A SELECTIVE THROMBOXANE SYNTHETASE INHIBITOR BLOCKS THE cAMP LOWERING ACTIVITY OF PGH_2

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SUMMARY

The thromboxane synthetase inhibitor, 9,11-azoprosta-5,13-dienoic acid, blocks both platelet aggregation and the cyclic AMP lowering activity of the prostaglandin endoperoxide PGH_2. These data indicate PGH_2 must be converted into thromboxane A_2 in order to lower cAMP or induce platelet aggregation.

INTRODUCTION

The initial report by Kloeze (1) that PGE_1 inhibited human platelet aggregation was followed by numerous papers that associated the PGE_1 inhibition with an elevation of platelet cAMP levels (2,3). Salzman hypothesized that agents that elevate cAMP inhibit platelet aggregation, while agents that decrease cAMP levels induce platelet aggregation (4). Willis (5) was the first to report a "labile aggregation-stimulating substance" (LASS) that was formed from arachidonic acid. Later, Hamberg and Samuelsson (6) and Nugteren and Hazelhof (7) isolated and identified structurally the prostaglandin endoperoxides. Hamberg et al. (8) later showed that the purified endoperoxides were potent inducers of human platelet aggregation, and that LASS was a mixture of PGG_2 and PGH_2. Since these initial observations of the biological activity of endoperoxides in platelets, another short-lived inter-

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mediate, formed from PGH_2, thromboxane A_2 (TXA_2) has also been identified by Hamberg et al. (9).

The relationships between the pro-aggregatory, activity of PGH_2, and cyclic nucleotide modulation were clarified by Miller and Gorman (10) who reported that the endoperoxides were potent inhibitors of PGE_1-stimulated cAMP accumulation in human platelets. Subsequent work from our laboratory has shown that, on a molar basis, TXA_2 is both a more potent inducer of platelet aggregation and inhibitor of PGE_1-stimulated cAMP accumulation than PGH_2 (11). Recently we reported a "reciprocal regulation" of cAMP levels in human platelets by prostacyclin (PGI_2) and the PGH_2-TXA_2 system. The balance between the cAMP lowering activity of the PGH_2-TXA_2 system and the adenylate cyclase stimulating activity of prostacyclin appears to control human platelet aggregation (12).

It has been reported that the conversion of PGH_2 into TXA_2 is not obligatory for the induction of human platelet aggregation (13,14). Our discovery of a potent thromboxane synthetase inhibitor, the enzyme that converts PGH_2 to TXA_2, has allowed us to study this problem directly (15). The inhibitor, 9,11-azoprosta-5,13-dienoic acid (Azo analog I), is an inhibitor of arachidonic, PGH_2, and collagen-induced platelet aggregation as well as the second wave of epinephrine and ADP-induced aggregation. Although Azo analog I inhibits aggregation, it does not elevate platelet cAMP (15). We now report that Azo analog I blocks the cAMP lowering activity of PGH_2, which suggests that PGH_2 must be converted to TXA_2 in order to either inhibit cAMP accumulation or induce human platelet aggregation.

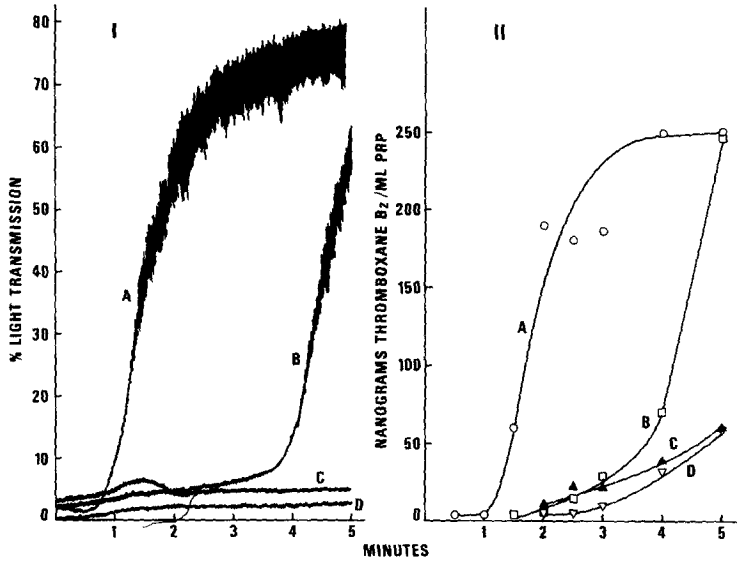

Fig. 1. Effect of Azo analog I on arachidonic acid-induced platelet aggregation, and TXB_2 formation. Arachidonic acid (400 µg/ml) was incubated with 2.0 ml of PRP and the platelet aggregations monitored. (Panel I). A - Control aggregation with arachidonic acid alone. B, C, D - Arachidonic acid added to platelets that had been preincubated for 2 min with 1.4, 2.8 or 5.6 µM Azo analog I. (Panel II). At the specified time intervals, 0.1 ml aliquots of PRP were removed from the aggregations shown in Panel I and were analyzed for TXB_2 formation by RIA as described previously (16).

METHODS

All aggregation experiments and cAMP measurements were done with fresh human platelet rich plasma (PRP) that was prepared by withdrawing blood directly into 3.8% (v/v) trisodium citrate, followed by centrifugation at 200 xg for 10 min at room temperature.

Platelet cAMP levels were measured by radioimmunoassay (RIA) according to Steiner et al. (16), with the incorporation of the acetylation-modification of Harper and Brooker (17). All samples were tested at two dilutions, and all of the immunodetectable cAMP could be destroyed by beef heart phosphodiesterase. The levels of cAMP were the same with or without column separation and purification prior to radioimmunoassay (10).

Human platelet microsomes (HPM) were prepared as described previously (11) by a slight modification of the method of Needleman et al. (18). PGH_2 was prepared bio-

synthetically according to Gorman et al. (19), and thromboxane B_2 was measured by RIA according to Fitzpatrick et al. (20).

RESULTS AND DISCUSSION

The potency of Azo analog I as a thromboxane synthetase inhibitor and antagonist of arachidonic acid-induced platelet aggregation is shown in Fig. 1. In this experiment, the addition of 400 µg/ml of arachidonic acid to PRP results in a rapid platelet aggregation IA, which is antagonized by 1.4 µM Azo analog I and completely blocked by incubation with the inhibitor at 2.8 or 5.6 µM IB, IC and ID, respectively. Coincident with the onset of aggregation, TXB_2, the stable hydrolysis product of TXA_2 is synthesized, IIA. However, in the presence of Azo analog I, there is a dose-dependent inhibition of TXB_2 (e.g. TXA_2) formation that parallels the observed aggregation patterns IIB, C, and D.

The effects of Azo analog I on both human platelet aggregation induced by PGH_2 and prostacyclin-stimulated cAMP levels are summarized in Fig. 2.

An analysis of the cAMP levels shows an inverse relationship between cAMP levels and the intensity of platelet aggregation.

Reading left to right, the basal level of cAMP (no additions) is not changed by 5.6 µM Azo analog I. The addition of platelet microsomes does not change the cAMP level, but induces a small aggregatory response. Azo analog I does not alter either the small aggregation due to microsomes, or change the basal cAMP levels. Prostacyclin 0.028 µM induces a dramatic rise in cAMP, which is not antagonized by 5.6 µM Azo analog I. In this experiment, 0.56 µM PGH_2 induces a violent platelet aggregation, without altering

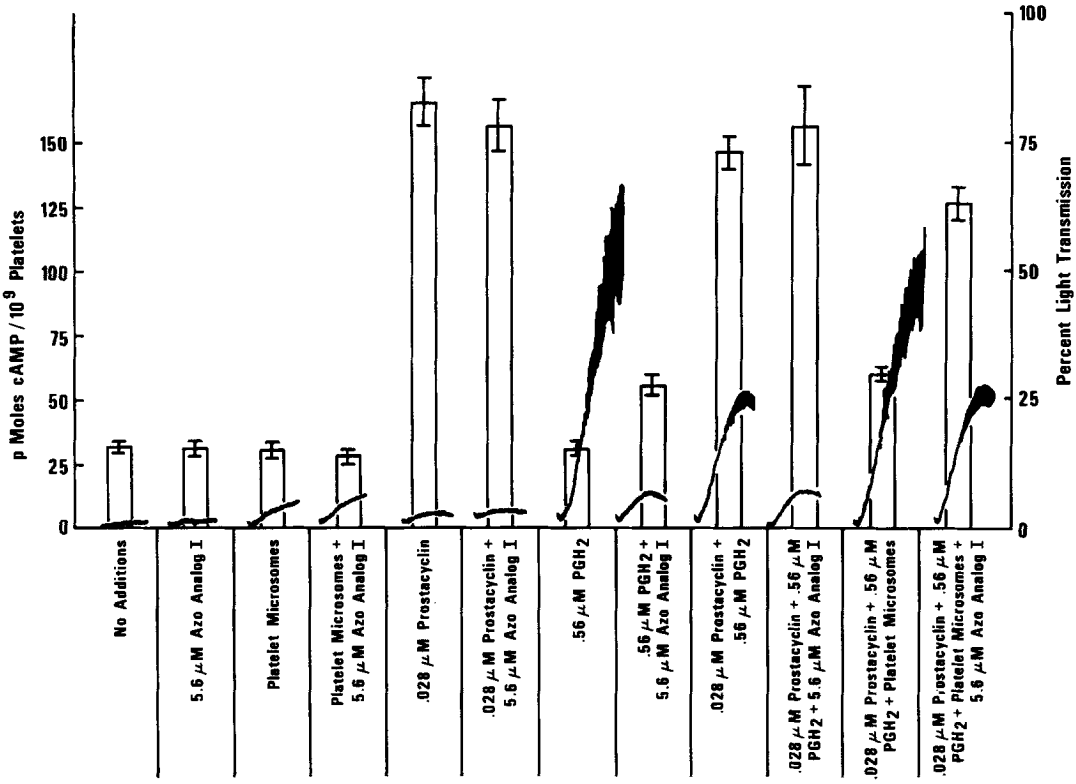

Fig. 2. Effect of Azo analog I on the cAMP lowering activity of PGH_2. One ml aliquots of PRP (0.68 x 10^9 platelets/ml) were preincubated for 2 min at 37°, and then the appropriate compounds were added and platelet aggregation monitored for 1 min. For the samples that contained Azo analog I, both the platelets and the microsomes were preincubated for 2 min with the inhibitor. At the end of 1 min, the aggregations were stopped by the addition of 0.7 ml of 10% trichloroacetic acid and freezing in liquid nitrogen. The samples were subsequently extracted with ether and the cAMP contents measured as described in the text. Twenty µl of the platelet microsomal preparation used to generate TXA_2 contained 80 µg of protein. Data presented as Mean ± S.E.M. of triplicate samples.

the basal level of cAMP, but 5.6 µM Azo analog I completely blocks the PGH_2-induced aggregation with only a small increase in cAMP levels, which is probably due to PGE_2 and PGD_2 formation from the endoperoxide. Coincubation of

prostacyclin and 0.56 μM PGH_2 in this series of experiments results in a partial inhibition of the PGH_2-induced aggregation with a modest decrease in the prostacyclin-induced cAMP accumulation. Azo analog I suppresses the PGH_2-induced aggregation further without a significant change in cAMP levels. However, if the same concentration of PGH_2 is incubated with 20 μl of platelet microsomes (and thromboxane A_2 formed) there is a potent aggregation, even in the presence of 0.028 μM prostacyclin. The generation of TXA_2 results in an essentially complete inhibition of the prostacyclin-stimulation of cAMP levels. The addition of 5.6 μM Azo analog I, which blocks TXA_2 synthesis, inhibits both the aggregation and the cAMP lowering activity due to TXA_2 generation.

Our previous work has shown that TXA_2 was a more potent inhibitor of PGE_1-stimulated cAMP accumulation and inducer of platelet aggregation than PGH_2 (11). These experiments with Azo analog extend our previous observations, and suggest that both aggregation and the cAMP lowering activity of PGH_2 are dependent upon its conversion to TXA_2.

Recent reports that imidazole can inhibit TXB_2 synthesis without blocking PGH_2-induced platelet aggregation suggested that PGH_2 and not TXA_2 is the active platelet aggregator (14). However these studies were done with washed platelets which can give erroneous results. We have studied imidazole and found that it does inhibit both the proaggregatory and the cyclic AMP lowering activity of PGH_2 when the experiments are done in PRP (Gorman and Fitzpatrick, unpublished experiments). Support for our data is found in a recent report where another thromboxane

synthetase inhibitor (2-isopropyl-3-nicotinyl-indole) also was found to block arachidonate-induced aggregation (22).

The ability of the thromboxane system to lower cAMP levels, and the prostacyclin system to stimulate adenylate cyclase, offers a novel regulatory mechanism. Both TXA_2 and prostacyclin share a common precursor molecule PGH_2. In the cells where the prostacyclin synthetase predominates, synthesis of endoperoxide will result in an increase in cAMP. However, if the thromboxane synthetase is dominant, a lowering of cAMP will result. This "reciprocal regulation" of cAMP by TXA_2 and prostacyclin offers a unifying theme for the mechanism of action of prostaglandins.

It is possible that Azo analog I is a receptor level antagonist of PGH_2, as well as a thromboxane synthetase inhibitor. However, our ability to duplicate our results using imidazole in place of Azo analog I, makes this alternative mechanism unlikely.

The discovery and use of compounds such as Azo analog I will allow study of the various parts of the endoperoxide-thromboxane system independently, and offer exciting new tools for the study of the products of arachidonic acid metabolism.

In addition, Azo analog I may prove to be a valuable antithrombotic drug. Unlike agents that inhibit the prostaglandin cyclo-oxygenase, it is capable of antagonizing endoperoxide-thromboxane mediated aggregations while leaving intact the synthesis of classical prostaglandins.

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