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

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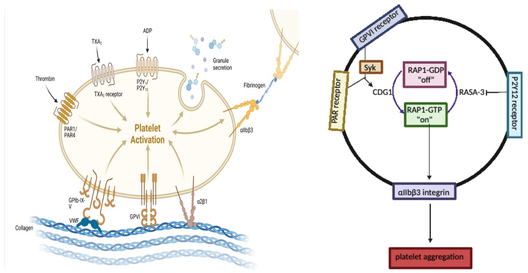
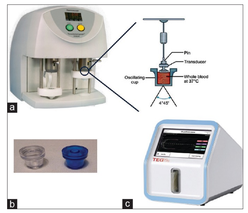
Background

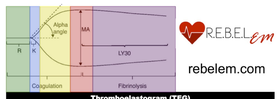
Platelet activation signaling



- Platelet signaling triggered by receptor activation (PARs, GPVI) via thrombin or collagen
- Rap1 GTPase is dually turned on by activation of CalDAG-GEFI and inhibition of Rasa3 via P2Y12 binding.
- Integrin inside-out activation results in platelet aggregation
- Integrin outside-in signaling mediates clot contraction
- Impairment in PAR activation or Rap1 signaling causes bleeding
- Lesser impact of GPVI deficiency or impaired outside-in signaling on platelet-mediated hemostasis

Thromboelastography (TEG)





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Thromboelastogram (TEG)				
Components	**Definition**	**Normal Values**	**Problem with...**	**Treatment**
R Time	Time to start forming clot	5 – 10 minutes	Coagulation Factors	FFP
K Time	Time until clot reaches a fixed strength	1 – 3 minutes	Fibrinogen	Cryoprecipitate
Alpha angle	Speed of fibrin accumulation	53 – 72 degrees	Fibrinogen	Cryoprecipitate
Maximum Amplitude (MA)	Highest vertical amplitude of the TEG	50 – 70 mm	Platelets	Platelets and/or DDAVP
Lysis at 30 Minutes (LY30)	Percentage of amplitude reduction 30 minutes after maximum amplitude	0 – 8%	Excess Fibrinolysis	Tranexemic Acid and/or Aminocaproic Acid

- How does it work?
  - Small benchtop unit measures the dynamics of clot development (initiation of clot formation, stability ,etc.) using either whole blood or plasma
- What does it measure?
  - R (time to start of clotting)
  - K (time till clot reaches fixed strength)
  - α (rate of clot formation)
  - MA (maximal clot strength)
- What is it used for?
  - Theoretically, TEG –guided transfusions are thought to reduce the use of blood products (platelets, FFP, TXA etc.) by preventing unnecessary transfusions in surgery
  - However, the use of TEG in surgery is not always associated with improved patient outcomes.

- What is missing?
  - In the TEG assay, it is assumed that platelet activation is mediated by thrombin and activation of the PAR receptors.
  - Specific receptors and signaling pathways required for platelet contribution to TEG parameters have not been determined
  - Novel ligand-receptor interactions have not been investigated
    - GPVI and fibrin(ogen) interaction
- **Goal:** to better define platelet activation mechanisms in TEG and correlate TEG results with *in vivo* platelet function during hemostasis.
- **Approach**: to investigate 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

- Samples
  - Citrated mouse blood collected from anesthetized mice via retroorbital plexus
  - Human whole blood collected with a 21-G needle vacutainer butterfly
  - Both mouse and human samples were collected into 3.2% citrated tubes.
- TEG
  - TEG 5000 (Haemonetics)
  - If applicable, inhibitors added
  - Samples recalcified (20 µL CaCl2, coagulation initiated with kaolin (16 µL kaolin), 384 µL whole blood added to cups
  - Run for 1 hour
- Parameters
  - R, α, MA
  - Flow cytometry used to quantify platelet counts and confirm platelet activation//inhibition when applicable

Results

Figure 1: Role of platelets and platelet contraction in TEG

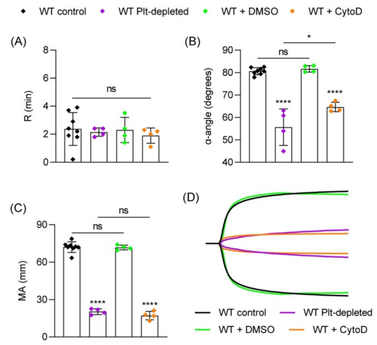


Fig 1: TEG clotting parameters were assessed in citrated whole blood samples via retroorbital bleed from wild-type (WT) or WT mice with depleted platelets (WT Plt-depleted) via injection of anti-GPIb antibody. To test platelet-mediated contraction, WT samples were incubated with DMSO or cytochalasin D (5 µg/mL) for 10 minutes prior to running TEG assay. Statistical significance was determined using either unpaired Student's t-test or one-way ANOVA. Symbols directly over bars represent significant results compared to WT control *P<.05, ****P<0.0001.

Figure 2: Role of αIIbβ3 integrin activation and ligand binding in TEG

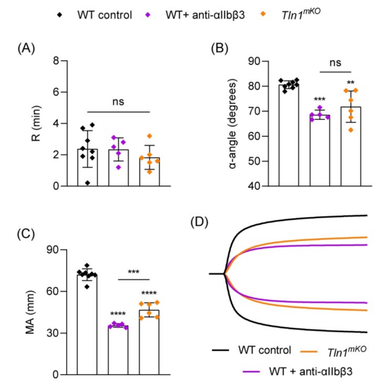


Fig 2: Citrated blood samples were analyzed from WT mice or mice with a megakaryocyte/platelet-specific deletion of Talin1 (*Tln1^{mKO})*. Anti-αIIbβ3 antibody was added to WT samples in order to inhibit αIIbβ3 ligand binding. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test. Symbols directly over bars represent significant results compared to WT control *P<.01, P***<.001 ****P<0.0001.

Figure 3: Role of Rap1 GTPase signaling in TEG

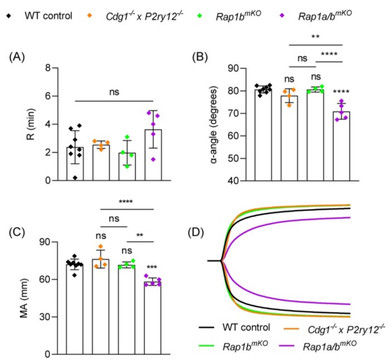


Fig 3: Citrated whole blood samples were analyzed from WT mice with combined global deficiency in CalDAG-GEFI and P2Y12 ("*Cdg1$^{-/-}$ x P2ry12$^{-/-}$*") or from mice lacking the Rap1b isoform in megakaryocytes and platelets (*Rap1b^{mKO}*) or both Rap1a and Rap1b *(Rap1a/b^{mKO}*). Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test. Symbols directly over bars represent significant results compared to WT control *P<.01, P***<.001 ****P<0.0001.

Results (continued)

Figure 4: Role of platelet PAR4 and GPVI in TEG

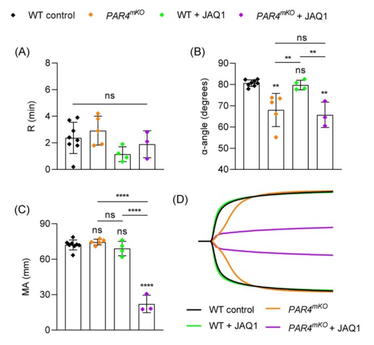


Fig 4: Citrated whole blood samples were analyzed from WT mice with megakaryocyte/platelet-specific of PAR4 receptor. Anti-GPVI antibody was used to deplete GPVI on circulating platelets in WT mice (JAQ1) or JAQ1-treated *PAR4^{mKO}* mice. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test. Symbols directly over bars represent significant results compared to WT control *P<.01, ****P<0.0001.

Figure 5:Role of Syk tyrosine kinase signaling in TEG

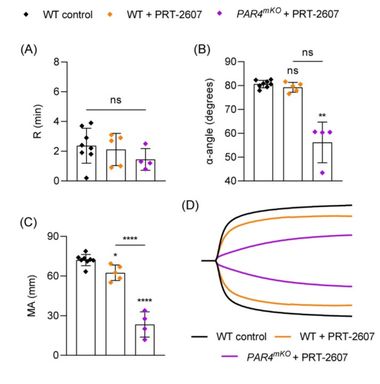


Fig 5: Citrated whole blood samples were analyzed from WT mice or *PAR4^{mKO}* mice treated with a Syk inhibitor (PRT-2607). Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test. Symbols directly over bars represent significant results compared to WT control *P<.01, ****P<0.0001.

Figure 6: Role of PAR1/PAR4 and Syk in human blood TEG

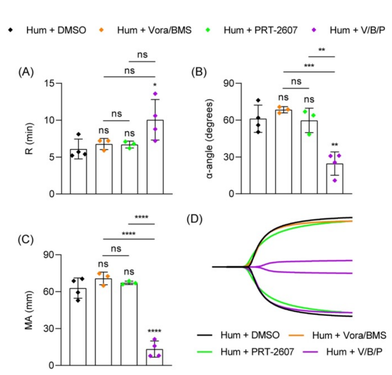


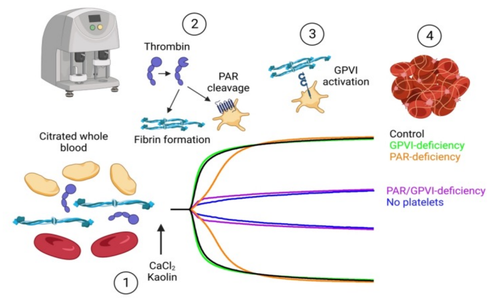
Fig 6: Volunteer blood samples were analyzed with the addition of DMSO, vorapaxar (Vora), BMS-986120, and PRT-2607 to inhibit PAR1, PAR4, and Syk, respectively. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparison test. Symbols directly over bars represent significant results compared to control *P<.05, **P<.01,****P<0.0001.

Summary

- Platelet depletion and cytochalasin D samples showed a significant decrease in α and MA but no change in R time.
- Significant reductions in α and MA were seen in WT blood treated with blocking antibody to αIIbβ3 integrin and in Tln1^{mKO} mice.
- Mice lacking both isoforms of Rap1 (*Rap1 a/b^{mKO}*) had significant reductions in α and MA (but were also thrombocytopenic), while individual deletion of the Rap1b isoform or of the two major pathways for Rap1 activation (*Cdg1$^{-/-}$ x P2ry12$^{-/-}$*) appeared to have no impact on parameters.
- PAR4^{mKO} mice + JAQ1 antibody had a similar reduction in α as untreated PAR4^{mKO} mice and a drastic reduction in MA. WT mice treated with anti-GPVI antibody (JAQ1)had no reductions in R, α, or MA.
- *PAR4^{mKO}* samples + Syk inhibition with PRT-2607 had extreme reductions in MA but individual inhibition of Syk appeared to only minimally affect the MA.
- Human samples only showed a marked reduction in α and MA when both PAR receptors and Syk were simultaneously inhibited – individual blockage of PAR4/PAR1 receptors or Syk appeared to have no impact on parameters.

Conclusions

- There is a disconnect between platelet function in TEG versus platelet function during hemostasis in vivo
  - Platelet activation does not require RAP1 GTPase signaling which is critical *in vivo* for platelet integrin activation and aggregation
  - PAR4 and GPVI seem to play the same role for platelet dependent parameters (alpha angle, MA) for both mice and humans (not species specific)
  - TEG can effectively identify platelet contraction defects but does not seem to require the signaling pathways critical for integrin inside-out activation and platelet hemostatic function
- Standard TEG uses kaolin to initiate clotting and drive robust activation of the contact pathway, so all prothrombin is converted to thrombin
  - In vivo, platelets at the sties of injury do not experience such a strong thrombin activation



Future Directions

- In trauma associated hemorrhage, a defect in the MA would typically call for platelet transfusions
  - According to our findings, substantial platelet dysfunction (multiple defective inside-out signaling pathways, integrin outside-in signaling, or direct αIIbβ3 inhibition) would be needed to show a reduction in MA
  - A patient with a normal MA in standard TEG may have platelet defects that are masked in the TEG assay
- We would like to further investigate the ability of TEG to assess the hemostatic efficacy platelet transfusions for patients with inherited platelet disorders

Rudran T, Antoniak S, Flick MJ, Ginsberg MH, Wolberg AS, Bergmeier W, Lee RH, Protease activated receptors and glycoprotein VI cooperatively drive the platelet component in thromboelastography, Journal of Thrombosis and Haemostasis (2023), doi: https://doi.org/10.1016/ j.jtha.2023.04.008.

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