EFFECTS OF THROMBOXANE SYNTHETASE INHIBITION ON ARACHIDONATE METABOLISM AND PLATELET BEHAVIOUR

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- 1 The effects of the thromboxane synthetase inhibitor dazoxiben (UK 37248) on arachidonic acid and collagen-induced platelet aggregation and arachidonate acid metabolism were studied both *in vitro* and *ex vivo* in the presence and absence of sources of prostaglandin I₂ synthetase.
- 2 In platelets activated by exogenous arachidonic acid, the anti-aggregatory activity of dazoxiben was weak compared with indomethacin, despite comparable inhibition of TXB₂ production. This was due to the accumulation of pro-aggregatory metabolites, principally endoperoxides.
- 3 The anti-aggregatory activity of dazoxiben, both *in vitro* and *ex vivo*, was higher and more consistent when platelets were stimulated by collagen, threshold levels of which resulted in an endoperoxide accumulation only 2–3% of that achieved with exogenous arachidonic acid.
- 4 The anti-aggregatory activity of dazoxiben is enhanced if drug equilibration is facilitated by prolonging the preincubation time from 2 to 15 minutes.
- 5 Incubation of platelets with pig aortic microsomes, which act both as aggregant and a source of PGI₂ synthetase, facilitates the conversion to PGI₂ of some endoperoxides accumulated after dazoxiben, resulting in augmented anti-aggregatory activity.
- 6 Leukocytes as well as blood vessels have the capacity to generate PGI₂ from platelet derived endoperoxides. This was demonstrated by the increases in 6-keto-PGF_{1α} accompanying decreased TXB₂ production in clotted whole blood from volunteers treated with dazoxiben.
- 7 It was concluded that a closer approach to *in vivo* conditions allowing a fuller expression of the mechanism of action of dazoxiben could be achieved *in vitro* by stimulating platelets with a pathophysiological activator such as collagen in the presence of a source of PGI₂ synthetase.

Introduction

Most pathophysiological activators of platelets can mediate aggregation through both arachidonic acid (AA)-dependent and AA-independent mechanisms, the relative contributions of which depend on the type and level of activator (Harlan & Harker, 1981). Thus, AA-dependent processes are responsible for the irreversible aggregation responses to ADP, adrenaline and low levels of collagen and thrombin (Gerrard & White, 1976), whereas AA-independent mechanisms dominate the responses to high levels of collagen and thrombin (Kinlough-Rathbone *et al.*, 1977), platelet-activating factor (Chignard *et al.*, 1979) and primary reversible aggregation with ADP and adrenaline (Gerrard & White, 1976).

Agents which interfere with the production or action of pro-aggregatory metabolites of AA – thromboxane A₂ (TXA₂) (Hamberg *et al.*, 1975) or PGH₂ (Hamberg *et al.*, 1974) – would not be expected to influence AA-independent processes unless they facilitated the production of prostacyclin (PGI₂) which, by elevating cyclic AMP (Gorman *et al.*, 1977), exhibits universal antiplatelet activity (Moncada *et al.*, 1976; Bussolino & Camussi, 1980). Of

such agents – cyclo-oxygenase inhibitors, TXA₂-receptor antagonists and thromboxane synthetase inhibitors (TXSI) – only the last have the potential for enhancing PGI₂ biosynthesis by increasing the availability of the cyclic endoperoxide substrates, particularly, but not necessarily exclusively, from platelets (Needleman *et al.*, 1979; Marcus *et al.*, 1980). Although endoperoxides are weaker aggregating agents than TXA₂ (Hamberg *et al.*, 1975), sufficient may accumulate in *in vitro* platelet studies, particularly with AA as aggregating agent, to produce normal aggregation responses in some cases despite a high level of thromboxane synthetase inhibition – the so called “non-responders” (Heptinstall *et al.*, 1980; Bertele *et al.*, 1981).

The data now presented demonstrates that this apparently paradoxical platelet behaviour with exogenous AA is less likely to occur after stimulation of platelets with a more pathophysiological activator such as collagen, where release of endogenous AA results in much less accumulation of endoperoxides. Utilization of the latter by sources of PGI₂ synthetase, which would be available *in vivo*, can improve further the anti-aggregatory activity of a TXSI.

Methods

Preparation of platelet-rich plasma (PRP)

Blood, obtained by venepuncture (man) or cardiac puncture under halothane anaesthesia (rabbit), was transferred to tubes containing 0.1 volume 3.8% (w/v) trisodium citrate. PRP was prepared by centrifugation either at 150g for 15 min (rabbit, Tables 2, 5 and Figure 1; human, Table 2), 300g for 10 min (Table 1; Heptinstall *et al.*, 1980), 120g for 5 min (Table 4; Knudsen, J.B., personal communication).

Platelet aggregation in the absence of a PGI_2 source

In all aggregation studies PRP was stirred (1100 r.p.m.) at 37°C. For *in vitro* studies, unless otherwise stated, PRP was preincubated with or without the thromboxane synthetase inhibitor dazoxiben (UK 37248) for 2 min before addition of the aggregating agent (AA or collagen). Aggregation was monitored for 5 min after which time the platelets were sedimented by centrifugation and the platelet-poor plasma (PPP) stored at -20°C before analysis of AA metabolites.

Ex vivo platelet aggregation studies were carried out after oral administration of dazoxiben 100 mg to 16 patients with severe atherosclerotic heart disease. The amounts of collagen required to produce threshold aggregation responses were established before and 1, 2, 3, 4, 6 and 24 h after drug administration (Knudsen, J.B., personal communication).

Platelet aggregation in the presence of a PGI_2 source

Pig aortic microsomes (PAM), prepared as described previously (Randall *et al.*, 1981), were used both as aggregating agent (probably collagen according to the aggregation profile) and as a source of PGI_2 synthetase (Moncada *et al.*, 1976). The latter was largely inactivated for control measurements by preincubation for 30 min in ice with 10 μ M 15-hydroperoxy-5,8,11,14-tetraenoic acid (15-HPETE), a selective inhibitor of PGI_2 synthetase (Gryglewski *et al.*, 1976), prepared as described previously (Randall *et al.*, 1981). The PAM preparations were shown to be free of cyclo-oxygenase activity by the absence of any detectable production of 6-keto-PGF $_{1\alpha}$ following incubation with AA.

Rabbit PRP was preincubated for 5 min, with or without dazoxiben (5 μ M) before initiating aggregation with either untreated or 15-HPETE-treated PAM. After monitoring aggregation for 10 min samples were removed and centrifuged to prepare PPP, which was stored at -20°C before analysis of AA metabolites.

Studies using whole blood

In a volunteer study (Tyler *et al.*, 1981), blood samples taken before and at various times (0.5–8 h) after oral administration of dazoxiben 5–200 mg were allowed to clot in plain glass tubes at 37°C for 1 h (Patrono *et al.*, 1980). Serum was separated by centrifugation and stored at -20°C until analysed for AA metabolites.

Analysis of AA metabolites

The major stable spontaneous breakdown products of TXA $_2$ (TXB $_2$), PGE $_2$ (6-keto-PGF $_{11}$) and cyclic endoperoxides (PGI_2 ; Nugteren & Hazelhof, 1973) were each measured with specific radioimmunoassays using either a secondary antibody for human samples (Heptinstall *et al.*, 1980; Randall *et al.*, 1981) or 50% ammonium sulphate for rabbit samples (Randall *et al.*, 1981) to separate free and antibody-bound [3 H]-ligands. Apart from the analysis of high levels of TXB $_2$ in human serum or AA-activated PRP, where samples were diluted after deproteinization with ethanol, all other analyses were performed using appropriate dilutions of native PPP or serum. Any non-specific effects with such samples were controlled by the addition to standards of plasma or serum prepared after pretreatment for 3 d with aspirin 700 mg three times daily in man or 300 mg daily in rabbit to deplete cyclo-oxygenase products.

In all the studies involving drug administration to man, informed consent was obtained from volunteers and patients and the studies were approved by the appropriate Ethical Committee.

Results

Platelet studies in the absence of a PGI_2 source

Aggregation of human PRP by suprathreshold concentrations of AA (Heptinstall *et al.*, 1980) results in the production of comparable high levels of TXB $_2$ (Table 1) with both a "non-responder" (donor 1) and a "responder" (donor 2).

When comparable levels of TXB $_2$ were generated

Table 1 Production of TXB $_2$ from AA by human PRP *in vitro*

Donor	TXB $_2$ production (ng/ml)
1	1373
2	1696
	[AA]=1.7 mM

Data from Heptinstall *et al.* (1980).

Table 2 Effects of dazoxiben and indomethacin on AA-induced aggregation, and on levels of TXB₂ and PGE₂ in rabbit PRP *in vitro*

Drug	Drug concentration (μM)	Metabolite levels (ng/ml)		% Inhibition of aggregation
		TXB ₂	PGE ₂	
Dazoxiben	0	1512±128*	58±16.9	–
	100	41±6.4	922±107	31±10
Indomethacin	0	1271±346	79±42.7	–
	5	40±22.4	18±18	100

*Mean±s.e.mean, n=3. [AA]=0.33 mM.

Table 3 Effects of dazoxiben and indomethacin on aggregation of human PRP *in vitro* by threshold levels of collagen

Drug	Inhibition of aggregation IC ₅₀ (μM)
Dazoxiben	4.8±2.1 (4)
Indomethacin	3.1±1.7 (3)

[Collagen]=80–360 ng/ml

from AA in rabbit PRP (Table 2), inhibition of thromboxane synthetase by dazoxiben produced high levels of PGE₂, reflecting a large accumulation of endoperoxides. Inhibition of aggregation, however, was both weaker and more variable than with indomethacin. The latter, though reducing TXB₂ to the same level as with dazoxiben, also inhibited the production of endoperoxides as indicated by the marked reduction in PGE₂ levels.

When aggregation is induced in human PRP by collagen the production of TXB₂ (50.2±2.4 ng/ml, n=7; collagen 5 μg/ml) and consequently the capacity for endoperoxide accumulation after inhibition of thromboxane synthetase is some 30–40 times lower than that achieved with AA. When tested, using threshold levels of collagen, dazoxiben consistently exhibited a potent antiaggregatory effect comparable with that of indomethacin (Table 3).

In addition to using threshold levels of aggregating agent to account for variations in platelet sensitivity between individuals, it is important also for *in vitro* studies with inhibitors to ensure that drug equilibration has occurred. Structural features of dazoxiben, notably its carboxylic acid group, prevent its entering platelets very rapidly. This is illustrated in Figure 1, where preincubation of dazoxiben with PRP for 2 min before initiating aggregation with collagen results in only a small inhibition of both TXB₂ production and aggregation. However, inhibition of both parameters increased markedly with more prolonged preincubation.

Dazoxiben also inhibits collagen aggregation *ex vivo* in man, where drug equilibration has been achieved. In a study involving 16 patients with atherosclerotic heart disease (Knudsen *et al.*, personal communication) it was shown (Table 4) that over a 6-h period after administration of a single oral dose of dazoxiben 100 mg, PRP from an increasing number of patients required collagen 10 μg/ml to initiate aggregation, whereas only 2 μg/ml was sufficient in all cases both before and 24 h after drug treatment.

Platelet studies in the presence of a PGI₂ source

None of the preceding studies completely reflect conditions *in vivo*, where sources of PGI₂ synthetase are

Figure 1 Effect of preincubation time on inhibition by dazoxiben 10 μM of aggregation response (○) and TXB₂ production (●) by rabbit PRP stimulated by collagen (5 μg/ml). TXB₂ production in controls was 38.3±1.2 ng/ml.

present potentially to utilize any endoperoxides which accumulate after inhibition of thromboxane synthetase. As an approach to such conditions, rabbit PRP was incubated with or without dazoxiben in the presence of cyclo-oxygenase-free PAM, which served both as aggregating agent and as a source of PGI₂ synthetase. The representative data in Table 5 show that in the presence of PAM, both with PGI₂ synthetase intact and severely reduced by 15-HPETE treatment, dazoxiben markedly inhibited TXB₂ production and increased PGE₂ levels, the latter reflecting accumulated endoperoxides. In the presence of PAM having a severely reduced PGI₂ synthetic capacity, dazoxiben facilitated little PGI₂ production and its effect on aggregation was small. In contrast, with PGI₂ synthetase intact there was a much greater production of PGI₂ in the presence of dazoxiben, which was reflected in an almost complete inhibition of aggregation.

Evidence that leukocytes as well as blood vessels could act as a source of PGI₂ was obtained in a study where blood samples taken from normal volunteers before and at various times after oral administration of dazoxiben 5–200 mg were allowed to clot at 37°C (Tyler *et al.*, 1981). In the absence of dazoxiben, high levels of TXB₂ (497 ± 69 ng/ml, $n=6$) and 6-keto-PGF_{1 α} (2.76 ± 0.14 ng/ml, $n=6$) were obtained in serum, reflecting the activation of platelets and leukocytes by the thrombin generated during clotting. The data in Figure 2 show that after the ad-

ministration of dazoxiben, the decreasing levels of TXB₂ in serum were mirrored by increases in 6-keto-PGF_{1 α} . Whereas the latter increased four-fold after complete inhibition of TXB₂ production, a doubling in 6-keto-PGF_{1 α} could be achieved with only a modest (20%) reduction in TXB₂ levels.

Discussion

Platelet responses to AA, whether exogenous or released by various activators, are likely to reflect the net effect of the levels achieved, sensitivity to and interactions between the various pro- and anti-aggregatory metabolites of AA. In the absence of inhibitors platelets will synthesize TXA₂ as a major enzymic product of AA, with relatively little accumulation of endoperoxides (Needleham *et al.*, 1979), PGE₂ or PGD₂ (Hamberg *et al.*, 1974) and no PGI₂ (Blackwell *et al.*, 1978). After inhibition with thromboxane synthetase, there is an accumulation of endoperoxides (Needleman *et al.*, 1979; Marcus *et al.*, 1980). The latter are transformed subsequently, both enzymically but predominantly non-enzymically, largely to PGE₂ (Nugteren & Hazelhof, 1973), but also to PGD₂ (Hamberg & Fredholm, 1976; Oelz *et al.*, 1977). The pro- and anti-aggregatory activities of the endoperoxides and PGD₂, respectively, are weaker than those of TXA₂ (Hamberg *et al.*, 1975) and PGI₂ (Moncada *et al.*, 1976).

The effects of PGE₂ on platelet behaviour are complex. Relatively high levels (100 ng/ml) of PGE₂ potentiate aggregation (Westwick, 1976), with even higher concentrations (1–3 µg/ml) required to inhibit aggregation (Bruno *et al.*, 1974; Westwick, 1976) via selective binding to the PGE₁/PGI₂, but not the PGD₂, platelet receptor (Miller & Gorman, 1979; Bonne *et al.*, 1981). The reported antagonism by PGE₂ of the anti-aggregatory effects of PGE₂, PGI₂ and PGD₂ (Bonne *et al.*, 1981) seems to result from both selective antagonism at the PGE₁/PGI₂ receptor and non-selective antagonism of adenylate cyclase.

The very high levels of TXA₂ generated when platelets are activated by exogenous AA (Tables 1 and 2) will be replaced after thromboxane synthetase inhibition by comparable levels of endoperoxides. The latter, having a half-life of about 5 min at 37°C (Hamberg *et al.*, 1974), are likely to remain in significant amounts during the period of aggregation, despite a gradual isomerization to PGE₂ and, to a lesser extent, PGD₂. It has been suggested (Heptinstall *et al.*, 1980) that in platelets from "non-responders" the net balance between pro-aggregatory metabolites (endoperoxides, residual TXA₂ and possibly PGE₂) and anti-aggregatory PGD₂ favours the former, whereas the converse may be true in "responders". A similar situation may

Figure 2 Relationship between serum TXB₂ and 6-keto-PGF_{1 α} levels in healthy volunteers at various times (0.5–8 h) after oral administration of dazoxiben 5–200 mg. Predose levels were TXB₂: 497 ± 69 ng/ml; 6-keto-PGF_{1 α} : 2.76 ± 0.14 ng/ml. Data fits equation $x = -0.02 y^2 + 4.73y + 2.81$, where $x=6\text{-keto-PGF}_{1\alpha}$ and $y=\text{TXB}_2$. Coefficient of determination=0.9477.

occur with rabbit platelets (Table 2) where, despite identical residual levels of TXB₂ after indomethacin and dazoxiben, only with the latter, where endoperoxides accumulated, was inhibition of aggregation weak and variable.

That the amount of TXA₂ generated with exogenous AA is far in excess of that required for full aggregation is illustrated when platelets are aggregated with the more pathophysiological activator, collagen, where production of TXA₂ is only 2–3% of that with AA (Table 3). Although the responder/non-responder distribution obtained with AA may represent the extremes of a spectrum, platelet responsiveness to other activators, including collagen, is also likely to vary between individuals. Such variation can be limited by using threshold levels of collagen. In such conditions, functionally active levels of endoperoxides are less likely to be achieved after thromboxane synthetase inhibition, as reflected by the comparable anti-aggregatory activities of dazoxiben and indomethacin *in vitro* (Table 3), and the increased level of collagen required to initiate aggregation *ex vivo* in man (Table 4). In order to approximate *ex vivo* conditions in *in vitro* studies, it is necessary to allow sufficient time

for drug equilibration, rather than using an arbitrarily chosen preincubation time. With dazoxiben, whose carboxylic acid function reduces lipophilicity, extending preincubation time results in a marked increase in activity (Figure 1).

Although all of these manoeuvres go some way towards reflecting pathophysiological conditions, in no instance is there present a capacity for diverting accumulated endoperoxides to PGI₂ synthesis. When platelets are incubated with a vascular source of PGI₂ synthetase (PAM), dazoxiben facilitates the production of sufficient PGI₂ to achieve a much greater inhibition of aggregation than that obtained with PAM containing little PGI₂ synthetic capacity (Table 5). Similar effects have been demonstrated *ex vivo* with dazoxiben (Knudsen *et al.*, 1981a;b) where inhibition of ADP aggregation occurred only in the presence of vascular endothelial cells.

The activities of TXSIs in whole blood systems suggest that leukocytes as well as blood vessels could act as a potential source of PGI₂. Such a source could be of particular significance in atherosclerotic conditions, where the vascular PGI₂ synthetic capacity may be reduced (Johnson *et al.*, 1979; Silbauer *et al.*, 1979). The capacity of leukocytes to generate sig-

Table 4 Effect of dazoxiben on aggregation to collagen *ex vivo* in man

Time after dazoxiben 100 mg orally (h)	Number of patients* with aggregation responses to collagen at:	
	2 µg/ml	10 µg/ml
0	16	0
1	12	4
2	9	7
3	6	10‡
4	5	11§
6	4	12§
24	11	0

*Sixteen patients with severe atherosclerotic heart disease.

†100 mg orally.

‡*P* < 0.02; §*P* < 0.01 (Wilcoxon signed rank test).

Data from Knudsen, J.B. (personal communication).

Table 5 Effects of dazoxiben on AA metabolites and aggregation in rabbit PRP±source of PGI₂ synthetase

Additions to PRP			Metabolite levels		% Inhibition of aggregation
PGI ₂ Source	Dazoxiben (5 µM)	TXB ₂	(ng/ml) PGE ₂	6-keto-PGF _{1α}	
—*	—	121.4	6.0	0.3	—
—	+	37.2	51.3	3.5	17
+°	—	155.2	5.4	3.2	—
+	+	15.6	41.5	14.0	85

*Pig aortic microsomes pretreated with 15-HPETE.

†Untreated pig aortic microsomes.

nificant amounts of PGI₂ from accumulated endoperoxides in clotting blood was demonstrated clearly after administration of dazoxiben both to normal volunteers (Fig. 2) and angina patients (Parry *et al.*, 1982). In the latter study, it was also shown that aspirin decreased PGI₂ production. Similar increases in PGI₂ production have been demonstrated with dazoxiben both *in vitro* (Defreyn *et al.*, 1982) and *ex vivo* (Vermylen & Deckmyn, 1982) using citrated whole blood stimulated with collagen. Furthermore, as shown with 1-butylimidazole, such an increase in PGI₂ was sufficient to produce a much greater inhibition of aggregation than could be achieved in PRP (Flower & Cardinal, 1979). This effect may also reflect a potentiation of the anti-aggregatory activity of PGI₂, as has been demonstrated with dazoxiben (Bertele *et al.*, 1982).

In conclusion, since dazoxiben exhibits good activity in several animal models of thrombosis (Randall & Wilding, 1982), including protection from AA-induced mortality (Randall *et al.*, 1981; Lefer *et al.*, 1981), it seems reasonable to suggest that platelet responses to TXSIs *in vitro* may not always provide a reliable index of antiplatelet activity *in vivo*. However, greater concordance can be achieved in conditions considered to approach those present *in vivo*. Such conditions, which would allow a more complete expression of the mechanism of action of a TXSI, include: aggregation of platelets with pathophysiological activators using a range of concentrations, including threshold, to account for individual variations in sensitivity; allow sufficient time for drug equilibration to occur and approach the *in vivo* milieu of platelets by inclusion of a source of PGI₂ synthetase.

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