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Activated thrombin-activatable fibrinolysis inhibitor (TAFIa) attenuates fibrin-dependent plasmin generation on thrombin-activated platelets

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Abstract

Background: Thrombin-activated platelets can promote fibrinolysis by binding plasminogen in a fibrinogen-dependent manner and enhancing its activation by tissue-type plasminogen activator (t-PA). Whether t-PA also binds to activated platelets and the mechanism for regulation of platelet-dependent fibrinolysis remain unknown.

Objectives: Determine the mechanism of plasminogen and t-PA binding on thrombin-activated platelets and its regulation by activated thrombin-activatable fibrinolysis inhibitor (TAFIa).

Methods: Plasminogen and t-PA binding with or without TAFIa treatment was quantified using flow cytometry. Plasmin generation on platelets was quantified using a plasmin-specific substrate. Mass spectrometry analyses identified fibrinogen as a potential target of TAFIa. Thrombus formation was studied in mice lacking fibrinogen ($Fg^{-/-}$) using intravital microscopy.

Results: Plasminogen and t-PA bind to platelets activated by thrombin but not by other agonists, including protease-activated receptor agonists (PAR-AP). Plasminogen binds via its kringle

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AUTHOR CONTRIBUTIONS

RN performed all flow cytometry and in vivo experiments, analyzed data, and wrote the manuscript. CW and SEC performed the activation experiments and edited the manuscript. MADN, HN, and MJF generated the data with fibrinogen-deficient platelets and edited the manuscript. PLG and JIW analyzed the data and edited the manuscript. PYK devised the experiments, analyzed the data, and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

domains because ε-aminocaproic acid eliminates binding, whereas t-PA binds via its finger and kringle domains. Plasminogen binding is fibrinogen-dependent because it is abolished on (a) $Fg^{-/-}$ platelets, and (b) thrombi in $Fg^{-/-}$ mice. Binding requires thrombin-mediated fibrinogen modification because addition of batroxobin to PAR-AP activated platelets has no effect on plasminogen binding but induces t-PA binding. TAFIa reduces plasminogen and t-PA binding to thrombin-activated platelets and attenuates plasmin generation in a concentration-dependent manner. Mass spectrometry identified K556 on the fibrinogen alpha-chain as a potential thrombin cleavage site that generates a TAFIa sensitive C-terminal lysine residue.

Conclusion: These findings provide novel mechanistic insights into how platelets activated by thrombin at sites of vascular injury can influence fibrinolysis.

Keywords

fibrinolysis; blood platelets; carboxypeptidase B2; intravital microscopy; plasminogen

1 | INTRODUCTION

Upon vascular damage, activated platelets localize to the injury site where they become activated and aggregate to form a platelet plug. Tissue factor exposed at the site of injury triggers thrombin generation, which leads to fibrin formation. Fibrin stabilizes the platelet plug and renders it an efficient barrier for prevention of blood loss. Once hemostasis is established, the thrombus must be degraded to restore blood flow and promote repair of the injured vessel. Tissue-type plasminogen activator (t-PA), which converts plasminogen to plasmin, initiates intravascular fibrinolysis. Plasminogen binds to fibrin via its lysine binding kringle domains, whereas t-PA binds to fibrin via its fibronectin-type I finger[1] and second kringle domains.[1,2] Co-localization of t-PA and plasminogen on the fibrin surface enhances plasminogen activation by several orders of magnitude.[3,4] Thus, efficient hemostasis depends on the dynamic balance between clot formation and degradation.

Thrombin-activatable fibrinolysis inhibitor (TAFI), also known as procarboxypeptidase (proCP) U, proCPB2 or plasma proCPB, down-regulates fibrinolysis. TAFI circulates in plasma in a latent form and is activated to TAFIa by the thrombin-thrombomodulin complex (thrombin-TM).[5] TAFIa suppresses fibrinolysis by releasing the C-terminal lysine residues on degrading fibrin that serve as plasminogen and t-PA binding sites. Therefore, TAFIa is an important inhibitor of plasminogen activation and downstream fibrinolysis.

Recent studies suggest that platelets modulate fibrinolysis under flow conditions, but the mechanism of such regulation is largely undefined.[6] Fibrinogen binds to the surface of thrombin-activated platelets via $\alpha_{IIb}\beta_3$.[7,8] Plasminogen binds to platelet-bound fibrinogen in a lysine-dependent manner.[4,9–12] t-PA also binds to platelets,[2,13,14] but it is uncertain if (a) this interaction is fibrin(ogen)- or activation-dependent, and (b) whether TAFIa regulates platelet-dependent fibrinolysis. Here, we investigated the mechanistic underpinnings of platelet-dependent fibrinolysis by testing the hypothesis that by binding plasminogen and t-PA, thrombin-modified fibrin(ogen) on the platelet surface promotes plasmin generation; a reaction that is suppressed by TAFIa.

2 | METHODS

2.1 | Materials

Recombinant TAFI was isolated as described previously.[15,16] 5-iodoacetamidofluorescein (5IAF) was purchased from Marker Gene Technologies Inc (Eugene, OR, USA). AlexaFluor (AF)488-C_5-maleimide and AF647-C_2-maleimide were purchased from ThermoFisher Scientific (Waltham, MA, USA). A plasminogen derivative with a S741C mutation was expressed and labeled with 5IAF (5IAF-Pg).[17] t-PA was a generous gift from Genentech. Reteplase,[18] a truncated variant of t-PA lacking the finger domain, was a generous gift from Centocor Inc (Malvern, PA, USA). R-phycoerythrin (PE)-tagged mouse anti-human P-selectin antibody (AC1.2) was purchased from Becton Dickinson Biosciences (San Jose, CA, USA). 5IAF-labeled rat anti-mouse P-selectin antibody (Wug.E9) and Dylight 649-labeled rat anti-mouse GPIbβ (X649) were purchased from Emfret Analytics (Eibelstadt, Germany). An antibody against the human plasminogen receptor Plg-R_{KT} (anti-Plg-R_{KT}) was purchased from Atlas Antibodies (Bromma, Sweden). Phe-Pro-Arg-chloromethylketone (FPR-ck) and Val-Phe-Lys-chloromethylketone (VFK-ck) were purchased from Millipore (Billerica, MA, USA). Human α-thrombin was purchased from Enzyme Research Laboratories (South Bend, IN, USA). Batroxobin, a thrombin-like enzyme isolated from the venom of *Bothrops moojeni* that only releases fibrinopeptide A from fibrinogen, was purchased from Pentapharm (Basel, Switzerland). Hirudin was purchased from EMD Chemicals, Inc (Gibbstown, NJ, USA). Recombinant soluble TM (Solulin) was a generous gift from Paion GmbH (Berlin, Germany). Potato tuber carboxypeptidase inhibitor (PTCI), a TAFIa-specific inhibitor, protease inhibitor cocktail, and ε-aminocaproic acid (εACA), a lysine analogue, were purchased from Sigma-Aldrich Canada (Oakville, CA). Protease-activated receptor (PAR)-1 agonist peptide (PAR1-AP; SFLLRN) and S-2251, a plasmin-directed chromogenic substrate, were purchased from Diapharma (West Chester, OH, USA). PAR-4 agonist peptide (PAR4-AP; AYPGKF) and Gly-Pro-Arg-Pro (GPRP), an inhibitor of fibrin polymerization, were purchased from Bachem (Bubendorf, Switzerland). Collagen was purchased from Nycomed Pharma (Ismaning, Germany). Integrilin, a synthetic $\alpha_{IIb}\beta_3$ inhibitor, was purchased from Kingston General Hospital Pharmacy (Kingston, Canada). t-PA was labeled with AF488-C_5-maleimide (AF488-t-PA) or AF647-C_2-maleimide (AF647-t-PA) using its free cysteine as described previously.[19]

2.2 | Preparation of washed platelets

After obtaining signed informed consent in accordance with the Hamilton Integrated Research Ethics Board, blood from 10 healthy human donors was collected into 97.9 mmol/L sodium citrate, 78.1 mmol/L citric acid, and 2% glucose (ACD) in a 9:1 ratio (v/v). Platelet-rich plasma was obtained by centrifugation at 180 *g* for 20 minutes. Platelet-poor plasma was excluded by centrifugation at 1000 *g* for 10 minutes in the presence of 4 μmol/L prostacyclin and platelet pellets were then washed with calcium-free Tyrode's buffer containing 5 mmol/L glucose and 0.35% bovine serum album in the presence of 2 μmol/L prostacyclin and 12% (v/v) ACD. Washed human platelets were resuspended in Tyrode's buffer and allowed to rest at room temperature for 30 minutes before use.

Mouse platelets were isolated from age-matched 6 to 12 weeks old C57BL/6J wild-type mice (Jackson Laboratory) or mice deficient in fibrinogen ($Fg^{-/-}$)[20] with or without von Willebrand factor ($Fg^{-/-}$:$vWF^{-/-}$)[21] on a C57BL/6 background weighing at least 20 g. Blood was collected via carotid cannulation into ACD and platelets were isolated as described previously.[22] The platelet count was determined using a hemocytometer. Murine studies were carried out according to Canadian Council of Animal Care Guidelines and all animal utilization protocols were approved by the Animal Research Ethics Board at McMaster University or the Animal Care Committee at St. Michael's Hospital, Toronto, Ontario, Canada.

2.3 | Flow cytometry analyses of plasminogen and t-PA binding to activated platelets

Platelets (1×10^6) were activated with 0.1, 3.4 or 38.2 U/mL of thrombin, 17 μmol/L PAR1-AP, 400 μmol/L PAR4-AP, 20 μg/mL collagen, a combination of 17 μmol/L PAR1-AP and 400 μmol/L PAR4-AP, or a combination of 0.1 or 3.4 U/mL thrombin plus 20 μg/mL collagen, all in the presence of 2 mmol/L $CaCl_2$ for 10 minutes at room temperature. No additional fibrinogen was added. Platelet activation was assessed by measuring surface-expressed P-selectin with AC1.2 for human platelets and Wug.E9 for murine platelets. Binding of the labeled antibody was monitored and quantified by flow cytometry (Becton Dickinson, San Jose, USA) and the fluorescence signal values were expressed as Geometric Mean Fluorescence Intensity (GMFI). Resting platelets were used as a control from which GMFI values were subtracted.

Platelets were also incubated with 0.4 μmol/L 5IAF-Pg or AF488-t-PA for 10 minutes at room temperature in the absence or presence of 20 mmol/L εACA or reteplase (0 to 2 μmol/L). Reteplase was used to demonstrate the specificity of plasminogen binding via its kringle domain, which is further validated by the use of εACA. To determine the effect of TAFIa on plasminogen and t-PA binding, resting or activated platelets were incubated with TAFIa at concentrations up to 18.15 nmol/L for 10 minutes prior to the addition of 5IAF-Pg or AF488-t-PA; studies were done in the absence or presence of 20 μmol/L PTCI. Binding was monitored and quantified using flow cytometry as described above.

To determine if plasminogen and t-PA compete for the same binding sites on platelets, each was titrated against the other. To determine the impact of t-PA on plasminogen binding, 0.4 μmol/L 5IAF-Pg was incubated with platelets in the presence of AF647-t-PA at concentrations up to 1 μmol/L. Conversely, to determine the impact of plasminogen on t-PA binding, 0.4 μmol/L AF647-t-PA was incubated with platelets in the presence of 5IAF-Pg in concentrations up to 1 μmol/L.

2.4 | Quantification of plasmin generation on platelets

Plasmin generation on resting or thrombin-activated platelets (1×10^8 platelets/mL) was determined in the presence of 9 μmol/L integrilin. Platelets suspended in 0.02 mol/L HEPES, 0.15 mol/L NaCl, pH 7.4, and 0.01% Tween 80 (v/v) (HBST) containing 5 mmol/L $CaCl_2$ were incubated with 0.67 μM plasminogen and 400 μmol/L S-2251. Reactions were initiated by addition of 2.5 nmol/L t-PA to resting or no platelets, or 1 nmol/L t-PA to activated platelets to compensate for the faster reaction observed on activated platelets.

Plasmin generation was quantified by monitoring S-2251 hydrolysis every minute at 405 nm at 25°C using a SpectraMax M2 spectrophotometer (Molecular Devices, Sunnyvale, USA). Time-squared analyses[23] were performed to estimate the rates of plasmin generation. Absolute rates of plasmin generation were calculated by determining the specific activity of plasmin in the presence of platelets in separate experiments. The specific activity values with activated or resting human platelets were 1.458 OD/min/μmol/L and 1.785 OD/min/μmol/L, respectively. The specific activity values with activated or resting mouse platelets were 2.468 OD/min/μmol/L and 1.862 OD/min/μmol/L, respectively.

2.5 | Influence of fibrin polymerization on plasminogen or t-PA binding

To determine the contribution of fibrin polymerization to plasminogen or t-PA binding to platelets, binding studies were performed in the absence or presence of 5 mmol/L GPRP as described previously.[6,24] In addition, to bypass thrombin-mediated proteolysis, platelets were activated with a combination of 17 μmol/L PAR1-AP and 400 μmol/L PAR4-AP, and fibrin polymerization was induced with 5 U/mL batroxobin.

2.6 | Identification of TAFIa targets on activated platelets by mass spectrometry

After activating platelets with 3.4 U/mL thrombin, a 5-fold molar excess of FPR-ck and VFK-ck was added to inhibit thrombin and plasmin, respectively. Platelets were then exposed to 9.07 nmol/L TAFIa after which they were subjected to lysis using an equal volume of RIPA lysis buffer containing 2% NP40 (v/v) and 4% protease inhibitor cocktail for 30 minutes at 4°C. After centrifugation at 10 000 *g*, the supernatant was harvested and snap frozen at −80°C until analyzed. Peptides were identified at the SPARC Biocentre at the Hospital for Sick Children (Toronto, ON, CA). Data were searched using PEAKS 8.0 (Bioinformatics Solutions Inc, Waterloo, ON, Canada), and identified peptides were manually searched for loss of C-terminal lysine residues.

2.7 | Laser-induced injury of isolated cremaster muscle and intravital microscopy

Vessel injury and analysis of thrombus formation was carried out as described previously[22,25,26] with minor modifications. Once the surgery was completed, mice were infused with 120 μg 5IAF-Pg and 100 ng/g X649 via jugular cannulation. Once visible thrombi formed, a three-dimensional stack comprising 180 slices taken 0.5 μm apart was captured every 20 seconds using fast-confocal microscopy (Intelligent Imaging Innovations, Denver, CO, USA) and the fluorescence of plasminogen and platelets as reflected by 5IAF-Pg and X649, respectively, was quantified using Slidebook (v6.0) and summed over the duration of the experiment.

2.8 | Statistical analyses

GraphPad Prism (v4.0) was used for statistical analyses. Data shown represent the mean ± standard error of the mean. The comparisons between two groups were performed using a 2-tailed Student's *t* test. Dose response was analyzed using Analysis of Variance. Results were considered statistically significant at $P < .05$ ($*P < .05$, $**P < .01$, $***P < .001$).

3 | RESULTS

3.1 | Plasminogen and t-PA only bind to platelets activated with thrombin

Stimulation of platelets with well characterized agonists (PAR1-AP, PAR4-AP, PAR1/4-AP, and thrombin with or without collagen) induced platelet activation as evidenced by P-selectin expression on their surface (Figure 1A). Plasminogen (Figure 1B) and t-PA (Figure 1C) binding to platelets only occurred with thrombin stimulation ($P < .01$). The extent of thrombin-stimulated binding was dependent on thrombin concentration ($P < .0001$) and stimulation with 3.4 U/mL thrombin produced saturable binding of plasminogen and t-PA (not shown).

TAFIa reduced plasminogen (Figure 2A) and t-PA (Figure 2B) binding to platelets activated with 3.4 U/mL thrombin by 70% ($P = .005$) and 33% ($P < .0005$), respectively. εACA almost completely eliminated plasminogen binding ($P < .005$) suggesting that plasminogen binding is solely via its kringle domains (Figure 2A). In contrast, εACA had the same effect on t-PA binding as TAFIa treatment ($P > .3$) (Figure 2B). These data suggest that (a) t-PA binds primarily through its finger domain, and (b) the lysine-dependent binding of t-PA favours C-terminal lysine residues. Some values appear to be slightly below zero. However, because the apparent zero is an arbitrary baseline reflecting the background signal with resting platelets, these small changes are unlikely to be physiologically relevant.

3.2 | Plasminogen and t-PA compete for the same lysine binding sites on thrombin-activated platelets

To determine whether t-PA and plasminogen compete for the same binding sites, a fixed concentration of plasminogen was incubated with increasing concentration of t-PA. A biphasic plasminogen binding response was observed such that low t-PA concentrations enhanced plasminogen binding, whereas higher concentrations reduced it (Figure 2C). In contrast, t-PA binding to activated platelets under the same conditions was unaffected ($P = .2$) by the presence of plasminogen (Figure 2D).

When the conditions were reversed and the t-PA concentration was kept constant, increasing concentrations of plasminogen were un-able to displace platelet-bound t-PA (Figure 2E). The presence of t-PA resulted in an overall reduction in plasminogen binding to activated platelets ($P = .0002$) (Figure 2F). Reteplase, a t-PA variant that lacks the finger domain, displaced plasminogen ($P = .0001$) but not t-PA, thereby confirming that plasminogen binds solely via its kringle domains whereas t-PA binding is mainly finger domain-dependent (Figure 2G).

3.3 | TAFIa down regulates plasminogen and t-PA binding and plasminogen activation on thrombin-activated platelets

Platelets activated with PAR1-AP or 0.1 U/mL thrombin displayed little to no enhancement in plasminogen binding and TAFIa had no effect under these conditions (Figure 3A). In contrast, when platelets were activated with 3.4 U/mL thrombin, plasminogen binding was significantly enhanced and TAFIa attenuated plasminogen binding in a concentration-dependent manner but did not eliminate it. The concentration of TAFIa required for half-

maximal reduction in plasminogen binding was ~1 nmol/L, which is consistent with its half-maximal concentration for attenuating fibrinolysis.[5] The decrease in plasminogen binding was TAFIa-dependent because PTCI reversed its activity ($P < .001$) (Figure 3B).

There was little t-PA binding when platelets were activated with PAR1-AP. In contrast, t-PA binding was near maximal when platelets were activated with either 0.1 or 3.4 U/mL thrombin (Figure 3C). TAFIa decreased t-PA binding by about 33%, a decrease like that observed with εACA (Figure 2B), with half-maximal binding reduction achieved with ~1 nmol/L TAFIa. PTCI significantly reduced this minimal TAFIa-dependent decrease (Figure 3D).

Consistent with its effect on plasminogen and t-PA binding, TAFIa reduced plasminogen activation on thrombin-activated platelets (Figure 3E). The concentration of TAFIa required for half-maximal effect decrease in plasminogen activation was approximately 1 nM.

3.4 | Proteolytic modification of fibrin is essential for enhanced plasminogen binding

Activation of platelets with PAR1-AP and/or PAR4-AP did not enhance plasminogen binding to the same extent as activation with thrombin (Figures 1 and 4A). Likewise, inhibition of fibrin polymerization with GPRP also attenuated plasminogen binding to thrombin-activated platelets ($P < .0001$), suggesting that fibrin polymerization is important for enhanced plasminogen binding consistent with previous reports.[6] However, plasminogen binding was not enhanced when fibrin polymerization on PAR1/4-activated platelets was induced with batroxobin. These data confirm previous reports that enhanced plasminogen binding requires thrombin-induced fibrin polymerization.

Although activation of platelets with PAR1/4-AP did not promote t-PA binding (Figure 4B), t-PA binding was enhanced with batroxobin addition ($P = .0005$). Under these conditions, t-PA binding was sensitive to GPRP, but not to TAFIa (Figure 4B) or εACA (not shown), demonstrating that batroxobin-induced fibrin polymerization is sufficient to promote t-PA binding on platelets activated with PAR-APs, while implying that fibrin polymerization alone is insufficient to promote plasminogen binding.

3.5 | Plasminogen and t-PA binding and plasmin generation on activated platelets is fibrin-dependent

To confirm that binding of plasminogen and t-PA to platelets is fibrinogen-dependent, we used platelets isolated from mice deficient in fibrinogen. With mouse platelets, a higher thrombin concentration was used to ensure full platelet activation as measured by P-selectin expression. Compared with washed platelets from wild-type mice activated with 38.2 U/mL[6] thrombin, activated platelets from fibrinogen-deficient mice demonstrated 80% less plasminogen binding (Figure 5A) and no t-PA binding (Figure 5B). Plasminogen binding to thrombin-activated platelets from fibrinogen-deficient mice was significantly reduced by εACA ($P = .005$), suggesting that this small proportion of plasminogen is able to bind activated platelets via a fibrinogen-independent manner.

Plasmin generation was enhanced only with thrombin-activated platelets from wild-type mice in a TAFIa-dependent manner (Figure 6A) but not resting wild-type or resting/activated

fibrinogen-deficient platelets (Figure 6B), confirming the fibrinogen-dependence of plasminogen/t-PA binding enhancement and subsequent activation.

To extend our in vitro findings to an in vivo model system, we evaluated plasminogen accumulation within arterial thrombi whose formation was triggered by laser injury. Plasminogen accumulation at the site of arterial injury was observed in thrombi in wild-type mice (Figure 7A) but not in thrombi in fibrinogen-deficient mice (Figure 7B). The plasminogen signal co-localized with the platelet marker (yellow), but some was also seen close to the injury where there were no detectable platelets. Both platelet and plasminogen accumulation were quantified over time by monitoring X649 and 5IAF-Pg fluorescence, respectively (Figure 7C,D), and the sums of their respective fluorescence intensities over the time course were determined (Figure 7E). Compared with thrombi in wild-type mice, thrombi in fibrinogen-deficient mice exhibited a 55% decrease in total platelet accumulation ($P < .05$) and no plasminogen accumulation ($P < .0001$). These data are consistent with our in vitro findings and support the concept that plasminogen binding near or on platelets that are activated at sites of vessel injury is fibrin(ogen)-dependent.

3.6 | Cleavage of fibrinogen alpha-chain at K556 provides a C-terminal lysine residue necessary for promoting plasminogen binding to activated platelets

Mass spectrometry was used for analysis of protein in lysates of activated platelets but not resting platelets that possess C-terminal lysine residues removed by TAFIa. A peptide fragment of fibrinogen alpha-chain (ESSSHHPGIAEFPSRGK) was identified whereby its lysine residue at position 556 was lost after treating the activated platelet lysate with TAFIa. However, there was no apparent cleavage of the alpha-chain when purified fibrinogen was incubated with thrombin and subjected to SDS-PAGE analysis under reducing conditions (not shown). These data suggest that enhancement of plasminogen and t-PA binding to thrombin activated platelets requires fibrinogen modification on the activated platelet surface.

4 | DISCUSSION

Our findings provide new insights into the role of platelets in fibrinolysis. We provide evidence that (a) t-PA and plasminogen bind to fibrin deposited on the surface of thrombin-activated platelets, (b) platelet-bound fibrin potentiates plasminogen activation by t-PA, and (c) binding of t-PA and plasminogen, and activation of plasminogen by t-PA are attenuated by TAFIa. These are illustrated in our working model of fibrinolysis on activated platelets (Figure 8). Although previous studies showed that plasminogen binding to platelets is activation- and fibrin-dependent,[6,24] we are the first to show that (a) t-PA binding also is activation and fibrin-dependent, and (b) TAFIa attenuates plasminogen and t-PA binding on thrombin-activated platelets, thereby down regulating plasmin generation. In addition, the concentrations of TAFIa required to achieve half-maximal down regulation of plasminogen binding and activation were both ~1 nmol/L, which is the similar to the TAFIa concentration needed to produce half-maximal clot lysis.[5] Taken together, these data suggest that TAFIa regulates plasmin generation on thrombin-activated platelets and on fibrin in a similar manner.

Fibrin(ogen) polymerization is postulated to be a prerequisite for plasminogen binding to thrombin-activated platelets in an $\alpha_{IIb}\beta_3$-dependent manner. This was confirmed in experiments using GPRP,[6] including our data shown in Figure 4. To delineate the requirement of thrombin specifically as the driver of fibrin polymerization and downstream binding of plasminogen and t-PA, we compared their binding to thrombin-activated platelets with that to platelets activated with PAR1/4-AP with fibrin polymerization mediated by batroxobin. Although PAR1/4-AP activates platelets as evidenced by P-selectin expression, neither PAR1/4-AP alone nor in combination with batroxobin as the driver of fibrin polymer formation promoted plasminogen binding. In contrast, the combination of PAR1/4-AP and batroxobin was able to promote t-PA binding in a lysine-independent manner, thus supporting the concept that t-PA binding mainly requires fibrin polymer formation but not thrombin activity *per* se. The importance of fibrin polymerization is further highlighted by the results of studies with GPRP. When GPRP was added to inhibit fibrin polymerization, the apparent binding of t-PA to PAR1/4-AP/batroxobin-activated platelets was reduced, suggesting that fibrin polymerization increases the total number of lysine-independent t-PA binding sites. Addition of GPRP does not completely eliminate plasminogen and t-PA binding to platelets activated with PAR1/4-AP or thrombin. This may reflect the fact that although GPRP inhibits fibrin polymerization, it does not eliminate the fibrin-platelet interaction, which is likely mediated via $\alpha_{IIb}\beta_3$. Furthermore, when platelets are activated with PAR1/4-AP, GPRP addition appears to reduce plasminogen and t-PA binding by a small amount. Although this small reduction is statistically significant, it is unlikely to be biologically relevant.

Almost no binding of plasminogen or t-PA was observed on thrombin activated platelets isolated from mice lacking fibrinogen, suggesting that their binding is fibrin specific. We used platelets from fibrinogen-deficient mice rather than inhibitors of $\alpha_{IIb}\beta_3$ because agents such as integrilin and tirofiban do not eliminate fibrinogen binding to platelets.[27,28] Because over 99.5% of plasminogen binding to activated platelets is fibrin(ogen)-dependent in vivo (Figure 7A), it is unlikely that fibrinogen-independent plasminogen receptors (eg Plg-R_{KT}[29]) play a major role in promoting plasminogen binding to platelets.

Brzoska et al[30] also used intravital microscopy to investigate plasminogen accumulation after laser-induced injury to the mesenteric vein. They demonstrated plasminogen accumulation on the endothelium toward the centre of the thrombus, where it appeared to colocalize with phosphatidylserine expression. In contrast, with arteriolar injury, our studies suggest that plasminogen binds close to the site of the injury near the vessel wall. This difference may reflect the higher shear environment of cremaster arterioles than the lower shear found in the mesenteric vein. Nonetheless, Rand et al[31] demonstrated phosphatidylserine expression near the injury site in cremaster arterioles, which is consistent with where we observed plasminogen accumulation (Figure 7).

Although all concentrations of thrombin that were tested activated platelets as evidenced by similar levels of P-selectin expression, thrombin concentrations of at least 3.4 U/mL (~30 nmol/L) were required to induce maximal binding of both plasminogen and t-PA, and enhancement of fibrinolysis. Whyte *et al* have shown that platelets activated with very high levels of thrombin (100 nmol/L)[6] display an altered shape when compared with platelets that

are activated with a thrombin concentration ~0.1 U/mL (0.9 nmol/L),[32] which is widely viewed as being generous for activating platelets.[33–35] Low or high thrombin concentrations may lead to weak or strong platelet stimulation, respectively, which may lead to differences in their granule secretion[36] and subsequent plasminogen/t-PA binding. Although our studies did not evaluate platelet morphology, it is possible that the 'cap'-formation where plasminogen binding localizes[6] requires at least 3.4 U/mL of thrombin during activation whereas lower thrombin concentration does not afford this 'cap'-formation to promote localized plasminogen/t-PA binding and consequent plasmin formation.

Our studies identified K556 on the fibrinogen αC domain as one potential novel C-terminal lysine residue that is generated upon thrombin stimulation of platelets. Although direct evidence is lacking, it is likely that K556 is involved in plasminogen binding because no other C-terminal lysine residues have been identified after thrombin modification of fibrinogen. The identity of the protease responsible for this cleavage remains to be determined. Although plasmin is a potential candidate, VFK-ck failed to inhibit enhanced plasminogen binding on thrombin-activated platelets, rendering it unlikely that plasmin is responsible. Furthermore, we used a plasminogen derivative with its active site serine replaced with a cysteine, which would generate a catalytically inactive form of plasmin. Our studies indicate that thrombin is required, but it is uncertain whether this cleavage is mediated by thrombin itself, thrombin in complex with additional cofactors (eg thrombomodulin), or by an enzyme released from thrombin-activated platelets. A recently published unbiased screen of thrombin substrate specificity identified the GKS peptide sequence, such as that surrounding K556 (PSRGKSS), as being favorable for thrombin recognition.[37] That study also showed that serine at the P4 position ablates cleavage past arginine at P3. These observations are consistent with the observed TAFIa dependence for regulating plasminogen binding, which requires C-terminal lysine residues. Such modification of platelet-bound fibrin(ogen) is necessary since neither the initial thrombin-mediated conversion of fibrinogen to fibrin by the release of fibrinopeptides A and B, nor polymerization of fibrin monomers results in the generation of C-terminal lysine residues on fibrin. Furthermore, the lack of enhanced plasminogen binding when platelets are activated with PAR1/4-AP and fibrin polymerization is initiated by batroxobin suggests that polymerization and PAR1-/PAR4-dependent stimulation are insufficient to promote plasminogen binding.

Cleavage of fibrinogen at K556 by human tryptase from mast cells has been reported previously by Prieto-Garcia et al[38] Cleavage at this site appeared to inhibit the procoagulant properties of fibrin(ogen), thereby acting as a down-regulator of coagulation. Because of its location in the αC-domain, cleavage of K556 may alter fibrin structure.[39] Although a role for this cleavage in fibrinolysis has never been reported, mutation of K556 to alanine resulted in a bleeding diathesis in a patient described by Sumitha et al[40] More information is needed to determine the clinical consequence of this mutation, and whether this mutation leads to protection against TAFIa-mediated modulation of fibrinolysis.

Collectively, we have identified a novel mechanism by which fibrinolysis is modulated on thrombin-activated platelets and demonstrated fibrin(ogen)-dependent plasminogen accumulation on platelet-rich thrombi in vivo. Persistence of occlusive platelet-rich thrombi

on top of disrupted atherosclerotic plaques can lead to heart attack, stroke, or acute limb ischemia. Platelet-dependent fibrinolysis may contribute to clot degradation, thereby identifying a novel mechanism that can be modulated to enhance the degradation of platelet-rich arterial thrombi.

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Essentials

- Plasminogen (Pg) and tissue plasminogen activator bind to activated platelets in a fibrinogen-dependent manner
- Pg binding is enhanced by generation of new C-terminal lysine residues by thrombin on platelet-bound fibrin
- Activated thrombin-activatable fibrinolysis inhibitor (TAFIa) inhibits Pg activation on activated platelets
- Lysine 556 on alpha-chain of fibrinogen becomes a new C-terminal lysine that is cleaved by TAFIa

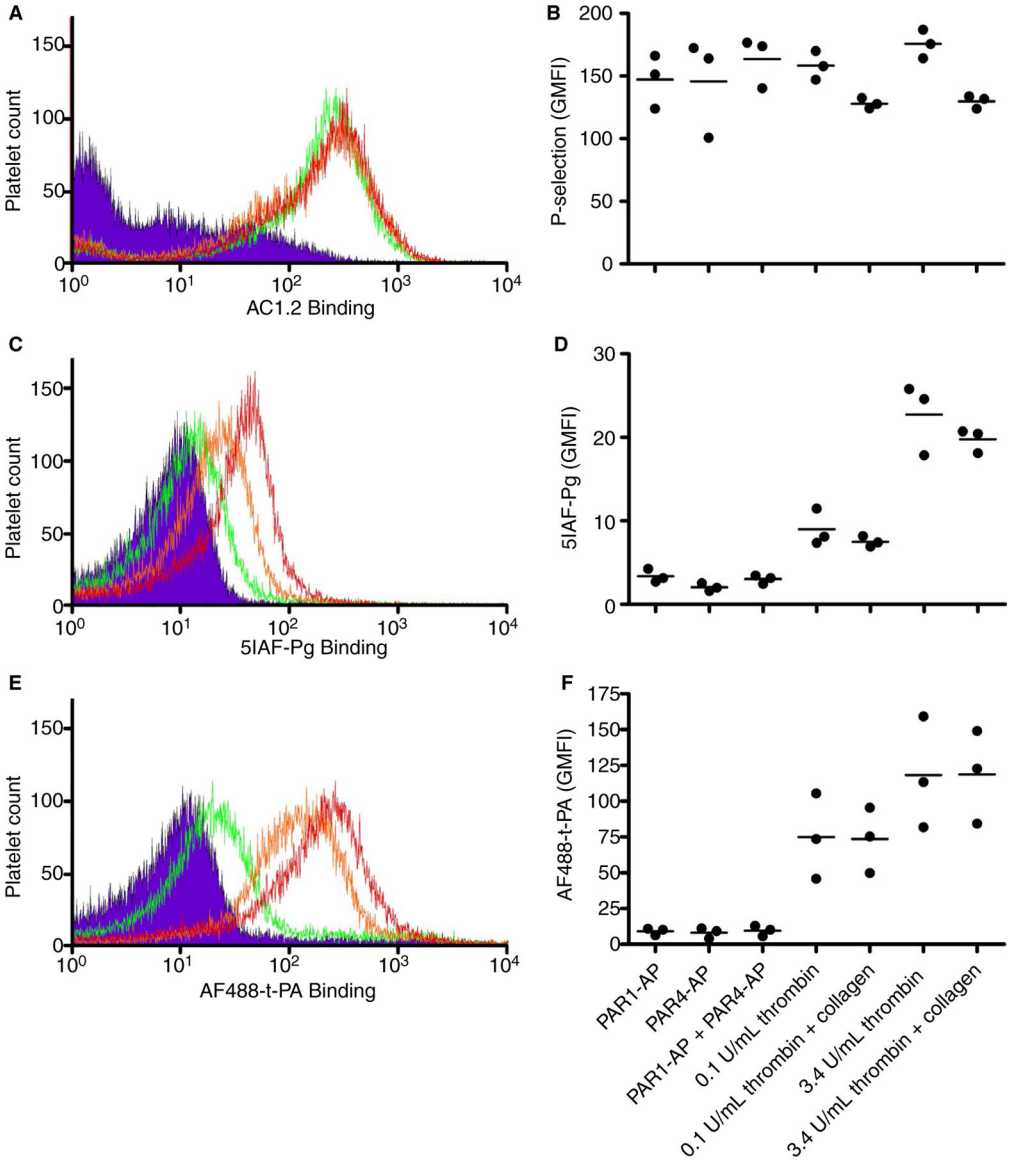

FIGURE 1.

Platelet activation by various agonists and the binding of plasminogen or t-PA. Washed platelets were activated by PAR1-AP (17 μmol/L), PAR4-AP (400 μmol/L), both PAR1-AP and PAR4-AP, thrombin at varying concentrations (0.1 or 3.4 U/mL), with or without collagen (20 μg/mL). On platelets, either resting (*shaded*) or activated with PAR1/4-AP (*green*), 0.1 U/mL thrombin (*orange*), or 3.4 U/mL thrombin (*red*), (A) P-selectin expression, (C) plasminogen (5IAF-Pg) binding, or (E) t-PA (AF488-t-PA) binding were measured using flow cytometry. The fluorescence values were quantified and its values are expressed as Geometric Mean Fluorescence Intensity (GMFI) in (B), (D), and (F), respectively, after normalization using the background values obtained from resting platelets. (N = 3)

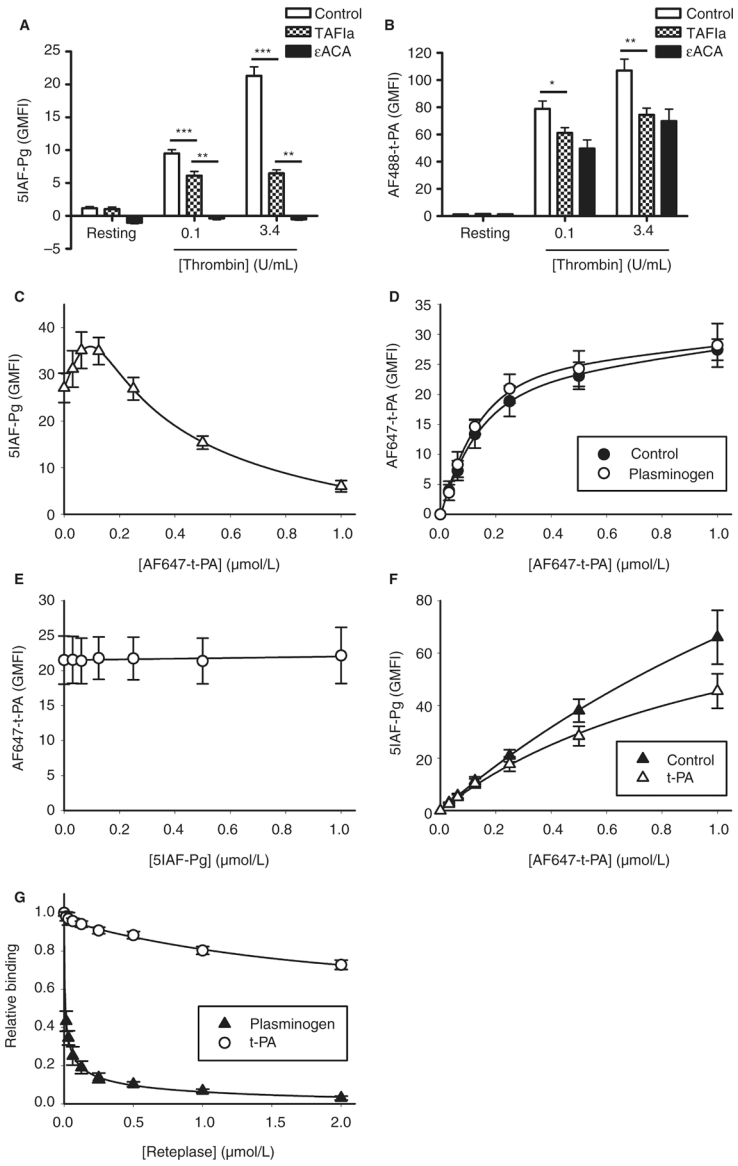

FIGURE 2.

Binding of plasminogen and t-PA on activated platelets. Washed platelets were activated by 0.1 or 3.4 U/mL thrombin and (A) plasminogen or (B) t-PA binding was quantified in the absence or presence of either 9.07 nmol/L TAFIa (*grid bars*) or 20 mmol/L ɛACA (*solid bars*). Competition between plasminogen and t-PA were investigated on platelets activated with thrombin (3.4 U/mL). Fixed 5IAF-Pg (0.4 μmol/L) was incubated with AF647-t-PA at varying concentrations (0 to 1 μmol/L), and the levels of bound (C) plasminogen, and (D) t-PA were quantified. Conversely, AF647-t-PA (0.4 μmol/L) was incubated with 5IAF-Pg at varying concentrations (0 to 1 μmol/L), and the levels of bound (E) t-PA, and (F) plasminogen were quantified. (G) Plasminogen or t-PA binding to activated platelets with reteplase as a competitor. Binding is shown as Geometric Mean Fluorescence Intensity (GMFI) or relative binding, whereby the GMFI values were normalized to the values obtained without reteplase. (N = 4 to 10) **P* < .05, ***P* < .01, ****P* < .001

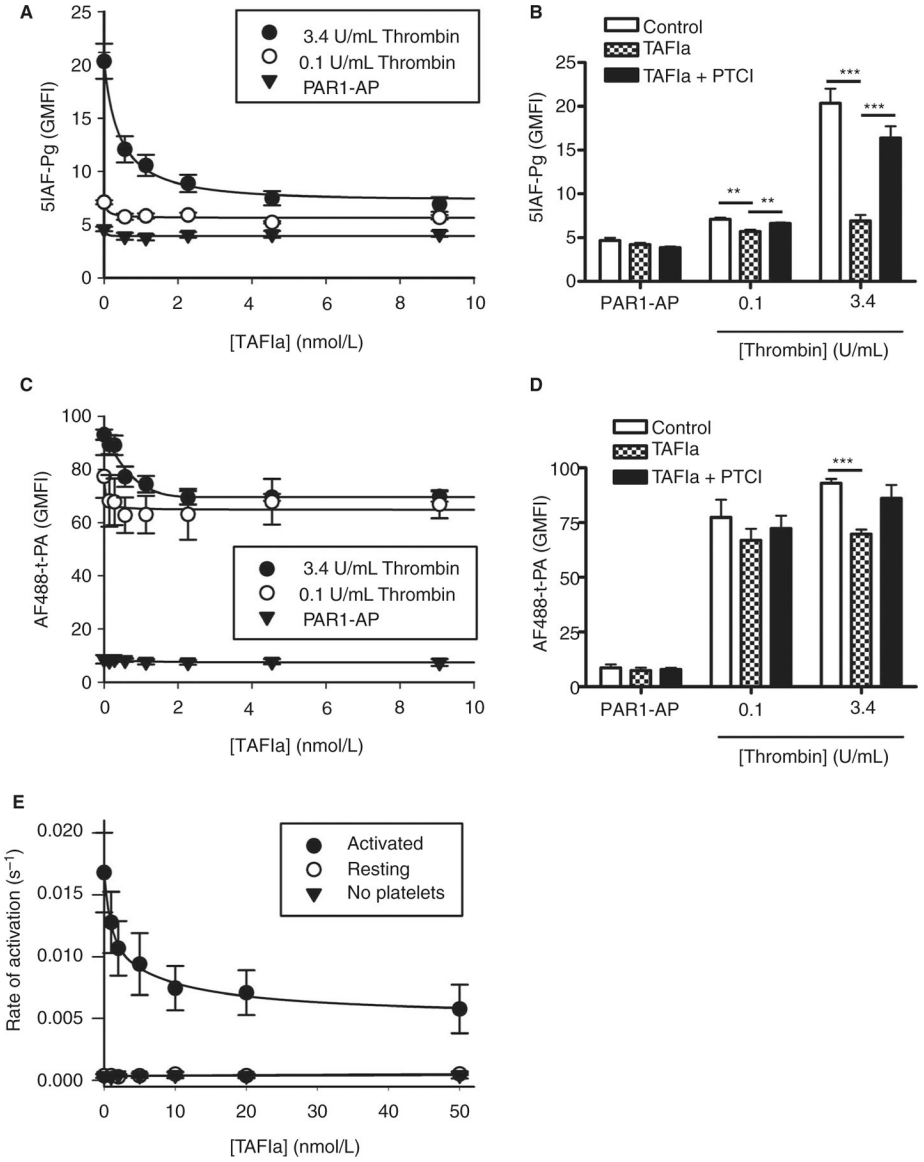

FIGURE 3.

The effect of TAFIa on plasminogen/t-PA binding and plasmin generation on activated platelets. Washed platelets were activated by PAR1-AP (17 μmol/L), or thrombin (0.1 or 3.4 U/mL), treated with TAFIa at varying concentrations (0 to 18.15 nmol/L) and (A) plasminogen (5IAF-Pg) or (C) t-PA (AF488-t-PA) binding was quantified. PTCI (20 μmol/L) was also added to demonstrate TAFIa-specificity towards (B) plasminogen or (D) t-PA binding on activated platelets. Binding was quantified by flow cytometry with the values shown as GMFI after normalization using the background values obtained from resting platelets. (E) Plasminogen (0.67 μmol/L) activation was performed in the presence or absence of thrombin-activated or resting platelets (1×10^8 platelets/mL) using S-2251 (400 μmol/L). Reactions were initiated by the addition of t-PA (2.5 nmol/L for resting or in the absence of platelets, or 1 nmol/L for activated platelets). The reactions were monitored at

405 nm at 25°C, and plasmin generation was quantified using a time-squared analysis. (N = 3 to 6) *$P < .05$, **$P < .01$, ***$P < .001$

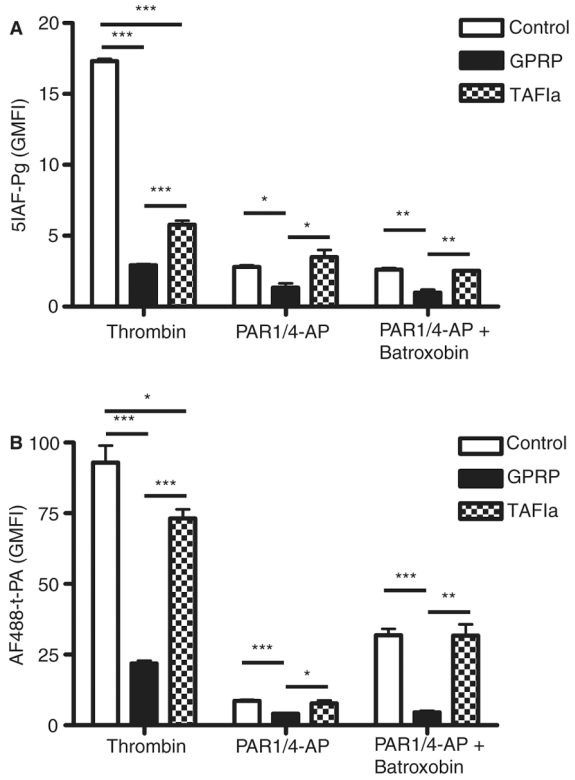

FIGURE 4.

Binding of (A) plasminogen and (B) t-PA to washed platelets activated with thrombin or a combination of PAR1-AP and PAR4-AP, with or without batroxobin. Washed platelets were activated with human thrombin (3.4 U/mL) or a mixture of PAR1-AP (17 μmol/L) and PAR4-AP (400 μmol/L), with or without batroxobin (5 U/mL), in the presence or absence of GPRP (5 mmol/L). TAFIa (9.07 nmol/L) was then added. The binding of plasminogen or t-PA was quantified using flow cytometry and the values are expressed as GMFI after normalization using the background values obtained from resting platelets. (N = 3) $*P < .05$, $**P < .01$, $***P < .001$

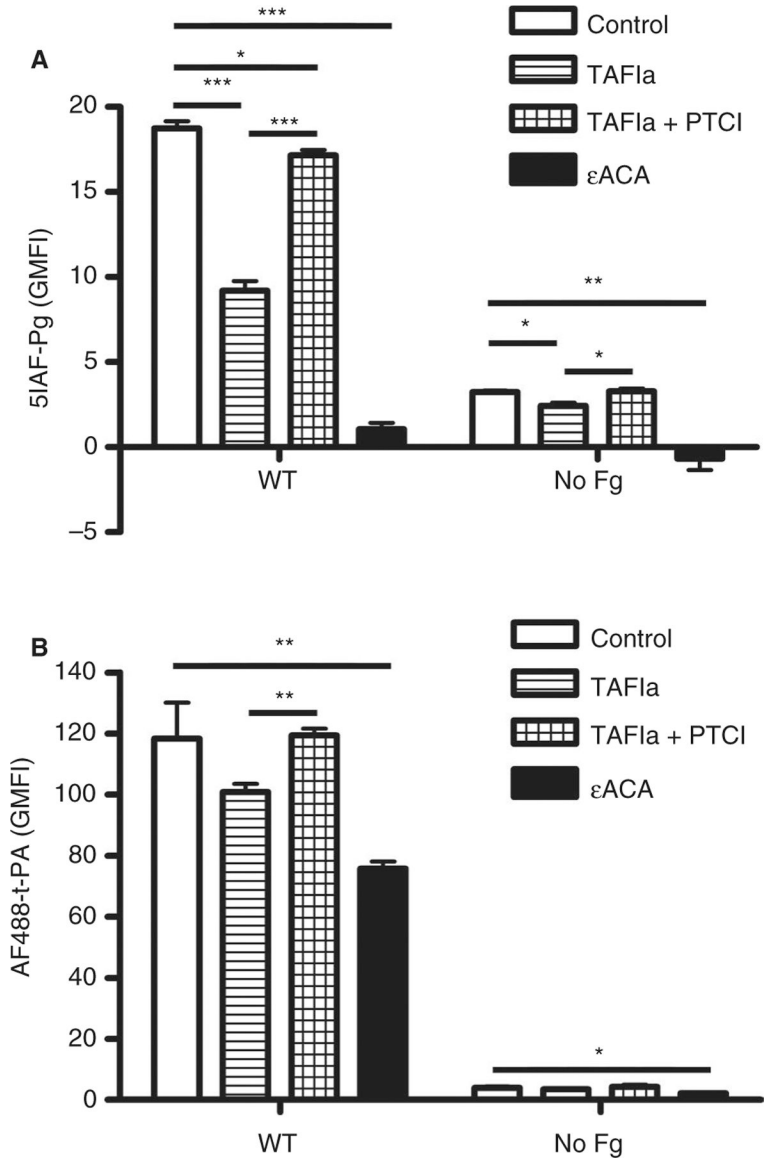

FIGURE 5.

Binding of (A) plasminogen and (B) t-PA to platelets isolated from the wild-type mice or mice deficient in fibrinogen (No Fg). Washed mouse platelets were activated with human thrombin (38.2 U/mL). TAFIa (18.15 nmol/L), with or without PTCI (20 μmol/L) or εACA (20 mmol/L) were also tested to verify lysine-dependence of plasminogen and t-PA binding. The binding was quantified using flow cytometry and the values are expressed as GMFI after normalization using the background values obtained from resting platelets. (N = 3 to 4) **P* < .05, ***P* < .01, ****P* < .001

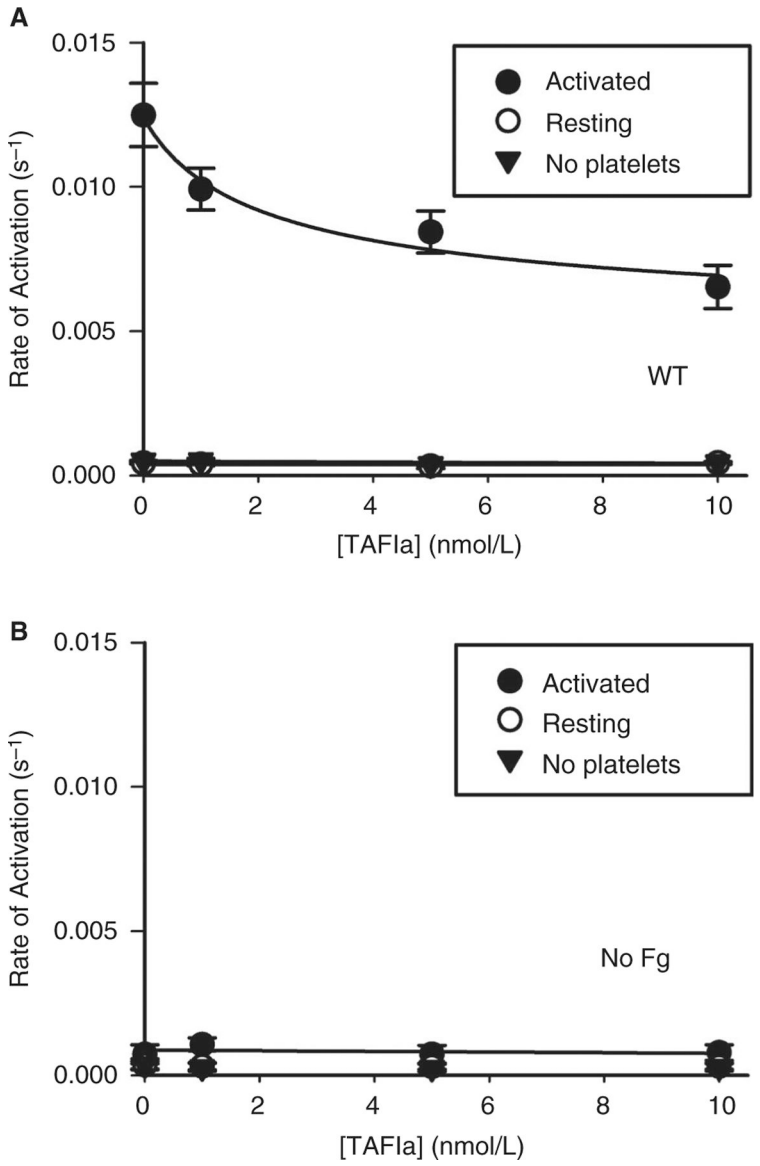

FIGURE 6.

Plasmin generation on platelets isolated from (A) wild-type or (B) mice deficient in fibrinogen (No Fg). Human Glu-plasminogen (0.67 μmol/L) activation was performed in the presence or absence of thrombin-activated or resting platelets (1×10^8 platelets/mL) using S-2251 (400 μmol/L). Reactions were initiated by the addition of t-PA (2.5 nmol/L for resting or in the absence of platelets, or 1 nmol/L for activated platelets). The reactions were monitored at 405 nm at 25°C, and plasmin generation was quantified using a time-squared analysis. (N = 3 to 4)

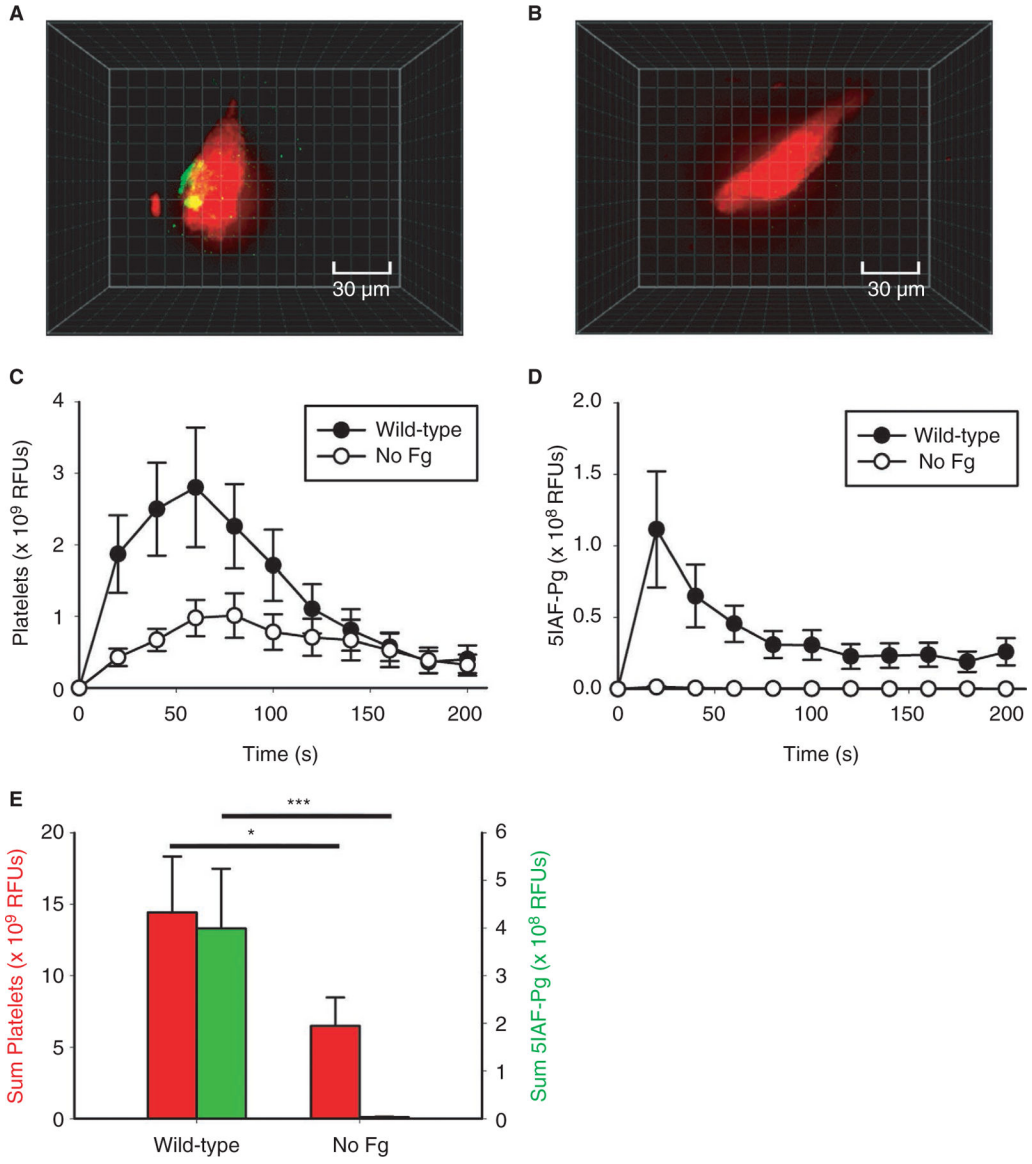

FIGURE 7.

Plasminogen binding in vivo in (A) wild-type or (B) fibrinogen-deficient (No Fg) mice. Using laser-induced injury on isolated cremaster muscle of mice, (C) platelet (red/yellow) or (D) plasminogen (green/yellow) accumulation was observed and quantified by intravital microscopy. (E) The relative fluorescence signals were then added (area under the curve) and compared. (N = 11 to 21 thrombi in at least 3 mice) $*P < .05$, $**P < .01$, $***P < .001$

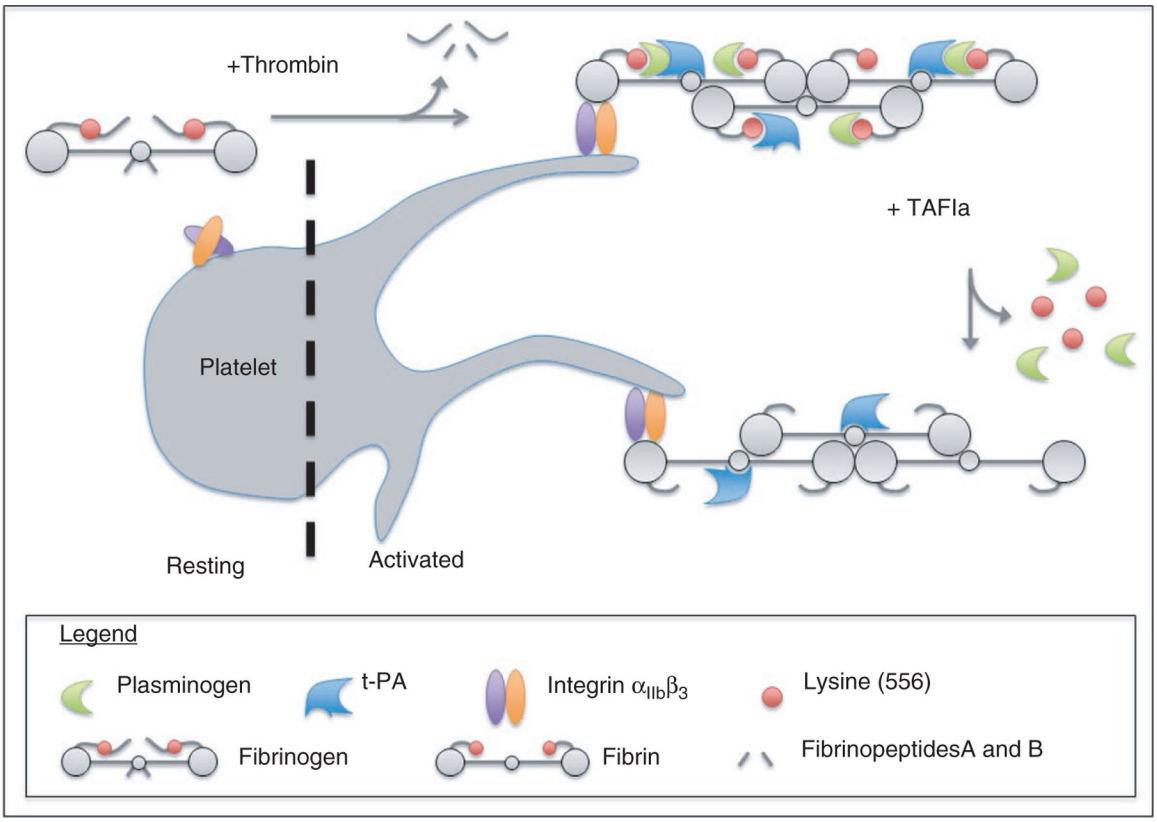

FIGURE 8.

Model of fibrinolysis regulation on thrombin-activated platelets. Thrombin (A) activates platelets, which then result in the activation of $\alpha_{IIb}\beta_3$, and (B) cleaves FPA and FPB to convert fibrinogen to fibrin. On platelets, thrombin presence also leads to the cleavage of the α-chain of fibrin(ogen) after K556, which exposes K556 as the new C-terminal lysine that could possibly bind plasminogen and/or t-PA. TAFIa removes K556, which we hypothesize results in the loss of fibrin-dependent plasminogen and/or t-PA binding on platelets, and overall reduced plasmin generation