Thrombin Pretreatment of Human Platelets Impairs Thromboxane A₂ Synthesis From Endogenous Precursors in the Presence of Normal Cyclooxygenase Activity

By Hans-Joachim Reimers, Rüdiger E. Scharf, and R. Kendall Baker

Exposure of horse platelets to thrombin has been reported to cause nearly complete inactivation of cyclooxygenase within 30 sec. This contrasts with the observation that human platelets, depleted of their granule constituents by stimulation with thrombin, still aggregate in response to arachidonic acid, a reaction presumably mediated by thromboxane A₂ (TxA₂) formation. Because of this conflicting evidence, TxA₂ formation was measured by radioimmunoassay in washed human platelets depleted of their alpha- and dense-storage granule constituents by prior stimulation with thrombin. These platelets aggregated in response to adenosine diphosphate (ADP), collagen, arachidonic acid, and thrombin, and formed TxA₂. However, collagen- and thrombin-induced TxA₂ formation by these platelets was reduced in comparison to control platelets that had not been depleted of their storage granule constituents by prior thrombin stimulation. In contrast, arachidonic acid-induced TxA₂ formation was not significantly different in thrombin-depleted and control platelets. These results demonstrate that thrombin can induce degranulation of platelets without concomitant inactivation of cyclooxygenase.

Cyclooxygenase converts arachidonic acid that is liberated from phospholipids during platelet activation to the labile endoperoxides prostaglandin G₂ and H₂. Thromboxane A₂, the major product of the platelet cyclooxygenase pathway, is then formed from the endoperoxides by thromboxane synthetase. Its stable metabolite, thromboxane B₂ (TxB₂), is easily measured by radioimmunoassay. Thromboxane A₂ is a potent vasoconstrictor and inducer of platelet aggregation, and thus promotes hemostasis.

It has been reported that exposure of horse platelets to arachidonic acid or thrombin inactivates platelet cyclooxygenase. The observation of rapid thrombin-induced inactivation of horse platelet cyclooxygenase contrasts with reports that human or rabbit platelets degranulated with thrombin still undergo shape change and aggregation upon incubation with arachidonic acid. Moreover, arachidonic acid-induced aggregation of thrombin-degranulated platelets is inhibited by prostaglandin E₁, indomethacin, and acetysalicylic acid. These drugs are known to inhibit thromboxane A₂ formation from arachidonic acid. Lapetina and Cuatrecasas has suggested, therefore, that arachidonic acid may induce platelet aggregation by a mechanism other than its metabolism via cyclooxygenase. As there is presently no supportive evidence for such an assumption, we decided to directly measure thromboxane A₂ production of thrombin-degranulated platelets incubated with arachidonic acid. We report that human platelets treated with thrombin so as to release almost completely their α- and dense-granule constituents are still able to produce large amounts of TxB₂ from exogenous arachidonic acid. However, synthesis of TxB₂ from endogenous arachidonic acid upon stimulation of these platelets with thrombin or collagen is impaired.

MATERIALS AND METHODS

Preparation of Washed Platelets From Humans

Suspensions of washed platelets from human volunteers were prepared according to the method of Mustard et al., with the modification that 10 μM prostaglandin E₁ (PGE₁; Upjohn Co., Kalamazoo, MI) was included in the washing solution. For aggregation and release experiments, platelets were finally resuspended at a concentration of 500,000/μl in Tyrode-albumin solution containing 3.5 g/liter human albumin (Behring-Werke A.G., Marburg/Lahn, West Germany), 5 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid) and apyrase (Sigma Chemical Co., St. Louis, MO) at a concentration capable of degrading 1.3 nmole adenosine diphosphate (ADP) per milliliter in a 10 μM ADP solution within 60 sec at 37°C. Inclusion of apyrase and PGE₁, in the washing solution and of apyrase in the final suspending medium were shown not to impair thrombin-induced TxB₂ production of the final platelet preparation. Apyrase was omitted from the final platelet suspending medium when the release of adenosine triphosphate (ATP) and ADP was to be determined. For the preparation of thrombin-degranulated platelets, the platelet suspension was adjusted to approximately 1.5 x 10⁶ platelets/μl.

Preparation of Thrombin-Degranulated Platelets

Thrombin-degranulated platelets were prepared by a method similar to those published previously. Ten milliliters of platelet suspension was incubated with 2 U/ml thrombin (bovine thrombin, specific activity 200 U/mg; Behring-Werke A.G., Marburg/Lahn,

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Supported in part by Grants Re 555/Scha 358, from Deutsche Forschungsgemeinschaft, a grant from the American Heart Association, Missouri Affiliate Inc., and Institutional Grant BRSG 562 of St. Louis University.

Submitted March 28, 1983; accepted October 18, 1983.

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obtained from AB Kabi and treated with diisopropylfluorophosphate (DFP) before use.18 Platelet aggregation was recorded in an aggregation module (Fa. Braun, Melsungen, West Germany) coupled to a photometer (Eppendorf GmbH, Hamburg, West Germany) during a 5-min period after the addition of the aggregating stimulus. Collagen was obtained from Hormon-Chemie (Munich, West Germany) and reconstituted according to the manufacturer’s instructions. Twenty microfilters of this working solution, yielding 4 μg collagen/ml platelet suspension, was used in the aggregation and release studies. Adenosine diphosphate and thrombin were dissolved in 150 mM NaCl. The ionophore, A23187 (Eli Lily Co., Indianapolis, IN), was dissolved in dimethyl sulfoxide (DMSO) and added in a 1:100 dilution. Arachidonic acid (Sigma Chemical Co.) was prepared as described previously.5 Human fibrinogen used in the aggregation experiments was obtained from AB Kabi and treated with disopropylfluorophosphate (DFP) before use.18

Release Studies

The release of platelet constituents [ATP, ADP, β-thromboglobulin (β-TG)] into the suspending medium was measured 5 min after the addition of the aggregation- and release-inducing stimulus. Release of platelet constituents and thromboxane B₂ (TxB₂) formation were stopped by addition of 10 μM PGE, and 8 μM indomethacin (Serva GmbH & Co., Heidelberg, West Germany) and subsequent centrifugation (Eppendorf centrifuge; 9,000 g for 1 min). ATP and ADP in the supernatant, as well as total platelet ATP and ADP, were determined in EDTA-ethanol extracts by the luciferin-luciferase assay.16 β-TG was determined using a commercially available kit (Boehringer Mannheim GmbH, Mannheim, West Germany). β-Glucuronidase was determined according to Fishman18 in Triton X100 extracts of platelet suspension.18

RESULTS

It was the aim of these studies to examine the thromboxane A₂-forming capacity of human platelets that had released practically all their granule constituents upon stimulation with thrombin but were still responsive to physiologic stimuli.

Depletion of Platelet Granule Constituents by Thrombin

Treatment of washed human platelets with thrombin for 20 sec induced the release of approximately 97% of the α-granule constituents, as indicated by the amount of β-thromboglobulin left in platelets after thrombin treatment (Table 1). Repeat centrifugation and resuspension of the platelets had no significant effect on the β-thromboglobulin content. The β-thromboglobulin content of the control platelets decreased during these procedures from 61.8 ± 35.6 μg/10⁹ platelets to 58 ± 31.3 μg/10⁹ platelets (n = 7; p < 0.9). The ATP + ADP and Ca²⁺ content of thrombin-treated platelets was reduced by about 50%-60% (Table 1). These compounds are partially stored in the dense bodies (δ-granules) together with serotonin. The serotonin content of thrombin-treated platelets was diminished by approximately 73% in three platelet preparations.

Table 1. Content of Various Constituents in Thrombin-Treated and Control Platelets

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Control Platelets*</th>
<th>Thrombin-Treated Platelets*</th>
<th>Significance of Difference‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Granule constituent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Thromboglobulin (μg/10⁹ platelets)</td>
<td>58 ± 31 (7)</td>
<td>1.5 ± 1.5 (7)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>δ-Granule constituent</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP† (μ mole/10¹¹ platelets)</td>
<td>5.0 ± 0.5 (6)</td>
<td>2.3 ± 0.5 (6)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>ADP† (μ mole/10¹¹ platelets)</td>
<td>2.4 ± 1.2 (6)</td>
<td>0.5 ± 0.3 (6)</td>
<td>p &lt; 0.005</td>
</tr>
<tr>
<td>Ca²⁺† (μ mole/10¹¹ platelets)</td>
<td>22.2 ± 1.7 (3)</td>
<td>9.8 ± 0.6 (3)</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>5HT† (nmole/10¹⁰ platelets)</td>
<td>90.3 ± 25.5 (3)</td>
<td>24.6 ± 15.5 (3)</td>
<td>p &lt; 0.02</td>
</tr>
<tr>
<td>Lyosomal granule constituent</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>β-Glucuronidase (U/10⁹ platelets)</td>
<td>15.5 ± 0.8 (4)</td>
<td>9.7 ± 0.6 (4)</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Plasma membrane constituent</td>
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<td></td>
</tr>
<tr>
<td>γ-Glutamyl-transpeptidase (U/10¹¹ platelets)</td>
<td>3.9 ± 1.0 (6)</td>
<td>4.5 ± 1.8 (7)</td>
<td>p &lt; 0.5</td>
</tr>
</tbody>
</table>

*Values are given as mean ± SD (number of different platelet preparations).† It is recognized that these compounds are also present in other platelet compartments.‡ Student’s t test.
preparations in which the serotonin content was measured fluorometrically (Table 1). In these experiments, serotonin reuptake was not prevented by drugs such as imipramine. Thus, reaccumulation of serotonin after thrombin-induced release could occur as described previously in rabbit platelets and as observed during the course of the present experiments in platelet preparations in which platelets had been prelabeled with 14C-serotonin (data not shown). Nevertheless, almost complete depletion (i.e., >90%) of the β-granule adenine nucleotides in thrombin-treated platelets is likely, as these platelets released only small amounts of ATP and ADP upon subsequent stimulation with collagen, arachidonic acid, the ionophore A23187, or thrombin (Table 2). β-Glucuronidase was measured as a marker for lysosomal granule release. It was found that the β-glucuronidase content of thrombin-treated platelets was reduced by about one-third as compared to the control platelets (Table 1).

Platelet γ-glutamyl-transferase (γ-GT), a sialylated glycoprotein, is an ectoenzyme of the plasma membrane. Its activity was determined to evaluate loss of parts of the platelet plasma membrane subsequent to thrombin stimulation. However, γ-GT activity of control and thrombin-treated platelets was not found to be significantly different (Table 1).

Thus, the adenine nucleotide, calcium, serotonin, and β-thromboglobulin content of thrombin-degranulated platelets is similar to that found in the α-, β-granule defect of congenital storage pool disease.

Aggregation Behavior of Thrombin-Treated Platelets

Thrombin-degranulated platelets aggregated in response to micromolar concentrations of ADP, as well as in response to collagen, the ionophore A23187, thrombin, and arachidonic acid (Fig. 1). Aggregation induced by collagen, arachidonic acid, and ADP usually required addition of fibrinogen. The extent of ADP-induced aggregation was similar in thrombin-treated and control platelets (Fig. 1). However, collagen and low concentrations of arachidonic acid consistently caused a smaller aggregation response of thrombin-treated platelets as compared to that of control platelets. In most experiments, thrombin induced aggregation of thrombin-pretreated platelets in the absence of added fibrinogen (Fig. 1). However, thrombin-induced aggregation of thrombin-pretreated platelets in the absence of added fibrinogen was consistently less than that of control platelets (Fig. 1). Addition of fibrinogen increased the aggregation response of these platelets (data not shown).

Thromboxane B2 Formation by Thrombin-Treated Platelets

Washed human platelets that had released practically all of the α- and β-granule constituents in response to thrombin were able to synthesize thromboxane A2 in response to collagen or upon a second stimulation with thrombin as determined by measurement of the stable end-product, TxB2. However, the amounts formed were significantly smaller than those formed by the control platelets (Table 3). This was not due to inactivation of cyclooxygenase after thrombin-stimulation of platelets, as demonstrated in experiments in which thrombin-pretreated platelets were incubated with arachidonic acid. Table 3 shows that the amount of TxB2 formed from exogenous arachidonic acid in thrombin-pretreated platelets was not different from that formed in the control platelets. This behavior was demonstrable over a range of arachidonic acid concentrations (Fig. 2). In these experiments, thrombin-degranulated platelets were exposed to arachidonic acid about 1–2 hr after the initial thrombin stimulation. Therefore, in another set of

<table>
<thead>
<tr>
<th>Table 2. Release of ATP, ADP, and β-Thromboglobulin From Thrombin-Treated and Control Platelets</th>
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<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td>ATP (μmole/10^11 platelets)</td>
</tr>
<tr>
<td>Control platelets</td>
</tr>
<tr>
<td>Thrombin-treated platelets</td>
</tr>
<tr>
<td>Significance of difference*</td>
</tr>
<tr>
<td>ADP (μmole/10^11 platelets)</td>
</tr>
<tr>
<td>Control platelets</td>
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<tr>
<td>Thrombin-treated platelets</td>
</tr>
<tr>
<td>Significance of difference*</td>
</tr>
<tr>
<td>β-Thromboglobulin (μg/10^8 platelets)</td>
</tr>
<tr>
<td>Control platelets</td>
</tr>
<tr>
<td>Thrombin-treated platelets</td>
</tr>
<tr>
<td>Significance of difference*</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD (number of different platelet preparations).

*Student’s t test.
experiments, it was determined whether or not exposure of platelets to thrombin may cause transient, short-lived inactivation of platelet cyclooxygenase. Figure 3 shows that addition of 450 μM arachidonate to platelets previously exposed to thrombin (2 U/ml) for 15 sec up to 10 min induced the synthesis of large amounts of TxB₂ (curve C). However, overall synthesis of TxB₂ in these experiments was less than expected from the numerical addition of the value for TxB₂ formation in aliquots of the platelet suspensions that had been stimulated with either thrombin (curve A) or arachidonic acid (curve B) alone.

**DISCUSSION**

Lapetina and Cuatrecasas⁴ have proposed that platelet cyclooxygenase is nearly completely inactivated within 30 sec following thrombin-induced platelet stimulation. However, there is indirect evidence that platelets still possess cyclooxygenase activity after thrombin-induced degranulation. Thrombin-degranu-
Table 3. Effect of Thrombin-Pretreatment of Washed Human Platelets on Thromboxane B₂ Formation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control Platelets (nmole/10⁶ Platelets)</th>
<th>Thrombin-treated Platelets* (nmole/10⁶ Platelets)</th>
<th>Significance of Difference†</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP (4.5 μM)</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Thrombin (1.8 U/ml)</td>
<td>2.16 ± 1.24 (5)</td>
<td>0.32 ± 0.35 (5)</td>
<td>p &lt; 0.02</td>
</tr>
<tr>
<td>Collagen (4 μg/ml)</td>
<td>0.81 ± 0.32 (5)</td>
<td>0.40 ± 0.22 (5)</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>Arachidonic acid (90 μM)</td>
<td>0.54 ± 0.57 (5)</td>
<td>0.58 ± 0.50 (5)</td>
<td>p &lt; 0.9</td>
</tr>
<tr>
<td>Arachidonic acid (450 μM)</td>
<td>8.39 ± 5.42 (5)</td>
<td>10.59 ± 8.23 (5)</td>
<td>p &lt; 0.7</td>
</tr>
</tbody>
</table>

Values are given as mean ± SD (number of different platelet preparations).
* Thrombin-treated platelets had formed 2.19 ± 1.12 nmole TxB₂/10⁶ platelets during their preparation.
† Student's t test.

...lated platelets were shown to aggregate in response to arachidonic acid, and this aggregation response was inhibited by acetylsalicylic acid or indomethacin. These nonsteroidal antiinflammatory drugs interfere with cyclooxygenase activity and thereby prevent the synthesis of thromboxane A₂. Lapetina and Cuatrecasas have considered that arachidonic acid may have induced aggregation of thrombin-degranulated platelets by an unknown mechanism unrelated to thromboxane formation. The present experiments demonstrate that platelets stimulated with a high concentration of thrombin for a period of time sufficient to induce practically complete release of α- and δ-granule constituents (and are subsequently recovered as single platelets, washed, and resuspended in fresh medium), are still capable of forming as much thromboxane A₂ as unstimulated control platelets upon incubation with exogenous arachidonic acid. Thus, thrombin treatment of washed human platelets does not inactivate cyclooxygenase irreversibly. In these experiments, degranulated platelets were exposed to arachidonic acid about 1–2 hr after the initial thrombin stimulation.

Transient inactivation of cyclooxygenase, upon exposure to thrombin of washed human platelets suspended in an albumin-containing medium, was excluded in experiments in which arachidonic acid was added to the platelet suspension at short time intervals (15 sec–10 min) after the addition of thrombin. However, in these experiments, it was observed that the overall TxB₂ production by thrombin plus 450 μM arachidonate was less than expected from control experiments in which platelets were stimulated with thrombin or arachidonic acid alone. A possible explanation for these findings is that the critical arachidonate concentration beyond which inactivation of platelet...
cyclooxygenase occurs\textsuperscript{4,24} was exceeded earlier (in the experiments in which thrombin-treated platelets were not washed before addition of arachidonate) due to thrombin-induced liberation of arachidonate from platelet phospholipids.\textsuperscript{9} This critical arachidonic acid concentration is likely to depend on the albumin concentration in the suspending medium, as well as on the fatty acid content of the albumin\textsuperscript{25,26} and the metabolic capabilities of platelets to cope with oxidant stress. Taken together, the present observations are compatible with those of Baenziger and coworkers,\textsuperscript{27,28} who did not find any evidence of irreversible or transient inactivation of cyclooxygenase after histamine-, thrombin-, or bradykinin-induced prostaglandin synthesis in endothelial or fibrosarcoma cells. In contrast to thrombin or other physiologic stimuli, incubation of platelets or endothelial cells with high (but not low) doses of exogenous arachidonic acid may inactivate cyclooxygenase for prolonged periods of time or irreversibly.\textsuperscript{4,15,24,29}

Although platelet cyclooxygenase was not inactivated by prior thrombin treatment, thrombin-pretreated platelets were not able to produce as much thromboxane $A_2$ from endogenous sources of arachidonic acid as control platelets, either upon a second stimulation with thrombin or upon stimulation with collagen. It is of interest that the thrombin-induced thromboxane formation in these platelets was inhibited less (about 50\%) than thrombin-induced thromboxane formation (about 85\%). This is similar to the observations of Becherer et al.\textsuperscript{28} in fibrosarcoma cells. In the experiments of these investigators, PGE\textsubscript{2} production was impaired more when these cells were challenged the second time with the same agonist as during the first stimulation than when challenged with an agonist known to interact with a different cell surface receptor. Collagen-induced platelet aggregation appears to be dependent on released ADP and formation of arachidonic acid metabolites.\textsuperscript{8,30,31} Thus, collagen-induced aggregation of degranulated platelets is largely determined by their residual capacity to synthesize thromboxane $A_2$. This may explain their reduced and sometimes variable aggregation response to this aggregating agent, as observed during the course of these studies, as well as by other investigators.\textsuperscript{30,31}

There are a number of possible explanations for the reduced thromboxane formation from endogenous arachidonic acid in thrombin-pretreated platelets. First, these platelets are “unspecifically damaged” due to extensive in vitro manipulation resulting in impaired metabolism and function. This appears unlikely, however. Thrombin-pretreated platelets aggregated in response to all agents tested. Furthermore, we have shown previously\textsuperscript{32} that rabbit platelets treated simi-
their original arachidonate content. In this context, it is of interest that thrombin-treated fibrosarcoma cells require about 3–5 hr after thrombin neutralization to regain their full potential for PGE₁-forming capacity. It is unknown, however, whether this time is required for remodeling of phospholipids or any other processes. The hypothesis that insufficient remodeling of platelet phospholipids after thrombin-induced degranulation in artificial media in vitro is responsible for reduced thromboxane formation requires examination in experiments in which thrombin-pretreated platelets are exposed to exogenous arachidonic acid or phospholipids for prolonged periods of time. Transitory, or partial but permanent, blockade of a thrombin-specific step in receptor–response coupling other than phospholipase inactivation or inhibition of arachidonic acid metabolism remains an additional important consideration, however, as thrombin-induced thromboxane formation was inhibited more than collagen-induced thromboxane formation in these experiments.

ACKNOWLEDGMENT

We are very grateful to E. Freiberger and A. Dimic for excellent technical assistance and to T. Ripperda for preparing the manuscript.

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TxA₂ SYNTHESIS IN PREACTIVATED PLATELETS


Thrombin pretreatment of human platelets impairs thromboxane A2 synthesis from endogenous precursors in the presence of normal cyclooxygenase activity

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