MATERIALS AND METHODS

Mice. The generation of NOX1 and NOX2 knockout mice was previously described.[1, 2] Heterozygous female $NOX1^{+/-}$ mice were bred with male C57BL/6 mice, and the corresponding male $NOX1^{+/Y}$ and $NOX1^{-/Y}$ offspring were used as control wild-type (WT) and NOX1 KO ($NOX1^{-/Y}$), respectively. $NOX2^{-/-}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME). $NOX1^{-/Y}$ and $NOX2^{-/-}$ mice were maintained on a C57BL/6 background. Animal usage and protocol were approved by the institutional animal care committee of the University of Illinois at Chicago. To genotype NOX1 mice, three primers were used: 5'-ACGGGCACATGTGTAAGACTCACC-3', 5'-CTACCAGGC CAATCTCTCTGTTTCCA-3', 5'-GCCTGCAACTCCCCTTATGGTCA-3'. To genotype NOX2 mice, three primers were used: 5'-AAGAGAAACTCCTCTG CTGTGAA-3', 5'-CGCACTGGAACCCCTGAGAAAGG-3', and 5'-GTTCTAATTCCATCAGAAGCTTATCG -3'. The mutant NOX1 and NOX2 alleles are detected at 667 and 195 base pairs, respectively. The WT NOX1 and NOX2 alleles are detected at 806 and 240 base pairs, respectively.

Preparation of washed mouse platelets. Washed mouse platelets were prepared as previously described.[3, 4] Briefly, blood was collected from isoflurane anesthetized mice from the inferior vena cava using one-seventh volume of ACD (85 mM trisodium citrate, 83 mM dextrose, and 21 mM citric acid). Platelets were isolated by differential centrifugation, washed twice with CGS buffer (0.12 M sodium chloride, 0.0129 M trisodium citrate, and 0.03 M glucose, pH 6.5) and resuspended in N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)-buffered Tyrode's solution containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. They were then allowed to recover to resting state at 25°C for 1 to 2 hours.

RT-PCR

Mouse platelet RNA was extracted from isolated $NOX1^{-/Y}$ and $NOX2^{-/-}$ mouse platelets and their respective littermate controls using the PureLink® RNA Mini Kit (Life technologies). RNA concentrations were determined using a Thermo Scientific NanoDrop 1000. cDNA synthesis and PCR were combined using SuperScript® One-Step RT-PCR with Platinum® Taq (Invitrogen). 1 μg template RNA was used for each cDNA synthesis/PCR reaction. Mouse NOX3 and NOX4 cDNA was amplified over 40 cycles with a forward 5′ ATG CCG GTG TGC TGG ATT CTG′ and reverse 5′ CTA GAA GTT TTC CTT GTT GTA′ primer for mouse NOX3; and with a forward 5′ ATG GCG GTG TCC TGG AGG AGC 3′ and reverse 5′ TCA GCT GAA GGA TTC TTT ATT GTA 3′ for mouse NOX4. HUVEC NOX3 and NOX4 cDNA was amplified over 40 cycles with a forward 5′ ATG ATG GGG TGC TGG ATT TTG 3′ and reverse 5′ CTA GAA GCT CTC CTT GTT GTA ATA 3′ primer for human NOX3; and with a forward 5′ ATG GCT GTG TCC TGG AGG AGC TGG 3′ and reverse 5′ TCA GCT GAA AGA CTC TTT ATT GTA 3′ for human NOX4. PCR products were separated on a 1% agarose gel containing GelRedTM nucleic acid stain and then directly visualized and photographed while under UV light. Control PCR reactions were performed with the same RNA preparations using primers specific for GAPDH.

Immunoblotting. Rabbit anti-NOX1 (Santa Cruz), mouse anti-gp91^{phox} antibodies (BD Biosciences), anti-NOX4 antibodies (Santa Cruz), anti-phospho Syk (Cell Signaling Technology), anti-phospho PLCγ2 (Cell Signaling Technology), anti-phospho P38 (Cell Signaling Technology), anti-phospho ERK (Cell Signaling Technology) and anti-tubulin antibody (Sigma-Aldrich) were used for western blot detection of their respective antigen.

Platelet aggregation assay. Platelet aggregation and secretion of granule ATP was determined in a Lumi-Aggregometer (Chrono-log, Havertown, PA) at 37°C with stirring (1000 rpm) after addition of the luciferin-luciferase reagent and platelet agonists.[3]

Calcium mobilization. Calcium mobilization was monitored using the FLIPR calcium 5 assay kit (Molecular Devices). Platelets, 1x10^8/mL, were used and the calcium response was monitored using the FlexStation plate reader (Molecular Devices).

Detection of intracellular ROS. Intracellular ROS was measured essentially as previously described with modifications.[5] Briefly, platelets suspensions (1x10^8/mL) were incubated with the global intracellular ROS indicator 10 µM 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Carboxy-H_2DCFDA, Invitrogen) for 15 minutes. Agonist was added to platelet suspensions (1x10^8/mL) and DCF fluorescence was measured as a function of time using an Accuri C6 flow cytometer to obtain kinetic changes in intracellular ROS. Total DCF fluorescence was determined by integrating the area under the curve during the kinetics of DCF production. Detailed methods are provided in the “Supplemental Material” and Figure I.

Fluorescence intravital microscopy. Intravital microscopy was performed as described previously.[6] Littermate WT control and NOX1$^{-/Y}$ mice, or WT (C57BL/6) and NOX2$^{-/-}$ mice (male, 6-8 weeks old) were anesthetized via intraperitoneal injection of ketamine and xylazine and placed on a thermo-controlled blanket (37ºC). The cremaster muscle was exteriorized and superfused with bicarbonate-buffered saline throughout the experiment. Fluorescence and brightfield images were recorded using an Olympus BX61W microscope with a 60 x/1.0 NA water immersion objective and a high speed camera (Hamamatsu C9300) through an intensifier (Video Scope International). Fluorescence images were captured at 20 frames per second, and data were analyzed using Slidebook v5.5 (Intelligent Imaging Innovations). Arteriolar wall injury was induced with a micropoint laser ablation system (Photonics Instruments). Platelet accumulation was visualized by infusion of Dylight 649-labeled anti-mouse CD42c antibodies (0.05 µg/g body weight). Ten thrombi were generated in 2-4 different arterioles with a diameter of 30-45 µm in one mouse, with new thrombi formed upstream of earlier thrombi. Data were collected for 4 minutes following laser injury. The kinetics of platelet accumulation was analyzed by integrated median fluorescence intensities of the antibodies as a function of time.

Infusion of WT and NOX2$^{-/-}$ platelets into thrombocytopenic mice. For platelet depletion, WT mice were treated by iv injection of rat anti-mouse GPIbα antibodies (0.5 µg/g BW, R300, Emfret Analytics) 30 minutes prior to the experiment.[7] WT and NOX2$^{-/-}$

platelets were isolated and labeled with calcein AM (1 µg/mL). The labeled platelets (10^8 cells/100 µL) were infused into thrombocytopenic mice. Endogenous platelets were visualized by infusion of Dylight 649-conjugated anti-CD42c antibodies. Two-three thrombi were induced by laser injury and accumulation of infused and endogenous platelets were monitored. Subsequently, the same mouse was treated with the labeled platelets and 2-3 additional thrombi were induced. Data were analyzed as described in "fluorescence intravital microscopy".

Tail bleeding time. 10-15 week-old mice were anesthetized with isoflurane. The tail was excised at a fixed distance of 5 mm from the tip, and the remaining tail was immersed in 37°C, 0.15 M NaCl. Bleeding was followed visually. The time to stable cessation of bleeding was recorded and determined when recurrent bleeding did not occur within 1 minute of the time of cessation[8, 9].

Statistics. Data analysis was performed using the statistical software GraphPad Prism 5. Statistical significance was assessed by ANOVA (analysis of variance) or Kruskal-Wallis test for comparison of multiple groups or Student t-test for comparison of 2 groups.

References

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