Arteriosclerosis, Thrombosis, and Vascular Biology

BASIC SCIENCES

Shear and Integrin Outside-In Signaling Activate NADPH-Oxidase 2 to Promote Platelet Activation

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OBJECTIVE: Despite the importance of reactive oxygen species (ROS) and NOX (nicotinamide adenine dinucleotide phosphate [NADPH] oxidase) 2 in platelet activation and in vivo thrombosis, it is unclear how ROS and NOX2 play a role in platelet activation and why NOX2 deficiencies in humans and mice do not affect hemostasis. Outside-in signaling of integrin $\alpha_{IIb}\beta_3$ mediates platelet response to shear stress, secondary platelet activation, and thrombus expansion and is critical to thrombosis but dispensable for hemostasis. We studied the mechanisms of platelet ROS generation, ROS-mediated platelet response, and the role of ROS in integrin $\alpha_{IIb}\beta_3$ outside-in signaling.

APPROACH AND RESULTS: ROS generation in activated platelets was low and slow without shear but was robust under shear. Shear-enhanced ROS generation and activation of p47phox, an important regulatory subunit of NOX2, were diminished by the integrin antagonist integrilin or β_3 knockout, and by $G\alpha_{13}$ knockout or blocking the $G\alpha_{13}$-β_3 interaction. Resting platelets spreading on integrin ligand fibrinogen also $G\alpha_{13}$-dependently stimulated ROS generation and p47phox activation. Hence, $G\alpha_{13}$-mediated outside-in signaling induces NOX2 activation and ROS generation which is greatly enhanced by shear. Outside-in NOX2 activation requires Src, phosphoinositide 3-kinase and Akt downstream of $G\alpha_{13}$. Importantly, NOX2-knockout platelets showed defective ROS generation, reduced platelet spreading without shear, and reduced platelet adhesion and thrombus volume on collagen and VWF (von Willibrand factor) under shear, whereas ROS inhibition diminished activation of tyrosine kinase Syk.

CONCLUSIONS: Outside-in signaling activates the mainly NOX2-mediated ROS generation, which mediates Syk-dependent secondary platelet activation, adhesion, and thrombosis with minimal effect on hemostasis.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: glycoprotein ■ integrin ■ reactive oxygen species ■ platelet activation ■ thrombosis

Platelets are activated following vascular injury and play critical roles in hemostasis and thrombosis. Reactive oxygen species (ROS) have been recognized as an important mediator of vascular injury and thrombosis. Although there are numerous sources of ROS following vascular injury,[1–3] a long-recognized source of ROS that promotes platelet activation are platelets themselves, which produce superoxide anion (O_2^-), hydroxyl radicals (OH•), and hydrogen peroxide (H_2O_2).[1,2,4–6] Recent studies further support the importance of ROS and ROS-generating enzymes in platelet activation and thrombosis.[5,7–10]

The NOX (nicotinamide adenine dinucleotide phosphate [NADPH] oxidase) family of enzymes transfers electrons to generate superoxide anion (O_2^-).[11] The enzymatic activity of the prototypical NOX, NOX2, is activated only after being induced to form a complex with multiple regulatory subunits including p22phox, p40phox, p47phox, p67phox, and the small GTPase Rac.[11] Of these subunits, p47phox is an important regulator of the assembly of the multisubunit complex once it is activated by phosphorylation at critical serine residues,[12–14] which occurs during platelet activation.[15] The importance of NOX2 in platelet ROS

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For Sources of Funding and Disclosures, see page 1651.

Nonstandard Abbreviations and Acronyms

CRP	collagen-related peptide
FcRγ	Fc receptor γ chain
FcγRIIA	Fcγ receptor IIA
GP	glycoprotein
ITAM	immunoreceptor tyrosine-based activation motif
MAPK	mitogen-activated protein kinase
NADPH	nicotinamide adenine dinucleotide phosphate
NOX	NADPH oxidase
PKC	protein kinase C
ROS	reactive oxygen species
SFK	Src family kinase

Highlights

- Agonist-stimulated platelet reactive oxygen species generation is robustly enhanced by shear stress.
- Gα13-dependent integrin outside-in signaling mediates shear-dependent and shear-independent platelet reactive oxygen species generation.
- Outside-in signaling mediates reactive oxygen species generation by stimulating p47phox phosphorylation and NOX (nicotinamide adenine dinucleotide phosphate [NADPH] oxidase) 2 activation via the Gα13-SFK (Src famly kinase)- and likely PI3K-Akt signaling pathway.
- Reactive oxygen species is important in facilitating outside-in signaling-induced activation of Syk-ITAM (immunoreceptor tyrosine-based activation motif) signaling pathway in platelets, leading to amplification of platelet activation.

generation and platelet activation is supported by multiple independent studies.[8,10,16] Moreover, NOX2 deficiency in mouse platelets inhibited thrombosis and platelet-leukocyte interaction in vivo,[8,17,18] demonstrating its importance in thrombosis and inflammation. Also, the role of NOX2 in human platelets is supported by the observation that platelets from X-linked chronic granulomatous disease patients have defective agonist-induced platelet ROS generation and CD40 ligand expression.[10] Controversially, Sonkar et al[19] reported that NOX2 deficiency did not affect arterial thrombosis using the Rose-Bengal injury model of thrombosis. However, Rose-Bengal releases large amount of ROS upon photoactivation, enough to cause severe vascular injury/thrombosis and likely to overwhelm any effect of platelet-generated ROS, resulting in negative data. Nevertheless, this group reported no significant ROS increase in platelets stimulated under static conditions. Interestingly, NOX2 deficiency in both human granulomatous disease patients and knockout mice[8] do not show significant bleeding, apparently paradoxical to the important roles of NOX2 in thrombosis. Thus, it is important to resolve these apparent paradoxes and controversies.

Integrin $\alpha_{IIb}\beta_3$, upon activation by agonists through inside-out signaling, binds to fibrinogen or other ligands to mediate platelet adhesion/aggregation.[20] Ligand binding to $\alpha_{IIb}\beta_3$ induces the binding of $G\alpha_{13}$ to the β_3 cytoplasmic domain[21,22] and subsequent activation of SFK (Src family kinase) to transmit outside-in signaling.[23,24] Outside-in signaling inhibits RhoA, leading to platelet spreading.[22,24–28] Outside-in signaling also greatly stimulates secondary platelet activation and aggregation especially under flow conditions, which is important in thrombus expansion and thrombosis. Previous studies demonstrated that $G\alpha_{13}$ is dispensable for inside-out integrin activation[22,29] but important for outside-in signaling.[21,22] Interestingly, $G\alpha_{13}$-dependent early phase outside-in signaling appears to be dispensable or less important in hemostasis under experimental conditions in vivo as blocking $G\alpha_{13}$-β_3 interaction inhibited thrombosis without affecting bleeding time.[21,30] Importantly, $\alpha_{IIb}\beta_3$ serves as a shear force sensor activating outside-in signaling and platelet procoagulant activity.[31] However, the pathways of integrin outside-in signaling leading to secondary platelet responses as well as to platelet response to shear are unclear. Previous studies suggest that the role of outside-in signaling in platelet activation requires activation of Syk,[28,32,33] a critical kinase in the immunoreceptor tyrosine-based activation motif (ITAM) signaling pathway, and that, in human platelets, $\alpha_{IIb}\beta_3$ is associated with FcγRIIA (Fcγ receptor IIA), an ITAM receptor.[26] However, it remains unclear how outside-in signaling activates Syk and ITAM signaling.

In this study, we demonstrate that agonist-stimulated platelet ROS generation is greatly enhanced by shear, requires outside-in signaling and is mainly mediated by NOX2. We further demonstrate that $G\alpha_{13}$-dependent outside-in signaling is the major mechanism responsible for p47phox/NOX2 activation, leading to generation of ROS, which promotes Syk activation, platelet aggregation and thrombus expansion. These data address the controversy about ROS generation during platelet activation, explains why NOX2 is important for thrombosis but dispensable for hemostasis. Importantly, they suggest a novel ROS-dependent outside-in signaling pathway leading to Syk-dependent platelet activation and thrombus expansion.

MATERIALS AND METHODS

The authors declare that all supporting data are available within the article and the Data Supplement.

Mice

The generation of NOX2 knockout mice was described as before.[34] NOX2 knockout mice and integrin $\beta_3^{-/-}$ were purchased

from The Jackson Laboratory (Bar Harbor, ME) and were maintained on a C57BL/6J background. Both male ($NOX2^{-/y}$) and female ($NOX2^{-/-}$) mice were used and are referred to as NOX2 knockout.[8] $G\alpha_{13}^{flox/flox}$ mice were gifts obtained from Dr Stefan Offermanns' lab, Max-Planck-Institute for Heart and Lung Research, Germany.[29] Platelet specific $G\alpha_{13}$ knockout mice were generated by breeding of $G\alpha_{13}^{flox/flox}$ and PF4-Cre mice[35] and confirmed by genotyping and Western blot analysis. The in vitro platelet function data used pooled platelets from 5 mice, including both male and female. It is not practical currently to reliably and in-detail analyze platelet aggregation of individual mice due to small blood volume. Also, there is no evidence of sex variation in ROS and ROS-dependent platelet functions in our studies. Animal usage and protocol were approved by the institutional animal care committee of the University of Illinois at Chicago.

Isolation of Platelets

Washed mouse platelets were prepared as previously described.[21,36] Briefly, fresh blood was drawn from mouse inferior vena cava and anti-coagulated with ACD (85 mmol/L trisodium citrate, 83 mmol/L dextrose, and 21 mmol/L citric acid).[36] Platelets were isolated by differential centrifugation of whole blood with 0.1 μg/mL prostaglandin E1 and 1 U/mL apyrase (Sigma-Aldrich, St Louis, MO), washed twice with CGS buffer (sodium chloride 0.12 mol/L, trisodium citrate 0.0129 mol/L, D-glucose 0.03 mol/L, pH 6.5) and resuspended in N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid-buffered Tyrode solution, and 1 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$ was added. They were then allowed to rest at room temperature for at least 1 hour. Studies using human blood were approved by the institutional review board at the University of Illinois at Chicago, and informed consent was obtained from all donors. Washed human platelets from healthy donors who have not taken medication within 2 weeks before donation and platelets were prepared as described previously and resuspended in modified Tyrode buffer.[22]

Detection of Intracellular ROS

Intracellular ROS was measured as previously described.[8] Briefly, washed platelets (1×10^8/mL) in Tyrode buffer were incubated with an intracellular ROS dye that reacts with H_2O_2, 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (Carboxy-H_2DCFDA, Invitrogen) 20 μmol/L, for 10 minutes in the dark. Platelets, resting or agonist-stimulated for increasing times, were analyzed for fluorescent ROS signals using an Accuri C6 flow cytometer (BD). Mean fluorescence intensity in activated platelets at a time point was subtracted by the baseline mean fluorescence intensity of resting platelets and plotted to show time-dependent ROS changes. Total ROS production was estimated by integrating the area under the mean fluorescence intensity curve. In some experiments, a cone-plate rheometer (Rheostress 1 or HAAKE MARS, Thermo-HAAKE, Paramus, NY) was used to induce shear stress (800/s) to the platelets, also as previously described.[37]

Detection of H_2O_2 Release

Reaction solution of Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen; 100 μL) was preincubated at 37 °C for 10 minutes, and then mixed with 20 μL of washed platelet suspension (3×10^8/mL). The mixture was placed into a platelet lumi-aggregometer with or without stirring at 1000 rpm and stimulated with agonists. Platelet aggregation was monitored and then stopped at indicated time points. The fluorescence intensity was immediately measured using the FlexStation plate reader (Molecular Devices).

Immunoblotting

Western blotting was essentially performed as described previously.[38] Washed platelets (3×10^8/mL) were preincubated with 10 μmol/L PP2 (Calbiochem), 15 μmol/L piceatannol (Calbiochem), 2 μmol/L BAY 61-3606 (santa cruz),15 μmol/L SH-6 (Calbiochem), 10 μmol/L Akt inhibitor X (Calbiochem), 5 μmol/L LY294002 (Calbiochem), 200 nmol/L wortmannin (EMD Chemicals), 10 μmol/L SB203580 (Calbiochem), 250 nmol/L U0126 (Calbiochem), 50 nmol/L Gö6976 (Calbiochem), or vehicle control (0.1% dimethyl sulfoxide) and placed into a platelet lumi-aggregometer with stirring at 1000 rpm. Platelet aggregation was induced for 2 minutes, and the reaction stopped by addition of equal volume of 2× sodium dodecyl sulfate sample buffer (2% sodium dodecyl sulfate, 0.1 mol/L Tris, 2% glycerol, 2 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L Na_3VO_4, 2 mmol/L NaF, and Complete Protease Inhibitor Cocktail [Roche Molecular Biochemicals, IN]). When platelets were spreading on immobilized fibrinogen or incubated in suspension for control, they were lysed with the 2× sodium dodecyl sulfate buffer at the indicated time points. Proteins were separated by sodium dodecyl sulfate-PAGE on 4% to 15% polyacrylamide gels as previously described,[39] transferred to PVDF (polyvinylidene difluoride) membranes and immunoblotted with antiphosphorylated p47phox at Serine 304 (Fisher Scientific: PA5-36773), antiphosphorylated p47phox at Serine 328 antibody (Fisher Scientific: PA5-38777), anti-p47phox (Fisher Scientific: PA5-31169), and anti-α tubulin (Sigma) antibodies. To test Syk activation, washed platelets were preincubated with 500 μmol/L apocynin (Sigma), 1 mmol/L N-acetylcysteine (Calbiochem), 100 μmol/L mP6 peptide, or scrambled control peptide for 3 minutes, aggregation was induced in a lumi-aggregometer for 1, 2, or 5 minutes. Proteins were immunoblotted with antiphosphorylated Syk at Tyrosine 525/526 (cell signaling: 2710), anti-Syk (cell signaling: 2712), and anti-α tubulin (Sigma) antibodies.

Detection of ROS in Platelets Spreading on Immobilized Fibrinogen

Microscope cover glasses (Fisher Scientific) were coated with 100 μg/mL fibrinogen (Enzyme Research Laboratories) in 0.1 mol/L $NaHCO_3$ (pH 8.3), placed into 12-well plates, and blocked with 5% BSA in PBS. Washed mouse platelet suspensions (1×10^7/mL) were then aliquoted into fibrinogen-coated wells (300 μL/well) and incubated at 37 °C for 60 minutes. Ten micromolar H_2DCFDA (Invitrogen) was then added to the suspension, which was incubated for another 30 minutes. Platelet suspension was then aspirated to remove nonadherent platelets and washed with PBS 6 times. ROS production was

immediately observed using Zeiss LSM8 META confocal microscope. Quantification of the total ROS production per platelet was performed using National Institutes of Health ImageJ software.

Platelet Spreading on Immobilized Fibrinogen

Platelet spreading was performed essentially as previously described.[22,38] Microscope cover glasses (Fisher Scientific) were coated with 100 μg/mL fibrinogen (Enzyme Research Laboratories) in 0.1 mol/L $NaHCO_3$ (pH 8.3), placed into 12-well plates and blocked with 5% BSA in PBS. Washed mouse platelet suspensions (1×10^7/mL) were then aliquoted into the 12-well plates (300 μL/well) and incubated at 37 °C for the indicated length of time. Platelet suspension was then aspirated to remove nonadherent platelets and fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% triton X-100 in PBS, and stained with Alexa-Fluor FITC conjugated phalloidin (Invitrogen). Adherent platelets were observed with a Leica DM IRB fluorescence microscope (Leica Microsystems). Images were acquired using a Cool SNAP HQ CCD camera (Photometrics) and processed with National Institutes of Health ImageJ software.

Platelet Thrombus Formation Under Shear Using the Flow Chamber Assay

The flow chamber assay was performed as described previously.[40] Briefly, glass coverslips were coated with rat tendon fibrillar type I collagen or purified human VWF (von Willibrand factor) and connected to a parallel plate flow chamber (Glycotech, Rockville, MD). Blood drawn from wild type and NOX2 knockout mice was treated with 50 mmol/L PPACK (D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone) and perfused through the chamber for 1 minute at 37 °C under 1000 s^{-1}. The coverslip was then taken out and washed, and adherent platelet thrombi were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 0.1% BSA, and stained with 0.1 μg/mL rhodamine- or Alexa Fluor 488-conjugated phalloidin (ThermoFisher Scientific). To measure the surface coverage, fluorescence images were obtained using a Nikon microscope equipped with 100×/1.3 NA oil objective lens as described above. Confocal images were also obtained using a laser scanning confocal microscope (LSM 510 META, Zeiss) equipped with a 100×/1.3 NA oil immersion objective lens. Analyses of adherent and aggregated platelets were made in randomly selected 10 fields proximal to the inlet of the chamber. Thrombus volume in an area of 0.006 mm^2 was calculated from confocal microscopy images using Image J.

Statistics

Prism/Graphpad software was used for statistical analysis, in which Shapiro-Wilks and D'Agostino-Pearson tests are used for normality, and F test or Bartlett test for variances. For parametric data, statistical significance was analyzed using Student *t* test (or Welch *t* test for samples with nonequal variances; 2 groups) or 1-way ANOVA with Tukey multiple comparison (multi-groups). For nonparametric data, statistical significance was determined using Mann-Whitney test or Kruskal-Wallis test with post hoc Dunn multiple comparisons. $P<0.05$ was considered as significant difference.

RESULTS

The Integrin $\alpha_{IIb}\beta_3$-Mediated Response to Shear Is a New and Significant Stimulator of ROS in Platelets

To investigate ROS production during platelet activation, mouse platelet suspensions were incubated with the fluorescent oxidative stress indicator carboxy-H_2DCFDA and analyzed using flow cytometry.[8] Under static conditions, thrombin-stimulated ROS production was low and slow (minimal in the first 3 minutes; Figure 1A and 1B), which was partially inhibited by the integrin $\alpha_{IIb}\beta_3$ antagonist, integrilin, suggesting that the slow ROS production induced by thrombin under static conditions was partially integrin-dependent (Figure 1A). In contrast, when thrombin-stimulated mouse platelets were subjected to shear (800/s) using a cone-plate rheometer, ROS production was remarkably fast and significantly enhanced (Figure 1B and 1C), indicating that shear is a previously unrecognized but important factor in flowing blood that greatly accelerates and enhances ROS production in activated platelets. Integrilin almost totally abolished ROS production in the presence of shear in thrombin- or CRP (collagen-related peptide)-stimulated platelets (Figure 1B–1D), indicating that $\alpha_{IIb}\beta_3$ plays a critical role in enhancing ROS in platelets activated by these agonists signaling pathways under shear. Similar to platelets subjected to shear in the cone-plate rheometer, ROS generation in mouse platelets stimulated with thrombin or CRP under stirring flow shear in a platelet aggregometer was also inhibited by integrilin (Figure I in the Data Supplement). To validate the effects of integrilin, we further showed that agonist-induced ROS generation was similarly diminished in $\beta_3^{-/-}$ platelets under stirring flow in a platelet aggregometer (Figure 1E), indicating that integrin $\alpha_{IIb}\beta_3$ is critical for intracellular ROS generation during platelet activation under flow shear. To exclude possible artifact caused by using carboxy-H_2DCFDA, and to determine whether $\alpha_{IIb}\beta_3$ is important for ROS generation/release in the extracellular compartment, supernatants from platelet suspensions activated with thrombin in the presence of vehicle or integrilin were incubated with amplex red, which, in a way similarly to carboxy- H_2DCFDA, H_2O_2-dependently generates fluorescent oxidation product (resorufin). Stirring of human or mouse platelets caused only a slight elevation of H_2O_2. Stimulation of human or mouse platelets with thrombin or CRP without stirring did not induce extracellular H_2O_2 increase. When human or mouse platelets were stimulated with thrombin or CRP under constant stirring flow, H_2O_2 secretion was remarkably increased (Figure 1F–1I). These data indicate that activated platelets generate a significant amount of H_2O_2 under flow shear and suggest that H_2O_2 is released into extracellular compartment under flow shear. Generation of

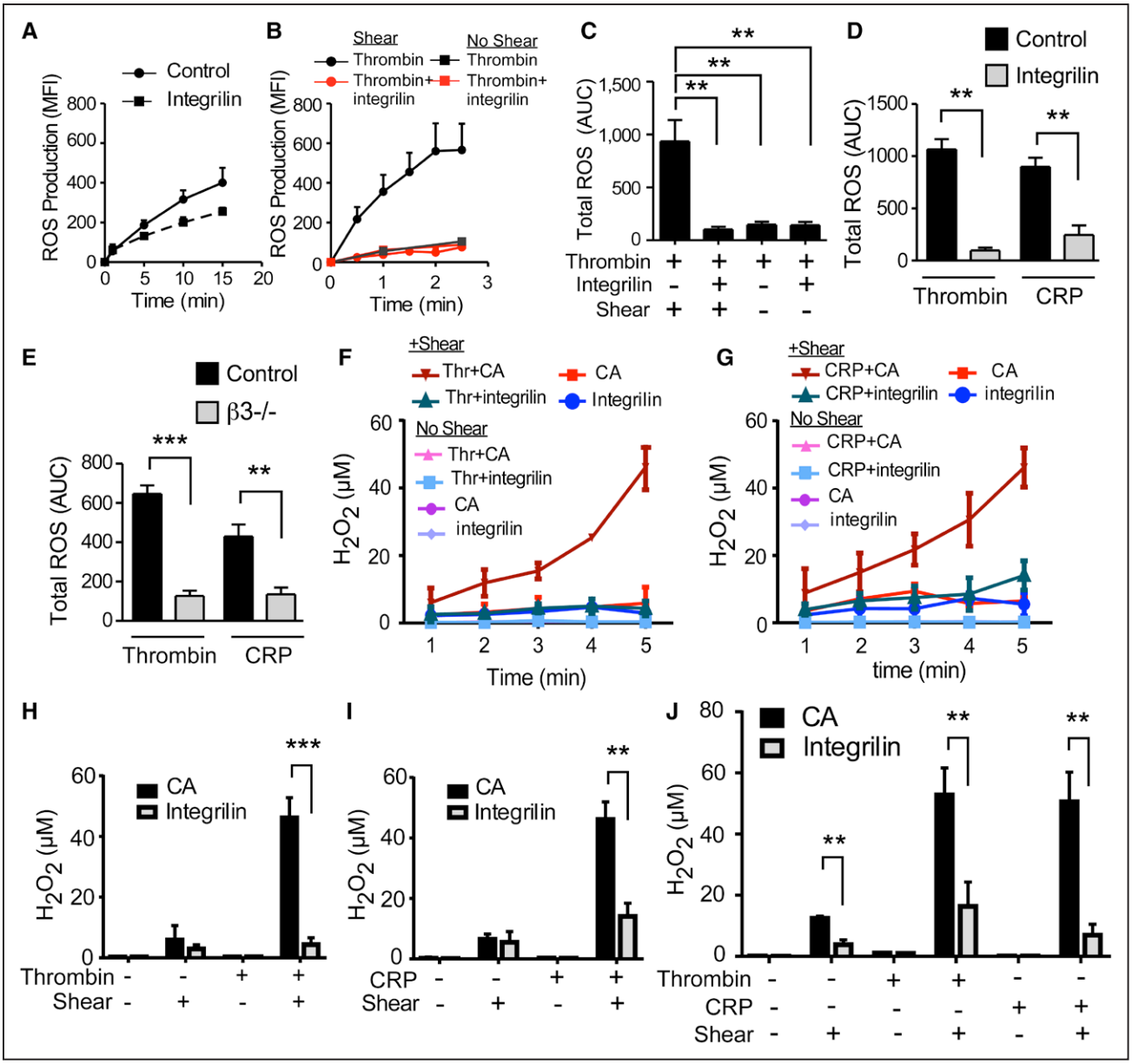

Figure 1. Effect of shear and importance of integrin $\alpha_{IIb}\beta_3$ in reactive oxygen species (ROS) production.
A–**E**, Washed wild-type (WT) mouse platelet suspension, treated with vehicle control (2.63 mg% citric acid [CA]) or integrilin (10 μg/mL; **A**–**D**), and $\beta_3^{-/-}$ or WT platelets (**E**), were preincubated with carboxy-H_2DCFDA and stimulated with 0.025 U/mL thrombin or 1 μg/mL CRP (collagen-related peptide) with or without subjecting to shear at 800/s. Mean fluorescence intensity (MFI) was measured over time using flow cytometry and total ROS production was calculated by integrating the area under the curve (AUC) of ROS production. **A**, ROS under static conditions over 15 min. **B**, ROS production under shear or no shear conditions over 150 seconds. **C**–**E**, Quantification of the total platelet ROS production (mean±SEM, 1-way ANOVA, Tukey multiple comparison test (**C**), Student *t* test (**D** and **E**), n=3). **F** and **G**, Washed mouse platelet suspension, treated with vehicle control (CA, 5.25 mg%) or integrilin (20 μg/mL) for 3 min, mixed with preincubated Amplex Red mixture and then stimulated with 0.025 U/mL thrombin (Thr; **F**) or 1 μg/mL CRP (**G**) with or without subjecting to flow shear at 1000 rpm in aggregometer. Fluorescence was measured over time using flexstation. H_2O_2 production was calculated according to standard curve. Note that H_2O_2 concentrations in the four no shear groups in both (**F**) and (**G**) were near zero and minimally different among these groups. Thus their curves overlap with *x* axis. **H** and **I**, Quantification of H_2O_2 production at 5 min in mouse platelets (mean±SEM, Student *t* test, n=3). **J**, Quantification of H_2O_2 production at 5 min in human platelets (mean±SEM, Student *t* test, n=3). **P*<0.05, ***P*<0.01, and ****P*<0.001.

extracellular H_2O_2 was enhanced with increasing thrombin concentrations but peaked at 0.25 U/mL thrombin. Further increase in thrombin concentration resulted in the detection of less H_2O_2 generation (Figure II in the Data Supplement). Integrilin inhibited H_2O_2 generation in thrombin- or CRP-stimulated platelets under flow at all agonist concentrations (Figure 1F–1I and Figure II in the Data Supplement), indicating a critical role for $\alpha_{IIb}\beta_3$ in enhancing platelet ROS generation both in the intracellular and extracellular compartments. Agonist-stimulated, shear-dependent ROS generation occurs similarly in mouse and human platelets (Figure 1J).

β_3 Integrin Is Important for Phosphorylation of p47phox During Platelet Activation Under Shear

Shear-dependent enhancement of ROS production in activated platelets is not agonist-selective, similar to NOX2-mediated generation of ROS during platelet activation.[8] To investigate the possible role of NOX2 in agonist-stimulated ROS generation under shear, we tested NOX2 activation by analyzing the phosphorylation of p47phox at S^{304}, which is important in the assembly of active NOX2 complex,[12,41–43] by immunoblotting with phosphor-S^{304}-specific anti-p47phox antibody in thrombin- or CRP-activated platelets. In agreement with ROS generation, flow shear alone did not induce p47phox phosphorylation (Figure III in the Data Supplement). When platelets were activated by thrombin or CRP under shear, however, not only does p47phox become phosphorylated, its phosphorylation was markedly inhibited by β_3 knockout or integrilin treatment (Figure 2A–2D), indicating that ligand binding to $\alpha_{IIb}\beta_3$ is important for p47phox activation during platelet activation under shear. Please note that the effect of β_3 knockout on p304 phosphorylation is unlikely to be associated with changes in the expression levels of other major adhesion receptors, because GP (glycoprotein) VI and GPIbα, the functional subunit of glycoprotein Ib-IX complex, were similar between β_3 knockout mice and wild type mice (Figure IV in the Data Supplement).

The Outside-in Signaling Pathway Stimulates p47phox Activation and ROS Generation in Platelets Without Requiring Prior Platelet Activation

Our data that $\alpha_{IIb}\beta_3$ and agonist stimulation are both required for platelet p47phox activation and ROS generation, especially under shear stress, suggest the following possibilities: (1) outside-in signaling is required to facilitate agonist-induced ROS activation signaling but is not sufficient to stimulate ROS production without agonist-stimulated platelet activation; or (2) outside-in signaling is the major mechanism that activates ROS production, whereas agonist-induced platelet activation signal is important mainly because it induces ligand binding to $\alpha_{IIb}\beta_3$ and thus outside-in signaling. To differentiate these possibilities, we analyzed ROS production in platelets spreading on the immobilized integrin ligand fibrinogen under static conditions. Unlike soluble fibrinogen binding to $\alpha_{IIb}\beta_3$, binding of $\alpha_{IIb}\beta_3$ to immobilized fibrinogen and platelet adhesion does not require inside-out signaling and prior platelet activation. Platelet spreading under this condition, however, requires integrin ligand interaction and outside-in signaling. Figure 3 demonstrates that platelet spreading on fibrinogen leads to a slow but significant increase in ROS production relative to control platelets treated with the ROS inhibitor N-acetylcysteine (Figure 3A and 3B). Platelet spreading on fibrinogen also lead to an increase in p47phox phosphorylation (Figure 3C and 3D), which was minimal in $\beta_3^{-/-}$ platelets (Figure 3E and 3F). These data indicate that $\alpha_{IIb}\beta_3$ outside-in signaling stimulates activation of p47phox and ROS production without requiring prior platelet activation and shear stress. Activation of p47phox during platelet spreading was accelerated by either $MnCl_2$ (which promotes ligand binding to integrins independent of inside-out signaling) or CRP (which induces inside-out signaling leading to integrin activation but may also induce integrin-independent p47phox phosphorylation; Figure 3C–3F). Together, these data suggest that $\alpha_{IIb}\beta_3$ outside-in signaling is a major signaling mechanism responsible for NOX2 activation and ROS generation, while platelet agonist thrombin mainly serves to induce inside-out activation of $\alpha_{IIb}\beta_3$. Our previous data suggest that applying pulling force (eg, shear) via integrin-bound ligand, greatly stimulates outside-in signaling,[31] which explains why shear greatly enhanced integrin-dependent p47phox activation and ROS production.

The Critical Role of $G\alpha_{13}$ in Outside-in Signaling-Stimulated Activation of p47phox and ROS Generation

We demonstrated that early phase $\alpha_{IIb}\beta_3$ outside-in signaling requires the direct interaction between the β_3 cytoplasmic domain and $G\alpha_{13}$. To determine whether $G\alpha_{13}$-β_3 interaction is required for activating p47phox and ROS production in platelets, we tested the effect of mP6, a β_3-derived peptide that inhibits $G\alpha_{13}$-β_3 interaction,[21] on $\alpha_{IIb}\beta_3$-dependent phosphorylation of p47phox and ROS generation. As expected, mP6 inhibited outside-in signaling as indicated by platelet spreading (Figure V in the Data Supplement). mP6 blocked ROS production (Figure 4A and 4B) and p47phox activation (Figure 4C and 4D) in platelets adherent on fibrinogen with or without adding thrombin, suggesting a dominant role of the outside-in signaling pathway in ROS production in adherent platelets. We also examined the effect of mP6 on ROS production in agonist-stimulated platelet suspensions under shear. Thrombin-stimulated platelet ROS production under shear was also diminished by mP6 (Figure 4E). To exclude the possible nonspecific effect of this inhibitor, we further tested the effect of $G\alpha_{13}$ knockout[29] on agonist-stimulated platelet ROS production under shear stress. $G\alpha_{13}$ knockout platelets were defective in agonist-stimulated ROS production under shear (Figure 4F). Together, these data demonstrate that the early phase of $\alpha_{IIb}\beta_3$ outside-in signaling mediated by $G\alpha_{13}$ interaction is a major mechanism that stimulates activation of p47phox and generation of ROS in platelets.

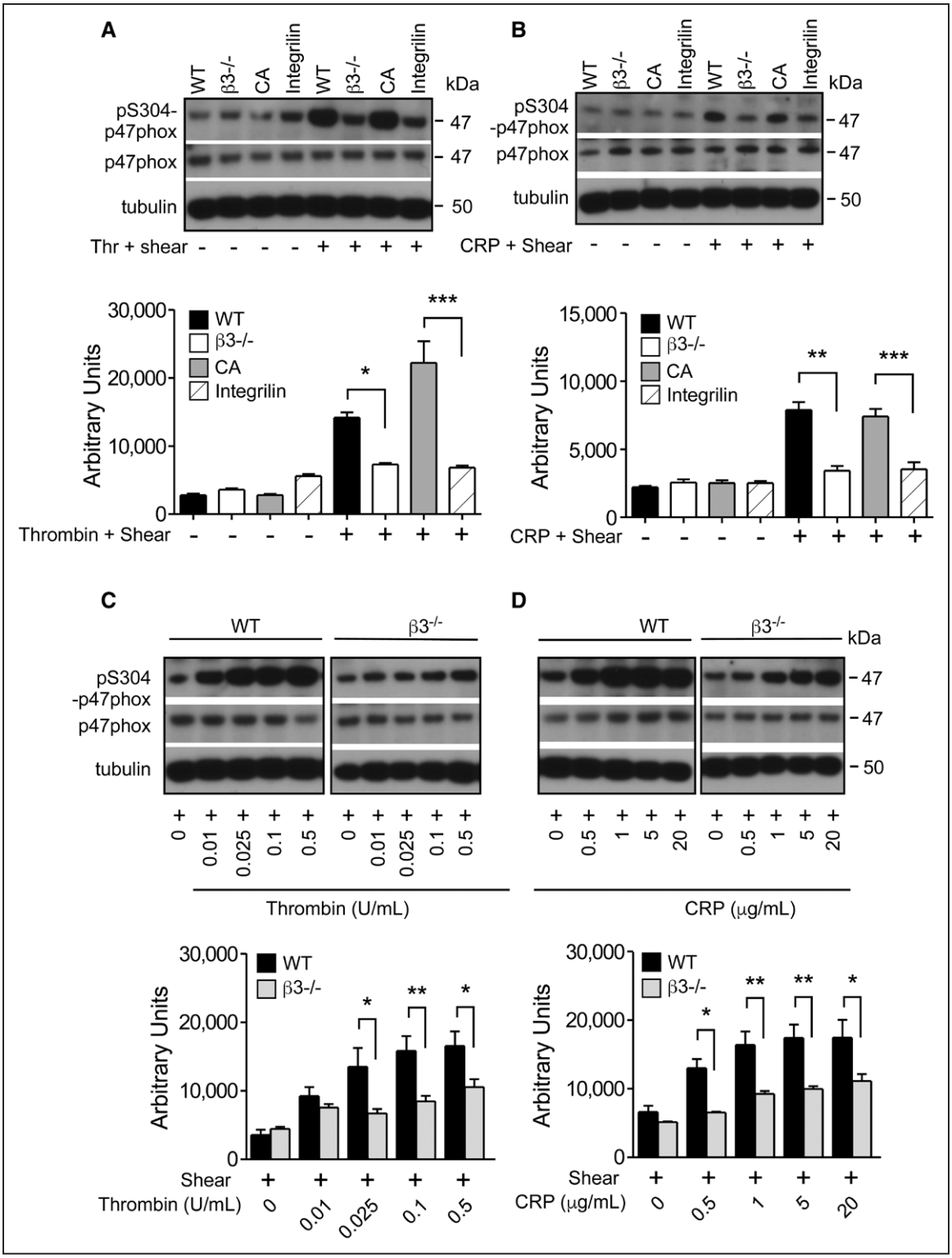

Figure 2. Effect of integrilin and importance of integrin $\alpha_{IIb}\beta_3$ in activation of p47phox during platelet activation.
A and **B**, Washed wild-type (WT) or $\beta_3^{-/-}$ mouse platelets, or WT mouse platelets treated with vehicle control (citric acid [CA]) or integrilin (10 μg/mL), were stimulated with or without thrombin (0.025 U/mL; **A**), CRP (collagen-related peptide; μg/mL; **B**) under shear, and were lysed and analyzed via SDS-PAGE and Western Blot for phosphorylated p47phox at Ser^{304} (pS304-p47phox), total p47phox, or α-tubulin as loading control. **C** and **D**, Lysates of washed WT and $\beta_3^{-/-}$ mouse platelets stimulated with increasing doses of thrombin (**C**) or CRP (**D**) were probed for phosphorylated p47phox at Ser^{304} (pS304-p47phox), total p47phox or α-tubulin as loading control. Lower parts of **A**–**D** are quantifications of Western blot results. Data plotted as mean±SEM (n=3, **P*<0.05, ***P*<0.01, and ****P*<0.001, Student *t* test).

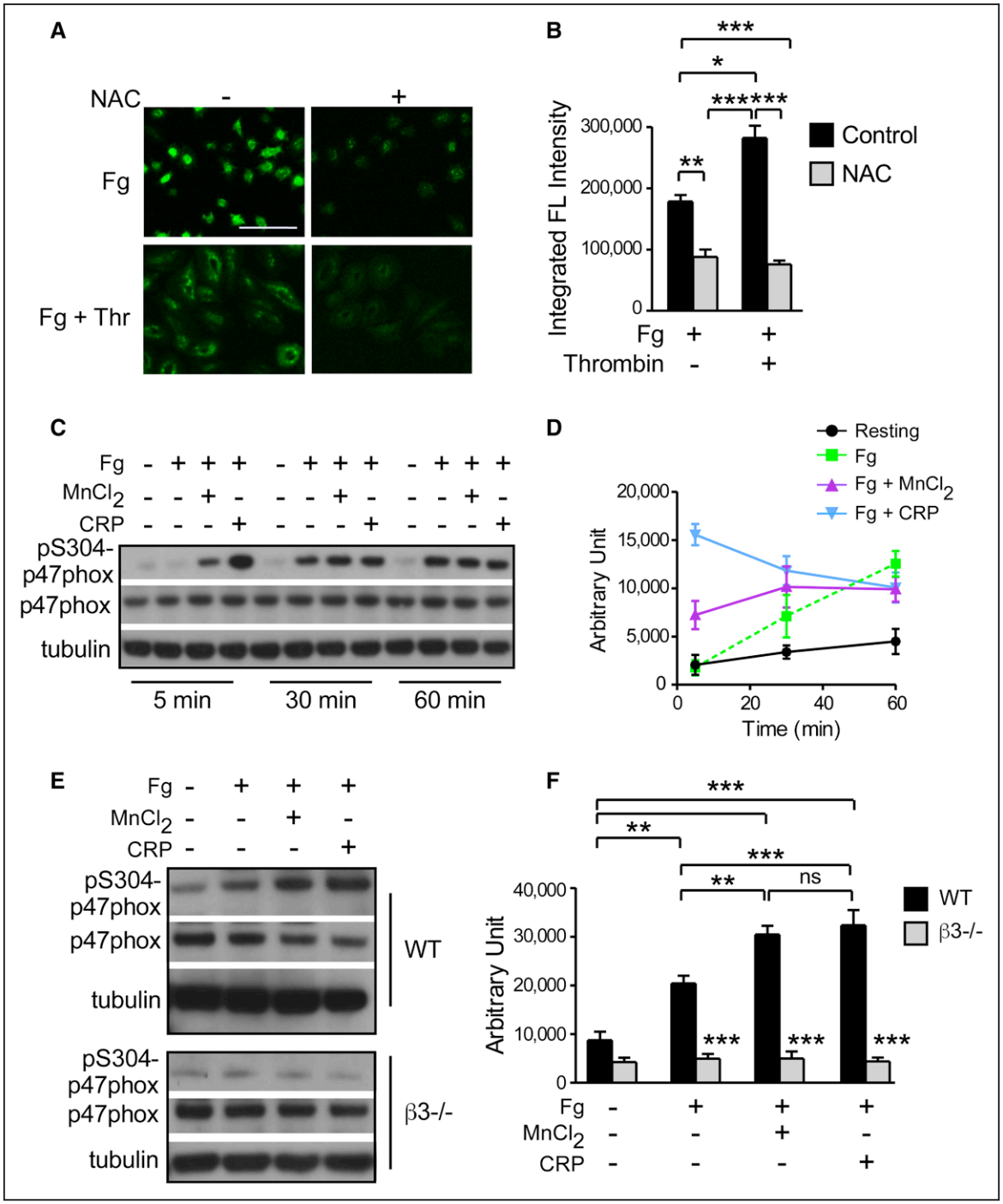

Figure 3. Reactive oxygen species (ROS) production and p47phox activation during platelet spreading on integrin ligand fibrinogen.

A, Images of DCF fluorescence in mouse platelets loaded with 10 µmol/L carboxy-H_2DCFDA and allowed to spread on fibrinogen, with or without 0.01 U/mL thrombin. N-acetylcysteine (NAC)-treated platelets was used as negative control (scale bar, 10 µm). **B**, Quantification of the total ROS production per cell was calculated by integrating the fluorescence (FL) intensity (mean±SEM, *P<0.05, ***P<0.001, 1-way ANOVA with Dunn multiple comparisons (Fg, –NAC: n=75, Fg, +NAC: n=19; Fg+thrombin, –NAC n=95, Fg + thrombin, +NAC n=47). **C**, Washed mouse platelets spreading on fibrinogen in the absence or presence of $MnCl_2$ (0.5 mmol/L) or (collagen-related peptide [CRP]; 1 µg/mL) were lysed at the indicated times and analyzed via SDS-PAGE and Western Blot for phosphorylated p47phox at Ser^{304} (pS304-p47phox), total p47phox, or α-tubulin as loading control. **D**, Quantification of the Western blot results for phosphorylated p47phox at Ser^{304} (pS304-p47phox; mean±SEM, n=4). **E**, Washed wild type (WT) and $\beta_3^{-/-}$ mouse platelets, either resting or spreading on fibrinogen in the absence or presence of $MnCl_2$ (0.5 mmol/L) or CRP (1 µg/mL), were lysed after 30 min and Western Blot for phosphorylated p47phox at Ser^{304} (pS304-p47phox), total p47phox, or α-tubulin as loading control. **F**, Quantification of (**E**), plotted as mean±SEM (*P<0.05, **P<0.01, ***P<0.001, 1-way ANOVA with Tukey multiple comparison test, n=4).

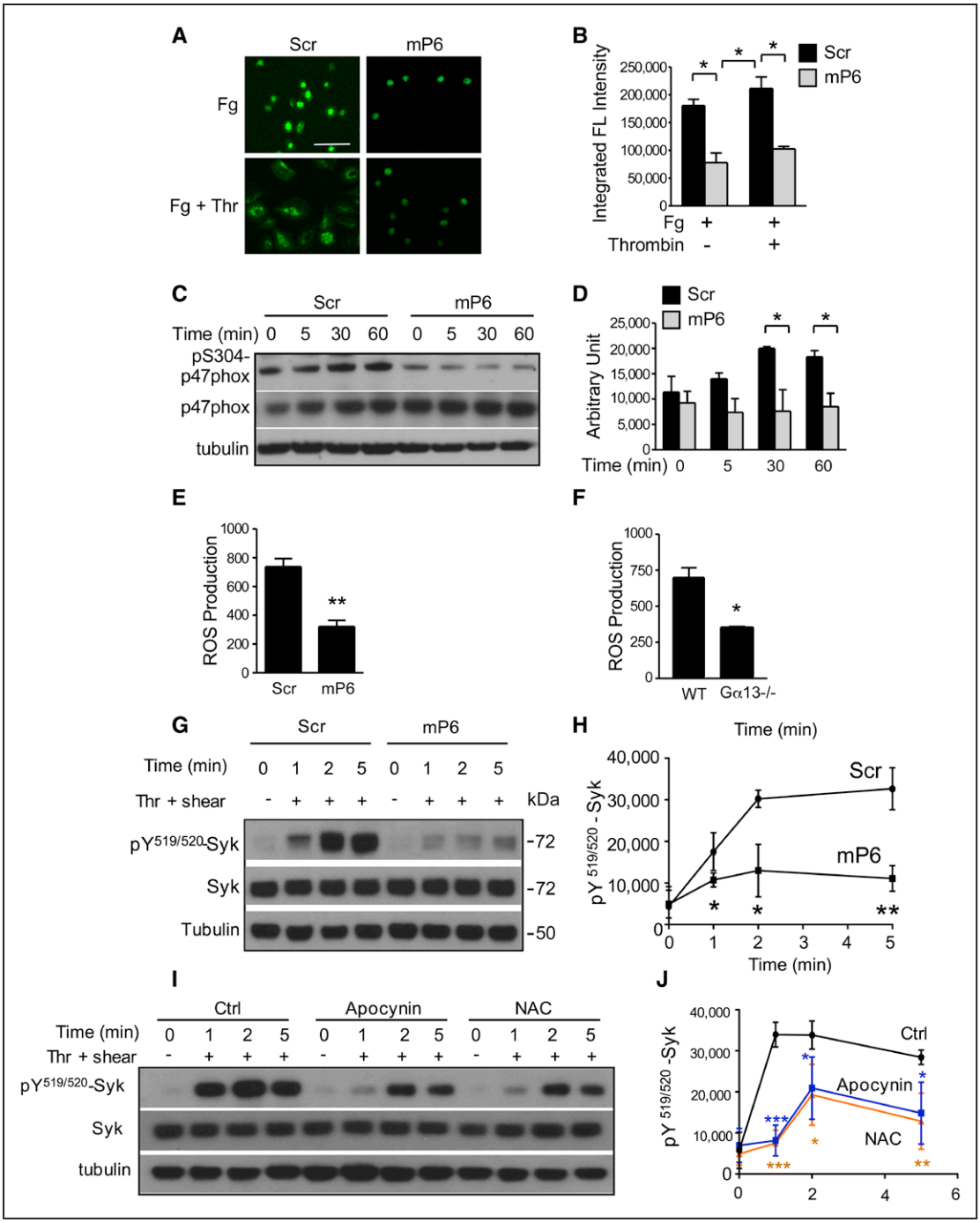

Figure 4. Gα13-mediated integrin outside-in signaling stimulates reactive oxygen species (ROS) production, p47phox activation and Syk phosphorylation.

A, Images of DCF fluorescence in mouse platelets loaded with 10 µmol/L carboxy-H_2DCFDA, treated with 100 µmol/L scrambled control peptide or mP6, and allowed to spread on fibrinogen with or without 0.01 U/mL thrombin (scale bar, 10 µm). **B**, Quantification of the total ROS production per cell in (**A**) was calculated by integrating the fluorescence (FL) intensity (mean±SEM, Kruskal-Wallis test with Dunn multiple comparisons, Fg −thrombin, Scr: n=100, Fg −thrombin, mP6: n=6; Fg+thrombin, Scr: n=45, Fg+thrombin, mP6 n=9). **C** and **D**, Washed mouse platelets were treated with 100 µmol/L scrambled control peptide or mP6, allowed to adhere and spread on fibrinogen, and lysed for (**C**) SDS-PAGE and Western blot for phosphorylated p47phox at Ser^{304} (pS304-p47phox), total p47phox or α-tubulin as loading control. **D**, Quantification of (**C**), plotted as mean±SEM, Student *t* test, n=4). **E** and **F**, Washed mouse platelets, treated with 100 µmol/L scrambled control peptide or mP6 (**E**), or wild type and $G\alpha_{13}^{-/-}$ platelets (**F**), were preincubated with 10 µmol/L carboxy-H_2DCFDA and stimulated with 0.025 U/mL thrombin under the shear rate of 800/s, and total ROS production is shown as mean±SEM (Student *t* test, n=3). **G** and **H**, Mouse platelets treated with scrambled control peptide or mP6 were stimulated with thrombin (0.025U/mL) for increasing lengths of time and then immunoblotted for Syk phosphorylated at $Tyr^{519/520}$, total Syk and tubulin. **G**, A typical blot; (**H**) quantification (n=3). **I** and **J**, Mouse platelets treated with ROS inhibitors apocynin (500 µmol/L) and NAC (1 mmol/L) were stimulated with thrombin (0.025 U/mL) for increasing lengths of time and then immunoblotted for Syk phosphorylated at $Tyr^{519/520}$, total Syk, and tubulin. **I**, A typical blot; (**J**) quantification (mean±SEM, *t* test, n=3). **P*<0.05, ***P*<0.01, and ****P*<0.001.

ROS Is Important for $\alpha_{IIb}\beta_3$-Dependent Syk Activation During Platelet Aggregation

Syk is activated not only by ITAM receptors such as GPVI-FcRγ complex but also by outside-in signaling.[28,32] We demonstrated that Shear- and thrombin induced Syk activation as indicated by Syk phosphorylation at $Y^{519/520}$ (Figure 4G and 4H). By contrast, Shear only is not sufficient to induce Syk phosphorylation (Figure VI in the Data Supplement). Thrombin- and shear-induced Syk phosphorylation is abolished by mP6, indicating the importance of $G\alpha_{13}$-β_3 interaction and outside-in signaling in Syk activation during platelet activation under shear (Figure 4G and 4H). We further showed that 2 different ROS inhibitors, apocynin and N-acetylcysteine, both significantly inhibited thrombin-induced Syk activation under shear (Figure 4I and 4J). Thus, ROS plays an important role in outside-in signaling-mediated Syk activation in mouse platelets.

NOX2 Is Important for $\alpha_{IIb}\beta_3$-Dependent ROS Production and Functional Platelet Responses

To further determine whether NOX2 is required for outside-in signaling-dependent ROS generation, washed control or NOX2 knockout mouse platelets were loaded with fluorescent ROS indicator H_2DCF and allowed to adhere and spread on immobilized fibrinogen. Knockout of NOX2 significantly but incompletely inhibited ROS generation in adherent platelets (Figure 5A and 5B). Likewise, agonist-induced ROS production under shear was also significantly but incompletely inhibited in NOX2-knockout platelets (Figure 5C and 5D) and to a lesser extent under static conditions (Figure VII in the Data Supplement). These data thus demonstrate that NOX2 is important for ROS production mediated by $\alpha_{IIb}\beta_3$ outside-in signaling, either during platelet spreading or under shear. These data also suggest the presence of possible additional mechanism(s) of ROS generation induced by outside-in signaling, which will be explored in future studies.

Previously, we have shown that NOX2 knockout platelets are defective in the second wave platelet aggregation and in aggregation stability.[8] To further determine whether the NOX2-dependent ROS generation is important in functional consequences of outside-in signaling, we showed that NOX2 knockout platelets spread significantly slower on immobilized fibrinogen than wild-type platelets (Figure 5E and 5F). We further studied platelet adhesion and thrombus formation under shear in collagen or VWF-coated flow chamber perfused with PPACK-treated blood. Stable platelet adhesion and sizes of platelet thrombi on immobilized collagen (Figure 5G and 5H) or VWF (Figure 5I and 5J) under shear was both reduced in NOX2 knockout platelets. These data demonstrate that both stable platelet adhesion and thrombus expansion under shear requires outside-in signaling-dependent NOX2 activation and ROS generation.

Differential Roles of SFKs and Syk in Agonist-Induced p47phox Activation

To further explore how p47phox is regulated downstream of $G\alpha_{13}$, we tested the effects of inhibitors of SFK and Syk known to be important in outside-in signaling downstream of $G\alpha_{13}$ binding to β_3.[22,28] As expected, SFK inhibitor PP2 but not control compound PP3 almost abolished p47phox phosphorylation at S^{304} and S^{328} induced by both thrombin and CRP (Figure 6A and 6B, Figure VIII in the Data Supplement) in mouse and human platelets under shear stress. In contrast, Syk inhibitors, piceatannol (Figure 6C and 6D), and Bay 61-3606 (Figure VIIIA and VIIIB in the Data Supplement), selectively inhibited CRP-induced p47phox S^{304} and S^{328} phosphorylation without affecting thrombin-induced p47phox phosphorylation in both human and mouse platelets under shear. These results are consistent with the importance of Syk in ITAM signaling, but not in thrombin-induced, integrin-dependent p47phox S^{304} and S^{328} phosphorylation (see graphic abstract). Thus, Syk does not appear to be involved in the outside-in signaling pathway regulating p47phox S^{304} and S^{328} phosphorylation, although we do not exclude the possibility that Syk may still be important in regulating NOX2 activity and ROS generation via other mechanisms.

Platelet p47phox May be Regulated by the PI3K/Akt Pathway

The PI3K-Akt pathway, PKC (protein kinase C), and MAPK (mitogen-activated protein kinase) have all been shown to be important in integrin outside-in signaling.[38,44] Akt, PKC, and MAPK are also reportedly involved in signaling leading to phosphorylating p47phox.[45–50] To explore the potential roles of these molecules in regulating p47phox during platelet activation under shear, platelets were treated with inhibitors of PKC (Gö6976), PI3K (wortmannin and LY294002), Akt (SH-6 and AktX), or the MAPK pathways (p38 inhibitor SB203580, MEK inhibitor U0126), stimulated with thrombin or CRP in a platelet lumi-aggregometer and assessed for phosphorylation of p47phox by Western Blot. In both thrombin- or CRP-stimulated human and mouse platelets, phosphorylation of p47phox at S^{304} and S^{328} was significantly inhibited by various inhibitors of PI3K and Akt (Figure VIIIA, IXA, and IXB in the Data Supplement), suggesting that a potential role for the PI3K/Akt signaling pathway in p47phox activation. Inhibition of p38 MAPK and ERK1/2 had no effect on p47phox phosphorylation at S^{304} or S^{328} (Figure IXC and IXD in the Data Supplement), indicating they are dispensable for integrin-dependent p47phox S^{304} or S^{328} phosphorylation. Inhibition of PKC selectively blocked CRP- but not thrombin-induced phosphorylation at S^{304}

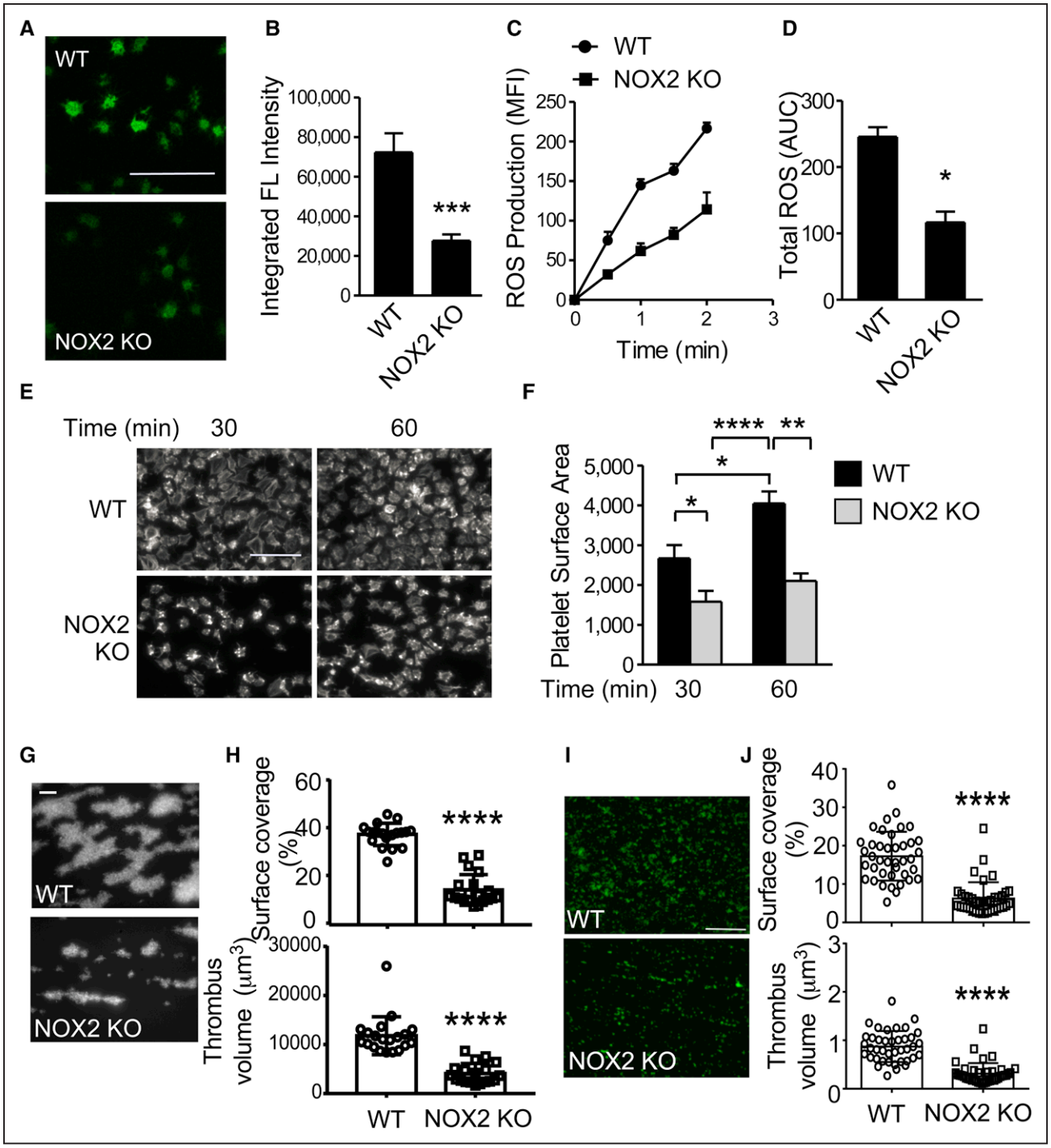

Figure 5. NOX (nicotinamide adenine dinucleotide phosphate [NADPH] oxidase) 2 is important for integrin- and shear-dependent reactive oxygen species (ROS) generation.

A and **B**, DCF fluorescence in 10 µmol/L carboxy-H_2DCFDA-loaded wild type (WT) and NOX2 knockout (KO) mouse platelets spreading on fibrinogen was measured via fluorescence microscopy. **A**, Images of DCF fluorescence (scale bar, 10 µm). **B**, Quantification of the total ROS production per cell calculated by integrating the fluorescence (FL) intensity (mean±SEM, ***P<0.001, Student t test, WT: n=46, NOX2 KO: n=35). **C** and **D**, Washed WT and NOX2 KO mouse platelets were preincubated with 10 µmol/L carboxy-H_2DCFDA, stimulated with 0.025 U/mL thrombin under a shear rate of 800/s, and flow cytometry was used to (**C**) measure DCF mean fluorescence intensity (MFI) over time mean±SEM (n=4) and (**D**) determine total ROS production (area under the curve [AUC]; mean±SEM, *P<0.05, Student t test, n=4). **E** and **F**, Washed WT and NOX2 KO mouse platelets spreading on fibrinogen were fixed, permeabilized, and stained with phalloidin. Fluorescence microscopy used to observe spreading. **E**, Images of platelet spreading (scale bar, 10 µm). **F**, Quantification of platelet spreading shown as platelet surface area (mean±SEM, ***P<0.001, Kruskal-Wallis test with Dunn multiple comparisons, n≥40). **G**–**J**, Blood from WT and NOX2 KO mice was perfused over collagen-coated surfaces (**G** and **H**), or VWF (von Willibrand factor)-coated surfaces (**I** and **J**) at a wall shear rate of 1000 s^{-1} for 1 min, adherent platelet aggregates stained with rhodamine-conjugated phalloidin and analyzed via fluorescence microscopy. **G** and **I**, Representative images (scale bar, 10 µm). **H** and **J**, Surface coverage and thrombus volume were measured and presented as mean±SD (n=25). ***P<0.001, Student t test.

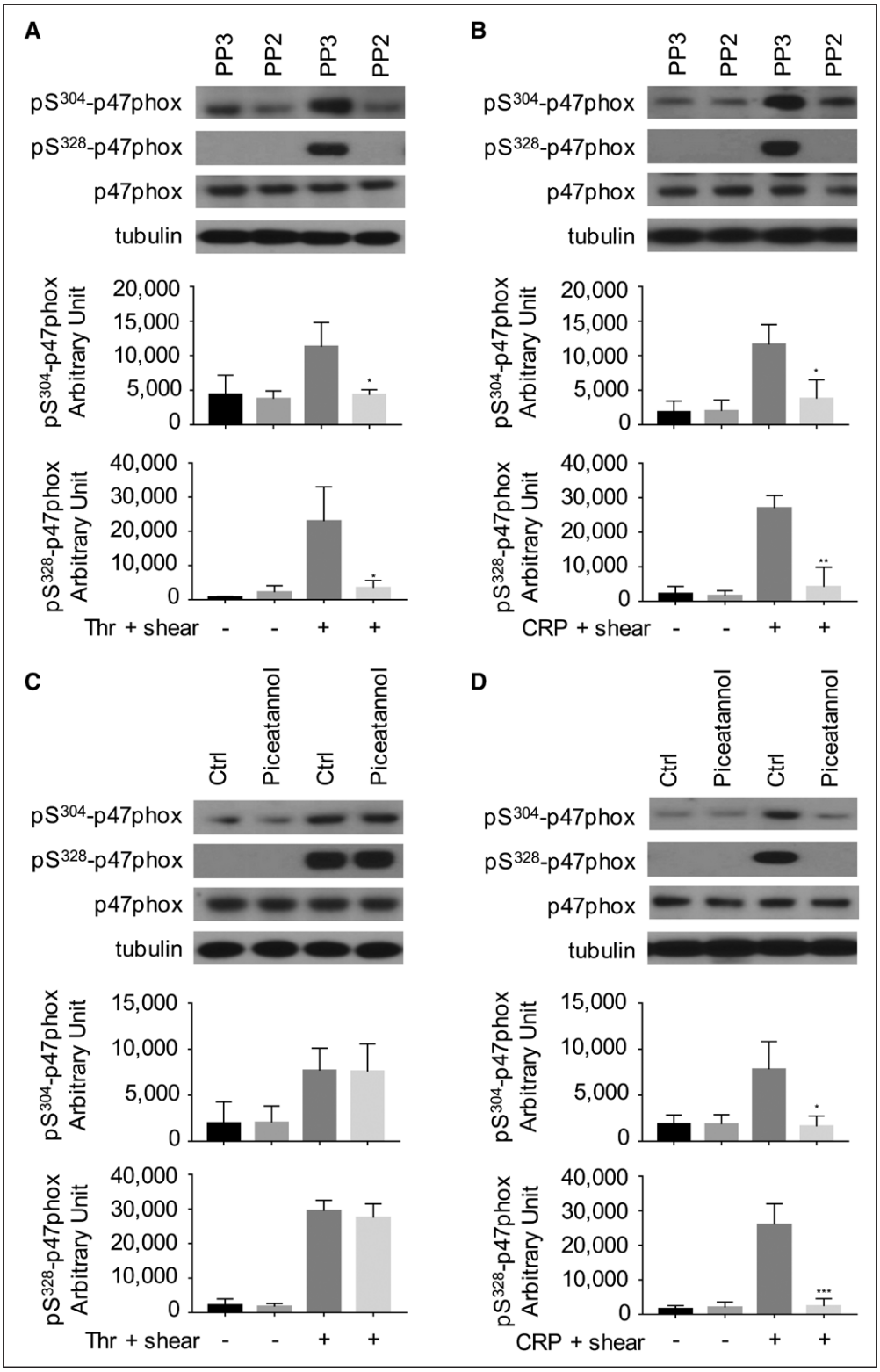

Figure 6. Differential roles of SFKs (Src family kinases) and Syk in agonist-induced p47phox phosphorylation.
A and **B**, Washed mouse platelets were treated with the SFK inhibitor PP2 (10 µmol/L) or negative control compound PP3(10 µmol/L), and stimulated with (**A**) 0.025 U/mL thrombin or (**B**) 1 µg/mL CRP (collagen-related peptide) to induce aggregation. **C** and **D**, Washed mouse platelets were treated with DMSO control (ctrl) or the Syk inhibitor piceatannol (15 µmol/L) and stimulated with (**C**) 0.025 U/mL thrombin or (**D**) 1 µg/mL CRP to induce aggregation. After 2 min, platelets were lysed and analyzed via SDS-PAGE and Western Blot for phosphorylated p47phox at Ser^{304} (p-p47phox S^{304}) or Ser^{328} (p-p47phox S^{328}), total p47phox or α-tubulin as loading control. The blots were scanned and quantified using National Institutes of Health Image J and plotted as mean±SEM (*P<0.05, **P<0.01, and ***P<0.001, Student t test comparing inhibitor-treated samples with controls, n=3). FL indicates fluorescence.

and S^{328} of p47phox (Figure IXC and IXD in the Data Supplement). Considering the known importance of PKC to the ITAM pathway, it is likely that PKC, like Syk, is also dispensable for outside-in signaling-dependent p47phox S^{304} and S^{328} phosphorylation. These data suggest that the PI3K-Akt pathway—but neither MPAKs nor PKC—may be important in mediating integrin-dependent NOX2 activation by phosphorylating S^{304} and S^{328}. Although, we do not exclude the possibility that PKC and MAPK may regulate NOX2 by phosphorylating other targets during integrin outside-in signaling.

DISCUSSION

In this study, we demonstrate that $G\alpha_{13}$-dependent early phase $\alpha_{IIb}\beta_3$ outside-in signaling is a major mechanism responsible for stimulating platelet ROS production during platelet adhesion and activation. We further discovered that blood flow shear is an important new factor that greatly accelerates and enhances the outside-in signaling-mediated ROS production both intracellularly and extracellularly. Our data further suggest a $G\alpha_{13}$-SFK- and PI3K-Akt-dependent pathway mediating $\alpha_{IIb}\beta_3$-dependent p47phox phosphorylation and NOX2 activation and an important but nonexclusive role of NOX2 in platelet ROS generation induced by outside-in signaling. Furthermore, our data reveal a novel ROS-mediated outside-in signaling pathway leading to activation of Syk and Syk-mediated secondary platelet activation promoting platelet spreading and thrombus expansion in blood flow. Our data also explains why NOX2 deficiency affect arterial thrombosis but not hemostasis in vivo. Thus, these findings represent a significant advancement in platelet biology.

ROS generation during platelet activation and its role in facilitating platelet activation were discovered in the 1970s by Marcus[1] and by Handins,[2] 2 great contributors to the field of platelet biology and hemostasis of that era. These early reports are convincingly supported by in vitro and in vivo studies of an increasing number of research groups.[7,8,17,51–56] However, ROS production in agonist-stimulated platelets under static conditions is slow and almost insignificant during early minutes of platelet activation, which explains the apparent controversy regarding the significance of ROS generation in platelet activation.[19] We show here that agonist-stimulated ROS production becomes robust under the shear stress present in flowing blood, and have thus uncovered a critical new factor that greatly enhances platelet ROS production. This new finding has important in vivo implications in studying the mechanisms of platelet adhesion and activation in that platelets are normally exposed to the shear stress of blood flow and are most efficient in mediating thrombus formation under relatively high shear stress and that integrin-dependent secondary platelet aggregation requires the shear force generated by blood flow in vivo and stirring in vitro. This brings in the further question of how ROS may play roles in thrombosis. Extracellularly, the ability of platelets to adhere to subendothelial matrix under shear stress has been known to require the interaction between collagen-bound VWF with its platelet receptor, GPIb-IX, to form a shear-resistant adhesive bond in flow. It has been shown that the affinity of VWF binding to GPIb-IX is greatly enhanced by oxidation of key residues in VWF.[57] Interestingly, our data shows a large amount of H_2O_2 is released from activated platelet under shear and that platelet adhesion to VWF are significantly reduced in NOX2 knockout mice (Figure 4). Thus, it would be interesting to further study whether extracellular ROS generated by NOX2 in adherent platelets would be an important mechanism for VWF oxidation. Intracellularly, ROS has been shown to play an important role in regulating tyrosine phosphatase activity and tyrosine kinases,[58,59] which controls the activation and inactivation of Syk, a critical tyrosine kinase both in the ITAM signaling pathway and integrin signaling pathway. We have shown that ROS inhibitors (Figure 4I and 4J) and NOX2 knockout[8] significantly inhibited Syk activation and reduced the sizes of thrombi on collagen and VWF surfaces (Figure 5). Previously, we demonstrated that NOX2-knockout platelets are defective in forming occlusive thrombus in the laser-induced arterial thrombosis in vivo.[8] Thus both extracellular and intracellular ROS may serve as shear-induced mechanisms that greatly promote platelet adhesion and amplify platelet activation leading to thrombosis.

Not only have we discovered the important role of flow shear in platelet ROS production, we also identified integrin outside-in signaling as the molecular mechanism responsible for converting shear force into intracellular signaling leading to ROS production. The early phase of outside-in signaling is critical for platelet spreading on $\alpha_{IIb}\beta_3$ ligands and for the secondary waves of platelet responses following primary platelet adhesion and aggregation, which greatly stabilizes and amplifies platelet thrombi.[21,24] We previously showed that ligand binding to $\alpha_{IIb}\beta_3$ induces $G\alpha_{13}$-β_3 interaction, which is required for activation of SFK and SFK-dependent RhoA inhibition mediating platelet spreading,[22] and demonstrated a PI3K-Akt-dependent activation pathway facilitating platelet spreading.[38] However, the mechanism of outside-in signaling leading to secondary platelet activation and thrombus expansion has not been clear. Brugge and Shattil groups[28,32,33] have shown that the key ITAM kinase Syk is activated and important for outside-in signaling. Boylan et al[26] reported that, in human platelets, $\alpha_{IIb}\beta_3$ is associated with an ITAM receptor FcγIIA, suggesting that outside-in signaling via SKF may mediate ITAM-dependent activation of Syk leading to amplification of platelet activation and thrombus expansion. However, mouse platelets do not express FcγIIA and mouse platelets deficient in another classic ITAM receptor the

GPVI-associated FcRγ (Fc receptor γ chain) showed minimal effect on arterial thrombosis induced by laser injury[60] in contrast to the dramatic inhibition by blocking $G\alpha_{13}$-β_3 interaction or $G\alpha_{13}$ knockout.[21,31] Furthermore, Gao et al[33] suggest that there may be an additional ITAM receptor-independent mechanism of Syk activation via outside-in signaling. Here we found that outside-in signaling, via a $G\alpha_{13}$/SFK- and likely PI3K/Akt-dependent mechanisms leads to NOX2 activation and ROS generation, which activates Syk in platelets. Thus, our study provides a conceptual advance as we have discovered that ROS generation is a missing link between the integrin outside-in signaling and the Syk-dependent platelet activation pathway in platelets and provides a novel pathway of integrin outside-in signaling-mediated amplification of platelet activation and thrombus expansion (Graphic abstract). Furthermore, we previously demonstrated that this ligand binding-induced outside-in signaling mechanism serves as a mechanical force-signaling converter to stimulate platelet responses to shear stress in a $G\alpha_{13}$-dependent manner, but the mechanism for this role of integrin outside-in signaling remains unclear.[31] Our finding of a ROS-dependent platelet activation mechanism initiated by $G\alpha_{13}$-β_3 interaction and shear-dependent stimulation of ROS generation thus provide a novel mechanism explaining how integrin transmits shear-dependent signals leading to great amplification of platelet activation.[61]

Not only have we found that outside-in signaling activates ROS generation, but we also show that NOX2 plays an important role in the outside-in signaling-mediated ROS generation. There has been increasing amount of data from multiple groups showing that NOX2 plays an important role in platelet activation,[8,10,16] platelet-leukocyte interaction and arterial thrombosis,[8,17,18] Whereas both NOX1 and NOX2 are important in platelet activation,[8,62] we demonstrated that NOX2 knockout primarily affects the stability and secondary platelet activation, and that NOX2 knockout significantly reduced Syk activation.[8] However, it remains unclear how NOX2 is stimulated, and how it plays roles during platelet activation. This study demonstrates that NOX2 is primarily activated by integrin outside-in signaling and that NOX2-mediated ROS generation serves to link $\alpha_{IIb}\beta_3$ outside-in signaling to the Syk-dependent platelet activation signaling pathway (although we do not exclude the possible involvement of NOX1 or other ROS generation mechanisms). The $G\alpha_{13}$-dependent early phase outside-in signaling of $\alpha_{IIb}\beta_3$ occurs after the $\alpha_{IIb}\beta_3$-mediated primary platelet adhesion and aggregation and is dispensable for normal primary hemostasis,[21] but $G\alpha_{13}$-dependent outside-in signaling is important for occlusive thrombosis.[21,31] Thus the findings that NOX2-mediate ROS generation is a part of the outside-in signaling pathway explains why NOX2 is important in platelet activation and thrombosis,[8,17] but NOX2 deficiency in human granulomatous disease patients and NOX2 knockout in mice do not bleed excessively. These findings support the concept that the $G\alpha_{13}$-dependent early phase outside-in signaling and the NOX2-ROS pathway is an excellent drug target for blocking thrombosis without affecting hemostasis.

In conclusion, our new finding of activation of $\alpha_{IIb}\beta_3$- and NOX2-dependent ROS generation mediated by the $G\alpha_{13}$-SFK- and PI3K-Akt-dependent pathway and its enhancement by shear force provide significant conceptual advances concerning how integrin outside-in signaling promotes platelet adhesion and secondary platelet activation under shear stress, reveals a novel mechanism with regarding to how platelet ROS generation is activated and play a role in platelets and have addressed current controversy with regarding to the importance of platelet ROS generation. These findings also provide significant mechanistic insight to our understanding of human granulomatous disease and our development of new antithrombotic drug targets and new drugs for treating thrombosis with minimal effects on bleeding.[24,63]

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Disclosures

University of Illinois holds patents related to the Gα13 inhibitor peptide mP6. X. Du has ownership interests in DMT (Dupage Medical Technology), Inc, which licences the University patents. The other authors report no conflicts.

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