Hydrogen Peroxide Promotes Aging-Related Platelet Hyperactivation and Thrombosis

Running title: Dayal et al.; Platelet activation and thrombosis in aging

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Abstract:

Background—The incidence of thrombotic events increases during aging but the mechanisms are not well understood. To investigate the prothrombotic role of oxidative stress during aging, we tested the hypothesis that aged mice overexpressing the antioxidant enzyme glutathione peroxidase-1 (Gpx1) are protected from experimental thrombosis.

Methods and Results—Susceptibility to carotid artery thrombosis was first examined in wild-type C57BL/6J mice. After photochemical injury of the carotid artery, the time to stable occlusion was significantly shorter in 12 and 18 month old mice compared with 4 month old mice (P<0.01). Unlike wild-type mice, transgenic mice overexpressing Gpx1 (Gpx1 Tg) did not exhibit shortened times to occlusion of the carotid artery at 12 or 18 months of age. Wild-type mice also exhibited increased susceptibility to venous thrombosis after inferior vena cava ligation at 12 or 18 months of age (P<0.05 vs. 4 months of age). Gpx1 Tg mice were protected from this aging-related enhanced susceptibility to venous thrombosis. Age-dependent platelet hyperactivation, evidenced by increased hydrogen peroxide, fibrinogen binding, and activation of fibrinogen receptor αIIbβ3, were observed in thrombin-activated platelets from wild-type but not Gpx1 Tg mice (P<0.05). Enhanced platelet activation responses in aged mice were also prevented by PEG-catalase or apocynin, an inhibitor of NADPH oxidase. Aged mice displayed increased intra-platelet expression of p47phox and superoxide dismutase-1, suggesting a mechanistic pathway for increased H2O2 generation.

Conclusions—Our findings demonstrate that hydrogen peroxide is a key mediator of platelet hyperactivity and enhanced thrombotic susceptibility in aged mice.

Key words: aging, platelet, oxidative stress
Introduction

Thrombotic events such as stroke, myocardial infarction, deep vein thrombosis, and pulmonary embolism are common causes of morbidity and mortality in the elderly.\textsuperscript{1-3} Despite the well-established clinical associations between aging and thrombosis, surprisingly little is known regarding the mechanisms by which aging increases susceptibility to thrombotic events.

Several lines of evidence indicate that aging is accompanied by a generalized increase in oxidative stress and that increased generation of reactive oxygen species (ROS) may contribute to cardiovascular events including thrombosis. A marked increase in oxidative stress has been observed with aging in multiple tissues in humans\textsuperscript{4, 5} and experimental animals.\textsuperscript{6, 7} Increased oxidative stress has been mechanistically implicated in several of the cardiovascular consequences that are also associated with aging, such as myocardial dysfunction,\textsuperscript{8, 9} myocardial ischemia-reperfusion injury,\textsuperscript{10} hypertension,\textsuperscript{11} and endothelial dysfunction.\textsuperscript{12, 13} Moreover, there is abundant evidence that ROS regulate several components of thrombotic processes,\textsuperscript{14-16} including platelet activation.\textsuperscript{17-19} To date, however, no studies have directly investigated the mechanistic contribution of ROS to aging-related thrombosis.

The increased ROS production associated with aging has been attributed, in part, to decreased expression and activity of antioxidant enzymes such as glutathione peroxidase,\textsuperscript{20-23} which reduces and detoxifies peroxides such as hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). Glutathione peroxidase-1 (Gpx1) is the most abundant and widely expressed isoform of glutathione peroxidase in most tissues. In a prospective human study, it was demonstrated that the risk of cardiovascular events was inversely associated with increasing quartiles of erythrocyte Gpx1 activity.\textsuperscript{24} This observation suggests that peroxides may contribute to arterial thrombotic vascular events and that Gpx1 may be protective. It remains unclear, however, whether peroxides or Gpx1
play a role in the increased thrombotic susceptibility of aging.

In the present study, we compared thrombotic responses to arterial or venous injury in young vs. aged mice and examined the protective effects of overexpression of Gpx1. Our findings suggest that, in aging, peroxides mediate enhanced arterial and venous thrombosis as well as platelet hyperactivity. Our data also suggest that strategies to lower platelet H2O2 levels abrogate the enhanced activation of platelets from aged mice, providing mechanistic insights and a potential therapeutic approach to prevent aging-related thrombosis.

Methods

Mice

C57BL/6 mice were purchased from Jackson Laboratory at the age of 3 months and maintained in the animal care facilities of the University of Iowa. Gpx1 Tg mice were obtained from Dr. Yi Shi Ho25 and bred at least 15 generations with C57BL/6 mice before study. Genotyping for the Gpx1 transgene was performed using real-time PCR.26 All animal protocols were approved by the University of Iowa Animal Care and Use Committee. Both male and female mice were included in the study. Mice at ages 4, 12, and 18 months were used for studies. These age groups were chosen in accordance with accepted principles for experiments on the biology of aging in mice.27 Since the mean life span of C57BL/6 mice is around 27-29 months, the 4, 12 and 18 month old mice are roughly equivalent to young adult (18-20 years), middle age (around 45-50 years) and older (>70 years) humans, respectively.

Carotid artery thrombosis

Carotid artery thrombosis was induced by photochemical injury as described previously.28 Mice were anesthetized with sodium pentobarbital (70-90 mg/kg intraperitoneally) and ventilated
mechanically with room air and supplemental oxygen. To induce photochemical injury to endothelial layer, the right common carotid artery was dissected free, transilluminated continuously with a 1.5-mV, 540-nm green laser (Melles Griot, Carlsbad, CA) from a distance of 6 cm, and rose Bengal (35 mg/kg) was injected via a femoral vein catheter. Blood flow was monitored continuously for 90 minutes or until stable occlusion occurred at which time the experiment was terminated. Stable occlusion was defined as the time at which blood flow remained absent for $\geq 10$ minutes.

**Inferior vena cava (IVC) thrombosis**

Susceptibility to thrombosis in the venous system was measured as previously with minor modifications.$^{29}$ Briefly, mice were anesthetized using ketamine/xylazine (87.5 mg/kg ketamine and 12.5 mg/kg xylazine, intraperitoneally). A midline laparotomy was made, and the IVC was exposed directly via blunt dissection. The IVC was ligated inferiorly to the left renal vein using a 6.0 silk suture and mice were allowed to recover. Two days later, the IVC was harvested for measurement of the length and weight of thrombus.

**Platelet activation**

Washed platelets were isolated and resuspended in modified Tyrode’s buffer (134 mmol/L NaCl, 2.9 mmol/L KCl, 2.9 mmol/L CaCl$_2$, 0.34 mmol/L Na$_2$HPO$_4$, 12 mmol/L NaHCO$_3$, 20 mmol/L HEPES, 1.0 mmol/L MgCl$_2$, 5.0 mmol/L glucose, 0.05% (w/v) fatty acid-free bovine serum albumin, pH 7.35) as described previously.$^{30}$ To assess platelet activation, washed platelets were activated with human thrombin (0.5 U/mL; Haematological Technologies, Essex Junction, VT), for 2 minutes at 37$^\circ$C, incubated for 10 minutes with either FITC-conjugated sheep anti-human fibrinogen antibody (Novus Biologicals, Littleton, CO), FITC-conjugated rat anti-mouse CD62P antibody for P-selectin (BD Biosciences) or PE-conjugated JON/A for activated $\alpha$IIb$\beta$3 (Emfret
40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The comparative threshold cycle (ΔΔC_{T}) method was used for quantification with values normalized to 18S and expressed relative to levels in 4 month old wild-type (Wt) mice. Validation experiments were performed to confirm equal amplification efficiency for all primers sets. Platelet purity was confirmed by two methods: 1) complete blood counting; and 2) real-time PCR with primers for CD45, a marker for leukocytes. All samples used for real-time PCR had undetectable levels of CD45 mRNA, and the
leukocyte and RBC counts were equivalent to background.

**Platelet count**

Blood was collected by retro-orbital bleeding into a 20 μl EDTA-coated glass capillary tube and immediately diluted 1:10 into PBS with 5% BSA and analyzed by a laser-based Bayer Advia 120 whole blood analyzer.

**Statistical analysis**

One way analysis of variance (ANOVA) with the Tukey test for multiple comparisons was used to compare occlusion time, baseline blood flow, platelet counts, platelet activation responses and mRNA levels in C57BL/6 or Wt mice of different ages. Two-way ANOVA with the Tukey test for multiple comparisons was used to compare time to occlusion, thrombus length and weight, H2O2 levels, platelet activation responses, platelet count and baseline blood flow in Gpx1 Tg mice and Wt littermate control mice. The paired Student’s t-test was performed to compare platelet activation responses before and after treatment with inhibitors. All data were normally distributed except for the data in Figure 1; therefore, one-way ANOVA was performed on log-transformed values for this data set. Statistical significance was defined as a P value <0.05. All the values are reported as mean±SE.

**Results**

**Susceptibility to carotid artery thrombosis is increased in C57BL/6 mice with advancing age**

We first examined the relationship between age and susceptibility to arterial thrombosis in Wt C57BL/6 mice (Figure 1). Following photochemical injury of the carotid artery, the time to stable occlusion in 4 month old mice was 52.5±9.3 min, whereas the time to develop stable
occlusion was significantly shorter for both 12 month old and 18 month old mice (15.9±3.2 and 15.6±3.7 min, respectively, P<0.01). No differences in baseline carotid artery blood flow were observed between 4, 12, and 18 month old mice (Table 1). Platelet count did not differ significantly between 4 and 12 month old mice but was approximately 30% higher in 18 month old mice (P<0.05 vs. 4 and 12 month old mice). These findings demonstrate that C57BL/6 mice exhibit increased susceptibility to carotid artery thrombosis with aging.

**Overexpression of Gpx1 protects aged mice from accelerated arterial and venous thrombosis**

We next examined the role of oxidative stress in accelerated carotid artery thrombosis in aging using Gpx1 Tg mice and their Wt littermates at 4, 12, and 18 months of age (Figure 2).

Consistent with the findings in C57BL/6 mice, the time to develop stable occlusion of the carotid artery was significantly shorter in 12 and 18 month old Wt mice as compared to 4 month old Wt mice (17.7±2.8, 16.9±4.2, and 56.2±1 min, respectively, P<0.05). At 4 months of age, the time to stable occlusion was similar in Wt and Gpx1 Tg mice (56.2±10 min and 41.7±9.6 min, respectively, P=0.35), suggesting that at a young age overexpression of Gpx1 does not influence susceptibility to arterial thrombosis. However, the 12 and 18 month old Gpx1 Tg mice were protected from the age-induced shortening of the time to stable occlusion (37.6±8.6 and 56.3±11.4 min respectively, compared to age-matched Wt littermates, P<0.05). Of note, the times to stable occlusion in the 12 and 18 month old Gpx1 Tg mice did not differ significantly from 4 month old Gpx1 Tg mice (P=0.9 and 0.4, respectively). There was a significant overall interaction between age and genotype by two-way ANOVA (P<0.017), which reflects the lack of effect of the Gpx1 Tg genotype on stable occlusion in 4 month old mice.

No significant differences in baseline carotid artery blood flow were observed between 4,
12, and 18 month old Wt or Gpx1 Tg mice (Table 2). Platelet counts were again found to be higher in 18 month old Wt or Gpx1 Tg mice compared with 4 or 12 month old mice of the same genotype (P<0.05). There were no significant differences in platelet count between Wt and Gpx1 Tg mice at any age.

We next performed IVC ligation to examine whether Gpx1 overexpression provides protection from stasis-induced venous thrombosis (Figure 3). At 4 months of age, Wt and Gpx1 Tg mice developed thrombi of similar length and weight (P>0.8). This observation suggests that overexpression of Gpx1 at a young age does not affect susceptibility to venous thrombosis. In contrast, the 12 and 18 month old Wt mice developed significantly larger and heavier IVC thrombi compared to 4 month old Wt mice (P<0.05). Overexpression of Gpx1 protected against this age-dependent effect, as evidenced by the significantly smaller thrombi developed in 12 and 18 month old GPx1 Tg mice compared to age-matched Wt littermates (P<0.05). Collectively, these findings demonstrate that mice develop increased susceptibility to both arterial and venous thrombosis with advancing age, and overexpression of Gpx1 protects from these adverse effects.

Platelets from aged mice exhibit increased platelet adhesion during thrombus formation in vivo

Since H₂O₂ has been reported to contribute to platelet activation responses, we hypothesized that the protective effect of overexpression of Gpx1 may be mediated through an effect on platelets. To address this hypothesis, platelets isolated from 4 month or 18 month old mice were differentially fluorescently labeled and infused into 4 week old recipient mice. Thrombus formation in mesenteric arterioles was initiated with 5% ferric chloride and the relative adhesion of young vs. old platelets was quantitated in real-time by intra-vital microscopy. The results (Supplemental Figure 1A and C and Supplemental Video S1) demonstrate that platelets from
18 month old mice exhibit increased adhesion during thrombus formation in vivo compared to platelets from 4 month old mice (P=0.017). There was also a trend towards decreased adhesion of infused platelets from aged (18 month old) Gpx1 Tg mice compared with platelets from 18 month old Wt mice (Supplemental Figure IB and D and Supplemental Video S2).

Activated platelet from aged mice generate increased levels of H2O2

To further investigate the role of H2O2 in platelet activation during aging, platelets were stimulated with thrombin in vitro. We found that the intracellular concentration of H2O2 in thrombin-stimulated platelets was significantly higher in 18 month old Wt mice than in 4 month old Wt mice (Figure 4, P<0.05). In contrast, platelet H2O2 levels in 18 month old Gpx1 Tg mice were similar to those in 4 month old Gpx1 Tg mice (P>0.8). These data suggest that aging produces an elevation in levels of H2O2 within activated platelets, and that overexpression of Gpx1 protects from this response. To confirm that Gpx1 is overexpressed in platelets from Gpx1 Tg mice, we measured levels of Gpx1 mRNA and Gpx1 protein in isolated platelets by qPCR and western blotting, respectively. Regardless of age, Gpx1 Tg mice had a large increase in both Gpx1 mRNA and Gpx1 protein levels in platelets compared to Wt littermates (P<0.05, Supplemental Figure IIA, B, and C).

Aging is associated with H2O2-dependent inside-out platelet activation

To determine if increased generation of platelet H2O2 causes enhanced platelet activation in aged mice, we next examined surface P-selectin expression, fibrinogen binding, and integrin αIIbβ3 activation in thrombin-stimulated platelets from 4 and 18 month old mice. No effects of advancing age or Gpx1 overexpression on P-selectin expression were observed (Supplemental Figure III), which suggests that the increased levels of platelet H2O2 in aged mice do not influence platelet granule release. By contrast, we observed an age-dependent increase in
fibrinogen binding and αIIbβ3 activation in platelets from Wt mice (Figure 5A and B, P<0.05). The increases in fibrinogen binding and αIIbβ3 activation were absent in 18 month Gpx1 Tg mice (P<0.05), suggesting a peroxide-dependent mechanism. To confirm the role of H2O2 vs. other peroxides that are reduced by Gpx1, platelets from 4 month old and 18 month old Wt mice were treated with PEG-catalase, which selectively reduces H2O2. We found that PEG-catalase attenuated thrombin-stimulated fibrinogen binding and αIIbβ3 activation at both 4 months and 18 months of age (P<0.05) and completely abolished the age-dependent increased platelet activation in mice (Figure 5C and D). Taken together, these findings suggest that H2O2 is a critical mediator of increased inside-out activation of integrin αIIbβ3 (the major receptor for fibrinogen) leading to hyperactivation of platelets in aged mice.

A potential mechanistic pathway leading to H2O2–mediated platelet hyperactivation

To identify potential mechanisms of H2O2–mediated platelet hyperactivation, we performed quantitative RT-PCR to measure mRNA levels of NADPH oxidase subunits and superoxide dismutase in platelets. We found that platelets express significant levels of mRNA for the NADPH oxidase catalytic subunit Nox2 (Figure 6A) but do not express detectable levels of Nox1 or Nox4 mRNA at either 4 months or 18 months of age (data not shown). We also observed a significant increase in platelet mRNA levels of the NADPH oxidase regulatory subunit p47phox (Figure 6B, P<0.05) in 18 month old mice compared with 4 month old mice. Furthermore, we observed an increase in superoxide dismutase-1 (Sod1) mRNA in platelets from aged mice (Figure 6C, P<0.05). These findings suggest a potential mechanistic pathway, involving NADPH oxidase and SOD1, for increased generation of H2O2 in platelets from aged mice. To further assess the potential role of NADPH oxidase in aging-related platelet hyperactivation, we treated platelets from 4 month and 18 month old Wt mice with the NADPH oxidase inhibitor
APOCYNIN. WE FOUND THAT APOCYNIN INHIBITED THE INCREASE IN BOTH FIBRINOGEN BINDING AND ACTIVATION OF αIIbβ3 IN PLATELETS FROM 18 MONTH OLD MICE (FIGURE 7A, B). IN CONTRAST, NO INHIBITION OF PLATELET ACTIVATION WAS OBSERVED IN THE PRESENCE OF THE NITRIC OXIDE SYNTHASE INHIBITOR, L-NAME, WHICH SUGGESTS THAT NITRIC OXIDE SYNTHASE IS NOT A SOURCE OF ROS LEADING TO PLATELET HYPERACTIVATION IN AGING.

**Discussion**

In the present study we examined the role of H₂O₂ in the mechanism of increased susceptibility to arterial and venous thrombosis with aging. The main findings from this study are: 1) with advancing age, C57BL/6 mice become increasingly susceptible to both arterial and venous thrombosis; 2) overexpression of the peroxide-reducing enzyme Gpx1 protects from the accelerated thrombosis of aging; and 3) platelet activation responses are increased in aging and platelet hyperresponsiveness is mediated by H₂O₂. Taken together, these findings suggest a mechanism in which increased H₂O₂ production leads to platelet hyperactivity and enhanced susceptibility to thrombosis in aging.

ONE ADVANTAGE OF ANIMAL MODELS OF THROMBOSIS, AS OPPOSED TO HUMAN ASSOCIATION STUDIES, IS THAT THE CONTRIBUTION OF AGING CAN BE ASSESSED INDEPENDENTLY OF OTHER CARDIOVASCULAR RISK FACTORS, SUCH AS HYPERCHOLESTEROLEMIA, HYPERTENSION, OBESITY, AND DIABETES. IN ACCORDANCE WITH ACCEPTED PRINCIPLES FOR EXPERIMENTS ON THE BIOLOGY OF AGING IN MICE, WE UTILIZED A SYSTEMATIC STUDY DESIGN WITH A CONTROL GROUP OF 4 MONTH OLD MICE TO AVOID POTENTIAL CONFOUNGING FROM STUDYING YOUNGER MICE STILL IN THE DEVELOPMENTAL STAGE. WE INCLUDED TWO EXPERIMENTAL GROUPS OF 12 AND 18 MONTH OLD MICE. FINALLY, WE EXAMINED SUSCEPTIBILITY TO BOTH ARTERIAL AND VENOUS THROMBOSIS. IN AGREEMENT WITH A PREVIOUS STUDY, WE DEMONSTRATED INCREASED SUSCEPTIBILITY TO...
venous thrombosis in mice at 12 and 18 months of age compared with 4 months of age. We also observed increased susceptibility to carotid artery thrombosis in mice at 12 and 18 months of age using the rose bengal photochemical injury method of experimental thrombosis. In a prior study, Stämpfli and colleagues did not observe increased susceptibility to rose bengal-induced carotid artery thrombosis in mice at 15 or 24 months of age compared to 11 weeks of age.34 Potential reasons for the discrepant results between our study and that of Stämpfli et al.34 include Stämpfli’s use of mice under 4 months of age as the control group, a higher dose of rose bengal to initiate carotid artery injury, and a protocol that did not include mechanical ventilation to avoid acidosis and altered carotid artery blood flow.28 Overall, the consistent effects of age on both venous and arterial thrombosis in our study suggest that this murine model is an appropriate one for mechanistic studies of aging-associated thrombosis.

One novel finding of our study is that aged mice overexpressing Gpx1 were protected from enhanced thrombotic susceptibility, which implicates H2O2 and/or lipid peroxides in the prothrombotic phenotype of aging. Previous studies in humans have suggested that prothrombotic conditions such as diabetes are associated with increased platelet H2O2 production and enhanced platelet activation responses.35,36 In concordance with these previous studies in humans, we found that intra-platelet H2O2 levels were elevated in activated platelets from aged mice with increased thrombotic susceptibility. We also found that platelets from aged mice exhibited increased adhesion to injured mesenteric arterioles in an in vivo model of thrombosis and enhanced activation responses to thrombin in vitro in conjunction with increased accumulation of H2O2. Platelet hyperactivation was overcome not only by overexpression of Gpx1 but also by treatment with PEG-catalase, which implicates platelet H2O2 rather than lipid peroxides in the mechanism of enhanced platelet activation with aging.
We explored several possible mechanisms for the increased accumulation of H$_2$O$_2$ in activated platelets from aged mice. We first considered decreased Gpx1 expression in platelets as a potential mechanism because decreased expression of Gpx1 has been reported to be associated with advancing age in several other tissues$^{20,21,23,37}$ and decreased erythrocyte Gpx1 activity is associated with increased cardiovascular risk in patients with coronary artery disease.$^{24}$ However, we did not observe any differences in platelet Gpx1 mRNA or protein levels between young and old mice (Supplemental Figure II). We also did not observe a decrease in the expression of catalase mRNA in platelets from aged mice (data not shown). These findings suggest that the increase in platelet H$_2$O$_2$ in aged mice is not caused by decreased expression of Gpx1 or catalase.

We next considered the potential roles of NADPH oxidase and superoxide dismutase as enzymatic sources of elevated H$_2$O$_2$ in platelets from aged mice. NADPH oxidase-dependent generation of superoxide has been reported to regulate platelet integrin αIIbβ3 activation.$^{31}$ Additionally, platelets from patients with chronic granulomatous disease due to deficiency of the NADPH oxidase catalytic subunit Nox2 have almost complete suppression of platelet superoxide production.$^{38,39}$ Since H$_2$O$_2$ can be generated from superoxide by superoxide dismutase, we hypothesized that increased generation of H$_2$O$_2$ in platelets from aged mice results from increased NADPH oxidase-dependent production of superoxide that is subsequently converted to H$_2$O$_2$ by superoxide dismutase. In agreement with this hypothesis, we found that pre-incubation of platelets from aged mice with the NADPH oxidase inhibitor apocynin resulted in a significant decrease in thrombin-induced activation of αIIbβ3 and surface fibrinogen binding. We also observed increased expression of mRNA for the NADPH oxidase regulatory subunit, $p47^{phox}$, as well as $Sod1$, in platelets from aged mice. Furthermore, we considered the possibility that
platelet NADPH oxidase might directly generate H₂O₂, since isoforms of NADPH oxidase that contain the Nox4 catalytic subunit can generate significant amounts of H₂O₂.⁴⁰,⁴¹ We consider this to be unlikely, however, because we did not detect any significant expression of Nox4 mRNA in platelets from young or old mice. Unlike Nox4-containing NADPH oxidases, Nox2-containing NADPH oxidases usually generate superoxide with little or no direct generation of H₂O₂.⁴¹ Future studies using mice deficient in p47phox or Nox2 might provide additional mechanistic insights into the role of platelet NADPH oxidases in platelet hyperactivity and thrombosis.

Interestingly, previous work by Loscalzo and colleagues has demonstrated that deficiency of plasma glutathione peroxidase (Gpx3) causes platelet hyperactivation in both humans and mice.⁴²,⁴³ The proposed mechanism was that deficiency of Gpx3 may lead to increased extracellular levels of H₂O₂, resulting in decreased bioavailability of nitric oxide (NO) and decreased NO-mediated inhibition of platelet activation. Our findings suggest that regulation of intracellular H₂O₂ by Gpx1 also affects platelet activation responses. The protective effect of Gpx1 is likely independent of platelet-derived NO, since we did not observe any inhibition of platelet activation in the presence of the NO synthase inhibitor, L-NAME (Figure 7). Although our data suggest a direct, NO-independent effect of aging on H₂O₂-mediated platelet activation, we recognize that aging is also associated with increased oxidative stress in the vessel wall and that decreased endothelium-derived NO may contribute in part to increased thrombotic susceptibility. In addition, other vascular cell-derived thrombotic and inflammatory factors, such as plasminogen activator inhibitor-1, P-selectin, and tissue factor²⁹ also may play a role in the mechanism by which increased oxidative stress enhances susceptibility to thrombosis during aging.
Given the well-established role of platelets in arterial thrombosis as opposed to venous thrombosis, it is perhaps surprising that we observed similar effects of aging and Gpx1 overexpression in experimental models of both arterial and venous thrombosis. However, the paradigm that platelets are more important in arterial than venous thrombosis has been challenged by recent work in mouse models\textsuperscript{44,45} and by human studies in which aspirin has proven to have benefit in the prevention of recurrent venous thromboembolism.\textsuperscript{46,47} Our work presented here is consistent with these recent findings and suggests a possible mechanism for enhanced platelet-dependent thrombosis in both arterial and venous systems during aging.

Finally, although there was a modest but significant increase in the platelet count in mice at 18 months of age compared to 4 or 12 months of age (Tables 1 and 2), it is unlikely that this difference contributed appreciably to the increased thrombotic susceptibility with aging, for two reasons: 1) the platelet count was significantly elevated only at 18 months of age, whereas increased susceptibility to arterial and venous thrombosis was apparent at both 12 and 18 months of age; 2) a similar elevation of the platelet count was observed in Wt and Gpx1 Tg mice at 18 months of age (Table 2), and Gpx1 Tg mice were nevertheless protected from platelet hyperactivation and thrombosis. We previously reported that a similar increase in platelet count was not associated with increased thrombotic susceptibility in mice with hyperhomocysteinemia.\textsuperscript{48} We conclude, therefore, that the prothrombotic phenotype of aged mice is likely to be mediated by platelet hyperactivity rather than thrombocytosis.

In summary, we demonstrate herein that aged mice develop increased susceptibility to both arterial and venous thrombosis, and that H2O2-mediated platelet hyperactivation is a likely mechanism leading to this prothrombotic phenotype. These findings suggest that therapeutic strategies targeted toward lowering platelet H2O2 levels may have the potential to decrease
thrombotic complications of aging. One potential strategy is to identify drugs that increase platelet glutathione peroxidase or catalase activity, perhaps by upregulating or allosterically increasing their activity. Another approach might be to develop inhibitors of the platelet NADPH oxidase/SOD1 pathway. Inhibition of SOD1 may prove to be problematic, however, because SOD1 inhibitors might be expected to not only decrease H2O2 but also increase superoxide, which also may produce platelet hyperactivation. Targeting NADPH oxidase may prove to be more a promising approach, since several small molecule and peptide-based inhibitors of Nox2-containing NADPH oxidases subunits are currently in development.49, 50

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Conflict of Interest Disclosures: None.

References:


2006;18:69-82.


Table 1. Baseline carotid artery blood flow and platelet count in C57BL/6 mice.

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<th>Age group</th>
<th>4 months</th>
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<th>18 months</th>
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<td>Platelet count (x 1000/μL)</td>
<td>1393±66</td>
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*P<0.05 compared to 4 months by one-way ANOVA.
8-12 mice were studied in each group.

Table 2. Baseline carotid artery blood flow and platelet count in wild-type and Gpx1 Tg littermates.

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<th>18 months</th>
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<td>Baseline blood flow (ml/min)</td>
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<td>Wild-type</td>
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*P<0.05 compared to 4 and 12 months by two-way ANOVA. 6-11 mice were studied in each group.

Figure Legends:

Figure 1. Susceptibility to carotid artery thrombosis increases with age. The time to stable occlusion of the carotid artery following photochemical injury was measured in 4, 12, and 18 month old wild-type C57BL/6 mice. 7-12 mice were studied in each group. Values are mean±SE. *P<0.05 compared with 4 month old mice by one-way ANOVA.

Figure 2. Overexpression of Gpx1 protects from age-associated increased susceptibility to carotid artery thrombosis. The time to stable occlusion of the carotid artery following photochemical injury was measured in 4, 12, and 18 month old Gpx1 Tg mice (Tg) or wild-type
(Wt) littermate controls. 5-10 mice were studied in each group. Values are mean±SE. *P<0.05 compared with 4 month old Wt mice; #P<0.05 compared with age matched Wt littermates by two-way ANOVA.

**Figure 3.** Overexpression of Gpx1 protects from age-associated increased susceptibility to venous thrombosis. The weight (A) and length (B) of thrombi that developed in the IVC 48 hours following ligation was measured in 4, 12, or 18 month old wild-type (Wt) or Gpx1 Tg (Tg) mice. 10-11 mice were studied in each group. Values are mean±SE. *P<0.05 compared with 4 month old Wt mice; #P<0.05 compared with age-matched Wt littermates by two-way ANOVA.

**Figure 4.** Accumulation of H$_2$O$_2$ is increased in platelets from aged mice, and Gpx1 overexpression is protective. Platelets from 4 or 18 month old wild-type (Wt) or Gpx1 Tg (Tg) mice were activated with thrombin (0.5 U/ml), and then levels of intracellular H$_2$O$_2$ were measured as the PEG-catalase-inhibitable fluorescent signal formed due to oxidation of DCFH by H$_2$O$_2$. Platelets from 7-8 mice were studied in each group. Values are mean±SE. *P<0.05 compared with 4 month old Wt mice; #P<0.05 compared with age-matched Wt littermates by two-way ANOVA.

**Figure 5.** Aging is associated with increased fibrinogen binding and activation of $\alpha$IIb$\beta$3, which are prevented with Gpx1 overexpression or PEG-catalase treatment. Fibrinogen binding (A) and activation of $\alpha$IIb$\beta$3 (B) were examined in thrombin-activated platelets from either wild-type (Wt) or Gpx1 Tg (Tg) mice at 4 or 18 months of age. Platelets from 4 or 18 month old Wt mice were treated with or without PEG-catalase (500 U/ml) and fibrinogen binding (C) and activation...
of αIIbβ3 (D) were measured following thrombin activation. Platelets from 10-11 mice were studied in each group. Values are mean ± SE. *P<0.05 compared with 4 month old Wt mice; #P<0.05 compared with age-matched Wt littermates by two-way ANOVA for (A) and (B) and by paired t-test for (C) and (D).

Figure 6. P47phox and Sod1 are upregulated in platelets from aged mice. Platelet mRNA levels of Nox2 (A), p47phox (B), and Sod1 (C) were measured by real-time PCR. Values were normalized to 18S mRNA and are expressed as percent of the control values observed in 4 month old wild-type mice. Values are mean ± SE. Platelets from 9-15 mice were studied in each group. *P<0.05 compared with 4 month old mice by one-way ANOVA.

Figure 7. Activation of platelets from aged mice is mediated in part by NADPH oxidase. (A) Platelets from 4 or 18 month old wild-type (Wt) mice were treated with or without L-NAME (100 μM) or apocynin (600 μM) followed by activation with thrombin (0.5 U/ml), and then fibrinogen binding was measured by flow cytometry. (B) Platelets from 4 or 18 month old Wt mice were treated as in (A) and activation of αIIbβ3 was measured by flow cytometry. Values are mean ± SE. Platelets from 7-8 mice were studied in each group. *P<0.05 compared with 4 month old mice without apocynin by one-way ANOVA, and #P<0.05 compared with age-matched mice without apocynin by paired t-test.
Figure 1

Time to occlusion (min)

4 months 12 months 18 months

* *
Figure 2

The figure shows a bar graph comparing the time to occlusion (min) for mice of different genotypes and ages. The x-axis represents the age of the mice in months (4, 12, and 18), and the y-axis represents the time to occlusion in minutes. The graph compares two genotypes: Wt (wild type) and Tg (transgenic). The bars indicate the mean time to occlusion with error bars representing standard deviation. The symbol * represents a statistically significant difference compared to the Wt group, and the symbol # represents a significant difference compared to the 4-month Tg group.

- At 4 months, the time to occlusion is similar for both Wt and Tg groups.
- At 12 months, the time to occlusion is significantly longer for the Tg group compared to the Wt group.
- At 18 months, the time to occlusion is significantly longer for both the Wt and Tg groups, but the Tg group shows a significantly longer occlusion time compared to the Wt group.

**Note:** The asterisks (*) and hash signs (#) indicate statistical significance.
Figure 3

(A) Thrombus weight (g)

(B) Thrombus length (mm)

4 months 12 months 18 months

Thrombus weight (g)

Thrombus length (mm)

Wt Tg

* #

Figure 3
Figure 4

Platelet H$_2$O$_2$ (% Positive)

Wt

Tg

4 months 18 months

* #

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Figure 5

A) Fibrinogen binding (% Positive) at 4 months and 18 months for Wt and Tg groups.

B) Activated αIIbβ3 (% Positive) at 4 months and 18 months for Wt and Tg groups.

C) Fibrinogen binding (% Positive) at 4 months and 18 months for w/o catalase and with catalase groups.

D) Activated αIIbβ3 (% Positive) at 4 months and 18 months for w/o catalase and with catalase groups.

**Significance:** *

- Wt: Wild-type
- Tg: Transgenic
- w/o catalase: Without catalase
- with catalase: With catalase
Figure 6
**Figure 7**

(A) Fibrinogen binding (% Positive) over time (4 months and 18 months) with different treatments: Thrombin, Thrombin + L-NAME, and Thrombin + Apocynin. The graph shows a significant increase in fibrinogen binding with the combination of Thrombin + L-NAME compared to Thrombin alone (*). Thrombin + Apocynin also shows an increase, but it is not as significant as Thrombin + L-NAME.

(B) Activated αIIbβ3 (% Positive) over time (4 months and 18 months) with different treatments: Thrombin, Thrombin + L-NAME, and Thrombin + Apocynin. The graph shows a significant increase in activated αIIbβ3 with the combination of Thrombin + L-NAME compared to Thrombin alone (*). Thrombin + Apocynin also shows an increase, but it is not as significant as Thrombin + L-NAME.

Note: Thrombin, Thrombin + L-NAME, and Thrombin + Apocynin treatments are represented by different bars in each graph.
SUPPLEMENTAL MATERIAL

Hydrogen peroxide promotes aging-related platelet hyperactivation and thrombosis

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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 (Complete™ Mini EDTA-free, Roche, USA). Immunoblotting was performed as described previously. 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. The host mouse was anesthetized with avertin, and differentially labeled platelets (2.5 x 10⁹ platelets/kg) were infused retro-orbitally. Mesenteric
arterioles (between 80-100 μm in diameter) of the host mouse were topically injured with 5% FeCl₃. 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±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
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. *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 ± SE.
Supplemental References


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
recipient C57Bl6/J mice, mesenteric arterioles were injured with 5% FeCl$_3$ and relative platelet adhesion during initial thrombus formation was assessed by intravital microscopy.