Platelet interaction with von Willebrand factor is enhanced by shear-induced clustering of glycoprotein Ibα

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ABSTRACT

Initial platelet arrest at the exposed arterial vessel wall is mediated through glycoprotein Ibα binding to the A1 domain of von Willebrand factor. This interaction occurs at sites of elevated shear force, and strengthens upon increasing hydrodynamic drag. The increased interaction requires shear-dependent exposure of the von Willebrand factor A1 domain, but the contribution of glycoprotein Ibα remains ill defined. We have previously found that glycoprotein Ibα forms clusters upon platelet cooling and hypothesized that such a property enhances the interaction with von Willebrand factor under physiological conditions. We analyzed the distribution of glycoprotein Ibα with Förster resonance energy transfer using time-gated fluorescence lifetime imaging microscopy. Perfusion at a shear rate of 1,600 s^{-1} induced glycoprotein Ibα clusters on platelets adhered to von Willebrand factor, while clustering did not require von Willebrand factor contact at 10,000 s^{-1}. Shear-induced clustering was reversible, not accompanied by granule release or αIIbβ3 activation and improved glycoprotein Ibα-dependent platelet interaction with von Willebrand factor. Clustering required glycoprotein Ibα translocation to lipid rafts and critically depended on arachidonic acid-mediated binding of 14-3-3ζ to its cytoplasmic tail. This newly identified mechanism emphasizes the ability of platelets to respond to mechanical force and provides new insights into how changes in hemodynamics influence arterial thrombus formation.

Introduction

Platelet adhesion to subendothelial matrices in the damaged vessel wall is the prime event in the arrest of bleeding. Recruitment of platelets to sites of vascular injury is hampered by the rapid flow of blood in arteries and arterioles. In these vessels, the interaction between von Willebrand factor (VWF), a multimeric plasma glycoprotein, and the platelet glycoprotein (GP) Ib-IX-V receptor complex is critical for initial platelet adhesion.[1] This interaction requires unfolding of the VWF A1 domain and allows platelets to decelerate until they attach firmly in a process assisted by platelet integrins.[1,2] Defects in both the GPIb-IX-V complex (Bernard Soulier syndrome) and VWF (von Willebrand disease) result in a bleeding diathesis, which underscores the importance of this interaction in hemostasis.[3,4]

The GPIb-IX-V complex consists of four transmembrane subunits; GPIbα, GPIbβ, GPIX and GPV that are expressed in a 2:4:2:1 stoichiometry.[5] Each platelet contains approximately 25,000 copies of GPIbα, the subunit that binds to the VWF A1 domain.[6] The extracellular domain (residues 1-485) of GPIbα consists of an N-terminal flank, seven leucine-rich repeats, a C-terminal flank, a sulphated region and a highly glycosylated macroglycopeptide domain. Residues 486-514 form the transmembrane domain and the cytoplasmic tail consists of 96 amino acid residues (residues 515-610),[7-9] which contain binding sites for multiple intracellular proteins, including filamin A[10] and the adaptor protein 14-3-3ζ.[11] The region that interacts with the VWF A1 domain resides within the concave face of the leucine-rich repeat domain of GPIbα.[12,13] Despite the fundamental importance in initiating platelet adhesion, the molecular mechanism regulating the VWF-GPIbα interaction remains incompletely understood.

Binding of VWF to GPIbα requires the dynamic conditions of flowing blood. The unique biomechanical properties of VWF and GPIbα allow the interaction to strengthen upon increasing hemodynamic drag.[14] An explanation for this counterintuitive finding is that VWF needs to change its conformation to allow GPIbα access to the A1 domain. Elevated shear force and immobilization on a surface trigger this conformational change *in vivo*, a process mimicked by the antibiotic ristocetin *in vitro*.[15] The interaction between VWF and GPIbα is also regulated through changes in GPIbα. This molecule's adhesive properties depend on translocation to cholesterol-rich membrane domains known as lipid rafts,[16,17] which may increase the local density of GPIbα receptors and stimulate their signaling properties. Indeed, studies with Chinese hamster ovary cells in which GPIbα was artificially dimerized have suggested that receptor clustering increases the overall strength of the VWF-GPIbα interaction.[18,19]

During efforts to optimize the storage conditions of platelet concentrates used for transfusion, we recently demonstrated that GPIbα clusters in lipid rafts when platelets are kept at low temperature.[20] Analysis of Förster resonance energy transfer

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(FRET) by fluorescence lifetime imaging microscopy (FLIM) revealed that cooling of platelets triggers [GPIbα-GPIbα] associations in lipid rafts within a range of 1-10 nm. In the present study, we assessed whether clustering of GPIbα occurs under physiological conditions, investigated its influence on VWF interaction and identified the responsible molecular mechanism.

Methods

The patient

Citrated blood (10.9 mM f.c.) was obtained from a patient with von Willebrand disease type 3. Permission was obtained from the local medical ethics committee. The patient had no detectable plasma VWF (<0.1%), 1% plasma factor VIII, <1% factor VIII activity, no ristocetin-induced platelet aggregation, and a normal platelet count and volume.[21]

Materials, antibodies, platelet preparation and incubations

A detailed description of the materials, antibodies, platelet preparation and incubations used in this study can be found in the *Online Supplementary Methods*.

Platelet adhesion and rolling under flow conditions

A parallel plate perfusion chamber[22] was used to investigate platelet adhesion and rolling. Further details are available in the *Online Supplementary Methods*.

Exposure to shear force

Platelets were exposed to shear force by perfusion through a microcapillary (inner diameter 760 μm, blocked with 4% bovine serum albumin). Washed platelets were resuspended in HT buffer ($2.5x10^{11}$ cells/L, pH 7.3) supplemented with 4% human albumin. Platelet suspensions were prewarmed to 37°C for 5 min and perfused through the microcapillary at indicated shear rates for 5 sec. The length of the microcapillaries was matched with the shear rate, which means that the platelet suspensions had similar shear exposure times at different shear rates. Indicated shear rates are the maximal shear rates to which platelets were exposed near the wall of the microcapillary. The wall shear rate (γ_w) inside a microcapillary is described as

$$wall\ shear\ rate\ (\gamma_w) = \frac{4Q}{\pi r^3}$$

Where Q is the volumetric flow rate and r is the inner radius of the microcapillary.

Agglutination

Platelet agglutination was measured in a Chrono-log Lumi-Aggregometer (model 700, Chrono-log Corporation, Haverton, PA, USA) with Aggrolink 8.0 software. Washed platelets in HT buffer were pre-incubated with the prostacyclin (PGI_2) analog iloprost and dRGDW (5 min, 37°C) and stimulated with VWF (10 μg/mL) and ristocetin (0.3 mg/mL) while stirring (900 rpm). Data are expressed as percentage of maximal agglutination, with light transmission through HT buffer set at 100%.

Flow cytometric analysis, immunoprecipitations and western blots

A detailed description of the flow cytometric analysis, immunoprecipitations and western blots can be found in the *Online Supplementary Methods*.

Analysis of GPIbα distribution by Förster resonance energy transfer and fluorescence lifetime imaging microscopy

GPIbα distribution was analyzed by FRET/FLIM as described elsewhere.[20] In brief, 6B4-Fab fragments conjugated to either Alexa Fluor-488 or Alexa Fluor-594 (6B4-488 and 6B4-594, respectively) were incubated with fixed platelet samples under conditions in which each Fab labeled ~50% of the total number of receptors. GPIbα translocation to lipid rafts was determined by labeling GPIbα with 6B4-488 and monosialo-tetrahexosylganglioside (GM1) with Cholera toxin subunit B conjugated to Alexa Fluor-594 (CTB-594; 5 μg/mL). The fluorescence lifetimes of the donor fluorophore (6B4-488) were determined in the absence and presence of acceptor fluorophore (6B4-594 or CTB-594) and used to calculate the FRET efficiency, defined as

$$FRET\ Efficiency = \frac{\tau_D - \tau_{D/A}}{\tau_D} \times 100\%$$

where τ is the donor fluorophore's lifetime in nanoseconds in the absence (τ_D) and presence ($\tau_{D/A}$) of the acceptor fluorophore. To determine variation in FRET efficiency, the lifetimes of three randomly chosen quadrants were quantified.

Statistical analysis

Data are means ± SEM. Statistical analysis was based on GraphPad Prism 5 (San Diego, CA, USA). Differences between control platelets and incubations were analyzed by the Mann-Whitney test. *P*-values less than 0.05 (* or $\underline{*}$) and between incubations (|–*–|)) were considered statistically significant.

Results

Platelet adhesion to von Willebrand factor under conditions of flow triggers GPIbα clustering

Platelet adhesion at shear rates above 1,000 s^{-1} depends on the interaction between surface-bound VWF and GPIbα.[1] We analyzed the effect of this interaction on the spatial distribution of GPIbα on the platelet plasma membrane with FRET/FLIM. Whereas GPIbα molecules were dispersed in resting platelets (Figure 1A,B), indicated by a FRET efficiency of 0.9±0.2%, GPIbα clustered upon adhesion to VWF (FRET efficiency 10.3±0.9%). Clustering was not caused by close contact between adjacent platelets, as FRET efficiency did not differ between single platelets and platelets that adhered as small aggregates (*Online Supplementary Figure S1A,B*). As the platelet-VWF interaction is influenced by flow conditions, we analyzed the GPIbα distribution of platelets adhered to VWF at different shear rates. Adhesion to VWF at 300 s^{-1} left GPIbα dispersed, but perfusion at 750 s^{-1} and higher induced clustering (Figure 1C). The observed increase in clustering was not the result of more efficient adhesion, as the number of platelets binding to VWF was similar at each shear rate (*Online Supplementary Figure S1C*). To investigate whether changes in GPIbα distribution were specific for adhesion to VWF, platelets were perfused over collagen. Adhesion to collagen at a low shear rate (300 s^{-1}) in the presence or absence of VWF resulted in FRET efficiencies similar to those observed in resting platelets (Figure 1D). Perfusion over collagen at 1,600 s^{-1} in the absence of VWF had little effect on GPIbα distribution. In contrast, addition of VWF prior to perfusion at 1,600 s^{-1} increased FRET efficiency to 8.3±0.6%, indicating that clustering of GPIbα requires the presence of VWF.

Exposure to high shear leads to reversible von Willebrand factor-independent GPIbα clustering

The change in GPIbα distribution measured on surface-attached platelets might be the result of shear, of rolling/attachment or both. To understand the contribution of shear, we perfused platelets in VWF-free buffer through a microcapillary tube at different shear rates in the absence of an adhesive surface. FRET/FLIM analysis showed that a shear rate of 300 s^{-1} left GPIbα dispersed. Exposure to 1,600 s^{-1} had a minor effect on GPIbα distribution, whereas a shear rate of 10,000 s^{-1} increased FRET efficiency to 9.1±0.6% (Figure 2A). Addition of exogenous VWF prior to perfusion at a shear rate of 10,000 s^{-1} did not further increase clustering. Platelet α-granules also contain VWF,[23] which might be released during platelet isolation and thereby influence GPIbα clustering. To investigate this possibility, experiments were repeated using a nanobody against VWF which prevents its binding to GPIbα. Exposure of platelets to shear force in the presence of this nanobody led to the same increase in FRET efficiency as control platelets (Figure 2B). GPIbα clustering induced by a shear rate of 10,000 s^{-1} was also similar in platelets from a patient with VWD type 3 (*Online Supplementary Figure S2A*), indicating that GPIbα clusters independently of the presence of VWF.

GPIbα clustering was reversible, as exposure to shear followed by incubations under static conditions resulted in a gradual decline to the range found in resting platelets (Figure 2C). Platelet exposure to a shear rate of 10,000 s^{-1} did not result in P-selectin expression, αIIbβ3 activation, VWF binding (Figure 2D), cytoskeleton reorganization or altered whole protein tyrosine phosphorylation (*Online Supplementary Figure S2B,C*). Conversely, clustering was not induced by stimulation with cross-linked collagen-related peptide (CRP), thrombin receptor activating peptide (TRAP) or the thromboxane A_2 receptor (TPα) agonist U46619 under static conditions (Figure 2E). Ristocetin-induced VWF binding did induce clustering of GPIbα. Transient GPIbα clustering did not affect the ability of platelets to respond to agonists, because stimulation with TRAP or CRP before and after exposure to shear resulted in similar levels of P-selectin expression and αIIbβ3 activation (Figure 2F).

Platelet interaction with von Willebrand factor is stimulated by clustered GPIbα

To clarify whether changes in GPIbα distribution contributed to platelet responsiveness to VWF, agglutination was measured in platelets with shear-induced clustered GPIbα. VWF and a suboptimal concentration of ristocetin were used and aggregation was prevented by pre-incubation with iloprost, a stable analog of prostacyclin, and

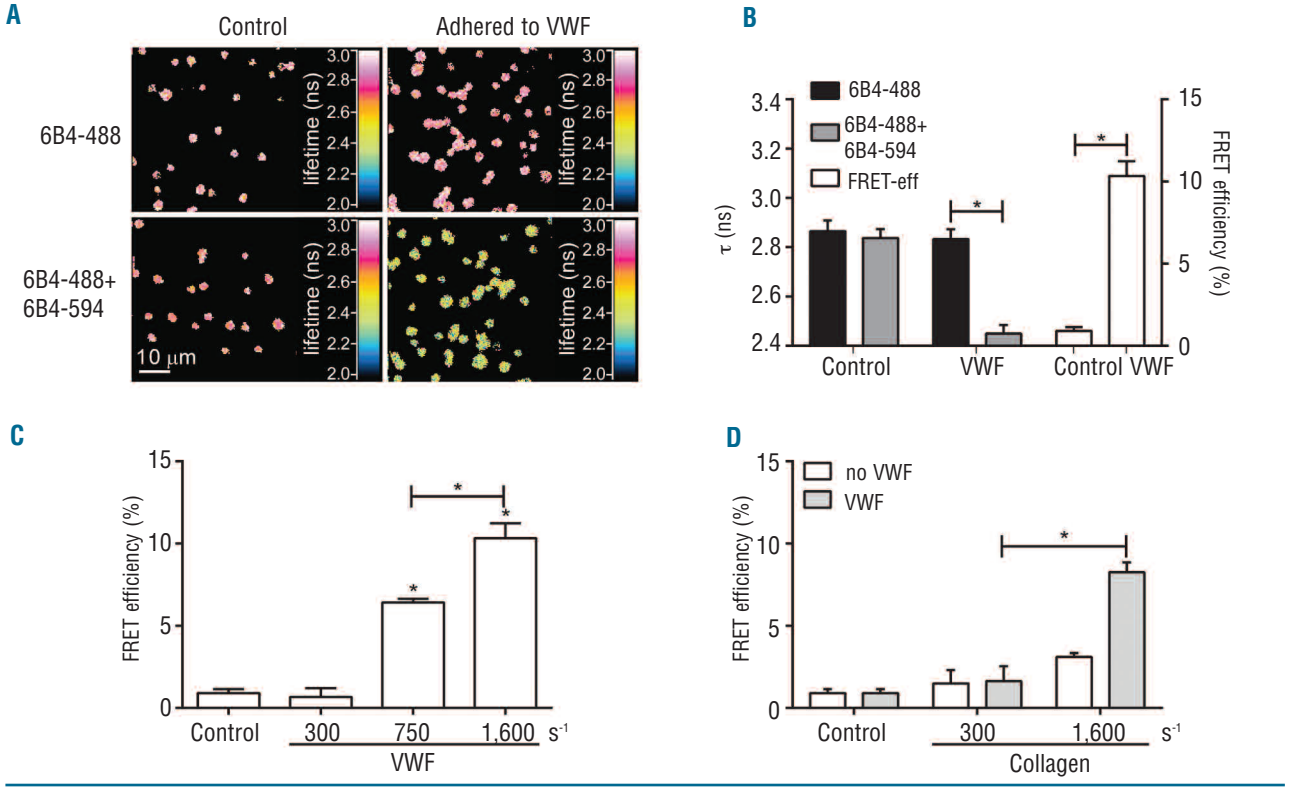

Figure 1. Platelet adhesion to VWF induces clustering of GPIbα. (A) Freshly isolated resting platelets (control; left panels) or platelets adhered to VWF after whole blood perfusion (1 min, 37°C) at a shear rate of 1,600 s^{-1} (right panels) were analyzed for GPIbα distribution by FRET/FLIM. Platelets were fixed with 2% paraformaldehyde and stained with 1 μg/mL 6B4-488 (donor) in the absence (top panels) and presence of 1 μg/mL 6B4-594 (acceptor; bottom panels). The fluorescence lifetimes in nanoseconds (ns) are shown in false color images. (B) Quantification of fluorescence lifetime values of donor probe in the absence and presence of acceptor probe of platelets treated under the conditions of (A). Corresponding FRET efficiencies are calculated as described in the *Methods*. (C-D) FRET/FLIM analysis of platelets adhered to VWF after whole blood perfusion (C) or to collagen after reconstituted blood perfusion (D) at indicated shear rates (1 min, 37°C). Reconstituted blood over collagen was performed in the absence (open bars) and presence (gray bars) of 10 μg/mL VWF (n=4).

dRGDW. Neither agent affected shear-induced GPIbα clustering (*data not shown*). Maximal agglutination of platelets with pre-clustered GPIbα was four-fold higher than with controls (Figure 3A,B).

VWF enables platelets to roll over the damaged vessel wall until they attach firmly in an integrin-dependent manner. The effect of GPIbα clustering was measured by platelet perfusion over a VWF-coated surface in the presence of dRGDW to block αIIbβ3-mediated attachment. Induction of GPIbα clustering prior to perfusion reduced the rolling velocity by 40% (Figure 3C,D). These data show that GPIbα clustering facilitates the platelet-VWF interaction.

GPIbα translocates to lipid rafts and forms clusters through 14-3-3ζ binding

Platelet binding to VWF depends on reallocation of GPIbα in membrane domains enriched in sphingomyelin and cholesterol, known as lipid rafts.[16,17] To understand the role of raft allocation in GPIbα clustering, GPIbα was labeled with 6B4-488 (donor) and the raft marker GM1 with CTB conjugated to Alexa Fluor-594 (CTB-594; acceptor). The surface of resting platelets showed little co-localization but adhesion to VWF or exposure to high shear (10,000 s^{-1}) induced GPIbα-GPIbα as well as GPIbα-GM1 associations, suggesting that clustering and raft translocation go hand in hand (Figure 4A). Disruption of lipid rafts by cholesterol depletion with methyl-β-cyclodextrin (mβCD) effectively abrogated GPIbα clustering. The time-dependent decay of shear-induced GPIbα clusters closely followed raft translocation, again suggesting a tight inter-relationship (Figure 4B).

Binding of the adaptor protein 14-3-3ζ to the cytoplasmic tail of GPIbα is essential for platelet interaction with VWF.[24,25] To assess the role of 14-3-3ζ in GPIbα clustering, we pre-incubated platelets with MPαC. This membrane permeable peptide represents the critical 14-3-3ζ binding site on GPIbα, which includes the constitutively phosphorylated Ser-609 residue on its cytoplasmic tail.[24,26,27] As expected, the peptide prevented VWF-induced 14-3-3ζ binding to GPIbα (*Online Supplementary Figure S2*). Figure 4C shows that pre-incubation with MPαC had little effect on lipid raft translocation induced by platelet adhesion to VWF, but completely abrogated clustering of GPIbα. Control peptide MαC, which lacks phosphorylation at Ser-609, had no effect on GPIbα redistribution. FRET/FLIM analysis of platelets exposed to a shear rate of 10,000 s^{-1} procuded similar results (Figure 4D), demonstrating that 14-3-3ζ binding to the cytoplasmic tail of GPIbα is essential for its clustering. To elucidate the importance of 14-3-3ζ-induced clustering, we determined the rolling velocity of platelets perfused over VWF in the presence of MPαC (Figure 4E). While control peptide MαC had no effect, the rolling velocity of platelets pre-incubated with MPαC increased more than two-fold. Moreover, inhibition of 14-3-3ζ-induced GPIbα clustering impaired stable adhesion to VWF during whole blood perfusion (Figure 4F).

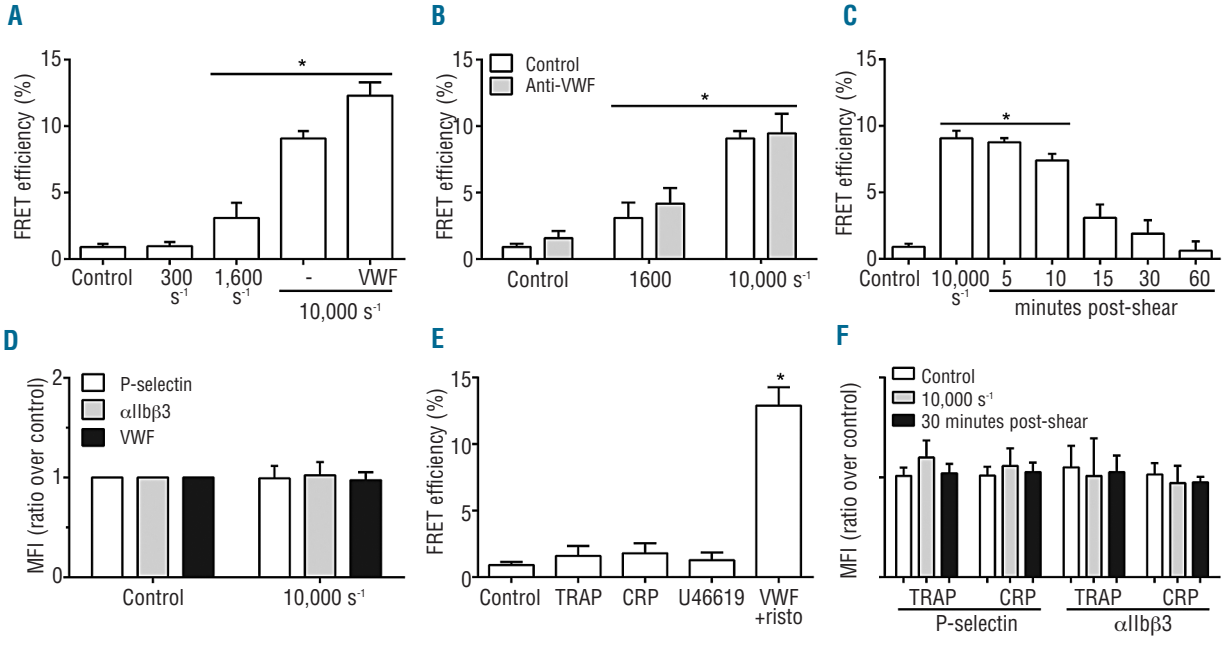

Figure 2. High shear force induces reversible GPIbα clustering in the absence of VWF. (A) Platelets resuspended in HT buffer (pH 7.3) supplemented with 4% albumin were exposed to indicated shear rates in the absence or presence of VWF (10 μg/mL) by perfusion (37°C) through a microcapillary tube for 5 s and analyzed for GPIbα distribution by FRET/FLIM. (B) Shear-induced GPIbα clustering occurs in the absence of VWF. Platelets in the absence or presence of a nanobody against VWF (10 μg/mL) that prevents its interaction with GPIbα were exposed to indicated shear rates and analyzed by FRET/FLIM. (C) Shear-induced GPIbα clustering is reversible. Platelets exposed to 10,000 s^{-1} were monitored for clustering at indicated time intervals post-shear, which after 10 min decreased to control levels. (D) Platelets were analyzed by FACS for expression of P-selectin, αIIbβ3 activation and VWF binding after exposure to shear. (E) FRET/FLIM analysis of platelets stimulated by CRP (1 μg/mL), TRAP-6 (20 μM), U46619 (10 μM) or ristocetin (0.3 mg/mL) and VWF (10 μg/mL) for 5 min at 37°C in the absence of shear. (F) The effects of reversible GPIbα clustering on platelet responsiveness were analyzed by stimulating platelets with TRAP or CRP 0 and 30 min post-shear. FACS analysis revealed that agonist-induced surface expression of P-selectin or αIIbβ3 activation was similar to that of control platelets. Data are presented as the ratio of mean fluorescence intensity (MFI) of treated platelets over control platelets (n=4).

Arachidonic acid mediates 14-3-3ζ-induced GPIbα clustering

Platelet interaction with VWF or incubation at low temperature activates the stress kinase P38-mitogen-activated protein kinase (P38MAPK), which liberates arachidonic acid (AA) from membrane phospholipids through cytosolic phospholipase A_2.[20,28,29] Incubation with inhibitors at 37°C indicated that P38MAPK-mediated AA release might support GPIbα-GPIbα interactions during exposure to shear. The P38MAPK inhibitor SB203580 and the cytosolic phospholipase A_2 inhibitor $AACOCF_3$ inhibited the rise in FRET efficiency induced by high shear. The low FRET efficiency observed under static conditions increased 12-fold upon addition of AA. The intracellular accumulation of free AA might therefore contribute to GPIbα clustering (Figure 5A). The FRET efficiency of shear-treated platelets decreased upon subsequent incubations under static conditions (Figure 2C).

In platelets, liberated AA is metabolized by cyclo-oxygenase-1 and lipo-oxygenase to thromboxane A_2 and other eicosanoids, which could account for the reversibility of shear-induced GPIbα clustering. Indeed, accumulation of AA by inhibition of these enzymes with indomethacin and 5,8,11-eicosatriynoic acid prevented GPIbα clusters from dispersing after exposure to high shear (Figure 5B). AA release triggered by platelet stimulation with CRP or TRAP in the presence of indomethacin and 5,8,11-eicosatriynoic acid also induced clustering of GPIbα (Figure 5C). Control studies confirmed that SB203580 blocked shear-induced P38MAPK phosphorylation/activation whereas the other treatments left the enzyme undisturbed (Figure 5D). Treatments that inhibited AA release prevented shear-induced 14-3-3ζ-GPIbα association and blockade of AA degradation and the separate addition of AA preserved the complex (Figure 5E). These data indicate that liberated AA binds 14-3-3ζ and facilitates its translocation to the cytoplasmic tail of GPIbα.[20,28]

The inhibitors of AA release and degradation affected platelet rolling velocity over a VWF surface. Inhibition of AA release induced by shear increased the rolling velocity, whereas treatments that preserved accumulation of AA resulted in reduced velocities (Figure 5F). These data indicate that the AA-mediated transfer of 14-3-3ζ to the GPIbα cytoplasmic tail induces GPIbα clustering which supports platelet interaction with VWF at high shear.

Discussion

Our results demonstrate that the exposure of platelets to high shear leads to clustering of GPIbα and enhances the interaction with VWF. Shear-induced clustering is reversible and not associated with granule release or activation of αIIbβ3. Clustering requires lipid raft translocation and critically depends on AA-mediated 14-3-3ζ binding to the cytoplasmic tail of GPIbα (Figure 6).

Previous studies found a role for receptor clustering in the GPIbα-VWF interaction.[18,19] Experiments with Chinese hamster ovary cells showed that intracellular dimerization of a modified GPIbα construct increases the overall bond strength with VWF. We found that GPIbα formed clusters in platelets adhered to VWF after perfusion at a shear rate of 750 s^{-1} or higher. At these shear rates, platelet adhesion strongly depends on the interaction between GPIbα and VWF, because only this interaction is sufficiently fast and strong to withstand the associated hemodynamic drag.[1] Our results indicate that GPIbα clustering contributes to GPIbα-mediated platelet adhesion to VWF, as inhibition of clustering strongly attenuated both platelet rolling velocity and adhesion.

Initial adhesion to VWF increases the hemodynamic drag on platelets substantially and results in the formation

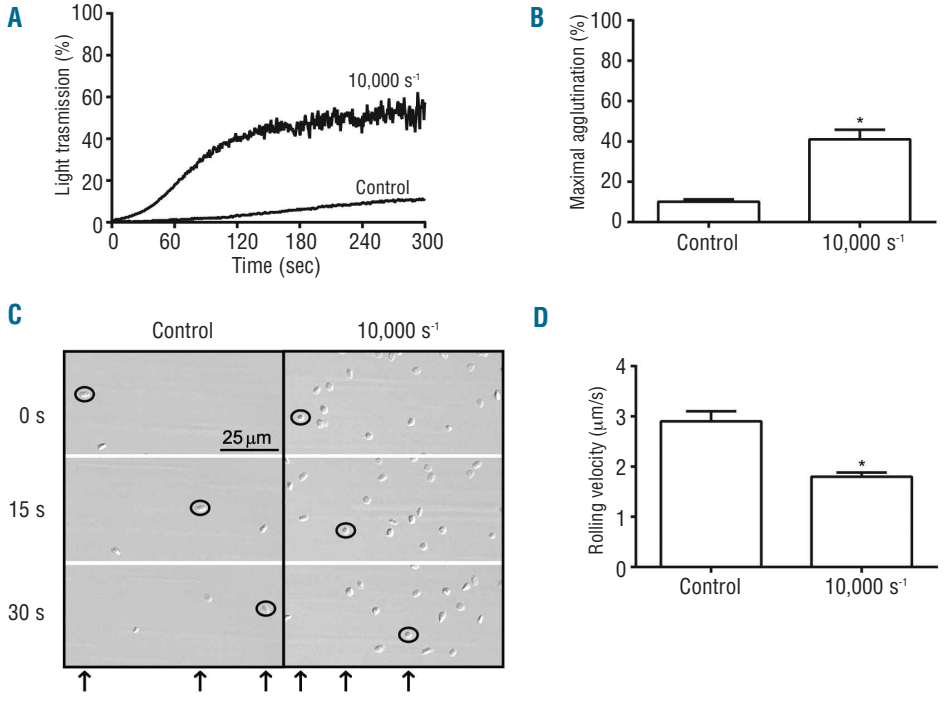

Figure 3. GPIbα clustering improves platelet interaction with VWF. Resting platelets (control) or platelets exposed to 10,000 s^{-1} were pre-incubated with dRGDW and iloprost for 5 min at 37°C. Platelet agglutination and rolling velocity experiments were performed immediately after shear exposure. (A-B) Platelets were stimulated with suboptimal concentrations of ristocetin (0.3 mg/mL) and VWF (10 μg/mL) and agglutination (A) was measured at 37°C while stirring. (B) Quantification of mean maximal agglutination (n=3). (C-D) Analysis of platelet rolling velocity over VWF at a shear rate of 1,600 s^{-1}. (C) Single platelets (black circles) were tracked over distance in time from which the rolling velocity was determined. Black arrows indicate the location of a single platelet at 0, 15 and 30 s. (D) Quantification of rolling velocity in μm/s. Rolling velocity was significantly reduced after pre-exposure to a shear rate of 10,000 s^{-1} (n=5).

of membrane tethers that are pulled from the cell surface.[30,31] Upon adhesion to VWF, clustering was observed at a shear rate of 750 s^{-1} or higher. In the absence of an adhesive surface, GPIbα clustering required exposure to 10,000 s^{-1}, a shear rate found in stenotic arteries. By definition, only those platelets nearest to the vessel wall are subjected to this shear rate. Shear exposure is, therefore, probably limited to part of the platelet population perfused through a microcapillary. Nevertheless, this short exposure to shear stress induced clustering of GPIbα, illustrating the high sensitivity of platelets to mechanical stress. The combined force of shear exposure and tensile stress exerted on GPIbα when bound to VWF apparently cooperate in facilitating GPIbα clustering. Shear-induced clustering did not coincide with platelet activation, which is in line with earlier reports of VWF-dependent adhesion[32] or aggregation of discoid platelets[33] at high shear rates.

The effects of clustering on the interaction with VWF probably reflect avidity modulation, where an increased local density of GPIbα molecules increases the number of ligand-receptor bonds. Based on crystal structure studies, it is less likely that GPIbα clustering facilitates binding of two GPIbα molecules to a single A1 domain.[12,34] Under the influence of elevated hemodynamic drag, VWF-bound GPIbα can subsequently undergo a conformational change that further strengthens the interaction.[14]

Disruption of lipid rafts by cholesterol depletion strongly impairs platelet adhesion to VWF under conditions of flow, indicating that GPIbα localization to these regions is essential for its function.[16] Lipid rafts are viewed as platforms that can physically concentrate receptors, adaptor proteins and effector enzymes, which lead to amplification of signaling events. We observed little GPIbα localization in rafts on the surface of resting platelets, which increased about three-fold upon ligation to immobilized VWF or exposure to high shear in solution. Disruption of rafts prevented GPIbα from clustering. Although translocation was essential for this process, clustering depended critically on the interaction between 14-3-3ζ and GPIbα. The importance of the [14-3-3ζ-GPIbα] association for the interaction of platelets with VWF is well established,[24,25] but the exact mode of action remains poorly defined. It has been suggested that this association participates in αIIbβ3 integrin activation in GPIb-IX-expressing Chinese hamster ovary cells.[35,36] We show that inhibition of 14-3-3ζ binding to GPIbα impaired adhesion to VWF and increased rolling velocity. The presence of iloprost and dRGDW excluded involvement of αIIbβ3 integrin in platelet rolling on VWF. Together, these data indicate that the 14-3-3ζ association with GPIbα directly improves

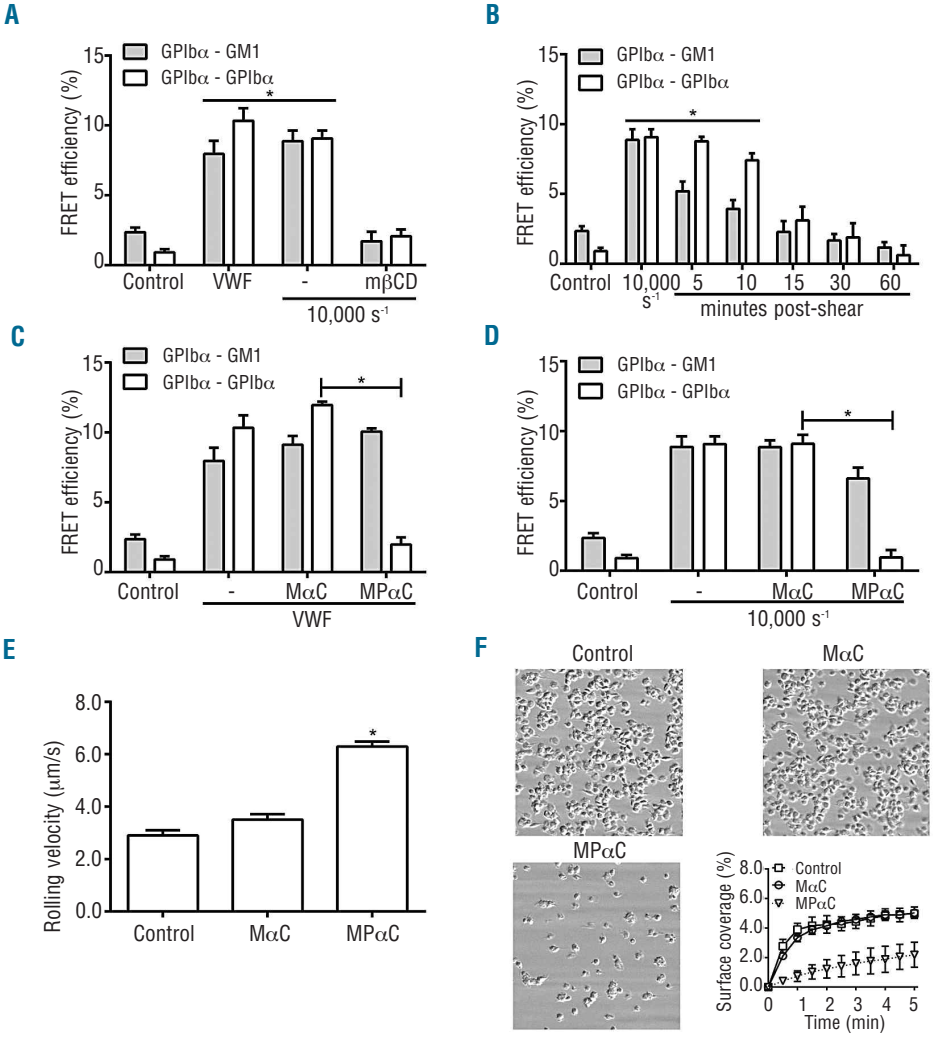

Figure 4. Clustering of GPIbα requires translocation to lipid rafts and 14-3-3ζ binding to its cytoplasmic tail. GPIbα translocation to lipid rafts was measured by labeling GPIbα with 6B4-488 (donor, 1 μg/mL) and raft-specific GM1 ganglioside with CTB-594 (acceptor; 5 μg/mL). (A-D) FRET/FLIM analysis of [GPIbα-GM1] and [GPIbα-GPIbα] associations. (A) GPIbα distribution of platelets adhered to VWF by whole blood perfusion at 1,600 s^{-1} (VWF) or washed platelets perfused through a microcapillary at 10,000 s^{-1}. Disruption of lipid rafts by cholesterol depletion with mβCD (10 mM, 30 min at 37°C) prior to perfusion prevented changes in GPIbα distribution. (B) GPIbα translocation to lipid rafts induced by shear force is reversible after 10 min. (C-D) GPIbα clustering requires 14-3-3ζ binding to its cytoplasmic tail. Binding of 14-3-3ζ to GPIbα was prevented by pre-incubation with the cell-permeable peptide MPαC (100 μM, 5 min at 37°C). FRET/FLIM analysis of platelets bound to VWF by perfusion (C) or exposed to shear (D) in the presence of control peptide MαC or MPαC. (E-F) Platelet interaction with VWF is impaired by prevention of 14-3-3ζ binding to GPIbα. Analysis of platelet rolling velocity over VWF at 1,600 s^{-1} (E) and quantification of platelet surface coverage during whole blood perfusion (F) in the presence of MαC or MPαC. (F) Snapshots were taken after 5 min of perfusion (n=4).

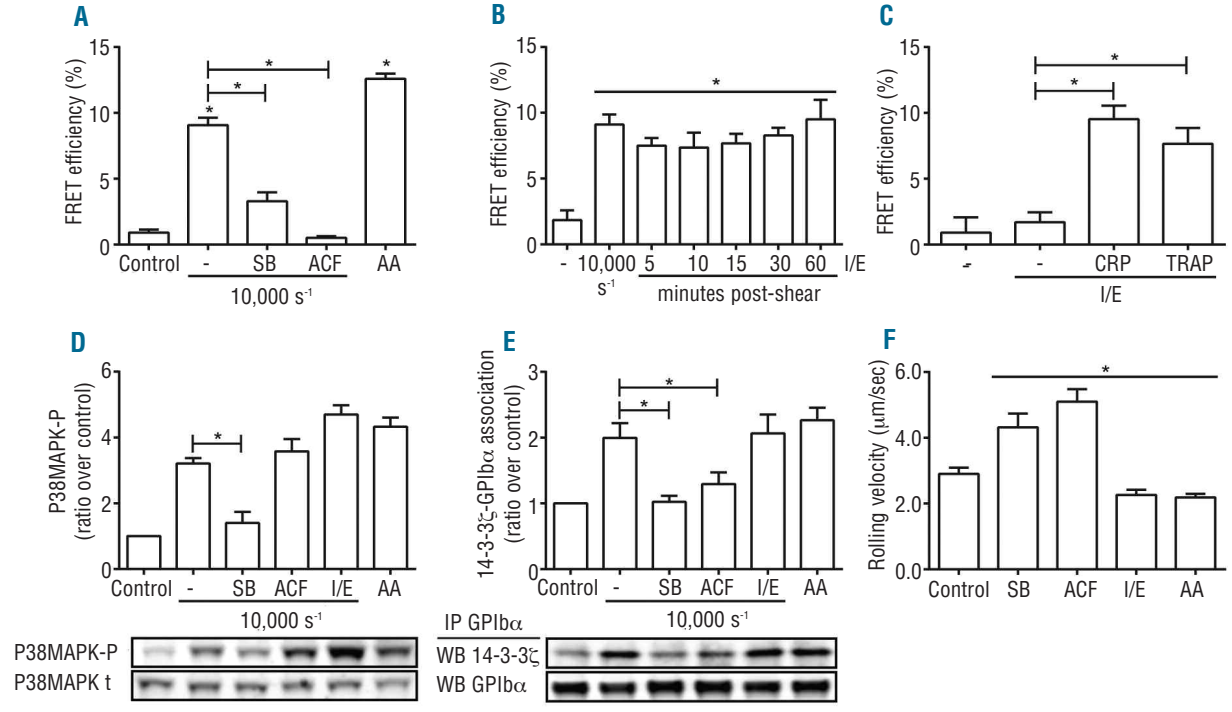

Figure 5. Binding of 14-3-3ζ to GPIbα is regulated by AA. (A) Clustering of GPIbα is prevented by inhibitors of upstream regulators of intracellular AA release. Pre-incubation (10 min at 37°C) with P38MAPK inhibitor SB203580 (SB; 10 μM) or cytosolic phospholipase A_2 inhibitor $AACOCF_3$ (ACF; 20 μM) abolished shear-induced clustering of GPIbα. Platelet incubation with 10 μM AA (5 min at 37°C) in the presence of TPα antagonist SQ30741 (25 μM) induced GPIbα clustering. (B) Shear-induced clustering of GPIbα is maintained in the presence of inhibitors of AA metabolism. Inhibitors were against cyclooxygenase-1 (indomethacin; 30 μM) and lipo-oxygenase (5, 8, 11-eicosatriynoic acid, ETI; 25 μM), abbreviated as I/E. (C) Platelet stimulation with CRP or TRAP in the presence of indomethacin and ETI induces clustering of GPIbα. (D-E) Analysis of shear-induced phosphorylation of P38MAPK (D) and [14-3-3ζ-GPIbα] complex formation (E) under conditions described for (A) and (B). (F) Platelet rolling velocity increases in the presence of SB203580 and AACOCF3 and is reduced in the presence of AA metabolism inhibitors (I/E) or exogenous AA, under conditions described for (A) and (B) (n=4).

platelet interaction with VWF by allowing receptor clustering. The dimeric nature of 14-3-3ζ supports this finding.[37] Indeed, a similar mechanism has been described in muscle cells, in which clustering of the acetylcholine receptor depends on 14-3-3ζ.[38]

P38MAPK is a kinase that is responsive to stress stimuli, including alterations in thermal[28] and shear conditions.[39] We found that platelet exposure to shear in solution leads to P38MAPK phosphorylation. Phosphorylated P38MAPK subsequently activates cytosolic phospholipase A2 to release AA from membrane phospholipids.[40] Our study reveals a central role for AA in GPIbα clustering. Inhibition of AA release during exposure to shear prevented the transfer of 14-3-3ζ to the cytoplasmic tail of GPIbα. Moreover, addition of AA enhanced clustering and inhibition of AA metabolism resulted in irreversible clustering. The findings that AA binding to 14-3-3ζ induces 14-3-3ζ multimerization[41] and that AA-bound 14-3-3ζ directly associates with GPIbα,[28] both support the concept that the adaptor protein provides a platform for GPIbα clustering. In addition, lipid rafts are enriched in AA,[42] which may explain the dependence of GPIbα cluster formation on these membrane domains. Rolling experiments established the importance of AA-mediated GPIbα clustering, as inhibitors of AA release reduced platelet interaction with VWF, while its accumulation enhanced this initial step in adhesion.

Aspirin, a widely used antithombotic drug, also interferes with AA conversion by inhibiting COX-1 activity. Our studies suggest that the use of aspirin may prolong

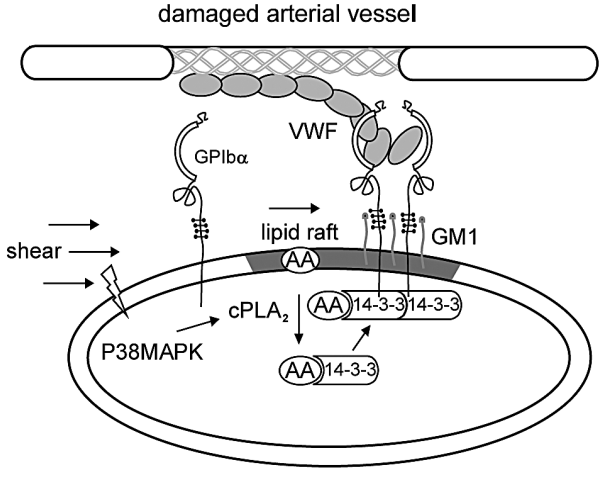

Figure 6. Schematic representation of initial platelet adhesion to VWF at the damaged arterial vessel wall. Shear triggers GPIbα translocation to lipid rafts and activates P38MAPK. This stress kinase subsequently activates cytosolic phospholipase A_2 ($cPLA_2$), which liberates AA from membrane phospholipids. GPIbα clusters upon AA-mediated 14-3-3ζ binding to its cytoplasmic tail, leading to enhanced interaction with VWF.

the presence of GPIbα clusters, which contradicts the antithrombotic effects of this drug. However, the inhibitory effects of aspirin are attributed to inhibition of thromboxane A_2-enhanced platelet activation,[43] which is important for more advanced steps in thrombus forma-

tion. Interestingly, several studies have demonstrated that GPIbα-dependent platelet adhesion actually increases upon aspirin intake.[44,45] These unexplained findings may be the result of aspirin-enhanced GPIbα clustering.

In conclusion, we have defined a central role for GPIbα clustering in platelet interaction with VWF under conditions of flow. Clustering of GPIbα requires translocation to lipid rafts and AA-mediated 14-3-3ζ binding to its cytoplasmic tail. These findings illustrate the mechanosensitive properties of platelets and give a new perspective on the molecular mechanism of arterial thrombus formation.

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Authorship and Disclosures

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