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Procoagulant activity of platelet arachidonic acid metabolites

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1 Arachidonate metabolites have been extracted from indomethacin-treated human platelets after incubation with arachidonic acid.

2 After separation from platelet phospholipids, the extracts promoted the generation of large amounts of thrombin in normal plasma, but not in plasma devoid of lipoproteins.

3 The procoagulant activity was associated with a minor component of the mixture, which was active at concentrations below 10 $\mu g\,ml^{-1}$.

4 The activity was similar to that of autoxidised arachidonic acid previously described.

5 Platelet arachidonic acid metabolites could play a role in the coagulation system.

Introduction

Platelet arachidonic acid metabolism can follow two main enzymatic pathways. The cyclo-oxygenase enzyme system gives rise to unstable thromboxane A_2 (TXA_2), a powerful inducer of platelet aggregation and a potent vasoconstrictor, via the cyclic endoperoxides, prostaglandin G_2 (PGG_2) and PGH_2. TXA_2 breaks down non-enzymatically to stable TXB_2 (Hamberg *et al.*, 1975). Lipoxygenase, the second enzyme system involved in arachidonic acid metabolism by platelets, is now known to synthesize a number of products. These include 12-hydroperoxyeicosatetraenoic acid (12-HPETE) and its degradation product, 12-hydroxyeicosa-tetraenoic acid (12-HETE) (Nugteren, 1975) and 15-HPETE, a more recently discovered product of platelet lipoxygenase (Wong *et al.*, 1985). Two trihydroxyeicosatrienoic acids, collectively termed THETA, and an epoxy-hydroxyeicosatrienoic acid (EPHETA, shown to exert a chemotactic influence on rabbit heterophils) have also been described as lipoxygenase products from washed human platelets and washed platelets from several other species (Jones *et al.*, 1979; Walker *et al.*, 1980). However, the physiological role of the platelet lipoxygenase system remains uncertain.

Hydroperoxyacids such as 12-HPETE can also be produced by free-radical-induced lipid peroxidation (Pryor *et al.*, 1976; Porter *et al.*, 1979) and lipid peroxides produced in this manner have been shown to have potent biological activities (Schauenstein, 1967). Previous work from our laboratory showed that products from autoxidation of arachidonic acid are highly active in promoting thrombin generation in platelet-free plasma (Barrowcliffe *et al.*, 1975) and that this procoagulant activity requires the presence of triglyceride-rich lipoproteins (Barrowcliffe *et al.*, 1984).

In the present study, we have investigated the procoagulant properties of arachidonic acid metabolites produced by platelets and compared them with those of lipid peroxides produced by autoxidation of arachidonic acid.

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Methods

Platelets

Platelets were kindly provided by the North London Blood Transfusion Centre, Edgware, Middx., in the form of platelet concentrates which had just passed their expiry date for clinical use. They were centrifuged then washed twice with a solution containing: 0.15 M NaCl, 4 mM Na_2 EDTA, 4 mM KCl, 10 mM, HEPES, pH 6.2 (Menashi *et al.*, 1981), then resuspended in phosphate buffered saline, pH 7.4, to a concentration of approximately $6 \times 10^8\,ml^{-1}$.

Preparation and extraction of arachidonate metabolites

To 30 ml of the platelet suspension were added 15 mg arachidonic acid (99%, Sigma, Poole, Dorset), 5 μCi [^{14}C]-arachidonic acid and 300 μg indomethacin dissolved in 60 μl ethanol. The suspension was incubated at 37°C for 30 min. Enzyme activity was arrested by addition of 150 ml of ice-cold ethanol. The ethanolic solution was evaporated to dryness under reduced pressure, and the residue taken up in 100 ml phosphate/citric acid buffer, pH 4.0, then partitioned twice with equal volumes of ethyl acetate. The ethyl acetate was evaporated and the residue dissolved in 70% v/v ethanol. The aqueous ethanol was partitioned twice with equal volumes of petroleum ether BP, 60–80°C, to remove neutral lipids and unreacted arachidonic acid. The ethanolic solution was evaporated to dryness and the residue dissolved in 1.0 ml methanol for coagulation testing. In control experiments, indomethacin, or indomethacin and arachidonic acid, were omitted from the incubation mixtures, which were then extracted in exactly the same way.

Thin layer chromatography

System 1 The F VI solvent system (Andersen, 1969), originally described for the separation of classical prostaglandins, was found to be a convenient system for the preliminary separation of arachidonic acid metabolites from platelet phospholipids. Samples were applied in methanol and the chromatogram was developed with ethyl acetate:acetone: acetic acid (90:10:1). Plates were precoated silica, $60F_{254}$ (Merck, Darmstadt, F.R.G.). The plate was divided into two areas: the origin (including 1 cm above), which contains most of the phospholipid, and the rest of the plate, which contains the arachidonic acid metabolites. After scraping off the silica, material in these two fractions was eluted with methanol, dried out and reconstituted in a known volume of methanol for testing.

System 2 Samples were applied in methanol and the chromatogram developed with diisobutyl ketone: acetic acid:H_2O (40:25:5) (Nicholls, 1963). Plates were as for system 1. One cm fractions were scraped off and material from each fraction was eluted with 1 ml methanol for coagulation testing.

Clotting tests

The activated partial thromboplastin time (APTT) was performed as previously described (Barrowcliffe *et al.*, 1975). The generation of thrombin over a period of up to 20 min after recalcification of plasma in the presence of the test sample was followed by subsampling into fibrinogen and measurement of clotting time (Barrowcliffe *et al.*, 1984). Normal plasma, kindly provided by the North London Blood Transfusion Centre, Edgware, Middx., was centrifuged at 100,000 *g* before storage at −40°C. Lipoprotein-free plasma was prepared by density-gradient ultracentrifugation (Barrowcliffe *et al.*, 1984).

In all clotting tests, the activity of the arachidonate metabolites was compared with that of a standard procoagulant phospholipid preparation, NIBSC 83/555, extracted from bovine brain (Barrowcliffe *et al.*, 1982).

Organic phosphorus was estimated by the method of Raheja *et al.* (1973).

Results

In initial experiments, extracts were tested without t.l.c. separation and high activity was seen in all clotting tests, both with the platelets and arachidonic acid extracts and with the platelet control (i.e. no added arachidonic acid). It seemed likely that some platelet phospholipid was being carried over in the extracts, and this was confirmed in a subsequent set of experiments in which the extracts were separated into two fractions, the origin and remaining fraction, by the t.l.c. system 1, using the F VI solvent system. Table 1 shows the results of organic phosphorus and clotting assays on these two fractions.

The material remaining at the origin had a high phospholipid content and behaved essentially as a procoagulant phospholipid, producing large amounts of thrombin in both normal and lipoprotein-free plasma and shortening the APTT. By contrast, the fraction from the remainder of the t.l.c. plate, though generating large amounts of thrombin in normal plasma was virtually inactive in lipoprotein-free plasma and did not shorten the APTT.

Figure 1 shows a typical thrombin generation curve: in three separate experiments, the average peak thrombin was 23.1 units ml^{-1}, after subtracting the blank value. The average % conversion of arachidonic acid was 17%. Most of the material was recovered in the arachidonate metabolite fraction, and the average yield was estimated at 2.5 mg.

The material from one experiment was further fractionated by t.l.c. system 2. Figure 2 shows radioactivity measurements and procoagulant activity in the thrombin generation test. Virtually all the activity was recovered in a single fraction, which represented only a minor portion of the total radioactivity, with an estimated amount of 113 μg (4% of the total). As shown in Table 2, this fraction was active down to a plasma concentration of 4.5 μg ml^{-1}.

Table 1 Phospholipid content and procoagulant activities of t.l.c. system 1 fractions from arachidonic acid metabolite extracts

	Origin	*Remaining fraction*	*Buffer*	*PL*
Phospholipid ($\mu g\ ml^{-1}$)	5.5	0.4	—	0.03
APTT (S)	63.1	161.6	149.8	38.0
Peak thrombin generated ($iu\ ml^{-1}$), normal plasma	23.2	18.7	3.5	16.5
Peak thrombin generated ($iu\ ml^{-1}$), LPF plasma	18.7	4.3	2.0	18.3

Abbreviations used: APTT = activated partial thromboplastin time, PL = standard procoagulant phospholipid, LPF = lipoprotein-free

Discussion

In any studies of organic solvent extracts of platelets, it is important to eliminate the contribution of platelet phospholipids, which are highly procoagulant (Zwaal & Hemker, 1982). Although ethyl acetate extraction of prostaglandins is commonly used (Greenwood & Kerry, 1979), the extracts were contaminated with phospholipid, as shown from initial clotting results and subsequent organic phosphorus analyses. As shown in Table 1, the t.l.c. system used was effective in removing more than 90% of this phospholipid, but the remaining material still had potent procoagulant activity in the thrombin generating system. However, unlike the phospholipid material remaining at the origin, the fraction containing arachidonate metabolites was virtually inactive in lipoprotein-free plasma and did not substitute for phospholipid in the APTT. Thus this arachidonate fraction behaves exactly like the autoxidised arachidonic acid previously studied (Barrowcliffe *et al.*, 1984), in that it requires plasma lipoproteins for its procoagulant activity. However, the platelet enzyme fraction is produced within 30 min incubation of arachidonic acid with

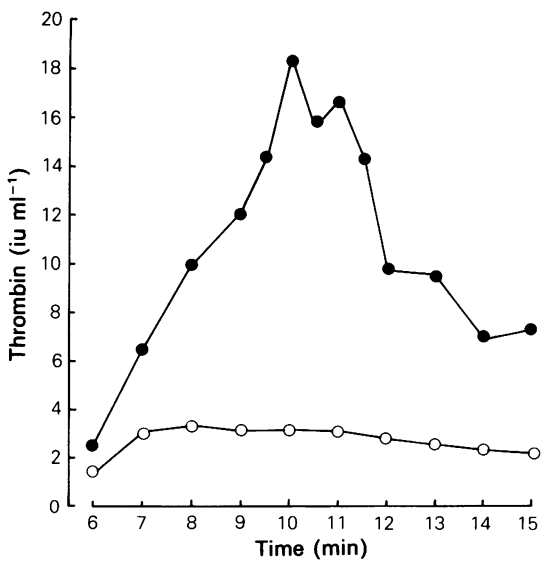

Figure 1 Thrombin generating activity of arachidonic acid metabolites (●) from t.l.c. system 1. (○) Represents control response to addition of buffer. One hundred μl of the methanolic solution (containing approximately 240 μg material) were dried down and reconstituted in 20 μl methanol and 480 μl 0.15 M NaCl. Plasma 0.5 ml and 25 mM $CaCl_2$ 0.5 ml were added and the thrombin generated recorded by subsampling into fibrinogen. Clotting times were converted into international units (iu) by comparison with the international standard for thrombin. No thrombin was generated between 0 and 6 min in these experiments.

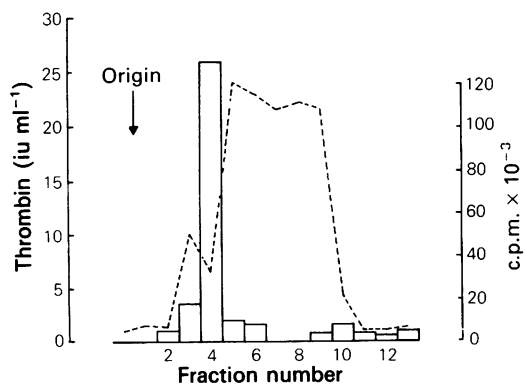

Figure 2 Procoagulant activity of t.l.c. system 2 fractions. Fractions were eluted with 1 ml methanol and 300 μl of each fraction tested in the thrombin generation system as described for Figure 1. The columns represent the thrombin generated and the broken line the amount of radioactivity.

Table 2 Procoagulant activity (thrombin generation) in Fraction 4 from t.l.c. separation (system 2) of arachidonic acid metabolites.

Amount tested (μl)	*Plasma conc.* (μg ml^{-1})	*Peak, thrombin* (iu ml^{-1})
300	67.8	30.0
100	22.6	28.3
50	11.3	24.1
20	4.5	16.6
0	0	3.8

platelets, whereas autoxidation requires four days for development of full procoagulant activity (Barrowcliffe *et al.*, 1975; 1984).

The concentration of total material in the arachidonic acid metabolite fraction was fairly high, but preliminary t.l.c. analysis indicated that the active fraction was a minor component of the mixture. Since cyclo-oxygenase was blocked by indomethacin in our experiments, it seems likely that the active material is one or more platelet lipoxygenase products. Although the major lipoxygenase metabolite of arachidonic acid is 12-HETE, several biological activities have been ascribed to minor metabolites such as EPHETA (Walker *et al.*, 1980) and 15-lipoxygenase products (Bild *et al.*, 1978; Vanderhoek *et al.*, 1980). Detailed structural analysis will be required to determine whether the procoagulant activity is associated with any of these products.

Although we have not identified the active component(s), our results show that platelets can develop potent procoagulant activity by metabolism of arachidonic acid, distinct from that due to phospholipids. These results could indicate a possible role for platelet arachidonic acid metabolites in the coagulation system.

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