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

Jung-Hae Shin[1] · **Hyuk-Woo Kwon**[2]

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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 (αIIb/β3)-mediated signaling events, including platelet adhesion, granule secretion, thromboxane A_2 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 αIIb/β3 affinity · Ca^{2+} mobilization · cAMP and cGMP · Clot retraction · Euchrestaflavanone A

Hyuk-Woo Kwon (✉)
E-mail: kwonhw@kdu.ac.kr

[1]Department of Biomedical Laboratory Science, Catholic Kwandong University, Gangneung 25601, Republic of Korea

[2]Department of Biomedical Laboratory Science, Far East University, Eumseong 27601, Republic of Korea

Introduction

In normal circulation, collagen fibers cannot bind to platelets. However, when endothelial walls are injured, exposed collagen fibers can bind to integrin α2β1 and glycoprotein VI on platelet receptors, leading to platelet activation. After activation, phospholipase $C\gamma_2$ within the cytosol hydrolyzes phosphatidylinositol 4,5-bisphosphate into inositol diacylglycerol and 1,4,5-trisphosphate (IP_3), which then mobilizes calcium from the endoplasmic reticulum [1,2]. These signaling events in activated platelets cause conformational changes in glycoprotein IIb/IIIa (integrin αIIb/β3), 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 B_2 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 (IP_3RI), phospho-cytosolic phospholipase A_2 ($cPLA_2$), phospho-p38 mitogen-activated protein kinase ($p38^{MAPK}$), phospho-Akt, and â-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 5×10^8/mL.

Platelet aggregation

For platelet aggregation, human platelet suspensions (10^8/mL) were pre-incubated for 3 min in the presence or absence of EFA (25 to 150 μM) along with 2 mM $CaCl_2$ at 37 °C, followed by stimulation with agonists. Collagen (2.5 μg/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 (10^8/mL) were incubated with different concentrations of EFA (25 to 150 μM) 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 μM) and PRP mixture was pre-incubated at 37 °C for 60 min, and then the human platelet suspension (10^8/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 μg/mL collagen in the presence of 2 mM $CaCl_2$. To calculate the $[Ca^{2+}]_i$ values, Fura 2-AM fluorescence was measured according to the Grynkiewicz method [11] using a spectrofluorometer (Hitachi F-2700; Tokyo, Japan).

Measurement of thromboxane B_2

Since thromboxane A_2 (TXA_2) is unstable and transforms into thromboxane B_2 (TXB_2) quickly, TXA_2 generation was measured by detecting TXB_2 production. After platelet activation, the reaction was stopped by adding 0.2 mM indomethacin with 5 mM EDTA. TXB_2 levels were measured using an ELISA reader (TECAN, Salzburg, Austria) and a TXB_2 ELISA kit.

Measurement of serotonin

Human platelet suspensions (10^8/mL) were pre-incubated for 3 min at 37 °C with EFA and then stimulated with 2.5 μg/mL collagen in the presence of 2 mM $CaCl_2$ 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 μg 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 αIIb/β3

Human platelet suspensions (10^8/mL) were incubated with EFA (25 to 150 μM) and treated with 30 μg/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 αIIb/β3 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/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 μg/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/mL) were pre-incubated for 3 min at 37 °C with or without EFA (25 to150 μM) in the presence of 2 mM $CaCl_2$, and then stimulated with 2.5 μg/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 to150 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 ± 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, xanthones 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 μg/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 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).

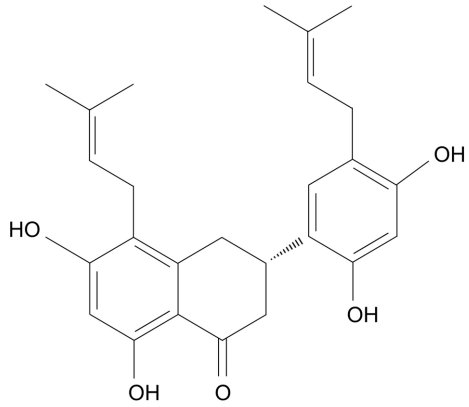

Fig. 1 Chemical structure of euchrestaflavanone A (EFA)

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

Intracellular ($[Ca^{2+}]_i$) is an essential factor for platelet activation; thus, we focused on the effect of EFA on $[Ca^{2+}]_i$ mobilization. As shown in Fig. 3A, collagen elevated $[Ca^{2+}]_i$ levels from 110.2 ± 0.7 nM to 770.5±6.5 nM. However, 25-150 μM EFA reduced the increased $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ mobilization. As shown in Fig. 3C, collagen increased IP_3RI phosphorylation was increased by EFA (25 to 150 μM).

Effects of EFA on $[Ca^{2+}]_i$ 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 μg/mL collagen elevated $cPLA_2$ and $p38^{MAPK}$ phosphorylation, 25-150 ìM 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.

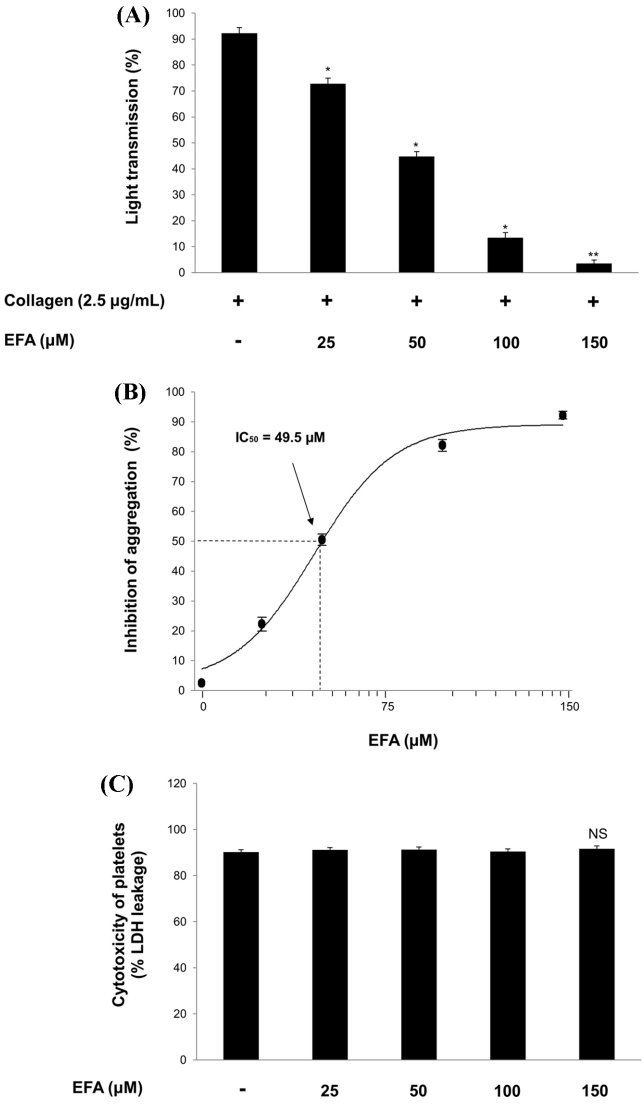

Fig. 2 Effects of EFA on platelet aggregation, half maximal inhibitory concentration, and cytotoxicity (A) Effect of EFA on collagen-induced human platelet aggregation. (B) Half maximal inhibitory concentration (IC_{50}) value of EFA in collagen-induced human platelet aggregation. (C) Effect of EFA on 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

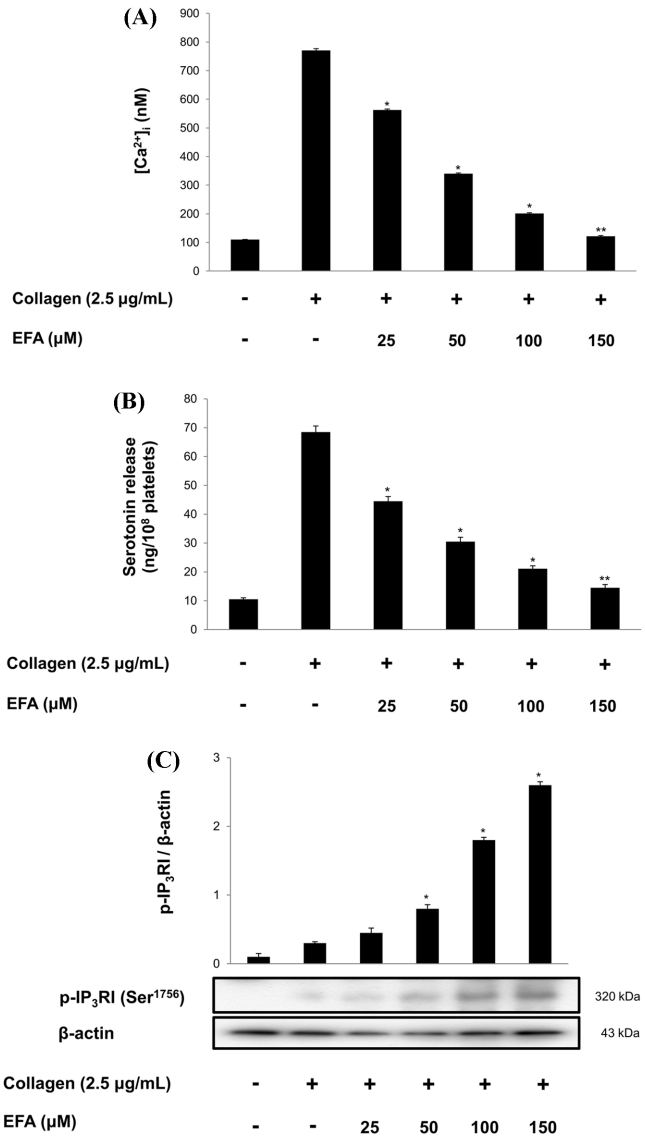

Fig. 3 Effects of EFA on $[Ca^{2+}]_i$ mobilization, Serotonin release and IP_3RI phosphorylation (A) Effect of EFA on collagen-induced $[Ca^{2+}]_i$ mobilization. (B) Effect of EFA on collagen-induced serotonin release. (C) Effect of EFA on collagen-induced IP_3RI (Ser^{1756}) phosphorylation. Measurement of $[Ca^{2+}]_i$ 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

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).

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 Ser^{157} (Fig. 6A) and dephosphorylated Akt at Ser^{473} (Fig. 6B). We further investigated the effect of EFA on cAMP and cGMP production in platelets and found that EFA elevated cAMP production dose-

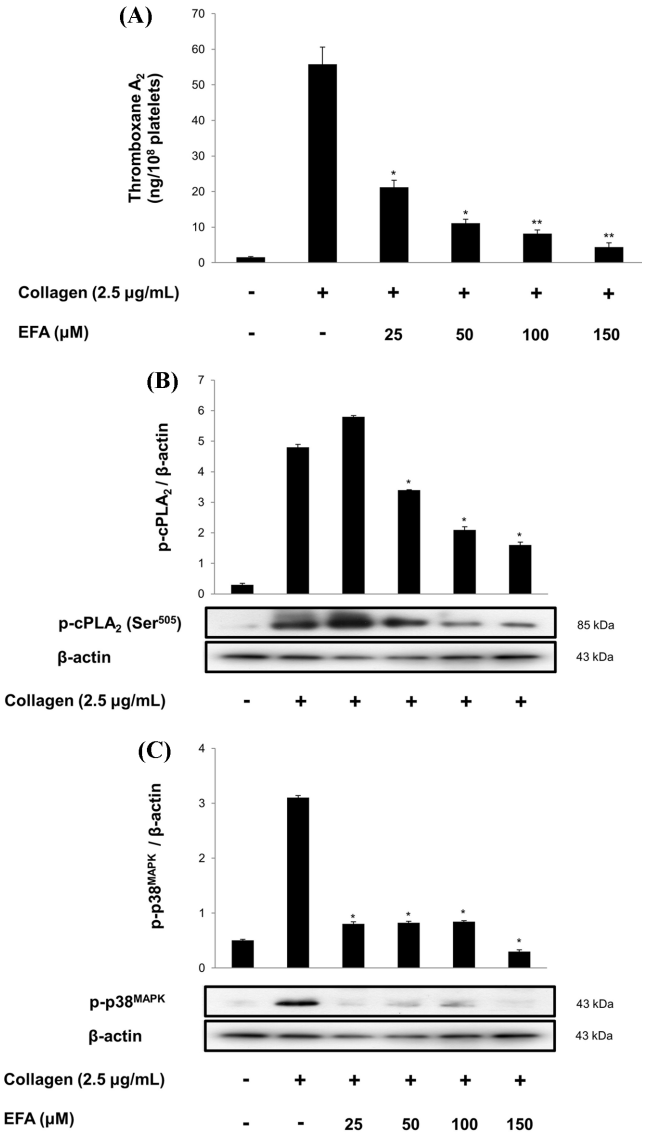

Fig. 4 Effects of EFA on TXA_2 generation and $cPLA_2$ and p38 ^{MAPK} phosphorylation. (A) Effects of EFA on collagen-induced TXA_2 generation. (B) Effect of EFA on collagen-induced $cPLA_2$ (Ser^{505}) phosphorylation. (C) Effect of EFA on collagen-induced $p38^{MAPK}$ phosphorylation. Measurement of TXA_2 generation and 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

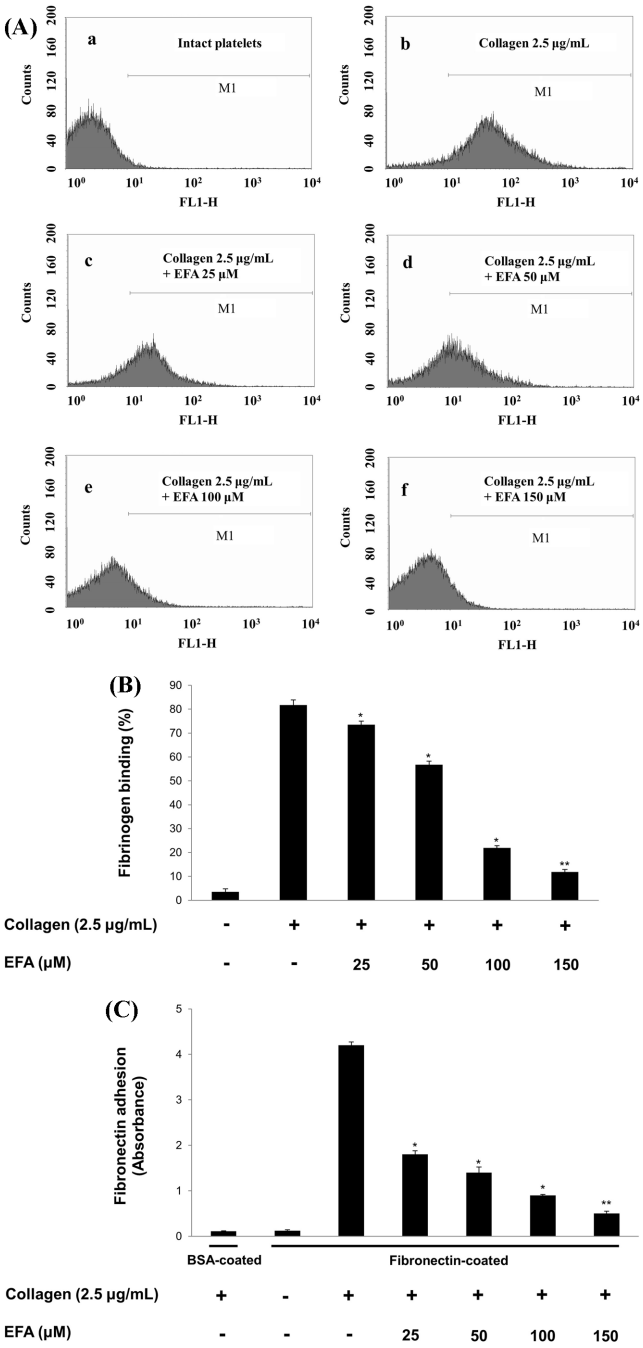

Fig. 5 Effects of EFA on fibrinogen binding to αIIb/β3 and Fibronectin adhesion (A) The flow cytometry histograms on fibrinogen binding. (B) Effects of EFA on collagen-induced fibrinogen binding (%). (C) Effects of EFA on collagen-induced fibronectin adhesion. Measurement of fibrinogen binding and fibronectin adhesion was carried out as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n=4). *p <0.05, **p <0.01 versus the collagen-stimulated human platelets

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

Effects of EFA on clot retraction

Activated integrin αIIb/β3 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 25-150 μM EFA (Fig. 7B). Y27632 (5 μM), a positive control, delayed fibrin clot formation by 37.0% compared with unstimulated platelet-rich plasma.

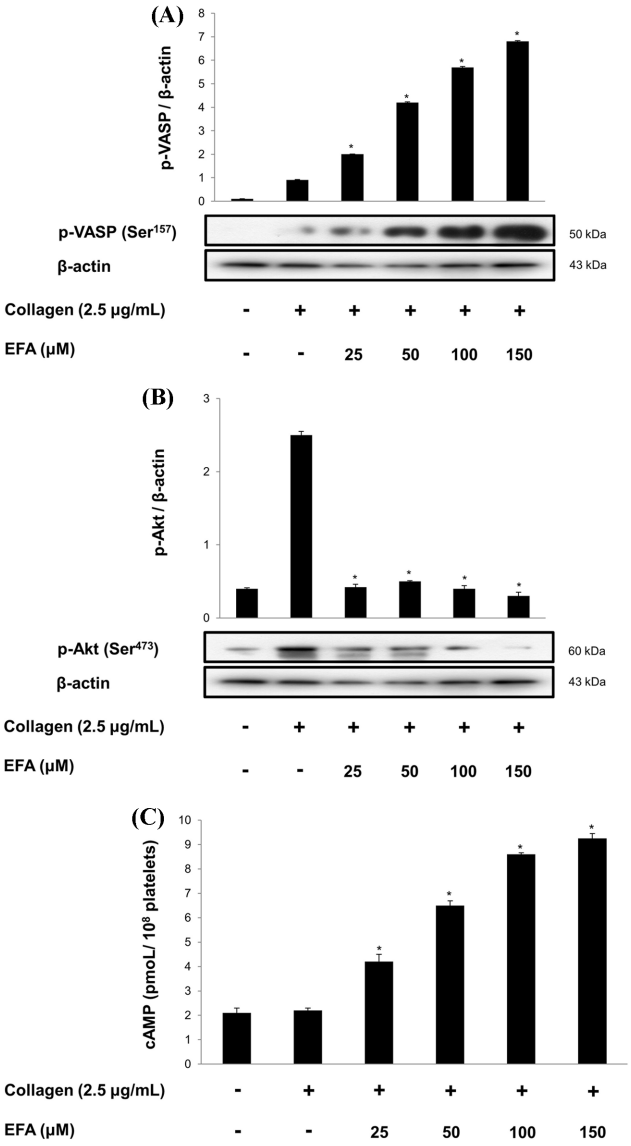

Fig. 6 Effects of EFA on VASP, Akt phosphorylation and cAMP. (A) Effect of EFA on collagen-induced VASP (Ser^{157}) phosphorylation. (B) Effect of EFA on collagen-induced Akt (Ser^{473}) phosphorylation. (C) Effect of EFA on collagen-induced cAMP production. Measurement of Western blot and cAMP level 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

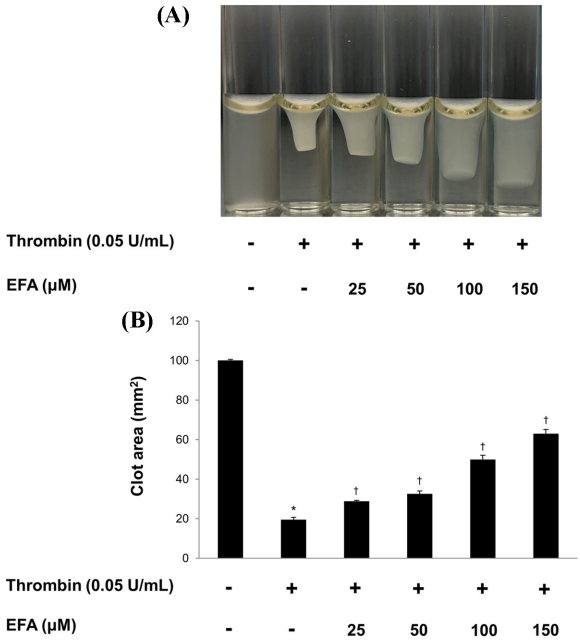

Fig. 7 Effects of EFA on fibrin clot retraction. (A) Photographs of fibrin clot (B) Effects of EFA on thrombin-retracted fibrin clot (%). Quantification of fibrin clot retraction was performed as describe in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n =4).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

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. $[Ca^{2+}]_i$ mobilization is the most critical event in the inside-out signaling pathway. IP_3RI, located on the endoplasmic reticulum surface, induces $[Ca^{2+}]_i$ mobilization by binding with inositol 1,4,5-trisphosphate [12]. Increased $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ level and serotonin release. IP_3RI is a major substrate of protein kinase A and protein kinase G in platelets, but its phosphorylation (Ser^{1756}) leads to the inhibition of $[Ca^{2+}]_i$ mobilization [13,14]. Therefore, this result indicates that the inhibition of $[Ca^{2+}]_i$ levels by EFA is due to the elevation of IP_3RI phosphorylation (Fig. 3C). TXA_2 acts as a powerful autacoid that activates other platelets. Therefore, TXA_2 production inhibitors are useful as anti-platelet substances; for example, aspirin, ozagrel, and indomethacin block TXA_2 production by inhibiting cyclooxygenase-1 or TXA_2 synthase [15]. $cPLA_2$ activation and $[Ca^{2+}]_i$ mobilization is essential for TXA_2 production. Intracellular Ca^{2+} binds to $cPLA_2$, and activated $cPLA_2$ is translocated from the cytosol to the membrane. Subsequently, $p38^{MAPK}$ phosphorylates $cPLA_2$ for full enzyme activity [16,17]. Therefore, inhibition of $[Ca^{2+}]_i$ mobilization and $p38^{MAPK}$ phosphorylation leads downregulation of TXA_2 production and we confirmed the relevance using specific inhibitors in a previous study [18,19]. EFA suppressed collagen-induced TXA_2 production (Fig. 4A). This result indicates that the inhibition of TXA_2 production by EFA is due to the decrease in $[Ca^{2+}]_i$ levels

and dephosphorylation of $cPLA_2$ and $p38^{MAPK}$ (Fig. 4B, C).

αIIb/β3 is the most abundant integrin on the platelet surface and acquires the ability to bind to ligands such as fibrinogen, vitronectin, and fibronectin. αIIb/β3 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 αIIb/β3 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 αIIb/β3 affinity caused by EFA is due to VASP (Ser^{157}, Ser^{239}) phosphorylation and Akt (Ser^{473}) 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 αIIb/β3 activity and $[Ca^{2+}]_i$ 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 (Ser^{157}) and IP_3RI (Ser^{1756}). 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 αIIb/β3 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 anti-platelet agent through thrombus formation inhibition.

In conclusion, we confirmed that EFA decreases calcium mobilization, TXA_2 production, fibrinogen binding to αIIb/β3, 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.

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