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THROMBOXANE SYNTHASE INHIBITION POTENTIATES WASHED PLATELET ACTIVATION BY ENDOGENOUS AND EXOGENOUS ARACHIDONIC ACID

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Abstract—The effect of the thromboxane (TX) synthase inhibitors dazoxiben and imidazole on platelet activation by endogenous and exogenous arachidonic acid (AA) was tested with human washed platelets. Dazoxiben (1–20 μM) inhibited the formation of TXB_2 and markedly enhanced the shape change, aggregation, and (^{3}H)serotonin release induced by added AA or when prostaglandin synthesis from endogenous AA was triggered by collagen, hydrogen peroxide or methyl mercury chloride (methyl-Hg). Platelet activation by hydrogen peroxide (20–1200 μM) or methyl-Hg (1–5 μM) was entirely dependent on endogenous prostaglandin (PG) synthesis since acetylsalicylic acid (ASA), indomethacin or the cyclic endoperoxide/TXA_2-antagonist BM 13.177 counteracted these stimulants with and without dazoxiben. Apparently, the potentiation is due to accumulating cyclic endoperoxides which during TX synthase inhibition reach greater platelet-activating potency than TXA_2. Albumin or human platelet-poor plasma inhibited the platelet activation by hydrogen peroxide and methyl-Hg and suppressed the potentiation by dazoxiben. The latter effect of albumin may result from its PGD isomerase activity which redirects the cyclic endoperoxide metabolism to the platelet-inhibitory PGD_2. The results show that non-platelet factors such as albumin are necessary to prevent a potentiating effect of TX synthase inhibitors on platelet activation.

Exogenous arachidonic acid or endogenous arachidonic acid released from platelet phospholipids is rapidly converted by platelet cyclooxygenase into the cyclic endoperoxides PGG_2 and PGH_2 and by thromboxane synthase into thromboxane A_2 (TXA_2). The cyclic endoperoxides [1] and TXA_2 [2] are potent platelet agonists which induce the shape change, aggregation and the release reaction via a common receptor on platelets [3]. The cyclooxygenase-dependent platelet responses can be suppressed by acetylsalicylic acid (ASA) or other cyclooxygenase inhibitors producing a general inhibition of prostaglandin synthesis [4]. A more specific inhibition of the TX-dependent processes is expected from selective TX synthase inhibitors such as the imidazole derivative dazoxiben [5]. However, although TXA_2 has greater agonist potency than cyclic endoperoxides [6] sufficient endoperoxides may accumulate from the arachidonic acid metabolism to cause normal aggregation response in some cases ("non-responders") despite a high level of TX synthase inhibition [7–9]. It has been suggested [7] that in platelets from "non-responders" the net balance between pro-aggregatory metabolites (cyclic endoperoxides, residual TXA_2 and possibly PGE_2) and anti-aggregatory PGD_2 [10] favours the former, whereas the opposite may be true in "responders" [7]. This paradoxical platelet behaviour is particularly observed with exogenous arachidonic acid as aggregating agent [11]. It has been claimed [11] that much less pro-aggregatory endoperoxides can accumulate when platelets are stimulated with collagen, where endogenous arachidonic acid is released and metabolized in the platelets [11]. At threshold levels of collagen, dazoxiben and indomethacin exhibited comparable anti-aggregating activity [11]. In contrast to those studies on platelet-rich plasma, the present investigation describes the effects of the TX synthase inhibitors dazoxiben [5] and imidazole [12] on washed platelets stimulated by several inducers of TXA_2 synthesis from endogenous arachidonic acid and by added arachidonic acid. It is demonstrated that in the absence of plasma and at low albumin concentrations, inhibition of TX synthase strongly enhances platelet activation by these stimulants. This implicates an essential requirement of plasma components or other non-platelet factors for an inhibition of platelet activity by inhibitors of TX synthase.

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MATERIALS AND METHODS

Biochemicals. Human albumin was kindly supplied by DRK Blutspendezentrale (Baden-Baden, F.R.G.). Bovine albumin, essentially fatty acid-free, No. A 7511, apyrase grade I No. A 6132, arachidonic acid grade I No. 4006, indomethacin No. I 7378 were from Sigma GmbH (Taufkirchen, F.R.G.); ADP and BM 13.177 (4-[2-(benzenesulfonamido)-ethyl]-phenoxyacetic acid) were from Boehringer Mannheim GmbH (Mannheim); hydrogen peroxide and imidazole were from Merck (Darmstadt, F.R.G.); collagen was from Hormonchemie, (Munich, F.R.G.); methyl mercury chloride

(methyl-Hg) was from ICN K&K Labs Div. (Plainview, NY); dazoxiben (4-[2-(1*H*-imidazol-1-yl)-ethoxy]benzoic acid hydrochloride) was from Pfizer Central Res. (Sandwich, Kent, U.K.); U 46619 ((15*S*)-Hydroxy-11alpha,9alpha-(epoxymethano)-prosta-5Z,13E-dienoic acid) from Upjohn Co. (Kalamazoo, MI); human fibrinogen from Behringwerke, Marburg, F.R.G.

Human washed platelets. Washed platelets were prepared from human venous blood anticoagulated with 1/7 vol ACD NIH-formula A (0.8% citric acid, 2.2% sodium citrate, 2.45% hydrous dextrose) by my previously described method, i.e. washing in acid citrate [13]. The platelet concentrate was labelled with 1.6 μM (^{3}H)serotonin creatinine sulphate. For some test series, the platelet-rich plasma or the platelet concentrate was treated with 5 mM acetylsalicylic acid (ASA) for 30 min at 22° (for details see [14]). The washed platelets were finally suspended with NaCl 98 mM, KCl 5 mM, $CaCl_2$ 1 mM, $MgCl_2$ 0.1 mM, glucose 5 mM, human albumin 0.5 mg/ml, apyrase 50 μg/ml, sodium citrate 1 mM, sodium phosphate 1 mM, Tris/HCl-buffer pH 7.4 45 mM.

Platelet function tests. Platelet shape change and aggregation were followed turbidimetrically with two dual-channel aggregometers from LABOR GmbH (Hamburg, F.R.G.) at 37° [13, 14]. The stirring speed was 1000 rpm in aggregation and 400 rpm in shape change experiments. The latter were performed with added EDTA 2 mM to prevent aggregation. The percentage of shape change was calculated as follows: % = 100 × (amplitude of the increase in absorbance/amplitude of the increase in absorbance during full shape change with a high agonist concentration). In aggregation experiments, fibrinogen 300 μg/ml was added. The release of (^{3}H)serotonin was calculated as the percentage of the total platelet-bound radioactivity released. To obtain the platelet supernatant, the samples were rapidly cooled to ~ 0° and centrifuged at 0° for 30 sec (Eppendorf 3200 centrifuge). The inhibitors dazoxiben, imidazole, indomethacin, BM 13.177 and additional albumin were added 1 min prior to the platelet stimulants, if not otherwise indicated. Arachidonic acid, methyl mercury chloride and U 46619 were dissolved in dimethyl sulfoxide and 2 μl of these solutions were added to a 500 μl sample volume. The other agents were used as aqueous solutions in buffered saline added to the platelet suspension in volumes of 1/50 or less. Albumin at 50 mg/ml was dissolved in the suspending medium before addition of platelets. In some experiments, the washed platelet concentrate was diluted with various portions (1/5–9/10 vol) of platelet-poor plasma obtained from platelet-rich plasma by centrifugation (Eppendorf 3200 centrifuge) for 5 min at 0°. Threshold concentrations of platelet stimulants were concentrations just high enough to produce the desired platelet response. A sub-threshold concentration was just below an effective concentration.

TXB_2 assay. TXB_2 is the stable hydrolysis product of TXA_2 [2] and was used as a measure of the amount of TXA_2 produced during platelet stimulation. TXB_2 was determined by a commercially available (^{125}I)radioimmunoassay kit (New England Nuclear, No. NEK-024A). Platelet supernatants were prepared by centrifugation (Eppendorf 3200 centrifuge) for 30 sec at 0°, diluted with assay buffer and used for the assay or stored at −70°. Matrix effects of the diluted specimens on TXB_2 standards were not observed. In the figures and tables, the net formation of immunoreactive TXB_2 is noted, i.e. TXB_2 in the experiment minus TXB_2 in the non-stimulated control platelets. The latter was in the range between 0.4 and 0.8 ng/ml in the different platelet batches.

RESULTS

In human washed platelets, indomethacin as well as pre-treatment with ASA or the TX receptor antagonist BM 13.177 suppressed the platelet activations by hydrogen peroxide (Table 1), methyl-Hg, and arachidonic acid (AA) and strongly reduced the stimulating effects of collagen. However, the imidazole derivative dazoxiben (5–20 μM) or imidazole (1 mM) enhanced the shape change (Fig. 1) and potentiated aggregation and (^{3}H)serotonin release (Fig. 2) induced by collagen, hydrogen peroxide, methyl-Hg and AA. This potentiation was observed with all platelet batches prepared from 23 donors. Dazoxiben concentration-dependently reduced the TXB_2 formation whereas aggregation was increased (Fig. 3). Imidazole 1 mM produced the same increase in the rate of the collagen-induced shape change as dazoxiben 20 μM (Fig. 1). However, dazoxiben and imidazole did not alter the shape change, aggregation and (^{3}H)serotonin release induced by the TXA_2-mimetic U 46619 and the ADP-induced shape change and aggregation in ASA-treated washed platelets.

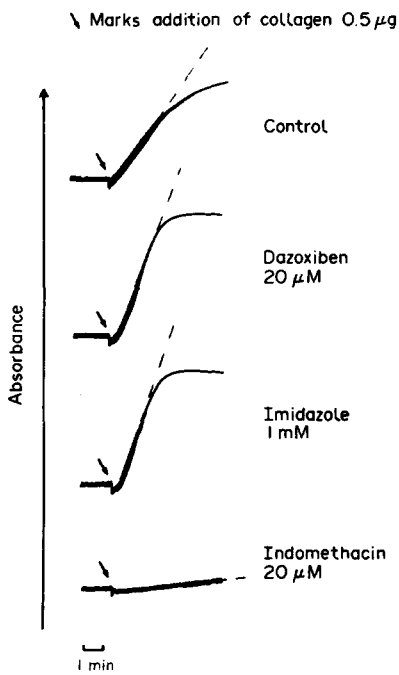

Fig. 1. Stimulation by dazoxiben and imidazole of rate and extent of collagen-induced shape change and inhibition by indomethacin. Dazoxiben, imidazole, and indomethacin were added 2 min prior to collagen 0.5 μg/ml applied at the arrows.

Table 1. Differential effects of indomethacin, dazoxiben, and BM 13.177 on shape change and TXB_2 formation induced by hydrogen peroxide

	Shape change $\bar{x} \pm$ S.E.M. (N) (%)	TXB_2 $\bar{x} \pm$ S.E.M. (N) (ng/ml)*
Hydrogen peroxide		
20 μM	27 ± 3 (3)	1.46 ± 0.75 (3)
100 μM	95 ± 2 (3)	3.38 ± 0.70 (3)
100 μM + indomethacin 20 μM	2 ± 1 (3)	0.20 ± 0.04 (3)
100 μM + dazoxiben 10 μM	98 ± 2 (3)	0.35 ± 0.15 (3)
100 μM + BM 13.177 10 μM	5 ± 2 (3)	3.81 ± 0.48 (3)

* Platelet concentration: 2×10^8/ml. The samples for TXB_2 determinations were taken 3 min after addition of hydrogen peroxide.

The TX receptor antagonist BM 13.177 and the TX synthase inhibitor dazoxiben showed opposite effects on the platelet activation and TXB_2 formation induced by arachidonic acid (Fig. 4) and hydrogen peroxide (Table 1). BM 13.177 did not inhibit the TXB_2 formation but suppressed the platelet activation in the presence or absence of dazoxiben, whereas dazoxiben did not reduce the activation although it inhibited the TXB_2 formation.

The platelet-stimulating effects of collagen, hydrogen peroxide (Table 2), methyl-Hg (Table 2), and AA but not the ADP-induced shape change and primary aggregation were inhibited by albumin. At 5 mg/ml instead of 0.5 mg/ml albumin, the former stimulants were equi-effective in inducing shape change and aggregation at about 2 instead of 1 μg/ml of collagen, 1 instead of 0.2 mM of hydrogen peroxide (Table 2), 4 instead of 2 μM of methyl-Hg (Table 2), and 30 instead of 10 μM of arachidonic acid. With hydrogen peroxide and methyl-Hg, the TXB_2 level was found to be about the same at the equi-effective agonist concentrations (Table 2). The potentiating effect of dazoxiben on stimulations by threshold concentrations of these stimulants was also strongly decreased at 5 mg/ml of albumin (Table 3), although dazoxiben inhibited the TXB_2 formation independent of the albumin concentration (Table 2). At a physiological albumin concentration of 50 mg/ml or when the washed platelets were resuspended with platelet-poor plasma, dazoxiben or imidazole did not enhance the stimulations by these agents (Table 3). Some series of experiments were performed with washed platelets suspended with fatty acid-free bovine albumin 0.5 mg/ml. Compared with a platelet suspension with "normal" human albumin 0.5 mg/ml, the activating effects of hydrogen peroxide or methyl-Hg were diminished but the potentiation by dazoxiben occurred in both preparations. Five mg/ml of fatty acid-free albumin inhibited and 10 mg/ml abolished the potentiating effect of dazox-

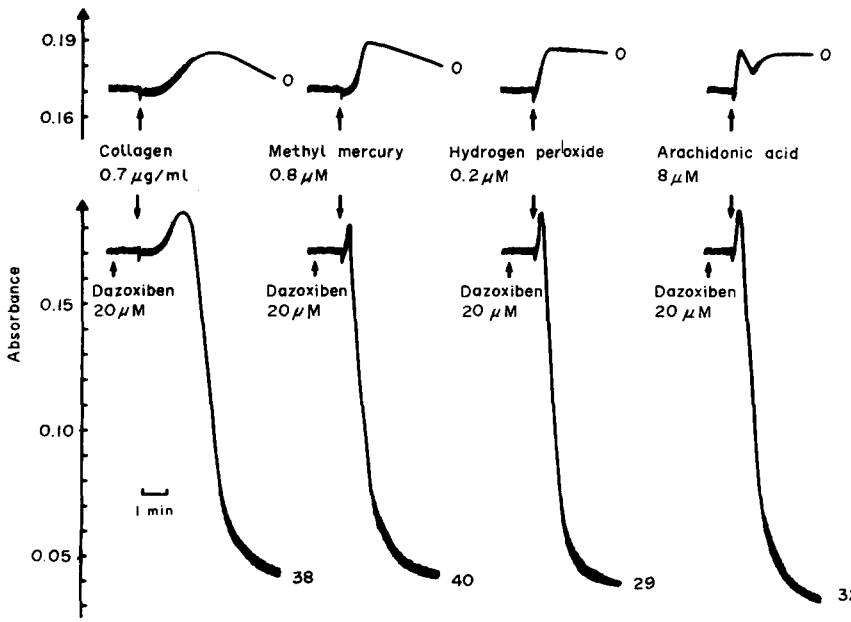

Fig. 2. Potentiation by dazoxiben of aggregation and (^{3}H)serotonin release induced by sub-threshold concentrations of collagen, methyl mercury, hydrogen peroxide and arachidonic acid. Percentage release is indicated by the numbers at curve ends.

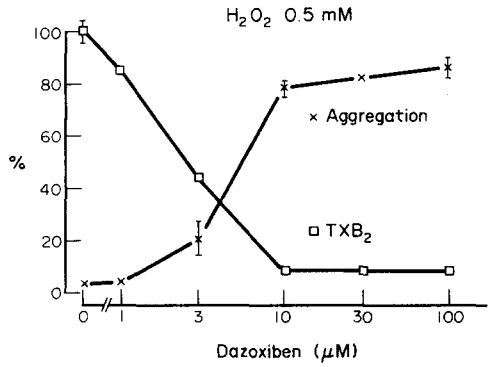

Fig. 3. Concentration-related inverse effects of dazoxiben on TXB_2 formation and aggregation of washed platelets stimulated with a threshold concentration of hydrogen peroxide (H_2O_2). TXB_2 formation and aggregation were measured in the same samples (N = 4). Extent of aggregation is given as percent decrease of absorbance at the aggregation maximum. The TXB_2 formation in the control (= 100%) was 8.6 ± 1.2 ng/ml ($\bar{x}$ ± S.E.M.). Symbols and bars indicate mean ± S.E.M. Where bars are lacking S.E.M. was less than the symbols.

iben on platelet shape change, aggregation and (^{3}H)serotonin release induced by arachidonic acid or methyl-Hg.

Table 2. Formation of immunoreactive TXB_2 at threshold concentrations of hydrogen peroxide and methyl-mercury (methyl-Hg) producing a strong aggregation of washed platelets. Comparison at two albumin concentrations*

	TXB_2 $\bar{x}$ ± S.E.M. (N) (ng/ml)†
Albumin 0.5 mg/ml	
Hydrogen peroxide:	
0.2 mM	12.4 ± 2.7 (4)
0.2 mM + dazoxiben 10 μM	0.6 ± 0.1 (3)
Methyl-Hg:	
2 μM	18.5 ± 6.1 (4)
2 μM + dazoxiben 10 μM	0.8 ± 0.2 (3)
Albumin 5.0 mg/ml	
Hydrogen peroxide:	
1 mM	9.2 ± 2.8 (5)
1 mM + dazoxiben 10 μM	0.9 ± 0.2 (5)
Methyl-Hg	
4 μM	14.1 ± 2.2 (6)
4 μM + dazoxiben 10 μM	0.5 ± 0.3 (3)

* At the higher albumin concentration, higher agonist concentrations were necessary to produce the same control aggregation at even slightly reduced levels of immunoreactive TXB_2. Dazoxiben suppressed TXB_2 formation under all conditions without reducing the aggregation.

† Platelet concentration: 2×10^8/ml. The samples for TXB_2 determinations were taken 3 min after addition of hydrogen peroxide or methyl-Hg.

DISCUSSION

This investigation focuses on human platelet stimulation that depends on cyclic endoperoxide and TXA_2 synthesis. At the concentrations used, hydrogen peroxide [15], methyl-Hg [16], and arachidonic acid (AA) were platelet agonists which essentially, and collagen for its major effect, required cyclic endoperoxide and TXA_2 formation. This was indicated by the ability of indomethacin, acetylsalicylic acid and the thromboxane receptor-antagonist BM 13.177 [14] to prevent the effects of hydrogen

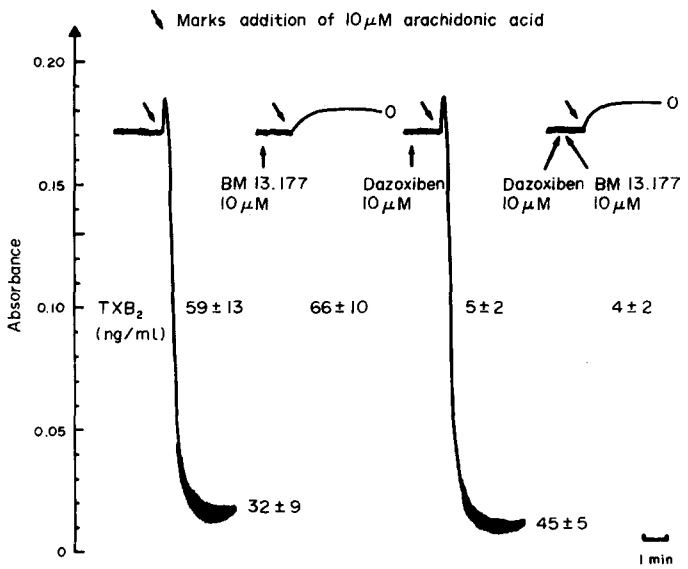

Fig. 4. Opposite effects of dazoxiben and BM 13.177 on TXB_2 formation, aggregation and serotonin release induced by arachidonic acid in washed platelets. Dazoxiben produces reduction of TXB_2, no inhibition of aggregation and increase in release; whereas BM 13.177 does not reduce TXB_2 and blocks aggregation and release with and without dazoxiben. The TXB_2 values are given as $\bar{x}$ ± S.E.M. (N = 3). Percentage release is indicated as $\bar{x}$ ± S.E.M. (N = 6) at curve ends. Platelet concentration: 2×10^8/ml.

Table 3. Inhibitory effect of albumin and of platelet-poor plasma (PPP) on the potentiation by dazoxiben of aggregation induced by methyl mercury (methyl-Hg)

		Aggregation $\bar{x} \pm$ S.E.M. (N) (%)		Effect ratio*
		Control	Dazoxiben 20 μM	
Albumin (mg/ml)	Methyl-Hg (μM)			
0.5	1.3 (1–1.5)‡	4.4 ± 1.2 (19)	83.7 ± 1.6 (19)	19†
5.0	6.2 (4–10)‡	14.6 ± 4.6 (11)	68.5 ± 3.9 (11)	4.7
10	13.1 (10–15)‡	31.1 ± 11 (6)	63.2 ± 7.5 (6)	2.0
50	115 (50–150)‡	43.8 ± 9.3 (6)	43.6 ± 8.1 (6)	1.0
PPP (%)	Methyl-Hg (μM)			
2	5.7 (4–10)‡	10.2 ± 5.6 (6)	78.6 ± 4.7 (6)	7.7
40	12.2 (10–20)‡	23.4 ± 5.7 (13)	56.6 ± 7.5 (13)	2.4
90	81.8 (50–150)‡	51.2 ± 6.2 (17)	41.1 ± 6.9 (17)	0.8

* The effect ratio (= aggregation with dazoxiben/aggregation control) is a measure of potentiation.

† Notes the degree of potentiation in the control suspension with albumin 0.5 mg/ml.

‡ Means (and range) of agonist concentrations are given. Various control effects were chosen in order to discriminate with high sensitivity between different degrees of potentiation.

peroxide, methyl-Hg, and AA and to inhibit the major effect of collagen. Platelet stimulation induced by collagen is known to involve also pathways independent of cyclooxygenase products [17]. In contrast, the activations by hydrogen peroxide or methyl-Hg provide models of platelet activation depending entirely on prostaglandin/thromboxane synthesis from endogenous arachidonic acid. The lowest amount of endogenously formed, immunoreactive TXB_2 that was measured during a significant shape change in the washed platelets was ~ 4 nM.

Three major conclusions can be reached based on the experiments with dazoxiben and imidazole. First, a blockade of thromboxane formation in platelets leads to the accumulation of platelet-stimulating cyclooxygenase products, probably cyclic endoperoxides, with greater platelet-stimulating potency than TXA_2. The potentiating effect of TX synthase inhibitors is independent of whether exogenous or endogenous AA is metabolized. Dazoxiben does not influence the platelet-activating effects of the TXA_2-mimetic U 46619 and of ADP in ASA-treated platelets, showing that its potentiating effect is limited to platelet activation which involves PG synthesis. The cyclic endoperoxides PGG_2 and PGH_2 are believed to be less potent platelet stimulants but have an approximately tenfold longer half life than TXA_2 in physiological solutions [6]. Thus PGH_2 (and PGG_2) can be expected to reach and maintain higher agonist concentrations than TXA_2. Since they already achieved a greater platelet-stimulating potency than TXA_2 when TX formation was only halved, their intrinsic activity is not very different from TXA_2 in washed platelets. It is also reasonable to assume that a portion of PGH_2 is converted into PGE_2 [18]. PGE_2 is not a direct platelet agonist but effectively supports platelet activation by other agonists [19].

Second, albumin or plasma not only inhibits cyclooxygenase-dependent platelet stimulation but also suppresses the potentiating effect of TX synthase inhibitors. Albumin also exhibited these effects during platelet stimulations by endogenous AA, suggesting that endogenously mobilized AA or its metabolites may escape from platelets and interact with external albumin. Binding of AA or of the prostanoic agonists to albumin [20] most likely causes the inhibitory effect of albumin and platelet-poor plasma on the cyclooxygenase-dependent platelet activations. It is notable that at a higher albumin concentration higher concentrations of hydrogen peroxide or methyl-Hg were necessary to produce the same level of immunoreactive TXB_2 and platelet response. An interaction of cyclic endoperoxides with albumin is also involved in the ability of albumin to abolish the potentiating effect of dazoxiben. Albumin either binds more cyclic endoperoxides than TXA_2 or catalyzes the conversion of cyclic endoperoxides into platelet-inhibitory products. The demonstration of PGD isomerase activity in human serum albumin [21, 22] underlines the latter conclusion. It obtains direct support from the recent observation of Gresele *et al.* [23] that exogenously added serum albumin increases the production of the anti-aggregatory PGD_2 from cyclic endoperoxides in dazoxiben-treated platelet-rich plasma and makes "non-responder" platelets to be inhibited by dazoxiben.

Third, inhibition of platelet activation by inhibitors of TX synthase requires non-platelet factors such as albumin or plasma to divert the cyclic endoperoxide metabolism to anti-aggregatory products. The dif-

ferentiation between "responders" and "non-responders" to dazoxiben, as based on inhibition experiments with platelet-rich plasma [7], describes different effects of plasma components instead of a varying platelet response in different individuals. Thus, in order to profit from a diversion of the cyclic endoperoxide metabolism by TX synthase inhibitors *in vivo*, it appears rational to make sure that cyclic endoperoxides cannot substitute for TXA_2 as platelet agonists. This can be achieved by combination with a thromboxane receptor antagonist [14, 24] which recently became available for use in humans.

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