



Research article

Vasodilator-stimulated phosphoprotein-phosphorylation by ginsenoside Ro inhibits fibrinogen binding to α IIb/ β ₃ in thrombin-induced human platelets



Jung-Hae Shin^{1,☆}, Hyuk-Woo Kwon^{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, Korea

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

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

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ABSTRACT

Background: Glycoprotein IIb/IIIa (α IIb/ β ₃) is involved in platelet adhesion, and triggers a series of intracellular signaling cascades, leading to platelet shape change, granule secretion, and clot retraction. In this study, we evaluated the effect of ginsenoside Ro (G-Ro) on the binding of fibrinogen to α IIb/ β ₃. **Methods:** We investigated the effect of G-Ro on regulation of signaling molecules affecting the binding of fibrinogen to α IIb/ β ₃, and its final reaction, clot retraction.

Results: We found that G-Ro dose-dependently inhibited thrombin-induced platelet aggregation and attenuated the binding of fibrinogen to α IIb/ β ₃ by phosphorylating cyclic adenosine monophosphate (cAMP)-dependently vasodilator-stimulated phosphoprotein (VASP; Ser¹⁵⁷). In addition, G-Ro strongly abrogated the clot retraction reflecting the intensification of thrombus.

Conclusion: We demonstrate that G-Ro is a beneficial novel compound inhibiting α IIb/ β ₃-mediated fibrinogen binding, and may prevent platelet aggregation-mediated thrombotic disease.

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1. Introduction

Activation of platelets by various agonists (i.e., adenosine diphosphate, collagen, thrombin) causes shape change, granule secretion, and platelet aggregation. These signaling events are mediated by the activation of integrins such as glycoprotein IIb/IIIa (α IIb/ β ₃). Activated α IIb/ β ₃ interacts with its ligands (i.e., fibrinogen, fibronectin), then causes Ca²⁺ mobilization, granule secretion, and clot retraction [1–3], and subsequently augments the formation of thrombus.

Vasodilator-stimulated phosphoprotein (VASP) in platelets is associated with actin filament dynamics and focal adhesions, but its phosphorylated-forms (Ser¹⁵⁷, Ser²³⁹) weaken the affinity of VASP for actin filaments to block the binding of adhesive proteins to α IIb/ β ₃ [4,5]. Accordingly, phosphorylation of VASP could be used to appreciate the binding of adhesive proteins to α IIb/ β ₃, and

contribute to estimating the antithrombotic effect of a certain compound. For instance, antiplatelet compounds such as p-terphenyl curtsian E and quercetin lead to VASP phosphorylation [6,7]. In addition, abciximab, eptifibatid, tirofiban, and lamifiban are known to inhibit the activation of α IIb/ β ₃ [8,9].

Ginseng, the root of *Panax ginseng* Meyer, has been used frequently in traditional Oriental medicine. Ginsenoside Ro (G-Ro; Fig. 1), an oleanane-type saponin, in *P. ginseng* Meyer [10,11], is known to inhibit fibrin formation [12,13], and has no inhibitory effect on collagen-elevated platelet aggregation [14]. Until now, there has been no report on the antiplatelet mechanism of G-Ro. In this study, we found that G-Ro stimulates VASP (Ser¹⁵⁷) phosphorylation in a cyclic adenosine monophosphate (cAMP)-dependent manner, which attenuates the binding of fibrinogen to α IIb/ β ₃, and clot retraction in thrombin-activated human platelets.

* Corresponding author. Department of Biomedical Laboratory Science, College of Biomedical Science and Engineering, Inje University, 197, Inje-ro, Gimhae, Gyungnam 50834, Korea.

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

☆ These two authors contributed equally to this work.

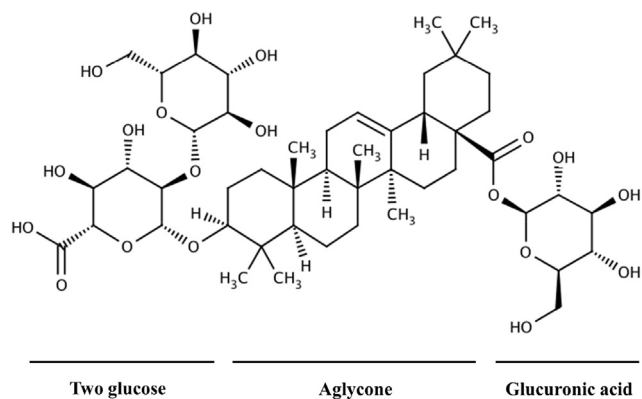


Fig. 1. Chemical structure of ginsenoside Ro. Ginsenoside Ro (G-Ro), an oleanane-type saponin, is contained in *Panax ginseng* Meyer [10,11], and is composed of oleanolic acid as aglycone, and two glucose and one glucuronic acid as sugar component [10].

2. Materials and methods

2.1. Materials

G-Ro was obtained from Ambo Institute (Daejeon, Korea). Thrombin was obtained from Chrono-Log Corporation (Havertown, PA, USA). Anti-VASP, anti-phosphor-VASP (Ser¹⁵⁷), anti-phosphor-VASP (Ser²³⁹), anti-rabbit IgG-HRP-horseradish peroxidase conjugate (HRP), and lysis buffer were purchased from Cell Signaling (Beverly, MA, USA). The α IIb/ β 3 inhibitor eptifibatide, GR 144053, and anti- β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinylidene difluoride membrane was purchased from GE Healthcare (Piscataway, NJ, USA). Enhanced chemiluminescence solution was purchased from GE Healthcare (Chalfont St. Giles, UK). cAMP and cyclic guanosine monophosphate (cGMP) enzyme immunoassay kits were purchased from Cayman Chemical (Ann Arbor, MI, USA). An A-kinase inhibitor Rp-8-Br-cAMPS, an A-kinase activator 8-(4-chlorophenylthio)-cAMP (pCPT-cAMP), and a G-kinase activator 8-Br-cGMP were purchased from Sigma Chemical Corporation (St. Louis, MO, USA). Fibrinogen Alexa Fluor 488 conjugate was obtained from Invitrogen Molecular Probes (Eugene, OR, USA).

2.2. Preparation of washed human platelets

Human platelet-rich plasma with acid-citrate-dextrose solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose) was supplied from Korean Red Cross Blood Center (Changwon, Korea). To remove red blood cells and white blood cells, it was centrifuged for 10 min at 250g and 10 min at 1,300g. The platelets were washed twice using washing buffer (138mM NaCl, 2.7mM KCl, 12mM NaHCO₃, 0.36mM NaH₂PO₄, 5.5mM glucose, and 1mM Na₂EDTA, pH 6.5), then resuspended in suspension buffer (138mM NaCl, 2.7mM KCl, 12mM NaHCO₃, 0.36mM NaH₂PO₄, 0.49mM MgCl₂, 5.5mM glucose, 0.25% gelatin, pH 6.9) to a final concentration of 5×10^8 /mL. All of the above procedures were performed at 25°C to maintain platelet activity. Approval (PIRB12-072) for these experiments was received from the National Institute for Bioethics Policy Public Institutional Review Board (Seoul, Korea).

2.3. Determination of platelet aggregation

Washed human platelets (10^8 /mL) were preincubated with or without G-Ro in the reaction system containing 2mM of CaCl₂ for 3 min at 37°C, then stimulated with thrombin (0.05 U/mL). The

aggregation was performed for 5 min using an aggregometer (Chrono-Log Corporation, Havertown, PA, USA) at a constant stirring speed of 1,000 rpm. Each aggregation rate was determined as an increase in light transmission. G-Ro was dissolved in platelet suspension buffer (pH 6.9), and suspension buffer was used as the reference (transmission 0)

2.4. Western blot for analysis of VASP-phosphorylation

The platelet aggregation was terminated by adding an equal volume (250 μ L) of lysis buffer (20mM Tris-HCl, 150mM NaCl, 1mM Na₂EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM serine/threonine phosphatase inhibitor β -glycerophosphate, 1mM ATPase, alkaline, and acid phosphatase, and protein phosphotyrosine phosphatase inhibitor Na₃VO₄, 1 μ g/mL serine and cysteine protease inhibitor leupeptin, and 1mM serine protease and acetylcholinesterase inhibitor phenylmethanesulfonyl fluoride, pH 7.5). Protein contents were measured using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Proteins (15 μ g) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (6%, 1.5 mm), then polyvinylidene difluoride membrane was used for protein transfer from the gel. The dilutions for anti-VASP, anti-phosphor-VASP (Ser¹⁵⁷), anti-phosphor-VASP (Ser²³⁹), and anti-rabbit IgG-HRP were 1:1,000, 1:1,000, 1:1,000, and 1:10,000, respectively. The membranes were visualized using enhanced chemiluminescence solution. The degrees of protein phosphorylation were analyzed using the Quantity One, version 4.5 (BioRad, Hercules, CA, USA).

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

The platelet aggregation was conducted 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 cold phosphate-buffered saline (pH 7.4), and the aforementioned procedures were implemented under dark conditions. The assay of fibrinogen binding was carried out using flow cytometry (BD Biosciences, San Jose, CA, USA), and its degree was determined with CellQuest software (BD Biosciences).

2.6. Assay of platelet-mediated fibrin clot retraction

Human platelet-rich plasma, 250 μ L, was preincubated with or without G-Ro (300 μ M) for 10 min at 37°C, and incubated with thrombin (0.05 U/mL) for 10 min and 20 min at 37°C. Photographs of the fibrin clot were taken by a digital camera, and its area (at 20 min) was measured by NIH Image J Software (version 1.46, National Institutes of Health, Bethesda, Maryland, USA). Percentage of clot retraction was calculated as follows:

$$\text{retraction (\% by thrombin)} = \frac{(\text{control area} - \text{thrombin area})}{\text{control area}} \times 100. \quad (1)$$

2.7. Measurement of cAMP and cGMP

After platelet aggregation, 80% ice cold ethanol was added to terminate the reaction, and cAMP and cGMP were extracted three times with 80% ice cold ethanol. The extracts tubes were dried by nitrogen gas, and subsequently dissolved with assay buffer (Cayman Chemical, Ann Arbor, MI, USA). The level of cAMP and

cGMP was determined with Synergy HT Multi-Model Microplate Reader (BioTek Instruments, Winooski, VT, USA).

2.8. Statistical analysis

The experimental results are indicated as the mean \pm standard deviation accompanied by the number of observations. Data were determined by analysis of variance. If this analysis showed significant differences among the group means, then each group was compared by the Newman-Keuls method. Statistical analysis was carried out 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 G-Ro on thrombin-induced human platelet aggregation

Because 0.05 U/mL of thrombin maximally aggregated human platelets [15], this concentration was used to investigate the anti-platelet effect of G-Ro (Fig. 1). In unstimulated platelets, the light transmission in response to various concentrations of G-Ro (50 μ M, 100 μ M, 200 μ M, 300 μ M) was $1.3 \pm 0.6\%$ (at 50 μ M of G-Ro), $1.7 \pm 0.6\%$ (at 100 μ M of G-Ro), $1.3 \pm 0.6\%$ (at 200 μ M of G-Ro), and $1.7 \pm 0.6\%$ (at 300 μ M of G-Ro), which were not significantly different from that ($1.0 \pm 0.0\%$) in resting platelets without G-Ro (Fig. 2). Thrombin increased light transmission and the aggregation rate was $90.7 \pm 1.2\%$ (Fig. 2). However, G-Ro dose-dependently (50 μ M, 100 μ M, 200 μ M, 300 μ M) reduced thrombin-elevated light transmission, meaning G-Ro inhibits thrombin-induced platelet aggregation (Fig. 2).

3.2. Effects of G-Ro on VASP phosphorylation

In intact platelets, 46 kDa dephosphoprotein only of VASP was observed (Fig. 3A, Lane 1). Thrombin weakly increased the phosphorylation of VASP (Ser¹⁵⁷) at 50 kDa phosphoprotein of VASP (Fig. 3A, Lane 2), and the ratio of p-VASP (Ser¹⁵⁷) to β -actin (Fig. 3B). This means that 46 kDa dephosphoprotein of VASP (46 kDa + 50 kDa) was weakly shifted to 50 kDa phosphoprotein by thrombin, and thrombin is involved in a feedback inhibition by elevating p-VASP (Ser¹⁵⁷, Ser²³⁹) [16]. Because G-Ro dose (100, 200, 300 μ M)-dependently inhibited up to $26.9 \pm 0.6\%$ (by 100 μ M G-Ro), $56.0 \pm 2.8\%$ (by 200 μ M G-Ro), and $88.4 \pm 1.7\%$ (by 300 μ M G-Ro) in thrombin-induced platelet aggregation (Fig. 2), we investigated whether G-Ro has dose (100, 200, 300 μ M)-dependent effects on VASP (Ser¹⁵⁷ or Ser²³⁹) phosphorylation in thrombin-activated

platelets. G-Ro dose-dependently increased p-VASP (Ser¹⁵⁷; Fig. 3A, Lanes 3–5), and the ratio of p-VASP (Ser¹⁵⁷)-50 kDa to β -actin in thrombin-activated platelets (Fig. 3B). However, G-Ro did not affect phosphorylation of VASP (Ser²³⁹; Fig. 3C), even though G-kinase activator 8-Br-cGMP, a positive control, phosphorylated VASP (Ser²³⁹; Fig. 3C, Lane 6). This reflects the result that G-Ro does not increase the cGMP level (Table 1), and subsequently involve in phosphorylation of VASP (Ser²³⁹) in thrombin-activated platelets. Because G-Ro increased the cAMP level (Table 1), it is thought that G-Ro-increased VASP (Ser¹⁵⁷) phosphorylation would be decreased by the A-kinase inhibitor. Accordingly, to observe an apparent inhibitory mechanism of cAMP/A-kinase on G-Ro-phosphorylated VASP (Ser¹⁵⁷), we examined the effect of the A-kinase inhibitor on VASP (Ser¹⁵⁷) phosphorylation by 300 μ M of G-Ro that potentially phosphorylated VASP (Ser¹⁵⁷; Fig. 3B). The A-kinase inhibitor Rp-8-Br-cAMPS (Fig. 3D, Lane 4) potentially decreased G-Ro (300 μ M)-phosphorylated VASP (Ser¹⁵⁷; Fig. 3D, Lane 3), and the A-kinase activator pCPT-cAMP, a positive control, also phosphorylated VASP (Ser¹⁵⁷; Fig. 3D, Lane 5). The results mean that G-Ro increases cAMP level (Table 1), and subsequently phosphorylates VASP (Ser¹⁵⁷) in thrombin-activated platelets.

3.3. Effects of G-Ro on the production of cAMP and cGMP

Because it is well established that cAMP and cGMP stimulate VASP phosphorylation [17,18], we investigated the effect of G-Ro on the production of cAMP and cGMP in thrombin-induced platelet aggregation. As shown in Table 1, G-Ro increased the cAMP level in thrombin-induced platelet aggregation, but did not increase the level of cGMP.

3.4. Effects of G-Ro on fibrinogen binding to α IIb/ β ₃

Because VASP (Ser¹⁵⁷) phosphorylation is involved in inhibition of fibrinogen binding, and G-Ro increased VASP (Ser¹⁵⁷) phosphorylation (Fig. 3A), it is thought that G-Ro may decrease fibrinogen binding to α IIb/ β ₃. G-Ro dose-dependently (100 μ M, 200 μ M, 300 μ M) activated the phosphorylation of VASP (Ser¹⁵⁷) in thrombin-activated platelets (Fig. 3B). Therefore, we investigated whether G-Ro has dose-dependent (100 μ M, 200 μ M, 300 μ M) inhibitory effects on fibrinogen binding to α IIb/ β ₃ in thrombin-activated platelets. Thrombin elevated fibrinogen binding to α IIb/ β ₃ (Figs. 4A–b, 4B) and its degree was $95.3 \pm 0.7\%$ (Table 2). However, G-Ro attenuated the fibrinogen binding achieved by thrombin in dose-dependent manner (Figs. 4A–c–f, 4B), and the inhibitory degree by G-Ro (300 μ M) was 88.9% (Table 2) as compared with that ($95.3 \pm 0.7\%$) by thrombin. α IIb/ β ₃ Inhibitors (eptifibatid, GR 144053), positive controls, inhibited thrombin-induced fibrinogen binding to α IIb/ β ₃ (Figs. 4A–g, –h, 4B). Their inhibitory degrees were $38.4 \pm 3.4\%$ (at eptifibatid 50 μ M) and $35.7 \pm 2.6\%$ (at GR144053 50 μ M), which were almost equal to that ($37.6 \pm 0.6\%$) by G-Ro (200 μ M; Fig. 4B). As G-Ro increased the cAMP level (Table 1) and VASP (Ser¹⁵⁷) phosphorylation (Fig. 3B), if the inhibition of fibrinogen binding by G-Ro (Fig. 4B) resulted from cAMP/A-kinase-mediated VASP (Ser¹⁵⁷) phosphorylation (Fig. 3B), it is thought that G-Ro-decreased fibrinogen binding would be increased by the A-kinase inhibitor. To confirm that cAMP/A-kinase had an inhibitory effect on G-Ro-blocked fibrinogen binding, we examined the effect of the A-kinase inhibitor on inhibition of fibrinogen binding by 300 μ M of G-Ro (Fig. 4B) that potentially inhibited fibrinogen binding. The A-kinase inhibitor Rp-8-Br-cAMPS increased G-Ro-inhibited fibrinogen binding to α IIb/ β ₃ in thrombin-activated platelets (Figs. 5A, 5B), and its degree was increased to 179.2% as compared with that ($10.6 \pm 1.3\%$) by G-Ro (300 μ M) plus thrombin (Table 2).

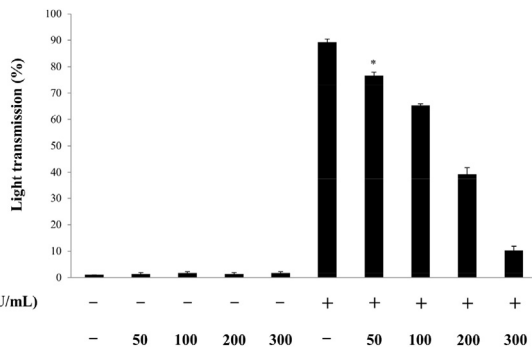


Fig. 2. Effects of ginsenoside Ro (G-Ro) on thrombin-induced human platelet aggregation. Measurement of platelet aggregation was carried out as described in the "Materials and methods" section. Data are presented as mean \pm SD ($n = 4$). * $p < 0.05$ versus the thrombin-stimulated human platelets.

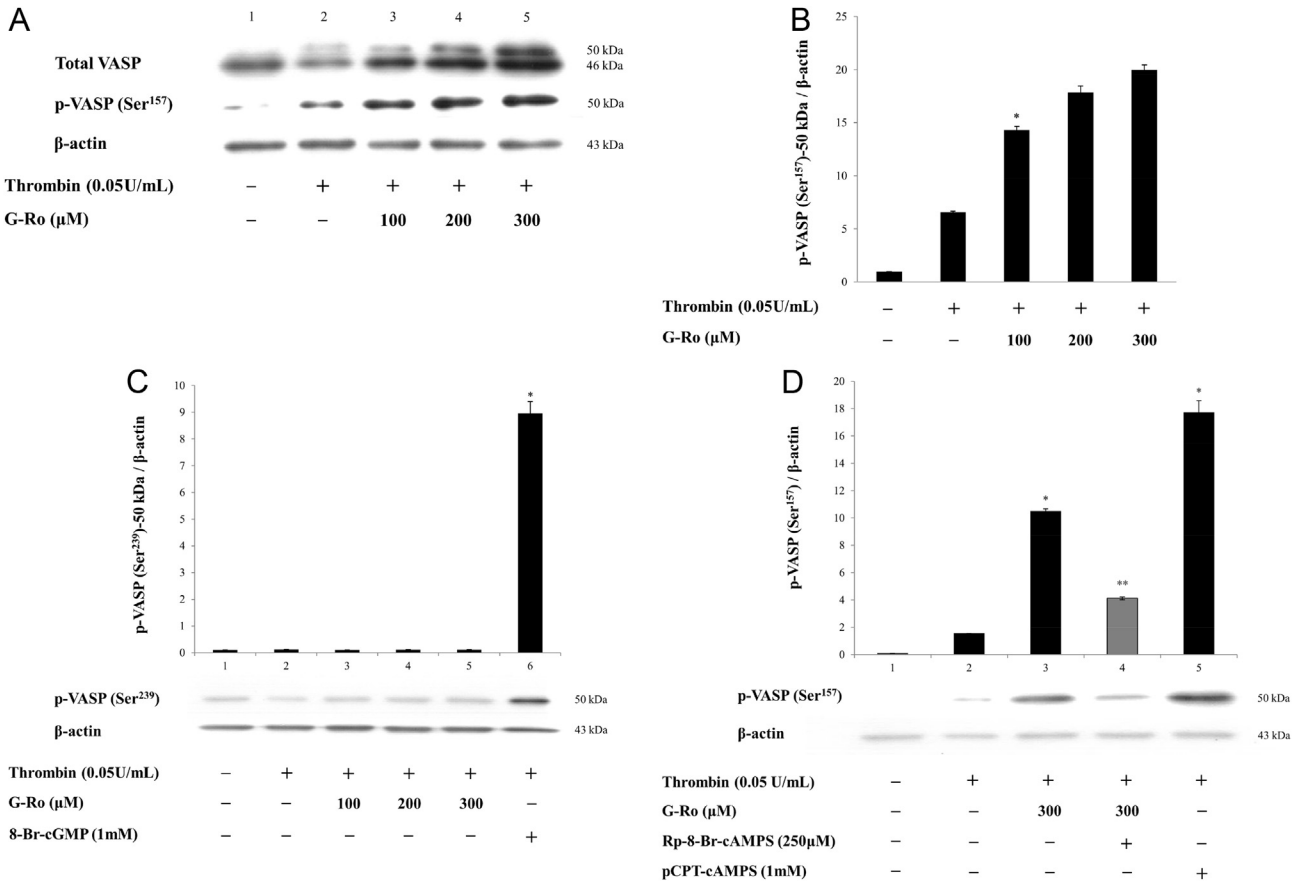


Fig. 3. Effects of ginsenoside Ro (G-Ro) on vasodilator-stimulated phosphoprotein (VASP) phosphorylation. (A) Effects of G-Ro on VASP phosphorylation. Lane 1, intact platelets (base); Lane 2, thrombin (0.05 U/mL); Lane 3, thrombin (0.05 U/mL) + G-Ro (100µM); Lane 4, thrombin (0.05 U/mL) + G-Ro (200µM); and Lane 5, thrombin (0.05 U/mL) + G-Ro (300µM). (B) The ratio of phosphorylation of VASP (Ser¹⁵⁷)-50 kDa to β-actin by G-Ro. Its ratio is from Fig. 3A. (C) Effects of G-Ro on VASP (Ser²³⁹)-50 kDa phosphorylation. Lane 1, intact platelets (base); Lane 2, thrombin (0.05 U/mL); Lane 3, thrombin (0.05 U/mL) + G-Ro (100 µM); Lane 4, thrombin (0.05 U/mL) + G-Ro (200µM); Lane 5, thrombin (0.05 U/mL) + G-Ro (300 µM); and Lane 6, thrombin (0.05 U/mL) + 8-Br-cGMP (1mM). (D) Effects of G-Ro on VASP (Ser¹⁵⁷)-50 kDa phosphorylation in the presence of an A-kinase inhibitor (Rp-8-Br-cAMPS). Lane 1, intact platelets (base); Lane 2, thrombin (0.05 U/mL); Lane 3, thrombin (0.05 U/mL) + G-Ro (300µM); Lane 4, thrombin (0.05 U/mL) + G-Ro (300µM) + Rp-8-Br-cAMPS (250 µM); and Lane 5, thrombin (0.05 U/mL) + pCPT-cAMP (1mM). Western blotting was performed as described in the “Materials and methods” section. Data are presented as mean ± SD (n = 4). * p < 0.05 versus the thrombin-stimulated human platelets. ** p < 0.05 versus the thrombin-stimulated human platelets in the presence of G-Ro (300µM).

3.5. Effects of G-Ro on retraction of fibrin clot

The binding of fibrinogen is known to stimulate clot retraction to intensify the formation of thrombus [19–21]. Thus, we investigated whether G-Ro inhibits clot retraction. Thrombin reaction time-dependently (10 min and 20 min) accelerated the clot retraction (Fig. 6A), and its degree (at 20 min) was increased to 90% compared to that (55.4 ± 1.3 mm²) without thrombin, control

(Fig. 6B). However, G-Ro very potently inhibited clot retraction by thrombin (Fig. 6A), which was attenuated up to 740% against that (5.5 ± 0.8 mm²) by thrombin (Fig. 6B).

4. Discussion

Intracellular cAMP and cGMP phosphorylate inositol 1,4,5-triphosphate receptor type I (IP₃RI) and VASP. The

Table 1
Effects of ginsenoside Ro (G-Ro) on cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) production¹⁾

	cAMP (pmol/10 ⁸ platelets)	cGMP (pmol/10 ⁸ platelets)
Basal	4.9 ± 0.3	3.0 ± 0.1
Thrombin (0.05 U/mL)	4.6 ± 0.1	3.0 ± 0.1
Thrombin (0.05 U/mL) + G-Ro (300µM)	10.9 ± 0.6*	2.1 ± 0.1

Data are presented as mean ± standard deviation (n = 4)
*p < 0.05 versus the thrombin-stimulated human platelets
¹⁾ Determination of cAMP and cGMP was carried out as described in the “Materials and methods” section

Table 2
Effects of ginsenoside Ro (G-Ro) on changes of fibrinogen binding¹⁾

	Fibrinogen Binding (%)	Δ(%)
Intact platelets	2.4 ± 0.4	—
Thrombin (0.05 U/mL)	95.3 ± 0.7	—
G-Ro (300µM) + thrombin (0.05 U/mL)	10.6 ± 1.3	-88.9 ²⁾
G-Ro (300µM) + thrombin (0.05 U/mL) + Rp-8-Br-cAMPS (250µM)	29.6 ± 3.1	+179.2 ³⁾

¹⁾ Data presented are from Fig. 5B
²⁾ Δ (%) = [(G-Ro + thrombin) - thrombin]/thrombin × 100
³⁾ Δ (%) = [(G-Ro + thrombin + Rp-8-Br-cAMPS) - (G-Ro + thrombin)]/(G-Ro + thrombin) × 100

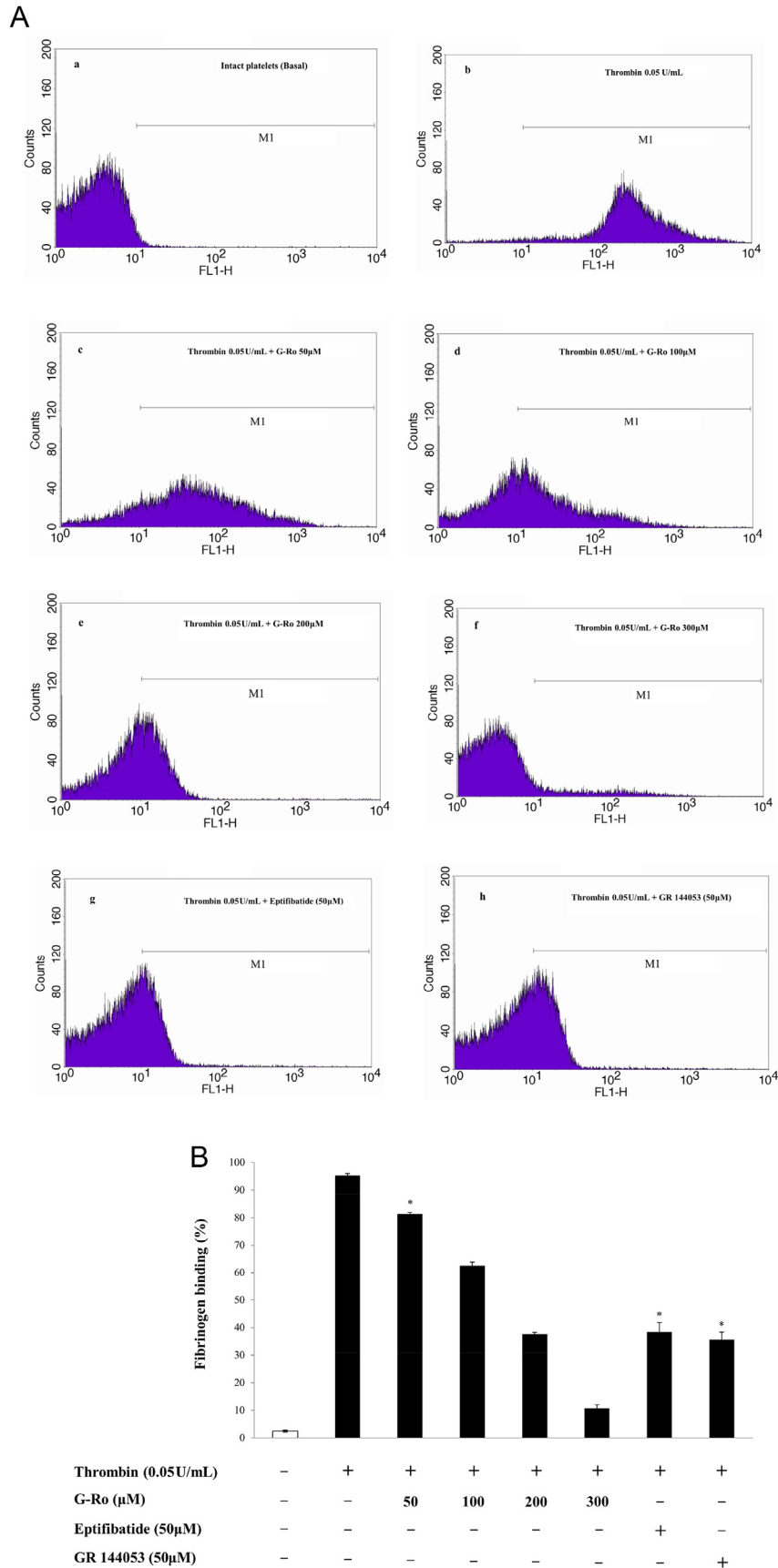


Fig. 4. Effects of ginsenoside Ro (G-Ro) on thrombin-induced fibrinogen binding. (A) The flow cytometry histograms on fibrinogen binding. a, intact platelets (base); b, thrombin (0.05 U/mL); c, thrombin (0.05 U/mL) + G-Ro (50µM); d, thrombin (0.05 U/mL) + G-Ro (100 µM); e, thrombin (0.05 U/mL) + G-Ro (200µM); f, thrombin (0.05 U/mL) + G-Ro (300 µM); g, thrombin (0.05 U/mL) + eptifibatide (50µM); and h, thrombin (0.05 U/mL) + GR 144053 (50µM). (B) Effects of G-Ro on thrombin-induced fibrinogen binding. Its binding degree (%) is from Fig. 4A. Determination of fibrinogen binding to glycoprotein IIb/IIIa (α IIb/ β_3) was carried out as described in the “Materials and methods” section. Data are presented as mean \pm standard deviation ($n = 4$). * $p < 0.05$ versus the thrombin-stimulated human platelets. FL1-H, fluorescent light-1 height.

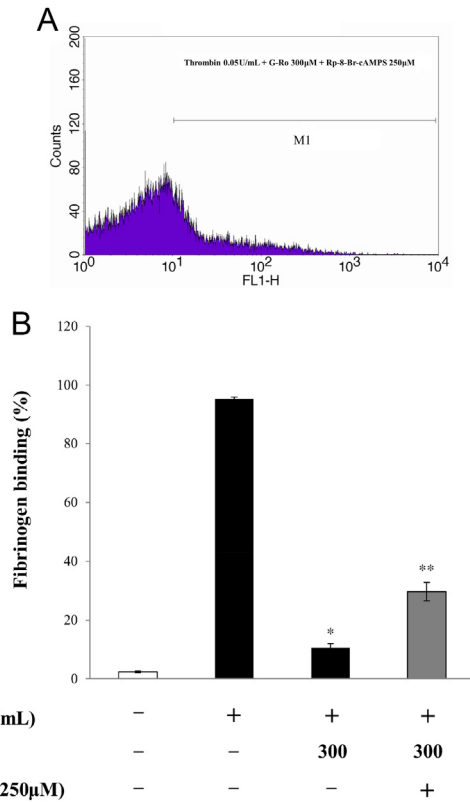


Fig. 5. Effects of ginsenoside Ro (G-Ro) on thrombin-induced fibrinogen binding in the presence of an A-kinase inhibitor (Rp-8-Br-cAMPS). (A) The flow cytometry histograms on fibrinogen binding. Thrombin (0.05 U/mL) + G-Ro (300µM) + Rp-8-Br-cAMP (250µM). (B) Effects of G-Ro on thrombin-induced fibrinogen binding in the presence of the A-kinase inhibitor (Rp-8-Br-cAMPS). Its binding degree (%) is from Figs. 4A, 5A. Determination of fibrinogen binding to α IIb/ β ₃ was carried out as described in the “Materials and methods” section. Data are presented as mean \pm SD (n = 4). * p < 0.05 versus the thrombin-stimulated human platelets. ** p < 0.05 versus the thrombin-stimulated human platelets in the presence of G-Ro (300µM). FL1-H, fluorescent light-1 height.

phosphorylation of IP₃RI is connected to inhibition of [Ca²⁺]_i mobilization by IP₃, and the phosphorylation of VASP contributes to inhibition of fibrinogen binding to α IIb/ β ₃. In a previous report, we found that G-Ro inhibits thrombin-elevated [Ca²⁺]_i mobilization by phosphorylating IP₃RI in a cAMP-dependent manner [22]. This means that G-Ro may be involved in inhibition of fibrinogen binding to α IIb/ β ₃ by decreasing [Ca²⁺]_i, and increasing cAMP. It is well established that intracellular Ca²⁺ activates α IIb/ β ₃, and subsequently stimulates the binding of fibrinogen to α IIb/ β ₃ [23]. If so, G-Ro that increases the level of the Ca²⁺-antagonistic molecule cAMP might be involved in inhibition of fibrinogen binding to α IIb/ β ₃ via cAMP-dependent VASP phosphorylation. VASP (Ser¹⁵⁷) is phosphorylated by cAMP/A-kinase, and VASP (Ser²³⁹) is phosphorylated by cGMP/G-kinase [17,18]. In reality, G-Ro cAMP-dependently stimulated the phosphorylation of VASP (Ser¹⁵⁷), but not phosphorylation of VASP (Ser²³⁹), which is evidenced as G-Ro increased the level of cAMP, but not cGMP. In addition, this is also supported from the results that the A-kinase inhibitor Rp-8-Br-cAMPS decreased G-Ro-increased VASP (Ser¹⁵⁷) phosphorylation, and elevated G-Ro-attenuated fibrinogen binding to α IIb/ β ₃.

The fibrinogen binding to α IIb/ β ₃ intensifies the formation of thrombus [19] by stimulating the retraction of the fibrin clot, which accelerates the progression of atherosclerosis [24,25]. Therefore, it is natural that G-Ro, inhibiting the fibrinogen binding, potently inhibits the retraction of the fibrin clot.

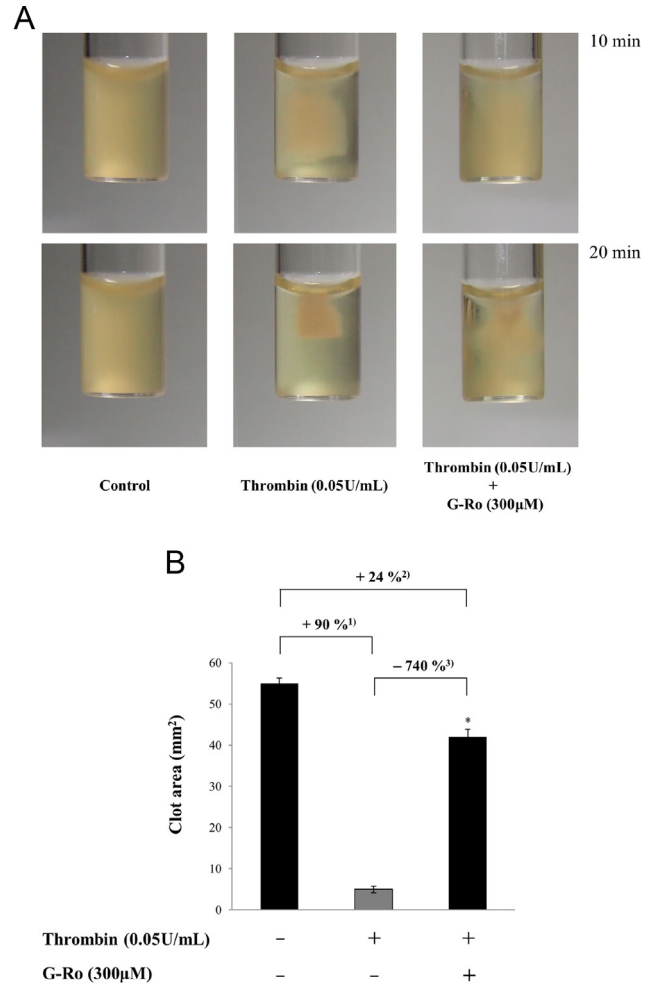


Fig. 6. Effects of ginsenoside Ro (G-Ro) on fibrin clot retraction. (A) Photographs of fibrin clot. (B) Effects of G-Ro on thrombin-retracted fibrin clot. Quantification of fibrin clot retraction was performed as described in the “Materials and methods” section. ¹⁾ (control - thrombin)/control \times 100. ²⁾ [control - (thrombin + G-Ro)]/control \times 100. ³⁾ [thrombin - (thrombin + G-Ro)]/thrombin \times 100. Data are presented as mean \pm standard deviation (n = 4). * p < 0.05 versus the thrombin-stimulated human platelets.

Platelet aggregation is connected to inflammation, a cause of atherosclerosis, and its associated proteins (platelet-derived growth factor, vascular endothelial growth factor, p-selectin, interleukin 1 β , etc.) are secreted out of α -granules [26–33]. Even though G-Ro phosphorylates VASP (Ser¹⁵⁷), it inhibits fibrinogen binding to attenuate thrombin-induced platelet aggregation, and fibrin clot retraction. If G-Ro dose not attenuate inflammation by leukocytes, antiplatelet effects by G-Ro would be doubtful. It is known that p-selectin is released by Ca²⁺, and subsequently interacts with monocyte to trigger inflammation [34]. G-Ro in a Ca²⁺-antagonistic action inhibited thrombin-induced expression of p-selectin [22], which means that the decrease of Ca²⁺ level by G-Ro might involve in inhibition of inflammation by suppressing thrombin-induced p-selectin secretion. This is also evidenced by results that G-Ro had anti-inflammatory activity *in vivo* and *in vitro* [35,36]. These reports [35,36] indicate that G-Ro may protect against thrombosis and atherosclerosis without inflammation.

The concentrations of G-Ro with antiplatelet effects (50–300 µM) are very low as compared with that 1mM of G-Ro attenuated arachidonic acid-induced platelet aggregation [12]. It is reported that G-Ro (10–50 mg/kg body weight-rat) administration activates fibrinolysis, an index of inhibition in fibrin thrombi [13]. Even

though 300 μM (about 287 mg/kg) of G-Ro (MW. 957.1) has the anticlot retraction effect, it is unknown whether thrombin-induced clot retraction would also be inhibited in vivo through administration. However, because thrombin stimulates platelet aggregation and fibrin formation, it is thought that G-Ro 300 μM (287 mg/kg) would be involved in attenuation of fibrin clot retraction.

In conclusion, we found inhibitory effects of G-Ro on fibrinogen binding to $\alpha\text{IIb}/\beta_3$, and clot retraction, which is mediated by cAMP-dependent phosphorylation of VASP (Ser¹⁵⁷). From this study, we suggest that G-Ro is a novel compound of *P. ginseng* which inhibits fibrinogen binding to $\alpha\text{IIb}/\beta_3$.

Conflicts of interest

The authors declare no conflicts of interest.

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