SUPPLEMENTARY APPENDIX

Aerobic glycolysis fuels platelet activation: small-molecule modulators of platelet metabolism as anti-thrombotic agents

Paresh P. Kulkarni,[1†] Arundhati Tiwari,[1†] Nitesh Singh,[1] Deepa Gautam,[1] Vijay K. Sonkar,[1] Vikas Agarwal[2] and Debabrata Dash[1]

[1]Department of Biochemistry, Institute of Medical Sciences and [2]Department of Cardiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

[†]PPK and AT contributed equally to this work.

 doi:10.3324/haematol.2018.205724

Received: September 3, 2018.

Accepted: October 30, 2018.

Pre-published: October 31, 2018.

Correspondence: *DEBABRATA DASH*

ddash.biochem@gmail.com

Supplemental Information

Aerobic glycolysis fuels platelet activation: small-molecule modulators of platelet metabolism as anti-thrombotic agents

Paresh P. Kulkarni[1†], Arundhati Tiwari[1†], Nitesh Singh[1], Deepa Gautam[1], Vijay K. Sonkar[1], Vikas Agarwal[2], Debabrata Dash[1*]

1 Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, Uttar Pradesh, India

2 Department of Cardiology, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, Uttar Pradesh, India

† PPK and AT contributed equally to this work

***Correspondence**: Prof. Debabrata Dash, Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221005, Uttar Pradesh, India, Tel: +91-9336910665, Fax: +91-542-2367568, E-mail: ddash.biochem@gmail.com

Supplemental Information includes

Legends to Supplementary Videos 1-4

METHODS

Figures: Figure S1 to Figure S9

Legends to Supplementary Videos 1-4

Supplementary Video 1. Video showing ferric chloride-induced mesenteric arteriolar thrombosis in mice pre-administered with vehicle (control). Platelets were fluorescently labeled with Dylight 488 anti-GPIbβ antibody (0.1 μg/g body weight).

Supplementary Video 2. Video showing ferric chloride-induced mesenteric arteriolar thrombosis in mice pre-administered with DCA (200 mg/kg). Platelets were fluorescently labeled with Dylight 488 anti-GPIbβ antibody (0.1 μg/g body weight).

Supplementary Video 3. Video showing ferric chloride-induced mesenteric arteriolar thrombosis in mice pre-administered with DHEA (50 mg/kg). Platelets were fluorescently labeled with Dylight 488 anti-GPIbβ antibody (0.1 μg/g body weight).

Supplementary Video 4. Video showing ferric chloride-induced mesenteric arteriolar thrombosis in mice pre-administered with DASA (40 mg/kg). Platelets were fluorescently labeled with Dylight 488 anti-GPIbβ antibody (0.1 μg/g body weight).

METHODS

Materials

Potassium dichloroacetate, dehydroepiandrosterone, ethylene glycol tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), sodium orthovanadate, acetylsalicylic acid, antimycin A, oligomycin, thrombin, fibrinogen, xyalzine, and bovine serum albumin were purchased from Sigma. DASA-58 was procured from Cayman Chemicals. Ketamine was from Neon Laboratories Ltd. and reagents for electrophoresis were from Merck. Polyvinylidene fluoride (PVDF) membranes and enhanced chemiluminescence (ECL) detection kit were from Millipore. Antibodies against PKM1, PKM2 and phospho(Tyr-105)-PKM2 were procured from Cell Signaling Technology. FITC-labeled PAC-1, PE-annexin V, PE-anti-CD62P and FACSFlow sheath fluid were from BD Biosciences. Sources of other antibodies used were as follow: Alexa fluor 488-fibrinogen (Invitrogen), anti-phospho(Ser-293)-PDH E1-α (Abcam), anti-GLUT3 (R&D Systems), anti-β-actin (Sigma), Alexa Fluor 488-goat anti-mouse IgG (H+L) (Thermo Fisher Scientific), DyLight 488-labled anti-GPIbβ antibody (Emfret Analytics), Horseradish peroxidase (HRP)-labeled anti-rabbit IgG (Bangalore Genei). Collagen, epinephrine and Chronolume luciferin-luciferase reagent were procured from Chrono-log. All other reagents were of analytical grade. Type I deionized water (18.2 MΩ▪cm, Millipore) has been used throughout the experiments.

Platelet preparation

Platelets were isolated from fresh human blood by differential centrifugation. Briefly, peripheral venous blood was collected from consenting healthy volunteers under aseptic precautions, and centrifuged at 200 × g for 10 min. Platelet-rich plasma (PRP) thus obtained was incubated with 1 mM acetylsalicylic acid for 15 min at 37^{o} C. After addition of EDTA (5 mM), platelets were sedimented by centrifugation at 600 ×

g for 10 min. Cells were washed in buffer A (20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 0.36 mM NaH_2PO_4, 1 mM EGTA, 5.5 mM glucose, pH 6.2) and were finally resuspended in buffer B (pH 7.4), which was the same as buffer A but without EGTA. The cell count was determined using Beckman Coulter Multisizer 4, and adjusted to 2-4 × 10^8/ml. The study methodologies conformed to the standards set by the Declaration of Helsinki. Precautions were taken for asepsis and to maintain the cells in resting condition.

High-resolution respirometry for mitochondrial respiration

Mitochondrial respiration was measured using a high-resolution respirometer (Oxygraph-2k, Oroboros Instruments) at 37°C under stirring conditions (750 rpm). Washed human platelets (in buffer B containing 5.5 mM glucose) with or without pre-treatment were transferred into oxygraph chamber. Respiration was first allowed to stabilize at the routine state, i.e., in the physiological coupling state controlled by cellular energy demands for oxidative phosphorylation. Then, platelets were activated with thrombin (0.5 U/ml), and changes in oxygen flux were recorded in real time at high resolution (sampling at 2 sec intervals). In other experiments, cells were pretreated with either oligomycin (1 µg/ml) or antimycin A (1 µg/ml) to study leak respiration or residual/extra-mitochondrial oxygen consumption, respectively. Further, the maximal capacity of electron transport system was determined by titrating the dose of protonophore CCCP until there was no further increase in oxygen flux. Calibration at air saturation was performed each day before starting experiments by letting Millipore water/buffer B stir with air in the oxygraph chamber until equilibration and a stable signal was obtained. All experiments were performed at an oxygen concentration in the range of 100–205 µM O_2. Data were recorded and analyzed using DatLab 5.1 software (Oroboros Instruments).

Platelet α-granules secretion

Secretion from platelet α-granules was evaluated by quantifying surface expression of P-selectin. Washed human platelets were incubated at 37° C for 5 min

without stirring in presence of thrombin (0.1 U/ml). FITC-labeled anti-CD62P antibody (5% v/v) was added to each sample and incubated for 30 min in the dark at room temperature. Platelets were fixed for 30 min with equal volume of 4% paraformaldehyde. Cells were washed twice in phosphate-buffered saline (PBS; pH 7.4), resuspended in sheath fluid, and analyzed on a flow cytometer (FACSCalibur, BD Biosciences). Forward and side scatter voltages were set at E00 and 273, respectively, with a threshold of 52 V. An amorphous region (gate) was drawn to encompass platelets separate from noise and multi-platelet particles. All fluorescence data were collected using 4-quadrant logarithmic amplification for 10,000 events in the platelet gate from each sample and analyzed using CellQuest Pro software.

Study of platelet surface integrin activation

Platelet stimulation induces change in surface integrins $\alpha_{IIb}\beta_3$ to an open conformation that allows high-affinity binding of fibrinogen and platelet aggregation. PAC-1 antibody specifically recognizes the open conformation of $\alpha_{IIb}\beta_3$. Washed human platelets were incubated at 37°C for 5 min without stirring in the presence of thrombin (0.1 U/ml). Either FITC-labeled PAC-1 antibody (final 1.25 µg/ml) or Alexa 488-conjugated fibrinogen (final 10 ug/ml) was then added to each sample and incubated for 30 min in dark at room temperature. Platelets were fixed with equal volume of 4% paraformaldehyde, washed and resuspended in sheath fluid. Samples were analyzed on a flow cytometer as described above.

Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS was determined using a redox-sensitive cell-permeable dye, H_2DCF-DA. Washed human platelets were incubated at 37°C for 5 min without stirring in the presence of thrombin (0.1 U/ml). H_2DCF-DA (1 µM) was added to each sample and incubated for 30 min in the dark at room temperature. Platelets were fixed for 30 min with equal volume of 4% paraformaldehyde. Cells were washed twice in PBS, resuspended in sheath fluid, and analyzed by flow cytometry as

described above. Hydrogen peroxide (1%) was added to platelet suspension as positive control.

Analysis of phosphatidylserine (PS) exposure on platelet surface

Externalization of PS, a measure of surface procoagulant activity, in stimulated platelets was assessed from annexin V binding. Washed platelets were incubated with thrombin (0.5 U/ml) at 37° C for 5 min without stirring. PE-labeled annexin V (5% v/v) was added to each sample along with 5 mM $CaCl_2$ and incubated for 30 min in the dark at room temperature. Cells were analyzed by flow cytometry as described above.

Immunoblotting

Platelet proteins were separated on 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gels and electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane by using a semidry blotting system (BioRad). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (10 mM Tris-HCl and 150 mM NaCl, pH 8.0) containing 0.05% Tween-20 (TBST) for 1 h at room temperature. Blots were incubated overnight with respective primary antibodies (anti-pPDH, 1:10000; anti-pAMPK, 1:1000 in 5% BSA; anti-pPKM2, 1:1000; anti-PKM2, 1:1000; anti-PKM1, 1:1000; anti-β-actin, 1:5000), followed by 3 washings with TBST for 5 min each. Membranes were then placed in HRP-labeled anti-rabbit IgG diluted in blocking buffer or TBST for 1 h. Blots were similarly washed, and antibody binding was detected using enhanced chemiluminescence. Images were acquired on a multispectral imaging system (BioSpectrum 800 Imaging system, UVP) and quantified using VisionWorks LS software (UVP).

Study of extracellular vesicle (EV) release by Nanoparticle Tracking Analysis

Washed human platelets were incubated with thrombin (0.5 U/ml) for 5 min at 37° C without stirring. Cells were sedimented by centrifugation. EVs present in supernatant were fixed with paraformaldehyde and a beam from solid-state laser (638 nm) was allowed to pass through the sample. Light scattered by rapidly moving particles in

suspension in Brownian motion at room temperature was observed under 20X microscope. This revealed hydrodynamic diameters of particles, calculated using Stokes-Einstein equation, within range of 10 nm to 1 μm and concentration between 10^7–10^9/ml. The average distance moved by each EV in x and y directions were captured by CCD camera (30 frames per sec) attached to the microscope. Both capture and analysis were performed with NanoSight LM10 (Malvern) employing NTA 2.3 analytical software, which provides an estimate of the particle size versus concentration in sample.

LDH Leak Assay

Platelets treated with vehicle (control), DCA (20 mM), DHEA (200 μM), DASA (100 μM) or digitonin for different periods of time, were pelleted by centrifugation at 800g for 10 min. Supernatants were preserved. The reaction was initiated by the addition of platelet supernatant (40 μl) to reaction mixture (100 μl) containing 0.168 mM NADH and 32.52 mM sodium pyruvate at 30 °C. LDH activity was assayed from the time course of decrease in NADH absorbance at 340 nm. Platelets incubated with vehicle (control) for 1h or digitonin for 5 min served as negative and positive control, respectively, for calculating percent LDH release.

Imaging thrombus formation in mice mesenteric arterioles by intravital microscopy

Swiss albino mice (5-6 weeks old, 8-12 g each) were anesthetized with intraperitoneal injection of ketamine/xylazine cocktail (100 mg/kg ketamine and 10 mg/kg xylazine). DyLight 488-labled anti-GPIbβ antibody diluted in 50 μl sterile PBS was injected into retro-orbital vein of mice at 0.1 μg/g body weight. A mid-line incision was made through the abdomen and the exposed mesentery was kept moist and warm by superfusion with warm (37° C) and sterile PBS. Mesenteric arterioles of diameter 100-150 μm were isolated and focused under epifluorescence inverted video microscope (Nikon model Eclipse Ti-E) equipped with a monochrome CCD cooled camera. Whatman filter paper saturated with ferric chloride (10%) solution

was applied topically for 3 min and thrombus formation in the injured vessel was monitored in real time. Thrombus formation was recorded by using a high-speed camera for 40 min or until occlusion. Movies were subsequently analyzed with Nikon image analysis software to determine (a) the time required for formation of first thrombus (>20 μm in diameter), (b) time required for occlusion of the vessel (i.e. time required after injury till blood stopped flowing for 30 sec), and (c) thrombus growth rate (growth of thrombus of 30 μm diameter over period of 3 min). Fold increase was calculated by dividing the diameter of thrombus at given time (n) by the diameter of the same thrombus at time (0). Time 0 was defined as the time point at which the thrombus diameter first reached approximately 30 μm.

Tail bleeding assay

8-12 week old mice weighing 20-25 g were anesthetized with ketamine/xylazine cocktail (100 mg/kg ketamine and 10 mg/kg xylazine). A 3 mm tip of the tail was cut with a fresh sharp scalpel. The tail was immediately placed into PBS maintained at 37^{o}C, and bleeding was monitored. 'Bleeding time' was recorded as time required till cessation of bleeding for at least 10 sec. Experiment was terminated in case bleeding continued till 20 min after injury. Quantum of bleeding was evaluated by measurement of hemoglobin bled into warm PBS employing Drabkin's reagent.

Collagen-epinephrine induced pulmonary thromboembolism

8-12 week old mice weighing 25-35 g were anesthetized with ketamine/xylazine cocktail (100 mg/kg ketamine and 10 mg/kg xylazine). Deep vein thrombosis and pulmonary embolism was induced by intravenously administering a cocktail of collagen (1 mg/kg) plus epinephrine (10 μg/kg). The mice were euthanized after observation for a maximum of 30 min by administering an overdose of anesthesia. Lungs were perfused with cold saline, dissected out, and immediately fixed in 10% formalin for 24 h. Histological sections were prepared from paraffin-embedded lung tissue, stained with hematoxylin and eosin (H&E), observed under a light microscope

(Nikon Eclipse Ti-E), and images acquired at 100X magnification. Number of thrombosed pulmonary vessels per low power field of pulmonary sections from each mouse was counted.

Statistical analysis

All statistical analyses were performed using Graphpad Prism 7 software. One-tailed Student's t-test (for two groups) or One-way Analysis of Variance (ANOVA) with Dunnett's multiple comparison test (for three or more groups) was used for evaluating significance in difference of means between groups, and values of $p<0.05$ were considered significant. Linear regression analysis was performed, and the slopes from best-fit were used to arrive at rates in time-lapse experiments.

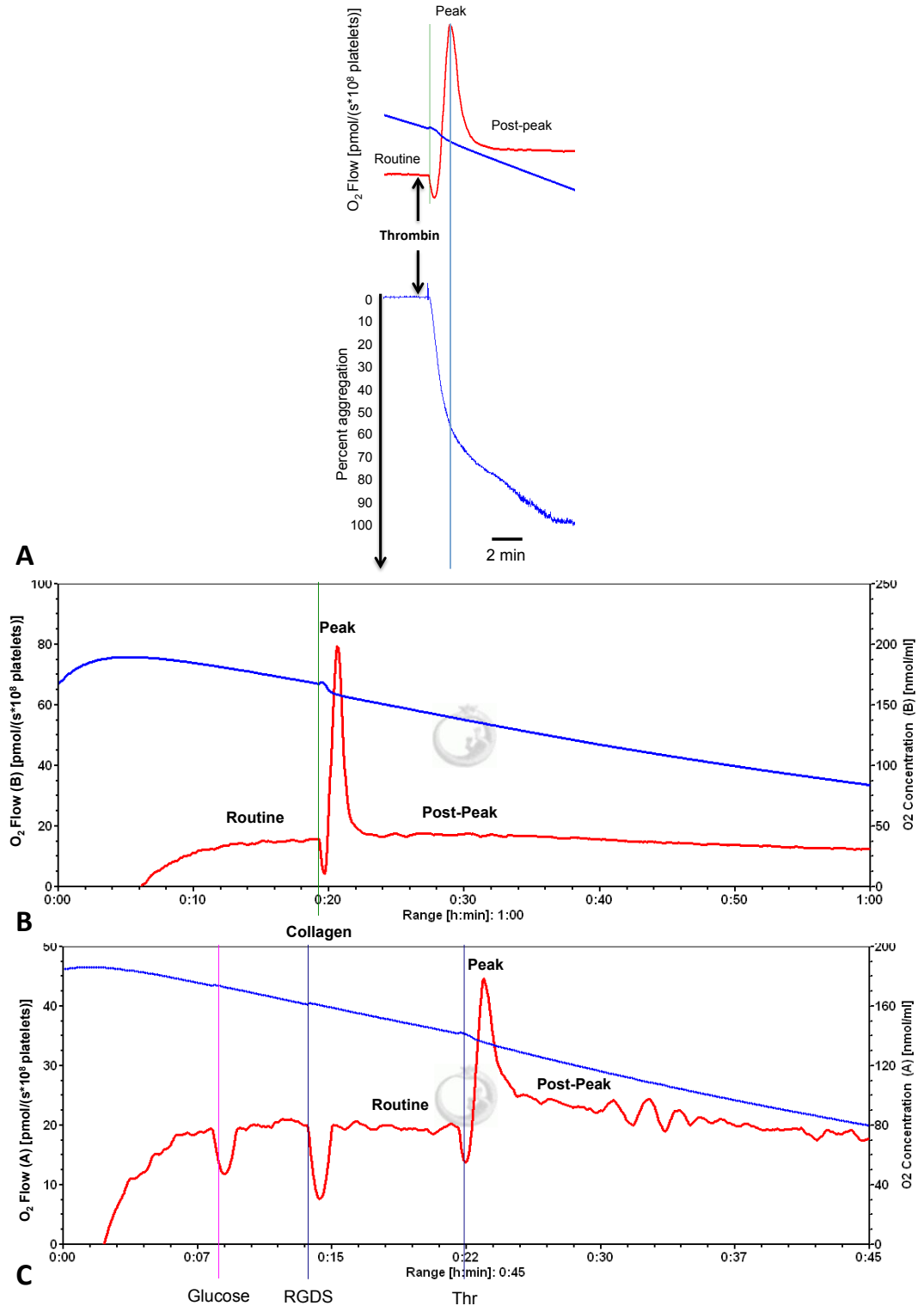

Figure S1. Related to Figure 1. A) Platelets were stimulated with thrombin (Thr, 1U/ml) under stirring. Extent of platelet aggregation (increase in light transmittance recorded by light transmittance aggregometry) was compared with changes in oxygen flux (rate of oxygen consumption by platelets measured by high-resolution

respirometry) on same time scale. B and C) Oxygen flux measured in (B) collagen (5 µg/ml)-stimulated platelets and (C) thrombin (Thr, 1 U/ml)-stimulated platelets following addition of RGDS peptide, respectively. Polarograms are representative of 3 independent experiments.

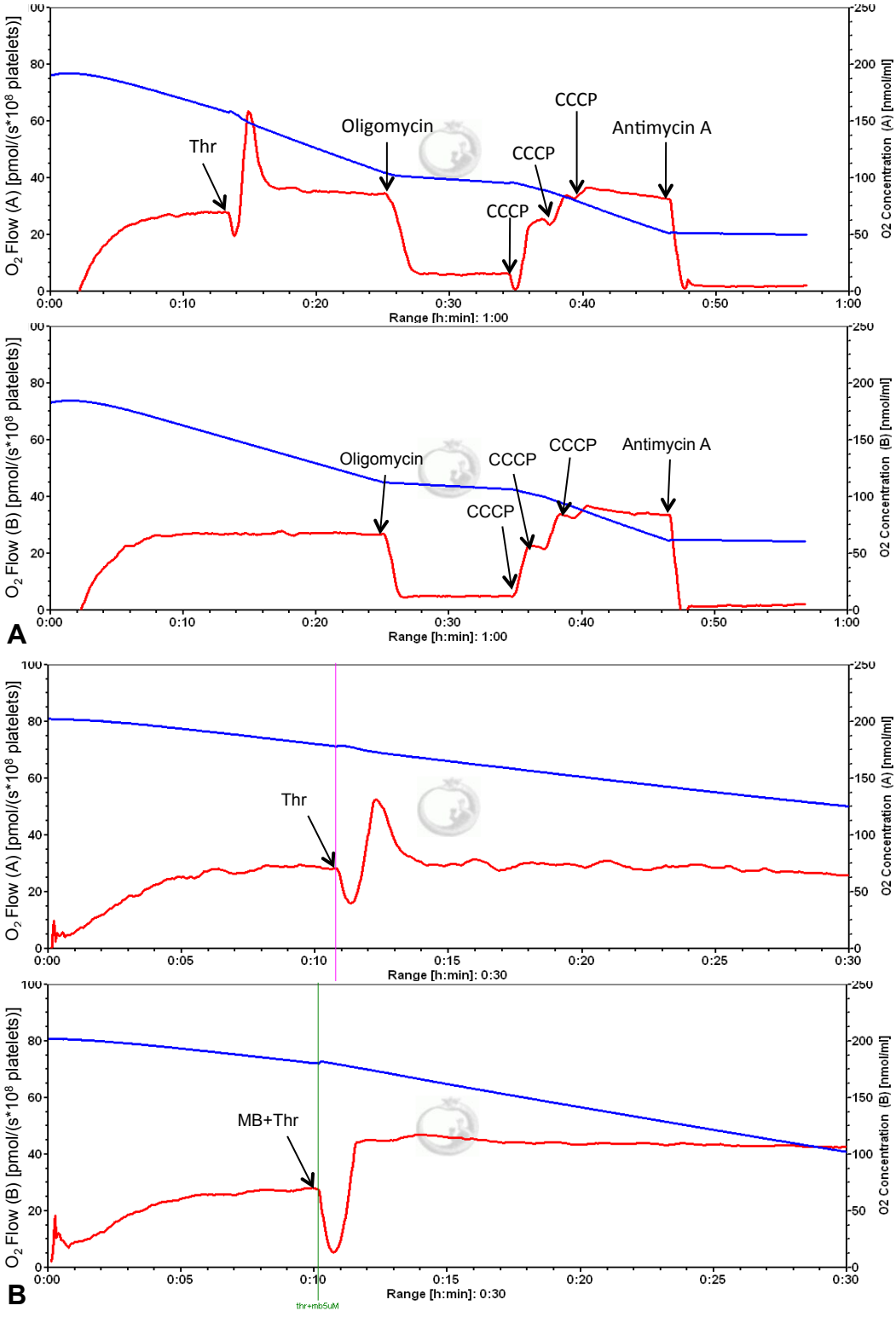

Figure S2. Related to Figure 1. A, Oxygen flux measured in thrombin (Thr, 1 U/ml)-stimulated (upper panel) as well as control (unstimulated) (lower panel) platelets following sequential addition of oligomycin (inhibitor of ATPase, 1 µg/ml), carbonyl cyanide m-chlorophenyl hydrazine (CCCP) (uncoupler, titrated in steps of 0.5 µM till

no further rise in oxygen flux was observed) and antimycin A (inhibitor of respiratory complex III, 1 µg/ml). B, Oxygen flux measured in thrombin (Thr, 1 U/ml)-stimulated platelets with or without pre-treatment of cells with methylene blue (MB, 10 µM). Polarograms are representative of 3 independent experiments.

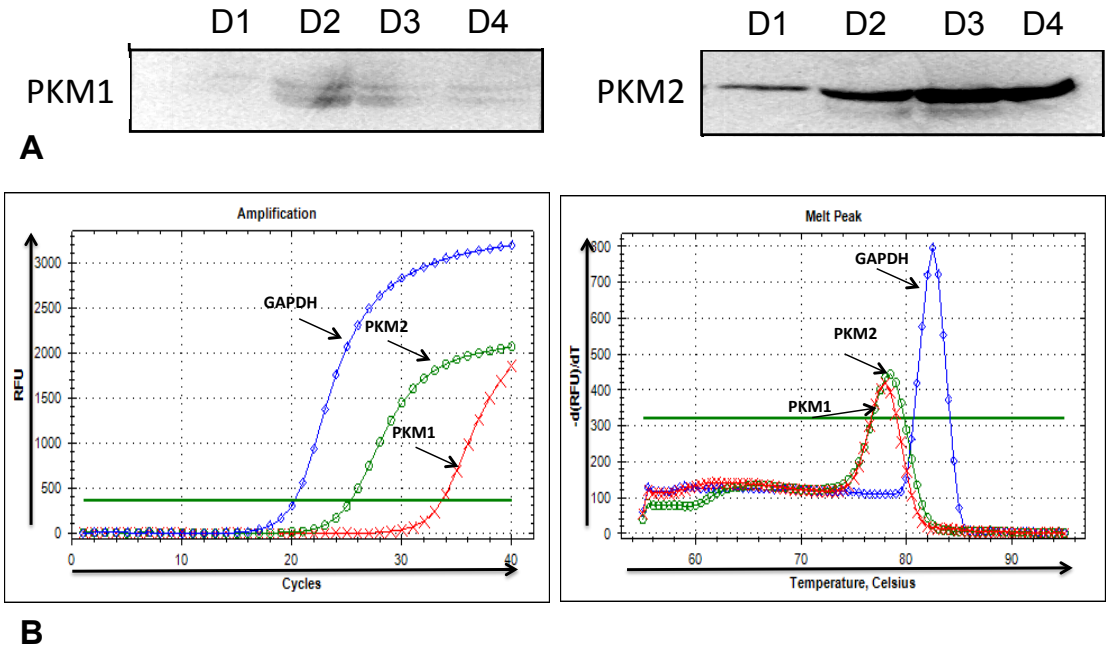

Figure S3. Related to Figure 2. A, Immunoblots showing expression of PKM1 and PKM2 in four different individuals (designated D1 to D4) B) Total RNA in platelets was reverse transcribed and subjected to qRT-PCR for expressions of PKM1, PKM2 and GAPDH. Left panel, amplification chart representing Cq of corresponding mRNAs. Right panel, melt peak analyses indicative of lack of formation of by-products during amplification reaction. Figures are representative of 3 independent experiments.

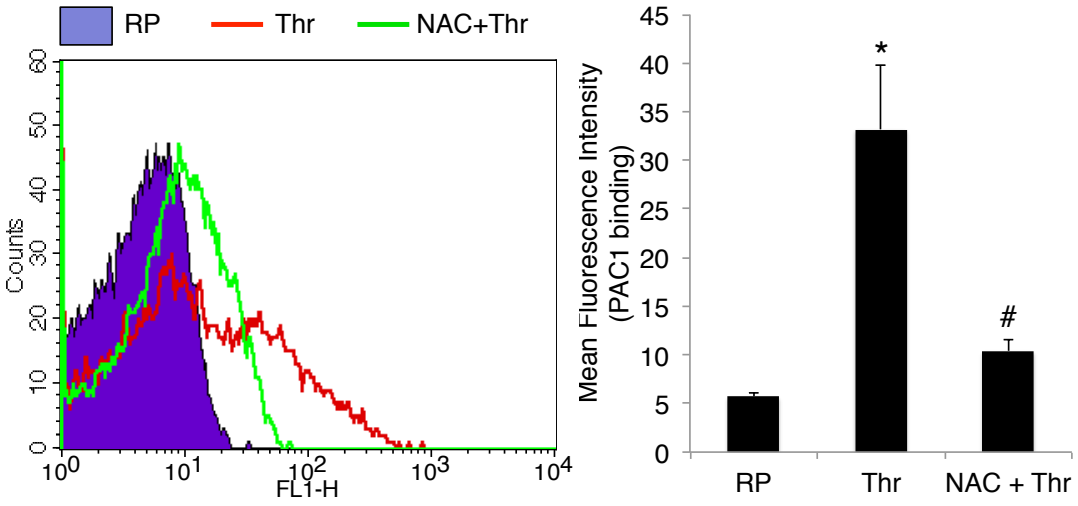

Figure S4. Related to Figure 3. Histogram and corresponding bar diagram showing flow cytometric analysis of PAC1 binding to resting (RP) or thrombin-stimulated platelets with or without N-acetyl cysteine (10 mM) pretreatment. Data are presented as mean ± SEM (n=3). *P<0.05 as compared to resting platelets; #P<0.05 as compared to thrombin-stimulated platelets.

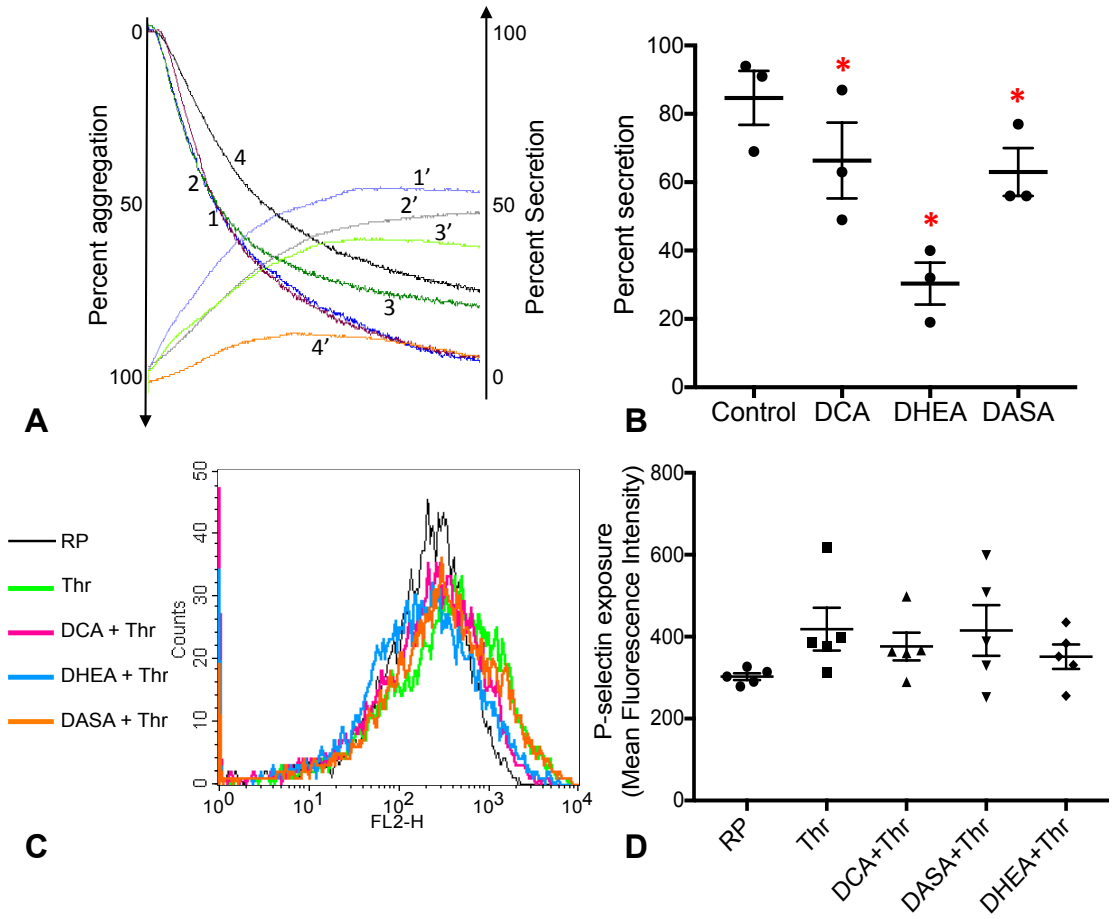

Figure S5. Related to Figure 3. A, Aggregation (tracings 1, 2, 3 and 4) and dense granule secretion (tracings 1', 2', 3' and 4') of platelets induced by thrombin (0.5 U/ml) in the presence of vehicle, DHEA (200 μM), DCA (20 mM) or DASA (200 μM), respectively. B, Scatter dot plot showing thrombin-induced dense granule secretion in the presence of vehicle, DHEA (200 μM), DCA (20 mM) or DASA (200 μM). C and D, Histogram and corresponding scatter dot plot showing P-selectin exposure on platelets treated with various reagents as indicated. Data are presented as mean ± SEM. Each dot represents an independent observation. (*P<0.05 as compared to vehicle-treated control platelets)

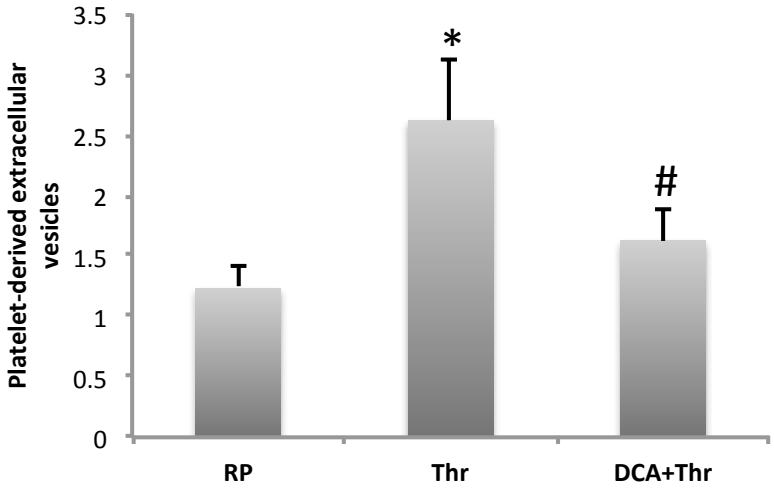

Figure S6. Related to Figure 3. Extracellular vesicles released from thrombin (0.5 U/ml)-stimulated platelets with or without DCA pre-treatment measured by NTA. Bar diagram averaging independent experiments (n=3) (mean ± SEM). (*P<0.05 as compared to resting platelets (RP); #P<0.05 as compared to thrombin-stimulated platelets)

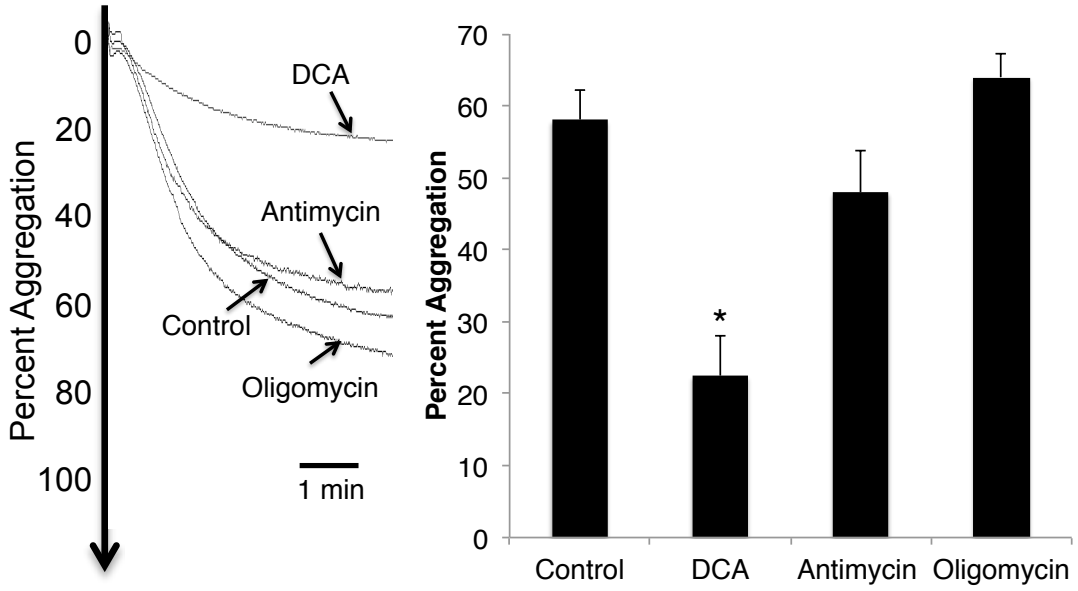

Figure S7. Related to Figure 3. Tracings and corresponding bar diagram showing percent aggregation of platelets induced by thrombin (0.2 U/ml) in the presence of vehicle (control) (n=7), DCA (20 mM) (n=4), antimycin (1 μg/ml) (n=3) or oligomycin (10 μg/ml) (n=3), as indicated. Data are presented as mean ± SEM. *P<0.05 as compared to control platelets.

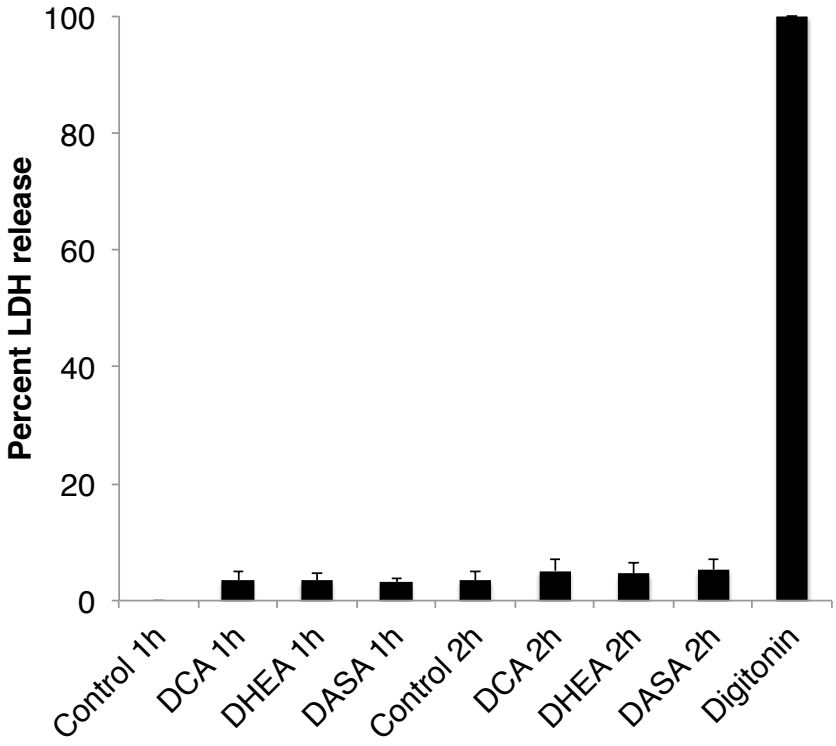

Figure S8. Bar diagram showing percent LDH activity leaking out from platelets incubated with vehicle (control), DCA (20 mM), DHEA (200 μM), DASA (100 μM) or digitonin for different periods of time, as indicated. Platelets incubated with vehicle (control) for 1h or digitonin for 5 min served as negative and positive control, respectively. Data are presented as mean ± SEM (n=3).

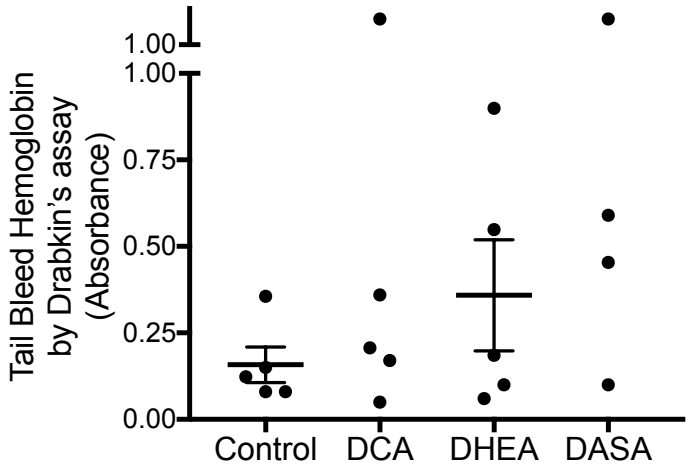

Figure S9. Related to Figure 5. Scatter dot plot showing estimates of hemoglobin in tail bleeds by Drabkin's assay in mice administered with vehicle (n=5), DCA (n=5), DHEA (n=5) or DASA (n=4). Data are presented as mean ± SEM. Each dot represents an independent observation.