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Inhibition of Prostaglandin H Synthase and Activation of 12-Lipoxygenase by 8,11,14,17-Eicosatetraenoic Acid in Human Endothelial Cells and Platelets

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ABSTRACT. The effects of the marine fatty acid 20:4n-3, an isomer of arachidonic acid (20:4n-6), have been compared to that of 20:5n-3 on 20:4n-6 oxygenation in human platelets and endothelial cells. In platelets, 20:4n-3 added along with 20:4n-6 was as potent as 20:5n-3 in inhibiting prostaglandin H synthase (PGH synthase) activity. From 2.5- to 10 μM of 20:4n-6, the synthesis of thromboxane B_2 and 12-hydroxy-5,8,10-heptadecatrienoic acid, reflecting the PGH/thromboxane synthase activity, was lowered by 5 and 10 μM of both fatty acids. In contrast, 20:4n-3, but not 20:5n-3, strongly stimulated the lipoxygenase activity at each concentration of 20:4n-6 used whatever the amount of 20:4n-3 added. The effects of both n-3 polyunsaturated fatty acids on endothelial cell PGH/prostacyclin synthases were compared after 2- and 24-hr incubation with the cells, leading to moderate (2 hr) and high (24 hr) concentrations of these fatty acids in membrane phospholipids. The incorporation of 20:4n-3 and 20:5n-3 occurred mostly in phosphatidylcholine and phosphatidylethanolamine and did not alter the 20:4n-6 level of phospholipid classes after 2-hr supplementation, whereas it was drastically decreased after 24 hr. The synthesis of prostacyclin obtained after cell stimulation by 0.1 U/mL thrombin was unaffected by the fatty acid modifications induced after 2-hr supplementation, whereas it was strongly depressed after 24 hr. It was concluded that 20:4n-3 is not an agonist for platelet activation, despite its close structural analogy with 20:4n-6, and is as potent as 20:5n-3 in inhibiting PGH synthase activities, showing that the double bond at C5 is not necessary for inhibition. In contrast, the oxygenation of 20:4n-6 by 12-lipoxygenase was stimulated by 20:4n-3 but not by 20:5n-3, which might be related to the efficient oxygenation of 20:4n-3 by this enzyme compared with 20:5n-3. BIOCHEM PHARMACOL **57**;6:631–638, 1999.

KEY WORDS. polyunsaturated fatty acids; n-3 fatty acids; arachidonic acid; phospholipids; dioxygenases

Eicosapentaneoic acid (5,8,11,14,17-20:5, 20:5n-3) is one of the n-3 PUFA§ contained in marine lipids which appear to have a protective effect against coronary heart disease and thrombosis [1, 2]. These actions are the combined results of effects on plasma lipids and lipoproteins [3, 4] and on eicosanoid production, especially in blood platelets [5]. Hyperactivity of these cells, through the synthesis of thromboxane A_2 (TXA_2) and prostaglandins, has been related to the formation of atherogenic lesions [6]. In contrast, the vascular endothelium metabolizes arachidonic acid (5,8,11,14-20:4, 20:4n-6) into PGI_2, a potent inhibitor of platelet aggregation and vasodilator agent [7]. 20:5n-3 reduces platelet aggregation by its incorporation into phospholipids, where it decreases the 20:4n-6 content of the intracellular pools [8]. It also inhibits the conversion of 20:4n-6 into TXA_2 via PGH synthase/TX synthase, presumably by substrate analogy [9]. Similarly, 20:5n-3 reduces the capacity of endothelium to produce PGI_2 by replacing 20:4n-6 in EC phospholipids and also by competing with 20:4n-6 for access to PGH synthase [10]. Marine lipids also contain 8,11,14,17-eicosatetraenoic acid (8,11,14,17-20:4, 20:4n-3) [11], a minor n-3 PUFA which is a position isomer of 20:4n-6. 20:4n-3 is not converted into prostanoids, although platelet cyclooxygenase produces 12-hydroxy-8,11,14-heptadecatrienoic acid, but it appears to be a fairly good substrate of 12-lipoxygenase [12]. It is not known whether the close structural analogy of 20:4n-3 with 20:4n-6 confers a modulatory effect on 20:4n-6 metabolism. In the present work, the effects of 20:4n-3 on 20:4n-6 oxygenation by the platelet and EC dioxygenases were compared to that of 20:5n-3.

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§ *Abbreviations:* EC, endothelial cells; HHT, heptadecatrienoic acid; HETE, hydroxyeicosatetraenoic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PGH, prostaglandin H; PGI_2, prostacyclin; PUFA, polyunsaturated fatty acids; and TX, thromboxane.

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MATERIALS AND METHODS

Purification of 20:4n-6

A preparation of fatty acid ethyl esters containing 20:4n-3 ethyl ester (27 mol%), 20:4n-6 ethyl ester (64 mol%), and 4,7,10,13,16,19-22:6 (22:6n-3) ethyl ester (9 mol%) was obtained from fish oil purification by preparative HPLC (Separex). Ethyl esters were further separated by HPLC using a 0.46 × 25 cm Merck ODS column and acetonitrile/H_2O (8/2, v/v) as a mobile phase, at a flow rate of 2 mL/min [13]. Ethyl esters were detected by monitoring at 210 nm and eluted at retention times of 25 min (22:6n-3 ethyl ester), 29 min (20:4n-3 ethyl ester), and 32 min (20:4n-6 ethyl ester). 20:4n-3 ethyl ester was collected and the free fatty acid was released by acid hydrolysis, according to Aveldano and Horrocks [14]. HCl (10 M) was added to the collected HPLC effluent to obtain a 0.5 M HCl solution in acetonitrile/H_2O (8/2, v/v), and the mixture was heated in screw-capped tubes for 45 min at 100° after flushing with N_2. The free fatty acid released was extracted by $CHCl_3$/H_2O and the organic phase, separated by centrifugation, was backwashed three times with H_2O. 20:4n-3 and 20:5n-3 (Sigma) were spotted on TLC plates and eluted with hexane/diethyl ether/acetic acid (60/40/1, v/v/v), as mobile phase. The fatty acids were extracted from the silica gel by diethyl ether, taken to dryness, and dissolved in ethanol prior to quantification by GC. An aliquot of both fatty acids was submitted to derivatization with diazomethane in presence of 17:0 and 19:0 methyl esters. The concentration of fatty acids was determined by GC analyses, relative to the known amount of added 17:0 and 19:0 [12]. 20:4n-3 was 99% pure and did not contain any trace of 20:4n-6.

Platelets

Platelets were prepared as previously described [15]. Blood from healthy volunteers who had not taken any medication for 10 days prior to donation was collected into a one-ninth volume of anticoagulant (citrate/phosphate/dextrose). Platelet-rich plasma, obtained by centrifugation of the blood at 100 *g* for 15 min, was acidified to pH 6.4 with citric acid and again centrifuged for 15 min at 900 *g*. The resulting platelet pellet was diluted in a Tyrode-Hepes buffer solution (137 mM NaCl, 2.6 mM KCl, 11.9 mM $NaHCO_3$, 0.46 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM Hepes, 5.5 mM dextrose; pH 7.35). To measure the PGH synthase and lipoxygenase activities, [1-^{14}C]-20:4n-6 (57 mCi/mmol, NEN) was incubated with 0.4 mL of platelet suspension (around 300,000 platelets/μL) magnetically stirred at 37° in a Chrono-Log dual aggregometer (Coulter) [16]. After 2 min of preincubation, 4, 2 or 1 nmol of [1-^{14}C]-20:4n-6 was added either in the presence of appropriate concentrations of 20:4n-3 or 20:5n-3 or with vehicle alone, for 4 min. In each assay, the substrates were added in 0.5% ethanol. Incubations were terminated by adding 1.2 ml ethanol and lipids were extracted by 2.4 mL of $CHCl_3$ containing butylated hydroxytoluene as an antioxidant (5×10^{-5} M final concentration). Oxygenated metabolites of labeled 20:4n-6 were measured after submitting lipid residues to TLC. A first elution with the hexane/ether/acetic acid mixture (60/40/1, v/v/v) separated 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) from HHT. A second elution with the ether/methanol/acetic acid mixture (90/1/2, v/v/v) separated TXB_2 from the other compounds. After each run, a quantitative chromatogram was performed with a Berthold TLC linear analyzer. Total radioactivity represented the initial amount of the labeled substrate, and the integrated peaks, calculated as the percentage of total radioactivity, could then be quantified in nanomoles according to the specific activity of 20:4n-6 [16]. Platelet aggregation was measured in isolated platelets using the turbidimetric method of Born [17] in a dual channel aggregometer. The extent of platelet aggregation was expressed by recording the changes in light transmission for 5 min after the addition of the fatty acids.

Endothelial Cells

Primary cultures of EC from fresh human umbilical veins were obtained according to Jaffe *et al.* [18] in 75-cm^2 flasks. Briefly, the perfused vein was washed with PBS and cells, detached by a collagenase treatment (0.1% in PBS, 12 min at 37°), were centrifuged and resuspended in culture medium. All culture glasswares were coated with 1% gelatin. Culture medium composed of M199 was supplemented with 20% of human AB serum, 100 U/mL streptomycin, 100 μg/mL penicillin, 0.25 μg/mL amphotericin B and with 80 μg/mL of an EC growth supplement (ECGS) prepared as described by Maciag *et al.* [19]. Growing EC were subcultured by brief treatment with 0.1% collagenase in PBS. These cells incubated in the culture medium without ECGS, typically plated at 50×10^3/cm^2 in 96-well plates for 6-oxo-prostaglandin $F_{1\alpha}$ measurements or in 60-mm diameter Petri dishes for lipid analysis, reached confluency after 3 days of culture. N-3 fatty acids (final concentration, 25 μM) were incubated under a nitrogen atmosphere for 12 hr at 37° in 0.2 μm filtered M199 containing 25 μM free fatty acid human albumin. Confluent EC monolayers, washed twice with M199, were incubated for 2 or 24 hr at 37° with each fatty acid solution. Control cells were incubated with M199 containing albumin alone. After 2 washes with M199, the cells in 96-well plates were stimulated for 10 min with 0.125 U/mL thrombin in M199. The basal formation of PGI_2 was measured by taking an aliquot of supernatants 5 min after placing the EC in fresh medium. Supernatants were collected and treated for radioimmunoassay of 6-oxo-$PGF_{1\alpha}$ [20], each experiment being carried out in duplicate. Cell monolayers in 60-mm diameter Petri dishes were rinsed with PBS and then scraped off twice with a rubber policeman in the presence of methanol/water (5/2, v/v), final volume 3.5 mL. They were harvested in 1 mL $CHCl_3$ containing 1,2-diheptadecanoyl-glycero-3-phosphocholine (8 μg) and 1,2-diheptadecanoyl-glycero-3-phosphoethanolamine (5 μg), as internal standards. Lipids

were extracted by adding 1.5 mL of $CHCl_3$ and 1.5 mL of 9‰ NaCl. The organic phase was removed and the upper phase was reextracted with 2.5 mL of $CHCl_3$. Each organic solvent used for extraction contained butylated hydroxytoluene 5×10^{-5} M as an antioxidant.

Lipid Analysis

EC phospholipid classes were purified by TLC migration of the lipid extracts on silica gel 60 plates, with $CHCl_3$/methanol/40% aqueous methylamine (60/20/5, v/v/v), as the mobile phase after the plate was predevelopped with $CHCl_3$/methanol (80/8, v/v) [12]. Lipid classes were scraped off the plate and treated with 5% H_2SO_4 in methanol for 90 min for fatty acid methyl ester preparation. Fatty acid methyl esters were quantified by GC with a DI200 Delsi chromatograph model equipped with a Ross injector and an SP-2380 capillary column (30 m × 0.32 mm, Supelco). The oven temperature was held at 145° for 5 min and raised to 215° at 2°/min. The absolute amount of fatty acid methyl esters was determined relative to the known amount of added 17:0 [13].

Statistical Analysis

Mean comparisons between control and fatty acid-treated cells were analyzed using analysis of variance and the Fisher's LSD post hoc test. *P* values lower than 0.05 were considered significant.

RESULTS

Effects of 20:4n-3 and 20:5n-3 on Platelet PGH/Tx Synthase and Lipoxygenase Activities

The effects of the two n-3 PUFA on 20:4n-6 metabolism were compared by measuring the formation of TXB_2 and HHT as a reflection of the PGH synthase/Tx synthase activities, and the synthesis of 12-HETE, the end-product of the lipoxygenase activity. The substrate and the n-3 PUFA were added exogenously to platelets, since the main purpose of this study was to assess the potential of the two fatty acids to interfere with 20:4n-6 conversion. Three increasing concentrations of 20:4n-6 were used to mimic the physiological situation where the amount of 20:4n-6 made available for oxygenases upon cell stimulation varies greatly according to the nature and concentration of the agonist. It can be seen in Fig. 1 that the synthesis of the three oxygenated metabolites increased linearly with the increasing concentrations of substrate. At 2.5 μM 20:4n-6, the PGH synthase/Tx synthase activities were not antagonized by adding 2.5 μM n-3 PUFA, but they were strongly inhibited by increasing the n-3 PUFA concentrations to 5 μM (Fig. 1A). At 5 μM of substrate, the formation of HHT and TXB_2 was weakly but significantly inhibited by 2.5 μM n-3 PUFA, this inhibition increasing with the n-3 PUFA concentration (Fig. 1B). When equal concentrations of substrate and n-3 PUFA were added to platelets, the strongest inhibition of the prostanoid pathway was obtained at the highest 20:4n-6 concentration (10 μM) (Fig. 1C). Although the inhibitory effect of 20:5n-3 was always slightly higher than that of 20:4n-3, there was no significant difference between the amount of HHT and TXB_2 produced in the presence of either n-3 fatty acid. Adding 20:4n-3 or 20:5n-3 to the platelet suspension along with 20:4n-6 inhibited the aggregation when at least 60% of the TXB_2 formation was inhibited, and there was no significant difference between the two PUFA (not shown). 20:4n-3 alone did not induce any platelet aggregation, up to a concentration of 20 μM.

In contrast, 20:4n-3 but not 20:5n-3 strongly stimulated the 12-HETE production (Fig. 1, A, B, and C) at each concentration of the 12-HETE precursor (20:4n-6) whatever the amount of 20:4n-3 added. The highest stimulation was obtained with 10 μM 20:4n-6 in presence of 10 μM 20:4n-3. In contrast, no effect of 20:5n-3 was observed on the formation of 12-HETE, whatever the concentrations of 20:4n-6 used.

Incorporation of 20:4n-3 and 20:5n-3 in EC Lipids

After a 2-hr incubation, the total amount of phospholipids, as calculated from their fatty acid content measured as fatty acid methyl esters, was not modified by adding the n-3 fatty acids into the incubation medium (Table 1, 2). A slight but not significant decrease in PC and PE concentrations was observed after supplementing the cells for 24 hr instead of 2 hr. This was presumably due to minor losses of EC during the 24-hr culture without serum. Moreover, the 24-hr incubation of the n-3 PUFA loaded on albumin did not significantly modify the amount of PC and PE. When the cells were supplemented with 20:4n-3 or 20:5n-3 for 2 hr, the fatty acid composition of the phospholipid classes was weakly altered. Despite a substantial incorporation of both n-3 PUFA, the arachidonic acid level did not change in PC, PE, PI, or PS (Tables 1–3). In contrast, drastic modifications in PC, PE, PI, and PS fatty acid composition were observed by increasing the incubation time to 24 hr. After incubation with 20:4n-3, it reached 29.4, 16.7, 20.5, and 12.8 mol % in PC, PE, PI, and PS, respectively, these incorporations inducing a significant increase in 20:5n-3 in each class of phospholipid. Adding 20:5n-3 to the medium raised its proportion to 10.4, 10.2, 13.7, and 2.5 mol % in PC, PE, PI, and PS, respectively, and induced a large increase in 22:5n-3 in all phospholipid classes. In contrast, it did not change the 22:6n-3 level in PC and significantly decreased it in PE and PS. This was also observed after 20:4n-3 incorporation. Both n-3 PUFA drastically altered the arachidonic acid level in all phospholipid classes. The fatty acids most replaced were monoenoic acids, 18:2n-6, and 22:4n-6, although differently depending on the phospholipid class. An increase in the proportion of 16:0, but not in 18:0, was observed only in PI and PS.

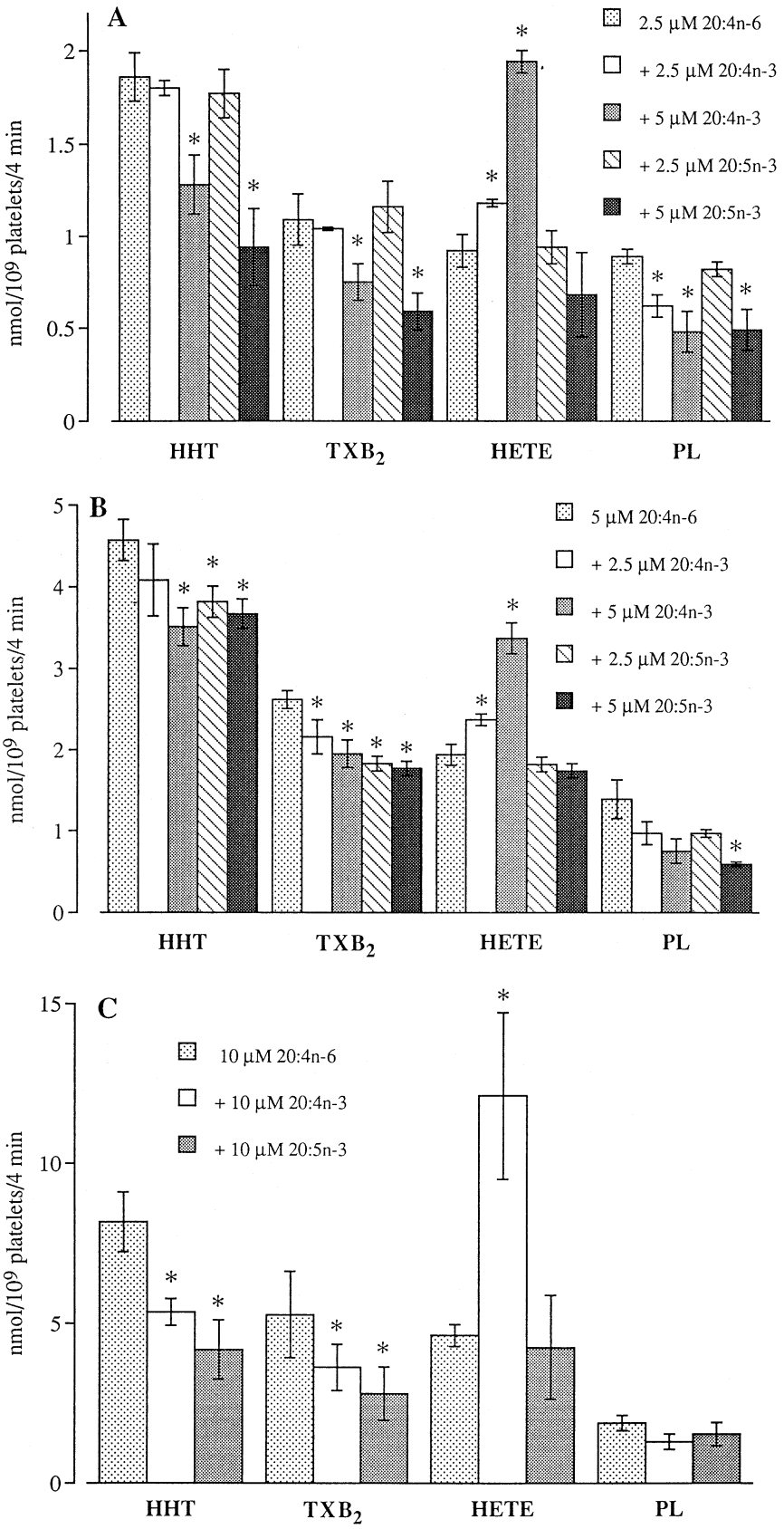

FIG. 1. Effect of 20:4n-3 and 20:5n-3 on [1-^{14}C]-20:4n-6 oxygenation in human platelets. (A) Platelets were incubated with 2.5 μM [1-^{14}C]-20:4n-6 for 4 min at 37° in an aggregometer cuvette in the presence of 2.5 or 5 μM n-3 PUFA or vehicle alone. The metabolites produced were separated by TLC and quantified by radiochromatography. (B) Platelets were incubated with 5 μM [1-^{14}C]-20:4n-6 in the presence of 2.5 or 5 μM n-3 PUFA or vehicle alone. (C) Platelets were incubated with 10 μM [1-^{14}C]-20:4n-6 in the presence of 10 μM n-3 PUFA or vehicle alone. Results are means ± SEM of 4 experiments. *: values differed significantly from controls at least by $P < 0.05$ according to Fisher's LSD post hoc test. HHT and TXB_2: PGH synthase and thromboxane metabolites. HETE: 12-lipoxygenase end-product. PL: incorporation in phospholipids.

TABLE 1. The fatty acid composition of human endothelial cell phosphatidylcholine after 2- and 24-hr incubation with 20:4n-3 and 20:5n-3

	Control (2 hr)	20:4n-3 (2 hr) (mol %)	20:5n-3 (2 hr)	Control (24 hr)	20:4n-3 (24 hr) (mol %)	20:5n-3 (24 hr)
16:0	34.43 ± 1.64	30.68 ± 3.95	30.48 ± 2.09	33.79 ± 1.26	28.46 ± 1.86	30.02 ± 3.53
18:0	8.75 ± 0.31	8.96 ± 0.45	9.07 ± 0.64	8.28 ± 0.39	6.45 ± 0.35*	8.22 ± 0.57
16:1n-7	0.73 ± 0.07	0.63 ± 0.15	0.55 ± 0.14	1.02 ± 0.16	0.58 ± 0.18*	0.81 ± 0.30
16:1n-9	0.97 ± 0.06	0.96 ± 0.23	0.84 ± 0.13	1.52 ± 0.45	0.74 ± 0.11	0.98 ± 0.42
18:1n-7	3.49 ± 0.08	3.08 ± 0.21	3.27 ± 0.26	3.81 ± 0.20	1.98 ± 0.26*	2.71 ± 0.22*
18:1n-9	18.64 ± 0.95	17.92 ± 0.87	18.47 ± 0.53	19.87 ± 1.30	10.70 ± 0.17*	16.74 ± 0.70*
20:1n-9	0.47 ± 0.06	ND	0.52 ± 0.06	0.68 ± 0.38	ND	0.13 ± 0.07*
18:2n-6	17.78 ± 0.52	16.80 ± 0.97	17.46 ± 0.74	16.20 ± 1.13	9.12 ± 1.20*	13.37 ± 1.58*
20:2n-6	1.64 ± 0.34	1.61 ± 0.69	1.59 ± 0.47	1.46 ± 0.22	0.26 ± 0.16*	1.53 ± 0.37
20:3n-6	1.97 ± 0.13	2.06 ± 0.37	2.00 ± 0.15	1.92 ± 0.22	1.46 ± 0.14*	1.54 ± 0.30
20:4n-3	ND	6.92 ± 0.63*	ND	ND	29.45 ± 3.67*	ND
20:4n-6	6.97 ± 0.66	6.45 ± 0.54	6.61 ± 0.49	6.89 ± 0.21	4.61 ± 0.23*	3.58 ± 0.43*
20:5n-3	0.43 ± 0.08	0.73 ± 0.05	4.42 ± 0.69*	0.40 ± 0.07	2.23 ± 0.37*	10.43 ± 1.66*
22:4n-6	1.88 ± 0.11	2.07 ± 0.44	1.87 ± 0.19	2.17 ± 0.23	1.02 ± 0.04*	1.33 ± 0.28*
22:5n-3	0.86 ± 0.18	0.97 ± 0.14	1.88 ± 0.14	0.82 ± 0.09	0.94 ± 0.06	7.77 ± 1.21*
22:6n-3	1.10 ± 0.15	1.44 ± 0.23	1.14 ± 0.13	0.94 ± 0.08	0.87 ± 0.18	0.65 ± 0.10
TOTAL ($\mu g/10^5$ cells	**31.83 ± 1.56**	**31.41 ± 1.97**	**30.24 ± 1.80**	**27.99 ± 2.31**	**23.95 ± 1.78**	**25.23 ± 1.72**

Endothelial cells were incubated with 25 μM free fatty acids bound to albumin in M199 for 2 and 24 hr. Lipids were extracted by $CHCl_3$/methanol/H_2O and separated by TLC. Fatty acid methyl esters were obtained by transmethylation with 5% H_2SO_4 in methanol and analyzed by GC. Results are means ± SEM of 3–4 separate cultures.

*Values differed significantly from controls at least by $P < 0.05$ by Fisher's LSD. ND: not detectable.

Effect of 20:4n-3 or 20:5n-3 Incubation on Endothelial PGI_2 Production

The capacity of fatty acid-modified EC to synthesize PGI_2 was tested after 2- and 24-hr supplementation, corresponding to a moderate (2 hr) and marked incorporation (24 hr) of these fatty acids in cell lipids. The basal PGI_2 release, measured in the medium prior to thrombin stimulation, was not affected in EC supplemented for 2 hr, whereas 76 and 77% inhibition was observed in 20:4n-3 and 20:5n-3 cells, respectively, enriched for 24 hr (Fig. 2). When cells were

TABLE 2. The fatty acid composition of human endothelial cell phosphatidylethanolamine after 2- and 24-hr incubation with 20:4n-3 and 20:5n-3

	Control (2 hr)	20:4n-3 (2 hr) (mol %)	20:5n-3 (2 hr)	Control (24 hr)	20:4n-3 (24 hr) (mol %)	20:5n-3 (24 hr)
16:0	7.10 ± 0.37	8.60 ± 1.15	8.80 ± 0.77	9.51 ± 1.48	9.25 ± 1.30	9.55 ± 1.08
18:0	17.25 ± 1.27	16.48 ± 1.21	17.99 ± 1.35	16.36 ± 1.31	14.15 ± 0.85	16.64 ± 0.54
16:1n-7	0.29 ± 0.12	0.12 ± 0.07	0.04 ± 0.04	0.35 ± 0.05	0.38 ± 0.18	0.15 ± 0.09
16:1n-9	0.32 ± 0.11	1.03 ± 0.22	0.89 ± 0.20	0.85 ± 0.27	1.26 ± 0.43	1.24 ± 0.44
18:1n-7	1.58 ± 0.62	1.70 ± 0.57	1.16 ± 0.70	2.52 ± 0.49	1.87 ± 0.45	2.19 ± 0.23
18:1n-9	10.62 ± 0.63	10.01 ± 0.99	10.90 ± 1.23	12.84 ± 0.14	8.82 ± 0.09*	11.77 ± 0.53
16:0DMA	8.21 ± 0.26	8.55 ± 1.20	8.64 ± 0.39	8.34 ± 1.03	7.65 ± 0.38	7.90 ± 0.75
18:0DMA	4.87 ± 0.23	5.34 ± 0.15	4.27 ± 0.38	4.62 ± 0.43	4.42 ± 0.32	4.13 ± 0.21
18:1n-9DMA	2.37 ± 0.34	2.11 ± 0.38	1.91 ± 0.30	2.41 ± 0.21	2.55 ± 0.32	2.02 ± 0.28
18:2n-6	8.40 ± 0.45	7.98 ± 0.31	8.88 ± 0.52	7.62 ± 0.61	5.35 ± 0.40*	7.25 ± 0.71
20:2n-6	0.84 ± 0.38	1.04 ± 0.22	1.09 ± 0.25	0.42 ± 0.42	0.74 ± 0.28*	0.57 ± 0.38
20:3n-6	1.56 ± 0.13	1.41 ± 0.11	0.99 ± 0.33	1.42 ± 0.15	1.16 ± 0.23	1.24 ± 0.09
20:4n-3	ND	1.98 ± 0.28*	ND	ND	16.63 ± 0.47*	ND
20:4n-6	18.40 ± 0.49	16.88 ± 0.70	16.49 ± 0.82	16.54 ± 1.33	10.69 ± 0.26*	8.93 ± 0.24*
20:5n-3	0.58 ± 0.15	0.77 ± 0.10	2.27 ± 0.26*	0.68 ± 0.16	2.53 ± 0.31*	10.22 ± 1.20*
22:4n-6	6.37 ± 0.68	5.69 ± 0.44	4.81 ± 0.35	5.63 ± 0.65	4.57 ± 0.77	3.55 ± 0.27*
22:5n-3	3.41 ± 0.24	3.38 ± 0.48	3.67 ± 0.20	3.12 ± 0.52	4.10 ± 0.43	9.71 ± 1.09*
22:5n-6	0.44 ± 0.11	0.30 ± 0.09	0.26 ± 0.10	0.66 ± 0.09	0.97 ± 0.49	0.13 ± 0.08*
22:6n-3	7.24 ± 0.32	7.00 ± 0.60	6.76 ± 0.65	5.89 ± 1.02	2.89 ± 0.16*	2.84 ± 0.23*
TOTAL ($\mu g/10^5$ cells)	**18.01 ± 2.13**	**15.35 ± 1.22**	**17.80 ± 2.00**	**15.64 ± 2.13**	**14.86 ± 2.95**	**14.75 ± 1.49**

Results are means ± SEM of 3–4 separate cultures.

*Values differed significantly from controls at least by $P < 0.05$ by Fisher's LSD. ND: not detectable.

TABLE 3. Fatty acid composition of human endothelial cell phosphatidylinositol and phosphatidylserine after 2- and 24-hr incubation with 20:4n-3 and 20:5n-3

	Control (2 hr)	20:4n-3 (2 hr) (mol %)	20:5n-3 (2 hr)	Control (24 hr)	20:4n-3 (24 hr) (mol %)	20:5n-3 (24 hr)
			PHOSPHATIDYLINOSITOL			
16:0	11.99 ± 3.52	9.56 ± 1.60	8.52 ± 0.55	10.35 ± 0.92	15.58 ± 2.29*	14.44 ± 1.33*
18:0	42.65 ± 1.09	40.05 ± 0.85	39.26 ± 1.68	40.26 ± 1.46	30.68 ± 1.81*	35.49 ± 2.85
18:1n-9	8.82 ± 1.11	9.26 ± 1.11	9.33 ± 1.04	11.08 ± 1.54	8.58 ± 0.83	10.88 ± 2.21
18:2n-6	3.07 ± 0.55	3.32 ± 0.40	3.41 ± 0.38	3.47 ± 0.45	5.18 ± 0.56*	3.53 ± 0.54
20:4n-3	ND	3.52 ± 0.44*	ND	ND	20.54 ± 2.13*	ND
20:4n-6	29.95 ± 4.53	30.56 ± 1.87	30.81 ± 1.25	31.69 ± 1.52	13.15 ± 1.21*	17.67 ± 1.92*
20:5n-3	0.29 ± 0.13	0.90 ± 0.50	4.64 ± 0.62*	0.24 ± 0.08	3.33 ± 1.36*	13.67 ± 1.60*
22:4n-6	1.49 ± 0.34	1.53 ± 0.08	1.70 ± 0.22	1.73 ± 0.28	0.71 ± 0.24*	1.19 ± 0.27
22:5n-3	0.38 ± 0.03	0.44 ± 0.07	1.19 ± 0.21	0.31 ± 0.12	0.76 ± 0.27	2.69 ± 0.75*
			PHOSPHATIDYLSERINE			
16:0	10.64 ± 1.86	11.18 ± 7.71	12.46 ± 2.92	10.72 ± 1.21	14.09 ± 1.29*	11.78 ± 0.92
18:0	38.65 ± 1.30	39.25 ± 0.80	40.67 ± 1.04	40.65 ± 0.29	35.23 ± 2.26	39.56 ± 3.55
22:0	0.50 ± 0.13	0.83 ± 0.17	0.72 ± 0.13*	0.63 ± 0.08	0.53 ± 0.07	0.57 ± 0.36
24:0	0.89 ± 0.06	0.95 ± 0.11	0.89 ± 0.09	1.05 ± 0.18	ND	0.89 ± 0.11
16:1n-9	1.01 ± 0.14	0.71 ± 0.55	0.82 ± 0.25	0.93 ± 0.42	2.21 ± 0.92*	0.78 ± 0.23
18:1n-9	16.72 ± 1.07	16.04 ± 0.69	17.03 ± 0.71	16.90 ± 0.16	13.40 ± 0.89*	16.49 ± 1.48
24:1n-9	0.53 ± 0.07	0.41 ± 0.21	0.40 ± 0.05	0.53 ± 0.08	0.53 ± 0.29	0.55 ± 0.18
18:2n-6	8.67 ± 0.61	8.37 ± 0.96	9.05 ± 0.22	8.57 ± 0.48	6.35 ± 0.50*	7.24 ± 0.49
20:3n-6	3.02 ± 0.07	3.19 ± 0.37	2.83 ± 0.47	3.58 ± 0.58	2.62 ± 0.51	2.35 ± 0.19
20:4n-3	ND	1.29 ± 0.18*	ND	ND	12.77 ± 0.29*	ND
20:4n-6	3.26 ± 0.13	3.20 ± 0.53	2.54 ± 0.62	3.29 ± 0.49	1.66 ± 0.15*	1.44 ± 0.20*
20:5n-3	ND	ND	0.80 ± 0.05*	ND	1.19 ± 0.79*	2.47 ± 0.35*
22:4n-6	4.40 ± 0.22	5.56 ± 0.75	4.85 ± 1.11	6.30 ± 0.84	3.63 ± 0.25*	3.86 ± 0.45*
22:5n-3	3.09 ± 0.64	3.80 ± 1.13	2.85 ± 0.58	3.16 ± 0.53	2.75 ± 0.23	8.78 ± 1.03*
22:6n-3	5.02 ± 0.99	6.04 ± 1.75	4.08 ± 0.63	4.35 ± 0.92	1.89 ± 0.23*	2.35 ± 0.23*

Results are means ± SEM of 3–4 separate cultures.
*Values differed significantly from controls at least by $P < 0.05$ by Fisher's LSD. ND: Not detectable.

stimulated for 4 min by 0.125 U/mL of thrombin, the PGI_2 release was not significantly decreased after 2-hr enrichment with either n-3 PUFA, whereas it was strongly inhibited after 24 hr.

DISCUSSION

Earlier studies reporting on the properties of n-3 PUFA have stated that their inhibitory effects on eicosanoid formation may be mediated by decreasing the substrate availability [21] and/or by inhibiting the dioxygenases [22]. The mechanism by which n-3 PUFA interfere with 20:4n-6 oxygenation is not fully clear. However, it has been observed that exogenous 20:5n-3 acts as a competitive inhibitor of the conversion of 20:4n-6 to prostanoids at the level of PGH synthase [23, 24]. In this work, we have shown that 20:4n-3 is not an agonist for platelet aggregation, despite its close structural analogy with 20:4n-6. In accordance with this observation, 20:4n-3 added along with 20:4n-6 to platelets was as potent as 20:5n-3 in inhibiting the PGH synthase activity, as shown by the synthesis of HHT and TXB_2 in the presence and absence of these PUFA. Thus, the fact that 20:4n-3 has four double bonds as 20:4n-6 acid does not increase its inhibitory effect as compared to 20:5n-3. Moreover, the double bond at C_5 is not necessary for inhibition. 20:5n-3 is metabolized into TXA_3 and 12-hydroxy-5,8,10,14,17-20:5 [25], whereas 20:4n-3 does not lead to any cyclic products but is metabolized into 12-hydroxy-8,10,14-heptadecatrienoic acid [12]. This difference in the metabolism of the two n-3 PUFA by the PGH/Tx synthases does not influence their inhibitory effect. It is, however, noteworthy that 20:4n-3 strongly stimulated the 12-lipoxygenase activity, whereas 20:5n-3 had no effect on this enzyme. The reason for this may be related to their different metabolism through the 12-lipoxygenase pathway. This enzyme transforms fatty acids having the 1,4-cis,cis pentadiene configuration, but its substrate affinity varies greatly according to the fatty acids [26]. 20:5n-3 is poorly transformed to 12-hydroxy-5,8,10,14,17-20:5 by the platelet 12-lipoxygenase, as it needs peroxide to stimulate its oxygenation [27, 28]. Conversely, Careaga and Sprecher [12] have reported that 20:4n-3 is an excellent substrate for platelet lipoxygenase and is actively metabolized into 12-hydroxy-8,10,14,17-20:4. Its transformation does not reach saturation level, even at 100 μM substrate. Platelet lipoxygenase is an autocatalytic enzyme which is stimulated by its own metabolites, such as 12-hydroperoxy-5,8,10,14,17-20:4 (12-HPETE), and by other peroxides [28]. It is likely that 12-HPETE is formed at too low a concentration in the presence of 20:5n-3 as a substrate to stimulate its lipoxygenation, whereas the 12-hydroperoxy derivative from 20:

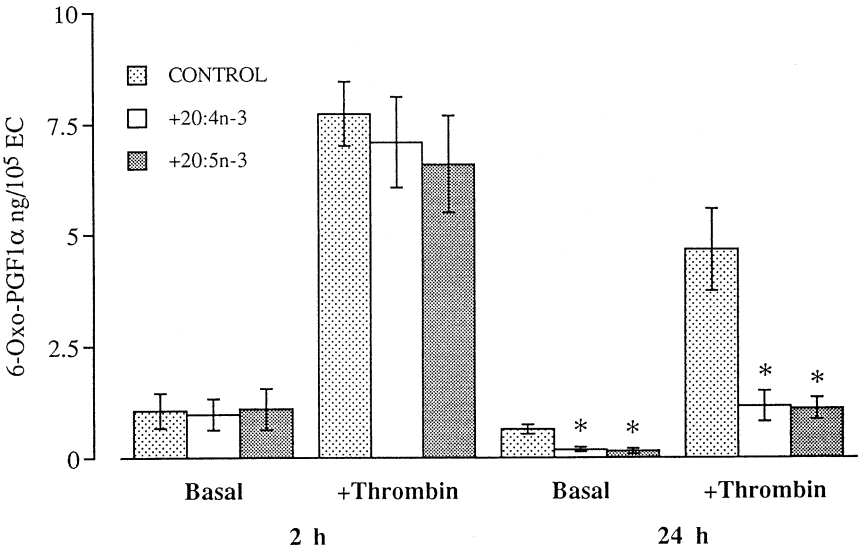

FIG. 2. Synthesis of PGI_2 by the human endothelial cells after incubation with 20:4n-3 and 20:5n-3, for 2 and 24 hr. Confluent endothelial cell monolayers were incubated for 2 and 24 hr at 37° with 25 μM each n-3 PUFA bound to albumin. Control cells were incubated with M199 plus albumin alone. The cells were stimulated for 10 min with 0.125 U/mL thrombin in M199. Supernatants were collected and treated for radioimmunoassay. The basal PGI_2 represents its release in M199 prior to stimulation. Results are means ± SEM of 5 different cultures. *: values differed significantly from controls at least by $P < 0.05$ in Fisher's LSD post hoc test.

4n-3 efficiently potentiates the 12-lipoxygenase, especially for the conversion of 20:4n-6.

The effects of both n-3 PUFA on the endothelial PGH/PGI_2 synthases were compared after their incorporation at moderate and high concentrations in the membrane phospholipids. The specificity of incorporation was quite comparable for both fatty acids in terms of the amount of fatty acids esterified in phospholipids and of distribution within the phospholipid pools. The appearance of 20:5n-3 after incorporation of 20:4n-3 shows that this fatty acid is a substrate for the EC Δ5-desaturase and that its rate of incorporation corresponds to the sum of these two fatty acids present in each phospholipid. Similarly, 20:5n-3 was elongated into 22:5n-3 and its rate of incorporation is the sum of these two fatty acids. 20:4n-3 and 20:5n-3 had the highest affinity for PC and PI at 2 hr, whereas after 24-hr incubation, their esterification in PE and PS increased relative to PC and PI. The relative amount of 20:4n-6, which was not altered after 2-hr incubation with the two PUFA, was similarly decreased after 24 hr. Fatty acid modification induced by 2-hr incubation did not alter PGI_2 synthesis, whereas it was strongly inhibited at 24 hr. Thus, as both n-3 PUFA presented the same pattern of incorporation and similarly altered PGI_2 production, it is concluded that the double bond at C_5 is not crucial for these fatty acids to be substrates of acylCoA transferases and transacylases nor for the inhibition of PGH/PGI_2 synthase activities. Interestingly, the n-3 PUFA incorporation occurring in each phospholipid class after 2-hr incubation did not displace 20:4n-6 in these pools. These fatty acid modifications did not alter the PGI_2 synthesis induced by thrombin stimulation, nor the basal PGI_2 formation resulting from the weak endogenous cell stimulation. In contrast, a strong inhibition of PGI_2 production occurred when the high n-3 PUFA incorporation displaced 20:4n-6 from phospholipids. It should also be noted that after 2-hr incubation, the subsequent incorporation of n-3 PUFA, which did not alter the 20:4n-6 proportion, was not able to inhibit the PGI_2 production, even in the case of very weak stimulation, as for the basal stimulation. Therefore, the weak n-3 PUFA enrichment did not affect the PGH/PGI_2 synthase activities by itself, but these activities might rather have been altered by displacement of the substrate 20:4n-6 and/or by high concentrations of n-3 PUFA. This might, then, be beneficial for the TX/PGI_2 balance under fish oil feeding *in vivo*, where the extent of n-3 PUFA incorporation at the expense of 20:4n-6 is generally moderate [29].

In conclusion, 20:4n-3 and 20:5n-3 had a comparable inhibitory effect on platelet and endothelial prostanoid synthesis, despite the additional double bond at C_5 of 20:5n-3. The 12-lipoxygenase pathway was stimulated by 20:4n-3 but not by 20:5n-3, and this might be due to their different metabolism by the enzyme.

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