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Metabolism of arachidonic acid by platelets: utilization of arachidonic acid by human platelets in presence of linoleic and dihomo-γ-linolenic acids

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With 3 figures and 6 tables

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Several physical and chemical stimuli induce irreversible platelet aggregation which is accompanied by the synthesis of prostaglandins E_2 and $F_{2\alpha}$ (24, 25). These end-products in prostaglandin synthesis do not themselves induce platelet aggregation (14) but their precursor, arachidonic acid (AA) and intermediate compounds (prostaglandin endoperoxides, PGG_2 and PGH_2) in prostaglandin biosynthesis do so (10, 23). As a matter of fact arachidonic acid owes its aggregation activity to prostaglandin endoperoxides which are derived from it. Recently a new dimension has been added to the understanding of platelet aggregation due to the discovery of thromboxanes, a new group of biologically active compounds derived from prostaglandin endoperoxides (9). Both the endoperoxides and thromboxanes are formed in human platelets from arachidonic acid released from platelet membrane phospholipids due to the action of activated phospholipase A_2 in response to stimuli and these compounds (PGG_2, PGH_2 and thromboxane A_2, TXA_2) are extremely potent aggregating agents (9, 10, 23). Thromboxane B_2 (TXB_2) formed non-enzymatically by incorporation of a molecule of water into TXA_2 is biologically inactive.

Arachidonic acid in its free form is present in platelets only in negligible amounts (3), but is present in fairly large amounts in the bound form in platelet membrane phospholipids (16). Usually the rate limiting factor of prostaglandin synthesis in a biological system is the availability of free percursor acid and platelet is no exception to this. When platelets are exposed to a stimulus, as for example collagen, prostaglandin endoperoxides and thromboxane A_2 are formed in sufficient amounts to induce

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Melvian et al. (Prostaglandins [abstr.], April 1978, vol. 15, No. 4, p. 706) have examined the alteration of profile and amount of prostaglandins and thromboxanes produced by platelets and adipocytes from rats that were fed 8 levels of dietary linoleate varying from 0–30% of total calorie intake for 1–6 months. The following results were obtained: PGE_2 with all linoleate levels increased 2-fold; TXB_2 increased 3-fold between 20–30% linoleate calories; and PGE_1, PGE_2, and $PGF_{2\alpha}$ increased linearly in adipocytes from rats fed 1–20% linoleate calories. These results thus indicate a complex relationship between dietary fat and prostaglandin and thromboxane synthesis.

irreversible platelet aggregation. An antagonist in prostaglandin synthesis inhibits prostaglandin formation by platelets (25) and also the aggregation associated with it (22) but it does not inhibit aggregation induced by prostaglandin endoperoxides (10, 23). It is thought that thromboxane A_2 is the true mediator of platelet aggregation and release (9), but some recent data of *Smith* and his group cast doubt on this supposition. These workers have shown that endoperoxides rearrange in platelet-rich plasma (PRP) into PGE_2 and prostaglandin D_2 (PGD_2) without appreciable formation of thromboxane B_2 and yet are able to cause platelet aggregation (26). This observation goes strongly in favour of the suggestion that endoperoxides may have their own activity in platelet aggregation.

This paper gives some data on the incorporation of radioactive AA into platelet phospholipids, its release in response to thrombin and effects of linoleic and dihomo-γ-linolenic acid on the platelet utilization of arachidonic acid with the aim to explain the antithrombotic activity of these two essential fatty acids.

Materials and methods

Arachidonic acid ($1-^{14}C$) (specific activity 60.2 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, dihomo-γ-linolenic acid ($1-^{14}C$) (specific activity 55 mCi/mmol) was purchased from New England Nuclear (NEN Chemicals) and linoleic and dihomo-γ-linolenic acids were purchased from Nu Check Prep, Inc., P. O. Box 172, Elysian, Minnesota. Prostaglandins E_2 and $F_{2\alpha}$ were obtained as generous gifts respectively from Prof. *D. A. van Dorp* of the Unilever Research, Vlaardingen, The Netherlands, and Dr. *Kazuo Sano* of the Ono Pharmaceutical Co. Ltd., Osaka, 541 Japan.

Preparation of platelets

Blood from healthy donors who had not taken aspirin at least two weeks before donating blood was collected into 3.8% sodium citrate containing 1 mM EDTA, blood and anticoagulant ratio being 9 : 1 by volume. Platelets were isolated from other blood particles by differential centrifugation at 250 × g for 10 min at room temperature. Siliconized glass ware was used throughout.

Preparation of salts of acids

Arachidonic, dihomo-γ-linolenic and linoleic acids were used as sodium salts prepared by adding 100 mM sodium carbonate and mixing under nitrogen to yield a water-clear solution.

Preparation of labelled platelet suspension

Platelet-rich plasma (PRP) was prepared as described earlier (3). In all 40 ml PRP was used for labelling the platelets by incubating PRP with 0.25 μCi of $1-^{14}C$ arachidonic acid dissolved in 40 μl ethanol for 1 h at 37 °C. The incubation mixture was cooled to 4 °C and sodium ethylenediamine tetraacetate (EDTA), pH 7.4, was added to a concentration of 1 mM followed by centrifugation at 4 °C for 20 min at 2000 × g. The supernatant containing excess of arachidonic acid was discarded, the platelet pellet was washed with saline and resuspended in tris-saline EDTA buffer (1 mM EDTA, 5 mM D-glucose, 0.134 M sodium chloride, 15 mM Tris. HCl, pH 7.4). The platelets were recentrifuged at 2000 × g for 20 min at 4 °C and finally resuspended in Tris-saline buffer (0.134 M sodium chloride, 5 mM D-glucose, 15 mM Tris. HCl, pH 7.4) (3) to a final concentration of 1×10^9 platelets per ml buffer.

Incubation

(A). Platelets were incubated either as platelet-rich plasma or as washed platelets. Blank determinations were conducted with identical platelet preparations preincubated with 2×10^{-4} M aspirin. After incubation, 50 µg of the prostaglandins were added followed by acidification to pH 3, with formic acid. In the case of endoperoxide determination no acidification was necessary on account of the formation of hydrochloric acid due to the hydrolysis of stannous chloride in aqueous medium.

(B). Platelets labelled with trace amounts of arachidonic acid and later suspended in Tris-saline buffer (1 ml suspension) were incubated with thrombin in saline at a final concentration of 5 U/ml for 5 min. Control was run at the same time by incubating labelled platelet suspensions only with saline.

Lipid extraction

Incubation mixtures containing plasma were extracted by adding an equal volume of saline followed by addition of formic acid to obtain a pH of 3. To this acidified mixture were added 2 volumes of absolute ethanol and then extracted three times with four volumes of chloroform. The mixed chloroform phase, after washing with water in order to remove formic acid, was separated by centrifugation and evaporated to a residue under nitrogen (29).

Incubation mixtures containing platelet suspensions free of plasma proteins were extracted with chloroform-methanol (2 : 1, v/v) after acidification to pH 3. The organic phase was separated from the aqueous phase. The aqueous phase was re-extracted with chloroform. The organic phase was mixed and evaporated to a residue under nitrogen. For the separation of the organic phase from the aqueous phase, centrifugation was employed.

Thin-layer chromatography (TLC)

(i) The extracts were subjected to TLC in the following succession: solvent (I) ethylacetate/iso-octane/acetic acid/water (110 : 20 : 10 : 100, v/v equilibrated for 1 h before using the organic phase) in which $PGF_{2\alpha}$ and PGE_2 moved with R_f values 0.34 and 0.49 respectively (4). The material of these two zones was extracted quantitatively into methanol and subjected to a further TLC using solvent (II) consisting of benzene/dioxane/acetic acid (20 : 20 : 1, v/v) in which $PGF_{2\alpha}$ and PGE_2 separated with R_f values 0.48 and 0.63 respectively (7). The material from these two zones was extracted with methanol and subjected to a third TLC separation using solvent (III) consisting of chloroform/methanol/acetic acid/water (90 : 9 : 1 : 0.65 v/v) in which $PGF_{2\alpha}$ and PGE_2 moved with R_f values 0.20 and 0.34 respectively (19). The R_f values of thromboxane B_2 (TXB_2) in the first two solvents were respectively 0.60 and 0.72 thus clearly separating itself from PGE_2 and $PGF_{2\alpha}$ *(ii)*. For the separation of other oxygenated products of arachidonic acid a different scheme was used. The incubation extract was subjected to a first TLC on silica gel G using solvent (IV) consisting of hexane/diethylether/acetic acid (80 : 20 : 1, v/v) in which all the prostaglandins and thromboxane B_2 remained at the application line (R_f 0.00) while hydroxy acids (HHT and HETE) moved to R_f value 0.1–0.13. Excess arachidonic acid (R_f 0.30) was separated from these compounds (15). The prostaglandins and thromboxane B_2, and hydroxy acids were further resolved separately on silica gel G and silver nitrate coated silica gel G [solvents (V) chloroform/methanol/acetic acid, 90 : 5 : 5 v/v; (VI) ethylacetate/acetic acid/iso-octane/water, 80 : 20 : 50 : 100 v/v respectively). In solvent (V), TXB_2 moved discretely between PGE_2 and $PGF_{2\alpha}$ (11). HHT and HETE were resolved by argentation TLC (27).

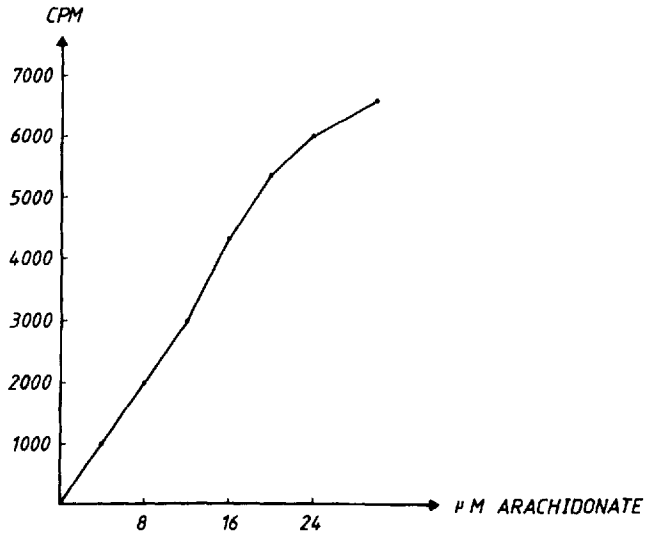

Fig. 1. Human platelet prostaglandin E_2 (PGE_2) synthesis as a function of arachidonate concentration. Mean of triplicate experiments. Platelets 0.5×10^9, reduced glutathione (GSH) 1 mM, total incubation volume 1.2 ml, incubated for 30 min at 37 °C in Tris/saline buffer, pH 7.4.

Results

Distribution of radioactivity in phospholipids

There is a difference in the way arachidonic acid is utilized by platelets. With trace amounts of arachidonic acid in presence of plasma, i.e., incubating trace amounts of arachidonic acid with PRP, the radioactivity was mainly confined to phospholipids. Oxygenation products of arachidonic acid were not shown to be present in the supernatant plasma by standard TLC procedure. Examination of the platelet extract for the distribution of radioactivity showed that almost all (ca. 97%) of the uptaken radioactivity was present in the phospholipid zone whose further analysis showed that most of the radioactivity was confined to phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine and phosphatidylinositol – representing respectively 52.7, 18.1, 9.9 and 16.5% (Table 1).

Effect of thrombin on release of radioactivity from labelled platelet preparation

When arachidonic acid labelled platelet suspensions were treated with thrombin for 5 min at 37 °C, decrease in radioactivities were observed mainly in the PC and phosphatidylserine/phosphatidylinositol fractions. Of the total loss of radioactivity, 68% was from PC and 21.6% from PI and 10% from PE fractions. Phosphatidylserine seemed to lose no radioactivity in presence of thrombin. Incubation of such platelet preparations with prostaglandin synthetase inhibitor-aspirin did not affect the release (Table 1).

Table 1. Radioactive arachidonic acid incorporation into platelet phospholipids and its loss induced by thrombin and accounted for mainly by the generation of oxygenation products of arachidonic acid by the action of platelet prostaglandin synthetase. Labelled platelet suspension (1 ml containing 1×10^9 platelets) were treated with either saline or thrombin and incubated for 5 min at 37 °C. The lipids from incubation medium were extracted, subjected to TLC and radioactivity of various fractions measured. Three determinations were made in each case.

Lipids	Radioactivity (CPM) mean ± SD				
	Control (Saline)	Thrombin	Loss	Gain	Aspirin*)
Phosphatidylcholine	15678 ± 645	12480 ± 936	3198		12638
Phosphatidylinositol	5375 ± 186	4359 ± 293	1016		4124
Phosphatidylserine	2961 ± 179	3117 ± 431	–		3241
Phosphatidylethanolamine	4925 ± 271	4436 ± 429	489		4568
			total loss 4703		
Arachidonic acid	96 ± 22	276 ± 86		180	1046
HHT	286 ± 52	1869 ± 356		1583	415
HETE	232 ± 95	1350 ± 161		1118	2978
Other compounds	216 ± 140	1684 ± 253		1468	318
				total gain 4369	

*) Preincubated for 5 min before addition of thrombin, mean of two determinations.

HHT 17 C-(ω6) hydroxy acid; HETE 20 C-(ω9) hydroxy acid; other compounds constitute together prostaglandins, thromboxane B_2 and other unknown products (see ref. 27).

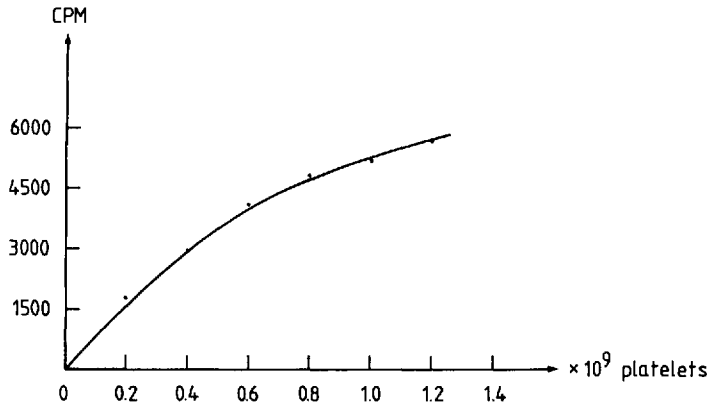

Fig. 2. Human platelet PGE_2 synthesis as a function of platelet concentration. Mean of triplicate experiments. GSH 1 mM, arachidonate 16 μM, incubation volume 1.5 ml, incubated for 30 min at 37 °C in Tris/saline buffer, pH 7.4.

Platelet PGE_2 synthesis as a function of arachidonate concentration, platelet concentration and pH

The results are shown in figures 1, 2 and 3. As expected the amount of PGE_2 synthesized depended on the amount of the substrate and platelet concentration. Maximum PGE_2 synthesis was observed at pH 8.5. The identity of the PGE_2 biosynthesized in human platelets was established by treating the material obtained from the PGE_2 zone from the third and the last TLC with alkali and subjecting the reaction products to standard TLC

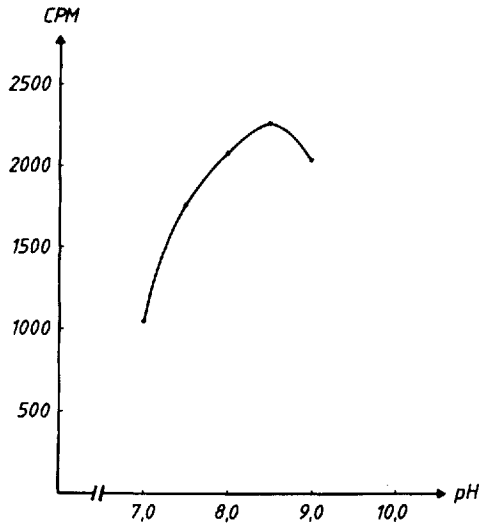

Fig. 3. Human platelet PGE_2 synthesis as a function of pH. Mean of triplicate experiments. Platelets 0.5 × 10^9, GSH 1 mM, arachidonate 8 μM, incubation volume 1.2 ml, incubated for 30 min at 37 °C in Tris/saline buffer.

Table 2. Conversion of prostaglandin E_2 (PGE_2) into prostaglandin B_2 (PGB_2) by treatment with a base.

TLC separation	Radioactivity (CPM)	
	PGE_2 zone	PGB_2 zone
Reaction product (I) → (II) → (III)	6796	315
Material present in the PGE zone (III) treated with KOH	104	6432

Platelets 0.5×10^9, reduced glutathione 1 mM, arachidonate 25 µM, total incubation volume 1.2 ml, incubated for 30 min at 30 °C in Tris/saline buffer, pH 7.4. The solvent systems used in the TLC separations are indicated in parentheses.

procedure. A major portion of the radioactivity was confined to the PGB_2 zone as shown in Table 2.

Effect of linoleate on the utilization of arachidonic acid by human platelets

With washed human platelet suspensions effect of linoleate on the metabolization of arachidonic acid was examined. Various linoleate concentrations (0.1–1.6 mM) were used. No effect of linoleate was observed on the formation of $PGF_{2\alpha}$, PGD_2 and thromboxane B_2 at any of its concentrations used except only at 1.6 mM linoleate concentration at which the quantity of TXB_2 synthesized was found to be reduced. While the formation of HHT and HETE remained unaffected at from 0.1 to 0.4 mM linoleate concentrations, the quantity of HHT was reduced and that of HETE increased at 0.8 and 1.6 mM linoleate concentrations (Table 3a). In another experiment done previously washed platelet preparations and platelets as in PRP were used to see the effect of linoleate on the PGE_2 synthesis from exogenous arachidonate. Results are shown in Tables 3b, 3c.

Assay of prostaglandin endoperoxides formed by human platelets from added arachidonate in presence of linoleate

From Tables 4a, 4b it is obvious that endoperoxide formation remained unaffected at lower linoleate concentrations (0.1–0.4 mM) but its formation was reduced at higher concentrations (0.8 and 1.6 mM).

Effect of dihomo-γ-linolenate on the utilization of arachidonic acid by human platelets

15 min incubation. When the ratios of arachidonate to dihomo-γ-linolenate were 1 : 2 and 1 : 4, there was found reduced synthesis of PGE_2 (by ca. 50 and 80%), thromboxane B_2 (by ca. 40 and 70%) and $PGF_{2\alpha}$ (by ca. 54 and 82%). However, when these two prostaglandin precursor acids were present in equimolecular proportions, dihomo-γ-linolenate did not affect the synthesis of these compounds (Table 5).

45 seconds incubation. When these acids were present in 1 : 4 ratio, reduced synthesis of these compounds was observed. But when present in 1 : 1 and 1 : 2 ratios, no change was noticed (Table 5).

Table 3a. Effect of linoleic acid on platelet utilization of arachidonic acid. Washed platelets were prepared as described under Methods (ref. 3). 140 µl platelet suspension (1.4×10^8 platelets) were incubated with various concentrations (0.1–1.6 mM) of linoleate for 20 min followed by addition of 25 µl of a mixture of radioactive + non-radioactive (1 : 10) arachidonate (final concentration in the incubation medium 0.44 mM) and incubated for an additional 10 min. Incubations were done at 37 °C. Total incubation volume was 200 µl. Reaction was terminated by adding 1 ml 0.9% sodium chloride and 20 µl N HCl. Extraction was done twice with 2 volumes of ether. Final extraction was done by adding 1 volume of absolute ethanol and the mixture extracted with 2 volumes of chloroform twice. HCl was removed by a minimum amount of water. Organic phases were mixed and residue obtained by evaporation under N_2.

Conditions	Compounds (CPM)					
	PGE_2	$PGF_{2\alpha}$	PGD_2	HHT	HETE	TXB_2
Control (linoleate absent)	992 ± 70	252 ± 22	275 ± 32	33878 ± 2383	29426 ± 1604	31370 ± 1817
+0.1 mM linoleate	894 ± 181	188 ± 11[a]	×	31239 ± 1826	31149 ± 1897	30363 ± 1269
+0.2 mM linoleate	905 ± 90	196 ± 19[a]	×	32247 ± 1363	28556 ± 1059	30543 ± 591
+0.4 mM linoleate	1343 ± 148[a]	247 ± 40	×	31582 ± 738	30673 ± 1074	30920 ± 1417
+0.8 mM linoleate	1956 ± 196[e]	236 ± 31	×	27909 ± 2554[a]	33712 ± 2218[a]	30083 ± 1919
+1.6 mM linoleate	2475 ± 267[f]	×	225 ± 41	19146 ± 2443[c]	39295 ± 1750[c]	26442 ± 441[d]

× counts below 150 CPM a = $p < 0.05$ b = $p < 0.025$ c = $p < 0.01$ d = $p < 0.025$ e = $p < 0.0125$ f = $p < 0.005$

Table 3b. Formation of PGE_2 by human platelets from added arachidonate in absence and presence of linoleate.

Washed platelet incubation

Conditions	CPM (PGE_2)
Blank (linoleate not present)	3429 ± 177
0.1 mM linoleate	3204 ± 137
0.2 mM linoleate	2987 ± 155[a]
0.4 mM linoleate	3596 ± 337
0.8 mM linoleate	4747 ± 380[b]
1.6 mM linoleate	8592 ± 969[c]

Platelets 1.0×10^9, GSH 1 mM, arachidonate 0.55 mM (radioactive : not radioactive = 1 : 10), incubation volume 1.2 ml. Samples were first incubated with linoleate in appropriate concentrations for 30 min at 37 °C followed by addition of GSH and arachidonate and incubated for an additional 30 min. Mean of three determinations.

a = $p < 0.025$ b = $p < 0.01$ c = $p < 0.005$

Utilization of arachidonic and dihomo-γ-linolenic acid by human platelets

In incubations where platelets were incubated separately with either of these prostaglandin precursors, different ways of utilization of these acids became obvious. As shown in Table 6 endoperoxides and thromboxane formation was many times more from arachidonate compared to the corresponding compounds formed from dihomo-γ-linolenate. On the contrary the amounts of PGE_1, $PGF_{1\alpha}$ and PGD_1 formed were 2–3 times more than similar compounds of the 2 series.

Discussion

It is well established that when prostaglandin precursor acids are added to platelet preparations, either as in PRP or in washed suspensions, they are converted into a variety of products. The initially reported pro-

Table 3c. Formation of PGE_2 by human platelets from added arachidonate in absence and presence of linoleate.

PRP incubation

Conditions	CPM (PGE_2)
Blank (linoleate absent)	562 ± 59
0.1 mM linoleate	463 ± 62
0.2 mM linoleate	533 ± 68
0.4 mM linoleate	605 ± 86
0.8 mM linoleate	815 ± 65 $p < 0.025$

PRP 1 ml (2.32×10^8 platelets), GSH 1 mM, arachidonate 0.55 mM (radioactive : not radioactive = 1 : 10), incubation volume 1.2 ml. Samples were first incubated with linoleate in appropriate concentrations for 30 min at 37 °C followed by addition of GSH and arachidonate and incubated for an additional 30 min. Mean of three determinations.

Table 4a. Assay of prostaglandin endoperoxides formed in human platelets from added arachidonate in presence of linoleate.

Conditions	CPM ($PGF_{2\alpha}$)
Blank (linoleate absent)	469 ± 38
0.25 mM linoleate	457 ± 66
0.40 mM linoleate	422 ± 86

Platelet (suspension) 0.2×10^9, arachidonate 0.29 mM (radioactive : not radioactive = 1 : 6), incubation volume 1.2 ml. Samples were first incubated with linoleate in appropriate concentrations for 30 min at 37 °C followed by incubation with arachidonate for 45 seconds and addition of 5 ml stannous chloride (0.5%) in ethanol. Mean of three determinations.

ducts, PGE_2 and $PGE_{2\alpha}$ constitute a small fraction when compared with the quantities of other products formed. When platelets are exposed to free fatty acids, they are rapidly taken up by them (1, 6). These fatty acids are usually incorporated into the membrane phospholipids. Free fatty acids in very small amounts accumulate in the platelets.

There is a difference in the way arachidonic acid is utilized by platelets and this is determined by the nature of the suspending medium and the quantity of the acid added. Thus added in small amounts to platelet-rich plasma, almost all arachidonic acid taken up by the platelets is confined to membrane phospholipids and is not accessible to the platelet prostaglandin synthetase system. This is in accordance with the observations of others that plasma or albumin inhibits the metabolism of arachidonic acid by platelets (2, 21). This could be due to the binding of prostaglandin precursors to albumin in plasma which prevents them from entering platelets. This may explain why high concentrations of arachidonate (0.5–1 mM) are needed to induce platelet aggregation in platelet-rich plasma (21). From this it would imply that when platelets are activated by a stimulus, phospholipase A_2 becomes active and cleaves the precursor acid from the membrane phospholipids, the precursor thus remaining within the platelets to be utilized by the platelet prostaglandin synthetase. When arachidonic acid is added to a washed platelet suspension free of plasma proteins and thus also free of albumin, prostaglandin synthesis (8,

Table 4b. Assay of prostaglandin endoperoxides formed in human platelets from added arachidonate in presence of linoleate.

Conditions	CPM ($PGF_{2\alpha}$)
Blank (linoleate absent)	668 ± 23
0.8 mM linoleate	526 ± 110[a]
1.6 mM linoleate	360 ± 59[b]

Platelet (suspension) 0.36×10^9, arachidonate 0.29 mM (radioactive : not radioactive = 1 : 6), incubation volume 1.2 ml. Condition of incubation and termination of reaction as above.

a = $p < 0.05$ b = $p < 0.0025$

Table 5. Effect of dihomo-γ-linolenic on the utilization of arachidonic acid by human platelets. To 1 ml of washed platelet suspension containing 0.3×10^9 platelets was added a mixture of sodium salts of radioactive arachidonic and dihomo-γ-linolenic acid in appropriate proportions. The control and other tubes contained 20 μM arachidonate. Total incubation volume was 1.1 ml. Incubation was done either for 15 min or for 45 seconds and reaction terminated followed by extraction and separation as described in the text.

Compounds	Radioactivity (CPM) Mean ± SD							
	15 min				45 seconds			
	Control	1 : 1*	1 : 2*	1 : 4*	Control	1 : 1*	1 : 2*	1 : 4*
PGE_2	8653 ± 590	8549 ± 696	4137[b] ± 621	1775[b] ± 206	2088 ± 67	2047 ± 171	2125 ± 167	1014[b] ± 159
TXB_2	38553 ± 1582	37540 ± 4074	22746[d] ± 2562	10794[b] ± 1270	25188 ± 1041	26314 ± 1058	24841 ± 858	15915[a] ± 1577
$PGF_{2\alpha}$	3268 ± 110	3148 ± 340	1438[c] ± 213	621[b] ± 73	2076 ± 301	2185 ± 261	1983 ± 294	952[a] ± 73

a = $p < 0.025$ b = $p < 0.001$ c = $p < 0.0025$ d = $p < 0.0125$
* ratio of arachidonic acid to dihomo-γ-linolenic acid.

Table 6. Different utilization of arachidonic and dihomo-γ-linolenic acids by human platelet prostaglandin synthetase. 140 μl washed platelet suspension were incubated separately with a mixture of radioactive + not radioactive (1 : 10) of sodium arachidonate or sodium dihomo-γ-linolenate (final concentration in the incubation medium 0.44 mM) and incubated for 10 min. Reaction termination and other procedures were followed as described in Table 3a. In the case of endoperoxide assay, incubation was allowed only for 30 seconds and reaction was terminated by addition of a 5 ml of a 5% ethanolic stannous chloride solution.

Radioactivity (CPM)									
Arachidonic acid incubation					Dihomo-γ-linolenic acid incubation				
PGG_2/PGH_2	TXB_2*	PGE_2*	PGD_2*	$PGF_{2\alpha}$*	PGG_1/PGH_1	TXB_1	PGE_1	PGD_1	$PGF_{1\alpha}$
1342	31370	992	275	252	418	2946	2648	567	696

* data taken from Table 3a

17) β-oxidation (3) and lipoxygenation (8, 17) takes place. Arachidonic acid is different from other fatty acids in the way that it does not enter into triglycerides while other acids do so in the platelets as is our experience with linoleic and palmitic acids (unpublished data).

There are two independent reports (3, 20) on the incorporation of radioactive arachidonic acid into platelet phospholipids and its release from these on treatment with thrombin. *Bills* et al. (3) have reported that incorporation of arachidonic acid into phospholipids follows the following order: PC > PI > PE. This has not been found to be so by *Schoene* and *Iacono* (20) who found arachidonic acid incorporation to take place in the following order: PE > PI > PC > PS.

Bills et. al. (3) have reported that on treatment with thrombin a major portion of arachidonic acid comes from PC followed by PS + PI; PE seems to contribute nothing. On the contrary *Schoene* and *Iacono* (20) have reported that most of the arachidonic acid released comes from PI fraction, PE contributing no arachidonic acid. Our results approximate those of *Bills* et al. (3). Aspirin showed no effect on the release of arachidonic acid from the labelled platelet phospholipids (Table 1).

A comparison of the data on the incorporation of arachidonic acid into platelet phospholipids and the proportion of endogenous arachidonic acid in platelet phospholipids suggests that there is a difference between the two in that in the endogenous phospholipids, PE comes next to PC and also sphingomyelin contributes to about 15% of the total. It should be noted, however, that the phospholipids which incorporate arachidonic acid most, that is PC and PI, are the chief source of release of this acid when labelled platelets are challenged with thrombin; PC losing the greatest amount of labelled arachidonic acid.

Effects of linoleic and dihomo-γ-linolenic acids on platelet prostaglandin synthesis from added arachidonic acid together with the utilization of the latter (AA) has been examined with the aim to see in what way these two acids behave antiaggregatory. These two acids seem to act differently from this points of view.

Linoleic acid at its various concentrations (0.1–1.6 mM) did not show any effect on the synthesis of $PGF_{2\alpha}$ and PGD_2. At 0.8 mM linoleate concentration, significantly more PGE_2 was synthesized. As $PGF_{2\alpha}$ and PGE_2 do not themselves induce platelet aggregation, a change in their synthetic rate has no significance in this context. PGD_2 is strongly antiaggregatory and its synthesis remained unaffected in presence of linoleate. Two other oxygenation products of AA which are of significance in platelet aggregation and its control are prostaglandin endoperoxides and thromboxan A_2 (measured in the present study as TXB_2). Linoleate does not seem to affect the synthesis of endoperoxides at its lower concentrations (0.1–0.4 mM) while it significantly reduced the synthesis of these compounds at 0.8 and 1.6 mM concentrations. Although the formation of TXB_2 remained unchanged at from 0.1 to 0.8 mM linoleate concentrations, at 1.6 mM linoleate concentration it was found to be reduced. But this may not be of any significance because its reduced synthesis could be due to reduction in the quantities of endoperoxides synthesized at this concentration. Thus the present *in vitro* study points out that linoleic acids'

antithrombotic activity could *partly* be due to reduced endoperoxide synthesis, i.e. by its direct action on the platelet cyclo-oxygenase.

That high amounts of linoleic acid intake helps in decreasing thrombosis tendency in rats has been demonstrated by *Vergroesen* (30). He introduced a loop-shaped polyethylene cannula, (aorta loop) designed by *Honstra* (13), into the abdominal aorta of male rats and observed the production and growth of a fibrin-poor, platelet-rich mural thrombus induced by endothelial damage and flow disturbances which occluded the cannula after 5 days (obstruction time, OT = 120 h). When rats received fat-free diet, a low thrombosis tendency was observed (OT = 175). In these rats EFA-deficiency was observed, as determined by water vapour loss *in vivo* (28) and the fatty acid pattern of total serum lipids (12). But when the diet of these rats was supplemented by 5 cal% sunflower seed oil or (3 cal% linoleic acid), EFA-deficiency was cured, but this increased the thrombosis tendency (OT = 95 h). When the dietary linoleic acid content was gradually increased from 3 to 40 cal%, the OT was raised from 95 to 175 h. This strongly suggests that linoleic acid might behave antithrombotic in high dietary amounts.

From the data obtained on the synthesis of PGE_2 and other oxygenation products formed by platelets from added arachidonate in the presence of dihomo-γ-linolenate, it appears that these two acids compete with each other as substrates for the platelet prostaglandin synthetase. Thus a control in the generation of thromboxane A_2 and PGG_2 and PGH_2, compounds responsible for platelet aggregation and release reaction, may be exercised due to the simultaneous presence of dihomo-γ-linolenate. This might be assisted in another way in that thromboxane A_1 and PGG_1 and PGH_1 formed from this acid are not aggregatory and also that PGE_1 formed from it is a potent antiaggregatory substance. As oral ingestion of dihomo-γ-linolenate has been shown to enrich plasma lipids and platelet phospholipids at the expence of other fatty acids including arachidonic acid in rats (5) and in rabbits (18), *Willis* et al. (32) have suggested that for the prevention of arterial thrombosis (heart attack and stroke) in man, ingestion of dihomo-γ-linolenate in gram quantities may be undertaken. The substrate competition observed in *in vitro* studies and also similar results obtained by others (31) support the hypothesis of *Willis*. The encouraging part is that *Willis* and co-workers (32) did not observe any gross toxic effects in any of the animal studies with dihomo-γ-linolenate. In a way dihomo-γ-linolenate combines the anti-thrombotic effects of aspirin and PGE_1 with the disadvantages of neither.

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Summary

In vitro human platelet prostaglandin synthesis has been studied from added radioactive arachidonic acid (i) as function of substrate concentration, (ii) as function of platelet concentration and (iii) as function of pH. Platelets, as in platelet rich plasma when labelled with arachidonic acid, washed and treated with thrombin, released radioactivity mainly from phosphatidylcholine and phosphatidylinositol. The released radioactivity was mostly accounted for by the formation of the

previously identified oxygenation products of arachidonic acid. Platelet utilization of arachidonic acid was also studied in presence of linoleic and dihomo-γ-linolenic acids, the two essential fatty acids known for antithrombotic effect. At its high concentrations linoleic acid decreased platelet cyclo-oxygenase activity as seen by a decreased formation of endoperoxides from arachidonic acid. Dihomo-γ-linolenic acid was found to be a mutually competitive substrate with arachidonic acid for the platelet prostaglandin synthetase thus causing reduced utilization of arachidonic acid as shown by measuring the various oxygenation products of arachidonic acid. These two acids were utilized differently by platelet prostaglandin synthetase.

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