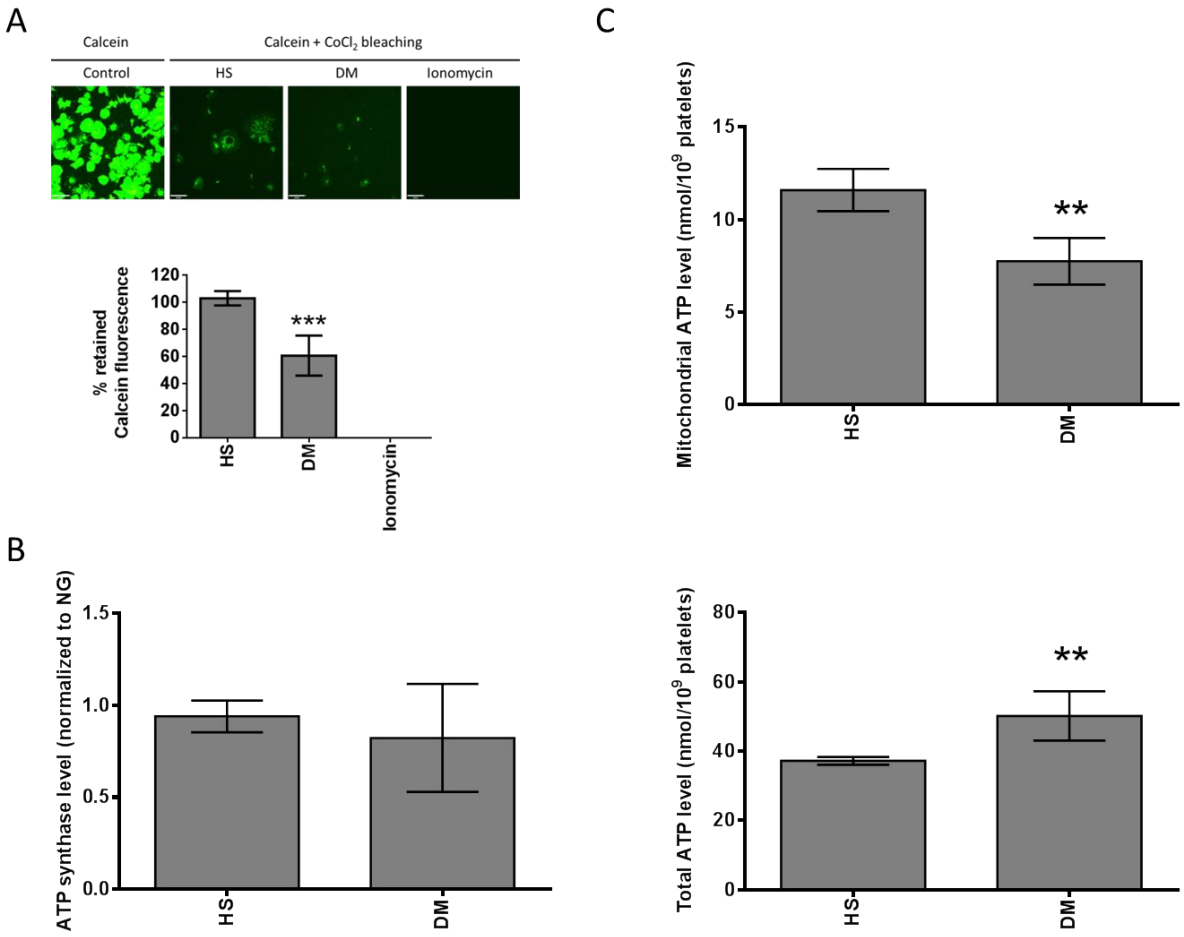
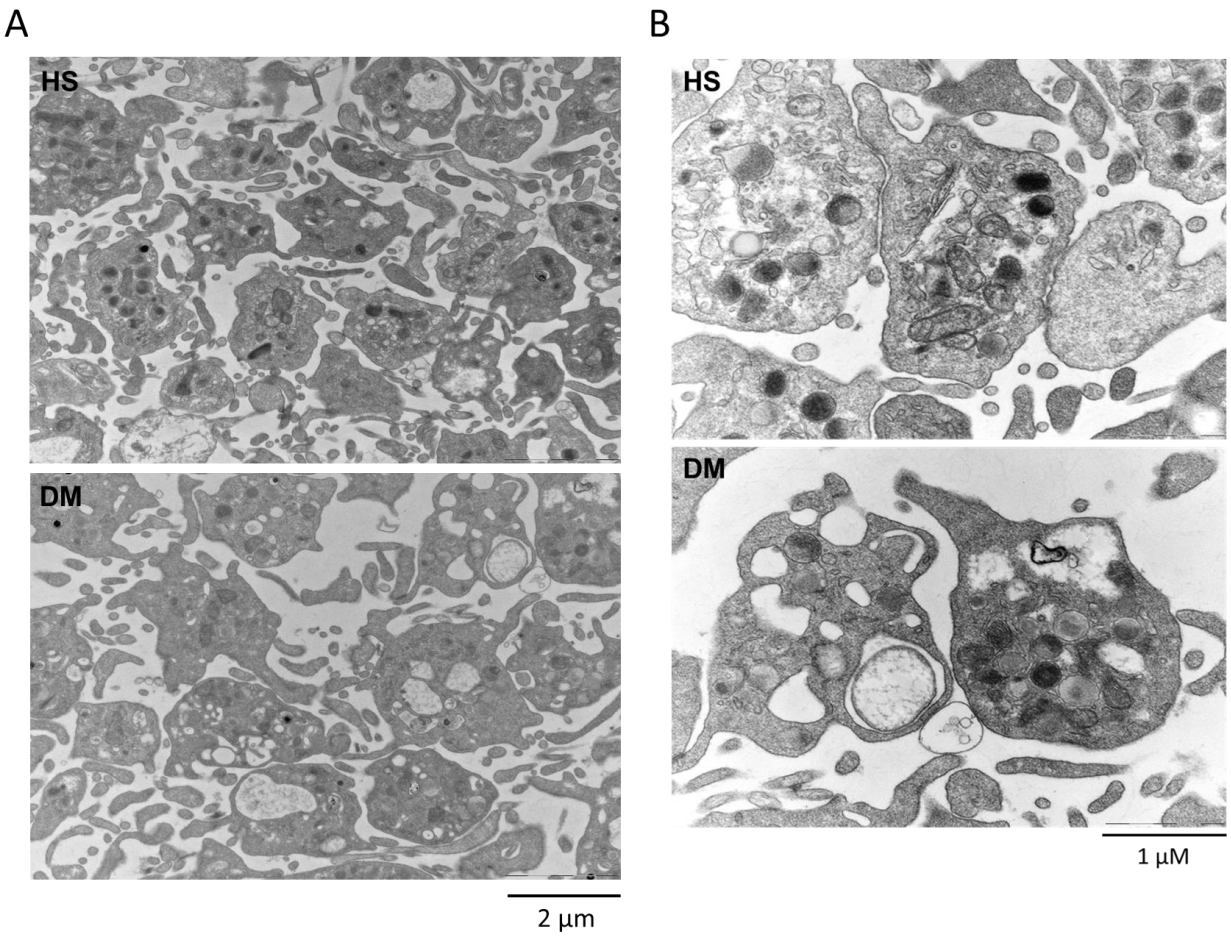
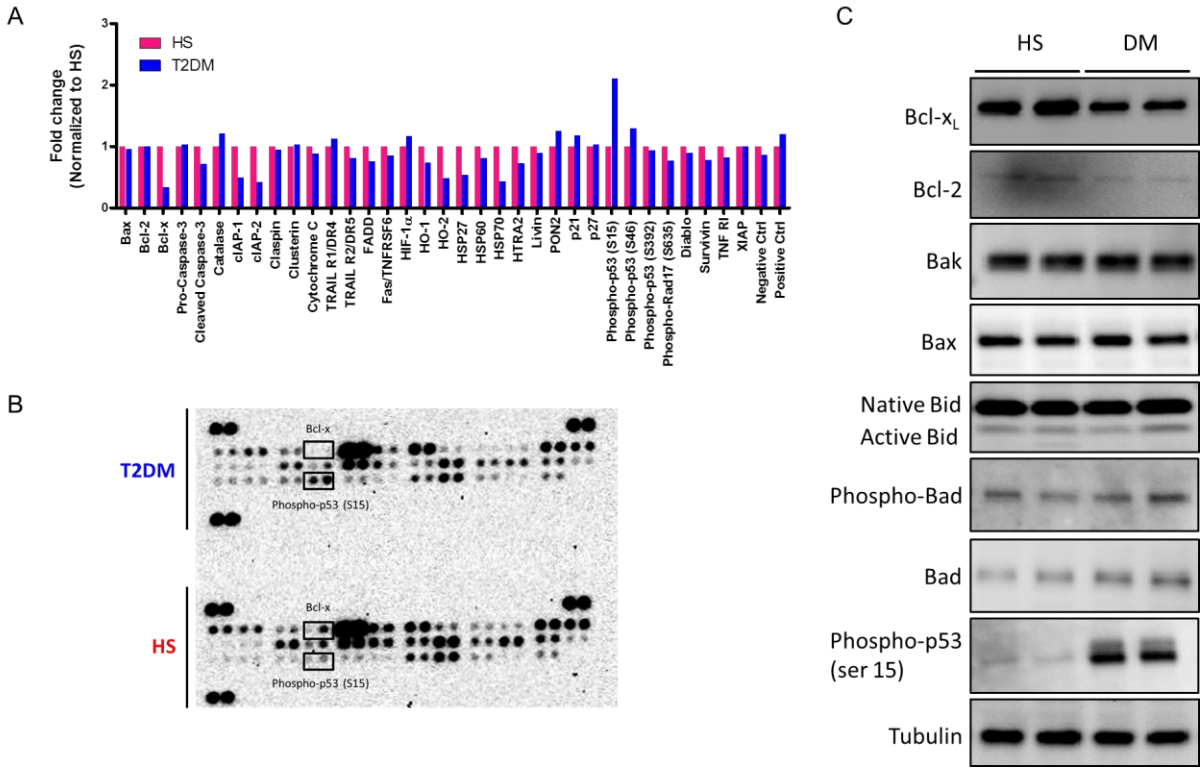
Supplemental Material



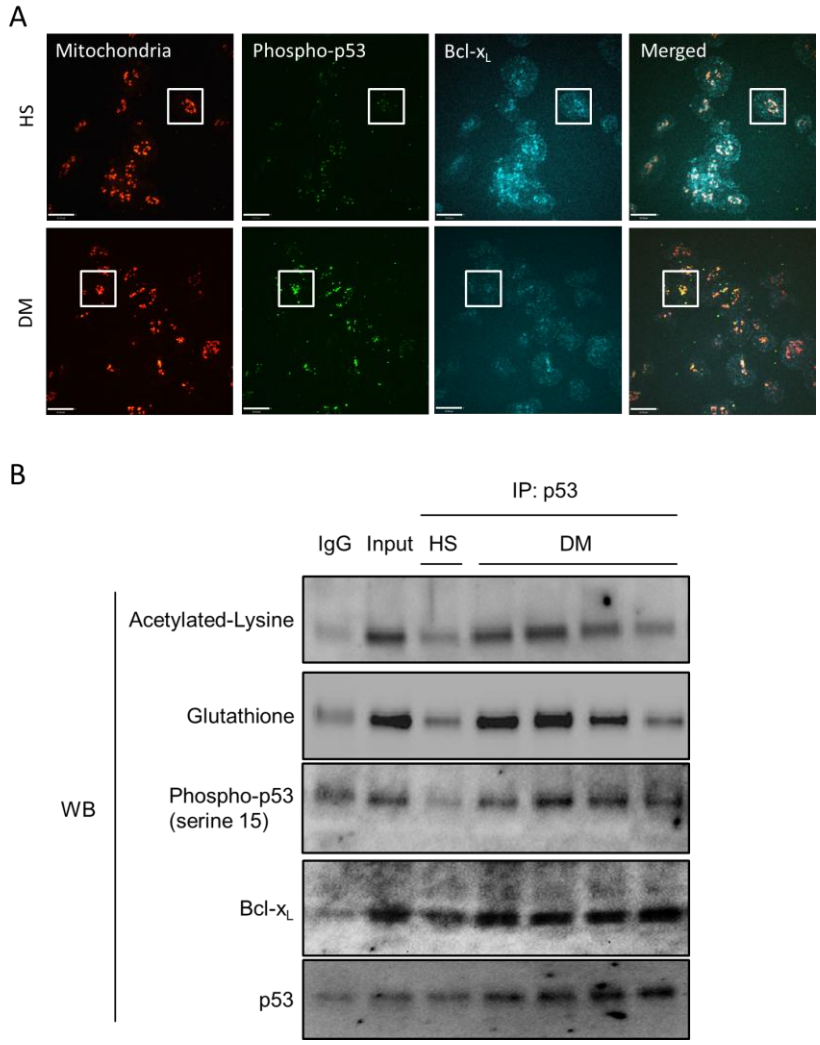
Supplemental Figure 1: (A) Activation of mitochondrial permeability transition pores (MPTP) was assessed by loss of retained calcein fluorescence. (mean fluorescence ± SD, n=3; bar=10 µm). ****P*<0.001 compared with HS. **(B)** The activity of ATP synthase in platelets from healthy subjects (HS) and diabetic patients (DM). Data are presented as mean ± SD (for HS, n=3; For DM, n=4). **(C)** The mitochondrial and total ATP levels in HS and DM platelets. Data are presented as mean ± SD (For HS, n=5; For DM, n=4). ***P*<0.01 compared with HS.



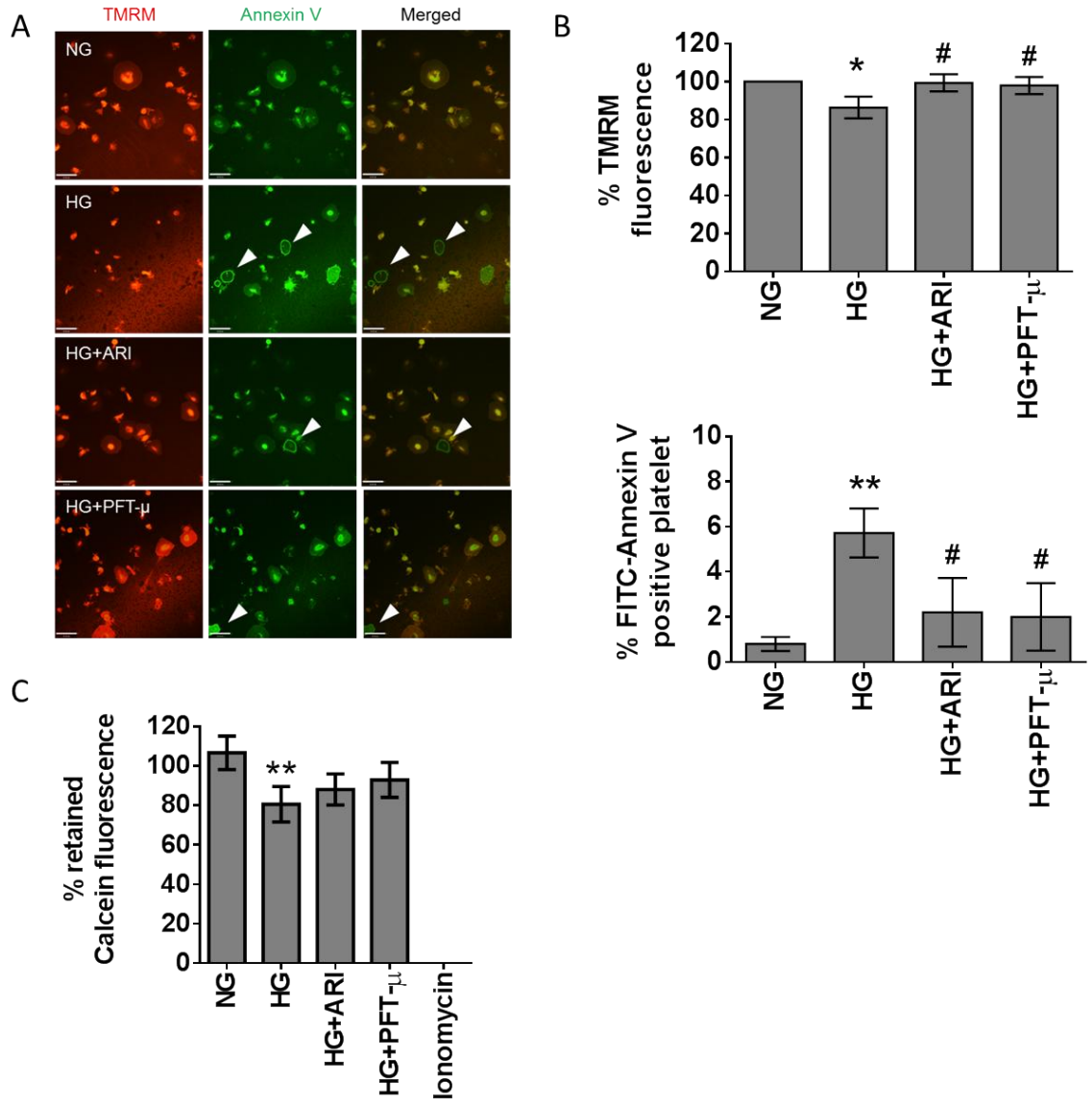
Supplemental Figure 2: (A) Representative EM field demonstrating a number of platelets from a healthy subject (HS) or a diabetes mellitus subject (DM). **(B)** Representative higher power EM field demonstrating a small number of platelets from a healthy subject (HS) or a diabetes mellitus subject (DM).



Supplemental Figure 3: Expression profiles of apoptosis-related proteins in platelets from DM patients. Washed platelets were isolated from healthy subjects (HS) & type 2 (T2DM) DM patients. Platelets from eight different patients in each group were pooled. **(A)** The relative expressions of 35 apoptosis-related proteins were measured using a human apoptosis array (R&D Systems). The intensity of the dot blots were analyzed by Image Lab software, and normalized to HS. Data are presented as fold change. **(B)** The changes of Bcl-2 family proteins and phosphorylated p53 in platelet from HS and DM patients were further assessed by Western blotting. Representative blots are shown (n=4).

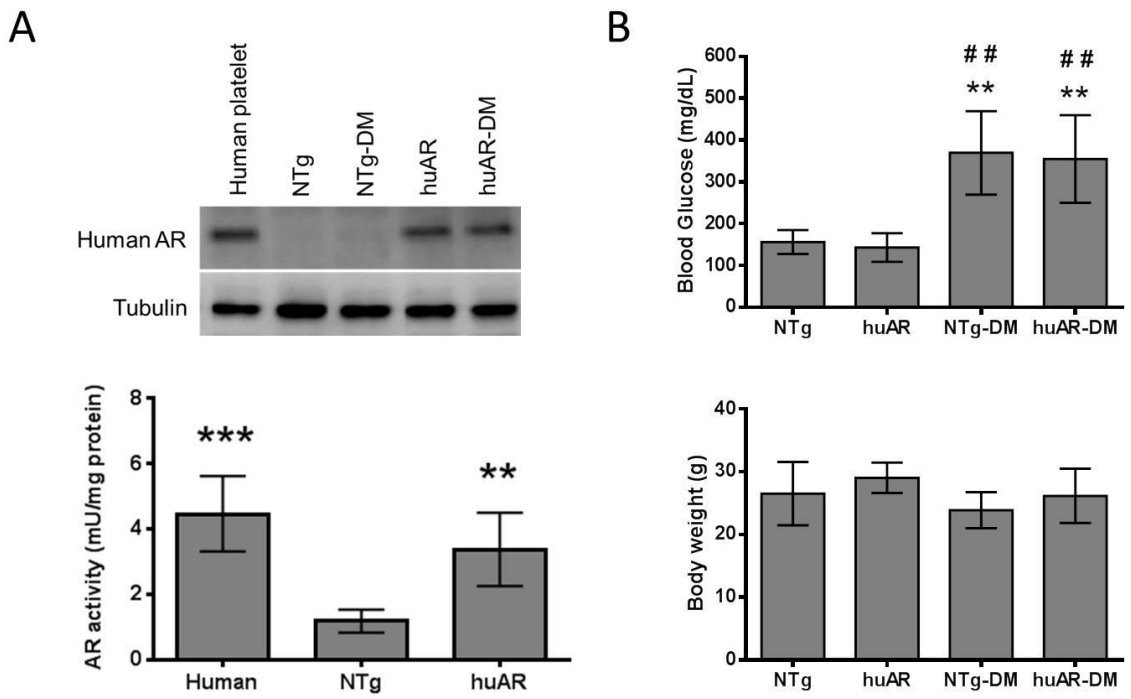


Supplemental Figure 4: (A) Lower powered confocal field from where the single platelets in Figure 3B was taken (bar=10 µm). **(B)** The level of acetylation, glutathionylation and phosphorylation of p53 in DM platelets (n=4).



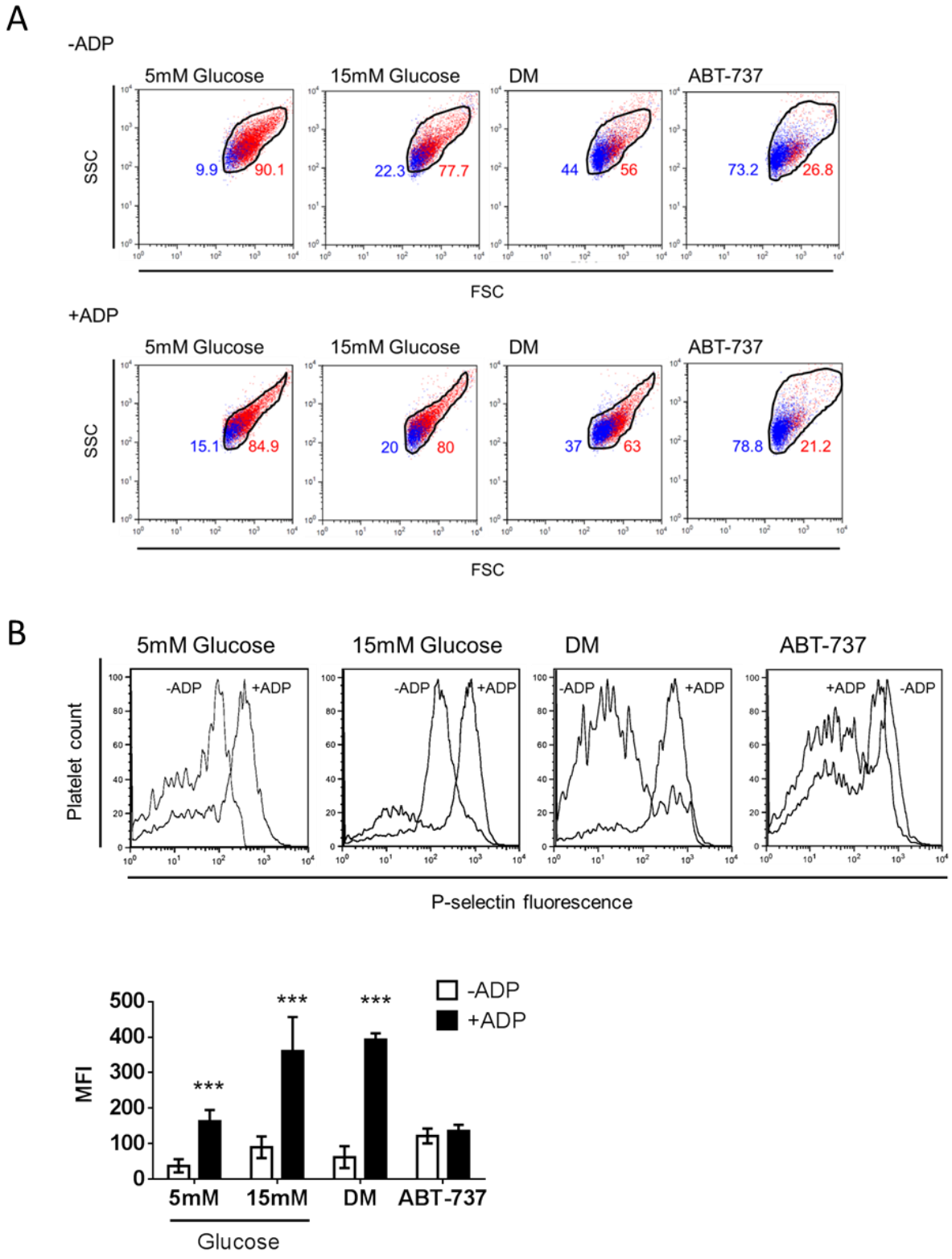
Supplemental Figure 5: The role of AR in hyperglycemia-induced mitochondrial dysfunction in human platelets. Platelets were isolated from healthy subjects (HS), washed, and incubated with 25 mM glucose (HG) for 2 hours. **(A)** Platelet $\Delta\Psi_m$ was measured by staining with 1 μM Tetramethylrhodamine methyl ester (TMRM) under NG and HG conditions, and PS externalization was assessed by labeling with Annexin-V. Platelets incubated with HG were either pre-treated with 10 μM epalrestat for 30 min (HG+ARI) or 20

μM Pifithrin-μ (HG+PFT-μ). Representative images are shown (n=8; bar=10 μm). **(B)** $\Delta\Psi_m$ and PS externalization were also measured by FACS. Quantification of $\Delta\Psi_m$ was presented as mean fluorescence ± SD, and PS externalization was presented as percentage of Annexin-V positive platelets ± SD (n=8). $^{**}P<0.01$ & $^{*}P<0.05$ compared with NG; $^{\#}P<0.05$ compared with HG. **(C)** MPTP opening was assessed by loss of retained calcein fluorescence in mitochondria using FACS. Quantification of data was presented as mean fluorescence ± SD (n=3). $^{**}P<0.01$ compared with NG.



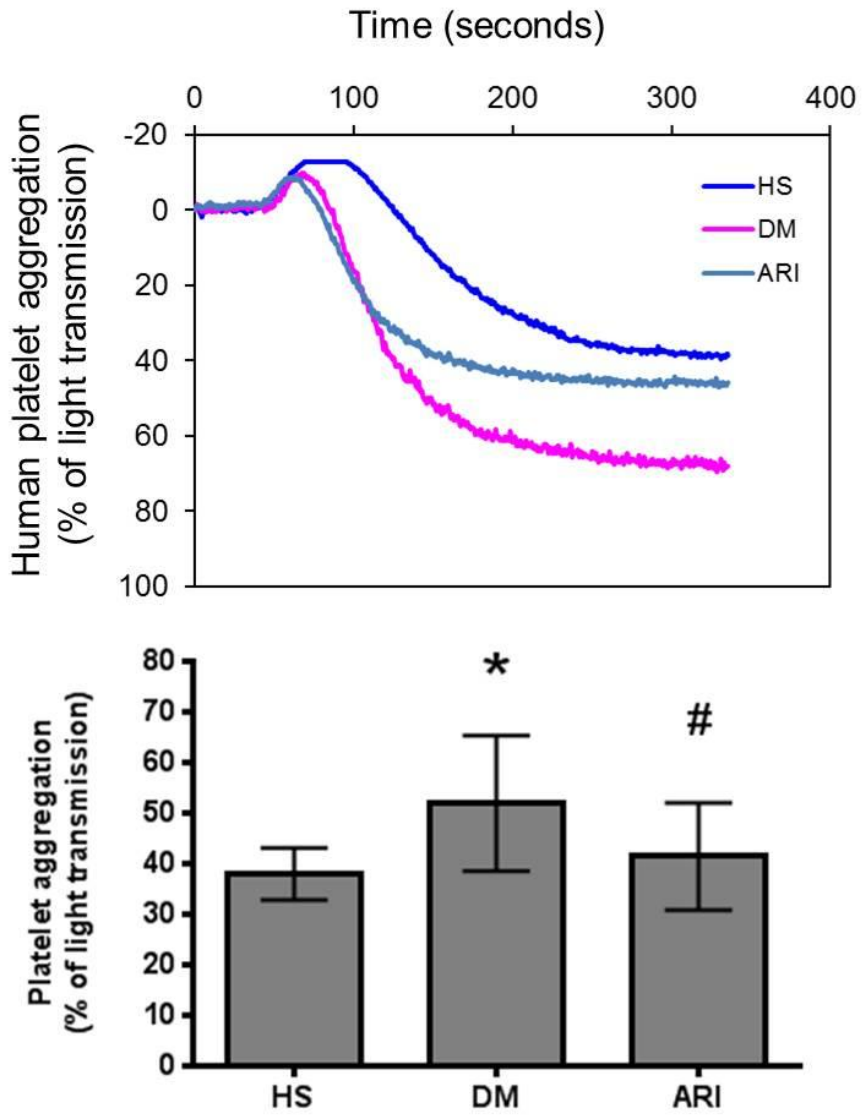
Supplemental Figure 6: Human aldose reductase expressed in diabetic mouse platelets.

(A) The expression and activity of aldose reductase in platelets isolated from non-transgenic (NTg) and huAR-transgenic (huAR) mouse with or without STZ-induced DM. Representative blots are shown (n=4). AR activity was defined as the μmole of NADPH consumption/minute/mg protein. Data were presented as mean ± SD (n=4). ****P*<0.001 & ***P*<0.01 compared with NTg. **(B)** Fasting blood glucose level (upper panel) and body weight (lower panel) were assessed. The mice were starved for 6 hours before measuring the blood glucose level (mean ± SD, n=8 ***P*<0.01 compared with NTg; ##*P*<0.01 compared with huAR).



Supplemental Figure 7: Effect of hyperglycemia-induced mitochondrial dysfunction on mouse platelet reactivity. (A) Platelets of non-DM and DM huAR mice were isolated.

Non-DM platelets were further incubated with either 5 mM or 15 mM glucose for 2 hours. Platelets treated with 10 μM ABT-737 served as a control. Murine platelets were gated by TMRM fluorescence into two populations, TMRM positive (red) and TMRM negative (blue). Flow cytometry of isolated murine platelets showing FSC vs SSC in the absence (top) and presence (bottom) of 1μM ADP. **(B)** Representative data showing P-selectin expression in the resting and ADP-stimulated murine platelets. Data was presented as mean fluorescence intensity (MFI) of FITC-P-selectin ± SD (n=4). ***P<0.001 compared with the resting platelet in the corresponding groups.



Supplemental Figure 8: Inhibition of AR attenuates platelet hyperaggregability in DM patients. Percentage of light transmission, an index of platelet aggregation, was measured in both HS and DM platelet suspension in response to 1 μg/ml collagen for 10 min. Platelets isolated from DM patients were treated with 10 μM epalrestat (ARI) for 30 min before the experiments. Quantification of data is presented as percentage of light transmission. Data are expressed as mean ± SD (n=9 for both HS and DM). **P*<0.05 compared with HS; #*P*<0.05 compared with DM.

	Healthy control	T2DM
	n=8	n=66
Age (years ± SD)	41.5±6.3	57.5±12.6
Gender (Males/Females)	8	40/26
BMI (kg/m^2 ± SD)	21.8±2.0	34.1±8.6
Blood glucose (mg/dL ± SD)	103.8±4.7	170.7±66.8
HbA1c (% ± SD)	5.2±0.3	7.2±1.8
Systolic blood pressure (mmHg ± SD)	114.1±5.8	134.6±14.7
Diastolic blood pressure (mmHg ± SD)	77.4±3.5	74.7±12.7
Hypertension (%)	0/8 (0%)	47/66 (71.2%)
CAD (%)	0/8 (0%)	17/66 (25.8%)
Medications (%)		
Aspirin	0/8 (0%)	32/66 (48.5%)
Clopidogrel	0/8 (0%)	3/66 (4.5%)
Warfarin	0/8 (0%)	4/66 (6.1%)
Statin	0/8 (0%)	41/66 (62.1%)
Beta-blocker	0/8 (0%)	30/66 (45.5%)
Angiotensin converting enzyme inhibitor	0/8 (0%)	26/66 (39.4%)
Angiotensin receptor blocker	0/8 (0%)	14/66 (21.2%)
Insulin	0/8 (0%)	30/66 (45.5%)
Metformin	0/8 (0%)	21/66 (31.8%)
Other anti-diabetic drugs	0/8 (0%)	8/66 (12.1%)
Diuretic	0/8 (0%)	21/66 (31.8%)

Supplemental Table 1: Clinical characteristics of healthy control and patients with type 2 diabetes mellitus (T2DM).

Supplemental Methods:

Human platelet preparation

Platelet rich plasma was prepared by differential centrifugation of 27 ml of blood drawn by venipuncture into 3 ml of 3.8% trisodium citrate (w/v). Platelet-poor plasma (PPP) was obtained by centrifugation at 1400 *g* (25°C for 10 min). PRP was adjusted with PPP to 2-3 × 10^8 platelets/ml. For washed platelets, the platelets were washed twice and resuspended at 2-3 × 10^8 platelets/ml.

Development of mouse model for DM with human AR expression

To develop mice that mimic human subjects with high cardiovascular risk, huAR-LDLR$^{-/-}$ mice with human AR (huAR) transgene expression on a C57BL/6J background (B6.Cg-*Ldlr*^{tm1Her}Tg(H2-K-AKR1B1)1Tj/J; stock no. 006877) were purchased from Jackson Labs. To study the effects of recurrent episodes of hyperglycemia on platelets rather than single high glucose treatment, we induced DM in mice using streptozotocin (STZ). Eight week old mice were divided into 2 groups; one half were injected with STZ (50 mg/kg) intraperitoneally for 5 days to induce recurrent episodes of acute hyperglycemia (DM), and the other half were used as non-DM controls. Four weeks after STZ administration, DM and non-DM mice were maintained on high cholesterol diet (HCD) for 12 weeks, fasted 6 hours and sacrificed for blood sampling. Glucose was measured from the tail-tip with a glucometer.

The Institutional Animal Care and Use Committee at Yale University approved all animal protocols (IACUC #11413 & #11539).

Murine platelet preparation

Blood (0.7-1 mL) was directly aspirated from the right cardiac ventricle into 1.8% sodium citrate (pH 7.4). Citrated blood from several mice of identical genotype was pooled, and diluted with equal volume of HEPES/Tyrode's buffer. PRP was prepared by centrifugation at 100 *g* for 10 min and then used for measuring platelet aggregation in response to 10 μg/ml of collagen. Washed platelets were prepared from PRP by centrifugation at 5000 *g* for 2 mins. The platelet pellets were resuspended in HEPES/Tyrode's buffer in the presence of 3 μg/mL apyrase.

Ligation of mouse carotid artery

The left common carotid artery was partially ligated near the carotid bifurcation. All mice recovered from surgery and showed no symptoms of stroke. Mice were euthanized seven days after carotid artery ligation. Left and right arteries were fixed in 4% paraformaldehyde. Arteries were embedded in OCT compound, and serial sections (5 μm thick) were cut for analysis by hematoxylin-eosin staining and immunofluorescent staining. Five sections spanning most of the vessel segments from each mouse were analyzed for

morphometry.

Confocal microscopy

Washed platelets were allowed to settle on glass-bottom dishes for 30 min before fixing for 15 mins with 4% paraformaldehyde in PBS. After fixation, platelets were permeabilized for 10 minutes using 0.5% triton-X 100 in PBS with 3% BSA, and incubated with different antibodies, including mouse anti-OxPhos Complex V (Invitrogen), rabbit anti-Bcl-x_L (Cell Signaling), Alexa Fluor 488 conjugated anti-p53 phosphorylated at serine 15 (Cell Signaling), or rabbit anti-cleaved Caspase-3 antibodies at 1:250 dilution in PBS with 10% BSA at 4^{o}C overnight. The platelets were then washed and incubated with Alexa Fluor 546 conjugated anti-mouse or Alexa Fluor 647 conjugated anti-rabbit antibodies (Invitrogen).

Determination of mitochondrial membrane potential ($\Delta\Psi_m$), phosphatidylserine (PS) externalization and mitochondrial superoxide production

To assess the $\Delta\Psi_m$ and PS externalization simultaneously, platelet suspensions (5×10^6 platelets/ml) were incubated with 40 nM TMRM at 37^{o}C for 30 mins, followed by staining with 4 µg/ml annexin V at room temperature for 15 mins. To measure mitochondrial superoxide production, platelets were stained with 5 µM MitoSOX^{TM} Red for 10 mins at 37 ^{o}C. The fluorescent intensity was detected by flow cytometry (LSRII). Platelets were identified and gated by their characteristic forward and side scatter properties. 10,000

platelets were analyzed from each sample.

Western blotting, subcellular fractionation and immunoprecipitation

The protein samples were subjected to SDS-PAGE, and the proteins were transferred to nitrocellulose membrane and probed with the indicated antibodies. Primary antibodies against the following proteins were used according to the manufacturer's instructions: Bcl-x_L, Bcl-2, Bax, Bak, Bid, Bad, phosphorylated Bad, p53, phosphorylated p53 (serine 15), p38α, phosphorylated p38α, cleaved caspase-3 and cytochrome C (Cell Signaling). The blots were reprobed with a β-tubulin antibody (Santa Cruz Biotechnology) for normalization. For subcellular fractionation, the blots were re-probed with antibodies against COX IV (Cell Signaling), a mitochondrial marker, and β-tubulin (Santa Cruz Biotechnology), a cytoplasmic marker for normalization. Quantification was performed using Image Lab software. For immunoprecipitation, platelet lysates were incubated with agarose conjugated p53 antibody (Calbiochem) at 4 ^{o}C overnight, and agarose conjugated mouse IgG was used as control. After washing, the immunoprecipitated proteins were eluted in SDS loading buffer for Western analysis. Quantification of Bcl-x_L binding to p53 was performed using Image Lab software.