Supplemental Data

Nox2 NADPH oxidase is dispensable for platelet activation or arterial thrombosis

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Supplemental Methods, References, Table and Figures

Supplemental Methods

Neutrophil isolation

Neutrophils were isolated as described[1] with minor modifications. Briefly, mice were euthanized by CO_2 asphyxiation, bone marrow was isolated from the femur and tibia into RPMI media supplemented with 10% fetal bovine serum, 1% streptomycin/penicillin and 2 mM EDTA. The cell suspension was filtered with 70 μm cell strainer and centrifuged (300 ***g*** for 7 min, 4 °C). The cell pellet was resuspended in 1 mL Ca-Mg free HBSS followed by addition of 3 mL of each Histopaque-10771 and Histopaque-11191 very gently to the bottom of tube, centrifuged at 1000 ***g*** for 30 min at RT. After centrifugation, the middle layer containing neutrophils was collected, washed and resuspended in suspension buffer (HBSS supplemented with 1% human serum albumin and 0.1% glucose).

Superoxide and hydroxyl radical quantification using EPR spin trapping

For detection of superoxide and hydroxyl radical by EPR, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was used as a spin trapping agent. Neutrophils (2×10^6/mL) or bead isolated platelets (4×10^8/mL) were incubated with 0.5 mM diethylenetriaminepentaacetic acid (as a transition metal chelator) and 50 mM DMPO (Dojindo Molecular Technologies, Inc. Rockville, MD USA) for 5 min. After incubation, cells were activated with phorbol 12-myristate 13-acetate (PMA) or thrombin-convulxin, and immediately transferred into a flat cell and examined by EPR spectroscopy (Bruker EMX spectrometer, TM cavity with TM flat cell; Bruker BioSpin, Billerica, MA). Instrument settings were: modulation frequency of 100 kHz; nominal microwave power of 20 mW; modulation amplitude of 1.0 G; scan rate 80 G/81 s; receiver gain $10^4 – 10^6$; six sets with 10 additive scans for each spectrum in the set.

Real-time PCR

Levels of mRNA for *Nox2, Nox4, Nox1, p47phox* and *18s* in mouse platelets (bead purified) and lung samples were measured by quantitative real-time PCR as described previously.[2] Total RNA was isolated from platelets using Trizol reagent (Invitrogen, Carlsbad, CA), and reverse transcribed using Taqman reverse transcriptase and random hexamer primers. PCR primers and 6-carboxy fluorescein-labeled probes for *18s* (Mm03928990), *Nox1* (Mm00549170), *Nox4* (Mm00479246) and *p47Phox* (Mm00447921) were purchased from Applied Biosystems. The primers to detect *Nox2* mRNA was designed by us and prepared by Applied Biosystems (5' CAG TGC TGA CCC AAG GAG TT 3', and 5' GGG AAC TGG GCT GTG AAT GA 3'). Reverse transcribed cDNA was incubated with TaqMan Universal PCR mix (Applied Biosystems) and PCR primers and probes at 50 °C for 2 min and then at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min using the Applied Biosystems 7700 sequence detection system. For quantitative analysis of mRNA, the comparative threshold cycle ($\Delta\Delta C_T$) method[3] was used, with values normalized to *18s* rRNA and expressed relative to levels in *Cybb*+/y mice. Validation experiments were performed to confirm equal amplification efficiency for all primers sets.

Western Blotting

Bead-purified platelets were lysed with RIPA buffer (50 mM Tris pH-8.0**,** 150 mM NaCl**,** 1% NP-40, 0.5% Na-deoxycholate**,** 1%SDS**,** 5 mM EDTA, supplemented with protease and phosphatase inhibitor cocktail). Samples containing 40 µg protein were mixed with 2x Laemmli sample buffer (1:1, v/v) and denatured at 95 °C for 10 min. The proteins were separated by SDS-PAGE and transferred to Immobilon-P polyvinylidene fluoride (PVDF) membranes, blocked with 5% non-fat milk and incubated with primary antibodies; Nox1(1:500, Sigma), Nox4 (1:1000, Novus Biologicals), p47Phox (1:500, Santa Cruz Biotechnology) and beta actin (1:5000, Abcam). The immuno-decorated proteins were

detected with HRP- conjugated secondary antibodies using SuperSignal West Femto (Thermo).The proteins were visualized using gel documentation system (Bio-Rad) and quantified using densitometer.

Dense granule secretion

Release of ATP from dense granules was measured using Chrono-lume luciferin-luciferase reagent (Chrono-log, stock concentration, 0.2 μM luciferase/luciferin) using Optical/Lumi-Aggregometer (model 700-2) as described previously.[4,5] Briefly, washed platelets (280 μL of 2 x10^8/mL) were incubated with 20 μL of Chrono-lume reagents for 2 min at 37 °C. After incubation, thrombin (0.02 U/mL) was added under stirring condition (1200 rpm) to induce platelet aggregation. Dense granule secretion (ATP release) was monitored in parallel to aggregation.

Supplemental References

1. Canobbio I, Visconte C, Momi S, et al. Platelet amyloid precursor protein is a modulator of venous thromboembolism in mice. *Blood*. 2017;130(4):527-536.

2. Dayal S, Wilson KM, Leo L, Arning E, Bottiglieri T, Lentz SR. Enhanced susceptibility to arterial thrombosis in a murine model of hyperhomocysteinemia. *Blood*. 2006;108(7):2237-2243.

3. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 2001;25(4):402-408.

4. Sonkar V, Kulkarni PP, Chaurasia SN, et al. Plasma Fibrinogen Is a Natural Deterrent to Amyloid Beta-Induced Platelet Activation. *Mol Med*. 2016;22:224-232.

5. Nayak MK, Dhanesha N, Doddapattar P, et al. Dichloroacetate, an inhibitor of pyruvate dehydrogenase kinases, inhibits platelet aggregation and arterial thrombosis. *Blood Adv*. 2018;2(15):2029-2038.

Table S1. DHE oxidation products in buffer.

pmol analyte/500 µL of buffer						
Time	**Thrombin & Convulxin**	**2-OH-E^+**	**DHE**	**E^+**	**E^+-dimer**	**DHE-dimer**
0 min	-	242 ± 42	14000 ± 600	5490 ± 660	113 ± 20	3439 ± 45
30 min	-	252 ± 34	13810 ± 990	6600 ± 360	116 ± 20	3401 ± 4
30 min	+	300 ± 64	17290 ± 550	5870 ± 980	85 ± 21	3395 ± 2

There is little, if any, oxidation of DHE in buffer in the time frame of the experiments in the presence or absence of thrombin and convulxin. DHE (25 µM) was incubated in Tyrode's buffer with or without thrombin (0.05 U/mL) and convulxin (50 ng/mL), as indicated above. The oxidation products of DHE: 2-hydroxyethidium (2-OH-E^+), ethidium (E^+), ethidium-dimer (E^+-dimer), and dihydroethidium-dimer (DHE-dimer) as well as left over DHE (substrate), were quantitated. Values are mean ± SEM. n = 3 - 4 in each group.

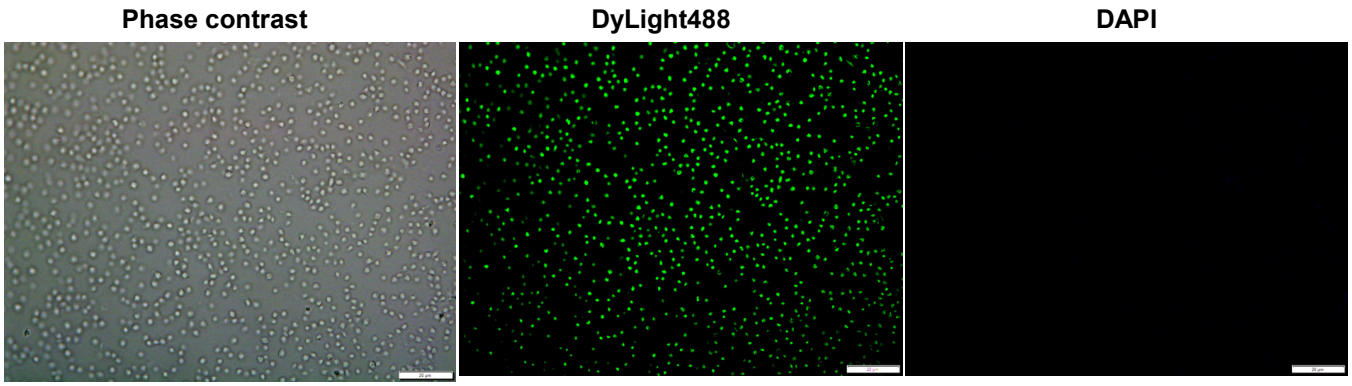

Figure S1. Bead-purified washed murine platelet preparations are free of leukocyte contamination: Washed mouse platelets (2.5 x10^8 /mL) were incubated with 35 µL of Ter-119 and CD45 microbeads for 20 min in the dark. RBC- and leukocyte-bound beads were separated on precalibrated MACS column as per manufacturer instructions. Bead-purified washed platelets were stained with a platelet specific antibody (GpIbβ labeled with Dylight488, Emfret Analytics) and nuclear stain (DAPI) for 15 min at RT. Samples were plated on poly-L-lysine coated slides and analyzed under fluorescence microscope at 100X magnification with oil emersion objectives. Platelet images were taken in different modes, as indicated in the figure. Manual cell counting in 25 different image fields showed no nucleated cells/10^5 platelets counted.

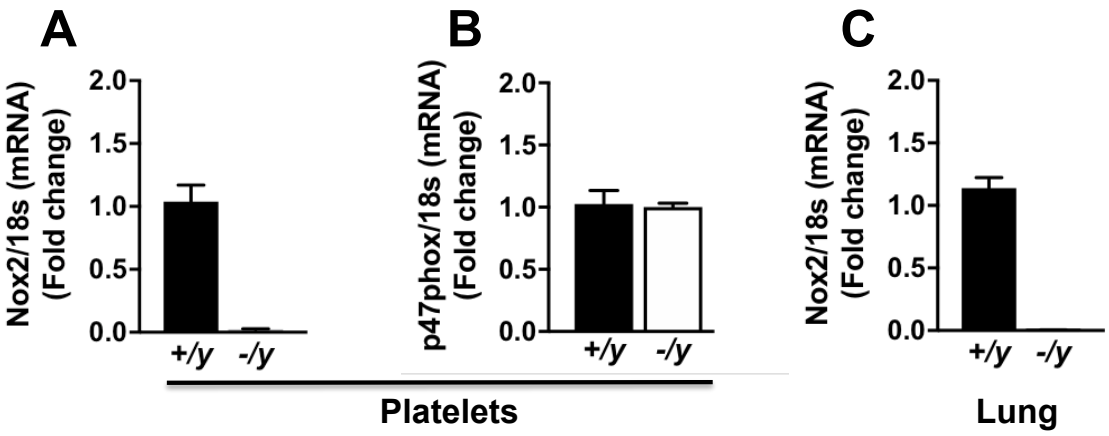

Figure S2. Expression of mRNA for NADPH oxidase subunits in murine platelets and lung tissue. Levels of **(A)** *Nox2* mRNA in platelets, **(B)** *p47phox* mRNA in platelets, and **(C)** *Nox2* mRNA in lung were measured by real-time qPCR in male mice. Values are normalized to *18s* rRNA and are expressed as fold change relative to the signal in wild type mice. Data are presented as mean ± SE and analyzed using t-test. n = 6 - 8 in each group.

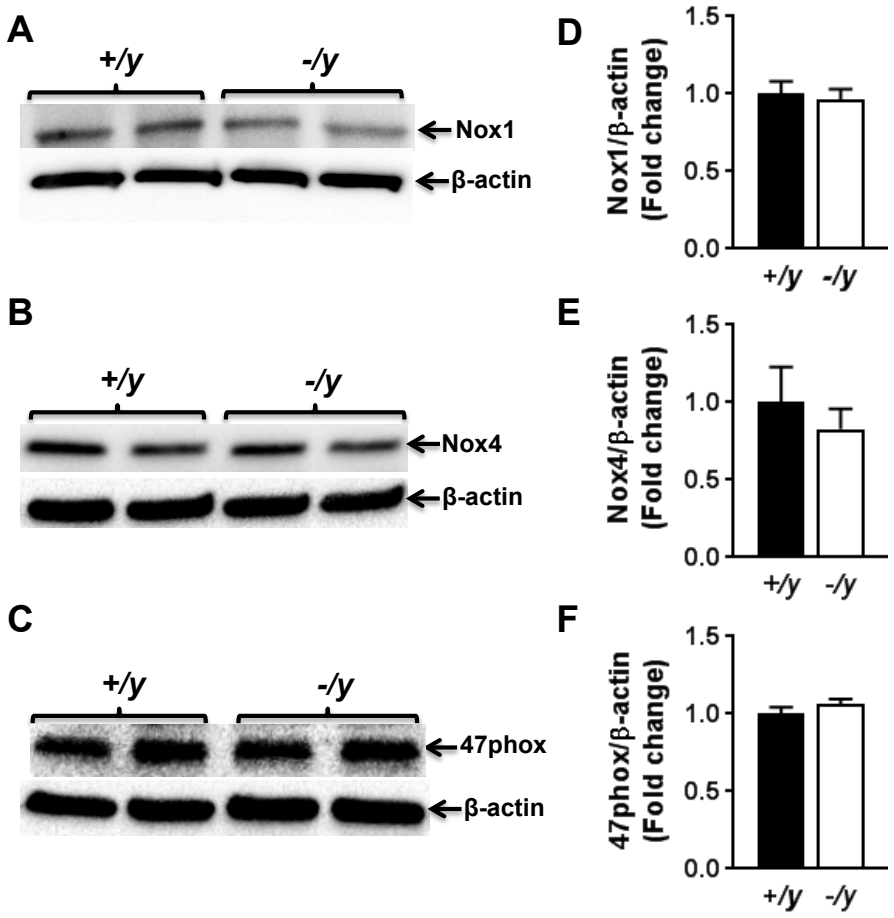

Figure S3. Proteins levels for NADPH oxidase subunits in murine platelets. Platelet lysates from male wild type *(+/y)* or Nox2-deficient *(-/y*) mice were separated on SDS-PAGE followed by western blotting to determine the levels of Nox1, Nox4, or 47phox subunits. **A-C:** Representative western blot images for protein expression for Nox1, Nox4 and p47phox subunits of NADPH oxidase and β-actin in platelets. D-**F:** Quantification of protein levels was performed by densitometry and values are presented as ratio to β-actin and analyzed using t-test. $n = 4$ in each group.

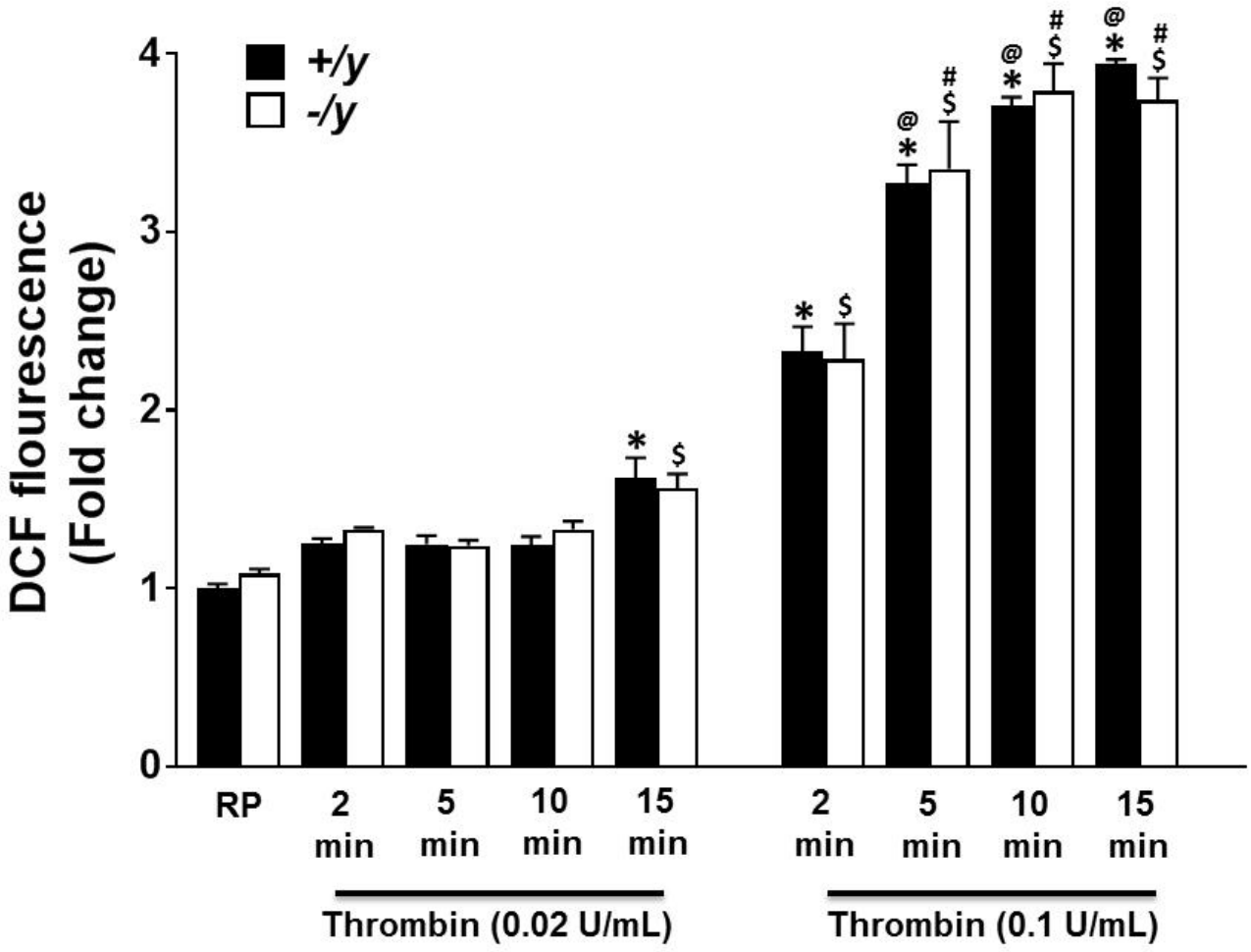

Figure S4. Time dependent platelet ROS generation in murine platelets. CM-H_2DCF-DA loaded platelets from male wild type *(+/y)* or Nox2-deficient *(-/y*) mice were either unactivated (resting platelets, RP) or activated with thrombin (0.02 U/mL or 0.1 U/mL) and at different time intervals (as indicated) samples were diluted with 1X DPBS (v/v) and analyzed immediately on flow cytometer. The fluorescent signal is presented as fold change over the signal observed with platelets from WT mice without thrombin. Data are presented as mean ± SE and analyzed using two-way ANOVA followed by Tukey test for multiple comparisons. n = 3 in each group. * P< 0.05 *vs*. *+/y* (RP); $^{\$}$ P< 0.05 *vs*. *-/y (*RP); $^{@}$ P< 0.05 *vs*. *+/y* (2 min, thrombin (0.01 U/mL); $^{\#}$ P< 0.05 *vs*. *-/y* (2 min, thrombin (0.01 U/mL).

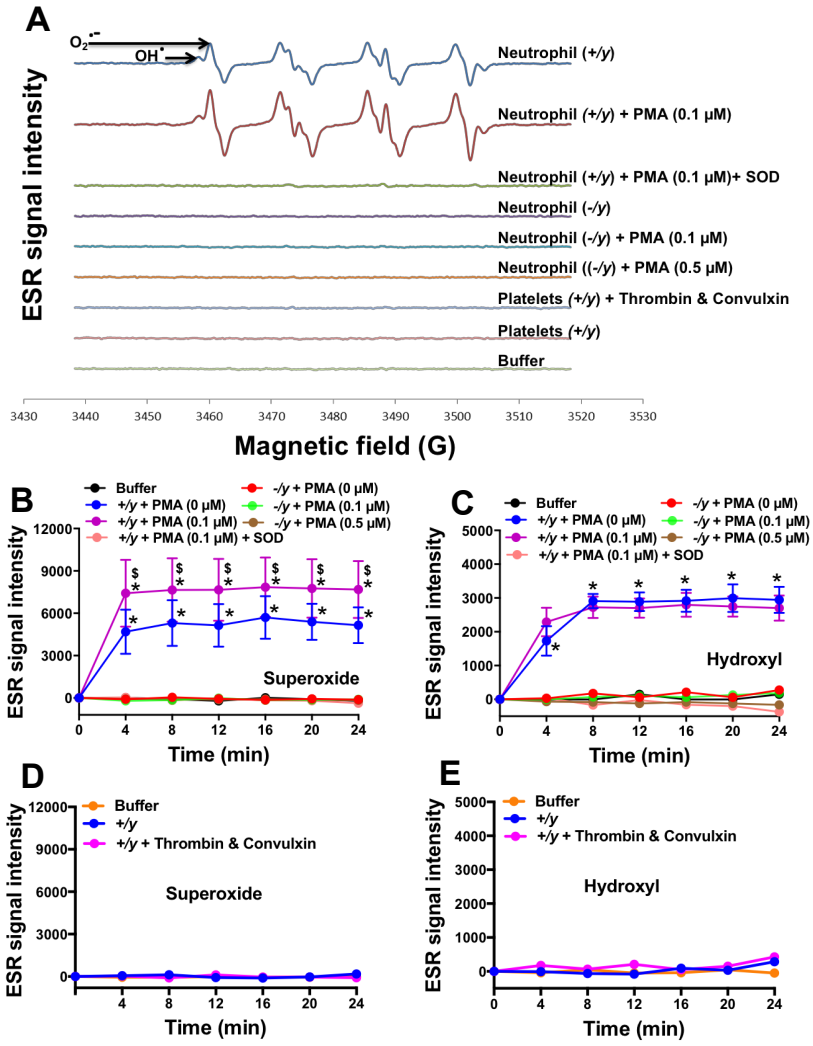

Figure S5. Extracellular generation of superoxide by neutrophils and platelets from wild type *(+/y)* or Nox2-deficient *(-/y)* mice. Neutrophils or bead purified platelets were activated with PMA or thrombin + convulxin (0.05 U/mL + 50 ng/mL), respectively, and ESR spectra for the DMPO-superoxide and DMPO-hydroxyl radical spin adducts were acquired. **(A)** Representative ESR spectra from neutrophils and platelets. (**B & C**) Quantification of ESR signal intensity for superoxide and hydroxyl radical spin adducts from neutrophils. (**D & E**) Quantification of ESR signal intensity for superoxide and hydroxyl radical spin adducts from platelets. $^{*}P < 0.01$ *vs*. *-/y* with PMA (0.5 µM), $^{\$}P < 0.01$ *vs*. *+/y* with PMA (0 µM). Data are presented as mean ± SE and analyzed using two-way ANOVA repeated measures with Tukey test for multiple comparisons. $n = 3$ in each group.

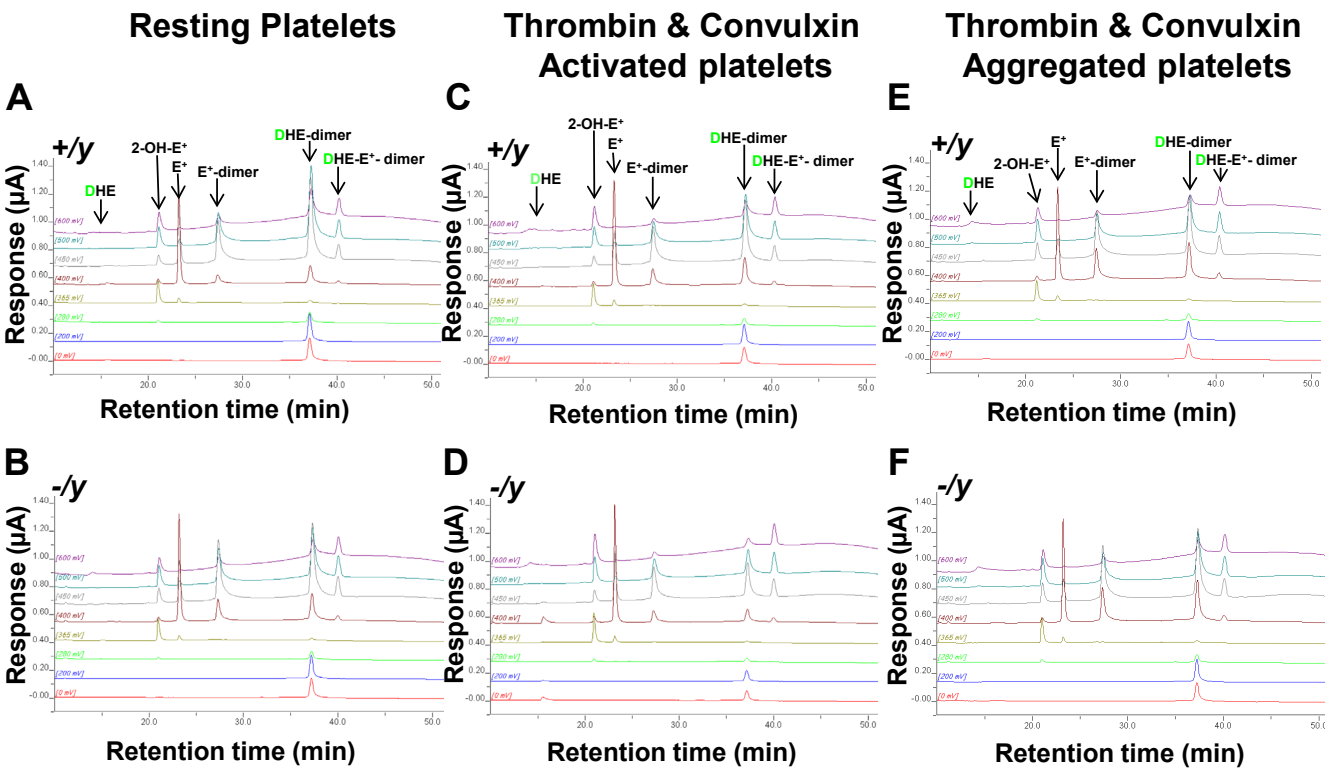

Figure S6. Representative HPLC chromatograms of DHE oxidation products from resting, activated, and aggregated murine platelets. Platelets from male wild type *(+/y)* or Nox2-deficient *(-/y*) mice were incubated with DHE, followed by stimulation with thrombin (0.05 U/mL) and convulxin (50 ng/mL). Representative chromatograms for resting (**A & B**); activated (**C & D**); and aggregated platelets (**E & F**). See data quantification in Table 1 and supplemental Table S1.

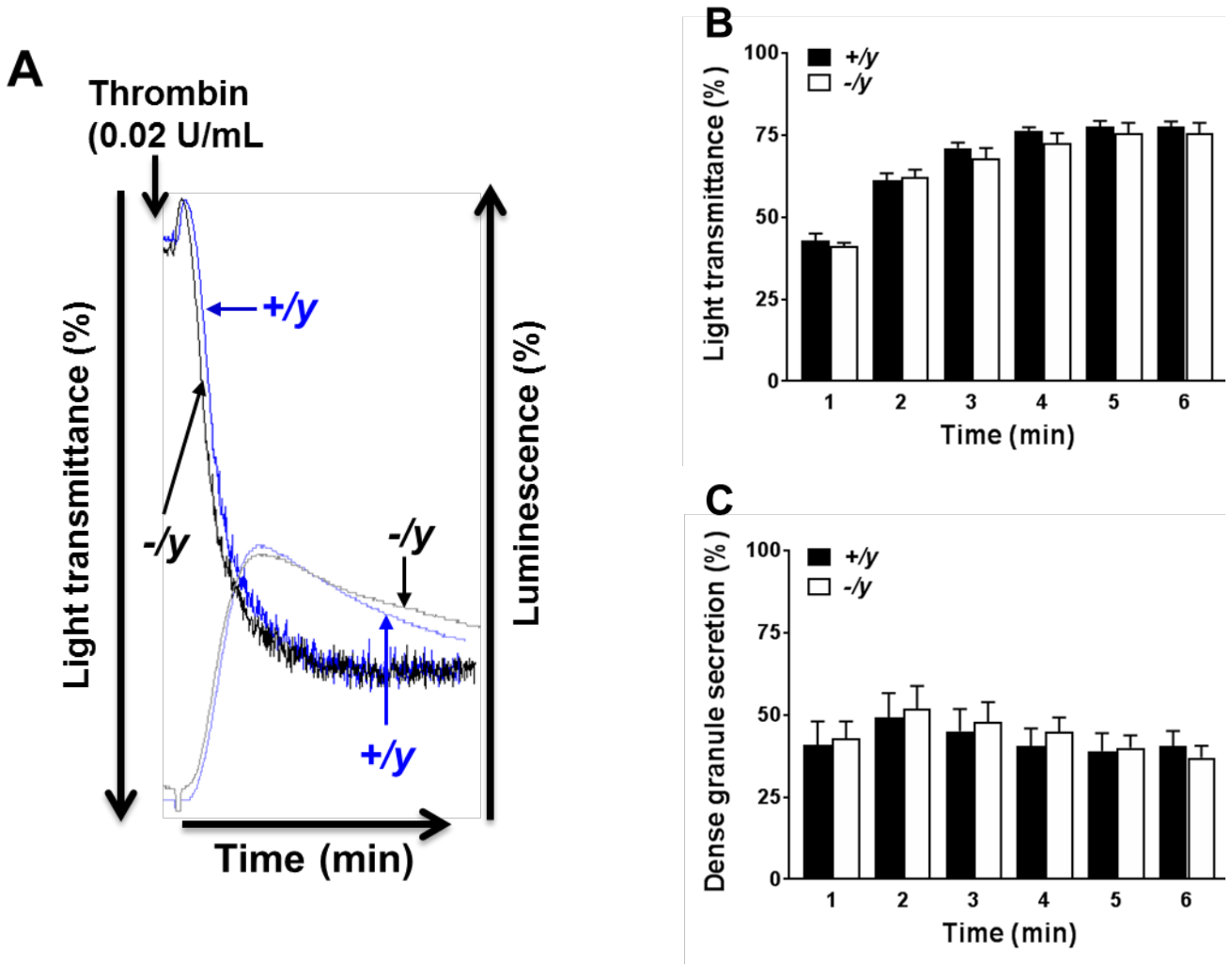

Figure S7. Secretion of ATP from dense granules was similar in both WT **and Nox2-deficient mice**. Washed platelets were incubated with chrono-lume reagent, then activated with thrombin (0.02 U/mL and monitored simultaneously for dense granule secretion and aggregation. **(A)** Representative tracings for secretion and aggregation from WT and Nox2-deficient platelets. The bar graphs show quantitative data for aggregation **(B)** and dense granule secretion **(C)** at different time points. Data are presented as mean ± SE and analyzed using multiple t-tests. n = 4 - 6 in each group.

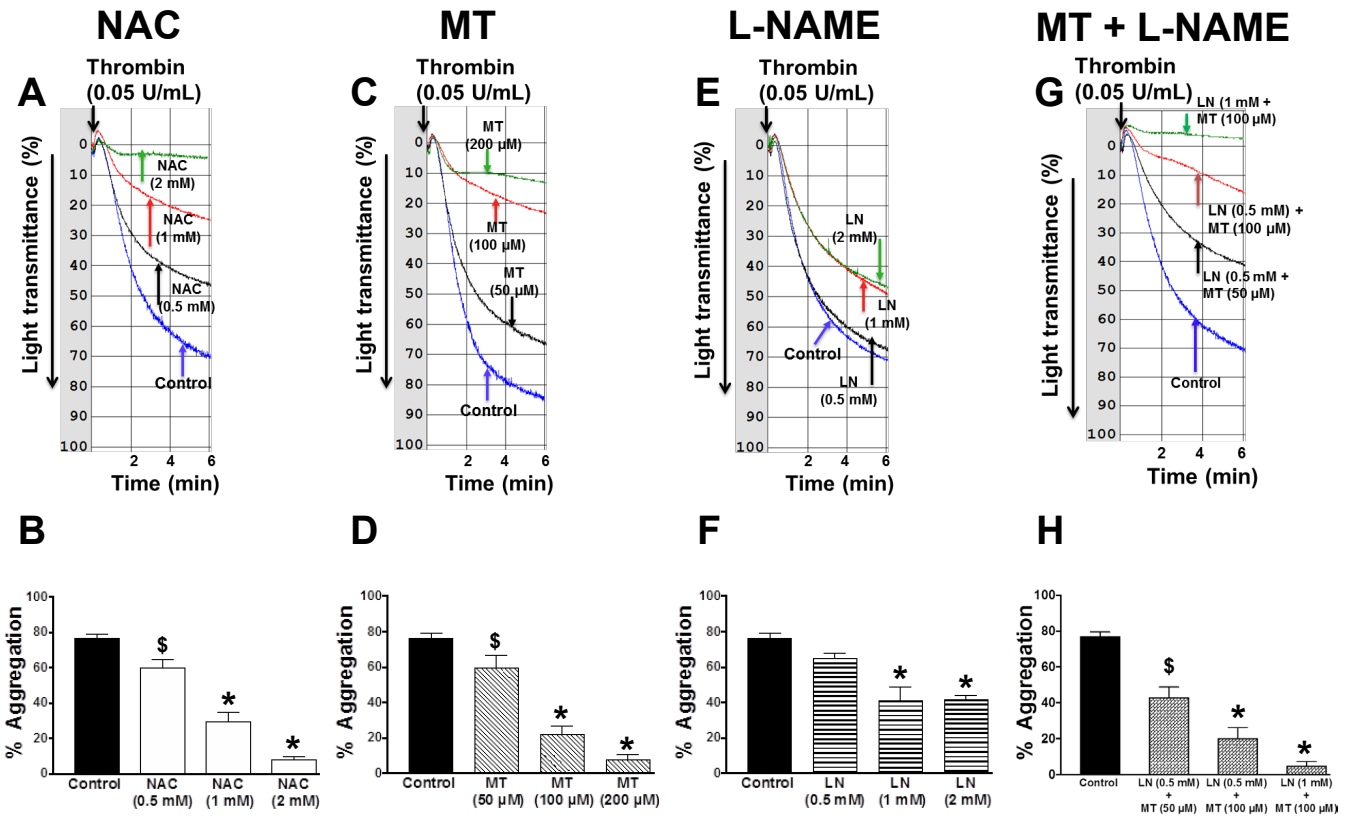

Figure S8. Platelet aggregation in healthy subjects is dependent on mitochondrial ROS and endothelial nitric oxide synthase. Washed platelets from healthy human subjects were untreated or pretreated with the indicated inhibitors (at indicated concentrations) for 15 min at 37 °C and aggregation was induced by thrombin (0.05 U/mL). Representative aggregation responses and percent aggregation are shown in absence (control) or presence of **(A & B)** N-acetylcysteine (NAC), **(C & D)** MitoTEMPO **(**MT**), (E & F)** L-NAME (LN), and **(G & H)** MT+LN. Data are presented as mean ± SE and analyzed using one way ANOVA with Tukey test for multiple comparisons. n = 4 - 6 in each group. * $P < 0.01$ *vs*. healthy control, $ $P < 0.05$ *vs*. healthy control.

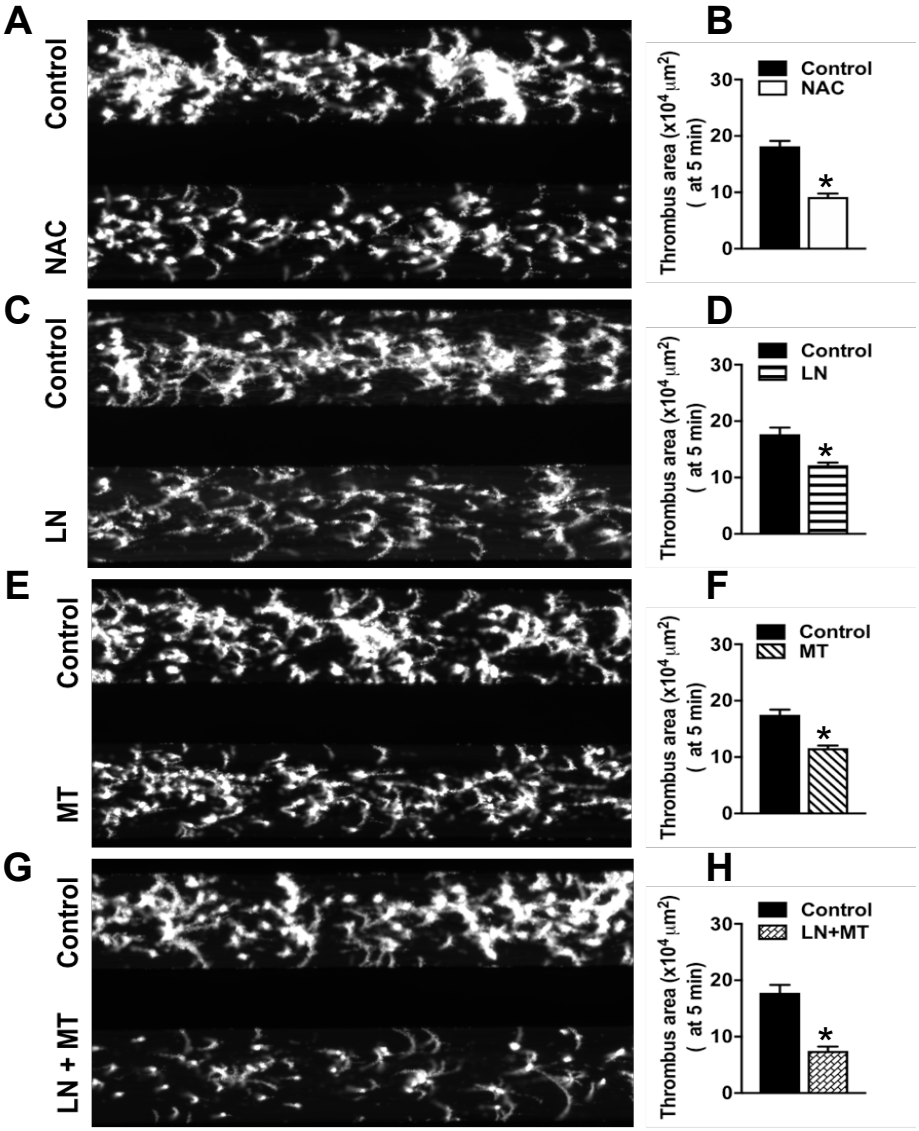

Figure S9. Platelet thrombi formation in healthy subjects is dependent on mitochondrial ROS and endothelial nitric oxide synthase. Washed platelets from healthy human subjects were studied for *ex vivo* thrombi formation. Accumulation of platelet thrombi over collagen was examined in a microfluidic flow chamber at a shear rate of 2000 s^{-1} in the presence or absence of various inhibitors at indicted concentrations. Representative images of platelet accumulation and quantification of total thrombus area at 5 min is presented under control condition or in presence of inhibitors such as **(A & B)** N-acetylcysteine (NAC), **(C & D)** L-NAME (LN), **(E & F)** MitoTEMPO (MT) or **(G & H)** LN+MT. Data are presented as mean ± SE and analyzed using t-test. * $P < 0.01$ *vs*. healthy control, $ $P < 0.05$ *vs*. healthy control. $n = 4$ in each group.

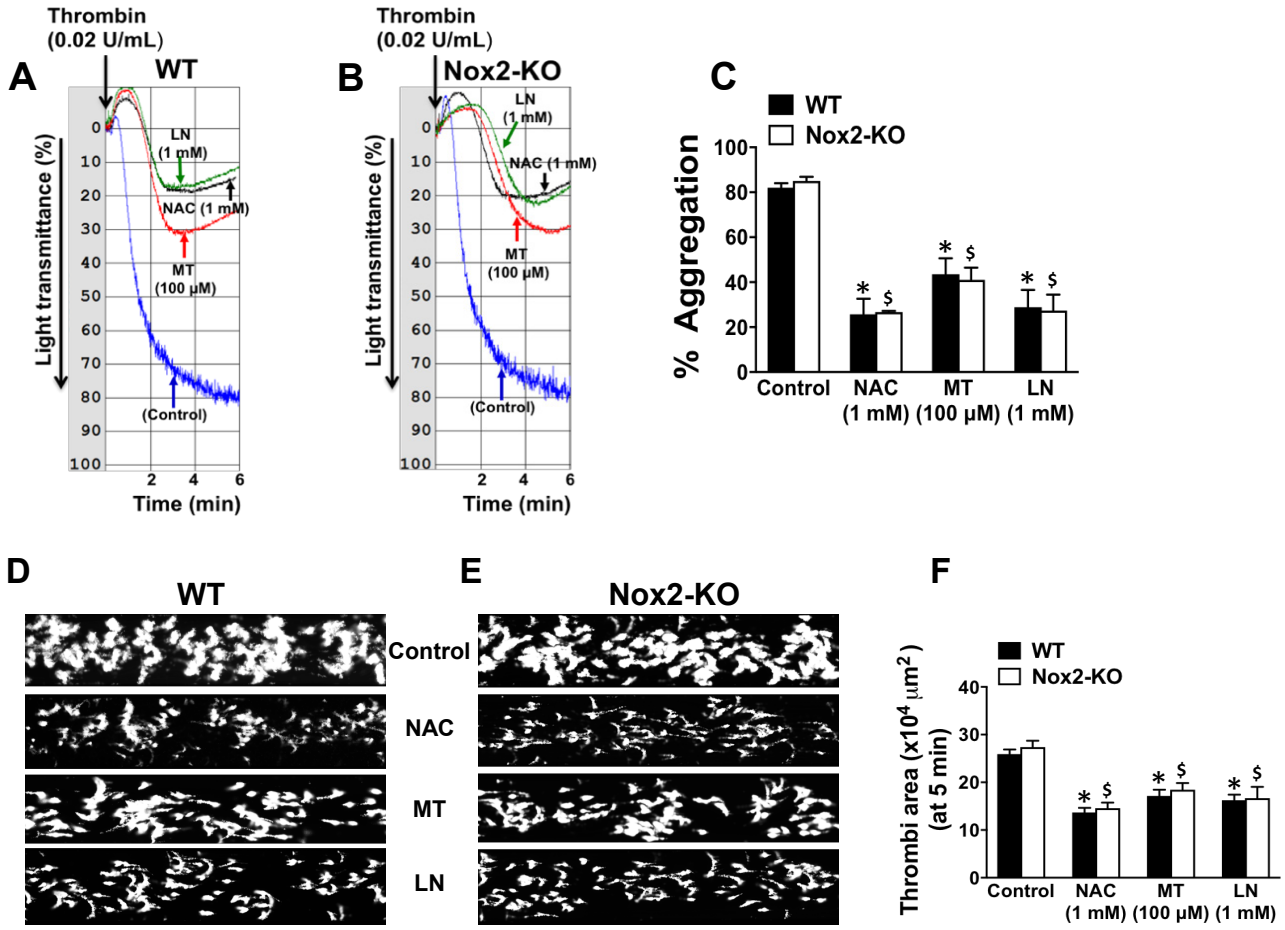

Figure S10. Aggregation and thrombi formation responses in murine WT or Nox2-deficient platelets are equally modulated by inhibitors of mitochondrial ROS and endothelial nitric oxide synthase. (A-C) Washed platelets from either wild type (WT) or Nox2-difficient (Nox2-KO) mice were either untreated (control) or pretreated with indicated inhibitors for 15 min at 37 °C and aggregation was induced by thrombin (0.02 U/mL). Representative aggregation responses and percent aggregation are shown for WT **(A & C)** and Nox2-deficient (**B & C**) platelets in control condition or in presence of N-acetylcysteine (NAC, 1 mM), MitoTEMPO **(**MT, 100 µM**),** or L-NAME (LN, 1 mM). **(D-F)** Accumulation of platelet thrombi over collagen was examined in a microfluidic flow chamber at a shear rate of 2000 s^{-1} in the presence or absence of N-acetylcysteine (NAC, 1 mM), MitoTEMPO (MT, 100 µM) or L-NAME (LN, 1 mM) for WT **(D)** or Nox2-KO **(E)** platelets. Quantification of total thrombus area was performed at 5 min. **(F)** Data are presented (from both male and female) as mean ± SE and analyzed using two-way ANOVA with Tukey test for multiple comparisons. * $P < 0.05$ *vs*. WT (control), $ $P < 0.05$ *vs*. Nox2-KO (control). $n = 3$ - 4 in each group.