Heliyon 9 (2023) e20286

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Myricetin as a promising inhibitor of platelet fibrinogen receptor in humans

Yi Chang [a,b,c,1], Chih-Wei Hsia [b,1], Wei-Chieh Huang [b], Thanasekaran Jayakumar [d], Chih-Hsuan Hsia [e], Ting-Lin Yen [f], Joen-Rong Sheu [b,g,*], Shaw-Min Hou [b,c,h,**]

[a] Department of Anesthesiology, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, 111, Taiwan
[b] Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, Taipei, 110, Taiwan
[c] School of Medicine, College of Medicine, Fu Jen Catholic University, New Taipei City, 242, Taiwan
[d] Department of Ecology and Environmental Sciences, Pondicherry University, Puducherry, 605014, India
[e] Translational Medicine Center, Shin Kong Wu Ho-Su Memorial Hospital, Taipei, 111, Taiwan
[f] Department of Medical Research, Cathay General Hospital, Taipei, 106, Taiwan
[g] Department of Pharmacology, School of Medicine, College of Medicine, Taipei Medical University, Taipei, 110, Taiwan
[h] Department of Cardiovascular Center, Cathay General Hospital, Taipei, 106, Taiwan

ARTICLE INFO

Keywords:
Arterial thrombosis
Fibrinogen
Human platelets
Integrin $\alpha_{IIb}\beta_3$
Myricetin
Occlusion time

ABSTRACT

Platelets play a vital role in the formation of dangerous arterial thrombosis. Platelets are activated by adhesive proteins or soluble agonists through their specific receptors. The receptor-mediated signaling pathways lead to common signaling events, which result in shape changes and inside–out signaling, leading fibrinogen binding to glycoprotein IIb/IIIa complex (integrin $\alpha_{IIb}\beta_3$). This interaction initiates integrin $\alpha_{IIb}\beta_3$-mediated outside-in signaling, subsequently culminating in granule secretion and aggregation. Myricetin is a flavonoid that occurs in a variety of plants. Although myricetin has been demonstrated to have several bioactive properties, its role in platelet activation has not been extensively studied. The present study demonstrated the ability of myricetin to inhibit platelet aggregation stimulated by collagen, thrombin, and U46619. Myricetin reduced the ATP-release, cytosolic Ca^{2+} mobilization, and P-selectin expression and the activation of PLCγ2/PKC, PI3K/Akt/GSK3β, and MAPK. Myricetin exerted a direct inhibitory effect on the activation of integrin $\alpha_{IIb}\beta_3$ by disrupting the binding between FITC-PAC-1 and the integrin. Moreover, myricetin suppressed integrin $\alpha_{IIb}\beta_3$-mediated outside–in signaling, such as integrin β_3, Src, and Syk phosphorylation on immobilized fibrinogen. In animal studies, myricetin significantly prolonged the occlusion time of thrombotic platelet plug formation in mesenteric microvessels without extending bleeding time. This study concludes that myricetin is a natural integrin $\alpha_{IIb}\beta_3$ inhibitor and a novel antithrombotic agent.

* Corresponding author. Graduate Institute of Medical Sciences, College of Medicine, Taipei Medical University, 250 Wu-Hsing St., Taipei, 11031, Taiwan.
** Corresponding author. Department of Cardiovascular Center, Cathay General Hospital, 280 Sec. 4, Jen-Ai Rd., Taipei, 106, Taiwan.
E-mail addresses: sheujr@tmu.edu.tw (J.-R. Sheu), cgh05414@cgh.org.tw (S.-M. Hou).
[1] Contributed equally to this work

https://doi.org/10.1016/j.heliyon.2023.e20286
Received 27 June 2023; Received in revised form 12 September 2023; Accepted 18 September 2023
Available online 21 September 2023

1. Introduction

Platelets, which are anucleated blood cells, play a pivotal role in the pathogenesis of cardiovascular diseases (CVDs) through the formation of blood clots within blood vessels [1]. The primary physiological role of platelets is the formation of hemostatic thrombi, serving to prevent hemorrhage and uphold vascular integrity. However, the development of pathological arterial thrombosis constitutes the principal mechanism behind cardiocerebrovascular diseases, including myocardial infarction, ischemic stroke, and peripheral arterial disease. These conditions represent the leading causes of mortality worldwide. Platelets are activated when they come into contact with a damaged blood vessel or exposed collagen fibers. Once activated, they release chemicals such as ADP and thromboxane A_2 (TxA_2) that attract more platelets to the site of injury, which leads to the formation of a clot [2]. Activated platelets then recruit circulating platelets by triggering fibrinogen to bind to glycoprotein IIb/IIIa complex (integrin $\alpha_{IIb}\beta_3$), which is mainly expressed on platelets and is necessary for the formation of blood clots. In their resting state, platelets exhibit a low activation level of integrin $\alpha_{IIb}\beta_3$, thereby constraining its capacity to engage with specific ligands, such as fibrinogen and fibronectin [3]. However, upon stimulation by agonists, platelets undergo conformational changes in integrin $\alpha_{IIb}\beta_3$, leading to its activation and subsequent interaction with ligands, ultimately triggering platelet aggregation [3,4].

Myricetin is a valuable naturally occurring flavonoid that is widely distributed in several plants, including berries, fruits (e.g., guava), vegetables (e.g., garlic), tea (e.g., black tea), and herbs [5]. It was reported to have potential therapeutic effects for the prevention and treatment of several chronic diseases, including CVDs, diabetes, and neurodegenerative diseases [6]. Myricetin exhibits anti-inflammatory activity that is beneficial for the treatment of inflammation-related disorders, such as arthritis and inflammatory bowel disease; it acts by inhibiting the expression of proinflammatory cytokines and enzymes, such as cyclooxygenase (COX)-2 and inducible nitric oxide synthase [6]. In addition, myricetin was discovered to exert anticancer effects through the inhibition of the growth and proliferation of cancer cells and induction of apoptosis or programmed cell death in several tumor cells [5]. Furthermore, myricetin can lower blood pressure, improve lipid profiles, and exert antiatherosclerosis effects by reducing low-density lipoprotein (LDL) oxidation and inhibiting the uptake of oxidized LDL by macrophages [7]. Moreover, it reduces ischemia/reperfusion injury, alters cardiac function, and reduces myocardial injury [8], all of which are risk factors for CVDs [5].

Despite being recognized for its pharmacological properties, the effects of myricetin on platelet activation have not been extensively studied. However, a notable investigation by Tseng et al. [9] revealed that myricetin effectively inhibits rabbit platelet aggregation triggered by collagen, platelet activating factor, and ADP. Additionally, myricetin inhibited calcium mobilization and TxB_2 formation by blocking COX in human platelets [10]. Studies on the effects of myricetin on human platelets are limited and fragmented. Therefore, the present study conducted a more comprehensive and systematic investigation of the mechanisms by which myricetin activates platelets in humans. The results obtained from our study demonstrate that myricetin displays antithrombotic activity by directly interfering with the function of integrin $_{\alpha IIb}\beta_3$. This novel mechanism of action positions myricetin as a promising and innovative antiplatelet agent.

2. Materials and methods

2.1. Materials

Myricetin (≥98%) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Aspirin, aprotinin, bovine serum albumin (BSA), collagen (type I), dimethyl sulfoxide (DMSO), ethylenediaminetetraacetate (EDTA), fibrinogen, heparin, leupeptin, luciferin–luciferase, thrombin, 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin (U46619), paraformaldehyde, PDBu, prostaglandin E_1 (PGE_1), phenylmethylsulfonyl fluoride, sodium pyrophosphate, sodium orthovanadate, and sodium fluoride, were all sourced from Sigma-Aldrich (St. Louis, MO, USA). Anti-phospho PLCγ2 monoclonal antibody (mAb) was obtained from Abcam (Cambridge, UK). Anti-phospho-integrin β_3 (Tyr^{759}) polyclonal antibody (pAb), and anti-phospho-GSK3α/β and anti-α-tubulin mAbs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-p38 MAPK (Thr^{180}/Tyr^{182}) pAb was purchased from Affinity (Cincinnati, OH, USA). Anti-phospho-p44/p42 extracellular signal–regulated kinase (ERK; Thr^{202}/Tyr^{204}), anti-phospho-*c*-Jun N-terminal kinase (Thr^{183}/Tyr^{185}), anti-phospho-phosphoinositide 3-kinase (PI3K) p85 (Tyr^{458})/p55 (Tyr^{199}), anti-phospho-(Ser) PKC substrate, phospho-Src family (Tyr^{416}; D49G4), and phospho-spleen tyrosine kinase (Syk; $Tyr^{525/526}$) pAbs were purchased from Cell Signaling (Beverly, MA, USA). Anti-phospho-Akt (Ser^{473}) pAb was purchased from BioVision (Mountain View, CA, USA), and a protein assay dye reagent concentrate was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Fura 2-AM was obtained from Molecular Probes (Eugene, OR, USA). CF™488A Dye and CF™405 M Dye were obtained from Biotium (Hayward, CA, USA). FITC mouse anti-human PAC-1 was purchased from BD Biosciences (San Jose, CA, USA). Fluorescein isothiocyanate (FITC)–anti-human CD42P (P-selectin) was purchased from BioLegend (San Diego, CA, USA). Horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G (IgG), and goat anti-mouse IgG, enhanced chemiluminescence Western blotting detection reagent, and hybond-P polyvinylidene difluoride membranes were obtained from Amersham (Buckinghamshire, UK). Myricetin was solubilized in 0.1% DMSO and preserved at 4 °C until utilization in ensuing experiments.

2.2. Preparation and aggregation of human platelets

The research protocol strictly adhered to the ethical guidelines outlined in the Declaration of Helsinki and received approval from the Institutional Review Board of Taipei Medical University (TMU-JIRB-N202112047). Informed consent was obtained from all human volunteers who participated in the study. Blood samples were obtained from adult volunteers who abstained from using any

medications or substances that could potentially influence the experiment for a minimum of 14 days prior to sample collection. The collected blood samples were combined with an acid-citrate-dextrose solution in a ratio of 9:1 (v/v). Subsequently, centrifugation was performed to obtain platelet-rich plasma, which heparin (6.4 IU/mL) and EDTA (2 mM) were added as supplements. The final suspension of washed human platelets was prepared using Tyrode's solution containing 3.5 mg/mL of BSA, and the final Ca^{2+} concentration in Tyrode's solution was 1 mM [11]. To assess platelet aggregation, platelets were preincubated with a solvent control (0.1% DMSO) and various concentrations of myricetin for a duration of 3 min, prior to the introduction of agonists such as collagen, thrombin, and U46619. Platelet aggregation was measured using a Lumi-Aggregometer (Payton, Scarborough, ON, Canada), and the results are presented as the percentage of platelet aggregation relative to the control group treated with 0.1% DMSO. For the ATP release assay, luciferin–luciferase was added 1 min before the collagen was added, and absorbance measurements were analyzed utilizing a Hitachi Spectrometer F-7000 (Tokyo, Japan).

2.3. Assessment of lactate dehydrogenase activity and cytosolic calcium ion mobilization

The current study investigated the cytotoxic impact of myricetin by analyzing the lactate dehydrogenase (LDH) level. Washed platelets at a concentration of 3.6×10^8 cells/mL were preincubated with different concentrations of myricetin or 0.1% DMSO as the solvent control for 20 min at 37 °C. Subsequently, a 10 μL sample of the supernatant was applied to a Fuji Dri-Chem slide LDH-PIII (Fuji, Tokyo, Japan), and the absorbance was measured at 540 nm using an ultraviolet–visible spectrophotometer (UV-160; Shimadzu, Japan). The maximal LDH level was determined by measuring triton-lysed platelets as a positive control. Additionally, to evaluate the cytosolic calcium concentration, platelet preparations were incubated with Fura 2-AM (5 μM). The absorbance readings were then taken using the Hitachi Spectrometer F-7000 (Tokyo, Japan) at excitation wavelengths of 340 nm and 380 nm, with an emission wavelength set at 500 nm [11].

2.4. Determination of surface P-selectin expression and activation of integrin $\alpha_{IIb}\beta_3$

The current study used fluorophore-labeled antibodies to detect platelet P-selectin expression and activation of integrin $\alpha_{IIb}\beta_3$. Washed platelets (3.6×10^8 cells/mL) were preincubated with myricetin (10 and 20 μM) and FITC-conjugated anti-P-selectin mAb (2 μg/mL) or FITC-conjugated PAC-1 mAb (2 μg/mL) for 3 min. Subsequently, the platelets were stimulated with collagen (1 μg/mL) for an additional 5 min. Fluorescein-labeled platelets were detected in the suspensions by using a flow cytometer (FACScan system, Becton Dickinson, San Jose, CA, USA). The data were collected from 10,000 platelets per experimental group, and the platelets were identified on the basis of their characteristic forward and orthogonal light-scattering profiles. To ensure reproducibility, all experiments were repeated at least four times.

2.5. Immunoblot analysis

Washed platelets at a concentration of 3.6×10^8 cells/mL were preincubated with solvent control (0.1% DMSO) or myricetin (10 and 20 μM) for 3 min. Subsequently, collagen was added to initiate activation. In another experiment, dishes with a 6 cm diameter were coated with fibrinogen (100 μg/mL) overnight and then blocked with 1% BSA. The washed platelets preincubated with solvent control (0.1% DMSO) or myricetin (10 and 20 μM) were added to the dishes, and the platelets were allowed to undergo adhesion and surface extension for a duration of 60 min. The platelets were lysed, yielding 80 μg of protein in the resulting supernatant, which was then subjected to separation by 12% sodium dodecyl sulfate gel electrophoresis. Protein concentrations were quantified using the Bio-Rad Bradford protein assay. The study then used respective primary antibodies to detect the targeted proteins, and the optical density of the protein bands was measured using a video densitometer and Bio-Profil Biolight software (version V2000.01; Vilber Lourmat, Marne-la-Vallée, France). The relative expression of the protein of interest was calculated after normalization to the expression of the total protein.

2.6. Confocal laser fluorescence microscopy

In brief, resting or collagen (1 μg/mL)-activated platelets at a concentration of 3×10^7 cells/mL were immobilized on poly-L-lysine-coated coverslips and fixed with 4% (v/v) paraformaldehyde for 1 h. Following fixation, the platelets were permeabilized with 0.1% Triton X-100 and then subjected to a 1-h incubation with 5% BSA in phosphate-buffered saline (PBS). after which they were stained with primary antibodies specific to the targeted proteins and incubated overnight. The platelets were washed with PBS and incubated with either goat anti-rabbit CF 488A Dye or goat anti-mouse CF 405 M Dye for 1 h. Fluorescence images were obtained using a Leica TCS SP5 microscope with a 100 × oil immersion objective lens (Leica, Wetzlar, Germany).

2.7. Assessment of sodium fluorescein-induced vascular thrombosis in mouse mesenteric microvessels

Prior to conducting in the vivo experiments, this study received approval from the Institutional Animal Care and Use Committee of Taipei Medical University (LAC-2021-0386). Male ICR mice were intraperitoneally administered either 50 μL of 0.1% DMSO or myricetin (5 and 10 mg/kg) prior to the intravenous injection of sodium fluorescein (15 μg/kg) via the lateral caudal vein, as previously detailed [12]. The mice were anesthetized using intraperitoneal injection of sodium pentobarbital (50 mg/kg). Microthrombi were produced in venules (30–40 μm) on irradiation at a wavelength of <520 nm, and the duration for which the thrombus blocked the

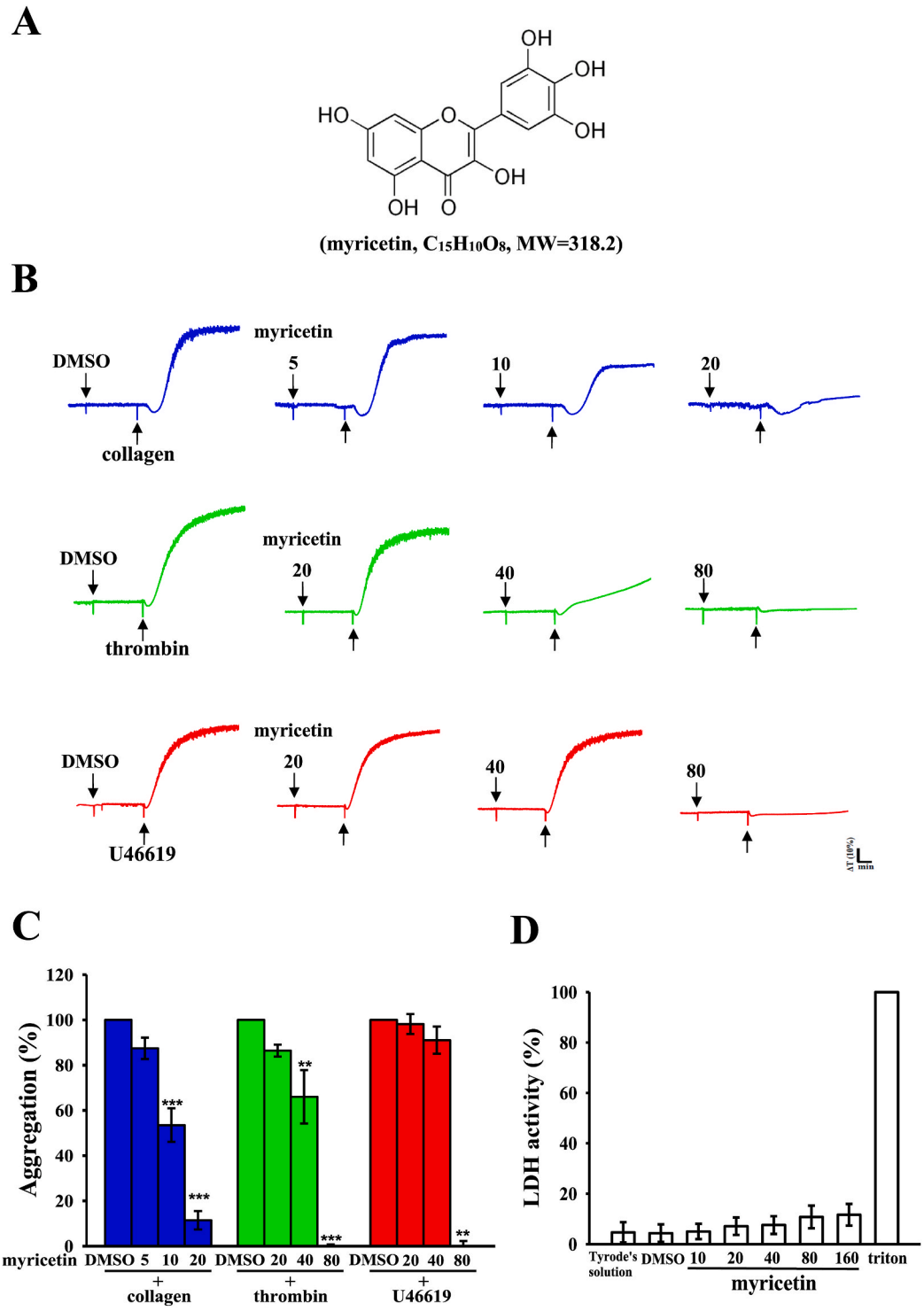


Fig. 1. Effect of myricetin on platelet aggregation and lactate dehydrogenase release stimulated by agonists in washed human platelets. (A) Chemical structure of myricetin ($C_{15}H_{10}O_8$). (B) Washed platelets (3.6×10^8 cells/mL) were preincubated with 0.1% DMSO (solvent control) or different concentrations of myricetin and then induced with collagen (1 μg/mL), thrombin (0.02 U/mL), or U46619 (1 μM). (C) Concentration-response histograms were constructed to depict the inhibitory impact of myricetin on platelet aggregation stimulated by the agonists, presented as percentages (%). (D) Additionally, washed human platelets were preincubated with myricetin (10–160 μM) for 20 min, and a 10-μL supernatant aliquot was deposited onto a Fuji Dri-Chem slide lactate dehydrogenase (LDH)-PIII. Data are presented as the mean ± standard error of the mean ($n = 4$). $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the 0.1% DMSO + agonist group.

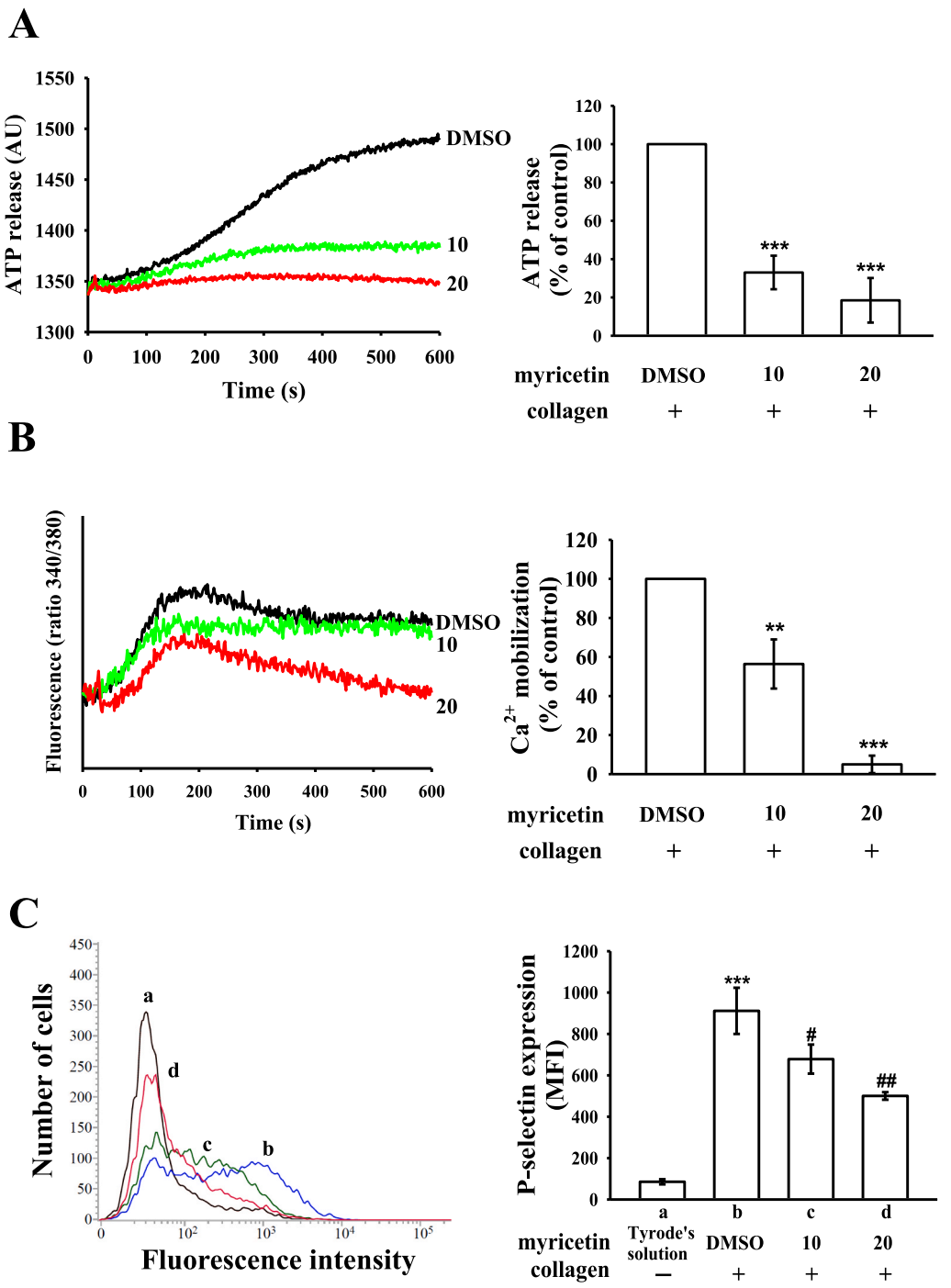


Fig. 2. Effect of myricetin on adenosine triphosphate release, relative intracellular Ca^{2+} mobilization, and surface P-selectin expression in human platelets. Washed platelets were preincubated with 0.1% DMSO or myricetin (10 and 20 μM) before the addition of collagen (1 μg/mL) to trigger (A) adenosine triphosphate (ATP) release (arbitrary unit [AU]), (B) relative intracellular Ca^{2+} ($[Ca^{2+}]i$) mobilization, and (C) surface P-selectin expression (mean fluorescence intensity [MFI]; a. Tyrode's solution, b. 0.1% DMSO + collagen, c. 10 μM myricetin + collagen, and d. 20 μM myricetin + collagen). Data are presented as the mean ± standard error of the mean ($n = 4$). (A and B) $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the 0.1% DMSO + collagen group. (C) $^{***}p < 0.001$ compared with the resting (Tyrode's solution) group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with the 0.1% DMSO + collagen group.

microvessel (referred to as “occlusion time”) was documented.

2.8. Assessment of tail vein bleeding time in mice

To measure bleeding time, we conducted tail vein transection in ICR mice that had been anesthetized with 50 μL of 0.1% DMSO or

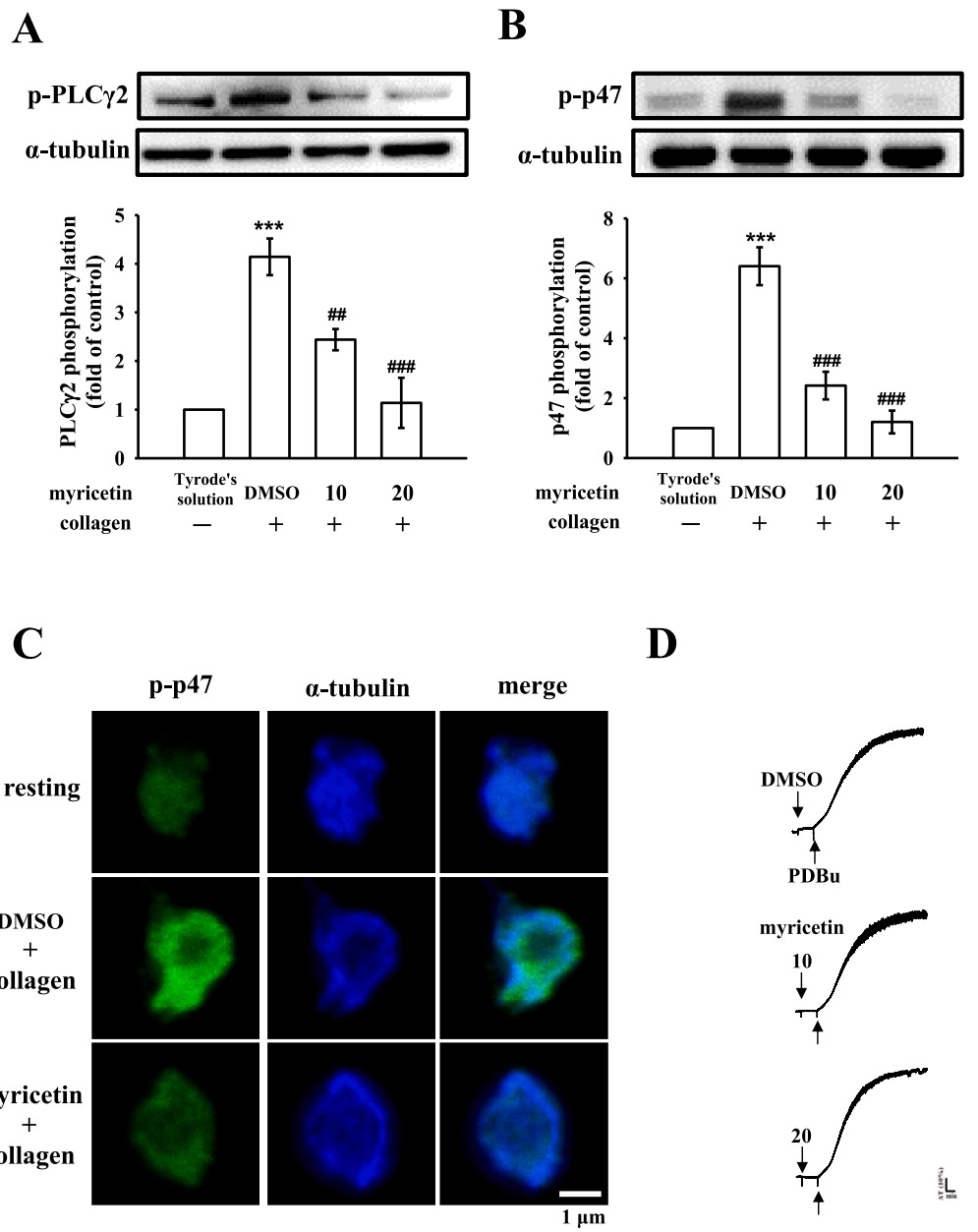


Fig. 3. Effect of myricetin on the activation of phospholipase Cγ2 and protein kinase C in platelets. Washed platelets were preincubated with 0.1% DMSO or myricetin (10 and 20 μM), and collagen (1 μg/mL) was subsequently added to trigger (A) phospholipase Cγ2 (PLCγ2) and (B) protein kinase C (PKC) activation (p-p47). (C) Green fluorescence, representing phosphorylated p47, and blue fluorescence, corresponding to α-tubulin, were examined via confocal microscopy using goat anti-rabbit CF 488A and anti-mouse CF 405 M Dyes, respectively. (D) Washed platelets were subjected to preincubation with either 0.1% DMSO or myricetin (10 and 20 μM), followed by the addition of phorbol 12,13-dibutyrate (PDBu, 150 nM) to induce platelet aggregation. Data are presented as the mean ± standard error of the mean ($n = 4$). $^{***}p < 0.001$ compared with the resting (Tyrode's solution) group; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared with the 0.1% DMSO + collagen group. Confocal images and aggregation profiles were obtained from four independent experiments.

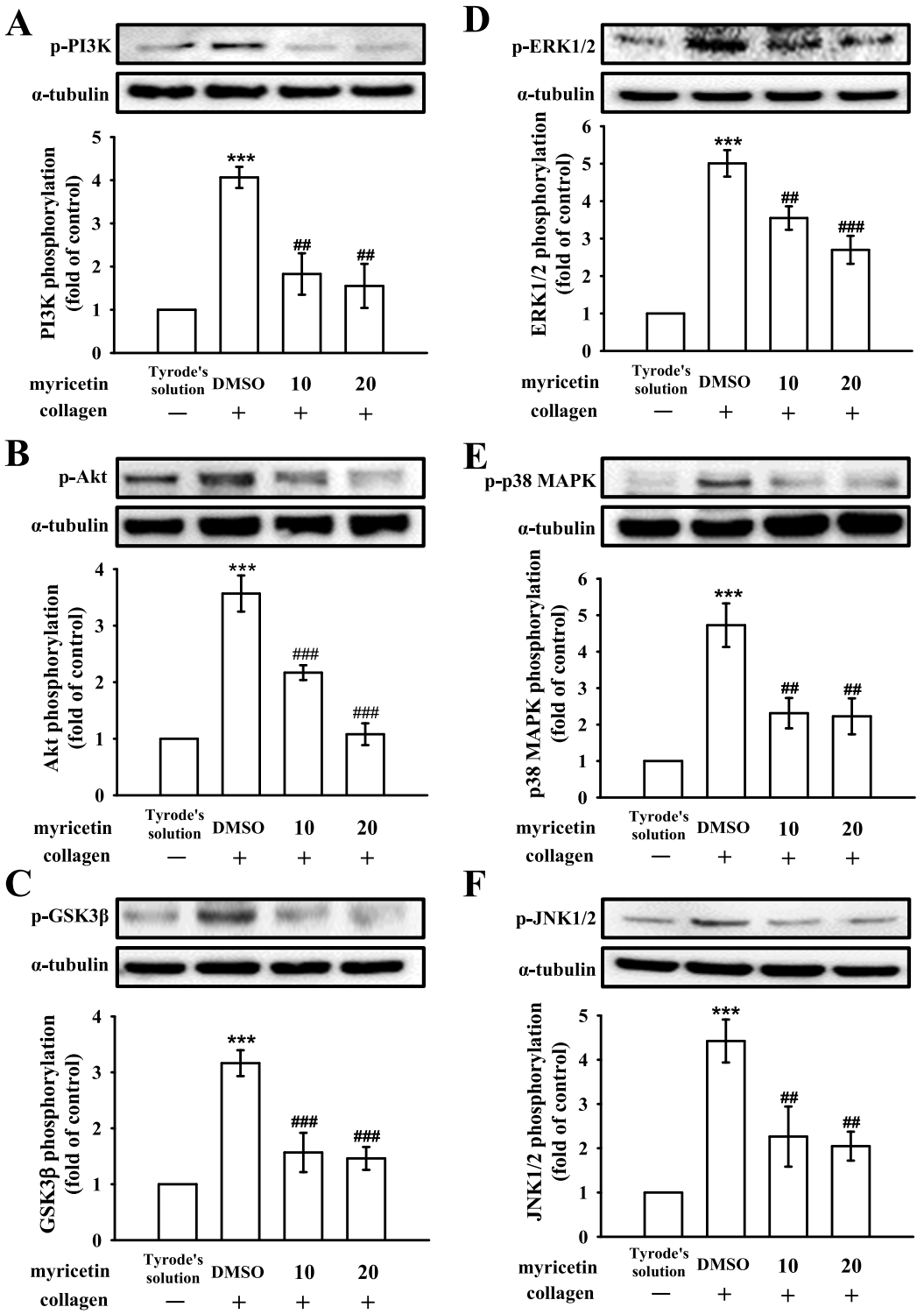


Fig. 4. Inhibitory effects of myricetin on the activation of phosphoinositide 3-kinase/Akt/glycogen synthase kinase 3β and mitogen-activated protein kinases in platelets. Washed platelets were preincubated with 0.1% DMSO or myricetin (10 and 20 μM), and collagen was subsequently added (1 μg/mL) to trigger the activation of (A) phosphoinositide 3-kinase (PI3K), (B) Akt, (C) glycogen synthase kinase 3β (GSK3β), (D) extracellular signal–regulated kinase 1/2 (ERK1/2), (E) p38 mitogen–activated protein kinase (p38 MAPK), and (F) c-Jun N-terminal kinase 1/2 (JNK1/2). Data are presented as the mean ± standard error of the mean ($n = 4$). $^{***}p < 0.001$ compared with the resting (Tyrode's solution) group; $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared with the 0.1% DMSO + collagen group.

myricetin (5 and 10 mg/kg) through intraperitoneal injection. After 30 min, the mouse tails were incised sharply at a distance of 3 mm from the tip and promptly submerged in a tube filled with 37 °C normal saline. The duration until complete cessation of bleeding was recorded.

2.9. Statistical analysis

The data are expressed as mean ± standard error of the mean. The value of *n* indicates the number of experiments conducted on blood samples obtained from different donors. To determine significant differences among the experimental groups, we employed one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls post hoc test, which effectively controls for family-wise type I error. A p value of <0.05 was considered statistically significant. All statistical analyses were performed using SAS software (version 9.2; SAS, Cary, NC, USA).

3. Results

3.1. Inhibitory effect of myricetin on platelet aggregation in humans

Myricetin ($C_{15}H_{10}O_8$; Fig. 1A) demonstrated its strongest inhibitory activity against collagen (1 μg/mL)-induced platelet aggregation within the concentration range of 5–20 μM, as presented in Fig. 1B and C. At elevated concentrations between 20 and 80 μM, myricetin exhibited a moderate inhibitory effect on platelet aggregation induced by thrombin (0.02 U/mL) and U46619 (1 μM), a prostaglandin endoperoxide. The calculated 50% inhibitory concentration (IC_{50}) for myricetin against collagen stimulation was approximately 10 μM. To assess the potential cytotoxicity of myricetin on human platelets, a LDH release assay was conducted. The results revealed that myricetin concentrations ranging from 10 to 160 μM had no significantly impact on LDH release, suggesting the non-cytotoxic nature of myricetin towards platelets (Fig. 1D). In comparison, aspirin exhibited concentration-dependent inhibition of collagen-induced platelet aggregation, with an IC_{50} of approximately 65 μM (data not shown). The solvent control (0.1% DMSO) showed no noticeable impact on platelet aggregation (Fig. 1B and C). Subsequent experiments focused on collagen as the agonist to uncover the mechanisms underpinning myricetin's inhibition of platelet aggregation.

3.2. Inhibitory effects of myricetin on ATP release, cytosolic Ca^{2+} mobilization, and surface P-selectin expression in activated platelets

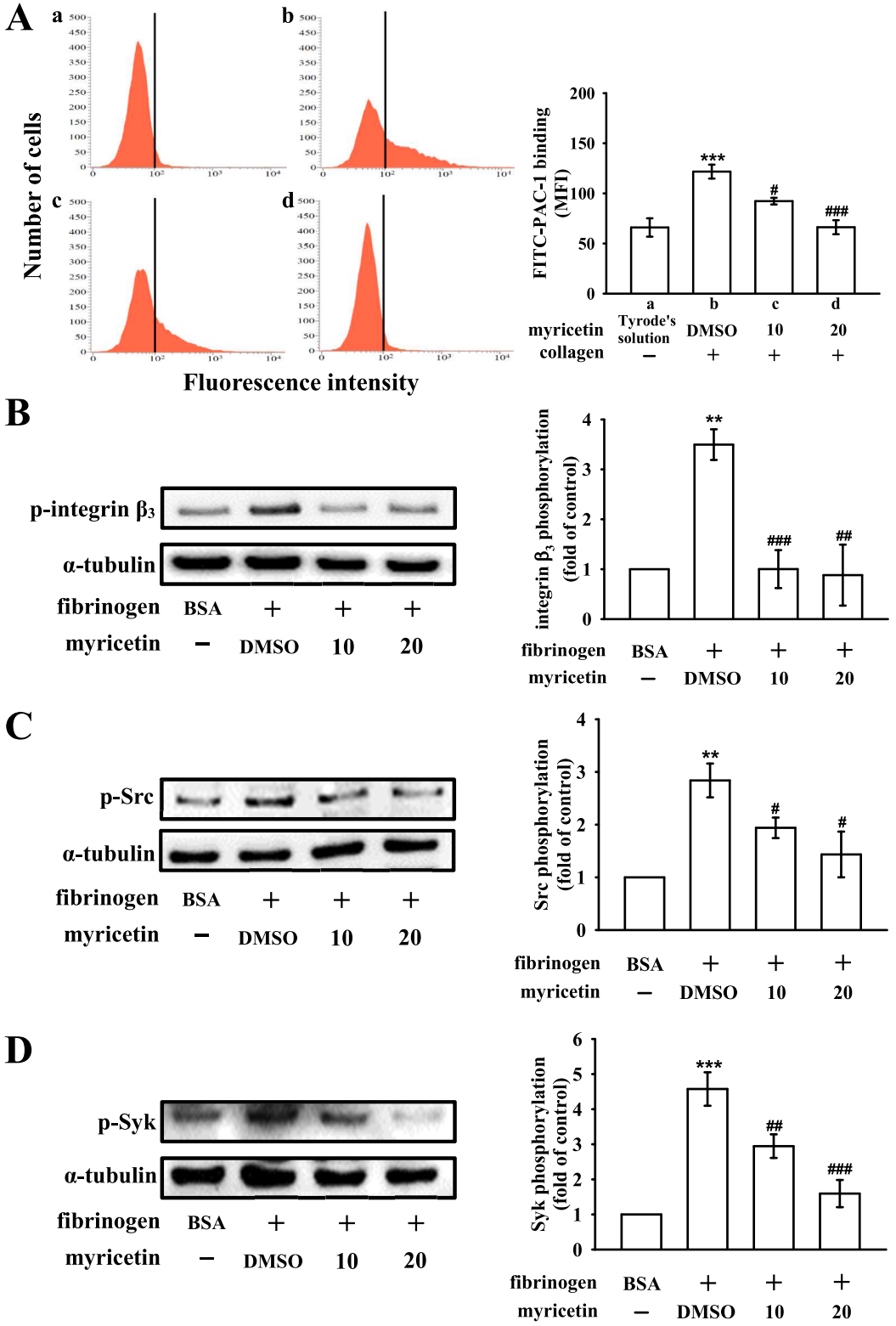
The assessment of platelet degranulation involved quantifying the increase in adenosine triphosphate (ATP) release and surface expression of P-selectin. These processes, which involve the release of ATP or Ca^{2+} from dense granules and P-selectin from α-granules, significantly contribute to the amplification of platelet aggregation. Our study findings unveiled a concentration-dependent inhibitory effect of myricetin (10 and 20 μM) on the release of ATP from collagen-activated platelets (Fig. 2A). In addition, platelet aggregation is triggered by an increase in intracellular calcium concentration ($[Ca^{2+}]i$), which was measured using fluorescence. The data indicated that 10 μM myricetin markedly reduced the $[Ca^{2+}]i$ increase by 40%, whereas 20 μM myricetin nearly inhibited this increase (Fig. 2B). In resting platelets, P-selectin is located on the inner wall of α-granules, and its expression on the platelet surface is negligible. However, upon collagen stimulation, a substantial upregulation of P-selectin expression was observed, which was significantly attenuated by myricetin treatment at concentrations of 10 and 20 μM (Fig. 2C).

3.3. Effect of myricetin on PLCγ2/PKC activation

Phospholipase C (PLC) and protein kinase C (PKC) are two vital proteins involved in cellular signaling pathways. PLC catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, generating diacylglycerol (DAG) and inositol trisphosphate (IP_3) as important secondary messengers. DAG activates protein kinase C (PKC), leading to the phosphorylation of a predominantly 47-kDa protein (pleckstrin or p47). This phosphorylation event ultimately facilitates granule secretion. IP_3 triggers the release of calcium ions from intracellular stores [13]. In the context of this research, myricetin (10 and 20 μM) notably reduced both PLCγ2 and PKC activation (p47 phosphorylation) in collagen-activated platelets (Fig. 3A and B). Furthermore, the confocal scanning fluorescence microscopy analysis provided insights into how myricetin hinders PLCγ2/PKC activation. The images showed green fluorescence, indicating PKC activation, and blue fluorescence corresponding to α-tubulin in both resting and collagen-activated platelets (Fig. 3C). The intensity of phosphorylated p47 fluorescence was discovered to be increased in collagen-stimulated platelets compared with resting platelets. However, when platelets were treated with myricetin (20 μM), the fluorescence intensity decreased. No significant disparity in the intensity of α-tubulin was detected among the groups. Furthermore, myricetin (10 and 20 μM) did not significantly affect platelet aggregation triggered by phorbol 12,13-dibutyrate (PDBu, a PKC activator; Fig. 3D). These findings indicate that myricetin exerts its antiplatelet activity by inhibiting the activation of PLCγ2 and PKC. However, it appears that myricetin primarily affects PLCγ2 activation, rather than directly influencing PKC.

3.4. Effect of myricetin on PI3K/Akt/GSK3β and MAPK activation

The phosphoinositide 3-kinase (PI3K)/Akt/glycogen synthase kinase (GSK) 3β signaling plays a crucial role in thrombus formation under conditions of high shear stress [14]. Akt is primarily regulated by PI3K, and platelet agonists activate the Akt pathway, which regulates platelet activation and hemostasis. The PI3K/Akt also regulates GSK3β in platelets [15]. Myricetin (10 and 20 μM)



(caption on next page)

Fig. 5. Regulation of myricetin on the activation of integrin $\alpha_{IIb}\beta_3$ and phosphorylation of integrin β_3, Src, and Syk on a surface coated with fibrinogen. (A) Platelets (3.6×10^8 cells/mL) were either at (a) resting (Tyrode's solution) or preincubated with (b) 0.1% DMSO or myricetin (c, 10; d, 20 μM) and anti-PAC-1 mAb (2 μg/mL) for 3 min, after which they were stimulated with collagen (1 μg/mL) for 5 min. The number of fluorescein-labeled platelets was measured using a flow cytometer. In another experiments, washed platelets were preincubated with solvent control (0.1% DMSO) or myricetin (10 and 20 μM) and subsequently stimulated using immobilized fibrinogen (100 μg/mL). Subcellular extracts of the platelets were analyzed to determine the levels of phosphorylation of (B) integrin β_3, (C) Src, and (D) Syk. Data are presented as the mean ± standard error of the mean ($n = 4$). $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the resting (Tyrode's solution) group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ compared with the 0.1% DMSO + collagen group.

significantly suppressed the activation of the PI3K/Akt/GSK3β pathway in platelets stimulated by collagen (as shown in Fig. 4A–C). Mitogen-activated protein kinases (MAPKs) are involved in the regulation of various cellular functions, such as differentiation, proliferation, and platelet activation. Platelets mostly contain MAPKs such as ERK1/2, JNK1/2, and p38 MAPK [16]. As indicated in Fig. 4D–F, myricetin (10 and 20 μM) reduced the phosphorylation of all three MAPKs. The statistical data corresponding to each figure is provided below.

3.5. Interference in integrin $\alpha_{IIb}\beta_3$ outside–in signaling by myricetin

Platelet aggregation relies on the interaction between fibrinogen and integrin $\alpha_{IIb}\beta_3$ [17]. To investigate the potential interference of myricetin with integrin $\alpha_{IIb}\beta_3$ activation, we assessed the binding of FITC–PAC-1 mAb to the activation-induced conformational epitope of integrin $\alpha_{IIb}\beta_3$ using flow cytometry. Our results revealed that myricetin at concentrations of 10 and 20 μM inhibited the binding of PAC-1 to integrin $\alpha_{IIb}\beta_3$ on collagen-activated platelets (Fig. 5A), demonstrating that myricetin disrupts the binding of fibrinogen to integrin $\alpha_{IIb}\beta_3$. In order to elucidate the underlying mechanism of myricetin's interference with the integrin $\alpha_{IIb}\beta_3$ outside-in signaling pathway, we conducted an analysis of key kinases involved, including integrin β_3, Src, and Syk, by assessing their phosphorylation status. Remarkably, our findings demonstrated a substantial decrease in the phosphorylation levels of these proteins upon exposure to immobilized fibrinogen in the presence of myricetin (Fig. 5B–D). The corresponding statistical data are presented below of each figure.

3.6. Evaluation of the antithrombotic activity of myricetin in vivo

We experimentally investigated the antithrombotic activity of myricetin by evaluating fluorescein-induced platelet plug formation in the mesenteric microvessels of mice. In normal saline- or 0.1% DMSO-treated mice, the occlusion time in the mesenteric microvessels was approximately 120 s after pretreatment with fluorescein sodium (15 μg/kg; Fig. 6Aa). Treatment with 5 mg/kg myricetin did not significantly increase the occlusion time compared with 0.1% DMSO (DMSO, 117 ± 11 s; 5 mg/kg myricetin, 129 ± 24 s; $n = 12$; $p > 0.05$, Fig. 6Aa). However, treatment with 10 mg/kg myricetin considerably increased the occlusion time compared with 0.1% DMSO (10 mg/kg myricetin, 314 ± 26 s, $n = 12$; $p < 0.001$). After irradiation, a thrombotic platelet plug formation was observed in the mesenteric microvessels at 200 s but not at 5 s in the groups treated with normal saline, 0.1% DMSO, or 5 mg/kg myricetin (Fig. 6Ab; white arrows). After pretreatment with 10 mg/kg myricetin, platelet plug formation was not observed at 5 or 200 s after irradiation (Fig. 6Ab). Moreover, we assessed the bleeding time by performing tail vein transection 30 min after administration intraperitoneal myricetin (5 and 10 mg/kg; Fig. 6B). The bleeding time nonsignificantly changed from 238 ± 25 s (0.1% DMSO treatment; $n = 12$) to 242 ± 21 s (5 mg/kg myricetin treatment; $p > 0.05$) and 243 ± 27 s (10 mg/kg myricetin treatment; $p > 0.05$. The mice were continuously observed for a duration of 15 min, even after the cessation of bleeding, to identify any instances of rebleeding. The results of our study demonstrated that myricetin, administered at an effective dose of 10 mg/kg, displayed antithrombotic activity without affecting bleeding time.

4. Discussion

Aspirin has been employed in clinical settings for the treatment and prevention of CVDs. Notably, myricetin exhibits an antiplatelet effect that is more than six times potent than aspirin when evaluated under comparable conditions. These findings underscore the considerable scientific potential of myricetin as a therapeutic agent for CVD treatment in clinical settings. Triflavin is a disintegrin that contains the Arg-Gly-Asp (RGD) sequence and acts as a specific antagonist of integrin $\alpha_{IIb}\beta_3$ by directly disrupting the interaction between fibrinogen and integrin $\alpha_{IIb}\beta_3$ [18]. Triflavin was reported to inhibit platelet aggregation induced by a variety of agonists, such as collagen, U46619, and thrombin [19]. Our findings align with prior research on triflavin. Our observations revealed that myricetin inhibits platelet aggregation induced by collagen, thrombin, and U46619. While myricetin exhibited a more substantial inhibitory effect on collagen-induced platelet aggregation compared to other agonists, myricetin appears to operate through a common inhibitory pathway rather than targeting the individual receptors of agonists.

In resting platelets, integrin $\alpha_{IIb}\beta_3$ maintains a state of low activation, which prevents its interaction with specific ligands, such as fibrinogen, von Willebrand factor, and fibronectin. Upon the activation of platelets by agonists, integrin $\alpha_{IIb}\beta_3$ undergoes a conformational change that enables it to bind to its ligands, resulting in platelet aggregation; this is the integrin $\alpha_{IIb}\beta_3$ inside–out signaling pathway [3]. In addition, when a ligand such as fibrinogen binds to integrin $\alpha_{IIb}\beta_3$, it not only causes platelet aggregation but also initiates a cascade of intracellular signaling events, which is known as the outside–in signaling pathway. This outside–in signaling pathway leads to the tyrosine phosphorylation of numerous proteins, such as integrin β_3, Src family kinases (SFKs), and Syk [3,20]. In

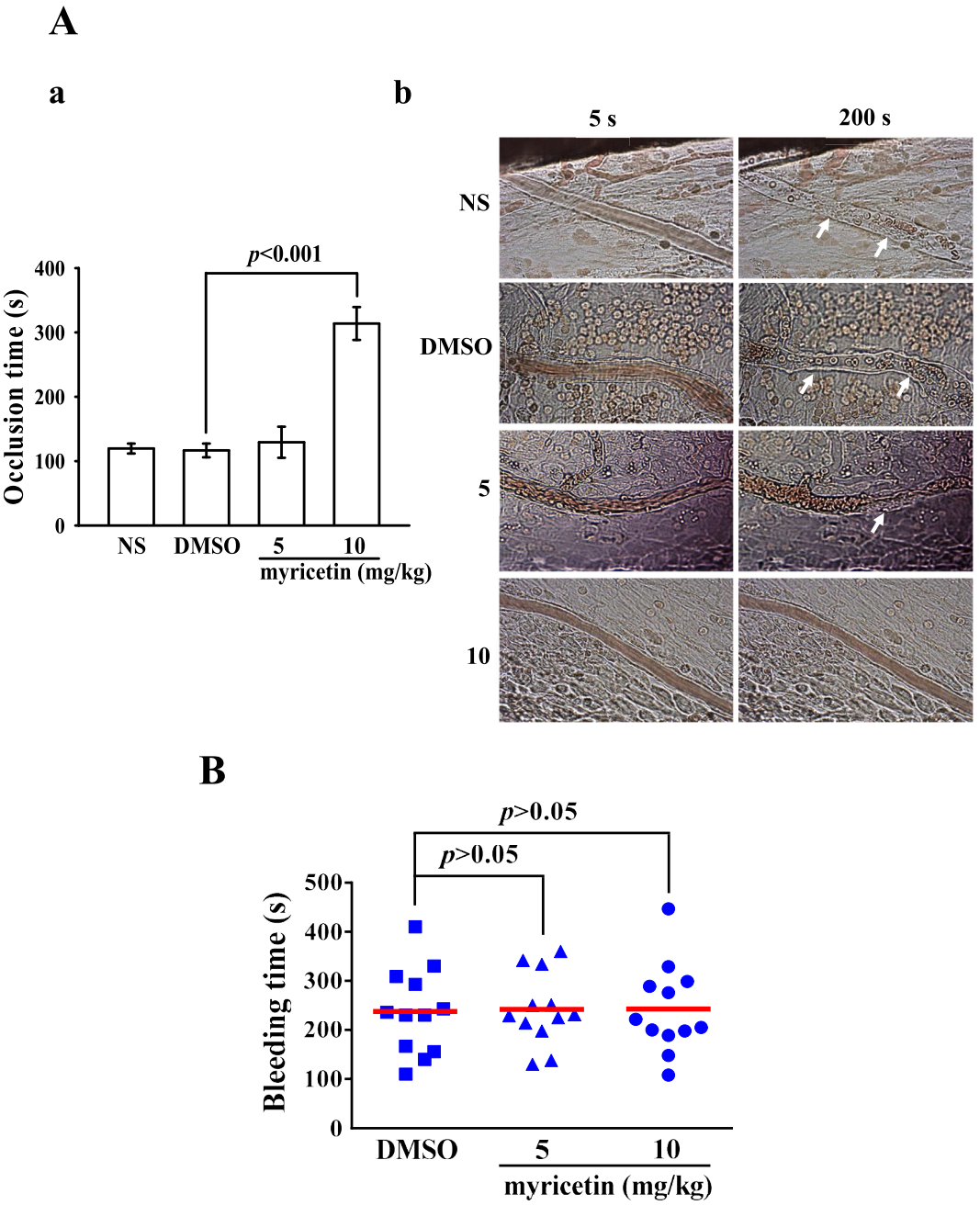


Fig. 6. Investigation of the role of myricetin in thrombotic platelet plug formation in mesenteric venules and the bleeding time of the tail veins of mice. Mice were intraperitoneally injected with either normal saline (NS), solvent control (0.1% DMSO), or myricetin (5 and 10 mg/kg), after which fluorescein-pretreated mesenteric venules were irradiated at a wavelength of <520 nm to induce microthrombus formation. Microscopic images were captured at 5 and 200 s post irradiation to observe platelet plug formation (white arrows; 400 × magnification). (B) Tail vein transection bleeding was performed following a 30-min of intraperitoneal administration of 0.1% DMSO and myricetin (5 and 10 mg/kg) to measure bleeding time. Data are presented as the mean ± standard error of the mean ($n = 12$). $p < 0.001$, compared with the 0.1% DMSO-treated group.

vivo studies have demonstrated the crucial role of the cytoplasmic tail of integrin β_3 at Tyr^{759} in platelets; its mutation can lead to bleeding disorders in vivo [21]. The activation of β_3 triggers SFKs activation, specifically β_3-bound Src, thereby initiating signaling pathways that are dependent on SFKs [22]. Syk plays a vital role in platelet activation vascular development, innate immune recognition, and cellular adhesion [23]. The activation of Syk by immobilized fibrinogen can subsequently modulate PLCγ2 activation [22]. In this study, we discovered that myricetin markedly reduced the phosphorylation levels of integrin β_3, Src, and Syk when platelets were adhered to immobilized fibrinogen. Furthermore, myricetin notably impeded the interaction between PAC-1 and

activated integrin $\alpha_{IIb}\beta_3$. Collectively, the results indicate that myricetin may act on integrin $\alpha_{IIb}\beta_3$, consequently blocking integrin $\alpha_{IIb}\beta_3$ outside–in signaling.

Platelets initially adhere to collagen, a pivotal adhesive protein abundantly present in the subendothelium. This interaction triggers a cascade of tyrosine kinase activation, leading to an elevation in intracellular calcium levels and subsequent granule secretion [24]. Among the platelet receptors directly involved in collagen interaction, integrin $\alpha_2\beta_1$ (glycoprotein [GP] Ia/IIa) and GP VI play predominant roles [25]. GP VI, a member of the immunoglobulin superfamily, forms a complex with the Fc receptor γ-chain containing immunoreceptor tyrosine-based activation motifs, and is recognized as a critical contributor to platelet aggregation on the collagen-coated surfaces within the bloodstream. Consequently, platelet activation comprises a coordinated interplay between the inside-out signaling of agonist-triggered integrin $\alpha_{IIb}\beta_3$ and the outside-in signaling of integrin $\alpha_{IIb}\beta_3$. Furthermore, immobilized fibrinogen activates Syk phosphorylation, triggering the activation of PLCγ2-PKC and PI3K-Akt-GSK3β signaling pathways, which subsequently facilitate platelet activation events such as granule secretion and mobilization of intracellular calcium ($[Ca^{2+}]i$) (Fig. 7). [26].

In the current study, myricetin effectively inhibited collagen-stimulated PLCγ2-PKC, PI3k-Akt-GSK3β, and MAPK activation. The family of PLCγ in platelets comprises two isozymes (1 and 2), with PLCγ2 playing a crucial role in collagen-dependent signaling [25]. The PKC family encompasses multiple isoforms and plays a crucial role in serine/threonine phosphorylation, exerting its regulatory function in various cellular contexts [13]. MAPKs represent essential signaling pathways that regulate a wide array of cellular processes. Prior research utilizing MAPK-specific inhibitors or knockout mouse models has provided evidence for the presence of ERK1/2, JNK1/2, and p38 MAPK in platelets, all of which contribute to the process of platelet activation [27]. PI3K activation is also vital for platelet activation, and it operates in response to various platelet receptors, such as GP VI or the ADP receptors P2Y12, as well as integrin $\alpha_{IIb}\beta_3$ [28]. All isoforms of PI3K (including α, β, γ, and δ) are expressed within platelets. PI3Kβ serves as a prominent isoform implicated in the signaling pathways of activated platelets. Akt, a key and widely expressed effector of PI3K, plays a crucial role in this context. Human platelets express three isoforms of Akt (Akt1, Akt2, and Akt3) [14], and studies involving Akt-deficient mice have exhibited compromised platelet aggregation and sustained adhesion in the course of bloodstream circulation [29]. Within platelets, the PI3K/Akt and MAPK pathways exhibit reciprocal activation, with PKC acting as an upstream regulator, as illustrated in Fig. 7 [30].

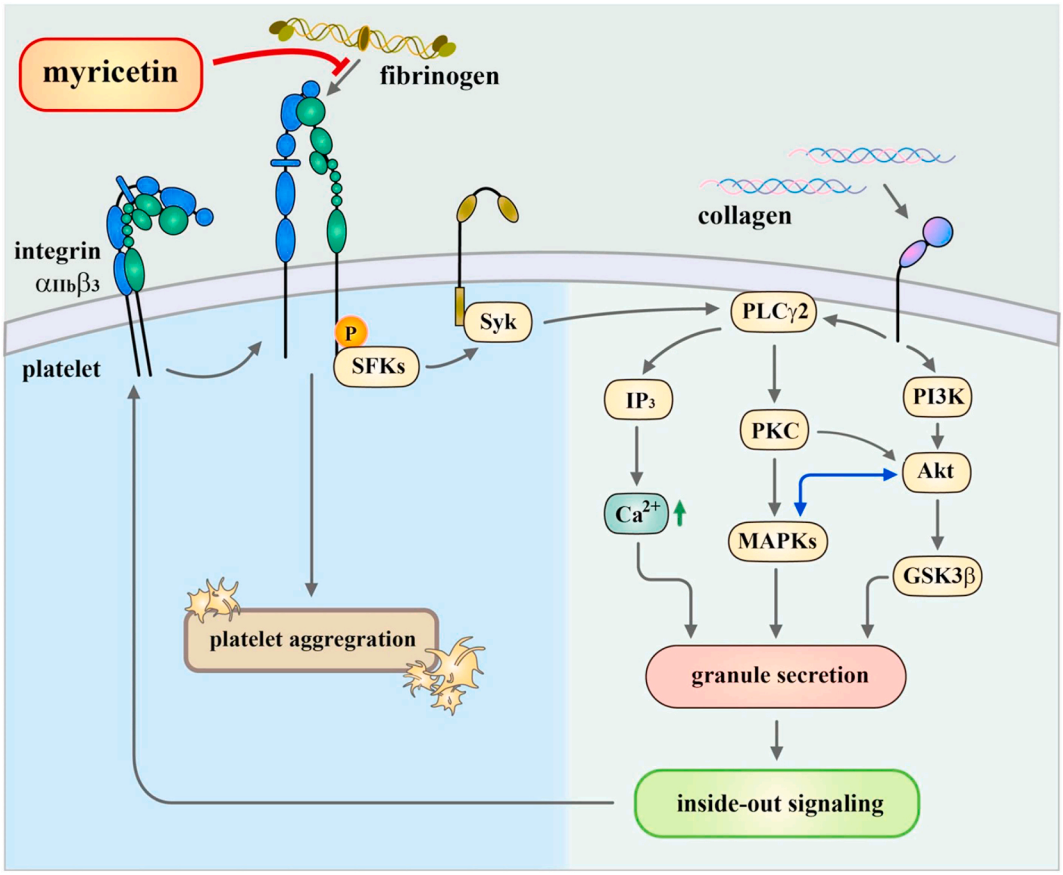


Fig. 7. Conceptual framework illustrating the potential mechanisms by which myricetin inhibits platelet activation. Myricetin initially disrupts the interaction between fibrinogen and integrin $\alpha_{IIb}\beta_3$. Consequently, it blocks integrin $\alpha_{IIb}\beta_3$ outside-in signaling, subsequently resulting in the inhibition of integrin $\alpha_{IIb}\beta_3$ inside-out signaling. Finally, this leads to the inhibition of platelet aggregation.

Nonetheless, it remains unclear whether the signaling cascades downstream of Akt directly participate in platelet activation, and further research is warranted to elucidate this aspect. Platelets express several downstream candidates of Akt, including GSK3 (α and β isoforms), with GSK3β being the most abundant protein [31,32]. Previous studies utilizing mice deficient in platelet-specific PI3Kβ have revealed the development of arterial thrombus instability, particularly under high shear stress conditions, due to impaired Akt/GSK3 activation within the growing thrombus [14]. Building upon these findings, we formulated the hypothesis that myricetin exerts its effects on integrin $\alpha_{IIb}\beta_3$, obstructing the outside-in signaling pathway and subsequently reducing the inside-out signaling triggered by agonists such as collagen.

Experimental models of arterial thrombosis have increasingly been employed to evaluate the efficacy of potential therapeutic interventions for vascular diseases. An ideal murine model should exhibit simplicity and reproducibility, enabling consistent and dependable outcomes. In a study on arterial thrombosis [30], mesenteric venules were continuously exposed to fluorescein throughout the experimental period, which caused considerable damage to endothelial cells. Treatment with myricetin at a dosage of 10 mg/kg resulted in a notable prolongation of occlusion time, indicating the critical involvement of platelet aggregation as a risk factor in the development of arterial thrombosis. To assess the impact of myricetin on bleeding time, we employed a tail transection mouse model and observed that even at a dose of 10 mg/kg, myricetin did not induce a significant prolongation of bleeding time compared to 0.1% DMSO. This finding demonstrates that myricetin inhibits platelet aggregation without affecting bleeding time in vivo.

5. Conclusions

Myricetin is a promising novel antiplatelet agent that can be used for preventing arterial thrombotic events, such as CVDs and ischemic stroke. Nevertheless, other unidentified mechanisms may contribute to its antiplatelet effects. Further research is warranted to gain a complete understanding of the mechanisms through which myricetin affects platelet activation and to identify potential clinical applications.

Author contribution statement

Yi Chang: Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Chih-Wei Hsia: Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Wei-Chieh Huang: Performed the experiments. Thanasekaran Jayakumar: Performed the experiments. Chih-Hsuan Hsia: Performed the experiments. Ting-Lin Yen: Analyzed and interpreted the data. Joen-Rong Sheu: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data, wrote the paper. Shaw-Min Hou: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Ethics statement

The study protocol received approval from the Institutional Review Board of Taipei Medical University (TMU-JIRB-N202112047) and adhered to the principles outlined in the Declaration of Helsinki. All animal experiments and care protocols complied with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals and were granted approval by the Institutional Animal Care and Use Committee of Taipei Medical University (LAC-2021-0386).

Data availability statement

All data generated or analyzed in this study are included in this article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was funded by the Ministry of Science and Technology of Taiwan (MOST111-2320-B-038-036-MY3), Shin Kong Wu Ho-Su Memorial Hospital (2020SKHADR029; 2021SKHADR027), Cathay General Hospital (CGH-MR-A11133), and Taipei Medical University (DP2-111-21121-01-N-08-03).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20286.

References

[1] M. Leslie, Cell biology. Beyond clotting: the powers of platelets, Science 328 (5978) (2010) 562–564, https://doi.org/10.1126/science.328.5978.562.
[2] J.R. Sheu, M.H. Yen, W.C. Hung, Y.M. Lee, C.H. Su, T.F. Huang, Triflavin inhibits platelet-induced vasoconstriction in de-endothelialized aorta, Arterioscler. Thromb. Vasc. Biol. 17 (12) (1997) 3461–3468, https://doi.org/10.1161/01.atv.17.12.3461.
[3] B. Payrastre, K. Missy, C. Trumel, S. Bodin, M. Plantavid, H. Chap, The integrin $\alpha_{IIb}\beta_3$ in human platelet signal transduction, Biochem. Pharmacol. 60 (8) (2000) 1069–1074, https://doi.org/10.1016/s0006-2952(00)00417-2.
[4] R.I. Litvinov, D.H. Farrell, J.W. Weisel, J.S. Bennett, The platelet integrin αIIbβ3 differentially interacts with fibrin versus fibrinogen, J. Biol. Chem. 291 (15) (2016) 7858–7867, https://doi.org/10.1074/jbc.M115.706861.
[5] G. Agraharam, A. Girigoswami, K. Girigoswami, Myricetin: a multifunctional flavonol in biomedicine, Curr. Pharmacol. Rep. 8 (1) (2022) 48–61, https://doi.org/10.1007/s40495-021-00269-2.
[6] M. Imran, F. Saeed, G. Hussain, A. Imran, Z. Mehmood, T.A. Gondal, A. El-Ghorab, I. Ahmad, R. Pezzani, M.U. Arshad, U. Bacha, M.A. Shariarti, A. Rauf, N. Muhammad, Z.A. Shah, G. Zengin, S. Islam, Myricetin: a comprehensive review on its biological potentials, Food Sci. Nutr. 9 (10) (2021) 5854–5868, https://doi.org/10.1002/fsn3.2513.
[7] Y. Qiu, N. Cong, M. Liang, Y. Wang, J. Wang, Systems pharmacology dissection of the protective effect of myricetin against acute ischemia/reperfusion-induced myocardial injury in isolated rat heart, Cardiovasc. Toxicol. 17 (3) (2017) 277–286, https://doi.org/10.1007/s12012-016-9382-y.
[8] S. Salvamani, B. Gunasekaran, N.A. Shaharuddin, S.A. Ahmad, M.Y. Shukor, Antiatherosclerotic effects of plant flavonoids, BioMed Res. Int. 2014 (2014), 480258, https://doi.org/10.1155/2014/480258.
[9] S.H. Tseng, D.C. Ko, P.N. Ko, C.M. Teng, Inhibition of platelet aggregation by some flavonoids, Thromb. Res. 64 (1) (1991), https://doi.org/10.1016/0049-3848(91)90208-e, 91-l00.
[10] R. Landolfi, R.L. Mower, M. Steiner, Modification of platelet function and arachidonic acid metabolism by bioflavonoids, Biochem. Pharmacol. 33 (9) (1984) 1520–1530, https://doi.org/10.1016/0006-2952(84)90423-4.
[11] D. Varga-Szabo, A. Braun, B. Nieswandt, Calcium signaling in platelets, J. Thromb. Haemost. 7 (7) (2009) 1057–1066, https://doi.org/10.1111/j.1538-7836.2009.03455.x.
[12] P.A. Laurent, S. Severin, M.P. Gratacap, B. Payrastre, Class I PI3-kinases signaling in platelet activation and thrombosis: PDK1/Akt/GSK3 axis and impact of PTEN and SHIP1, Adv. Biol. Regul. 54 (2014) 162–174, https://doi.org/10.1016/j.jbior.2013.09.006.
[13] B.D. Manning, L.C. Cantley, AKT/PKB signaling: navigating downstream, Cell 129 (7) (2007) 1261–1274, https://doi.org/10.1016/j.cell.2007.06.009.
[14] M. Cargnello, P.P. Roux, Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases, Microbiol. Mol. Biol. Rev. 75 (1) (2011) 50–83, https://doi.org/10.1128/MMBR.00031-10.
[15] J.M. Cosemans, B.F. Iserbyt, H. Deckmyn, J.W. Heemskerk, Multiple ways to switch platelet integrins on and off, J. Thromb. Haemostasis 6 (8) (2008) 1253–1261, https://doi.org/10.1111/j.1538-7836.2008.03041.x.
[16] J.R. Sheu, W.C. Hung, C.H. Wu, M.C. Ma, Y.C. Kan, C.H. Lin, H.N. Luk, M.H. Yen, Reduction in lipopolysaccharide-induced thrombocytopenia by triflavin in a rat model of septicemia, Circulation 99 (23) (1999) 3056–3062, https://doi.org/10.1161/01.cir.99.23.3056.
[17] T.F. Huang, J.R. Sheu, C.M. Teng, A potent antiplatelet peptide, triflavin, from Trimeresurus flavoviridis snake venom, Biochem. J. 277 (pt 2) (1991) 351–357, https://doi.org/10.1042/bj2770351.
[18] S.J. Shattil, P.J. Newman, Integrins: dynamic scaffolds for adhesion and signaling in platelets, Blood 104 (6) (2004) 1606–1615, https://doi.org/10.1182/blood-2004-04-1257.
[19] D.A. Law, F.R. DeGuzman, P. Heiser, K. Ministri-Madrid, N. Killeen, D.R. Phillips, Integrin cytoplasmic tyrosine motif is required for outside-in alphaIIbbeta3signaling and platelet function, Nature 401 (6755) (1999) 808–811, https://doi.org/10.1038/44599.
[20] Z. Li, M.K. Delaney, K.A. O'Brien, X. Du, Signaling during platelet adhesion and activation, Arterioscler. Thromb. Vasc. Biol. 30 (12) (2010) 2341–2349, https://doi.org/10.1161/ATVBAHA.110.207522.
[21] A. Mócsai, J. Ruland, V.L. Tybulewicz, The SYK tyrosine kinase: a crucial player in diverse biological functions, Nat. Rev. Immunol. 10 (6) (2010) 387–402, https://doi.org/10.1038/nri2765.
[22] D.A. Rubenstein, W. Yin, Platelet-activation mechanisms and vascular remodeling, Compr. Physiol. 8 (3) (2018) 1117–1156, https://doi.org/10.1002/cphy.c170049.
[23] F. Niedergang, A. Alcover, C.G. Knight, R.W. Farndale, M.J. Barnes, I.M. Francischetti, C. Bon, M. Leduc, Convulxin binding to platelet receptor GPVI: competition with collagen related peptides, Biochem. Biophys. Res. Commun. 273 (1) (2000) 246–250, https://doi.org/10.1006/bbrc.2000.2940.
[24] Y. Liu, T. Liu, K. Ding, Z. Liu, Y. Li, T. He, W. Zhang, Y. Fan, W. Ma, L. Cui, X. Song, Phospholipase Cγ2 signaling cascade contribute to the antiplatelet effect of notoginsenoside Fc, Front. Pharmacol. 9 (2018) 1293, https://doi.org/10.3389/fphar.2018.01293.
[25] X. Fan, C. Wang, P. Shi, W. Gao, J. Gu, Y. Geng, W. Yang, N. Wu, Y. Wang, Y. Xu, X. Chen, L. Zhang, K. Wang, B. Su, J. Liu, Platelet MEKK3 regulates arterial thrombosis and myocardial infarct expansion in mice, Blood Adv 2 (12) (2018) 1439–1448, https://doi.org/10.1182/bloodadvances.2017015149.
[26] M.P. Gratacap, J. Guillermet-Guibert, V. Martin, G. Chicanne, H. Tronchère, F. Gaits-Iacovoni, B. Payrastre, Regulation and roles of PI3Kβ, a major actor in platelet signaling and functions, Adv. Enzym. Regul. 51 (1) (2011) 106–116, https://doi.org/10.1016/j.advenzreg.2010.09.011.
[27] H. Yin, A. Stojanovic, N. Hay, X. Du, The role of Akt in the signaling pathway of the glycoprotein Ib-IX induced platelet activation, Blood 111 (2) (2008) 658–665, https://doi.org/10.1182/blood-2007-04-085514.
[28] T. Jayakumar, W.F. Chen, W.J. Lu, D.S. Chou, G. Hsiao, C.Y. Hsu, J.R. Sheu, C.Y. Hsieh, A novel antithrombotic effect of sulforaphane via activation of platelet adenylate cyclase: ex vivo and in vivo studies, J. Nutr. Biochem. 24 (6) (2013) 1086–1095, https://doi.org/10.1016/j.jnutbio.2012.08.007.
[29] D.S. Woulfe, Akt signaling in platelets and thrombosis, Expert Rev. Hematol. 3 (1) (2010) 81–91, https://doi.org/10.1586/ehm.09.75.
[30] D. Li, S. August, D.S. Woulfe, GSK3beta is a negative regulator of platelet function and thrombosis, Blood 111 (7) (2008) 3522–3530, https://doi.org/10.1182/blood-2007-09-111518.
[31] J.R. Sheu, C.R. Lee, C.H. Lin, G. Hsiao, W.C. Ko, Y.C. Chen, M.H. Yen, Mechanisms involved in the antiplatelet activity of staphylococcus aureus lipoteichoic acid in human platelets, Thromb. Haemostasis 83 (5) (2000) 777–784.
[32] T.L. Shih, K.H. Lin, R.J. Chen, T.Y. Chen, W.T. Kao, J.W. Liu, H.H. Wang, H.Y. Peng, Y.Y. Sun, W.J. Lu, A novel naphthalimide derivative reduces platelet activation and thrombus formation via suppressing GPVI, J. Cell Mol. Med. 25 (19) (2021) 9434–9446, https://doi.org/10.1111/jcmm.16886.