Critical role of peroxisome proliferator-activated receptor α in promoting platelet hyperreactivity and thrombosis under hyperlipidemia

Li Li,[1,2*] Jiawei Zhou,[3*] Shuai Wang,[1,2*] Lei Jiang,[4] Xiaoyan Chen,[1,2] Yangfan Zhou,[1,2] Jingke Li,[1,2] Jingqi Shi,[1,2] Pu Liu,[5] Zheyue Shu,[6,7] Frank J. Gonzalez,[8] Aiming Liu[9#] and Hu Hu[1,2,10#]

[1]Department of Pathology and Pathophysiology and Bone Marrow Transplantation Center of the First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China; [2]Institute of Hematology, Zhejiang Engineering Laboratory for Stem Cell and Immunotherapy, Zhejiang University, Hangzhou, China; [3]State Key Laboratory of Diagnosis and Treatment for Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China;[4]Department of Pathology, Zhejiang Provincial Key Laboratory of Pathophysiology, Ningbo University School of Medicine, Ningbo, China; [5]Department of Pathology of the First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China; [6]Division of Hepatobiliary and Pancreatic Surgery, Department of Surgery, First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China; [7]Key Laboratory of Combined Multi-Organ Transplantation, Ministry of Public Health, Hangzhou, China; [8]Laboratory of Metabolism, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD, USA; [9]Ningbo University School of Medicine, Ningbo, China; [10]Key Laboratory of Disease Proteomics of Zhejiang Province, Hangzhou, China.

[*]LL, JZ and SW contributed equally as co-first authors.
[#]AML and HH contributed equally as co-senior authors.

Correspondence:

Hu Hu
huhu@zju.edu.cn

Aiming Liu
liuaiming@nbu.edu.cn

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SUPPLEMENTAL DATA

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Supplemental methods

Material

F2/thrombin (T6884), H2DCF-DA (D8665), mepacrine (quinacrinedihydrochloride) (Q3251), SynthChol (C3045) were from Sigma Aldrich. Collagen (385) and luciferase (395) were from Chrono-Log Corporation. PAPC (1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine, 15101-100) and KODiA-PC (1-(palmitoyl)-2-(5-keto-6-octene-dioyl) phosphatidylcholine, 62945-500) were purchased from Cayman. The following reagents were used, p38 inhibitor SB203580 (Sigma, S8037), AKT III inhibitor SH-6 (Sigma, 124009), Carnitine palmitoyl transferase I (CPT-I) inhibitor etomoxir (Sigma, E1905), xanthine oxidase inhibitor allopurinol (Sigma, PHR1377), NADPH oxidase (NOX) inhibitor VAS2870 (MedChemExpress, 722456-31-7), PPARα agonist WY14643 (Selleck, S8029), PPARα antagonist GW6471 (Selleck, S2798). The following antibodies were used, anti-phospho-Akt Ser^{473} (Cell Signaling Technology, 4060), anti-phospho-Akt Thr^{308} (Cell Signaling Technology, 13038), anti-phospho-p38 Thr^{180}/Tyr^{182} (Cell Signaling Technology, 9216), anti-phospho-ERK1/2

Thr^{202}/Tyr^{204} (Cell Signaling Technology, 9101), anti-phospho-Src Tyr^{418} (Bioworld, BS4675), anti-phospho-p40phox (Santa Cruz, sc-33403), anti-Akt (Cell Signaling Technology, 4685), anti-p38 (Cell Signaling Technology, 8690), anti-ERK1/2 (Cell Signaling Technology, 4695), anti-Src (Cell Signaling Technology, 2109), PPARα (GeneTex, GTX101098), anti-β-actin (Santa Cruz, sc-47778), anti-GAPDH (Sungenebiotech, KM9002). Alexa Fluor 488- and 546-tagged secondary antibodies for immunostaining were from Molecular Probes.

Hematologic analysis

Complete blood count and hematocrit were conducted by Animal hospital affiliated to Zhejiang University with ProCyteDx™ hematology analyzer (IDEXX Laboratories, Westbrook,Maine, USA).

Tail-bleeding time

As previously described,[1] tails of anesthetized mice were cut 0.5 cm from the tip and immediately immersed in saline (37°C). The time taken for the bleeding to stop (no blood flow for 1 minute) was recorded. Tail-bleeding assays were stopped at 900 seconds if the bleeding did not stop.

$FeCl_3$-induced murine carotid artery thrombosis model

As previously described,[2, 3] 8- to 10-week-old mice were anesthetized, a midline incision was made in the neck, and the right carotid artery was isolated from surrounding tissues. Carotid artery thrombosis was induced by treating the exposed artery with a piece of filter paper (1 × 2

mm) soaked in 10% $FeCl_3$ for 3 minutes, and blood flow through the carotid artery was monitored with a laser-speckle blood flow imaging system (RFLSI III; RWD Life Science, Shenzhen, China). The end points were: 1) blood flow perfusion was less than 50 for >1 minute; or 2) occlusion is not seen after 15 minutes of FeCl3 injury. In this case, the time will be recorded as 15 minutes for statistical analysis.

$FeCl_3$-induced mesenteric arteriole thrombosis model

Fluorescently labeled platelets (10^8 platelets) were injected through the tail vein. The intestines were exposed, and the mesentery was spread on the translucent stage of the fluorescence microscope. Injury of mesenteric arterioles (60-100 μm in diameter) was induced by topical application of 10% $FeCl_3$. Arterioles were monitored for 60 minutes or until complete occlusion occurred (blood flow stopped for > 1 minute).

Flow restriction model

Mice were anesthetized and placed in a supine position. After gentle separation from aorta, IVC was ligated by a 6.0 polypropylene suture immediately below the renal veins to obtain complete blood stasis. For partial flow restriction (stenosis), IVC ligation was performed over a 30-gauge needle and then the needle was removed. The needle was placed outside the vessel so that piercing or any other injury to the IVC wall was completely avoided. After surgery, peritoneum and skin were closed by monofilament absorbable suture and 6.0 silk, respectively. Mice were euthanized after 48 h, and thrombi developed in the IVC below the suture (toward the tail) were taken for analysis.[4]

In vitro thrombus formation under flow conditions

Thrombus formation was evaluated in a microfluidic whole-blood perfusion assay on a fibrillar collagen matrix under arterial shear conditions (a shear rate of 1000 s^{-1}) using a Bioflux-200 system (Fluxion, South San Francisco, CA). Bioflux plates were coated with fibrillar collagen at 50 μg/mL overnight, and after blocking with 0.5% bovine serum albumin in PBS for 15 minutes, plates were placed on an inverted microscope and 0.4 mL of mepacrine-labeled blood applied to the inlet well. A shear force of 40 dynes/cm^2 was applied, and the platelets were allowed to adhere to collagen for 5 minutes. Adherent platelets were viewed with an inverted fluorescence microscope using an S Plan Fluor lens (× 10/0.4 numerical aperture objectives). Images were acquired with a Nikon DS-Qi1-U3 CCD camera. The platelet-covered area was measured using Bioflux software (Fluxion, South San Francisco, CA).

Electron microscopy

Washed wild-type (WT) or *Pparα*$^{-/-}$ platelets were fixed with 2.5% glutaraldehyde in modified Tyrode buffer. After fixation, staining, and dehydration, the platelets were infiltrated in embedding medium. Thin sections were stained with uranyl acetate and lead citrate. Samples were examined at 80 kV using a Tecnai 10 transmission electron microscope (Tecnai 10, FEI, Hillsboro, OR, USA), and images were captured with an ES500W (782) camera (Gatan) using Digital micrograph software (Gatan).

Flow cytometric analysis

WT or *Pparα*$^{-/-}$ platelets (10^6) were labeled for 10 minutes at room temperature with different specific antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD41 monoclonal antibody (mAb) (BD Biosciences, MWReg30), FITC-conjugated anti-mouse CD42b mAb (Emfret Analytics GmbH & Co. KG, Xia.B2), FITC-conjugated anti-mouse GPVI mAb (Emfret Analytics, JAQ1), PE-conjugated CD62P (M130-2, Emfret Analytics, Germany), PE-conjugated JON/A (M023-2, Emfret Analytics, Germany).The samples were analyzed with a flow cytometer (EPICSXL; Beckman Coulter).

Platelet aggregation and ATP secretion

Platelet aggregation and secretion were measured using a lumi-aggregometer (Chrono-Log) at 37°C with stirring (1200 rpm). Washed platelets (2×10^8/mL) in modified Tyrode buffer were stimulated with thrombin, collagen, U46619, and ADP (in the presence of fibrinogen). Platelet secretion was monitored in parallel with aggregation as adenosine triphosphate (ATP) release with the addition of luciferin/luciferase reagent to the platelet suspension. Inhibitor was incubated with the platelets for 10 minutes prior to stimulation.

Platelet spreading on fibrinogen

Glass coverslips were coated with 20 μg/mL fibrinogen in 0.1 M NaHCO3 (pH 8.3) at 4°C overnight. Washed platelets (2×10^7/mL, 200 μL) or platelets preincubated with 1 U/mL apyrase or 1 μM ADP for 10 minutes at 37°C were allowed to spread on the fibrinogen-coated surfaces at 37°C for 60 minutes. After 3 washes with phosphate-buffered saline (PBS), the platelets were fixed, permeabilized, and stained with fluorescein-labeled phalloidin (Molecular

Probes, Eugene, OR). Adherent platelets were viewed with an inverted fluorescence microscope (Nikon Ti-S, Japan). Images were acquired using a Nikon DS-Qi1-U3 camera. The platelet-covered area was measured using NIS-D software (Nikon).

Clot retraction

Murine platelets were resuspended using citrated human platelet-depleted plasma to a concentration of 4×10^8/mL. Recombined plasma was induced to coagulate by stimulation with thrombin (0.4 U/mL). The clots were allowed to retract at room temperature and photographed at various time points.

Immunoblotting

Washed WT, *Pparα*$^{+/-}$ or *Pparα*$^{-/-}$ platelets (250 μL; 2×10^8/mL) were stimulated with or without collagen and thrombin in a Chrono-Log aggregometer. After 5 minutes of stimulation, the reaction was stopped by adding 4 × sodium dodecyl sulfate (SDS) sample buffer (100 mM Tris-HCl, pH 6.8, 3.2% SDS, 16% glycerol, 400 mM dithiothreitol, 0.016% bromophenol blue, 4 mM sodium fluoride, 4 mM sodium metavanadate) and boiled for 5 minutes. Proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes, which were incubated with primary antibodies. After incubation with the corresponding secondary antibodies (horseradish peroxidase-conjugated immunoglobulin Gs; Jackson Immunol Research Laboratories), proteins were visualized by enhanced chemiluminescence and imaged using a Syngene G: BOX Chemi XR system and Gene Snap software (Syngene, Frederick, USA).

Measurement of intracellular ROS

Intracellular ROS was measured according to a previously reported method.[5] Briefly, washed platelets (1×10^8/mL) were incubated for 15 minutes at 37 °C with fluorogenic probe 2',7'-dichlorofluorescein diacetate (H2DCF-DA, 50 μM) in the dark, then pretreated with or without designated inhibitors for 10 minutes, followed by stimulation with convulxin or thrombin for 10 minutes at 37 °C in the darkness. Samples were diluted with 10-fold PBS containing 50 μM H2DCF-DA and analyzed by flow cytometry immediately.

Serum lipid profile analysis

Serum lipid profile (TC and TG) was evaluated by enzymatic analysis using four commercial kits (Nanjing Jiancheng Bioengineering Institute, China).

Platelets culture

As previously described,[6] washed platelets were resuspended at 10^8/mL in serum-free M199 medium, placed in round-bottom polypropylene tubes (BD Biosciences, USA), and cultured in a 37°C humidified incubator (HF90, Heal Force, China).

Cell Culture

The human megakaryocytic cell line (Meg-01) was cultured in RPMI-1640 medium (Gibco) supplemented with 1% heat-inactivated fetal bovine serum, 20 mM L-glutamine for at least 5 passages allowing for their adaptation prior to use. Cells were treated in medium with

cholesterol or fatty acid (oleic acid (OA, 400 μM) and palmitic acid (PA, 200 μM)) for 24 hours. For experiments with pharmacological inhibition of intracellular reactive oxygen species (ROS) or NF-κB, 1 mM *N*-acetyl-L-cysteine (NAC), 1 mM DL-dithiothreitol (DTT) or 10 μM BAY 11-7082 was added. At the end of the treatment, the cells were collected and processed for RNA and protein extraction.

RNA extraction and real-time polymerase chain reaction (PCR)

Total RNA was extracted from megakaryocytes using TRIzol reagent (Invitrogen, 15596026), following the manufacturer's instructions. RNA samples (1 μg) were subsequently reverse-transcribed into cDNA with a Reverse Transcription Reagent Kit (TOYOBO, FSQ-101), and the resulting cDNA was amplified by semi-quantitative RT-PCR using SYBR Green Mix (CWBIO, CW0957M). PCR products were confirmed by a single band of the expected size on 2% agarose gels. All the primers used for RT-PCR are shown in Supplemental table 4.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed using the Simple ChIP enzymatic chromatin immunoprecipitation kit (Beyotime Biotechnology) according to the manufacturer's protocol with minor modification. Briefly, Meg-01 cells were cross-linked with 1.5% (v/v) formaldehyde for 10 minutes and then stopped with 0.125 M glycine. Subsequently, nuclei were collected and chromatin sonicated to desired chromatin length (300bp-500bp). Sonicated lysates were then diluted to 2 mL, with protease inhibitor cocktail, and 20 μL of this solution was removed for PCR analysis (Input). After preclearing with Protein A/G Plus Agarose (Beyotime

Biotechnology) for 1 hour at 4°C, antibody specific to nuclear factor NF-κB p65 subunit was added and incubated at 4°C overnight on a rocking platform. Immune complexes were collected by the addition of Protein A/G Plus Agarose for 1 hour at 4°C, and the agarose beads were extensively washed with solutions of increasing ionic strength. Bound immune complexes were eluted and cross-links were reversed by incubating at 65°C for 4 hours. Samples were then treated with proteinase K, and DNA was purified using DNA purification spin columns. DNA was amplified for detection of the binding motif of transcription factors in PPARα regulation region by PCR using the primers in Supplemental table 5.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical significance was evaluated with Paired *t*-test, 2-tailed Mann-Whitney U tests and 2-way ANOVA tests using statistical software GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Supplemental tables

Supplemental table 1. Hematologic analysis of WT and *Pparα*$^{-/-}$ mice

	WT (n = 9)	*Pparα*$^{-/-}$(n = 9)	*P*
RBC, ×10^{12}/L	9.64 ± 0.29	9.21 ± 0.17	0.2330
WBC, ×10^{9}/L	4.28 ± 0.33	3.97 ± 0.51	0.3757
Platelet, ×10^{9}/L	591.10 ± 64.67	605.60 ± 44.72	0.7572
Hematocrit, %	42.33 ± 1.21	39.00 ± 0.65	0.0569
Hemoglobin, g/L	144.40 ± 4.75	136.70 ± 2.36	0.1881
MPV, fL	4.77 ± 0.06	4.63 ± 0.06	0.1960

No abnormalities or significant differences between WT and *Ppara*$^{-/-}$ mice were found for hematologic parameters (2-tailed Mann-Whitney test). Values are expressed as means ± SEM. RBC, red blood cell; WBC, white blood cell; MPV, mean platelet volume; WT, wild type.

Supplemental table 2. Serum TG and TC in control diet and high-fat diet fed *Pparα*$^{+/+}$/*Apoe*$^{-/-}$ and *Pparα*$^{-/-}$/*Apoe*$^{-/-}$ mice.

	Control diet		High fat diet	
	***Pparα*$^{+/+}$/*Apoe*$^{-/-}$**	***Pparα*$^{-/-}$/*Apoe*$^{-/-}$**	***Pparα*$^{+/+}$/*Apoe*$^{-/-}$**	***Pparα*$^{-/-}$/*Apoe*$^{-/-}$**
	(n = 12)	**(n = 9)**	**(n = 10)**	**(n = 11)**
TG (mg/dL)	46.08 ± 6.60	60.84 ± 18.40	74.24 ± 16.91	80.62 ± 7.27**
TC (mg/dL)	668.40 ± 44.63	720.70 ± 84.89	1392 ± 105.80***	1376 ± 107.30***

Plasma lipids levels were detected in mice at 8 weeks (2-tailed Mann-Whitney test). Values are expressed as means ± SEM. TG, triglycerides; TC, total cholesterol. $^{**}P < 0.01$; $^{***}P < 0.001$ compared to the values of control diet fed *Pparα*$^{+/+}$/*Apoe*$^{-/-}$ mice.

Supplemental table 3. Characteristics of study population

Variable	Control (n = 24)	HTG (n = 34)	Control (n = 16)	HTC (n = 16)
Age (year)	45 ± 2	47 ± 2	50 ± 2	52 ± 2
Sex (M/F)	15/10	27/9	7/9	7/9
TG (mM/L)	0.94 ± 0.07	2.47 ± 0.10***	0.89 ± 0.06	1.73 ± 0.32**
TC (mM/L)	4.13 ± 0.10	4.75 ± 0.10***	4.24 ± 0.13	6.13 ± 0.23***
HDL (mM/L)	1.25 ± 0.05	0.98 ± 0.04***	1.37 ± 0.08	1.34 ± 0.09
LDL (mM/L)	2.41 ± 0.10	2.75 ± 0.10*	2.44 ± 0.12	4.01 ± 0.21***
VLDL (mM/L)	0.46 ± 0.02	1.02 ± 0.09***	0.43 ± 0.02	0.79 ± 0.20
Glu (mM/L)	4.62 ± 0.08	4.72 ± 0.12	4.56 ± 0.12	4.63 ± 0.08

Numeric variables were analyzed with the 2-tailed Mann-Whitney test. Sex distribution was analyzed with the Fisher's exact test. Values are expressed as means ± SEM. HTG, hypertriglyceridemia; HTC, hypercholesterolemia; M, male; F, female; TG, total triglycerides; TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; Glu, glucose. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to the values of control subjects.

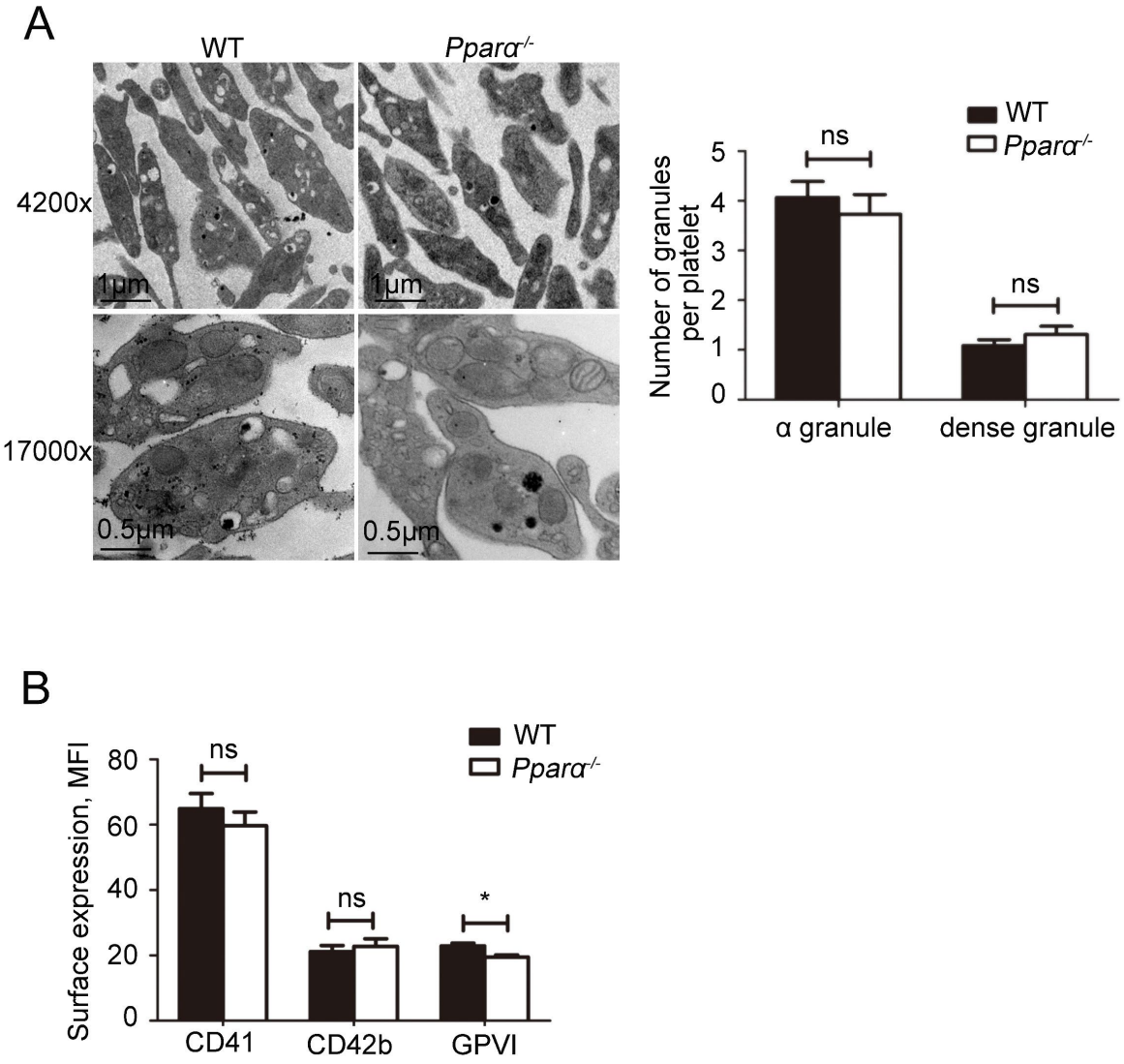
Supplemental table 4. The primers for real-time PCR detection of *Pparα* and *Gapdh*

Target gene	Primer 5'→3'	Product (bp)
Pparα	GACGAATGCCAAGATCTGAGAAAGC CGTCTCCTTTGTAGTGCTGTCAGC	946
Gapdh	GTCCACTGGCGTCTTCACCA GTGGCAGTGATGGCATGGAC	261

Supplemental table 5. The primers for ChIP analysis

Target gene	Primer 5'→3'	Product (bp)
Pparα-Region 1	CCCTTTGTGACAGTTGCTCC CAGGCGTCCTGAGGGTAGAA	199
Pparα-Region 2	TTGCGTAGGCACAAAGTCAG GGCTCAGAAGTGCGTAGGG	297
Pparα-Region 3	CTAGACCGGCTCATCGCAC CTGAATTCGAGGCGGGGAC	133
Pparα-Region 4	ATCGAGGAGGCAGGAGGG GGAAACTGAGGCCGGAGAG	191
Gapdh	GTCCACTGGCGTCTTCACCA GTGGCAGTGATGGCATGGAC	261

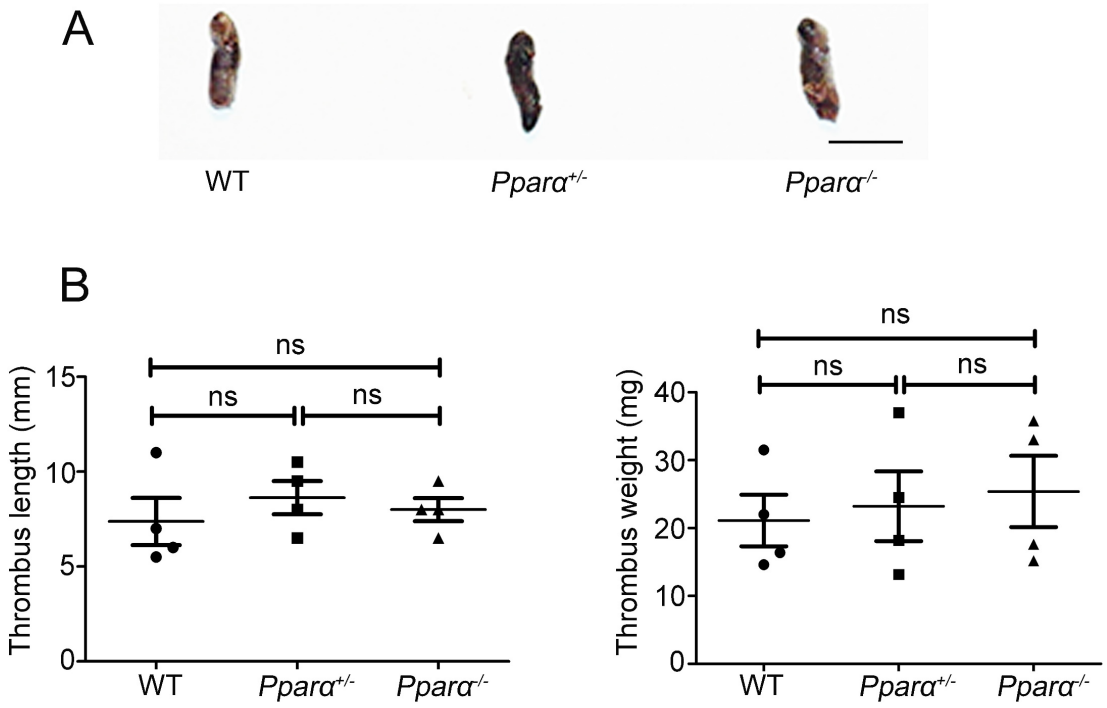
Supplemental figures



Supplemental figure 1. Ultrastructural morphology and surface receptor expression of *Pparα*$^{-/-}$ platelets.

(A) Representative electron microscopic images of WT and *Pparα*$^{-/-}$ platelet ultrastructure. Scale bars, 1 μm (top panel) and 0.5 μm (bottom panel). Quantification of α-granules and dense granules of WT (black bars) and *Pparα*$^{-/-}$ (white bars) platelets. Under × 17000 magnification, α-granules and dense-granules in 116 platelets (71 from WT and 45 *Pparα*$^{-/-}$ each) were counted. Statistical significance was evaluated with 2-tailed Mann-Whitney test (ns, no significance). The data were expressed as mean ± SEM.

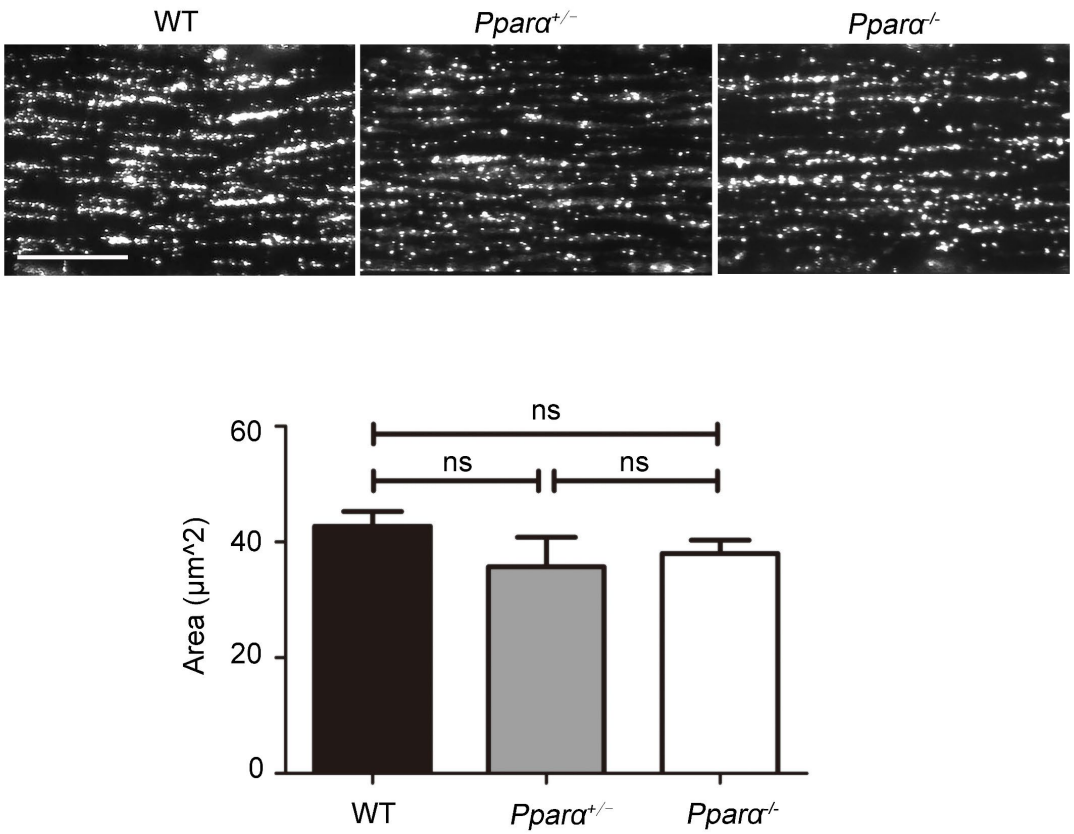
(B) Surface expression of CD41, CD42b, and GPVI was determined by flow cytometry. Results are expressed as mean fluoresce intensity (MFI). Statistical significance was evaluated with 2-tailed Mann-Whitney test ($^{*}P < 0.05$; ns, no significance).



Supplemental figure 2. Deep venous thrombosis was not affected by PPARα deficiency.

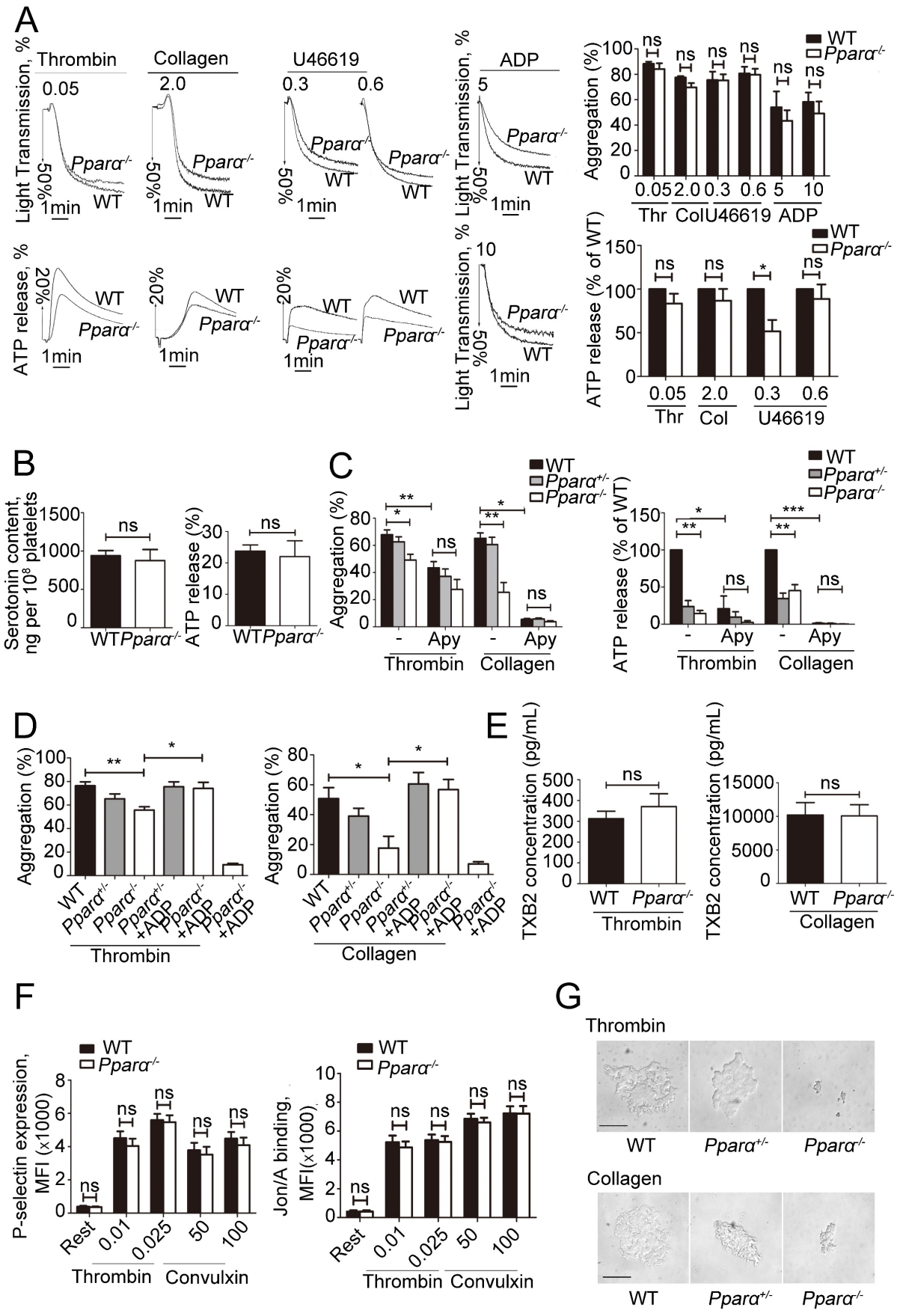
(A) Thrombus length and weight were measured in DVT model. Representative images and values for weight and length of the thrombi after 48 h in WT, *Pparα*$^{+/-}$ and *Pparα*$^{-/-}$ mice. Scale bars, 5 mm.

(B) Dot plot showing thrombus weight and length in WT (●, n = 4), *Pparα*$^{+/-}$ (■, n = 4) and *Pparα*$^{-/-}$ mice (▲, n = 4) after DVT induction. Statistical significance was evaluated with 2-tailed Mann-Whitney test (ns, no significance).



Supplemental figure 3. Adhesion of platelets to collagen was not affected by PPARα deficiency.

Calcein-labeled platelets (2×10^7/mL) were added to red blood cells (hematocrit 45%) with 2 mM $CaCl_2$. The reconstituted blood was then perfused through fibrillar collagen-coated bioflux plates at a shear rate of 40 dynes/cm^2 for 5 min. Statistical significance was evaluated with 2-tailed Mann-Whitney test (ns, no significance). Original magnification × 10. Scale bars, 50 μm.


A
Thrombin
Collagen
U46619
ADP
Light Transmission, %
ATP release, %
Aggregation (%)
ATP release (% of WT)
WT
Pparα-/-
ns
1min
Thr
Col
U46619
ADP
B
Serotonin content, ng per 10^8 platelets
ATP release (%)
C
Pparα+/-
Apy
Thrombin
Collagen
D
E
TXB2 concentration (pg/mL)
F
P-selectin expression, MFI (x1000)
Jon/A binding, MFI(x1000)
Convulxin
G
Thrombin
Collagen

Supplemental figure 4. Impaired aggregation in *Pparα*$^{-/-}$ platelets is caused by the reduced ADP secretion.

(A) Platelets were stimulated with thrombin, collagen, U46619 and ADP with a Chrono-log lumiaggregometer under stirring at 1200 rpm for 5 minutes. Traces are representative of at least 3 in dependent experiments. Statistical significance was evaluated with 2-tailed Mann-Whitney test and Paired *t* test ($^{*}P < 0.05$; ns, no significance). Results are expressed as mean ± SEM.

(B) Serotonin content was detected in WT and *Pparα*$^{-/-}$ platelets. ATP release of washed WT and *Pparα*$^{-/-}$ platelets stimulated with thrombin (0.5 U/mL). Statistical significance was evaluated with 2-tailed Mann-Whitney test (ns, no significance). Results are expressed as mean ± SEM.

(C) Aggregation and ATP release of washed WT, *Pparα*$^{+/-}$ and *Pparα*$^{-/-}$ platelets stimulated with thrombin (0.025 U/mL) or collagen (0.8 µg/mL) in the presence of vehicle or apyrase (1 U/mL) incubated for 5 minutes. Statistical significance was evaluated with 2-tailed Mann-Whitney test and Paired *t* test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, no significance). Results are expressed as mean ± SEM.

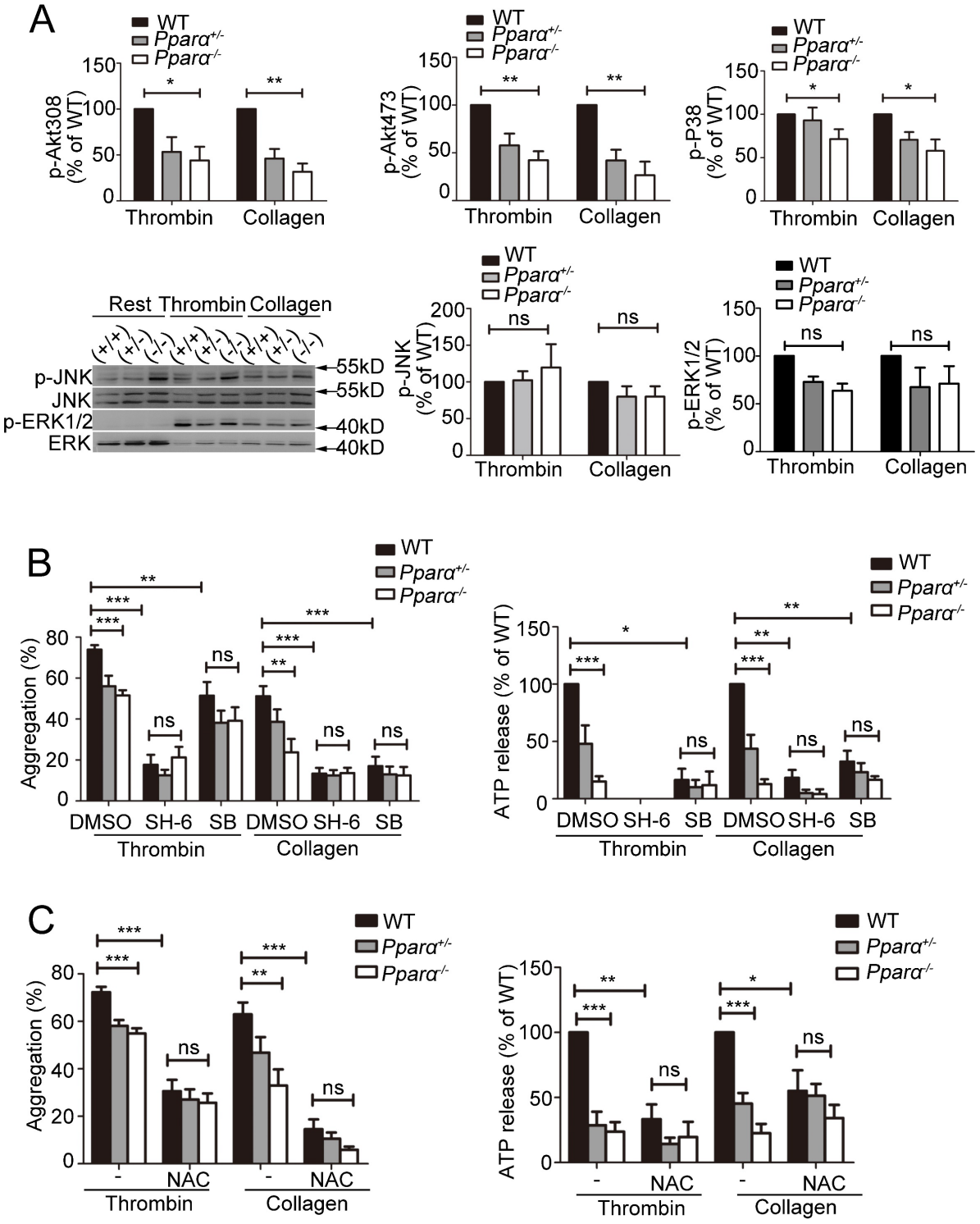
(D) Aggregation and ATP release of washed WT, *Pparα*$^{+/-}$ and *Pparα*$^{-/-}$ platelets stimulated with thrombin (0.025 U/mL) or collagen (0.8 µg/mL) in the presence of a low concentration of ADP (1 µM) for 5 minutes. Statistical significance was evaluated with 2-tailed Mann-Whitney test ($^{*}P < 0.05$; $^{**}P < 0.01$). Results are expressed as mean ± SEM.

(E) Accumulation of TXB2 in the supernatant of WT (black bars) and *Pparα*$^{-/-}$ (white bars) platelets stimulated with thrombin (0.025 U/mL) and collagen (0.8 µg/mL) for 5 min (n = 7). Statistical significance was evaluated with 2-tailed Mann-Whitney test (ns, no significance).

Results are expressed as mean ± SEM.

(F) Flow cytometric analysis of P-selectin exposure and integrin αIIbβ3 activation. Washed WT and *Pparα*$^{-/-}$ platelets were stimulated with thrombin (0.01 U/ml, 0.025 U/ml) and convulxin (50 ng/ml, 100 ng/ml) in the presence of FITC-labeled rat anti–mouse P-selectin mAb (RB40.34) and phycoerythrin-labeled rat anti–mouse integrin αIIbβ3 mAb (Jon/A). After fixation, samples were measured in flow cytometer. Results are expressed as mean fluoresce intensity (MFI). Statistical significance was evaluated with 2-tailed Mann-Whitney test (ns, no significance).

(G) Platelets aggregates formed when stimulated with low doses of thrombin and collagen. Washed WT, *Pparα*$^{+/-}$ and *Pparα*$^{-/-}$ platelets were stimulated with thrombin (0.025 U/mL) and collagen (0.8 μg/mL) with a Chrono-log lumiaggregometer under stirring at 1200 rpm for 5 minutes. Platelets were then fixed with 1.5% paraformaldehyde for 30 minutes at room temperature, and observed by differential interference contrast (Nikon TE-2000S, × 20 objective, DS-2MBWc-U1 CCD camera). Scale bars, 100 μm. Data are representative of 4 independent experiments.



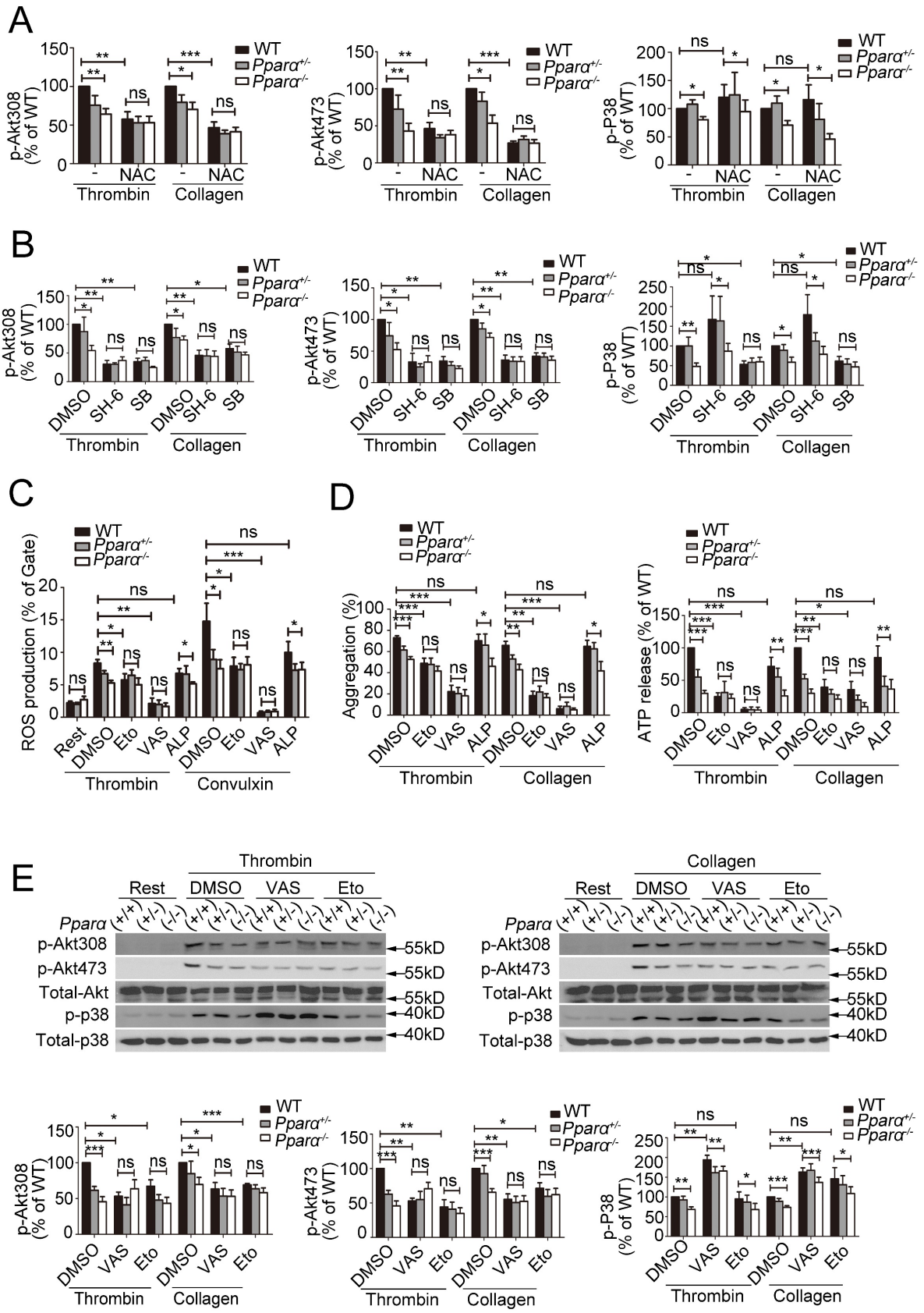
Supplemental figure 5. PPARα regulates platelet activation through a signal axis of p38/ ROS/Akt rather than through JNK or ERK1/2 signaling.

(A) Immunoblot analysis of WT, *Pparα*$^{+/-}$ and *Pparα*$^{-/-}$ platelets, stimulated with thrombin

(0.025 U/mL) and collagen (0.8 μg/mL) for 5 minutes, with antibodies recognizing phosphorylated Akt Thr^{308}, phosphorylated Akt Ser^{473}, total Akt, phosphorylated p38 Thr^{180}/Tyr^{182} (T180/Y182), total p38, phosphorylated JNK Thr^{183}/Tyr^{185}(T183/Y185), total JNK, phosphorylated ERK1/2 Thr^{202}/Tyr^{204}(T202/Y204) and total ERK1/2. Statistical significance was evaluated with Paired *t* test ($^{*}P < 0.05$; $^{**}P < 0.01$; ns, no significance). Results were quantified and presented as mean ± SEM.

(B) Aggregation and ATP release of WT, *Pparα*$^{+/-}$ and *Pparα*$^{-/-}$ platelets stimulated with thrombin (0.025 U/mL) or collagen (0.8 μg/mL) incubated in the presence of DMSO, SH-6 (10 μM), SB203580 (10 μM) for 10 minutes. Statistical significance was evaluated with 2-tailed Mann-Whitney test and Paired *t* test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, no significance). Results were quantified and presented as mean ± SEM.

(C) Aggregation and ATP release of WT, *Pparα*$^{+/-}$ and *Pparα*$^{-/-}$ platelets stimulated with thrombin (0.025 U/mL) or collagen (0.8 μg/mL) incubated in the absence or presence of NAC (2 mM) for 10 minutes. Statistical significance was evaluated with 2-tailed Mann-Whitney test and Paired *t* test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, no significance). Results were quantified and presented as mean ± SEM.


A
p-Akt308 (% of WT)
p-Akt473 (% of WT)
p-P38 (% of WT)
WT
Pparα+/-
Pparα-/-
NAC
Thrombin
Collagen
B
DMSO
SH-6
SB
C
ROS production (% of Gate)
Rest
Eto
VAS
ALP
Convulxin
D
Aggregation (%)
ATP release (% of WT)
E
Thrombin
Collagen
Pparα (+/+) (+/-) (-/-)
p-Akt308
p-Akt473
Total-Akt
p-p38
Total-p38
55kD
40kD

Supplemental figure 6. ROS from NOX and mitochondrial fatty acid β-oxidation may constitute the important sources of ROS for PPARα regulated platelet activation.

(A) Immunoblot analysis of WT, *Pparα*$^{+/-}$ and *Pparα*$^{-/-}$ platelets, stimulated with thrombin (0.025 U/mL) and collagen (0.8 μg/mL) for 5 minutes in the absence or presence of NAC (2 mM) for 10 minutes, with antibodies recognizing phosphorylated Akt Thr^{308}, phosphorylated Akt Ser^{473}, total Akt, phosphorylated p38 Thr^{180}/Tyr^{182} (T180/Y182), total p38. Statistical significance was evaluated with Paired *t* test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, no significance). Results were quantified and presented as mean ± SEM.

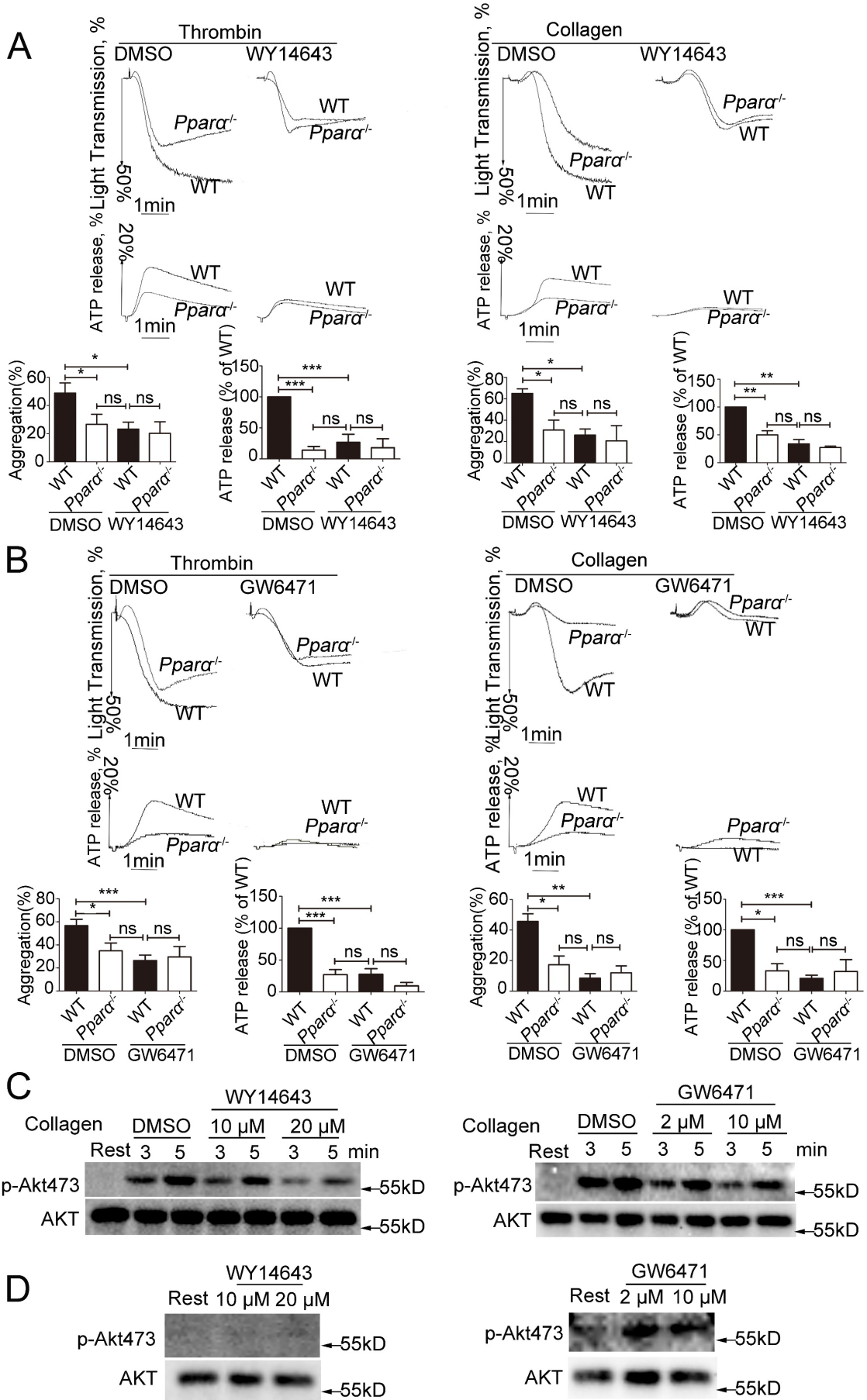
(B) Immunoblot analysis of WT, *Pparα*$^{+/-}$ and *Pparα*$^{-/-}$ platelets, stimulated with thrombin (0.025 U/mL) and collagen (0.8 μg/mL) for 5 minutes in the presence of DMSO, SH-6 (10 μM), SB203580 (10 μM) for 10 minutes, with antibodies recognizing phosphorylated Akt Thr^{308}, phosphorylated Akt Ser^{473}, total Akt, phosphorylated p38 Thr^{180}/Tyr^{182} (T180/Y182), total p38. Statistical significance was evaluated with Paired *t* test ($^{*}P < 0.05$; $^{**}P < 0.01$; ns, no significance). Results were quantified and presented as mean ± SEM.

(C) H2DCFDA-loaded (50 μM) WT, *Pparα*$^{+/-}$ and *Pparα*$^{-/-}$ platelets were incubated with DMSO, etomoxir (25 μM), VAS2870 (10 μM), or ALP (200 μM) stimulated with thrombin (0.025 U/mL) or convulxin (50 ng/mL) for 10 minutes. Samples were analyzed immediately. Statistical significance was evaluated with 2-tailed Mann-Whitney test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, no significance).

(D) Aggregation and ATP release of WT, *Pparα*$^{+/-}$ and *Pparα*$^{-/-}$ platelets stimulated with thrombin (0.025 U/mL) and collagen (0.8 μg/mL) incubated in the presence of DMSO, etomoxir (25 μM) or VAS2870 (10 μM), ALP (200 μM) for 10 minutes. Statistical significance

was evaluated with 2-tailed Mann-Whitney test and Paired *t* test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, no significance). Results were quantified and presented as mean ± SEM.

(E) Immunoblot analysis of WT, *Pparα*$^{+/-}$ and *Pparα*$^{-/-}$ platelets, stimulated with thrombin (0.025 U/mL) and collagen (0.8 μg/mL) for 5 minutes in the presence of DMSO, etomoxir (25 μM) or VAS2870 (10 μM) for 10 minutes, with antibodies recognizing phosphorylated Akt Thr^{308}, phosphorylated Akt Ser^{473}, total Akt, phosphorylated p38 Thr^{180}/Tyr^{182} (T180/Y182), total p38. Statistical significance was evaluated with Paired *t* test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, no significance). Results were quantified and presented as mean ± SEM.


A
Thrombin
DMSO
WY14643
Collagen
Light Transmission, %
ATP release, %
50%
20%
1min
WT
Pparα-/-
Aggregation(%)
ATP release (% of WT)
ns
DMSO
WY14643
B
GW6471
C
WY14643
Collagen
DMSO
10 μM
20 μM
Rest 3 5 3 5 3 5 min
p-Akt473
AKT
55kD
GW6471
2 μM
10 μM
D

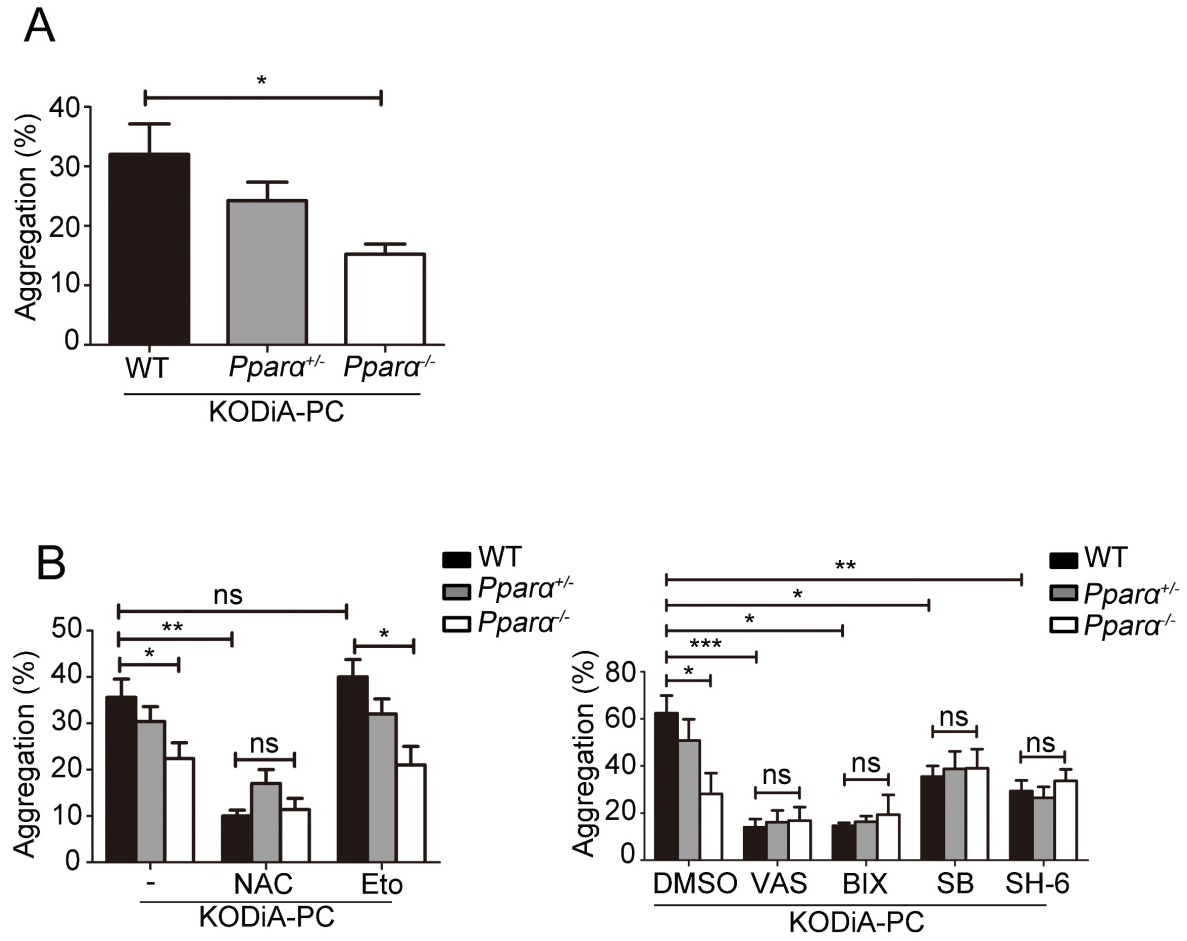
Supplemental figure 7. PPARα agonist WY14643 and antagonist GW6471 inhibit platelet aggregation and ATP release.

(A) Aggregation and ATP release of washed WT and PPARα$^{-/-}$ platelets were stimulated with thrombin (0.025 U/mL) or collagen (0.8 µg/mL) in the presence of DMSO or WY14643 (10 µM) incubated for 10 minutes. Aggregation and ATP release was assessed with a Chrono-log lumiaggregometer under stirring at 1200 rpm. Traces are representative of at least 3 independent experiments. Statistical significance was evaluated with 2-tailed Mann-Whitney test and Paired *t* test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

(B) Aggregation and ATP release of washed WT and PPARα$^{-/-}$ platelets were stimulated with thrombin (0.025 U/mL) or collagen (0.8 µg/mL) in the presence of DMSO or GW6471 (2 µM) incubated for 10 minutes. Aggregation and ATP release was assessed with a Chrono-log lumiaggregometer under stirring at 1200 rpm. Traces are representative of at least 3 independent experiments. Statistical significance was evaluated with 2-tailed Mann-Whitney test and Paired *t* test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

(C) Immunoblot analysis of WT platelets stimulated with collagen (0.8 µg/mL) for 3 and 5 minutes in the presence of DMSO, WY14643 (10 µM, 20 µM) or GW6471 (2 µM, 10 µM) for 10 minutes, with antibodies recognizing phosphorylated Akt Ser^{473} and total Akt. Representative immunoblots from at least three independent experiments.

(D) Immunoblot analysis of WT platelets incubated with WY14643 (10 µM, 20 µM) or GW6471 (2 µM, 10 µM) for 10 minutes, with antibodies recognizing phosphorylated Akt Ser^{473} and total Akt. Representative immunoblots from at least three independent experiments.

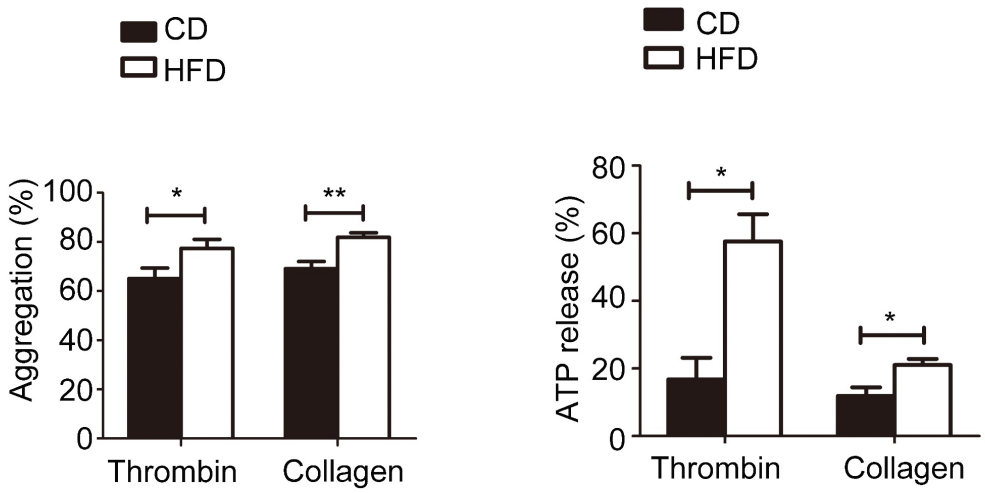


Supplemental figure 8. PPARα is important in oxLDL-induced platelet activation.

(A) Aggregation of WT, *Pparα*$^{+/-}$ and *Pparα*$^{-/-}$ platelets were stimulated with 1-(palmitoyl)-2-(5-keto-6-octene-dioyl) phosphatidylcholine (KODiA-PC, 15 μM) with a Chrono-log lumiaggregometer under stirring at 1200 rpm for 5 minutes. Statistical significance was evaluated with 2-tailed Mann-Whitney test ($^{*}P < 0.05$). Results were quantified and presented as mean ± SEM.

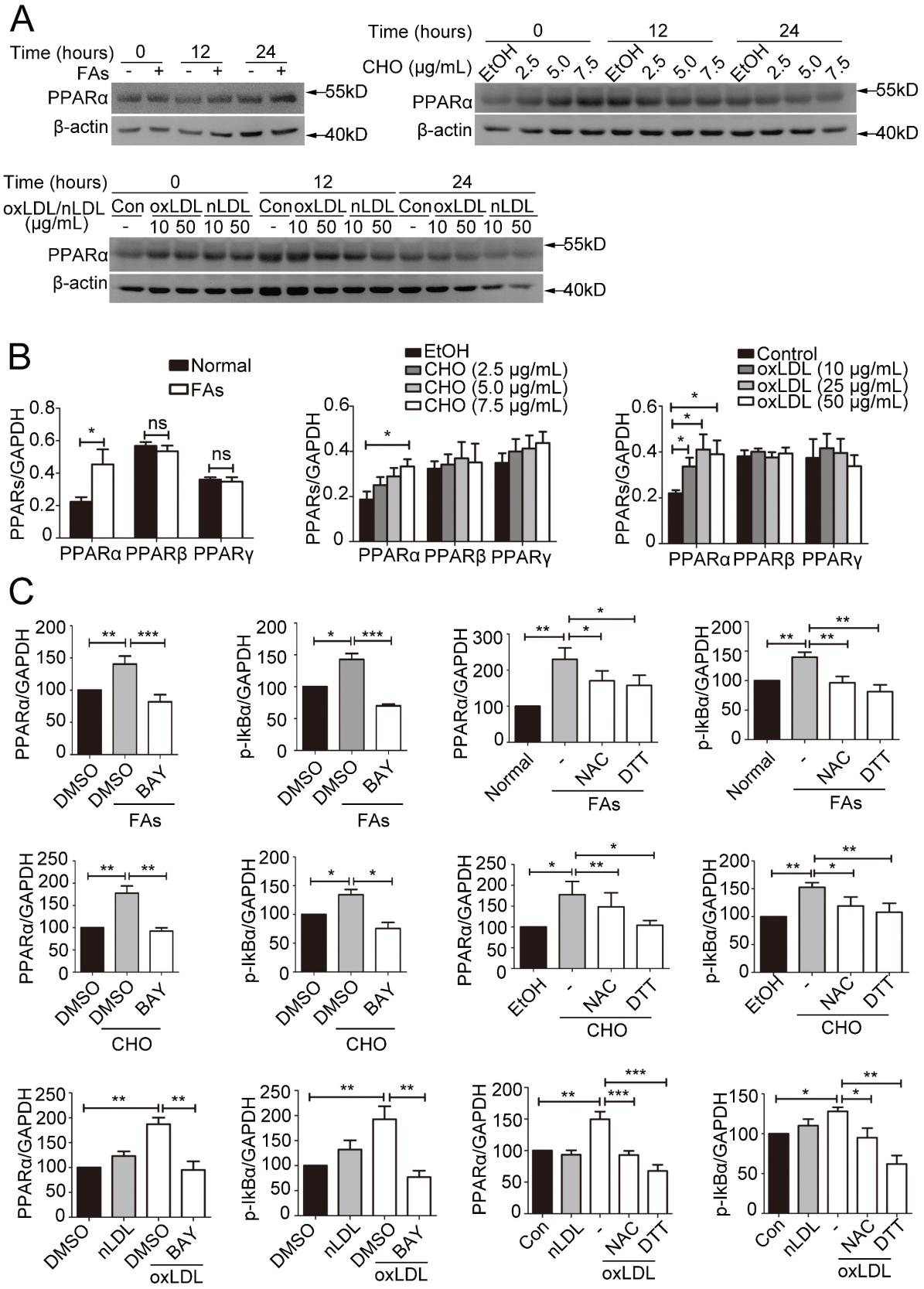
(B) Aggregation of WT, *Pparα*$^{+/-}$ and *Pparα*$^{-/-}$ platelets stimulated with KODiA-PC (15 μM) incubated in the absence or presence of NAC (2 mM), etomoxir (25 μM), DMSO, VAS2870 (10 μM) or BIX02188 (10 μM), SB203580 (10 μM), SH-6 (10 μM) for 10 minutes. Statistical

significance was evaluated with 2-tailed Mann-Whitney test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, no significance). Results were quantified and presented as mean ± SEM.



Supplemental figure 9. Increased platelet hyperactivity in hyperlipidemic mice.

Aggregation and ATP release of platelets from *Apoe*$^{-/-}$ mice fed with HFD or CD stimulated with thrombin (0.015 U/mL) and collagen (0.6 μg/mL). Statistical significance was evaluated with 2-tailed Mann-Whitney test ($^{*}P < 0.05$; $^{**}P < 0.01$). Results were quantified and presented as mean ± SEM.


A
Time (hours)
FAs
PPARα
β-actin
55kD
40kD
CHO (μg/mL)
EtOH
oxLDL/nLDL (μg/mL)
Con
oxLDL
nLDL
B
Normal
FAs
EtOH
CHO (2.5 μg/mL)
CHO (5.0 μg/mL)
CHO (7.5 μg/mL)
Control
oxLDL (10 μg/mL)
oxLDL (25 μg/mL)
oxLDL (50 μg/mL)
PPARs/GAPDH
PPARα PPARβ PPARγ
C
PPARα/GAPDH
p-IkBα/GAPDH
DMSO
BAY
NAC
DTT
Normal
nLDL
CHO
oxLDL

Supplemental figure 10. oxLDL and lipids upregulate megakaryocyte- but not platelet-PPARα.

(A) Immunoblot analysis of PPARα in human platelets incubated with fatty acids (OA, 400 μM and PA, 200 μM), cholesterol (CHO, 2.5 μg/mL, 5.0 μg/mL, 7.5 μg/mL) or oxLDL (10 μg/mL, 50 μg/mL) for 12 hours or 24 hours with PPARα antibody. Representative immunoblots from at least three independent experiments.

(B) Immunoblot analysis of PPARs in Meg-01 cells incubated with fatty acids (OA, 400 μM and PA, 200 μM), cholesterol (CHO, 2.5 μg/mL, 5.0 μg/mL, 7.5 μg/mL) or oxLDL (10 μg/mL, 50 μg/mL) for 24 hours with PPARα, PPARβ and PPARγ antibody. Statistical significance was evaluated with 2-tailed Mann-Whitney test ($^{*}P < 0.05$; ns, no significance).

(C) Immunoblot analysis of PPARα and phosphorylated IκBα level in Meg-01 cells cultured with fatty acids (OA, 400 μM and PA, 200 μM), cholesterol (CHO, 2.5 μg/mL, 5.0 μg/mL, 7.5 μg/mL) or oxLDL (10 μg/mL, 50 μg/mL) in the absence or presence of BAY11-7082 (10 μM), NAC (1 mM) or DTT (1 mM) for 24 hours. Statistical significance was evaluated with Paired *t* test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

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