

SUPPLEMENTAL MATERIAL

Hydrogen peroxide promotes aging-related platelet hyperactivation and thrombosis

Sanjana Dayal, PhD, Katina M. Wilson, BS, David G. Motto, MD, PhD, Francis J. Miller, Jr.,
MD, Anil K. Chauhan, PhD, and Steven R. Lentz, MD, PhD.

Supplemental Methods

Platelet immunoblotting

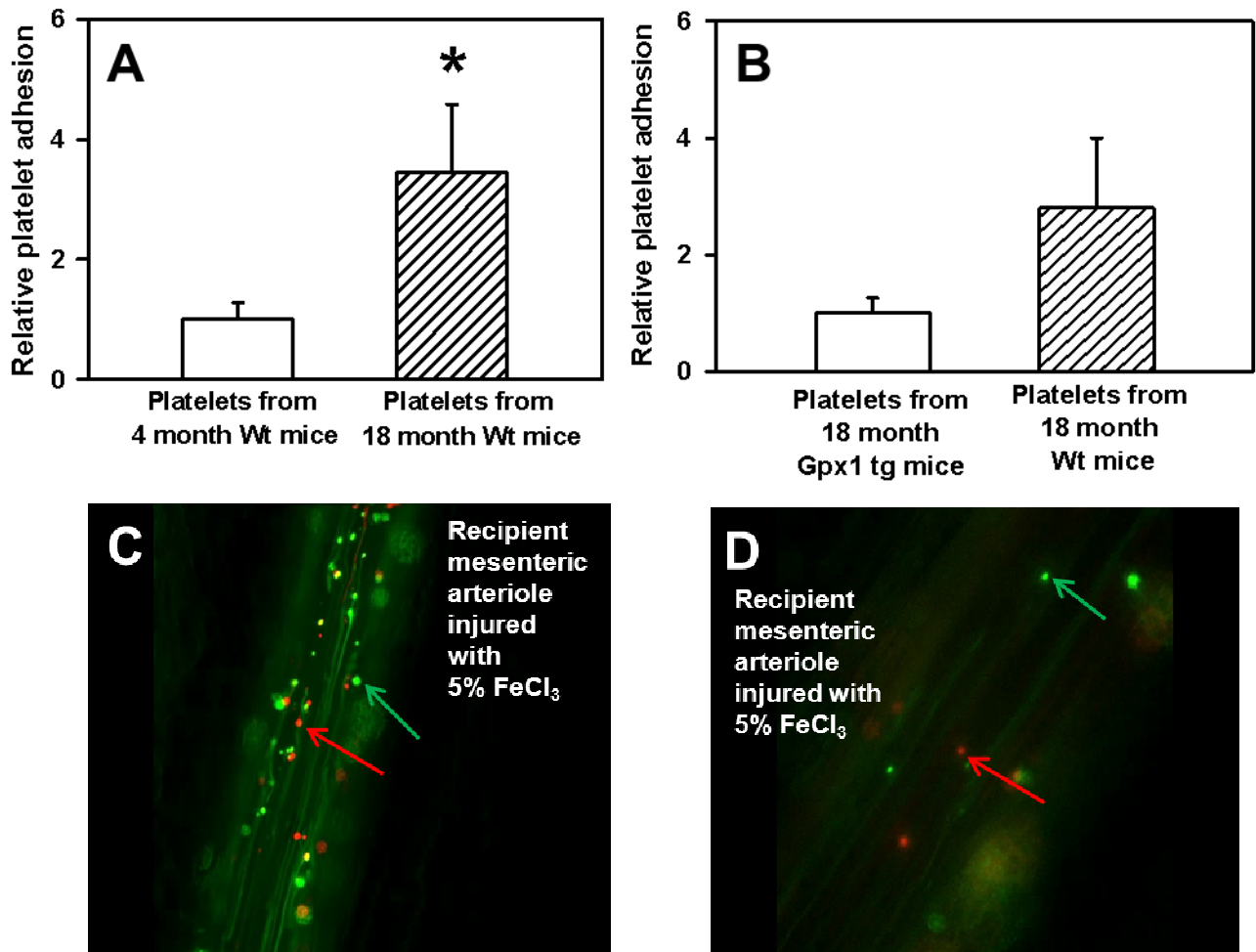
Platelets were isolated from citrated blood and washed in modified Tyrode's buffer. Platelet lysates were prepared in 1M Tris buffer (pH 7.4) containing 1% triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA (pH 8.0), 0.5% NP-40, and protease inhibitor cocktail (CompleteTM Mini EDTA-free, Roche, USA). Immunoblotting was performed as described previously.¹ Briefly, 30 µg of protein was run on a 12.5 % Tris-HCl reducing gel (Bio-Rad), and membranes were probed with either 1.0 µg/ml rabbit polyclonal mouse anti-Gpx1 (ab22604, Abcam, Cambridge, MA) or 0.5 µg/ml of mouse monoclonal anti-β-actin as a loading control (ab8226, Abcam, Cambridge, MA). Immunoreactive bands were visualized using Supersignal West femto (Pierce, IL, USA) detection system. Results were quantified with NIH Image J, and expressed as percent of levels observed in 4 month old wild-type (Wt) mice. Two-way ANOVA was used for comparison between the groups.

Platelet adhesion during thrombus formation *in vivo*

The relative adhesion of isolated platelets from donor mice (4 vs. 18 month old Wt mice, or 18 month old Wt vs. 18 month old Gpx1 Tg mice) was assessed during ferric chloride-induced thrombosis formation in mesenteric arterioles of 4 week old Wt recipient mice. Blood was collected from the retro-orbital venous plexus of donor mice into a polypropylene tube containing 0.3 mg/ml enoxaparin. Platelets were isolated, washed, and differentially fluorescently labeled with acetomethoxy (AM) derivative of calcein green or calcein red-orange (Invitrogen) as previously described.² The host mouse was anesthetized with avertin, and differentially labeled platelets (2.5×10^9 platelets/kg) were infused retro-orbitally. Mesenteric

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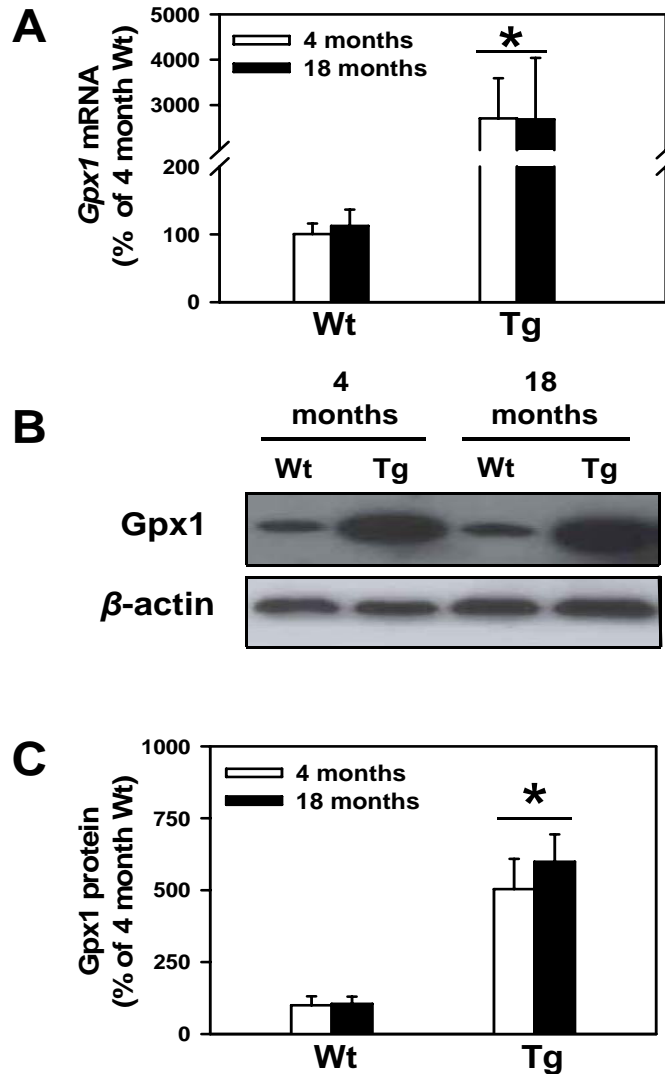
arterioles (between 80-100 μm in diameter) of the host mouse were topically injured with 5% FeCl_3 . Real-time imaging was performed using intravital microscopy to record the number of fluorescent platelets adhering to the injured vessel wall during the initial 3 minutes of thrombus formation. One to three mesenteric arterioles were studied in each recipient mouse. At the completion of each experiment, blood was collected and the percentage of circulating fluorescently labeled platelets was determined by flow cytometry. Relative platelet adhesion was defined as the number of adherent fluorescent platelets, normalized to the percentage of circulating fluorescent platelets, and presented relative to either 4 month old Wt mice or 18 month old Gpx1 Tg mice. One-way analysis of variance (ANOVA) was performed on log-transformed values to compare the relative adhesion. Statistical significance was defined as a P value <0.05 . Values are reported as mean \pm SE.



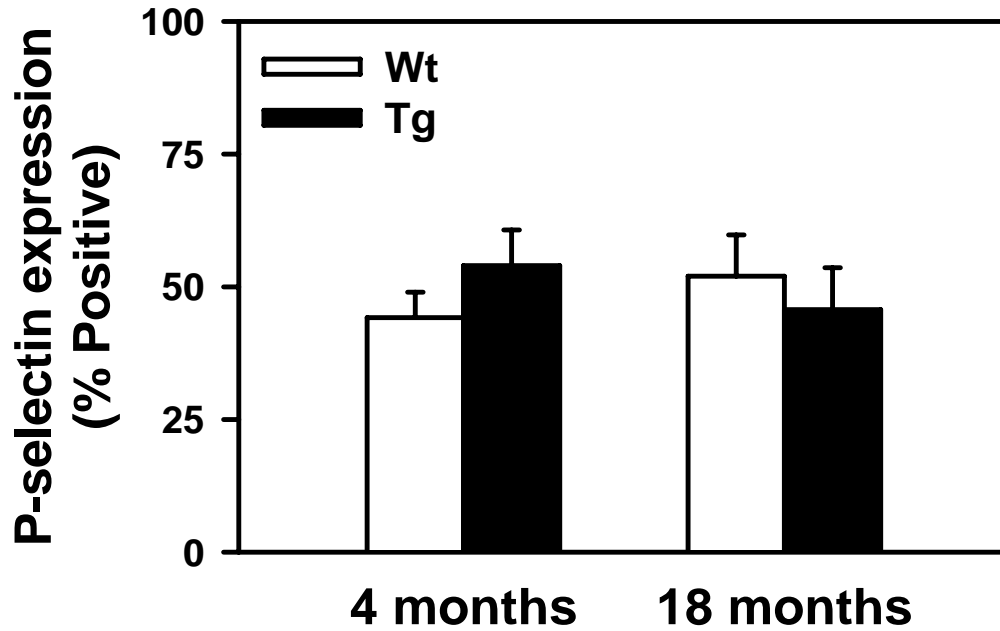
Supplemental Figure I. Platelet adhesion during thrombus formation is increased with aged platelets. Differentially fluorescently labeled platelets were infused into 4 week old recipient C57Bl6/J mice, mesenteric arterioles were injured with 5% FeCl₃ and relative platelet adhesion during initial thrombus formation was assessed by intravital microscopy. (A) Relative adhesion of platelets from 4 month old vs. 18 month old Wt mice. (B) Relative adhesion of platelets from 18 month old Gpx1 Tg vs. 18 month old Wt mice. 12-20 vessels were examined in 5-8 host mice per group. Values are mean ± SE. *p<0.05 vs. 4 month old C57Bl6/J mice by one-way ANOVA. (C) Representative image of adhesion of platelets from 4 month old (calcein red-orange AM-labeled; red arrow) vs. 18 month old (calcein green AM-labeled; green arrow) Wt

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mice. Also see Supplemental Video S1. (D) Representative image of adhesion of platelets from 18 month old Gpx1 Tg (calcein red-orange AM- labeled; red arrow) vs. 18 month old Wt (calcein green AM-labeled; green arrow) mice. Also see Supplemental Video S2.



Supplemental Figure II. Gpx1 Tg mice have higher Gpx1 mRNA and protein levels in platelets. *Gpx1* mRNA and protein levels were measured in platelets from 4 or 18 month old wild-type (Wt) or Gpx1 Tg (Tg) mice by real time PCR and Western blotting. (A) Platelet mRNA levels of *Gpx1* expressed as the percent of *Gpx1* levels observed in 4 month old Wt mice. Platelets from 9-15 mice were studied in each group. (B) Representative immunoblot of platelet Gpx1 protein levels. (C) Summary of quantitation of 5-9 immunoblotting experiments, with Gpx1 protein levels normalized to β-actin and expressed as percent of levels observed in 4 month old Wt mice. *P<0.05 compared with age-matched Wt littermates by two-way ANOVA.



Supplemental Figure III. Expression of P-selectin on platelet surface is not influenced by aging or Gpx1 genotype. Expression of P-selectin was examined in thrombin-activated platelets from either wild-type (Wt) or Gpx1 Tg (Tg) mice at 4 or 18 months of age using flow cytometry. Platelets from 6-8 mice were studied in each group. Values are mean \pm SE.

Supplemental References

1. Dayal S, Rodionov RN, Arning E, Bottiglieri T, Kimoto M, Murry DJ, Cooke JP, Faraci FM, Lentz SR. Tissue-specific downregulation of dimethylarginine dimethylaminohydrolase in hyperhomocysteinemia. *Am J Physiol Heart Circ Physiol*. 2008;295:H816-825.
2. Chauhan AK, Motto DG, Lamb CB, Bergmeier W, Dockal M, Plaimauer B, Scheiflinger F, Ginsburg D, Wagner DD. Systemic antithrombotic effects of ADAMTS13. *J. Exp. Med*. 2006;203:767-776.

Video legends

Supplemental Video S1: Relative adhesion of platelets from 4 and 18 month old C57Bl6/J mice. Representative video of adhesion of platelets from 4 month old (calcein red-orange AM-labeled) vs. 18 month old (calcein green AM-labeled) C57Bl6/J mice. Differentially fluorescently labeled platelets from 4 and 18 months old C57Bl6/J mice were infused into 4 week old recipient C57Bl6/J mice, mesenteric arterioles were injured with 5% FeCl₃ and relative platelet adhesion during initial thrombus formation was assessed by intravital microscopy.

Supplemental Video 2. Relative adhesion of platelets from 18 month old wild-type (Wt) and Gpx1 Tg (Tg) mice. Representative video of adhesion of platelets from 18 month old Tg (calcein red-orange AM-labeled) vs. Wt (calcein green AM-labeled) mice. Differentially fluorescently labeled platelets from 18 months old Wt or Tg mice were infused into 4 week old

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recipient C57Bl6/J mice, mesenteric arterioles were injured with 5% FeCl₃ and relative platelet adhesion during initial thrombus formation was assessed by intravital microscopy.