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Superoxide Dismutase 2 is dispensable for platelet function

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Summary

Increased intracellular reactive oxygen species (ROS) promote platelet activation. The sources of platelet-derived ROS are diverse and whether or not mitochondrial derived ROS, modulates platelet function is incompletely understood. Studies of platelets from patients with sickle cell disease, and diabetes suggest a correlation between mitochondrial ROS and platelet dysfunction. Therefore, we generated mice with a platelet specific knockout of superoxide dismutase 2 (SOD2-KO) to determine if increased mitochondrial ROS increases platelet activation. SOD2-KO platelets demonstrated decreased SOD2 activity and increased mitochondrial ROS, however total platelet ROS was unchanged. Mitochondrial function and content were maintained in non-stimulated platelets. However SOD2-KO platelets demonstrated decreased mitochondrial function following thrombin stimulation. *In vitro* platelet activation and spreading was normal and *in vivo*, deletion of SOD2 did not change tail-bleeding or arterial thrombosis indices. In pathophysiological models mediated by platelet-dependent immune mechanisms such as sepsis and autoimmune inflammatory arthritis, SOD2-KO mice were phenotypically identical to wild-type controls. These data demonstrate that increased mitochondrial ROS does not result in platelet dysfunction.

Keywords

Thrombosis; ROS; platelet physiology; mitochondria

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Author contributions

TPF, performed experiments, analyzed data, and wrote the manuscript. JR, CA, RS, KD, and LB participated in *in vitro* and *in vivo* experiments. EB, ASW, and EDA conceived and supervised the project and wrote the manuscript.

Conflicts of interest

None declared.

Introduction

Incubations of platelets with exogenous superoxide anion induce platelet activation and following thrombin stimulation facilitate a synergistic increase in activation (1–3). Upon activation, platelets generate large quantities of superoxide anions, and hydrogen peroxide. *In vitro* administration of antioxidants completely abolishes agonist-mediated platelet activation. Thus, reactive oxygen species (ROS) are an essential component of intracellular signal transduction pathways that facilitate platelet activation (4–7). In addition, when intracellular ROS are increased, platelets demonstrate a decreased threshold for agonist-mediated activation (8, 9).

Multiple enzymes facilitate agonist-mediated platelet ROS production and clearance (5, 7, 8). In particular, NADPH oxidase 1 and 2 (NOX1 and NOX2) and cyclooxygenase (COX) significantly contribute to agonist-mediated ROS production (5, 7). Conversely, catalase, glutathione peroxidase (Gpx), and total superoxide dismutase (SOD) have been implicated in ROS clearance and changes in their activity results in altered platelet function. These regulatory mechanisms largely govern the levels of cytosolic ROS and have all been implicated in various disease states.

The contribution of mitochondrial derived ROS to platelet function is unknown. Platelet activation increases mitochondrial respiration two-fold (10), and administration of the mitochondrial uncoupler cyanide m-chlorophenylhydrazine (CCCP) and the complex II inhibitor thenoyltrifluoroacetone (TTFA) decreased ROS and platelet activation (9), but these inhibitors also impair mitochondrial ATP production. Although this relationship may reflect differences in mitochondrial ATP generation, a contribution of respiration-derived mitochondrial superoxide cannot be excluded (11). Platelets express two SOD enzymes SOD1, which is cytosolic and SOD2 that is localized in the mitochondrial matrix. This spatial organization results in SOD2 specifically converting mitochondrial derived superoxide anions to hydrogen peroxide and water (11). SOD2 is essential for life, as whole-body knockout is neonatally lethal (12). In addition deletion of SOD2 specifically in cardiomyocytes leads to oxidative stress, mitochondrial dysfunction and increased apoptosis (13).

Increased mitochondrial ROS correlates with increased platelet activation in patients with sickle cell disease (14) and type II diabetes mellitus (15). Platelet protein lysates from patients with type II diabetes mellitus displayed increased mitochondrial antioxidant proteins SOD2 and thioredoxin-dependent peroxide reductase 3 content (16). Platelet mitochondrial ROS is elevated in hypothermia-mediated platelet apoptosis (17). In sepsis, platelet mitochondrial respiration and membrane potential correlates with survival outcomes (18). The multifactorial pathophysiology renders it challenging to discern the extent to which mitochondrial ROS contribute to the platelet dysfunction.

To modulate mitochondrial ROS, we generated mice that lack SOD2 specifically in platelets, to determine if mitochondrial ROS contributes to platelet activation. Here we demonstrate that increased mitochondrial ROS does not modulate platelet activation *in vitro*. Moreover,

in vivo deletion of SOD2 does not alter thrombosis, hemostasis, or outcomes in platelet-dependent immune disorders.

Materials and methods

Animals

All animals were generated on the C57Bl6J background and housed under normal light and temperature conditions. Pf4 Cre transgenic mice were obtained from Jackson laboratories (Bar Harbor, ME). SOD2 floxed mice were genotyped as previously described (19). SOD2-KO mice were generated by crossing mice harboring homozygous SOD2 floxed alleles to mice expressing a Pf4 driven cre recombinase. All experiments were conducted on mice between the ages of 7–15 weeks. All animal studies were approved by the institutional animal care and use committees at the University of Utah and University of Iowa.

Platelet isolations

Blood was isolated from isoflurane-anesthetized mice into 1:20 acid-citrate-dextrose solution. Samples were then diluted with pipes-saline-glucose (PSG) buffer and centrifuged at $120g \times 10$ minutes. Platelet rich plasma was then diluted with PSG and prostaglandin E1 (PGE_1) (10nM) and centrifuged at $378g \times 10$ minutes, and repeated for a total of two washes. Platelets were resuspended in DMEM with 5mM glucose, or the indicated assay buffer and allowed to rest for 30 minutes prior to functional studies. Washed platelet counts were determined using Cellometer Auto M10 (Nexcelom Bioscience, Lawrence, MA). Complete blood counts were determined via ADVIA 120 (Siemens, Germany).

Immunoblot analysis

Platelets were lysed in RIPA buffer with protease and phosphatase inhibitors and protein quantification was determined by bicinchoninic acid (BCA) analysis. Anti-SOD2 antibody (ADI-SOD-111-D) was purchased from Enzo Biochem Inc. (Farmingdale, NY), Anti-Tubulin (T6199) was purchased from (Sigma Aldrich, St. Louis MO). Cytochrome C (11940) was purchased from (Cell signaling technology, Danvers MA). Densitometry was analyzed using ImageJ.

SOD2 activity assay

Superoxide dismutase activity was determined using an assay kit purchased from Cayman Chemical (Ann Arbor, MI) and normalized to protein content. Briefly, platelet lysates were incubated in the presence of xanthine oxidase and tetrazolium salt. SOD activity was determined as inhibition of superoxide anion production over 30 minutes. SOD1 activity was inhibited by addition of 3mM potassium cyanide, to specifically determine SOD2 activity. SOD1 activity was determined as total SOD activity minus SOD2 activity.

ROS evaluation

Washed platelets in DMEM with 5mM glucose, 1mM glutamate, and 1mM pyruvate were incubated with 5 μ M MitoSOX or 5 μ M DCFDA for 30 minutes in the presence or absence of the indicated agonist, then diluted 1:20 with PBS and immediately analyzed using flow

cytometry (LSR II, Beckman Dickson, San Jose, CA). For N-Acetyl-Cysteine (NAC) studies, platelets were pre-incubated for 30 minutes with 1mM NAC prior to staining. GSH and GSSG were determined using the GSH/GSSG-Glo Assay (V6611) (Promega, Madison WI), normalizing to total platelet number.

Catalase activity

Washed platelets were incubated in the presence of 20mM 3-amino-1,2,4-triazol. Platelets were sampled every five minutes for 20 minutes, washed three times with HBSS and flash frozen in liquid nitrogen. Samples were then suspended in HBSS, sonicated 30 seconds, and placed in a spectrophotometer. H_2O_2 absorbance was then monitored at 240nm. Following baseline measurements, 30mM H_2O_2 was added to cuvettes and the disappearance of H_2O_2 was monitored every 10 seconds for 180 seconds. Rates of disappearance were recorded and steady state H_2O_2 concentrations were then determined (20, 21).

8-Isoprostane ELISA

4×10^8 platelets/mL were incubated in 500 μL DMEM, and then treated in the presence or absence of 500 μM arachidonic acid for 10 minutes and reactions were stopped by addition of ethanol. Samples were purified with a C-18 solid phase extraction cartridge, followed by ELISA analysis for 8-isoprostane (516319), Cayman Chemical (Ann Arbor, MI).

Seahorse flux analysis

Washed platelets were further purified by incubation with Ter119 and CD45 microbeads (Miltenyi Biotec, Auburn CA) to remove red blood cells and leukocytes respectively. Platelets were then negative depleted and resuspended in 25mM DMEM with 1mM pyruvate, and 2mM glutamate. Platelets were seeded at 1×10^8 platelets/well into Seahorse XF24 analyzer plates (Agilent Technologies, Santa Clara, CA). Analysis was conducted as previously described (22). Platelets were treated with 0.5U/mL thrombin (final concentration), 10 minutes prior to each run where indicated. Data were normalized to platelet counts.

Mitochondrial membrane potential

Washed platelets suspended in DMEM with 5mM glucose, 1mM pyruvate, and 2mM glutamate were incubated with 100nM tetramethylrhodamine methyl ester (TMRM) for 15 minutes at room temperature. Samples were then diluted with 1:20 PBS and analyzed using flow cytometry. 1 μM CCCP was then added to diluted platelets for 10 minutes and samples were analyzed once more using flow cytometry, to determine non-mitochondrial staining. TMRM values reported were determined as initial-CCCP Geo. MFI values.

Transmission electron microscopy

Washed platelets were fixed with 4 % glutaraldehyde and processed for transmission electron microscopy as previously described (23). Organelle density was determined by analysis of 20 platelets/mouse. Investigators were blinded to genotype.

Platelet activation

Washed platelets were incubated in the presence of the indicated agonist, JONA-PE (M130–1, Emfret, Germany), CD62p-FITC (M023–3, Emfret, Germany), or CD41-APC (17–0411–82, eBioscience, San Diego, CA) for 10 minutes at 37°C. Reactions were stopped by addition of FACs lysis buffer and analyzed using flow cytometry.

Aggregation

Washed platelets were suspended at 4×10^8 platelets/mL in HEPES Tyrode's buffer, with 1mM CaCl_2 , and 0.02U/mL Apyrase (Sigma Aldrich, St. Louis, MO) and allowed to rest for 30 minutes. Platelets were then analyzed using a CHRONO-LOG Model 700 Optical lumi-aggregometer (Havertown, PA). Platelets were stimulated with thrombin 0.02U/mL, Collagen type 1 2 μ g/mL, or 4 μ M ADP, using agonist volumes less than 2 μ L. ATP release was determined using chrono-lume reagent (CHRONO-PAR, Havertown, PA).

Annexin V

Washed platelets were incubated in DMEM with 5mM glucose, 1mM pyruvate, and 2mM glutamate for 30 minutes. Platelets were then stained with annexin V-APC, and 100nM TMRM in the presence or absence of 1U/mL thrombin (Sigma Aldrich, St. Louis, MO) plus 700ng/mL convulxin (Santa Cruz, Dallas TX) or 1 μ M Ionomycin (Sigma Aldrich, St. Louis, MO) for 15 minutes. Samples were then diluted 1:20 in PBS and immediately analyzed using flow cytometry.

Spreading

Washed platelets in DMEM were incubated on fibrinogen (50–230–4925, Fisher Scientific, Waltham, MA) coated chamber slides for 1 hour under static conditions. Spreading was then stopped by the addition of 2 % paraformaldehyde. Slides were then washed, permeabilized and stained with WGA-Alexa Fluor 555 (W32464, ThermoFisher, Waltham, MA), and phalloidin-Alexa Fluor 488 (A12379, ThermoFisher), and imaged.

In vivo thrombosis

Isoflurane-anesthetized mice were assessed for tail bleeding. Tails were excised 3mm from the tip and submerged in 37° C saline. Bleeding cessation was determined as the time when bleeding stopped for 10 seconds.

FeCl_3 -mediated arterial thrombosis: 0.5mm $\times$ 0.5mm whatman paper was saturated with 10 % FeCl_3 , and applied to exposed right carotid arteries for 3 minutes. Time to occlusion was then monitored using a VisualSonics 2100 ultrasound (FUJIFILM Visual-Sonics, Inc., Toronto, Canada).

CLP model of sepsis

Ketamine/Xylazine (100 mg/kg and 10mg/kg, respectively) anaesthetized mice were subjected to cecal ligation and puncture (CLP) as previously described (24). Mice received subcutaneous sterile isotonic saline (1 mL) for fluid resuscitation immediately after the surgery. Mice were allowed free access to standard chow and water after CLP surgery.

Sham-operated mice were subjected to identical procedures except that ligation and puncture of the cecum were omitted. Survival of mice subjected to CLP or sham injury were followed for 7 days after the surgical procedure to determine survival.

Autoimmune inflammatory arthritis

K/BxN mice were generated by breeding KRN mice with non-obese diabetic mice (NOD) mice, and the arthrogenic serum from K/BxN mice was obtained by terminal bleeding as described (25). To induce arthritis, recipient mice were injected i.p. with 150 μ l K/BxN serum at day 0 and day 2. Ankle thickness, measured at the malleoli with the ankle in a fully flexed position using a spring-loaded dial caliper (DGI Supply, Montreal, Canada), and clinical index were monitored daily (25, 26). Investigators were blinded to genotype for data collection and analysis. All these experiments were approved by the CHU de Quebec-Universite Laval animal welfare committee.

Statistics

Data are represented as mean $\pm$ standard error of the mean. Statistical analysis was conducted using GraphPad 6 and/or Microsoft office Excel 2011. T-test, and ANOVA analysis were utilized when appropriate. Multiple comparisons were evaluated using a Tukey's analysis. Survival studies utilized a log rank (Mantel-Cox test) analysis. $P < 0.05$ were considered significant.

Results

Deletion of SOD2 increases mitochondrial ROS

To investigate the contribution of SOD2 to platelet function, we generated mice lacking SOD2 specifically in platelets by crossing mice harboring SOD2 floxed alleles to mice expressing a PF4 driven Cre recombinase. Immunoblot analysis of lysates from SOD2 knockout (SOD2-KO) platelets demonstrated decreased SOD2 protein content compared to littermate controls (Figure 1A). Furthermore SOD2-KO platelets displayed significantly reduced SOD2 activity (Figure 1B). No differences were observed in SOD1 activity (Figure 1C). SOD2 is localized to mitochondria; therefore we monitored mitochondrial ROS using mitoSOX. SOD2-KO platelets demonstrated a ~20 % increased mitoSOX geometric mean fluorescence (Geo. MFI) (Figure 1D), consistent with increased mitochondrial superoxide.

SOD2-mediated dismutation of superoxide anions generates H_2O_2 which is degraded in mitochondria by glutathione peroxidase, whereas in the cytosol, catalase mediates the catalytic degradation of H_2O_2 . Therefore, to determine if deletion of SOD2 results reduced total levels of cellular H_2O_2 , we monitored steady state H_2O_2 concentrations using an assay based upon 3-amino-1,2,4-triazol-mediated inhibition of catalase (20, 21). Under basal conditions total platelet H_2O_2 was unchanged in SOD2-KO platelets (Figure 1E). In addition, ratios of oxidized to reduced glutathione were unchanged (Figure 1F). To further determine if SOD2 deletion altered total cellular ROS we evaluated DCFDA staining in basal, N-Acetyl Cysteine (NAC), and agonist stimulated platelets. Under all conditions, SOD2-KO platelets demonstrated similar DCFDA levels relative to controls (Figure 1G). 8-Isoprostane is produced by non-enzymatic oxidation of free-fatty acids, largely via NOX-

derived (cytosolic) ROS, and is an indicator of oxidative stress. Under baseline conditions no differences in 8-isoprostane production was observed (Figure 1H). 500 μ M arachidonic acid administration increased 8-isoprostane formations in platelets between 40–120 fold although values between SOD2-KO and control platelets were unchanged (Figure 1H). Thus despite increased mitochondrial ROS, total cellular ROS was unchanged in SOD2-KO platelets under basal and stimulated conditions.

Mitochondrial function and structure in SOD2 deficient platelets

SOD2 activity modulates mitochondrial function in multiple cell types. We therefore monitored platelet mitochondrial oxygen consumption rates (OCR) using the Seahorse XF24 flux analyzer. Under non-stimulated conditions, SOD2-KO and control platelets demonstrated equivalent basal, and maximal OCR (Figure 2A). In human platelets, thrombin stimulation increases mitochondrial respiration (27). Consistent with these observations, thrombin increased mitochondrial respiration in control platelets. However, no increase was observed in SOD2-KO platelets (Figure 2A). Mitochondrial membrane potential was unchanged under basal conditions in SOD2-KO platelets (Figure 2B). Quantification of electron micrographs revealed equivalent numbers of mitochondria, α -granules, and δ -granules in SOD2-KO platelets relative to littermate controls (Figure 2C–D). Levels of the mitochondrial protein cytochrome C were equivalent in SOD2-KO and controls (Figure 2E–F).

In vitro function of SOD2-KO platelets is normal

ROS plays an essential role in platelet activation, thus *in vitro* platelet activation was determined. There were no differences in baseline GPIIb/IIIa activation marked by JonA Geo. MFI. and CD62p surface translocation marked by CD62p Geo. MFI in washed platelets from SOD2-KO mice (Figure 3A–B). There were no differences in GPIIb/IIIa activation or CD62p surface translocation between SOD2-KO and control platelets following stimulation with thrombin, Ionomycin, the thromboxane A₂ analog U46619, PAR4 peptide or the GPVI agonist convulxin (Figure 3A–B). Washed platelet aggregation analysis indicated SOD2-KO displayed normal aggregation in response to thrombin, collagen, and ADP (Figure 3C–E). In addition, dense granule release monitored by ATP release analyzed in parallel with aggregation studies indicated that SOD2-KO platelets degranulate to the same extent as controls (Figure 3F–H). The ability of SOD2-KO platelets to spread on a fibrinogen matrix under static conditions were also unchanged relative to controls (Figure 3I). Stimulation of platelets with potent agonists induces mitochondrial depolarization leading to exposure of phosphatidylserine (PS) on the outer leaflet of the plasma membrane. This PS exposure can be monitored via annexin v binding. Relative to controls, annexin v binding at baseline was equivalent in SOD2-KO platelets, and increased to similar extents following stimulation with thrombin plus convulxin or with ionomycin (Figure 3J).

In vivo function of SOD2 deficient platelets is normal

There were no differences in circulating platelet counts (Table 1). In a tail-bleeding model, SOD2-KO mice had similar bleeding time relative to littermate controls (Figure 4A). Evaluation of SOD2-KO mice using a ferric chloride induced arterial thrombosis model,

demonstrated equivalent degrees of thrombosis, relative to controls (Figure 4B). Platelets also modulate inflammatory responses and mitigate sepsis disease progression, as mice depleted of platelets exhibit decreased survival (28). Thus we evaluated survival in the CLP model of sepsis. Mice with SOD2 deficient platelets displayed survival rates that were equivalent to controls (Figure 4C). Platelets also mediate disease progression in certain forms of autoimmune mediated arthritis (26). SOD2-KO mice subjected to autoimmune mediated arthritis, displayed equivalent disease progression as measured by clinical index and ankle thickness (Figure 4D–E).

Discussion

The present study revealed that deletion of SOD2 increases mitochondrial ROS, without increasing total cellular ROS. Increased mitochondrial ROS neither promoted platelet activation nor increased *in vivo* thrombosis, or platelet-mediated immune activation. Thus a constitutive increase in mitochondrial ROS does not influence platelet function.

In the absence of SOD2, mitochondrial superoxide anion concentrations were increased. Superoxide anions maintain a short half-life, and are unlikely to traverse the mitochondrial membrane. Thus if any increase in mitochondrial superoxide would contribute to cellular oxidative stress, this would likely derive from the conversion of superoxide anions to H₂O₂ (29). Using multiple independent measures of total cellular ROS in addition to direct estimations of cellular H₂O₂ content we observed no increase in H₂O₂ or ROS, indicating that the contribution of SOD2 (mitochondrial)-mediated H₂O₂ production is not a major regulator of total cellular H₂O₂ production. As expected, arachidonic acid (AA) incubation markedly increased 8-isoprostane formation in wildtype platelets. The fold increase in deficient platelets was similar, which is consistent with cytosolic ROS from NADPH Oxidases representing the major mediator of this phenomenon.

We observed evidence of defective mitochondrial respiratory capacity following stimulation of SOD2-KO platelets with thrombin, suggesting reduced mitochondrial respiratory capacity. Previous studies revealed that *in vitro* administration of inhibitors of mitochondrial respiration impairs platelet activation (31). We were therefore surprised that SOD2-KO platelets demonstrated no impairment in platelet activation. It is possible that the degree of agonist-mediated mitochondrial dysfunction in SOD2 deficient platelets is not sufficient to impair platelet activation. Alternatively, a compensatory increase in glycolysis could offset impaired mitochondrial metabolism to sustain normal levels of platelet activation in SOD2-KO platelets.

Platelet activation may partially retard sepsis disease progression, as mice depleted of platelets display decreased survival (28). In sepsis, ROS is elevated in plasma and multiple cell types (32). Studies of patients with sepsis indicate increased platelet mitochondrial respiration may correlate with adverse outcomes (18). SOD2 KO platelets did not exhibit increased basal oxygen consumption, that might have occurred if mitochondria were uncoupled. Thus, absence of greater mortality in SOD2-KO mice in a CLP-mediated sepsis model suggests that increased mitochondrial ROS in the absence of overt or severe mitochondrial dysfunction does not impair platelet function sufficiently to accelerate

mortality in sepsis. We did not directly measure total or mitochondrial levels of ROS in platelets of septic animals, but the absence of any changes in survival suggests that cellular platelet responses are likely similar. Platelets also play an essential role in autoimmunemediated arthritis (26). Increased ROS is believed to contribute to disease progression in autoimmune-mediated arthritis, and our results suggest that platelet mitochondrial ROS does not contribute to disease progression.

Although we were unable to determine any impact of SOD2 deficiency on *in vitro* or *in vivo* parameters of platelet function, the possibility remains that other functions could be altered or that a more severe increase in mitochondrial ROS generation might be needed to induce platelet dysfunction. For example, in diabetes mellitus, increased mitochondrial ROS has been observed that correlates with platelet dysfunction. However, it is likely in this context that increased platelet activation in diabetes also reflects extra-mitochondrial changes in signaling. Deletion of SOD2 in drosophila and mammals, reduces lifespan (33). Thus, as animals age, reliance of platelets on SOD2 might increase, which will be addressed in future studies. In conclusion, platelet SOD2 is dispensable for platelet function, and mitochondrial ROS does not play a major role in platelet activation or immune function in young mice.

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What is known about this topic?

- Increased total platelet ROS results in increased platelet activation.
- Increased mitochondrial ROS correlate with increased platelet activation.

What does this paper add?

- Increased mitochondrial ROS does not result in increased total platelet ROS.
- Increased mitochondrial ROS alone does not result in increased platelet activation.

Figure 1. Deletion of SOD2 increases mitochondrial ROS

(A) Immunoblot analysis of SOD2 and tubulin in platelet lysates, n=8. (B) SOD2 activity normalized to protein content, n=5 Control n=4 SOD2-KO; (C) SOD1 activity normalized to protein, n=5 Control, n=4 SOD2-KO. (D) MitoSOX Geo. MFI in platelets, normalized to control, n=6. (E) Steady State H_2O_2 concentrations in platelets incubated with 3-Amino-1,2,4-triazol, n=5. (F) GSH/GSSG ratio normalized to absolute platelet number, n=6. (G) DCFDA Geo. MFI in platelets following administration of the indicated agonist, n=3. (H) 8-isoprostane production in the presence or absence of arachidonic acid (AA), n=5. Data are represented as mean $\pm$ SEM. *P<0.05, **P<0.01; 2-way ANOVA followed by Tukey's multiple comparison post hoc test (G and H); Student's t test (B-F).

Figure 2. Basal mitochondrial function is maintained in SOD2-KO mice

(A) Seahorse analysis of oxygen consumption rates (OCR) in platelets in the presence or absence of 0.5U/mL Thrombin (IIa), n=5. (B) TMRM geo. MFI of nonstimulated platelets normalized to control, n=6. (C) Representative transmission electron micrographs of non-stimulated platelets; (D) organelle quantification of electron micrographs, n=4. (E) Immunoblot analysis of cytochrome C; (F) quantification of cytochrome c normalized to ponceau S staining, n=9. Data are represented as mean $\pm$ SEM. *P<0.05,

P<0.01,*P<0.001; 2-way ANOVA followed by Tukey's multiple comparison post hoc test (A and D); Student's t test (B and F).

Figure 3. Deletion of SOD2 does not impair *in vitro* platelet function

(A) Platelets were stimulated in the presence of the indicated agonist and monitored for GPIIb/IIIa activation (JonA geo. MFI), and (B) Cd62p surface translocation (CD62p Geo. MFI), n=6. Washed platelet aggregometry analysis of platelets stimulated with (C) 0.02U/mL thrombin, n=5, (D) 2µg/mL collagen, n=5, and (E) 4µM ADP, n=4 when indicated by the arrowhead. Analysis of ATP release was conducted in parallel with aggregometry studies following agonist stimulation (F-H). (I) Platelet spreading on fibrinogen under static conditions stained with WGA (red) and phalloidin (Green), n=4. (J)

Platelets were monitored for annexin v binding under basal conditions and following stimulation with 1U/mL thrombin + 700ng/mL Convulxin, or 1 μ M Ionomycin, n=6, (Ionomycin n=3). Data are represented as mean $\pm$ SEM. Groups were compared by 2-way ANOVA followed by Tukey's multiple comparison post hoc test (A, B, and J)).

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Figure 4. Loss of SOD2 does not impair platelet function *in vivo*

(A) Time to bleeding cessation in a tail-bleeding model, n=10. (B) Time to occlusion in a FeCl₃-mediated arterial thrombosis model, n=10. (C) Percent survival in a CLP model of sepsis, n=50. (D) Change in ankle thickness and (E) clinical index following administration of KBx/N serum, n=5. Data are represented as mean ± SEM. Groups were compared by 2-way ANOVA followed by Tukey's multiple comparison post hoc test (D and E)); Student's t test (A and B) Log-rank (Mantel-Cox) test (C).

Table 1

Whole blood analysis by Advia 120 (n=7). P-value based on Student t-test.

Sample	Control (Mean $\pm$ SD)	SOD2-KO (Mean $\pm$ SD)	P-value
WBC (10^3 Cells/ μ L)	7.8 $\pm$ 1.7	9.9 $\pm$ 2	0.07
RBC (10^3 Cells/ μ L)	10.2 $\pm$ 0.8	9.9 $\pm$ 0.9	0.45
Hemoglobin (g/dL)	13.7 $\pm$ 1.3	13.1 $\pm$ 0.9	0.35
Hematocrit (%)	49.7 $\pm$ 3.5	48.7 $\pm$ 4.8	0.66
MCV (fL)	48.9 $\pm$ 1.5	49.4 $\pm$ 1.5	0.57
MCH (pg)	13.3 $\pm$ 0.4	13.4 $\pm$ 0.4	0.65
MCHC (g/dL)	27.3 $\pm$ 0.7	27.2 $\pm$ 1	0.83
CHCM (g/dL)	28.5 $\pm$ 0.4	28.6 $\pm$ 0.7	0.85
CH(pg)	13.9 $\pm$ 0.4	14.1 $\pm$ 0.2	0.34
RDW(%)	16.0 $\pm$ 2.5	16.3 $\pm$ 1.6	0.80
HDW (g/dL)	2.0 $\pm$ 0.1	2 $\pm$ 0.1	0.42
Platelets (10^3 Cells/ μ L)	1274.3 $\pm$ 121	1332.9 $\pm$ 125.4	0.39
Mean Platelet Volume	7.6 $\pm$ 0.2	7.4 $\pm$ 0.3	0.28
Neutrophils (10^3 Cells/ μ L)	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.44
Lymphocytes (10^3 Cells/ μ L)	7.4 $\pm$ 1.6	9.3 $\pm$ 1.9	0.06
Monocytes(10^3 Cells/ μ L)	0	0	0.15
Eosinophils (10^3 Cells/ μ L)	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1	0.64
PDW(%)	43.7 $\pm$ 2.1	43.4 $\pm$ 3.1	0.85
MPC (g/dL)	19.5 $\pm$ 0.4	19.9 $\pm$ 0.7	0.20
MPM (pg)	1.3 $\pm$ 0.1	1.3 $\pm$ 0.1	0.45