Malonaldehyde Formation in Intact Platelets is Catalysed by Thromboxane Synthase

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Imidazole and compound L8027 (selective inhibitors of thromboxane synthase) produced parallel inhibition of malonaldehyde and thromboxane B₂ secretion induced by collagen or thrombin in gel-filtered suspensions of human platelets. Comparing the effects of these inhibitors and aspirin on secretion of granule constituents indicated that platelet degranulation depends mainly on thromboxane production; prostaglandin endoperoxides contributed little.

Platelet-aggregation responses associated with the secretion of platelet granule constituents (secondary aggregation) are modulated by prostaglandins and thromboxane A₂ (for review see Smith & Silver, 1976). Agents that induce secondary aggregation activate a phospholipase A₂, which liberates from membrane phospholipids arachidonic acid, the major substrate for the platelet enzyme cascade known as prostaglandin synthase (Blackwell *et al.*, 1977). Arachidonic acid is then metabolized by fatty acid cyclo-oxygenase (EC 1.14.99.1) to prostaglandin endoperoxides (prostaglandins G₂ and H₂), which are unstable in aqueous media and are rapidly converted into prostaglandins D₂, E₂ and F₂α, into thromboxane A₂, and into malonaldehyde plus 12-L-hydroxyheptadeca-5,8,10-trienoic acid (Willis *et al.*, 1974; Hamberg *et al.*, 1975). Prostaglandin endoperoxides and thromboxane A₂ are powerful inducers of platelet aggregation and secretion, but the relative importance of these endogenously produced labile substances in mediating platelet responses to other stimuli is under debate (Hamberg *et al.*, 1975; Needleman *et al.*, 1977; Fitzpatrick & Gorman, 1978).

The involvement of arachidonate metabolites in platelet function has led to the search for a convenient indicator of platelet prostaglandin synthase activity. Malonaldehyde formation can be readily assayed colorimetrically (Stuart *et al.*, 1975; Smith *et al.*, 1976; Macfarlane *et al.*, 1977) or fluorimetrically (McMillan *et al.*, 1977), but a major disadvantage of using this product as an assay for prostaglandin biosynthesis is that the pathway of malonaldehyde formation from arachidonic acid has not been elucidated. Diczfalussy *et al.* (1977) isolated and purified thromboxane synthase from human platelet homogenates and reported that this enzyme was responsible for the formation of malonaldehyde (and hydroxyheptadecatrienoic acid) as well as throm-

boxane B₂. There is, however, no evidence for this in intact cells. We have now investigated malonaldehyde and thromboxane B₂ production by gel-filtered platelets and studied the effects of selective inhibitors of fatty acid cyclo-oxygenase and of thromboxane synthase. In addition, by comparing the effects of these selective inhibitors on platelet degranulation responses, we have estimated the relative importance of prostaglandin endoperoxides and thromboxane A₂ in modulating platelet secretion.

Methods

Human blood was obtained by antecubital venepuncture from volunteers who had ingested no drugs for at least 7 days. One vol. of trisodium citrate (0.13 M) was added to 9 vol. of blood, and platelet-rich plasma was prepared by centrifugation (1500g at 20°C for 1 min). Plasma-free suspensions of platelets in calcium-free Tyrode buffer were prepared by gel filtration with Sepharose 2B (Salzman *et al.*, 1976). Platelet aggregation was measured photometrically (Born, 1962) in 0.1 ml or 0.15 ml samples (Gordon & Drummond, 1974). Platelets were counted with a Coulter model B electronic particle counter.

For measurement of secretion, platelet-rich plasma was preincubated at 37°C for 30 min with 1 μM-5-hydroxy[side chain-2-¹⁴C]tryptamine creatinine sulphate ([¹⁴C]serotonin) before gel filtration. Platelet aggregation, in 0.1 ml samples of gel-filtered platelets, was terminated by the addition of 0.4 ml of ice-cold 0.4% (w/v) EDTA in iso-osmotic saline and rapid centrifugation (14 700g for 30 s). Subsamples of cell-free supernatant were removed for fluorimetric measurement of lysosomal enzyme release (Gordon, 1975) or transferred into scintillation vials for determination of [¹⁴C]serotonin release (MacIntyre *et al.*, 1978).

For measurement of arachidonate metabolites platelet aggregation in 0.15 ml samples of gel-filtered platelets was terminated by the addition of 0.45 ml of 100% ethanol and rapid centrifugation. Subsamples of the cell-free supernatant were removed for fluorimetric measurement of malonaldehyde (McMillan *et al.*, 1977) or for radioimmunoassay of thromboxane B₂ (Sors *et al.*, 1978).

Materials

Bovine thrombin and tendon collagen were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. 5-Hydroxy-[side chain-2-¹⁴C]tryptamine creatinine sulphate (sp. radioactivity 58 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Aspirin was obtained from Addenbrookes Hospital Pharmacy, Cambridge, U.K., as acetylsalicylic acid powder and imidazole was from Koch-Light Laboratories, Colnbrook, Bucks., U.K.

2-Isopropylindol-3-yl 3-pyridyl ketone (compound L8027) was kindly given by Dr. M. Descamps, Labez Research Centre, Brussels, Belgium. Thromboxane B₂ was kindly given by Dr. J. E. Pike, Upjohn Co., Kalamazoo, MI, U.S.A. Thromboxane B₂ antiserum and tracer were a generous gift from Dr. H. Sors, Hopital Laennec, Paris, France.

Results and Discussion

Fig. 1 shows the effect of compound L8027 and imidazole on malonaldehyde and thromboxane B₂ production by gel-filtered platelets stimulated with thrombin and collagen. Both drugs produced parallel inhibition of malonaldehyde and thromboxane B₂ synthesis, whether induced by thrombin or collagen, although the absolute potencies of the drugs differed markedly. Inhibition of fatty acid cyclo-oxygenase, the initial enzyme of the prostaglandin synthesis pathway, blocks the formation of all products derived from prostaglandin endoperoxides, and therefore cyclo-oxygenase inhibitors such as aspirin (Rome *et al.*, 1976) produce the same pattern of inhibition as that shown in Fig. 1. However, at the concentrations used in the present study, compound L8027 and imidazole act selectively on thromboxane synthase and do not affect fatty acid cyclo-oxygenase (Gryglewski *et al.*, 1977; Needleman *et al.*, 1977). These results therefore indicate that in whole platelets, as in microsomal preparations, malonaldehyde and thromboxane B₂ are formed by the same enzyme.

Although the studies cited above showed that compound L8027 and imidazole do not act on cyclo-oxygenase, we wished to confirm this under our experimental conditions by establishing that these drugs were not acting like aspirin. Therefore, the

Fig. 1. Inhibition of thromboxane B₂ and malonaldehyde formation in intact platelets

Malonaldehyde (closed symbols) and thromboxane B₂ (open symbols) formation were measured in gel-filtered suspensions of human platelets as described in the text. Platelets were stimulated (a) by thrombin (1 unit/ml) or (b) by collagen (20 µg/ml). The amounts of arachidonate metabolites formed in control samples ranged from 0.1 to 0.67 nmol of malonaldehyde/10⁸ cells and 0.07 to 0.44 nmol of thromboxane B₂/10⁸ cells. Inhibitors were preincubated in the platelet suspensions for 2 min. Points shown are means for triplicate determinations, expressed as a percentage of control responses (i.e. with no inhibitor present). Symbols; ○ and ●, effect of compound L8027; △ and ▲, effect of imidazole.

Table 1. *Inhibition of platelet responses by drugs affecting arachidonate metabolism*

Thromboxane B₂ synthesis and secretion of [¹⁴C]serotonin and β -N-acetylglucosaminidase were measured in gel-filtered suspensions of human platelets as described in the text. Platelets were stimulated by collagen (20 μ g/ml). When present, drugs were added 2 min before collagen. Values are means $\pm$ S.E.M. ($n = 4$), expressed as a percentage of control responses (i.e. with no drug present). In control samples, 1.5–4.0 nmol of β -N-acetylglucosaminidase/10⁸ cells were secreted, and 0.12–0.38 nmol of thromboxane B₂/10⁸ cells were formed. Of the [¹⁴C]serotonin incorporated (about 0.25 nmol/10⁸ cells) 51–67% was secreted.

Response	Treatment...	Inhibition (% of control)			
		Control	Aspirin (1 mM)	Compound L8027 (1 μ M)	Imidazole (500 μ M)
Thromboxane synthesis		100 $\pm$ 5.8	1.8 $\pm$ 0.1	0.3 $\pm$ 0.05	0.2 $\pm$ 0.04
[¹⁴ C]Serotonin secretion		100 $\pm$ 6.7	21.3 $\pm$ 3.5	41.8 $\pm$ 4.6	40.3 $\pm$ 5.9
β -N-Acetylglucosaminidase secretion		100 $\pm$ 5.6	19.2 $\pm$ 4.1	42.5 $\pm$ 5.1	36.4 $\pm$ 4.2

effects of the three drugs on collagen-induced synthesis of thromboxane B₂, secretion of [¹⁴C]serotonin and secretion of lysosomal enzymes were compared to see if their pharmacological profiles were identical. All three drugs abolished thromboxane B₂ formation, but aspirin produced greater inhibition of the secretory responses than did compound L8027 or imidazole (Table 1). This is consistent with blockade of thromboxane synthase by compound L8027 and imidazole and blockade of cyclo-oxygenase by aspirin, because cyclo-oxygenase inhibition prevents the formation of both types of endogenous pro-secretory agent (prostaglandin endoperoxides and thromboxane A₂), whereas thromboxane synthase inhibition does not affect prostaglandin endoperoxide formation.

By comparing the inhibition of platelet secretion by cyclo-oxygenase and thromboxane synthase inhibitors, it is possible to estimate the relative importance of individual arachidonate metabolites. Compound L8027 produced approx. 60% inhibition of [¹⁴C]serotonin and lysosomal enzyme secretion, which presumably represents the component of the secretory response mediated by thromboxane A₂. Aspirin inhibited secretion by approx. 80%. The additional inhibition by aspirin presumably represents the maximal contribution of the prostaglandin endoperoxides to the degranulation processes.

The results of the present study support the concept that, in intact platelets, malonaldehyde and thromboxane B₂ are both derived via thromboxane synthase. Moreover we have demonstrated that both prostaglandin endoperoxides and thromboxane A₂ contribute to platelet secretion induced by collagen and that secretion dependent on thromboxane A₂ generation exceeds that due to prostaglandin endoperoxide generation. Malonaldehyde production has been proposed as a suitable marker for platelet prostaglandin synthase activity (Stuart *et al.*, 1975; Smith *et al.*, 1976), but our present results indicate that this premise is not justified: platelet malonaldehyde production apparently reflects thromboxane

synthase activity accurately, but is not an adequate indicator of the cyclo-oxygenase that is the initial step in the platelet prostaglandin-synthetic pathway. It remains to be determined whether malonaldehyde is formed from thromboxane synthase in other cells, most of which synthesize little thromboxane, but much more prostaglandins, when stimulated, compared with platelets.

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