Deubiquitinases Modulate Platelet Proteome Ubiquitination, Aggregation, and Thrombosis

Nilaksh Gupta, Wei Li, Thomas M. McIntyre

Objective—Platelets express a functional ubiquitin–proteasome system. Mass spectrometry shows that platelets contain several deubiquitinases, but whether these are functional, modulate the proteome, or affect platelet reactivity are unknown.

Approach and Results—Platelet lysates contained ubiquitin–protein deubiquitinase activity hydrolyzing both Lys$_{48}$ and Lys$_{63}$ polyubiquitin conjugates that was suppressed by the chemically unrelated deubiquitinase inhibitors PYR41 and PR619. These inhibitors acutely and markedly increased monoubiquitination and polyubiquitination of the proteome of resting platelets. PYR41 (intravenous, 15 minutes) significantly impaired occlusive thrombosis in FeCl$_3$-damaged carotid arteries, and deubiquitinase inhibition reduced platelet adhesion and retention during high shear flow of whole blood through microfluidic chambers coated with collagen. Total internal reflection microscopy showed that adhesion and spreading in the absence of flow were strongly curtailed by these inhibitors with failure of stable process extension and reduced the retraction of formed clots. Deubiquitinase inhibition also sharply reduced homotypic platelet aggregation in response to not only the incomplete agonists ADP and collagen acting through glycoprotein VI but also to the complete agonist thrombin. Suppressed aggregation was accompanied by curtailed procaspase activating compound-1 binding to activated IIb/IIIa and inhibition of P-selectin translocation to the platelet surface. Deubiquitinase inhibition abolished the agonist-induced spike in intracellular calcium, suppressed Akt phosphorylation, and reduced agonist-stimulated phosphatase and tensin homolog phosphatase phosphorylation. Platelets express the proteasome-associated deubiquitinases USP14 and UCHL5, and selective inhibition of these enzymes by b-AP15 reproduced the inhibitory effect of the general deubiquitinase inhibitors on ex vivo platelet function.

Conclusions—Remodeling of the ubiquitinated platelet proteome by deubiquitinases promotes agonist-stimulated intracellular signal transduction and platelet responsiveness. (Arterioscler Thromb Vasc Biol. 2015;35:2657-2666. DOI: 10.1161/ATVBAHA.115.306054.)

Key Words: blood platelets ■ proteasome endopeptidase complex ■ thrombosis ■ ubiquitin ■ ubiquitin-specific protease

Platelet activation underlies thrombotic cardiovascular disease, responsible for significant death and disability in the developed world, and modulation of platelet activation is an established therapeutic target for these diseases. Platelets are anucleate and lack transcription, so their activation is transduced by the proteome delivered to them during thrombopoiesis, although this is augmented by limited de novo protein synthesis. Conversely, inhibitors show that platelets also possess a limited ability to reduce their proteome through the ubiquitin–proteasome proteolytic system that participates in their production during thrombopoiesis and contributes to the functions of activated cells. Analysis of the platelet proteome by quantitative mass spectrometry not only identifies the expected components of the ubiquitin ligase system but also identifies deubiquitinases at high copy number. These enzymes might modify the pattern of ubiquitin chains conjugated to the platelet proteome, but this is unstudied.

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Covalent modification of proteins with ubiquitin is dynamic and reversible with 6 families of evolutionarily conserved deubiquitinases hydrolyzing these monomeric and polymeric ubiquitin–protein adducts. Deubiquitinases are isopeptidases that play pivotal roles in ubiquitin-mediated signaling pathways, and deubiquitinase inhibitors alter diverse cellular functions, as anticipated from the range of processes using ubiquitin addition. Accordingly, some deubiquitinase inhibitors have therapeutic potential. The general deubiquitinase inhibitor PR619 promotes autophagy, protein aggregation, and the unfolded protein response in nucleated cells. A small-molecule inhibitor of E1 ubiquitin–activating enzyme,
The novel small-molecule inhibitor PYR41 and PR619 (Figure 1C) polyubiquitin chains. The deubiquitinase inhibitors, PYR41 and PR619, protected some of the short K48 polymers from proteolysis but not the K63 polymer. Treating intact platelets for 30 minutes with either PYR41 or PR619 increased the content of polyubiquitin-conjugated proteins relative to pattern of conjugation found in quiescent platelets (Figure 1E). The increase in decorated proteins was apparent for most of the protein bands but was prominent in the more slowly migrating proteins that no longer appeared as distinct bands. Thus, inhibition of deubiquitinase activity shows that quiescent platelets must rapidly and continuously cycle ubiquitin through polymeric ubiquitin chains adducting select proteins.

### Deubiquitination of the Platelet Proteome Promotes Thrombosis

We investigated whether deubiquitinase inhibition altered platelet function during arterial thrombosis created by injuring the wall of murine carotid arteries in situ with FeCl3. This damage results in rapid platelet accretion with formation of a platelet-rich occlusive barrier at the site of injury. Typically, complete cessation of flow through the artery occurred 12 minutes after the brief exposure to ectopic FeCl3 in animals treated with the dimethyl sulfoxide vehicle (Figure 2A). However, disruption of ubiquitin metabolism by intravenous injection of PYR41 15 minutes before vessel injury significantly lengthened the time to occlusion to 26 minutes, consistent with the delay induced by inhibition of the platelet proteasome.

We modeled platelet accretion ex vivo by flowing whole human blood through a collagen-coated microfluidic channel that generates high shear. Fluorescently labeled platelets in whole blood were immobilized along the length of the chamber, as shown in a typical video frame captured at the distal end of the chamber after 3 minutes of flow (Figure 2B; Video I in the online-only Data Supplement). Preincubating blood with either PYR41 or PR619 reduced the number of platelets adhering in the collagen-coated chamber by 80% (Figure 2C), and this difference was significant (P<0.05). These outcomes show that the adhesive phenotype of activated platelets depends on rapid deubiquitination of their proteome.

### Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

### Results

#### Platelets Express Functional Deubiquitinases

The platelet proteome is decorated with polyubiquitin chains that direct select proteins to the proteasome for degradation after activation, suggesting that the pattern of ubiquitin conjugation should be dynamic. We treated platelet lysates with HA-tagged ubiquitin vinyl sulfone, which covalently adds the active deubiquitinases during hydrolysis of this isopeptidase substrate, to determine whether platelets contain enzymes that actively modify conjugated polyubiquitin chains. We found that platelets contain ≤10 differently sized proteins labeled by this mechanism-based deubiquitinase inhibitor (Figure 1A). The general deubiquitinase inhibitor PYR41 and a structurally unrelated deubiquitinase inhibitor PR619 decreased catalytic labeling of each of these active platelet deubiquitinases. We directly assayed polyubiquitin deconjugating enzymatic activity in platelet lysates to find whether platelets expressed ubiquitin conjugate isopeptidase enzymatic activity that was inhibited by PYR41 and PR619 (Figure 1B).

Polyubiquitin chains most commonly polymerize through K48, and in general, these proteins are then recognized by the proteasomal system. Alternatively, polymerization through K63 generally alters protein distribution. Deubiquitinases are selective for these isomeric ubiquitin chain linkages, and platelet lysates catalyzed rapid disassembly of heptameric K48-linked (Figure 1C) and K63-linked (Figure 1D) polyubiquitin chains. The deubiquitinase inhibitors, PYR41 and PR619, protected some of the short K48 polymers from proteolysis but not the K63 polymer. Treating intact platelets for 30 minutes with either PYR41 or PR619 increased the content of polyubiquitin-conjugated proteins relative to pattern of conjugation found in quiescent platelets (Figure 1E). The increase in decorated proteins was apparent for most of the protein bands but was prominent in the more slowly migrating proteins that no longer appeared as distinct bands. Thus, inhibition of deubiquitinase activity shows that quiescent platelets must rapidly and continuously cycle ubiquitin through polymeric ubiquitin chains adducting select proteins.

### Deubiquitinase Inhibition Impairs the Function of Isolated Platelets

We purified and washed human platelets and then assayed a range of functions to determine whether deubiquitinase inhibitors directly act on platelets to alter their function and not just nucleated cells of the vasculature or blood. Aggregometry showed that aggregation induced by the incomplete agonists ADP (Figure 3A) or collagen (Figure 3B) was strongly suppressed by PYR41 and abolished after pretreatment with PR619. These 2 inhibitors had an identical effect on the more robust aggregation induced by the complete agonist thrombin (Figure 3C). These effects were stimulus-dependent because neither compound by itself promoted aggregate
formation (Figure 3D). The reduction of stimulated adhesion by each deubiquitinase inhibitor was statistically significant (Figure 3E–3G). Both PYR41 and PR619 suppressed platelet adhesion and the formation and extension of pseudopods in response to thrombin stimulation (Video 2 in the online-only Data Supplement). The inhibitory effect of PYR41 and PR619 on deubiquitinase activity in platelet lysates was concentration dependent and correlated with their level of thrombin-induced aggregation (Figure IA and IB in the online-only Data Supplement). The inhibitory effect of PYR41 and PR619 was optimal at 0.2 U of thrombin and was lost at higher concentrations (data not shown).

Outside-in signaling induces cytoskeletal rearrangement, with extension of filopodia and lamellapodia, when platelets interact with a glass substrate. Thrombin stimulation enhances these responses, which was apparent in individual video frames captured by total internal reflection microscopy of fluorescently labeled platelets (Figure 3H; Video II in the online-only Data Supplement). Both PYR41 and PR619 abolished the adhesion and spreading of unstimulated platelets, while significantly (Figure 3I) reducing these responses after thrombin stimulation.

Agonist Activation of Glycoprotein αIIbβ3 Is Reduced by Deubiquitinase Inhibition

Agonist stimulation alters the conformation of the platelet β3 integrin αIIbβ3 glycoprotein complex that enhances platelet–platelet and platelet–endothelial cell interactions. 22 The antibody procaspase activating compound-1 (PAC-1) recognizes a neoeptope in activated αIIbβ3, and thrombin stimulation greatly increased PAC-1 binding to the surface of washed human platelets compared with quiescent control cells (Figures 4A). Pretreating platelets with PYR41 for 15 minutes suppressed PAC-1 binding to such an extent that αIIbβ3 activation was not statistically distinct from unstimulated platelets (Figure 4B). PR619 also effectively reduced thrombin-stimulated expression of PAC-1 (Figure 4E and 4F). Inhibition of deubiquitinase activity by either PYR41 (Figure 4C and 4D) or PR619 (Figure 4G and 4H) also fully suppressed thrombin stimulation of α-granule secretion detected by the translocation of P-selectin from these granules to the platelet surface. Thrombin at the concentration used above activates platelets primarily through the protease-activated receptor-1, but activation of platelets through a second G-protein–coupled receptor, the P2Y12 receptor for ADP, was also abolished by
Deubiquitinase inhibition (Figure II in the online-only Data Supplement). ADP stimulation increased binding of PAC-1 to platelets (Figure IIA in the online-only Data Supplement), although not to the extent induced by thrombin, and PYR41 effectively reduced the extent of \( \alpha_{IIb}\beta_{3} \) activation after ADP activation to nearly quiescent levels (Figure IIB in the online-only Data Supplement). PYR41 pretreatment also suppressed P-selectin exocytosis after ADP stimulation (Figure IC in the online-only Data Supplement), and this reduction was statistically significant (Figure IID in the online-only Data Supplement). PR619 was equally effective in suppressing platelet integrin activation (Figure IIE and IIF in the online-only Data Supplement).

Proteasome-Associated Deubiquitinases Regulate Platelet Activation

Platelets not only contain several cytoplasmic deubiquitinas but also abundantly express the proteasome-associated deubiquitinases USP14 and UCHL5 at 3100 and 1500 copies per platelet, respectively.\(^7\) Specific inhibitors of USP14 and UCHL5 have been developed since the levels of these 2 enzymes are upregulated in many cancers\(^23\) and are considered to be targets for potential anticancer therapies. A small-molecule inhibitor, b-AP15, was recently identified\(^17\) that specifically inhibited just these 2 proteasome-bound deubiquitinases without affecting the catalytic activities of soluble deubiquitinases. This inhibitor is potent and available to intracellular enzyme with b-AP15 inhibiting hydrolysis of fluorogenic ubiquitin substrate by the 19S subunit of the proteasome with an IC50 of just 6.5 µmol/L.\(^24\) We treated washed platelets with b-AP15 to determine whether this inhibitor induced the accumulation of high–molecular weight ubiquitin-conjugated proteins (Figure 5A). We confirmed that platelets contained both USP14 and UCHL5 targets of b-AP15 by recovering platelet proteasomes by high-speed centrifugation and then Western blotting the proteins of the isolated proteasome (Figure 5B).

Next, we determined whether USP14 and UCHL5 regulated platelet function to find whether b-AP15 inhibited thrombin- (Figure 5C), collagen- (Figure 5D), and ADP- (Figure 5E) induced aggregation. The inhibitory effect of b-AP15 on agonist-induced platelet aggregation was significant for each of these agonists (Figure 5F), and the data show that platelet aggregation was not affected by b-AP15 treatment alone. The small-molecule inhibitor b-AP15 also inhibited thrombin-induced \( \alpha_{IIb}\beta_{3} \) activation detected by PAC-1 binding (Figure 5G), as well as P-selectin exocytosis from platelet \( \alpha \)-granules (Figure 5H). Pretreatment with b-AP15 also blocked ADP-induced \( \alpha_{IIb}\beta_{3} \) activation (Figure IIIA in the online-only Data Supplement). USP14 and UCHL5 inhibition had a more profound effect on collagen-induced aggregation.

Figure 2. Deubiquitinase inhibitors suppress platelet activation and thrombosis. A, Deubiquitinase inhibitor PYR41 prolongs the time to vascular occlusion. Mice were injected with PYR41 or dimethyl sulfoxide (DMSO), and thrombosis was induced by application of \( \text{FeCl}_3 \) 15 minutes later to a surgically exposed murine carotid artery as described in Methods section. Time to complete cessation of blood flow in the murine carotid artery was determined using intravital microscopy (n=5 experimental and 3 control; \( ** P \leq 0.01 \)). B, PYR41 or PR619 pretreatment blocked platelet adhesion to collagen at high shear. Calcein AM–labeled blood, treated or not with PYR41 or PR619, was perfused over immobilized type 1 collagen fibrils (150 µg/mL) at 67.5 dyne/cm\(^2\) for 3 minutes. Images are representative fields taken from 3 independent experiments that yielded similar results (n=3). C, Area of platelet attachment after PYR41 or PR619 treatment. Platelet area in (B) was quantified by ImagePro plus software, and results are plotted as area of platelet adhesion in square microns (n=3; \( *** P \leq 0.001 \)).
than the general deubiquitinase inhibitors, so we tested the effect of inhibition of these enzymes on platelet adhesion to collagen under static conditions. b-AP15 pretreatment reduced the number of platelets adhering to a exposed collagen-coated surface (Figure IIIB in the online-only Data Supplement), suggesting a vital role of proteasome-associated deubiquitinases in mediating platelet interactions with exposed collagen.

**Inhibition of Platelet Deubiquitinases Modulates Signaling Downstream of Thrombin and Collagen Receptors**

Akt (protein kinase B) is a serine/threonine kinase that is an established downstream effector of phosphatidylinositol 3-kinase (PI3K). Platelets express 2 Akt isoforms, Akt1 and Akt2, where targeted deletion of Akt1 diminishes responses to both thrombin and collagen, whereas Akt2 deletion suppresses secretion and thrombosis. We determined whether deubiquitinase activity modulated Akt activation. Stimulation with thrombin resulted in time-dependent increase in Akt phosphorylation at both serine473 and threonine308 residues, which was reduced by pretreatment with either PYR41, PR619, or b-AP15 (Figure 6A). Pretreating platelets with any of the 3 deubiquitinase inhibitors resulted in equivalent reduction of collagen-induced phosphorylation of Akt serine473 and threonine308 (Figure 6B), although the inhibition of threonine308 phosphorylation by either agonist was more profound than the reduction at serine473. In platelets, Akt activation is both PI3K dependent and independent, but sustained Akt activation is PI3K dependent.
PI3K isoform-β is essential to promote PLCγ-2 activation downstream of glycoprotein VI–FcγRIIγ receptor that leads to intracellular calcium release critical for ex vivo clot retraction and consolidation in vivo. Collagen-induced intracellular calcium release was inhibited when platelets were preincubated with deubiquitinase inhibitors before stimulation by collagen (Figure 6C). Deubiquitinase inhibitors also significantly reduced fibrin clot retraction (Figure 6D), suggesting impaired inside-out signaling from integrin αIIbβ3.

Phosphatase and tensin homolog (PTEN) on chromosome 10 is a negative regulator of PI3K/Akt signaling that reduces collagen-dependent platelet activation. PTEN phosphatase activity is negatively regulated in nucleated cells by phosphorylation at serine-380, although this has not been extended yet to platelets. We observed that both thrombin and collagen increased PTEN phosphorylation at this residue (Figure 6E) and that pretreatment with deubiquitinase inhibitors reduced agonist-dependent increase in PTEN phosphorylation and inactivation.

**Discussion**

We found that protein ubiquitination in platelets is a dynamic process where acute inhibition of deubiquitinase activity caused a significant increase in ubiquitin modification of the proteome of quiescent cells within just a few minutes. This change occurred both with general deubiquitinase inhibitors and in response to b-AP15 inhibition of UCHL5 and USP14 of the platelet proteasome. Because these changes were apparent in the absence of agonist stimulation and intracellular signaling, we conclude that the proteome of quiescent platelets undergoes a continuous, and rapid, cycle of ubiquitination and deubiquitination. Notably, deubiquitinase inhibition was without detectable effect on the total content of platelet proteins (beyond release of filamin A to the soluble fraction). We found that protein deubiquitination promotes platelet reactivity. The general deubiquitinase inhibitors PYR41 or PR619 blocked inside-out activation of αIIbβ3 integrin, homotypic

**Figure 4.** Inhibitors of platelet deubiquitinases suppress protease-activated receptor-mediated αIIbβ3 activation and degranulation. A, PYR41 pretreatment suppressed stimulated αIIbβ3 activation. Flow cytometry using procaspase activating compound-1 (PAC-1) antibody, that binds active αIIbβ3, on platelets treated or not with PYR41 stimulated or not with thrombin (0.2 U; n=3). B, Mean fluorescence intensity (MFI) of PAC-1 binding to platelets in panel A (n=3; *P≤0.05). C, PYR41 pretreatment reduced P-selectin surface expression upon activation. Flow cytometry using phycoerythrin (PE)-conjugated anti–P-selectin antibody to platelets treated or not with PYR41 with or without thrombin (0.2 U; n=3) stimulation. D, Mean fluorescence intensity of anti–P-selectin binding to platelets in panel C (n=3; *P≤0.05). E, PR619 pretreatment suppressed thrombin-stimulated αIIbβ3 activation. Flow cytometry using PAC-1 antibody binding to platelets treated or not with PR619 with or without 0.2 U thrombin (n=3). F, Mean fluorescence intensity of PAC-1 binding to platelets in panel 6E (n=3; **P≤0.01). G, PR619 pretreatment reduced P-selectin surface expression after thrombin activation. Flow cytometry using PE-conjugated anti–P-selectin antibody binding to platelets treated or not with PR619 with or without thrombin (0.2 U; n=3). H, Mean fluorescence intensity of anti–P-selectin binding to platelets in (G) (n=3; **P≤0.01).
aggregation, and inhibited adhesion to collagen under flow at high shear. PYR41 additionally blocked the formation of occlusive platelet-rich thrombi in damaged carotid arteries. Inhibition of deubiquitinase activity suppressed the extension of membrane protrusions and cell spreading, and deubiquitinase inhibition blocked α-granule release and hence blocked the agonist-stimulated increase of P-selectin on the platelet surface. Continual remodeling and turnover of ubiquitin modification of platelet proteins is thus a positive element in the transition from quiescent cells to activated platelets that is necessary for thrombosis. We conclude that the widespread suppression of stimulated platelet function by deubiquitinase inhibition means that ubiquitin modification of the proteome must maintain platelets in an inactive state and that either restructuring of the existing polyubiquitin decoration of the platelet proteome or that recycled ubiquitin is produced to allow its addition to new targets—Western blotting shows that intracellular free ubiquitin is limiting—releases tonic ubiquitin inhibition of platelet signaling and activation.

Figure 5. Pharmacological inhibition of the proteasome-associated deubiquitinases USP14 and UCHL5 reduces platelet responsiveness to agonists. A, b-AP15 pretreatment increased ubiquitination of the platelet proteome. Immunoblot of platelet lysates, treated or not with b-AP15, probed with antiubiquitin antibody (P4D1, n=3). B, Platelet proteasomes contained UCH14 and UCHL5. Western blot of platelet proteasomes recovered by high-speed centrifugation (178,000g; 2 hours). C, Proteasome-associated deubiquitinases promote stimulated platelet aggregation. b-AP15 pretreatment inhibited thrombin- (C; n=3), collagen- (D; n=3), and ADP-induced aggregation (E; n=3). F, b-AP15-mediated inhibition of stimulated platelet aggregation is statistically significant (n=3; **P<0.01, ***P<0.001, and *P<0.05). G, b-AP15 pretreatment suppressed stimulated αIIbβ3 activation. Flow cytometry using procaspase activating compound-1 (PAC-1) antibody binding to platelets treated or not with b-AP15 and stimulated or not with 0.2 U of thrombin (n=3). H, b-AP15 pretreatment reduced P-selectin surface expression on activation. Flow cytometry using phycoerythrin (PE)–conjugated anti–P-selectin antibody binding to platelets treated or not with b-AP15 with or without 0.2 U of thrombin (n=3).
Deubiquitinase inhibitors affected signaling downstream of G-protein-coupled receptors and the glycoprotein VI receptor for collagen. Thrombin- and collagen-induced Akt phosphorylation was reduced not only by the pan deubiquitinase inhibitors PYR41 and PR619 but also by the specific inhibitor of proteasome-associated deubiquitinase activity, b-AP15. Activation of PI3K and its effector, Akt, is essential in most aspects of collagen-induced platelet activation and thrombin- and collagen-induced $\alpha_{IIb}\beta_3$ integrin outside-in signaling. The effect of PI3K/Akt axis on thrombin-induced aggregation is only seen at lower thrombin concentrations and is overcome at higher agonist concentrations.\(^\text{33,40}\) Akt activation in platelets is both PI3K dependent and PI3K independent\(^\text{41}\); however, PI3K is isoform- $\beta$ is mandatory for glycoprotein VI–Fcr$\gamma$ receptor–mediated Akt activation, platelet aggregation, intracellular calcium release, and degranulation.\(^\text{42}\) We found that ubiquitin remodeling is an integral component of this cascade.

We attribute at least part of the reduction in phosphorylated Akt after pretreatment with deubiquitinase inhibitors to the upregulation of PTEN phosphatase activity. PTEN negatively regulates Akt activation, which negatively regulates collagen-induced platelet activation.\(^\text{47}\) by converting phosphatidylinositol 3,4,5-trisphosphate to phosphatidylinositol 4,5-bisphosphate. Phosphoinositide-dependent protein kinase-1 is activated by an increase in phosphatidylinositol 3,4,5-trisphosphate after PI3K activation, and activated phosphoinositide-dependent protein kinase-1 phosphorylates Akt at residue threonine $\text{Thr}^{308}$.\(^\text{43}\) Platelet-specific knockdown of phosphoinositide-dependent protein kinase-1 and subsequent loss in Akt phosphorylation reduces thrombin-induced platelet aggregation, clot retraction, and thrombosis.\(^\text{48}\) This central...
phosphorylation cascade, we have found, includes ubiquitin chain remodeling.

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Disclosures
None.

References
Platelet proteins are modified by ubiquitination, which is enhanced in stimulated platelets. Platelets express functional deubiquitinase activity, whose inhibition rapidly increased the ubiquitination of the platelet proteome and blocked agonist-stimulated adhesion, spreading, and stimulated aggregation. Intravascular injection of a deubiquitinase inhibitor slowed the formation of occlusive platelet thrombi in damaged carotid arteries. General deubiquitinase inhibitors, or selective inhibition of proteasome-associated deubiquitinases, reduced signaling through the kinase cascade and blocked the increase in intracellular Ca++ necessary for platelet activation. Therefore, deubiquitination is necessary for platelet activation and is a drugable target that would reduce thrombosis.

**Significance**
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Supplemental Material

Deubiquitinases modulate platelet proteome ubiquitination, aggregation, and thrombosis

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Supplemental Figure I. Concentration response of deubiquitinase inhibition and platelet aggregation

(A) Platelet deubiquitinase activity was assayed using luminogenic substrates in lysates from untreated platelets and platelets treated with different concentrations of PYR41 and PR619 (0, 3, 10, and 25 (PYR41) or 30 (PR619) µM (n=2) (B) Effect of dose dependent inhibition of thrombin (0.2 U) induced aggregation was assessed at indicated concentrations of deubiquitinase inhibitors (n=3).
Supplemental Figure II. Deubiquitinase inhibitors diminish ADP induced platelet αIIbβ3 activation and degranulation

(A) PYR41 pretreatment suppressed stimulated αIIbβ3 activation. Flow cytometry using PAC-1 antibody, that binds active αIIbβ3, on platelets treated or not with PYR41 and with or without 25 µM ADP (n=4). (B) Mean fluorescence intensity (MFI) of PAC-1 binding on platelets in panel A (n=4; *p ≤ 0.01). (C) PYR41 pretreatment reduced P-selectin surface expression upon activation. Flow cytometry using PE-conjugated anti-P-selectin antibody on platelets treated or not with PYR41 and with or without 25 µM ADP (n=3). (D) Mean fluorescence intensity (MFI) of P-selectin binding on platelets in panel C (n=3; *p ≤ 0.05). (E) PR619 pretreatment suppressed stimulated αIIbβ3 activation. Flow cytometry using PAC-1 antibody on platelets treated or not with PR619 and with or without 25 µM ADP (n=3). (F) Mean fluorescence intensity (MFI) of PAC-1 binding on platelets in panel E (n=3; *p ≤ 0.05).
Supplemental Figure III. b-AP15 reduce ADP-induced αIIbβ3 activation and platelet adhesion to collagen
(A) Preincubation with b-AP15 suppressed ADP-induced αIIbβ3 activation. Flow cytometry using PAC-1 antibody of platelets treated or not with b-AP15 and with or without 25 µM ADP (n=3). (B) Proteasome-associated deubiquitinases mediate platelet adhesion to collagen-coated surface. Microscopic images of platelet adhesion on collagen under static conditions pre-treated or not with the b-AP15 inhibitor of proteasome-associated deubiquitinase activity (n=2).

Supplemental Figure IV. Deubiquitinase inhibitors do not change the platelet proteome. Washed human platelets were treated with the stated deubiquitinase inhibitors for 30 min as in “Methods,” the cells were pelleted by centrifugation, lysed in
RIPA buffer, and the proteins resolved by gradient gel electrophoresis. The transferred proteins were detected by Coomassie blue staining and photographed.

**Platelet deubiquitinases reduce GPVI-mediated collagen responses**

Collagen binds and activates platelets through two major platelet surface receptors, α2β1 and GPVI, with GPVI being the primary signaling receptor for collagen. The three deubiquitinase inhibitors by themselves did not promote platelet aggregation (Supplementary Figure V A), but PYR41, PR619, and b-AP15 each suppressed aggregation initiated by convulxin, a snake venom toxin that activates platelets via the GPVI receptor (Supplementary Figure V B). Convulxin stimulated PAC-1 binding to platelets, a marker of αIIbβ3 activation, and pretreatment with PYR41 (Supplementary Figure V C), PR619 (Supplementary Figure V D), or b-AP15 (Supplementary Figure V E) reduced this convulxin-induced increase in PAC-1 binding. This suggest that the platelet deubiquitinase activities are critical for GPVI-mediated transduction of collagen signaling in addition to interfering with G protein coupled receptor signaling.

**Supplemental Figure V. Deubiquitinase inhibitors alter collagen responses via the GPVI receptor.** (A) Neither the general deubiquitinase inhibitors PYR41 and PR619, nor b-AP15 stimulated platelet aggregation in the absence of agonists. (B) Deubiquitinase
inhibitors inhibited GPVI specific platelet responses. Platelets were pretreated with PYR41, PR619, or b-AP15 before initiating platelet aggregation with convulxin (n=3). (C) Convulxin-mediated increase in PAC-1 binding was assessed using flow cytometry in platelets pretreated with PYR41 (n=3), (D) PR619 (n=3), or (E) b-AP15 (n=3).
Supplemental Video I, II

Platelet adhesion under flow was assessed ex vivo by flowing whole blood through a collagen-coated microfluidic channel in a Cellix VenaFluor8+ plate that generates high shear. Here the shear was 67.5 dynes for 3 minutes, with imaging at the distal end of the chamber. Preincubating blood with either PYR41 or PR619 reduced the number of platelets adhering in the collagen-coated chamber. The first clip examines control platelets, the second clip captures PYR41 treated blood and the third examines PR619-treated cells.

Supplemental Video II

Formation and extension of pseudopods in response to thrombin stimulation. Platelets were imaged by total internal reflection microscopy, as in Fig. 3, individual frames of this microscopy shown in Fig. 3.

Discussion

The proteome of platelets is constitutively modified by single ubiquitin molecules and by conjugated ubiquitin polymeric chains, with the abundance of both modifications increasing after agonist stimulation. Here we present evidence that ubiquitin decoration of platelet proteins is dynamic, and that this turnover aids agonist-induced signal transduction and activation. Ubiquitin polymerizes through isopeptide bond formation, commonly either at lysine48 or lysine63, with hydrolysis and chain remodeling catalyzed by a small subfamily of proteases with isopeptidase activity. These enzymes are considered to be novel targets for cancer therapy, which has driven development of specific inhibitors. Quantitative ITAM mass spectrometry of platelet proteins that numerically and unequivocally identifies the platelet proteome shows platelets contain members of the deubiquitinase family, but also reveals that these cells have only a limited complement of the nearly 100 such enzymes. Proteins post-translationally modified with lysine48-conjugated polyubiquitin chains are targeted for proteasomal degradation, with the ubiquitin being deconjugated and re-activated for reuse. The regulatory 19S component of the proteasome of cells with nuclei contains three such deubiquitinases, USP14, UCHL5, and RPN11, while the proteasome of platelets contain just the first two enzymes. In contrast, polyubiquitin chains can be conjugated through their lysine63 residues, but this modification alters protein distribution and trafficking, and does not target these proteins to the proteasome for degradation. Whether proteasome-associated enzymes or cytoplasmic deubiquitinases modify ubiquitin chains in platelets, or whether this process modulates platelet function has been undefined.

The compound PYR41 was identified as an inhibitor of E1 activity, which catalyzes the essential step of ubiquitin activation necessary for polymerization. However, PYR41 did not interfere with ubiquitin activation in platelet lysates (not shown) and, like b-AP15 and PR619, PYR41 caused a rapid and massive increase in ubiquitin decoration of soluble platelet proteins. This result is incompatible with E1 inhibition. Potentially this outcome indicates ubiquitin activation through the second E1 enzyme, although this protein is not detected in the platelet proteome. In any case, both the general deubiquitinase inhibitor PR619 and specific deubiquitinase inhibitor b-AP15 demonstrate
that inhibiting the deubiquitinase family of proteases suppressed platelet signaling and function, and so the similar effects of PYR41 suggest its action in platelets is as a deubiquitinase inhibitor.

Deubiquitinase remodeling of ubiquitinated platelet proteins does not result in either rapid or global changes to the platelet proteome (Supplementary Figure IV), suggesting ubiquitin remodeling does not primarily act by changing the content of platelet proteins. This conclusion is strengthened by finding\(^5\) that direct inhibition of the Ubiquitin-Proteasome System by proteasome inhibition also does not alter the platelet proteome (other than filamin A solubilization), so platelet proteins are not transient and are not constitutively turned over (Supplementary Figure VI). In contrast, the Ubiquitin-Proteasome System does impact intracellular signal transduction and platelet signaling by crossing over to modulate phosphorylation of platelet proteins and altering Ca\(^{++}\) homeostasis (Supplementary Figure VI). The target(s) of deubiquitination necessary to allow platelet activation is unknown, but could be at the level of the receptors themselves, an unidentified component of signaling complexes, or members of the signal transduction cascade themselves. Notably exogenous polyubiquitin, supplied either by polyubiquitinated exosomes or as recombinant protein, suppressed platelet reactivity and CD36 expression.\(^5\)\(^5\) Conversely, deubiquitination may be required to release sufficient ubiquitin to allow modification of chronically unmodified proteins since most ubiquitin is sequestered in the platelet proteome. Suppression of the agonist-induced spike in intracellular calcium by b-AP15, Akt activation, and phosphorylation of the PTEN phosphatase all indicate that proteasome-associated deubiquitinases contribute more to platelet reactivity than just degradation of ubiquitin-targeted proteins by the proteasome. Rather, proteasome-associated deubiquitinases are essential components of platelet reactivity that unshackle signal transducing enzymes and mediators from constitutive inactivation. Overall, these data indicate that platelet deubiquitinases modulate platelet reactivity and thrombosis, and that deubiquitinase inhibition might represent a novel class of antithrombotic agents.

References:


Supplemental Material

Deubiquitinases modulate platelet proteome ubiquitination, aggregation, and thrombosis

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Materials and Methods

Chemicals and reagents

Chemicals and reagents were purchased from: sterile filtered HBSS and sterile tissue culture plates (Falcon Labware); endotoxin-free human serum albumin (25% human albumin solution, Baxter Healthcare); PYR41 (Biogenova), PR619 (Life Sensors), b-AP15 (Sigma-Aldrich); polyubiquitin chains (Ub$_{2-7}$) K48 or K63 linked, and HA-Ubiquitin-Vinyl Sulfone (Boston Biochem); anti-ubiquitin antibody (P4D1; Santa Cruz Biotechnology, Inc.); anti-Ub K48 antibody (Millipore); anti-USP14, Cell Signaling; anti-UCHL5, Thermo Scientific; Calcein-AM (Molecular Probes®, Life Technologies); DUB-GLO™ Protease Assay kit (Promega); anti-Akt, anti-pAkt ser$_{473}$, anti-pAkt thr$_{308}$ and anti-pPTEN antibody (Cell Signaling). Other chemicals were from Sigma-Aldrich or Biomol Research Laboratories.

Platelet preparation

Human blood was drawn into acid-citrate-dextrose and centrifuged (200 x g, 20 min) without braking to obtain platelet-rich plasma in a protocol approved by the Cleveland Clinic Institutional Review Board. Purified platelets were prepared as before \textsuperscript{1}. Briefly, platelet-rich plasma was filtered through two layers of 5-µm mesh (BioDesign) to remove nucleated cells and recentrifuged (500 x g, 20 min) in the presence of 100 nM PGE$_1$. The pellet was resuspended in 50 ml PIPES/saline/glucose (5 mM PIPES, 145 mM NaCl, 4 mM KCl, 50 µM Na$_2$HPO$_4$, 1 mM MgCl$_2$, and 5.5 mM glucose) containing 100 nM PGE$_1$. These cells were centrifuged (500 x g, 20 min) and recovered platelets were centrifuged again before resuspension in 0.5% human serum albumin in HBSS.

Proteasomes were prepared from platelets ($5 \times 10^9$) mechanically lysed in 1 ml HANK’s buffer containing 10% glycerol, 1 mM ATP to stabilize proteasome structure, and protease inhibitor mix. Organelles and the cytoskeleton were cleared by centrifugation (21,000 x g, 30 min) before proteasomes were recovered by centrifugation (178,000 x g, 2h). This pellet was solubilized in RIPA buffer, and the proteins resolved in a 4 to 20% gradient gel.
Western blotting

Washed platelets (4 X 10^8/ml) were treated with 25 µM PYR41, 30 µM PR619, 10 µM b-AP15, or buffer for 30 min before platelets were pelleted (2000 X g, 10 min) and lysed using radioimmunoprecipitation assay (RIPA) lysis buffer [150 mM NaCl, 25 mM Tris (pH 7.6), 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 2 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 100 µM phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL pepstatin, and 10 µg/mL leupeptin]. Samples were kept on ice for 30 min with occasional vortexing to ensure complete lysis before centrifugation (21,000 × g, 10 min) to clear insoluble debris. Reducing SDS sample buffer was added to the resulting supernatants before the proteins were resolved by SDS-PAGE.

Deubiquitination Assay and Ubiquitin chain disassembly

DUB-Glo™ protease assay system (Promega) assayed hydrolysis of luminogenic substrates for the deubiquitinating enzymes (DUBs). Washed platelets (4 X 10^8/ml) were pretreated with or without 25 µM PYR41 or 30 µM PR619 for 30 min, pelleted and lysed in ice cold DUB buffer (50 mM Tris-HCl, pH 7.5, 0.1% NP-40, 5 mM MgCl_2, 150 mM NaCl, 10 mM DTT, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 100 µM PMSF, 1 µg/ml pepstatin, and 10 µg/µl leupeptin) and incubated with luminogenic substrate for 30 min before luminescence was measured. Ubiquitin chain disassembly was assessed by incubating recombinant polymerized heptameric K48 or K63 linked-ubiquitin chains with lysates (20 µg) from untreated, PYR41-(25 µM) or PR619-(30 µM) treated platelets for 30 min at 30 ºC followed by boiling in reducing sample buffer and resolution by SDS-PAGE. The extent of chain disassembly was assessed by western blotting using P4D1 antibody to recognize both free and polymeric ubiquitin.

Activity-based deubiquitinase labeling used washed platelets (4 X 10^8/ml) treated or not with 25 µM PYR41 or 30 µM PR619 for 30 min before lysis in ice cold DUB buffer. Lysates (20 µg) cleared by centrifugation were incubated with 200 ng HA-Ubiquitin vinyl sulfone (Boston Biochem) for 30 min at 30 ºC, boiled in reducing sample buffer, and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted with anti-HA antibody to detect covalently labeled proteins.

In vivo thrombosis

Twelve week old C57B/6 mice were anesthetized with ketamine (90 mg/kg)/xylazine (15 mg/kg) and the right jugular vein and the left carotid artery were exposed via a middle cervical incision. Platelets were labeled by injecting 100 µl of rhodamine 6G (0.5 mg/ml) in saline into the right jugular vein. PYR41 (25 µM in DMSO; 4.7 mg/kg) or DMSO vehicle (10.5 µl) was added to the above solution, and the drug was allowed to circulate for 15 min before FeCl_3 injury 2, 3. Thrombosis was induced in the left carotid artery by stripping the adventitia and placing a piece of black plastic under the vessel to reduce background fluorescence. A 1 X 2 mm piece of filter paper saturated with 7.5% FeCl_3 was applied to the carotid artery for 1 minute, the filter paper was removed, and the vessel rinsed with saline. Fluorescent thrombus formation was observed in real-time under a water immersion objective at 10 X magnification. Time to occlusive thrombosis was determined offline using video images captured with a QImaging Retigo Exi 12-bit mono digital camera (Surrey, Canada) and Streampix version 17.2 software (Norpix, Montreal, Canada). The end points were set as either cessation of blood flow for >30 seconds or no occlusion after 30 minutes (three times longer than the average occlusion time), in which case the time was recorded as 30 minutes for statistical comparison.
In vitro thrombus formation:
Microfluidic experiments were performed using the Cellix Microfluidics System (Cellix Ltd., Dublin, Ireland). Each micro channel of a Vena8 Fluoro+ biochip was coated with 15 µl of type 1 collagen (150 µg/ml) and the biochip was then placed in a humidified box, and incubated overnight at 4ºC. Each channel of the Vena8Fluoro+ biochip was washed with 1X PBS using the Mirus Nanopump before placing the biochip on the microscope stage. Images were collected using an HC Plan Apo 20X/0.7NA lens was on a Leica DMI6000 inverted microscope equipped with an environmental chamber and a Hamamatsu ImagEM cooled CCD camera. Whole blood collected from healthy volunteers was fluorescently tagged with Calcein-AM and was pretreated with or without 25 µM PYR41 or 30 µM PR619 for 30 min. After the incubation, blood was drawn through the channel at a shear rate of 67.5 dynes for 3 minutes. Images of platelets adhering to the collagen coating were captured every 2 seconds during that time. At the end of the experiment, the tube containing the blood was removed and the 1X PBS in the biochip reservoir was drawn through the channel at 5 dynes. Ten images were captured along the length of the channel during that time.

Flow cytometry
Platelet rich plasma (2 X 10^8/ml) was pretreated with either 25 µM PYR41, 30 µM PR619, or 10 µM b-AP15 for 15 min. After pretreatment, this plasma was incubated with 25 µM ADP and stained simultaneously with PAC1-FITC or CD62P-PE and isotype control antibody (BD Biosciences) for 20 min room temperature. Washed platelets (2 X 10^8/ml) were pretreated with 25 µM PYR41, 30 µM PR619, 10 µM b-AP15, or buffer for 30 min and then were incubated with 0.2 U thrombin or 100 ng convulxin. These cells were then stained simultaneously with PAC1-FITC and CD62P-PE or with isotype control for 20 min room temperature. The samples were then analyzed using flow cytometry.

Total internal reflection fluorescence (TIRF) microscopy
Washed platelets (2 X 10^8/ml) were labeled with Calcein-AM for 30 min and were incubated with 25 µM PYR41, 30 µM PR619, or buffer for 30 min before addition onto a drop of 0.2 U thrombin placed in a glass bottomed microwell (MatTek) dish. Imaging was performed at 100X with a 1.46 N.A. objective in a Leica AM TIRF MC System (Leica Microsystems, Wetzlar, Germany) equipped with an ImageEM C9100-13 EMCCD camera (Hamamatsu, Bridgewater, N.J). The 488 nm 10-mW diode laser was used for excitation and the penetration depth was set to 70 nm. Within 10 seconds of being placed on the stage, the sample was focused and a time-lapse series was initiated to collect images every 3 seconds for 5 minutes. At the end of the series, five static images were collected in both TIRF and differential interference contrast (DIC) modes of five different fields in the sample.

Aggregation
Platelet (2 X 10^8/ml) rich plasma was pretreated with either 25 µM PYR41, 30 µM PR619, or 10 µM b-AP15 for 15 min. After pretreatment, the plasma was stimulated with 5 µM ADP (Chronolog). Washed platelets (2 X 10^8/ml) were pretreated with either 25 µM PYR41, 30 µM PR619, or 10 µM b-AP15 for 30 min before stimulation with 0.2 U thrombin, 2 µg/ml Collagen or 100 ng convulxin. Platelet aggregation was measured by transmittance (Chronolog) with stirring (900 rpm).
Expression of data and statistics

All experiments were performed at least three times with cells from different donors, and all assays were performed in triplicate. The standard errors of the mean from all experiments are presented as error bars. Figures and statistical analyses were generated with Prism4 (GraphPad Software). A value of \( p \leq 0.05 \) was considered statistically significant.

REFERENCES

