SUPPLEMENTAL MATERIAL

Detailed Methods

Detection of intracellular ROS.

Intracellular ROS was measured using methods adapted from previously described methods.[1] Washed platelet suspensions ($1x10^8$/mL) were incubated with 10 µM of the global intracellular ROS indicator 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (Carboxy-H_2DCFDA, Invitrogen) for 15 minutes in the dark. After incubation in the absence of light, an Accuri C6 flow cytometer was used to measure DCF fluorescence over time. A gate was placed on single platelets on SSC-A versus FSC-A plots and the DCF fluorescence was acquired from single platelets at different time points to obtain kinetic changes in intracellular ROS. The baseline was acquired first, agonist was then added to the suspension, and more time points were acquired. There was no difference in baseline fluorescence between different genotypes and controls (Figure IA). In order to quantify ROS production, the Mean Fluorescence Intensity (MFI) of the DCF fluorescence of single platelets was determined at different time points, and the baseline MFI was subtracted from the MFI at each time point. The increase in DCF fluorescence was then plotted (Figure IB) as a function of time to obtain a kinetic of ROS production. Total ROS production was then determined by integrating the area under the curve (AUC) (Figure IC), where the AUC is representative of the total ROS produced with a given dose of agonist.

Supplemental Figures

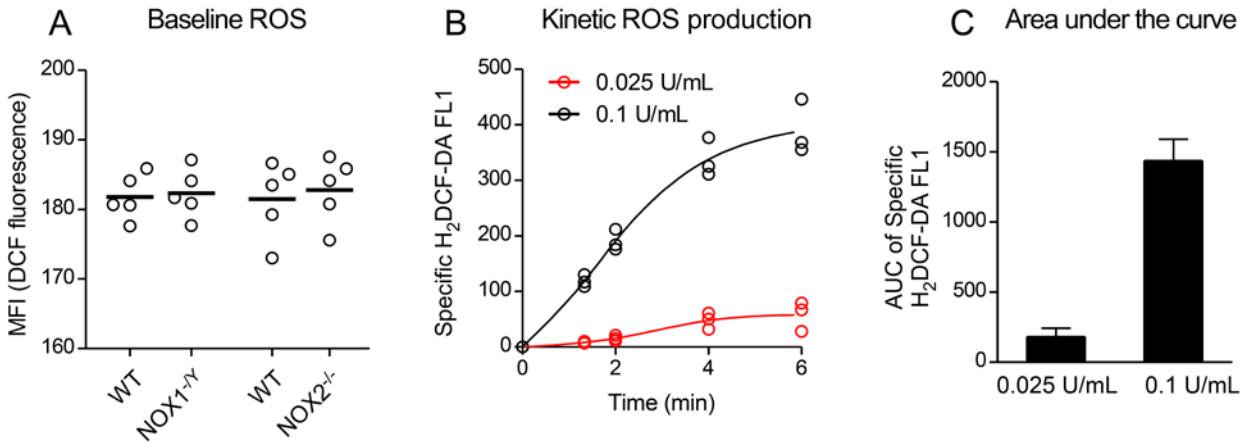

Figure I. Detection of ROS in platelets using flow cytometry. (A) The baseline intracellular ROS levels of washed mouse platelets determined using flow cytometry. The Mean Fluorescence Intensity (MFI) of the DCF fluorescence (Carboxy-H_2DCF-DA) was measured and the mean of the MFIs (line) plotted. WT versus NOX1$^{-/Y}$ and WT versus NOX2$^{-/-}$ are shown and no difference was observed. (B) Graph showing the kinetic increase in DCF fluorescence over time in thrombin-stimulated mouse platelets (0.025 and 0.1 U/mL) determined using a flow cytometer. The MFI of DCF fluorescence was measured and the baseline subtracted to provide the specific DCF fluorescence, which is plotted over time. (C) The area under the curve was calculated from the kinetics of ROS production in order to determine the total ROS produced.

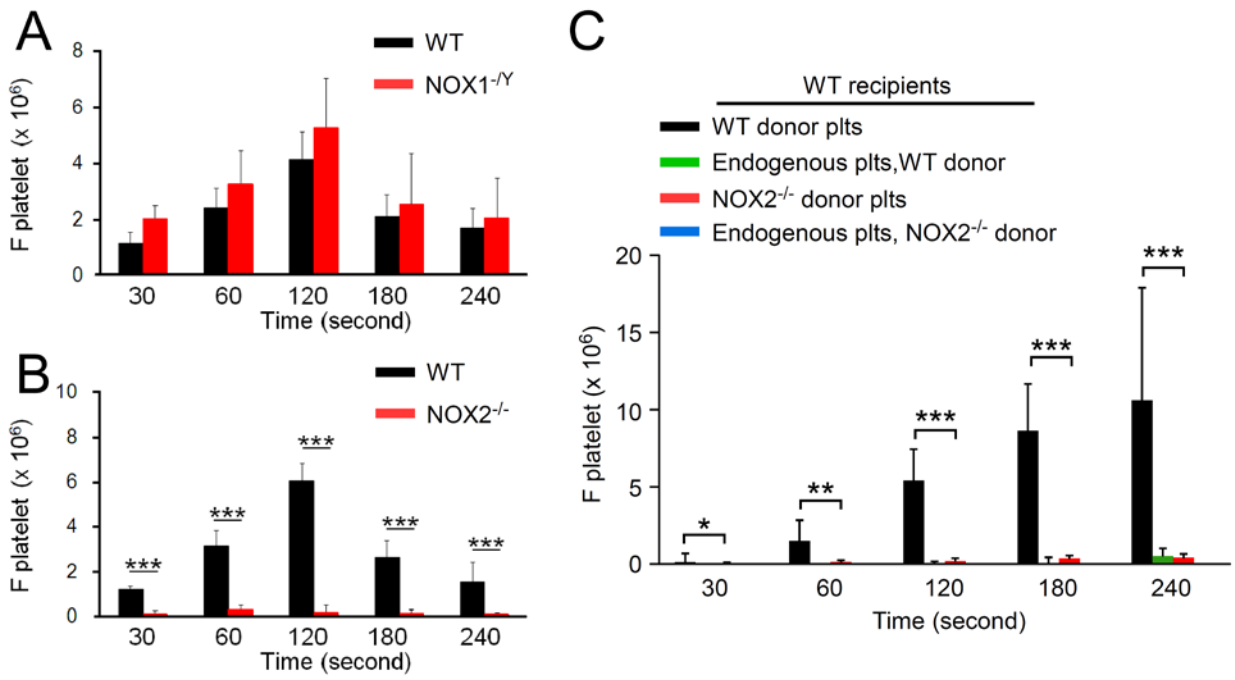

Figure II. The effects of NOX2 or NOX1 deficiency on *in vivo* thrombosis and hemostasis. Laser-induced arterial thrombosis and intravital microscopy was performed as described in Methods. (A-B) The median integrated fluorescence intensities of anti-CD42c antibodies (F platelet) were obtained from 28-30 thrombi in 3 WT versus 3 NOX1$^{-/Y}$ mice (A) and 3 WT versus 3 NOX2$^{-/-}$ mice (B). The fluorescence intensities are shown at 30, 60, 120, 180, and 240 seconds after vascular injury. *** represents P<0.001 versus WT control mice after Student's *t*-test. (C) The median integrated fluorescence intensities of Dylight 649-conjugated anti-CD42c antibodies (endogenous WT platelets) and calcein-AM labeled WT or NOX2$^{-/-}$ platelets (donor platelets) injected into WT thrombocytopenic mice were obtained from 17-24 thrombi in 5 mice per group and are presented as a function of time. The fluorescence intensities are shown at 30, 60, 120, 180, and 240 seconds after vascular injury. ***, ** and * represents P<0.001, P<0.01 and P<0.05, respectively as determined by Student's *t*-test comparing WT donor platelets to NOX2$^{-/-}$ donor platelets in WT recipients.

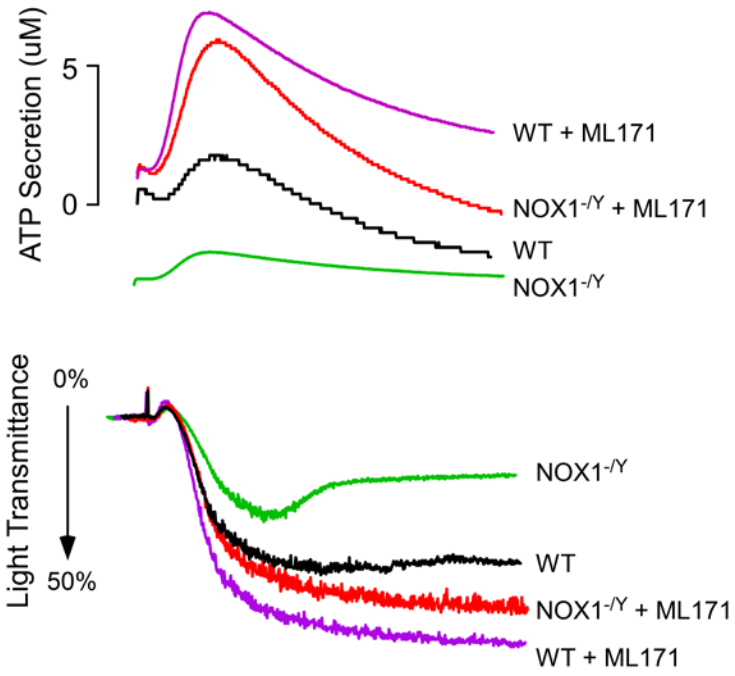

Figure III. The effects of ML171 on mouse platelet aggregation and secretion. Washed WT and NOX1$^{-/Y}$ platelets (3 x 10^8/mL) were incubated with vehicle control or 4 μM of the NOX1 inhibitor ML171. Platelets were stimulated with 0.01 U/mL thrombin and platelet aggregation and secretion was monitored in a platelet lumi-aggregometer.

Supplemental References

1. Arthur JF, Qiao J, Shen Y, Davis AK, Dunne E, Berndt MC, Gardiner EE, Andrews RK. Itam receptor-mediated generation of reactive oxygen species in human platelets occurs via syk-dependent and syk-independent pathways. *J Thromb Haemost*. 2012;10:1133-1141