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Protease activated receptors and glycoprotein VI cooperatively drive the platelet component in thromboelastography

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Summary

Background: Thromboelastography (TEG) is used for real-time determination of hemostatic status in patients with acute risk of bleeding. Thrombin is thought to drive clotting in TEG through generation of polymerized fibrin and activation of platelets through PARs. However, the specific role of platelet agonist receptors and signaling in TEG has not been reported.

Objectives: Here we investigated the specific receptors and signaling pathways required for platelet function in TEG using genetic and pharmacological inhibition of platelet proteins in mouse and human blood samples.

Methods: Clotting parameters (R, α, MA) were determined in recalcified, kaolin-triggered citrated blood samples using a TEG 5000 analyzer.

Results: We confirmed the requirement of platelets, platelet contraction, and αIIbβ3 integrin function for normal α-angle (α) and maximal amplitude (MA). Loss of the integrin adaptor Talin1 in megakaryocytes/platelets (*Talin1^mKO^*) also reduced α and MA, but only minimal defects were observed in samples from mice lacking Rap1 GTPase signaling. *PAR4^mKO^* samples showed impaired α but normal MA. However, impaired TEG traces similar to platelet-depleted samples were observed with samples from *PAR4^mKO^* mice depleted of GPVI on platelets or with addition of a Syk inhibitor. We reproduced these results in human blood with combined inhibition of PAR1, PAR4, and Syk.

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Author contributions

TR conducted experiments and analyzed data. SA, MJF, MHG and ASW provided critical reagents and assisted with data interpretation. WB and RHL designed experiments, analyzed and interpreted data, and wrote the manuscript. All authors reviewed and provided revisions of the manuscript.

Conflict of Interest

The authors report no conflicts of interest.

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Conclusions: Our results demonstrate that standard TEG is not sensitive to platelet signaling pathways critical for integrin inside-out activation and platelet hemostatic function. Furthermore, we provide first evidence that PARs and GPVI play redundant roles in platelet-mediated clot contraction in TEG.

Keywords

Thromboelastography; blood platelets; protease-activated receptor; fibrin; bleeding

Introduction

Thromboelastography (TEG) is used clinically at point-of-care to determine the risk of bleeding and/or thrombosis [1,2]. The assay accounts for the contribution of coagulation factors, fibrinogen, and platelets to clot formation, and generates parameters representing the speed and strength of clot formation. The primary clinical application is to rapidly determine a patient's hemostatic status and guide administration of blood products [3]. Clotting parameters include R time (time to first inflection of the trace), α-angle (angle between the midline and the developing trace), and MA (maximum amplitude-maximum force transmitted to the pin). The R parameter is dependent on soluble coagulation factors, while α and MA are dependent on fibrin(ogen) and platelets, and these associations are used to guide transfusion of blood products [3]. TEG-guided transfusions can reduce the use of blood products by preventing unnecessary transfusions in the setting of surgery (cardiac, liver transplant) and trauma [4–7]; however, the impact on clinical outcomes (re-exploration, morbidity/mortality) is often limited [5,8,9] and unfortunately, low quality evidence (low numbers, poor methodology) often hampers analysis of the real impact of viscoelastic tests on patient outcomes [4,10].

Platelets mediate both hemostasis and thrombosis, and express agonist receptors and signaling molecules to rapidly respond to damaged blood vessels. Von Willebrand factor (VWF) recruits platelets to the site of injury via glycoprotein (GP)Ibα, and platelets are subsequently activated by collagen via GPVI and by thrombin via protease activated receptors (PARs). Receptor stimulation induces Ca^{2+} influx and activation of CalDAG-GEFI, a guanine nucleotide exchange factor (GEF) for the small GTPase Rap1. Ultimately, Rap1 and Talin1 drive integrin αIIbβ3 into an active conformation, leading to fibrinogen binding and platelet aggregation [11]. Thrombin also cleaves fibrinogen which assembles into polymerized fibrin, binds αIIbβ3, and induces platelet outside-in signaling to mediate clot contraction [12]. Recently, GPVI has also been shown to bind fibrinogen and fibrin, although the consequences of this interaction on hemostasis in vivo are not entirely understood [13,14]. Importantly, loss of PAR, CalDAG-GEFI, Rap1, Talin1, or αIIbβ3 lead to severe bleeding in mice [15–18], and humans [19,20].

In TEG, MA is sensitive to thrombocytopenia and inhibition of αIIbβ3 integrin and platelet contraction [21,22]. It is assumed that platelet activation is mediated by activation of PARs by thrombin [23]. However, there is little information on the receptors and signaling proteins that mediate platelet function in TEG. Prostacyclin, which prevents platelet activation by inducing inhibitory signaling, has no impact on MA [24]. Inhibition of PAR1 with vorapaxar

does not modify TEG parameters, even in the presence of dual antiplatelet therapy [25,26]. Viscoelastic data on patients lacking CalDAG-GEFI/Rap1 signaling has not been reported. Additionally, the importance of the novel interaction between GPVI and fibrin(ogen) to platelet-dependent viscoelastic parameters has not yet been assessed. Here, we investigated the specific receptors and signaling pathways required for platelet function in TEG.

Methods

Mice:

The following strains were included in this study: C57BL/6J, *Caldaggef1*$^{-/-}$ [16], *P2ry12*$^{-/-}$ [27], *Rap1b*$^{fl/fl}$ × *Pf4-Cre*$^{+}$ (*Rap1b*^{mKO}) [17], *Talin1*$^{fl/fl}$ × *Pf4-Cre*$^{+}$ (*Talin1*^{mKO}) [18], *Par4*$^{fl/fl}$ × *Pf4-Cre*$^{+}$ (*Par4*^{mKO}) [15], *Talin1*$^{fl/R35E,R118E}$ × *Pf4-Cre*$^{+}$(*Tln1*^{mR35E,R118E}) [11], and Fibrinogen γ chain $^{5/\ 5}$ (*Fgn*$^{\gamma\ 5/\gamma\ 5}$) [28]. All mice were bred in-house and are on a C57BL/6/J background. Male and female mice were used as blood donors between 8–20 weeks of age, but males were used whenever possible due to greater blood volume. All procedures were approved by the Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Mouse blood collection:

Blood was collected from anesthetized mice via retroorbital plexus (RO) or inferior vena cava (IVC). RO collection was performed by puncturing the retroorbital plexus with a non-heparinized capillary tube, wasting the first two drops of blood to limit tissue factor contamination and then dripping blood into a microcentrifuge tube containing 3.2% citrate (9:1 volume ratio). IVC collection was performed by exposing the IVC and gently drawing blood through a 25-G needle into a 10 mL syringe containing 3.2% citrate (9:1 volume ratio). For platelet-depleted samples, donor mice were injected intravenously with 1 mg/kg anti-GPIbα antibody (Emfret Analytics, clone R300) 3 hours prior to blood collection. For GPVI-deficient samples, donor mice were injected intravenously with 50 μg of the anti-GPVI antibody JAQ1 (Emfret Analytics) 5 days before blood collection [29]. Cytochalasin D (5 μg/ml, Sigma) [21], anti-αIIbβ3 antibody (75 μg/ml, Emfret Analytics, clone Leo.H4), and PRT-062607 (20 μM, Selleck Chem) were added directly to whole blood samples prior to starting TEG. PRT-2607 was used at a supratherapeutic concentration of 20 μM to achieve complete inhibition of whole blood platelet aggregation induced by a high concentration of the GPVI-specific agonist convulxin; 20 μM PRT-2607 did not affect PAR4-mediated platelet aggregation (not shown).

Human blood collection:

Whole blood was collected from healthy subjects (male and female subjects between the ages of 20–50 who had not taken aspirin/NSAIDs within two weeks) using a 21-G needle vacutainer butterfly into 3.2% citrate tubes (BD). For inhibition of αIIbβ3, PAR1, PAR4, or Syk, samples were treated with abciximab (20 μg/ml, provided by Dr. Rick Stouffer), vorapaxar (5 μM, Med Chem Express), BMS-986120 (10 μM, Cayman Chemical), or PRT-062607 (20 μM), respectively. Vorapaxar and BMS-986120 were used at concentrations which completely prevented thrombin-induced human platelet aggregation when combined (not shown). Blood collection from healthy donors was performed with informed consent in

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accordance with a protocol approved by the Institutional Review Board at the University of North Carolina at Chapel Hill.

Flow cytometry analysis:

Platelet counts were determined in 2 μL whole blood stained with anti-GPIX antibody (Emfret Analysis), and diluted samples in PBS were analyzed on an Accuri C6 Plus flow cytometer (BD). To confirm GPVI deficiency by JAQ1 administration, platelets in whole blood were diluted in modified Tyrode's buffer containing 1 mM Ca^{2+} and activated with the GPVI agonist convulxin (Cvx) for 10 minutes in the presence of JON/A-PE (Emfret Analytics) and anti-P-selectin-AlexaFluor647 (BD) antibodies to measure activated αIIbβ3 integrin and α-granule secretion, respectively.

TEG analysis:

All samples underwent TEG analysis (TEG 5000 Thrombelastograph Hemostasis Analyzer System, Haemonetics) using a citrated kaolin assay following manufacturer instructions. Blood samples were allowed to rest at room temperature for 15–20 mins prior to TEG analysis. 16 μL of kaolin reagent was pipetted from the vial and added to 384 μL of citrated mouse or human blood (after addition of inhibitors when applicable) in a microcentrifuge tube and mixed by inversion. Then, 340 μL blood was pipetted into a plain cup containing 20 μL of $CaCl_2$ (0.2 M) and inserted into the TEG machine. Samples were run for approximately one hour for calculation of R time (mins), α-angle (degrees), and MA (mm). Representative traces for each condition are shown overlaid in figures; for groups of data in which R times were not significantly different, traces were aligned so that the first inflection point of all traces are superimposed for a more direct comparison of α-angle and MA.

Statistical analysis:

Data are presented as mean ± standard deviation (SD). Normality of data was determined by Shapiro-Wilk test. Parametric data were analyzed using a 2-tailed student's t-test for two groups or a 1-way ANOVA with Tukey's post-hoc test for three or more groups. Non-parametric data were analyzed by Mann-Whitney test for two groups or Kruskal-Wallis test with Dunn's post-hoc test for three or more groups. Analyses were performed using GraphPad Prism 9 software. A P-value of 0.05 or less was considered significant.

Results

Thromboelastography (TEG) was performed with mouse citrated whole blood samples collected via retroorbital (RO) blood collection, and clotting was initiated with kaolin using a TEG 5000 analyzer. For this study we analyzed 3 parameters: R time (R), α-angle (α), and maximum amplitude (MA). K time (a secondary measure of clot kinetics) was not included because it is not calculated for traces with MA <20, and LY30 (extent of fibrinolysis at 30 mins) was not included because mouse blood samples did not undergo spontaneous fibrinolysis. Using wild-type (WT) C57BL/6J mice, we did not observe any differences in parameters between males and females (Figure S1). Although inferior vena cava (IVC) blood collection was demonstrated to be ideal for TEG when studying coagulation factor deficiencies in mice [30] and may be more comparable to human venipuncture, direct

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comparison of IVC vs RO blood collection methods with WT mice showed significant but only small differences in R and α, and no difference in MA (Figure S2). We therefore used RO blood draws for subsequent studies as this method is non-terminal but should be comparable to clinical sample collection for TEG.

Platelets are required for clot formation [31], and thrombocytopenia impairs viscoelastic clotting parameters [22]. To determine the contribution of platelets to TEG parameters under our experimental conditions, we analyzed blood from WT mice depleted of virtually all circulating platelets by administration of an anti-GPIbα antibody which reduced platelets counts by >99%. Platelet depletion did not alter the R value but significantly reduced α and MA (Figure 1). Pre-treatment of whole blood with cytochalasin D, an inhibitor of actin-mediated platelet contraction [32], reduced α and MA to a similar degree as in platelet-depleted samples without affecting R (Figure 1), suggesting the platelet contribution to α and MA is predominantly dependent on platelet contraction. This was also supported by normal TEG parameters in blood samples from mice expressing a mutant fibrinogen ($Fgn^{\gamma\ 5/\gamma\ 5}$) which impairs platelet aggregation but leaves platelet contraction intact [28] (Figure S3). The ability of platelets to interact with fibrin and contract the fibrin clot is dependent on integrin αIIbβ3 [33]. R was not affected by integrin inhibition, but consistently, significant reductions in α and MA were observed in WT blood treated with a blocking antibody to αIIbβ3 integrin and in blood from mice with megakaryocyte(MK)/platelet-specific deficiency in the integrin adaptor protein Talin1 (*$Tln1^{mKO}$*) (Figure 2). Although the requirement for αIIbβ3 integrin in TEG was known [21], the requirement for inside-out signaling has not been directly demonstrated. Therefore, we next determined if platelet intracellular signaling upstream of Talin is critical in TEG. The small GTPase Rap1 is a key regulator of αIIbβ3 activation [17,34]. Mice lacking the major isoform of Rap1 (*$Rap1b^{mKO}$*) [34] or the two major pathways for Rap1 activation (*$Cdg1^{-/-}$* × *$P2ry12^{-/-}$*) [35] had normal TEG parameters (Figure 3). Mice lacking both isoforms of Rap1 (*$Rap1a/b^{mKO}$*) [17] showed significant reductions in α and MA (Figure 3), however these mice also have significant thrombocytopenia (<50% of WT; Figure S4) [17]. It was recently demonstrated that a direct interaction between Rap1 and Talin1 is critical for αIIbβ3 activation in platelets [11]. TEG parameters were also normal in $Tln1^{mR35E,R118E}$ mice lacking this interaction (Figure S5), demonstrating a primarily Rap1-independent but talin/integrin-dependent function of platelets in TEG.

Thrombin is assumed to activate platelets in TEG [23], but the requirement for thrombin-mediated platelet activation has not been directly demonstrated. Interestingly, PAR1 inhibition on human platelets with vorapaxar had no effect on TEG parameters [25,26]. Mouse platelets express 2 thrombin receptors, protease activated receptor (PAR) 4 and 3. PAR4 is the main activating receptor and deletion of PAR4 completely eliminates the platelet response to thrombin [15,36]. However, when we tested blood from mice lacking PAR4 in MKs/platelets (*$PAR4^{mKO}$*), we only observed a significant reduction in α, while R and MA were comparable to controls (Figure 4). We confirmed the absolute requirement for thrombin activity by treating WT samples with hirudin and observed no initiation of clotting up to 60 minutes (R >60 min; not shown). Based on these findings, we considered whether a different ligand-receptor interaction can drive platelet contraction in the absence of PAR4. One candidate we considered was GPVI, which has recently been identified as an important

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receptor for fibrin on platelets [13,14], and this interaction can initiate platelet intracellular signaling [14]. We tested blood from mice treated with an anti-GPVI antibody (JAQ1) which depletes GPVI from the surface of all circulating platelets [29]. GPVI deficiency alone had no impact on R, α, or MA (Figure 4). However, when we tested blood from JAQ1-treated *PAR4*^{mKO} mice, which have platelets that are unresponsive to both PAR4 and GPVI agonists (Figure S6), α was reduced to a similar extent as in *PAR4*^{mKO} samples while MA was dramatically reduced (Figure 4). In fact, MA was reduced to a similar extent observed with platelet depletion or inhibition of platelet contraction (Figure 1). The tyrosine kinase Syk is critical for GPVI-mediated platelet activation [37] and also plays a role in αIIbβ3 outside-in signaling [38]. Inhibiting Syk with PRT-2607 (PRT) slightly reduced MA compared to controls but had no effect on R or α (Figure 5). However, *PAR4*^{mKO} samples treated with PRT displayed the near-maximal reduction in MA observed in *PAR4*^{mKO} + JAQ1 samples, as well as a significant reduction in α (Figure 5).

Human platelets express PAR4 but also express PAR1, both of which induce platelet signaling independently but can also heterodimerize [39]. To characterize the contribution of thrombin signaling to TEG in human blood, we simultaneously inhibited both PAR1 and PAR4 with the specific inhibitors vorapaxar (Vora) and BMS-986120 (BMS), respectively. Vora/BMS-treated human blood samples were comparable to DMSO vehicle samples in all parameters (Figure 6), demonstrating that thrombin activation through PAR1/4 is not the only mechanism that drives the platelet component in TEG. Similar results were observed in samples treated with PRT to inhibit Syk (Figure 6). As a positive control, αIIbβ3 ligand binding was blocked with an anti-αIIbβ3 antibody (abciximab), which significantly reduced MA (Figure S7). However, when human samples were simultaneously inhibited with Vora/BMS/PRT, we observed a marked reduction in α and MA (Figure 6). Collectively, these data reveal functional redundancy in mouse and human PARs and GPVI that drives platelet activation and contraction in TEG (Figure 7).

Discussion

The primary clinical utility of TEG is to guide transfusion of blood products in patients with active hemorrhage. Platelet Mapping TEG, which generates fibrin independently of thrombin and directly activates platelets [40], can identify defects in COX-1 and P2Y12 [41], but identifying other forms of platelet dysfunction relies on standard TEG. While TEG utilization may reduce unnecessary transfusions and costs, the impact on patient outcomes has not been universally demonstrated [3]. Regarding platelet transfusions, the lack of universal improvements in outcomes may be due to a disconnect between platelet function in TEG versus platelet function during hemostasis in vivo, and an incomplete understanding of the platelet contribution to TEG parameters. Our study uncovers several novel aspects of platelet activation and function in TEG: 1) platelet activation largely bypasses Rap1 GTPase signaling, which is critical for platelet integrin activation and aggregation under flow conditions, 2) PAR4 and GPVI play redundant roles in driving the platelet-dependent parameters α-angle and MA, and 3) standard TEG can effectively identify platelet contraction defects. We also observed redundant roles for PAR1/PAR4 and GPVI in human blood samples, demonstrating that this is not a species-specific phenomenon. While this study exclusively utilized TEG we anticipate that similar mechanisms would mediate the

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ROTEM equivalent of MA when using INTEM (maximum clot firmness, MCF), although this requires future investigation.

Integrin inside-out signaling through the Rap1/Talin1/αIIbβ3 axis is critical for platelet aggregation and hemostasis at sites of injury and deficiencies in this pathway are associated with moderate to severe bleeding in mice and humans [17,18,42]. For example, humans with mutations in CalDAG-GEFI can suffer from severe epistaxis and bleeding after dental extractions despite intact P2Y12 signaling [42]. However, we found that MA is driven by Talin1 and αIIbβ3 but not Rap1 signaling. TEG parameters were normal in mice with various Rap1 signaling defects, although *Rap1a/b*^{mKO} mice had reduced α-angle and MA. However, *Rap1a/b*^{mKO} mice also suffer from substantial thrombocytopenia [17], which may contribute to reduced parameters either independently or in conjunction with the platelet function defect in these mice. When activated with high concentrations of thrombin or PAR4p, platelets with defective Rap1 signaling demonstrate residual aggregation and clot contraction [11,17], and this may be sufficient to generate normal TEG values. In standard TEG, clotting is initiated with kaolin to drive rapid and robust activation of the contact pathway and virtually all prothrombin is converted to thrombin, so all platelets will be exposed to high concentrations of thrombin. In contrast to TEG, platelets at sites of injury in vivo during hemostasis and thrombosis undergo heterologous spatiotemporal activation by various agonists such as collagen, thrombin, and ADP [43]. Brass and Stalker described thrombi as containing a “core” of highly activated, degranulated platelets activated by thrombin and collagen surrounded by a “shell” of less strongly activated platelets with some platelets remaining discoid, heterogenous degranulation and activation by feedback mediators rather than primary agonists [44]. In our saphenous vein laser injury model, “core” platelets can be found around the edge of the injury whereas “shell” platelets bridge the center of the plug (unpublished observation), which is sensitive to antiplatelet therapy [45,46]. A similar arrangement of platelet phenotypes was reported in a larger venous puncture model [47]. In this context, platelet function in TEG is representative of a subset of highly activated core platelets, but it does not give a picture of overall platelet function.

Knowing that TEG is driven entirely by thrombin and that *PAR4*^{mKO} platelets are unresponsive to this agonist [15], we expected to see markedly reduced MA in *PAR4*^{mKO} samples, yet only observed a mild reduction in α. This was also unexpected as we and others have shown a critical role for PAR4 in hemostasis in mice [15,48]. The recent identification of a GPVI-fibrin(ogen) interaction [13,14] led us to investigate a potential role for GPVI in platelet activation and contraction during TEG. We found that GPVI/Syk can compensate for the lack of PAR4 in mouse platelets or inhibition of both PAR1 and PAR4 in human platelets. The literature describing GPVI-fibrin(ogen) interaction and function continues to evolve and is not lacking in controversy [49]. GPVI has been shown to bind fibrinogen [50], fibrin [13,14], and fibrin degradation products [51] with some species differences [50]. Candidate binding sites for GPVI on fibrin(ogen) include the αC and D regions [51–53]. Interestingly, one study suggested that GPVI can bind fibrin formed from recombinant fibrinogen but not from blood-sourced fibrinogen [54], and another demonstrated a lack of GPVI dimer binding to fibrin [55]. Studies to address whether the requirement for GPVI in TEG is indeed due to binding on fibrin will require specific tools to disrupt this interaction without affecting fibrin(ogen) structure or expression levels [56].

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The finding that GPVI has a redundant role with PARs was surprising to us, as the activation of GPVI by fibrin(ogen) binding has not been described as a primary pathway for platelet integrin activation, but rather for supporting thrombus stability and thrombin generation [13,51,57]. We do not suspect that platelet-mediated thrombin generation is critical in kaolin TEG as we observed no differences in TEG parameters in samples from mice lacking cyclophilin D (unpublished observation), which mediates platelet procoagulant activity [58]. However, we expect the majority of fibrinogen to be converted to fibrin, and therefore the platelets are exposed to an extremely dense and abundant fibrin network which may drive GPVI clustering and greater activation [52]. However, the relevance of this interaction during hemostatic plug formation is unknown. Fibrin is mainly generated at the periphery or extravascular face of platelet plugs [45,47] and may only contact a subset of platelets. GPVI does play a minor supporting role during hemostasis in $PAR4^{mKO}$ mice [15], but whether this is due to GPVI interaction with collagen or fibrin(ogen) has not been determined. There may also be a role for active regulation of the platelet cytoskeleton by PARs and GPVI. PAR4 activates the heterotrimeric Gα13 protein which manipulates the cytoskeleton through regulation of Rho GTPases [59]. Engagement of PAR4 and GPVI triggers release of thromboxane A2, and the TxA2 receptor also connects to G13/Rho signaling [60].

Our results demonstrate that the platelet contribution to MA is almost entirely mediated through platelet contraction, as demonstrated by maximal reduction in MA with cytochalasin D. Interestingly, MA was not reduced to the maximal extent observed with platelet depletion or cytochalasin D when we inhibited αIIbβ3 function, suggesting receptors other than αIIbβ3 may contribute to clot contraction. Platelet contractile forces are critical for hemostasis and reduced platelet forces strongly associate with bleeding risk [61], but our data suggest that these defects may be masked in standard TEG due to robust thrombin activation and multiple redundant pathways for activation. Importantly, TEG is a static assay and does not account for defects in platelet function under flow conditions. Platelet interaction with VWF via GPIbα is a critical first step in the sequence of platelet adhesion and activation under flow, and platelet surface expression of GPIbα as well as GPVI may be altered in trauma [62].

We expect that our findings are relevant to clinical scenarios where kaolin (standard) TEG is used to determine the necessity for platelet transfusion. First, in trauma-associated hemorrhage, transfusion algorithms include MA thresholds which would trigger platelet transfusion [63]. Taken in the context of our findings, significant reductions in standard TEG MA would require substantial platelet dysfunction, affecting either multiple inside-out signaling pathways, integrin outside-in signaling, or a direct effect on αIIbβ3 itself. However, we and others have shown that platelet transfusion is ineffective in reversing platelet integrin dysfunction when platelet counts are within the normal range unless enough units are given to achieve specific transfused to endogenous platelet ratios [64]. Second, a patient with a normal MA by standard TEG may have platelet defects that contribute to ongoing hemorrhage but are masked in the TEG assay. The risk of missing these patients can be mitigated, at least in part, by the use of platelet mapping TEG; however, that assay is still specific to platelet dysfunction affecting ADP and thromboxane activation pathways. Inclusion of a GPVI function-blocking antibody as a TEG reagent to remove the GPVI/fibrin interaction may help unmask underlying platelet dysfunction and increase the sensitivity

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of TEG to additional pathological mechanisms. Interestingly, the GPVI blocking antibody fragment glenzocimab was recently shown to inhibit both collagen and fibrin-dependent GPVI activation [65].

Overall, our findings are in agreement with the literature that standard TEG is sensitive to thrombocytopenia and direct inhibition of αIIbβ3, but we demonstrate that multiple redundant pathways mediate platelet integrin activation and contraction in TEG. Moreover, these data suggest that TEG outcomes have little correlation with platelet function in vivo. We also demonstrate a novel role for GPVI in TEG, suggesting that further research is needed on the impact of GPVI-fibrin(ogen) interaction in various clotting assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Essentials

- Thromboelastography (TEG) helps evaluate bleeding risk at point-of-care and guide transfusion.
- The platelet receptors and signaling pathways mediating TEG have not been fully investigated.
- TEG is largely insensitive to inside-out signaling defects associated with major bleeding.
- The receptors PAR1/4 and GPVI play redundant roles in the platelet contribution to TEG parameters.

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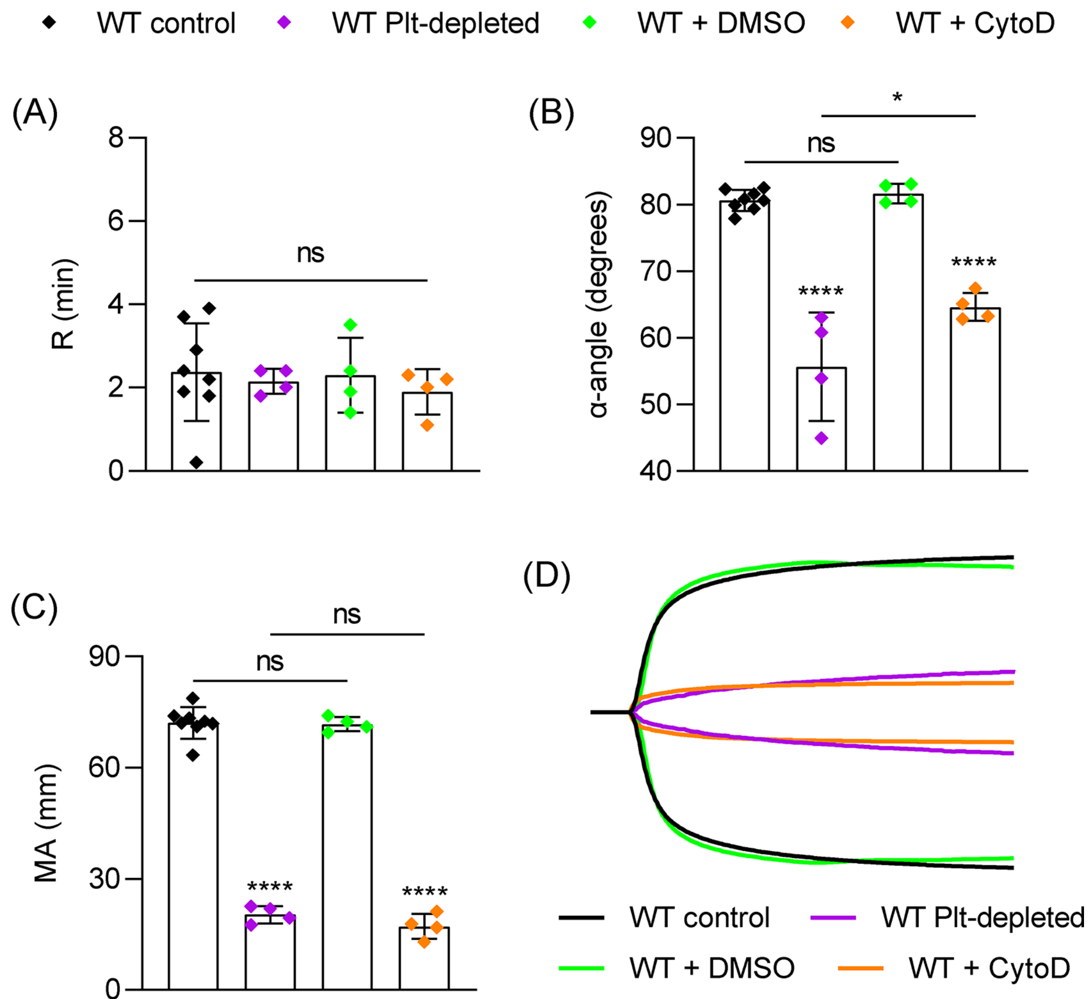

Figure 1:

Role of platelets and platelet contraction in TEG. Citrated blood samples were collected by retroorbital bleed (RO) from wild-type (WT) mice (n=8) or WT mice depleted of circulating platelets (WT Plt-depleted) by injection of an anti-GPIbα antibody (R300, 1 mg/kg, n=4). For inhibition of platelet-mediated contraction, WT samples were treated with DMSO (n=4) or cytochalasin D (5 μg/ml, n=4) for 10 mins prior to TEG assay. Samples were mixed with $CaCl_2$ and kaolin in plastic TEG cup and run immediately in a TEG 5000 analyzer and recorded for 1 hour. (A-C) TEG parameters: R time (A), α-angle (B) and MA (C). (D) Representative TEG traces. Data shown as mean ± SD. Statistical significance was determined by unpaired Student's t-test (A) or one-way ANOVA with Tukey's multiple comparison test. Symbols directly over bars represent significance compared to WT control. *P < .05, ****P < .0001.

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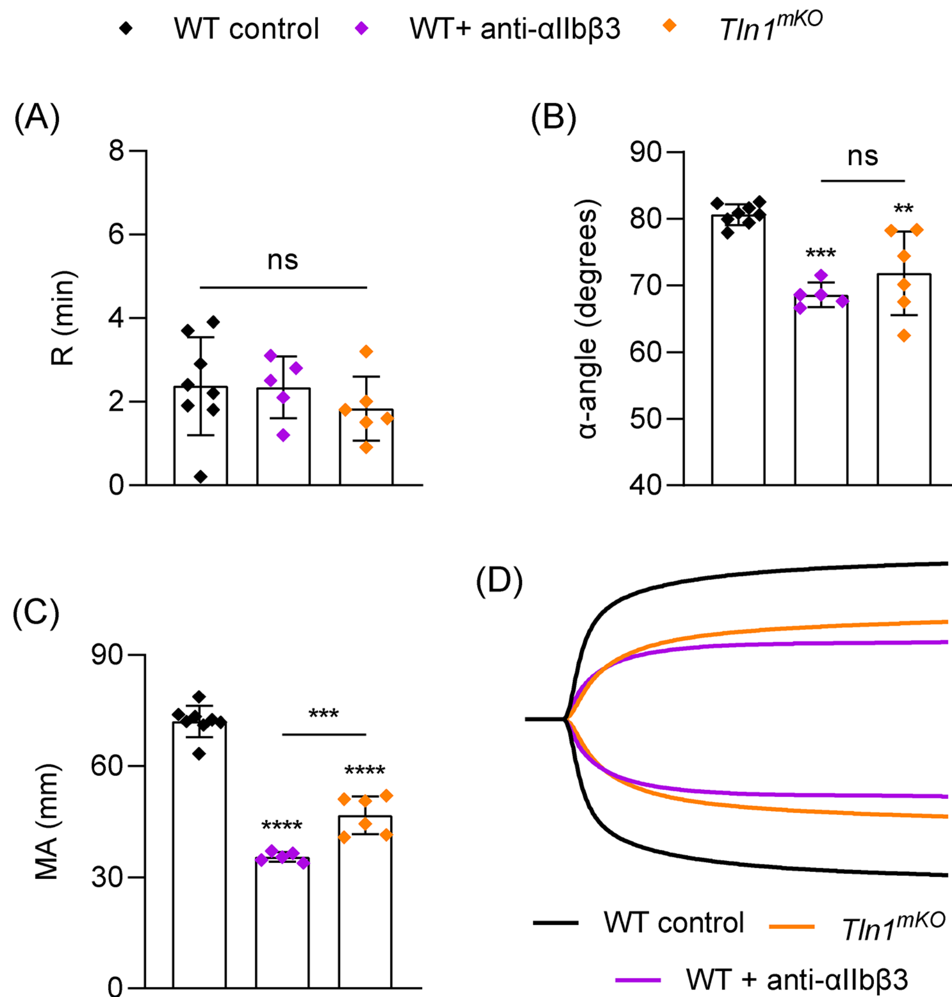

Figure 2:

Role of αIIbβ3 integrin activation and ligand binding in TEG. Blood samples were analyzed from WT mice (n=8) or mice with megakaryocyte/platelet-specific deletion of Talin1 (*Tln1*^{mKO}, n=5). αIIbβ3 ligand binding was inhibited by treating WT samples with anti-αIIbβ3 antibody (Leo.H4, 75 μg/ml, n=6) for 10 mins prior to TEG assay. (A-C) TEG parameters: R time (A), α-angle (B) and MA (C). (D) Representative TEG traces. Data shown as mean ± SD. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test. Symbols directly over bars represent significance compared to control. **P< .01, ***P<0.001, ****P < .0001.

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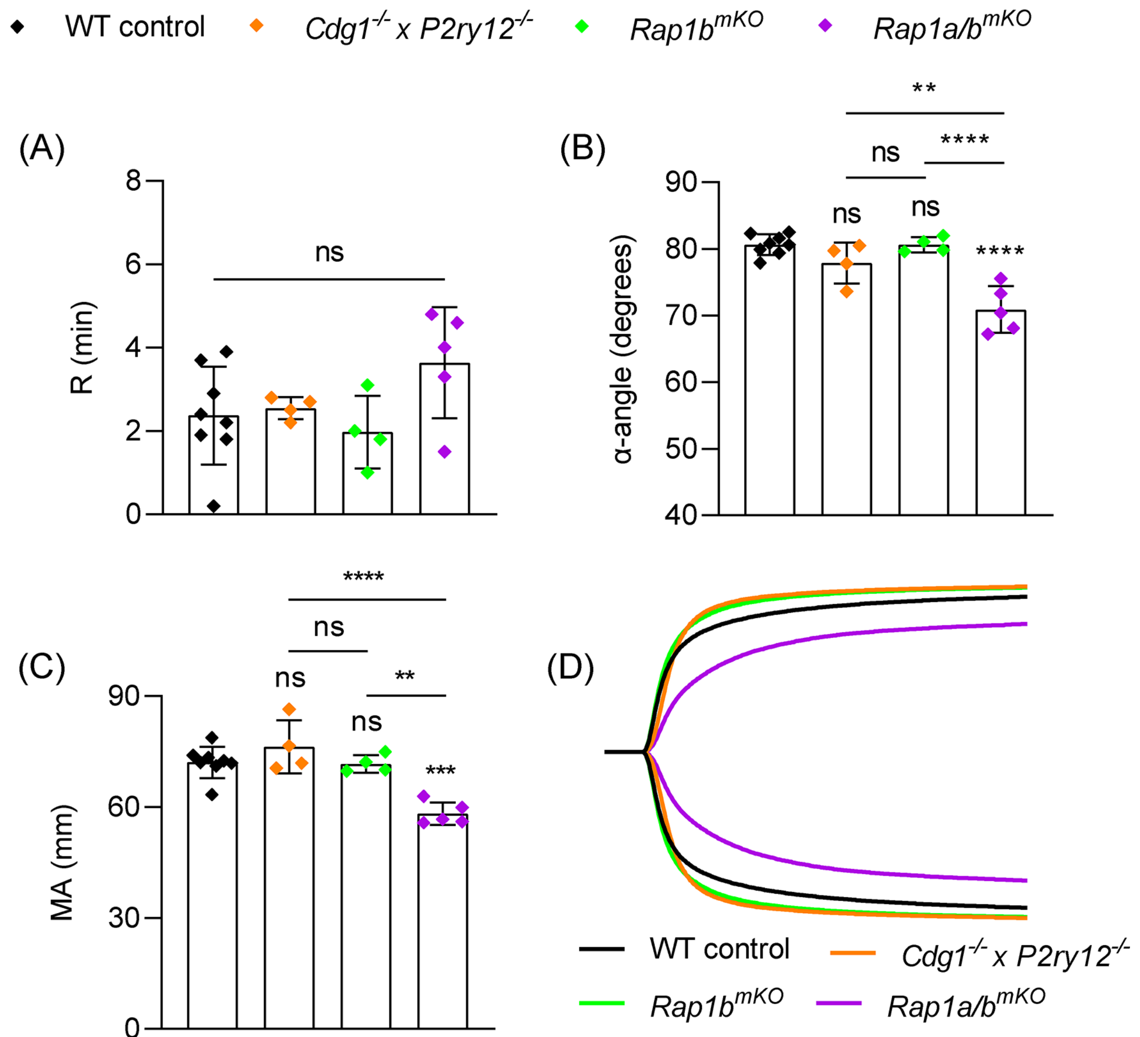

Figure 3:

Role of Rap1 GTPase signaling in TEG. Blood samples were analyzed from WT mice (n=8), mice with global deficiency in CalDAG-GEFI and P2Y12 ($Cdg1^{-/-} \times P2ry12^{-/-}$, n=4) or mice with megakaryocyte/platelet-specific deletion of Rap1b alone ($Rap1b^{mKO}$, n=4) or both Rap1a and Rap1b ($Rap1a/b^{mKO}$, n=5). (A-C) TEG parameters: R time (A), α-angle (B) and MA (C). (D) Representative TEG traces. Data shown as mean ± SD. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test. Symbols directly over bars represent significance compared to control. **P< .01, ***P<0.001, ****P < .0001.

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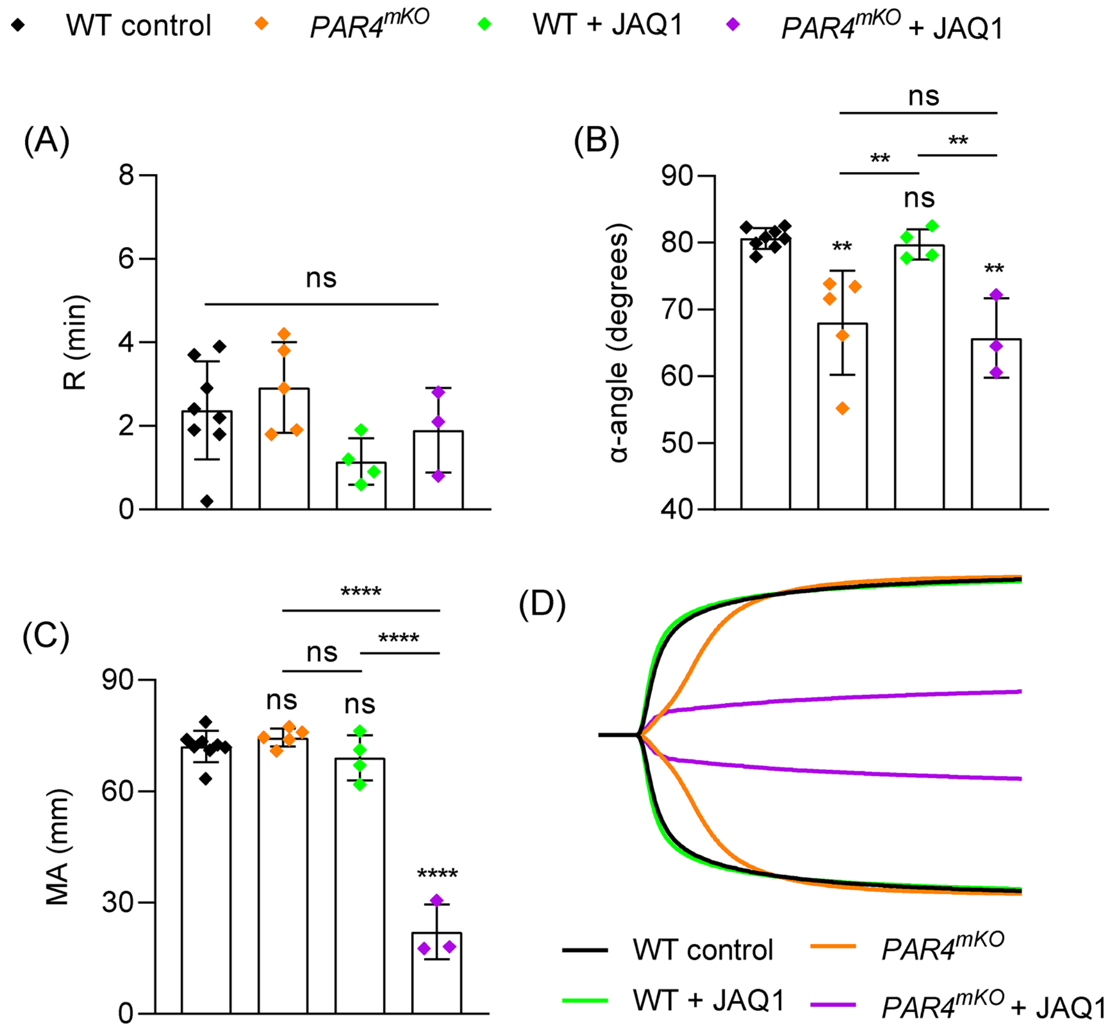

Figure 4:

Role of platelet PAR4 and GPVI in TEG. Blood samples were analyzed from WT mice (n=8), mice with megakaryocyte/platelet-specific deletion of PAR4 (*PAR4*^{mKO}, n=5), WT mice treated with anti-GPVI antibody to deplete GPVI on circulating platelets (JAQ1, 50 μg/mouse, n=4), or JAQ1-treated *PAR4*^{mKO} mice (n=3). (A-C) TEG parameters: R time (A), α-angle (B) and MA (C). (D) Representative TEG traces. Data shown as mean ± SD. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test. Symbols directly over bars represent significance compared to control. **P< .01, ****P < .0001.

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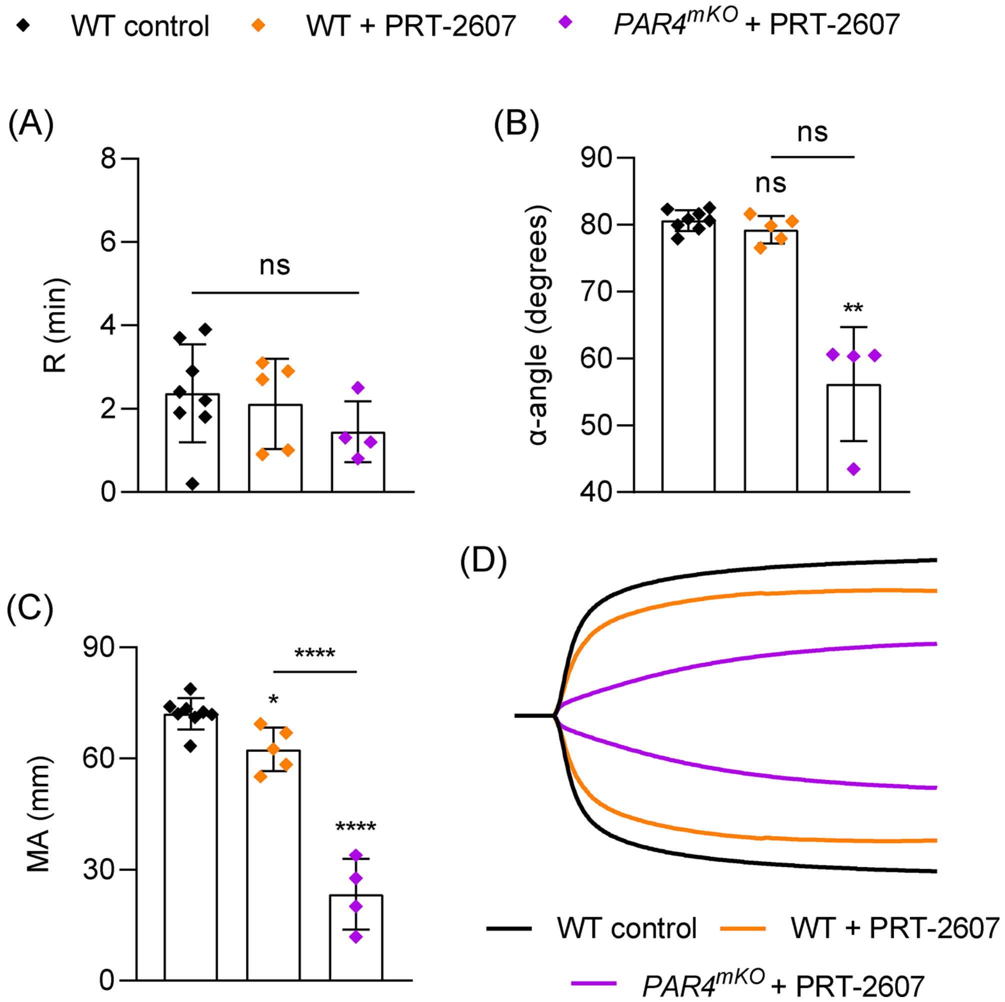

Figure 5:

Role of Syk tyrosine kinase signaling in TEG. Blood samples were analyzed from WT mice (n=8), or WT (n=5) or *PAR4*^{mKO} (n=4) mice with addition of the Syk inhibitor PRT-2607 (20 μM) 10 mins prior to TEG assay. (A-C) TEG parameters: R time (A), α-angle (B) and MA (C). (D) Representative TEG traces. Data shown as mean ± SD. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test (A,C) or Kruskal-Wallis test with Dunn's multiple comparison (B). Symbols directly over bars represent significance compared to control. **P < .01, ****P < .0001.

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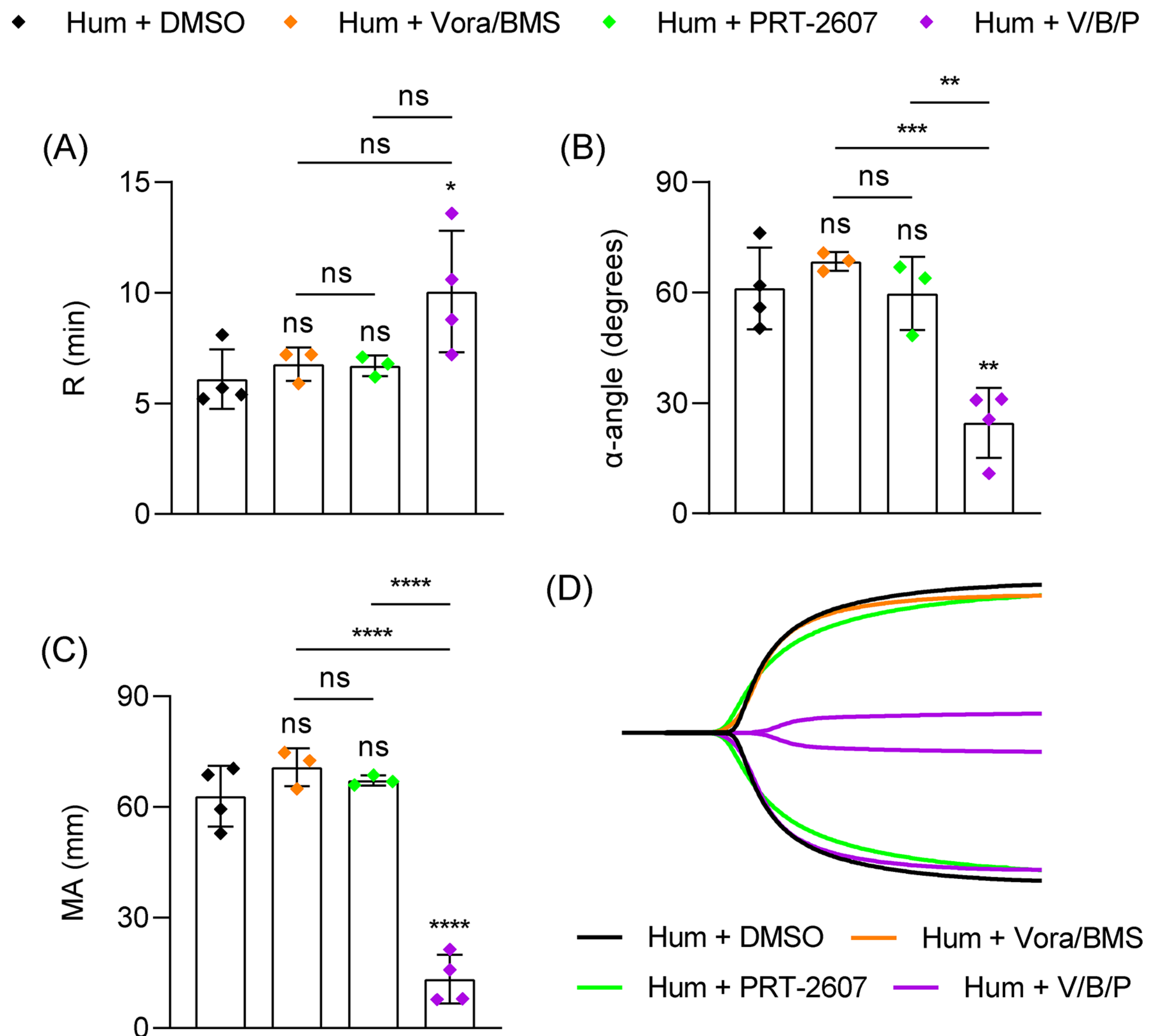

Figure 6:

Role of PAR1/PAR4 and Syk in human blood TEG. Healthy volunteer blood samples were analyzed with addition of DMSO (n=4), vorapaxar (Vora, 5 μM, n=3), BMS-986120 (BMS, 10 μM, n=3) and PRT-2607 (PRT, 20 μM, n=4) to inhibit PAR1, PAR4 and Syk, respectively, 10 mins prior to TEG assay. (A-C) TEG parameters: R time (A), α-angle (B) and MA (C). (D) Representative TEG traces. Data shown as mean ± SD. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test. Symbols directly over bars represent significance compared to control. *P < .05, **P< .01, *** P<0.001, ****P < .0001.

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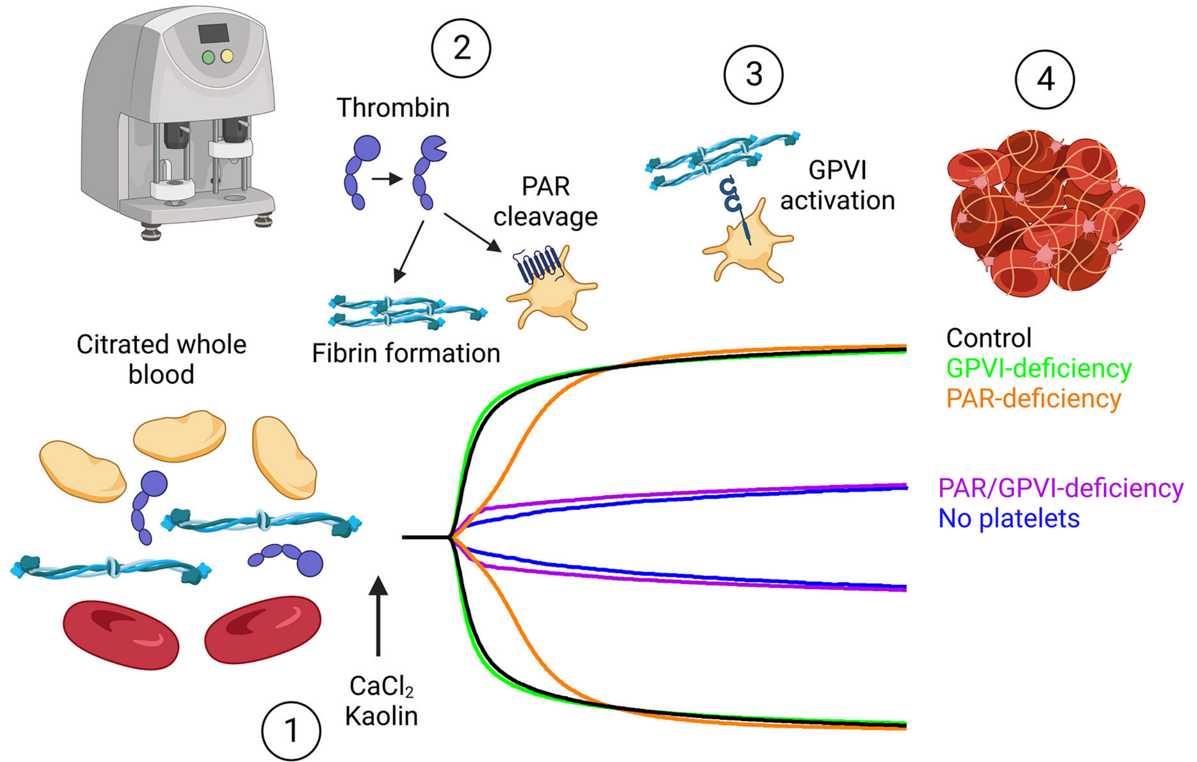

Figure 7:

PAR1/4 and GPVI play redundant roles in platelet activation and contraction in TEG. (1) Analysis of clot formation speed and clot strength in TEG is performed by recalcifying citrated whole blood and activating with kaolin to initiate coagulation. (2) Within several minutes, thrombin generation initiates fibrinogen cleavage to fibrin, which polymerizes and crosslinks. Additionally, thrombin activates platelets through protease activated receptors (PARs) (PAR4 on mouse platelets, PAR1 and PAR4 on human platelets). Both fibrin polymerization and platelet activation contribute to the speed of clot formation (α-angle). (3) As more fibrin is generated, platelets can also bind fibrin via GPVI for additional activation signaling, resulting in robust αIIbβ3 integrin activation and platelet-mediated contraction of the fibrin clot. Clot strength (MA) is entirely dependent on platelet contraction. (4) The end result is a tightly contracted whole blood clot with contracting platelets bound to fibrin. While loss of either platelet PARs or GPVI alone has limited impact on TEG parameters, loss of both leads to TEG traces similar to platelet-depleted blood samples. Created with BioRender.com.