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Quantitative loss of individual eicosapentaenoyl-relative to arachidonoyl-containing phospholipids in thrombin-stimulated human platelets

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Abstract The thrombin-dependent losses of eicosapentaenoate (EPA) from the various phospholipids of platelets derived from human subjects ingesting a fish lipid concentrate (MaxEPA) were quantitatively assessed and studied in relation to arachidonate (AA). The net loss of AA and EPA from the total phospholipid, phosphatidylcholine (PC) + phosphatidylethanolamine (PE) + phosphatidylserine (PS) + phosphatidylinositol (PI) (loss from phosphatidylinositol *minus* accumulated phosphatidate), amounted to 44.4 and 7.3 nmol/2 × 10^9 platelets (mean values, n = 4 subjects), respectively, in response to thrombin (2 units/ml). The phosphatidylcholine, phosphatidylethanolamine (including alkenylacyl), phosphatidylserine, and phosphatidylinositol contributed 46, 17, <5, and 33%, respectively, of the AA loss; in contrast to these distributions, the corresponding phospholipid contributions to the net loss of EPA were 71, 27, <1, and <2%, respectively. Furthermore, the inhibition of AA- and EPA-phospholipid degradation by trifluoperazine indicated that almost all of the release of EPA occurs from PC and PE (>95% of total EPA loss) upon thrombin stimulation and is mediated predominantly via phospholipase A_2 activity with almost no contribution from PI. Similarities in the molar ratios of AA/EPA in the PC (3.9) or PE (3.7) which were degraded with those in the corresponding phospholipids from resting platelets suggested no marked selectivity by the phospholipase A_2 in intact thrombin-stimulated human platelets in the hydrolysis of AA-PC (or AA-PE) versus EPA-PC (or EPA-PE). Quantitation of the newly released free AA and EPA was determined in the presence of BW755C, a dual cyclooxygenase/lipoxygenase inhibitor which was found not to influence the degradation of individual AA- and EPA-containing phospholipids. The AA/EPA molar ratio in the accumulated free fatty acids (thrombin-dependent) was 6.0; this value was identical to the corresponding ratio in the summed net losses of AA and EPA from the various phospholipids. These results indicate that dietary regulation of the AA and EPA contents of individual membrane phospholipids can control the amounts and types of eicosanoid precursors (as newly released free fatty acids) and their corresponding products in agonist-stimulated platelets. **Mahadevappa, V. G., and B. J. Holub.** Quantitative loss of individual eicosapentaenoyl-relative to arachidonoyl-containing phospholipids in thrombin-stimulated human platelets. *J. Lipid Res.* 1987. **28:** 1275–1280.

Supplementary key words thrombin-stimulated • phosphatidylcholine • phosphatidylethanolamine • phosphatidylserine • phosphatidylinositol • phospholipase A_2 • eicosanoids

It has been documented recently that the consumption of fish/fish oils containing eicosapentaenoic acid (EPA), 20:5(n-3), can diminish platelet–vessel wall interactions and platelet aggregation in response to various agonists (1–4). The biochemical mechanism(s) by which dietary EPA appears to reduce the risk of arterial thrombosis in human subjects via its platelet-mediated effects is of considerable interest. The consumption of fish oils containing EPA results in an altered fatty acid composition of human platelet phospholipids such that the decrease of esterified arachidonic acid (AA), 20:4 (n-6), is compensated for by the marked rise in EPA and, to a lesser extent, by docosahexaenoic acid (DHA), 22:6 (n-3), another fatty acid found in fish/fish oils which is also formed from EPA via fatty acid elongation and desaturation. The AA/EPA ratios vary markedly across the individual platelet phospholipids of human subjects ingesting fish oil containing EPA (5–7).

The aforementioned results are of interest since the agonist-induced release of free AA from various platelet phospholipids provides its availability for metabolism via the cyclooxygenase complex to prostaglandins of the 2-series including thromboxane A_2(TxA_2), a potentiator of platelet aggregation (8). The thrombin-stimulated degradation of phosphatidylcholine (PC) via phospholipase A_2 activity is considered as an important source of free AA for eicosanoid formation in human platelets based on experiments with platelets prelabeled with [^{14}C] arachidonic acid in their phospholipid components (9). Lipid phosphorus measurements have indicated (10) the deacylation

Abbreviations: EPA, eicosapentaenoic acid; AA, arachidonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine (including alkenylacyl); PI, phosphatidylinositol; PS, phosphatidylserine; DHA, docosahexaenoic acid; DG, 1,2-diacylglycerol; PA, phosphatidic acid; MG, monoacylglycerol; TFP, trifluoperazine.

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of PC and phosphatidylethanolamine (PE) in thrombin-stimulated human platelets as well as a loss of phosphatidylinositol (PI) but not phosphatidylserine (PS). The breakdown of PI upon exposure of intact platelets to thrombin is mediated predominantly by the combined action of phospholipase C and A_2 activities (11-14) with minimal apparent contribution from PI phosphorylation to PI 4-P and PI 4,5-P_2 (15). The intermediary 1,2-diacylglycerol (DG) can be rapidly phosphorylated to form phosphatidic acid (PA) or hydrolyzed via the DG/monoacylglycerol (MG) lipase pathway to release free AA. It has been suggested that dietary EPA may decrease platelet aggregability by reducing the formation of TxA_2 (1-4); in vitro studies have shown that both free EPA and DHA can competitively inhibit AA conversion at the level of the cyclooxygenase (16-18). Furthermore, some synthesis of the weakly active derivative TxA_3 from EPA has been reported in human platelets (19).

In view of the dramatic differences observed in the distributions of radiolabeled AA versus the endogenous mass of AA amongst individual human platelet phospholipids (20), recent work has employed direct mass analyses (21, 22) to elucidate and quantitatively estimate the phospholipid origins of the released AA in activated platelets. Although the loss of ^{14}C-labeled EPA from the phospholipids of prelabeled human platelets has been reported (23, 24) upon platelet exposure to thrombin, absolute amounts of EPA lost from the various platelet phospholipids of human platelets via the different pathways has not been assessed alone or in relation to AA loss.

The purpose of the present work was to directly quantitate the losses of AA and EPA from the different phospholipids upon thrombin stimulation of human platelets from fish oil consumers and to assess the contributions of the various phospholipids and phospholipase-mediated pathways to the released AA and EPA.

MATERIALS AND METHODS

Materials

MaxEPA capsules were obtained from Seven Seas Health Care Ltd., Marfleet, UK. Lipid standards, silica gels G and H, and thrombin were all purchased from the Sigma Chemical Company, St. Louis, MO. Commerical silica gel H precoated plates were purchased from Merck, Darmstadt, FRG. Monopentadecanoin and standard fatty acid methyl esters (for gas-liquid chromatography) were obtained from Nu-Chek Prep Inc., Elysian, MN. Trifluoperazine (TFP) was kindly provided by Smith, Kline, and French, Canada, Ltd. 3-Amino-1-(3-trifluoromethylphenyl)-2-pyrazoline hydrochloride (BW755C) was donated by the Wellcome Research Laboratories, Kent, UK. All organic solvents employed were of analytical grade.

Isolation of EPA-enriched platelets from supplemented human volunteers

Four healthy human volunteers, who denied taking any antiinflammatory drugs including aspirin at least 2 weeks prior to entering this study, were recruited. The subjects gave informed consent for their participation in the study. Volunteers were instructed to take 20 MaxEPA capsules per day (equivalent to 3.6 g of EPA and 2.4 g of DHA) with no restriction on their daily food consumption; each capsule provided 1 mg of vitamin E, 0.18 g of EPA, and 0.12 g of DHA. During this supplementation period, the subjects were asked to abstain from taking any medication. After 22 days of supplementation, blood was drawn from the antecubital vein into bags containing acid-citrate-dextrose. The platelet-rich plasma was removed and the platelet suspensions were prepared as described previously (25). The platelet concentration was adjusted to 2×10^9/ml in 5 mM glucose, 0.15 M NaCl, 0.02 M Tris-HCl (pH 7.4) by phase-contrast microscopy and/or protein determination (26) prior to incubation.

Incubation of platelet suspensions

The platelet suspension (2×10^9 cells/ml) was warmed at 37°C in a siliconized glass cuvette with constant stirring at 900 rpm for 2 min in an aggregometer (Payton Associates Ltd., Scarborough, Ont.) and then preincubated with or without BW755C (100 μM) for 1 min. Pretreated platelets were then exposed to thrombin (2 units/ml) for 4 min when the reaction was terminated. In incubations where TFP was used, platelets were warmed at 37°C for 2 min, and preincubated first with TFP for 2 min, and then with BW755C for 1 min followed by stimulation with thrombin for 4 min (2 units/ml). Platelet lipids were immediately extracted by the method of Bligh and Dyer (27).

Chromatography of phospholipids, phosphatidic acid, and free fatty acids

The major phospholipids including phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylethanolamine (PE) were separated on precoated silica gel H plates developed with chloroform-methanol-water-acetic acid 50:37.5:3.5:2 (v/v/v/v). Elution of the PE region containing phosphatidic acid (PA) and the fraction above (free fatty acid (FFA) plus neutral lipid) and subsequent thin-layer development as outlined (21) provided for the isolation of PE, PA, and FFA.

Quantitation of EPA and AA in individual phospholipids, PA, and FFA

The various lipids were transmethylated in the presence of the gel scrapings and a known amount of monopentadecanoin (internal standard) as previously described (28).

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The derived fatty acid methyl esters were extracted with petroleum ether, redissolved in carbon disulfide, and injected into a Hewlett-Packard 5840A gas chromatograph (Hewlett Packard Co., Palo Alto, CA) fitted with a flame ionization detector and glass columns packed with 10% Silar 10C on 100/200 Gas-Chrom Q (Supelco Inc., Bellefonte, PA). Corresponding blank regions of the thin-layer plates were also methylated as above in the presence of internal standard and analyzed by gas-liquid chromatography as control (blank) samples.

RESULTS AND DISCUSSION

Our quantitative data on AA-phospholipid are in good general agreement with previous reports (21, 22, 29). For example, the level of AA in resting platelet PI, which incorporates very minor amounts of EPA, was found to be 39.8 nmol/2 × 10^9 platelets (mean value) as compared to the value of 35.3 from the data of Mauco, Dangelmaier, and Smith for normal volunteers (29). It is apparent from the data in **Table 1** that the mass distributions of EPA amongst the various platelet phospholipids of dietary EPA-consumers differed considerably from those of AA. Whereas PE represented a single major reservoir of both esterified fatty acids in resting human platelets (45% of total AA and 52% of EPA) followed by PC, PI accounted for 15% of the total AA-phospholipid but $<2\%$ of the EPA-phospholipid. In agreement with previous work (6), the EPA/AA molar ratios varied greatly across individual phospholipids of EPA-consumers and were 0.29, 0.22, 0.02, and 0.05 for PC, PE, PI, and PS, respectively, as compared to 0.19 for the total phospholipid. The PC, PE, PI, and PS contained 10.6, 18.2, 0.1, and 1.6 nmol, respectively, of docosahexaenoate (DHA)/2 × 10^9 platelets (mean values); Fisher et al. (30) have reported that human platelets do not release significant amounts of ^{14}C-labeled DHA from phospholipid when stimulated ex vivo with thrombin.

As seen in **Table 2**, thrombin stimulation produced a significant degradation of both AA- and EPA-containing PC. The magnitude of the AA and EPA loss from phospholipid was not significantly affected by the presence of BW755C with the level of thrombin employed (2 units/ml) in agreement with other work (21, 31). The latter compound, a combined cyclooxygenase/lipoxygenase inhibitor (31), was used to test for the release of measurable free EPA and AA (see Table 5). A progressive reduction in the amount of AA-PC and EPA-PC which was degraded was found with increasing levels of TFP (Table 2), a compound employed as a phospholipase A_2 inhibitor in thrombin-stimulated platelets (32); no significant thrombin-dependent breakdown of PC was found at the highest level of TFP. These results indicate that the thrombin-dependent degradation of PC was predominantly mediated via phospholipase A_2 activity. The transfer of ^{3}H-labeled AA from PC to alkenylacyl PE has been reported in thrombin-stimulated platelets (20); Purdon and Smith (33) observed that the increase in ^{3}H-labeled AA in alkenylacyl PE was not apparent until 3–5 min after thrombin exposure and the ^{3}H-labeled AA accumulating by 10 min was a minor proportion of that lost from PC. The molar ratio of AA/EPA in the PC that was degraded upon thrombin exposure (3.9) in our work (Table 2) was very similar to that in the PC from resting platelets (3.5). Although an exclusive (9) or preferential degradation (34) of AA- over oleoyl- or linoleoyl-PC has been suggested upon thrombin stimulation of human platelets, the present mass data indicates no marked selectivity by the phospholipase A_2 in the agonist-dependent hydrolysis of AA-PC versus EPA-PC.

Some thrombin-dependent loss of both AA and EPA from platelet PE (including alkenylacyl) was also observed **(Table 3)**; no effect of BW755C addition on the breakdown was observed. Increasing levels of added TFP blocked this degradation thereby indicating the involvement of phospholipase A_2. The similarity of the molar ratio for the PE which was lost upon thrombin exposure (3.7) with that in the PE of resting platelets (4.5) suggests that both

TABLE 1. Relative distribution of AA and EPA among individual phospholipids of platelets from EPA-subjects

Phospholipid	% of Total AA	% of Total EPA
PC	28.6	43.2
PE	45.0	52.1
PS	11.6	3.3
PI	14.9	1.4

Absolute amount in total phospholipid (PC + PE + PS + PI): AA = 268 nmol/2 × 10^9 platelets and EPA = 51 nmol/2 × 10^9 platelets (mean of four separate subjects).

TABLE 2. Effects of thrombin stimulation, BW755C, and TFP on the absolute amounts of AA and EPA in PC from EPA-enriched platelets

Conditions	nmol/2 × 10^9 Platelets[a] AA	nmol/2 × 10^9 Platelets[a] EPA
Resting platelets	76.5 ± 0.4	22.1 ± 4.1
Stimulated (– BW755C)	56.2 ± 0.5	16.9 ± 3.2
Stimulated (+ BW755C)	57.5 ± 0.5	17.7 ± 3.5
Stimulated (+ BW755C + 40 μM TFP)	62.2 ± 0.8	18.4 ± 3.6
Stimulated (+ BW755C + 80 μM TFP)	71.7 ± 1.1	20.5 ± 4.0
Stimulated (+ BW755C + 120 μM TFP)	75.6 ± 1.8	22.4 ± 4.0
Net loss (thrombin-dependent)[b]	20.3	5.2

[a]Values are given as means ± SEM for four separate subjects.
[b]Net loss represents difference between mean values from thrombin-stimulated (– BW755C) and resting platelets.

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TABLE 3. Effects of thrombin stimulation, BW755C, and TFP on the absolute amounts of AA and EPA in PE from EPA-enriched platelets

Conditions	nmol/2 × 10^9 Platelets[a]	
	AA	EPA
Resting platelets	120.5 ± 1.9	26.6 ± 5.4
Stimulated (– BW755C)	113.1 ± 1.0	24.6 ± 4.8
Stimulated (+ BW755C)	113.2 ± 1.1	24.3 ± 4.6
Stimulated (+ BW755C + 40 μM TFP)	117.6 ± 3.9	25.3 ± 4.4
Stimulated (+ BW755C + 80 μM TFP)	118.2 ± 4.0	25.7 ± 5.3
Stimulated (+ BW755C + 120 μM TFP)	121.5 ± 1.9	26.6 ± 4.0
Net loss (thrombin-dependent)[b]	7.4	2.0

[a]Values are given as means ± SEM for four separate subjects.
[b]Net loss represents difference between mean values from thrombin-stimulated (– BW755C) and resting platelets.

AA-PE and EPA-PE are subject to phospholipase A_2 attack with no marked preferential hydrolysis of one species over the other.

Considerable degradation (approx. 50%) of the AA-containing PI was observed in thrombin-stimulated platelets from the EPA-consuming subjects **(Table 4)**; very little EPA was present in PI (< 1 nmol/2 × 10^9 platelets) or lost from it. This loss of AA-PI was not significantly affected by the addition of BW755C. In contrast to the PC (Table 2) and PE (Table 3) results, increasing the level of TFP (to 80 or 120 μM) was able to effect only a 30–45% inhibition of the thrombin-dependent breakdown of PI. These latter results are consistent with the reported involvement of both phospholipase A_2 and C activities in the degradation of PI in thrombin-stimulated platelets (11–14). The phospholipase A_2-mediated release of free AA from PI can be mediated via the direct hydrolysis of PI (13) and indirectly by hydrolysis of the derived PA (35).

TABLE 4. Effects of thrombin stimulation, BW755C, and TFP on the absolute amounts of AA and EPA in PI from EPA-enriched platelets

Conditions	nmol/2 × 10^9 Platelets[a]	
	AA	EPA
Resting platelets	39.8 ± 1.6	0.7 ± 0.1
Stimulated (– BW755C)	18.1 ± 1.2	0.5 ± 0.1
Stimulated (+ BW755C)	20.1 ± 0.5	0.4 ± 0.1
Stimulated (+ BW755C + 40 μM TFP)	18.1 ± 0.1	0.4 ± 0.1
Stimulated (+ BW755C + 80 μM TFP)	24.6 ± 0.7	0.4 ± 0.1
Stimulated (+ BW755C + 120 μM TFP)	27.8 ± 1.1	0.4 ± 0.1
Loss (thrombin-dependent)[b]	21.7	0.2
Net loss (thrombin-dependent)[c]	14.8	0.1

[a]Values are given as means ± SEM for four separate subjects.
[b]Loss represents difference between mean values from thrombin-stimulated (– BW755C) and resting platelets.
[c]Net loss from PI represents loss of AA and EPA from PI minus the absolute amounts of the corresponding fatty acids (6.9 and 0.1 nmol/2 × 10^9 platelets for AA and EPA, respectively) accumulated in newly formed PA.

By means of dual-isotope prelabeling experiments, both ^{3}H-labeled- and ^{14}C--labeled EPA-containing species of PI were found to readily form PA, presumably via phospholipase C plus 1,2-diacylglycerol (1,2-DG) kinase activities, in thrombin-stimulated human platelets (36). Although insufficient material was available to accurately quantitate the AA and EPA in PI 4,5-P_2 (PIP_2) in this work, Broekman (22) found from mass analyses that the net change in AA in PIP_2 upon thrombin stimulation represented only 3.9% of the measured loss of AA from PI. The net release of free AA/EPA from PI likely reflects phospholipase A_2 action on PI/PA plus DG lipase hydrolysis of intermediary 1,2-DG. There is evidence to indicate that the majority of the 1,2-DG formed during platelet exposure to thrombin is derived from PI directly with a much lesser contribution from the polyphosphoinositides (15, 37). Mauco et al. (29) have reported that the transient accumulated 1,2-DG and associated AA represent approx. 3% of the corresponding mass loss of PI after 60 sec of human platelet stimulation with thrombin.

No significant thrombin-stimulated loss of AA- or EPA-PS was observed in the absence or presence of BW755C or TFP. No significant degradation of PS in thrombin-stimulated human platelets has been reported previously (10, 25). In the present work, the AA and EPA residing in the platelet PS of the MaxEPA subjects represented 31.6 ± 1.6 and 1.7 ± 0.1 nmol/2 × 10^9 platelets (mean ± SEM, n = 4). Less than 2 nmol of AA and 0.1 nmol of EPA were lost (per 2 × 10^9 platelets) from PS upon thrombin stimulation.

A marked accumulation of both free AA and EPA was observed in the presence of BW755C in thrombin-stimulated platelets **(Table 5)**. The amount of free AA and EPA appearing in response to thrombin was progressively diminished by adding increasing amounts of TFP; these results indicate that most of the AA and EPA was released from phospholipid via phospholipase A_2 activity. The AA/EPA molar ratio in the accumulated free

TABLE 5. Effects of thrombin stimulation, BW755C, and TFP on the absolute amounts of accumulated AA and EPA as free fatty acids

Conditions	nmol/2 × 10^9 Platelets[a]	
	AA	EPA
Resting platelets	0.4 ± 0.1	nd
Stimulated (– BW755C)	2.4 ± 0.5	0.5 ± 0.1
Stimulated (+ BW755C)	26.8 ± 1.5	4.4 ± 0.7
Stimulated (+ BW755C + 40 μM TFP)	21.3 ± 2.6	3.4 ± 0.5
Stimulated (+ BW755C + 80 μM TFP)	4.7 ± 1.5	1.3 ± 0.2
Stimulated (+ BW755C + 120 μM TFP)	0.7 ± 0.1	0.3 ± 0.1
Net Accumulation[b]	26.4	4.4

[a]Values are given as means ± SEM for four separate subjects; nd, not detectable.
[b]Net accumulation represents difference between mean values from thrombin-stimulated (+ BW755C) and resting platelets.

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TABLE 6. Relative contribution of individual phospholipids to the thrombin-dependent loss of AA/EPA

Phospholipid	% of Total AA	% of Total EPA
PC	46	71
PE	17	27
PS	<5	<1
PI	33	<2

Values are given as the means from four separate subjects. Each value represents the percentage contribution of the stated phospholipid to the net loss of AA or EPA from the total phospholipid (PC + PE + PS + PI).

fatty acids (thrombin-dependent) was 6.0 (Table 5); this value is identical to the corresponding ratio in the summed net losses of AA and EPA from PC, PE, PI, and PS.

From the mass data provided, the phospholipid origins of the free AA and EPA that are released in response to thrombin can be determined **(Table 6)**. In the case of PI, the 'net loss' values for AA and EPA (Table 4) (loss from PI minus that accumulated in newly formed PA) were used in this calculation. Thus, PC is a major source of the released AA (46% of total) followed by PI (33%) and PE (17%). The percentage contribution of PI to the net loss of AA is in good agreement with the data (60 sec) of Broekman (22) where 32% of the AA loss from platelet phospholipid upon thrombin activation was phosphoinositide-derived. The slightly higher percentage contribution of PI to the total AA loss which we have observed using platelets from EPA-consumers may reflect the partial replacement of EPA for AA in both PC and PE with little such substitution occurring in PI. In contrast to our data on AA loss, PC and PE accounted for 71 and 27%, respectively, of the released EPA with almost no significant contribution from PI (<2%).

From these results, it may be concluded that the release of the cyclooxygenase inhibitor and eicosanoid precursor, EPA, occurs predominantly from PC and PE (>95% of total) upon thrombin stimulation (at 2 units/ml) and is mainly via phospholipase A_2 activity with almost no contribution from PI. The phospholipid origins of the released AA exhibited very different patterns from those for EPA with respect to their quantitative contributions. EPA- and AA-containing phospholipids appeared to be degraded to similar extents based on mass analyses. These results indicate that dietary regulation of the AA and EPA compositions of individual membrane phospholipids and their degradations may play important roles in controlling the amounts and types of both eicosanoid precursors (as newly released free fatty acids) and their respective products in agonist-stimulated platelets.

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