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PLATELET THROMBOXANE A_2/PROSTAGLANDIN H_2 RECEPTORS IN HUMAN VOLUNTEERS ON LOW DOSES OF ASPIRIN

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Abstract—Administration of aspirin (81 mg/day for 2–3 weeks) in nine healthy volunteers (out of an initial ten subjects, only nine qualified) resulted in a >95% decrease of thromboxane B_2 production by thrombin-stimulated platelets. At the same time, ligand binding studies with a thromboxane A_2 antagonist, ^{125}I-PTA-OH, measurements of shape change, and aggregation of platelets stimulated with U46619, a prostaglandin H_2 analogue, indicated that administration of aspirin to normal human subjects does not result in the up-regulation of platelet thromboxane A_2/prostaglandin H_2 receptors.

The arachidonic acid metabolites, prostaglandin H_2 (PGH_2) and thromboxane A_2 (TXA_2) have been shown to be potent inducers of platelet aggregation and secretion [1, 2]. It is well established that aspirin inhibits PGH_2 and TXA_2 production in platelets through an irreversible inhibition of cyclooxygenase [3, 4], and it is generally accepted that this mechanism is critical for the therapeutic effects of aspirin in thromboembolic and cardiovascular disease [5–7]. However, the effect of aspirin administration on TXA_2/PGH_2 receptors on the platelet surface has not been elucidated.

Desensitization of platelet TXA_2/PGH_2 receptors has been shown to occur *in vitro*. Exposure of washed human platelets to the TXA_2 agonist, U46619, involves uncoupling of the receptor from its G protein followed by a decrease in the number of binding sites for the TXA_2 agonist, [^{3}H]U46619, or its antagonists, [^{3}H]SQ 29548 and ^{125}I-PTA-OH [8, 9]. Since aspirin prevents TXA_2/PGH_2 production by the platelets and megakaryocytes, we hypothesized that it may up-regulate thromboxane receptors on the surface of these cells.

In this study, ten healthy volunteers were given 81 mg aspirin per day for 14–21 days. TXA_2/PGH_2 receptors were assessed both by ligand binding studies and by functional studies before and after aspirin treatment. The ability of the platelets to generate thromboxane when stimulated by thrombin was also tested before and after aspirin treatment.

MATERIALS AND METHODS

Reagents. I-PTA-OH and ^{125}I-PTA-OH, stable TXA_2/PGH_2 receptor antagonists, were gifts of Dr. Perry Halushka (Medical University of South Carolina) and Dr. R. Garlick (New England Nuclear, Boston, MA). [^{3}H]Thromboxane B_2 for the thromboxane B_2 (TXB_2) radioimmunoassay was also purchased from New England Nuclear. The anti-TXB_2 antiserum was a gift from Dr. J. Bryan Smith (Temple Unversity, Philadelphia, PA). U46619, a stable PGH_2 analogue, was purchased from the Cayman Chemical Co. (Denver, CO). Prostaglandin E_1, γ-globulin, thrombin, ADP, apyrase grade IV, bovine serum albumin, glucose and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer were purchased from the Sigma Chemical Co. (St. Louis, MO). All other reagents were purchased from the Fisher Scientific Co. (King of Prussia, PA).

Volunteers. Ten healthy volunteers, four females and six males, ages 22- to 35-years-old, participated in this study after informed consent was obtained. All subjects were aspirin-free for 14 days and non-steroidal anti-inflammatory drug-free 48 hr prior to testing. Over the next 2 days (days 15 and 16), 180 mL of blood was drawn (90 mL/day). The volunteers were instructed to take 81 mg of aspirin per day for the next 14 days. On days 30 and 31, 180 mL of blood was drawn again (90 mL/day).

Binding of ^{125}I-PTA-OH to washed platelets. Binding studies were performed according to the procedure previously described by Mais *et al.* [10]. Tris buffer used in the experiments was supplemented with 4 mM EDTA.

K_d and B_{max} were estimated by fitting the binding data to a two-parameter model, and using an iterative simplex computer program for non-linear regression analysis [11]. K_d is the dissociation constant for I-PTA-OH, and it was expressed in nM. B_{max} is the maximum binding expressed as fmol/10^7 platelets, and it was used in calculating the number of binding sites.

Platelet isolation for functional studies. At each sample collection, 90 mL of blood was drawn from a forearm vein. The blood was collected into tubes containing the anticoagulant, acid citrate dextrose (ratio 1:8). Platelets were isolated by using the method developed by Mustard *et al.* [12] and modified by Morinelli *et al.* [13].

Aggregation studies. Aggregation studies were carried out in a Payton dual chamber aggregometer. Platelets were suspended in Tyrode's albumin buffer containing 10 mM Hepes (pH 7.35). A final assay

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Table 1. Effect of administration of aspirin on the platelet TXA_2/PGH_2 receptors and on the platelet responses to U46619

	TXB_2 (ng/mL)	Number of ^{125}I-PTA-OH binding sites per platelet	Binding affinity, K_d (nM)	Platelet aggregation, EC_{50} (μM)	Platelet shape change, EC_{50} (μM)
Before aspirin	19.2 ± 6.8 (9)	2226 ± 642 (5)	32.8 ± 11.5 (5)	0.422 ± 0.058 (6)	0.064 ± 0.012 (9)
After aspirin	0.22 ± 0.04* (9)	2168 ± 746 (5)	34.2 ± 9.2 (5)	0.414 ± 0.058 (6)	0.056 ± 0.008 (9)

Values are means ± SD (the number of experiments is given in parentheses).
* Statistically different from the value before aspirin ($P < 0.01$).

volume of 500 μL was maintained by adding 490 μL of washed platelets (conc. 250,000–400,000 platelets/μL), which were allowed to equilibrate for at least 1 min, and then stimulating them with 10 μL of the appropriate agonist concentration. The aggregatory response was monitored for at least 3 min, and the rate of this response was determined as the slope of the line drawn through the primary aggregation curve for each of the agonist concentrations. The EC_{50}, which is the concentration (in μM) at which half the maximal response occurs, was estimated according to Tallarida and Murray [14].

Shape change. The Payton aggregometer was also used to determine shape change; however, the sensitivity of the machine was turned up to augment the shape change recording [13]. Again, a final assay volume of 500 μL was maintained. A baseline was established by allowing 490 μL of washed platelets to stir at 500 rpm for approximately 30 sec or until equilibrium was reached. A volume of 10 μL was added, and the decrease in light transmission was measured in arbitrary units, LTUs (light transmission units). A concentration–response curve was constructed by adding U46619 starting at 1 μM and decreasing the concentration of this agonist until no further shape change was observed. The EC_{50} was calculated as described for platelet aggregation.

Thromboxane B_2 determination. A sample of platelet-rich plasma (PRP) (375 μL) was stimulated with 4 units of thrombin and allowed to incubate at room temperature for 5 min. The samples were then spun for 2 min in a Fisher Scientific Microcent (Model No. 2350), and the supernatant fraction was drawn off and frozen at −70°. The radioimmunoassay of TXB_2 was performed as described by Lewy *et al.* [15].

Statistical analysis. Paired Student's *t*-tests were performed on all results to determine the significant differences between pre- and post-treatment groups. For this software package, the *Manual of Pharmacological Calculations with Computer Programs* was employed [14].

RESULTS

Aspirin was administered to volunteers as described in Materials and Methods. Adherence to the schedule was determined by the maximum platelet TXB_2 production performed both before and after aspirin treatment. Only those subjects whose platelet thromboxane production was inhibited more than 95% and whose pre-aspirin TXB_2 production was at least 2 ng/mL were qualified for the remaining experiments. These qualifications were met by nine out of ten volunteers. The results of all experiments are listed in Table 1. Nine subjects averaged a pre-aspirin PRP TXB_2 level of 19.2 ± 6.8 ng/mL and a post-aspirin treatment TXB_2 level of 0.22 ± 0.04 ng/mL. This difference was statistically significant ($P < 0.01$). The pre-aspirin values of TXB_2 were slightly lower than those reported by Fitzgerald *et al.* [16].

In the platelet shape change experiments with U46619, the mean EC_{50} of nine of the volunteers ($N = 9$) before aspirin therapy (0.064 ± 0.012 μM) was not significantly different from the mean EC_{50} after aspirin therapy (0.056 ± 0.008 μM).

In six volunteers, the EC_{50} values for platelet aggregation before and after aspirin therapy were 0.422 ± 0.058 and 0.414 ± 0.058 μM respectively. The difference was not statistically significant.

For the binding studies, complete results from five volunteers were used to tabulate the mean values for K_d and the number of binding sites. There was no significant difference in K_d before (32.8 ± 11.5 nM) or after (34.2 ± 9.2 nM) aspirin therapy, nor in the number of binding sites (2226 ± 642 sites/platelet vs 2168 ± 746 sites/platelet).

DISCUSSION

Previous investigations from our laboratory suggested that there are two types of platelet TXA_2/PGH_2 receptors: high-affinity receptors governing platelet shape change and myosin light chain phosphorylation and low-affinity receptors governing serotonin release from the platelets and platelet aggregation [13, 17]. Binding studies with ^{125}I-PTA-OH can detect only high-affinity receptors, whereas analysis of binding isotherms of [^{3}H]U46619 to platelets suggests the presence of both low- and high-affinity receptors [13].

It can be concluded from our experiments on ligand binding and shape change of platelets stimulated with U46619 that high-affinity TXA_2/PGH_2 receptors are not regulated following administration of low doses of aspirin to normal volunteers. The lack of regulation of the TXA_2 receptors under these conditions is not unexpected given the low level of TXA_2 formation. It is not excluded that alteration in the receptors may occur in subjects with increased TXA_2 formation. The release of a high concentration

of TXA_2 locally at the site of vessel injury may also regulate platelet TXA_2/PGH_2 receptor function without a demonstrable systemic effect.

Although I-PTA-OH requires only a single site, the platelet aggregation studies suggest that low-affinity receptors are also not altered in volunteers on low doses of aspirin.

A number of investigators [16, 18–20] observed that there was no correlation between TXB_2 level in serum and degree of inhibition of platelet aggregation in individuals treated with various doses of aspirin. It appears that platelets of some individuals may show a slight decreased sensitivity to aspirin as assayed by means of platelet aggregation. This may be due to the differences in the turnover of megakaryocytes or the turnover in the cyclooxygenase of the endothelial cell which is known to synthesize prostacyclin, a potent inhibitor of platelet aggregation [21], rather than to the regulation of platelet TXA_2/PGH_2 receptors.

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