Induction of Human Platelet Fibrinogen Receptors by Epinephrine in the Absence of Released ADP

By Ellinor I. Peerschke

The ability of epinephrine to expose platelet fibrinogen receptors independent of released ADP was assessed using aspirin-treated, gel-filtered platelets. Similar to ADP-induced aggregation, platelet aggregation in response to epinephrine was accompanied by fibrinogen binding. Ten micromolar epinephrine induced a maximum number of platelet fibrinogen receptors in the absence of significant $^{13}$C-serotonin release. As indicated by Scatchard analysis, receptors exposed by both epinephrine and ADP had similar affinities for fibrinogen, but epinephrine induced approximately 30% fewer receptors than did ADP. This appears to correlate with the lesser degree of primary aggregation observed with this agent. Studies using phentolamine, a specific $\alpha$-adrenergic antagonist, apyrase, or creatine phosphate/creatine kinase indicate that the exposure of platelet fibrinogen receptors by epinephrine was specific for platelet $\alpha$-adrenergic receptor stimulation and was not the result of released ADP.

EPINEPHRINE induces both primary and secondary aggregation of human blood platelets in vivo. Secondary aggregation is thought to be mediated by adenosine diphosphate (ADP) released from platelet dense granules and/or products of arachidonic acid metabolism, whereas primary aggregation seems to result from epinephrine stimulation of platelet $\alpha_2$-adrenergic receptors. Aggregation induced by both ADP and epinephrine requires exogenous fibrinogen. While the ability of ADP to expose fibrinogen receptors has been well documented, there is controversy as to whether epinephrine induces exposure of fibrinogen receptors directly or via released ADP.

In the previous studies, the effects of the thromboxane-\(A_2\)-mediated release reaction were not investigated, and correlations between the amount of fibrinogen binding and the extent of epinephrine-induced primary aggregation were not made. The latter is important since epinephrine is known to induce lesser aggregation than ADP at optimal doses, and thus, if fibrinogen receptor occupancy actually is the controlling factor in the extent of aggregation, one would anticipate that epinephrine would induce either a smaller number of receptors or receptors with lower affinity than ADP.

The current study was designed to investigate the effects of the arachidonate metabolites on epinephrine-induced fibrinogen receptor exposure by making use of aspirin’s known inhibitory effect on cyclooxygenase and also to try to correlate fibrinogen receptor occupancy with the extent of epinephrine induced primary aggregation.

**MATERIALS AND METHODS**

All studies were performed at room temperature using the following stock solutions prepared in isotonic saline: 1 mM acetyl-salicylic acid (Mallinkrodt, Pa.) and 1.0 mM ADP (Sigma Chemical Co., St. Louis, Mo.) stored at –20°C; 10 mM creatine phosphate (Sigma) stored at –20°C; 10 mg/mL potato apyrase (Sigma) and 1 mg/mL creatine kinase (Sigma) prepared fresh daily. A 1.0 M stock solution of phentolamine was also prepared daily. Phentolamine was a generous gift from CIBA Geigy, Summit, N.J.

**Platelet Preparation**

Blood was obtained from healthy volunteers and collected into 0.1 vol. 3.2% sodium citrate, 0.05 vol 1 mM acetyl salicylic acid, and 0.001 vol 5-hydroxy (side chain-2-$^{14}$C) tryptamine creatinine sulfate (Amersham Corp., Arlington Heights, Ill.; diluted to 10 $\mu$Ci/ml with isotonic saline). Platelet-rich plasma (PRP) was prepared and incubated with 0.1 $\mu$M PGE, as described previously. Platelets were concentrated by centrifugation and resuspension, and gel-filtered through Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with 0.01 M HEPES-buffered modified Tyrode’s solution containing 0.2% bovine serum albumin (HBMT) and either 0.1 mg/mL apyrase or the combination of 0.065 mM creatine phosphate and 0.21 U/ml creatine phosphokinase (CPK) as indicated. Gel-filtered platelets (GFP) were diluted to a count between 300,000 and 500,000/μl with the appropriate buffer.

**Fibrinogen Preparation**

Purified band 1 fibrinogen, characterized previously, was iodinated to a specific activity between 3000 and 4000 cpm/ng with iodine-125 using chloramine-T. Following iodination, the fibrinogen preparations were between 90% and 93% clottable with thrombin, and 99% of the radioactivity precipitated in 10% trichloracetic acid. The same labeled fibrinogen preparation was used for all binding studies described herein.

**Platelet Aggregation**

Aggregation studies with GFP or PRP were performed at room temperature using a dual channel aggregometer (Chrono-Log Corp., Havertown, Pa.).
Fibrinogen Binding

Binding studies were performed essentially as described7 using GFP in HBMT, HBMT containing 0.1 mg/ml apyrase (apyrase-HBMT), or HBMT containing 0.065 mM CP/0.21 U/ml CPK (CP-CPK-HBMT). Nonspecific binding was assessed as described previously7 and subtracted from the total binding observed.

Characterization of Epinephrine-Induced Fibrinogen Binding

Experiments were conducted using GFP in apyrase-HBMT. The concentration of epinephrine resulting in exposure of the maximum number of fibrinogen receptors was determined by incubating GFP for 1 min with trace concentrations (1-2 μg/ml) of 125-I-fibrinogen and 1, 2, 5, and 10 μM epinephrine. The time course of epinephrine-induced fibrinogen binding was examined by stimulating gel-filtered platelets in apyrase-containing HBMT with 10 μM epinephrine in the presence of 5 μg/ml of labeled fibrinogen. Binding was measured after 1, 5, 7.5, 10, and 20 min. After 1 and 20 min, excess unlabeled fibrinogen (2 mg/ml) was added to determine the reversibility of binding.

The saturation of epinephrine (10 μM) induced fibrinogen receptors was compared to that of receptors exposed by ADP (10 μM) at fibrinogen concentrations ranging from 50 to 2000 μg/ml. Fibrinogen preparations with specific activities from 3000 to 6000 cpm/μg were prepared by diluting iodinated fibrinogen with unlabeled material. Specific binding was analyzed by the method of Scatchard.16

Inhibition of Fibrinogen Binding

Inhibition of 10 μM epinephrine-induced fibrinogen binding by apyrase (1 mg/ml), 2 mM CP/6.6 U/ml CPK, 5 mM EDTA, or 10 μM phenolamine16 was compared to inhibition of 10 μM ADP-induced binding by these compounds using GFP suspended in HBMT, apyrase HBMT, or CP/CPK-HBMT. The inhibitors were added just prior to the addition of the aggregating agent, and their effect on fibrinogen binding was determined at trace fibrinogen concentrations (10-20 μg/ml).

Reversal of Platelet Aggregation and Fibrinogen Binding

GFP in apyrase-HBMT were stimulated with ADP (10 μM) or epinephrine (10 μM) in the presence of trace iodinated fibrinogen (10-20 μg/ml). After 1 min, aliquots were removed to assess fibrinogen binding. Subsequently, phenolamine (10 μM) was added to both ADP- and epinephrine-treated platelet suspensions and binding measured after 2, 5, and 10 min.

The effect of 10 μM phenolamine or 5 mM EDTA on the reversal of epinephrine- and ADP-induced aggregation was determined by adding these inhibitors to platelet suspensions in the aggregometer cuvette after maximal primary aggregation had occurred (3 min). Aggregation was monitored for an additional 3 min.

Potentiation of Epinephrine-Induced Aggregation and Fibrinogen Binding

The ability of trace concentrations of ADP (1 μM) to enhance epinephrine-induced aggregation and exposure of fibrinogen receptors was determined using GFP in apyrase HBMT or CP/CPK-HBMT with 10-20 μg/ml of iodinated fibrinogen. (For aggregation studies, 0.1 mg/ml of unlabeled fibrinogen were added to platelet suspensions.) Platelets were stimulated with 10 μM epinephrine, 10 μM epinephrine plus 1 μM ADP, 10 μM ADP or 10 μM epinephrine plus 1 μM ADP. Binding was assessed after 1 min and primary aggregation monitored for 3 min. The effect of adding apyrase (1 mg/ml) or 2 mM CP/6.6 U/ml CPK immediately before platelet stimulation on the potentiation of epinephrine-induced fibrinogen binding, and aggregation by ADP was evaluated as described under Inhibition of Fibrinogen Binding.

14C-Serotonin Release

GFP in HBMT containing 0.1 mg/ml fibrinogen and 5 μM imipramine17 were stimulated with 10 μM epinephrine or 10 μM ADP. After 1 min, aliquots of unstirred samples were fixed in 1% formalin (Fisher, Scientific Co., Springfield, N.J.), centrifuged (5 min, 1000 g, 4°C), and an aliquot of the supernatant counted in a liquid scintillation counter using a Toluene-ethanol based scintillation fluid. The percent of released 14C-serotonin was calculated by comparing the percent of radioactivity in the supernatants of stimulated platelet preparations to the total amount of radioactivity present in the initial GFP suspensions. The amount of radioactivity in the supernatants of unstimulated platelet suspensions was subtracted from that observed following stimulation.

RESULTS

GFP prepared without 0.10 mg/ml apyrase or 0.065 mM CP/0.21 U/ml CPK bound similar amounts of

<p>| Table 1. Comparison of ADP- and Epinephrine-Induced Fibrinogen Binding* in Various Buffer Media |</p>
<table>
<thead>
<tr>
<th>Additions</th>
<th>HBMT</th>
<th>Apyrase HBMT</th>
<th>CP/CPK-HBMT</th>
<th>Apyrase HBMT Plus 1 μM ADP</th>
<th>CP/CPK-HBMT Plus 1 μM ADP</th>
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<td>EPI</td>
<td>ADP</td>
<td>EPI</td>
<td>ADP</td>
<td>EPI</td>
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<tr>
<td>None</td>
<td>12.783±</td>
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<tr>
<td>Apyrase</td>
<td>1.720 ± 2.995</td>
<td>536 ± 8.528</td>
<td>735 ± 2.173</td>
<td>2.639 ± 3.190</td>
<td>5.018 ± 3.978</td>
</tr>
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<td>(1 mg/ml)</td>
<td>532 ± 156</td>
<td>671 ± 238</td>
<td>814 ± 125</td>
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<tr>
<td>CP/CPK</td>
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<td>321±</td>
<td>3.840±</td>
<td>545±</td>
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<tr>
<td>(2.0 mM/6.6 U/ml)</td>
<td>979 ± 144</td>
<td>1.395 ± 320</td>
<td>181 ± 227</td>
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</table>

*Binding assessed with 10-20 μg/ml iodinated fibrinogen.
†n = 4.
*±1−10Indicate significant differences between counts using the Student's t test (p > 0.10).
iodinated fibrinogen whether stimulated with 10 μM ADP or 10 μM epinephrine. Adding 1.0 mg/ml apyrase or 2.0 mM CP/6.6 U/ml CPK, however, inhibited most of this binding, although platelets stimulated with epinephrine consistently bound more fibrinogen following addition of these enzymes than their ADP-treated counterparts (Table 1).

No significant release of 14C-serotonin was noted following stimulation of GFP with either ADP or epinephrine (p > 0.05). Platelets stimulated with ADP released 1.86% ± 2.03% (SD), n = 6, of their 14C-serotonin, while epinephrine-stimulated platelets released 1.29% ± 1.80% (n = 6) above the background release of unstimulated platelets.

The amount of 14C-serotonin found in the supernatants of unstimulated platelet preparations, however, was high (13.58% ± 7.30%, n = 6) considering platelets were gel-filtered after their initial incubation with serotonin. This should have separated free radioactivity from that taken up by platelet dense granules unless leakage from these granules was occurring. Since serotonin release correlates with release of ADP, experiments were performed to compare epinephrine- and ADP-induced fibrinogen binding of platelets gel-filtered in the presence and absence of 0.10 mg/ml apyrase or 0.065 mM CP/0.21 U/ml CPK.

In contrast to platelets prepared without ADP scavengers, platelets gel-filtered in the presence of 0.1 mg/ml apyrase or 0.065 mM CP/0.21 U/ml CPK bound less iodinated fibrinogen when stimulated with epinephrine than with ADP (Table 1). Furthermore, addition of 1 mg/ml apyrase or 2 mM CP/6.6 U/ml CPK to the latter suspensions did not alter fibrinogen binding in response to epinephrine. This suggested that trace amounts of ADP were not responsible for the observed epinephrine-induced exposure of a limited number of fibrinogen receptors. The ability of 1.0 mg/ml apyrase or 2.0 mM CP/6.6 U/ml CPK to degrade ADP was demonstrated by the fact that under similar conditions, 10 μM ADP-induced binding was reduced to 3.04% ± 0.04% of binding noted in the absence of these enzyme systems.

Since platelets prepared without low concentrations of ADP scavengers bound similar amounts of fibrinogen whether stimulated with epinephrine or ADP (Table 1), and because small amounts of 14C-serotonin were detected in the suspending buffers following gel-filtration, the effect of trace concentrations of ADP on the potentiation of epinephrine-induced fibrinogen binding was examined. As is evident from Table 1, stimulating platelets in apyrase or CP/CPK containing buffers with a combination of 1 μM ADP plus 10 μM epinephrine enhanced fibrinogen binding significantly. This effect of trace concentrations of ADP was blocked by further apyrase or CP/CPK as indicated (Table 1). One micromolar ADP alone had little effect on fibrinogen binding since it was rapidly degraded by the 0.1 mg/ml apyrase or 0.065 mM CP/0.21 U/ml CPK in the HBMT.

The effects of proteolytic contaminants in the apyrase or CPK preparations on fibrinogen binding was considered negligible, as ADP-induced fibrinogen binding in buffers containing these enzymes was similar to that noted previously.

Five millimolar EDTA inhibited epinephrine- and ADP-induced fibrinogen binding to the same extent (91.5% ± 12.8% and 85% ± 4.2%, respectively; n = 6, p > 0.05). However, phentolamine, a specific α-adrenergic blocking agent, selectively inhibited epinephrine-
induced fibrinogen binding. The latter was 72.3% ± 18.9% inhibited, whereas ADP-induced binding was almost unaffected (8.8% ± 1.3%, n = 3).

Epinephrine-induced fibrinogen binding correlated well with platelet aggregability. Figure 1 shows that addition of 10 μM epinephrine to aspirin-treated PRP produced much less aggregation than 10 μM ADP, and the same pattern was observed with platelets gel-filtered in the presence of apyrase or CP/CPK. Further addition of apyrase or CP/CPK selectively inhibited ADP-induced aggregation, whereas addition of phentolamine inhibited aggregation in response to epinephrine (Fig. 1). EDTA reversed aggregation induced by both agents, while phentolamine selectively disaggregated platelet samples stimulated with epinephrine (Fig. 2).

The kinetics of epinephrine-induced fibrinogen binding were found to be similar to those reported elsewhere for ADP. Binding was maximal using 10 μM epinephrine and was complete by 1 min (Fig. 3). Epinephrine-induced binding of labeled fibrinogen could be displaced with excess unlabeled material in 5–10 min (Fig. 4).

Binding was similarly reversed by phentolamine [81.0% ± 15%, (n = 3)] of bound fibrinogen dissociated within 2 min]. ADP-induced binding, however, was unaffected 2 min after exposure to phentolamine, but decreased slowly with time due to the 0.1 mg/ml apyrase in the buffer.

Epinephrine-induced fibrinogen binding approached saturation at fibrinogen concentrations of
Fig. 5. Comparison of epinephrine (○) and ADP (●) induced fibrinogen binding to GFP in apyrase-HBMT at increasing fibrinogen concentrations. One of five similar experiments.

Fig. 6. Representative Scatchard analysis of fibrinogen binding. (A) Binding induced by 10 μM epinephrine using GFP suspended in apyrase-HBMT. (B) Binding induced by 10 μM ADP. The dashed lines represent high and low affinity binding assuming the presence of two distinct classes of binding sites. One of five similar experiments.
Similar concentrations of ADP were found to potentiate epinephrine-induced fibrinogen binding by virtue of its divalent structure\(^8\)\(^9\) or to modify platelet membrane glycoproteins, perhaps glycoproteins IIb and III which have been reported to interact with complementary molecules on adjoining platelet membranes to induce aggregation.\(^1\)

The mechanism by which platelet fibrinogen receptors are exposed, however, is not clear. Conflicting evidence exists regarding the ability of low concentrations of epinephrine and thrombin to induce receptors in the absence of released ADP.\(^7\)\(^1\)\(^0\)\(^2\)\(^3\)\(^4\) If fibrinogen binding indeed serves as the common mechanism of platelet aggregation by these agents, agents like epinephrine and thrombin, which are capable of inducing primary aggregation in the absence of this nucleotide,\(^1\)\(^2\)\(^2\)\(^2\) ought to promote platelet fibrinogen interactions independently.

The controversy in the literature regarding the ability of epinephrine to expose fibrinogen receptors independently of released ADP may be explained by the observation that small amounts of ADP were released during gel-filtration, as had been reported by others.\(^2\)\(^4\) The amount of released ADP corresponded to a final concentration of at least 1 \(\mu M\), calculated on the basis of the observed 13% \(^1\)\(^4\)\(^\text{C}\)-serotonin release in unstimulated GFP suspensions, and 35 nmole of ADP being present in the dense granules of 10\(^1\)\(^2\) platelets.\(^2\)\(^5\) Similar concentrations of ADP were found to potentiate epinephrine-induced fibrinogen binding and aggregation. Thus, the observation by Plow and Marguerie,\(^9\) that enzymes that destroy ADP significantly decrease epinephrine-induced fibrinogen binding and aggregation may relate to the presence of small amounts of ADP leaked into platelet suspensions during gel-filtration. Potentiation of epinephrine-induced platelet response by the presence of these trace concentrations of ADP could also explain findings by Bennett and Vilaire\(^6\)\(^7\) that epinephrine exposes the same number of fibrinogen receptors as does ADP.

Similar to ADP-induced fibrinogen binding, the amount of fibrinogen bound in response to epinephrine was variable. It has been suggested that these differences may relate to the age and size distribution of platelets.\(^2\)\(^3\) It may also result from as yet undefined effects of platelet washing techniques on platelet physiology.\(^7\)

Several groups of investigators have reported the existence of only a single class of platelet fibrinogen receptors,\(^6\)\(^9\)\(^2\)\(^0\)\(^2\)\(^1\) while others\(^7\)\(^8\) suggest heterogeneity of receptors or negative cooperativity among receptors. This report confirms previous observations by the author and others\(^7\)\(^8\) suggesting platelet fibrinogen receptors are either heterogeneous with respect to their affinity for ligand or interact with apparent negative cooperativity.

In conclusion, the present study illustrates that platelets treated with aspirin to prevent the thromboxane-\(A_2\)-mediated release reaction,\(^1\)\(^4\) bind fibrinogen when stimulated with epinephrine in buffers containing enzymes that would destroy any contaminating ADP. This binding is specific for the epinephrine-induced stimulation of platelet \(\alpha\)-adrenergic receptors, since phenotamine, a specific \(\alpha\)-adrenergic antagonist, but not apyrase or CP/CPK, inhibits both epinephrine-induced primary aggregation and fibrinogen binding.

These data are in agreement with recent findings\(^2\)\(^6\) suggesting that aspirin and indomethacin significantly decrease fibrinogen binding in response to epinephrine, presumably by preventing release of dense granule ADP and/or the formation of products of arachidonate metabolism that may potentiate the platelet response.

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