Blocking the Protease-Activated Receptor 1-4 Heterodimer in Platelet-Mediated Thrombosis

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Background—Thrombin is the most potent agonist of platelets and plays a critical role in the development of arterial thrombosis. Human platelets express dual thrombin receptors, protease-activated receptor (PAR) 1 and PAR4; however, there are no therapeutic strategies that effectively target both receptors.

Methods and Results—Platelet aggregation studies demonstrated that PAR4 activity is markedly enhanced by thrombin–PAR1 interactions. A combination of bivalirudin (hirulog) plus a novel PAR4 pepducin antagonist, P4pal-i1, effectively inhibited aggregation of human platelets to even high concentrations of thrombin and prevented occlusion of carotid arteries in guinea pigs. Likewise, combined inhibition of PAR1 and PAR4 with small-molecule antagonists and pepducins was effective against carotid artery occlusion. Coimmunoprecipitation and fluorescence resonance energy transfer studies revealed that PAR1 and PAR4 associate as a heterodimeric complex in human platelets and fibroblasts. PAR1-PAR4 cofactoring was shown by acceleration of thrombin cleavage and signaling of PAR4 on coexpression with PAR1.

Conclusions—We show that PAR1 and PAR4 form a stable heterodimer that enables thrombin to act as a bivalent functional agonist. These studies suggest that targeting the PAR1-PAR4 complex may present a novel therapeutic opportunity to prevent arterial thrombosis. (Circulation. 2006;113:1244-1254.)

Key Words: carotid arteries ■ thrombin ■ platelets ■ receptors ■ thrombosis

Arterial thrombosis is the leading cause of mortality in Western nations and remains a prevalent, postintervention complication in patients undergoing percutaneous coronary interventions. Thrombin-bound thrombin is the primary mediator of platelet activation and aggregation, which have been shown to be heightened in the setting of angioplasty and stenting. Thrombin activates platelets by cleaving the G protein–coupled, protease (proteinase)-activated receptors PAR1 and PAR4. Chronic stimulation of PAR1 on endothelium, smooth muscle cells, and other mesenchymal cells leads to inflammation and intimal hyperplasia, whereas inhibition of PAR1 attenuates restenosis in animal models. However, blockade of only PAR1 may not be sufficient to prevent acute thrombin-mediated platelet aggregation and thrombosis because of the presence of PAR4, the other thrombin receptor present on platelets.

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The unique and enigmatic physiological role of the PAR4 thrombin receptor has recently begun to be understood. PAR1 and PAR4 signal with considerably different kinetics and appear to have distinct functions in platelet aggregation. PAR1 and PAR4 do not produce redundant signals during platelet activation but instead complement each other by evoking 2 distinct waves of intracellular calcium. The signal from PAR1 is fast and is quickly shut off and is followed by a prolonged signal that arises from PAR4 receptors that are more slowly activated by thrombin. Interestingly, unlike PAR1, PAR4 leads to irreversible platelet aggregation even in the absence of an adenosine 5'-diphosphate–autocrine response.

The marked differences in the rates of activation of PAR1 and PAR4 largely reflect differences in the association of thrombin with their respective exodomains. The PAR1 exodomain harbors a hirudin-like sequence element, Y52EPF55 (Hir), that interacts with exosite I of thrombin and is essential for rapid association to thrombin. PAR4 is cleaved more slowly than PAR1 mainly because it lacks a functional Hir sequence. To compensate for the lack of exosite I binding, PAR4 has optimized its cleavage sequence to provide high-affinity interactions with the active site and uses an anionic cluster to slow dissociation from the cationic thrombin. The cleaved PAR1 exodomain retains the Hir motif and binds to...
exosite I, suggesting that thrombin may remain tethered to the surface of human platelets via its association with the cleaved PAR1 receptor (Figure 1A).

A recent nuclear magnetic resonance structure of the thrombin-cleaved PAR1 exodomain indicates that PAR1 bound to exosite I leaves the active site of thrombin free to potentially interact with other large macromolecules such as PAR4. This accessible active site is interesting in light of studies that have shown that mouse PAR3 serves as a Hir-containing thrombin-binding site that assists in the cleavage of adjacent PAR4 receptors. Murine platelets lack PAR1 and instead have a high-affinity murine PAR3 (mPAR3) receptor that pairs with the lower-affinity mPAR4 thrombin receptor. Unlike in humans, in whom PAR1 (mPAR3) receptor that pairs with the lower-affinity mPAR4 receptor, PAR2 by thrombin-cleaved PAR1 on human umbilical vein endothelial cells and in recombinant systems. In many cases, G protein–coupled receptor (GPCR) dimerization has been shown to modulate ligand affinity, signaling, endocytosis, and receptor stability with significant implications for the pathophysiology of cardiovascular disease.

In the present study we show that PAR1 serves as a thrombin-enhancing cofactor for PAR4 on the surface of human platelets. We demonstrate that PAR1 and PAR4 form stable heterodimeric complexes on platelets and other cells and that PAR1 assists in the activation and cleavage of PAR4. Combined inhibition of PAR1 and PAR4 with small-molecule antagonists and pepducins effectively prevented occlusion of carotid arteries in animals. Likewise, blocking PAR4 with a cell-penetrating i1-loop pepducin in combination with bivalirudin was highly effective in the acute thrombosis model. Targeting the PAR1-PAR4 complex on the surface outside and/or inside surfaces may present a novel therapeutic opportunity to prevent arterial thrombosis.

**Methods**

**Pepducins**

P4pal-i1 (palmitate-NH-ATGAPRLPST) and P1pal-7 (palmitate-NH-K7KSARALF) were synthesized with C-terminal amides by standard 9-fluorenylmethyloxycarbonyl solid-phase methods. In the present study we show that PAR1 serves as a thrombin-enhancing cofactor for PAR4 on the surface of human platelets. We demonstrate that PAR1 and PAR4 form stable heterodimeric complexes on platelets and other cells and that PAR1 assists in the activation and cleavage of PAR4. Combined inhibition of PAR1 and PAR4 with small-molecule antagonists and pepducins effectively prevented occlusion of carotid arteries in animals. Likewise, blocking PAR4 with a cell-penetrating i1-loop pepducin in combination with bivalirudin was highly effective in the acute thrombosis model. Targeting the PAR1-PAR4 complex on the surface outside and/or inside surfaces may present a novel therapeutic opportunity to prevent arterial thrombosis.

**Chemotaxis Assays**

Migration of human embryonic kidney (HEK) cells transiently transfected with pcDEF3-PAR1 and/or pcDEF3-PAR4 toward chemotactic gradients of PAR1 or PAR4 peptide agonists was measured with the use of a 48-blindwell microchemotaxis chamber (Neuro Probe, Gaithersburg, Md) equipped with 8-µm pores. Data are expressed as chemotaxis index, which is the ratio between the distance of migration toward chemotaxtractants versus Roosevelt Park Memorial Institute (RPMI) medium alone.

**Guinea Pig Carotid Artery Injury Model**

Two- to 4-week-old Hartley guinea pigs (weight, 170 to 260 g) were purchased from Charles River Laboratories (Wilmington, Mass). A 0.61-mm-diameter polyethylene catheter was introduced into the left jugular vein for delivery of hirulog (bivalirudin), P4pal-i1, P1pal-7, and/or RWJ-56110 in 200-µL volumes of 10% dimethylformamide/90% sterile saline (vehicle) 5 minutes before injury. Right carotid arteries were injured for 20 minutes with a 24-mm2 piece of Bio-Rad Trans-Blot paper soaked in 20% FeCl3. Arterial flow 5 mm distal to the site of injury was measured with a 0.5-V Doppler probe (Transonic Systems, Ithaca, NY). An arterial occlusion was defined as a flow rate of <0.05 mL/min for >5 minutes. Doppler measurements were terminated 30 minutes after injury. Animal experiments were performed in accordance with the National Institutes of Health guidelines and approved by the Tufts–New England Medical Center Institutional Animal Care and Use Committee.

**Fluorescence Resonance Energy Transfer**

Template DNA encoding pcDEF3-PAR1 was amplified by polymerase chain reaction to introduce a XhoI site at the 5′ end of PAR1 and PAR4 and a HindIII site at the promoter corresponding to amino acid 396 or 377 of PAR1 and amino acid 348 of PAR4. The PAR genes were cloned into the XhoI and HindIII sites in the pEYFP-N1 and pEFYP-N1 vectors (Clontech, Palo Alto, Calif) to generate PAR1Δ377-CFP, PAR1Δ377-YFP, PAR4Δ348-CFP, and PAR4Δ348-YFP. COS7 fibroblasts were transiently transfected by the diethylaminoethanol-dextran method. Scanning fluorometry of transfected COS7 cells was performed on a Perkin-Elmer LS50B fluorescence spectrophotometer to analyze expression levels of cyan fluorescent protein (CFP)- and yellow fluorescent protein (YFP)-tagged proteins and to detect fluorescence resonance energy transfer (FRET) interactions between donor and acceptor receptors. Transfected cells expressing both PAR1Δ377-CFP and PAR4Δ348-YFP were excited at 425 nm, and the emission spectrum was collected from 450 to 650 nm. This spectrum consisted of CFP donor fluorescence, YFP acceptor fluorescence due to cross-excitation at 425 nm, and FRET between donor and acceptor. FRET was calculated by subtracting 2 components as previously described. The first subtracted component was the emission spectrum of cells expressing CFP (donor) normalized to the CFP emission maximum of the cells coexpressing donor and acceptor. The second subtracted component was the emission spectrum of YFP (acceptor) only via cross-excitation by 425 nm at the same expression level as YFP in the cells coexpressing donor and acceptor receptors.

**Immunoprecipitation of PAR1 and PAR4**

PAR4 was tagged at its N-terminus with a T7-epitope (underlined) and/or RWJ-56110 in 200-µL volumes of 10% dimethylformamide/90% sterile saline (vehicle) 5 minutes after transfection. Doppler measurements were terminated 30 minutes after injury. Animal experiments were performed in accordance with the National Institutes of Health guidelines and approved by the Tufts–New England Medical Center Institutional Animal Care and Use Committee.

**Platelet Aggregation**

In accordance with informed consent procedures approved by the Tufts–New England Medical Center institutional review board, whole blood was drawn from healthy volunteers (n = 15) with an 18-gauge needle into a 30-µL syringe containing 3 µL of 4% sodium citrate (0.4% vol/vol final). Platelets were then isolated by gel filtration chromatography of platelet-rich plasma with the use of Sepharose 2B (Pharmacia, Piscataway, NJ) in modified PIPES buffer and adjusted to 1.5 × 10^10/µL in PBS. Platelet aggregation was measured with the use of a Chronolog 506VS/490-2D aggregometer as described. Thrombin (α, γ) was from Hematologic Technologies (Essex Junction, Vt).
Figure 1. PAR4-dependent platelet aggregation is inhibited upon blocking thrombin–PAR1 interactions. A, Sequential model of thrombin activation of the PAR1–PAR4 heterodimeric complex. B, RWJ-56110 and the PAR1-Ab completely block PAR1 Ca^{2+} signaling. TFLLRN (50 \mu mol/L) is a selective PAR1 agonist, and AYPGKF (500 \mu mol/L) is a selective PAR4 agonist. CCRF-CEM cells were preincubated for 20 minutes with 37 \mu g/mL PAR1-Ab or 5 minutes with 1 \mu mol/L RWJ-56110 and treated with thrombin (T). Ca^{2+}/fura 2 fluorescence was measured at 25°C. C, RWJ-56110 does not affect thrombin cleavage of PAR1. COS7 cells transiently expressing T7-PAR1 were treated with thrombin in the presence and absence of RWJ-56110. Release of the T7 epitope tag over 1 minute was measured by FACS as described. D, PAR4 expressed on SW620 colon adenocarcinoma cells is not inhibited by RWJ-56110 or the PAR1-Ab (74 \mu g/mL). Thrombin (T) was used at 50-nmol/L concentration. E, Human platelets were preincubated with 1 \mu mol/L RWJ-56110 or RWJ-56110 plus 74 \mu g/mL PAR1-Ab before the addition of thrombin. F, Human platelets were preincubated for 2 minutes with buffer (untreated), 1 \mu mol/L RWJ-56110, or 1 \mu mol/L RWJ-56110 plus 74 \mu g/mL PAR1-Ab before the addition of thrombin.
1 mL. Agarose beads were collected by centrifugation and washed 3 times in ice-cold IP buffer followed by a single wash of IP buffer containing 0.1% (wt/vol) SDS. Immunoprecipitated protein was eluted either by direct addition to SDS loading buffer (α-T7 agarose) or incubation in 0.2 mol/L glycine, pH 3.4 (α-Flag agarose) and then neutralized in 300 mmol/L Tris-HCl, pH 7.4. Glycosidase reactions consisted of 5 to 45 μg COS7 or platelet lysate protein, 1× glycosidase reaction buffer, and 500 U PNGaseF (New England Biolabs, Beverly, Mass). Immunoprecipitation of human platelet PAR4 was performed with the PAR4 antibody20 coupled to CNBr-activated Sepharose beads (Amersham, Uppsala, Sweden) with a procedure similar to that described above except that platelet lysates were prepared with IP buffer containing 0.1% Triton (vol/vol), and platelet lysates and PAR4-Ab beads were incubated for 3 hours at 4°C.

Statistical Analysis
The data are expressed as mean±SD. Statistical significance was determined with the use of Excel 2001 by tailed Student t test and considered significant at P<0.05.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results
PAR4 Is Efficiently Activated by Thrombin on Human Platelets
To study the activation of platelet PAR4 by thrombin, we eliminated the PAR1 signal with RWJ-56110, a competitive antagonist of the PAR1 tethered ligand.23 Using cell lines that individually express either PAR1 or PAR4, we showed that RWJ-56110 completely inhibits the PAR1 thrombin signal (Figure 1B) without preventing thrombin cleavage of PAR1 (Figure 1C). RWJ-56110 was selective for PAR1 and did not nonspecifically block thrombin activation of PAR4 (Figure 1D).

We showed that platelet PAR4 is strongly activated at concentrations of thrombin >3 nmol/L. Thus, blockade of PAR1 with RWJ-56110 did not inhibit platelet aggregation to concentrations of thrombin >5 nmol/L (Figure 1F; Figure I in the online-only Data Supplement). Addition of RWJ-56110 shifted the thrombin EC50 for platelet aggregation from 2 to 4 nmol/L. This indicates that PAR4 activation occurs with only 2-fold lower apparent affinity for thrombin relative to the combined PAR1-PAR4 response on platelets, consistent with previous observations.8,10 This result is in stark contrast to thrombin activation of PAR4 in recombinant systems, where the apparent difference in cleavage efficiency between PAR1 and PAR4 is much greater.9,12 To explain the enhanced activation of platelet PAR4 by thrombin, we tested the model of Figure 1A that PAR1 was acting as a cofactor for PAR4 on the surface of human platelets.

PAR1 Assists in Thrombin Activation of PAR4 on Platelets
To determine whether PAR1 was acting as a thrombin-enhancing cofactor for PAR4, we blocked the interaction of thrombin with the PAR1 Hir motif by 2 independent methods. First, we used a PAR1-blocking antibody (PAR1-Ab) raised against the PAR1 sequence, which contains the Hir motif.10,20 Using cell lines that express either PAR1 or PAR4, we showed that the PAR1-Ab completely blocks thrombin (20 to 200 nmol/L) activation of PAR1 (Figure 1B) without nonspecifically blocking thrombin activation of PAR4 (Figure 1D). We tested whether blocking this region on PAR1 with the PAR1-Ab would indirectly affect thrombin activation of platelet PAR4. Platelets were treated with RWJ-56110 to inhibit the PAR1 signal and then activated with thrombin. Addition of the PAR1-Ab greatly delayed the slope and extent of PAR4-dependent aggregation at 7 to 9 nmol/L thrombin (Figure 1E). A full thrombin titration in the presence of RWJ-56110 showed that the PAR1-Ab shifted the PAR4 thrombin response by an additional 2-fold from an EC50 of 4 to 8 nmol/L. (Figure 1F). A control E2-Ab raised against the first extracellular loop of PAR1, residues S164–A181, had no effect on the thrombin EC50 for PAR4-dependent platelet aggregation (data not shown). The PAR1-Ab alone gave a nearly identical thrombin EC50 for platelet aggregation as RWJ-56110 plus the PAR1-Ab (Figure I in the online-only Data Supplement). Thus, activation of platelet PAR4 requires higher concentrations of thrombin when the PAR1 Hir motif is blocked, suggesting that PAR1 assists in PAR4 activation.

Blocking PAR4 With a Cell-Penetrating i1-Loop Pepducin
Previous work in our laboratory demonstrated that cell-penetrating lipopeptides based on the third intracellular loop (i3) of PAR1, PAR4, and several other GPCRs inhibit their cognate receptor in vitro and in animals.7,18,24,25 The i3-loop pepducin derived from PAR4, P4pal-10, completely blocks PAR4-dependent aggregation; however, it can also partially inhibit SFLLRN (PAR1 peptide ligand) activation of PAR1 possibly because it binds at the interface between PAR1 and PAR4.7 We developed a more selective PAR4 pepducin based on the first intracellular loop (i1) of the receptor. Because the i1 loop is on the opposite side of the receptor relative to the i3 loop, we anticipated that an i1-loop pepducin based on PAR4 would not cross-inhibit PAR1. PAR4 was expressed on HEK cells singly or in combination with PAR1. The PAR4-i1 pepducin, P4pal-i1, completely blocked the chemotactic response of PAR4 on HEK cells and prevented platelet aggregation to its peptide ligand, AYPGKF (Figure 2A to 2C). P4pal-i1 was selective for PAR4 and did not inhibit the chemotactic response of PAR1, nor did it appreciably inhibit platelet aggregation to the PAR1 peptide ligand SFLLRN. P4pal-i1 did not inhibit PAR1 even on coexpression with PAR4, indicating that if PAR1 and PAR4 form a complex, the bound i1 pepducin of PAR4 does not prevent signaling from PAR1. Likewise, when used alone, P4pal-i1 had only a minor effect on platelet aggregation to 3 nmol/L thrombin because thrombin also activates PAR1 (Figure 2D). However, when used in combination with the PAR1 antagonist RWJ-56110, P4pal-i1 greatly inhibited aggregation to 3 nmol/L thrombin. Thus, targeting either PAR1 or PAR4 alone has a limited effect on thrombin aggregation, whereas simultaneous inhibition of both PAR1 and PAR4 is effective in blocking the response to thrombin.

Effect of Bivalirudin on Platelet PAR1 and PAR4 Responses
Next, we assessed the efficacy of inhibiting thrombin–PAR1 interactions in combination with PAR4 blockade on platelet aggregation. To prevent thrombin from binding to the Hir site
of PAR1, we used bivalirudin (hirulog, angiomax), which binds to thrombin with nanomolar affinity.26 Despite its widespread use in treating patients with acute coronary syndromes,27 the effects of bivalirudin on PAR1- and PAR4-dependent platelet activation have not been determined. We found that 200 nmol/L bivalirudin alone or bivalirudin plus RWJ-56110 gave a similar EC50 (10 nmol/L) for H9251-thrombin activation of PAR4-dependent aggregation in the presence of the PAR1-blocking antibody (Figure 3A). At concentrations >10 nmol/L, α-thrombin regained full activation of PAR4-dependent aggregation, even when RWJ-56110 was supplemented with the PAR1-blocking antibody plus bivalirudin. This result was somewhat surprising because bivalirudin is bifunctional and should also directly inhibit the active site of thrombin and prevent PAR4 cleavage (eg, competitive inhibition12). Alternatively, the binding of bivalirudin to exosite I and/or active site may result in noncompetitive inhibition (ES, EI, and ESI species) with substrates such as PAR4, which interact with the active site and not exosite I.11 To test these possibilities, we conducted kinetic studies of the inhibition of bivalirudin on thrombin cleavage of the active-site binding peptide (N Ac-Pro45-Ala-Pro-Arg48-pNA) from PAR4.12 We found that bivalirudin exhibited mixed noncompetitive inhibition with the PAR4 peptide (Figure II in the online-only Data Supplement). This indicates that 1 molecule of thrombin can simultaneously bind to the PAR4 cleavage sequence at the active site while bound to bivalirudin at exosite I. This result is also consistent with previous studies26 that demonstrated that bivalirudin is a noncompetitive inhibitor with the active site–binding ligand p-aminobenzamidine. Complexation of bivalirudin with thrombin occurred in several steps; the first one consisted of a rapid and tight association with exosite I, followed by slower and weaker intramolecular binding to the active site.26 However, if very high levels of bivalirudin are added, the noncompetitive kinetic model would predict that bivalirudin would prevent cleavage at the active site. Indeed, we did find that addition of higher amounts of bivalirudin (0.25 to 10 μmol/L) was able to completely inhibit 10 nmol/L α-thrombin activation of platelets (Figure 3B). To show that the inhibitory effects of bivalirudin on thrombin activation of platelets required exosite I of thrombin, we used P4pal-i1-thrombin, which has a defective exosite I. As shown in Figure 3A, bivalirudin had no effect on P4pal-i1-thrombin activation of platelets required exosite I of thrombin, we used γ-thrombin, which has a defective exosite I. As shown in Figure 3A, bivalirudin had no effect on γ-thrombin activation of platelets, confirming that the inhibitory effects of bivalirudin are dependent on its ability to interact with exosite I of thrombin.

We then tested whether blocking PAR4 with the P4pal-i1 pepducin was able to synergize with IC50 concentrations (200 nmol/L) of bivalirudin. Indeed, addition of P4pal-i1 to bivalirudin-treated platelets was able to greatly delay and inhibit the extent of aggregation even to very high thrombin.
concentrations (12 to 16 nmol/L) (Figure 3C). Collectively, these data are consistent with the model of Figure 1A, in which thrombin docked to the PAR1 Hir motif enhances activation of PAR4; however, at high enough thrombin concentrations, PAR4 will become activated by thrombin unless blocked downstream with the PAR4 pepducin or by a large molar excess of bivalirudin.

Effect of Combined Treatment of Bivalirudin, PAR1, and/or PAR4 Antagonists on Arterial Thrombosis in Guinea Pigs

We used a standard carotid artery injury model to assess the efficacy of simultaneous administration of bivalirudin and the PAR4 pepducin on arterial thrombosis in guinea pigs. Unlike mice, which lack PAR1 on their platelets, guinea pigs share functional similarity with human platelets and express both PAR1 and PAR4.6,28 Consistent with earlier results using hirudin,6 bivalirudin alone (0.18 mg/kg) prolonged the mean arterial occlusion time from 13 to 20 minutes (Figure 4A). P4pal-i1 (0.13 mg/kg) prolonged the occlusion time to a similar extent, although this was not significant. Strikingly, coadministration of bivalirudin plus P4pal-i1 caused a significant ($P<0.001$) protection against acute arterial occlusion compared with vehicle treatment (Figure 4A, 4B) and was also significantly better than bivalirudin alone ($P=0.027$). Therefore, additional blockade of PAR4 confers a benefit beyond that of inhibition of thrombin in this guinea pig carotid artery injury model. As was shown previously with RWJ-58259,6 blockade of PAR1 alone with the PAR1 pepducin P1pal-718,24 caused only partial protection of arterial thrombosis. In comparison with P4pal-i1, supplementation of P1pal-7 with bivalirudin gave no additional prolongation of the arterial occlusion time.

We then determined whether combined PAR1 plus PAR4 blockade in the absence of thrombin inhibition may be efficacious in preventing acute thrombosis. Whereas monotherapy with the PAR1 antagonist 0.5 mg/kg RWJ-56110 did not appreciably affect the mean occlusion time, dual blockade of PAR1 and PAR4 with RJW-56110 plus P4pal-i1 resulted in a highly significant protection against occlusion (Figure 4A). These data demonstrate for the first time that combined antagonism of PAR1 and PAR4 is effective in preventing

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Figure 3. Combination of bivalirudin plus a PAR4 pepducin blocks thrombin-dependent aggregation. A, Human platelets were preincubated for 2 minutes with buffer (untreated), bivalirudin (200 nmol/L), RWJ-56110 (1 μmol/L), and/or PAR1-Ab (74 μg/mL for 20 minutes) as indicated before the addition of 200 PM-300 nmol/L α-thrombin or γ-thrombin. B, Platelets were preincubated for 2 minutes with 4 nmol/L to 10 μmol/L bivalirudin before the addition of 10 nmol/L thrombin. C, Platelets were preincubated for 2 minutes with 200 nmol/L bivalirudin plus or minus 5 μmol/L P4pal-i1 before the addition of the indicated concentrations of thrombin (T).

Figure 4. Combination of bivalirudin plus a PAR4 pepducin or PAR1 and PAR4 blockade inhibits occlusion of carotid arteries in guinea pigs. A, Guinea pigs were treated with bivalirudin, P4pal-i1, P1pal-7, and/or RWJ-56110 for 5 minutes before injury of the carotid artery with FeCl3 as described in Methods. Probability values for each treatment group (n=3 to 6) relative to vehicle-treated group are indicated at the bottom. **$P<0.01$, ***$P<0.005$. B, Representative carotid artery occlusion tracings for vehicle vs bivalirudin plus P4pal-i1–treated guinea pigs from A.
PAR1 and PAR4 Form a Complex in Human Platelets

The assistance of PAR1 in thrombin activation of PAR4 on platelets suggests that PAR1 and PAR4 reside within close proximity on the platelet surface. We directly tested whether PAR1 and PAR4 might form a stable heterodimeric complex in human platelets. We first had to generate an antibody that would bind PAR4 on platelets. Polyclonal antibodies prepared against PAR4 exodomain epitopes G34-R47 and S41-C54 failed to recognize PAR4 on human platelets. To improve steric accessibility, we generated a polyclonal antibody that would react with the freshly cleaved PAR4 exodomain, residues G48YPGQVCANDSDTLELPD65 (GYP-Ab). As before, the GYP-Ab did not detect PAR4 from resting platelets; however, thrombin treatment of platelets produced a newly reactive broad band migrating at 32 to 38 kDa that was not present in untreated platelets (Figure 5A). Its heterogeneous appearance suggested that the putative thrombin-cleaved PAR4 species was differentially glycosylated. We treated the platelet lysates with PNGaseF to determine whether we could collapse the broad band to a single species. Treatment with PNGaseF yielded a homogenous band migrating at 29 kDa, consistent with removal of N-linked sugars from platelet PAR4. The 29-kDa species was 7 kDa smaller than expected for cleaved, nonglycosylated PAR4, as also occurs with PAR1.20,29

To confirm the identity of the various monomeric, dimeric, and glycosylated species of PAR4, we expressed a glycosylation-deficient mutant, T7-PAR4-N56S, in COS7 cells. Recombinant wild-type T7-PAR4 expressed in COS7 migrates as several species including 2 bands at 32 to 35 kDa, several bands around 60 to 64 kDa, and a band at 81 kDa (Figure 5B). Mutation of the sole N-linked N56DS58 glycosylation site generated PAR4 monomers that migrate at 32 to 35 kDa (NGM) and dimer that migrates at 70 kDa (NGD), similar to treatment of T7-PAR4 with PNGaseF (Figure 5B). Addition of PNGaseF did not further reduce the molecular mass of the N56S mutant, confirming that N56 is the sole site of N-linked glycosylation in PAR4. Cleavage of the N56S PAR4 species was more apparent on Western blots (Figure 5B). The N56S mutant migrated at a lower molecular mass than its wild-type counterpart, consistent with the absence of N-linked glycosylation (Figure 5B). The results of the experiments in Figure 5B suggest that PAR1 and PAR4 form a complex in human platelets.

Figure 5. PAR1 is physically associated with PAR4 in human platelets. A, D, Washed human platelets were treated with PBS or 100 nmol/L thrombin for 15 minutes at 37°C. Lysates from thrombin (T)-treated platelets were incubated with Sepharose beads covalently coupled to either rabbit IgG or the cleavage-sensitive PAR4-Ab (α-GYP). Platelet lysates and immunoprecipitation eluates were treated with PNGaseF overnight at 4°C. Platelet proteins were separated by 12% SDS-PAGE, and Western blot analysis was conducted with the use of the cleavage-sensitive PAR4-Ab, PAR1-Ab, or a β3-Ab against the α6β1 integrin subunit. B and C, COS7 fibroblasts were transiently transfected with WT T7-PAR1 or T7-N56S-PAR4. Lysates from transfected COS7 were treated with PBS or 100 nmol/L thrombin for 15 minutes at 37°C, and lysates were then incubated with PNGaseF for 60 minutes at 37°C. Proteins were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes, and Western analysis was conducted with the use of the T7-Ab or the cleavage-sensitive PAR4-Ab. GD indicates glycosylated dimer; NGD, nonglycosylated dimer; GM, glycosylated monomer; NGM, nonglycosylated or minimally glycosylated monomer; D, nonglycosylated dimer; M, nonglycosylated monomer; UC, uncleaved PAR4; and C, cleaved PAR4.
mutant with thrombin caused loss of the N-terminal T7 epitope (Figure 5B) but exposed the GYPGQV neoepitope of PAR4 (Figure 5C). The thrombin-cleaved N56S PAR4 nonglycosylated monomer (C) migrated at the identical molecular mass as PNGase/thrombin-treated PAR4 on human platelets (Figure 5A). Likewise, treatment of the PAR4 immunoprecipitate revealed monomeric and dimeric PAR4 as well as nonglycosylated monomer (Figure 6A, lanes 1, 3), and treatment with PNGaseF reduced the molecular mass of the bands to 35 and 65 kDa (data not shown). The 40- and 75-kDa PAR1 species therefore most likely represent monomeric and dimeric forms of the receptor, as previously described.29

To detect PAR1-PAR4 complexes in fibroblasts, we immunoprecipitated full-length (noncleaved) T7-PAR4 using agarose beads covalently coupled to a T7-Ab. After stringent washing with 1% Triton X-100 and 0.1% SDS detergents, PAR1 Western blots of the T7-PAR4 immunoprecipitate demonstrated abundant bound PAR1 (Figure 6A, lane 6). Conversely, FLAG-PAR1 was immunoprecipitated with the use of agarase-FLAG-Ab beads. PAR4(T7) Western blots of the PAR1 immunoprecipitate revealed monomeric and dimeric PAR4 as well as nonglycosylated monomer (Figure 6B, lane 6). Therefore, PAR1 and PAR4 stably associate as hetero-oligomers when expressed in recombinant form. Moreover, PAR4 does not need to be cleaved by thrombin to form stable complexes with PAR1.

As an independent method to detect PAR1-PAR4 complexes, we conducted FRET experiments in live cells using PAR1-CFP as the fluorescent donor and PAR4-YFP as the fluorescent acceptor. A FRET signal occurs when fluorescent donors and acceptors are in close molecular proximity (<100 Å). PAR1-CFP and PAR4-YFP signaling to Gq (InsP accumulation) was not impaired relative to the parent constructs (data not shown). As shown in Figure 6C, a FRET signal was observed when PAR1-CFP and PAR4-YFP were coexpressed in COS7 fibroblasts. A titration of increasing PAR4-YFP acceptor expression with the same level of PAR1-CFP donor was performed. The signal was saturable, indicating that the FRET occurring between PAR1 and PAR4 was a consequence of specific binding rather than the result of random collisions (Figure IIIA in the online-only Data Supplement). By comparison, no FRET signal occurred when PAR1-CFP

Figure 6. PAR1 and PAR4 form a stable complex in transfected COS7 fibroblasts. A, COS7 fibroblasts were transiently transfected with WT PAR1 and/or T7-PAR4. Lysates from transfected COS7 were incubated with T7-agarose beads, proteins in lysates and immunoprecipitation eluates were separated by 10% SDS-PAGE, and Western blot analysis was performed. B, COS7 fibroblasts were transiently transfected with Flag-PAR1, T7-PAR4, or both. Lysates were prepared from transfected COS7 and incubated with agarose beads coupled to Flag (M2)-Ab. Proteins in lysates and immunoprecipitation eluates were separated by 10% SDS-PAGE, and Western blot analysis was performed. C, FRET between PAR1Δ377-CFP and PAR4Δ346-YFP was determined with the use of COS7 cells expressing both receptors expressed together, whereas the control "separate cells FRET" was determined with the use of a mixture of cells expressing PAR1Δ377-CFP and PAR4Δ348-YFP individually. Fluorescence measurements were conducted with transfected cells at a concentration of 0.5 × 10⁶ cells/mL with excitation at 425- and 10-nm slit widths.

PAR1 and PAR4 Form Stable Complexes in COS7 Fibroblasts

Next, we tested whether PAR1 and PAR4 form complexes when coexpressed in COS7 fibroblasts. Recombinant PAR1 appears as 2 clusters of bands centered at 40 and 75 kDa (Figure 6A, lanes 1, 3), and treatment with PNGaseF reduced the molecular mass of the bands to 35 and 65 kDa (data not shown). The 40- and 75-kDa PAR1 species therefore most likely represent monomeric and dimeric forms of the receptor, as previously described.29

To detect PAR1-PAR4 complexes on platelets, we immunoprecipitated PAR4 using Sepharose beads covalently coupled to the cleavage-sensitive PAR4-Ab. The PAR4-affinity beads enriched the same 32- to 38-kDa glycosylated species as had been observed in lysates of thrombin-treated human platelets (Figure 5A). Likewise, treatment of the PAR4 immunoprecipitate with PNGaseF produced the nonglycosylated PAR4 band as well. PAR4 was not detected in the PAR1 immunoprecipitate (Figure 6D). These results demonstrate for the first time that PAR1 and PAR4 associate as a stable heterodimeric complex in human platelets.
and PAR4-YFP were expressed on separate cells that were subsequently mixed together (Figure 6C). Therefore, a PAR1-PAR4 FRET signal could only be elicited when the 2 protease receptors were expressed on the same cell membrane. Confocal FRET microscopy was then used to determine whether the FRET signal was arising from the plasma membrane or intracellular membranes of transfected COS7 cells. We found that a significant PAR1-PAR4 FRET signal emanated from the plasma membrane with a smaller FRET signal from intracellular compartments (Figure IIIB in the online-only Data Supplement).

Coexpression of PAR1 with PAR4 Enhances PAR4 Cleavage and Activation

We determined whether coexpression of PAR1 with PAR4 enhanced thrombin cleavage and signaling of PAR4. We used the proteolytically dead PAR1 F43A, which is defective in ligand residue Phe-43.22-24 PAR1 F43A stimulated ≤5% InsP activity on thrombin treatment but still fully signaled on addition of exogenous peptide ligand, TFLLRN (Figure 7A). We expressed PAR1 F43A in the PAR1-null cell line, SW620, which contains endogenous PAR4 (Figure 1D). SW620 cell lines stably transfected with either the PAR1 F43A mutant (A42) or vector control (A21) were isolated. Both cell lines had equal levels of PAR4 expression and produced nearly identical Ca2+ responses to the PAR4 ligand AYPGKF (Figure 7B). The PAR1 F43A-expressing cell line A42 gave a typical spike calcium response upon addition of the PAR1 agonist TFLLRN, whereas the control cell line A21 was unresponsive to TFLLRN because of the lack of endogenous PAR1 (data not shown). Strikingly, the PAR4 thrombin response was enhanced in the A42 cell line, which harbored the PAR1 F43A mutant. As shown in Figure 7B, thrombin gave a much more robust Ca2+ response (314%) in the A42 cells compared with the A21 cells that expressed PAR4 alone.

The initial rates of calcium mobilization at early times (0 to 1 minute) were obtained over a full range of thrombin concentrations for the A42 versus the A21 cell lines. Similar to the results in human platelets, the thrombin EC50 for PAR4-dependent Ca2+ flux was shifted by 2.9-fold in the presence of coexpressed PAR1 F43A (Figure 7C) but not in a negative control cell line with coexpressed CCR5 (data not shown). This enhanced signaling capacity was not due to ectopic expression of wild-type PAR1 or spurious activation of PAR1 F43A because addition of the PAR1 antagonist RWJ-56110 did not attenuate the heightened thrombin response (data not shown). Furthermore, in the presence of PAR1, the EC50 for thrombin cleavage of PAR4 was enhanced from 5.2 to 2.6 nmol/L (Figure 7D). This helper effect by PAR1 is nearly identical to that observed in human
platelets. Moreover, these data demonstrate that PAR1 is able to promote the cleavage and activation of PAR4 in cells other than human platelets.

**Discussion**

The present study shows that PAR1 acts as a cofactor for thrombin activation of PAR4 on human platelets and other cells and provides a mechanistic basis to understand PAR1-PAR4 synergy. By selectively ablating the PAR1 signal with a potent inhibitor of the PAR1 tethered ligand, we determined that PAR4 is activated at surprisingly low concentrations of thrombin on human platelets. By inhibiting the ability of thrombin to associate with PAR1, we show that PAR1 plays a critical helper function in assisting PAR4 activation by thrombin. A cleavage-sensitive PAR4-Ab was used to demonstrate that PAR1 and PAR4 exist as a complex on human platelets. Stable hetero-oligomerization between PAR1 and PAR4 was also observed in recombinant systems and did not require prior cleavage by thrombin.

The present work supports earlier observations with PAR1 and PAR4 pepducin antagonists and blocking antibodies that targeting only PAR1 and not PAR4 may have a partial therapeutic effect. Thus, a combination of a PAR1 small-molecule antagonist, RWJ-56110, plus a PAR4 pepducin, P4pal-i1, was highly effective in preventing acute occlusion of carotid arteries in guinea pigs. Blockade with direct PAR1 and PAR4 antagonists would have the advantage of preventing thrombin platelet aggregation without inhibiting the ability of thrombin to cleave fibrinogen and thus potentially avoiding bleeding complications.

As an alternative therapeutic strategy, preventing the interaction of the PAR1 Hir motif with thrombin would have the dual benefit of directly inhibiting PAR1 and indirectly inhibiting PAR4. In this regard, we show that the widely used antithrombotic agent bivalirudin was efficacious in blocking thrombin activation of both PAR1- and PAR4-dependent platelet aggregation. In combination with a PAR4 pepducin but not a PAR1 pepducin, bivalirudin was able to prevent acute arterial thrombosis in guinea pigs. In a baboon arteriovenous shunt model, bivalirudin (0.2 mg/kg per minute infusion) inhibited platelet thrombus deposition onto thrombogenic surfaces by 50%, providing indirect evidence that bivalirudin may also block thrombin receptor activation in primates. It will be interesting to determine whether bivalirudin, at the currently used dosage (0.75 mg/kg IV, 4-hour 1.75 mg/kg per hour infusion) for percutaneous coronary intervention, blocks PAR1 and PAR4 to any appreciable extent in patients with acute coronary syndromes. PAR4 responses have been shown to vary greatly in patients with acute coronary syndromes undergoing percutaneous coronary intervention. It is conceivable that this could be due to different PAR4 expression levels or the extent of interaction of PAR4 with PAR1 or other thrombin-binding accessory molecules such as GPb-V-IX. Surplus PAR4 may not exist in complex with PAR1 and would not be expected to be blocked by agents that prevent interactions of thrombin with PAR1. Therefore, the combination of bivalirudin plus a PAR4 antagonist such as the P4pal-i1 or P4pal-10 pepducin might provide efficient blockade of thrombin-mediated platelet activation in diverse populations of patients being treated for acute coronary syndromes.

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**Disclosures**

None.

**References**


Blocking the Protease-Activated Receptor 1-4 Heterodimer in Platelet-Mediated Thrombosis

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