Proteasome Proteolysis Supports Stimulated Platelet Function and Thrombosis

Nilaksh Gupta, Wei Li, Belinda Willard, Roy L. Silverstein, Thomas M. McIntyre

Objective—Proteasome inhibitors used in the treatment of hematologic cancers also reduce thrombosis. Whether the proteasome participates in platelet activation or function is unclear because little is known of the proteasome in these terminally differentiated cells.

Approach and Results—Platelets displayed all 3 primary proteasome protease activities, which MG132 and bortezomib (Velcade) inhibited. Proteasome substrates are marked by ubiquitin, and platelets contained a functional ubiquitination system that modified the proteome by monoubiquitination and polyubiquitination. Systemic MG132 strongly suppressed the formation of occlusive, platelet-rich thrombi in FeCl₃-damaged carotid arteries. Transfusion of platelets treated ex vivo with MG132 and washed before transfusion into thrombocytopenic mice also reduced carotid artery thrombosis. Proteasome inhibition reduced platelet aggregation by low thrombin concentrations and ristocetin-stimulated agglutination through the glycoprotein Ib-IX-V complex. This receptor was not appropriately internalized after proteasome inhibition in stimulated platelets, and spreading and clot retraction after MG132 exposure also were decreased. The effects of proteasome inhibitors were not confined to a single receptor as MG132 suppressed thrombin-stimulated, ADP-stimulated, and lipopolysaccharide-stimulated microparticle shedding. Proteasome inhibition increased ubiquitin decoration of cytoplasmic proteins, including the cytoskeletal proteins Filamin A and Talin-1. Mass spectrometry revealed a single MG132-sensitive tryptic cleavage after R₁₇₄₅ in an extended Filamin A loop, which would separate its actin-binding domain from its carboxy terminal glycoprotein Ibα–binding domain.


Key Words: cell-derived microparticles • cytoskeletal proteins • proteasome endopeptidase complex • thrombin

Proteasome inhibitors are widely used for the treatment of hematologic cancers, specifically relapsed/refractory multiple myeloma and mantle cell lymphoma; however, little is known of their impact on platelet function and hemostasis. Potentially, these agents may also target platelets because a cyclic course of the proteasome inhibitor bortezomib (Velcade) induces significant cyclic thrombocytopenia, and proteasome inhibition hastens platelet death and turnover. Bortezomib therapy also associates with reduced thrombosis. Beyond this, there is little evidence of proteasome action on the platelet proteome, platelet activation, or thrombosis.

Platelets are recruited to the vascular wall at high shear through the glycoprotein Ib (GPIb)-IX-V receptor complex, where the GPIbα subunit binds von Willebrand factor and its C-terminal cytoplasmic domain interacts with the actin-binding protein Filamin A with high affinity. This interaction tethers the receptor complex to the platelet cytoskeleton, maintaining the cytoskeletal architecture of resting platelets and those adhering in vessels at high shear rates. The GPIbα subunit also contains a high-affinity binding site for thrombin that contributes to platelet activation when thrombin concentrations are low. These interactions are essential for thrombosis. Thrombotic platelet deposition is modeled in mice by FeCl₃–induced injury of carotid arteries, resulting in rapid platelet adhesion and formation of an occlusive platelet-rich thrombus at the site of injury.

Nucleated cells remove proteins from their proteome by proteasome-catalyzed proteolysis. This multimeric complex consists of a 20S catalytic core of noncatalytic α-subunits and 3 distinct β-subunits that hydrolyze peptide bonds of unfolded protein substrates by β1 caspase-like hydrolysis, β2 tryptic hydrolysis, and β5 chymotryptic cleavage. The proteasome is capped by a 19S regulatory subunit that conducts substrate recognition, deubiquitination, unfolding, and protein translocation into the 20S core. Substrates for proteasome hydrolysis are recognized by the 19S core through the covalent conjugation of monomeric or polymeric chains of the ≈8 kDa ubiquitin to the targeted protein.
Newly released reticulated platelets express a richer proteome than older, dense platelets. In addition, platelets contain several components of the 20S proteasome core and possess at least the chymotryptic activity of the proteasome.

Here, we show the expression of a functional ubiquitin/proteasome system in platelets. By investigating the impact of proteasome inhibition on a well-established mouse model of thrombosis and on a range of ex vivo activities, we conclusively demonstrate that the platelet proteasome contributes to cellular activation and function. Therapeutic proteasome inhibition in platelets produces a hypotrombotic state, but also might augment antiplatelet therapy.

Materials and Methods

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

Results

The Platelet Proteasome Aids Occlusive Thrombosis

Human platelets expressed all 3 primary proteolytic activities of the proteasome, effectively hydrolyzing peptides through chymotryptic, tryptic, and caspase-like cleavage (Figure 1A). The luminescent product was the same for each luminogenic proteasome substrate, so the caspase-like activity was 4–times more efficient than either the tryptic or the chymotryptic activity. The trimeric leucinyl proteasome inhibitor MG132 at 30 µmol/L completely blocked platelet chymotryptic and caspase-like activities and reduced the tryptic activity by half. MG132 at 30 µmol/L is just optimally inhibitory (Figure I in the online-only Data Supplement). The structurally unrelated specific boron-based proteasome inhibitor, bortezomib, inhibited both chymotryptic and caspase-like activities but not the tryptic activity (not shown). Proteasome activity was retained in murine platelets that hydrolyzed these 3 substrates at equivalent ratios (Figure I in the online-only Data Supplement).

We determined whether proteasome inhibition affected thrombosis by systemically injecting MG132 into BL6 mice to achieve an estimated initial circulating concentration of 30 µmol/L along with rhodamine dye to fluorescently label circulating platelets. We initiated occlusive thrombosis 15 minutes later in a surgically exposed external carotid artery by a brief ectopic application of 7.5% FeCl₃. This oxidative insult to the vascular wall resulted in the deposition of fluorescently labeled platelets along the damaged vessel wall that increased over several minutes (Figure 1B). Typically, complete occlusion of the vessel occurred by 10 minutes after FeCl₃ treatment; however, in animals previously injected with MG132, occlusion was significantly delayed to 25 minutes (Figure 1C).

The delay in thrombosis after systemically administering MG132 need not solely reflect participation of platelet proteasomes in thrombosis. We therefore isolated and washed platelets from wild-type BL6 mice and treated these isolated cells with 30 µmol/L MG132 or buffer, washed the cells by centrifugation, and transfused these cells into mice previously treated with 7.5% FeCl₃. MG132 pretreatment of platelets prolongs occlusion time after transfusion into irradiated thrombocytopenic mice. Platelets from donor mice were first pretreated for 30 min with MG132 and were washed before transfusion into normal BL6 mice and FeCl₃-induced thrombosis of carotid arteries initiated 15 min later (n=4 experimental, 3 control; P<0.05).
rendered thrombocytopenic by γ irradiation. We induced carotid artery thrombosis with ectopic FeCl₃, as before and found that control platelets were fully functional and occluded the carotid artery by 10 minutes (Figure 1D). We again found occlusive thrombosis was delayed in mice reconstituted with platelets treated with MG132 ex vivo. Thus, the platelet proteasome participates in arterial thrombosis.

Platelets Contain a Stimulatable Ubiquitination System

Modification of platelet function by MG132 suggests that platelets may designate proteins as substrates for proteolysis by ubiquitination, as in nucleated cells. We determined whether platelets contained E1, E2, and E3 enzymes that sequentially conjugate ubiquitin to target proteins. To ensure that we assessed only platelet proteins, we also isolated highly purified, negatively selected platelets that were essentially free of nucleated cells (<1 monocyte in 10⁹ platelets). Western blotting showed that these platelets contained the E1 activating enzyme UBE1, E2, and E3 ligases, and Rpn2 and the 11S α-subunit of the 19S regulatory complex (Figure 2A).

We used in vitro ubiquitination of exogenous p53, because platelets lack this transcription factor, to determine whether platelet UBE1 was functional. Incubation of recombinant p53 with recombinant E1 and appropriate recombinant E2 and E3 enzymes along with ubiquitin and ATP resulted in robust p53 ubiquitination with the formation of numerous slowly migrating adducts (Figure 2B). Substitution of a platelet lysate for recombinant E1 also promoted in vitro ubiquitination of p53, although the higher-molecular-weight ladder was less prominent than produced by recombinant E1 (Figure 2B).

We next determined whether endogenous platelet proteins were modified by ubiquitin. Western blotting with FK2 antibody that recognizes both monoubiquitin and polyubiquitin chains showed that the proteome of quiescent cells contained multiple ubiquitinated proteins (Figure 2C). The intensity of ubiquitination increased after thrombin activation, although the pattern and position of the adducted bands was unaltered compared with control cells. ADP stimulation also increased ubiquitination of platelet proteins, but this primarily reflected an increase in modification of very slowly migrating proteins (Figure 2C).

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Figure 2. Platelets contain a functional ubiquitination system. A, Platelets contain components of ubiquitin–proteasome system. Immunoblots of platelet E1 activating enzyme, E2 and E3 conjugating enzymes, and proteasome regulatory cap proteins (n=3). B, Platelet E1 is functional. Platelet lysates substituted for recombinant E1 and together with recombinant E2, E3, ubiquitin, and ATP-ubiquitinated recombinant p53 (n=3). C, Agonist stimulation increases platelet proteome ubiquitination. Lysates of platelets stimulated with buffer, thrombin (thr), or ADP were immunoblotted with FK2 antibody against monoubiquitinated and polyubiquitinated proteins (n=3). D, MG132 increases proteome ubiquitination. Lysates of platelets, treated or not with MG132, were stimulated or not stimulated before immunoblotting with FK2 (n=3). E, MG132 increases polyubiquitination of platelet proteins (n=3). Western blotting of platelet lysates with FK1 antibody (n=3).
We investigated whether inhibiting the platelet proteasome altered the amount of ubiquitinated proteins. Pretreatment with MG132 increased both the abundance and the levels of ubiquitinated proteins as detected with the antiubiquitin antibody (Figure 2D). In fact, MG132 was significantly more effective than thrombin or ADP stimulation in enhancing decoration of the proteome with ubiquitin, and the combination of agonist stimulation and MG132 was not different from the effect of MG132 alone (Figure 2D). We observed a similar increase in ubiquitinated proteins after treatment with bortezomib (not shown). Probing the platelet proteome with FK1 antibody that recognizes only polyubiquitinated proteins revealed less abundant modification in unstimulated cells (Figure 2E). This was increased by agonist stimulation and was again greatly enhanced by MG132 treatment. Comparison of FK2 (Figure 2D) and FK1 (Figure 2E) immunoblots showed distinct patterns of monoubiquitination and polyubiquitination of the platelet proteome.

**Filamin A Is Ubiquitinated, and Truncated by the Proteasome**

Filamin A links the GPIb-IX-V complex to actin filaments of the cytoskeleton to modify cytoskeletal shape. Filamin A was present in the soluble fraction of quiescent platelets as a 225-kDa fragment of the 280-kDa native protein (Figure 3A). MG132 inhibition of the proteasome decreased the amount of this smaller band and increased the amount of intact Filamin A. Capture of ubiquitinated platelet proteins by a sushi domain column followed by immunoblotting using anti-Filamin A antibody showed that the fragment of Filamin A in quiescent cells was constitutively modified with ubiquitin (Figure 3B). This approach also showed that MG132 increased the amount of Filamin A ubiquitination and increased the apparent size of the ubiquitinated Filamin A (Figure 3B). That Filamin A was ubiquitinated and that a larger, more extensively ubiquitinated protein accumulated after MG132 treatment was confirmed by the converse experiment in which Filamin A was immunoprecipitated from platelet lysates and then probed for ubiquitin (Figure 3C) using FK2 antibody.

The cytoskeletal protein Talin-1 participates in cell spreading, and similar experimental approaches of Western blotting (Figure 3D), sushi domain capture of ubiquitin adducts (Figure 3E), and immunoblotting of captured Talin-1 for monoubiquitin and polyubiquitin chains (Figure 3F) showed that Talin-1 was present in the cytosol in a rapidly migrating ubiquitinated form. Again, MG132 treatment increased its apparent size and ubiquitin content.

**Figure 3.** MG132 protects cytoskeletal protein cleavage. A, Filamin A is ubiquitinated and its length increased by MG132. Western blot of Filamin A in the soluble fraction of platelets before and after MG132 exposure (n=3). B, Ubiquitination and size of Filamin A are increased by MG132. Western blot of Filamin A in eluates of ubiquitinated platelet proteins captured with sushi columns (n=3). C, Filamin A ubiquitination and size are increased by MG132. FK2 Western blot of monoubiquitinated and polyubiquitinated proteins immunoprecipitated with anti-Filamin A. D, Talin-1 is ubiquitinated and its size increased by MG132. Western blot of Talin-1 before and after MG132 exposure (n=3). E, Ubiquitination and size of Talin-1 are increased by MG132. Talin-1 in ubiquitinated platelet proteins captured by sushi domain chromatography (n=3). F, Talin-1 ubiquitination and size are increased by MG132. FK2 Western blot of monoubiquitinated and polyubiquitinated proteins immuno precipitated by anti-Talin-1 (n=3). G, MG132 increases high-molecular-weight proteins. Coomassie-stained gel of resolved platelet cytoplasmic proteins. H, Mass spectrometer determination of the m/z 126/127 ratio of tandem mass spectrometry tag–labeled peptides along the Filamin A sequence (n=2). I, Pictogram of Filamin A structure.
Coomassie blue staining of soluble proteins showed that MG132 treatment did not alter overall platelet protein mass or composition, but there were 2 abundant exceptions (Figure 3G). Two new slowly migrating bands appeared in the soluble fraction of platelets treated with MG132. Mass spectrometry showed Filamin A to be present in the new band 1, and Talin-1 was present in bands 1 and 2 of the resolved soluble proteins (data not shown), corresponding to the altered mobility detected by Western blotting.

We used tandem mass spectrometry tags—chemically identical tags differentially substituted with heavy atoms that enable multiplexed analysis—to quantitatively compare the amount of Filamin A peptides in tryptic digests of control and MG132-treated platelets. Untreated (band 3) and MG132-treated (band 1) platelets were separately digested with trypsin, and their primary amines were exhaustively modified with isobaric tandem mass spectrometry tags (m/z 126 and m/z 127, respectively) before combined analysis by mass spectrometry. The peptides confirmed that both bands contained Filamin A, and the tags showed that the abundance of Filamin A tryptic peptides in band 1 and band 3 uniformly gave an m/z 126/127 ratio of 1.9. This ratio plunged to 0.08 after the trypsic peptide containing residues 1718 to 1745 (Figure 3H), demonstrating increased abundance of the full-length protein after MG132 treatment. Chymotryptic digestion of band 3 isolated from control cells generated a distinct peptide map, and selected reaction monitoring identified a new Filamin A peptide with S1746 as its amino terminus (not shown). This identifies the R1745–S1746 bond as the site of tryptic-like and is distinct from the previously determined region of Filamin A (Figure 3I). Notably, this cleavage is unique to control cells, which maps into the unfolded hinge domain of Filamin A (Figure 4B).

Proteasome Inhibition Selectively Reduces Aggregation Stimulated by Low Concentrations of Thrombin

Thrombin stimulates platelets by cleaving surface protease-activated receptor 1 (PAR1) to create a self-stimulatory terminal SFLLRN peptide; however, at low concentrations, thrombin activates platelets through the high-affinity GPIb-IX-V receptor. Both MG132 and bortezomib reduced homotypic platelet aggregation at low concentrations of thrombin (Figure 5A).

The effect of proteasome inhibitors was on the high-affinity GPIb-IX-V complex because the SZ2 antibody against GPIbα (CD42b) fully blocked aggregation at a low concentration of thrombin (Figure 5A). The PAR1 agonist SFLLRN induced platelet aggregation that was completely blocked by a PAR1-specific antagonist (RWJ56110; Figure 5B). However, neither MG132 (Figure 5B) nor bortezomib (not shown) suppressed aggregation induced by SFLLRN. Aggregation induced by a low submaximal amount of SFLLRN also was unimpeded by MG132 (Figure IV in the online-only Data Supplement). Thus, the inhibition observed at low thrombin concentration by proteasome inhibitors is independent of PAR1. MG132 also failed to affect aggregation induced by AYPGKF stimulation of PAR4 (Figure IV in the online-only Data Supplement). Furthermore, neither MG132 nor bortezomib inhibited platelet aggregation stimulated by a higher thrombin concentration that acts through PAR1 (Figure 5C). The modifiers SZ2, MG132, RWJ56110, or bortezomib (not shown) by themselves did not induce aggregation (Figure 5D).

Proteasome protease activity was required by the high-affinity thrombin receptor because epoxydocin inhibited platelet aggregation by low thrombin concentrations but not by high thrombin concentrations that stimulate PAR1 (Figure V in the online-only Data Supplement). Epoxydocin is a fungal metabolite, structurally unrelated to either MG132 or bortezomib, that specifically inhibits proteasome catalytic activity and blocks platelet aggregation.

The GPIb-IX-V complex is displayed on the surface of quiescent cells and is downregulated after thrombin stimulation. We found that both MG132 (Figure 5E) and bortezomib (Figure 5F) stabilized the GPIb-IX-V complex on
the surface of stimulated cells and enhanced surface GPIbα (CD42b) expression. Ristocetin increases the affinity of GPIbα for von Willebrand factor, agglutinating unactivated cells, and pretreatment of quiescent platelets with MG132 (Figure 5G) or bortezomib (Figure 5H) reduced ristocetin-induced and botrocetin-induced (not shown) platelet agglutination. Thus, despite the increased surface expression of the GPIb-IX-V complex after proteasome inhibition, the complex is less able to interact with von Willebrand factor than in control platelets.

**Discussion**

Multiple myeloma is associated with an increased incidence of venous and arterial thrombotic disease, and bortezomib therapy suppresses these thrombotic states. Platelets display proteasome chymotryptic activity that is greatly stimulated by soluble agonists. Additionally, bortezomib represses ADP-induced aggregation, and platelets isolated from patients receiving bortezomib are hyporesponsive to other stimuli. A second proteasome inhibitor, PSI, suppresses thrombosis in hypertensive animals.

We extend these observations by showing that platelets contain an intact and functional ubiquitin/proteasome system that participates in agonist-stimulated responses, especially those aided by cytoskeletal rearrangement. We found that inhibition of the proteolytic activity of the proteasome reduced thrombosis at sites of oxidative damage to murine carotid arterial walls. Because MG132 was introduced into the circulation in this experiment, protection need not have been a direct effect of MG132 on platelet function, but the target of MG132 in platelet is in fact the proteasome. We treated purified platelets with MG132 ex vivo, washed them, and transfused these cells into mice rendered thrombocytopenic by previous γ irradiation. This platelet-specific exposure to MG132 interfered with the in vivo function of platelets because MG132-treated platelets were deficient in their occlusion of a FeCl3-damaged carotid artery. Remarkably, this ex vivo exposure exactly mimicked systemic exposure to MG132. This establishes a role for the platelet proteasome in thrombosis.

Platelets contained a functional ubiquitin system that modified cellular proteins to mark them as proteasome substrates. The proteome of quiescent platelets contained numerous...
ubiquitin–protein conjugates whose adduction was increased on stimulation, consistent with the previous observation that collagen activation stimulates ubiquitination of platelet Syk kinase through the E3 ligase Cbl-b. Two of the proteins decorated with ubiquitin in quiescent platelets were Filamin A and Talin-1. Agonist stimulation modestly increased the amount of ubiquitin esterified in the platelet proteome, but blockade of the proteasome was far more effective than agonist stimulation for this, with the result that heavily ubiquitinated Filamin A and Talin-1 accumulated in the cytoplasm of cells with diminished proteasome proteolytic activity.

Filamin A is organized into an actin-binding domain, tightly compacted 4.1, ezrin, radixin, moesin immunoglobulin-like repeats connected with short linking sequences, and 2 unstructured hinge regions. Cytoplasmic Filamin A migrated more quickly than the intact protein during gel electrophoresis, suggesting that it primarily was a fragment. Quantitative comparison of tryptic peptides using tandem mass spectrometry tags showed that MG132 protected Filamin A from cleavage to the smaller 225-kDa fragments. Identification of Filamin A peptides present only after MG132 treatment and identification of the new amino terminal peptide after chymotryptic digestion of the new amino terminal peptide after chymotryptic digestion by multiple reaction monitoring showed that cleavage of the Filamin A protein occurred between R1745 and S1746. This is in a hinge region that is not compacted into FERM domains. Cleavage at this site is distinct from the previously determined calpain cut site and shows that the protease was tryptic because it occurred after an arginine residue. The proteasome subunits functionally interact with one another to degrade proteins to amino acids and small peptides, but the proteasome also processes large proteins to functional fragments, as shown by nuclear factor-κB p105 proteolysis to the p50 transcription factor. We now identify the longer unstructured Filamin A loop as another site of limited proteasome proteolysis, but our result is associative so we cannot ascribe this proteolysis as causal in MG132 inhibition of platelet adhesion or spreading.

Platelets pretreated with MG132 or bortezomib aggregated less in response to low concentrations of thrombin. Adhesion, spreading, microparticle shedding, and the ability to generate the force to retract formed clots were also reduced by proteasome inhibition. Cytoskeletal interactions aid microparticle shedding from stimulated platelets, and proteolysis by...
activated calpain\textsuperscript{6,9,10} and caspase-3\textsuperscript{5} promotes microparticle shedding through cytoskeletal proteolysis.\textsuperscript{90} The reduction of microparticle shedding is additionally revealing because MG132 suppressed shedding after stimulation by lipopolysaccharide activation of Toll-like receptor 4, thrombin activation of PAR1, and ADP activation of P2Y12. So, MG132 interference with stimulated platelet function is not restricted to GP\textsubscript{IIb-IX-V} activation by thrombin.

We establish that platelets, like nucleated cells, express a functional ubiquitin/proteasome system that enables them to ubiquitinate their proteome. This decoration increases on stimulation and modulates an array of responses from several receptors that all engage the cytoskeleton. The cytoskeletal proteins Filamin A and Talin-1 are targets of ubiquitination and proteasome-mediated proteolysis. The ubiquitin/proteasome system of platelets affects their response to thrombotic stimuli, and proteasome inhibition effectively delayed arterial thrombosis. Bortezomib is already in clinical practice for multiple myeloma patients that all engage the cytoskeleton. The cytoskeletal proteins Filamin A and Talin-1 are targets of ubiquitination and proteasome-mediated proteolysis. The ubiquitin/proteasome system of platelets affects their response to thrombotic stimuli, and proteasome inhibition effectively delayed arterial thrombosis. Bortezomib is already in clinical practice for multiple myeloma.

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**Disclosures**

None.

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**Significance**

Proteasome inhibitors are widely used to treat hematologic cancers; however, little is known of their impact on platelet function and hemostasis. We found that platelets have a complete ubiquitin/proteasomal system that participates in cytoskeletal-dependent platelet processes. Proteasome inhibition inhibited platelet aggregation to low-dose thrombin and suppressed occlusive thrombus formation in FeCl₃-damaged carotid arteries. Therapeutic proteasome inhibition may reduce coagulation and augment antiplatelet therapy.
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Supplemental Figure I: Concentration dependence of proteasome inhibition. Proteasome proteolytic activities were individually assayed using luminogenic substrates for indicated catalytic activities in lysates from untreated platelets and platelets treated with different concentrations of MG132 (10, 20, 30 and 40 µM) (A) Chymotryptic activity (n=6; ***p< 0.001) (B) Tryptic activity (n=6; ***p< 0.001, **p< 0.01). (C) Caspase-like activity (n=6; ***p<0.001). D. Mouse platelets express a functional proteasome. Proteasome proteolytic activities were individually assayed in mouse platelet lysates using luminogenic substrates for the indicated catalytic activities (n=4).
Supplemental Figure II. Proteasome inhibitors suppresses agonist-induced shedding of procoagulant microparticles. (A) Annexin V positive microparticle shedding by platelets stimulated with 0.2 U thrombin with or without MG132 (n=3). p≤ 0.05 (B) Bortezomib (n=3). p≤ 0.05. (C) Annexin V positive microparticle shedding by platelets stimulated with lipopolysaccharide (LPS, n=3). p≤ 0.05. (D) Tissue factor procoagulant activity in platelet microparticles stimulated with 0.2 U thrombin with or without MG132 (n=3). p≤ 0.05.
Supplemental Figure III. MG132 does not reduce surface GPVI and CD36 after stimulation with low dose thrombin. (A) MG132 pretreatment did not suppress GPVI surface expression. Flow cytometry of surface GPVI expression on platelets treated or not with MG132 and stimulated or not with 0.025 U thrombin (n=3). (B) MG132 pretreatment did not suppress CD36 surface expression. Flow cytometry of surface CD36 expression on platelets treated or not with MG132 and stimulated or not with 0.025 U thrombin (n=3). (C) Mean fluorescence intensity (MFI) of GPVI expression on platelets treated or not with MG132 and stimulated or not with 0.025 U thrombin (n=3; ns= not significant). (D) Mean fluorescence intensity (MFI) of CD36 expression on platelets treated or not with MG132 and stimulated or not with 0.025 U thrombin (n=3; ns= not significant). (E) MG132 did not suppress intracellular calcium release at low (0.025 U) or high (0.2 U) thrombin concentrations. MG132 pretreatment did not reduce Ca²⁺ flux induced by thrombin. Platelets loaded with FURA-2 and pretreated with MG132 or buffer failed to suppress intracellular calcium release induced by either 0.025 U or 0.2 U thrombin (thr; n=3) (F) Proteasome inhibition prevents thrombin induced GP1ba downregulation. Mean fluorescence intensity (MFI) of surface CD42b (GP1ba) expression on platelets treated either with MG132 or bortezomib and stimulated or not with 0.025 U thrombin (n=3). p≤ 0.05.
Supplemental Figure IV. MG132 does not affect PAR1- or PAR4-induced aggregation. PAR4. MG132 failed to reduce aggregation stimulated by the PAR4 agonist peptide AYPGKF at both 200 µM and 150 µM concentrations (n=3). (B) MG132 inhibits high avidity thrombin stimulation. Pretreatment with 40 µM MG132 completely blocked aggregation induced by 0.025 U thrombin. Notably, proteolytic activity was maximally inhibited by 30 µM MG132, suggesting non-specific effects above this level (n=5) (C) MG132 concentration relationship. Aggregation induced by 0.025 U thrombin (thr) after treatment with the stated concentration of MG132 (0 µM; n=5). (D) MG132 (30 µM) pretreatment minimally reduces aggregation stimulated by low concentrations of the PAR1 agonist peptide SFLLRN (n=3).
Supplemental Figure V. Epoxomicin reduces aggregation in response to low dose thrombin. Epoxomicin suppress platelet aggregation induced through the high affinity thrombin receptor, GP1b-IX-V. (A) Aggregation induced by 0.025 U thrombin (thr) was reduced in platelets pretreated with 1 µM epoxomicin for 30 min (n=3). (B) Quantitative representation of percentage maximum (Max) aggregation (n=3). **p ≤ 0.01. (C) Epoxomicin pretreatment did not suppress aggregation induced by high dose thrombin (Thr, 0.1 U).
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); endotoxin-free PBS, phenol-extracted LPS (Escherichia coli O111:B4) free of lipoprotein contamination (List Biological Laboratories); recombinant soluble CD14, recombinant lipopolysaccharide-binding protein (LBP), APC-anti-human vWF (R&D Systems); anti-polyubiquitin antibodies FK1 and FK2, anti-UBE1, UbiQapture™-Q kit (Enzo lifesciences); anti-Filamin A, anti-Talin1(C45f1), anti-β-actin and anti-skp1 (Cell signaling); anti-Cul5 and anti-Asb2 (Abcam); anti-talin 1(YQ 16), anti-GPV, anti-p53 and SZ2 antibody (SCBT); p53 ubiquitination kit, anti-UBcH 1,5 and 7, anti-RPN2 and anti-11S-α subunit (Boston Biochem); MG132, AYPGKF (PAR4 agonist peptide), SFLLRN (PAR1 agonist peptide) and RWJ56110 (Tocris); Bortezomib (Eurasia); Antichrome TF® (American Diagnostica); PE-anti-human CD42b (Millipore); FITC-CD36 (Cayman Chemicals); Calcein AM and Fura-2 AM (Invitrogen™); Ristocetin and Botrocetin (Sigma-Aldrich). Other chemicals were from Sigma-Aldrich or Biomol Research Laboratories.

Platelet preparation
Human blood was drawn into acid-citrate-dextrose and centrifuged (200 × 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 stated. Briefly, platelet-rich plasma was filtered through two layers of 5-µm mesh (BioDesign) to remove nucleated cells and recentrifuged (500 × g, 20 min) in the presence of 100 nM PGE1. The pellet was resuspended in 50 ml PIPES/saline/glucose (5 mM PIPES, 145 mM NaCl, 4 mM KCl, 50 µM Na₂HPO₄, 1 mM MgCl₂, and 5.5 mM glucose) containing 100 nM PGE₁. These cells were centrifuged (500 × g, 20 min) and recovered platelets were centrifuged again before resuspension in 0.5% human serum albumin in HBSS. In some studies highly purified platelets were prepared by negative selection using an AutoMACS magnetic bead system decreased platelet responsiveness, with anti-CD45, anti-CD15, anti-CD14, and anti-glycophorin–coated magnetic beads as previously described.

Proteasome function
Proteasome-Glo™ 3-Substrate System (Promega) separately assayed hydrolysis of luminogenic substrates for the three proteasome activities [chymotryptic (Suc-LLVY-aminoluciferin), tryptic (Z-LRR-aminoluciferin), and caspase-like (Z-nLPnLD-aminoluciferin)]. Washed platelets (4 X 10⁸/ml) were pretreated with or without 30 µM MG132 for 30 min, pelleted and lysed using NP-40 lysis buffer (150 mM NaCl, 25 mM Tris [pH 7.6], 1% nonidet P-40, 2 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 100 µM PMSF, 1 µg/mL pepstatin, and 10 µg/mL leupeptin to inhibit non-proteasome activities) and incubated with luminogenic substrates for 30 min before luminescence was measured.

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. MG132 (30 µM in DMSO; 6 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 FeCl₃ injury. 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% FeCl₃ 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 real time video image capture with a QImaging Retigo Exi 12-bit mono digital camera (Surrey, Canada) and Streampix version 3.17.2 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.

p53 ubiquitination
Washed platelets (10⁹/ml) were lysed using NP-40 lysis buffer and incubated with components from a p53 ubiquitination kit (Boston Biochem), but lacking recombinant E1, for 60 min before the reaction was stopped with 2X reducing SDS sample buffer. The products were resolved by SDS-PAGE, and p53 ubiquitination status determined by western blotting with anti-p53 antibody.

Western blotting and liquid chromatography-mass spectrometry
Washed platelets (4 X 10⁸/ml) were treated 30 µM MG132 or buffer for 30 min before treatment with thrombin (0.2 U) or ADP (100 µM). After incubation, platelets were pelleted 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, 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 x g, 10 min). Reducing SDS sample buffer was added before the proteins were resolved by SDS-PAGE.

Three different LC-MS/MS experiments were performed, an identification experiment, truncation analysis with N-terminal tagging, and truncation analysis with chymotryptic digestion with multiple reaction monitoring (MRM). For all of these experiments the SDS-PAGE bands were excised from the Coomassie stained gels, washed, reduced and alkylated prior to in-gel digestion with either trypsin (identification and TMT N-terminal tagging experiments) or chymotrypsin. The N-terminal tagging was performed on extracted peptides by adding 100 µl of the tandem mass tags (TMT, Thermo scientific) in 200 mM triethyl ammonium bicarbonate for 1 hr. The reaction was quenched by 5% hydroxylamine for 15 min, combined, dried by speedvac, and dissolved in 1% acetic acid.

Two different LC-MS/MS systems were used in these experiments. Protein identification and MRM analysis was carried out on a LTQ linear ion trap equipped with an Eksigent nano-1D HPLC system. Peptides were resolved on a self-packed Phenomenex Jupiter C18 column (8 cm x 75 µm) with an acetonitrile/0.1% formic acid gradient. The instrument was operated in a either a data dependent mode in which a mass scan was followed by MS/MS scans on the most abundant ions or Multiple Reaction Monitoring (MRM) mode in which specific ions are fragmented and assessed over the entire course of the LC experiment. Proteins were identified by searching the LC-MS/MS against the human reference sequence database with the program Mascot. The TMT-Tagged samples were analyzed on an LTQ-Orbitrap-Velos hybrid mass spectrometer equipped with an Eksigent nano-1D HPLC system. Peptides were resolved on a PicoFrit column packed with ProteoPep 2 (10 cm x 75 µm) C18 with an acetonitrile/0.1% formic acid gradient. The instrument was operated in a data dependent mode in which all high resolution mass scans were obtained in the Orbitrap (resolution at 60,000) to identify the five most abundant ions. These ions were then subjected to five MS/MS scans in the LTQ for peptide identification, and five HCD (higher energy collisional dissociation) scans in the Orbitrap.
for TMT Tag quantitation. The resulting data was then searched against the human reference sequence database (http://www.ncbi.nlm.nih.gov/RefSeq/) using the program SEQUEST. The search parameters include 10 ppm parent ion tolerance, 0.8 Da MS/MS tolerance, full tryptic peptides, oxidized methionine as a variable modification, carbamidomethylated cysteines and TMT tag modifications as static modifications. Positive protein identifications were determined by setting the FDR rate to less than 1% and requiring at least 2 peptides per protein. Peptide TMT quantitation was performed by Proteomics Dynamics Software with an integration window tolerance of 20 ppm and monoisotopic masses for the tabs at 126.127 and 127.131 Da.

**TIRF microscopy**
Washed platelets (2 x 10⁸/ml) were treated with 30 µM MG132 or not for 30 min before addition onto a drop of 0.025 U thrombin placed in a glass bottomed microwell (MatTek) dish. Imaging was performed using 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 DIC modes of five different fields in the sample.

**Microparticle isolation and quantitation**
Washed platelets (10⁹/ml) were pretreated with 30 µM MG132, 40 µM bortezomib or buffer for 30 min before an overnight treatment with thrombin (0.2 U), ADP (100 µM) or 0.1 µg/ml LPS with addition of 0.1 µg/ml each of human recombinant CD14 and LPS binding protein (LBP). Platelets were removed twice by centrifugation (500 x g) before microparticles were collected by centrifugation (100,000 x g, 90 min). The microparticle pellet was resuspended in 0.5% human serum albumin/HBSS and defined numbers of 3-µm polystyrene latex beads (Sigma) were added as an internal standard for flow cytometry. Forward and side scatter boxes were established using 1-µm beads (Sigma) and particles until enumerated while 50,000 3-µm events were collected.

**Clot retraction**
Washed platelets (5 x 10⁸/ml) were incubated with MG132 (30 µM, 30 min) before fibrinogen was added to a final concentration of 500 µg/mL and dispensed (500 µl) into siliconized glass tubes. Clot retraction was initiated by the addition of human α-thrombin (2 U/mL) with clot volume imaged over time. The size of the retracted clot was determined at stated times using Imagepro plus software (Media Cybernatics, Bethesda,MD) to calculate retraction.

**Aggregation**
Washed platelets (2 x 10⁸/ml) were pretreated with combinations of 30 µM MG132, 40 µM bortezomib, 10 ng SZ2, 35 nM RWJ56110 (PAR1 receptor inhibitor) or buffer for 30 min before stimulation with low (0.025 U) or high concentrations of thrombin (0.1 U) or 50 µM SFLLRN (PAR1 specific agonist). Platelet aggregation was measured by transmittance (Chronolog) with stirring (600 rpm). For ristocetin or botrocetin induced agglutination, washed platelets (2 x 10⁶/ml) were resuspended in a buffer containing 50% HBSS-A and 50% platelet poor plasma (PPP) and were pretreated with 30 µM MG132, 40 µM bortezomib or buffer for 30 min. Agglutination was induced with either 650 µg/ml ristocetin or 300 ng/ml botrocetin and was measured by transmittance (Chronolog) with stirring (1000 rpm).
Flow cytometry
Washed platelets (2 X 10^6/ml) were pretreated with 30 µM MG132 or buffer for 30 min before stimulation with 0.025 U thrombin (30 min) followed by fixation with 2% paraformaldehyde. Anti-CD42b antibody or non-immune isotype control (Millipore) were used to determine the surface expression of GPIIbα by flow cytometry.

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≤0.05 was considered statistically significant.