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20(S)-ginsenoside Rg3 inhibits glycoprotein IIb/IIIa activation in human platelets

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Abstract The Panax ginseng Mayer is used in conventional medicine in Asia owing to its preventing effects on thrombosis, hypertension, atherosclerosis, vasorelaxation and myocardial infarction. Because platelets are crucial mediators of cardiovascular diseases, many studies have investigated its functions. The previous study showed the antiplatelet effects of crude ginseng fraction and two of its components, ginsenoside Rg3 (20S and 20R). In addition, ginsenoside Rg3-enriched fraction shows an inhibitory effect on collagen-activated rat platelets. However, the mechanism underlying this effect remains unclear. Thus, I investigated the inhibitory action of ginsenoside Rg3 (20S, G-Rg3) on the regulation of signaling molecules involved in αIIb/β3 activation. I found that G-Rg3, in a cyclic AMP dependent manner, inhibited thrombin-induced activation of human platelets and affinity of fibrinogen and fibronectin with αIIb/β3. Thus, in the present study, G-Rg3 showed an inhibitory effect on glycoprotein IIb/IIIa (αIIbβ3) activation, suggesting its potential use for preventing platelet-mediated thrombotic disease.

Keywords 20(S)-ginsenoside Rg3 · Clot retraction · Cyclic AMP · Fibrinogen · Fibronectin

Introduction Platelets are activated at a site of vascular damage by adenosine diphosphate, collagen and thrombin. Platelet aggregation is important for the maintenances of normal hemostasis. Platelet activation and platelet aggregation are due to the action of glycoprotein IIb/IIIa (αIIbβ3). The αIIbβ3 located on platelet surface binds with plasma proteins such as fibrinogen, fibronectin, thrombospondin, and vitronectin, causing Ca2+ mobilization, shape change, degranulation [1], and ultimately thrombus formation.

In normal circulation, vascular endothelial cells release both prostaglandin I2 and nitric oxide, which facilitate the 3’,5’-cyclic adenosine monophosphate (cyclic AMP) and 3’ 5’-cyclic guanosine monophosphate (cyclic GMP) synthesis. Elevated cyclic AMP and cyclic GMP levels induce the protein kinase A (PKA) and protein kinase G (PKG), respectively, which downregulate platelet functions [2]. Vasodilator-stimulated phosphoprotein (VASP) is a major substrate of PKA and PKG in the platelet. VASP supports the regulation of actin filament dynamics and αIIbβ3 activation, but VASP Ser157 phosphorylation or VASP Ser239 phosphorylation leads to the inhibition of actin filament elongation and suppression of αIIbβ3 activation [3,4]. αIIbβ3, the most abundant binding molecule on the platelet surface, is important in the formation of fibrin-platelet mesh and in the interaction between platelet and monocytes. Activated αIIbβ3 undergoes a rapid shape change allowing the platelet membrane to bind to vascular adhesion molecules. Therefore, VASP phosphorylation could be used to examine the anti-thrombotic effect of a compound.

G-Rg3 suppresses blood platelet aggregation [5], thromboxane A2 release, [Ca2+] mobilization and ATP release [6]. Furthermore, an Rg3 derivative, dihydroxyginsenoside Rg3, decreases mitogen-activated protein kinases [7]. Rg3-enriched fraction also shows inhibitory effects on collagen-induced rat platelets [8]. In previous report, I confirmed the inhibitory action of G-Rg3 on thrombin-activated platelets [9]. However, the suppressive mechanism on the platelets is not fully understood. Therefore, in the present investigation, I characterized the modulatory mechanism of G-Rg3 on human platelet αIIbβ3.
Materials and Methods

Materials
I purchased 20(S)-ginsenoside Rg3 from the Ambo Institute (Daejon, Korea). Human fibrinogen connected-Alexa Flour 488 was purchased from Invitrogen Molecular Probes (Eugene, OR, USA). I purchased thrombin and all materials for platelet aggregation from Chrono-Log (Havertown, PA, USA) and purchased eptifibatide, GR 144053, anti-rabbit IgG-HRP, anti-beta-actin from Santa Cruz (Santa Cruz, CA, USA). The cyclic AMP and cyclic GMP EIA kits were purchased from Cayman (Ann Arbor, MI, USA). I purchased all materials for buffer solution and Rp-8-Br-cAMPS, pCPT-cAMP, 8-Br-cGMP from Sigma (St. Louis, MO, USA). Antibodies for Western blotting such as Anti-VASP, anti-phosphor-VASP (Ser157, Ser239), and lysis buffer were purchased from Cell Signaling (Beverly, MA, USA), and the Cell Adhesion Assay (Colorimetric Format) Kit were purchased from Cell Biolabs (San Diego, CA, USA).

Preparation of washed human platelets
Human platelet rich plasma (PRP) was obtained from the Korean Red Cross Blood Center (Changwon, Korea). The PRP centrifuged for 10 min at 1,300×g and the platelet-containing pellets were washed twice with washing buffer solution (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), and resuspended in suspension buffer solution (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). The platelet concentration was adjusted to a final concentration of 5×10⁸/mL. All procedures for platelet suspension were performed at 25°C. Experimental approval (PIRB12-072) was obtained from the Public Institutional Review Board at the National Institute for Bioethics Policy (Seoul, Korea).

Measurement of platelet aggregation
Washed human platelets (10⁸/mL) were preincubated with various concentrations of G-Rg3 in 2 mM CaCl₂ for 2 min at 37°C and then facilitated by thrombin (0.05 U/mL). The platelet aggregation assay was conducted for 5 min and the aggregation percentage was determined as an increase in light transmission. G-Rg3 was dissolved in 0.1% dimethyl sulfoxide (DMSO).

Measurement of cyclic AMP and cyclic GMP concentration
Platelet aggregation with various concentrations of G-Rg3 was terminated by adding 80% ethanol to the platelet suspension. Next, cyclic nucleotides were extracted three times from the suspension by using 80% ethanol, and were dried by nitrogen gas. The dried pellet was dissolved in an assay buffer from a cyclic AMP/GMP EIA kit. The cyclic AMP and cyclic GMP concentration was determined using Synergy HT Multi-Model Microplate Reader (BioTek Instruments, Winooski, VT, USA).

Measurement of VASP-phosphorylation
Platelet aggregation with various concentrations of G-Rg3 was terminated and dissolved by 1× lysis buffer. The platelet lysates were adjusted to 15 µg of protein for Western blot using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The protein samples were separated through 6% SDS-PAGE, and transferred onto PVDF membranes. The dilutions for the primary and secondary antibodies were 1:1,000 and 1:10,000, respectively. The protein bands were visualized using the ECL reagent (General Electric Healthcare, Buckinghamshire, UK).

Measurement of fibrinogen binding to αIIb/β3
The platelet aggregation assay was conducted with various concentrations of G-Rg3 in the presence of human fibrinogen (30 µg/mL) connected Alexa Flour 488. The platelets were fixed by adding 0.5% paraformaldehyde in PBS and all procedures were conducted in darkness. The fibrinogen binding assay was conducted by a flow cytometry (BD Biosciences, San Jose, CA, USA).

Measurement of fibronectin adhesion
The adhesion kit is coated with fibronectin or, as a negative control, bovine serum albumin (BSA). Washed platelets (10⁷/mL) were preincubated on the plates for 60 min at 37°C in the presence of thrombin (0.05 U/mL) and various concentrations of G-Rg3. Next, the plates were washed three times with PBS, and incubated with cell staining solution at RT for 10 min. The platelets were washed again, extraction solution was used. Fibronectin adhesion was measured using Synergy HT Multi-Model Microplate Reader (BioTek Instruments, Winooski, VT, USA).

Measurement of platelet-mediated fibrin clot retraction
Human PRP (250 µL) was preincubated in a tube with G-Rg3
(300 µM) for 10 min at 37 °C, and facilitated by 0.05 U/mL of thrombin. Clot pictures were taken at 15 min, and Clot area was calculated using the Image J Software. The percentage of clot retraction was calculated as follows: Retraction (%) by thrombin = (basal area – thrombin area) / basal area × 100.

**Statistical analyses**

Experimental data are indicated as the mean ± standard deviation accompanied by the number of observations. Significant differences among the groups were determined by analysis of variance (ANOVA) and further analyzed by using the Newman-Keuls method. Statistical analysis was performed by using SPSS 21.0.0.0 software (SPSS, Chicago, IL, USA). *p <0.05 was considered as statistically significant.

**Results**

**Inhibitory action of G-Rg3 on platelet aggregation**

In previous report, thrombin at 0.05 U/mL induces the optimum aggregation of human platelets [9]. In present study, the aggregation rate of thrombin induced platelets was 91.5±1.3% (Fig. 2). However, the aggregation rates of platelets treated with G-Rg3 (50, 100, 200, and 300 µM) were significantly reduced (inhibitory degrees were 15.7, 38.1, 74.7, and 88.2%, respectively), indicating that G-Rg3 inhibited the platelet aggregation in a dose-dependent manner. DMSO 0.1% did not influence the aggregation (Fig. 2).

**Inhibitory action of G-Rg3 on cyclic AMP and cyclic GMP concentration**

Next, I investigated the cyclic AMP and cyclic GMP concentration by G-Rg3 during platelet aggregation. During thrombin-stimulated platelet aggregation, G-Rg3 (300 µM) increased cyclic AMP concentration but did not affect the cyclic GMP concentration (Table 1).

**Inhibitory action of G-Rg3 on VASP-phosphorylation**

Following phosphorylation, the molecular mass of VASP shifted from 46 to 50 kDa [9,10]. As G-Rg3 showed a dose-dependent inhibitory effect on agonist-induced platelet aggregation (Fig. 2), I further investigated the effect of G-Rg3 on VASP (Ser157, Ser239) phosphorylation in human platelets. G-Rg3 significantly increased VASP Ser157 phosphorylation (Fig. 3A) in a dose-dependent manner. On the contrary, G-Rg3 did not affect VASP Ser239 phosphorylation (Fig. 3B) suggesting that G-Rg3 did not affect cyclic GMP level (Table 1). Since G-Rg3 stimulated cyclic AMP level (Table 1), I hypothesized that G-Rg3-elevated VASP Ser157 phosphorylation may be inhibited by Rp-8-Br-cAMPS, a A-kinase inhibitor. To test this hypothesis, I examined the effect of Rp-8-Br-cAMPS on G-Rg3 (300 µM)-induced VASP Ser157 phosphorylation. The Rp-8-Br-cAMPS significantly decreased G-Rg3-induced VASP Ser157 phosphorylation, whereas the pCPT-cAMP, a A-kinase activator, increased VASP Ser157 phosphorylation (Fig. 3C). These results indicated the involvement of elevated cyclic AMP level in G-Rg3-induced VASP Ser157 phosphorylation.

**Inhibitory action of G-Rg3 on binding between fibrinogen and αIIb/β3**

Since G-Rg3 increased VASP phosphorylation (Fig. 3A), I examined the binding affinity fibrinogen with αIIb/β3 in human platelets using G-Rg3. Thrombin increased the binding between fibrinogen and αIIb/β3 (Fig. 4A-b, 4B), with a rate of 94.8±0.5% (Table 2) but, G-Rg3 decreased the binding affinity of fibrinogen with αIIb/β3 (Fig. 4A-d~g, 4B), with an inhibitory degree of

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**Table 1 Inhibitory effects of G-Rg3 on cyclic AMP and cyclic GMP**

|                      | cyclic AMP (pmoL/10^8 platelets) | Δ (%) | cyclic GMP (pmoL/10^8 platelets) | Δ (%) |
|----------------------|----------------------------------|-------|----------------------------------|-------|
| Thrombin (0.05 U/mL) | 4.6±0.3                          | -     | 3.0±0.1                          | -     |
| +DMSO 0.1%           | 5.8±0.3                          | 24.9  | 2.2±0.1                          | 26.7  |
| +G-Rg3 (300 µM)      | 11.3±0.1                         | 145.2 | 2.2±0.1                          | 26.7  |

Δ (%) = [(thrombin+G-Rg3–thrombin)/thrombin×100. The data are expressed as the mean ± standard deviation (n =4)
81.4% (Table 2). The eptifibatide and GR 144053, αIIb/β3 inhibitors, were used as a positive control and inhibited thrombin-induced affinity of fibrinogen with αIIb/β3, with inhibitory degrees of 59.5 and 62.3%, respectively. Moreover, I tested the effect of Rp-8-Br-cAMPS, an A-kinase inhibitor, on G-Rg3-suppressed the affinity of fibrinogen with αIIb/β3. The Rp-8-Br-cAMPS increased G-Rg3-reduced the affinity (Fig. 4A-a, 4B) with an inhibitory degree of 171.0% higher than that of G-Rg3 (300 μM) alone (17.6±1.9%) (Table 2).

### Table 2

Inhibitory effects of G-Rg3 on changes of fibrinogen binding

| Fibrinogen Binding (%) | Δ (%) |
|------------------------|-------|
| Intact platelets       | 81±0.3 | -   |
| Thrombin (0.05 U/mL)   | 94.8±0.5 | -   |
| G-Rg3 (300 μM) + Thrombin | 17.6±1.9 | -81.4 |
| G-Rg3 (300 μM) + Rp-8-Br-cAMPS (250 μM) | 47.7±0.7 | +171.0 |

Data are from Fig. 4B and 5B. 1) Δ (%)=[(G-Rg3+Thrombin–Thrombin)/Thrombin]*100, 2) Δ (%)=[(G-Rg3+Thrombin+Rp-8-Br-cAMPS)–(G-Rg3+Thrombin)]/[G-Rg3+Thrombin]*100

Inhibitory action of G-Rg3 on adhesion between fibronectin and αIIb/β3

The integrin αIIb/β3 on platelet surface served as a receptor for platelet adhesion. I examined whether G-Rg3 suppressed fibronectin adhesion to αIIb/β3. The platelets did not adhere to BSA coated well, but adhered to fibronectin coated well under thrombin stimulation. G-Rg3 (50 to 300 μM) decreased fibronectin adhesion to αIIb/β3 dose-dependent manner. Moreover, eptifibatide and GR 144053, αIIb/β3 inhibitors, attenuated fibronectin adhesion to αIIb/β3 as a positive control. Next, I investigated whether G-Rg3 inhibited αIIb/β3 adhesion to fibronectin by increasing cyclic AMP.
The Rp-8-Br-cAMPS increased G-Rg3-induced inhibition of fibronectin adhesion to αIIbβ3 (Fig. 6B). Moreover, the pCPT-cyclic AMP also inhibited fibronectin adhesion to αIIbβ3 (Fig. 6B), indicating that the elevated cyclic AMP concentration involved in the adhesion action.

Inhibitory action of G-Rg3 on fibrin clot retraction

The activation of αIIbβ3 by platelet agonists increased the affinity of fibrinogen and fibronectin with αIIbβ3 leading to the outside-in αIIbβ3 signaling and containing clot retraction. Thus I investigated the effect of G-Rg3 on the retraction of thrombin-induced fibrin clots, an index of outside-in αIIbβ3 signaling. In Fig. 7A shows result showed that thrombin stimulated fibrin clot formation and retraction. In Fig. 7B, thrombin potently increased fibrin clot retraction, and this increase was 90% higher than that observed without thrombin (55±1.3 mm²). However, G-Rg3 attenuated fibrin clot retraction, and its percentage was 664% as compared with thrombin stimulated fibrin clot retraction (22±1.1 mm²) (Fig. 7B).
Cardiovascular disease, which includes all diseases of the heart and blood vessels, is a leading cause of morbidity and mortality. Its major underlying pathology is atherosclerosis. Many studies have suggested that platelets have an important pro-inflammatory function in atherosclerosis [12]. The α-granules within the platelets contain abundant chemokines, which increase vascular inflammatory responses [13]. Moreover, activated platelets bind with monocyte and vascular endothelium, elevating monocyte recruitment and accelerating atherosclerotic lesions development [14,15]. Therefore, therapeutic agents that inhibit platelets may mitigate the development of early stage atherosclerosis.

Our previous reports showed that Korean red ginseng extract increases cyclic AMP level in thrombin-activated rat platelets [16]. I also previously reported that the ginsenosides [Ra1, Rb1, Rb2, Rh3, Re, Rd, and Rh2 (20R)] of the protopanaxadiol saponin group, as well as ginsenosides [Re, Rf, Rg1, Rg2 (20R), and Rh1 (20S)] of the protopanaxatriol saponin group, do not affect human platelet aggregation. However, G-Ro and G-Rg3 (20S) exert suppression of human platelet aggregation [9]. Thus, in the present study, I first examined the effect of G-Ro on cyclic AMP concentration in human platelets and verified its cyclic AMP-elevating effect [17]. The current study focused on the G-Rg3-induced increase in cyclic AMP level. G-Rg3 attenuated the fibrinogen binding and fibronectin adhesion to αIIb/β3 through VASP phosphorylation. VASP Ser157 and Ser239 are phosphorylated by cyclic AMP/A-kinase and cyclic GMP/G-kinase, respectively [10,11]. It was therefore concluded that G-Rg3 stimulated VASP Ser157 phosphorylation in a cAMP-dependent manner, but did not
influence on Ser239 phosphorylation, as G-Rg3 influenced only the cyclic AMP levels (Table 1).

Clot retraction is the most crucial step in the repair and healing process of damaged blood vessels [18,19]. The clot formed by coagulation factors and activated platelets, is composed of fibrin and platelets. In the coagulation phase, both extrinsic and intrinsic pathways result in the generation of thrombin, which converts fibrinogen into the fibrin. At the same time, the activated platelets are accumulated in the damaged blood vessel, forming a fibrin-platelet mesh. Additional circulatory platelets and blood cells are trapped in mesh, accelerating clot formation. The clot, which seals off the damaged vessel, then begins to undergo retraction over a period of 30-60 minutes, pulling the cut edges of the vessel together. Since G-Rg3 strongly suppressed thrombin-induced clot retraction, it was suggested that the downregulation of αIIb/β3 by G-Rg3 leads to the extension of clot retraction.

The G-Rg3 enriched faction from red ginseng inhibits collagen-induced granule secretion, p-selectin expression and fibrinogen binding [8]. The mechanism of G-Rg3 inhibitory effect on αIIb/β3 function was not clear, but our results suggested that this inhibitory effect was attributed cAMP/VASP Ser157 signaling pathway. Furthermore, it has been reported that antiplatelet compounds, such as Korean red ginseng extract, ginsenoside Rp1 and ginsenoside Ro increase cyclic AMP levels [16-17,20]. These reports also suggest that the inhibitory action of ginseng saponins on platelet activation might be caused by increased cyclic AMP levels. Recently, it has been reported that newly isolated ginseng saponins and metabolites (Rk1, Rk3, Rg5, Rg6, Rh4, Rs3, Rs4, Rs5, and F4) exert antiplatelet activities [21-23], but the mechanism is still unclear. Therefore, it will be investigated whether the new ginseng compounds on cyclic AMP level in human platelets.

In conclusion, our present study suggested that the affinity of fibrinogen and fibronectin with αIIb/β3 was inhibited by G-Rg3 via cyclic AMP-dependent VASP Ser157 phosphorylation. Therefore, G-Rg3 may be used as a therapeutic agent for prevention of thrombosis and other platelet-mediated cardiovascular diseases.

Fig. 6 Inhibitory effects of G-Rg3 on thrombin-induced fibronectin adhesion. (A) Inhibitory effects of G-Rg3 on fibronectin adhesion. (B) Effects of G-Rg3 on thrombin-induced fibronectin adhesion in the presence of Rp-8-Br-cAMPS. Measurement of fibronectin adhesion was described in “Materials and Methods” section. BSA, bovine serum albumin (negative control). 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 G-Rg3 (300 µM).
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Conflict of interest
The authors declare no conflict of interest.

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