



Research article

Total saponin from Korean Red Ginseng inhibits binding of adhesive proteins to glycoprotein IIb/IIIa via phosphorylation of VASP (Ser¹⁵⁷) and dephosphorylation of PI3K and Akt



Hyuk-Woo Kwon^{1,☆}, Jung-Hae Shin^{1,☆}, Hyun-Jeong Cho², Man Hee Rhee³,
Hwa-Jin Park^{1,*}

¹ Department of Biomedical Laboratory Science, College of Biomedical Science and Engineering, Inje University, Gyungnam, Republic of Korea

² Department of Biomedical Laboratory Science, College of Medical Science, Konyang University, Daejeon, Republic of Korea

³ Laboratory of Veterinary Physiology and Signaling, College of Veterinary Medicine, Kyungpook National University, Daegu, Republic of Korea

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ABSTRACT

Background: Binding of adhesive proteins (i.e., fibrinogen, fibronectin, vitronectin) to platelet integrin glycoprotein IIb/IIIa (α IIb/ β ₃) by various agonists (thrombin, collagen, adenosine diphosphate) involve in strength of thrombus. This study was carried out to evaluate the antiplatelet effect of total saponin from Korean Red Ginseng (KRG-TS) by investigating whether KRG-TS inhibits thrombin-induced binding of fibrinogen and fibronectin to α IIb/ β ₃.

Methods: We investigated the effect of KRG-TS on phosphorylation of vasodilator-stimulated phosphoprotein (VASP) and dephosphorylation of phosphatidylinositol 3-kinase (PI3K) and Akt, affecting binding of fibrinogen and fibronectin to α IIb/ β ₃, and clot retraction.

Results: KRG-TS had an antiplatelet effect by inhibiting the binding of fibrinogen and fibronectin to α IIb/ β ₃ via phosphorylation of VASP (Ser¹⁵⁷), and dephosphorylation of PI3K and Akt on thrombin-induced platelet aggregation. Moreover, A-kinase inhibitor Rp-8-Br-cyclic adenosine monophosphates (cAMPs) reduced KRG-TS-increased VASP (Ser¹⁵⁷) phosphorylation, and increased KRG-TS-inhibited fibrinogen-, and fibronectin-binding to α IIb/ β ₃. These findings indicate that KRG-TS interferes with the binding of fibrinogen and fibronectin to α IIb/ β ₃ via cAMP-dependent phosphorylation of VASP (Ser¹⁵⁷). In addition, KRG-TS decreased the rate of clot retraction, reflecting inhibition of α IIb/ β ₃ activation. In this study, we clarified ginsenoside Ro (G-Ro) in KRG-TS inhibited thrombin-induced platelet aggregation via both inhibition of [Ca²⁺]_i mobilization and increase of cAMP production.

Conclusion: These results strongly indicate that KRG-TS is a beneficial herbal substance inhibiting fibrinogen-, and fibronectin-binding to α IIb/ β ₃, and clot retraction, and may prevent platelet α IIb/ β ₃-mediated thrombotic disease. In addition, we demonstrate that G-Ro is a novel compound with antiplatelet characteristics of KRG-TS.

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1. Introduction

Platelet aggregation is caused by “inside-out signaling” and “outside-in signaling”, which is absolutely essential for the formation of a hemostatic plug when normal blood vessels are injured.

However, platelet aggregation can also cause circulatory disorders, such as thrombosis, atherosclerosis, and myocardial infarction [1].

Various agonists [i.e., collagen, thrombin, adenosine diphosphate (ADP)] induce “inside-out signaling” to bind adhesive proteins (i.e., fibrinogen, fibronectin, vitronectin) to glycoprotein IIb/

* Corresponding author. Department of Biomedical Laboratory Science, College of Biomedical Science and Engineering, Inje University, 197, Inje-ro, Gimhae, Gyungnam 621-749, Republic of Korea.

E-mail address: mhsjpark@inje.ac.kr (H.J. Park).

☆ These two authors contributed equally to this work.

IIIa (α IIb/ β ₃) and platelet membrane integrin, then “outside-in signaling” is subsequently processed to cause a series of signal events (i.e., Ca^{2+} mobilization, granule secretion, clot retraction) [2–4]. Through these signaling events, intravascular platelets finally are aggregated at the site of vascular wall injury, which contributes to intensity of the formation of thrombus.

Vasodilator-stimulated phosphoprotein (VASP) phosphorylation inhibits VASP affinity for contractile protein filamentous actin, and fibrinogen binding to α IIb/ β ₃ to inhibit the final common pathway for platelet aggregation [5,6]. On the contrary, phosphatidylinositol 3-kinase (PI3K)/Akt phosphorylation involves α IIb/ β ₃ activation [7]. Therefore, the upregulation of VASP phosphorylation and the downregulation of PI3K/Akt phosphorylation are paramount important indexes to understand α IIb/ β ₃ activation, and are very useful for evaluating the antiplatelet effect of substances or compounds. For instance, a major catechin analogue, (-)-epigallocatechin-3-gallate from green tea, and caffeic acid are known to produce cAMP via adenylate cyclase activation and subsequently phosphorylates VASP (Ser¹⁵⁷) to inhibit [Ca^{2+}]_i mobilization [8,9]. In addition, abciximab, etifibatide, tirofiban, and lamifiban are known as α IIb/ β ₃ antagonists [10,11].

Ginseng, the root of *Panax ginseng* Meyer, has been used frequently in traditional Oriental medicine, and is known to have various pharmacological activities such as anti-inflammatory action, antioxidation, antitumor, antidiabetes, and antihepatotoxicity effects [12,13]. It was recently reported that Korean Red Ginseng has an effect on cardiovascular disease, which is characterized with regard to reduction of blood pressure and arterial stiffness by inhibition of Rho kinase [14], anticoagulation by prolonged prothrombin and activated partial thromboplastin time [15], endothelium relaxation by nitric oxide-cyclic guanosine monophosphate (cGMP) pathway [16], and inhibition of hypercholesterolemia-induced platelet aggregation [17]. In our previous report, we demonstrated that total saponin from Korean Red Ginseng (KRG-TS) is a beneficial traditional Oriental medicine in platelet-mediated thrombotic disease via suppression of cyclooxygenase-1 (COX-1) and thromboxane A₂ (TXA₂) synthase to inhibit production of thromboxane A₂ [18]. In addition, KRG-TS is involved in increase of cAMP levels and subsequent reduction of [Ca^{2+}]_i mobilization in thrombin-induced rat platelet aggregation [19]. With regard to the effects of ginsenosides on platelet aggregation, it is well known that ginsenoside Rg3 (G-Rg3) and its chemical derivatives (dihydroxyginsenoside Rg3, ginsenoside Rp1) have antiplatelet effects by regulating the aggregation-inhibiting molecule cAMP, and aggregation-stimulating molecules [20,21]. In this study, we investigated the novel effects of KRG-TS on the phosphorylation of VASP and dephosphorylation of PI3K and Akt affecting on fibrinogen and fibronectin binding to α IIb/ β ₃. In addition, we found that ginsenoside Ro (G-Ro), an oleanane type saponin, in KRG-TS has a potent antiplatelet effect.

2. Materials and methods

KRG-TS was obtained from R&D Headquarter, Korea Ginseng Corporation (Daejeon, Korea). Ginsenoside Ro was purchased from Ambo Institute (Daejeon, Korea). Thrombin was purchased from Chrono-Log Corporation (Havertown, PA, USA). A CytoSelect 48-well cell adhesion assay kit (Fibronectin-Coated, Colorimetric Format) was purchased from Cell Biolabs (San Diego, CA, USA). A kinase inhibitor Rp-8-Br-cAMPS, G-kinase inhibitor Rp-8-Br-cGMPS, A-kinase activator 8-(4-chlorophenylthio)-cAMP (pCPT-cAMP), and G-kinase activator 8-Br-cGMP and 2-acetoxymethyl (Fura 2-AM) were obtained from Sigma Chemical Corporation (St. Louis, MO, USA). PI3K inhibitor wortmannin and cAMP enzyme immunoassay (EIA) kit were obtained from Cayman Chemical (Ann

Arbor, MI, USA). Anti-phosphor-VASP (Ser¹⁵⁷), anti-phosphor-VASP (Ser²³⁹), anti-PI3K, anti-phosphor-PI3K (Tyr⁴⁵⁸), anti-Akt, anti-phosphor-Akt (Ser⁴⁷³), and anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase conjugate (HRP), and lysis buffer were obtained from Cell Signaling (Beverly, MA, USA). α IIb/ β ₃ inhibitor eptifibatide, GR 144053, and anti- β -actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinylidene difluoride (PVDF) membrane was obtained from GE Healthcare (Piscataway, NJ, USA). Enhanced chemiluminescence solution (ECL) was obtained from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Fibrinogen Alexa Fluor 488 conjugate was obtained from Invitrogen Molecular Probes (Eugene, OR, USA).

2.1. Preparation of washed human platelets

Human platelet-rich plasma (PRP) anticoagulated with acid-citrate-dextrose solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose) was obtained from Korean Red Cross Blood Center (Changwon, Korea). The PRP was centrifuged for 10 min at 125 g to remove a few red blood cells, and was centrifuged for 10 min at 1,300 g to obtain the platelet pellets. The platelets were washed two times with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.36 mM NaH₂PO₄, 5.5 mM glucose, and 1 mM Na₂EDTA, pH 6.5). The washed platelets were then resuspended in suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.36 mM NaH₂PO₄, 0.49 mM MgCl₂, 5.5 mM glucose, 0.25% gelatin, pH 6.9) to a final concentration of 5×10^8 /mL. All of the aforementioned procedures were carried out at 25°C to avoid platelet aggregation from any effect of low temperatures. The Korea National Institute for the Bioethics Policy Public Institutional Review Board (Seoul, Korea) approved these experiments (PIRB12-072).

2.2. Measurement of platelet aggregation

Washed human platelets (10^8 /mL) were preincubated for 3 min at 37°C in the presence of 2mM exogenous CaCl₂ with or without substances, then stimulated with thrombin (0.05 U/mL) for 5 min. Aggregation was monitored using an aggregometer (Chrono-Log Corporation, Havertown, PA, USA) at a constant stirring speed of 1,000 rpm. Each aggregation rate was calculated as an increase in light transmission. The suspension buffer was used as the reference (transmission 0). KRG-TS was dissolved in the platelet suspension buffer (pH 6.9).

2.3. Western blot for analysis of VASP-, PI3K-, and Akt-phosphorylations

Washed human platelets (10^8 /mL) were preincubated with or without substances in the presence of 2mM CaCl₂ for 3 min and then stimulated with thrombin (0.05 U/mL) for 5 min at 37°C in an aggregometer at a constant stirring speed of 1,000 rpm. The reactions were terminated by adding an equal volume (250 μ L) of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1 mM serine/threonine phosphatase inhibitor β -glycerophosphate, 1 mM adenosine triphosphatase, alkaline and acid phosphatase, and protein phosphotyrosine phosphatase inhibitor Na₃VO₄, 1 μ g/mL serine and cysteine protease inhibitor leupeptin, and 1 mM serine protease and acetylcholinesterase inhibitor phenylmethanesulfonyl fluoride, pH 7.5). Platelet lysates containing the same protein (15 μ g) were used for the analysis. Protein concentrations were measured using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The effects of substances on VASP-, PI3K, and Akt-phosphorylation were analyzed using Western blotting. A 6-8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis

(1.5 mm gel) was used for electrophoresis and a PVDF membrane was used for protein transfer from the gel. The dilutions for anti-phosphor-VASP (Ser¹⁵⁷), anti-phosphor-VASP (Ser²³⁹), anti-PI3K, anti-phosphor-PI3K (Tyr⁴⁵⁸), anti-Akt, anti-phosphor-Akt (Ser⁴⁷³), and anti-rabbit IgG-HRP were 1:1,000, 1:1,000, 1:1,000, 1:1,000, 1:1,000, 1:1,000, and 1:10,000, respectively. The membranes were visualized using ECL. The blots were analyzed using the Quantity One, version 4.5 (BioRad, Hercules, CA, USA).

2.4. Determination of fibrinogen binding to α IIb/ β ₃

Washed human platelets (10⁸/mL) were preincubated for 3 min at 37°C with or without substances in the presence of 2mM CaCl₂ and then stimulated with thrombin (0.05 U/mL) in the presence of Alexa Fluor 488-human fibrinogen (30 μ g/mL) for 5 min at 37°C. The reaction was stopped by the addition of 0.5% paraformaldehyde in phosphate buffered saline, and the samples were placed in the dark. Alexa Fluor 488-fibrinogen binding to platelets was determined using flow cytometry (BD Biosciences, San Jose, CA, USA) and fibrinogen binding to α IIb/ β ₃ was analyzed using cellQuest software (BD Biosciences, San Jose, CA, USA).

2.5. Determination of fibronectin adhesion

Adhesion assay was performed with the CytoSelect 48-well cell adhesion assay kit (Cell Biolabs, San Diego, CA, USA). The plates were coated with fibronectin or bovine serum albumin (BSA) as a negative control. Washed human platelets (10⁸/mL) were incubated for 60 min at 37°C in the presence of thrombin (0.05 U/mL) with or without various concentrations of KRG-TS. After five times washing with phosphate buffered saline, cell stain solution was added and the plates were incubated at room temperature for 10 min. After washing five times, extraction solution was added and the plates were incubated for 10 min. Each sample was transferred to a 96-well microtiter plate and measured with Synergy HT Multi-Model Microplate Reader (BioTek Instruments, Winooski, VT, USA) at an optical density of 560 nm.

2.6. Assay of platelet-mediated fibrin clot retraction

Human PRP 250 μ L were transferred into a polyethylene tube to avoid clot adherence, then were preincubated with or without KRG-TS (150 μ g/mL) for 10 min at 37°C, and subsequently stimulated with thrombin (0.05 U/mL) for 5 min at 37°C. Pictures of fibrin clot were taken at 5 min using a digital camera, and its quantification was carried out by measurement of clot area using the Image J Software (version 1.46, National Institutes of Health, Bethesda, MD, USA). Percentage of clot retraction was calculated as follows:

$$\text{Retraction (\%)} \text{ by thrombin} = (\text{basal area} - \text{thrombin area}) / \text{basal area} \times 100.$$

2.7. Determination of cytosolic-free Ca²⁺ ([Ca²⁺]_i)

Human PRP was incubated with 5 μ M Fura 2-AM at 37°C for 60 min. Because Fura 2-AM is light sensitive, the tube containing the PRP was covered with aluminum foil during loading. The Fura 2-loaded washed platelets were prepared using the procedure described previously and platelets (10⁸/mL) were preincubated for 3 min at 37°C with or without G-Ro (200 μ M) in the presence of 2mM CaCl₂, then stimulated with thrombin (0.05 U/mL) for 5 min for evaluation of [Ca²⁺]_i. Fura 2 fluorescence was measured with a spectrofluorometer (SFM 25, BioTeck Instrument, Milan, Italy) with

an excitation wavelength that was changed every 0.5 s from 340 nm to 380 nm; the emission wavelength was set at 510 nm. The [Ca²⁺]_i values were calculated using the method of Grynkiewicz et al [22].

2.8. Measurement of cAMP

Washed human platelets (10⁸/mL) were preincubated for 3 min at 37°C with or without G-Ro (200 μ M) in the presence of 2 mM CaCl₂, then stimulated with thrombin (0.05 U/mL) for 5 min for platelet aggregation. The aggregation was terminated by the addition of 80% ice-cold ethanol. cAMP was measured with Synergy HT Multi-Model Microplate Reader (BioTek Instruments) using a cAMP EIA kit.

2.9. Statistical analyses

The experimental results are expressed as the mean \pm standard deviation accompanied by the number of observations. Data were assessed by analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared by the Newman-Keuls method. Statistical analysis was performed according to the SPSS 21.0.0.0 (SPSS Inc., Chicago, IL, USA). A *p* value < 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of KRG-TS on thrombin-induced human platelet aggregation

The concentration of thrombin-induced maximal human platelet aggregation was \sim 0.05 U/mL (Fig. 1A). Therefore, thrombin (0.05 U/mL) was used as the human platelet agonist in this study. In intact platelets, the light transmission in response to various concentrations of KRG-TS (25 μ g/mL, 50 μ g/mL, 100 μ g/mL, 150 μ g/mL) was 1.3 \pm 0.6% (at 25 μ g/mL of KRG-TS), 1.3 \pm 0.6% (at 50 μ g/mL of KRG-TS), 1.3 \pm 0.6% (at 100 μ g/mL of KRG-TS), and 1.3 \pm 0.6% (at 150 μ g/mL of KRG-TS), respectively, which were not significantly different from that (1.0 \pm 0.0%) in resting platelets without KRG-TS (Fig. 1B). When washed human platelets (10⁸/mL) were activated with thrombin, the aggregation rate was increased up to 87.8 \pm 5.7%. However, various concentrations of KRG-TS (25–150 μ g/mL) significantly reduced thrombin-stimulated platelet aggregation in a dose-dependent manner (Figs. 1B, C), and the half-maximal inhibitory concentration (IC₅₀) was \sim 45 μ g/mL (Fig. 1D). This IC₅₀ is low as compared with that (81.1 μ g/mL) from rat platelets [19]. In addition, 150 μ g/mL of KRG-TS inhibited to 95.9% thrombin-induced human platelet aggregation (87.8 \pm 5.7%).

3.2. Effects of KRG-TS on VASP phosphorylation

Because 150 μ g/mL of KRG-TS (Fig. 1B) significantly inhibited thrombin-induced platelet aggregation, we used 150 μ g/mL of KRG-TS to evaluate its effect on various signaling molecules. KRG-TS activated the phosphorylation of VASP (Ser¹⁵⁷), an A-kinase substrate (Fig. 2A, lane 3). Next we investigated whether VASP (Ser¹⁵⁷) phosphorylation by KRG-TS is dependent on cAMP/A-kinase pathway. A-kinase inhibitor Rp-8-Br-cAMPS (Fig. 2A, lane 4) potentially decreased KRG-TS (150 μ g/mL)-induced VASP (Ser¹⁵⁷) phosphorylation. A-kinase activator pCPT-cAMP stimulated also VASP (Ser¹⁵⁷) phosphorylation, indicating dependence on cAMP (Fig. 2A, lane 5). Although G-kinase activator 8-Br-cGMP stimulated VASP (Ser²³⁹) phosphorylation, indicating dependence on cGMP (Fig. 2B, lane 5), KRG-TS (150 μ g/mL) did not increase the VASP (Ser²³⁹) phosphorylation (Fig. 2B, lane 3).

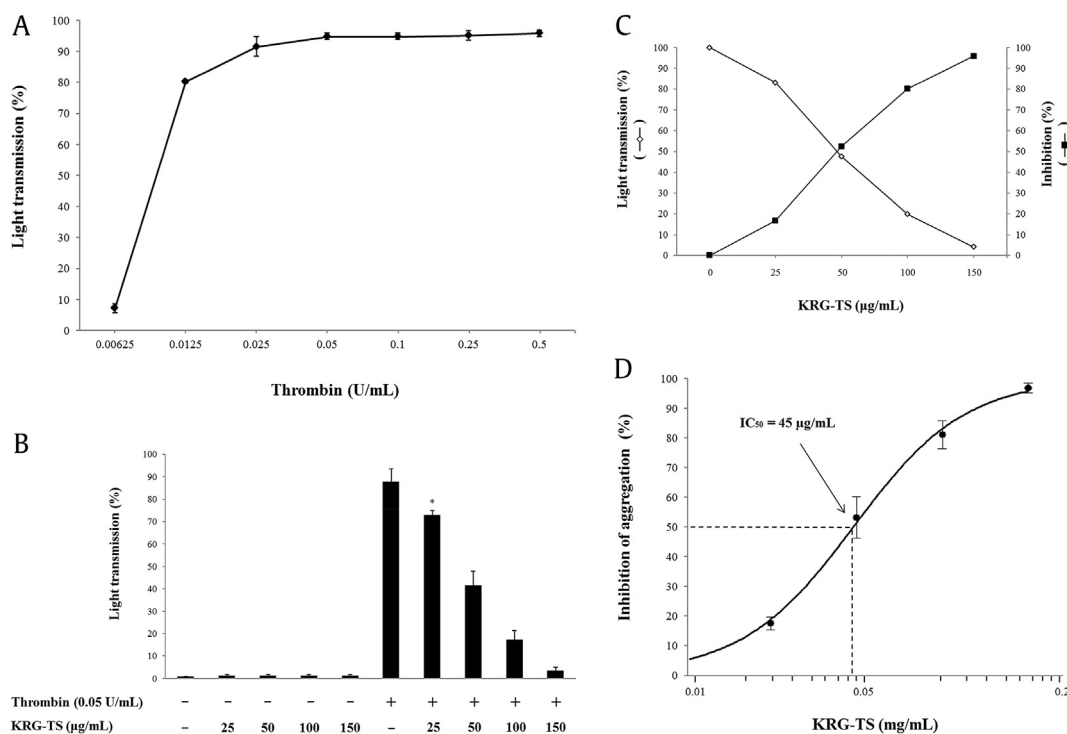


Fig. 1. Effects of total saponin Korean Red Ginseng (KRG-TS) on thrombin-induced human platelet aggregation. (A) The concentration threshold of thrombin on human platelet aggregation. (B) Effects of KRG-TS on thrombin-induced human platelet aggregation. (C) The inhibitory effects of KRG-TS on thrombin-induced human platelet aggregation. (D) The half maximal inhibitory concentration (IC_{50}) value of KRG-TS in thrombin-stimulated human platelet aggregation. Measurement of platelet aggregation was carried out as described in the *Materials and methods* section. The rate of inhibition by KRG-TS was recorded as the percentage of the thrombin induced aggregation rate. The IC_{50} value of KRG-TS was calculated according to the four-parameter log fit method. The data are expressed as the mean \pm standard deviation ($n = 4$). * $p < 0.05$ versus the thrombin-stimulated platelets.

3.3. Effects of KRG-TS on fibrinogen binding to $\alpha\text{IIb}/\beta_3$

Next, we investigated whether the VASP (Ser¹⁵⁷) phosphorylation by KRG-TS was involved in inhibition of fibrinogen binding to $\alpha\text{IIb}/\beta_3$. As shown in Fig. 3A, thrombin activated the fibrinogen binding to $\alpha\text{IIb}/\beta_3$ (Figs. 3A, B), and increased to $92.1 \pm 2.1\%$ fibrinogen binding to $\alpha\text{IIb}/\beta_3$ (Figs. 3A and B) from that ($1.7 \pm 0.2\%$) of intact platelets, control (Table 1). However, KRG-TS dose (25–150 $\mu\text{g}/\text{mL}$) dependently inhibited thrombin-activated fibrinogen binding to $\alpha\text{IIb}/\beta_3$ (Figs. 3A–c–f, 3B), and KRG-TS (150 $\mu\text{g}/\text{mL}$) inhibited to 91.6% fibrinogen binding as compared with that ($92.1 \pm 2.1\%$) by thrombin (Table 1). Although it is known that cAMP- and cGMP-increasing compounds are involved in inhibition of $\alpha\text{IIb}/\beta_3$ via cAMP/A-kinase, and cGMP/G-kinase pathway [23,24], in this study, because KRG-TS did not phosphorylate VASP (Ser²³⁹) (Fig. 2B), we investigated whether the inhibition of fibrinogen binding to $\alpha\text{IIb}/\beta_3$ by KRG-TS resulted from cAMP/A-kinase pathway. A-kinase activator pCPT-cAMP inhibited thrombin-induced fibrinogen binding to $\alpha\text{IIb}/\beta_3$ (Figs. 4A–b, B), which indicates that cAMP/A-kinase pathway involves inhibition of fibrinogen binding to $\alpha\text{IIb}/\beta_3$ in thrombin-induced platelet aggregation. KRG-TS (150 $\mu\text{g}/\text{mL}$)-inhibited fibrinogen binding to $\alpha\text{IIb}/\beta_3$ was elevated by A-kinase inhibitor Rp-8-Br-cAMPS (Figs. 4A–a, B), and its stimulatory degree was 350% as compared with that (7.7%) by both KRG-TS and thrombin (Table 1).

3.4. Effects of KRG-TS on PI3K/Akt phosphorylation

As apposed to the phosphorylated VASP, PI3K/Akt phosphorylation stimulates $\alpha\text{IIb}/\beta_3$ activation and fibrinogen binding [25,26]. Thus, we investigated the effect of KRG-TS on phosphorylation of PI3K and its downstream molecule Akt. Thrombin potently phosphorylated PI3K (Fig. 5, lane 2) as compared with that (Fig. 5, lane 1) of intact

platelets. However, KRG-TS (Fig. 5, lanes 3 and 4) inhibited thrombin-induced PI3K phosphorylation. Moreover, PI3K inhibitor wortmannin, negative control, suppressed thrombin-induced PI3K phosphorylation (Fig. 5, lane 5). Thrombin elevated the phosphorylation of PI3K target molecule Akt (Fig. 6, lane 2). However, KRG-TS inhibited thrombin-induced Akt phosphorylation (Fig. 6, lanes 3 and 4).

3.5. Effects of KRG-TS on adhesion to fibronectin

Integrin $\alpha\text{IIb}/\beta_3$ also served as a fibronectin receptor on platelets and occupancy of this receptor is essential for platelet adhesion. Therefore, we determined whether KRG-TS interferes with fibronectin binding to $\alpha\text{IIb}/\beta_3$. As shown in Fig. 7, thrombin did not adhere platelets to bovine serum albumin (BSA), negative control, but thrombin potently adhered platelets to fibronectin. These mean that thrombin induced fibronectin adhesion to $\alpha\text{IIb}/\beta_3$ of platelets, but not BSA (Fig. 7A). KRG-TS (25–150 $\mu\text{g}/\text{mL}$) dose dependently decreased thrombin-induced fibronectin adhesion, and $\alpha\text{IIb}/\beta_3$ inhibitors (eptifibatid and GR 144053) also inhibited thrombin-induced fibronectin adhesion. Next, we investigated whether fibronectin inhibition by KRG-TS was resulted from cAMP/A-kinase activation. A-kinase inhibitor Rp-8-Br-cAMPS increased KRG-TS-decreased fibronectin binding to $\alpha\text{IIb}/\beta_3$ (Fig. 7B). Moreover, A-kinase activator pCPT-cAMP inhibited thrombin-induced fibronectin binding to $\alpha\text{IIb}/\beta_3$ (Fig. 7B), which means that cAMP/A-kinase activation is involved in inhibition of fibronectin adhesion.

3.6. Effects of KRG-TS on retraction of fibrin clot

Activation of $\alpha\text{IIb}/\beta_3$ (inside-out signaling) by platelet agonists induces fibrinogen binding to $\alpha\text{IIb}/\beta_3$, and leads to outside-in $\alpha\text{IIb}/\beta_3$ signaling, containing clot retraction, platelet spreading, stable adhesion, granule secretion, protein phosphorylation, platelet

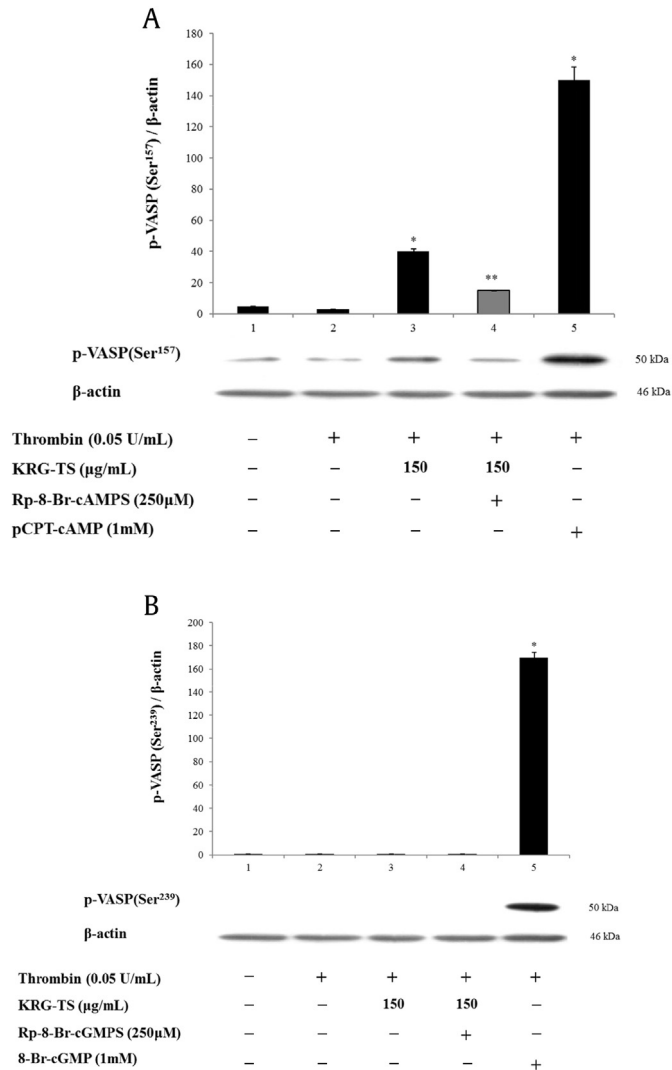


Fig. 2. Effects of total saponin Korean Red Ginseng (KRG-TS) on vasodilator-stimulated phosphoprotein (VASP) phosphorylation. (A) Effect of KRG-TS on VASP (Ser¹⁵⁷) phosphorylation. Lane 1, Intact platelets (base); Lane 2, Thrombin (0.05 U/mL); Lane 3, Thrombin (0.05 U/mL) + KRG-TS (150 µg/mL); Lane 4, Thrombin (0.05 U/mL) + KRG-TS (150 µg/mL) + Rp-8-Br-cAMPS (250 µM); and Lane 5, Thrombin (0.05 U/mL) + pCPT-cAMP (1mM). (B) Effect of KRG-TS on VASP (Ser²³⁹) phosphorylation. Lane 1, Intact platelets (base); Lane 2, Thrombin (0.05 U/mL); Lane 3, Thrombin (0.05 U/mL) + KRG-TS (150 µg/mL); Lane 4, Thrombin (0.05 U/mL) + KRG-TS (150 µg/mL) + Rp-8-Br-cGMPS (250µM); and Lane 5, Thrombin (0.05 U/mL) + 8-Br-cGMP (1mM). Western blotting was performed as described in the *Materials and methods* section. The data are expressed as the mean \pm standard deviation ($n = 4$). * $p < 0.05$ versus the thrombin-stimulated platelets. ** $p < 0.05$ versus the thrombin-stimulated platelets in the presence of KRG-TS (150 µg/mL). cAMP = cyclic adenosine monophosphate; cGMP = cyclic adenosine monophosphate; pCPT-cAMP = 8-(4-chlorophenylthio)-cAMP

contraction, $[Ca^{2+}]_i$ mobilization, and calpain activation [27–29]. Here, we investigated the effect of KRG-TS on retraction of thrombin-induced fibrin clot, an index of outside-in $\alpha IIb\beta_3$ signaling. Photographs in Fig. 8A show that thrombin stimulated the formation of fibrin clot, and the retraction of fibrin clot. The degree of retraction was determined from quantitation of fibrin clot area by Image J Software (version 1.46, National Institutes of Health, Bethesda, MD, USA). As shown in Fig. 8B, thrombin potently retracted fibrin clot, and increased to 60% the rate of clot retraction as compared with that without thrombin intact ($55 \pm 1.3 \text{ mm}^2$). However, KRG-TS suppressed thrombin-retracted fibrin clot, and its inhibitory degree was 82% as compared with that by thrombin ($22 \pm 1.1 \text{ mm}^2$; Fig. 8B).

3.7. Effects of G-Ro on platelet aggregation, $[Ca^{2+}]_i$ mobilization, and cAMP production

In our previous report [30], we showed that protopanaxadiol type saponins (G-Ra1, G-Rb1, G-Rb2, G-Rb3, G-Rc, G-Rd, G-Rh2), and protopanaxatriol type saponins (G-Re, G-Rf, G-Rg1, G-Rg2, G-Rh1) have no antiplatelet effect on thrombin-induced human platelet aggregation. In this study, however, we found that G-Ro, an oleanane type saponin, has a potent antiplatelet effect. When human platelets ($10^8/\text{mL}$) were stimulated by thrombin (0.05 U/mL), platelet aggregation was potently increased (Table 2). However, G-Ro inhibited thrombin-induced platelet aggregation, its inhibitory degree was 52.0% as compared with that by thrombin ($86.7 \pm 1.5\%$; Table 2). G-Ro increased to 89.1% the production of Ca^{2+} -antagonistic cAMP, and decreased to 77.3% the level of aggregating-molecule $[Ca^{2+}]_i$ in thrombin-induced platelet aggregation (Table 2).

4. Discussion

Of several aggregation inhibiting molecules, cAMP and cGMP are essential for inhibition of platelet aggregation. These cyclic nucleotides generate various biological functions (i.e., VASP phosphorylation, IP₃R phosphorylation) via cAMP/A-kinase- or cGMP/G-kinase-pathway.

A downstream pathway of both cAMP/A-kinase and cGMP/G-kinase is involved in VASP phosphorylation to inhibit $\alpha IIb\beta_3$ activity. Ser¹⁵⁷ at 50 kDa of VASP [VASP (Ser¹⁵⁷)] is phosphorylated by the cAMP/A-kinase pathway, whereas Ser²³⁹ at 50 kDa of VASP [VASP (Ser²³⁹)] is phosphorylated by the cGMP/G-kinase pathway [24,31]. KRG-TS markedly phosphorylated A-kinase substrate VASP (Ser¹⁵⁷), but did not phosphorylate G-kinase substrate VASP (Ser²³⁹) in thrombin-induced platelet aggregation. These results suggest that the VASP (Ser¹⁵⁷) phosphorylation by KRG-TS is possibly achieved by stimulating cAMP/A-kinase pathway, as evidenced by the fact that KRG-TS elevated cAMP only of cAMP and cGMP in thrombin-induced platelet aggregation [19]. Otherwise, A-kinase inhibitor Rp-8-Br-cAMPS would not decrease KRG-TS-elevated VASP (Ser¹⁵⁷) phosphorylation. In addition, A-kinase inhibitor Rp-8-Br-cAMPS increased KRG-TS-inhibited fibrinogen binding to $\alpha IIb\beta_3$. In this study, we have established that the inhibitory effect by KRG-TS on thrombin-induced $\alpha IIb\beta_3$ activation is due to cAMP/A-kinase-dependent VASP (Ser¹⁵⁷) phosphorylation, as evidenced by the fact that cAMP/A-kinase pathway involves suppression of $\alpha IIb\beta_3$ activation [3,4,32]. cAMP-elevating agents (i.e., cilostamide, cilostazole, and forskolin) are known to inhibit PI3K- and Akt-phosphorylation in thrombin-induced platelet aggregation [33]. Therefore, KRG-TS that increases cAMP level [19] may be involved in inhibition of PI3K/Akt phosphorylation, and subsequently could participate in suppression of $\alpha IIb\beta_3$ activation.

Platelet adhesion is the first step in the hemostatic response and various adhesive proteins such as von Willebrand factor, collagen, and fibronectin can mediate as substrates for platelet adhesion [34]. Fibronectin adhesion is mediated by both $\alpha_5\beta_1$ and $\alpha IIb\beta_3$, and subsequently is involved in platelet spreading, plug formation, and a series of events of outside-in signaling [34,35]. We clearly showed that KRG-TS strongly attenuated fibronectin binding to $\alpha IIb\beta_3$ in a cAMP/A-kinase-dependent manner, as verified that A-kinase inhibitor Rp-8-Br-cAMPS increased KRG-TS-reduced fibronectin binding in thrombin-induced platelet aggregation.

Fibrinogen binding to $\alpha IIb\beta_3$ is involved in fibrin clot retraction, which is an index of thrombus intensification [27], and is known to be involved in promotion of atheroprogession [36,37]. If so, inhibition of fibrinogen-, fibronectin-binding and clot retraction is a

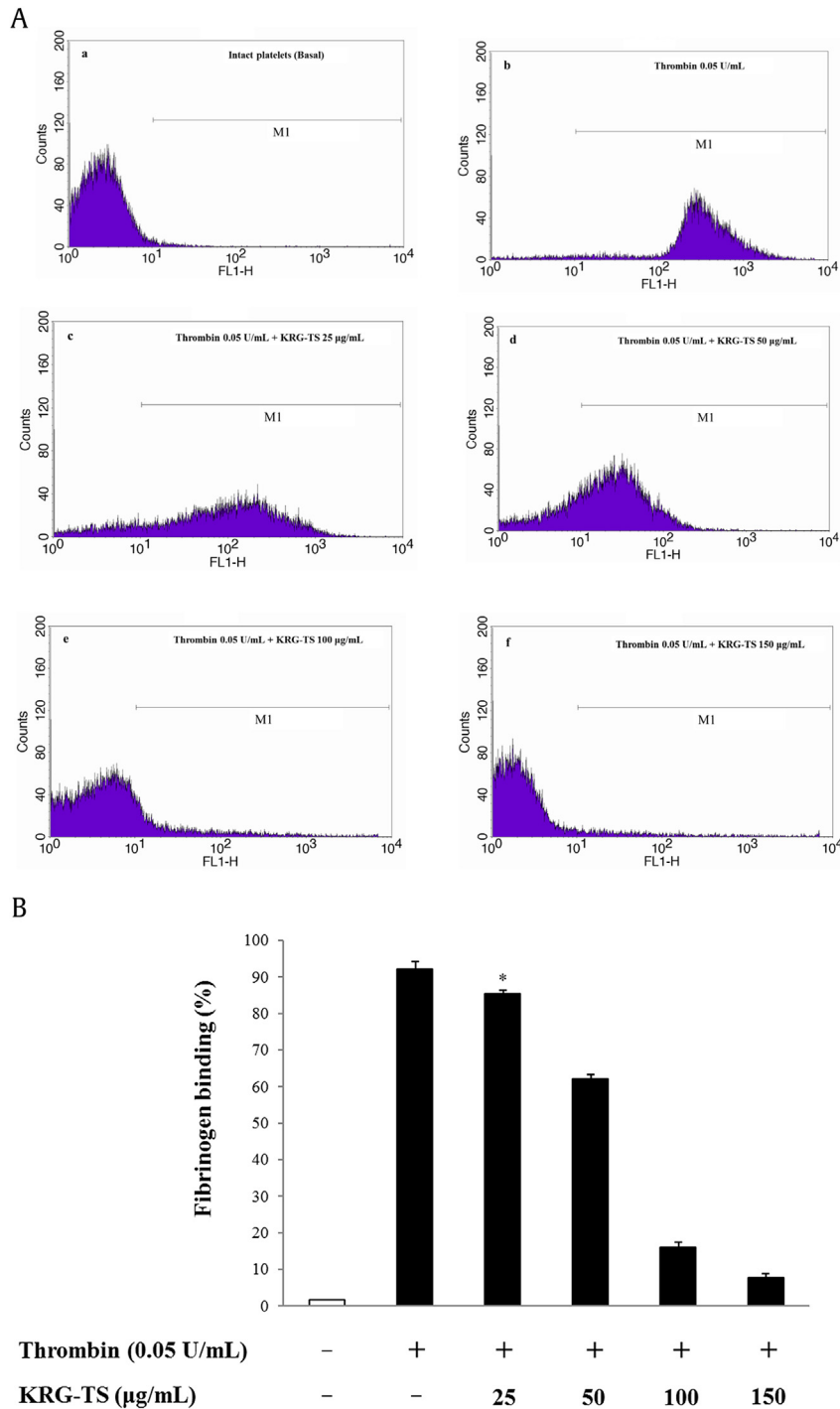


Fig. 3. Effects of total saponin Korean Red Ginseng (KRG-TS) on thrombin-induced fibrinogen binding. (A) The flow cytometry histograms on fibrinogen binding: a, intact platelets (base); b, thrombin (0.05 U/mL); c, thrombin (0.05 U/mL) + KRG-TS (25 µg/mL); d, thrombin (0.05 U/mL) + KRG-TS (50 µg/mL); e, thrombin (0.05 U/mL) + KRG-TS (100 µg/mL); and f, thrombin (0.05 U/mL) + KRG-TS (150 µg/mL). (B) Effects of KRG-TS on thrombin-induced fibrinogen binding (%). Determination of fibrinogen binding to $\alpha\text{IIb}/\beta_3$ was carried out as described in the *Materials and methods* section. The data are expressed as the mean \pm standard deviation ($n = 4$). * $p < 0.05$ versus the thrombin-stimulated platelets.

clear evidence that KRG-TS might protect platelet-mediated thrombotic disease.

Platelet aggregation is generated at the site of vascular wall injury, and is involved in the formation of thrombus. During the formation of thrombus, platelets release cell growth proteins such as platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) in their α -granule [38,39]. It is well established that PDGF and VEGF induce the proliferation of fibroblast,

vascular smooth cells, and epithelial cells, and subsequently enhance the rate of atherosclerosis lesion progression [40–44]. The progression of atherosclerosis is strongly induced by inflammatory cells such as monocyte/macrophage, and neutrophil [45]. Although KRG-TS has antiplatelet effects, if KRG-TS does not inhibit inflammation by leukocytes, the progression of atherosclerosis lesion would be generated at the site of vascular wall injury, and a question regarding antiplatelet effects of KRG-TS might be raised. Byeon

Table 1
Effects of Rp-8-Br-cAMPS on fibrinogen binding¹⁾

	Fibrinogen Binding (%)	Δ(%)
Intact platelets	1.7 ± 0.2	—
Thrombin (0.05 U/mL)	92.1 ± 2.1	—
KRG-TS (150 µg/mL) + Thrombin (0.05 U/mL)	7.7 ± 0.2	-91.6 ²⁾
KRG-TS (150 µg/mL) + Thrombin (0.05 U/mL) + Rp-8-Br-cAMPS (250µM)	34.8 ± 0.4	+350 ³⁾

cAMP, cyclic adenosine monophosphate; KRG-TS, total saponin Korean Red Ginseng

¹⁾ Data from Figs. 3B, 4B

²⁾ Δ (%) = [(KRG-TS + Thrombin) - Thrombin]/Thrombin × 100

³⁾ Δ (%) = [(KRG-TS + Thrombin + Rp-8-Br-cAMPS) - (KRG-TS + Thrombin)] / (KRG-TS + Thrombin) × 100

et al. [46] reported that saponin fraction inhibits lipopolysaccharide (LPS)-induced inflammation, and it is well reviewed that ginsenosides have anti-inflammatory effects by inhibiting the production of various pro-inflammatory mediators [i.e., prostaglandin E₂, nitric oxide (NO)] [47]. Recently, it was reported that Korean Red Ginseng saponin fraction downregulates LPS-induced proinflammatory mediators (i.e., NO, interleukin-1β) [48]. In addition, Yang et al. [49] clarified that protopanaxadiol saponin fraction inhibits inflammatory actions via suppression of p38-, JNK2-, and TANK [tumor necrosis factor receptor-associated factor (TRAF) family member-associated nuclear factor-κ-B activator]-binding kinase-1-linked pathway and their corresponding transcription factors (i.e., activation transcription factor 2, interferon regulatory transcription factor 3). Considering these previous reports [46–49], it is thought that KRG-TS may have antithrombotic, and antiatherosclerotic effects without generation of inflammation and progression of atherosclerotic lesion at the site of vascular wall injury. Therefore, KRG-TS is highlighted as a nontoxic antiplatelet compound, and

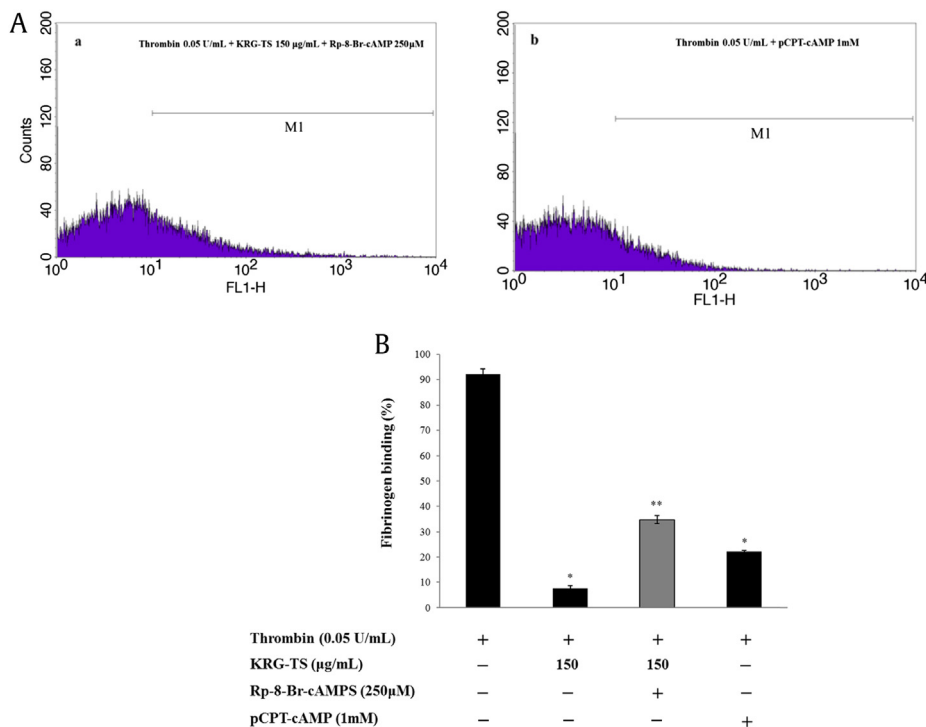


Fig. 4. Effects of total saponin Korean Red Ginseng (KRG-TS) on thrombin-induced fibrinogen binding in the presence of A-kinase inhibitor (Rp-8-Br-cAMPS). (A) The flow cytometry histograms on fibrinogen binding: a, thrombin (0.05 U/mL) + KRG-TS (150 µg/mL) + Rp-8-Br-cAMPS (250µM) and b, thrombin (0.05 U/mL) + pCPT-cAMP (1mM). (B) Effects of KRG-TS on thrombin-induced fibrinogen binding (%). Determination of fibrinogen binding to αIIb/β₃ was carried out as described in the *Materials and methods* section. The data are expressed as the mean ± standard deviation (n = 4). * p < 0.05 versus the thrombin-stimulated platelets. ** p < 0.05 versus the thrombin-stimulated platelets in the presence of KRG-TS (150 µg/mL). cAMP = cyclic adenosine monophosphate; pCPT-cAMP = 8-(4-chlorophenylthio)-cAMP

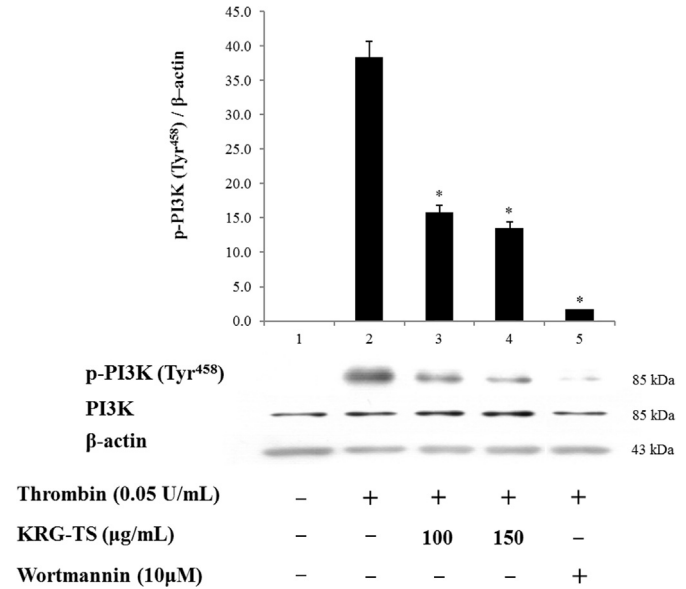


Fig. 5. Effects of total saponin Korean Red Ginseng (KRG-TS) on PI3K phosphorylation. Lane 1, Intact platelets (base). Lane 2, Thrombin (0.05 U/mL). Lane 3, Thrombin (0.05 U/mL) + KRG-TS (100 µg/mL). Lane 4, Thrombin (0.05 U/mL) + KRG-TS (150 µg/mL). Lane 5, Thrombin (0.05 U/mL) + wortmannin (10µM). Western blotting was performed as described in the *Materials and methods* section. The data are expressed as the mean ± standard deviation (n = 4). * p < 0.05 versus the thrombin-stimulated platelets.

could be clinically applied to the prevention of platelet-mediated thrombosis. This is supported from reports that Korean Red Ginseng has protective effects on rat carotid artery thrombosis *in vivo* [50], and both ginseng and ginsenoside are beneficial candidates for prevention of cardiovascular disease [51].

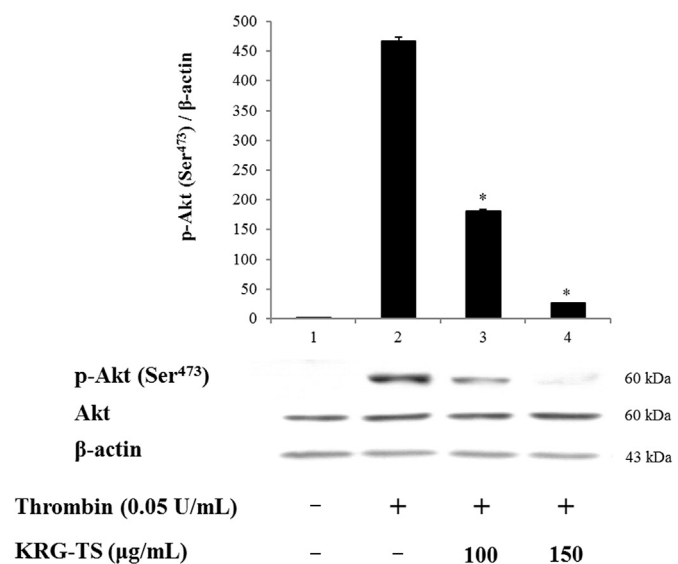


Fig. 6. Effects of total saponin Korean Red Ginseng (KRG-TS) on Akt phosphorylation. Lane 1, Intact platelets (base). Lane 2, Thrombin (0.05 U/mL). Lane 3, Thrombin (0.05 U/mL) + KRG-TS (100 μg/mL). Lane 4, Thrombin (0.05 U/mL) + KRG-TS (150 μg/mL). Western blotting was performed as described in the *Materials and methods* section. The data are expressed as the mean \pm standard deviation ($n = 4$). * $p < 0.05$ versus the thrombin-stimulated platelets.

Long-term (4–5 years) intake of red ginseng products (i.e., water extract, tea, drink) is known to inhibit platelet aggregation, blood coagulation, and hyperlipidemia such as indexes of thrombotic diseases and atherosclerosis, and their effects were also well sustained in the patients who have obesity, hyperlipidemia, and hypertension [52,53].

Hwang et al. [17] reported that dietary water-extract of Korean Red Ginseng (KRG-WE) inhibited collagen-induced rabbit platelet aggregation under hypercholesterolemia causing atherosclerosis such as cardiovascular disease, and KRG-WE had a strong antiplatelet effects as compared with those caused by lovastatin, an anticholesterolemic drug. Oral administration (250–500 μg/kg-body weight-rat) of KRG-WE significantly inhibited agonists (i.e., ADP, collagen)-induced platelet aggregation, and KRG-WE (300 μg/mL, 500 μg/mL) inhibited agonists (i.e., ADP, collagen, etc.)-induced washed rabbit platelet aggregation *in vitro* [49]. If these are physiological concentrations (300 μg/mL, 500 μg/mL) of KRG-WE to inhibit rat and rabbit platelet aggregation *in vitro* and *in vivo*, because it is thought that we used KRG-TS (150 μg/mL) is low concentration as compared with that of KRG-WE *in vitro* (300 μg/mL, 500 μg/mL), in this study, it is unknown whether *in vitro* KRG-TS (150 μg/mL)-mediated antiplatelet effects are also expressed *in vivo*. These should be studied in the future.

With regard to the G-Ro, Sanada et al. [54] reported that G-Ro is contained in *Panax ginseng*, Choi [55] reviewed that G-Ro (0.045 w/w %), and G-Rg3 (20S, 0.006 w/w %) and G-Rg3 (20R, 0.014 w/w %) are contained in *Panax ginseng*, but not in *Panax notoginseng* (Sanchi ginseng), and *Panax quinquefolius* (American ginseng). It is known that G-Ro (1mM) inhibited arachidonic acid-induced platelet aggregation and fibrin formation *in vitro* [56], and G-Ro (10–50 mg/kg body weight-rat) administration activated fibrinolysis, indicating the inhibition of fibrin thrombi [57]. Accordingly, when antiplatelet-mediated 200μM (about 191.4 mg/kg) of G-Ro (MW. 957.1) is administered to animals, it is unknown whether thrombin-induced platelet aggregation would also be inhibited *in vivo*. However, considering thrombin is involved in platelet aggregation and fibrin formation, it is thought that G-Ro 200μM (191.4 mg/kg)

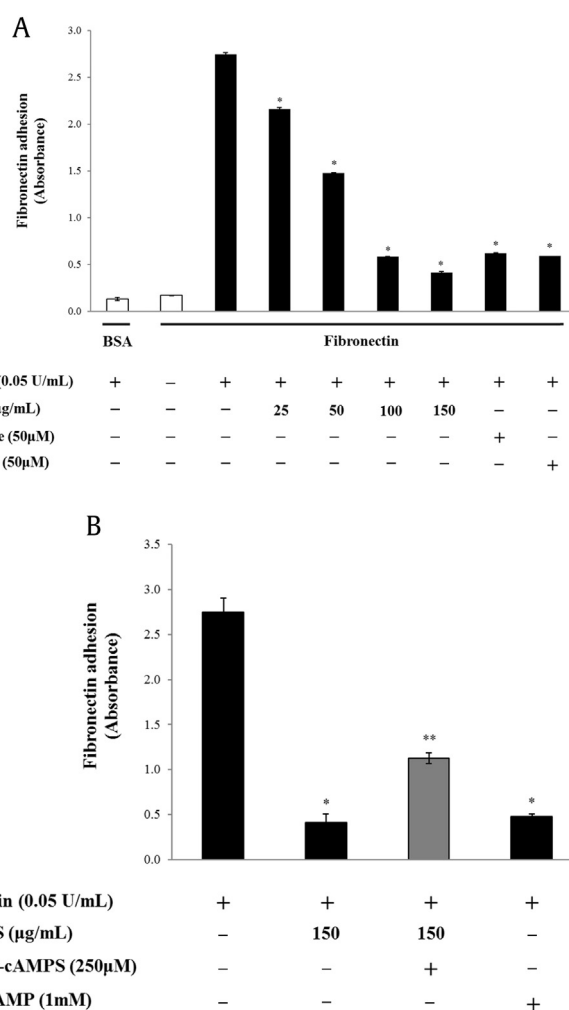


Fig. 7. Effects of total saponin Korean Red Ginseng (KRG-TS) on thrombin-induced fibronectin adhesion. (A) Inhibitory effects of KRG-TS on fibronectin adhesion. (B) Effects of KRG-TS on thrombin-induced fibronectin adhesion in the presence of A-kinase inhibitor (Rp-8-Br-cAMPS). Measurement of fibronectin adhesion was carried out as described in the *Materials and methods* section. BSA = bovine serum albumin (negative control). The data are expressed as the mean \pm standard deviation ($n = 4$). * $p < 0.05$ versus the thrombin-stimulated platelets. ** $p < 0.05$ versus the thrombin-stimulated platelets in the presence of KRG-TS (150 μg/mL).

would be involved in inhibition of platelet aggregation and fibrin formation. Currently, G-Ro is known to have anti-inflammatory effects [58]. If so, it is thought that G-Ro may also have a protective effect on thrombosis through inhibition of platelet aggregation and inflammation.

It is known that G-Ro does not inhibit collagen-induced $[Ca^{2+}]_i$ mobilization [59], however, in this study, we confirmed that G-Ro inhibits $[Ca^{2+}]_i$ mobilization by increasing Ca^{2+} -antagonistic cAMP in thrombin-induced platelet aggregation. Therefore, it is thought that G-Ro-elevated cAMP also may be involved in VASP (Ser¹⁵⁷) phosphorylation in the same manner that KRG-TS activated cAMP-dependent VASP (Ser¹⁵⁷) phosphorylation.

G-Rg3 (20S, 20R) is known to have antiplatelet effects by regulating various aggregating molecules [20,21]. Recently, we also reported that only G-Rg3 (20S, 20R) of protopanaxadiol saponin have inhibitory effects on thrombin-induced platelet aggregation [30]. Antiplatelet effects of G-Ro and G-Rg3 indicated that *Panax ginseng* would have an excellent antithrombotic effect by inhibiting platelet aggregation than that by any ginsengs, and G-Ro and G-Rg3 (20S, 20R) are major compounds in KRG-TS only of *Panax ginseng*.

Table 2
Effects of G-Ro on platelet aggregation, $[Ca^{2+}]_i$ mobilization, and cyclic adenosine monophosphate production in thrombin-induced human platelet activation

	Aggregation (%)	Δ (%)	cAMP (pmol/ 10^8 platelets)	Δ (%)	Ca^{2+} (nM)	Δ (%)
Basal (control)	-	—	4.8 ± 0.3	—	102.4 ± 0.5	—
Thrombin (0.05 U/mL)	86.7 ± 1.5	—	4.6 ± 0.1	0	655.7 ± 38.7	$+540.3^{2)}$
G-Ro (200 μ M) + Thrombin (0.05 U/mL)	$41.7 \pm 2.5^*$	$-52.0^{1)}$	$8.7 \pm 1.0^*$	$+89.1^{1)}$	$149.0 \pm 3.8^*$	$-77.3^{1)}$

The data are expressed as the mean \pm standard deviation ($n = 4$)

* $p < 0.05$ versus the thrombin-stimulated platelets

cAMP = cyclic adenosine monophosphate

¹⁾ Δ (%) = $[\text{Thrombin} - (\text{G-Ro} + \text{Thrombin})] / \text{Thrombin} \times 100$

²⁾ $(\text{Basal} - \text{Thrombin}) / \text{Basal} \times 100$

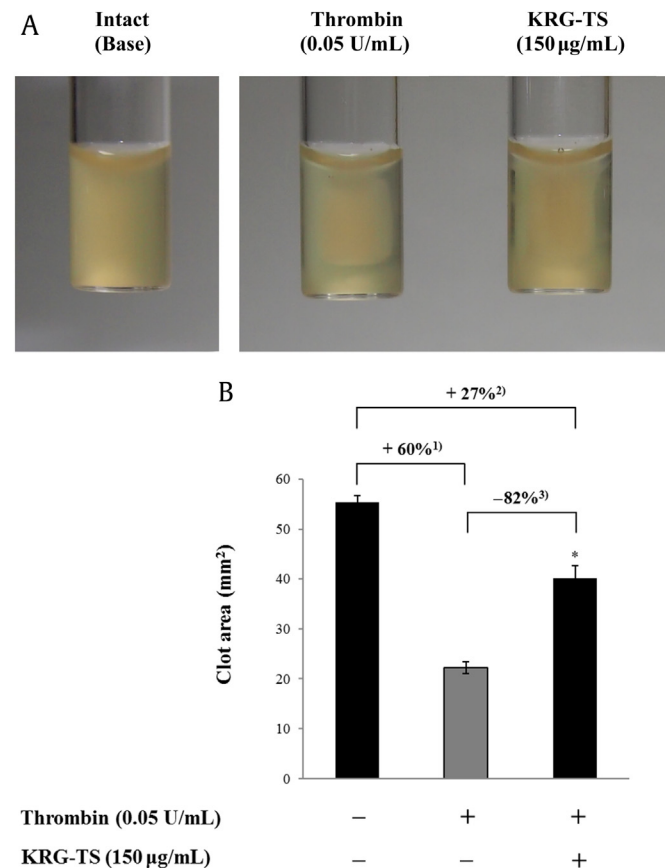


Fig. 8. Effects of total saponin Korean Red Ginseng (KRG-TS) on fibrin clot retraction. (A) Photographs of fibrin clot. (B) Effects of KRG-TS on thrombin-retracted fibrin clot. Quantification of fibrin clot retraction was performed as described in the *Materials and methods* section. (1) = $(\text{base} - \text{thrombin}) / \text{base} \times 100$, (2) = $[\text{base} - (\text{thrombin} + \text{KRG-TS})] / \text{base} \times 100$, (3) = $[\text{thrombin} - (\text{thrombin} + \text{KRG-TS})] / \text{thrombin} \times 100$. The data are expressed as the mean \pm standard deviation ($n = 4$).

* $p < 0.05$ versus the thrombin-stimulated platelets.

In conclusion, the most important result of this study is that KRG-TS significantly inhibits binding of adhesive proteins (i.e., fibrinogen, fibronectin) to α IIb/ β 3 by activating the phosphorylation of VASP (Ser¹⁵⁷) and dephosphorylation of PI3K and Akt, which contribute to inhibition of thrombotic formation. Therefore, we suggest that KRG-TS may be a physiologically effective negative regulator in thrombosis, atherosclerosis, and myocardial infarction via inhibition of platelet aggregation.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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