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Ginsenoside F4 inhibits platelet aggregation and thrombus formation by dephosphorylation of IP_3RI and VASP

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Abstract The root of *Panax ginseng* is used in ethnomedicine throughout eastern Asia and various recent studies have proved that *Panax ginseng* has inhibitory effects on cardiovascular disease. Each factor causing cardiovascular disease is known to have a very complex process which is achieved by a diverse number of mechanisms. Among these factors, platelets are the most important because they directly participate in thrombogenesis. Therefore, inhibiting the activity of platelets is an essential element for prevention of cardiovascular diseases. Our previous study showed the antiplatelet effects of Korean red ginseng extract and two of its components, ginsenoside Rg3 and ginsenoside Ro. However, the inhibitory mechanism of other ginsenosides remains unclear. Therefore, we investigated the inhibitory mechanism of ginsenoside F4 (G-F4) from Korean red ginseng on the regulation of signaling molecules involved in human platelet aggregation. With the use of G-F4, collagen-induced human platelet aggregation was inhibited in a dose-dependent manner, and it suppressed collagen-induced elevation of $[Ca^{2+}]_i$ mobilization through elevated phosphorylation of inositol 1, 4, 5-triphosphate receptor I (Ser^{1756}). In addition, G-F4 inhibited fibrinogen binding to $\alpha IIb/\beta_3$ during collagen-induced human platelet aggregation. Thus, in the present study, G-F4 showed an inhibitory effect on human platelet activation, suggesting its potential use as a new natural medicine for preventing platelet-mediated cardiovascular diseases.

Keywords Clot retraction · Ginsenoside F4 · Inositol 1, 4, 5-triphosphate receptor I (Ser^{1756}) · Vasodilator-stimulated phosphoprotein (Ser^{157})

Introduction

An area of damaged vascular wall activates platelets through a series of physiological molecules, such as ADP, thrombin, and collagen. Platelet aggregation is essential for the maintenance of normal hemostasis, but it can also cause thrombosis at the same time. The production of thrombosis is a critical factor for patients and people who have risks for cardiovascular disease. Therefore, drugs that can inhibit the activity of platelets are needed [1]. However, despite numerous investigations into the discovery and development of more effective antiplatelet and antithrombotic drugs, the effect of these drugs on mortality rates is still weak. In addition, some drugs can lead to serious complications [2]. Therefore, further research is needed for the development of more effective and safer drugs to ensure better treatment and prevention of cardiovascular disease.

Platelet activation and aggregation are due to the activation of intracellular calcium and integrin glycoprotein IIb/IIIa ($\alpha IIb/\beta_3$). Intracellular Ca^{2+} ($[Ca^{2+}]_i$) is a platelet aggregation-inducing molecule, and influences activation of aggregation associated molecules. The increased $[Ca^{2+}]_i$ activates the Ca^{2+}/calmodulin-dependent phosphorylation of the myosin light chain to trigger the inside-out signaling pathway leading to platelet activation. Activated platelets affect the structural change of $\alpha IIb/\beta_3$ on the platelet surface, which allows for interaction with its plasma ligands (i.e. fibrinogen, fibronectin, and vitronectin), causing activation of the outside-in signaling pathway, and thrombus formation [3,4].

Elevation in $[Ca^{2+}]_i$ level by agonists is dependent on its

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mobilization from the endoplasmic reticulum and influx from extracellular spaces. Thrombin, a platelet agonist, is known to bind to the Gq-coupled proteinase-activated receptor and activate phospholipase C_β (PLC_β), stimulating the aggregation of platelets. The activated PLC_β hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP_2) into diacylglycerol and inositol 1, 4, 5-trisphosphate (IP_3) [5]. IP_3 mobilizes free cytosolic Ca^{2+} from the endoplasmic reticulum by binding to IP_3 receptor type I (IP_3RI). The increased Ca^{2+} stimulates granule secretion and platelet aggregation. Another way to increase $[Ca^{2+}]_i$ level is through an influx of Ca^{2+} from extracellular spaces. Depletion of the intracellular Ca^{2+} store by IP_3 is known to be associated with the influx of extracellular Ca^{2+} [6].

During normal circulation, nitric oxide and prostaglandin I_2 are both released by vascular endothelial cells facilitating the production of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) in the platelets. Elevated cGMP and cAMP levels induce the activation of protein kinase G (PKG) and protein kinase A (PKA), respectively. Both PKA and PKG then phosphorylate substrate protein inositol triphosphate receptor type I and vasodilator-stimulated phosphoprotein (VASP) [7]. IP_3RI becomes inactive after phosphorylation, leading to inhibition of $[Ca^{2+}]_i$ mobilization [8,9]. In the platelet, a major substrate of PKA and PKG is vasodilator-stimulated phosphoprotein and it supports the regulation of actin filament dynamics as well as $\alpha IIb/\beta_3$ activation. However, the phosphorylation of VASP Ser^{157} counters these actions by suppressing $\alpha IIb/\beta_3$ activation and inhibiting actin filament elongation [10,11]. Therefore, the phosphorylation of IP_3RI can be very useful for evaluating the Ca^{2+}-antagonistic effect, whereas VASP phosphorylation is important for evaluating $\alpha IIb/\beta_3$-inhibition effect.

Panax ginseng C.A. Meyer has been well known to induce various biological activities through a number of different ginsenosides. The ginsenosides are produced of different composition via the processing method. Ginsenoside Rb1, Rc, Rd, Re, and Rg1 are major components of white and red ginseng, while unique ginsenoside constituents such as Rg3, Rg5, Rk1, and F4 are found specifically from red ginseng [12]. Ginsenoside F4 (G-F4) is a protopanaxatriol group which has two sugar residues, glucose-rhamnose, connected with the position of carbon-6 (Fig. 1) [12,13]. It has been reported that G-F4 has an inhibitory effect on human lymphocytoma JK cells by inducing apoptosis [14], and it is able to block cartilage breakdown in rabbit cartilage tissue culture [15]. However, a study for the effect on platelets by G-F4 has yet to be investigated. Therefore, we characterized the modulatory mechanism of G-F4 on human platelet activation.

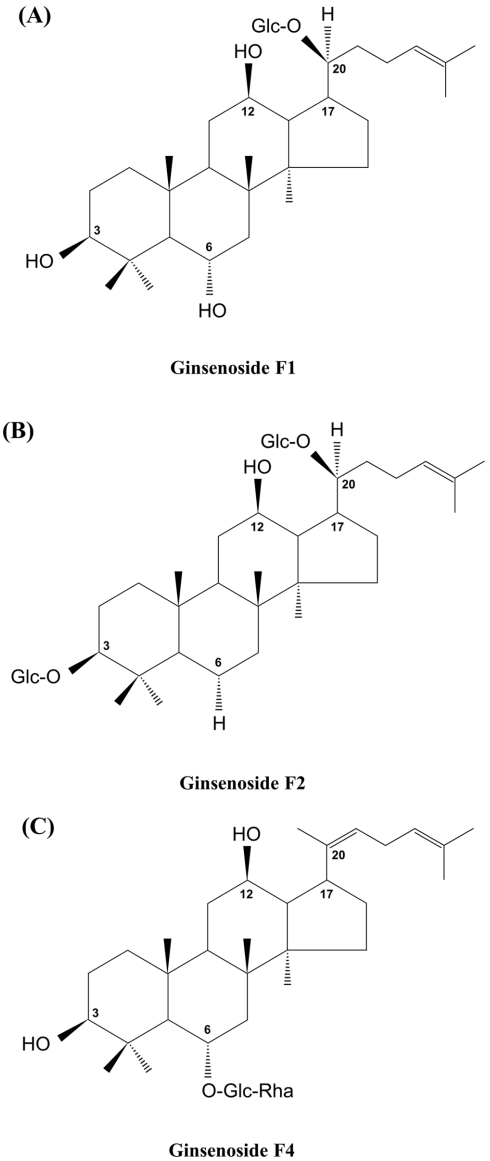

Fig. 1 Chemical structure of ginsenoside F1, F2 and F4. (A) Ginsenoside F1, a protopanaxatriol, isolated from the Korean Red Ginseng. (B) Ginsenoside F2, a protopanaxdiol, isolated from the Korean Red Ginseng. (C) Ginsenoside F4, a protopanaxatriol, isolated from the Korean Red Ginseng. Glc, glucose; Rha, rhamnose

Materials and Methods

Materials

Ginsenoside F1, F2, and F4 (G-F1, G-F2, and G-F4) were obtained from the Ambo Institute (Daejeon, Korea). Chrono-Log Corporation (Havertown, PA, USA) provided the collagen. 2-acetoxymethyl (Fura 2-AM) was obtained from Invitrogen (Eugene, OR, USA). Thapsigargin was obtained from Cayman Chemical (Ann Arbor, MI, USA). The lysis buffer, Anti-phosphor-VASP (Ser^{157}), anti-phosphor-inositol-3-phosphate receptor type I (Ser^{1756}), anti-β-actin, and anti-rabbit IgG-HRP-conjugate were bought from Cell Signaling (Beverly, MA, USA). Polyvinylidene difluoride (PVDF) membrane and enhanced chemiluminescence solution (ECL) were purchased from General Electric Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Invitrogen Molecular

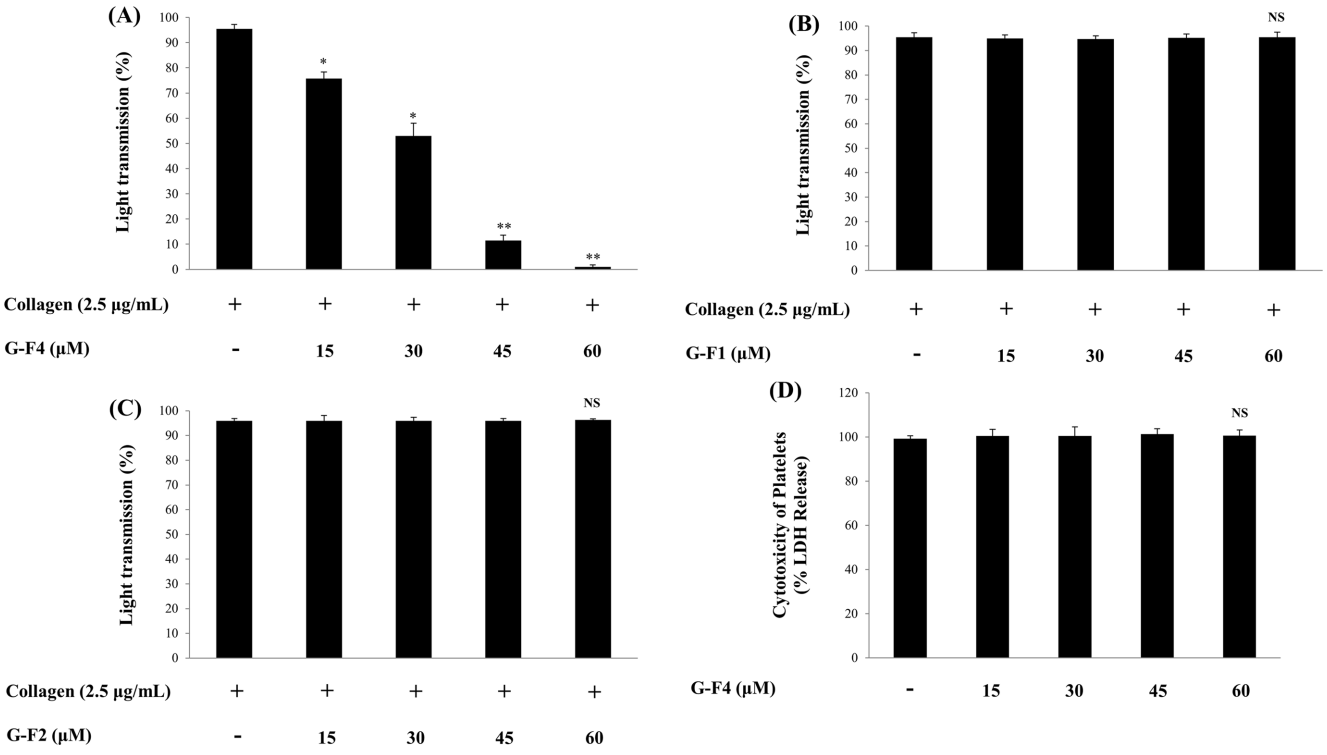

Fig. 2 Inhibitory effects of G-F1, F2 and F4 on collagen-induced human platelet aggregation. (A) Effects of G-F4 on collagen-induced human platelet aggregation. (B) Effects of G-F1 on collagen-induced human platelet aggregation. (C) Effects of G-F2 on collagen-induced human platelet aggregation. (D) Cytotoxicity of G-F4 on human platelets. Measurement of platelet aggregation was 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

Probes (Eugene, OR, USA) provided the Fibrinogen Alexa Fluor 488 conjugate.

Preparation of washed human platelets

The Korean Red Cross Blood Center (Changwon, Korea) provided the Human platelet-rich plasma (PRP). The plasma was centrifuged at 1,300×g for 10 min. The platelet-containing pellet was then washed twice with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.5 mM glucose, and 1 mM Na_2EDTA, pH 6.5), and a suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 0.49 mM $MgCl_2$, 5.5 mM glucose, 0.25% gelatin, pH 6.9) was then used to resuspend them. The final concentration of platelets was adjusted to 5×10^8/mL. All aforementioned procedures were performed at 25 °C. The Public Institutional Review Board at the National Institute for Bioethics Policy (Seoul, Korea) provided experimental approval (PIRB-).

Platelet aggregation

Platelets (10^8/mL) were preincubated in separate samples with or without G-F4 in 2 mM $CaCl_2$ for 3 min at 37 °C. Thrombin (0.05 U/mL) was then added to stimulate. An aggregometer (Chrono-Log Corporation) was used for 5 minutes to perform the platelet aggregation assay. An increase in light transmission was used to determine the platelet aggregation rate (%). A solution of 0.1% dimethyl sulfoxide (DMSO) was used to dissolve the G-F4.

Measurement of cytotoxicity

Cytotoxicity was determined through the leakage of lactate dehydrogenase (LDH) from cytosol. Human washed platelets (10^8 platelets/mL) were incubated for 2 hours at room temperature with various concentrations of substances and then centrifuged at room temperature for 2 min at 12,000 g. The supernatant was measured by LDH assay kit (Cayman Chemical) at an optical density of 490 nm.

Intracellular calcium concentration and calcium influx

PRP was incubated at 37 °C for 60 min with 5 μM of Fura 2-AM. The washed platelets (10^8/mL), loaded with Fura 2-AM, were prepared using the procedure described above, preincubated with G-F4 for 3 min at 37 °C in the presence of 2 mM $CaCl_2$. They were then stimulated with collagen (2.5 μg/mL) for intracellular calcium concentration ($[Ca^{2+}]_i$) and thapsigargin (1 μM) for Ca^{2+} influx. After thapsigargin stimulation, 2 mM of calcium was added at 3 min. A spectrofluorometer (SFM-25; BioTek Instruments, Italy) was used to measure Fura 2-AM fluorescence. The excitation wavelength was set starting at 340 nm and changed every 0.5 s until reaching 380 nm. An emission wavelength of 510 nm was

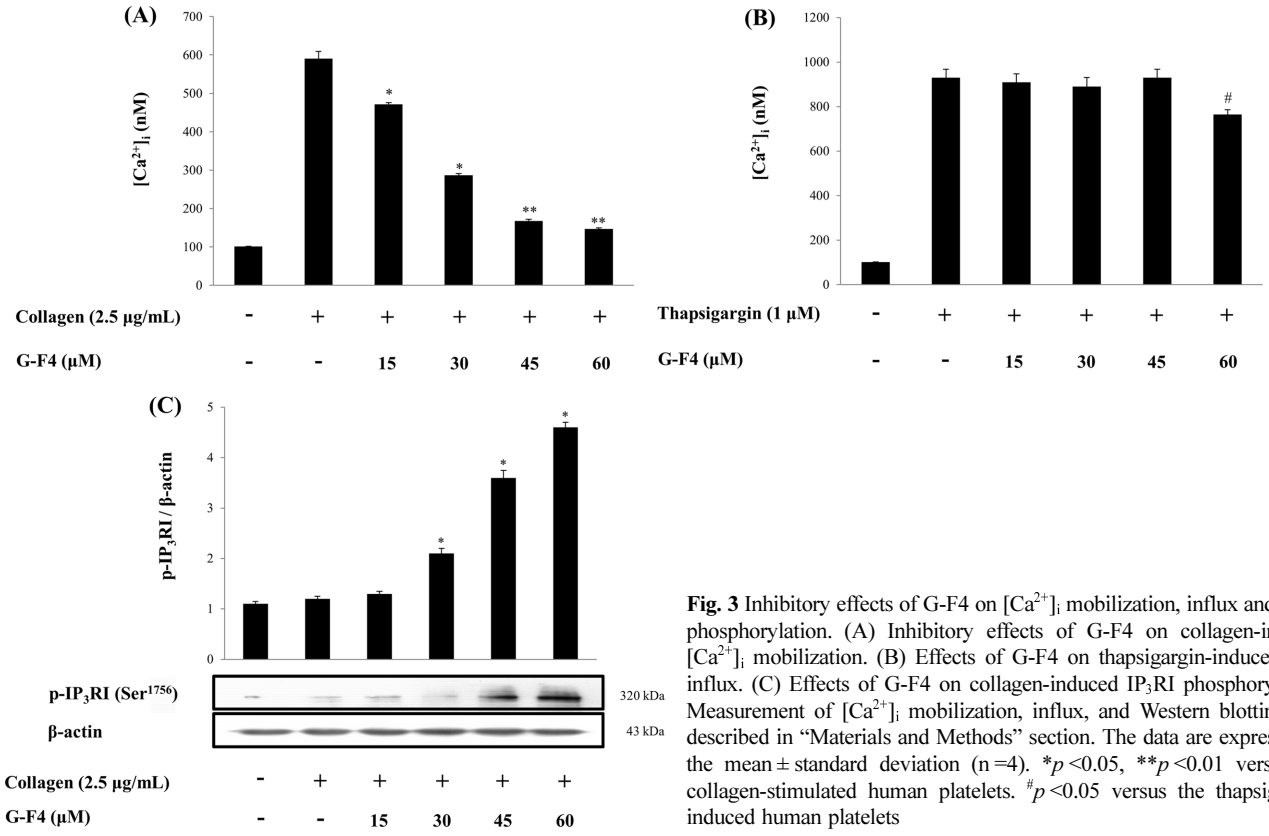

Fig. 3 Inhibitory effects of G-F4 on $[Ca^{2+}]_i$ mobilization, influx and IP_3RI phosphorylation. (A) Inhibitory effects of G-F4 on collagen-induced $[Ca^{2+}]_i$ mobilization. (B) Effects of G-F4 on thapsigargin-induced Ca^{2+} influx. (C) Effects of G-F4 on collagen-induced IP_3RI phosphorylation. Measurement of $[Ca^{2+}]_i$ mobilization, influx, and Western blotting was 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. $^{\#}p<0.05$ versus the thapsigargin-induced human platelets

used. The Grynkiewicz method [16] was used to calculate the $[Ca^{2+}]_i$ values.

Immunoblotting

Platelet aggregation was terminated by addition of 1× lysis buffer. A BCA protein assay kit (Pierce Biotechnology, IL, USA) was used to measure the total protein concentration of the platelet lysates. Proteins (15 μg) were separated through 8% SDS-PAGE, and transferred onto PVDF membranes. The primary and secondary antibodies were set at dilutions of 1:1,000 and 1:10,000, respectively. ECL reagent (General Electric Healthcare, Buckinghamshire, UK) was used to visualize protein bands.

Fibrinogen binding to $\alpha IIb/\beta_3$

Using Alexa Flour 488-human fibrinogen (30 μg/mL), the platelet aggregation assay was conducted at 37 °C for 5 min. To terminate the reaction, 0.5% paraformaldehyde in cold phosphate-buffered saline (pH 7.4) was added. The aforementioned procedures were conducted in darkness. The fibrinogen binding assay was performed using flow cytometry (BD Biosciences, San Jose, CA, USA), and analyzed with the CellQuest software (BD Biosciences).

Platelet-mediated fibrin clot retraction

Human PRP (500 μL) was transferred into a polyethylene tube to avoid adhesion, separate samples were preincubated with G-F4 (45, 60 μM) for 15 min at 37 °C and stimulated with thrombin (0.05 U/mL). Using a digital camera, pictures of fibrin clot were taken at 15 min. The Image J Software (v1.46, National Institutes of Health, Bethesda, MD, USA) was used to calculate the clot area.

Statistical analyses

Experimental data are indicated as the mean ± standard deviation included with the number of observations. Analysis of variance (ANOVA) was performed to determine significant differences among the groups and then further analyzed using the Tukey-Kramer method. SPSS 21.0.0.0 software (SPSS, Chicago, IL, USA) was used to perform statistical analysis. $p<0.05$ was considered as statistically significant.

Results

Effects of G-F4 on thrombin-induced human platelet aggregation

Collagen at 2.5 μg/mL induces the optimum aggregation of human platelets, and in the present study, the aggregation rate of collagen induced platelets was 95.5% (Fig. 2A). However, platelets treated with G-F4 (15, 30, 45, and 60 μM) had aggregation rates that were significantly reduced (20.7, 44.5, 88.0, and 99.0%, respectively) without cytotoxicity (Fig. 2D), indicating that collagen-induced platelet aggregation was inhibited in a dose-dependent manner. DMSO 0.1% did not affect collagen-induced platelet

aggregation. In addition, we tested ginsenoside F1 (G-F1) and ginsenoside F2 (G-F2), however, these compounds did not affect collagen-induced human platelet aggregation (Fig 2B, C).

Effects of G-F4 on elevation of $[Ca^{2+}]_i$ mobilization, calcium influx, and IP_3RI phosphorylation

Due to the fact that $[Ca^{2+}]_i$ is essential for platelet activation, we investigated the effect of G-F4 on Ca^{2+} antagonistic activity. As shown in Fig. 3A, $[Ca^{2+}]_i$ level was increased from 101.2±0.6 nM to 590.6±18.0 nM by collagen (2.5 μg/mL). However, G-F4 dose (15 to 60 μM)-dependently decreased collagen-elevated $[Ca^{2+}]_i$ level (Fig. 3A). The inhibition rate of G-F4 (60 μM) was 75.2%. Thapsigargin is an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase, and is used to trigger Ca^{2+} influx from extracellular spaces. After adding 2 mM $CaCl_2$, 1 μM of thapsigargin facilitated Ca^{2+} influx from 101.2±0.6 nM (the basal level) to 930.9±38.0 nM (Fig. 3B). However, only 60 ìM of G-F4 inhibited Ca^{2+} influx weakly (Fig. 3B). The inhibition rate of G-F4 (60 μM) was 17.8%.

Therefore, we expected that G-F4 affects calcium mobilization and evaluated the effect of phosphorylation of the calcium mobilization signaling molecule. As shown in Fig. 3C, G-F4 (15 to 60 μM) dose-dependently increased IP_3RI (Ser^{1756}) phosphorylation in collagen-induced human platelet aggregation. This shows that the decrease of intracellular calcium concentration by G-F4 resulted from IP_3RI (Ser^{1756}) phosphorylation.

Effects of G-F4 on VASP-phosphorylation

As G-F4 showed a dose-dependent inhibitory effect on collagen-induced platelet aggregation (Fig. 2A), we further investigated the effect of G-F4 on VASP Ser^{157} phosphorylation in collagen-activated platelets. G-F4 significantly increased VASP Ser^{157} phosphorylation (Fig. 4) in a dose-dependent manner. Conversely, G-F4 did not affect VASP Ser^{239} phosphorylation (data not shown) suggesting that G-F4 did not affect cGMP level.

Effects of G-F4 on fibrinogen binding to αIIb/β_3

Since VASP Ser^{157} phosphorylation on Ser^{157} inhibited fibrinogen binding and G-F4 increased VASP Ser^{157} phosphorylation (Fig. 4), we investigated the effect of G-F4 on fibrinogen binding to αIIb/β_3 in human platelets. Collagen increased the binding of fibrinogen to αIIb/β_3 (Fig. 5A-b, 5B), with a rate of 90.8±0.5%. However, G-F4 attenuated the fibrinogen binding in a dose-dependent manner (Fig. 5A-d~g, 5B). The inhibition rate of G-F4 (60 μM) was 80.0%.

Effects of G-F4 on fibrin clot retraction

Platelet agonists activate αIIb/β_3 increasing the binding of fibrinogen to αIIb/β_3. Eventually leading to the outside-in αIIb/β_3 signaling pathway and clot retraction. Thus, we investigated the effect of G-F4 on the retraction of thrombin-induced fibrin clots, which is an index of outside-in αIIb/β_3 signaling. Fig. 6A shows thrombin stimulated fibrin clot formation and retraction. However, the thrombin-retracted fibrin clot was suppressed by G-F4 (45, 60 μM), and these inhibitory degrees were 48.4, 25.7%, respectively as compared with that by thrombin (Fig. 6B).

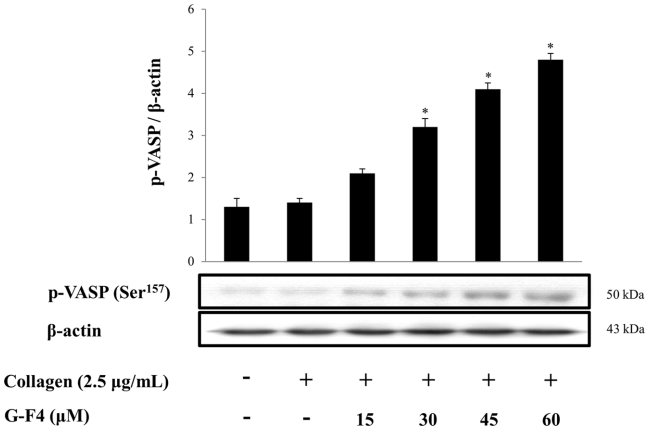

Fig. 4 Inhibitory effects of G-F4 on VASP phosphorylation. Western blotting was determined as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n =4). *p <0.05, **p <0.01 versus the collagen-stimulated human platelets

Discussion

Our previous reports showed that phosphorylation of IP_3RI (Ser^{1756}) and VASP (Ser^{157}) in human platelets is increased by the use of Korean red ginseng extract [17,18]. We also previously reported that the ginsenosides Rp1 show inhibitory effects through increased phosphorylation of VASP (Ser^{157}) [19]. Therefore, in the present study, we first examined the effect of G-F4 on cAMP/PKA-dependent phosphorylation in collagen-activated human platelets. Phosphorylation of the Ca^{2+}/calmodulin-dependent myosin light chain is involved in granule secretion and platelet aggregation. This phosphorylation is due to an increase in $[Ca^{2+}]_i$ which is triggered by a number of agonists including thrombin, collagen, and ADP. G-F4 showed inhibitory effect on $[Ca^{2+}]_i$; however, it did not affect thapsigargin-induced calcium influx which means that G-F4 has specific inhibitory effect on calcium mobilization from the endoplasmic reticulum without inhibition of calcium influx from extracellular space. This is clear evidence that the inhibition of $[Ca^{2+}]_i$ by G-F4 resulted from the elevation of IP_3RI (Ser^{1756}) phosphorylation.

VASP Ser^{157} and Ser^{239} are phosphorylated by cAMP/PKA and cGMP/PKG, respectively [20, 21]. G-F4 elevated VASP (Ser^{157}) phosphorylation, but did not affect VASP (Ser^{239}) phosphorylation. Since VASP (Ser^{157}) and IP_3RI (Ser^{1756}) are substrates of PKA, our results suggest that G-F4 increased VASP (Ser^{157}) and IP_3RI (Ser^{1756}) phosphorylation via cAMP/PKA dependent pathway in human platelets. Similar results were confirmed in our previous experiments. Ginsenoside Ro and Rg3 increased cAMP levels in human platelets, resulting in PKA-dependent phosphorylation and antiplatelet activity [22,23]. The results of G-F4 also showed the

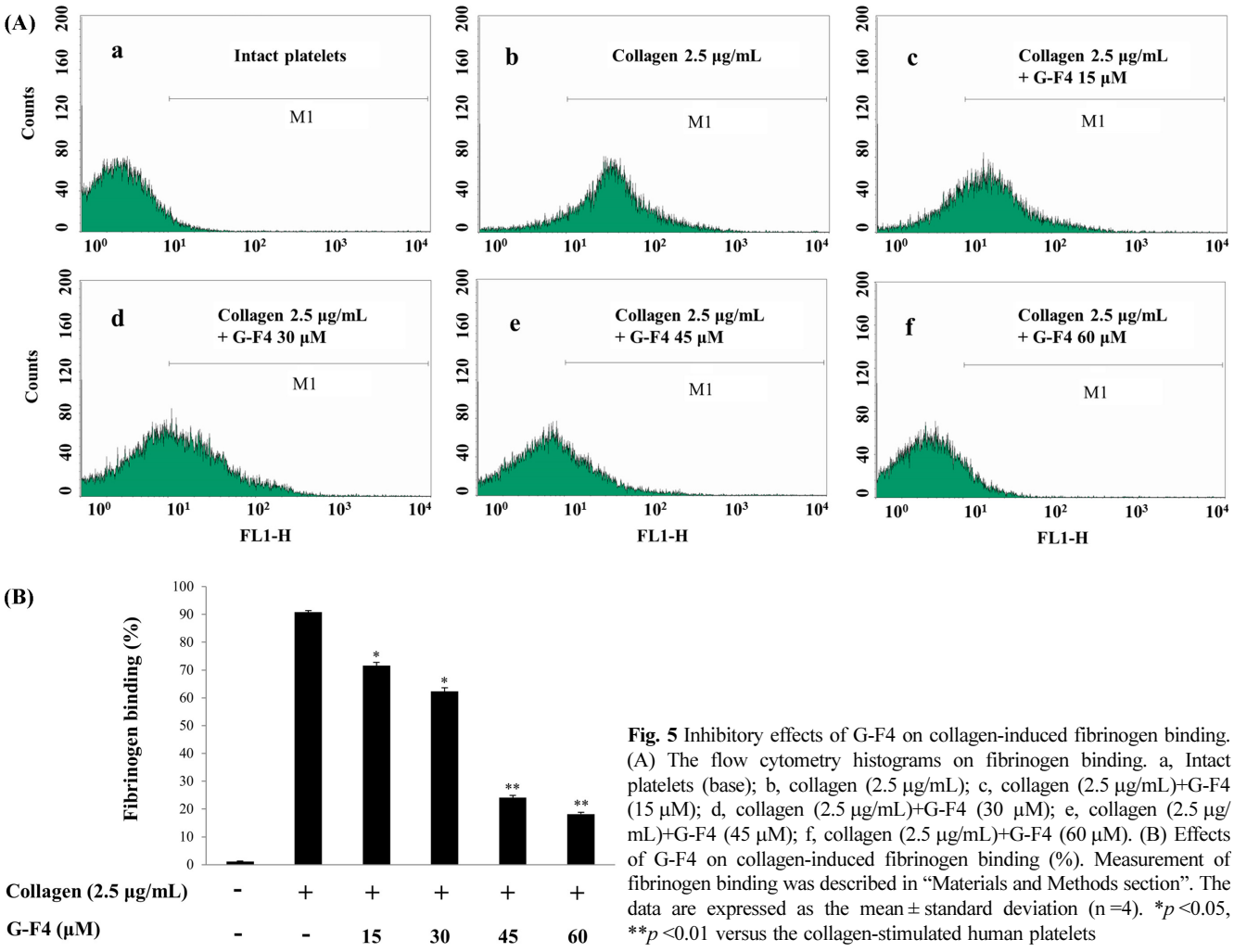

Fig. 5 Inhibitory effects of G-F4 on collagen-induced fibrinogen binding. (A) The flow cytometry histograms on fibrinogen binding. a, Intact platelets (base); b, collagen (2.5 μg/mL); c, collagen (2.5 μg/mL)+G-F4 (15 μM); d, collagen (2.5 μg/mL)+G-F4 (30 μM); e, collagen (2.5 μg/mL)+G-F4 (45 μM); f, collagen (2.5 μg/mL)+G-F4 (60 μM). (B) Effects of G-F4 on collagen-induced fibrinogen binding (%). Measurement of fibrinogen binding was 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

same inhibition pattern. G-F4 inhibited collagen-induced fibrinogen binding to αIIb/β3 and this result is mediated by increased VASP (Ser^{157}) phosphorylation.

Calpain is a calcium-dependent cysteine protease, which consists of calpain-1 and calpain-2 in platelets. Various calpain substrates have been identified in platelets such as Src, Syk, FAK, SHP-1, PTP1B, and inositol-4-phosphatase. These substrates consist of protein kinase or phosphatase; thus, calpain may regulate the balance between kinase and phosphatase activities [24]. Particularly, calpain has been implicated in the modulation of αIIb/β3-mediated outside-in signaling pathway. The platelets from calpain-1 knockout mice are defective in platelet aggregation in response to a number of agonists such as ADP, thrombin, and collagen [25]. Integrin αIIb/β3-mediated signaling generally results in the modification of the platelet cytoskeleton, therefore affecting platelet spreading and clot retraction. The clot retraction is the most important step during the repair of the damaged portion of the blood vessel. The damaged blood vessel accumulates activated platelets which develop into a fibrin-platelet meshwork. The clot formation that seals off the damaged vessel starts to retract during a time frame of around 30 to 60 minutes and pulls the cut edges together. The interaction between αIIb/β3 and fibrin is a key role for the clot formation and the αIIb/β3 inhibitors strongly suppress the clot retraction [26]. Our results showed that G-F4 decreased $[Ca^{2+}]_i$ through elevation of IP_3RI (Ser^{1756}) phosphorylation and inhibited $[Ca^{2+}]_i$ may be influenced by calcium-dependent cysteine protease. Therefore, G-F4 suppressed collagen-induced fibrinogen binding to αIIb/β3 and thrombin-stimulated clot retraction together with VASP (Ser^{157}) phosphorylation.

We previously reported that synthetic ginsenoside compounds Rp1 (G-Rp1), Rp3 (G-Rp3), Rp4 (G-Rp4), and natural compounds from Panax ginseng 20(S)-Rg3 (G-Rg3), Ro (G-Ro), have anti-platelet effects [17]. G-Rp1 has two glucose residues connected at carbon-3 position (Fig. 7A), and 20 μM of G-Rp1 fully inhibited rat platelet aggregation. G-Rp3 also has two glucose residues at carbon-6 position (Fig. 7B), and 50 μM of Rp3 shows maximal inhibitory effect on rat and human platelets. G-Rp4 has one glucose residue at carbon-6 position (Fig. 7C) which is structurally similar with G-Rp3. 50 μM of G-Rp3 showed inhibitory effect in rat platelets. In addition, 300 μM of G-Rg3 (Fig. 7D) and G-Ro

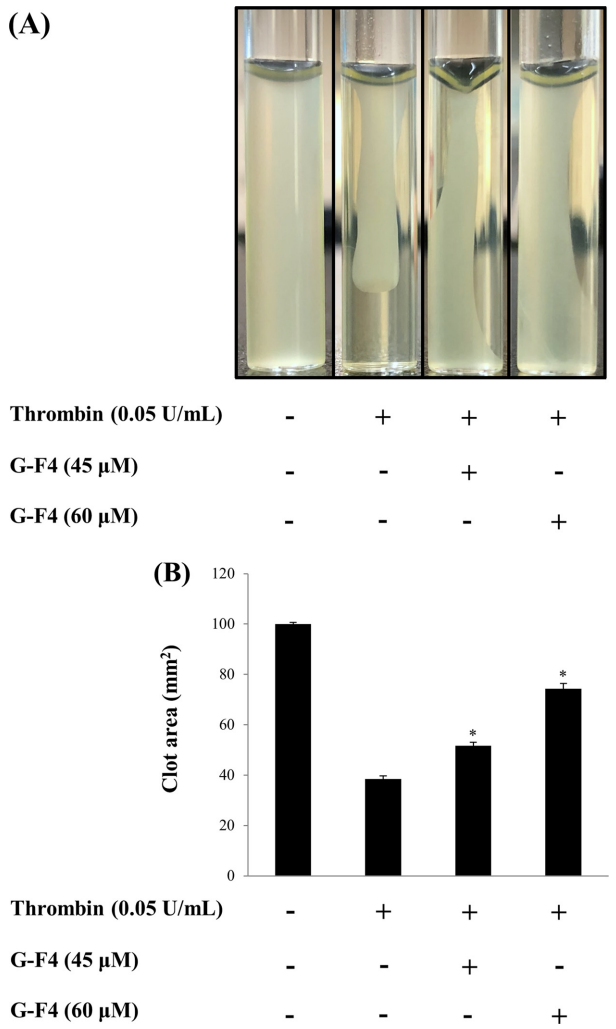

Fig. 6 Inhibitory effects of G-F4 on fibrin clot retraction. (A) Effects of G-F4 on thrombin-retracted fibrin clot photographs (B) Effects of G-F4 on thrombin-retracted fibrin clot area. Quantification of fibrin clot retraction was described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n =4). *p <0.05 versus the thrombin-stimulated platelets

(Fig. 7E) inhibited thrombin-induced human platelets. The structure of G-F4 is similar with G-Rp3 and G-Rp4 which has sugar residue at carbon-6 position. However, G-F1 do not have any sugar residue at carbon-3 and carbon-6 position, and G-F2 is connected one glucose residue at carbon-3 position, these did not inhibit on collagen-induced human platelets. Considering these structural differences, it is expected that the structure of ginsenoside, which have any sugar residue on carbon-6 position or two or more sugars on carbon-3 position, may be related to the specific ligands or integrins of platelets. In order to confirm this accurately, it is necessary to perform molecular docking analysis in the future.

In conclusion, we found G-F4 inhibited calcium mobilization, fibrinogen-binding to αIIb/β_3, and clot retraction, which are mediated by the phosphorylation of IP_3RI (Ser^{1756}) and VASP (Ser^{157}). Therefore, we suggest that G-F4 is a novel compound for prevention of thrombosis and other platelet-mediated cardiovascular diseases by inhibiting intracellular calcium mobilization and αIIb/β_3 activation.

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Conflict of interest The authors declare no conflict of interest.

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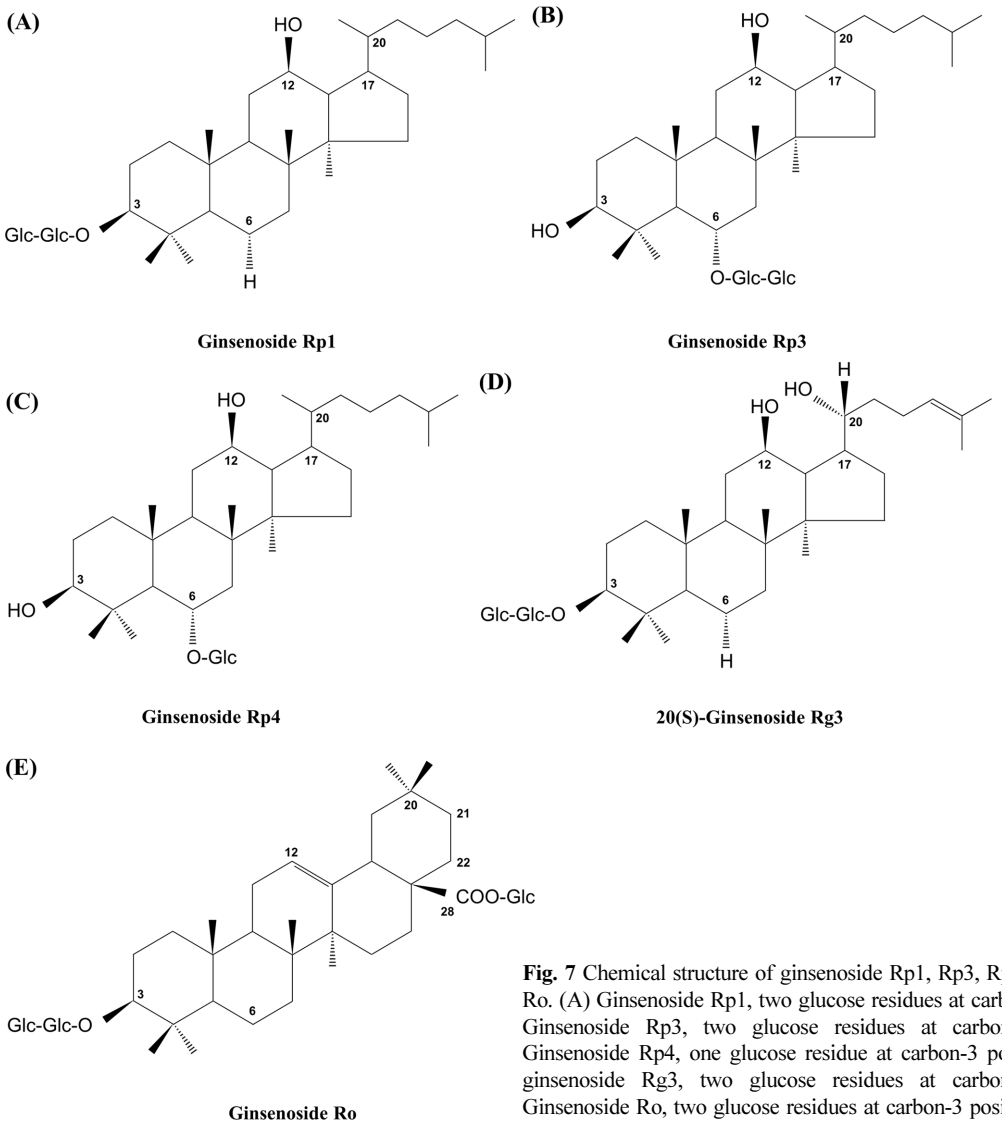

Fig. 7 Chemical structure of ginsenoside Rp1, Rp3, Rp4, 20(S)-Rg3 and Ro. (A) Ginsenoside Rp1, two glucose residues at carbon-3 position. (B) Ginsenoside Rp3, two glucose residues at carbon-6 position. (C) Ginsenoside Rp4, one glucose residue at carbon-3 position. (D) 20(S)-ginsenoside Rg3, two glucose residues at carbon-3 position. (E) Ginsenoside Ro, two glucose residues at carbon-3 position

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