Persistence of Thromboxane A_2-Like Material and Platelet Release-Inducing Activity in Plasma

J. B. SMITH, CAROL INGERMAN, and M. J. SILVER

From the Cardeza Foundation and the Department of Pharmacology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

ABSTRACT During the incubation of arachidonic acid with platelet-rich plasma, a persistent activity appeared that caused the release of [^{14}C]5-hydroxytryptamine from indomethacin-treated platelets. By the time-course of its appearance and disappearance, this release-inducing activity could be dissociated from prostaglandin endoperoxides and associated with thromboxane A_2-like material. This material persists in plasma because of its continued production and increased stability.

INTRODUCTION

The observation that arachidonic acid, a prostaglandin precursor, induces platelet aggregation and the platelet release reaction (1) initiated several investigations to elucidate the biochemical nature of these phenomena. It was found that prostaglandin endoperoxides (PGG_2 and PGH_2)[1] are potent inducers of platelet aggregation (2) and that they accumulate transiently during the incubation of arachidonic acid with platelet-rich plasma (3). Arachidonic acid-induced platelet aggregation, the platelet release reaction, and prostaglandin endoperoxide formation can all be prevented by the prostaglandin synthetase inhibitors aspirin and indomethacin, while endoperoxide-induced platelet aggregation cannot. In recent studies using washed human platelets resuspended in a physiological salt solution, it was shown that either arachidonic acid or PGG_2 is converted within 30 s into thromboxane A_2, a highly unstable intermediate with a $t_{\frac{1}{2}}$ at 37°C in aqueous medium of 32±2 s (4). Evidence was presented that thromboxane A_2 induces irreversible platelet aggregation and causes the release of serotonin from platelets pretreated with indomethacin. Previously, we had reported in a preliminary communication (5) that when arachidonic acid is incubated with platelets in the presence of plasma, the release-inducing activity that develops is maximal at 3 min and still present in appreciable amounts after 10 min. We now present evidence that this release-inducing activity is thromboxane A_2.

METHODS

Platelet preparations. Human blood was collected from volunteers, who denied having taken any drug within the previous week, into 0.05 vol of 0.1 M EDTA (pH 7.4) or 0.1 vol 3.8% trisodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation at 250 *g* for 15 min at 20–22°C. A part of the EDTA-PRP was further centrifuged at 1,000 *g* for 20 min to prepare platelet-poor plasma (PPP). The platelets sedimented in this manner were resuspended with a siliconized Pasteur pipette in either one-half or one-sixth vol of either PPP or a buffered saline solution (134 mM NaCl, 5 mM D-glucose, 15 mM Tris-HCl, pH 7.4). Platelet content was determined in a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.).

Release of 5-hydroxytryptamine. A portion of the PRP under study was incubated at 37°C for 15 min with [3-^{14}C]5-hydroxytryptamine creatinine sulphate, sp act 57 mCi/mmol (Amersham/Searle Corp., Arlington Heights, Ill.) at a final concentration of 0.5 μM and either aspirin (1 mM) or indomethacin (10 μM).[2] Samples (0.5 ml) of this PRP were placed in Eppendorf tubes (Eppendorf microfuge, Brinkmann Instruments, Inc., Westbury, N.Y.) and maintained at 20–22°C.

A portion of nonradioactive PRP, adjusted to contain about 10^9 platelets/ml by resuspension in 0.5 vol of plasma, was incubated at 37°C for different times with 1 mM arachidonic acid. Samples of 0.5 ml were transferred to and mixed with the PRP in the Eppendorf tubes, and incubation was continued at ambient temperature for 5 min. The sam-

Part of this work was presented at the Fall Meeting, American Oil Chemists Society, 1974, Philadelphia, Pa.

Received for publication 1 December 1975 and in revised form 23 July 1976.

[1] *Abbreviations used in this paper*: PG, prostaglandin; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

[2] We confirmed that these drugs do not inhibit the uptake of 5-hydroxytryptamine by platelets, and more than 90% of the radioactivity was incorporated into the platelets under these conditions. Less than 1% release of radioactivity from the platelets occurred after the direct addition of 1 mM arachidonic acid (99% pure, Nucheck Prep., Inc. Elysian, Minn.).

ples were centrifuged for 1 min at 15,000 *g*, and 0.2 ml of the supernatant fluid was taken to determine the percentage release of radioactivity. Controls made by incubating arachidonic acid with PPP and transferring to the PRP in the Eppendorf tubes were routinely subtracted. Percentage release was calculated by the formula: % release = cpm (test supernatant) − cpm (control supernatant)/cpm (total sample) − cpm (control supernatant)] × 100, where the radioactivity in the control supernatant was between 5 and 10% of that of the total sample.

This mixed system of aspirin-inhibited and untreated platelets for studying substances responsible for intracellular communication causing release and aggregation was introduced by White and Witkop (6).

Measurements of PGG_2, PGH_2, and thromboxane A_2. For the measurement of prostaglandin endoperoxides (PGG_2 and PGH_2), nonradioactive PRP containing about 1×10^9 platelets/ml was incubated at 37°C for different times with 1 mM arachidonic acid, and duplicate tubes were decanted into either 10 ml ethanol or 10 ml ethanol containing 50 mg stannous chloride. Prostaglandin $F_{2\alpha}$ was extracted, purified by column chromatography, and determined by radioimmunoassay as described previously (3). The difference in values of $PGF_{2\alpha}$ between reduced and unreduced samples was taken to be equivalent to the amount of endoperoxides. The amounts of $PGF_{2\alpha}$ in unreduced samples increased as a function of time to a maximum of 10 pmol/ml.

Since previous experiments (7) had demonstrated that plasma diminishes the oxygenation of archidonic acid by platelets, thromboxane A_2 determinations were performed with platelet suspensions adjusted to contain about 3×10^9 platelets/ml by resuspension in one-sixth vol of either buffered saline or PPP as described above. Thromboxane A_2 formation from arachidonic acid catalyzed by platelets resuspended in saline was determined as described elsewhere (4).

For the determination of thromboxane A_2 formed in plasma, 1-ml samples were incubated at 37°C for different times with 1 mM arachidonic acid containing 1 μCi [1-^{14}C]-arachidonic acid (Applied Science Labs, Inc., State College, Pa.). The samples were decanted into 5 ml methanol containing 25 mg stannous chloride and diluted with 4 ml saline. The stannous ion was precipitated by the addition of 1 M sodium hydroxide to pH 7, 0.1 ml formic acid was added, and the samples were extracted three times with 10 ml chloroform. The extracts were taken to dryness and applied to a silicic acid column (0.5 g). A fraction eluted with 10 ml chloroform was discarded, while a 12-ml fraction eluted with chloroform:methanol (95:5) was collected and subjected to thin layer chromatography using the solvent system: organic layer of ethyl acetate/2,2,4-trimethyl pentane/water/acetic acid (75:75:100:4 vol/vol).

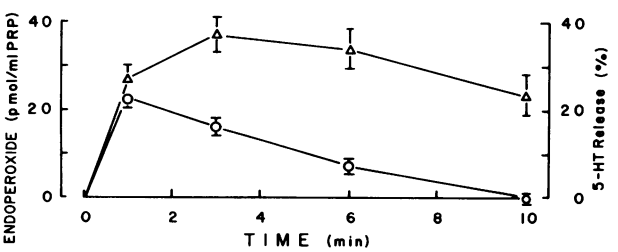

FIGURE 1 Appearance and disappearance of 5-hydroxytryptamine (5-HT)-releasing activity (△) and prostaglandin endoperoxides (○) during incubation of arachidonic acid with PRP. The experiments were performed as described in Methods, with EDTA-PRP, and the results shown are the mean (±SE) of duplicate determinations performed with PRP obtained from two donors.

RESULTS

When arachidonic acid was incubated with PRP, an activity was detected that caused the release of radioactive 5-hydroxytryptamine from aspirin or indomethacin-treated platelets. Since none of this activity (less than 2% release of 5-hydroxytryptamine) was detected when arachidonic acid was incubated with PPP, the release-inducing substance must have resulted from the interaction of arachidonic acid with the platelets not treated with aspirin. The time-course of the appearance and disappearance of this substance (Fig. 1) could be deduced from the percentage of radioactivity released in labeled, aspirin-treated PRP to which mixtures of arachidonic acid and PRP were transferred at different times. Maximal release of 5-hydroxytryptamine occurred when the transfer was made at 3 min, but appreciable release still occurred when the transfer was made at 10 min. The addition of frozen-thawed PRP to labeled aspirin-treated platelets did not induce the release of 5-hydroxytryptamine, suggesting that the release-inducing substance was not stored in the platelets, but rather formed from the added arachidonic acid.

It was possible that the release-inducing substance was a prostaglandin endoperoxide generated from arachidonic acid. Therefore, the amount of endoperoxide present at different times after addition of arachidonic acid to PRP was determined simultaneously with measurements of release-inducing activity (Fig. 1). Maximal amounts of the prostaglandin endoperoxides were present at 1 min as reported previously (3), and they were no longer detectable at 10 min. The substance was therefore dissociated from prostaglandin endoperoxides.

The time-course of appearance and disappearance of the release-inducing substance was similar with either EDTA-PRP or citrated PRP. However, when platelets in buffered saline solution were incubated with arachidonic acid, the activity appeared and disappeared very rapidly. Under these conditions, maximal amounts of the activity were present between 10 and 60 s, depending on the final concentration of the added arachidonic acid (1–0.01 nM). This difference between washed platelets and PRP in the response to arachidonic acid was not due to physical manipulation of the platelets, since they could be resuspended in plasma with restoration of the original time-course. Plasma could not be replaced by albumin or Cohn fractions I, II, III, IV-1, IV-4, IV-6, V, or VI, but could be replaced by serum.

The release-inducing substance was released from the platelets, since almost identical time-courses of

5-hydroxytryptamine release were obtained when incubates of arachidonic acid and EDTA-PRP were either transferred directly to labeled PRP or rapidly centrifuged (15,000 *g*, 15 s) and the supernatant transferred. The release-inducing activity of the supernatant remained essentially unchanged when kept at 0°C for 30 min but declined by about half when kept at 37°C for 5 min.

In view of the similarity of our studies using platelets in buffered saline to those recently reported by Hamberg and co-workers (4), who used a similar system and detected the presence of thromboxane A_2, it seemed possible that the release-inducing substance that peaked in plasma at 3 min was in fact thromboxane A_2. To determine if this was the case, radioactive arachidonic acid was incubated for different times with platelets resuspended either in saline or in plasma. The radioactive products were extracted and chromatographed as described in Methods. Two radioactive derivatives that were less polar than thromboxane B_2 (R_f values = 0.13, 0.18, and 0.04, respectively) and appear to be identical to the methanolic derivatives of thromboxane A_2 previously described (4) were detected. If the incubations were terminated by decanting into saline rather than methanolic stannous chloride before extraction and chromatography, the radioactivity in the thromboxane B_2 zone was greatly increased, and that in the zone corresponding to the methanolic derivatives was greatly decreased. The time-courses of appearance and disappearance of these substances and thromboxane B_2 in saline and plasma are shown in Figs. 2 and 3, respectively. Indomethacin (20 μM) added to PRP after 3 min of incubation with arachidonic acid caused no change in the time-course shown in Fig. 3.

When platelets were sedimented from plasma 3 min after addition of radioactive arachidonic acid and the plasma was maintained at 37°C, the half-life of thromboxane A_2-like material was estimated to be between 5 and 6 min. The half-life at room temperature was about 20 min. To demonstrate that thromboxane A_2 was still available to be converted into thromboxane B_2, the plasma was maintained at 37° for 10 min and then samples were decanted into either methanolic stannous chloride or saline. Treatment with saline resulted in an increase in thromboxane B_2 from 8.8 to 14.6 μM and a decrease in the methanolic derivatives from 5.2 to 1.2 μM. Similar results were obtained when 20 μM indomethacin was added to the PRP before sedimentation of the platelets.

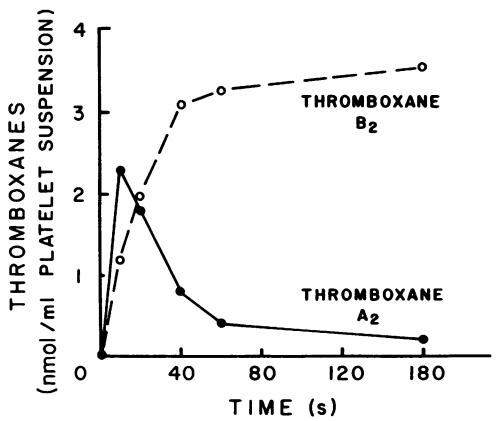

FIGURE 2 Appearance and disappearance of thromboxane A_2-like material (●) and thromboxane B_2 (○) during incubation of 22 μM arachidonic acid with a platelet suspension. The results shown are the mean of duplicate determinations in one experiment, representative of two, using platelets obtained from different donors. Material designated as thromboxane A_2 represents the combined radioactivity of the methoxy products. The specific activity of the added arachidonic acid was used to calculate the final concentrations of the products.

DISCUSSION

Since aspirin and indomethacin inhibit both platelet aggregation and the platelet release reaction induced by arachidonic acid, it is apparent that these phenomena depend on prostaglandin biosynthesis. The discovery that prostaglandin endoperoxides can induce aggregation (2) and the finding that these endoperoxides are rapidly and almost entirely metabolized by platelets to nonprostanoate compounds (8) suggested that PGG_2 might be the active mediator of both aggregation and release. Thus, PGG_2 was hypothesized to induce the release of ADP, which resulted in platelet aggregation (9). On the other hand, other evidence indicated that arachidonic acid-induced

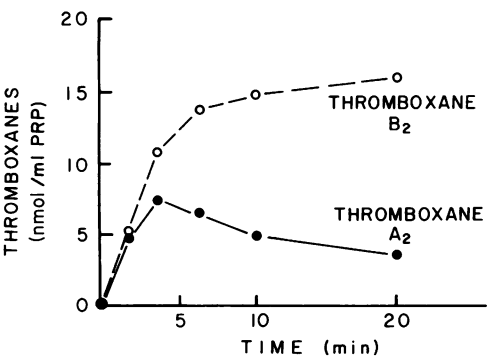

FIGURE 3 Appearance and disappearance of thromboxane A_2-like material (●) and thromboxane B_2 (○) during incubation of 1 mM arachidonic acid with PRP. The experiments were performed as described in Methods, with EDTA-PRP, and have been corrected for the background obtained after incubation of radioactive arachidonic acid with PPP. The results shown are the mean of duplicate determinations in one experiment, representative of three, with PRP obtained from different donors.

aggregation and the release reaction could be dissociated. For example, arachidonic acid was found to aggregate storage-pool deficient and thrombin-degranulated platelets (10) that have little or no releasable ADP.

It occurred to us that endoperoxide-induced platelet aggregation and the release reaction might be separate phenomena resulting from the action of different mediators. We therefore devised a system to test this hypothesis and found that when prostaglandin endoperoxides were no longer detectable in mixtures of arachidonic acid and PRP, release-inducing activity was still present (5). This activity was present in maximal amounts at 3 min and still present at 10 min (Fig. 1).

The results we obtained with washed platelets were similar to those recently described by Hamberg and co-workers (4). They found that aggregation of indomethacin-treated platelets caused by mixtures of arachidonic acid or PGG_2 and washed platelets resuspended in aqueous medium was greater than could be accounted for by PGG_2 alone. They also reported that when arachidonic acid was incubated with washed platelets, a substance that released radioactivity from indomethacin-treated platelets labeled with [^{14}C]serotonin was present at 30 s but not at 3 min. In sophisticated experiments, they demonstrated that PGG_2 is converted by platelets into thromboxane A_2, an unstable intermediate rapidly attacked by hydroxyl groups in aqueous medium to yield thromboxane B_2 (8-(1-hydroxy-3-oxopropyl)-9, 12L-dihydroxy-5, 10-hepatadecadienoic acid). The aggregating and release-inducing substances that they had detected had the same $t_{\frac{1}{2}}$ as thromboxane A_2 and it was proposed that they are identical to thromboxane A_2.

Thromboxane A_2 is converted into the two epimers of mono-O-methyl-thromboxane B_2 by treatment with methanol (4). We took advantage of this property to characterize it and followed the conversion of radioactive arachidonic acid into thromboxane A_2 and B_2 by thin layer chromatography. We confirmed that, in a washed platelet system, thromboxane A_2-like material is present in maximal amounts before 30 s and barely detectable at 3 min (Fig. 2). Little production of thromboxane B_2 occurred after 1 min. By contrast, in PRP we found that the production of thromboxane B_2 continued for at least 7 min, while the production of thromboxane A_2-like material continued for at least 3 min (Fig. 3). After 3 min the concentration of this material diminished in parallel with loss of the release-inducing activity.

The present study indicates that plasma markedly prolongs the persistence of thromboxane A_2 produced by platelets from arachidonic acid. One reason for this increased persistence is that plasma diminishes the oxygenation of arachidonic acid by platelets and this is associated with a more prolonged production of oxygenated products. However, material convertible into the methanolic derivatives of thromboxane A_2 (or into thromboxane B_2 by treatment with water) had a half-life of between 5 and 6 min in platelet-free plasma at 37°C, suggesting that plasma also stabilizes thromboxane A_2. This is in direct contrast to our recent findings that plasma accelerates the rearrangement of PGH_2 (11). The persistence of thromboxane A_2-like material in plasma dissociates it from PGG_2 and associates it with persistent release-inducing activity. The nature of the factor in plasma that appears to stabilize thromboxane A_2 has yet to be determined.

ACKNOWLEDGMENT

This work was supported in part by grant HL-14890 from the National Institues of Health.

REFERENCES

1. Silver, M. J., J. B. Smith, C., Ingerman, and J. J. Kocsis. 1973. Arachidonic acid-induced human platelet aggregation and prostaglandin formation. *Prostaglandins* **4:** 863–875.
2. Hamberg, M., J. Svensson, T. Wakabayashi, and B. Samuelsson. 1974. Isolation and structure of two prostaglandin endoperoxides that cause platelet aggregation. *Proc. Natl. Acad. Sci. U. S. A.* **71:** 345–349.
3. Smith, J. B., C. Ingerman, J. J. Kocsis, and M. J. Silver. 1974. Formation of an intermediate in prostaglandin biosynthesis and its association with the platelet release reaction. *J. Clin. Invest.* **53:** 1468–1472.
4. Hamberg, M., J. Svensson, and B. Samuelsson. 1975. Thromboxanes: A new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Natl. Acad. Sci. U. S. A.* **72:** 2994–2998.
5. Smith, J. B., C. M. Ingerman, and M. J. Silver. 1974. Propagation of platelet serotonin release by a factor generated in response to arachidonic acid. *J. Am. Oil Chem. Soc.* **51:** 525A, (Abstr.).
6. White, J. G., and C. J. Witkop. 1972. Effects of normal and aspirin platelets on defective secondary aggregation in the Hermansky-Pudlak syndrome. A test for storage pool deficient platelets. *Am. J. Pathol.* **68:** 57–66.
7. Smith, J. B., C. M. Ingerman, and M. J. Silver. 1975. Prostaglandins and precursors in platelet function. *In* Biochemistry and Pharmacology of Platelets. *Ciba Found. Symp.* **35:** 207–224.
8. Hamberg, M., and B. Samuelsson. 1974. Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. *Proc. Natl. Acad. Sci. U. S. A.* **71:** 3400–3404.
9. Malmsten, C., M. Hamberg, J. Svensson, and B. Samuelsson. 1975. Physiological role of an endoperoxide in human platelets: Hemostatic defect due to platelet cyclo-oxygenase deficiency. *Proc. Natl. Acad. Sci. U. S. A.* **72:** 1446–1450.
10. Reimers, H. J., J. P. Cazenave, A. F. Senyi, J. Hirsh, R. L. Kinlough-Rathbone, M. A. Packham, and J. F. Mustard. 1975. *In vivo* and *in vitro* functions of thrombin-treated rabbit platelets, Vth Congress on Thrombosis and Haemostasis, Paris, France, July, 1975, Abst. No. 445.
11. Smith, J. B., C. M. Ingerman, and M. J. Silver. 1976. Formation of Prostaglandin D_2 during endoperoxide induced platelet aggregation. *Thromb. Res.* In press.