*Supplementary Material*

# Sulforaphane Attenuates Glycoprotein VI-mediated Platelet Mitochondrial Dysfunction through Up-regulating cAMP/PKA Pathway in vitro and in vivo

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## 1. Supplementary Materials and Methods

### *1.1. Chemicals and reagents*

L-sulforaphane, thrombin, adenosine diphosphate (ADP), H89, SQ22536, ZM241385, MRS1754, cell membrane-permeable cGMP analog 8-pCPT-cGMP, forskolin, prostacyclin ($PGI_2$), N-acetyl-cysteine (NAC), chloral hydrate, and Erythro-9-(2 Hydroxy 3-Nonyl)adenine (EHNA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Convulxin was obtained from Cayman (Hamburg, Germany). Bay61-3606, PP2, milrinone, Ly294002, MK-2206 and Ro31-8220 were obtained from Selleck Chemical (Houston, Texas, USA). Collagen was obtained from Chrono-Log Corp. (Havertown, PA, USA). Phycoerythrin (PE)-conjugated anti-mouse CD62P

antibody and fluorescein isothiocyanate (FITC)-conjugated anti-human CD62P antibody were purchased from eBioscience (San Diego, CA, USA). Antibodies for Bax (CAT#2772), Bcl-xL (CAT#2764), cytochrome *c* (Cyto. *c*, CAT#4280), cleaved caspase-3 (17 kDa, CAT#9661), cleaved caspase-9 (37 kDa, CAT#7237), phospho-VASP at $Ser^{157}$ (CAT#84519), phospho-VASP at $Ser^{239}$ (CAT#3114), phospho-PKA substrate antibody (recognizing RRXS*/T*) (CAT#9624), Cox IV (CAT#4850), β-actin (CAT#3700) and GAPDH (CAT#5174) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody for phospho-PDE3A at $Ser^{312}$ (CAT#AF8501) was obtained from Affinity Biologicals (Ancaster, ONT, Canada).

### *1.2. Measurement of mitochondrial membrane potential (ΔΨm) in human and murine platelets*

The changes in ΔΨm in human platelets were detected by using an established red-fluorescent probe tetramethylrhodamine methyl ester (TMRM) staining, according to our previously described methods[1]. Healthy mitochondria sequester TMRM, which loses its fluorescent signal when ΔΨm is dissipated[1-2]. In brief, human gel-filtered platelets ($5\times10^6$/mL) pre-treated with SFN (1, 5, or 10 μM) or vehicle control (0.05% DMSO) for 40 min were stimulated with collagen (2 μg/mL) for additional 20 min in the presence or absence of H89 (10 μM). Platelets were then incubated with TMRM (40 nM) for 30 min at 37℃. TMRM-positive platelets were then recorded by using a CytoFLEX flow cytometer (Beckman Coulter Inc., California, USA).

The ΔΨm in murine platelets was measured by using a JC-1 assay kit (Solarbio Life Sciences, Beijing, China), according to the manufacturer's instructions. Briefly, murine gel-filtered platelets ($5\times10^6$/mL) prepared from LFD and BSE-

supplemented mice were stimulated by collagen (2 μg/mL) for 20 min at 37℃ in the presence or absence of H89 (10 μM). Platelets were incubated with JC-1 (5 μg/mL) for 20 min at 37 °C and then measured by using a FACSCalibur flow cytometer (BD Biosciences, CA, USA). The changes in platelet ΔΨm were determined by JC-1 red (JC-1 aggregates, FL3)/green (JC-1 monomers, FL1) fluorescence ratio as we previously described[3].

### *1.3. Measurement of PDE3A activity in human platelet*

PDE3A activity was measured by using Phosphodiesterase Assay Kit, according to the manufacturer's protocols (Abcam, Cambridge, UK) that have been previously described[4]. In brief, murine platelets or SFN-pretreated human platelets ($3\times10^8$ platelets/mL) were stimulated with collagen (2 μg/mL) or convulxin (50 ng/mL) for 20 min, in the presence or absence of PP2 (20 μM), Bay61-3606 (5 μM), Ro31-8220 (10 μM), Ly294002 (20 μM), or MK-2206 (10 μM). Platelet were pelleted and resuspended with PDE Assay Buffer for 10 min on ice. After centrifugation at $10000\times g$ for 10 min at 4℃, the supernatant was transferred to a fresh tube and protein concentrations were determined. PDE3A was immunoprecipitated from 500 μg of protein lysate using 1 μg of anti-PDE3A antibody or matched IgG control. The immunoprecipitate was incubated with PDE substrate at 37°C for 1 h. The levels of 5′-AMP production were measured at excitation/emission wavelength of 370 nm/450 nm. PDE3A activity was expressed as fmol AMP/min $1\times10^7$ platelets and normalized to control values to account for variations in basal activity between individual platelet donors.

### *1.4. Measurement of platelet aggregation in human and murine platelets*

Platelet aggregation in human and murine gel-filtered platelets ($2.5\times10^8$ platelets/mL) was carried out using a Techlink 400 aggregometer (Techlink Biomedical Technology Corp., Beijing, China) at 37°C with a sample stir speed of 1000 rpm. For *in vitro* study, human gel-filtered platelets were pre-treated with various concentrations of SFN (1, 5, or 10 μM) or the vehicle control for 40 min in the presence or absence of NAC (500 μM) or H89 (10 μM). For *in vivo* study, murine platelets were prepared from LFD and BSE-supplemented mice. Human and murine platelet aggregation was stimulated by 1 μg/mL collagen in the presence of 1 mM $CaCl_2$. Light transmission was monitored in real time over a period of at least 5 min. Results were expressed as a percentage of maximal light transmission[5-7].

### *1.5. Detection of platelet activation in human and murine platelets*

Platelet surface CD62P expression was used to determined platelet activation. Briefly, Human gel-filtered platelets ($5\times10^6$/mL) were pretreated with SFN (1, 5, or 10 μM) or the vehicle control for 40 min. Murine gel-filtered platelets ($5\times10^6$/mL) prepared from LFD and BSE-supplemented mice were with or without pretreatment of H89 (10 μM) for 20 min. Platelets were labeled with PE-conjugated or FITC-conjugated anti-mouse CD62P antibody for 20 min, and then stimulated by collagen (2 μg/mL) for 20 min in the presence of 1 mM $CaCl_2$. Samples were fixed with 1% paraformaldehyde and surface CD62P expression was detected by using a FACSCalibur flow cytometer or a CytoFLEX flow cytometer[5-7]. The data were analyzed by using FlowJo 7.6 software (Tree Star Inc., Ashland, OR, USA) or CytExpert 2.0 (Beckman Coulter, CA, USA).

### *1.6. Measurement of plasma SFN levels in mice*

SFN levels in the murine plasma were measured by using a liquid chromatography-mass spectrometry system (LC-MS), according to previously described methods[8]. Briefly, the murine plasma was mixed with cold acetonitrile and then centrifuged at 14000×*g* for 10 min at 4°C. The collected supernatant was passed through a 0.2 μm filter membrane (Millipore, USA). A 20-μL aliquot was injected into an automated LC with an analytical C18 column (2.0 mm × 150 mm; 3.5 μm bead size; Varian, Lake Forest, CA, USA). In the LC system, the ratio of mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) was 45:55 (v/v); flow rate was 0.2 mL/min. After loading of a 10-μL sample, the column was eluted and the eluant was directed to a mass spectrometer. The Ionspray needle was maintained at 5.5 kV under positive mode to generate the molecular ion $(M + H)^+$. The *m/z* for SFN was 178.

## 2. Supplementary References


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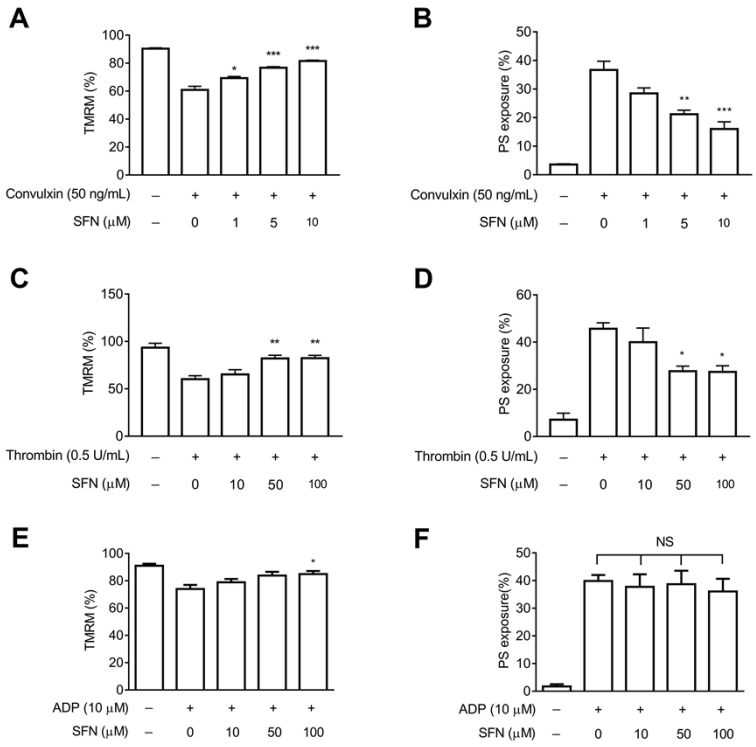
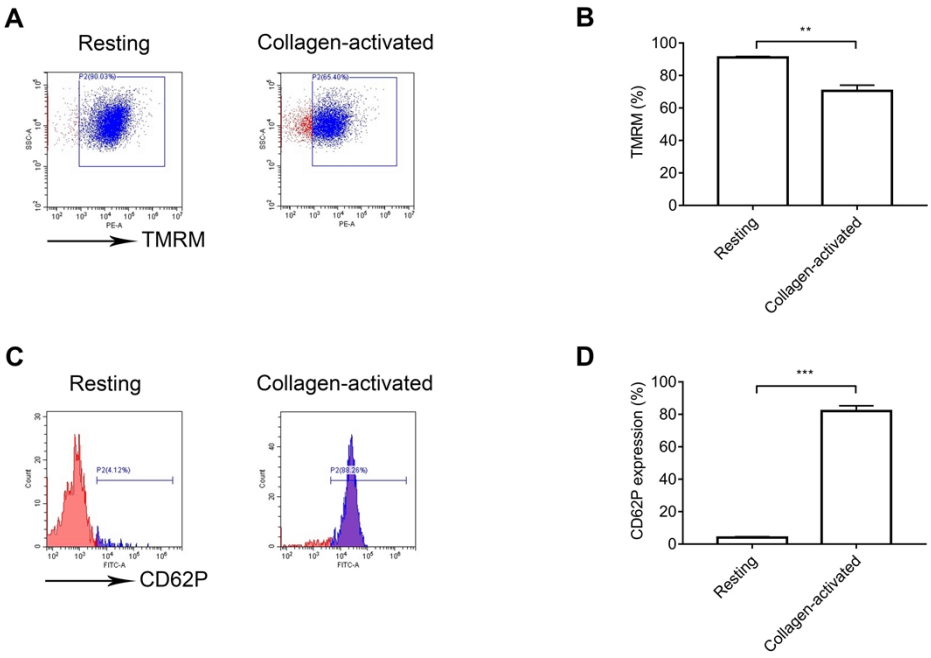
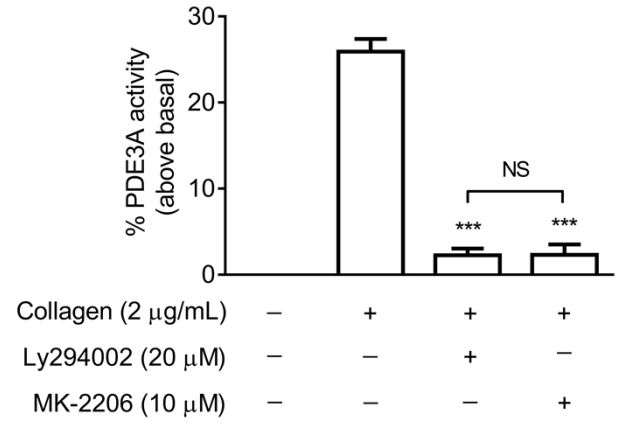
## 3. Supplementary Figures and Legends




**Supplementary Figure 1. Effects of SFN on the ΔΨm dissipation and PS exposure in human platelets in response to convulxin, thrombin and ADP. (A-B)** Human gel-filtered platelets were incubated with SFN (1, 5, or 10 μM) or the vehicle dimethyl sulfoxide (DMSO; control) for 40 min followed by the stimulation of convulxin (50 ng/mL) for additional 20 min, and platelet ΔΨm dissipation **(A)** and PS exposure **(C)** were then measured by flow cytometry. **(C-F)** Human gel-filtered platelets were incubated with SFN (10, 50, or 100 μM) or the vehicle control for 40 min followed by the stimulation of 0.5 U/mL thrombin **(C-D)** for additional 20 min, or human platelet-rich plasma (PRP) was incubated with SFN (10, 50, or 100 μM) or the vehicle control for 40 min followed by the stimulation of 10 μM adenosine diphosphate (ADP) for additional 20 min **(E-F)**, and then platelet ΔΨm dissipation **(C, E)** and PS exposure **(D, F)** were measured by flow cytometry. Data are expressed as the means ± SEM (n=3). **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 versus the vehicle control as assessed by a one-way ANOVA followed by Dunnett's *t*-test.




**Supplementary Figure 2. Effects of collagen on the ΔΨm dissipation and surface expression of CD62P in human platelets.** Human gel-filtered platelets were stimulated by collagen (2 μg/mL) for 20 min, and platelet ΔΨm dissipation **(A-B)** and surface expression of CD62P **(C-D)** were then measured by flow cytometry. ** $P < 0.01$ and *** $P < 0.001$ versus the resting platelets as assessed by unpaired Student's *t*-tests.




**Supplementary Figure 3. The role of PI3K/Akt signaling in the effects of collagen on PDE3A activity in human platelets.** Human gel-filtered platelets were pre-treated with either the PI3K specific inhibitor Ly294002 (20 μM) or Akt specific inhibitor MK-2206 (10 μM) for 20 min followed by the stimulation of collagen (2 μg/mL) for additional 20 min. PDE3A activity was determined. ***$P < 0.001$ versus the vehicle control as assessed by a one-way ANOVA followed by the Newman–Keuls test (n=3); NS, not significant difference.