# A comparative study of eicosapentaenoic acid metabolism by human platelets in vivo and in vitro

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**Abstract** During long-term dietary n-3 fatty acid supplementation, eicosapentaenoic acid (EPA) is not incorporated into phosphatidylinositol or -serine of human platelets in vivo and is not detectable in phosphatidic acid upon stimulation with thrombin. However, EPA is released from platelet phospholipids and metabolized to thromboxane $B_3$ ($TXB_3$). In contrast, in vitro, platelets incorporate [$^{14}$C]EPA into phosphatidylinositol, whether they contain endogenous EPA in their cellular lipids or not. Following platelet stimulation, [$^{14}$C]EPA appears in phosphatidic acid, as free fatty acid, and is transformed to $TXB_3$. We conclude that the fatty acid compositions of platelet phospholipid subclasses are regulated with a high degree of specificity in vivo. Qualitative differences exist between in vivo and in vitro uptake of EPA into platelet phospholipid subclasses. After in vivo incorporation, EPA is released by action of a phospholipase $A_2$. — **Schacky, C. v., W. Siess, S. Fischer, and P. C. Weber.** A comparative study of eicosapentaenoic acid metabolism by human platelets in vivo and in vitro. *J. Lipid Res.* 1985. **26:** 457–464.

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The modulation of eicosanoid formation and of eicosanoid-related cellular functions by n-3 polyunsaturated fatty acids is currently considered an attractive tool in the prevention of atherosclerosis (1–5). Eicosanoids derived from eicosapentaenoic acid (EPA) have spectra of biological activity that differ favorably from those derived from arachidonic acid (AA). Thromboxane $A_3$ ($TXA_3$) is less proaggregatory than thromboxane $A_2$ ($TXA_2$) (6) and leukotriene $B_5$ is less active as a chemotactic compound than leukotriene $B_4$ (7), whereas prostaglandin $I_3$ and prostaglandin $I_2$ are equally antiaggregatory (6). Cellular differences exist regarding action of cyclooxygenase and lipoxygenase on AA and EPA. For example, recently we obtained evidence for in vivo formation of prostaglandin $I_3$ in man without reduction of prostaglandin $I_2$ formation (8), which is in contrast to results from studies with animals (9) or with endothelial cell cultures (10, 11). Formation of $TXB_3$ by platelets from exogenous EPA has not been observed consistently in vitro (12, 13), whereas EPA incorporated into platelet phospholipids in vivo is transformed, to some extent, to $TXB_3$ following platelet stimulation (14, 15).

AA is released in stimulated platelets either by activation of phospholipase $A_2$ (16–19) or by the combined action of a phosphoinositide-specific phospholipase C and a 1,2 diacylglycerol-lipase (20–22). It is not known how EPA is released in human platelets. We therefore compared uptake, release, and cyclooxygenation of EPA and AA in human platelets after dietary enrichment with n-3 fatty acids and after in vitro incorporation. We found that EPA is not incorporated into PI under in vivo conditions. Following platelet stimulation, EPA is, however, released from other phospholipids and converted to $TXB_3$.

## MATERIALS AND METHODS

### Study protocol

Six healthy male volunteers (aged 26–36 years, weight 63–88 kg) took an average of 25 ml of cod liver oil (CLO) per day for 5 months. Blood was drawn on three occasions: the day before the CLO intake (before CLO); at the end of the 5-month period of CLO intake (during); and 5 months after the last CLO ingestion (after CLO). Fatty acid composition of platelet phosphatidylcholine (PC), -ethanolamine (PE), -serine (PS), and -inositol (PI) of unstimulated platelets was determined before, during, and after CLO intake. Fatty acid composition of phosphatidic acid (PA), free fatty acids, and $TXB_2$ and $TXB_3$ was measured from resting and stimulated platelets during and 5 months after CLO. Labeling studies of platelets with radioactive fatty acids were carried out during and after CLO intake.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; EPA, eicosapentaenoic (timnodonic) acid [C20:5 (n-3)]; AA, arachidonic acid [C20:4 (n-6)]; $TXA_2$, thromboxane $A_2$; $TXA_3$, thromboxane $A_3$; CLO, cod liver oil; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; DHA, docosahexaenoic acid [C22:6 (n-3)].

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## Materials

Cod liver oil was provided by Möller A/S, Oslo, Norway; composition (in %): saturated fatty acids, 17.3; monoenes, 50.4; C20:5 (n-3), 9.4; C22:6 (n-3), 13.8. Prostaglandin $D_2$, AA, α-linolenic acid, 2',7'-dichlorofluorescein, PA, PE, PS, PS, PI, PC, diacylglycerol, and thrombin were purchased from Sigma (Munich, FRG). Dimyristoyl phosphatidic acid, butylated hydroxytoluene, and reference fatty acid mixtures for gas-liquid chromatography (GLC) were from Serva (Heidelberg, FRG). Bondapak C18 reverse-phase high performance liquid chromatography columns and equipment were from Waters Associates, MA. Silica gel G thin-layer chromatography (TLC) plates DC 60 and all solvents were from Merck, Darmstadt, FRG. Silica gel NHR TLC plates without binding agent were from Macherey and Nagel, Düren, FRG. BW 755c was a gift from Burroughs-Wellcome, Research Triangle Park, NC. [$^{14}$C]AA (53–56 mCi/mmol) and [$^{14}$C]EPA (53–56 mCi/mmol) were from NEN, Dreieich, FRG. Ten percent SP 2340 on 100–120 mesh Sulpelcoport for GLC was purchased from Supelchem, Griesbach, FRG. $TXB_2$ was purchased from Upjohn, Kalamazoo, MI.

## Platelet preparation

Platelet-rich plasma was prepared as described previously (23) and was adjusted with autologous platelet-poor plasma to a final concentration of 250,000 platelets/μl. For the analysis of the fatty acid composition in resting platelet phospholipid subclasses (PE, PS, PI, PC) 8 ml of platelet-rich plasma was used. For the analysis of free fatty acids, fatty acids of PA, and thromboxanes, 30 ml of adjusted platelet-rich plasma was used. Platelets were washed twice in the presence of prostaglandin $I_2$ as described previously (23).

## Lipid analysis in unstimulated platelets

Platelet lipids were extracted (24) with butylated hydroxytoluene at a final concentration of 0.2% to prevent autoxidation. Lipids were partitioned on an open silicic acid column (0.5 g of Unisil, 100–120 mesh). Neutral lipids (containing free fatty acids) were eluted with 10 ml of chloroform; phospholipids and polar eicosanoids were eluted with 6 ml of methanol and 4 ml of methanol–water 99:1.

Phospholipid subclasses were separated with chloroform–methanol–acetic acid–water 75:45:12:3 (25) on silica gel NHR plates that had been activated for 12 hr at 130°C. Plates were dried under a gentle stream of nitrogen. The zones were identified via external standards and visualized under UV-light by spraying the plates with 0.05% 2',7'-dichlorofluorescein in methanol–water 50:50. The zones containing PE, PS, PI, and PC were scraped off and the fatty acid methyl esters were prepared by treating the phospholipids with methanol–concentrated $H_2SO_4$ (80°C, 2 hr).

Plasma free fatty acids were extracted and the methyl esters were formed as described previously (2).

## Gas-liquid chromatography

Fatty acid methyl esters were formed and analyzed on a Packard 419 gas chromatograph equipped with packed glass columns (2 m, 0.3 cm i.d.). The carrier gas was $N_2$; temperature was programmed at 2°C/min from 150–200°C. The data are presented as percentages of the given fatty acids (100%).

## Lipid analysis in stimulated platelets

For measurement of the release of free fatty acids and formation of PA in stimulated platelets, platelets were incubated in a cuvette in an aggregometer with BW755c (100 μM) for 2 min at 37°C to block metabolism by cyclooxygenase and lipoxygenase (26). Platelets were then exposed to isotonic saline (control) or thrombin (2 U/ml) for 60 sec. The reaction was stopped by transferring the samples into chloroform–methanol 1:2 (24), containing butylated hydroxytoluene (0.2%). C18:3 (n-3) and phosphatidic acid containing C14:0 in positions 1 and 2 were added as internal standards. After column partitioning (silicic acid), neutral lipids were separated with hexane–diethylether–acetic acid 90:15:1 (27) on silica gel (NHR) plates. PA was separated from other polar lipids with the organic phase of ethylacetate–2,2,4-trimethylpentane–acetic acid–water 90:50:20:100 (28) on silica gel (NHR) plates. Identification and visualization as described above. The zone containing the free fatty acids was eluted with chloroform–n-heptane–methanol 56:42:2. Free fatty acids were methylated in 75 μl of methanol with diazomethane; fatty acids of PA were esterified with methanol–$H_2SO_4$ (80°C, 2 hr). For measurement of $TXB_{2/3}$, platelet lipids were prepared, as described for PA, but in the absence of BW 755c. The zone containing $TXB_{2/3}$ was eluted with methanol and analyzed by combined capillary GLC–mass spectrometry as described previously (15). $TXB_2$ was quantified via an authentic external standard by multiple ion selection. Fragments m/z 558 ($M^+$ – 71) and 468 ($M^+$ – 90 – 71) of $TXB_2$-Me-MO-$Me_3Si$ were monitored. Formation of $TXB_3$, which eluted about 45 sec after $TXB_2$ from the capillary column, was assessed by quantitative comparison of the mass spectra.

## Labeling experiments with [$^{14}$C]AA and [$^{14}$C]EPA

Five ml of platelet-rich plasma (250,000 platelets/μl) was incubated in the presence of prostaglandin $D_2$ with either [$^{14}$C]AA or [$^{14}$C]EPA ($2 \times 10^6$ cpm) for 2 hr. The samples were washed twice (23) and then either exposed to saline (control) or to thrombin (1 U/ml) for 90 sec.

Lipid extraction and separation of PE, PS, PI, and PC were performed as described above. Phosphatidic acid, $TXB_{2/3}$, diacylglycerol, and free fatty acids were separated on silica gel G plates with the organic phase of ethylacetate–2,2,4-trimethylpentane–acetic acid–water 90:50:20:100 (28). Authentic standards were cochromatographed, zones were identified by staining with iodine vapor and were scraped into scintillation vials. After the addition of scintillation fluid, the samples were counted in a Beckman LS 330 counter (23). The data are given as percent of controls.

### Statistics

All data are presented as mean ± SD of six experiments from different blood donors. Two-tailed paired Student's *t*-test was applied where appropriate.

## RESULTS

Fatty acid patterns of PE, PC, PS, and PI in unstimulated platelets during the different dietary periods are shown in **Table 1**. During CLO ingestion, EPA increased significantly in PC and PE, whereas AA decreased in these subclasses. EPA was, however, not detectable in PI and PS. DHA (C22:6) increased about twofold in PC and PS, but was not detectable in PI (Table 1).

Free EPA in plasma increased from 0.55 to 5.76% ($P < 0.005$), AA remained at 4%, and linoleic acid decreased from 17.06 to 14.26% ($P < 0.01$) (**Table 2**).

In CLO-platelets stimulated with thrombin, EPA was not found in phosphatidic acid. Stearic acid and AA of phosphatidic acid increased 2- to 3-fold after stimulation. Free AA, EPA, and DHA were not detectable in unstimulated platelets. Upon stimulation with thrombin, EPA and AA were released in a ratio of 1/10, which is lower than the EPA/AA ratio in PC or PE (Table 1 and **Table 3**).

CLO-platelets ($3.75 \times 10^9$), stimulated with thrombin, formed 71 ± 27.6 ng of $TXB_3$ and 1413 ± 558 ng of $TXB_2$ (means ± SD, n = 3), a ratio of 1/20 (**Fig. 1**). In control platelets before CLO intake, $TXB_3$ was below the detection limit of our GLC–mass spectrometry method (about 1 ng per injected sample).

To investigate the effect of the fatty acid compositions of platelet phospholipids on the uptake and release of exogenous AA and EPA, control and CLO-platelets were labeled with [$^{14}$C]AA and [$^{14}$C]EPA. The overall uptake of [$^{14}$C]AA and [$^{14}$C]EPA was comparable during the control period and CLO-supplementation. The same was true for the incorporation of [$^{14}$C]AA and [$^{14}$C]EPA into the phospholipid subclasses (**Table 4**). During CLO, in thrombin-stimulated platelets, less [$^{14}$C]AA was liberated from PI, with a concomitant smaller formation of $^{14}$C-labeled PA, free fatty acids, and thromboxane, as compared to control platelets. In platelets labeled with

TABLE 1. The effect of cod liver oil intake (5 months, average 25 ml/day) on fatty acid composition of phospholipid subclasses of platelets

| Platelets | 16:0 | 18:0 | 18:1 (n-9) | 18:2 (n-6) | 20:4 (n-6) | 20:5 (n-3) | 22:6 (n-3) |
|---|---|---|---|---|---|---|---|
| PE | | | | | | | |
| Before | 13.60 (1.13) | 26.03 (1.44) | 9.17 (1.56) | 2.48 (1.04) | 43.28 (2.42) | 1.16 (0.16) | 4.22 (0.33) |
| During | 13.84 (1.94) | 23.11 (2.76) | 11.12 (1.47) | 2.82 (0.72) | 34.81 (1.86)[b] | 7.71 (0.86)[c] | 6.53 (2.01)[a] |
| After | 13.82 (2.29) | 21.43 (1.08) | 9.54 (0.92) | 2.74 (0.59) | 45.78 (3.52)[d] | 1.67 (0.50)[c] | 4.95 (1.21)[a] |
| PC | | | | | | | |
| Before | 32.47 (3.92) | 18.73 (0.67) | 26.51 (2.82) | 8.00 (2.32) | 12.79 (1.16) | 0.47 (0.14) | 1.12 (0.32) |
| During | 30.91 (3.25) | 16.58 (1.03) | 28.38 (2.50) | 9.90 (1.30) | 9.00 (2.71)[a] | 2.87 (0.68)[b] | 2.29 (0.43)[b] |
| After | 30.47 (2.70) | 16.77 (1.59) | 25.28 (2.23) | 10.45 (1.29) | 14.67 (2.46)[c] | 0.70 (0.27)[c] | 1.59 (0.42)[a] |
| PS | | | | | | | |
| Before | 10.57 (0.77) | 41.69 (6.95) | 23.82 (4.12) | trace | 21.93 (3.65) | n.d. | 1.93 (0.03) |
| During | 6.92 (1.11)[a] | 41.11 (7.66) | 23.31 (4.53) | trace | 24.12 (3.62) | n.d. | 4.49 (0.57)[b] |
| After | 16.95 (8.49) | 41.73 (4.48) | 15.90 (0.68) | trace | 22.69 (4.94) | n.d. | 2.69 (1.16)[a] |
| PI | | | | | | | |
| Before | 10.42 (1.53) | 39.45 (3.36) | 17.74 (3.85) | trace | 32.38 (4.03) | n.d. | n.d. |
| During | 10.09 (2.66) | 40.72 (4.28) | 16.68 (4.09) | trace | 32.47 (3.12) | n.d. | n.d. |
| After | 10.83 (1.01) | 37.52 (3.60) | 17.83 (2.96) | trace | 33.78 (4.64) | n.d. | n.d. |

PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol. Samples were analyzed before, during, and 5 months after withdrawal of cod liver oil (see Methods). Data are presented as percent of the given fatty acids, means ± SD from six experiments; detection limit for 20:5 (n-3) and C22:6 (n-3) was 2.5% of the amount of C20:4 (n-6).

[a] $P < 0.05$, [b] $P < 0.02$, [c] $P < 0.01$, [d] $P < 0.005$, [e] $P < 0.001$, paired *t*-test as compared to the results on the line above; n.d., not detectable.

TABLE 2. The effect of cod liver oil intake (5 months, average 25 ml/day) on plasma free fatty acid composition

| | 16:0 | 18:0 | 18:1 (n-9) | 18:2 (n-6) | 20:4 (n-6) | 20:5 (n-3) | 22:6 (n-3) |
|---|---|---|---|---|---|---|---|
| Before CLO | 30.78 (3.71) | 12.59 (1.53) | 34.13 (4.63) | 17.06 (2.66) | 3.79 (1.76) | 0.55 (0.29) | 1.24 (0.90) |
| During CLO | 27.05 (2.08) | 14.72 (1.59) | 29.93 (2.43) | 14.26 (2.43)[a] | 4.33 (1.31) | 5.76 (1.73)[b] | 3.88 (2.02)[c] |

Samples were analyzed before and during cod liver oil (CLO) ingestion (see Methods). Data are presented as percent of plasma free fatty acids, means ± SD from six experiments.
[a] $P < 0.01$, paired $t$-test; [b] $P < 0.005$, paired $t$-test; [c] $P < 0.05$, paired $t$-test.

[$^{14}$C]EPA, no such differences could be observed, except for FFA, which also decreased during CLO (**Table 5**).

## DISCUSSION

During long term dietary administration of CLO, EPA is neither incorporated into human platelet PI nor PS (Table 1), despite a large increase of EPA in plasma free fatty acids (Table 2). The same is true for PA from unstimulated and thrombin-stimulated platelets (Table 3). These data and similar results in previous short-term dietary studies (29, 30) are in contrast to animal experiments, where rats incorporated EPA into PI and PS of platelets after dietary fish oil (31, 32). The results differ also from in vitro labeling studies with human platelets since [$^{14}$C]EPA is readily incorporated into platelet PI in vitro (Table 4) (14, 32–35). Platelet preparation and washing as performed in this study leaves platelets in discoid shape (36). $PGD_2$ was added during labeling to prevent platelet activation; unstimulated platelets contained extremely small amounts of labeled PA and TX. This indicates that platelet activation during preparation and labeling of platelets is highly unlikely. Therefore, depletion of PI of AA or EPA in unstimulated platelets by activation of a phospholipase does not occur during this procedure. [$^{14}$C]EPA was also incorporated into PI of platelets isolated during the CLO period, therefore excluding a specific inhibition of EPA incorporation into platelets in vivo due to a change in plasma milieu. A high affinity incorporation of AA and EPA, probably via a specific acyl-CoA synthethase present in platelets (37), could explain the rapid uptake of [$^{14}$C]AA and [$^{14}$C]EPA into platelet phospholipids in vitro (Table 3) (14, 32–35). Such a mechanism, however, does not explain in vivo–in vitro differences of EPA incorporation into platelet phospholipid subclasses.

To investigate further the regulation of fatty acid composition in platelet phospholipid subclasses, plasma free fatty acids and the fatty acid composition of PI, PC, and PE, platelets were analyzed before and 2, 4, 8, and 24 hr after ingestion of 60 ml of CLO in a single dose. EPA in plasma free fatty acids remained at control values at 2 and 4 hr, increased to the levels of AA at 8 and 24 hr, but was not detectable in PI of platelets at any time, although it increased in PC and less so in PE of platelets at 8 and 24 hr (C. v. Schacky, W. Siess, S. Fischer, and P. C. Weber, unpublished observations). It is therefore very unlikely that, in vivo, ingested EPA might be incorporated into PI and subsequently transacylated to PC or PE.

Two regulatory mechanisms for membrane phospholipid fatty acid compositions have been suggested. *1*) Fatty acids are introduced into phospholipids during de novo synthesis in the endoplasmic reticulum and phospholipids are then transported to other organelle membranes where a first tailoring takes place. *2*) A "fine tuning" of the fatty acid compositions is supposed to be carried out by enzymes in the membranes. It is unclear at present to what

TABLE 3. Free fatty acids and fatty acid composition of phosphatidic acid in resting and stimulated platelets

| | 16:0 | 18:0 | 18:1 (n-9) | 20:4 (n-6) | 20:5 (n-3) | 22:6 (n-3) |
|---|---|---|---|---|---|---|
| | | | *μg ± SD* | | | |
| Free fatty acids | | | | | | |
| Saline | 33.29 (8.28) | 56.54 (18.48) | 2.08 (0.71) | n.d. | n.d. | n.d. |
| Thrombin | 44.37 (11.56) | 71.15 (11.59) | 3.15 (0.25) | 5.69 (2.29) | 0.60 (0.22) | n.d. |
| Fatty acids of phosphatidic acid | | | | | | |
| Saline | 5.29 (1.81) | 3.67 (0.86) | 3.39 (0.88) | 2.37 (0.69) | n.d. | n.d. |
| Thrombin | 5.26 (1.20) | 7.49 (1.57)[a] | 3.94 (1.19) | 6.04 (0.93)[b] | n.d. | n.d. |

Platelets ($3.75 \times 10^9$) were isolated from volunteers who were on supplementary cod liver oil for 5 months (25 ml/day). Platelets were preincubated with the cyclooxygenase and lipoxygenase blocking agent BW755c (100 μM) for 2 min at 37°C, and exposed to either thrombin (2 U/ml) or saline for 60 sec (see Methods). Detection limit for free fatty acids, 100 ng; for phosphatidic acid, 200 ng per sample.
[a] $P < 0.01$, paired $t$-test; [b] $P < 0.001$, paired $t$-test; n.d., not detectable.

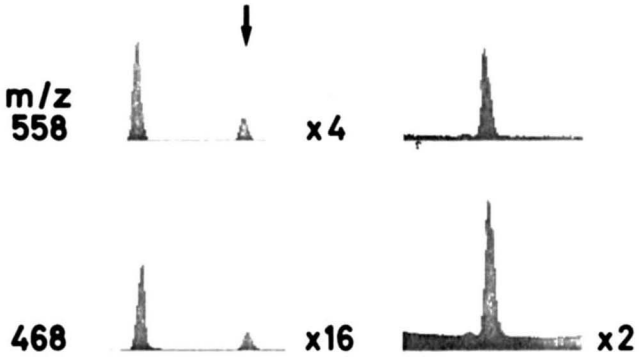


**Fig. 1.** Left, multiple ion selection tracings of TX (thromboxane) $B_2$ and $TXB_3$ (Me-MO-$Me_3$Si-derivatives) generated from endogenous arachidonic acid and eicosapentaenoic acid in washed human platelets stimulated with thrombin. $TXB_3$ (↓) elutes about 45 sec after $TXB_2$ from the capillary column. Common fragments m/z 558 and 468 were monitored. Sensitivity of the tracings is lowered by the factors indicated and is adjusted to the highest occurring peak. Right, multiple ion selection tracings of $TXB_3$ alone. Sensitivity of the tracings is not adjusted to the $TXB_2$ peaks.

extent which mechanism regulates the fatty acid composition in different platelet phospholipid subclasses in vivo (for review see reference 38). In platelets, at least one of these mechanisms seems to be ineffective in vitro.

In platelet PC and PE, EPA increased from a control value of 3% to 22% (PE) or even 32% (PC) of AA during CLO (Table 1). Free EPA appeared in washed platelets upon stimulation with thrombin in an amount of 10% of AA (Table 3), and $TXB_3$ was formed in an amount of about 5% $TXB_2$. Thus, EPA is not only released to a lesser extent than AA, but it also seems to be metabolized less readily. We have previously demonstrated the formation of $TXB_3$ in platelet-rich plasma upon stimulation with collagen after dietary n-3 fatty acids (15), but part of the $TXB_3$ in that study could have been derived from plasma EPA. In the present study, washed platelets were used, thus $TXB_3$ must be derived from EPA bound to platelet lipids.

PI in resting platelets and PA from stimulated platelets display a virtually identical fatty acid composition during control and CLO-supplementation (Tables 1 and 3, refs. 39, 40). As in PI, EPA does not appear in PA of stimulated platelets (Table 2). EPA is set free upon stimulation with thrombin and this clearly points to another source of EPA, such as PC or PE. Unsaturated 20 carbon fatty acids are located exclusively in the 2 position of the respective phospholipid subclass (40, 41). We therefore conclude that in vivo EPA is released via action of a phospholipase $A_2$ on either PC or PE, or both, and that PI is degraded most likely through a combined action of a phospholipase C and a diacylglycerol-kinase to phosphatidic acid (**Fig. 2**).

CLO had no influence on the overall in vitro incorporation of [$^{14}$C]AA and [$^{14}$C]EPA (Table 4); this is in agreement with published data (31). Uptake and release of [$^{14}$C]AA and [$^{14}$C]EPA are mainly observed in PC and PI; both display a higher metabolic activity than relative to their mass in the platelet membrane (40–42). PC is degraded during platelet stimulation at a slower rate than PI (4, 16, 43), thus the small decrease of radioactivity in PC could be explained by our short time (90 sec) of thrombin stimulation.

In vitro, [$^{14}$C]EPA is incorporated into all phospholipid subclasses, especially PI, released and metabolized to [$^{14}$C]$TXB_3$ upon stimulation with thrombin. The action of both a phosphoinositide-specific phospholipase C together with a diacylglycerol-lipase (20–22) and a phospholipase $A_2$ (16–19) could explain these findings and could be active in vitro. Since PI does not contain EPA after dietary supplementation, a combined action of phosphoinositide-specific phospholipase C and diacylglycerol-lipase cannot release EPA.

In platelets obtained during CLO ingestion and prelabeled with [$^{14}$C]AA, thrombin (1 U/ml) induced less degradation of [$^{14}$C]PI, and less formation of [$^{14}$C]PA (Table 5). The reduced formation of [$^{14}$C]$TXB_2$, as observed in this study, is in agreement with a previous study (33). PI degradation by phospholipase C upon high con-

TABLE 4. Uptake of [$^{14}$C]AA and [$^{14}$C]EPA into unstimulated platelets

| | Uptake | PE | PC | PS | PI | PA | TX | DG | FFA |
|---|---|---|---|---|---|---|---|---|---|
| | *cpm ± SD* | | | | *% incorporation ± SD* | | | | |
| [$^{14}$C]AA | | | | | | | | | |
| Control platelets[a] | 163,670 (44,290) | 12.2 (1.9) | 64.4 (2.0) | 2.3 (1.5) | 18.6 (3.3) | 1.0 (0.2) | 0.2 (0.0) | 0.8 (0.4) | 0.6 (0.2) |
| CLO platelets | 136,640 (40,040) | 11.6 (2.2) | 63.3 (4.4) | 2.3 (0.8) | 19.9 (5.8) | 1.1 (0.3) | 0.3 (0.1) | 0.9 (0.3) | 0.7 (0.4) |
| [$^{14}$C]EPA | | | | | | | | | |
| Control platelets | 60,600 (27,410) | 13.5 (1.3) | 67.3 (3.5) | 3.1 (1.5) | 13.1 (3.3) | 1.5 (0.2) | 0.4 (0.3) | 0.5 (0.4) | 0.8 (0.4) |
| CLO platelets | 65,870 (37,420) | 12.3 (2.1) | 68.0 (4.0) | 2.8 (1.4) | 13.7 (4.0) | 1.2 (0.5) | 0.4 (0.2) | 0.7 (0.3) | 0.9 (0.3) |

During control and dietary cod liver oil (CLO) supplementation, platelet-rich plasma was labeled for 2 hr in the presence of $PGD_2$ with [$^{14}$C]AA and [$^{14}$C]EPA ($2 \times 10^6$ cpm). Determinations were performed in washed, unstimulated platelets; data from six experiments.

[a]All comparisons between control and CLO not significant.

TABLE 5. Effect of dietary CLO supplementation (5 months, 25 ml/day) on the metabolism of [$^{14}$C]AA or [$^{14}$C]EPA in stimulated platelets

| | PE | PC | PS | PI | PA | TX | DG | FFA |
|---|---|---|---|---|---|---|---|---|
| | *% of unstimulated controls (= 100%) ± SD* | | | | | | | |
| [$^{14}$C]AA | | | | | | | | |
| Control platelets[a] | 106 (3.6) | 92 (3.8) | 110 (19.2) | 70 (6.8) | 807 (179) | 3902 (1413) | 170 (59) | 479 (80) |
| CLO platelets | 110 (15.2) | 93 (10.6) | 89 (21.3) | 83 (10.7)[a] | 587 (208)[b] | 2052 (1275)[c] | 193 (35) | 360 (98)[b] |
| [$^{14}$C]EPA | | | | | | | | |
| Control platelets | 117 (8.4) | 96 (6.1) | 117 (19.6) | 76 (9.4) | 319 (61) | 426 (121) | 116 (23) | 348 (37) |
| CLO platelets | 118 (20.8) | 88 (24.6) | 122 (41.0) | 75 (11.7) | 326 (98) | 442 (234) | 135 (5) | 234 (57)[b] |

Platelets were prelabeled with [$^{14}$C]AA and [$^{14}$C]EPA ($2 \times 10^6$ cpm), washed, and then stimulated with thrombin (1 U/ml) for 90 sec (see Methods); data from six experiments.
[a] $P < 0.02$, paired $t$-test; [b] $P < 0.05$, paired $t$-test; [c] $P < 0.001$, paired $t$-test. All other comparisons were not significantly different from each other.

centrations of thrombin (1–2 units/ml) is independent of cyclooxygenase metabolites (44). Thus a diminished formation of endoperoxides and $TXA_2$ leading to a reduced stimulation of phospholipase C and formation of phosphatidic acid cannot be invoked. AA and EPA compete for platelet cyclooxygenase, but the AA/EPA ratio necessary for a 50% inhibition of $TXB_2$ formation (as observed in our in vitro experiments, Table 5) was reported to be 1:1 (6). Thus, competition between AA and EPA for cyclooxygenase also cannot be the only explanation of the 50% inhibition of [$^{14}$C]$TXB_2$ formation in the EPA-enriched platelets. During CLO, less [$^{14}$C]AA or [$^{14}$C]EPA was detected in the free fatty acids of platelets after stimulation with thrombin (Table 5), suggesting that the coupling of the thrombin receptor to phospholipase C and $A_2$ might be impaired by n-3 fatty acids. Changes of physicochemical properties of membranes might contribute to this reduced aggregability as well. Changes in membrane fluidity, as observed in model membranes, occur with the degree of fatty acid unsaturation (38), and alterations in cell deformability have been observed in red cells after dietary n-3 fatty acids (45). As measured in platelets ex vivo, AA was reduced in PC and PE during CLO; this could partly explain a reduced $TXB_2$ formation and platelet aggregability ex vivo (2–4, 8, 15), but not our results in vitro, since the uptake of [$^{14}$C]AA was comparable during the CLO and the control periods.

We conclude that fatty acid incorporation into platelet membrane phospholipid subclasses occurs with a high degree of specificity in vivo. In vitro this specificity is different. Platelets, labeled endogenously with EPA, form $TXB_3$ upon stimulation with thrombin in spite of the absence of EPA in platelet PI, PA, and PS. This underlines a dominant role of a phospholipase $A_2$ for the release of eicosanoid precursor fatty acids during platelet activation at least with high doses of thrombin. In EPA-enriched platelets, formation of $TXB_2$ from cellular AA is reduced, and this is not simply explained by reduced AA content in phospholipids or competition of AA and EPA for cyclooxygenase.

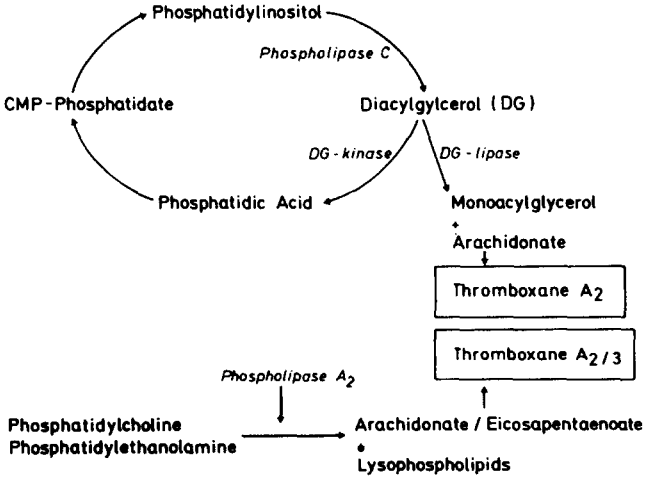


**Fig. 2.** Possible pathways for the biosynthesis of thromboxane $A_2$ and thromboxane $A_3$ by human platelets from endogenous precursor fatty acids during ingestion of an eicosapentaenoic acid-enriched diet.

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## REFERENCES

1. Dyerberg, J., H. O. Bang, E. Stofferson, S. Moncada, and J. R. Vane. 1978. Eicosapentaenoic acid and prevention of thrombosis and atherosclerosis. *Lancet.* **1:** 117–119.
2. Siess, W., P. Roth, B. Scherer, I. Kurzmann, B. Böhlig, and P. C. Weber. 1980. Platelet membrane fatty acids, platelet aggregation, and thromboxane formation during a mackerel diet. *Lancet.* **1:** 441–444.
3. Goodnight, S. H., W. S. Harris, W. E. Connor, and D. R. Illingworth. 1982. Polyunsaturated fatty acids, hyperlipidemia, and thrombosis. *Arteriosclerosis.* **2:** 87–113.
4. Lorenz, R., U. Spengler, S. Fischer, J. Duhm, and P. C. Weber. 1983. Platelet function, thromboxane formation and

blood pressure control during supplementation of the Western diet with cod liver oil. *Circulation.* **67:** 504–511.
5. Schacky, C. v., W. Siess, R. Lorenz, and P. C. Weber. 1984. Ungesättigte Fettsäuren, Eicosanoide und Atherosklerose. *Internist.* **25:** 268–274.
6. Needleman, P., A. Raz, M. S. Minkes, J. A. Ferrendelli, and H. Sprecher. 1979. Triene prostaglandins: prostacyclin and thromboxane biosynthesis and unique biological properties. *Proc. Natl. Acad. Sci. USA.* **76:** 944–948.
7. Lee, T. H., J. M. Mencia-Huerta, C. Shih, E. J. Corey, R. A. Lewis, and K. F. Austen. 1984. Characterization and biologic properties of 5,12-dihydroxy derivatives of eicosapentaenoic acid, including leukotriene $B_5$ and the double lipoxygenase product. *J. Biol. Chem.* **259:** 2383–2389.
8. Fischer, S., and P. C. Weber. 1984. Prostaglandin $I_3$ is formed in vivo in man after dietary eicosapentaenoic acid. *Nature.* **307:** 165–168.
9. Hornstra, G., E. Christ-Hazelhof, E. Haddeman, F. ten Hoor, and D. H. Nugteren. 1981. Fish oil feeding lowers thromboxane and prostacyclin production by rat platelets and aorta and does not result in the formation of prostaglandin $I_3$. *Prostaglandins.* **21:** 727–738.
10. Spector, A. A., T. L. Kaduce, P. H. Figard, K. C. Norton, J. C. Hoak, and R. L. Czervionke. 1983. Eicosapentaenoic acid and prostacyclin production by cultured human endothelial cells. *J. Lipid Res.* **24:** 1595–1604.
11. Morita, I., Y. Saito, W. C. Chang, and S. Murota. 1983. Effects of purified eicosapentaenoic acid on arachidonic acid metabolism in cultured murine aortic smooth muscle cells, vessel walls and platelets. *Lipids.* **18:** 42–49.
12. Hamberg, M. 1980. Transformation of 5,8,11,14,17-eicosapentaenoic acid in human platelets. *Biochim. Biophys. Acta.* **618:** 389–398.
13. Morita, I., R. Takahashi, Y. Saito, and S. Murota. 1983. Stimulation of eicosapentaenoic acid metabolism in washed human platelets by 12-hydroperoxyeicosatetraenoic acid. *J. Biol. Chem.* **258:** 10197–10199.
14. McKean, M. L., J. B. Smith, M. J. Silver, and D. Ahern. 1982. Metabolism of radioactive 5,8,11,14,17-eicosapentaenoic acid by human platelets. *Prog. Lipid Res.* **20:** 435–438.
15. Fischer, S., and P. C. Weber. 1983. Thromboxane $A_3$ ($TXA_3$) is formed in human platelets after dietary eicosapentaenoic acid (C20:5ω-3). *Biochem. Biophys. Res. Commun.* **116:** 1091–1099.
16. Bills, T. K., Smith, J. B., and M. J. Silver. 1977. Selective release of arachidonic acid from the phospholipids of human platelets in response to thrombin. *J. Clin. Invest.* **60:** 1–6.
17. Lapetina, E. G. 1982. Regulation of arachidonic acid production: role of phospholipases C and $A_2$. *Trends Pharmacol. Sci.* **3:** 115–118.
18. Lapetina, E. G., and W. Siess. 1983. The role of phospholipase C in platelet responses. *Life Sci.* **33:** 1011–1018.
19. Siess, W., and E. G. Lapetina. 1983. Properties and distribution of phosphatidylinositol-specific phospholipase C in human and horse platelets. *Biochim. Biophys. Acta.* **752:** 329–338.
20. Majerus, P. W., S. M. Prescott, S. L. Hofmann, E. J. Neufeld, and D. B. Wilson. 1983. Uptake and release of arachidonate by platelets. *Adv. Prostaglandin Thromboxane Leukotriene Res.* **11:** 45–52.
21. Majerus, P. W. 1983. Arachidonate metabolism in vascular disorders. *J. Clin. Invest.* **72:** 1521–1525.
22. Neufeld, E. J., and P. W. Majerus. 1983. Arachidonic acid release and phosphatidic acid turnover in stimulated human platelets. *J. Biol. Chem.* **258:** 2461–2467.
23. Siess, W., P. C. Weber, and E. G. Lapetina. 1984. Activation of phospholipase C is dissociated from arachidonate metabolism during platelet shape change induced by thrombin or platelet-activating factor. *J. Biol. Chem.* **259:** 8286–8292.
24. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
25. Skipski, V. P., R. F. Peterson, and M. Barclay. 1964. Quantitative analysis of phospholipids by thin-layer chromatography. *Biochem. J.* **90:** 374–378.
26. Higgs, G.A., R. J. Flower, and J. R. Vane. 1979. A new approach to antiinflammatory drugs. *Biochem. Pharmacol.* **28:** 1959–1961.
27. Bills, T. K., J. B. Smith, and M. J. Silver. 1976. Metabolism of $^{14}C$ arachidonic acid by human platelets. *Biochim. Biophys. Acta.* **424:** 303–314.
28. Lapetina, E. G., and P. Cuatrecasas. 1979. Stimulation of phosphatidic acid production in platelets precedes the formation of arachidonate and parallels the release of serotonin. *Biochim. Biophys. Acta.* **573:** 394–402.
29. Brox, J. H., J. E. Killie, S. Gunnes, and A. Nordoy. 1981. The effect of cod liver oil and corn oil on platelets and vessel wall in man. *Thromb. Haemostasis.* **46:** 604–611.
30. Galloway, J., B. E. Woodcock, V. Sawhney, M. Greaves, R. G. G. Russell, and F. E. Preston. 1983. A study of the fatty acid composition of platelet membrane phospholipids after dietary supplementation with a fish oil: failure of incorporation of eicosapentaenoic acid into phosphatidylinositol. *Thromb. Haemostasis.* **51:** 363 (abstract).
31. Ishinaga, M., M. Kakuta, H. Narita, and M. Kito. 1983. Inhibition of platelet aggregation by dietary linseed oil. *Agric. Biol. Chem.* **47:** 903–906.
32. Weiner, T. W., and H. Sprecher. 1984. Arachidonic acid and 5,7,11,14,17-eicosapentaenoic acid. Dietary manipulation of the levels of these acids in rat liver and platelet phospholipids and their incorporation into human platelet lipids. *Biochim. Biophys. Acta.* **792:** 293–303.
33. Hirai, A., T. Terano, T. Hamazaki, J. Sajiki, S. Kondo, A. Ozawa, T. Fujita, T. Miyamoto, Y. Tamura, and A. Kumagai. 1982. The effects of the oral administration of fish oil concentrate on the release and the metabolism of [$^{14}C$]arachidonic acid and [$^{14}C$]eicosapentaenoic acid by human platelets. *Thromb. Res.* **28:** 285–298.
34. Ahmed, A. A., V. G. Mahadevappa, and B. J. Holub. 1983. The turnover of [1-$^{14}C$]eicosapentaenoic acid among individual phospholipids of human platelets. *Nutr. Res.* **3:** 673–680.
35. Fischer, S., C. v. Schacky, W. Siess, T. Strasser, and P. C. Weber. 1984. Uptake, release and metabolism of docosahexaenoic acid (DHA, C22:6ω-3) in human platelets and neutrophils. *Biochem. Biophys. Res. Commun.* **120:** 907–918.
36. Siess, W., F. L. Siegel, and E. G. Lapetina. 1983. Arachidonic acid stimulates the formation of 1,2-diacylglycerol and phosphatidic acid in human platelets. *J. Biol. Chem.* **258:** 11236–11242.
37. Neufeld, E. J., D. B. Wilson, H. Sprecher, and P. W. Majerus. 1983. High affinity esterification of eicosanoid precursor fatty acids by platelets. *J. Clin. Invest.* **72:** 214–220.
38. Stubbs, C. D., and A. D. Smith. 1984. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochim. Biophys. Acta.* **779:** 89–137.
39. Broekman, M. J., J. W. Ward, and A. J. Marcus. 1981. Fatty acid composition of phosphatidylinositol and phos-

phatidic acid in stimulated platelets. *J. Biol. Chem.* **256:** 8271–8274.
40. Broekman, M. J., J. W. Ward, and A. J. Marcus. 1980. Phospholipid metabolism in stimulated human platelets. Changes in phosphatidylinositol, phosphatidic acid and lysophospholipids. *J. Clin. Invest.* **66:** 275–283.
41. Mahadevappa, V. G., and B. J. Holub. 1982. The molecular species composition of individual diacyl phospholipids in human platelets. *Biochim. Biophys. Acta.* **713:** 73–79.
42. Marcus, A. J., H. L. Ullman, and L. B. Safier. 1969. Lipid composition of subcellular particles of human blood platelets. *J. Lipid Res.* **10:** 108–114.
43. McKean, M. L., J. B. Smith, and M. J. Silver. 1984. Formation of lysophosphatidylcholine by human platelets in response to thrombin. *J. Biol. Chem.* **256:** 1522–1524.
44. Siess, W., P. Cuatrecasas, and E. G. Lapetina. 1983. A role for cyclooxygenase products in the formation of phosphatidic acid in stimulated human platelets. *J. Biol. Chem.* **258:** 4683–4686.
45. Terano, T., A. Hirai, T. Hamazaki, S. Kobayashi, T. Fujita, Y. Tamura, and A. Kumagai. 1983. Efect of oral administration of highly purified eicosapentaenoic acid on platelet function, blood viscosity and red cell deformability in healthy human subjects. *Atherosclerosis.* **46:** 321–331.