Euchrestaflavanone A can attenuate thrombosis through inhibition of collagen-induced platelet activation

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Abstract Euchrestaflavanone A (EFA) is a flavonoid found in the root bark of *Cudrania tricuspidata*. *C. tricuspidata* extract, widely used throughout Asia in traditional medicine, has been investigated phytochemically and biologically and is known to have anti-obesity, anti-inflammatory, and anti-tumor effects. It has been reported that *C. tricuspidata* extract also possesses anti-platelet effects; however, the mechanism of its anti-platelet and anti-thrombotic activities is yet to be elucidated. In this study, we investigated the effects of EFA on the modulation of platelet function using collagen-induced human platelets. Our results showed that EFA markedly inhibited platelet aggregation. Furthermore, it downregulated glycoprotein IIb/IIIa (α IIb/ β 3)-mediated signaling events, including platelet adhesion, granule secretion, thromboxane A₂ production, and clot retraction, but upregulated the cyclic adenosine monophosphate-dependent pathway. Taken together, EFA possesses strong anti-platelet and anti-thrombotic properties and is a potential therapeutic drug candidate to prevent platelet-related thrombosis and cardiovascular disease.

Keywords α IIb/ β 3 affinity · Ca²⁺ mobilization · cAMP and cGMP · Clot retraction · Euchrestaflavanone A

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Introduction

In normal circulation, collagen fibers cannot bind to platelets. However, when endothelial walls are injured, exposed collagen fibers can bind to integrin α 2 β 1 and glycoprotein VI on platelet receptors, leading to platelet activation. After activation, phospholipase C γ ₂ within the cytosol hydrolyzes phosphatidylinositol 4,5-bisphosphate into inositol diacylglycerol and 1,4,5-trisphosphate (IP₃), which then mobilizes calcium from the endoplasmic reticulum [1,2]. These signaling events in activated platelets cause conformational changes in glycoprotein IIb/IIIa (integrin α IIb/ β 3), leading to thrombus formation and platelet-mediated clot retraction [3]. Therefore, platelets are an essential factor for hemostasis, while simultaneously posing a risk for thrombosis. The underlying cause of cardiovascular disease (CVD) is pathological platelet overactivity, leading to thrombosis [4]. Pharmacological platelet suppression effectively reduces thrombosis, and many drugs are available for preventing CVD, with numerous anti-platelet and anti-thrombotic drugs being discovered. Nonetheless, these drugs have been largely unable to effectively decrease mortality rates [5].

Cudrania tricuspidata is widespread throughout Asia and used in ethnomedicine, particularly against eczema, mumps, and tuberculosis [6]. It has been reported that *C. tricuspidata* extracts have various physiological activities, including anti-inflammatory, anti-diabetic, neuroprotective, anti-obesity, anti-tumor, and anti-coagulant activities [7]. We have previously reported the anti-platelet effects of steppogenin and isoderrone isolated from *C. tricuspidata* on collagen-induced human platelets [8,9]. However, *C. tricuspidata* contains numerous components; thus, herein, we studied a new candidate, euchrestaflavanone A (EFA), for its anti-platelet effects.

Materials and Methods

Chemicals and reagents

ChemFaces (Wuhan, China) supplied EFA. The Chrono-Log corporation (Havertown, PA, USA) supplied platelet collagen. Cayman Chemical (Ann Arbor, MI, USA) supplied the cAMP EIA and thromboxane B₂ assay kits. Cell Signaling (Beverly, MA, USA) supplied the lysis buffer and antibodies against phospho-vasodilator-stimulated phosphoprotein (VASP), phospho-inositol-3-phosphate receptor type I (IP₃RI), phospho-cytosolic phospholipase A₂ (cPLA₂), phospho-p38 mitogen-activated protein kinase (p38^{MAPK}), phospho-Akt, and α -actin, and anti-rabbit secondary antibodies. Invitrogen (Eugene, OR, USA) provided Fura 2-AM- (2-acetoxymethyl-) and Alexa Fluor 488-conjugated fibrinogen. The fibronectin-coated cell adhesion kit was procured from Cell Biolabs (San Diego, CA, USA). The serotonin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Labor Diagnostika Nord GmbH & Co. (Nordhorn, Germany).

Preparation of human platelet suspension

The human platelet-rich plasma was procured from the Korean Red Cross Blood Center (Suwon, Korea), and study protocols were approved by the Public Institutional Review Board at the National Institute for Bioethics Policy (Seoul, Republic of Korea; PIRB-P01-201812-31-007). The platelet-rich plasma was centrifuged for 10 min at 1300×g and the pellet was washed twice using washing buffer (pH 6.5) and re-suspended in suspension buffer (pH 6.9) at room temperature according to the protocol published in a previous study [10]. The platelet suspension concentration was adjusted to 5×10⁸/mL.

Platelet aggregation

For platelet aggregation, human platelet suspensions (10⁸/mL) were pre-incubated for 3 min in the presence or absence of EFA (25 to 150 μ M) along with 2 mM CaCl₂ at 37 °C, followed by stimulation with agonists. Collagen (2.5 μ g/mL) triggers full platelet aggregation and was used as agonists for aggregation. The aggregation assay was conducted for 5 min under continuous stirring. An increase in light transmission converted into the platelet aggregation rate (%). A 0.1% dimethyl sulfoxide solution was used to dissolve the EFA.

Cytotoxicity assay

Cytotoxicity of EFA was examined by quantifying lactate dehydrogenase (LDH) leakage from the cytosol of platelets. Human platelet suspensions (10⁸/mL) were incubated with different concentrations of EFA (25 to 150 μ M) for 2 h and centrifuged for 2 min at 12,000×g. The supernatant was used to detect cytotoxic effects using an ELISA reader (TECAN, Salzburg, Austria).

Intracellular calcium concentration

The Fura 2-AM (5 μ M) and PRP mixture was pre-incubated at 37 °C for 60 min, and then the human platelet suspension (10⁸/mL) was washed with washing buffer. The platelets were then suspended in suspending buffer, pre-incubated with or without EFA for 3 min at 37 °C, and stimulated with 2.5 μ g/mL collagen in the presence of 2 mM CaCl₂. To calculate the [Ca²⁺]_i values, Fura 2-AM fluorescence was measured according to the Grynkiewicz method [11] using a spectrofluorometer (Hitachi F-2700; Tokyo, Japan).

Measurement of thromboxane B₂

Since thromboxane A₂ (TXA₂) is unstable and transforms into thromboxane B₂ (TXB₂) quickly, TXA₂ generation was measured by detecting TXB₂ production. After platelet activation, the reaction was stopped by adding 0.2 mM indomethacin with 5 mM EDTA. TXB₂ levels were measured using an ELISA reader (TECAN, Salzburg, Austria) and a TXB₂ ELISA kit.

Measurement of serotonin

Human platelet suspensions (10⁸/mL) were pre-incubated for 3 min at 37 °C with EFA and then stimulated with 2.5 μ g/mL collagen in the presence of 2 mM CaCl₂ to terminate serotonin release, followed by centrifugation (250 g). The supernatant was used for detecting serotonin release with an ELISA reader (TECAN, Salzburg, Austria) using a serotonin ELISA kit.

Immunoblotting

The platelets were aggregated for 5 min and stopped by adding lysis buffer. The protein concentration in the platelet lysates were calculated using a bicinchoninic acid protein assay kit (Pierce Biotechnology; IL, USA). For Western blotting, 15 μ g proteins from the platelet lysates were separated using SDS-PAGE (8%) and transferred onto PVDF membranes, which were then probed with the indicated primary (1:1,000) and secondary antibodies (1:10,000). The resulting bands were analyzed using the Quantity One, Ver. 4.5 (BioRad; Hercules, CA, USA).

Measurement of fibrinogen binding to α IIb/ β 3

Human platelet suspensions (10⁸/mL) were incubated with EFA (25 to 150 μ M) and treated with 30 μ g/mL Alexa Fluor 488-conjugated fibrinogen at 37 °C for 5 min. The interaction between platelet integrin and Alexa Fluor 488-conjugated human fibrinogen was stopped by adding 0.5% paraformaldehyde in cold PBS. All procedures were conducted in the dark. The binding of fibrinogen to integrin α IIb/ β 3 was examined by quantifying the fluorescence of fibrinogen using flow cytometry (BD Biosciences; San Jose, CA, USA), and data were analyzed using CellQuest software (BD Biosciences).

Fibronectin adhesion

Human platelet suspensions ($10^8/\text{mL}$) were pre-incubated with EFA (25 to 150 μM) and 2 mM CaCl_2 for 1 h at 37 °C in the presence of 2.5 $\mu\text{g/mL}$ collagen, washed 5 times with PBS, and incubated with cell stain solution for 10 min. The extraction solution was added after washing with PBS once to detach the adhesive platelet plaque from the fibronectin-coated well. Each sample was examined by measuring the absorbance using an ELISA reader (TECAN, Salzburg, Austria). Bovine serum albumin coated wells were used as a negative control.

Measurement of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) levels

Washed human platelets ($10^8/\text{mL}$) were pre-incubated for 3 min at 37 °C with or without EFA (25 to 150 μM) in the presence of 2 mM CaCl_2 , and then stimulated with 2.5 $\mu\text{g/mL}$ collagen for 5 min for platelet aggregation. The aggregation was terminated by adding 80% ice-cold ethanol. The cAMP and cGMP levels were measured using an EIA kit and an ELISA reader (TECAN, Salzburg, Austria).

Platelet-mediated fibrin clot retraction

Human platelet-rich plasma (300 μL) was poured into a polyethylene tube and pre-incubated in the presence or absence of various concentrations of EFA (25 to 150 μM) for 15 min at 37 °C. The clot retraction was triggered by adding 0.05 U/mL thrombin. The images of fibrin clots were captured using a digital camera at 15 min intervals. Image J Software was used to calculate the clot area (v1.46, National Institutes of Health; USA).

Statistical analyses

Experimental data are presented as the mean $\pm$ standard deviation included with various numbers of observations. To determine major differences among groups, analysis of variance (ANOVA) was performed followed by the Tukey-Kramer method. SPSS 21.0.0.0 software (SPSS; Chicago, IL, USA) was employed for statistical analysis, and $p < 0.05$ was considered statistically significant.

Results

Effects of EFA on human platelet aggregation and cytotoxicity

C. tricuspidata is a perennial plant of the family Moraceae, and its roots, leaves, bark, stems, and fruit contain diverse phytochemicals. Among various phytochemicals, xanthenes and flavonoids are the major constituents of *C. tricuspidata*. We searched for a new substance and investigated whether EFA (MW 408.49) (Fig. 1A) has an anti-platelet effect. To determine anti-platelet effects, 2.5 $\mu\text{g/mL}$ collagen was used for the optimum aggregation of human platelets (Fig. 2A). However, collagen-induced platelets treated with 25, 50, 100, and 150 μM EFA inhibited dose-dependently

Fig. 1 Chemical structure of euchrestaflavanone A (EFA)

and the half maximal inhibitory concentration (IC_{50}) was 49.5 μM (Fig. 2A, B). To investigate the cytotoxicity of EFA, we treated the platelets with 25–150 μM EFA, which did not affect LDH release (Fig. 2C).

Effects of EFA on $[\text{Ca}^{2+}]_i$ mobilization, serotonin secretion, and IP_3RI phosphorylation

Intracellular ($[\text{Ca}^{2+}]_i$) is an essential factor for platelet activation; thus, we focused on the effect of EFA on $[\text{Ca}^{2+}]_i$ mobilization. As shown in Fig. 3A, collagen elevated $[\text{Ca}^{2+}]_i$ levels from 110.2 ± 0.7 nM to 770.5 ± 6.5 nM. However, 25–150 μM EFA reduced the increased $[\text{Ca}^{2+}]_i$ levels dose-dependently. Intracellular calcium level is related to granule release; thus, we investigated whether EFA is involved in granule release from platelets. As shown in Fig. 3B, collagen-stimulated serotonin secretion was inhibited by 25–150 μM EFA dose-dependently. To confirm the effect of EFA on granule release, we investigated the associated signaling molecule, inositol 1,4,5-triphosphate receptor type I (IP_3RI), which regulates $[\text{Ca}^{2+}]_i$ mobilization. As shown in Fig. 3C, collagen increased IP_3RI phosphorylation was increased by EFA (25 to 150 μM).

Effects of EFA on $[\text{Ca}^{2+}]_i$ mobilization and cPLA_2 and p38^{MAPK} dephosphorylation

We next investigated whether EFA is involved in the inhibition of TXA_2 production to attenuate collagen-induced human platelet aggregation. Collagen-stimulated human platelet produced TXA_2 (determined as TXB_2) from 1.5 ± 0.2 nM to 55.8 ± 4.8 ng/ 10^8 . However, 25–150 μM EFA inhibited TXA_2 production dose-dependently (Fig. 4A). We next investigated the TXA_2 associated signaling molecules, cPLA_2 and p38^{MAPK} . While 2.5 $\mu\text{g/mL}$ collagen elevated cPLA_2 and p38^{MAPK} phosphorylation, 25–150 μM EFA inhibited cPLA_2 and p38^{MAPK} phosphorylation dose-dependently (Fig. 4B, C). These results suggest that the inhibition of TXA_2 production by EFA is due to cPLA_2 and p38^{MAPK} dephosphorylation.

Fig. 2 Effects of EFA on platelet aggregation, half maximal inhibitory concentration, and cytotoxicity (A) Effect of EFA on collagen-induced human platelet aggregation. (B) Half maximal inhibitory concentration (IC₅₀) value of EFA in collagen-induced human platelet aggregation. (C) Effect of EFA on cytotoxicity. Platelet aggregation and cytotoxicity were carried out as described in “Materials and Methods” section. The data are expressed as the mean ± standard deviation (n=4). **p* < 0.05, ***p* < 0.01 versus each agonist-stimulated human platelets. NS, not significant

Effects of EFA on fibrinogen binding to αIIb/β3 and fibronectin adhesion

We then investigated αIIb/β3 activation through fibrinogen binding to αIIb/β3. Collagen elevated the binding of fibrinogen to αIIb/β3 from 3.5±1.2% to 81.8±2.1 (Fig. 5A, B). However, EFA attenuated fibrinogen interaction with αIIb/β3 dose-dependently (Fig. 5A, B). Since αIIb/β3 also serves as a fibronectin binding molecule, we investigated fibronectin adhesion and found that 25–150 μM EFA inhibited fibronectin adhesion dose-dependently (Fig. 5C).

Fig. 3 Effects of EFA on [Ca²⁺]_i mobilization, Serotonin release and IP₃RI phosphorylation (A) Effect of EFA on collagen-induced [Ca²⁺]_i mobilization. (B) Effect of EFA on collagen-induced serotonin release. (C) Effect of EFA on collagen-induced IP₃RI (Ser¹⁷⁵⁶) phosphorylation. Measurement of [Ca²⁺]_i mobilization, serotonin release Western blot was performed as described in “Materials and Methods” section. The data are expressed as the mean ± standard deviation (n=4). **p* < 0.05, ***p* < 0.01 versus the collagen-stimulated human platelets

Measurement of VASP- and Akt-phosphorylation and cyclic nucleotides

Phosphorylated VASP is known to suppress αIIb/β3 activation, and phosphorylated Akt is known to increase αIIb/β3 activation. Thus, we examined whether EFA affects VASP- and Akt-phosphorylation. EFA strongly phosphorylated VASP at Ser¹⁵⁷ (Fig. 6A) and dephosphorylated Akt at Ser⁴⁷³ (Fig. 6B). We further investigated the effect of EFA on cAMP and cGMP production in platelets and found that EFA elevated cAMP production dose-

Fig. 4 Effects of EFA on TXA₂ generation and cPLA₂ and p38^{MAPK} phosphorylation. (A) Effects of EFA on collagen-induced TXA₂ generation. (B) Effect of EFA on collagen-induced cPLA₂ (Ser⁵⁰⁵) phosphorylation. (C) Effect of EFA on collagen-induced p38^{MAPK} phosphorylation. Measurement of TXA₂ generation and Western blot was performed as described in “Materials and Methods” section. The data are expressed as the mean $\pm$ standard deviation ($n=4$). * $p < 0.05$, ** $p < 0.01$ versus the collagen-stimulated human platelets

independently (Fig. 6C), but did not increase cGMP production (data not shown).

Effects of EFA on clot retraction

Activated integrin α IIb/ β 3 activates the signaling pathway that triggers fibrin clot retraction. Therefore, we investigated the inhibitory action of EFA on fibrin clot retraction. As shown in Fig. 7A, thrombin-induced fibrin clot formation contracted by 80.4% compared with that in unstimulated platelet-rich plasma. However, thrombin-stimulated contraction was dose-dependently delayed by

Fig. 5 Effects of EFA on fibrinogen binding to α IIb/ β 3 and Fibronectin adhesion (A) The flow cytometry histograms on fibrinogen binding. (B) Effects of EFA on collagen-induced fibrinogen binding (%). (C) Effects of EFA on collagen-induced fibronectin adhesion. Measurement of fibrinogen binding and fibronectin adhesion was carried out as described in “Materials and Methods” section. The data are expressed as the mean $\pm$ standard deviation ($n=4$). * $p < 0.05$, ** $p < 0.01$ versus the collagen-stimulated human platelets

25–150 μ M EFA (Fig. 7B). Y27632 (5 μ M), a positive control, delayed fibrin clot formation by 37.0% compared with unstimulated platelet-rich plasma.

Fig. 6 Effects of EFA on VASP, Akt phosphorylation and cAMP. (A) Effect of EFA on collagen-induced VASP (Ser¹⁵⁷) phosphorylation. (B) Effect of EFA on collagen-induced Akt (Ser⁴⁷³) phosphorylation. (C) Effect of EFA on collagen-induced cAMP production. Measurement of Western blot and cAMP level was performed as described in “Materials and Methods” section. The data are expressed as the mean ± standard deviation (n=4). **p*<0.05, ***p*<0.01 versus the collagen-stimulated human platelets

Discussion

Various agonists induce platelet-mediated hemostatic plaque by triggering granule secretion, platelet activation, and platelet aggregation. These agonists or adhesive proteins cause inside-out signaling pathways, which involve conformational changes in integrin αIIb/β3 structure and modulate fibrinogen binding to integrin. [Ca²⁺]_i mobilization is the most critical event in the inside-out signaling pathway. IP₃RI, located on the endoplasmic

Fig. 7 Effects of EFA on fibrin clot retraction. (A) Photographs of fibrin clot (B) Effects of EFA on thrombin-retracted fibrin clot (%). Quantification of fibrin clot retraction was performed as describe in “Materials and Methods” section. The data are expressed as the mean ± standard deviation (n=4). **p*<0.05 versus the unstimulated human PRP, †*p*<0.05 versus the thrombin-stimulated human PRP

reticulum surface, induces [Ca²⁺]_i mobilization by binding with inositol 1,4,5-trisphosphate [12]. Increased [Ca²⁺]_i causes phosphorylation of both the myosin light chain and pleckstrin to trigger δ- and α-granule secretion and P-selectin expression. Therefore, decreasing intracellular calcium levels leads to the inhibition of platelet activation and aggregation. As shown in Fig. 3A and 3B, EFA suppressed the collagen-induced [Ca²⁺]_i level and serotonin release. IP₃RI is a major substrate of protein kinase A and protein kinase G in platelets, but its phosphorylation (Ser¹⁷⁵⁶) leads to the inhibition of [Ca²⁺]_i mobilization [13,14]. Therefore, this result indicates that the inhibition of [Ca²⁺]_i levels by EFA is due to the elevation of IP₃RI phosphorylation (Fig. 3C). TXA₂ acts as a powerful autacoid that activates other platelets. Therefore, TXA₂ production inhibitors are useful as anti-platelet substances; for example, aspirin, ozagrel, and indomethacin block TXA₂ production by inhibiting cyclooxygenase-1 or TXA₂ synthase [15]. cPLA₂ activation and [Ca²⁺]_i mobilization is essential for TXA₂ production. Intracellular Ca²⁺ binds to cPLA₂, and activated cPLA₂ is translocated from the cytosol to the membrane. Subsequently, p38^{MAPK} phosphorylates cPLA₂ for full enzyme activity [16,17]. Therefore, inhibition of [Ca²⁺]_i mobilization and p38^{MAPK} phosphorylation leads downregulation of TXA₂ production and we confirmed the relevance using specific inhibitors in a previous study [18,19]. EFA suppressed collagen-induced TXA₂ production (Fig. 4A). This result indicates that the inhibition of TXA₂ production by EFA is due to the decrease in [Ca²⁺]_i levels

and dephosphorylation of cPLA₂ and p38^{MAPK} (Fig. 4B, C).

α IIb/ β 3 is the most abundant integrin on the platelet surface and acquires the ability to bind to ligands such as fibrinogen, vitronectin, and fibronectin. α IIb/ β 3 is expressed in a low-affinity state on resting platelets, and the inside-out signaling pathway initiates a conformational change within the receptor, which increases the affinity of α IIb/ β 3 to bind to ligands [3]. Upon fibrinogen binding, the receptor undergoes further shape changes, adhesion, and spreading. This process, termed outside-in signaling, results in platelet aggregation amplification. The effect of EFA upon fibrinogen binding was investigated using a fluorescence-activated cell sorter. EFA suppressed collagen-induced fibrinogen binding and fibronectin adhesion (Fig. 5A, C). These results indicate that the decrease in α IIb/ β 3 affinity caused by EFA is due to VASP (Ser¹⁵⁷, Ser²³⁹) phosphorylation and Akt (Ser⁴⁷³) dephosphorylation (Fig. 6A, B) [20–22].

Intracellular cAMP and cGMP are reported to reverse platelet aggregation and are regulated by the balance between adenylate/guanylate cyclase and phosphodiesterases [23]. These cyclic nucleotides can inhibit α IIb/ β 3 activity and [Ca²⁺]_i mobilization through protein kinase A and protein kinase G [24]. In our study, EFA increased cAMP concentration (Fig. 6C), which enhanced the phosphorylation of VASP (Ser¹⁵⁷) and IP₃RI (Ser¹⁷⁵⁶). Inside-out and outside-in signaling pathways lead to platelet activation and fibrin platelet meshwork development. Fibrin clot formation, which acts on the hemostasis of damaged blood vessels, creates a thrombus. The interaction between α IIb/ β 3 and fibrinogen or fibronectin plays an important role in fibrin clot formation. As shown in Fig. 7, the EFA suppressed thrombin-induced fibrin clot formation. These results show that EFA is a potential potent antiplatelet agent through thrombus formation inhibition.

In conclusion, we confirmed that EFA decreases calcium mobilization, TXA₂ production, fibrinogen binding to α IIb/ β 3, fibronectin adhesion, and clot retraction through cAMP-dependent phosphorylation. Therefore, we suggest that EFA extracted from the root and stems of *C. tricuspidata* would be a useful compound for thrombosis prevention.

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Conflict of interest The authors declare no conflict of interest.

References

- Chen H, Kahn ML (2003) Reciprocal signaling by integrin and nonintegrin receptors during collagen activation of platelets. *Mol Cell Biol* 23: 4764–4777
- Farndale RW (2006) Collagen-induced platelet activation. *Blood Cell Mol Dis* 36: 162–165
- Phillips DR, Nannizzi-Alaimo L, Prasad KS (2001) Beta3 tyrosine phosphorylation in α IIb β 3 (platelet membrane GP IIb-IIIa) outside-in integrin signaling. *Thromb Haemost* 86: 246–258
- Andrews RK, Berndt MC (2004) Platelet physiology and thrombosis. *Thromb Res* 114: 447–453
- Jackson SP (2011) Arterial thrombosis-insidious, unpredictable and deadly. *Nat Med* 17: 1423–1436
- Kim DC, Yoon CS, Quang TH, Ko W, Kim JS, Oh H, Kim YC (2016) Prenylated flavonoids from *Cudrania tricuspidata* suppress lipopolysaccharide-induced neuroinflammatory activities in BV2 microglial cells. *Int J Mol Sci* 17:255
- Xin LT, Yue SJ, Fan YC, Wu JS, Yan D, Guan HS, Wang CY (2017) *Cudrania tricuspidata*: an updated review on ethnomedicine, phytochemistry and pharmacology. *RSC advances* 7: 31807–31832
- Shin JH, Ha JY, Kwon HW (2020) Inhibitory Actions of Steppogenin on Platelet Activity Through Regulation of Glycoprotein IIb/IIIa and Ca²⁺ Mobilization. *Korean J Pharmacogn* 51: 100–106
- Shin JH (2020) Inhibitory Effects of Isoderrone on Platelet Aggregation through Regulation of Cyclic Nucleotides. *J Korean Soc Food Sci Nutr* 49: 796–802
- Shin JH, Kwon HW, Lee DH (2019) Ginsenoside F4 inhibits platelet aggregation and thrombus formation by dephosphorylation of IP₃RI and VASP. *J Appl Biol Chem* 62: 93–100
- Gryniewicz G, Poenie M, Tsien RY (1985) A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 260: 3440–3450
- VargaSzabo D, Braun A, Nieswandt B (2009) Calcium signaling in platelets. *J Thromb Haemost* 7: 1057–1066
- Quinton TM, Dean WL (1992) Cyclic AMP-dependent phosphorylation of the inositol-1,4,5-trisphosphate receptor inhibits Ca²⁺ release from platelet membranes. *Biochem Biophys Res Commun* 184: 893–899
- Cavallini L, Coassin M, Borean A, Alexandre A (1996) Prostacyclin and sodium nitroprusside inhibit the activity of the platelet inositol 1,4,5-trisphosphate receptor and promote its phosphorylation. *J Biol Chem* 271: 5545–5551
- Patrono C (1994) Aspirin as an antiplatelet drug. *N Engl J Med* 330: 1287–1294
- Kramer RM, Roberts EF, Um SL, Börsch-Haubold AG, Watson SP, Fisher MJ, Jakubowski JA (1996) p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A2 (cPLA2) in thrombin-stimulated platelets. evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA2. *J Biol Chem* 271: 27723–27729
- McNicol A, Shibou TS (1998) Translocation and phosphorylation of cytosolic phospholipase A2 in activated platelets. *Thromb Res* 92: 19–26
- Kwon HW (2018) Inhibitory Effects of PD98059, SB203580, and SP600125 on α - and δ -granule Release and Intracellular Ca²⁺ Levels in Human Platelets. *Biomed Sci Lett* 24: 253–262
- Shin JH, Kwon HW, Rhee MH, Park HJ (2019) Inhibitory effects of thromboxane A2 generation by ginsenoside Ro due to attenuation of cytosolic phospholipase A2 phosphorylation and arachidonic acid release. *J Ginseng Res* 43: 236–241
- Laurent V, Loisel TP, Harbeck B, Wehman A, Gröbe L, Jockusch BM, Carlier MF (1999) Role of proteins of the Ena/VASP family in actin-based motility of *Listeria monocytogenes*. *J Cell Biol* 144: 1245–1258
- Sudo T, Ito H, Kimura Y (2003) Phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) by the anti-platelet drug, cilostazol, in platelets. *Platelets* 14: 381–390
- Woulfe DS (2010) Akt signaling in platelets and thrombosis. *Expert review of hematology* 3: 81–91
- Schwarz UR, Walter U, Eigenthaler M (2001) Taming platelets with cyclic nucleotides. *Biochem Pharmacol* 62: 1153–1161
- Smolenski A (2012) Novel roles of cAMP/cGMP-dependent signaling in platelets *J Thromb Haemost* 10: 167–176