Online supplement

Dichloroacetate, an inhibitor of pyruvate dehydrogenase kinases, inhibits platelet aggregation and arterial thrombosis

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Materials

Potassium dichloroacetate (DCA), apyrase, EGTA (ethylene glycol tetraacetic acid), prostaglandin I_2 (PGI_2), thrombin and protease inhibitors were purchased from Sigma (St. Louis, MO, USA). Collagen, ADP and chronolume reagent were purchased from Chronolog Corporation (Havertown, PA, USA). Glucose uptake-Glo assay kit and Thromboxane B2 ELISA kit purchased from Promega and Abcam, respectively. Convulxin was purchased from Santa Cruz Biotech. Primary antibodies Phospho PLCγ2 (#3871, 1:1000), total PLCγ2 (#3872, 1:1000), Phospho Syk (# 2711, 1:1000), total Syk (#12358, 1:1000), and goat anti-rabbit IgG, HRP-linked secondary antibody (#7074, 1:2500) were from Cell Signaling Technologies. CD62P-FITC and Alexafluor 488 GP VI antibody was purchased from BD Biosciences and R&D systems, respectively. FITC Annexin V apoptosis detection kit I was purchased from BD Biosciences. Calcein-AM was from Molecular Probes (Invitrogen). Super Signal West Pico chemiluminescent substrate and PVDF membrane were the products from Thermo Scientific and Millipore, respectively. All other reagents were of analytical grade.

Methods

Mouse platelet isolation

Mouse platelets were prepared as described.[1-3] Blood from anesthetized mice was drawn from the retro-orbital plexus and collected in 1.5 mL polypropylene tubes containing 300 µL of enoxaparin (0.3 mg/mL; Sanofi-Aventis, US LLC). The blood was centrifuged at 100 *g* for 5 minutes, and the platelet-rich plasma (PRP) was collected in a fresh tube. To prevent platelet activation, PRP was incubated with PGI_2 (2 µg/mL) at 37°C for 5 minutes. PRP was further centrifuged at 600 *g* for 5 minutes. The obtained pellets were suspended in 1 mL modified Tyrode–*N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid (HEPES) buffer (137 mM NaCl, 0.3 mM Na_2HPO_4, 2 mM KCl, 12 mM $NaHCO_3$, 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid, 5 mM glucose, 0.35% bovine serum albumin, pH 7.2) containing PGI_2 (2 µg/mL) for 5 minutes at 37°C. The washing step was repeated twice with modified HEPES (no PGI_2) to remove PGI_2, and platelets were fluorescently labeled with calcein green, AM (2.5 µg/mL; Molecular Probes) for 10 minutes at 37°C.

Human platelet isolation

To obtain human platelets, blood was collected from healthy male volunteers, under informed consent, who was not on any antiplatelet medication for the past two weeks. Platelets were prepared as described.[4,5] Briefly, blood was collected in tubes containing anticoagulant citrate dextrose solution A and centrifuged at 180g for 20 min. After addition of apyrase (0.6 U/ml) to PRP, platelets were sedimented by centrifugation at 800g for 15 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 (ethylene glycol tetraacetic acid), supplemented with 5 mM glucose, and 0.6 ADPase units of

apyrase/mL, pH 6.2). Platelets were finally resuspended in buffer B (pH 7.4), which was the same as buffer 'A' but without EGTA and apyrase.

Static adhesion assay

Coverslips were coated with collagen (50 µg/ml) for one hour at room temperature, then blocked with denatured bovine serum albumin (BSA, 5 mg/ml) for one hour and subsequently washed with phosphate-buffered saline (PBS). The platelets were pre-incubated with 10 and 25 mM of DCA for 10 min at RT. Cells were allowed to attach to the collagen matrix for 20 min at RT followed by washing with PBS and fixing with 2% paraformaldehyde. Images were obtained using an Olympus IX-81 inverted microscope in differential interference contrast mode. Surface area was analyzed using Image J software from NIH (Bethesda, MD, USA).

Immunoblotting

Platelet proteins were separated on 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels and electrophoretically transferred to PVDF (polyvinylidene fluoride) membrane by using Bio-Rad western blotting system. Membranes were blocked with 5% BSA or skimmed milk in 10 mM Tris-HCl, 150 mM NaCl, pH 8.0 (TBS) containing 0.05% Tween-20 for one hour at room temperature. Blots were incubated for overnight with primary, followed by horseradish peroxidase-labeled secondary antibody for one hour. Blots were developed using enhanced chemiluminescence and quantified using Image J software from NIH (Bethesda, MD, USA).

Tail bleeding assay

Tail-transection bleeding time was measured as described.[6] Briefly**,** mice (approximately 8 weeks of age) were anesthetized with 100-mg/kg ketamine and 10-mg/kg xylazine and placed on a heating pad warmed at 37°C, and a 3 mm segment of the tail was amputated with a sharp scalpel blade. The tail was immediately immersed in saline (at 37°C), and the time taken for the stream of blood to stop for more than 30 seconds was defined as the bleeding time. If bleeding did not stop within 10 minutes, hemostasis was achieved by cauterizing the tail.

DCA preparation and calculation of dose for *in vivo* studies

For *in vitro* studies DCA was dissolved in deionized water. For *in vivo* studies, DCA was dissolved in 0.9% saline and administered via jugular vein by slow infusion over the time of 20 minutes at a dose of 600-mg/Kg body weight. A dose of 50 mg/Kg is used to treat lactic acidosis in humans.[7] The *in vivo* dose of 600 mg/Kg body weight was calculated based on animal equivalent dose (AED) calculation based on body surface area between human and mouse.[8] To convert human dose in mg/Kg to mouse in mg/Kg, human dose is either multiplied by 12.3 or divided by 0.08.[8] Controls were administered 0.9% saline via the jugular vein. *In vivo* thrombosis and tail transection assays were performed 10 minutes after completion of DCA or saline infusion by a person who was blinded to the drug and vehicle infusion.

Annexin V-affinity assay

Mouse and human washed platelets (1×10^8/ ml) were pre-incubated with different concentrations of DCA (10, 25, 50, 100 and 200 mM). The positive control was washed platelets activated with thrombin (0.5 U/ml) + collagen (10 µg/ml) at room temperature for 15 min in 1X Annexin V

binding buffer (BD Biosciences). Five µL of Annexin V-FITC antibody was added to each sample (100 µL), gently mixed, and incubated at room temperature for 15 minutes in the dark. After incubation 400 µL of 1X Annexin-binding buffer was added to each tube and analyzed by a Becton Dickinson FACSCalibur flow cytometer.

P-selectin expression

Platelets (1 x 10^8 cells in 100 µl) were pre-incubated for 10 min either in presence or absence of DCA (10 and 25 mM) then stimulated by collagen (0.46 µg/ml) without stirring, followed by addition of 100 µl of 4% paraformaldehyde for 30 min. Post fixation, platelets were labeled with 5 µl PE-labeled anti-CD61 antibody and 5 µl FITC conjugated anti-CD62P antibody as described.[9] The platelet samples were incubated for 30 min in the dark at room temperature and analyzed on the flow cytometer (FACS Calibur, Becton Dickinson). An amorphous region (gate) was drawn to differentiate the platelets from the noise and multi-platelet particles. After compensation for FITC and PE, CD61-positive 10,000 events were collected for each sample.

GP VI Assay by flow cytometry

Platelets (1 x 10^8 cells in 100 µl) were pre-incubated for 10 min either in presence or absence of DCA (10 and 25 mM). Cells were labeled with 5 µl PE-labeled anti-CD61 antibody and either 5 µl Alexafluor-488 GP VI antibody or isotype. The samples were incubated for 30 min at room temperature in the dark and analyzed on the flow cytometer (FACS Calibur, Becton Dickinson). To differentiate the platelets from the noise and multi-platelet particles an amorphous region (gate) was drawn. After compensation for FITC and PE, all fluorescence data were collected using four quadrant system. CD61-positive 10,000 events were collected for each platelet sample.

References

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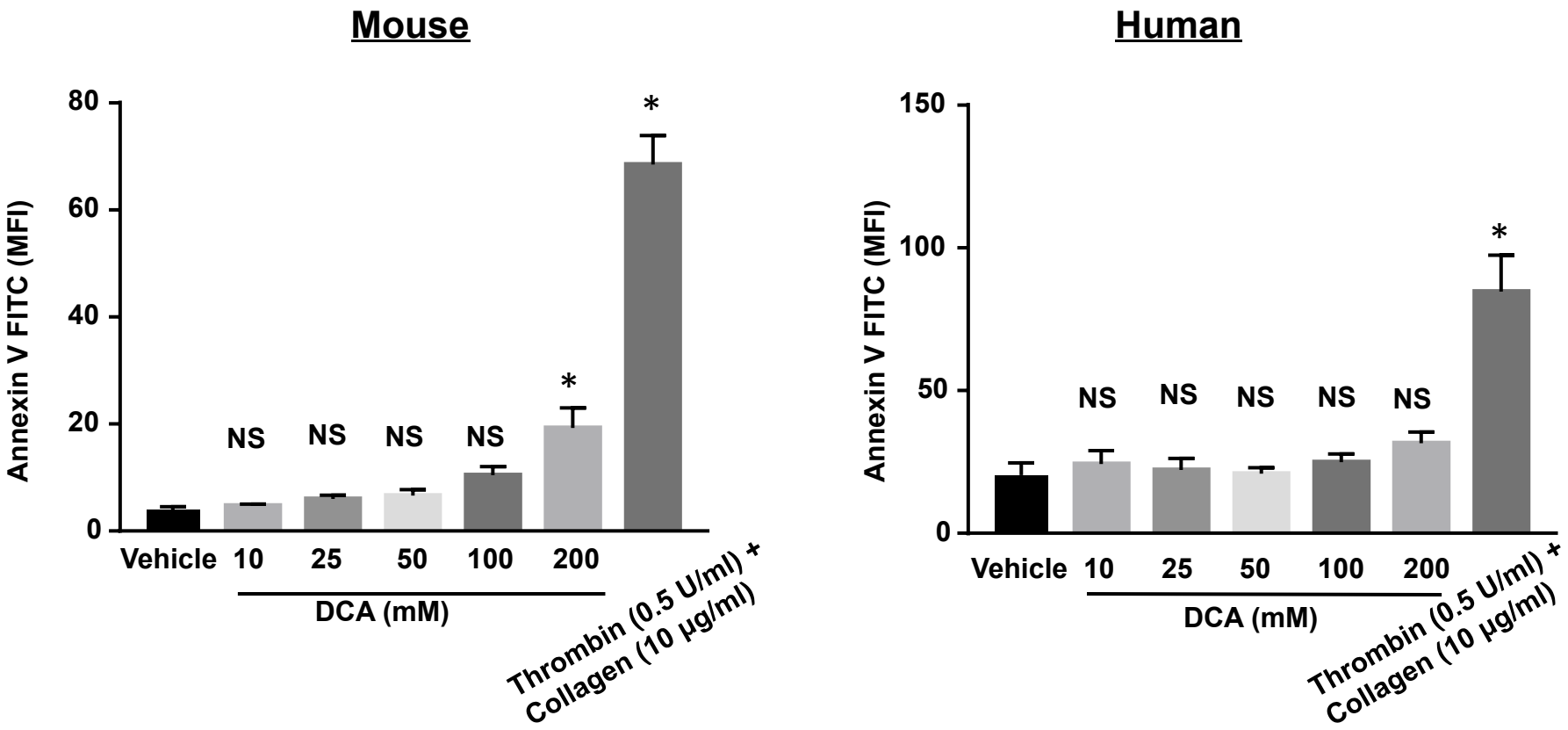
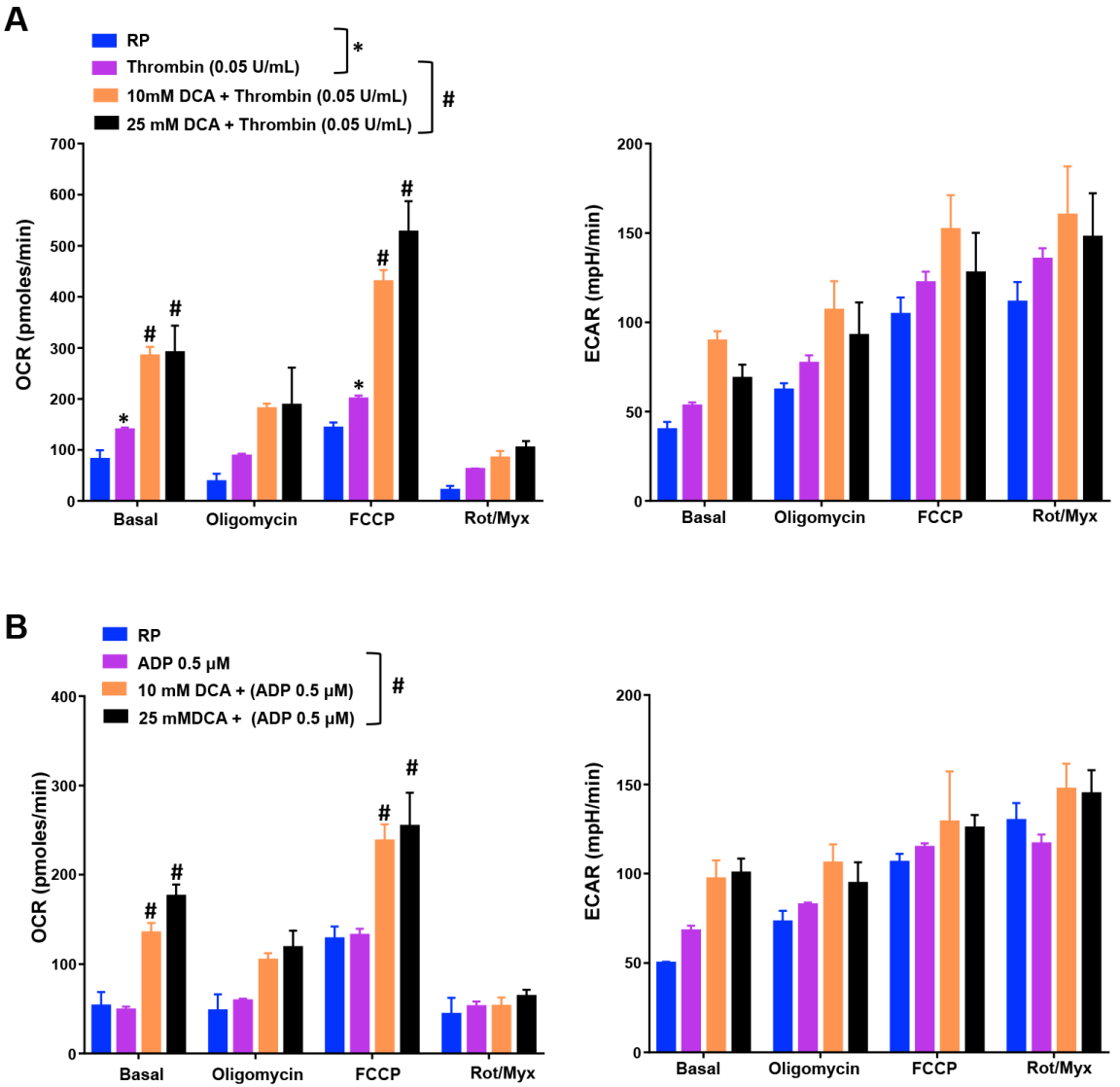
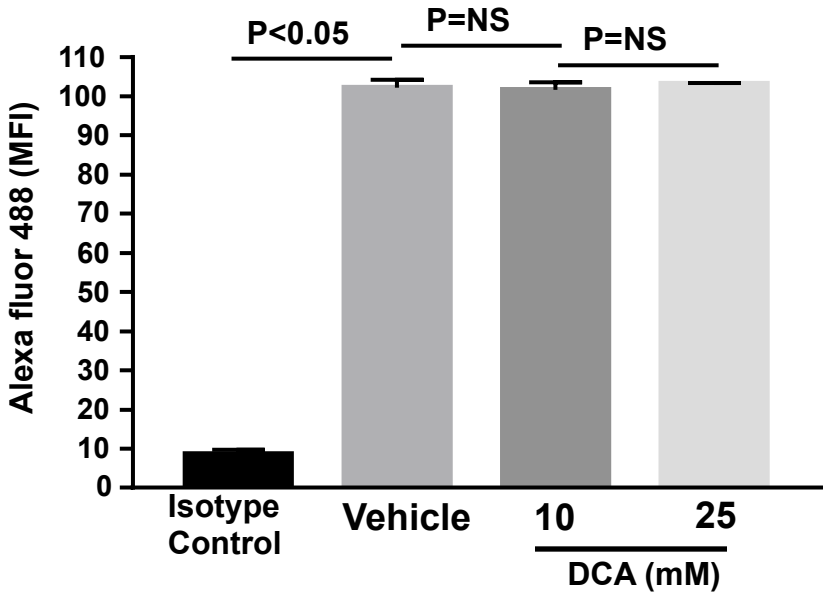


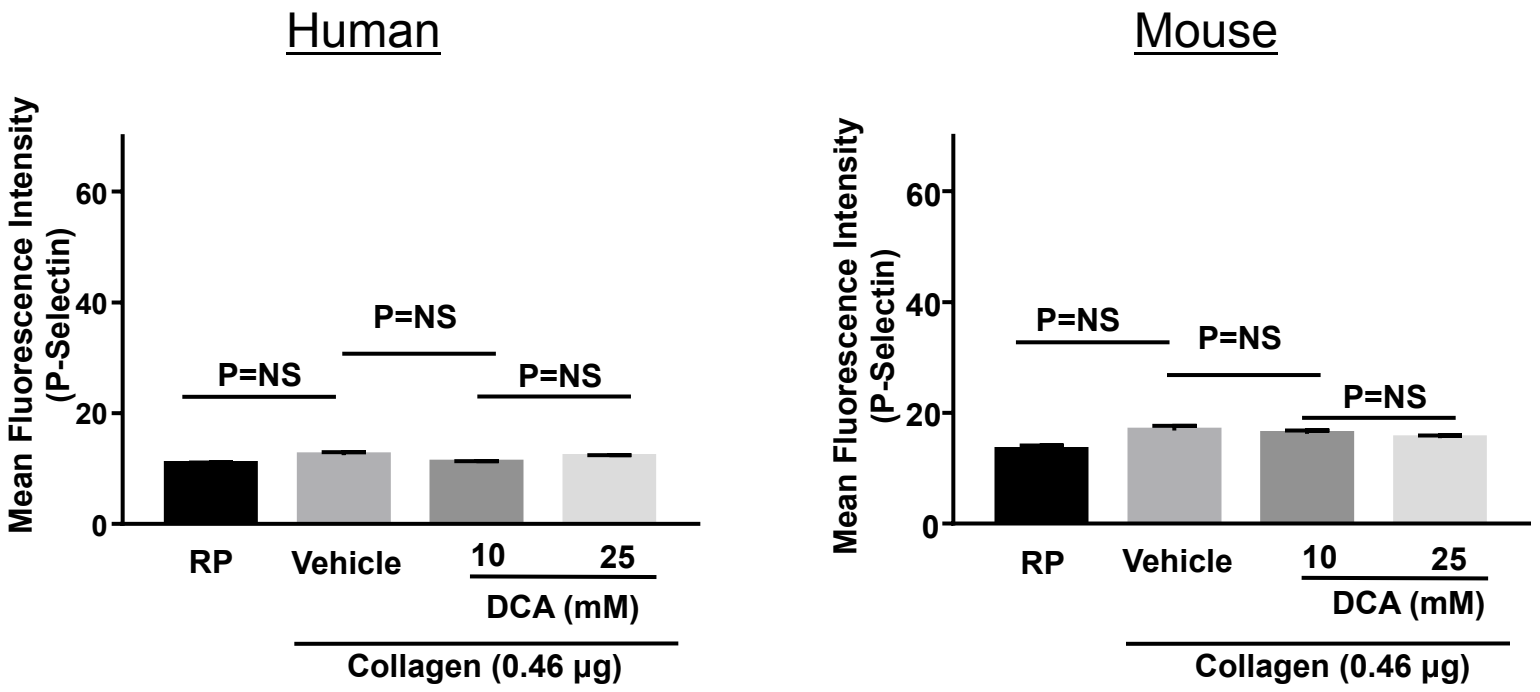
Supplementary Figure 1. Apoptotic effect of DCA on resting platelets. DCA up to a dose of 100 mM did not induce apoptosis in human and mouse platelets as determined by Annexin V assay. Activation with thrombin and collagen was used as positive control. For mouse studies, washed platelets were pooled from 3-4 mice. Values are represented as mean ± SEM, with n=3/group. *P<0.05 versus control (DCA untreated). NS=nonsignificant compared to control.



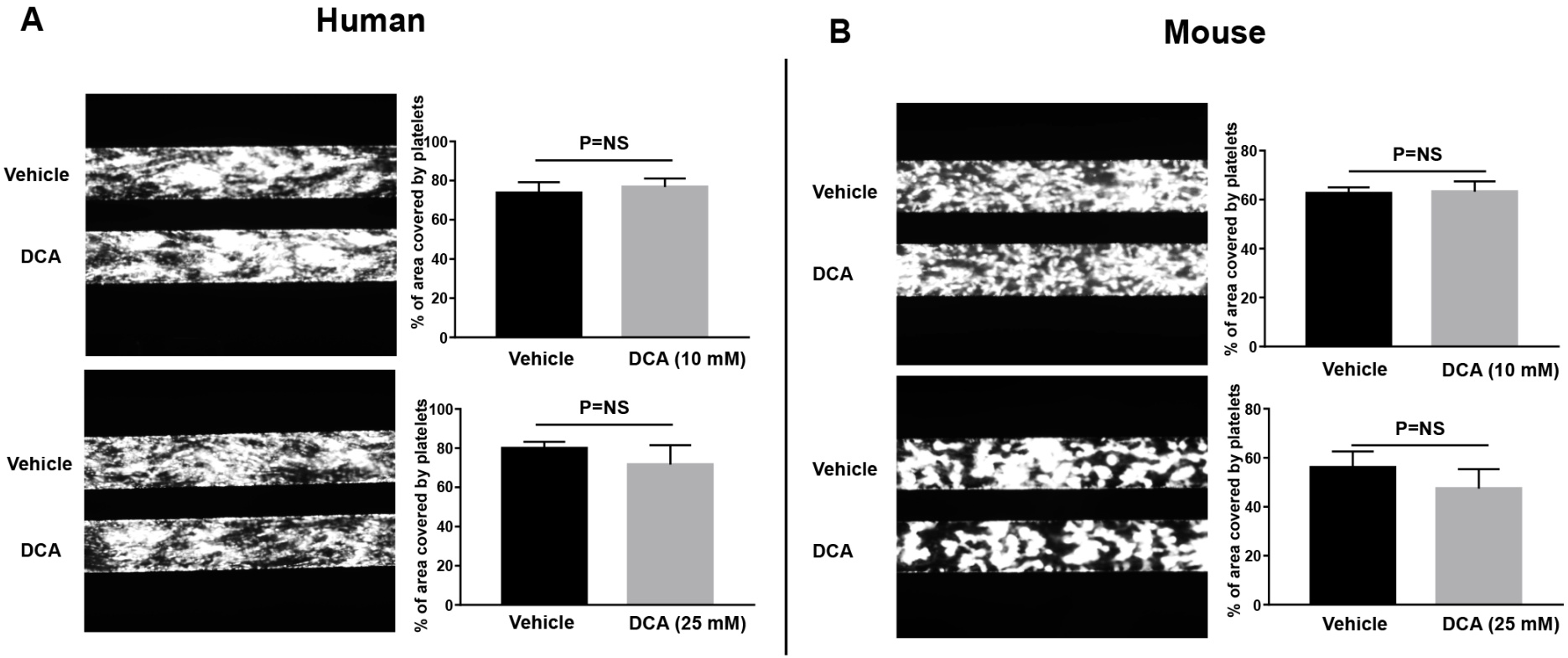
Supplementary Figure 2: DCA increases OCR but does not effect ECAR in platelets response to sub-optimal dose of thrombin and ADP. OCR and ECAR were measured in resting platelets (RP), and platelets in response to sub-optimal doses of thrombin (**A**) or ADP (**B**) in the presence or absence of DCA (10 and 25 mM) with sequential injections of 1 µg/ml oligomycin, 1 µM FCCP and 1 µM Rotenone/ 0.5 µM Myxothiazol. Thrombin was added at time zero just before the microplates were put in the Seahorse extracellular flux analyzer. All the bioenergetic measurements were normalized to protein content per well. Washed platelets were pooled from 5-6 mice/group. Values shown in bar graph are mean ± SEM, with n=9 values/group. *P<0.05 versus resting platelets (blue bar), #P<0.05 versus agonist-treated platelets (violet bar).



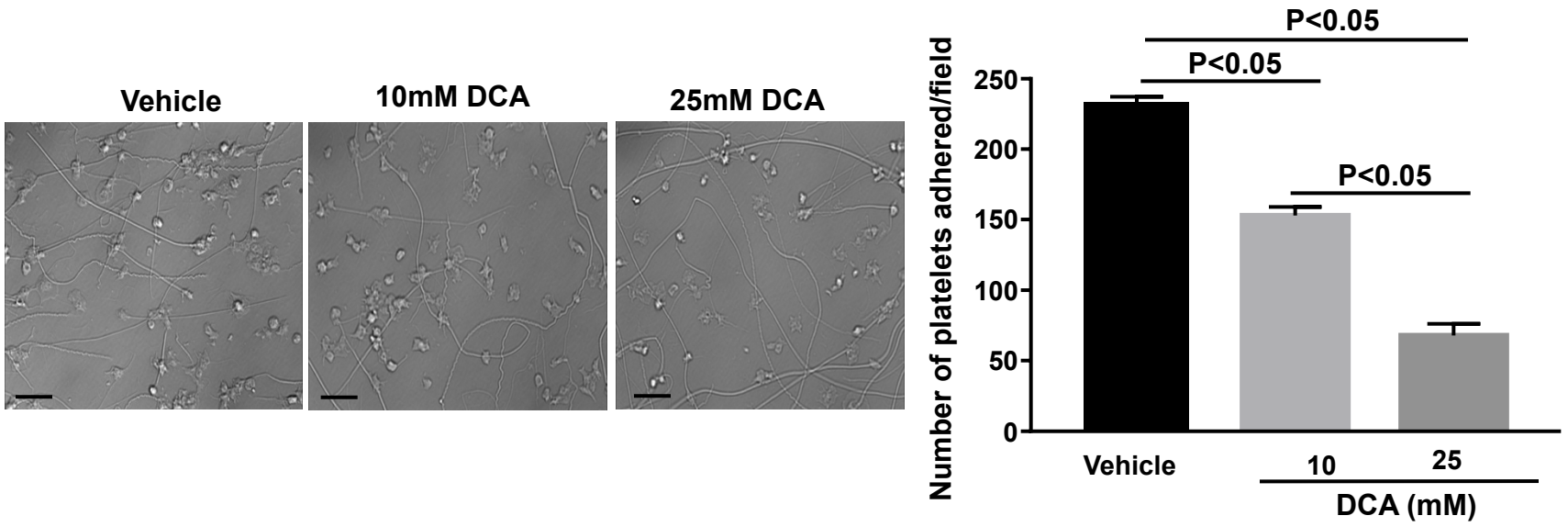
Supplementary Figure 3: Effect of DCA on surface expression of GP VI in platelets. Expression of GP VI on platelet membrane was studied by flow cytometry (as described in method). Mean fluorescence intensity (MFI) of Alexafluor 488 was calculated in resting platelets with or without DCA treatment in the presence of isotype control and Alexafluor 488 GP VI antibody. Washed platelets were pooled from 3-4 mice/group. Values shown in bar graph are mean ± SEM, with n=3 experiments/group..



Supplementary Figure 4: Effect of DCA on platelet alpha granule (assayed by P-selectin exposure) secretion measured by flow cytometric analysis. For mouse studies, PRP or washed platelets were pooled from 3-4 mice. Surface expression of P-selectin was studied (as described in methods) in quiescent, as well as collagen-stimulated platelets, either with or without DCA (10 and 25 mM) pretreatment. For mouse studies, washed platelets were pooled from 3-4 mice/group. Values shown in bar graph are mean ± SEM, with n=3 /group..



Supplementary Figure 5. Effect of DCA (10 and 25 mM) on thrombus formation under arterial shear on collagen-coated surfaces. . The analysis was done using flow chamber system from Bioflux microfluidics. Human (**A**) and mouse (**B**) whole blood pre-treated with 10 mM and 20 mM DCA (lower channels) or control (upper channels), was perfused over a collagen coated (150 µg/mL) surface for 10 min at a shear rate of $1500s^{-1}$. Left panels show representative images. Right panels show quantification of the surfaced area covered by fluorescent platelets after 10 min of perfusion. Four areas from different areas of flow chamber were analyzed from each blood sample. Data represent the mean percentage of surface area covered by fluorescence platelets ± SEM, with n=5/group for mice and 3/group for the human.



Supplementary Figure 6. DCA inhibits adhesion of platelets to the collagen matrix. Platelets were pretreated with DCA for 10 min at room temperature and then allowed to attach onto the collagen matrix (50 µg/mL) for 20 min at RT. Images were obtained using an Olympus IX-81 inverted microscope in differential interference contrast mode. The left panel shows representative images and right panel shows quantification of platelets attached/field. Ten fields from each slide were analyzed. Washed platelets were pooled from 3-4 mice/group. Data represent the mean of number of platelets attached/ field ± SEM, n=3-4/group. Scale bars=10 µm.