The Isothiocyanate Sulforaphane Modulates Platelet Function and Protects Against Cerebral Thrombotic Dysfunction

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ABSTRACT

Background and purpose

Platelet activation provides a critical link between inflammation and thrombosis. Sulforaphane (SFN), a naturally occurring isothiocyanate, has been shown to display both anti-inflammatory and anti-thrombotic actions in the systemic microvasculature. As inflammation promotes thrombosis and *vice versa*, this study investigates whether SFN is able to reduce inflammatory potentiation of thrombotic events, suppress platelet activation and thrombus formation in the cerebral microvasculature.

Experimental approach

Thrombosis was induced in the murine brain using the light/dye-injury model, in conjunction with lipopolysaccharide (LPS) treatment, with and without SFN treatment. *In vitro* and *in vivo* platelet assays (aggregation, flow and other functional tests) were also employed, using both human and murine platelets.

Key Results

SFN was found to reduce LPS mediated enhancement of thrombus formation in the cerebral microcirculation. In tail bleed experiments LPS treatment prolonged bleeding time, and SFN treatment was found to protect against this LPS-induced derangement of platelet function. SFN inhibited collagen-mediated platelet aggregation *in vitro* and *in vivo* and the associated adhesion and impaired calcium signalling. Furthermore, Glycoprotein VI (GPVI) is involved in the protective effects observed with SFN treatment.

Conclusions and Implications

Data presented here provides evidence for the use of SFN in preventing stroke in selected high-risk patient cohorts.

Abbreviations

LPS - Lipopolysaccharide

TNF-α - Tumor necrosis factor-alpha

vWF - von Willebrand factor

SFN - Sulforaphane

NF-κB - Nuclear factor -κB

AP-1 - Activator protein-1

ROS - Reactive oxygen species

ADP - Adenosine diphosphate

PRP - platelet rich plasma

CFSE - Carboxyfluorescein succinimidyl ester

CRP - Collagen-related peptide

AUC - Area under the curve

IgG – Immunoglobulin G

GPVI – Glycoprotein VI

PGE_1 – Prostaglandin E_1

CVX - Convulxin

INTRODUCTION

Platelet activation and thrombus formation are essential mechanisms to limit blood loss at sites of vascular injury. However, inappropriate or excessive platelet activation has a detrimental role in the pathophysiology of numerous human disorders, including atherosclerosis (Aukrust *et al*., 2010), sepsis (Woth *et al.,* 2011), sickle cell disease (Frelinger *et al.,* 2014) and stroke (Fateh-Moghadam *et al.,* 2007). Platelets may become activated and adhere to the damaged endothelium leading to vessel occlusion and ischemic injury in downstream vascular beds. Enhanced platelet activation and thrombus formation are particularly relevant in stroke, which accounts for 129,000 annual deaths in the US alone (Mozaffarian *et al.,* 2016).

Inflammatory and coagulation pathways are intimately linked and have been demonstrated to cross-activate (Fiusa *et al.,* 2015). Lipopolysaccharide (LPS) and inflammatory cytokines (e.g. tumor necrosis factor-alpha (TNF-α)) increase platelet P-selectin and von Willebrand factor (vWF) on endothelial cells, thus enhancing platelet adhesion (Lou *et al.,* 1997). The interdependence between inflammation and thrombosis is particularly evident in sepsis, where the systemic pro-inflammatory state favors a shift in the hemostatic balance towards a pro-coagulant state (Fiusa *et al.,* 2015). Systemic inflammation markedly increases platelet adhesion, fibrin deposition, and propensity of platelet-dependent thrombosis within capillaries (Secor *et al.,* 2010). Indeed, excessive platelet activation under inflammatory conditions can impair microvascular perfusion and propagate disseminated intravascular coagulation, tissue hypoxia and multiple organ dysfunction (Sakr *et al.,* 2004).

Sulforaphane (SFN), a naturally occurring isothiocyanate can be found in cruciferous vegetables e.g. broccoli and cabbage (Bai *et al.,* 2015). SFN has been demonstrated to have anti-inflammatory and anti-oxidative properties, exerting a beneficial modulation of nuclear factor (NF)-κB, activator protein-1 (AP-1) and initiation of anti-oxidative pathways (Zhang *et al.,* 1992). Reactive oxygen species (ROS) promote surface expression of P-selectin on platelets and endothelial cells enhancing platelet adhesion and coagulation. Thus, SFN may influence platelet activation and adhesion via both anti-inflammatory and anti-oxidative pathways. SFN inhibits human platelet aggregation in response to a number of receptor agonists by inhibiting PI3K/Akt pathways (Chaung *et al.,* 2013). Furthermore, SFN treatment also reduces mortality in adenosine diphosphate (ADP)-induced acute pulmonary

thromboembolism (Jayakumar *et al.*, 2013) and prolongs time to platelet plug formation in fluorescein sodium-induced platelet thrombi in murine mesenteric microvessels (Jayakumar *et al.*, 2013). Despite these beneficial anti-inflammatory and anti-thrombotic features of SFN in the systemic microvasculature, the effect on thrombosis in the cerebral microcirculation is unknown. Therefore, we used *in vitro* and *in vivo* assays to investigate potential therapeutic benefits of SFN treatment in cerebral thrombosis in the context of inflammation and platelet activation.

METHODS

Animals

Male mice aged 10-12 weeks were housed in controlled-temperature/humidity environment (22 ± 1°C, 60–70% relative humidity) in individual cages (five mice per cage, with wood shaving bedding and nesting material), with a 12 h light/dark cycle (lights on at 0700 h) and were had access to a standard chow pellet diet and tap water *ad libitum.* C57BL/6 mice were purchased from either Jackson Laboratory (Bar Harbor, ME, USA) or Harlan (Bicester, UK). Mice were allowed to acclimatize to their housing environment for at least 5 days prior to experimentation and to the experimental room for 1 h before experiments. All animal experiments complied with ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines and followed the European Union Directive (2010/63/EU) or were approved by the Louisiana State University Health Sciences Center Shreveport Institutional Animal Care and Use Committee and were in accordance with the guidelines of the American Physiological Society. All studies (animals and humans) were performed blinded and randomized, with a key system to identify which animal/sample had undergone which treatment. Furthermore, compounds administered were made by laboratory personnel other than the one performing the experiment. Animals were deeply anaesthetized, as determined by the absence of a pedal reflex, with ketamine (Ketaset, 150 mg/kg, Fort Dodge Animal Health, Southampton, UK) and xylazine (Rompun, 7.5 mg/kg, Bayer Healthcare, Newbury, UK) i.p. before being killed by cervical dislocation.

Drugs

The following drugs were used at the most effective doses/concentrations based on our previous findings and dissolved in sterile saline unless otherwise stated: SFN treatment or corn oil vehicle (Sigma-Aldrich St Louis, MO, USA); LPS (0.5 mg/kg, Sigma-Aldrich);

thrombin (Sigma-Aldrich); ADP (Sigma-Aldrich); collagen (Pharmaceuticals International, Linz, Austria); Convulxin (CVX, Cayman Chemical Company, MI, USA).

Photoactivation thrombosis model

Photoactivation of the cerebral microcirculation was performed in anesthetized animals, as previously described (Gavins *et al.,* 2011). Animals were randomly assigned to treatment groups (n = 6 mice/group. Gavins *et al.,* 2011). SFN treatment or corn oil vehicle (Sigma-Aldrich St Louis, MO, USA) was administered i.p. 24 h prior to photoactivation. LPS (0.5 mg/kg) or saline was administered 4 h prior to photoactivation. Briefly, a craniectomy was performed and artificial cerebrospinal fluid (NaCl 131.9 mM, $CaCl_2$ 1.26 mM, $CaCl_2.2H_2O$ 1.26 mM, KCl 2.95 mM, $MgCl_2.6H_2O$ 0.64 mM, $MgCl_2$ 0.5 mM, $(NH_2)_2CO$ 6.69 mM, $C_6H_{12}O_6$ 3.69 mM. Sigma-Aldrich) was placed on the cranial opening. The preparation was allowed to equilibrate for 30 min. 10mg/kg of 5% FITC dextran (150 000 mol wt; Sigma-Aldrich) was injected intraventricularly (i.v.) and allowed to circulate for 10 min before photoactivation, which was initiated (excitation, 495 nm; emission, 519nm) by exposing 100 μm of vessel length to epi-illumination with a 175-W xenon lamp (Lamda LS, Novato, CA, USA) and a fluorescein filter cube (HQ-FITC; Chroma, llows Falls, VT, USA) as described previously (Gavins *et al.,* 2011). Visualization of individual vessels and induction of thrombosis was performed using a 40x water-immersion objective attached to a Xanophot IVM microscope (HLX64610; Nikon, Melville, NY, USA). Epi-illumination was applied continuously, and time of blood flow cessation (≥60 sec duration) was recorded in both venules and arterioles (30-70 μm). Epi-illumination was discontinued once blood flow ceased in the respective vessel. Video recordings were made using a silicon-intensified target camera (C-2400; Hamamatsu Photonics, Tokyo, Japan). Typically, 2-4 thrombi were induced per mouse, and results averaged.

Analysis of bleeding time

Analysis of bleeding time in SFN, LPS or vehicle-treated animals was performed as previously described (Gavins *et al.,* 2011).

Platelet counts

Platelets from peripheral blood was stained with 3% citric acid and 10% crystal violet and manually counted using a hemocytometer.

Human platelet isolation

Written informed consent was obtained from human volunteers and blood was collected with LSUHSC-S Institutional Review Board approval (IRB STUDY00000261) immediately prior to performing experiments using venipuncture of the cubital fossa. All experimental procedures were reviewed and approved by LSUHSC-S Institutional Review Board approval (IRB STUDY00000261). Volunteers were healthy, aged 18-45 and non-steroidal anti-inflammatory drug (NSAID) free 2 weeks prior to blood collection. Blood was collected with the anti-coagulant acidified sodium dextrose (ACD, 1:9. Sigma-Aldrich) and washed platelets isolated as previously described (Jones *et al*., 2010).

In vitro aggregometry

Aggregations were run with wash platelets for 3 min following addition of thrombin (Sigma-Aldrich), ADP (Sigma-Aldrich) or collagen (Pharmaceuticals International) as previously described (Jones *et al*., 2010).

Platelet Velocity of Aggregation (low angle light scattering (LASCA) method).

Human venous blood (5-6 ml) was obtained from healthy donors into ACD (1:7) loaded 10 ml syringe. Platelet rich plasma (PRP) was collected immediately by centrifugation of blood sample at 300g for 5 min (RT). Platelet aggregation velocity of freshly isolated PRP (at final concentration of platelets ~1.25-1.67 x 10^7 cell/ml) in a cuvette loaded with platelet media (NaCl 140 mM, KCl 2 mM, HEPES 10 mM, CaCl2 2 mM, Glucose 5.5 mM, and MgCl 1 mM) was analyzed using laser particle analyzer LaSca (Lumex, LTD, Saint-Petersburg, Russia) as described previously (Gavins *et al.*, 2011b). Once a constant basal signal of platelet suspension was established (1-2 min), platelet aggregation was induced with CVX (1.7 ng/mL), LPS (7.5 μg/mL. 5 min incubation prior to CVX exposure) or a combination of CVX and LPS (1.7 ng/mL and 7.5 μg/mL respectively). Several samples of PRP were also preincubated with Vehicle or SFN treatment (60 μM) 30 min at RT prior to platelet aggregation being induced with CVX, LPS or LPS and CVX. No platelet preparation was exposed to more than one concentration of either CVX or LPS administration at any one time. Velocity of platelet aggregation was analyzed by LaScA method and normalized velocity of platelet aggregation was calculated using original software LaSca_32 as previously described (Gavins *et al.*, 2011b).

In vivo aggregation

We previously developed and characterized a model for measuring thromboembolic platelet aggregation *in vivo* (Tymvios *et al*., 2008). This model was developed as a refined model of thromboembolic models that use death as an end-point (DiMinno *et al*., 1983). Blood was collected from terminally anaesthetized donor mice by cardiac puncture and platelets isolated and radiolabelled with 1.8 MBq of indium-111 oxine (Tymvios *et al*., 2008). Radiolabelled platelets were infused *via* femoral vein into anaesthetized (Urethane 25% w/v, 10 μL/g) recipient mice. Platelet aggregation was measured as increase in platelet-associated counts in the pulmonary vasculature *via* a 1 cm diameter scintillation probe over the thoracic region following i.v. injection of non-lethal concentrations of collagen (50 μg/kg). Vehicle or SFN (10 mg/kg) were administrated i.p. 30 min before collagen.

Ca^{2+} signaling

Platelets from platelet rich plasma (PRP) and incubated at 37^{o}C with $CaCl_2$ (200μM) and the ionophore Fura2-AM (5μM. Sigma-Aldrich) for 30 min in the dark. PRP was supplemented with 5 μg/ml Prostaglandin E_1 (PGE_1) and 12 μl/ml ACD, and centrifuged (400 g, 10 min). Platelets were resuspended in THB+ at 2.5 x 10^8 cells/ml, and treated with SFN (1-100 μM) for 2 min prior to measurement of fluorescence at excitation wavelengths of 340 and 380 nm, and an emission wavelength of 500 nm using a Biotech Synergy H1 hybrid plate reader (BioTek, Winooski, VT, USA) at 37 ^{o}C with constant agitation.

Platelet Lactate Dehydrogenase (LDH) Assay

LDH release was detected as a measure of cytotoxicity in washed platelets (5 x 10^7 cells/ml) in response to varying concentrations of SFN, using the Roche cytotoxicity detection kit^{PLUS} (Roche, Nutley, NJ, USA) as per the manufacturer's instructions. Absorbance was read at 490 nm using a Biotech Synergy H1 hybrid plate reader (BioTek).

Platelet-collagen adhesion under flow

Washed platelets were labeled with 5 μM Carboxyfluorescein succinimidyl ester (CFSE) (Affymetrix, CA, USA) for 30 min. Platelets were centrifuged (800 g) and resuspended in tryrodes buffer with 3 mg/ml BSA and 5 mM glucose to 2.5 x 10^7 cells/ml, and mixed with erythrocytes to give a hemocrit of 40 %. Cells were then perfused over collagen coated IBIDI μ-Slide $VI^{0.4}$ flow chambers (IBIDI, Wisconsin, USA). To investigate platelet adhesion to collagen under venular shear rates, platelets were perfused at 1 dyne/cm^2 for 10 min. For

investigation of *vWF*-mediated collagen adhesion under high shear rates, IBIDI μ-Slide $VI^{0.4}$ flow chambers were pre-coated with vWF (2 μg/mL) in tyrodes buffer + BSA and glucose, at 5 dyne/cm^2 for 4 min followed by perfusion of tyrodes buffer alone, for 1 min. Platelets were perfused over vWF coated surface at 5 dyne/cm^2 for 5 min. Buffer alone was then perfused over the surface for 1 min before capturing images using an Olympus IX71 inverted microscope (Olympus, Saucon, PA, USA) with an Olympus DP70 camera and analyzed with ImageJ (NIH) software. Data are presented as % surface area coverage. SFN treatments were given 30 min before flow.

Murine Platelet Flow cytometry

JON/A-PE and P-selectin FITC antibodies (Emfret Analytics, Eibelstadt, Germany) were used to measure collagen-related peptide (CRP)-induced activation of αIIb β3 receptors and surface exposure of P-selectin in murine platelets using flow cytometry, as previously described (Yan *et al*., 2013). Immunoglobulin G (IgG) isotype antibodies were used as controls. A platelet pellet was obtained from PRP and resuspended in Tyrodes buffer. The platelets were treated with Fc block (15 min, rt) followed by SFN (60 μM) or vehicle for 30 min. The antibodies (1:8 dilution) were added before CRP (10 μg/mL, Collagen Toolkits, Cambridge, UK) was used to stimulate the platelets for 15 min. The activation was stopped by the addition of 450 μL of 1% paraformaldehyde. Platelets were identified by their light scattering using an LSRII flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) before two-color analysis of at least 10,000 events per sample was performed.

Human Platelet Flow cytometry

AlexaFluor647 conjugated anti-human CD41/CD61 antibody (BioLegend; clone PAC-1; Cat #362806) and Alexa Fluor 647 mouse IgM*k* Isotype control (BioLegend; clone MM-30; Cat #401618) were used in 1:100 dilution to determine % of αIIb β3 complex positive platelets. FITC conjugated anti-human CD62P (P-selectin) (Emfret; clone AK4; Cat# 304904) and FITC mouse IgG1*k* isotype control (Emfret; clone MOPC-21; Cat #400107) in 1:100 dilution was used to established P-selectin positive platelets. Human venous blood (5-6 mL) was obtained from healthy donors into ACD (1:7) loaded syringe. Platelet rich plasma (PRP) was collected immediately by centrifugation of blood sample at 300 g for 5 min (RT). Topical layer of PRP was transferred into new eppendorf and centrifugated at 3000 rpm for 10 min (Thermo Scientific Legend Micro 17 centrifuge). Supernatant was discarded and platelet

pellet was reconstituted with Tirode`s buffer (no $Ca^{2+}$). Fc block was used to inhibit non-specific binding accordingly with manufacture instruction (eBioscience). Platelets were reconstituted with Tyrode`s buffer with 1mM Ca^{2+} at final concentration $1x10^6$ cells per 25 µl and incubated with SFN (60 µM) for 30 min, LPS (7.5 µg/mL) for 5 min, and with combination with of SFN and LPS, stained with antibodies and isotopies. Selected platelet samples were stimulated with CVX (1.7 ng) for 15 min at 37^0C and platelet reactivity was stopped by adding 1% formaldehyde and platelet samples were assayed using flow cytometry assay with BD LSRII SORP immediately after fixation. 20000 events were recorded per sample and analyzed by DivaSoftware 8.0.1.

Data analysis and statistical procedures

All data were analyzed using GraphPad Prism 6 for MacOS X (Version 6). Data are expressed as mean ± SEM with n values given in the respective figure legends. When determining statistical significance between two groups an unpaired *t*-test was carried out and where appropriate, corrected for multiple comparisons using the Holm-Šídák method. Multiple groups were analyzed by one-way ANOVA or non-parametric Kruskal–Wallis test and *post-hoc* comparisons were performed by Bonferroni or Dunn's multiple comparison test respectively. *In vivo* platelet aggregation data were expressed as the percentage increase in maximal radioactive counts from the baseline recordings or area under the curve (AUC). A student *t*-test was used to compare mean values a. Differences were considered statistically significant if $p < 0.05$. The figures have been graphically presented on a range-specific axis. All data and statistical analysis comply with British Journal of Pharmacology guidelines (Curtis *et al.*, 2015).

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org,thecommon portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding *et al.*, 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander *et al.*, 2017).

RESULTS

SFN treatment inhibits thrombus formation in the inflamed cerebral microvasculature

Thrombus formation, assessed as time to blood flow cessation in murine cerebral arterioles and venules, was quantified using the light dye injury model and intravital fluorescence microscopy (Figure 1, A-C). Time required for complete flow cessation was longer in arterioles than venules, as reported previously (Gavins *et al*., 2011; Yan *et al*., 2013). LPS (0.5 mg/kg, 4 h) reduced time to flow cessation, demonstrating the pro-thrombotic environment within the inflamed cerebral microvasculature following systemic LPS exposure (Figure 1). We then assessed the influence of SFN pre-treatment on this accelerated thrombus formation. SFN treatment was found to have no effect on thrombus formation under non-inflammatory conditions. However, SFN (5 and 50 mg/kg administered 24 h prior to saline or LPS treatment) reversed the LPS-induced reduction in thrombotic time in both cerebral venules and arterioles (Figure 1).

SFN treatment reverses LPS-induced prolongation of bleeding *in vivo*

In order to investigate whether SFN could also influence coagulation following peripheral injury, tail-bleeding experiments were performed. LPS treatment prolonged tail-bleeding time and reduced the number of circulating platelets (Figure 2A and 2B). Whilst in the absence of inflammatory challenge SFN treatment had no effect on tail bleeding time, in LPS treated animals SFN was found to counteract the LPS-induced delay in cessation time (Figure 2A). No significant effect was observed in circulating platelet number in LPS treated animals with and without SFN (Figure 2B).

SFN treatment directly inhibits human platelet aggregation

Having implicated a protective role for SFN in reversing the LPS-induced pro-thrombotic cerebral microvascular phenotype *in vivo*, we next examined whether SFN may directly act on platelets to inhibit aggregation and thrombus formation in response to thrombin, ADP or collagen. Light transmission aggregometry experiments were performed on isolated human platelets. SFN (0.1-100 μM) did not induce any platelet aggregation above vehicle alone, while thrombin (0.1 U/ml) demonstrated a robust response (Figure 3A). When SFN treatments were given 2 min prior to activation with thrombin no influence over platelet aggregation was observed (*data not shown*). However, if SFN was administered 30 min prior to activation only the highest concentration (100 μM) tested was found induce a significant reduction of platelet

aggregation (Figure 3B). When given 2 min prior to activation the highest concentration of SFN tested, was able to inhibit platelet aggregation in response to ADP stimulation (*data not shown*), however all lower concentrations were ineffective. No effect was observed when SFN was administered 30 min prior to ADP stimulation (Figure 3C). SFN treatment did however show a robust inhibition of collagen-stimulated platelet aggregation, with both 60 μM and 100 μM significantly reducing collagen responses when given 2 min prior (*data not shown*). When given 30 min before activation, all but the lowest concentration of SFN were found to provide a significant inhibition of platelet aggregation in response to collagen (Figure 3D). Indeed, 60 μM and 100 μM treatments were found to completely abolish platelet collagen responses.

SFN treatment inhibits collagen-induced platelet aggregation *in vivo*

Having shown an effect of SFN on isolated human platelet aggregation, we explored the relevance of this *in vivo* using an established mouse model of radiolabeled platelet aggregation. Collagen (50 μg/kg i.v.) induced a transient increase in platelet-associated counts that returned to baseline (Figure 4). This has previously been shown to represent reversible aggregation of platelets (Jones *et al*., 2010). Treatment with SFN (10 mg/kg) for 30 min before injection of collagen had no effect on the amplitude of platelet aggregation response but significantly reduced the duration of the response measured as AUC (Figure 4).

SFN suppresses collagen induced Ca^{2+} signaling

Platelet intracellular calcium signaling is crucial for platelet aggregation and thrombus formation and is activated upon vWF and collagen binding. Thus, effects of SFN on calcium signaling were investigated. Consistent with the lack of aggregatory responses to SFN, treatment with SFN alone did not induce calcium signaling in isolated human platelets (Figure 5A). Collagen stimulation of human platelets was found to induce significant transient increases in intracellular calcium levels as detected by an increased ratio of 340/380 nm excitation emission of Fura 2AM dye. Treatments with 60 and 100 μM SFN, 2 min prior to stimulation, were found to significantly inhibit collagen-induced intracellular calcium signaling events (Figure 5B). In order to test ensure the observed reduction in platelet responses was not due to reduced cell viability at high SFN concentrations, an LDH assay was performed. Across all treatments tested, cells remained intact (Figure 5C).

SFN inhibits platelet adhesion under physiological flow

The above experiments demonstrate that SFN strongly inhibits collagen-induced platelet-platelet aggregation. *In vivo,* at sites of vascular injury, platelets come into contact with sub-endothelial collagen allowing them to adhere, triggering their activation and inducing subsequent thrombus formation. Thus, it is important to investigate levels of platelet adhesion to a collagen substrate under physiological flow conditions. CSFE labeled human platelets were perfused at shear rates physiologically relevant to venular shear stress, and platelet adhesion monitored. SFN treatment (100 μM) 30 min prior to flow was found to significantly reduce platelet adhesion to collagen, as represented by a reduced platelet coverage of collagen coated surface following 10 min of flow (Figure 6A + B).

While under static conditions and at low shear rates platelets may directly adhere to collagen, in conditions where shear rates are above 5-10 dynes/cm^2 vWF is a crucial determinant of thrombus formation. Circulating vWF is activated by immobilization at sites of vascular injury and the action of shear stress exposes binding sites in the vWF-A1 domain, allowing interaction with platelet GPIbα. As such, arteriole thrombosis in humans requires vWF interaction. To investigate vWF-mediated platelet adhesion to collagen, isolated human platelets were perfused over vWF-activated collagen IV at 5 dynes/cm^2 and platelet adhesion assessed. Unlike at the lower shear of 1 dyne/cm^2, in the absence of vWF very few platelets were found to adhere to collagen at 5 dyne/cm^2 within the time frame tested. However, perfusion of vWF for 4 min prior to the introduction of platelets resulted in a greater than 20-fold increase in platelet adhesion. Platelet pre-treatment with 1,40 and 100 μM (Figure 6C) SFN 30 min prior to flow was found to reduce the platelet-vWF-collagen adhesion (Figure 6D).

Effect of SFN is via a suppression of the GPVI pathway in murine platelets.

In light of the observed effects of SFN on collagen *in vivo* and *in vitro* under physiological flow conditions, and the fact that, in addition to vWF-mediated platelet-collagen interactions, GPVI is a platelet receptor crucial to the collagen-induced activation and aggregation, flow cytometry of isolated murine platelets was performed to investigate the influence of SFN on GPVI-mediated platelet activation. Activation status was measured by expression of the activated form of αIIb β3 using the JON/A antibody (DiMinno *et al*., 1983), and surface expression of P-selectin (Figure 7). While the GPVI-selective peptide CRP stimulated

platelets to increase levels of activated αIIb β3, SFN treatment (60 μM) was found to suppress these levels (Figure 7A+C). This anti-activation effect of SFN was further evidenced by the SFN-mediated reduction of CRP-induced P-selectin expression (Figure 7B+D).

SFN is via a suppression of the GPVI pathway in LPS induced inflammatory setting

Next, we wanted to translate these findings to the clinical setting and further tease out the mechanism of action of SFN in an inflammatory backdrop (i.e. LPS. We assessed the effect of SFN on platelet P-selectin and αIIb β3 expression following stimulation with the GPVI collagen receptor agonist convulxin (CVX). Figure 8 shows that CVX induced a marked increase in platelet activation (Figure 8A+B), which was suppressed by co-administration of SFN (60 μM). Stimulation with LPS (7.5 μg/ml) alone did not affect platelet αIIb β3 or P-selectin levels but it potentiated these markers of platelet activation when in the presence of CVX (which concurs with Lopes Pires *et al.,* 2017). SFN treatment (60 μM) was found to suppress these effects (Figure 8C+D), suggesting that SFN reduces platelet activation under inflammatory conditions via GPVI.

To analyze the effects of LPS on platelet aggregation velocity, platelet rich plasma was incubated with LPS (0, 1 and 7.5 μg/ml. Lopes Pires *et al.*, 2017) in the presence or absence of CVX. Whilst no effect of LPS alone was observed with platelet aggregation velocity (Supplemental Figure 1), in the presence of CVX, accelerated aggregation was observed (which concurs with previous findings (Lopes Pires *et al.,* 2017). Furthermore, these effects were reduced by the co-administration with SFN (Figure 8C+D), suggesting that SFN also reduces the velocity at which platelets aggregate in inflammatory conditions by interfering with GPVI signaling.

DISCUSSION

To evaluate the potential of SFN to reduce platelet thrombotic function during inflammation, we utilized two distinct *in vivo* models: tail bleeding time and cerebral intravascular occlusion in combination with LPS treatment to study the inflammatory perturbations of platelet activity. Consistent with the observation of sepsis-induced thrombocytopenia frequently observed in humans, we found LPS treatment in mice reduces the number of circulating platelets. In tail bleeding experiments LPS administration was found to prolong bleeding time, while in the cerebral microcirculation, light/dye induced thrombosis time was significantly reduced in LPS treated animals. These apparently contradictory findings are also evident in

sepsis, which is associated with an initial activation of coagulation and fibrinolysis with subsequent depletion of anticoagulant systems via endothelial disruption and glycocalyx degradation releasing heparin-like substances (Youn *et al*., 2010; Burgess *et al*., 2000; Patel *et al*., 2008). As such sepsis-induced disseminated intravascular coagulation (DIC) is characterized by simultaneously occurring thrombotic and bleeding problems (Gavins *et al*., 2011b), as has been observed here in LPS stimulated mice.

SFN treatment was found to counteract enhanced platelet activation and thrombus formation in the cerebral microcirculation by prolonging time to blood flow cessation. However, opposite effects were observed in the tail-bleeding model, in which SFN expedited time to cessation of bleeding. The bleeding assay is widely used as an *in vivo* assessment of hemostatic action of platelets (Zhang *et al*., 2009), hence the opposing effects are likely due to the mechanisms regulating these two processes of thrombosis and hemostasis not being identical, and effects being quantified outside (tail bleeding model) and inside the body (thrombosis model (Liu *et al*., 2012). Clinically, this is an important finding in that SFN protected against the pro-thrombotic disorder not only without increasing bleeding risk in our model, but actually alongside reducing bleeding. The influence of LPS on thrombotic function likely occurs through a number of mechanisms e.g. the direct influence of LPS on platelet function has been demonstrated through the activation of platelet cell surface TLR4, inducing platelet activation and secretion of dense and alpha granules and hence ATP release and P-selectin expression (Zhang *et al*., 2009). The ability of SFN to suppress TLR4 oligomerization in a thiol-dependent manner (Youn *et al*., 2010) along with other SFN anti-inflammatory mechanisms, may explain the lack of SFN's influence over *in vivo* thrombosis in the absence of pathological LPS challenge. In addition, some redundancy has previously been demonstrated in the tail bleed model between thrombin and collagen mediated platelet activation (Bynagari-Settipalli *et al*., 2014). Ablation of collagen signaling had previously been shown to have no effect on tail bleeding if thrombin signaling remains intact (Bynagari-Settipalli *et al*., 2014). Whilst *in vitro* we observed a robust inhibition of collagen induced aggregation with reduced intracellular signaling and collagen adhesion, relatively little effect was observed upon thrombin-induced activation. This preservation of thrombin signaling may explain the observed differences in SFN effects between the two models utilized.

In order to further study effects of SFN treatment on platelet function, we performed a number of *in vitro* studies using human platelets. We found SFN to have a small influence over ADP and thrombin-mediated platelet aggregation but found SFN to be most effective in reducing collagen-induced platelet activation. The observation that SFN has potent inhibitory actions on platelet aggregation is not unique to this study and has previously been demonstrated by others using *in vitro* collagen induced aggregation (Jayakumar *et al*., 2013; Kilkenny *et al*., 2010) and collagen-induced thromboembolic mortality *in vivo* (Kilkenny *et al*., 2010). Thus, in order to further investigate this phenomenon effects of SFN on collagen-mediated calcium signaling and platelet adhesive activity to collagen under physiological flow conditions were investigated. SFN treatment was found not only to reduce *in vitro* aggregation but also to inhibit both collagen-induced intracellular signaling and platelet binding/adhesion to collagen, suggesting SFN may interfere with collagen receptor binding. *In vivo* studies confirmed that the ability of SFN to inhibit collagen-induced platelet aggregation was relevant in an *in vivo* setting where platelets circulate in whole blood under the influence of negative mediators of platelet activation generated by the vascular endothelium. Therefore, effects of SFN on platelet aggregation were not an artifactual observation occurring only in isolated platelets. In contrast to earlier studies using thromboembolic mortality, effects of SFN here can conclusively be attributed to an effect on platelets rather than non-specific factors involved in the death of the animal.

Considering collagen-induced platelet aggregation has been demonstrated to be dependent on ADP (Gavins *et al*., 2011a), and that in the present study SFN treatment profoundly influenced platelet collagen responses but had little effect on ADP-induced platelet activation, these results suggest interference in platelet collagen signaling upstream of ADP, perhaps at the receptor level. While platelet adhesion to collagen is evident under static conditions or at low shear rates platelets, under levels of higher shear rates, relevant to conditions in arterioles vWF is crucial for platelet adhesion and thrombus formation. Thus, experiments were performed to assess the extent at which SFN may influence collagen-vWF-platelet interactions, finding SFN treatment to also inhibit vWF-mediated adhesion.

Both collagen activation and vWF binding of platelets are highly relevant to the pathology of atherosclerosis and stroke, where extracellular matrix exposure leads to platelet collagen binding via vWF. Elevated circulating levels of vWF have been demonstrated to remain

elevated for up to 3 months following stroke (Tang *et al*., 2014). The initial platelet-vWF-collagen interaction is via platelet GPIba and tethers the platelets to the endothelium. However, immobilization and subsequent activation of platelets requires direct binding of platelet GPVI to collagen, as evidenced by the failure of platelets from GPVI-deficient mice to respond to collagen (DiMinno *et al*., 1983). GPVI, via inside-out signaling, enhances the affinity of other integrins such as αIIb β3, ultimately leading to platelet adhesion. As such, GPVI plays a central role in thrombosis. Here, flow cytometry was utilized to investigate whether the anti-thrombotic effects observed with SFN was by interfering with GPVI signaling. By directly stimulating the GPVI pathway with CRP, we found that SFN was able to decrease activation of αIIb β3 and surface expression of P-selectin. Thus, GPVI is involved in the protective effects observed with SFN treatment. It is interesting to note that SFN has already been previously shown to significantly inhibit platelet–neutrophil aggregation (along with P-selectin and GPIIb–IIIa expression) in subjects with metabolic syndrome vs. healthy controls (Konić-Ristić *et al*., 2013). With respect to our study, whilst the effects of SFN on the interactions between leukocytes and platelets are not the main aim of our study, we found that SFN reduces expression of GPIIbIIIa and P-selectin on platelets which may reduce platelet-leukocyte interactions through P-selectin inhibition by increasing levels of cAMP followed by inhibiting intracellular signals (such as the PI3-kinase/Akt and PLCγ2-PKC-p47 cascades) and ultimately inhibiting platelet activation. Additionally, we found these effects of SFN on the markers of platelet activation (i.e. GPIIbIIIa and P-selectin) and furthermore also on platelet aggregation velocity to also hold true when platelets were assessed *in-vitro* under inflammatory conditions. Our findings suggest that SFN is able to shift the hemostatic balance towards an anti-coagulant state.

Here we have shown that SFN reduces LPS-mediated light/dye-induced thrombus formation within the cerebral microvasculature *in-vivo.* LPS effects the expression of GPVI, GP1b (vWF receptor) (Liu *et al*., 2013) and alpha2beta1 (type 1 collagen receptor) on platelets and collagen and vWF on endothelial cells (Echeverría *et al*., 2013). Both collagen and vWF activate platelet GPVI and GP1b, stimulating Ca^{2+} signaling in platelets and resulting in the overexpression of GPIIb/IIIa and alpha2beta1 on the surface of the platelet. These effects lead to accelerated platelet adhesion, activation, aggregation and exacerbated thrombus formation. Our findings show that the effect of SFN on LPS-mediated thrombotic responses may be dependent on the involvement of GPVI receptor on platelets as well as collagen. SFN may

also inhibit LPS-enhanced thrombosis by decreasing Ca^{2+} mobilization which may affect platelet activity, at least in part, through adenylate cyclase (AC)/cAMP followed by inhibiting intracellular signals (such as the PI3-kinase/Akt and PLCγ2-PKC-p47 cascades) and ultimately inhibiting platelet activation (Jayakumar *et al*., 2013). In addition, as SFN reduced thrombin-induced aggregation, it is highly likely that the effects of SFN are protease-activated receptor (PAR)3 and PAR4-mediated, but not through ADP-receptors (P2Y1 and P2Y12) (as demonstrated by a lack of response of SFN against ADP-induced aggregation). Thus, inhibition of the LPS-induced thrombotic responses by SFN in combination with effectiveness of SFN in suppressing collagen-induced Ca^{2+} mobilization, platelet adhesion underflow (GPVI) and platelet GPIIb/IIIa expression demonstrates the effectiveness of SFN in GPVI-mediated signaling as a potential therapeutic target and anti-thrombotic strategy.

In nucleated cells, SFN activates the transcription factor Nrf2 through binding cystine residues on KEAP1, although SFN has been shown to promiscuously bind to cysteine on thiol groups and thus influence protein function (Zhang *et al*., 1992). GPVI activation has been shown to involve receptor oligermization (Yan *et al*., 2013) stabilized through disulfide bonding between cysteinyl thiol groups (Yan *et al*., 2013). Thus, considering the prevalent role of SFN in binding thiol groups, it cannot be ruled out that SFN inhibits receptor dimerization through interaction with cystinyl-thiol groups. Furthermore, SFN has been previously shown to suppress oligomerization of TLR4 in a thiol-dependent manner, as mentioned above. Platelet thiol-disulfide balance is highly influential in regulating platelet function and upon activation 440% increase in surface protein thiol groups has been observed (Youn *et al*., 2010). Conformational changes in GPIb upon platelet activation have been shown to result in exposure of free thiols (Liu *et al*., 2012). Moreover, thiol groups are important in a number of other integrins such as $\alpha_{IIb}\beta_3$, which may indirectly interact with collagen via vWF. Thus, interaction with GPVI, as indicated by our experimental results, may occur through thiol groups. However, further investigations are needed to determine whether SFN modifies extra and/or intracellular thiol groups on GPVI

Efficacy of anti-platelet agents is often limited by bleeding complications. Thus, the present finding that SFN inhibited both the simultaneously LPS-induced elevated peripheral bleeding and increased propensity for intravascular thrombosis, whilst having no effect in saline treated

animals, is most promising for the treatment of thrombotic disorders and inflammatory related thrombotic complications.

The present study demonstrates the protective functions of SFN pretreatment in the brain by normalizing platelet thrombotic functions under inflammatory conditions, which is relevant to prophylactic strategies. The therapeutic benefit of SFN as an acute treatment to inhibit platelet activation, subsequent to thrombotic or inflammatory events, will require further investigation. However, the present findings are promising, in that SFN provides a modulation of platelet activation in the context of pathological inflammatory environment rather than an indiscriminant inhibition of platelet activation, thus potentially avoiding bleeding complications associated with current anti-thrombotic medications.

AUTHOR CONTRIBUTIONS

SG, PMH, SAV FR and KAT designed and performed experiments, interpreted results and helped write manuscript. FB provided scientific input, interpreted results and helped write manuscript. KYS designed experiments, provided scientific input and helped write manuscript. ME designed experiments, provided scientific input and interpreted results and helped write manuscript. FNEG designed and performed experiments, provided scientific input and interpreted results and helped write manuscript. All authors reviewed the manuscript.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

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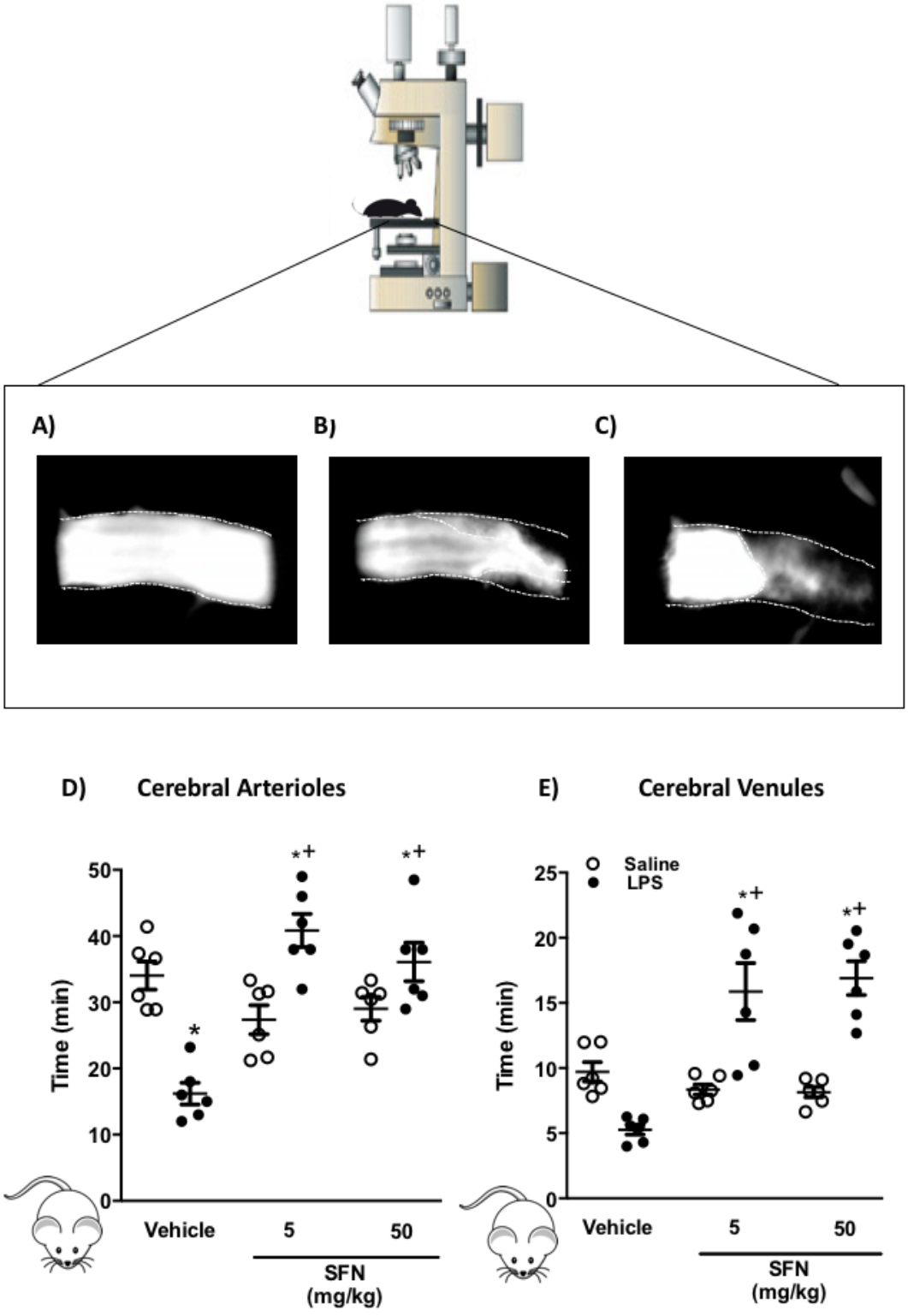

Figure 1

Figure 1. SFN treatment inhibits thrombosis formation in the inflamed cerebral microcirculation. Light/dye induced thrombosis was visualized in the brain and quantified in terms of time taken for complete blood flow cessation. Images taken of thrombus formation in the brain arterioles during light/dye thrombosis events at A) 0 min, B) 21 min and C) 29 min

when blood flow cessation occurred. Values represent time to flow cessation following light/dye injury in cerebral D) arterioles and E) venules. Animals were treated with SFN (5 or 50 mg/kg) or vehicle 24 h prior to inflammatory challenge with LPS (0.5 mg/kg). Bar = 10 μm. Data are mean±SEM of 6 mice/group with 1-3 vessels analyzed per mouse. $^{*}P<0.05$ vs. vehicle control. $^{+}P<0.05$ vs. LPS treated group.

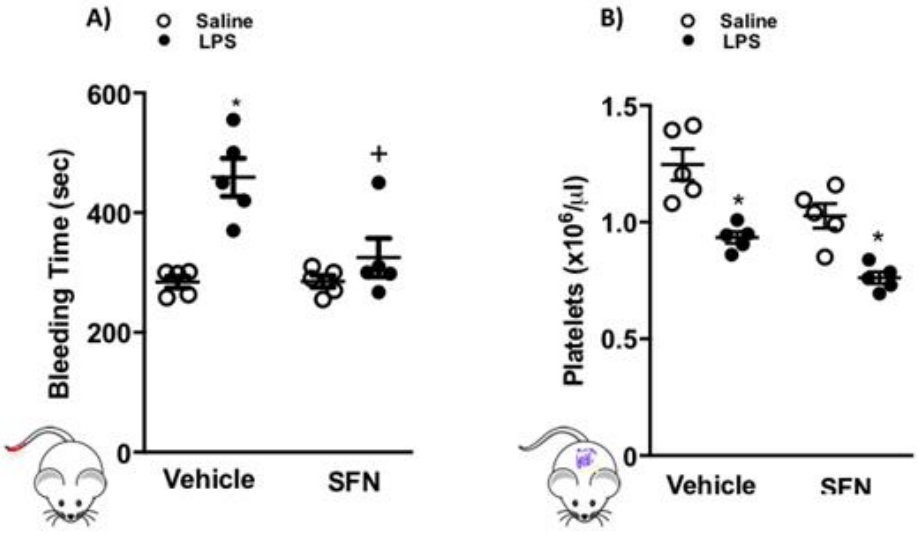

Figure 2

Figure 2. SFN treatment inhibits the LPS induced prolonged bleeding time A) Duration of tail bleeding time and B) peripheral blood platelet counts following SFN (50 mg/kg) or vehicle (saline) 24 h prior to inflammatory challenge with LPS (0.5 mg/kg). Data are mean±SEM of 5 mice/group. $^{*}P<0.05$ vs. corresponding vehicle group. $^{+}P<0.05$ vs. LPS treated group.

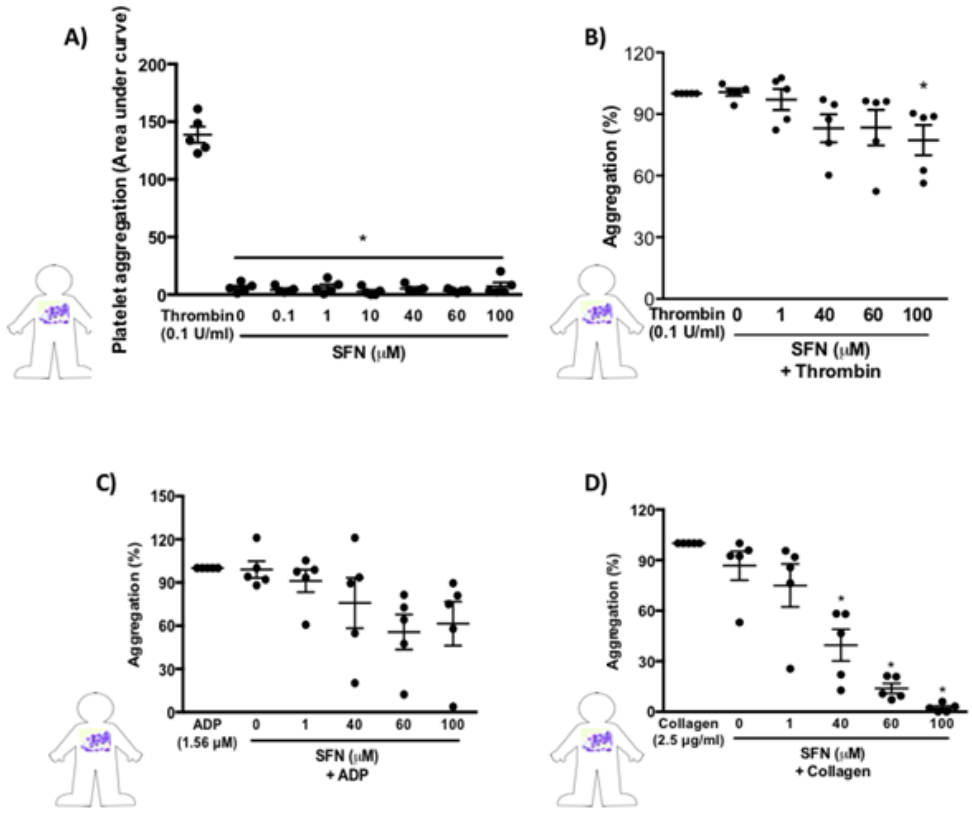

Figure 3

Figure 3. SFN treatment reduces human platelet aggregation in vitro. Light transmission aggregometry was used to investigate the influence of SFN treatment (0.1-100 μM) for 30 min on isolated platelet aggregation responses to a number of platelet agonists: A) no agonist, B) Thrombin, C) ADP and D) collagen. All aggregations were run for 3 min following addition of the platelet agonists. Aggregation responses are expressed as a percentage of aggregation achieved by platelet agonist alone. Data are mean±SEM of n=5 independent donors/group. $^{*}P<0.05$ vs. control.

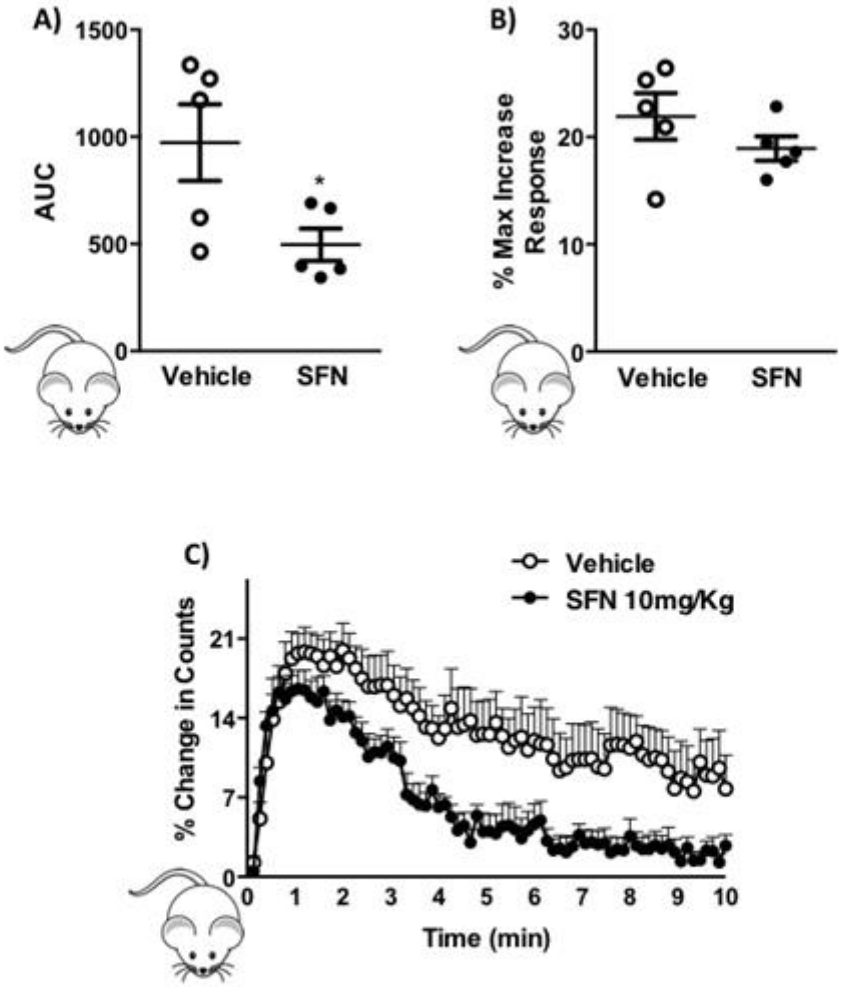

Figure 4

Figure 4. SFN reduces collagen-induced platelet aggregation *in vivo*. A thromboembolic *in vivo* model was used to measure platelet aggregation in mice treated with SFN (10mg/Kg) or vehicle saline (V) for 30 min prior to IV injection of collagen (50 µg/Kg). Platelet aggregation was measured by changes in platelet-associated radioactivity (10 min) and data are represented as a) maximum % increase in platelet counts, b) area under the curve (AUC) and c) % of change in platelet counts. Data are mean±SEM of n=5 mice/group; $^{*}P<0.05$ vs. saline vehicle treated group. $^{+}P<0.05$ vs. collagen stimulated vehicle group.

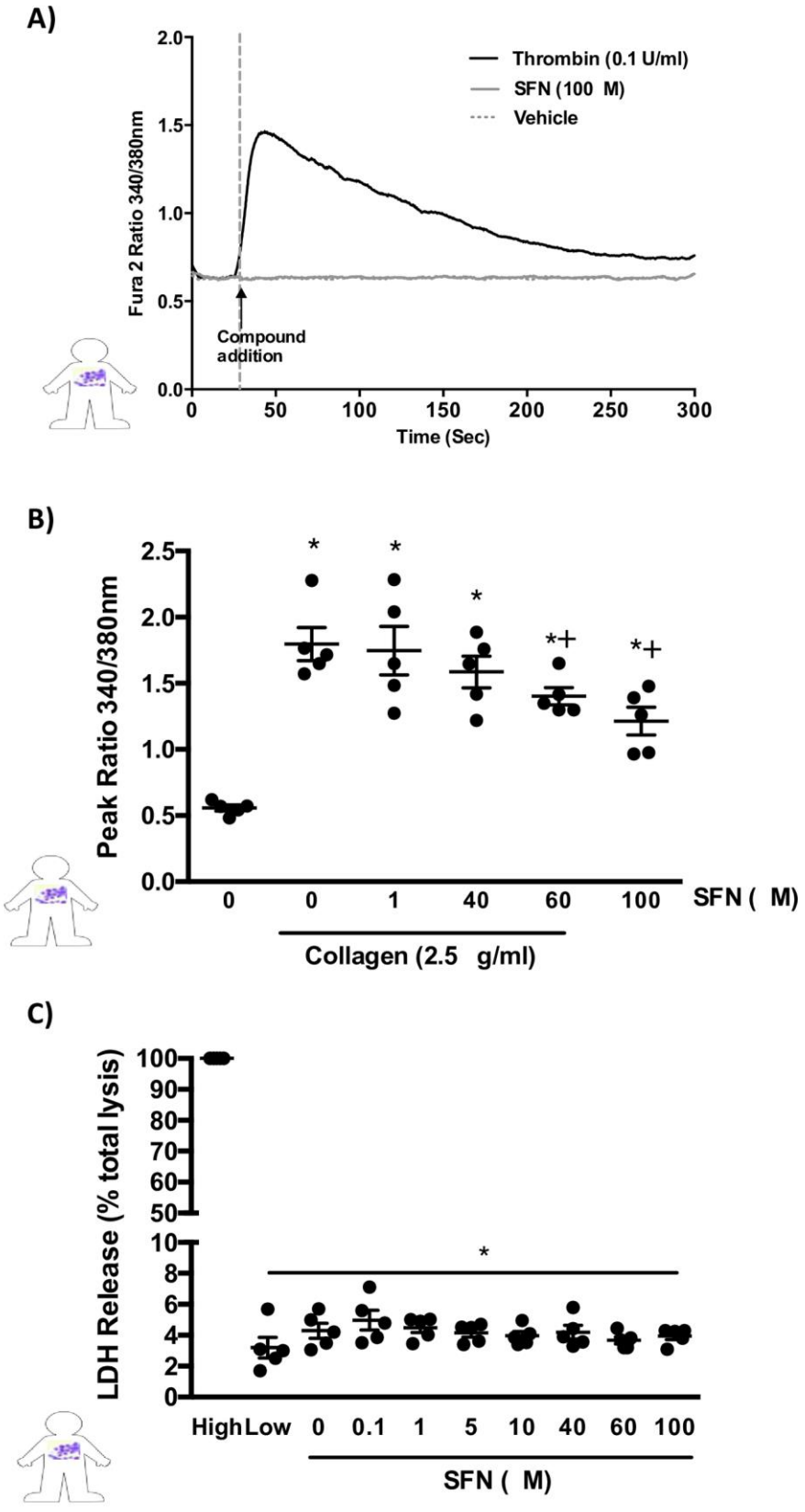

Figure 5

Figure 5. SFN influence over collagen induced Ca2+ signaling. Cytosolic calcium release was measured using Fura 2AM dye in human platelets. A) Representative trace showing temporal increase in Fura 2AM fluorescence following administration of the positive control

(0.1 U/ml Thrombin) but with no effect SFN treatments alone. B) Histogram of peak 340/380 nm emission, in platelets stimulated with 2.5 μg/ml collagen following SFN treatment. C) LDL release as a marker for cytotoxicity following SFN treatment, data presented as a % LDH release when compared to completely lysed cells. Data are mean±SEM of n=5 independent donors/group. $^{*}P<0.05$ vs. un-stimulated control. $^{+}P<0.05$ vs. collagen stimulated vehicle group.

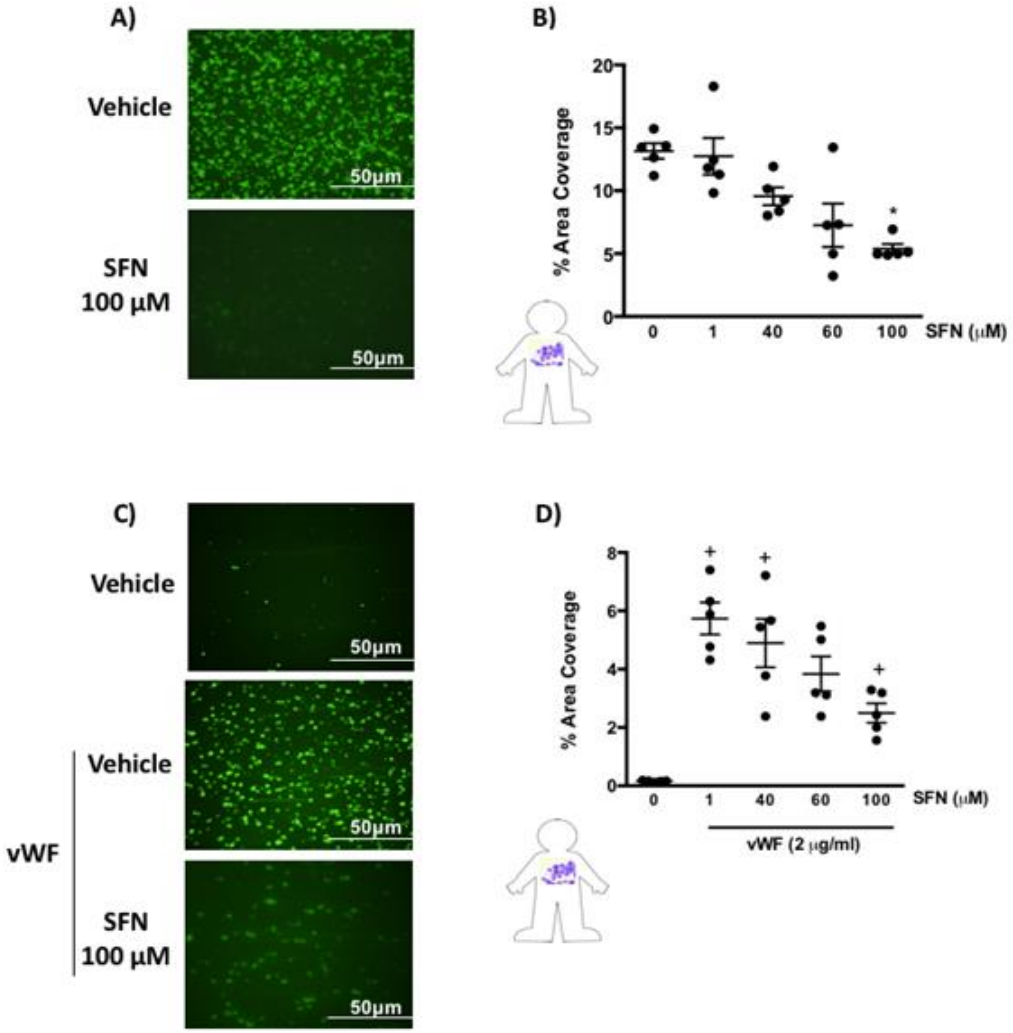

Figure 6

Figure 6. SFN treatment reduces collagen and vWF mediated collagen platelet adhesion under conditions of physiological flow. A) and B) human platelets were perfused over collagen-coated flow chambers at 1 dyne/cm^2 for 10 min before imaging, SFN treatments were given 30 min prior to flow. C) and D) collagen coated flow chambers were pre-treated with vWF (2µg/ml) before perfusion of platelets at 5 dyne/cm^2, and SFN given 30 min prior to flow. Data are mean±SEM of n=5 independent donors/group. $^{*}P<0.05$ vs. vehicle treated in the absence of vWF. $^{+}P<0.05$ vs. vWF treated vehicle group.

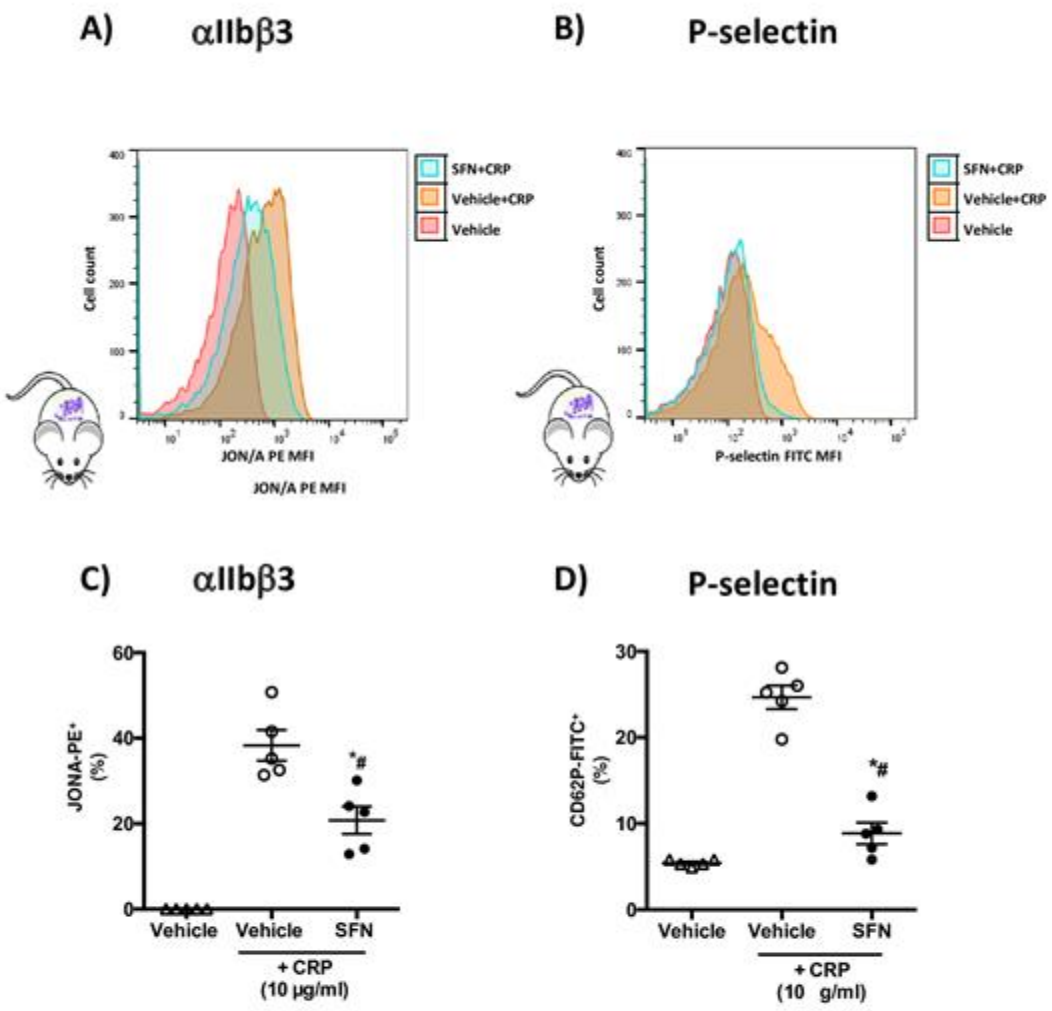

Figure 7

Figure 7. Platelet expression of cell surface receptors following CRP stimulation.
Activated αIIb β3 and P-selectin were quantified using flow cytometry in platelets following 15 min stimulation with collagen-related peptide (CRP@10 µg/mL). Vehicle or SFN treatment (60 µM) was given 30 min prior to activation. A+B) representative histograms of activated αIIb β3 and P-selectin expression on platelets treated with SFN/vehicle +/- CRP stimulation. Mean fluorescence intensities of platelet C) activated GPIIbIIIa and D) P-selectin. Data are mean±SEM of n=5 mice/group. $^{*}P<0.05$ vs. control (vehicle) and CRP group. $^{\#}P<0.05$ vs. CRP only control group.

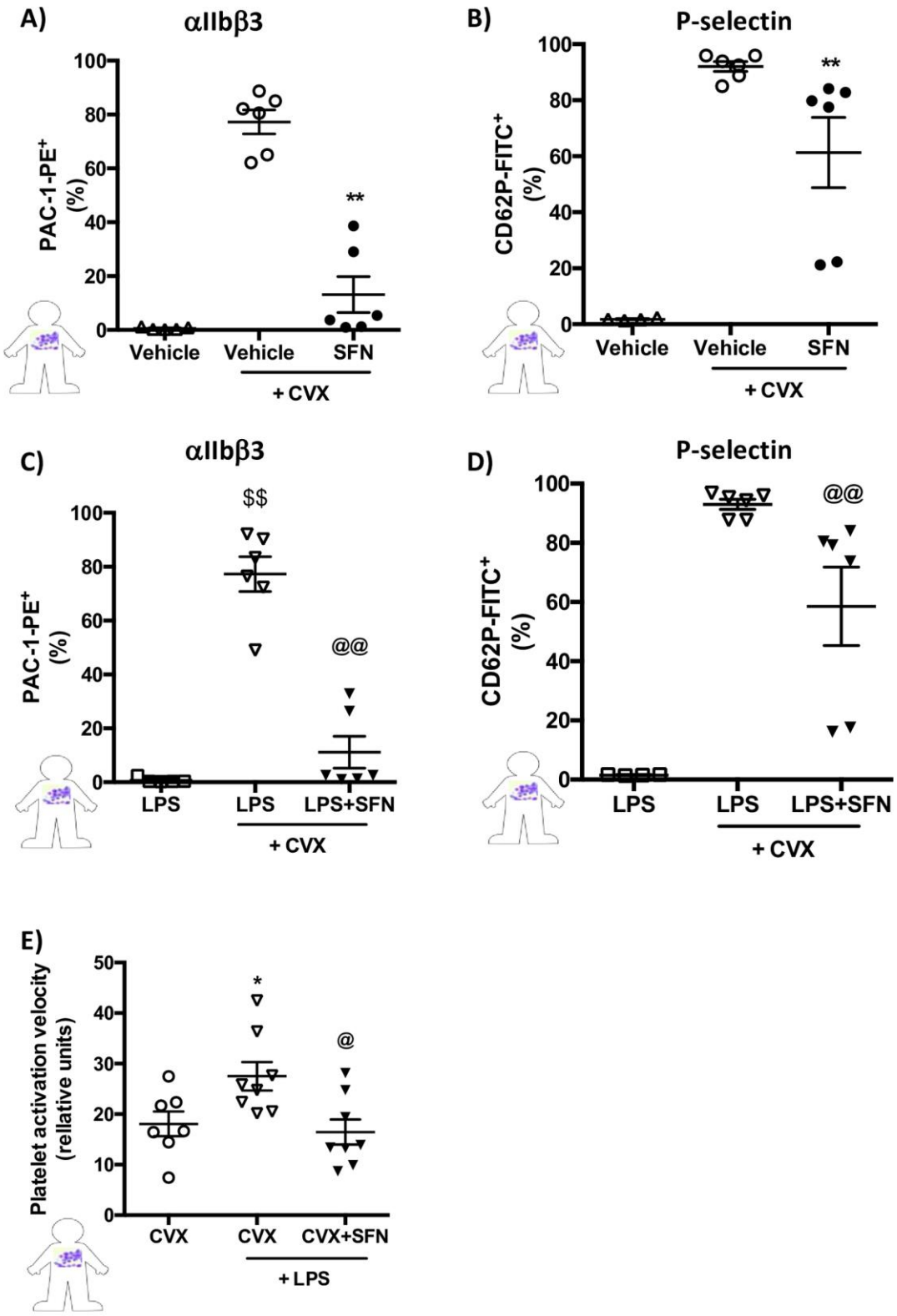

Figure 8

Figure 8. Human platelet expression of cell surface receptors post CVX stimulation.

Activated αIIb β3 and P-selectin were quantified using flow cytometry in platelets following 15 min stimulation with the GPVI collagen receptor agonist convulxin (CVX. 1.7 ng). Vehicle

or SFN treatment (60 μM) was given 30 min prior to activation, followed by vehicle (saline) or LPS (7.5 μg/ml for 5 min). Mean fluorescence intensities of platelet A+C) activated GPIIbIIIa and B+D) P-selectin. E) Velocity of aggregate formation was measured using low angle light scattering technique for platelets stimulated by CVX, CVX+LPS or CVX+LPS+SFN (concentrations as above). Data are mean±SEM of n=5-6 mice/group (n=4 for vehicle (P-selectin) only and LPS (P-selectin) only groups due to an outlier in each). $^{\#}P<0.05$ vs. control (vehicle) only group. $^{\$}P<0.05$ vs. LPS only group. $^{**}P<0.01$ vs. control (vehicle) and CVX group. $^{@}P<0.05$, $^{@@}P<0.01$ vs. LPS and CVX. group.