Synergistic Inhibition of Platelet Activation by Plasmin and Prostaglandin I₂

By Andrew I. Schafer, George B. Zavoico, Joseph Loscalzo, and Ann K. Maas

Endothelial cell prostacyclin (PGI₂) inhibits platelet activation by raising platelet cyclic AMP. Previously, platelet activation was also shown to be blocked by plasmin formed by endothelium-derived tissue plasminogen activator (TPA). We have now studied interactions between PGI₂ and plasmin in the control of platelet function. PGI₂ and plasmin cause synergistic inhibition of thrombin- and ADP-induced aggregation of washed platelets. Inhibition by PGI₂ is similarly potentiated by TPA added to platelet-rich plasma to generate plasmin. Thrombin-stimulated rise in platelet cytosolic Ca²⁺, measured by fura2 fluorescence, and thromboxane A₂ formation, measured by radioimmunoassay (RIA), are likewise synergistically inhibited by PGI₂ and plasmin. Plasmin neither increases nor potentiates PGI₂-stimulated increases in platelet cyclic AMP. Thus, PGI₂ and plasmin cause synergistic inhibition of platelet activation by both cyclic AMP-dependent and independent mechanisms. This interaction between two different endothelium-derived products may play an important role in maintaining the hemostatic plug at a site of vascular injury by preventing further thrombin-mediated accrual of platelets. © 1987 by Grune & Stratton, Inc.
Cyclic AMP radioimmunoassay. Reactions were stopped by the addition of an equal volume of ice-cold 10% trichloroacetic acid and extracted four times with water-saturated ether. Cyclic AMP in platelets was measured by radioimmunoassay (cyclic AMP [125I] RIA kit, New England Nuclear, Boston) and corrected for platelet counts.

Materials. The following reagents were obtained from commercial sources: fura2/AM from Calbiochem, La Jolla, CA; PGI2, PGE2, TXB2, and TXB2 antiserum from Upjohn, Kalamazoo, MI; [125I] TXB2 (100 to 150 Ci/mmol) from New England Nuclear, Boston; human thrombin from R.Q.P. Laboratories, South Bend, IN; ADP, aprotinin, and glycyl-L-prolyl-arginyl-L-proline from Sigma, St Louis; Sepharose 2B from Pharmacia Fine Chemicals, Uppsala, Sweden; and plasmin and S-2288 chromogenic substrate from Kabi, Stockholm. Recombinant human TPA was kindly provided by Genentech, South San Francisco.

Caseinolytic units (CU) of plasmin were defined according to Sgouris and colleagues: 1 CU equals 1.14 Committee on Thrombolytic Agents (CTA) units. Plasmin activity was determined in an S-2288 chromogenic substrate assay performed at 37°C in a spectrophotometer (Giford 2400, Oberlin, OH). One CU plasmin equals 0.45 nmol of plasmin. The purity of this human plasmin preparation was documented as previously reported: when subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), it produced two major bands with apparent mol wt of 70,000 and 28,000 under reducing conditions and a single band with an apparent mol wt of 81,000 under nonreducing conditions.

RESULTS

Human platelets washed in a modified Tyrode’s buffer were preincubated with either human plasmin for 10 minutes and/or PGI2 for 3 minutes at 37°C. Plasmin activity was neutralized by the addition of aprotinin, and platelet aggregation was initiated with either 0.2 U/mL of human thrombin or 10 μmol/L of ADP. As shown in Fig 1A, concentrations of plasmin and PGI2 were determined that individually caused only slight inhibition of thrombin or ADP-induced aggregation. When platelets were preincubated with both plasmin and PGI2 at the same concentrations as used individually, potentiation of the inhibitory effects was consistently noted. Similar experiments were performed with PRP instead of washed platelets: in this case, plasmin was generated in plasma by incubation with recombinant human TPA for 10 minutes instead of by the direct addition of plasmin. As shown in Fig 1B, preincubation of PRP with a combination of both TPA and PGI2 at threshold inhibitory concentrations caused potentiation of inhibition of platelet aggregation in response to both thrombin and ADP.

To show that in platelets plasmin and PGI2 exert their synergistic inhibitory effects not only on the aggregation response itself but also on the transduction of extracellular signals, we performed experiments to determine the interactions between plasmin and PGI2 on platelet Ca2+ mobilization and the release and metabolism of arachidonic acid induced by thrombin. As shown in Fig 2, preincubation of fura2/AM-loaded platelets with either 1.0 nmol/L of PGI2 for 3 minutes alone or 0.025 CU/mL of plasmin for 10 minutes alone at 37°C caused only a slight inhibition of thrombin-induced elevation in [Ca2+]. When platelets were preincubated with both PGI2 and plasmin at the same concentrations, however, marked inhibition of thrombin-induced Ca2+ mobilization occurred. Further addition of a fourfold higher concentration of thrombin did not overcome this synergistic inhibition by the combination of PGI2 and plasmin.

Because thrombin-activated phospholipase C stimulates the release of endogenous arachidonic acid, which is metabolized to the potent proaggregatory and vasoconstrictor eicosanoid TXA2 in platelets, we studied the effects of plasmin and PGI2 on inhibition of thrombin-stimulated TXA2 formation, measured by radioimmunoassay of its breakdown product, TXB2. As shown in Table 1, concentrations of plasmin and PGI2 were determined that individually caused only slight inhibition of thrombin-induced TXB2 production.
When platelets were preincubated with a combination of both plasmin and PGI2 at the same concentrations, marked potentiation of the inhibitory effect on TXB2 formation was noted. Although the synergism between the inhibitory effects of plasmin and PGI2 on platelet aggregation, \([\text{Ca}^{2+}]_{i}\), and TXB2 production was reproducibly and consistently observed, as yet unexplained individual variations in the magnitude of these platelet responses were found.

The potentiating effect of plasmin on inhibition of platelet activation by PGI2 was not mediated by cyclic AMP. As shown in Table 2, plasmin did not increase platelet cyclic AMP content. Furthermore, plasmin did not potentiate the PGI2-induced elevation of platelet cyclic AMP levels.

**DISCUSSION**

Our studies indicate that plasmin and PGI2 act synergistically to inhibit signal–response coupling in platelets. PGI2 exerts this effect by raising the intracellular concentration of cyclic AMP. The mechanism of platelet inhibition by plasmin is different and possibly more complex. Loss of platelet membrane glycoproteins following plasmin treatment\(^{2,3}\) might lead to loss of platelet receptors for thrombin\(^{4,13}\) and other agonists. We also recently showed that plasmin can activate platelet protein kinase C.\(^{11}\) Activation of protein kinase C by plasmin, detected by phosphorylation of the 47-kDa protein in 32P04-labeled platelets as previously described,\(^{11}\) did occur under the conditions of these experiments (data not shown). Activation of platelet protein kinase C, in turn, feeds back to inhibit extracellular signal transduction\(^{16,17}\) in response to thrombin and other agonists. Therefore, plasmin appears to exert its inhibitory effects on platelets, at least in part, by activation of protein kinase C. These results suggest that cyclic AMP and protein kinase C activation may synergistically inhibit the transduction of extracellular signals in platelets. This synergistic effect may be mediated by protein kinase C-induced phosphorylation and inactivation of the inhibitory guanine-nucleotide-binding regulatory component (N protein) of platelet adenylate cyclase.\(^{18,19}\)

Thus, PGI2 and plasmin act synergistically to inhibit platelet activation by blocking \(\text{Ca}^{2+}\) mobilization and the release and metabolism of arachidonic acid by both cyclic AMP and noncyclic AMP-mediated mechanisms. The potentiating inhibitory actions of plasmin and PGI2 on platelet activation described in this study may operate in vivo as an important antithrombotic mechanism. Thrombin generation at the site of vascular injury stimulates both fibrin formation and platelet aggregation to form a hemostatic plug. Surrounding the focus of injury, however, PGI2 and TPA are released from intact endothelial cells; thus, PGI2 and plasmin, possibly generated at locally amplified concentrations, may act synergistically as potent inhibitors of further thrombin-mediated accrual of platelets to the hemostatic plug beyond the site of vascular injury.

### Table 1. Potentiation of Inhibition of Thrombin-Induced Platelet TXA2 Production by Plasmin and PGI2

<table>
<thead>
<tr>
<th>Preincubations</th>
<th>TXB2 (pg/10^9 Platelets)</th>
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<tr>
<td>Control</td>
<td>196.0 ± 6.3</td>
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<tr>
<td>Plasmin</td>
<td>150.8 ± 5.7</td>
</tr>
<tr>
<td>PGI2</td>
<td>149.8 ± 25.6</td>
</tr>
<tr>
<td>Plasmin + PGI2</td>
<td>26.4 ± 0.8</td>
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**TXA2**, thromboxane A2; **TXB2**, thromboxane B2; **PGI2**, prostaglandin I2; CU, caseinolytic unit.

Washed platelets (1.5 x 10^8/mL) were preincubated at 37°C for 10 minutes with buffer blanks (control) or plasmin (0.25 CU/mL) for 10 minutes, with 10^-8 mol/L of PGI2 for 3 minutes, or with a combination of 0.2 CU/mL of plasmin for 10 minutes and 10^-8 mol/L of PGI2 for 3 minutes. Aprotinin was added to all samples following the preincubation period, and platelets were then activated with 0.37 U/mL of thrombin for 3 minutes. TXB2 was measured by radioimmunoassay of supernatants of the completed reactions. Data are means ± SE of triplicate determinations of two separate experiments.

### Table 2. Effects of Plasmin and Thrombin on PGI2-Stimulated Platelet Cyclic AMP

<table>
<thead>
<tr>
<th>Additions</th>
<th>Cyclic AMP (pmol/10^9 Platelets)</th>
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<tbody>
<tr>
<td>Control</td>
<td>96.0 ± 16.1</td>
</tr>
<tr>
<td>Thrombin</td>
<td>123.5 ± 17.7</td>
</tr>
<tr>
<td>Plasmin</td>
<td>124.6 ± 17.5</td>
</tr>
<tr>
<td>Plasmin + thrombin</td>
<td>118.2 ± 20.2</td>
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<tr>
<td>PGI2</td>
<td>837.4 ± 26.5</td>
</tr>
<tr>
<td>PGI2 + thrombin</td>
<td>373.1 ± 19.4</td>
</tr>
<tr>
<td>Plasmin + PGI2</td>
<td>871.9 ± 34.7</td>
</tr>
<tr>
<td>Plasmin + PGI2 + thrombin</td>
<td>569.4 ± 24.7</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1.

Platelets washed by gel filtration (~1.5 x 10^8/mL) were preincubated at 37°C for 10 minutes with buffer blank (control) or plasmin (0.25 CU/mL). Buffer blank or PGI2 (10^-8 mol/L) were then added for 30 seconds, followed by buffer blank or thrombin (0.75 U/mL) for an additional 2 minutes. Reactions were stopped by the addition of an equal volume of ice-cold 10% trichloroacetic acid. Cyclic AMP in platelets was measured by radioimmunoassay and corrected for platelet counts. Data are means ± SE of quadruplicate determinations of three separate experiments.

![Fig 2. Synergistic inhibition of thrombin-induced increase in cytoplasmic free Ca^{2+} concentration ([Ca^{2+}]_{i}) by plasmin and prostaglandin I2 (PGI2) in washed platelets. Tracings show fura2 fluorescence in response to the addition of 0.25 U/mL thrombin (T) at the point of each arrow. From left to right individual tracings show: no preincubation; preincubation with 1 nmol/L of PGI2 for 3 minutes; preincubation with 0.025 caseinolytic units (CU)/mL of plasmin for 10 minutes; preincubation with 0.025 CU/mL of plasmin for 10 minutes plus 1 nmol/L of PGI2 for 3 minutes. In the last tracing, an additional stimulus of 1 U/mL of T was introduced 3 minutes after the initial stimulus of 0.25 U/mL of T.]
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REFERENCES

Synergistic inhibition of platelet activation by plasmin and prostaglandin I2

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