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SERUM ALBUMIN ENHANCES THE IMPAIRMENT OF PLATELET AGGREGATION WITH THROMBOXANE SYNTHASE INHIBITION BY INCREASING THE FORMATION OF PROSTAGLANDIN D_2

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Abstract—Dazoxiben, a thromboxane synthase inhibitor, inhibits arachidonic acid induced aggregation in platelet-rich plasma from some donors only ("responders"). We have studied the effect of dazoxiben *in vitro* on platelet aggregation and prostaglandin (PG) metabolism and the influence of the incubation period and of exogenously added serum albumin (SA). SA, which increases the production of anti-aggregatory PGD_2 from cyclic endoperoxides, induced "non-responder" human platelets to respond. With rabbit platelets, however, that are insensitive to PGD_2, exogenous SA failed to potentiate dazoxiben-induced inhibition. The ratio between PGD_2 and $TXB_2 + PGE_2$ formed was crucial in determining the response of human platelets to dazoxiben: whenever this ratio was high, platelet aggregation was inhibited. SQ 22536, an adenylate cyclase inhibitor, and NO164, a PGD_2 antagonist, reversed the inhibition by dazoxiben in human platelet-rich plasma, stressing the importance of a PGD_2 mediated rise of cyclic AMP for the effectiveness of a thromboxane synthase inhibitor.

Thromboxane synthase inhibitors form a new class of antithrombotic drugs administrable to man; they have theoretical advantages over the classic anti-platelet agents, such as aspirin [1–3]. A selective inhibitor of thromboxane synthesis would not only inhibit production of TXA_2 but, unlike the non-steroidal anti-inflammatory agents that inhibit cyclo-oxygenase, would preserve or even enhance prostacyclin production. The cyclic endoperoxides, accumulating after thromboxane synthase inhibition, could indeed escape from platelets and be taken up and used as a substrate for the synthesis of prostacyclin (PGI_2) by endothelium and, in blood, by white blood cells. Furthermore, the metabolism of these endoperoxides would also be reoriented inside the platelets towards "classical" PGs, such as PGE_2, $PGF_{2\alpha}$ and PGD_2, that could also affect platelet function. These expectations have indeed been confirmed following the administration of dazoxiben, a thromboxane synthase inhibitor, to man [4–6]; the usefulness of thromboxane synthase inhibitors has however been questioned following the *in vitro* finding that these drugs inhibit aggregation in platelet-rich plasma from some donors only ("responders") [7–10].

To explain the inconstant effect of thromboxane synthase inhibitors, it has been proposed that prostaglandin endoperoxides suffice to mediate platelet aggregation in some individuals [8, 11] or that platelets from "responders" and "non-responders" have a different ability to produce antiaggregatory PGD_2 [7].

Normal plasma contains a factor stimulating the formation of PGD_2: this has been identified as serum albumin [12] which greatly enhances the ratio between the amounts of PGD_2 and PGE_2 formed from cyclic endoperoxides [13, 14]. If therefore the reorientation towards PGD_2 is critical for the efficacy of thromboxane synthase inhibitors, adding serum albumin should affect the response. We have further investigated the influence of the incubation period with dazoxiben, of an adenylate cyclase inhibitor and of a PGD_2-antagonist on the "responder" or "non-responder" behaviour. Rabbit platelets were also studied since they are hardly sensitive to PGD_2-induced inhibition of aggregation.

MATERIALS AND METHODS

Preparation of platelet-rich plasma (PRP). Blood samples were taken by clean venipuncture from 20 healthy volunteers, 12 males and 8 females aged 23–45 years, who had not taken any drugs during the previous two weeks, and transferred to tubes containing 0.1 vol 3.13% trisodium citrate. PRP was prepared by centrifugation at 150 *g* for 15 min.

For four experiments blood was collected from White New Zealand rabbits under light anesthesia through a cannula placed in the jugular vein, mixed with 0.1 vol 4% trisodium citrate and centrifuged for 15 min at 110 *g*.

Platelet-poor plasma (PPP) was prepared by centrifugation of blood remaining after removal of PRP at 1500 *g* for 20 min.

Platelet aggregation studies. Individual PRP samples were incubated for 1 min at 37° with 10 μM dazoxiben unless otherwise stated; then arachidonic acid, sodium salt (AA), at the threshold concentration, defined as the minimal concentration

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inducing full irreversible aggregation starting within 2 min, was added; aggregation was monitored by Born's method in an Elvi 840 aggregometer (Elvi Logos, Milan, Italy).

Subjects were defined as "responders" when no aggregation was observed within 2 min from AA addition (complete inhibition).

PRP from "non-responders" was further studied by prolonging the incubation time with dazoxiben to 10 min and/or by adding exogenous human or bovine serum albumin (SA). When SA was added, a new aggregating threshold for AA was determined since part of AA is bound to albumin and thus not available for platelet activation [15].

The effect of SQ 22536, an adenylate-cyclase inhibitor [16], and of NO164, a PGD_2 antagonist [17, 18], on platelet behaviour upon thromboxane synthase inhibition, were tested in "responders" or in platelets made "responders" by prolonged incubation or by exogenous SA.

A few experiments were performed as described above using collagen at the threshold concentration as aggregating stimulus. In this case a "responder" or "non-responder" behaviour could not be defined since this is characteristic for AA-induced aggregation only. The degree of inhibition was therefore taken as an endpoint.

Determination of platelet AA metabolites. PGE_2, PGD_2 and thromboxane B_2 (TXB_2), the stable breakdown product of TXA_2, were measured by specific radioimmunoassays on samples of plasma prepared by centrifuging PRP (12,000 *g* for 2 min) 4.5 min after adding the aggregating stimulus.

TXB_2 and PGE_2 were measured on unextracted plasma as previously described [6].

The PGD_2 assay was carried out as follows: samples were acidified to pH 3 with citric acid 0.1 N, extracted three times with diethylether and dried under a nitrogen stream. Dry residues were redissolved in Tris–buffer 50 mM pH 8.0 (recovery 53.3 ± 0.7%; N = 14), mixed with labelled PGD_2 and anti-PGD_2 antibody and incubated for 24 hr. Free and antibody-bound (^{3}H)-ligand were separated by precipitation with an equal volume of saturated $(NH_4)_2SO_4$, pH 7.5; samples were mixed and centrifuged at 12,000 *g* for 2 min and the supernatant was counted for radioactivity. All procedures were carried out at 4°.

Fifty percent binding for PGD_2 was at 2.27 nmole (22.7 nmole/ml). Cross reactivities were as follows: PGD_2 = 100%; 13,14-dihydro $PGF_{2\alpha}$ = 1.9%; $PGF_{2\alpha}$ = 1.5%; PGE_2 = 0.2%; 6-keto $PGF_{1\alpha}$ = 0.2%; TXB_2 = 0.18%; PGB_2 = 0.18%; 13,14-dihydro PGE_2 < 0.1%; PGA_2 < 0.1%.

Fifty percent binding and cross reactivities for TXB_2 and PGE_2 antibodies were as previously reported [6].

Chemicals. Dazoxiben (UK-37248 or 4-[2-(1H-imidazol-1-yl) ethoxy] benzoic acid hydrochloride) (Pfizer, Sandwich, U.K.) was dissolved in distilled water. SQ 22536 (9-[tetrahydro-2-furyl] adenine) (Squibb, Princeton, N.J., U.S.A.) was dissolved in Tris–buffer pH 7.5.

NO164 (sodium-*p*-benzyl-4-[1-oxo-2-(4-chlorobenzyl)-3-phenyl-propyl]phenyl phosphonate) (Nelson Research, Irvine, CA, U.S.A.) was first dissolved in a minimal amount of ethanol and then diluted to the desired concentration with Tris–buffer pH 7.5. Defatted lyophilized human and bovine serum albumin and polygeline (Haemacel®) were from Behringwerke AG (Marburg, West Germany), dextran 70 (Macrodex®) from Pharmacia AB (Uppsala, Sweden) arachidonic acid, sodium salt from Sigma (St. Louis, MO, U.S.A.), and collagen from Hormon-Chemie (München, West Germany). The antibody against TXB_2 was kindly donated by Dr. L. Levine (University Waltham, U.S.A.). The antibody against PGE_2 was from Miles-Yeda (Rehovot, Israel) and that against PGD_2 from Seragen (Boston, MA, U.S.A.).

Radiolabelled prostaglandins were from New England Nuclear (Boston, MA, U.S.A.). TXB_2, PGE_2 and PGD_2 standards were purchased from Upjohn (Kalamazoo, MI, U.S.A.).

All concentrations are expressed as final ones in the reaction mixtures.

Statistical evaluation. χ^2 Analysis was used to compare $PGD_2/TXB_2 + PGE_2$ ratios of responders and non-responders. Two tailed Student's *t*-test for paired data was applied to compare the actual amounts of platelet AA metabolites, produced in the presence and in the absence of dazoxiben with or without serum albumin added.

RESULTS

Platelet aggregation studies. AA-induced aggregation was inhibited in PRP preincubated for 1 min with 10 μM dazoxiben from 5 of 20 donors only.

However, 13 of the "non-responders" became "responders" when the incubation time was prolonged to 10 min. Aggregation in most of these subjects (N = 10) was also inhibited after 1 min incubation when exogenous SA was added. In 2 cases inhibition of platelet aggregation was obtained only by 10 min incubation with dazoxiben in the presence of exogenous SA.

An aspecific effect of SA was ruled out by control experiments with other macromolecular solutions: the preincubation for 1 or 10 min of PRP with dextran 70 or polygeline, resulting in comparable increases in osmolarity, did not turn "non-responders" into "responders" (Fig. 1).

Bovine SA was effective in our system at 2–4 times lower concentrations than human SA. No differences were found in albumin levels between "responders" (46.3 ± 0.37 mg/ml) and "non-responders" (49.7 ± 0.59 mg/ml).

Platelet AA metabolism. The prolongation of the incubation period in the presence of dazoxiben produced, together with an increase in the percentage of "responders", a further clear reduction of TXB_2 levels ($P < 0.005$) (Table 1). The effectiveness of dazoxiben in inhibiting platelet aggregation, however, does not seem to be attributable only to differences in the residual amounts of TXA_2 generated; these were indeed sometimes comparable or even higher in "responder" platelets or platelets made "responder" by the addition of exogenous SA than in "non-responder" platelets.

PGE_2 and PGD_2 were both produced in increased amounts after thromboxane synthase inhibition

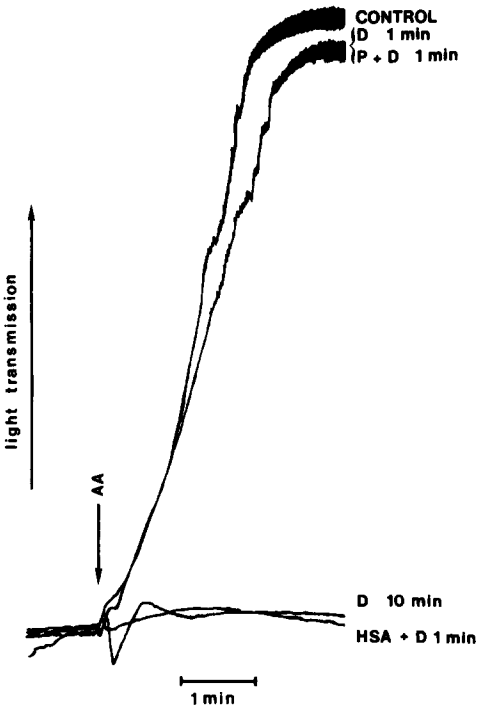

Fig. 1. Representative tracings of aggregation induced by AA in platelet-rich plasma preincubated (1 or 10 min) with dazoxiben (D) 10 μM in the absence and in the presence of added human serum albumin (HSA) or polygeline (P). In this particular case threshold AA concentrations were 0.4 and 0.65 mM in the absence and in the presence of HSA (15 μl of a 0.5 mM solution). Effective concentrations of HSA (5–15 μl of a 0.5 mM solution in a final volume of 300 μl) were about 3 times higher than those of bovine serum albumin.

($P < 0.025$ and $P < 0.05$ respectively). Addition of exogenous human and bovine SA induced a rise in the relative amount of PGD_2; this reached statistical significance ($P < 0.05$), however, only with bovine SA (Table 1).

The balance between the production of PGD_2 and that of $TXB_2 + PGE_2$ seemed to be crucial in determining the "responder" "non-responder" behaviour; in all instances (except 1) in which the $PGD_2/TXB_2 + PGE_2$ ratio was higher than 4, platelet aggregation was inhibited (Fig. 2).

The amount of PGD_2 produced in the absence of dazoxiben was not significantly different between "responders" and "non-responders".

Confirmatory evidence for the importance of PGD_2 in mediating dazoxiben induced inhibition. PGD_2 inhibits platelet aggregation through the stimulation of adenylate cyclase and the consequent increase in intraplatelet cAMP levels [19]. SQ 22536, an inhibitor of adenylate cyclase [16], at concentrations between 100 and 250 μM reversed the inhibition of AA-induced platelet aggregation ($N = 8$) induced by dazoxiben in "responders" or in platelets made "responder" by exogenous serum albumin (Fig. 3a). SQ 22536 alone, at the same concentrations, did not influence by itself platelet aggregation, and particularly no potentiation of aggregation induced by sub-threshold AA was observed. The inhibition of platelet aggregation by exogenous PGD_2 (8–10 nM) was instead counteracted by SQ 22536 in the concentration range 50–250 μM.

NO164, which at low concentrations is a selective PGD_2 antagonist [17, 18], was also able, in the 100–200 μM concentration range, to prevent the inhibitory effect of dazoxiben on platelet aggregation ($N = 3$) both in PRP from "responders" and in platelets made "responder" by the addition of serum albumin (Fig. 3b).

Rabbit platelets are poorly sensitive to PGD_2-induced inhibition [18]. In our experience the quantity of PGD_2 needed to get an inhibition of AA-induced aggregation is more than two orders of magnitude higher for rabbit PRP than for human PRP. The addition of human or bovine SA did not potentiate the inhibitory effect of dazoxiben on rabbit platelets ($N = 4$). SQ 22536 did not affect the inhibition induced by dazoxiben on rabbit platelets (Fig. 3c).

We also studied the effect of dazoxiben on collagen-induced aggregation in human PRP ($N = 4$). Although it is not possible to differentiate clearly between "responders" and "non-responders" when aggregation is induced by this agent, we could confirm that exogenous SA increases the inhibitory

Table 1. Effect of 1 or 10 min incubation with dazoxiben and exogenous human or bovine serum albumin on prostaglandin generation during aggregation of human PRP by threshold sodium arachidonate

D (μM)	HSA (mg/ml)	BSA (mg/ml)	Incubation time (min)	TXB_2 (nM)	PGE_2 (nM)	PGD_2 (nM)
0	0	0	—	*806 ± 36 (6)	11.4 ± 2.7 (6)	187 ± 48 (4)
10	0	0	1	394 ± 70 (6)	64.6 ± 16 (6)	269 ± 39 (4)
10	0	0	10	4.2 ± 0.4 (5)	20.3 ± 7.4 (5)	236 ± 47 (4)
40	0	0	1	11.9 ± 2.8 (5)	38.6 ± 7.8 (5)	285 ± 40 (4)
0	16.5	0	—	689 ± 50 (3)	12.6 ± 4.9 (3)	275 ± 8 (2)
10	16.5	0	1	330 ± 56 (3)	60.2 ± 23 (3)	346 ± 80 (2)
40	16.5	0	1	40.1 ± 14 (2)	20.0 ± 0.6 (2)	352 ± 56 (2)
0	0	16.5	—	743 ± 95 (4)	13.8 ± 1.9 (4)	476 ± 149 (3)
10	0	16.5	1	299 ± 60 (4)	65.8 ± 28 (4)	679 ± 165 (3)
40	0	16.5	1	30 ± 12 (2)	46.0 ± 7.7 (2)	484 ± 126 (2)

* Data are expressed as mean ± S.E.M.; between brackets: number of experiments.

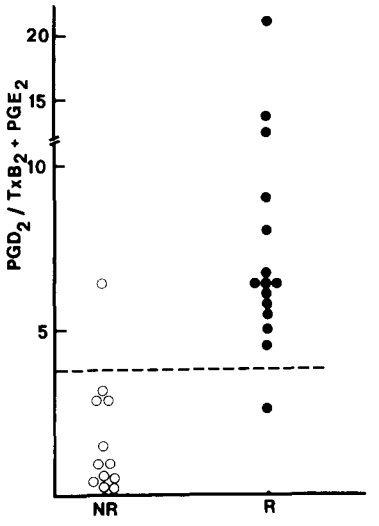

Fig. 2. Ratio between the production of PGD_2 and that of $TXB_2 + PGE_2$ after AA-induced aggregation in PRP from "responders" (R) and "non-responders" (NR). χ^2: $P < 0.001$.

effect of dazoxiben; this is reversed by SQ 22536 (Fig. 3d).

DISCUSSION

Radioimmunoassay has so far not been used frequently for measuring PGD_2 production by platelets. The values reported in two previous papers are lower than ours [12, 20]. However, it should be considered that in those studies platelets were stimulated with thrombin while we used arachidonic acid. During AA-induced aggregation, indeed, much more PGD_2 is produced [21].

Our results demonstrate that inhibition of platelet aggregation in plasma by dazoxiben depends on the nature of the endoperoxide metabolites that are formed instead of TXA_2. This conclusion is based in part on the effect of SA, that made "non-responder" platelets respond to the inhibitory action of dazoxiben and simultaneously increased the production of PGD_2. Bovine SA was more effective than human SA both in making "non-responder" platelets respond and in stimulating PGD_2 formation. This finding is in agreement with the previous observation

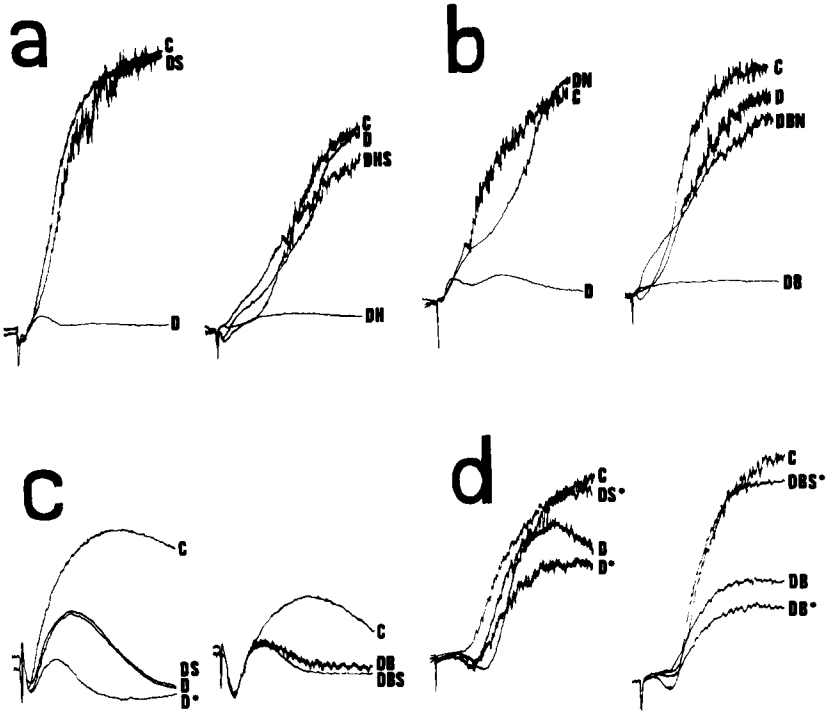

Fig. 3. Representative aggregation tracings giving further evidence for the role of PGD_2 in mediating dazoxiben-induced inhibition. (a) Effect of SQ 22536 (S) on dazoxiben-(D) (left) or dazoxiben + human serum albumin-(DH) (right) induced inhibition of AA-elicited aggregation. (b) Effect of NO164 (N) on dazoxiben (D) (left) or dazoxiben + bovine serum albumin-(DB) (right) induced inhibition of AA-induced aggregation. (c) Effect of dazoxiben, 1 min (D) or 10 min (D*) incubation time (left), and dazoxiben + bovine serum albumin (DB) (right) on AA-induced rabbit platelet aggregation, and the influence of SQ 22536 (S) on it. (d) Effect of dazoxiben, 1 min (D) or 10 min (D*) incubation time (left), and dazoxiben + bovine serum albumin (DB) (right) on collagen-induced human platelet aggregation, and the influence of SQ 22536 (S) on it. C means control. The concentrations used in the experiment shown were as follows: D, 40 μM except in (d) = 10 μM; H, 16.5 mg/ml; S, 250 μM except (a) (right) = 125 μM; B, 16.5 mg/ml; N, b (left) = 170 μM, b (right) = 200 μM.

in a purified system that bovine SA is five times more active than human SA in stimulating PGD_2 production from PG endoperoxides [13]. SA, instead, had no effect on PGE_2 generation consistent with the finding of Watanabe *et al.* [12]. PGE_2 is not directly proaggregatory but greatly potentiates the aggregatory activity of other stimuli [22] and reduces the antiaggregant activity of inhibitory prostaglandins [23]. Orchard *et al.* [24] have shown that human PRP, stimulated by collagen in the presence of dazoxiben, produces PGD_2 in a quantity which alone would inhibit platelet aggregation; however, the addition of PGE_2 in a quantity equal to that produced when dazoxiben is present neutralizes the inhibitory effect of PGD_2. It is therefore clear that the relative ratio between proaggregatory and antiaggregatory prostaglandins regulates the inhibitory activity of thromboxane synthase inhibitors in PRP. In our study, indeed, whenever the ratio between PGD_2 and $TXB_2 + PGE_2$ was high an inhibition of platelet aggregation was observed. Serum albumin is the plasmatic component catalyzing the conversion of PGH_2 to PGD_2, while PGE_2 formation does not need the presence of this plasma protein [12].

The "responder" "non-responder" behaviour of PRP from different individuals to thromboxane synthase inhibitors thus may well be influenced by serum albumin. In our series we could not find a correlation between serum albumin levels and the response to dazoxiben; however, the fatty acid content of serum albumin strongly influences its activity as PGH_2-PGD_2 isomerase [14] and this was not assayed in our subjects. An unfavourable reorientation of platelet PG metabolism could also help explain why platelets from diabetics do not respond *in vitro* to thromboxane synthase inhibitors [10]. Indeed, PGE_2 production is markedly increased in blood from diabetic patients [25] and a deranged modulation of platelet cAMP levels (the ultimate target of PGD_2 activity) has recently been reported in diabetes [26].

SQ 22536, an adenylate cyclase inhibitor [16], and NO164, a non cell-penetrating selective inhibitor of the effect of PGD_2 [17, 18], reversed the inhibition induced by dazoxiben on "responder" platelets or platelets made "responder" by the addition of exogenous SA. These observations confirm that the antiaggregatory effect of dazoxiben in human PRP is largely caused by a PGD_2-mediated rise of cAMP [27].

The present findings support our previous contention that PGD_2 plays an important role in eliciting the abnormal aggregation observed in congenital partial platelet deficiency of thromboxane synthase [28].

In rabbit PRP, which is insensitive to PGD_2 [18], the reduction of thromboxane production is probably of major importance in the inhibition of platelet function. Dazoxiben was indeed much more effective when preincubated with rabbit PRP for 10 instead of 1 min and this prolonged incubation was always accompanied, as in human PRP, by a further reduction of TXA_2 production. Due to its carboxylic group dazoxiben does not penetrate membranes easily and for this reason a prolonged incubation period is required for an optimal effect [29]. Furthermore, exogenous serum albumin did not augment the effect of dazoxiben on rabbit platelets nor did SQ 22536 reverse the inhibitory effect of the drug.

In conclusion, the effect of thromboxane synthase inhibitors on platelet aggregation is regulated by a complex balance between the relative levels of TXA_2, PGE_2, PGD_2 and perhaps other prostaglandins. *In vivo* and even *in vitro* in total blood reorientation towards PGI_2 could also be implicated [4, 6, 30, 31]. If the equilibrium between PGD_2 and PGE_2 is susceptible of modulation *in vivo*, this could affect the therapeutic potential of thromboxane synthase inhibitors.

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