PAR-4 Agonist AYPGKF Stimulates Thromboxane Production by Human Platelets

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Abstract—Previous reports have indicated that thrombin-induced thromboxane production by human platelets occurs through two types of interaction between thrombin and the platelet surface. One of these interactions is with protease activated receptor (PAR)-1, the first identified thrombin receptor. These studies were undertaken to determine whether stimulation of PAR-4 also results in thromboxane production. The results show that treatment of washed human platelets with the PAR-4 agonist AYPGKF stimulates a maximum of 40% to 60% of the thromboxane produced by 100 nmol/L thrombin. Maximal thromboxane production requires approximately 1.0 nmol/L AYPGKF, despite the observation that maximal aggregation is produced by 45 μmol/L AYPGKF. Thromboxane produced by the combined stimulation of PAR-1 and PAR-4 is additive. Pretreatment of platelets with 45 μmol/L AYPGKF partially desensitizes thromboxane production in response to higher concentrations of AYPGKF and thrombin but not to stimulation by SFLLRN. PAR-4–induced stimulation is also significantly inhibited by 60 μmol/L genistein. It is concluded that activation through either PAR-1 or PAR-4 results in thromboxane production, but that stimulation of neither receptor alone produces thromboxane equivalent to that produced by 100 nmol/L thrombin. Thus, these findings demonstrate the presence of two pathways for thrombin-induced thromboxane production by platelets as proposed previously.

Key Words: thromboxane ■ platelets ■ thrombin ■ PAR-1 ■ PAR-4

Thrombin plays a critical role in regulation of the thrombotic response, acting as the terminal protease of the coagulation cascade by initiating the conversion of fibrinogen to fibrin, activating platelets and limiting these responses through activation of Protein C. In contrast to classical ligand receptor–binding interactions, thrombin-induced platelet activation results from hydrolysis of a specific thrombin substrate, protease-activated receptor-1 (PAR-1), a G-protein–coupled 7-transmembrane domain receptor.1 Cleavage of the Arg41–Ser42 bond of PAR-1 forms a new amino terminus, which then serves as a tethered ligand. Thus, the receptor itself is the source of the ligand that binds and activates the receptor. Peptides with sequences derived from the amino terminus of the tethered ligand sequence also act as agonists for PAR-1.1 In 1998, a homologous G-protein–coupled receptor, designated PAR-4, which is also cleaved by thrombin and present on human platelets, was identified.2,3 However, the amino acid sequence surrounding the cleavage site in PAR-4 differs from that in PAR-1 in the lack of a down-stream hirudin-like domain for interaction with anion-binding exosite 1 of thrombin. As a consequence, PAR-4 requires higher thrombin concentrations than does PAR-1 for rapid activation.2,3 This feature could be an additional mechanism for regulating the thrombotic response. Like PAR-1, PAR-4 may also be stimulated by peptide agonists.

Although many actions of thrombin in the activation of platelets, including stimulation of a [Ca2+]i flux, platelet aggregation, and granule release, may be explained by the action of thrombin on PAR-1, other reports suggest that this receptor does not account for the total response of human platelets.4–10 Stimulation of PAR-4 also produces platelet aggregation and [Ca2+]i fluxes, but specific features of the latter response differ from those observed following PAR-1 stimulation.2,11

The present studies were undertaken to determine whether PAR-4 contributes to thromboxane production by human platelets and whether stimulation of this receptor accounts for the difference in thromboxane production observed for human platelets stimulated by thrombin compared with specific stimulation of PAR-1 by a peptide agonist.5 Because thromboxane, like thrombin, is a potent platelet agonist, understanding the mechanism by which it is produced may contribute to strategies for limiting its production and the prevention of thrombosis.

Methods

Reagents

The peptide agonist for PAR-1, SFLLRN, was obtained from BACHEM and was present at a final concentration of 85 μmol/L. For PAR-4 stimulation, the peptide agonists GYPGQV, GYPGKF, and AYPGKF12 were synthesized as the C-terminal amides by the
University of North Carolina Peptide Synthesis Laboratory (Chapel Hill, NC). Before use, GYPGKF was gel-filtered on Sephadex G-10 (Amersham Biosciences) with 0.15 mol/L NaCl as eluant. Peptide concentrations were determined by amino acid analysis (AAA Laboratory) or from a calculated molar extinction coefficient of 1198 at 280 nm for GYPGKF. Peptide stock solutions in 0.15 mol/L NaCl were stored in aliquots at −80°C. Human α-thrombin (thrombin) was prepared as described.13

**Platelets**

Blood was obtained by the two-syringe technique after obtaining informed consent from healthy, nonsmoking adults denying use of antiplatelet medication for 10 days before phlebotomy. Whole blood, 6 vol, was anticoagulated with 1 vol acid citrate dextrose A (748 mmol/L sodium citrate, 38 mmol/L citric acid, 136 mmol/L glucose). These studies were approved by the University and Medical Center Institutional Review Board at East Carolina University, and all procedures were in accordance with institutional guidelines. Platelets were prepared by differential centrifugation and washed three times essentially as described previously except that 1 U/mL heparin was included in the first wash.3 For aspirin-treated platelets, the initial platelet pellet was resuspended in buffer to which aspirin was added at a final concentration of 200 μmol/L, and the platelets were incubated at 37°C for 20 minutes. After the third wash, platelets were resuspended in platelet buffer containing (in mmol/L) HEPES 10, NaCl 137, KCl 2.7, NaH2PO4 0.36, MgCl2 1, glucose 5.6, pH 7.4, and 3.5 mg/mL bovine serum albumin. After counting, platelets were diluted in the same buffer, and 1.0 mol/L CaCl2 was added to yield a final concentration of 1.0 mmol/L Ca2+. Experiments were performed at final platelet counts of 2.6 to 3.1×10^8/mL.

Platelet aggregation was performed, in the absence of added fibrinogen, at 37°C with stirring at 1000 rpm, and light transmission was monitored with a Chrono-log Whole Blood Aggregometer, Model 560. For thromboxane determination, the platelet suspension was centrifuged for 1 minute at 16 000g, 5 minutes after addition of agonist to samples monitored for aggregation (without aspirin treatment). Platelet supernatants were stored at −80°C before assay. Thromboxane B2, the stable metabolite of thromboxane, was determined by competitive ELISA with reagents obtained from Neogen Corporation as described previously.3 In each experiment, responses were compared with that obtained for 100 nmol/L thrombin defined as 100%. For 5 minutes of incubation, this corresponded to 460±90 ng/mL thromboxane B2 for 10^10 platelets.

**Genistein Inhibition**

For studies of genistein inhibition, an 18 mmol/L stock solution in dimethyl sulfoxide (DMSO) was diluted into the platelet buffer. Platelets were preincubated for 2 minutes without stirring with either genistein at a final concentration of 60 μmol/L or with the platelet buffer, which contained DMSO. The final concentration of DMSO was 0.3%. For these studies, the incubation time for thromboxane production was 1.0 minute, followed by 1.0 minute of centrifugation. Thromboxane B2 was assayed as described above.

**Statistics**

All experiments were performed a minimum of three times with different platelet donors. Statistical significance was determined by t test with P<0.05 indicating significance.

**Results**

**PAR-4–Induced Aggregation**

Initial studies in our laboratory utilized the peptides GYPGQV, derived from the human sequence of PAR-4, and GYPGKF, derived from the murine sequence. The latter is slightly more potent than the human peptide in stimulating human PAR-4.2,3,14 With GYPGQV, platelet aggregation was not observed at concentrations up to 600 μmol/L. A concentration of 420 μmol/L GYPGKF (results not shown) induced aggregation, but not in all platelet preparations. Subsequently, from studies with a series of hexapeptide agonists for PAR-1 and PAR-4 acting on cell lines expressing one or the other of these human receptors, AYPGKF was identified as a specific and more potent agonist for PAR-4.12 Therefore, we have used this agonist in further studies. Shown in Figure 1 are platelet aggregation responses comparing the effects of the PAR-4 agonist AYPGKF, at 45 and 480 μmol/L, with those of 100 nmol/L thrombin and 85 μmol/L SFLLRN. There was no aggregation response after the addition of 0.15 mol/L NaCl to platelets.

**PAR-4–Induced Thromboxane Production**

To determine whether PAR-4 stimulation also results in thromboxane production, platelet supernatants were assayed for thromboxane B2. A dose-response curve for AYPGKF-induced thromboxane production is shown in Figure 2. These results suggest that maximal thromboxane production occurs at approximately 1 mmol/L AYPGKF, but that the maximal level of thromboxane produced is only about half of that obtained in response to 100 nmol/L thrombin. Thromboxane production in response to 1.0 mmol/L GYPGKF was less than 10% of that observed in response to 100 nmol/L thrombin. Figure 3 shows thromboxane production in response to the
individual agonists 85 \mu M SFLLRN and 480 \mu M AYPGKF compared with the results obtained for simultaneous addition of the two agonists. Although the sum of the effects of the PAR-1 and PAR-4 agonists appears to be less than that obtained on simultaneous addition of these two agonists, this difference is not statistically significant ($P > 0.05$). However, at these concentrations, the combined individual agonists do not yield a level of thromboxane equivalent to that obtained in response to 100 \mu M thrombin. The concentrations of 85 \mu M SFLLRN and 100 \mu M thrombin were selected to give near maximal thromboxane responses for each agonist, as reported previously.$^{5}$

**PAR-4 Desensitization**

To further characterize thromboxane production in response to PAR-4 stimulation, we determined whether this response could be desensitized by treatment with a low concentration of AYPGKF. Results of these studies, shown in Figure 4, indicate that the production of thromboxane in response to higher concentrations of AYPGKF or to thrombin is desensitized by a 40-minute preincubation of platelets with 45 \mu M AYPGKF, a concentration sufficient to produce maximum aggregation. However, there is no desensitization of thromboxane production when platelets pretreated with AYPGKF are subsequently treated with the PAR-1 agonist, 85 \mu M SFLLRN, indicating specificity in the responses initiated through PAR-1 and PAR-4.

**Genistein Inhibition of PAR-4–Induced Thromboxane Production**

Previously, we reported that PAR-1–independent thromboxane production was sensitive to inhibition by genistein.$^{5}$ This was examined directly for the agonist AYPGKF by pretreating platelets with 60 \mu M genistein for 2.0 minutes before addition of the agonist. Results of these studies, shown in Figure 5, indicate that the thromboxane production in response to all concentrations of AYPGKF is significantly inhibited by genistein. These findings suggest an essential role for a tyrosine kinase(s) in the stimulation of thromboxane production. However, identification of the specific site of inhibition by genistein awaits further investigation.

**Discussion**

**Two Thrombin Receptors Involved in Thromboxane Production**

In previous studies of human platelet activation in response to thrombin and the mutant thrombin, Thrombin Quick I, it was
aggregation, granule release, stimulation of \([\text{Ca}^{2+}]_i\), fluxes, and thromboxane production. \(\text{PAR}-1\) does not contribute to murine platelet activation and as the thrombin concentration within a developing thrombus increases, platelets stimulated initially through \(\text{PAR}-1\) at a low thrombin concentration (0.2 nmol/L) may continue to respond through \(\text{PAR}-4\), reaching a maximum response at higher concentrations (100 nmol/L). Although the physiological relevance of high thrombin concentrations might be questioned, 100 nmol/L thrombin represents the conversion of less than 10% of circulating prothrombin to thrombin. Because thrombin is generated at the platelet surface, the local concentration in the forming thrombus will be considerably higher than in the circulation where additional protective mechanisms prevent extension of thrombi. Thromboxane is an extremely potent platelet-aggregating agent, and the physiological importance of this prostaglandin is evidenced, at least in part, by the efficacy of aspirin therapy, which is widely prescribed for prevention of both primary and secondary thrombotic events.

**Densensitization of \(\text{PAR}-4\)**

Examination of receptor desensitization in response to agonists permits identification of the roles of multiple receptors. We have previously reported the desensitization of thromboxane production by pretreatment of platelets with a \(\text{PAR}-1\) agonist peptide at 20 \(\mu\text{mol/L}\), for either 2 or 10 minutes. Under these conditions, there was no further response to the \(\text{PAR}-1\) agonist at 100 \(\mu\text{mol/L}\), but the response to 100 \(\mu\text{mol/L}\) thrombin was nearly equivalent to that for platelets preincubated with only buffer. When platelets were preincubated with 5 nmol/L thrombin, desensitization was slower and a response to 100 nmol/L thrombin was still elicited after the 10-minute preincubation period. This incomplete desensitization to thrombin may be explained by the observation that down-regulation of the \(\text{PAR}-4\) receptor is slow, apparently because of the lack of a phosphorylation site in the C-terminal cytoplasmic domain. In separate experiments, we found that the aggregation response to 1.0 nmol/L (total concentration) GYPGKF was eliminated after a 40-minute preincubation with 500 \(\mu\text{mol/L}\) GYPGKF. In the studies presented here, there was only a minimal effect on the aggregation response to 480 \(\mu\text{mol/L}\) AYPGKF after a 40-minute preincubation with 45 \(\mu\text{mol/L}\) AYPGKF (results not shown). We also observed that activation of \(\text{PAR}-4\) does not desensitize the \(\text{PAR}-1\) receptor on platelets with respect to thromboxane production (Figure 4) or aggregation (results not shown). Thus, \(\text{PAR}-1\) and \(\text{PAR}-4\) display not only the previously reported differences in thrombin concentration dependence for stimulation, but also a differing pattern of desensitization, which we have confirmed here for thromboxane production. This difference in receptor desensitization would permit response through \(\text{PAR}-4\) for an extended period of time as the thrombin concentration increased in response to stimulation of prothrombinase activity at the platelet surface.

**Maximal Thromboxane Production**

The release of arachidonic acid from platelet phospholipids is mediated largely by cytoplasmic phospholipase \(\text{A}_2\) (cPLA\(_2\)). Previous work has shown that the amount of thromboxane produced in response to \(\text{PAR}-1\) peptide agonists is roughly half of the maximal levels obtained in response to

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**Figure 5.** Genistein inhibits thromboxane production by platelets stimulated with AYPGKF. The results shown indicate a marked shift to the right in the concentration dependence for thromboxane production by platelets preincubated for 2.0 minutes with 60 \(\mu\text{mol/L}\) genistein followed by stimulation with the indicated concentrations of AYPGKF. In these experiments, platelets were incubated with the agonist for 1.0 minute before preparation of the supernatant for thromboxane assay as described in Methods. Thromboxane produced by platelets treated with the genistein vehicle followed by 100 nmol/L thrombin is defined as 100%. Results shown are mean±SEM (when greater than size of symbol), \(n=3\). The lines represent third order polynomial fits of the data.
thrombin.\textsuperscript{5,9} Similarly, results presented here (Figure 2) show that the PAR-4 peptide agonist AYPGKF at 1 mmol/L also produces about half of the maximal amount of thromboxane observed in response to 100 nmol/L thrombin. Previously, 500 \(\mu\)mol/L AYPGKF was observed to be equivalent to 30 nmol/L thrombin in stimulating release of inositol phosphates from cultured cells expressing only the PAR-4 receptor.\textsuperscript{1,2} Our results, suggesting that neither PAR-1 nor PAR-4 stimulation is sufficient to induce maximal thromboxane production by platelets as well as the dependence of thromboxane production on agonist concentration, raise interesting questions concerning the regulation of eicosanoid production. The stimulation of either PAR-1 or PAR-4 appears to result not only in activation of thromboxane production, but also in the initiation of the attenuation of this response as well. This attenuation of response would serve to preserve the arachidonate-containing substrate for sustained release as the thrombin concentration increased. Although thromboxane production mediated by both PAR-1 and PAR-4 would appear to be dependent on cPLA\(_2\) activity, whether regulation of this response is at the level of cPLA\(_2\) or at a point proximal to signal initiation awaits further investigation of the role of other signaling intermediates. Because the activity of cPLA\(_2\) is at least partially dependent on Ca\(^{2+}\), attenuation of its activity may be associated with the decline in intracellular Ca\(^{2+}\) after cellular activation.

Results shown in Figure 3 indicate that the simultaneous addition of near maximal concentrations of PAR-1 and PAR-4 agonists results in approximately additive production of thromboxane compared with the addition of the individual agonists. However, in these experiments, the sum of these effects is not equivalent to that produced by 100 nmol/L thrombin. Possible explanations for this observation include 1) the concentrations of the peptide agonists used do not elicit the maximal response for these agonists and thus the sum is still less than that for 100 nmol/L thrombin, 2) there is still another thrombin-induced response, that is a third receptor, that results in thromboxane production, or more probably 3) there is a sequential effect in the stimulation of these two receptors such that products from the stimulation of PAR-1 enhance the stimulation of PAR-4. The first possibility provides at least a partial explanation for the levels of thromboxane observed. With respect to the second possibility, there is, at this time, an absence of additional evidence suggesting a third thrombin receptor on human platelets. The third possibility is supported by the previous observations that simultaneous addition of 2 mmol/L GYPGKF and 30 \(\mu\)mol/L SFLLRN does not elicit the same prolonged, plateau intracellular Ca\(^{2+}\) response as the addition of 20 nmol/L thrombin, suggesting a contribution from sequential activation of PAR-1 and PAR-4.\textsuperscript{11}

**Inhibition of Thromboxane Production by Genistein**

Genistein is a nonspecific inhibitor of tyrosine kinases. Thus, it is probable that the effects of genistein are mediated by interaction with more than one signaling intermediate. It has been shown previously that the tyrosine kinase inhibitor herbimycin A does not inhibit thromboxane production by platelets under conditions in which the tyrosine kinase c-src is inhibited,\textsuperscript{21} suggesting that the effects of genistein are not mediated by the inhibition of c-src, which is rapidly activated by thrombin.\textsuperscript{22} Our previous studies indicating that genistein was more effective in inhibition of PAR-1–dependent than PAR-1–dependent thromboxane production suggest that the two receptors have a differential dependence on tyrosine kinase signaling pathways.\textsuperscript{5} Identification of specific tyrosine phosphorylation events linking these two receptors to thromboxane production awaits further investigation.

We have demonstrated that the PAR-4 agonist peptide AYPGKF stimulates thromboxane production by human platelets with the maximal response to this agonist being approximately half of that observed after maximal thrombin stimulation. The response to the PAR-4 agonist is additive with that observed in response to a PAR-1 agonist, and the PAR-4 mediated response is genistein-sensitive. Preincubation of platelets for 40 minutes with a low concentration of the PAR-4 agonist partially desensitizes the thromboxane response to higher concentrations of the PAR-4 agonist or to thrombin stimulation, but not to the PAR-1 agonist peptide. Thus, PAR-1 and PAR-4 seem to account for the two previously proposed receptors that initiate thrombin-induced thromboxane production by human platelets.\textsuperscript{5}

**Acknowledgments**

Support for this study was received in part from the American Heart Association, North Carolina Affiliate and an East Carolina University School of Medicine Faculty Research Grant. V.K. Hanks received support from the Federal Work Study Program. Amy Morales and Yolanda Newton provided technical assistance. Figure 1 was prepared by Catherine Spruill. We also wish to acknowledge the contribution of numerous donors of fresh platelets.

**References**


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Arterioscler Thromb Vasc Biol. 2002;22:861-866; originally published online March 7, 2002; doi: 10.1161/01.ATV.0000014742.56572.25
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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