulatory events. Use of n-3 fatty-acid-enriched lipid infusions for parenteral nutrition results in micromolar concentrations of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DCHA) in the plasma-free fatty acid fraction. We investigated the influence of free EPA and DCHA on platelet thromboxane (Tx) A_2 and A_3 formation by using a recently developed high performance liquid chromatography–ELISA technique for separate quantification of the stable hydrolysis products TxB_2 and TxB_3. Washed human thrombocytes were incubated with free arachidonic acid (AA; 1 μM), A23187 (0.1 μM) or thrombin (5 U/mL) for stimulation; all regimens provoked large quantities of TxA_2 in the absence of TxA_3. Simultaneous admixture of free EPA or free DCHA to the incubation medium (concentration range, 0.01–50 μM) largely suppressed platelet TxA_2 generation in response to all stimuli used in a dose-dependent manner. The effective concentration with 50% influence of arachidonic acid was 4.2 μM, whereas the inhibitory concentration with 50% effect of EPA and DCHA were both in the same order of magnitude but differed with the nature of the agonist (0.2–7 μM). Platelet (co-)incubation with EPA, but not DCHA, provoked dose-dependent synthesis of n-3-lipid-derived thromboxane: kinetics of formation and absolute quantities of TxA_3 approximated 20% of the respective TxA_2 data upon stimulation with AA. Both EPA and DCHA dose-dependently suppressed U46619-provoked platelet aggregation. We conclude that EPA and DCHA are potent competitive inhibitors of TxA_2 generation by intact platelets, with EPA acting as poor substrate and DCHA being no substrate for the cyclooxygenase/thromboxane synthase complex. Enrichment of the plasma-free fatty acid fraction with n-3 lipids may offer a therapeutic regimen to suppress the synthesis of the potent proaggregatory and vasoconstrictory agent TxA_2. BIOCHEM PHARMACOL 52;8:1211–1217, 1996.

KEY WORDS. platelets; thromboxane A_2 and A_3; arachidonic acid; eicosapentaenoic acid; docosahexaenoic acid; post-HPLC-ELISA

Dietary enrichment of membrane phospholipid pools with n-3 fatty acids, in particular EPA‡ (20:5 n-3), has attracted attention as a putative therapeutic regimen for suppression of inflammatory and coagulatory events [1, 2]. Prolonged bleeding times have been observed in Eskimo populations using fish-oil-rich diets [3]. This finding has been related to the impact of n-3 fatty acids on the platelet cyclooxygenase pathway, and *in vivo* production of trienoic Tx was demonstrated in humans with dietary intake of substantial quantities of fish oil [4–6]. EPA was found to be less rapidly converted via the platelet cyclooxygenase/Tx synthase complex to TxA_3 than AA to TxA_2 [7]. The proaggregatory potency of TxA_3 ranges far below that of TxA_2 [7], and the trienoic Tx possesses only very limited or no vasoconstrictor potency [8]. Other suggested mechanisms of a platelet inhibitory effect of TxA_3 are (1) increased cAMP production, (2) suppression of phospholipase A_2 activity and (3) inhibition of thrombocyte signal transduction pathways [7, 9–12]. Moreover, some direct inhibitory effect of EPA on the TxA_2/prostaglandin H_2 receptor function has been reported [13].

In addition to being incorporated into membrane phospholipid pools, free extracellular AA may directly affect cell activation and mediator generation. Micromolar concentrations of nonesterified AA have been detected at sites of inflammatory events [14–16]. For organs composed of different cell types with inflammatory potencies such as the lung, evidence for intercellular exchange of free AA, which contributes to transcellular eicosanoid synthesis and is sus-

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‡ *Abbreviations:* AA, arachidonic acid; B/B_0, rate of antibody saturation; DCHA, docosahexaenoic acid; DMSO, dimethylsulfoxide; EC_{50}, effective concentration with 50% influence compared with maximum effect; EPA, eicosapentaenoic acid; IC_{50}, inhibitory concentration with 50% effect compared with no inhibition; PBS, phosphate-buffered saline; RP-HPLC, reversed-phase high performance liquid chromatography; TBA, tetrabutyl ammonium dihydrogenphosphate; Tx, thromboxane; GC/MS, gas chromatography/mass spectrometry.

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ceptible to modulations of extracellular free fatty acid contents, has been presented [17–21]. Moreover, infusion of lipid emulsions for parenteral nutrition was noted to cause a marked increase in plasma-free fatty acid levels: the lipid aggregates activate the endothelial lipoprotein lipase, including a translocation of this enzyme from its cellular binding sites into the vascular compartment, with a resultant increase in fatty acids due to escape from local cellular uptake mechanisms [22–25]. Free plasma AA concentrations surpassing 10 μM have been measured under these conditions [24–26].

Over the past few years, n-3 fatty-acid enriched (fish-oil-derived) lipid infusions have been developed for use under clinical conditions in an attempt to shift the AA/EPA ratio toward predominance of the latter lipid mediator precursor and thereby to combine parenteral nutrition and pharmacological intervention [27–31]. Plasma levels of free EPA were found to rise manifold upon use of n-3 lipid emulsions: concentrations of >10 μM were measured, which considerably surpassed those of free AA [26]. Even higher plasma concentrations of free DCHA (22:6 n-3) were reported from experimental studies with fish oil infusions in rats [24].

In the present study, we investigated the influence of free EPA and DCHA on platelet TxA_2 and TxA_3 formation and aggregation. We used a recently developed HPLC/ELISA technique that, in contrast with the very laborious GC/MS procedures, renders the analysis of series of experiments feasible. At micromolar concentrations, both fatty acids effected dose-dependent inhibition of platelet TxA_2 generation in response to exogenous AA and to receptor- and nonreceptor-operated release of endogenous AA. In parallel, substantial quantities of n-3–fatty-acid-derived thromboxanes were detected upon use of EPA, but not DCHA. Both free fatty acids decreased U46619-mediated platelet aggregation in the same dose range. We conclude that, at concentrations that may increase with use of n-3 lipid infusions, both alternate precursor fatty acids are potent inhibitors of platelet TxA_2 generation.

MATERIALS AND METHODS

Reagents

AA, EPA and DCHA were purchased from Sigma (Munich, Germany) at the highest purity available (99%, as checked by GC). All free fatty acids were initially aliquoted and stored at −20°C under nitrogen, and a fresh portion was used for each experiment. TxB_3 was a gift from C. O. Meese (Margarete Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany), and the monoclonal antibody against TxB_2 was generously supplied by K. Brune and J. Mollenhauer (Institute of Pharmacology, Erlangen, Germany). TxB_2 and bovine serum albumin (BSA) were delivered by Paesel and Lorei GmbH (Frankfurt/Main, Germany). ODS-Hypersil (3 μm) was obtained from Shandon GmbH (Frankfurt/Main, Germany). A23187 was received from Calbiochem (Giessen, Germany) and human thrombin (specific activity, 120 U/mg) from Boehringer Mannheim (Mannheim, Germany). The ionic pair reagent TBA (1 M solution) was purchased from Aldrich Chemie (Steinheim, Germany). Solid-phase extraction columns (C18; 3 cm^3) were obtained from Varian (Frankfurt/Main, Germany). Microtiter plates (immunoplate Maxisorp F96 with certificate) were supplied by Nunc (Wiesbaden, Germany). Biotinylated sheep anti-mouse antibody was purchased from Amersham Buchler GmbH (Braunschweig, Germany). Avidin biotin–horseradish peroxidase (AB complex) was from Dakopatts GmbH (Hamburg, Germany). 2.2′-Azino-di-[3-ethyl-benzthiazolinsulfonat(6)] (ABTS) was obtained from Boehringer GmbH (Mannheim, Germany). α-Tocopherol and Tween 20 were purchased from Sigma Chemie. High-purity HPLC and HPLC-column-packing solvents were obtained from Baker (Deventer, The Netherlands). All other biochemicals were obtained from Merck AG (Darmstadt, Germany).

Preparation of Washed Human Platelet Suspensions

Blood from healthy drug-free volunteers was collected in 3 mL plastic tubes containing 700 μL of a 7.5% EDTA solution. After centrifugation at 200*g* for 10 min, the platelet-rich plasma was decanted and spun again (1500*g*, 10 min). Pelleted platelets were washed with isotonic PBS (pH 7.4), recentrifuged and resuspended in Tris buffer (20 mM; pH 7.4; 132.8 mM NaCl, 4.3 mM KCl, 1.1 mM KH_2PO_4, 2.4 mM $CaCl_2$ and 1.3 mM $MgPO_4$; 240 mg/100 mL glucose). Platelet count was adjusted to 10^8/mL, and 1-mL aliquots were employed for *in vitro* stimulation. Recovery studies revealed that 86 ± 4% (mean ± sem, n = 15) of the whole blood platelets were contained in the washed platelet preparation.

Stimulation of Platelets

Platelets were incubated with different quantities of AA, EPA, DCHA, thrombin, A23187 or combinations of stimuli at 37°C. The vehicle for thrombin was PBS, DMSO for the fatty acids and A23187; the maximum DMSO concentration in the incubation medium never surpassed 2% (vol/vol), which was shown to exert no effect on platelet Tx generation and detection in the following control experiments: (1) DMSO up to 2% (vol/vol) did not provoke TxB_2 release in nonstimulated platelets, and (2) TxB_2 release from washed platelets (10^8/mL) exposed to 5 μM A23187 was 327.2 ± 18.38, 321.85 ± 7.61 and 308.78 ± 16.33 ng in the presence of 0.2, 0.5 and 2% (vol/vol) DMSO, respectively (mean ± SEM, n = 4 each). Incubation was terminated after different time periods by adding 500 μL TBA (1 M, pH 7.0) and 4 mL ice-cold Tris buffer. The supernatant was frozen (−80°C) and centrifuged at 1500*g* for 10 min after thawing prior to analysis.

Platelet Aggregation

Washed platelet suspensions with a platelet count of 10^8/mL were incubated in 0.5-mL portions in aggregometer

cuvettes with constant stirring (1000 rpm) at 37°C. After simultaneous addition of 1 μM U46619 and the respective fatty acid in DMSO (10 μL total volume), aggregation was monitored by light transmission in a Chronolog aggregometer. Responses after 5 min were normalised to the maximal extent of aggregation in controls incubated only with U46619.

Measurement of Thromboxane

TxA_2 and TxA_3 were measured as their stable hydrolysis products TxB_2 and TxB_3. The analytes were extracted from the buffer solution by solid-phase extraction, subjected to RP-HPLC separation and quantified by post-HPLC-ELISA as detailed elsewhere [32]. To avoid chemical decomposition of the analytes, an ionic pair reagent, an antioxidant and high buffer strength were used during critical steps of the analytical procedure. Briefly, buffer samples including zero controls and controls with known amounts of Txs were supplied with the ionic pair reagent TBA and submitted to solid-phase extraction with preconditioned C18 columns. This procedure was followed by elution with acetone/acetonitrile (50/50, v/v) into microreaction vessels provided with 10-fold concentrated PBS (pH 7.4) and α-tocopherol for protection of prostanoids in the following freeze-drying procedure in vacuum. Dried eluates were extracted with acetonitrile and submitted to RP-HPLC separation (C18 column: length 2 * 15 cm, 3-μm particles; mobile phase: 28/72 (v/v) acetonitrile/water, 0.5 mM TBA, pH 7.3, flow rate 1 mL/min) to separate 2- and 3-series Tx. Eluate fractions of 0.3 mL corresponding to the known retention times of TxB_2 and TxB_3 were collected, freeze dried, redissolved in water and subjected to ELISA. A monoclonal mouse antibody against TxB_2 with established cross reactivity with TxB_3 (74% at 50% B/B_0) was used.

RESULTS

Incubation of platelets with free AA caused dose-dependent release of marked quantities of TxB_2 in the absence of any TxB_3 liberation (Fig. 1), which is comparable to other findings [33]. Analysis of kinetics showed a plateau of the TxB_2 release reaction within 3–5 min after admixture of the fatty acid (Fig. 2). In a corresponding dose range, free exogenous EPA provoked predominant liberation of TxB_3 in connection with some minor (<15%) TxB_2 formation. At the highest EPA concentrations used (5 and 10 μM), the TxB_3 data detected approximately 20% of the TxB_2 quantities elicited by free AA in response to this precursor fatty acid. Relative to this reduced level of metabolite formation, the time course of EPA-induced TxB_3 formation, with plateauing of the response within 5 min, corresponded to that of dienoic Tx synthesis provoked by AA (Fig. 2). Free exogenous DCHA did not induce any detectable liberation of TxB_2 or TxB_3. Moreover, submitting the supernatant of DCHA-stimulated platelets to direct ELISA with the monoclonal anti-Tx antibody did not detect any "thromboxane-like" immunoreactivity.

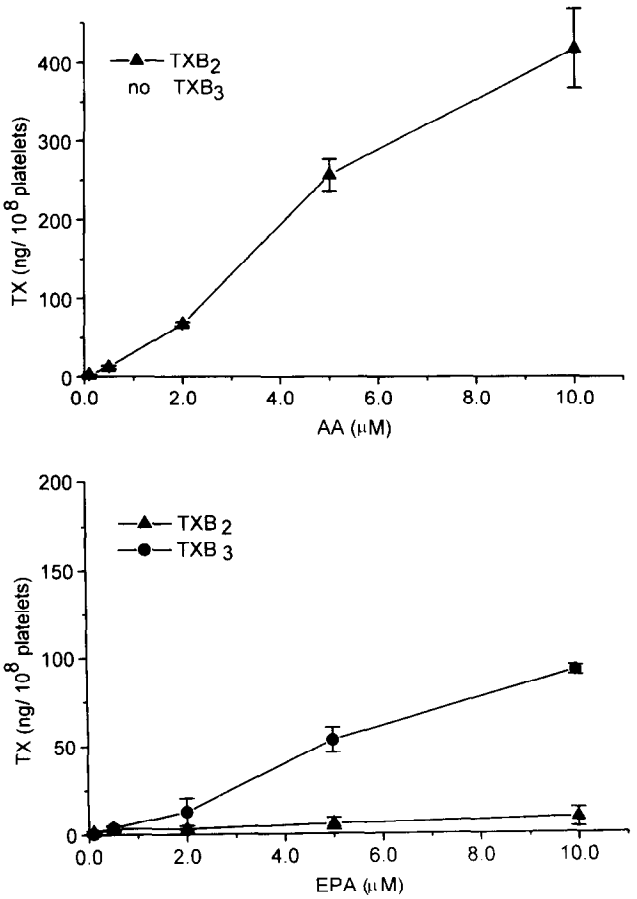

FIG. 1. Dose dependency of TxB_2 and TxB_3 liberation from human platelets incubated with free AA (top) and free EPA (bottom). The incubation period was 15 min for both fatty acids. Each data point represents the mean ± SEM of three independent experiments; error bars are not given when too small.

The liberation of TxB_2 in response to 1 μM free AA was dose-dependently inhibited by the simultaneous admixture of free EPA (effective concentrations, >1 μM) to the incubation medium (Fig. 3). At 5 μM EPA, AA-elicited TxB_2 release was approximately halved; at 10 μM, EPA was reduced to less than one-third of the control response. Concomitantly, substantial quantities of TxB_3 appeared, which ranged slightly below TxB_3 levels provoked by EPA in the absence of AA. Even stronger inhibitory efficacy on AA-induced TxB_2 generation was exerted by free DCHA: only 0.1 μM of this alternate precursor fatty acid sufficed to reduce TxB_2 data in response to 1 μM AA to ~60%; and at 10 μM DCHA, the release reaction was suppressed to <10%. Again, no TxB_3 was detectable in the supernatant of AA- and DCHA-incubated platelets.

Similar inhibitory efficacies of EPA and DCHA were noted with respect to thrombin- and A23187-elicited TxB_2 liberation (Figs. 4, 5). In the absence of either precursor fatty acid, TxB_2 levels in response to 5 U/mL thrombin and

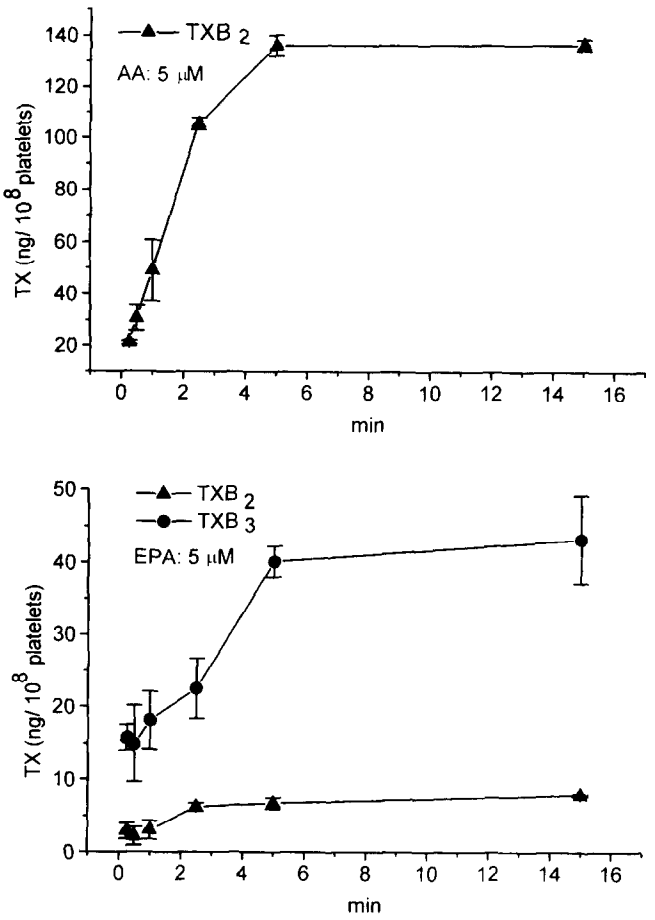

FIG. 2. Time course of TxB_2 and TxB_3 liberation in response to free AA (top) and free EPA (bottom) challenge. Both fatty acids were used at a concentration of 5 μM. Each data point represents the mean ± SEM of three independent experiments; error bars are not given when falling into the symbol.

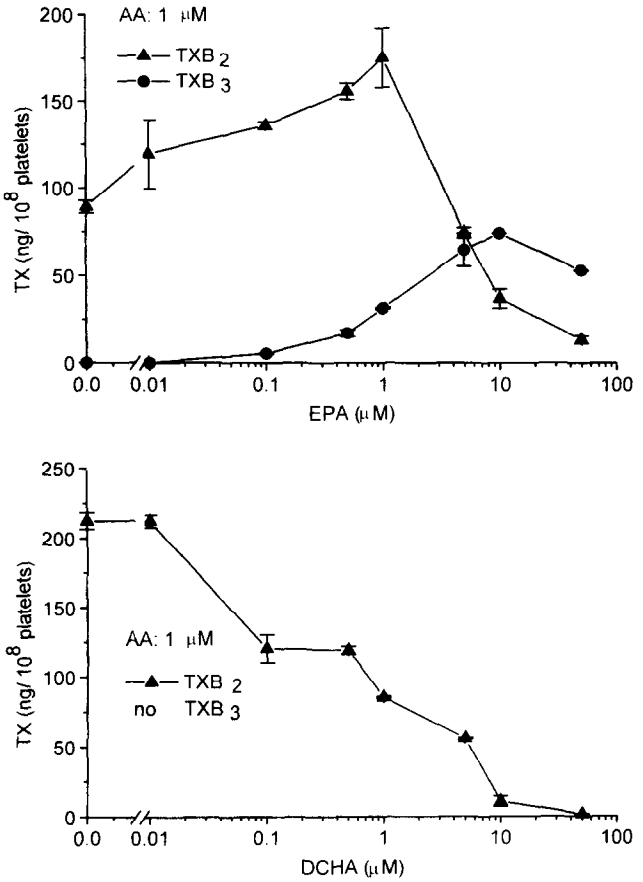

FIG. 3. Dose-dependent influence of free EPA (top) and DCHA (bottom) on AA-induced Tx release. AA was used at 1 μM throughout, and combined administration with EPA or DCHA was performed simultaneously. Incubation was terminated after 15 min in all experiments, and supernatants were analyzed for TxB_2 and TxB_3. Each data point represents the mean ± SEM of three independent experiments; error bars are not given when falling into the symbol.

0.1 μM A23187 approximated 50% and 70%, respectively, of the quantities of this metabolite provoked by maximum AA challenge (10 μM). At concentrations of ⩾1 μM, simultaneously applied free EPA suppressed thrombin- and A23187-elicited TxB_2 formation in a dose-dependent manner, with maximum reduction to <15% of control responses. This reduction was accompanied by dose-dependent TxB_3 liberation, the total quantities of which approximated those in response to administration of EPA alone. Inhibition of ionophore-provoked TxA_2 synthesis by DCHA commenced at 0.1 μM of the alternate precursor fatty acid and was nearly complete at 10–50 μM DCHA. The dose-inhibition curve on thrombin-elicited TxB_2 release commenced at >1 μM DCHA. No TxB_3 generation was measurable in platelets subjected to dual thrombin/DCHA and A23187/DCHA stimulation.

In a concentration range of 1–50 μM, both EPA and DCHA dose-dependently inhibited U46619-induced platelet aggregation, with DCHA displaying higher potency than EPA (Fig. 6).

DISCUSSION

The present study used a combined HPLC/ELISA technique [32], which allows a clear separation of TxB_2 from TxB_3 without the necessity of offering labeled exogenous fatty acids and/or of using prelabeled cells. Washed platelet suspensions were employed to circumvent interference of plasma-free fatty acid fractions, and stimulation was performed by admixture of exogenous AA and liberation of endogenous AA via calcium-ionophore application and ligand-operated cell activation. Platelet TxA_2 synthesis was found to be dose-dependently inhibited by the n-3 precursor fatty acids EPA and DCHA, the latter being the more potent suppressor agent. The inhibitory efficacy of EPA, but not that of DCHA, was accompanied by the appearance of n-3–fatty-acid-derived Tx generation.

The inhibitory effect of EPA on AA-induced TxA_2 synthesis in human platelets corresponds to previous results. Needleman *et al.* [7] demonstrated that the conversion of labeled arachidonate to TxB_2 was dose-dependently sup-

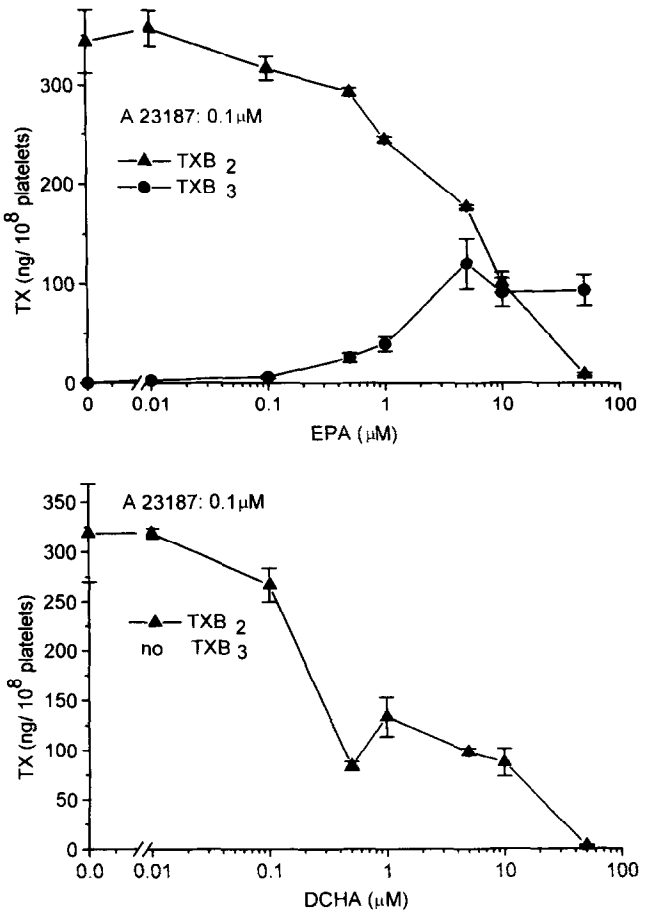

FIG. 4. Dose-dependent influence of free EPA (top) and DCHA (bottom) on thrombin-induced Tx release. Thrombin was used at 5 U/mL throughout, and combined administration with EPA or DCHA was performed simultaneously. Incubation was terminated after 15 min in all experiments, and supernatants were analyzed for TxB_2 and TxB_3. Each data point represents the mean ± SEM of three independent experiments; error bars are not given when falling into the symbol.

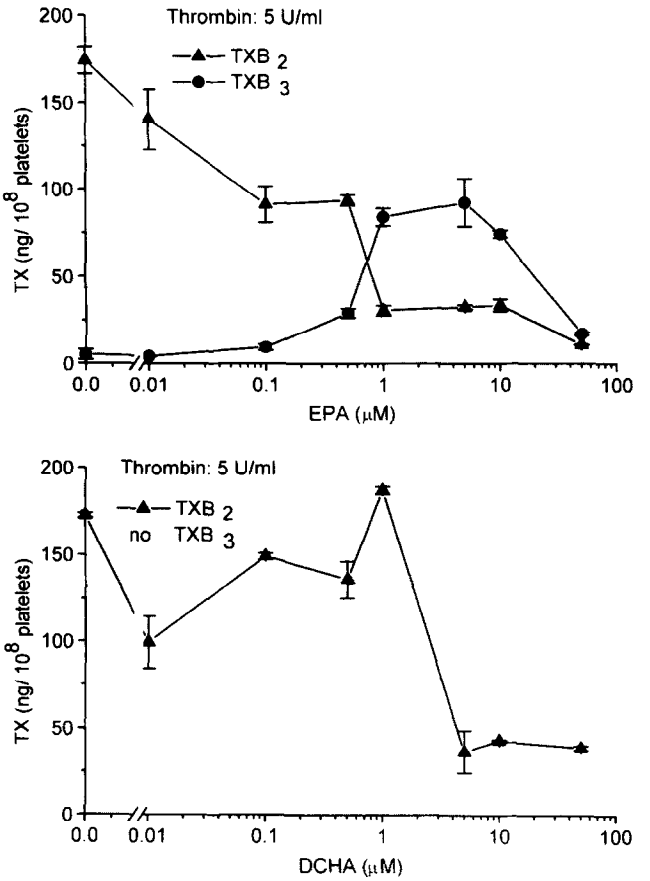

FIG. 5. Dose-dependent influence of free EPA (top) and DCHA (bottom) on A23187-induced Tx release. A23187 was used at 0.1 μM throughout, and combined administration with EPA or DCHA was performed simultaneously. Incubation was terminated after 15 min in all experiments, and supernatants were analyzed for TxB_2 and TxB_3. Each data point represents the mean ± SEM of three independent experiments; error bars are not given when falling into the symbol.

pressed by the simultaneous application of EPA, and EPA itself was found to be metabolized via platelet cyclooxygenase, although to a markedly lower extent than AA, in those experiments. These features characterize EPA as a competitive inhibitor of, but poorer substrate than, AA with regard to the cyclooxygenase/Tx synthase in intact thrombocytes. The present investigation extends these findings by showing that, similar to AA, A23187- and thrombin-induced TxA_2 synthesis is inhibited by exogenous EPA with virtually superimposable dose-inhibition characteristics. This finding suggests that free EPA has ready access to the platelet prostaglandin H synthase complex [7, 34], with kinetics at least comparable to A23187- and thrombin-induced signal transduction events because the current protocol used simultaneous application of both stimulus and EPA. Moreover, the enzymatic conversion of AA originating from endogenous (phospho)-lipid pools is apparently similarly affected by exogenous EPA and exogenous AA. Interestingly, application of EPA as sole agent provoked minor quantities of TxB_2 in addition to the predominant TxB_3. This observation suggests that the n-3 precursor fatty acid might substitute for AA in ongoing membrane remodeling processes, thereby resulting in some limited availability of free endogenous AA for oxygenation pathways. Incorporation of EPA into thrombocyte phospholipids has previously been demonstrated [7]. This phenomenon might explain why EPA concentrations in the threshold range (0.5–1 μM) even provoked an increase in TxB_2 release when applied simultaneously with the different stimuli (AA, thrombin, A2387), whereas the suppressive effect on AA cyclooxygenase metabolism with additional increase in the EPA dosage became predominant and TxA_2 generation declined sharply.

Free exogenous DCHA suppressed thrombin-elicited TxA_2 synthesis with dose-inhibition characteristics comparable to those of EPA. With respect to AA- and A23187-evoked TxB_2 liberation, DCHA possessed even higher inhibitory strength: only 0.1 μM of the n-3 fatty acid sufficed to reduce the formation of dienoic Tx by approximately

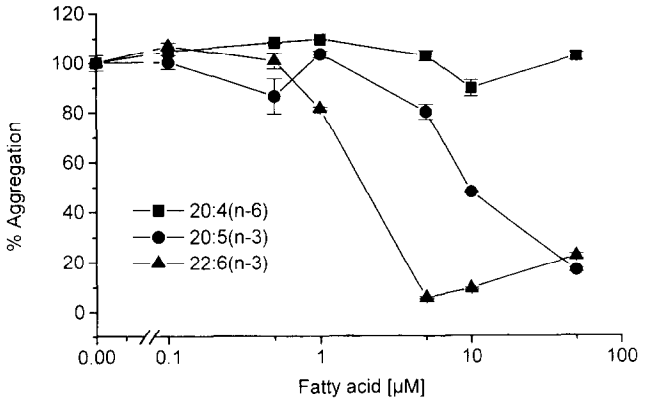

FIG. 6. Dose-dependent influence of free EPA and DCHA on U46619-mediated platelet aggregation. Maximal aggregation was achieved by 1 μM U46619 in all experiments. Data were obtained from change of light transmission and normalized to the maximal response. Each data point represents the mean ± SEM of three independent experiments; error bars are not given when falling into the symbol.

one-third. This finding compares favorably with the previous notion that DCHA is a strong competitive inhibitor of the enzymatic conversion of AA by isolated ram seminal vesicle and platelet cyclooxygenase [9, 11, 35]. GC/MS analysis in those experiments did not detect significant metabolic conversion of DCHA by the prostaglandin H synthase complex. Here, no Tx immunoreactivity was detectable in the experiments where only DCHA was applied. This finding indicates that the 22:6 fatty acid did not exert its effect through secondary metabolic conversion to EPA, with subsequent metabolism via the prostaglandin H synthase complex. We cannot fully exclude direct metabolic conversion of DCHA by the platelet cyclooxygenase. However, as the monoclonal antibody presently employed reacts with dienoic and trienoic Tx with similar efficacy and shows no reaction with the other primary prostaglandins [32], the recognized antigenic moiety may be the "TxB_X-ring" moiety, and some putative DCHA-derived Tx might also be expected to be recognized.

Both EPA and DCHA inhibited the stable Tx analogue U46619-mediated aggregation of washed platelets. This effect occurred in the same concentration range of free fatty acids in which interference with TxA_2 synthesis was noted. In the case of EPA, it is tempting to speculate that the increase in TxA_3 might substantially contribute to this antiaggregatory effect via competitive interaction with the stable TxA_2 analogue at the TxA_2/PGH_2 receptor. However, such an interpretation does apparently not hold true for DCHA, under the assumption that no Tx was generated from this precursor fatty acid. Alternatively, as previously suggested [13], EPA and in particular DCHA may directly interfere with the TxA_2/PGH_2 receptor, which would explain their inhibitory effects on U46619-elicited platelet aggregation.

We conclude that, at micromolar concentrations, the n-3 fatty acids EPA and DCHA are potent inhibitors of platelet TxA_2 generation in response to different stimuli. DCHA was revealed as an even more potent suppressor agent than EPA. The inhibitory efficacy of EPA, but not that of DCHA, was accompanied by the appearance of n-3–fatty-acid-derived Tx generation.

Previous studies in humans [36] have shown that dietary supplementation with either pure EPA or DCHA induced significant incorporation into plasma-free fatty acids and into phospholipids of plasma and platelets. In the case of DCHA, a partial retroconversion to EPA was detected. Both fatty acids reduced platelet aggregation in response to collagen, but only DCHA was able to reduce ADP-triggered aggregation. Because EPA levels in the platelets remained constant during DCHA intake, reduced platelet aggregation was not attributed to retroconversion to EPA and formation of inactive TxA_3. The mechanism of the effect was left open in this study. Formation of net $TxB_{2/3}$ in clotted whole blood was not changed, the relative amounts of n-6 and n-3 Tx being not determined. In support of our results, von Shacky and Weber [36] assigned a greater potency to DCHA than to EPA in reducing platelet aggregation, thus forming an important factor in the antithrombotic effect of fish oil rich in n-3 fatty acids. As our results suggest, DCHA acts like EPA via a diminished release of proaggregatory and vasoconstrictive TxA_2 but probably without formation of a related Tx-like metabolite.

Therapeutic use of n-3 lipid infusions by effecting availability of these alternate lipid precursors in the plasma-free fatty acid fraction even more than dietary supplementation may thus be anticipated to inhibit liberation and action of TxA_2 from platelet sources, thus interfering with the vasoconstrictive and proaggregatory consequences related to this potent lipid mediator.

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