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DIETS RICH IN n-9, n-6 AND n-3 FATTY ACIDS DIFFERENTIALLY AFFECT THE GENERATION OF INOSITOL PHOSPHATES AND OF THROMBOXANE BY STIMULATED PLATELETS, IN THE RABBIT

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Abstract—We have studied the effects of semi-synthetic diets rich in either n-9 (olive oil, OO) or n-6 (corn oil, CO), or n-3 (fish oil, FO, as MaxEPA) fatty acids on the levels of major PUFA in platelet lipids, on the generation of inositol phosphates by [^{3}H]inositol labelled platelets after stimulation with thrombin and of thromboxane B_2 (TxB_2) by platelet rich plasma (PRP) after stimulation with collagen. The predicted elevations of oleic (OA), linoleic (LA) and eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids were observed in platelet lipids of each animal group, but in the MaxEPA fed group accumulation of EPA was associated with depletion of linoleic acid (LA) rather than of arachidonic acid (AA). Basal levels of inositol-tris-phosphate (IP_3) in platelets were lowest in the OO group and highest in the CO group, whereas the increment after thrombin stimulation (1 unit/ml NIH) was maximal in the OO group and minimal in the FO group. Instead, when generation of TxB_2 by stimulated platelets was evaluated, no appreciable difference among the various groups could be detected, in accordance with the limited modifications of platelet AA content induced by the diets. The overall data indicate that dietary fatty acids modulate the pathway of inositol phosphate generation in rabbit platelets, independently of modifications of TxB_2 production.

Functional and biochemical parameters of circulating platelets have been shown by various studies in animals and humans to be affected by dietary fatty acids [1]. Changes of platelet responses to aggregating agents induced by manipulations of dietary fatty acids have been related to modifications of platelet lipid and fatty acid composition and especially to changes of the concentrations of eicosanoid precursor fatty acids [2, 3]. Modifications of the levels of arachidonic acid (AA), or the accumulation of eicosapentaenoic acid (EPA) in platelet lipids, induced by dietary fatty acids, result in altered production of thromboxane, the major cyclooxygenase metabolite generated by stimulated platelets [4]. Modulations of other parameters, such as the microviscosity of membranes, may contribute to the effects of dietary fatty acids on platelets and other cells [5].

An important step in triggering cell responses to receptor stimulation is the generation of active metabolites from polyphosphoinositides, through the activation of specific phosphohydrolases [6]. The production of water soluble inositol phosphates, especially of inositol-1,4,5-trisphosphate (IP_3) which is involved in intracellular calcium mobilization, is considered one of the early key steps in cell activation [7, 8]. In addition, the simultaneous generation of the lipid soluble metabolite diacylglycerol, rich in the eicosanoid-precursor AA, may contribute to the formation of the oxygenated metabolites of this fatty acid, through subsequent lipolytic steps [9]. On the other hand thromboxane generated during the platelet activation processes further modulates the phospholipases responsible for the formation of inositol phosphates [10].

The administration of diets rich in EPA has been shown in various studies to affect platelet function and the generation of platelet thromboxanes [1]. In addition, it has recently been reported [11] that incubation of rabbit platelets with EPA affects the generation of inositol phosphates. It appears of interest, at this stage, to assess whether the modulation of the inositol phosphate system in platelets is a general effect of dietary fats and whether these influences are related to changes of membrane fatty acids and eicosanoid production, in platelets. Aim of the present study was, thus, to comparatively evaluate the influences of diets enriched in fatty acids of the n-9, n-6 and n-3 series, respectively, on the formation of inositol phosphates and thromboxane by stimulated platelets and on the concentrations of the major fatty acids in platelet lipids.

MATERIALS AND METHODS

Animals and dietary treatments. Three groups of New Zealand male rabbits, of 10 animals each, of the average weight of 2.5 kg, were fed *ad lib.* semi-synthetic diets (Piccioni, Brescia, Italy) with optimal protein, carbohydrate, vitamin and salt contents and containing 5% by weight (12% of the energy) of either olive oil, or corn oil or fish oil (FO, as MaxEPA, Seven Seas, U.K.) for a period of 5 weeks. The fatty acid composition of dietary fats is presented in Table 1. Diets were stored at −20° and animals were fed with fresh diets, replaced every day.

Blood collection. At the end of treatments, animals

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Table 1. Fatty acid percentage composition of oil supplements

Fatty acids	OO	CO	FO
14:0	—	—	8.3
16:0	17.1	13.3	17.6
16:1	—	—	8.7
18:0	—	1.9	2.6
18:1	70.1	26.6	14.4
18:2	12.8	58.1	1.7
20:0	—	—	3.5
20:5	—	—	15.8
22:1	—	—	5.0
22:5	—	—	2.0
22:6	—	—	14.0

OO, olive oil; CO, corn oil; FO, fish oil.

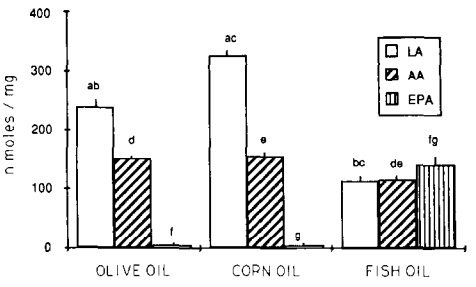

Fig. 1. Levels (nmol/mg total lipid) of major PUFA in platelets of rabbits fed different oils. Values (mean ± SE) sharing the same superscript are significantly different from each other at the $P < 0.005$ level.

were killed and 100 ml of blood was collected from the cannulated common carotid arteries under sodium thiopenthal (50 mg/kg) anaesthesia. Blood was drawn in plastic tubes using ACD 15% (v/v) as anticoagulant (citric acid 71 mM/Na citrate 85 mM dextrose 111 mM). Six animals for each dietary group were used for the preparation of platelets for inositol phosphate labelling and for thromboxane studies, whereas platelets prepared from the remaining four animals in each group were used for lipid analysis.

Platelet studies. Blood was subdivided in aliquots in plastic tubes and platelet rich plasma (PRP) was obtained by low speed centrifugation (200 *g* for 18 min). PRP was further centrifuged (800 *g* for 18 min) and the pellet was resuspended in modified Tyrode–HEPES buffer containing PGI_2 (5 μg/ml).

Labelling of platelet inositol phosphates was carried out by resuspending platelet pellets in 3 ml Tyrode–HEPES buffer and incubating the samples in the presences of 2-[^{3}H]myo-inositol (Amersham, Bucks, U.K.), 1 μCi/ml of blood, for 90 min at 37° without stirring. The platelet suspension was then centrifuged at 800 *g* for 18 min, the pellet was washed in 30 ml Tyrode–HEPES buffer containing PGI_2 (1 μg/ml) and the final pellet was resuspended in 3 ml of the same buffer, without EGTA and PGI_2 and containing LiCl 20 mM. Platelets were counted and diluted at the concentration of 2×10^9 cells/ml. Stimulation of inositol phosphate turnover was carried out on aliquots (0.5 ml) of platelet suspensions, incubated at 37° (stirring at 1000 rpm) in an ELVI aggregometer. After 1 min incubation, thrombin (Topostasin Roche 2 units NIH/ml) or saline (basal condition) were added to platelets and incubations were stopped at 10 and 90 sec after the addition of the stimulus, by quantitatively transferring the samples into tubes containing 1.88 ml chloroform/methanol/HCl (36% 100:200:2) [12]. After phase separation, the upper phase containing the water-soluble inositol phosphates was chromatographed on Dowex-1 anion exchange columns and products were eluted according to the methods of Berridge [13]. Recovery of total radioactivity from the columns was over 98%. The following ^{3}H labelled fractions were sequentially eluted: inositol, glycerophosphoryl inositol, inositol-1-phosphate (IP), inositol-1,4-bisphosphate (IP_2), inositol-1,4,5-trisphosphate (IP_3). Aliquots of eluates were counted with a scintillation counter using gel phase scintillation fluid.

Identification of eluted products was carried out with the use of authentic labelled reference compounds. Values for radioactivity determinations were expressed as dpm/10^9 cells. Preparation of samples for lipid analysis was carried out by further centrifuging washed platelet suspensions and subjecting the samples to osmotic shock in distilled water and to a final high speed configuration.

Lipid analysis. Lipids were extracted from platelet pellets with chloroform/methanol (2/1) containing 5 μg/ml of the antioxidant butylated hydroxytoluene (BHT). The total lipid contents of the extracts were quantified by microgravimetric procedures and fatty acid methyl esters were prepared by transmethylation using methanolic HCl (Supelco, Bellefonte, PA) and analysed by GLC on capillary columns (Supelcowax 10.30 m, 0.75 mm, i.d. 1.0 μm, film thickness df) and programmed temperature (140–210° at 2.5°/min increments). Quantitation of fatty acids was carried out by the use of internal standards and of calibration curves obtained with reference compounds.

Stimulation of thromboxane formation. Platelet thromboxane formation was evaluated by measuring the level of TxB_2 in PRP from the different animal groups at 2 min following stimulation with increasing concentrations of thrombin. TxB_2 determination was carried out by a specific RIA procedure [14].

RESULTS

In Fig. 1 the levels of LA, AA and EPA expressed as nmol/mg of total lipid, in platelets of the three groups of animals are shown. As expected, differences in fatty acid concentrations reflected the fatty acid composition of dietary lipids. LA levels were highest in platelets from the CO fed group and lowest in those from the FO group. Levels of AA were identical in the OO and CO groups and about 15% lower in the FO group, whereas EPA markedly accumulated only in platelets from the FO group. In the n-3 fed animals, accumulation of EPA appeared to replace LA more than AA.

The labelling of inositol phosphates under basal

Table 2. Incorporation of [^{3}H]myo-inositol in platelet inositol phosphates in basal conditions of incubation

Groups		dpm/10^9 platelets IP_3	IP_2	IP
Olive oil	(N = 6)	118 ± 9*	79 ± 8*	662 ± 72
Corn oil	(N = 6)	234 ± 51	218 ± 60	616 ± 85
Fish oil	(N = 6)	194 ± 41	151 ± 40	485 ± 75

Values are the average ± SE. Statistical significance of differences: * $P < 0.05$ vs IP_3 and IP_2 in the corn oil group.

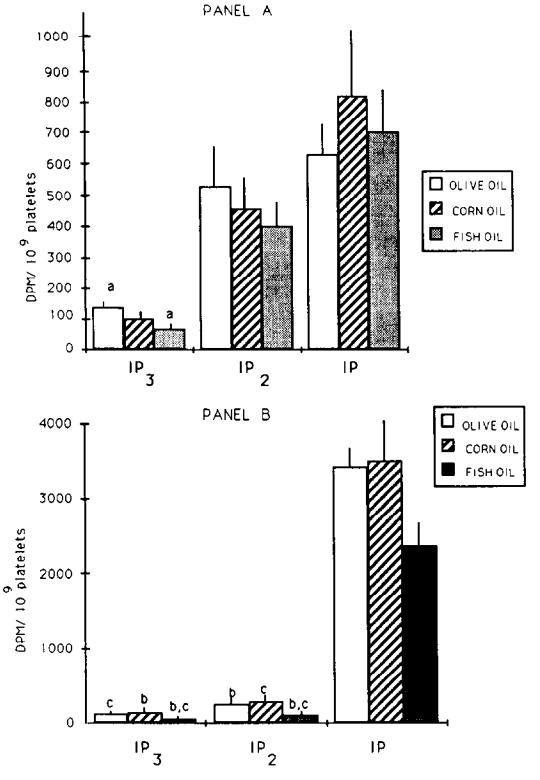

Fig. 2. Levels of inositol phosphate labelling in platelets from the three groups of animals at 10 sec (panel A) and at 90 sec (panel B) after stimulation. Data (mean ± SE) were obtained by subtracting basal values from those obtained after stimulation. Values sharing the same superscript are significantly different from each other at the following levels: a, $P < 0.0005$; b, $P < 0.005$; c, $P < 0.025$.

conditions is shown in Table 2. The highest incorporation of radioactivity was in IP, the lowest in IP_2. Comparing values among different groups, highest levels of IP_3 and IP_2 labelling were found in the CO group and lowest in the OO group.

In Fig. 2 (panels A and B) the increment of [^{3}H]myo-inositol incorporation in inositol phosphates at 10 and 90 sec after stimulation with thrombin, in respect of basal values, is presented. Labelling of all products progressively increased at the two subsequent time intervals, most of the increment at 10 sec concerning IP_2 and IP, and at 90 sec only IP. Labelling of IP_3 at 10 sec and of both IP_3 and IP_2 at 90 sec was lowest in the FO group.

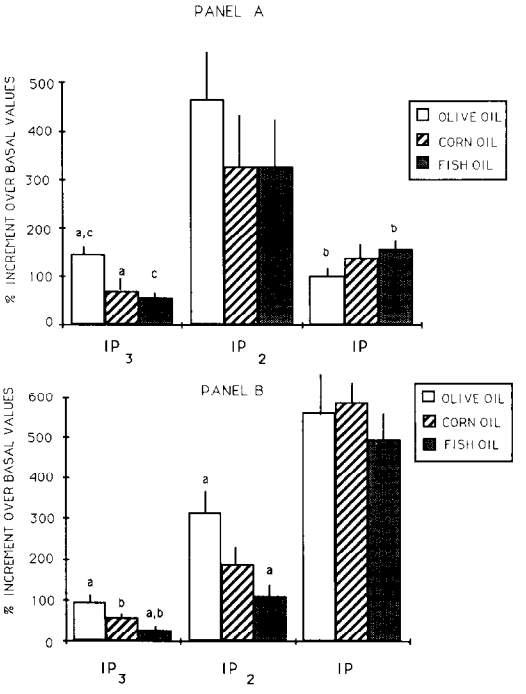

Fig. 3. Percentage increments of inositol phosphate labelling in platelets at 10 sec (panel A) and 90 sec (panel B) after stimulation. Values, (dpm after stimulation − dpm basal/dpm basal) × 100, are the mean ± SE. Values sharing the same letter are significantly different from each other at the following levels: a, $P < 0.025$; b, $P < 0.01$; c, $P < 0.002$.

When data were expressed as percentage increment of inositol phosphate labelling over basal values at 10 and 90 sec after stimulation (Fig. 3 panels A and B) the following was observed. At 10 sec, the highest increment was in IP_2, whereas at 90 sec IP was the major labelled product. When values among different groups were compared, the increment of IP_3 labelling was found significantly lower in the CO and FO vs the OO group, whereas the labelling of IP was highest in the FO group and lowest in the OO group. At 90 sec, increments of IP_3 and IP_2 values were lowest in the FO and highest in the OO group. Mean percentage increment in the CO group was significantly different from the other group of animals.

The generation of TxB_2 by PRP stimulated with increasing concentrations of thrombin is shown in Table 3. Concentration–response curves were obtained with samples from all groups of animals, but no difference in TxB_2 generation by platelets from the various groups of animals was observed.

DISCUSSION

The interest for the biological effects of dietary n-3 and n-6 fatty acids originates from the impact of these components of the diet not only on plasma lipids and lipoprotein, but also on multiple functional and biochemical aspects of cells involved in the thrombotic process. Several studies [1] have shown

Table 3. Levels of TxB_2 in PRP before and after stimulation with different concentrations of thrombin

Groups		Basal	Thrombin (units/ml NIH) 2.5	5.0	10.0
Olive oil	(N = 6)	1.1 ± 0.2	2.5 ± 0.7	10.3 ± 2.3	31.1 ± 5.3
Corn oil	(N = 6)	1.7 = 0.2	3.6 ± 0.8	12.4 ± 4.4	24.9 ± 4.1
Fish oil	(N = 6)	0.7 ± 0.3	3.6 ± 0.7	14.5 ± 3.7	26.2 ± 2.4

Values (ng/ml) are the mean ± SE.

that dietary induced manipulations of n-3 and n-6 fatty acid contents in platelet membranes result in significant reduction of the capacity of these cells to generate products of the cyclooxygenase pathway. These changes are interpreted as a consequence of the modifications of the precursor fatty acids in membrane lipids. It should be considered, however, that interactions among the multiple pathways involved in the generation of lipid derived mediators occur during cellular activation. Therefore changes of membrane lipid components may influence also the pattern of second messenger generation.

In this study we show that the administration of diets enriched with either oleic, or linoleic or with the long chain n-3 fatty acids EPA and DHA to rabbits, resulted not only in selected changes of polyunsaturated fatty acids in platelet lipids, but also in modified production of inositol phosphates by stimulated platelets, in a manner which appeared to be independent from corresponding modifications of TxB_2 generation. On the other side, it has been shown that TxA_2 stimulates receptor-mediated activation of phosphoinositide hydrolysis [10].

Analysis of the comparative effects of dietary fats on platelet fatty acids reveals that the administration of the diet containing CO, rich in LA, resulted in the expected elevation of LA concentrations in platelet lipids, when compared to those in the OO fed group, but AA levels were not affected. This observation confirms that levels of AA in platelet lipids depend on several factors, including e.g., deacylation–reacylation reactions, rather than exclusively on the conversion of the metabolic precursor LA, supplied with the diet. The administration of the diet rich in n-3 fatty acids resulted in accumulation of both EPA and DHA (not reported) in platelet lipids, associated with reduction of LA. It is of interest to note that in our feeding conditions, at variance to data reported in man and other animal species [1], the accumulation of n-3 fatty acids in platelets only marginally affected the absolute levels of AA. The comparative effects of the supplementation of the standard diet with MaxEPA on platelet lipids, in the rabbit were previously considered in a study by Vas Dias *et al.* [15]. These authors reported that supplementation of the diet with MaxEPA resulted in reduction of percentage levels of both LA and AA in platelets. However, in their study appreciable amounts of LA were supplied by the standard diet. Thus, under our conditions, the lack of LA in the diet of the MaxEPA fed animals resulted in selected depletion of this fatty acid in platelet lipids.

Platelets obtained from the three groups of rabbits incorporated [^{3}H]inositol in phospholipids and released labelled inositol phosphates. The pattern of distribution of the radioactivity among the various products showed that at 10 sec after stimulation, the major incorporation of the label occurred in IP and IP_2 and 90 sec in IP, indicating that in our conditions phosphatases were still quite active in spite of the presence of 20 mM LiCl. The shift in the proportion of the label in each inositol phosphate fraction, in all groups of animals, in the time period from 10 to 90 sec, indicates a time-related degradation of the polyinositol phosphates to inositol monophosphate.

The incorporation of radioactivity (dpm/number of cells) in platelet inositol phosphates and, especially, the increments of labelling after stimulation, in respect to basal values, differed significantly among the three groups of animals. Stimulation of IP_3 generation was definitely lower in the n-3 fed animals vs the other groups, at 10 sec, and, at 90 sec, IP_2 stimulation was also lowest in this group, the highest value occurring in the OO group. A similar inhibitory effect of fish oil administration has recently been reported [16], on IP_3 generation by epidermal cells in guinea-pigs.

In our study, in contrast with the effects of dietary manipulations on the phospholipase C-activated production of inositol phosphates, there was no apparent effect of dietary fatty acids, including the n-3, on the formation of TxB_2 by stimulated platelets. The last set of data, although somewhat at variance with results reported in the literature [1], is however in agreement with the concomitant observation that, in our feeding conditions, the levels of the thromboxane precursor AA in platelet phospholipids were not greatly modified by the diets. In previous studies [17, 18] it was shown that the administration to rabbits of diets containing 40 en% of an oil rich in n-3 fatty acids for 18 months resulted in no modification of the levels of AA in platelet phospholipids. However under such condition, thromboxane B_2 formation by collagen stimulated platelets was reduced. The discrepancy between our results and the above observations may be attributed to the much greater amounts of n-3 fatty acids in the diet combined with a considerably longer period of treatment used by these authors. In addition, it appears that the diet-induced modifications of the inositol-phosphate system were not dependent upon variations of platelet AA and/or of its metabolic cascade.

EPA has also been shown to inhibit inositol phosphate generation by stimulated platelet upon *in vitro* preincubation with this fatty acid [11]. The possible

comparative effects of other fatty acids, however, were not reported.

Although it is difficult to compare *in vitro* experiments, carried out by incubating platelets in the presence of concentrations of free EPA which are not comparable to those released from endogenous pools after stimulation, with the situation occurring in our conditions, it is of interest that also the *in vitro* effect of EPA on platelet inositol phosphates was apparently independent from the involvement of the cyclooxygenase. It is possible that modifications of the enzyme microenvironment, which might be detected as microviscosity changes in the lipid phase of platelet membranes, contribute to the observed modifications of the inositol phosphate system.

In conclusion, this study shows that membrane lipids, which are modulated by the diet, play a role in controlling early processes in cell activation, such as the stimulation of PLase C, in addition to or independently from affecting the subsequent stimulation of the eicosanoid system.

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