Redundancy and Interaction of Thrombin- and Collagen-Mediated Platelet Activation in Tail Bleeding and Carotid Thrombosis in Mice

Yamini S. Bynagari-Settipalli,* Ivo Cornelissen,* Daniel Palmer, Daniel Duong, Cherry Concengco, Jerry Ware, Shaun R. Coughlin

Objective—Current antiplatelet strategies to prevent myocardial infarction and stroke are limited by bleeding risk. A better understanding of the roles of distinct platelet-activating pathways is needed. We determined whether platelet activation by 2 key primary activators, thrombin and collagen, plays distinct, redundant, or interacting roles in tail bleeding and carotid thrombosis in mice.

Approach and Results—Platelets from mice deficient for the thrombin receptor protease-activated receptor-4 (Par4) and the collagen receptor glycoprotein VI protein (GPVI) lack responses to thrombin and collagen, respectively. We examined tail bleeding and FeCl₃-induced carotid artery occlusion in mice lacking Par4, GPVI, or both. We also examined a series of Par mutants with increasing impairment of thrombin signaling in platelets. Ablation of thrombin signaling alone by Par4 deficiency increased blood loss in the tail bleeding assay and impaired occlusive thrombus formation in the carotid occlusion assay. GPVI deficiency alone had no effect. Superimposing GPVI deficiency on Par4 deficiency markedly increased effect size in both assays. In contrast to complete ablation of thrombin signaling, 9- and 19-fold increases in EC₅₀ for thrombin-induced platelet activation had only modest effects.

Conclusions—The observation that loss of Par4 uncovered large effects of GPVI deficiency implies that Par4 and GPVI made independent, partially redundant contributions to occlusive thrombus formation in the carotid and to hemostatic clot formation in the tail under the experimental conditions examined. At face value, these results suggest that thrombin- and collagen-induced platelet activation can play partially redundant roles, despite important differences in how these agonists are made available to platelets. (Arterioscler Thromb Vasc Biol. 2014;34:2563-2569.)

Key Words: collagen • glycoprotein VI • hemostasis • mouse assays • platelets • protease-activated receptor-3 • protease-activated receptor-4 • thrombin • thrombosis

Platelet-dependent thrombi are a major cause of myocardial infarction and stroke.¹⁻⁴ The efficacy and use of established antiplatelet agents for preventing these events is limited by bleeding risk;²⁻⁹ Improved understanding of the relative importance and interactions of different pathways of platelet activation in hemostasis and thrombosis is needed to determine whether and how these processes can be better separated.

Thrombin and collagen are potent activators of platelets ex vivo.¹⁰⁻¹² Platelet activation by thrombin generated at sites of vascular injury and by vessel wall collagen exposed at such sites contributes to hemostasis and thrombosis in vivo. The extent to which these primary platelet activators serve independent, redundant, or interacting roles in vivo has not been directly examined.

Thrombin is arguably the most potent platelet activator.¹²,¹³ Mouse platelets use protease-activated receptor-3 (Par3) and Par4 (gene names F2rl3 and F2rl2, a.k.a. Par3 and Par4, respectively) to sense and respond to thrombin.¹¹,¹⁴ Mouse Par3 seems incapable of signaling by itself and instead serves as a thrombin-binding cofactor that promotes cleavage and activation of Par4 at low concentrations of thrombin.¹⁵ In accord, platelets from Par3-deficient mice require more thrombin than wild-type platelets for normal activation, and platelets from Par4-deficient mice fail to respond to even micromolar thrombin.¹¹,¹⁴ In the FeCl₃-induced carotid artery thrombosis assay, Par4-deficient mice are protected against thrombotic occlusion triggered by application of 250 mmol/L (4%) FeCl₃, compared with wild-type, but application of 1.25 mol/L (20%)...
FeCl₃, reliably causes thrombotic occlusion of carotid arteries in Par4-deficient mice (albeit more slowly than in wild-type). Par4-deficient mice also show increased blood loss in a tail bleeding assay compared with wild-type, but hemostasis is still reliably achieved and blood loss is modest compared with the maximal that can occur in this assay. Thus, mechanisms independent of thrombin-induced platelet activation are sufficient to drive formation of occlusive thrombi in the carotid and hemostatic clots in the tail.

Like thrombin, collagen is an effective primary activator of platelets. Collagen activates platelets initially by binding to platelet glycoprotein VI (GPVI, gene name Gp6), and platelets from GPVI-deficient mice fail to respond to collagen.

We used mice with different Par3 and Par4 genotypes to probe the effects of different levels of thrombin signaling in platelets as well as mice that lack thrombin signaling in platelets (Par4−/−). Collagen signaling in platelets (Gp6−/−), or both to determine whether thrombin- and collagen-induced platelet activation serve independent, redundant, or interacting roles in the tail bleeding and FeCl₃-induced carotid occlusion assays in mice. Under the moderate-to-high injury conditions explored, partial inhibition of thrombin signaling alone had no or modest effects, but complete ablation of thrombin signaling decreased thrombus formation in both assays even when GPVI was intact. By contrast, ablation of collagen signaling had no effect on tail bleeding or carotid occlusion when thrombin signaling was intact but had large effects when thrombin signaling was absent. These results suggest that thrombin- and collagen-induced platelet activation can independently support thrombus formation in these assays and that their contributions are partially redundant such that removal of both pathways produces outsized effects. Such redundancy is perhaps surprising given the different mechanisms by which these ligands become available to platelets. Implications for hypotheses regarding therapeutic strategies are discussed.

Materials and Methods

Results

Our previous studies revealed that subtle impairment of thrombin signaling in Par3−/− platelets protects against carotid artery thrombosis triggered by low (250 mmol/L; 4%) FeCl₃ without significantly increasing blood loss in the tail bleeding assay used here. To further explore the effect of different levels of impairment of thrombin-induced platelet activation and toward establishing conditions in which interactions with GPVI deficiency might be detected, we first characterized the concentration response to thrombin in platelets from mice homozygous or heterozygous for Par3 and Par4 alone and in combination as assessed by thrombin-induced P-selectin display measured by flow cytometry (Figure 1). EC₅₀ for thrombin-induced P-selectin display in platelets from wild-type, Par3+/+, Par4−/−, Par3−/−:Par4+/+, and Par3−/−:Par4−/− mice were 0.1, 0.3, 0.9, and 1.9 nmol/L, respectively (Figure 1A). Similar results were found when α₁β₃ activation was used as an end point (Figure 1B) and maximal α₁β₃ activation to thrombin was ≈20% decreased in Par3−/−:Par4−/− platelets (Figure 1C). Par4−/− platelets showed no responses to even 1000 nmol/L thrombin as previously described. Par3−/−:Par4+/+ mice were of special interest because, in addition to the 20% decrease in maximal response, platelets from these mice showed a 19-fold increase in the EC₅₀ for thrombin over wild-type, about twice the effect of isolated Par3 deficiency and in a range of partial impairment of thrombin signaling not previously explored. Accordingly, we next assessed the effect of these varying levels of loss of platelet responsiveness to thrombin in vivo.

In the tail bleeding assay (Figure 2), blood loss was increased ≈6-fold in Par4−/− mice compared with wild-type (≈0.1–0.6 AU), and this increase was independent of Par3 genotype as expected (Par4 deficiency alone is sufficient to ablate thrombin signaling). By contrast, neither Par3−/−:Par4+/+ nor Par3−/−:Par4−/− mice showed a statistically significant increase in tail bleeding compared with wild-type (Figure 2); the point estimate for mean hemoglobin loss was 0.2 AU in

Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<td>Gp6</td>
<td>glycoprotein VI gene</td>
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<tr>
<td>GPVI</td>
<td>glycoprotein VI protein</td>
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<td>Par</td>
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Par4 ≈ 80% of carotids never occluded (median ≈ 250 seconds after injury, whereas in otherwise wild-type background in which other platelet-activating pathways are intact, increasing the EC50 for thrombin-induced platelet activation from 0.1 nmol/L for wild-type to 0.9 nmol/L for Par3−/− or to 1.9 nmol/L for Par3−/−:Par4+/+− platelets produced detectable protection in the carotid occlusion assay. These changes in signaling did not produce statistically significant increases in tail bleeding, but point estimates suggested a positive trend. Ablation of thrombin signaling in Par4−/− mice in an otherwise wild-type background conferred more substantial protection in the carotid occlusion assay and increased blood loss in the tail bleeding assay.

We next examined the relative importance of GPVI in the same assays and its interactions with thrombin-induced platelet activation using mice homozygous and heterozygous for Gp6 and Par4 null alleles, alone and in combination. In contrast to the case with alterations in thrombin signaling via PARs, even ablation of collagen signaling via GPVI produced no detectable effect in an otherwise wild-type background.

In the tail bleeding assay (Figure 4), isolated Par4 deficiency caused an increase in hemoglobin loss from 0.1 AU
in *Gp6*+/+:*Par4*+/+ mice to 0.7 AU in *Gp6*+/+:*Par4*−/− mice, consistent with the previous experiment (Figure 2). In contrast to Par4 deficiency, GPVI deficiency in a *Par4*+/+ or *Par4*−/− background did not cause increased blood loss. Strikingly, however, superimposing GPVI deficiency on Par4 deficiency resulted in a marked increase in blood loss over and above that associated with Par4 deficiency alone. Mean hemoglobin loss in *Gp6*−/−:*Par4*−/− mice was 3.8 AU, corresponding to ≈15% of total blood volume and ≈5-fold the hemoglobin loss in *Gp6*+/+:*Par4*−/−. *Gp6* heterozygosity also had a significant effect in a *Par4*−/− background, with mean hemoglobin loss increasing from 0.7 AU in *Gp6*+/+:*Par4*−/− mice to 1.8 AU in *Gp6*+/+:*Par4*−/− mice.

In the carotid artery thrombosis assay, we showed that even at a low level of injury (0.25 mol/L (4%) FeCl₃), GPVI deficiency alone provided no protection against thrombosis, whereas under the same conditions, Par4 deficiency alone provided complete protection (not shown). These results are consistent with at least some published studies (see Discussion), in which Par4 and GPVI mutants were studied separately.16–18,26,29 Thus, in contrast to thrombin signaling, GPVI signaling was unnecessary for normal thrombus formation when other platelet signaling pathways were left intact.

To provide a system in which interactions of the kind found in the tail bleeding assay could be detected, we increased the concentration of FeCl₃ to a level at which *Par4*−/− mice were only partially protected (Figure 5). As expected, GPVI deficiency alone had no effect on the rate or frequency of occlusive thrombus formation in carotid arteries after injury with 1.25 mol/L (20%) FeCl₃; median time to occlusion was ≈200 s for wild-type, *Gp6*+/+ and *Gp6*−/− mice, and all arteries were occluded by ≈300 s (Figure 5A). Par4 deficiency alone caused a statistically significant delay in occlusion even at this level of injury (Figure 5B, curve A); median time to occlusion was ≈350 s in *Gp6*+/+:*Par4*−/− mice compared with ≈200 s in wild-type and in *Gp6*−/−:*Par4*+/+ and *Gp6*−/−:*Par4*+/− mice. Also in contrast to these genotypes, *≈10%* of vessels in Par4−/− mice remained open to 1200 s, the end of the protocol (Figure 5B, curve A).

Although GPVI deficiency alone had no effect in the thrombosis assay, superimposing GPVI deficiency on Par4 deficiency had a major effect. Compared with 10% in *Gp6*+/+:*Par4*−/− mice, ≈70% of vessels in *Gp6*−/−:*Par4*−/− remained open through the end of the protocol such that median time to occlusion was indeterminate (>1200 s; Figure 5B, curve E). Median time to occlusion in *Gp6*−/−:*Par4*−/− mice was intermediate between *Gp6*+/+:*Par4*−/− and *Gp6*−/−:*Par4*−/− mice (Figure 5B, curve B).

The results described above suggest that collagen-induced platelet activation via GPVI is not necessary for normal clot formation in the tail bleeding assay nor for occlusive thrombus formation in the carotid occlusion assay when thrombin signaling is intact. By contrast, thrombin-induced platelet activation is necessary for wild-type levels of hemostatic clot and arterial thrombus formation in these assays even when collagen signaling is intact. The fact that combined GPVI and Par4 deficiency had large effects compared with either deficiency alone suggests that collagen- and thrombin-induced platelet activation each make independent contributions that serve partially redundant functions in these assays.

**Discussion**

We report a comparison of the effects of ablating or reducing thrombin-induced platelet activation and ablating collagen-induced platelet activation, alone and in combination, on tail bleeding and thrombotic carotid occlusion in mice. A lack of an effect of isolated GPVI deficiency in these assays under conditions in which even modest changes in thrombin signaling (eg, Par3 deficiency) produced detectable effects suggests that, under the conditions examined, collagen-induced platelet activation via GPVI is not necessary for normal hemostasis in the tail bleeding assay nor for normal formation of occlusive thrombi in the carotid occlusion assay when thrombin signaling is intact. By contrast, the clear (albeit less than maximal) effect of isolated Par4 deficiency in the same assays suggests that thrombin-induced platelet activation is necessary for normal thrombus formation in these assays even when collagen signaling is intact. The large increase in bleeding and the marked decrease in carotid occlusion associated with combined GPVI and Par4 deficiency compared with either single deficiency suggests that collagen- and thrombin-induced platelet activation make substantially redundant contributions to thrombus formation in these assays, such that either pathway becomes critical in the absence of the other.

Previous studies have reported variable effects of loss of GPVI function in tail bleeding and carotid occlusion assays.10,18–29,33 Some variability might be because of different means of achieving GPVI loss of function, for example, Fcγ mutation and antibody-induced GPVI shedding might have effects beyond loss of GPVI signaling. Variability across studies of *Gp6* nulls may also be due in part to different genetic backgrounds.28 The *Gp6* and *Par4* mutant mice examined in this study had been backcrossed >7 times into a C57BL6 background, and littermates were compared. Different experimental conditions may also contribute to variability across studies.
in the literature. Indeed, the sensitivity of the tail bleeding and carotid occlusion assays can be tuned by varying the site of transection and concentration of FeCl₃ used, respectively.³⁷,³⁸ In the present study, assay conditions in which isolated GPVI or Par₄ deficiency had a modest effect were chosen to enable detection of additive or synergistic effects of combined deficiencies. Under conditions in which isolated Par₄ deficiency had a modest effect, isolated GPVI deficiency had no detectable effect. It is certainly possible that different results would have been observed under different experimental conditions or in a different genetic background.

Large effects of superimposing anticoagulation on defective collagen-induced platelet activation in tail bleeding, FeCl₃-induced carotid occlusion and other thrombosis assays have been described previously.²⁵,²⁸ Anticoagulants inhibit both fibrin formation and thrombin-induced platelet activation, and the effects of anticoagulation in these studies might be because of either or both. Indeed, combined loss of fibrin formation and thrombin-induced platelet activation in mice is incompatible with hemostasis and survival, whereas loss of either function alone has no or modest effects by comparison.³⁵ The current study focused specifically on platelet activation, using deficiencies of the receptors required for initial activation of mouse platelets by collagen and thrombin to directly probe the roles and interactions of platelet activation by these mechanisms. Expression of these receptors is relatively restricted to platelets, and effects of their deficiency in hemostasis and thrombosis assays can almost certainly be attributed to altered platelet function.²⁶,³⁷

Finding that collagen- and thrombin-induced platelet activation can have substantially redundant roles in thrombus formation is perhaps surprising, given that collagen is a preformed insoluble polymer that presumably can activate only those platelets that touch it at the damaged vessel wall, whereas thrombin is locally generated soluble mediator that can act on platelets in the growing thrombus.³¹ However, collagen signaling via GPVI does trigger release of soluble mediators like adenosine diphosphate (ADP) and thromboxane that can sustain and extend platelet activation in time and space,¹⁰ perhaps allowing collagen-induced platelet activation to partially mimic the ability of thrombin-induced platelet activation to drive growth of platelet thrombi at a distance from the vessel wall. Given the known dependence of collagen-induced platelet aggregation on ADP,¹⁰ the past observation that combined ablation of thrombin signaling via Par₄ and ADP signaling via P2Y₁₂ had generally additive effects in the assays used herein¹⁷ is consistent with this view.

Although combined loss of thrombin- and collagen-induced platelet activation was associated with remarkable protection against FeCl₃-induced carotid artery occlusion, 30% of vessels still clotted. Thrombus formation in these vessels might be driven by fibrin formation and by platelet-activating pathways independent of thrombin and collagen signaling.

At face value, the observation that superimposition of GPVI deficiency on Par₄ deficiency has large effects in the FeCl₃-induced carotid occlusion assay suggests that ligation of platelet GPVI by collagen occurs in this assay. A recent report suggested that relatively little subendothelial collagen is exposed by FeCl₃-induced carotid injury as assessed by scanning electron microscopy.³⁸ Whether collagen exposure sufficient to trigger an important level of GPVI-dependent platelet activation went undetected by SEM or whether GPVI can contribute to thrombus formation by collagen-independent mechanisms is not known. However, there is no need to invoke the latter possibility to explain the observation that superimposition of GPVI deficiency on Par₄ deficiency has large effects in the tail bleeding assay, in which mechanical disruption of vascular integrity likely provides both substantial collagen exposure and thrombin generation.

In the studies outlined above, we failed to separate effects on tail bleeding versus carotid occlusion by ablating collagen signaling, thrombin signaling, or both. Our past studies suggested that Par₃ deficiency, which produces a relatively subtle decrease in platelet responsiveness to thrombin, had no effect on tail bleeding under the conditions used here but protected against carotid occlusion at a low level of FeCl₃ injury, whereas Par₄ deficiency, which ablates thrombin signaling in platelets, increased both bleeding and protection.¹⁷,³⁹
By combining Par3 deficiency with Par4 heterozygosity, we obtained \textit{Par3}\textsuperscript{−/−};\textit{Par4}\textsuperscript{+/−} mice that provided a unique opportunity to probe the effect of a stable, \textit{=2}-fold further increase in the EC\textsubscript{50} for thrombin-induced platelet activation compared with \textit{Par3}\textsuperscript{−/−} mice. Like \textit{Par3}\textsuperscript{−/−} mice, \textit{Par3}\textsuperscript{−/−};\textit{Par4}\textsuperscript{+/−} mice showed protection against thrombosis without a statistically significant increase in bleeding compared with wild-type mice. This result is interesting as a point of contrast, in that even a partial decrease in thrombin signaling in platelets is associated with decreased carotid occlusion under conditions in which complete loss of collagen-induced responses has no detectable effect. Doubling the EC\textsubscript{50} for thrombin-induced platelet activation from 0.9 in \textit{Par3}\textsuperscript{−/−} mice to 1.9 nmol/L in \textit{Par3}\textsuperscript{−/−};\textit{Par4}\textsuperscript{+/−} did not produce a statistically significant increase in protection against carotid occlusion nor in tail bleeding, but point estimates suggested a trend in both assays. This result is perhaps not surprising, and limited power and small effect size may have prevented detection of real but modest effects of doubling the EC\textsubscript{50} for thrombin-induced platelet activation.

Important caveats must be weighed in considering whether hypotheses generated from these mouse studies might be relevant to human hemostasis and thrombosis. First, there are important species differences in PAR utilization between human and mouse platelets. As noted in the introduction, mouse platelets use Par3 and Par4, and rather than serving as an independent transmembrane signaling molecule, mouse Par3 functions as a cofactor that binds thrombin and promotes Par4 activation at low thrombin concentrations. By contrast, human platelets use PAR1 and PAR4 and these receptors signal independently; PAR1 is necessary for human platelet activation by low concentrations of thrombin and PAR4 contributes at higher concentrations. These and other species differences prevent direct extrapolation of studies of Par mutant mice to humans. Second, FeCl\textsubscript{3}-induced carotid occlusion and tail bleeding assays in mice do not recapitulate the complexity of human thrombosis and hemostasis. Although FeCl\textsubscript{3}-induced carotid occlusion in mice provides a tractable assay of thrombotic occlusion in an artery, it lacks important aspects of human pathophysiology, such as stenosis and exposure of atheromatous plaque contents after erosion or rupture. Similarly, although mouse tail bleeding provides an assay of hemostatic clot formation after transection of blood vessels under one set of conditions, it does not represent the different tissues, blood vessel and injury types, flow rates, pressures, and varying levels of tamponade involved in human hemostasis. Importantly, the tail bleeding and most other bleeding assays measure procedure-induced bleeding from normal vessels; bleeding that causes morbidity and mortality in humans is often spontaneous and may occur at sites of abnormal vascularisation (eg, neovessels, inflammation, vascular malformations, etc.), in which the relative importance of different contributors to clot formation may differ from normal vessels.

Notwithstanding the important caveats listed above, it is worth noting that in an absolute sense, the ability of inhibition of a pathway to increase tail bleeding or decrease carotid occlusion in mice has correlated with an ability to impair hemostasis and protect against thrombosis in humans.\textsuperscript{7,8,14,16,17,28,35,39,41–43} PARs mediate platelet activation by thrombin in mice and humans and, at least in some settings, can contribute to hemostasis and thrombosis in both species.\textsuperscript{11,15–17,35,40,44} GPVI mediates initial platelet activation by collagen in mice and humans and, at least in some settings, can contribute to hemostasis in both species.\textsuperscript{16–29,45} The role for GPVI in human thrombosis remains to be tested. New antiplatelet agents are often tested as an addition to standard of care, and use of antiplatelet agents in combination is growing.\textsuperscript{2,4–7,44} The findings of substantially redundant functions for protease-activated receptor- and GPVI-dependent platelet activation in tail bleeding and carotid occlusion assays in mice and of strong interactive effects of removing both functions might be considered in the design of future studies exploring these targets for antithrombotic therapy.

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Disclosures

None.

References


**Significance**

We demonstrated that thrombin- and collagen-dependent platelet activation via protease-activated receptors and glycoprotein VI protein have substantially redundant roles in mouse assays of hemostasis and thrombosis, such that removing both pathways had much larger effects than removing either alone. This redundancy is somewhat surprising given spatial and other differences in the way that thrombin and collagen become available to platelets in a growing thrombus. Testing of antplatelet agents is often accomplished in combination with established ones. The strong interactive effects seen with loss of protease-activated receptor and glycoprotein VI function in the *in vivo* hemostasis and thrombosis assays used here might be considered in further exploration of these receptors as targets for antithrombotic therapy.
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Materials and Methods

The Institutional Animal Care and Use Committee of University of California San Francisco approved these studies.

Materials

Par3 and Par4 knockout mice\(^1,2\) and Gp6 knockout mice\(^3\) were bred at least 7 generations into a C57BL6 background. Tail bleeding studies were performed on littermate offspring from crosses capable of generating offspring of different genotypes before genotyping was performed, and thrombosis assays were performed blind to genotype. Anti-CD62 (P-selectin) (Wug.E9 clone) and anti-\(\alpha_{IIb}\beta_3\) (JON/A clone) antibodies utilized in platelet activation studies were from Emfret analytics (Eibelstadt, Germany). The concentrations of ferric chloride (FeCl\(_3\)) solutions are specified by both molarity and % (weight per volume of anhydrous FeCl\(_3\)) for convenience in making comparisons across studies that utilize both nomenclatures.

Methods

Preparation of washed mouse platelets. Murine platelets were isolated as described previously\(^4\). Briefly, mice were anesthetized with ketamine (75 mg/kg), xylazine (15 mg/kg), and acepromazine (2.5 mg/kg) and blood from the inferior vena cava was withdrawn into a syringe containing 150 µL acid citrate dextrose (ACD) and mixed gently. Blood samples were further mixed with 500 µL PIPES (20 mM)-buffered saline (pH 6.5), and centrifuged at 100 × g for 20 minutes at 37°C without braking. The platelet-rich supernatant was removed and added to 500 µL low pH platelet wash buffer \[140 mM NaCl, 10 mM NaHCO\(_3\), 2.5 mM KCl, 0.34 Na\(_2\)HPO\(_4\), 1.0 mM MgCl\(_2\), 6.46 mM trisodium citrate, 0.1% weight/volume (w/v) dextrose, 0.35% w/v bovine serum albumin (BSA; Fraction V), pH 6.5\] supplemented with 5 U/mL Apyrase (Grade III). Platelets were pelleted by centrifugation at 500×g for 15 minutes with no braking. The platelets were resuspended in 400 µL calcium-free Tyrode’s HEPES (CFTH) buffer \(134 \text{ mM NaCl, } 12 \text{ mM NaHCO}_3, 2.9 \text{ mM KCl, } 0.34 \text{ mM Na}_2\text{HPO}_4, 1.0 \text{ mM MgCl}_2, 10 \text{ mM HEPES, } 0.9\% \text{ w/v dextrose, } 0.35\% \text{ w/v BSA, pH 7.4}\).  

Ex vivo platelet activation studies. Platelet activation was assessed by measuring agonist-induced P-selectin exposure and integrin \(\alpha_{IIb}\beta_3\) activation using flow cytometry\(^4\). Briefly, 23 µL of washed platelets at 40,000 platelets/µL concentration suspended in CFTH recalculated with 2 mM CaCl\(_2\) was used in assay. 1µL of Thrombin to a final concentration of 1pM to 1 µM together with 1µL each of FITC-conjugated anti-CD62 (P-selectin) antibody and PE-conjugated anti-\(\alpha_{IIb}\beta_3\) antibody was added to platelets. 15 minutes later, samples were diluted by adding 400 µL PBS and platelet-bound antibody was measured using C6 Accuri flow cytometer. 20,000 events with forward scatter channel threshold of ≤ 32,000 were analyzed for each sample. Mean fluorescence intensity for each antibody was plotted as a function of agonist concentration and EC\(_{50}\) values were calculated from those dose-response curves. Platelets from individual mice
were prepared and analyzed separately. Results reflect an average of 3 or more mice per genotype.

**In vivo tail bleeding assay.** Hemostasis in mice was assessed by measuring the amount of blood loss after transection of the tail tip. Briefly, 25-31 day-old mice were anesthetized with ketamine (100 mg/kg)/xylazine (10 mg/kg) mixture and maintained on heating pads. The tail was transected 2 mm from the tip and immediately immersed in 37 °C saline in a clear test tube for 10 minutes. Tails still bleeding at this time were cauterized and mice were allowed to recover from anesthesia. Blood cells collected in above test tubes were pelleted by centrifugation at 250 × g for 15 min and the re-suspended in 3 mL erythrocyte lysis buffer (8.3 g/L NH4Cl, 1.0 g/L KHCO3, and 0.037 g/L EDTA in water). Blood loss was determined by measuring Optical Density at 575 nM using a SPECTRAmax190 (Molecular Devices) and is expressed as arbitrary units (AU). In a standard curve constructed by spiking collection tubes with a known volume of blood, OD varied linearly with the volume of blood added over the range of the assay. The maximum blood loss observed in the assay resulted in an OD of 3.8 AU corresponded to 215 µL of whole blood or about 15% of blood volume. Tail tips were used for genotyping.

**In vivo carotid thrombosis assay.** Carotid thrombosis studies were performed as described. Briefly, 8- to 14-wk-old mice were anesthetized with ketamine (75 mg/kg), xylazine (15 mg/kg), and acepromazine (2.5 mg/kg). The left carotid artery was isolated and blood flow was continuously monitored using a perivascular flow probe (MA0.5PSB; Transonic Systems Inc.) connected to a TS420 flow meter (Transonic Systems Inc.) connected to an Adinstruments Powerlab 4/30 and Chart software. The surgical field was blotted dry and two 1 mm × 2 mm pieces of Whatman #3 filter paper soaked in 0.5 M (8%) or 1.25 M (20%) FeCl3 were applied to the artery (one above and one below) for 3 min or 5 min respectively. Filter paper was then removed and the surgical field was rinsed twice with saline to remove remaining FeCl3 solution. Flow was measured for the next 20 minutes. Time from removal of the FeCl3 paper to stable occlusion (defined as no flow for > 2 minutes) was recorded for each artery. Results are expressed as percent of arteries with continued blood flow as a function of time. For the purpose of calculating median time to occlusion, arteries that did not occlude after application of 0.5 M (8%) or 1.25 M (20%) FeCl3 injury were assigned occlusion times of 1200 seconds, the specified end of the protocol.

**Statistical analysis.** Data from carotid occlusion assay in Figures 3 and 5 were analyzed using log-rank test; the p-value for significance of follow-on individual comparisons was adjusted using Bonferroni's method. All other data were analyzed using two-way analysis of variance (ANOVA) followed by Tukey’s multiple-comparison test. The two group variables used were Par3 and Par4 genotype for the data in Figures 1 and 2, and Par4 and Gp6 genotype for the data in Figure 4. Figure 1 used a 2x2 group table (Par3+/+ and Par3-/- on one axis and Par4+/+ and Par4+-/ on the other). Figure 2 used a 3x3 group table with Par3+/+, Par3-/-, and Par3-/- on one axis and Par4+/+, Par4+-/ and Par4-/- on the other. Figure 4 used a 3x3 group table with Par4+/+, Par4+-/ and Par4-/- on one axis and Gp6+/+, Gp6+-/-, and Gp6+-/- on the other.
* indicates P<0.05, ** indicates P<0.01, ***indicates P<0.001, ****indicates P<0.0001.

References


