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PROSTAGLANDINS AND HUMAN PLATELET AGGREGATION

IMPLICATIONS FOR THE ANTI-AGGREGATING ACTIVITY OF THROMBOXANE-SYNTHASE INHIBITORS

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Abstract—Selective pharmacological blockade of thromboxane-synthase in human platelets by dazoxiben resulted in the reorientation of cyclic-endoperoxides towards PGE_2, PGD_2 and $PGF_{2\alpha}$. At concentrations which can be reached when thromboxane-synthase is inhibited, PGE_2 (100–500 nM) exerted a marked, concentration-dependent pro-aggregatory effect. This required the formation of endogenous or the addition of exogenous endoperoxides and was prevented by PGD_2 or 13-aza-prostanoic acid, a selective antagonist of PGH_2/TxA_2 receptors. The anti-aggregating effect of PGD_2 was evident at concentrations lower than those obtained in dazoxiben-treated platelets. It is proposed that in the absence of TxA_2 generation, a combination of endoperoxides and PGE_2 may result in normal aggregation. The latter may be inhibited by PGD_2. No interference of $PGF_{2\alpha}$ on platelet function could be shown.

Human platelets are aggregated by arachidonic acid. This fatty acid is metabolized via a cyclo-oxygenase enzyme to prostaglandin (PG) cyclic-endoperoxides, which in turn are transformed via thromboxane (Tx) A_2-synthase to TxA_2 [1]. Relatively small amounts of PGE_2, PGD_2 and $PGF_{2\alpha}$ too are formed from endoperoxides, but their role in arachidonic acid-induced platelet aggregation seems not to be relevant since none of these PGs, at the concentrations usually generated in stimulated platelets, could themselves induce platelet aggregation [1, 2].

PGE_2 sensitizes platelets to aggregating stimuli [3–5], but has also been shown to inhibit platelet function [6]. PGD_2 inhibits platelet aggregation through an adenylate-cyclase-mediated increase in cAMP [7, 8] but it is not known how this effect is related to the amount of endogenous platelet PGD_2. The effects of $PGF_{2\alpha}$ on platelet function are less defined and a moderate pro-aggregatory activity [5] and an anti-aggregating effect [9] have both been described.

The recent availability of selective inhibitors of TxA_2-synthase has renewed interest in the possible interaction of these prostaglandins with platelet aggregation. It has in fact been shown that pharmacological suppression of TxB_2 generation occurs concomitantly with re-orientation of PG endoperoxide metabolism towards PGE_2 [10, 11], $PGF_{2\alpha}$ and PGD_2 [12–15]. It has been suggested that accumulation of the endoperoxides (which are aggregating compounds themselves although less potent than TxA_2 [1, 12]) may to some extent explain the observation that AA-induced platelet aggregation occurs despite prevention of thromboxane synthesis [12, 16]. On the other hand, indirect evidence has been presented that newly formed PGD_2 could contribute to the anti-aggregating activity of TxA_2-synthase inhibitors [12, 17].

The purpose of this study was:

1. To quantitate the amounts of PGE_2, PGD_2 and $PGF_{2\alpha}$ formed in human platelets in concomitance with pharmacological blockade of thromboxane-synthase;
2. To investigate and characterize the effects on platelet aggregation of these three PGs within the range of concentrations determined in the previous experiments.

MATERIALS AND METHODS

Venous blood from healthy human volunteers who had taken no drugs during the previous 10 days was collected on 3.8% sodium citrate and platelet-rich plasma (PRP) and platelet-poor plasma were prepared as described [18].

Platelet aggregation was studied in PRP in a Born aggregometer (Elvi 840, Elvi Logos, Milan, Italy). Aliquots of 250 μl of PRP were pre-incubated at 37° for 3 min with the PGs or their vehicle at the concentrations specified in the Results, then the aggregating agents were added in microlitre amounts and aggregation was monitored for 3 min under continuous stirring. Selected experiments were performed on "aspirinated" PRP, obtained by incubating PRP with 100 μM aspirin (as the soluble lysine salt of acetylsalicylic acid, Flectadol, Maggioni, Italy) for 15 min at room temperature.

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The aggregating agents, sodium arachidonate (AA, Sigma, >99% pure), adenosine-5'-diphosphate (ADP, Sigma) and the stable endoperoxide analogue U-46619 (kindly provided by Dr. J. Pike, Upjohn, Kalamazoo, MI, U.S.A.) were dissolved as described [18, 19]. Stock solutions (at mmolar concentrations) of PGE_2, PGD_2 and $PGF_{2\alpha}$ (Upjohn) and 13-aza-prostanoic acid (13-APA, kindly provided by Dr. G. C. Le Breton, University of Illinois, Chicago, IL, U.S.A.) [20] were prepared in absolute ethanol and freshly diluted with isotonic saline. Dazoxiben (UK 37, 248-01, kindly obtained from Dr. H. M. Tyler, Pfizer, U.K.) was dissolved in Tris–HCl 0.15 M, pH 7.4. Prostaglandins were determined on PRP incubated for 3 min at 37° with dazoxiben or Tris–HCl and stimulated with threshold aggregating concentrations of AA [18]. The reaction was stopped 3 min later by adding to 500 μl PRP an equal volume of 0.001 N HCl. Protein precipitate was removed by centrifugation, and the pH of the supernatant was adjusted to 3.5 by HCl. Two acidified PRP samples were pooled and diluted with isotonic saline to a final volume of 10 ml.

PGE_2, PGD_2, $PGF_{2\alpha}$ and TxB_2 were detected simultaneously using high-resolution gas chromatography coupled to mass spectrometry in the selected ion monitoring mode, as described by Chiabrando *et al.* [21]. Briefly, this method is based on single-step extraction of PGs and TxB_2 from acidified PRP samples on C18 reversed phase cartridges (SEP-PAK C18, Waters) after addition of deuterated analogues as internal standards. This is followed by derivatization of functional groups and final analysis by high-resolution gas chromatography with selected ion monitoring using SE-54 WCOT persilanized Pyrex glass capillary columns (20 m long; internal diameter 0.35 mm) on an LKB 2091-051 gas chromatograph–mass spectrometer equipped with an LKB 2130 computer system. The detection limits (nM) were: TxB_2: 21; PGE_2: 45; PGD_2: 56 and $PGF_{2\alpha}$: 48.

RESULTS

Table 1 shows that preincubation of PRP with 40 μM dazoxiben before stimulation with threshold concentrations of AA (0.2–0.7 mM) resulted in more than 90% suppression of TxB_2 and concomitant increases of PGE_2, PGD_2 and $PGF_{2\alpha}$, PGE_2 being the most abundant. In the absence of dazoxiben, only PGE_2 could be detected besides TxB_2 in all samples. The amounts of PGs formed in dazoxiben-treated PRP samples were utilized to establish the concentrations of the three PGs in further experiments.

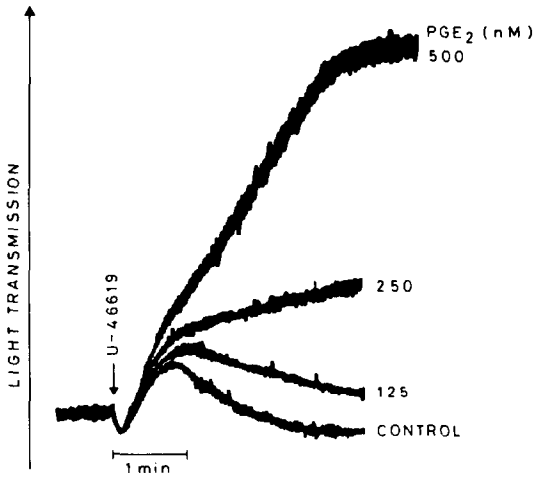

Fig. 1. Potentiation by PGE_2 of platelet aggregation induced by U-46619 (210 nM).

Platelets challenged with subthreshold concentrations of AA, the endoperoxide analogue U-46619 or ADP fully aggregated in the presence of PGE_2 (100–500 nM) but not of $PGF_{2\alpha}$ (up to 1 μM). Figure 1 shows, as an example, the potentiating effect of PGE_2 which was concentration-dependent within the range of concentrations tested (125–500 nM). The pro-aggregatory activity of PGE_2 could not be seen on "aspirinated" platelets challenged with AA but it was clearly apparent in "aspirinated" platelets challenged with subthreshold concentrations of the endoperoxide analogue U-46619. The first wave of ADP-induced aggregation was potentiated by PGE_2 but no second wave followed (Fig. 2).

The pro-aggregatory effect of PGE_2 was completely prevented by 13-APA, a selective endoperoxide/thromboxane receptor antagonist, when either AA or compound U-46619 were used as aggregating stimuli. 13-APA also prevented the second wave of ADP-PGE_2-induced aggregation but did not affect the pro-aggregatory effect of PGE_2 which was apparent on the first wave of ADP-induced aggregation (Fig. 2).

PGD_2 (2.5–50 nM) completely suppressed platelet aggregation induced by threshold concentrations of the three aggregating stimuli whereas neither PGE_2 nor $PGF_{2\alpha}$ inhibited platelet aggregation at concentrations as high as 1 μM (data not shown). The same range of concentrations of PGD_2 counteracted the pro-aggregatory effect of PGE_2 (Fig. 3). $PGF_{2\alpha}$ up to 1 μM was ineffective in all test systems.

Table 1. TxB_2 and PG levels (nM) in control and dazoxiben-treated (40 μM) platelets stimulated with threshold aggregating concentrations of arachidonic acid

	TxB_2	PGE_2	PGD_2	$PGF_{2\alpha}$
Control	437.8 ± 71.6	180.2 ± 10.3	n.d.	n.d.
Dazoxiben	36.4 ± 12.1	540.3 ± 60.8	202.6 ± 53.4	113.4 ± 42.5

n.d. = not detectable.
Figures are means ± S.E.M. (N = 6).

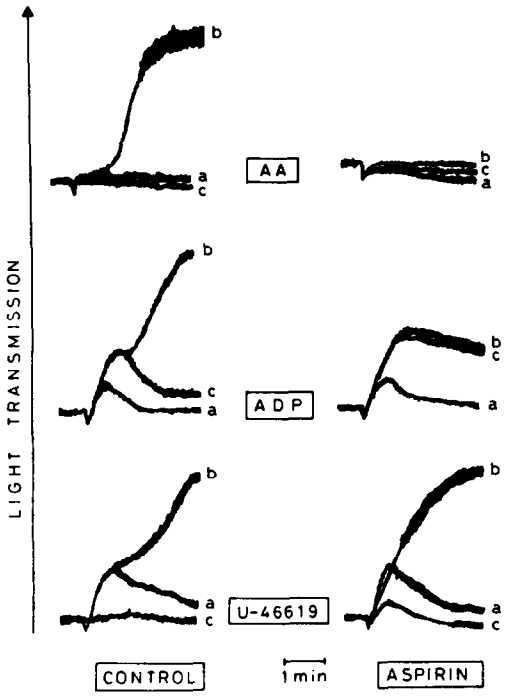

Fig. 2. Representative tracings of platelet aggregation in control and aspirin-treated (100 μM) PRP. Potentiation by PGE_2 (500 nM) and reversal by 13-APA (64 μM) of platelet aggregation induced by AA (0.4 mM), ADP (1 μM) and U-46619 (210 nM): (a) control; (b) PGE_2; (c) PGE_2+13-APA.

DISCUSSION

Dazoxiben, a thromboxane-synthase inhibitor, reoriented platelet cyclic-endoperoxide metabolism towards PGE_2, PGD_2 and $PGF_{2\alpha}$. High resolution capillary column gas-chromatography coupled with mass spectrometry permitted quantitative determination of the three prostaglandins formed. PGE_2 was the only one consistently measurable in control samples; its formation was increased by about 3 times in dazoxiben-treated samples and reached concentrations averaging 540 nM. Both PGD_2 and $PGF_{2\alpha}$ were formed by dazoxiben-treated platelets at concentrations about 3–5 times lower than PGE_2. None of the three prostaglandins at concentrations obtained in the presence of dazoxiben induced platelet aggregation themselves. However, PGE_2—but neither PGD_2 not $PGF_{2\alpha}$—showed marked pro-aggregatory activity when platelets were challenged with subthreshold concentrations of AA, the endoperoxide analogue U-46619 or ADP.

The observation that pretreatment of platelets with aspirin abolished potentiation by PGE_2 of AA—but not endoperoxide—induced platelet aggregation suggests that PGE_2 requires the presence of cyclic-endoperoxide/thromboxane (either endogenously formed or added as a stable analogue). This interpretation is supported by the observation that the pro-aggregatory activity of PGE_2 was counteracted by 13-APA, a selective antagonist of the endoperoxide/thromboxane platelet receptor.

This extends the original observation by Willis that the aggregation stimulating effect of endogenous endoperoxides was potentiated by PGE_2 [5].

The involvement of endoperoxide/thromboxane in the activity of PGE_2 is also shown by the finding that PGE_2-induced secondary aggregation in the presence of subthreshold concentrations of ADP was blocked both by aspirin and by 13-APA. However the first wave of ADP-induced aggregation was increased by PGE_2, independently of endoperoxide/thromboxane availability.

Although the pro-aggregatory properties of PGE_2 had already been reported [3–5], the requirement for endoperoxide/thromboxane had not been recognized. The potentiating effect of endoperoxide and PGE_2 at the concentrations formed in dazoxiben-treated platelets could explain why platelet aggregation may not be inhibited in platelet samples in which thromboxane-synthase has been pharmacologically blocked [17, 22–25].

PGD_2 counteracts the pro-aggregatory activity of PGE_2 on endoperoxide-induced platelet aggregation but is effective at concentrations (10–40 nM) well below those apparently formed in dazoxiben-treated samples (85–374 nM). The generation of such large amounts of PGD_2 in dazoxiben-treated PRP samples should completely prevent platelet aggregation. This is, however, not the case, since aggregation is not inhibited in all samples treated with dazoxiben. Thus the present study suggests that PGD_2 may not be the only factor implicated in the antiaggregating action of thromboxane-synthase inhibitors [12, 17, 22, 26–29]. The crucial role of PGD_2 was based on the observation that phosphodiesterase inhibitors potentiate the anti-aggregatory effect of dazoxiben and adenylate-cyclase inhibitor counteracts it. Both findings have been interpreted with respect to the well-known property of PGD_2 to activate adenylate-cyclase [7, 8]. Further studies are warranted to investigate whether all the PGD_2 formed in concomitance with thromboxane-synthase blockade is functionally available [27].

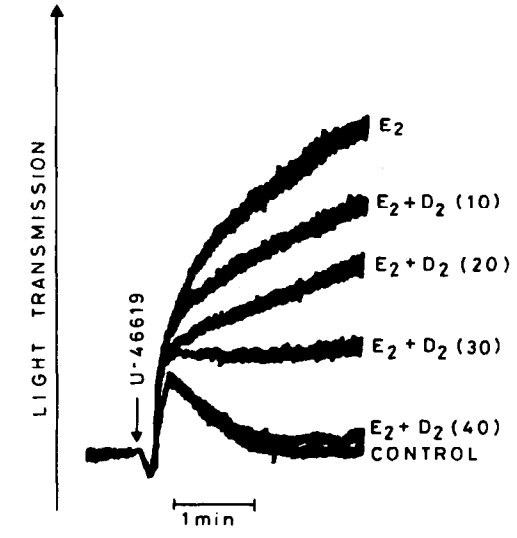

Fig. 3. Effect of different concentrations of PGD_2 on the potentiating effect of PGE_2 (500 nM) on platelet aggregation induced by U-46619 (210 nM).

Finally this study clearly shows that $PGF_{2\alpha}$ does not interfere with platelet function nor does it modify the effects of PGE_2 and PGD_2 up to 10 times the concentrations formed in dazoxiben-treated PRP. This supports the conclusion of Hung *et al.* [9] that the antagonism of $PGF_{2\alpha}$ (15 μM) against thromboxane-induced human platelet aggregation has no physiological relevance for platelet function.

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