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Functional plasminogen activator inhibitor 1 is retained on the activated platelet membrane following platelet activation

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ABSTRACT

Platelets harbor the primary reservoir of circulating plasminogen activator inhibitor 1 (PAI-1), but the reportedly low functional activity of this pool of inhibitor has led to debate over its contribution to thrombus stability. Here we analyze the fate of PAI-1 secreted from activated platelets and examine its role in maintaining thrombus integrity. Activation of platelets results in translocation of PAI-1 to the outer leaflet of the membrane, with maximal exposure in response to strong dual agonist stimulation. PAI-1 is found to co-localize in the 'cap' of phosphatidylserine-exposing platelets with its co-factor, vitronectin, and fibrinogen. Inclusion of tirofiban or Gly-Pro-Arg-Pro significantly attenuated exposure of PAI-1, indicating a crucial role for integrin $\alpha_{IIb}\beta_3$ and fibrin in delivery of PAI-1 to the activated membrane. Separation of platelets post stimulation into soluble and cellular components revealed the presence of PAI-1 antigen and activity in both fractions, with approximately 40% of total platelet-derived PAI-1 remaining associated with the cellular fraction. Using a variety of fibrinolytic models, we found that platelets produce a strong stabilizing effect against tissue plasminogen activator (tPA)-mediated clot lysis. Platelet lysate, as well as soluble and cellular fractions, stabilize thrombi against premature degradation in a PAI-1-dependent manner. Our data show for the first time that a functional pool of PAI-1 is anchored to the membrane of stimulated platelets and regulates local fibrinolysis. We reveal a key role for integrin $\alpha_{IIb}\beta_3$ and fibrin in delivery of PAI-1 from platelet α -granules to the activated membrane. These data suggest that targeting platelet-associated PAI-1 may represent a viable target for novel profibrinolytic agents.

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Introduction

The fibrinolytic system is primarily responsible for thrombus resolution *in vivo* thus maintaining vessel patency. The principal enzyme, is formed *via* cleavage of the inactive circulating zymogen plasminogen. The main plasminogen activators are tissue plasminogen activator (tPA) derived largely from endothelial cells¹⁻³ and urokinase (uPA), which is synthesized by cells of fibroblast morphology,⁴ epithelial cells, monocytes and macrophages.⁵ The activity of tPA is primarily regulated by one-to-one complex formation with the serpin inhibitor, plasminogen activator inhibitor-1 (PAI-1).^{6,7} PAI-1 is unusual amongst the family of serpin inhibitors, as in its free form it can exist in an active or latent state.⁸⁻¹⁰ The active form of secreted cellular PAI-1 has a relatively short half-life of around 30 minutes (min) in plasma^{8,11-14} but is stabilized by binding to the adhesive glycoprotein vitronectin (Vn), thereby prolonging its half-life 2-3-fold *in vivo*.¹⁵⁻¹⁷ Vn is crucial for PAI-1 function in fibrinolysis, acting as an intermolecular bridge between PAI-1 and fibrin,¹⁸ localizing PAI-1 within the fibrin clot.¹⁹ The primary reservoir of circulating PAI-1 resides within platelet α -granules,²⁰ however, it has been suggested that only 5-10% of platelet PAI-1 exists in an active configuration.²⁰⁻²²

Platelets play a crucial role in hemostasis and are the first to respond to vessel injury. Activation of platelets gives rise to multiple platelet subpopulations with diverse phenotypes and differential functions.^{23,24} Aggregating, or spread, platelets mediate clot retraction and are defined by expression of the active integrin $\alpha_{IIb}\beta_3$ and a lack of phosphatidylserine (PS) exposure.^{25,26} In contrast, PS-exposing

platelets demonstrate a characteristic balloon shape, increased cytosolic Ca^{2+} and enhanced ability to bind coagulation factors^{27,28} and promote thrombin generation.^{29,30} 'Coated' platelets are a subset of PS-exposing platelets, which harbor several procoagulant α -granule proteins on their surface, such as fibrinogen, factor V and von Willebrand factor.³¹⁻³⁴ It has been demonstrated that proteins are anchored via a transglutaminase-dependent mechanism and require integrin $\alpha_{\text{IIb}}\beta_3$ activation to permit anchoring of fibrin at the platelet surface.³⁵ PS-exposing platelets possess a protruding 'cap' on their membrane, also described as the platelet body,^{36,37} that is rich in aminophospholipids and harbors a number of platelet-derived and plasma proteins. Our laboratory has identified platelet FXIII-A and plasma-derived plasminogen within the 'cap' of PS-exposing platelets,^{38,39} that have the potential to direct fibrinolysis in platelet-rich areas of thrombi.

The susceptibility of a thrombus to fibrinolysis is influenced by platelet content and fibrin structure.⁴⁰ Platelets anchor to fibrinogen *via* the integrin $\alpha_{\text{IIb}}\beta_3$; this binding interaction stabilizes the forming thrombus and initiates the process of clot retraction.⁴¹ Outside-in signaling, initiated through engagement of $\alpha_{\text{IIb}}\beta_3$ by fibrin(ogen), stimulates contraction of the platelet intracellular cytoskeleton.⁴² This process reels in the fibrin network to create a tightly compacted clot with increased resistance to fibrinolysis.^{43,44} We have previously shown that the fibrin immediately adjacent to platelet aggregates is markedly more resistant to degradation under flow,³⁹ in agreement with observations under static conditions.^{39,45} In this study, we examine the fate of PAI-1 released from platelet α -granules. We provide the first evidence that a pool of platelet-derived PAI-1 is retained on the activated platelet membrane *via* a fibrin and integrin $\alpha_{\text{IIb}}\beta_3$ mechanism. Importantly, this pool of PAI-1 retains functional activity and directly participates in thrombus stability against fibrinolytic degradation.

Methods

Isolation of soluble and cellular fraction

Platelets were activated with 1 $\mu\text{g}/\text{mL}$ convulxin (CVX; Enzo Life Sciences) and 100 nM thrombin (Sigma-Aldrich). The soluble fraction was collected by centrifugation at 13,000xg for 4 minutes (min). The pellet, containing the cellular components, was re-suspended in HEPES buffer.

Flow cytometry analysis of platelets

Washed platelets (2×10^9 plt/mL) were stimulated with 1 $\mu\text{g}/\text{mL}$ CVX \pm 0.2 mM TRAP-6 (Sigma-Aldrich) or 100 nM thrombin in the presence of 2 mM CaCl_2 . In some cases, platelets were pre-treated for 30 min with 5 mM Gly-Pro-Arg-Pro (GPRP) (Sigma-Aldrich) or 1 $\mu\text{g}/\text{mL}$ tirofiban (Sigma-Aldrich). Fluorescently-labeled antibodies to either PAI-1 (5.8 $\mu\text{g}/\text{mL}$), fibrin(ogen) (37 $\mu\text{g}/\text{mL}$) or Vn (13 $\mu\text{g}/\text{mL}$) were added during stimulation. After 40 min Annexin A5-Alexa fluor 647 (AF647) (1/20) (BD Biosciences) was added in the presence of 2 mM CaCl_2 . Exposure of PAI-1 and PS were analyzed using a BD LSRII cytometer with FACS DIVA 6.1.3 software.

Fluorescence imaging of platelets

Ibidi μ -slide VI^{0.4} chambers were coated with collagen (20 $\mu\text{g}/\text{mL}$) (American Biochemical Pharmaceuticals) and throm-

bin (100 nM). Slides were blocked with 5% BSA before addition of washed platelets (0.5×10^8 plt/mL). In some cases, platelets were pre-treated with 5 mM GPRP or 1 $\mu\text{g}/\text{mL}$ tirofiban prior to activation. Fluorescently labeled antibodies to either PAI-1 (5.8 $\mu\text{g}/\text{mL}$), fibrin(ogen) (37 $\mu\text{g}/\text{mL}$) or Vn (13 $\mu\text{g}/\text{mL}$), P-selectin (1/20) or CD41 (1/20) were included during stimulation. After 30 min Annexin-A5 FITC or AF647 (1/20) was added in the presence of 2 mM CaCl_2 . At 45 min platelets were visualized using a x63 1.40 oil immersion objective and Zeiss 710 laser scanning confocal microscope.

Fluorescence imaging of platelet-rich plasma clots

Clots were formed from 30% platelet rich plasma (PRP) with 0.25 μM fibrinogen-Alexa fluor 546 (AF546) (Thermo Fisher Scientific) \pm a neutralizing antibody to PAI-1 (400 $\mu\text{g}/\text{mL}$). Clotting was initiated using 0.125 U/mL thrombin and 10 mM CaCl_2 . Annexin A5-AF647 and fluorescently-labeled rabbit polyclonal antibody to PAI-1 or Vn were incorporated. Clots were polymerized in Ibidi μ -slide VI0.4 chambers at 37°C for 2 hours (h) in a moist box. In some cases, 75 nM tPA (Genetech) was added to the edge of clot and lysis monitored by taking images every 10 seconds (s). Clots were imaged using a x63 1.40 oil immersion objective and Zeiss 710 laser scanning confocal microscope.

Chandler model thrombi

Thrombi were formed using the Chandler model.⁴⁶ Pooled normal plasma (PNP) thrombi containing 45 $\mu\text{g}/\text{mL}$ FITC-labeled fibrinogen and 10.9 mM CaCl_2 \pm a neutralizing antibody to PAI-1 (400 $\mu\text{g}/\text{mL}$) were rotated at 30 rpm for 90 min. Thrombi were removed and lysed in 1 $\mu\text{g}/\text{mL}$ tPA at 37°C and samples taken every 30 min for 4 h. The plate was read at excitation 485 nm and emission 525 nm using a BioTek FLx800 fluorescence reader and Gen5 software. Fluorescence release is directly proportional to the rate of fibrinolysis in the sample.

Ethical consent

Ethical approval was obtained from the University of Aberdeen College Ethics Review Board.

Further details of the methods used can be found in the *Online Supplementary Appendix*.

Results

Plasminogen activator inhibitor-1 is retained on the activated platelet membrane

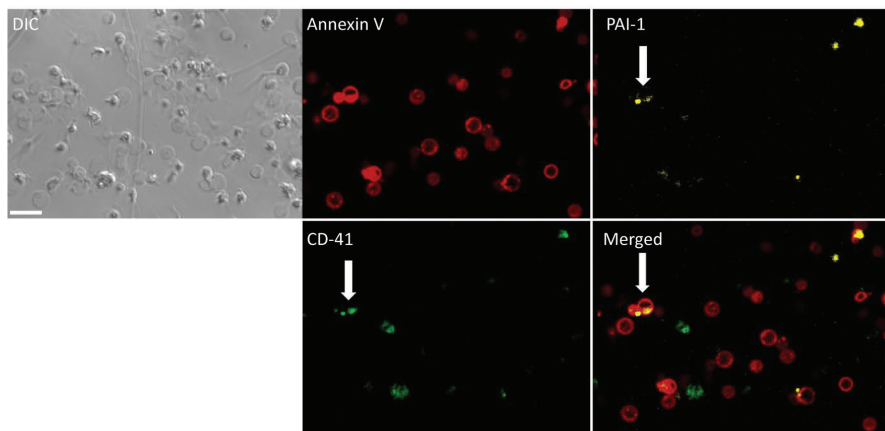
Plasminogen activator inhibitor-1 is abundant in platelet α -granules and is known to be a constituent of the platelet secretome. Here we address whether platelet-derived PAI-1 is retained on the surface of platelets. Using confocal microscopy we analyzed PAI-1 on the membrane of platelets stimulated on a collagen- and thrombin-coated surface for 45 min. The majority ($78.8 \pm 1.7\%$) of collagen- and thrombin-stimulated platelets were shown to be positive for PAI-1 (Figure 1). The serpin was found to be located within the 'cap' of PS-exposing platelets, which are characterized by Annexin V-AF647 staining and a characteristic balloon shape.^{27,39,47,48} P-selectin was included as a marker of platelet degranulation post stimulation and was found to co-localize with PAI-1 on the activated membrane of PS-positive platelets (*Online Supplementary Figure S1*). PAI-1 was found to co-localize with fibrin(ogen) and $\alpha_{\text{IIb}}\beta_3$ in the 'cap' of PS-exposing platelets (Figure 1A and B, arrows), with co-efficient (r) values of 0.92 and 0.57,

respectively. Platelet-derived Vn, a co-factor of PAI-1, was also found within the 'cap' region of PS-exposing platelets ($r=0.67$) (Figure 1C).

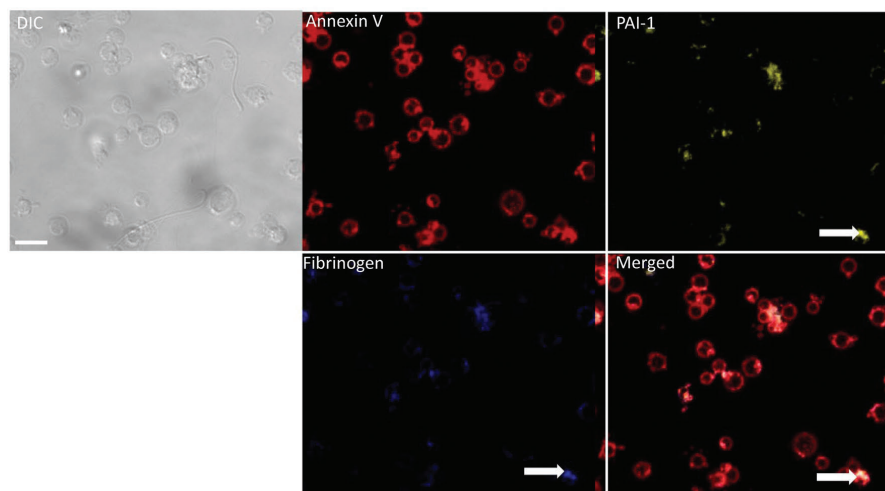
Flow cytometry analysis revealed a negligible amount of PAI-1 on the membrane of unstimulated platelets (Figure 2A and *Online Supplementary Figure S2A*). Following activation of platelets with CVX \pm TRAP-6 or thrombin there was a significant increase in the presence of PAI-1 compared to unstimulated platelets ($P<0.0001$). Maximum PAI-

1 exposure occurred following stimulation of platelets with CVX and thrombin, with a 35-fold increase in mean fluorescence intensity (MFI) compared to unstimulated platelets. Annexin V-AF647 staining revealed that the majority (93%) of PAI-1 was associated with PS-exposing platelets (*data not shown*). Similarly, maximal exposure of platelet-derived Vn and fibrinogen was observed in response to CVX and thrombin (Figure 2B and C, and *Online Supplementary Figure S2B and C*).

A



B



C

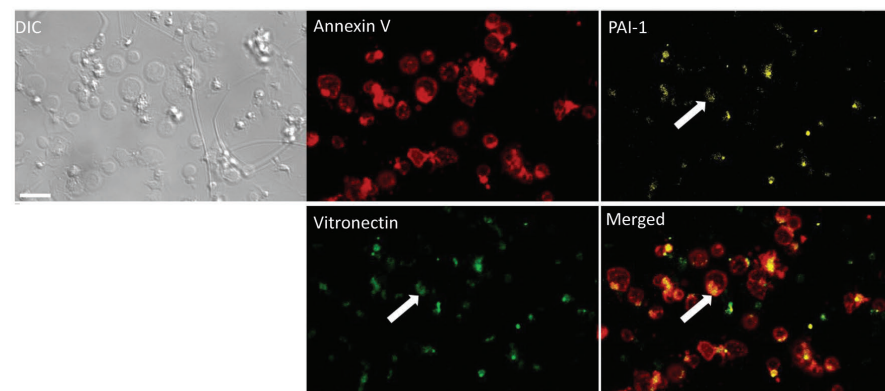


Figure 1. Platelet-derived plasminogen activator inhibitor 1 (PAI-1) co-localizes with fibrin(ogen), integrin $\alpha_{IIb}\beta_3$ and vitronectin (Vn) in the "cap" of phosphatidylserine-exposing platelets. Platelets (0.5×10^8 plt/mL), were adhered to a slide coated with collagen ($20 \mu\text{g/mL}$) and thrombin (100 nM) for 30 minutes (min) at ambient temperature. Annexin V (red) was added to stain phosphatidylserine and left for a further 15 min before imaging by confocal microscopy. (A) PAI-1 was detected using a rabbit polyclonal antibody labeled with DL550 (yellow) and integrin $\alpha_{IIb}\beta_3$ using a FITC-conjugated antibody to the CD41 subunit (green). (B) Fibrin(ogen) was analyzed using a rabbit polyclonal antibody labeled with DL405 (blue). (C) Vitronectin (Vn) was detected using a rabbit polyclonal antibody labeled with DL488 (green). Arrows highlight examples of co-localization. Images shown are representative of $n \geq 3$. Scale bars represent $2 \mu\text{m}$.

Plasminogen activator inhibitor-1 retention on platelets is dependent on $\alpha_{IIb}\beta_3$ and fibrin

We next analyzed the potential mechanism of retention of PAI-1 on the platelet membrane by blocking fibrin polymerization and the integrin $\alpha_{IIb}\beta_3$ with GPRP and tirofiban, respectively. A significant reduction in PAI-1 was observed on incorporation of tirofiban (2.3-fold; $P < 0.01$) or GPRP (2-fold; $P < 0.0001$) (Figure 3A). Interestingly, there was no change in association of Vn with the activated platelet membrane upon incorporation of tirofiban or GPRP (Figure 3B). Consistent with flow cytometry data confocal microscopy revealed significantly less membrane-associated PAI-1 upon inclusion of tirofiban or GPRP (Figure

3C). These data indicate that despite the clear co-localization of PAI-1 and Vn on the activated platelet membrane, the mechanism of retention on the activated platelet surface is different.

Distribution of platelet-derived plasminogen activator inhibitor-1 antigen and activity

Our data show for the first time that a pool of PAI-1 can be retained on the activated platelet membrane. The distribution of PAI-1 antigen and activity between the secretome and membrane fractions was then analyzed. Platelets were subjected to dual agonist stimulation to induce complete degranulation. The soluble fraction and

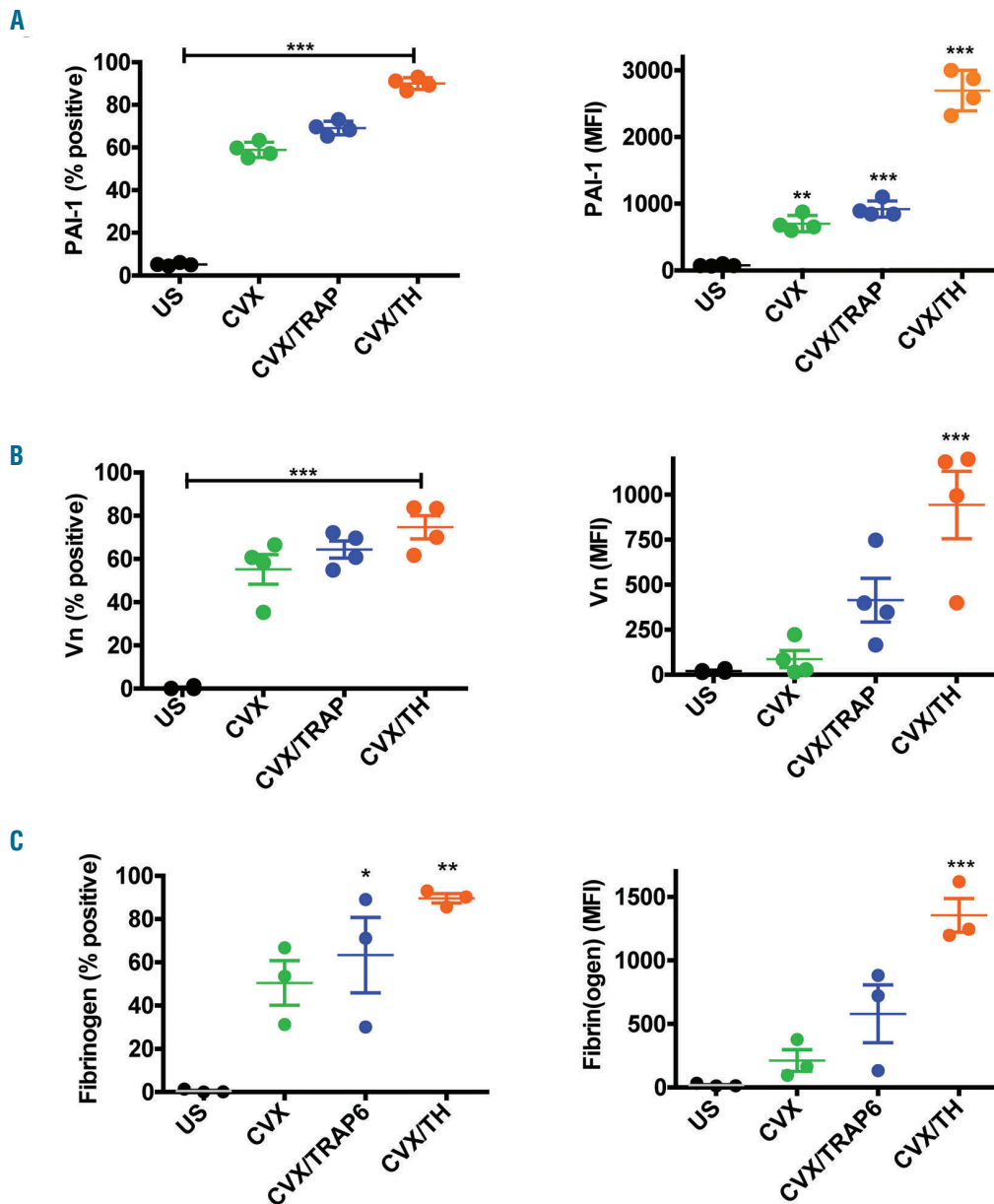


Figure 2. Exposure of platelet-derived plasminogen activator inhibitor 1 (PAI-1) on the activated membrane is maximized by strong dual agonist stimulation. Platelets (2×10^8 plt/mL), were unstimulated (US) or activated with convulxin (CVX) ($1 \mu\text{g/mL}$) \pm TRAP-6 (200 nM) or thrombin (TH; 100 nM) and analyzed using flow cytometry. (A) Platelet-derived PAI-1 was detected with a rabbit polyclonal antibody labeled with DL488. (B) Vitronectin (Vn) was detected with DL488 labeled rabbit polyclonal antibody. (C) Fibrin(ogen) was analyzed using a rabbit polyclonal antibody labeled with DL405. Percentage of platelets positive for PAI-1, Vn and fibrinogen and mean fluorescence intensity data are expressed as mean \pm standard deviation. $n \geq 4$. *** $P < 0.001$ versus ** $P < 0.01$, * $P < 0.05$ when comparing agonist treatment versus unstimulated.

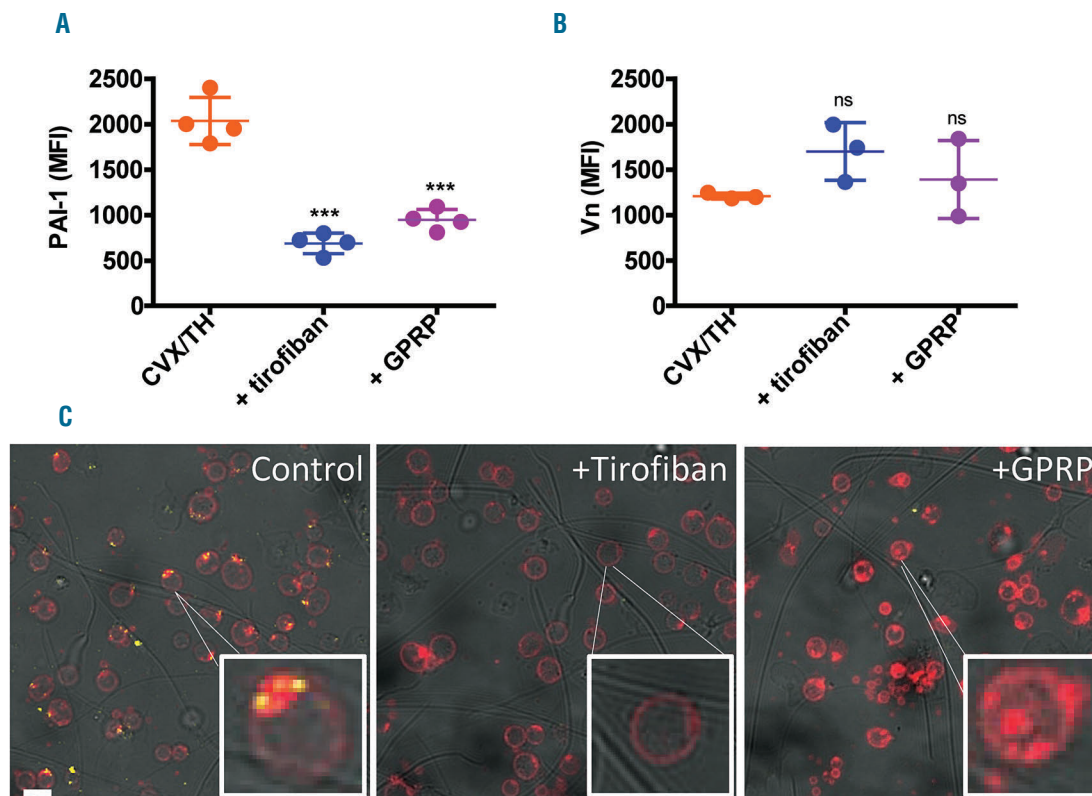


Figure 3. Plasminogen activator inhibitor 1 (PAI-1) is dependent on $\alpha_{\text{IIb}}\beta_3$ and fibrin for maximal exposure. Platelets (2×10^8 plt/mL), were pre-treated with tirofiban ($1 \mu\text{g/mL}$) or GPRP (5 mM) before activation with CVX ($1 \mu\text{g/mL}$) and thrombin (100 nM) and analyzed using flow cytometry. Platelet-derived (A) PAI-1 or (B) vitronectin (Vn) were detected using rabbit polyclonal antibodies labeled with DL488. Data are expressed as mean fluorescence intensity (mean \pm standard deviation). *** $P < 0.001$ versus untreated. $n \geq 3$. (C) Platelets (0.5×10^8 plt/mL), were pre-treated with tirofiban ($1 \mu\text{g/mL}$) or GPRP (5 mM) before adhering to a collagen ($20 \mu\text{g/mL}$) and thrombin (100 nM) coated slide. AnnexinV-AF647 (red) was used to stain phosphatidylserine and PAI-1 was detected using a rabbit polyclonal antibody labeled with DL550 (yellow). Images shown are representative of $n=3$. Scale bars represent $10 \mu\text{m}$ and magnified image of a single platelet. ns: not significant.

the remaining cellular fraction, consisting of the platelet internal and external membranes, were analyzed for PAI-1 antigen by ELISA and activity assay. PAI-1 antigen was more abundant in the soluble fraction ($19.2 \text{ ng}/10^9 \text{ plt}$), but almost a third of the total PAI-1 ($33.8 \text{ ng}/10^9 \text{ plt}$) remained associated with the cellular fraction ($10.5 \text{ ng}/10^8 \text{ plt}$) (Figure 4A). PAI-1 activity, determined by complex formation with tPA, revealed a similar distribution within the soluble and membrane fractions as the antigen (Figure 4B). These data indicate that a significant proportion of functional PAI-1 (~40%) is retained on the activated platelet surface where it can potentially regulate fibrinolysis.

Significant attenuation of PAI-1 antigen and activity in the soluble and cellular fraction was observed when $\alpha_{\text{IIb}}\beta_3$ or fibrin polymerization were inhibited (Figure 5A and B). These data indicate an essential role for functional $\alpha_{\text{IIb}}\beta_3$ and fibrin in translocation of PAI-1 from the platelet α -granules to both the activated platelet membrane and the secretome.

Platelet-derived PAI-1 localizes in platelet-dense areas and stabilizes thrombi

Platelet-derived PAI-1 staining in clots was localized to platelet-dense regions and emanated into the surrounding fibrin network (Figure 6). These data indicate that following platelet activation, the pool of PAI-1 is translocated from α -granules to the activated membrane and distally to

Table 1. Thrombodynamic analysis of clot formation and lysis.

Thrombodynamic parameter	Control	+ PAI-1 Ab	P
Lag time (min)	1.33 ± 0.41	0.60 ± 0.12	0.139
Rate of clot growth ($\mu\text{m}/\text{min}$)	15.80 ± 14.40	65.60 ± 6.55	0.0346
Clot density (a.u.)	14716 ± 906.5	7927 ± 123.4	0.0003
Full lysis time (min)	51.64 ± 2.01	24.19 ± 2.83	0.0002

Platelet-rich clots were formed \pm antibody to PAI-1 ($400 \mu\text{g/mL}$) and lysed with tPA (5 nM). Clot formation and lysis was monitored using a Hemacore thrombodynamic a.u. = arbitrary units, data represent mean \pm SEM, $n=4$. Statistical significance was determined by an unpaired Student's *t*-test; PAI-1: plasminogen activator inhibitor 1; tPA: tissue plasminogen activator.

platelet-associated fibrin. We also found evidence of co-localization of Vn with the fibrin network in the clot (Online Supplementary Figure S3).

The role of the platelet reservoir of PAI-1 in stabilization of thrombi has been a subject of debate as it reportedly chiefly exists in a latent inactive form.²¹ Here we analyze tPA-mediated lysis of platelet-rich clots using multiple static and flow-based models. Lysis of clots in real-time was visualized by confocal microscopy in the absence and presence of a neutralizing antibody to PAI-1. Inhibition of PAI-1 resulted in significantly faster lysis of clots ($5.7 \pm 0.8 \text{ min}$, $P < 0.001$ vs. $24 \pm 1.5 \text{ min}$) (Figure 6B and C, and Online Supplementary Video S1). Similarly, tPA-mediated lysis of PRP clots, monitored by change in absorbance, revealed sig-

nificantly faster 50% lysis times on inclusion of the neutralizing antibody to PAI-1 (96 ± 3.2 vs. 119 ± 3.5 min, respectively; $P < 0.001$, $n = 3$). A control polyclonal rabbit IgG had no effect (*data not shown*). Thrombodynamic analysis of PRP clots revealed a faster rate of clot formation and a reduction in clot density (Table 1). A significant enhancement of lysis was observed when PAI-1 was inhibited (Table 1 and *Online Supplementary Video S2*).

Lysates of activated platelets stabilized thrombi formed under arterial flow rates against premature lysis (Figure 7A).

Our activity data (Figure 4) revealed that there were two pools of functional PAI-1, therefore the contribution of the cellular and soluble fractions of platelets to thrombus stability were analyzed. Inclusion of soluble and cellular platelet fractions during thrombus formation resulted in a 2-fold and 2.7-fold reduction in lysis, respectively, compared to a 3-fold reduction on inclusion of whole platelet lysate (Figure 7A). Incorporation of a neutralizing antibody to PAI-1 completely attenuated the stabilizing effect of the platelet lysate and soluble fraction on thrombus lysis (Figure 7B and

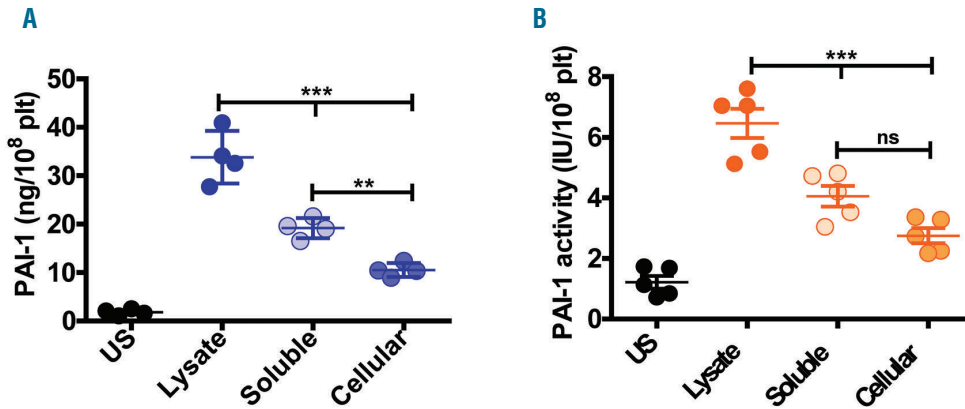


Figure 4. Active platelet-derived plasminogen activator inhibitor 1 (PAI-1) is retained within the cellular fraction of stimulated platelets. Platelets (2.5×10^8 plt/mL) were left unstimulated (US) or activated with $1 \mu\text{g/mL}$ CVX and 100 nM thrombin for 30 minutes at 37°C . Total platelet lysate or soluble and cellular fractions separated by centrifugation post-stimulation were analyzed for (A) PAI-1 protein ($n = 4$) or (B) PAI-1 activity ($n = 5$). Data are expressed as mean \pm standard deviation. $***P < 0.001$, $**P < 0.01$ versus whole platelet lysate; ns: not significant.

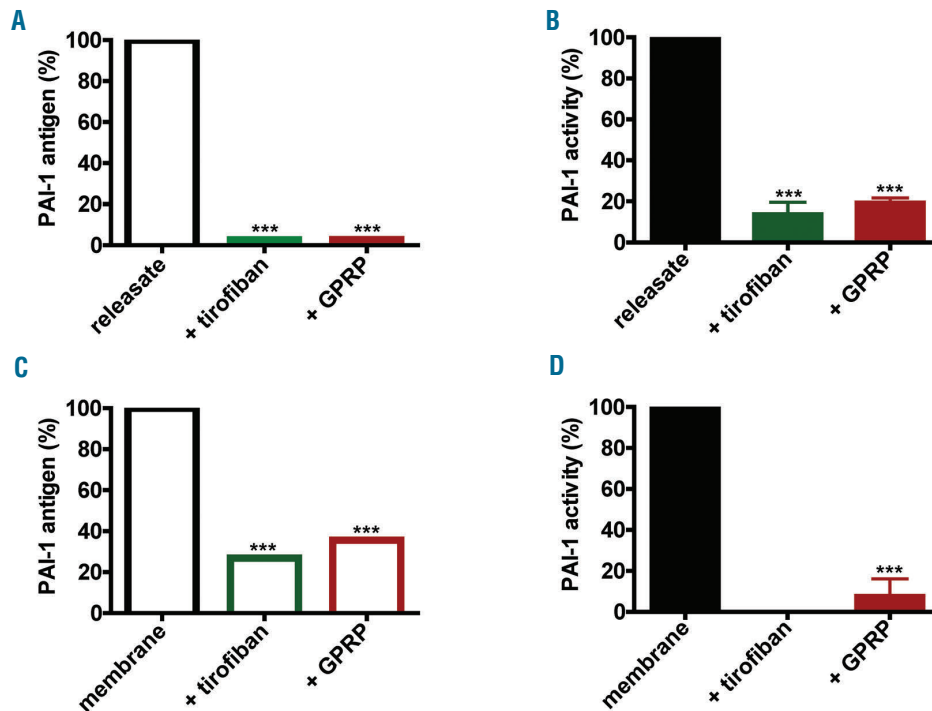


Figure 5. Plasminogen activator inhibitor 1 (PAI-1) release and retention on the activated platelet membrane is dependent on functional $\alpha_v\beta_3$ and fibrin. Platelets were activated with $1 \mu\text{g/mL}$ CVX and 100 nM thrombin for 30 minutes at $37^\circ\text{C} \pm$ pre-treatment with tirofiban ($1 \mu\text{g/mL}$) or GPRP (5 mM). Soluble and cellular fractions were isolated by centrifugation. PAI-1 antigen and activity levels were quantified by ELISA and activity assays. (A and B) Antigen and activity in the soluble fraction. (C and D) Antigen and activity in the cellular fraction. Data are expressed as mean \pm standard deviation. $n \geq 4$. $***P < 0.001$.

C). In contrast, the antibody only partially abrogated the stabilizing effect of the cellular fraction, suggesting that additional factors on the platelet membrane contribute to thrombus stabilization (Figure 7D).

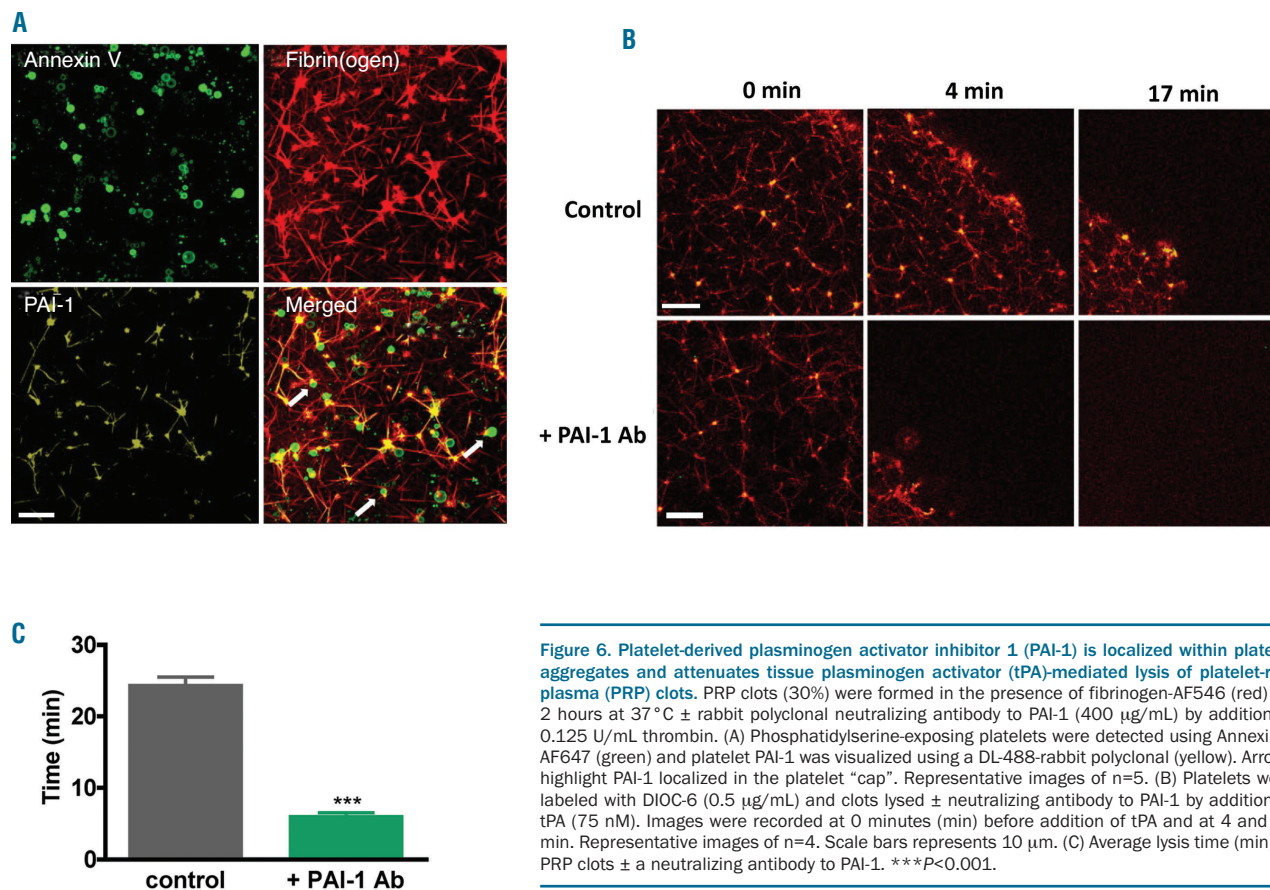
Discussion

Platelets are well known to be the primary circulating source of the fibrinolytic inhibitor PAI-1. Despite this the fate of the inhibitor following stimulation and degranulation of platelets is poorly defined. To our knowledge this is the first study to show that functional PAI-1 is retained on the activated platelet membrane following stimulation where it functions to regulate local fibrinolysis. Strong dual agonist stimulation of platelets maximizes PAI-1 exposure on the activated platelet membrane. PAI-1 was localized in the aminophospholipid-rich ‘cap’ of PS-exposing platelets, and over the granulome of spread platelets. There was evident co-localization of PAI-1 with its co-factor Vn and fibrinogen. Our data are also the first to show that the retention and release of platelet PAI-1 is dependent on integrin $\alpha_{IIb}\beta_3$ and fibrin, alluding to the importance of this inhibitor in fibrin stabilization. In accordance with this we have utilized several functional models of fibrinolysis to reveal a crucial role for platelet-derived PAI-1 in stabilizing thrombi against premature degradation.

Our lab and others have previously reported the accumulation of hemostatic and adhesive proteins within a small (~1 μm) concave cap area on PS-exposing platelets,

these include platelet-derived factor XIII-A,³⁸ plasminogen,³⁹ fibrinogen, thrombospondin⁴⁹ and coagulation factors such as prothrombin, factor V and factor X.⁵⁰ We have shown that PAI-1 was localized within the ‘cap’, alongside its co-factor Vn. Pre-treating platelets with tirofiban or GPRP to inhibit $\alpha_{IIb}\beta_3$ and fibrin polymerization, down-regulated PAI-1 but not Vn exposure on the activated platelet membrane. Interestingly, PAI-1 and Vn are reportedly not in complex within α -granules, instead PAI-1 is stabilized by calcium which is thought to mask the Vn binding site.⁵¹ These data imply that the PAI-1/Vn complex must form subsequent to platelet activation to permit PAI-1 interaction with fibrin.¹⁸

An important observation in this study is that only approximately 60% of total platelet-derived PAI-1 was released into the soluble fraction, often termed platelet releasate, while the remaining 40% was associated with the cellular fraction and was found to be functionally active. These data are consistent with findings that platelet-derived PAI-1 is more active than previously described.⁵² The discrepancy between our study and older literature^{20,21} is most likely accounted for by variations in experimental set-up, in particular the activation status of the platelets following strong dual agonist stimulation. Platelets harbor mRNA for PAI-1 and are thought to be capable of *de novo* synthesis of the inhibitor.⁵³ Interestingly, the rate of synthesis of platelet PAI-1 increases 25% over 24 h, post-stimulation with thrombin, and the serpin is found within an active conformation.⁵³ Inclusion of tirofiban and GPRP prior to platelet activation essentially abolished PAI-1 antigen and activity in the sol-



uble and cellular fraction, suggesting that release of active PAI-1 from α -granules and its retention on the platelet surface is dependent on $\alpha_{IIb}\beta_3$ and polymerized fibrin. This could arise due to an outside-in signaling mechanism whereby binding of extracellular fibrin(ogen) to $\alpha_{IIb}\beta_3$ mediates intracellular signaling events that trigger granule secretion and translocation of PAI-1 to the outer leaflet of the membrane.⁵⁴

We have clearly shown that addition of whole platelet lysate or soluble and cellular fractions, derived post-stimulation, stabilize thrombi formed under flow against lytic degradation. Neutralizing PAI-1 completely abolished the stabilizing effect of the soluble fraction, attributing it to PAI-1 inhibitory activity. The cellular fraction had a stronger stabilizing effect on thrombi which could not be completely alleviated by inhibition of PAI-1, indicating that additional factors on the platelet membrane contribute to thrombus resistance. Our work has previously shown that platelet-derived FXIII-A is retained on the activated platelet-membrane and stabilizes thrombi against premature degradation in an α 2AP-dependent manner.⁵⁸ Consistent with our results a recent study using a novel inhibitor, PAItrap, in a laser-induced vascular injury mouse model showed a significant reduction in platelet accumulation and thrombus formation but did not impact on global hemostasis.⁵⁵ Interestingly, in addition to the significant impact that neutralization of PAI-1 has on fibrinolysis we show using thrombodynamic analysis a trend toward altered clot growth. Studies in PAI-1 deficient mice reveal markedly prolonged time to occlusion in arterial and venous mouse models of injury.⁵⁶ A significant, but less pronounced effect, on occlusion was observed in Vn deficient mice. This sug-

gests that neutralization of PAI-1 during clot formation tilts the hemostatic balance toward fibrin lysis rather than fibrin formation. Collectively, these data highlight the huge potential of targeting fibrinolytic inhibitors in terms of modulating thrombus formation, propagation and stability.

It is now well documented that thrombi formed *in vivo* exhibit a hierarchical structure, with two distinct regions of platelet activation.^{26,57-60} The inner core is rich in fibrin(ogen) and thrombin and is comprised of tightly packed degranulated platelets. This is encapsulated by an outer shell of loosely packed platelets with minimal α -granule release.⁵⁷ A role for $\alpha_{IIb}\beta_3$ outside-in signalling has been described in consolidation of the platelet mass, indicating the key role of these signaling events in platelet packing, interplatelet molecular transport, agonist distribution, and subsequent platelet activation.⁶¹ Our studies reveal that PAI 1 exposure on platelets is highly dependent on $\alpha_{IIb}\beta_3$ and fibrin, suggesting that these signaling mechanisms may mediate solute transport of PAI-1 within the micro-environment of the thrombus.

There are currently no drugs in clinical trials that target fibrinolytic inhibitors, including PAI-1. Several approaches have been reported in the literature, including the use of a diabody directed against PAI-1 and TAFIa,⁶² monoclonal antibodies to PAI-1 and TAFIa,⁶³ PAItrap, an antagonist based on a variant of uPA,⁵⁵ and an inhibitory hexapeptide that corresponds to amino acids 350-355 of PAI-1.⁶⁴ These compounds demonstrate strong profibrinolytic capacity in various mouse models of ischemic stroke and thromboembolism without an increase in global bleeding. However, none have progressed further into phase II clinical trials. Our novel data

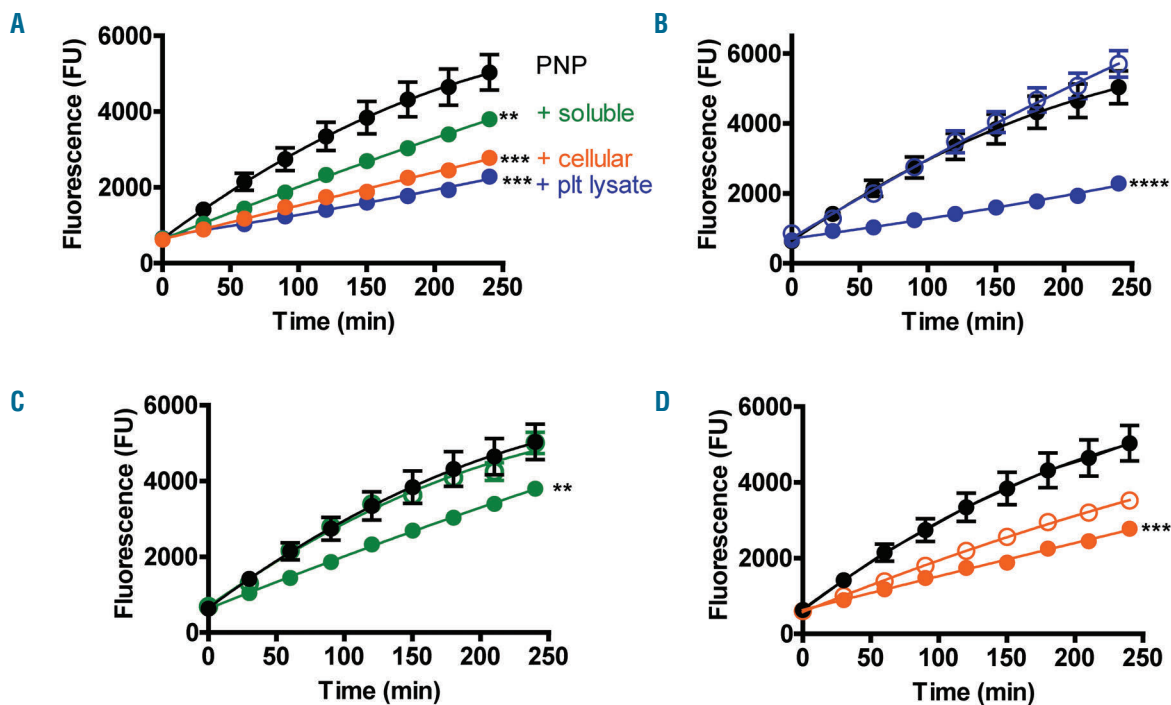


Figure 7. Platelet-plt)-derived plasminogen activator inhibitor 1 (PAI-1) stabilizes thrombi against premature fibrinolytic degradation. Platelets (2.5×10^9 plt/mL), were activated with thrombin (100 nM) and CVX (1 μ g/mL) for 30 minutes at 37 °C. Soluble and cellular fractions were isolated from stimulated platelets by centrifugation. Model thrombi were formed with pooled normal plasma (PNP) and FITC-labeled fibrinogen under arterial shear rates and lysis was subsequently induced by tissue plasminogen activator (tPA) (1 μ g/mL). Fluorescence release is directly proportional to the degree of fibrinolysis. (A) Pooled normal plasma (PNP) thrombi formed \pm whole platelet lysate (blue), soluble (green) or cellular (orange) fractions. (B-D) Thrombi were formed in the absence (closed symbols) and presence (open symbols) of a neutralizing antibody to PAI-1 (400 μ g/mL) with (B) whole platelet lysate (C) platelet soluble fraction, and (D) platelet cellular fraction. Data represent mean \pm standard error of mean. ** $P < 0.01$, *** $P < 0.001$; , **** $P < 0.0001$ versus plasma thrombi. n=5.

are the first to show that platelet-associated PAI-1 is functionally active and functions to maintain thrombus integrity. These results underscore the potential of PAI-1 as a target for novel profibrinolytic drugs to augment thrombus dissolution *in vivo*.

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