Online Supplement

Mitochondrial pyruvate dehydrogenase kinases contribute to platelet function and thrombosis in mice

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Running title: *Targeting PDKs inhibits platelet function*

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Materials

Prostaglandin I_2 (PGI_2), PAR4 peptide, Alexa-Fluor 488- conjugated phalloidin, and 3,3′-Dihexyloxacarbocyanine iodide (DiOC6) were purchased from Sigma (St. Louis, MO, USA). Collagen, ADP, and chronolume reagent were purchased from Chronolog Corporation (Havertown, PA, USA). CD62P-FITC 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. Primary Antibodies - PDK1 (ab207450), PDK3 (ab154549) and β-actin (ab8226) were from Abcam; PDK2 (Sc100534) was from Santacruz; PDK4 (1249-1-AP) was from ProteinTech; Anti-Phosphotyrosine Antibody, clone 4G10 (05-321) and Anti-phospho PDHE1-A type I (Ser300) Antibody (ABS194) was from Millipore Sigma; phosphoPDHα1 (Ser293) (#37115S), Total PDH (#3205S), phosphoPLCγ (#3871S), Total PLCγ (#3872S), PKC Substrate (#2261S), AMPKα (#5831), PKA C-α (#4782) and GAPDH (#5174S) were from Cell Signaling Technologies.

Alexa Fluor® 647 Annexin V was from Biolegend. Anti-hIL4R (clone 25463) was from R&D Systems. All other reagents were of analytical grade.

Methods

Mouse platelet isolation

The mice were bled from retro-orbital plexus in 1.5 mL tubes containing enoxaparin (0.3 mg/mL) or sodium citrate. The PRP/washed platelets were prepared as explained previously.[1,2] Briefly, the whole blood was centrifuged at 100 g for 5 mins at RT. Next, the supernatant containing platelet-rich plasma (PRP) was transferred to a fresh tube and incubated in PGI_2 (2 µg/mL) at 37°C for 5 minutes to prevent platelet activation. Next, the PRP was centrifuged at 600 g for 5 minutes at RT; the pellet was then rinsed by resuspending it in 1X modified Tyrode's buffer (1mM HEPES, 137 mM NaCl, 0.3 Mm Na_2HPO_4, 2 mM KCl, 12 mM NaHCO3, 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). The rinsing was repeated with Tyrode's buffer in the absence of PGI2 and finally resuspended in Tyrode's buffer (no PGI_2).

In vitro platelet aggregation and ATP secretion

Platelet-rich plasma (PRP) (2×10^8 cells/ml) was stirred (1200 rpm) at 37°C for 2 min in a whole blood/optical lumi-aggregometer (Chrono-log; model 700–2) before the addition of agonists (collagen, CRP-XL, PAR4 peptide, or ADP). Aggregation was measured as percent change in light transmission, where 100% refers to transmittance through the blank sample (PRP/Buffer). Platelet dense granule secretion was determined by measuring the release of ATP using a

luciferin-luciferase reagent. ATP release was performed in a lumi-aggregometer (Chrono-log; model 700–2) at 37°C.

Integrin αIIbβ3 activation and P-selectin exposure

Integrin αIIbβ3 activation and P-Selectin exposure were measured using PE-conjugated JonA and FITC-conjugated anti-CD62P antibody respectively. Mouse PRP ($2x10^8$ cells/ml were incubated with anti-CD62P or JonA antibody as described.[1,2] Following this, platelet were stimulated with CRP-XL or PAR-4 peptide for 10 minutes. Reactions were stopped by adding 0.2% (w/v) formyl saline. The samples were analyzed using a flow cytometer (FACS Calibur, Becton Dickinson) and data were collected from 10,000 events.

Annexin V binding assay

The phosphatidylserine exposure on the platelet surface was evaluated by measuring annexin V binding using flow cytometry. Washed platelets ($2x10^8$ cells/ml) from mouse were incubated with 2mM $CaCl_2$ for 5 minutes. Following this Alexa Fluor 647 conjugated Annexin V antibody was added. Platelets were stimulated with CRP-XL, PAR-4 peptide, or both for 10 minutes. Reactions were stopped by adding 0.2% (w/v) formyl saline. The samples were analyzed using a flow cytometer (FACS Calibur, Becton Dickinson) and data were collected from 10,000 events.

Clot retraction

Thrombin-stimulated fibrin clot retraction was measured in mouse PRP. First, Tyrode buffer was added to cuvettes and red blood cells to allow visualization of the clot. Following this, PAR was added to the cuvettes. Next, clot formation was initiated by adding thrombin (final concentration

1 U/ml) to the test tubes. Photographs were taken every 15 minutes, and the assay was terminated after 90 minutes when the clots were seen to have retracted completely. The clot area was analyzed using Image J software from NIH (Bethesda, MD, USA).

Platelet spreading

Platelet spreading was performed as described previously.[3,4] Glass coverslips were coated with collagen or fibrinogen (100 μg/ml each) (in modified PBS) for 1 hour. 1% (w/v) BSA was then added onto coverslips and incubated for 1 hour. Washed platelets were added onto coverslips at a density of $2x10^7$ cells/ml and incubated for 120 minutes at 37°C. Platelets were then fixed with 0.2% (w/v) formaldehyde for 10 minutes. Platelets were permeabilized with 0.2% (v/v) Triton-X-100 for 5 minutes. Alexa-Fluor 488 conjugated phalloidin was then added onto the coverslips for 1 hour. Coverslips were mounted onto slides with the addition of Prolong Gold Antifade mounting media. Samples were imaged, using a 100X oil immersion lens on an Olympus IX-81 Fluorescence Microscope.

Calcium Mobilization

The washed platelets (2×10^8 platelets) were loaded with 2.5 μm of Fura-2 LR/AM for 45 mins (37°C) to measure cytosolic Ca^{2+}.[3-5] Platelets were stimulated with CRP-XL in the presence of 1.3 mM $CaCl_2$. The baseline and CRP-XL-induced Ca^{2+} uptake was measured using a NOVOstar plate reader at 340 and 380nm excitation and 509 nm emission for Fura-2 LR/AM. The cytosolic Ca^{2+} was measured using the following formula $[Ca^{2+}] = Kd\beta(R-R_{min})/(R_{max}-R)$; where $R=F340/F380$, $R_{max,}$ and R_{min} are F340/F380 fluorescence ratios determined for Ca^{2+}-bound and Ca^{2+}-free forms of Fura-2, respectively; Kd is Ca^{2+} dissociation constants of Fura-2, and

$\beta = F_{min},380/F_{max},380$, where $F_{max},380$ and $F_{min},380$ are fluorescence intensities for λEx=380 nm determined for Ca^{2+}-bound and Ca^{2+}-free form of Fura-2, respectively.[11] The F_{min} was obtained by quenching the signal by adding 4mM EGTA. The F_{max} can be acquired by lysing the cells with Triton-X-100.

Glycolytic proton efflux rate (total and glycoPER) and oxygen consumption rate (OCR)

Platelets were diluted to a concentration of 2×10^7 platelets in XF Dulbecco's modified Eagle medium assay buffer (Dulbecco's modified Eagle medium with 1 mM pyruvate, 5.5 mM D-glucose, 4 mM L-glutamine, pH 7.4) and were seeded onto Cell-Tak coated XF24 microplates. The 24-well format Seahorse extracellular flux analyzer, XF24 (Seahorse Bioscience, Chicopee, MA), a service provided by Metabolic Core at the University of Iowa, was used to measure glycoPER and OCR as described.[1,6,7]

Bioflux flow chamber assay

In vitro thrombosis assays were performed using BioFluxTM 200 (Fluxion Biosciences, USA) microfluidics flow chamber. The channels were coated with Type I collagen (100 µg /ml) for one hour at room temperature and then blocked with 0.5% BSA for 30 minutes.
The whole blood was incubated with DiOC6 (5µM) for 30 minutes and perfused over the collagen-coated plate for 1 minute. The fluorescently labeled platelets/thrombi on the collagen-coated surface were analyzed using ImageJ software from NIH (Bethesda, MD, USA).

$FeCl_3$ injury-induced carotid thrombosis

Thrombus formation in the carotid artery after the $FeCl_3$ injury was assessed by intravital

microscopy as described previously.[1,2] Briefly, 10-12 weeks old mice were anesthetized using 100-mg/kg ketamine and 10-mg/kg xylazine. Platelets labeled with calcein green (2.5×10^9 platelets per kg) were infused through the retro-orbital plexus. The common carotid artery was carefully exposed and kept moist by super-fusion with warm (~37°C) saline. Whatman paper (0.5 x 1.5 mm) saturated with ferric chloride (5%) solution was applied topically for 2 mins and thrombus formation in the injured carotid vessel was monitored in real-time using a Nikon upright microscope (Plan Fluor 4X/0.2 objective), and thrombus growth overtime was recorded using a high-speed electron-multiplying camera for 40 minutes (the cut-off time point at which the experiment was terminated). The time to form an occlusive thrombus was considered as the time required for blood to stop flowing completely for >1 minute. Videos were evaluated offline using a Nikon computer-assisted image analysis program.

Laser injury-induced mesenteric artery thrombosis

As described previously, a micro-point laser ablation system (Andor Technology) was used to make injuries in the mesenteric arterioles.[1,2] Briefly, young mice [3- to 4-weeks (14-16 gm) old male)] were used to minimize fat surrounding the arterioles and facilitate the focusing of the laser. Fluorescent platelets labeled with calcein green (1.5×10^9 platelets per kg) were infused in anesthetized mice through the retro-orbital plexus. Infused platelets were isolated from adults (4-5 months) donor mice of the same genotype. Mesenteric arterioles having a diameter of approximately 80-100 μm (with shear rates of ~1300-1800 s-1) were used for the study. The specific illumination of the area of interest was carried out through the microscope eyepiece. The wavelength of light in the 365-400 nm range with a maximum output of 50–500 uJ was used for illumination. The power and frequency of pulses were regulated by software and empirically

defined. Thrombus growth in the injured vessel was monitored in real-time using a Nikon upright microscope with a Plan Fluor 10X/0.3 objective. Thrombus formation over time was recorded using a high-speed EM camera for 3-4 min. In our experimental setup with the laser injury model, the thrombus grows to its maximum size in approximately 1 min and then gradually disintegrates over time. Videos were evaluated, and mean fluorescence intensity was calculated using a Nikon computer-assisted image analysis program.

Tail bleeding assay

Tail-transection bleeding time was measured as described previously.[1,2] Briefly, mice (Approximately 10 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 bloodstream 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.

Platelet depletion assay in the hIL-4Rα/GPIbα-Tg mice

Anti-hIL4R antibody at a concentration of 2.5 μg/g body weight was infused through the retro-orbital plexus to deplete platelets from hIL-4Rα/GPIbα-Tg mice.[8] After 2 hours, 1.25×10^9 platelets from WT or PDK2/4$^{-/-}$ mice were injected through the retro-orbital plexus, and mice were subjected to FeCl3 injury-induced carotid thrombosis model.

Immunoblotting

Platelet proteins were separated on 4-20% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gradient gels and electrophoretic transferred to PVDF (polyvinylidene fluoride) membrane by using Bio-Rad western blotting system. Membranes were blocked with 5% BSA 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 overnight with the primary antibody, 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).

References

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Supplementary Table 1

	WT	PDK2/4$^{-/-}$	P-value
Body weight (g)	27.33 ± 0.33	26.00 ± 1.0	0.2346
WBC (10^3/µL)	7.7 ± 0.8	8.2 ± 1.3	0.7556
RBC (10^6/µL)	10.7 ± 0.1	11.2 ± 0.2	0.1172
HGB (g/dL)	14.5 ± 0.2	14.0 ± 0.4	0.2828
HCT (%)	53.8 ± 0.5	53.0 ± 0.7	0.4458
Platelets (10^3/µL)	1224 ± 46.5	1208 ± 83.8	0.8873
Neutrophils (10^3/µL)	0.4 ± 0.1	0.5 ± 0.1	0.3292

Table S1: Body weight and complete blood counts of WT and PDK2/4$^{-/-}$ were obtained using an automated veterinary hematology analyzer (ADVIA-120). Values are expressed as mean ± SEM. N=6 mice/group. Statistical analysis: Student t-test.

Supplementary figures

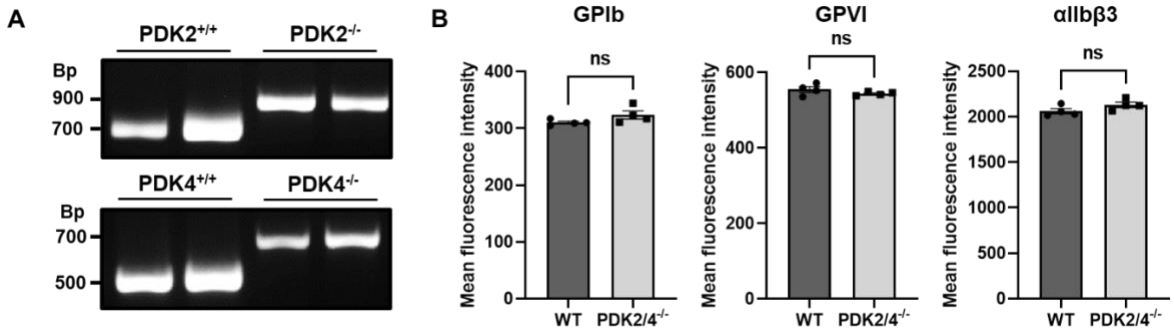

FIGURE S1. (A) The deletion of PDK2 and PDK4 was confirmed using a polymerase chain reaction. **(B)** Using flow cytometry, we analyzed the expression levels of GPIb, GPVI, and αIIbβ3 in resting platelets from WT or PDK2/4$^{-/-}$ mice. The results are expressed as mean fluorescence intensity. Values are mean ± SEM, n=4 mice/group. ns: non-significant. Statistical analysis: Mann-Whitney U test.

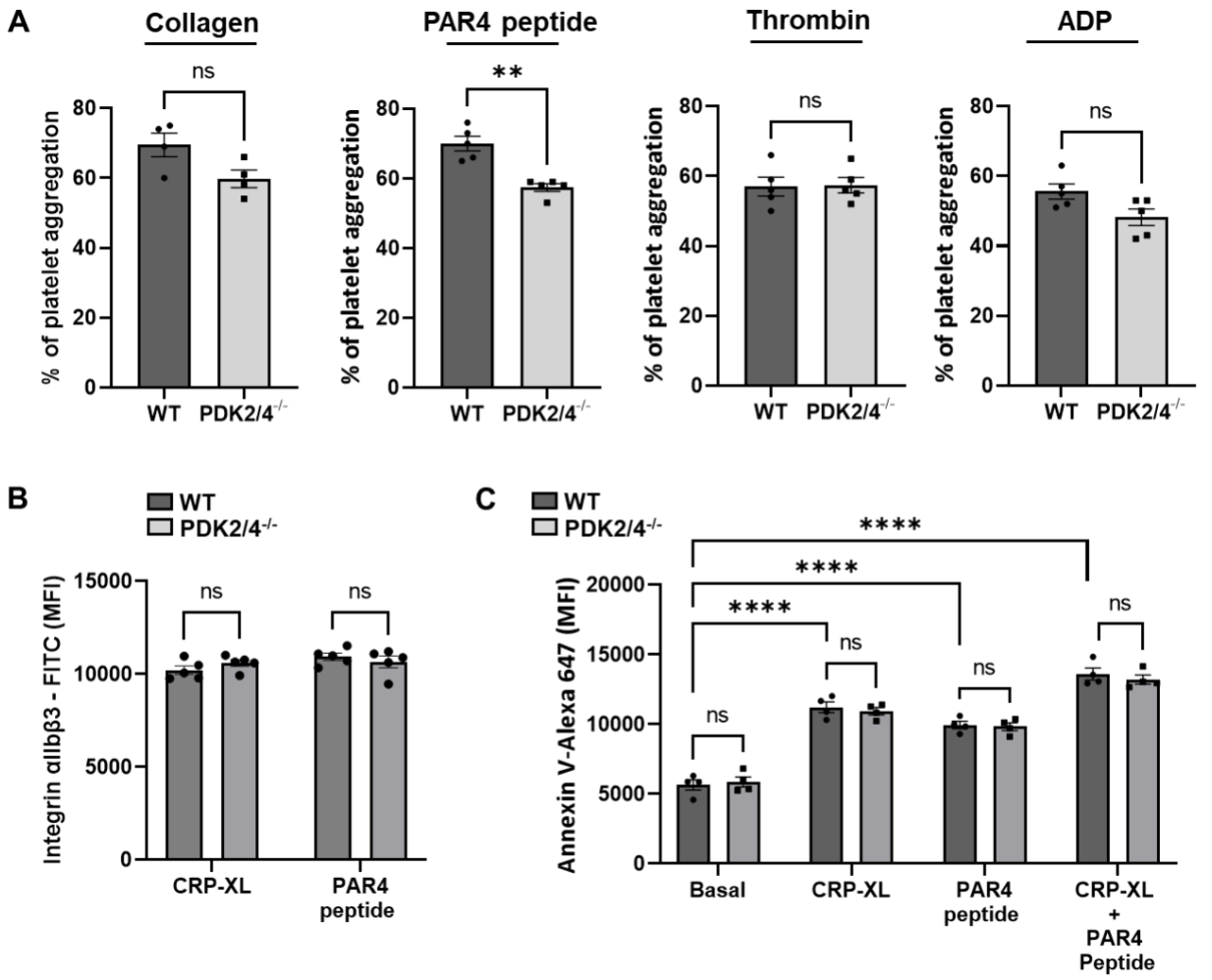

FIGURE S2. (A) Platelet-rich plasma (PRP) from WT or PDK2/4$^{-/-}$ mice was stimulated with collagen (0.5 µg/ml), PAR4 peptide (100 µM), Thrombin (0.04 U/ml) or ADP (0.5 µM). Results are expressed as the percent change in light transmission with respect to the blank (platelet-poor plasma/buffer without platelets), set at 100%. The representative aggregation curves are shown. Values are mean ± SEM, n=4-5 mice/group. Statistical analysis: Mann-Whitney U test, **P<0.01. **(B)** The expression levels of integrin αIIbβ3 in CRP-XL (0.1 µg/ml) or PAR4 peptide (100 µM) stimulated WT and PDK2/4$^{-/-}$ platelets was evaluated using flow cytometry. The results are expressed as mean fluorescence intensity. Values are mean ± SEM, n=5 mice/group. ns: non-significant. Statistical analysis: Two-way ANOVA followed by Tukey's multiple comparisons test. **(C)** Phosphatidylserine exposure on platelet surface was evaluated by measuring annexin V binding using flow cytometry in resting and CRP-XL (0.1 µg/ml) or PAR4 peptide (100 µM) stimulated WT and PDK2/4$^{-/-}$ platelets. Values are mean ± SEM, n=4 mice/group. ns: non-significant. Statistical analysis: Two-way ANOVA followed by Tukey's multiple comparisons test.

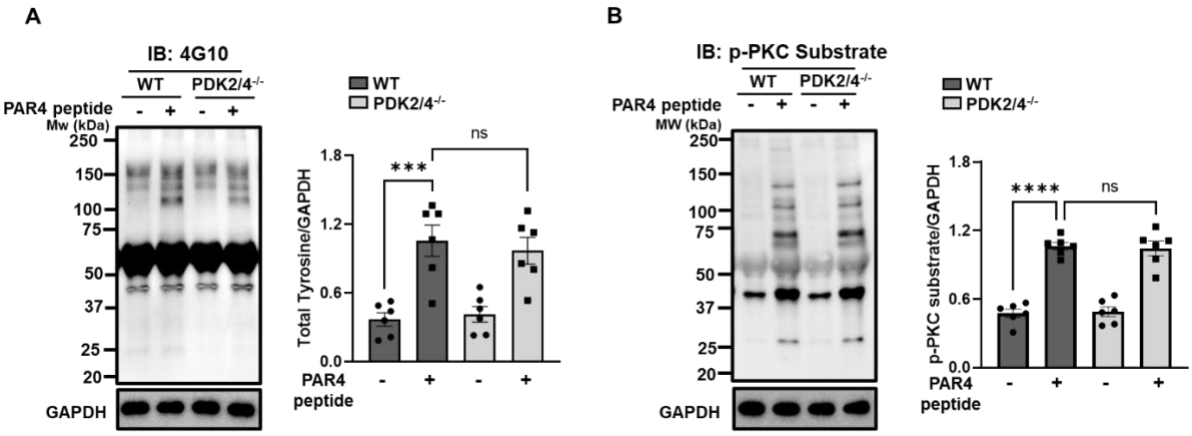

FIGURE S3. (A) Total tyrosine and **(B)** PKC substrate phosphorylation was measured in resting and PAR4 peptide (70 µM) stimulated WT and PDK2/4$^{-/-}$ platelets. Representative Western blot for total tyrosine and PKC substrate phosphorylation are shown. GAPDH was used as a loading control. The bar graphs show densitometry analysis of immunoblots. Values are mean±SEM, n=6 mice/group. ns: non-significant. Statistical analysis: Two-way ANOVA followed by Tukey's multiple comparisons test, ***P<0.001.

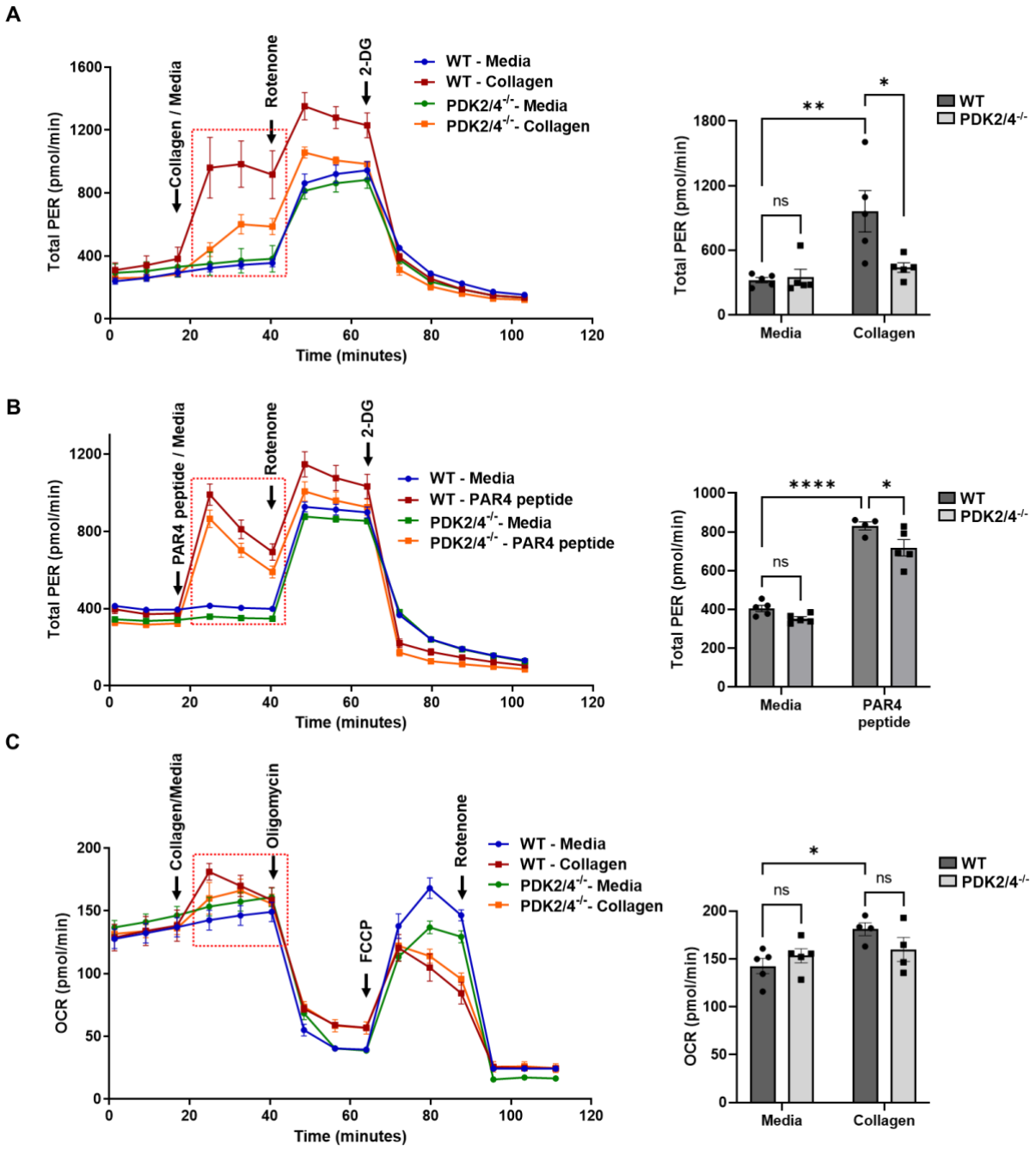

FIGURE S4. The total proton efflux rate (Total PER) was measured in WT or PDK2/4$^{-/-}$ platelets stimulated with **(A)** collagen (25 µg/ml) or **(B)** PAR4 peptide (50µM) using a Seahorse extracellular flux analyzer. **(C)** The oxygen consumption rate (OCR) was measured in WT or PDK2/4$^{-/-}$ platelets stimulated with collagen (25 µg/ml) using a Seahorse extracellular flux analyzer. The bar graph shows the quantified data (for the values present in the red box of the line graph). Values are mean ± SEM, with n = 4 to 5 mice/group. Statistical analysis: Two-way ANOVA followed by Tukey's multiple comparisons test, *P<0.05, **P<0.01 and ****P<0.0001.

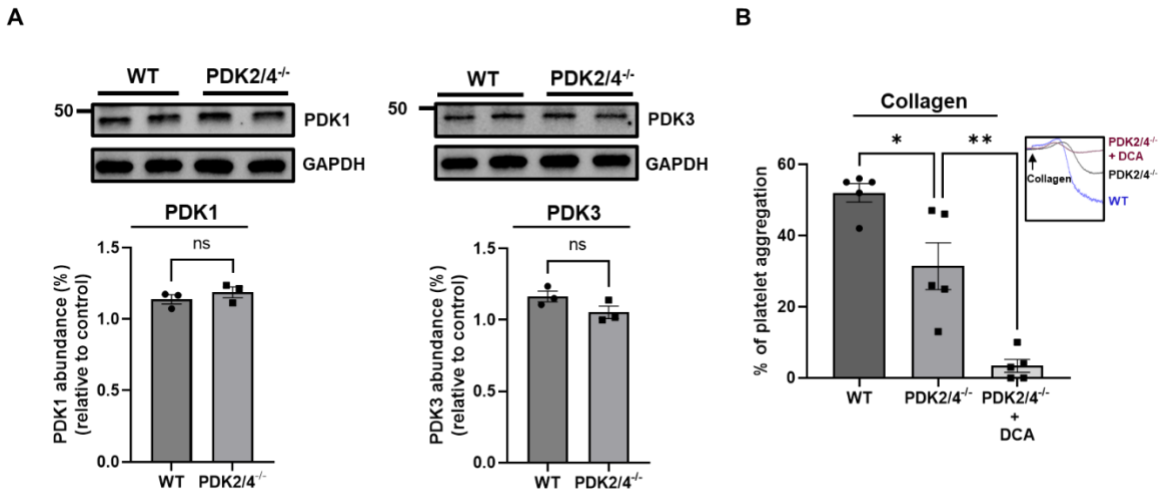

FIGURE S5. (A) The expression of PDK1 and PDK3 in WT and PDK2/4$^{-/-}$ mice was evaluated using a Western blot. Values are mean ± SEM, n=3 mice/group. Statistical analysis: Mann-Whitney U-test. **(B)** Aggregation was measured in collagen (0.4 μg/ml) stimulated PRP from WT or PDK2/4$^{-/-}$ platelets. Values are mean ± SEM, n=5 mice/group. Statistical analysis: One-way ANOVA followed by Tukey's multiple comparisons test *P<0.05 and **P<0.01.