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Effects of marine oil-enriched diets on guinea pig megakaryocyte and platelet lipids: effects on thromboxane synthesis and platelet function

Pauk K. Schick, Sunitha Menon and Carol Wojenski

Cardeza Foundation for Hematologic Research, Department of Medicine and Department of Pharmacology, Jefferson Medical College of the Thomas Jefferson University, Philadelphia, PA (U.S.A.)

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The effects of marine oil-enriched diets on the fatty acid composition of lipids in guinea pig megakaryocytes (MK) and platelets were studied to obtain a better understanding of the mechanisms for changes in platelet fatty acid composition and platelet function. Animals were fed 2%, 5% and 10% menhaden oil-enriched diets for up to 35 days. Platelets and MK were isolated and MK subpopulations at various stages of development were prepared. The diets did not cause a change in the cholesterol/phospholipid ratio in MK or platelets. The diets induced a dose related incorporation of eicosapentaenoic (20:5) and docosahexaenoic acid (22:6) and an associated decrease in linoleic acid (18:2) in both MK and platelets. However, there was a considerable greater depression of 20:4 in platelets than in MK. These changes were evident with 2% marine oil diets and maximal with 10% diets. Half maximal changes in fatty acid composition occurred after 3 days and maximal changes at 10 days after the initiation of the diets and no further changes occurred up to 35 days. Based on percent of total fatty acids in individual phospholipids, 20:5 had been primarily incorporated into phosphatidylethanolamine (PE) and phosphatidylinositol (PI) and 22:6 into PE and phosphatidylserine (PS) in both MK and platelets. 18:2 was decreased in all phospholipids. 20:4 was decreased only in PI in MK while 20:4 was decreased in PE, PI and PS in platelets. In animals on the 10% marine oil diet, more 20:5 and 22:6 were incorporated into mature than immature MK but the greatest amount of 20:5 and 22:6 had accumulated in platelets. Ingestion of marine oil-enriched diets did not cause thrombocytopenia or affect MK maturation based on the analysis of morphologic stage, ploidy or size. Marine oil-enriched diets caused a decrease in thromboxane synthesis in response to thrombin and calcium ionophore in platelets and MK at all stages of maturation. In platelet-rich plasma, collagen induced platelet aggregation, ATP secretion and thromboxane synthesis were decreased to a greater degree at 35 days than 10 days. Thus, the study indicates that the ingestion of marine oil-enriched diets resulted in the compartmentalization of 20:5 and 22:6 in acidic phospholipids in mature MK and platelets. The observation that marine oil-enriched diet induced maximal changes in lipid composition in MK and platelets within 10 days but caused progressive inhibition of platelet function for up to 35 days indicates that as yet undefined membrane and cellular changes may occur at later time points.

Introduction

Diets enriched with marine oils have been shown to modify the lipid composition and function of plasma lipoproteins, the vascular wall and other tissues and are thought to decrease the incidence of atherosclerosis [1,2]. Dietary supplementation with marine oils is known to alter platelet function as evidenced by the prolongation of the bleeding time, diminished platelet aggregation and secretion and a decrease in the production of platelet thromboxane A_2 [3–6]. The marine oil-related changes in platelet function are thought to be beneficial for avoiding cardiovascular disease since platelets under pathological conditions are involved in the development of thrombosis and atherosclerosis [1].

The mechanisms for the effect of marine oils on platelet function are not well-understood. Diets enriched with marine oils are known to cause an alteration in the fatty acid composition of platelet lipids [7–9]. However, it has been suggested that the incorporation of dietary eicosapentaenoic acid, $(20:5,n-3)$ into phospholipids differs in megakaryocytes and platelets [4]. The megakaryocyte is a more likely target for diets

Correspondence: P.K. Schick, Cardeza Foundation for Hematologic Research, Jefferson Medical College, 1015 Walnut Street, Philadelphia, PA 19107, U.S.A.

enriched with marine oils than platelets since megakaryocytes have a greater capacity for lipid synthesis than platelets [10–12]. For example, only megakaryocytes and not platelets can synthesize cholesterol [11], can desaturate eicosatrienoic acid for the synthesis of arachidonic acid [12] and can readily incorporate radiolabeled arachidonic acid (20 : 4, $n-6$) into all pools of endogenous arachidonic acid [12]. Our hypothesis is that marine oil-enriched diets initially alter lipid metabolism and fatty acid composition in megakaryocytes which ultimately leads to the perturbation of membrane organization and biological activities in both megakaryocytes and platelets. This paper describes the effects of marine oil-enriched diets on the fatty acid composition of platelets and of megakaryocytes at different stages of maturation and on the compartmentalization of fish oil fatty acids in acidic phospholipids. The study also demonstrates the relation of the changes in fatty acid composition to the ability of megakaryocytes and platelets to synthesize thromboxane and to the effects of these diets on platelet function. The guinea pig has been used as a source of megakaryocytes for biochemical studies because the yield, purity and viability of isolated megakaryocytes from guinea pigs are superior to those from other animal species and humans [10–13].

Materials and Methods

Male Fort Detrick Dunkin Hartley guinea pigs weighing between 300 to 350 g were used in the study. The animals were fed pellets of guinea pig chow enriched with menhaden oil and control animals were fed standard Purina guinea pig chow. The pellets were prepared by Bioserve (Frenchtown, NJ). Standard guinea pig chow contained a total of 4% fat. Fish oil-enriched diet contained either 2%, 5% or 10% menhaden oil with 4%, 7% or 12% total fat, respectively. The fatty acid content of the chow was analyzed by gas-liquid chromatography by methods described below. The analysis of the marine oil-enriched chow by Bioserve showed that the vitamin A and D content were negligible. Both standard guinea pig chow and chow enriched with marine oil had been fortified with vitamin E, an antioxidant. The food was kept at $-20\,^{\circ}C$ and used within one month of preparation. Deterioration of marine oil fatty acids present in the prepared chow was monitored by estimation of the presence of dienes [14] to detect peroxidation. Also, the content of 20 : 5 and docosahexaenoic acid (22 : 6, $n-3$) and other fatty acids in the chow was monitored during storage by gas liquid chromatography by methods described below.

Preparation of cells and plasma. Platelet-rich plasma (PRP) was prepared from blood collected in acid-citrate-dextrose by centrifugation at $200 \times g$ for 15 min. Platelets were washed twice in calcium- and magnesium-free Hanks' balanced salt solution (CMFH) at pH 6.5 [10–12]. Platelet poor plasma was prepared by centrifugation at $600 \times g$ for 20 min. Guinea pig megakaryocytes were isolated to about 85% purity by cell number and greater than 98% purity by cell volume using albumin density and velocity gradients as previously described [10–13]. Platelet counts were determined with a J.T. Baker MK-4/HC platelet counter (J.T. Baker Diagnostics, Allentown, PA), and megakaryocyte counts were determined under phase-contrast microscopy in a hemocytometer. Contamination of isolated megakaryocytes with other bone marrow cells and isolated platelets with other peripheral blood cells was determined under phase contrast microscopy. Contamination of washed platelets with erythrocytes and leukocytes was less than 0.0001%.

Megakaryocytes at different phases of maturation were isolated by a recently introduced Celsep procedure [15]. In this procedure, megakaryocytes are separated by size and collected in 18 fractions. Maturity was estimated by the assessment of morphologic stage and ploidy by previously described methods [13,15]. Fractions 2 to 13, which contained more than 92% mature megakaryocytes, were pooled to form a mature group. Fractions 14–18, which contained from 50 to 60% immature megakaryocytes, were pooled to form an immature group. The two groups of megakaryocytes were used for the analysis of the fatty acid composition of lipids in immature and mature megakaryocytes. For the study of thromboxane synthesis, the fractions were pooled into four groups, fractions 2–8 (group A), 9–13 (group B), 14–16 (group C) and 17–18 (group D), which contained different proportions of mature and immature megakaryocytes. Groups A and B contained about 95% and 80% mature megakaryocytes, respectively. Group C contained 50% mature megakaryocytes. Group D contained more than 75% immature megakaryocytes. The differences in the maturity of megakaryocytes separated by the Celsep procedure have been described in detail in a recent publication [15].

Platelet aggregation and secretion in response to collagen using PRP. The platelet count in the PRP was adjusted to $4 \cdot 10^8$/ml with autologous platelet poor plasma. Platelet aggregation in response to collagen (2 and 5 μg/ml) was studied by the extent of change in light transmittance at 3 minutes as previously described [16] using a Chronolog Lumi-Aggregometer (Havertown, PA). Platelet secretion in response to collagen was measured as released ATP by the luciferin/luciferase method 5 min after adding collagen [17]. Maximum release of ATP was defined as that released within 3 min in response to 10 U/ml thrombin. In order to determine thromboxane synthesis in response to collagen, platelets activated by collagen as described above were pelleted following the addition of 0.1 ml 0.1 M EDTA/0.5 ml plasma and the supernate plasma was

then stored at −20 C until assayed for thromboxane B2, the stable metabolite of thromboxane A2, by specific radioimmunoassay [18].

Thromboxane synthesis in washed megakaryocytes and platelets in response to thrombin and calcium ionophore. Isolated megakaryocytes or the four groups of purified subpopulations of megakaryocytes at different stages of maturation were pelleted by centrifugation and resuspended in Dulbecco's phosphate-buffered saline (DPBS) containing 0.1% dextrose and 0.2% fatty acid-free bovine serum albumin (BSA) (ICN, Lisle, IL). Platelets were washed once as described above and $1 \cdot 10^8$ washed platelets were added to DPBS with 0.1% dextrose and 0.2% fatty acid free BSA. Platelets were incubated for 10 min and megakaryocytes were incubated for 30 min at 37 °C with bovine thrombin (0.1 to 10 U/ml) or with calcium ionophore A23187 (0.1 to 10 μM) (Sigma Chem. Co., St. Louis, MO). Following the incubations, 0.1 ml 0.1 M EDTA was added per 1.0 ml incubation medium, the cells were pelleted and supernates were stored at −20 °C until assayed for thromboxane B2.

Lipids were extracted by the Bligh-Dyer method and lipid species were separated by high performance thin-layer chromatography (HPTLC) [10,12,19]: (1) Neutral lipids were separated on HPTLC chromatoplates (Analtech, Newark, RI) with petroleum ether/diethyl ether/glacial acetic acid (70 : 20 : 1, v/v); (2) phospholipids were separated on E. Merck HPTLC chromatoplates (No. 6541 E-M Sciences, Cherry Hill, NJ) with chloroform/methanol/glacial acetic acid/water (81 : 10 : 45 : 1, v/v).

Phospholipids were quantitated by the determination of lipid-phosphorus. Fatty acids were identified and quantitated as fatty acid methyl esters by gas-liquid chromatography using a SP2300 6 ft column as previously described [10,19]. Extracted lipids or phospholipids were subjected to acid methanolyis to produce fatty acid methyl esters. Cholesterol was quantitated by GLC using a SP 2100 2 ft column [10]. Internal and external standards were used to determine the recovery and to quantitate fatty acids and cholesterol.

Experimental design and statistical analysis. Control animals fed standard guinea pig chow were evaluated in each experiment. Megakaryocytes and platelets were studied from the same animal. The significance of differences in the data were evaluated with a Student's-*t* evaluation and the Mann-Whitney test, and $P < 0.05$ was considered to be significant.

Results

The fatty acid composition of the guinea pig chow enriched with 10% fish oil was found to be 24% palmitic (16 : 0), 3% stearic (18 : 0), 17% oleic (18 : 1), 1.6% linoleic, 0.9% linolenic acid (18 : 3, $n-3$), 16% 20 : 5, 9% 22 : 6, and 27% other fatty acids. Standard guinea pig chow contained 18% 16 : 0, 8% 18 : 0, 30% 18 : 1, 31% 18 : 2, and 5% 18 : 3. The fish oil-enriched guinea pig chow was stored at −20 °C for no longer than a month and food was left in the feeding tray for no longer than 24 h. There were no changes in the fatty acid composition of the fish oil-enriched guinea pig chow during storage under these conditions as determined by the assay of fatty acid content by gas chromatography. Significant peroxide formation was not detected when evaluated by the assay of dienes [14].

The diets were well-accepted. Weight gain was noted to be similar in guinea pigs fed standard chow and in animals fed marine oil-enriched diets for up to 6 weeks.

In experiments described below a 1/1000 ratio of the number of megakaryocytes were compared to platelets to compensate for the differences in the protein content and volume of the two cells as previously described [10–12].

There were no differences in the total phospholipid phosphorus and cholesterol content in megakaryocytes and platelets from animals on the marine oil diets compared to animals fed the control diet. The cholesterol/phospholipid ratio in megakaryocytes and platelets did not differ between the animals fed the marine oil diets and control animals. The total phospholipid phosphorus and cholesterol content and the cholesterol/phospholipid ratio in megakaryocytes and platelets of guinea pigs on the standard guinea pig chow diets have been previously described [10,19].

The effects of the concentration of the marine oils in the diet on the fatty acid composition of megakaryocyte and platelet total lipids are shown in Table I. The animals received the marine oil-enriched diet for 12 days prior to killing. Maximal uptake of 20 : 5 and 22 : 6 and changes in fatty acids occurred with diets enriched with 10% fish oils. Not shown was the observation that small amounts of docosatetraenoic acid (22 : 4) and docosapentaenoic acid (22 : 5), 2 and 3%, respectively, had been detected. 22 : 4 and 22 : 5 have been detected in platelets following dietary supplementation with marine oils in previous studies [9,20]. In animals on a 10% enriched marine oil diet, there was a 55% and 43.3% depression of 18 : 2 and 20 : 4 in platelets, respectively; a decrease of 55.7% and 19.6% of 18 : 2 and 20 : 4 in megakaryocytes, respectively. There were no changes in the content of other megakaryocyte and platelet fatty acids in animals fed the marine oil diet. The accumulation of 20 : 5 and 22 : 6 and the depression of 18 : 2 and 20 : 4 were evident in megakaryocytes and platelets with the diets enriched with 2% fish oil. In animals on the 5% marine oil-enriched diets, there was a marked depression of 18 : 2 in both megakaryocytes and platelets but 20 : 4 was only decreased in platelets.

The time course of the effects of the 10% menhaden oil-enriched diets on the fatty acid composition of megakaryocyte and platelet lipids are shown in Fig. 1.

TABLE I

The effect of diets of different percent of fish oil on 18:2, 20:4 and the incorporation of 20:5 and 22:6 in megakaryocytes and in platelets

Guinea pigs fed diets enriched with 2%, 5% or 10% menhaden oil and control animals on standard guinea pig chow were killed, megakaryocytes and platelets were isolated, lipids were extracted from these cells and subjected to acid methanolysis and fatty acid methyl esters were quantitated by gas-liquid chromatography. Only data on the effects of the diets on 18:2, 20:4, 20:5 and 22:6 are shown since the amounts of the other fatty acids were not changed by the diets. Internal and external fatty acid standards were used to identify and quantitate fatty acids. The mean ± S.D. of six experiments is shown. Values with the same superscripts were significantly different at $P < 0.01$. The data on the 2% marine oil diet represents the mean of two experiments.

	nmol per 10^5 megakaryocytes or per 10^8 platelets			
	18:2	20:4	20:5	22:6
Megakaryocytes				
Control	$7.9 \pm 1.2^{a,b}$	4.6 ± 0.6^{c}	0	0
2% diet	6.8	6.5	2.4	1.5
5% diet	4.6 ± 0.4^{a}	4.6 ± 0.4	3.4 ± 1.1	1.4 ± 0.1
10% diet	3.5 ± 0.3^{b}	3.7 ± 0.2^{c}	4.0 ± 0.2	1.2 ± 0.4
Platelets				
Control	$7.1 \pm 0.7^{d,e}$	$9.7 \pm 0.8^{f,g}$	0	0
2% diet	5.3	7.9	2.6	1.4
5% diet	4.3 ± 0.3^{d}	6.6 ± 1.3^{f}	3.4 ± 0.7	1.2 ± 0.1
10% diet	3.3 ± 0.4^{e}	5.5 ± 0.1^{g}	4.0 ± 0.2	1.5 ± 0.1

About 50% of maximal uptake of 20:5 and 22:6 into megakaryocytes and platelets had occurred at 3 days and maximal uptake occurred at 7 days. The only fatty acids that were depressed were 18:2 and 20:4. 18:2 was markedly reduced in 3 days and maximally reduced at 7 days in both megakaryocytes and platelets. 20:4 was markedly reduced at 3 days and maximally reduced at 7 days in platelets. However, there was considerably less depression of 20:4 in megakaryocytes than in platelets. As previously noted, there was twice as much 20:4 in control platelets as in megakaryocytes.

In order to determine whether the effects of marine oil-enriched diets differed on the fatty acid composition of megakaryocytes at different stages of maturation, purified subpopulations of immature and mature megakaryocytes were investigated. Table II demonstrates the effects of marine oil-enriched diets on the incorporation of 20:5 and 22:6 into each of the phospholipid species in platelets and in megakaryocytes at different stages of maturation. The greatest percent of 20:5 per total fatty acids in individual phospholipids was detected in PE and PI in megakaryocytes at all stages of maturation and in platelets. The greatest percent of 22:6 per total fatty acids in individual phospholipids was incorporated into PE and PS in megakaryocytes and platelets. No 22:6 was present in PI in either megakaryocytes or

TABLE II

Effects of fish oil diet on the fatty acid composition of phospholipids in megakaryocytes at different stages of maturation and on platelets

Guinea pigs fed diets enriched with 10% menhaden oil for 10 days and control animals on standard guinea pig chow were killed, megakaryocytes and platelets were isolated, mature and immature megakaryocytes were separated by the Celsep procedure, lipids were extracted from these cells and subjected to acid methanolysis and fatty acid methyl esters were quantitated by gas-liquid chromatography. Internal and external fatty acid standards were used to identify and quantitate fatty acids. Only data on the effects of the diets on 18:2, 20:4, 20:5 and 22:6 are shown since the percent of the other fatty acids were not changed by the diets. The mean ± S.D. of six experiments is shown. Values with superscripts except (g) were significantly different at $P < 0.01$ and (g) was significantly different at $P < 0.05$. Superscript (a) compares 18:2 and (b) compares 20:4 in data shown horizontally in controls and animals on diets; all other superscripts compare data vertically within each phospholipid species.

	Percent of total fatty acids (weight percent)					
	Control		Marine oil diet			
	18:2	20:4	18:2	20:4	20:5	22:6
PE						
Immature	11.1 ± 1.1^{a}	14.9 ± 3.1	3.6 ± 1.4^{a}	17.0 ± 3.3	13.5 ± 3.9^{d}	2.2 ± 2.0^{gh}
Mature	17.8 ± 2.1^{a}	17.8 ± 3.2	5.2 ± 0.6^{a}	15.0 ± 2.3	16.4 ± 1.6	4.5 ± 0.4^{g}
Platelet	12.9 ± 1.7^{a}	31.9 ± 3.9^{b}	4.1 ± 0.5^{a}	19.0 ± 1.8^{b}	18.4 ± 2.4^{d}	6.3 ± 1.5^{h}
PC						
Immature	14.1 ± 0.9^{a}	3.0 ± 1.1	7.0 ± 0.9^{a}	4.4 ± 1.9	4.3 ± 2.1	0.8 ± 1.1
Mature	19.1 ± 2.6^{a}	3.0 ± 1.9	9.5 ± 1.2^{a}	3.1 ± 1.4	4.4 ± 0.8	0.5 ± 0.7
Platelet	17.2 ± 3.2^{a}	4.2 ± 2.0	8.1 ± 0.8^{a}	4.0 ± 1.6	5.0 ± 1.1	0.2 ± 0.1
PS						
Immature	8.4 ± 0.4^{a}	5.8 ± 1.0	3.9 ± 0.4^{a}	3.9 ± 2.8	2.9 ± 0.8	0.3 ± 0.4^{i}
Mature	12.4 ± 1.1^{a}	5.7 ± 0.9	4.6 ± 0.6^{a}	5.8 ± 1.8	2.7 ± 1.6	3.2 ± 1.8^{ij}
Platelet	15.9 ± 2.0^{a}	12.8 ± 1.4^{b}	10.1 ± 1.2^{a}	4.0 ± 1.0^{b}	5.2 ± 1.4	6.8 ± 1.2^{j}
PI						
Immature	2.3 ± 0.5^{a}	30.3 ± 4.8^{b}	0.3 ± 0.7^{a}	21.1 ± 3.0^{b}	8.6 ± 1.8^{e}	0
Mature	4.9 ± 0.8^{a}	29.4 ± 2.2^{b}	0.2 ± 0.3^{a}	16.0 ± 2.2^{b}	13.6 ± 2.0^{ef}	0
Platelet	1.7 ± 1.1	37.6 ± 2.9^{b}	2.7 ± 0.8	23.0 ± 2.6^{b}	18.3 ± 1.9^{f}	0

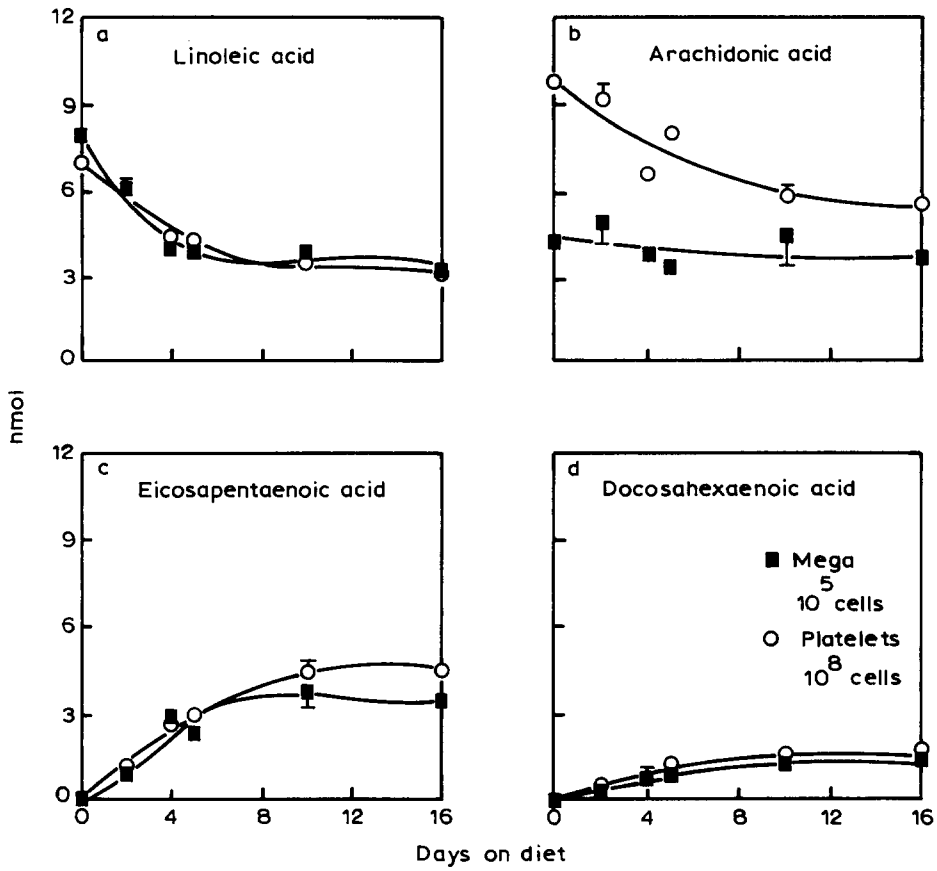

Fig. 1. (a–d) The time course of the effects of marine oil-enriched diets on the 20:5, 22:6, 20:4 and 18:2 composition of megakaryocytes and platelets. Guinea pigs fed 10% Menhaden oil diets and control animals on standard guinea pig chow were killed at different time points, megakaryocytes and platelets were isolated, lipids were extracted from these cells and subjected to acid methanolysis and fatty acid methyl esters were quantitated by gas-liquid chromatography. Internal and external fatty acid standards were used to identify and quantitate fatty acids. Fig. 1a demonstrates the effect of the diet on megakaryocyte and platelet linoleic acid; Fig. 1b shows the effect of the diet on arachidonic acid; Fig. 1c shows the uptake of eicosapentaenoic acid; and Fig. 1d shows the uptake of docosahexaenoic acid in megakaryocytes and platelets. The data were derived from six separate experiments. The mean ± S.D. of the data at 2, 10 and 16 days is shown.

platelets. The uptake of 20:5 into PI was greater in mature than immature megakaryocytes but the greatest uptake of 20:5 occurred in platelets. There were no differences in the amount of 20:5 that was incorporated into PC, PE or PS in megakaryocytes at different stages of maturation. Significantly more 22:6 was incorporated into PE and PS in mature than in immature megakaryocytes but platelets had the greatest capacity for the uptake of 22:6.

In the preceeding paragraph, the uptake of 20:5 was calculated per total fatty acids in PI and other phospholipids and based on this estimation, megakaryocyte and platelet PI contained from 2- to 3-times more 20:5 than PC. Nevertheless, 20:5 had been primarily incorporated into PC and not PI because the ratio of PC/PI in guinea pig megakaryocytes and platelets is about 6–7:1 [10]. Thus, 20:5 was primarily taken up into PC and PE in megakaryocytes and platelets in animals fed marine oil-enriched diets.

Table II also demonstrates the effects of marine oil-enriched diets on 18:2 and 20:4. There was a marked decrease of 18:2 in all megakaryocyte phospholipids and the decrease of 18:2 was greater in PE than in PC and PS. The 18:2 content of PI was virtually depleted in megakaryocytes from animals fed marine oil diets. 20:4 was decreased only in PI in megakaryocytes while 20:4 was decreased in PE, PS and PI in platelets.

There was no evidence of diet induced thrombocytopenia and the maturity of megakaryocytes as judged by morphologic stage, ploidy and size did not differ in control animals and animals that were fed diets enriched with marine oils for up to 6 weeks. Platelet counts were determined in most animals and the analysis of megakaryocyte morphology and ploidy was determined in five experiments.

The marine oil diets significantly affected thromboxane synthesis in megakaryocytes and platelets as shown in Table III. In the marine oil fed animals, thrombin induced thromboxane synthesis was reduced about 50% in megakaryocytes and ionophore induced thromboxane synthesis was reduced between 53% and 63% after 10 days. Continuing feeding for 35 days caused a signifi-

cant decrease in thrombin and calcium ionophore induced thromboxane synthesis in immature megakaryocytes but the reduction was only significant for a single group of immature cells. In platelets, synthesis of thrombin induced thromboxane synthesis was inhibited 60% after 10 days, similar to the effect on megakaryocytes, with no further decrease after 35 days. In contrast, platelet synthesis in response to ionophore was only decreased 31% after 10 days on the marine oil diet but 58% after 35 days.

TABLE III

Effect of diet on thromboxane synthesis by megakaryocytes (pmol/10^5 megakaryocytes and pmol/10^8 platelets)

Guinea pigs fed diets enriched with 10% menhaden oil or with standard guinea pig chow for 10 days of 35 days were killed, megakaryocytes and platelets were isolated and two groups of mature (A and B) and two groups of immature (C and D) megakaryocytes were separated by the Celsep procedure. Group A contained the most mature and group D contained the most immature megakaryocytes. Megakaryocytes and platelets were pelleted and resuspended in DPBS. Platelets were incubated with bovine thrombin or with calcium ionophore A23187 for 10 min at 37°C and megakaryocytes were incubated for 30 min at 37°C. Following the incubations, cells were pelleted and supernates were assayed for thromboxane B2 by specific radioimmunoassay. The values entitled 'Combined MK' represent thromboxane synthesis in megakaryocytes isolated by the standard method but not separated into groups by the Celsep procedure. The mean ± S.D. of (n), number of experiments, is shown. Values with the same superscript are significantly different at: (a, b, c, d) $P < 0.05$; (e, f, g, h, j) $P < 0.01$; (i) $P = 0.028$.

	Thrombin (1 unit/ml)		
	normal	marine oil	
		10 days	35 days
Megakaryocytes			
Mature A	89 ± 32 [a]	36 ± 20 [a]	26 ± 19
Mature B	75 ± 23 [b]	33 ± 14 [b]	21 ± 12
Immature C	31 ± 6 [c]	17 ± 6 [c]	8 ± 4
Immature D	9 ± 4	5 ± 2 [d]	1 ± 2 [d]
	(n = 4)	(n = 4)	(n = 3)
Combined MK	67 ± 15		
	(n = 4)		
Platelets	223 ± 50 [e]	87 ± 12 [e]	87 ± 27
	(n = 9)	n = 12)	(n = 6)
	A23187 (1 μM)		
	normal	marine oil	
		10 days	35 days
Megakaryocytes			
Mature A	278 ± 70 [f]	89 ± 28 [f]	138 ± 61
Mature B	211 ± 34 [g]	87 ± 19 [g]	94 ± 30
Immature C	86 ± 22 [h]	41 ± 8 [h,i]	28 ± 5 [i]
Immature D	28 ± 18	13 ± 3	9 ± 2
	(n = 4)	(n = 4)	(n = 3)
Combined MK	213 ± 68		
	(n = 4)		
Platelets	140 ± 32 [j]	96 ± 15 [j]	58 ± 30 [j]
	(n = 9)	(n = 9)	(n = 6)

TABLE IV

Effects of diet on the platelet response to collagen (2 μg/ml)

Guinea pigs fed diets enriched with 10% menhaden oil or with standard guinea pig chow for 10 or 35 days were studied. Blood was collected by cardiac puncture. Platelet aggregation and secretion were measured in platelet-rich plasma in a Lumiaggregometer. Thromboxane synthesis was measured as described in Methods. The mean ± S.D. of (n), the number of guinea pigs, is shown. Values with the same superscript are significantly different at: (a, c, d) $P < 0.05$; (b, e) $P < 0.01$.

	Normal	Marine oil	
		10 days	35 days
Aggregation (% at 3 min)	37 ± 16 (n = 8)	30 ± 13 [a] (n = 10)	12 ± 20 [a] (n = 6)
ATP release (% of maximum)	26 ± 7 [b] (n = 7)	16 ± 3 [b,c] (n = 8)	8 ± 8 [c] (n = 6)
Thromboxane synthesis (pmol/10^8 cells)	18 ± 8 [d] (n = 8)	11 ± 3 [d,e] (n = 10)	5 ± 4 [e] (n = 6)

In PRP, platelet ATP release and thromboxane synthesis were significantly reduced after 10 days of the marine oil diet but were further reduced after 35 days as shown in Table IV. Collagen-induced platelet aggregation was only significantly inhibited after 35 days.

Discussion

Most dietary studies have been performed in humans and some animal species such as rats and differences have been noted in the uptake of fish oils in human and rat platelets [1,21]. Obviously, the relevance of dietary studies in animals to human metabolism has been questioned. There were several considerations that made the guinea pig a very suitable system for investigating the effects of marine oils on megakaryocytes. Guinea pigs are the best available source of sufficient amounts of purified megakaryocytes that can be used for biochemical studies [10–13]. Also, we have introduced methodology that permits the assessment of biochemical characteristics in mature versus immature megakaryocytes [15]. There is evidence that platelet function in guinea pig and human platelets are similar. For example, thrombin, ADP, collagen and calcium ionophore can induce platelet aggregation, release and the production of thromboxanes in both guinea pig and human platelets [22]. The animals accepted the marine oil enriched diets, gained weight normally and thrived on these diets.

Both 20 : 5 and 22 : 6 the major polyunsaturated fatty acids (PUFA) in marine oils were incorporated into megakaryocytes and platelets. These changes were evident with 2% marine oil and maximal with 10% marine oil enriched diets. On 10% marine oil diets, the half-maximal amounts of 20 : 5 and 22 : 6 had accumulated after 3 days and maximal amounts had accumulated at

about 8 days. There were no additional changes in the fatty acid composition of lipids in the two cells when the animals had ingested the marine oil diets for up to 6 weeks. These observations indicate that significant changes in fatty acid composition occur with low concentrations of marine oils in diets and occur within a few days. However, the capacity for the diet related accumulation of 20 : 5 and 22 : 6 in megakaryocytes and platelets is limited since diets enriched with 10% marine oils produced maximal changes in the fatty acid composition of megakaryocytes and platelets. Also feeding animals marine oil diets for more than 10 days did not cause any additional changes.

There was a significant degree of compartmentalization of 20 : 5 and 22 : 6 in megakaryocytes at different stages of maturation and in individual phospholipid species in megakaryocytes and platelets in animals fed marine oil-enriched diets. The incorporation of 20 : 5 caused a greater displacement of fatty acids in PE and PI than in PC and PS as shown in Table II. However, more 20 : 5 was taken up by PC than PI since there is 6–7-times more PC and PI in megakaryocytes and platelets [10]. Thus, 20 : 5 was primarily taken into PE and PC which is consistent with the uptake of 20 : 5 into human platelet phospholipids in individuals on marine oil-enriched diets [20]. 22 : 6 was primarily incorporated into PS and PE in megakaryocytes and platelets. The uptake of 20 : 5 and 22 : 6 were considerably greater in mature versus immature megakaryocytes but were greatest in platelets which indicates that diets enriched with marine oil have a primary effect on mature megakarocytes. This compartmentalization of fish oil fatty acids in acidic phospholipids potentially may have a marked effect on cell physiology. PE, PI and PS are primarily located in the inner layer of the platelet plasma membrane [23]. The enrichment of these acidic phospholipids with polyunsaturated fatty acids in mature megakaryocytes and in platelets would be expected to alter the orientation and physical properties of these phospholipids, particularly those present in the inner membrane layer, and thus modify cellular biological activities. PS has been implicated in platelet coagulant activity and may be involved in other platelet functions [23,24]. PI and PC are major sources for arachidonic acid for the formation of eicosanoids [25] and the production of thromboxanes would be affected by the orientation of these lipids in membranes.

There was a decrease in 18 : 2 in both megakaryocytes and platelets from animals that had been on the marine oil diet. In general, the depression of 18 : 2 was proportional to the increased uptake of fish oils in individual phospholipids and the stage of megakaryocyte maturity. It should be noted that 18 : 2 was virtually depleted in PI in megakaryocytes and platelets in animals on the marine oil-enriched diet. A significant decrease in 20 : 4 occurred only in PI in megakaryocytes but occurred in PE, PS and PI in platelets. The total depression of 20 : 4 in megakaryocytes was considerably less than in platelets. The discrepancy between the effects of marine oil diets on 20 : 4 in megakaryocyte and platelet phospholipids most likely is due to differences in the activities of acyltransferases or transacylases in the two cells.

Thrombocytopenia or any alteration in megakaryocyte maturation based on the estimation of the ploidy, size and morphologic stages of megakaryocytes were not detected in animals that were fed the fish oil-enriched diets for up to 6 weeks. Thrombocytopenia has been noted to occur in humans ingesting diets containing large amounts of fish oils for prolonged periods [2,6] but thrombocytopenia has not been observed in most studies on the effect of dietary supplementation with marine oils [1]. Conceivably, diets enriched with greater than 10% fish oils may lead to thrombocytopenia and abnormal megakaryocyte maturation.

Although the marine oil-enriched diets did not alter megakaryopoiesis, the diets did significantly affect thromboxane synthesis in megakaryocytes and platelets and attenuated collagen-induced platelet aggregation and secretion. The study showed that the decrease in thromboxane synthesis in response to thrombin and calcium ionophore was not limited to platelets but was evident in megakaryocytes at all stages of maturation.

In platelet rich plasma, collagen induced platelet aggregation, ATP secretion and thromboxane synthesis were decreased to a greater degree at 35 days than 10 days. This was also true for ionophore induced thromboxane synthesis in washed platelets. In megakaryocytes, thromboxane production in response to ionophore and thrombin was only reduced to a greater extent at 35 days in a single group of immature megakaryocytes. In contrast, fatty acid changes in megakaryocytes and platelets were maximal after 10 days and no further changes were noted at 6 weeks. The discrepancy between the effects of the marine oil-enriched diet on the fatty acid composition and the function of platelets at 35 days indicates that as yet undefined membrane and cellular changes may occur at later time points.

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