Inhibitory Effects of Ginsenoside Ro on Clot Retraction through Suppressing PI3K/Akt Signaling Pathway in Human Platelets

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ABSTRACT: Glycoprotein IIb/IIIa (αIIb/β3) is the most abundant integrin on platelet surfaces, which is involved in interaction between platelets, and triggers an intracellular signaling cascade, platelet shape changes, granule secretion, and clot retraction. In this study, we evaluated the effect of ginsenoside Ro (G-Ro) on the binding of fibronectin and fibrinogen to αIIb/β3 and clot retraction. We found that G-Ro inhibited thrombin-induced platelet aggregation dose-dependently and attenuated the fibronectin-, and fibrinogen-binding to αIIb/β3 through the dephosphorylation of phosphoinositide 3-kinase p85 and Akt, which influence clot retraction, reflecting the intensification of thrombus. We observed that G-Ro is involved in αIIb/β3 in human platelets. These results suggest that G-Ro is beneficial, inhibiting fibronectin adhesion, fibrinogen binding, and clot retraction. Therefore, G-Ro in Panax ginseng may prevent platelet aggregation-mediated thrombotic disease.

Keywords: ginsenoside Ro, PI3K p85 (Tyr458), Akt (Ser473), fibrinogen-binding, fibronectin adhesion

INTRODUCTION

Platelets are activated at sites of vascular injury via several molecules, such as collagen, thrombin, and adenosine diphosphate. Full platelet aggregation is absolutely essential for normal hemostasis. Moreover, this physiological event can trigger circulatory disorders, such as thrombosis, atherosclerosis, and cardiovascular disease (1). Therefore, platelet function inhibition is a promising approach in preventing platelet-mediated circulatory disease. These signaling cascades are triggered by an inside-out signaling pathway and facilitate the activation of glycoprotein IIb/IIIa (αIIb/β3). The αIIb/β3 is abundant integrin at the platelet surface and acts as a receptor for adhesive proteins (i.e. fibronectin, fibrinogen, vitronectin, and thrombospondin). The activated αIIb/β3 induces Ca++ mobilization and granule secretion, and continuously intensifies the platelet aggregation and the formation of thrombus via outside-in signaling pathway (2). These two signaling-mediated molecules potentiate the formation of thrombus, the final reaction of platelets.

Human platelets contain class I phosphoinositide 3-kinase (PI3K) isoforms, which are subdivided into class IA and class IB. The class IA PI3Ks (PI3Kα, β, and δ) is composed of a regulatory subunit (p50, p55, and p85) with a catalytic subunit (p110α, β, and δ), and class IB PI3K (PI3Kγ) consists of a regulatory subunit (p101 and p84) with a catalytic subunit (p110γ) (3). The p85 protein is known as the regulatory subunit of class IA PI3Ks, which is activated by binding tyrosine phosphorylation sites (4), GTP-binding proteins (5) and its phosphorylation (6,7). The activated p85 directly binds to p110 that enables the activation of PI3K. Upon platelet stimulation by thrombin, phosphatidylinositol 3,4,5 triphosphate (PIP3) is accumulated by class I PI3Ks at the inner layer of the plasma membrane (8); then, a downstream effector Akt binds to PIP3 and is phosphorylated for full enzyme activity on Thr308 and Ser473 by phosphatidylinositol dependent kinases (9). Several studies have revealed that the PI3K and Akt are the most important mediators in human platelets leading to adhesive function, filopodia formation, platelet spreading, and αIIb/β3 activation (10,11). In addition, dephosphorylation of PI3K (p85 (Tyr545)) and Akt (Ser473) can suppress the binding of adhesive proteins to αIIb/β3. Thus, to determine if inhibition of PI3K/Akt signaling pathway contributes to the αIIb/β3 inhibitory action of ginsenoside Ro (G-Ro), PI3K/Akt inhibitors, wortmannin and miltefosine were used for fibronectin adhesion, fibrinogen binding to αIIb/β3, and clot retraction. The αIIb/β3 activation contributes to estimate the antithrombogenic and fibrinolytic activities.
botic effect of certain compounds (12,13). For instance, abciximab, etifibatide, tirofiban, and lamifiban have antithrombotic effects via suppression of the α\textsubscript{IIb}/β\textsubscript{3} (14).

Roots of Panax ginseng are used in traditional oriental medicine. G-Ro (Fig. 1) in Panax ginseng Meyer, is known to inhibit the fibrin formation (15), but the antiplatelet mechanism of G-Ro is not fully understand. Therefore, we investigated G-Ro inhibit-signaling pathways in human platelets using specific inhibitors.

### MATERIALS AND METHODS

#### Materials

G-Ro (molecular weight=957.1) was obtained from Ambo Institute (Daejon, Korea). Thrombin was obtained from Chrono-Log Corporation (Havertown, PA, USA). Anti-Pi3K, anti-phosphor-Pi3K p85 (Tyr458), anti-Akt, anti-phosphor-Akt (Ser473), anti-rabbit IgG-horseradish peroxidase, and lysis buffer were purchased from Cell Signaling (Beverly, MA, USA). Wortmannin (Pi3K inhibitor) and miltefosine (Akt inhibitor) were purchased from Cayman Chemical (Ann Arbor, MI, USA). CytoSelect 48 well cell adhesion assay kits (fibronectin-coated, colorimetric format) were purchased from Cell Biolabs (San Diego, CA, USA). Eptifibatide (e1lb/β\textsubscript{i} inhibitor, GR 144053 (e1lb/β\textsubscript{i} inhibitor), and anti-β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinylidene difluoride (PVDF) membrane and enhanced chemiluminescence solution (ECL) were purchased from General Electric Healthcare (Amersham, Buckinghamshire, UK). Fibrinogen Alexa Fluor 488 conjugate was obtained from Invitrogen Molecular Probes (Eugene, OR, USA).

#### Preparation of washed human platelets

Human platelet-rich plasma (PRP) was obtained from the Korean Red Cross Blood Center (Changwon, Korea) and centrifuged for 10 min at 1,300 g. The platelets were then washed twice with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO\textsubscript{3}, 0.36 mM Na\textsubscript{2}HPO\textsubscript{4}, 5.5 mM glucose, and 1 mM ethylenediaminetetraacetic acid disodium salt dihydrate, pH 6.5), and resuspended in suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO\textsubscript{3}, 0.36 mM Na\textsubscript{2}HPO\textsubscript{4}, 0.49 mM MgCl\textsubscript{2}, 5.5 mM glucose, and 0.25% gelatin, pH 6.9). The platelet concentrations were adjusted to a final concentration of 5×10\textsuperscript{8}/mL. All aforementioned procedures for platelet activity 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).

#### Determination of platelet aggregation

Platelets (10\textsuperscript{9}/mL) were preincubated with or without substances in the 2 mM CaCl\textsubscript{2} for 3 min at 37°C followed by thrombin stimulation (0.05 U/mL). The aggregation was performed for 5 min using an aggregometer (ChronoLog Corporation). Platelet aggregation rate was determined as an increase in light transmission. Pi3K/Akt inhibitors were dissolved in dimethyl sulfoxide to a final concentration of 0.1%.

#### Western blots for analyzing of Pi3K- and Akt-phosphorylation

Platelet aggregation was stopped by adding 1×lysis buffer. The platelet lysates were then measured using a bicinechonic acid protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Proteins (15 μg) were analyzed via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6%, 1.5 mm), and PVDF membranes were used for protein transfer. The dilutions for the primary and secondary antibodies were 1:1,000 and 1:10,000, respectively. The membranes were visualized using the ECL solution.

#### Determination of fibronectin adhesion

The plates were coated with fibronectin or bovine serum albumin (BSA) as a negative control. Washed human platelets (10\textsuperscript{9}/mL) were incubated for 60 min at 37°C in the presence of thrombin (0.05 U/mL) with or without various concentrations of G-Ro. After five times washing with phosphate buffered saline (PBS), 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 absorbance measured with a Synergy HT Multi-Model Microplate Reader (BioTek Instruments, Winooski, VT, USA) at 560 nm.
Determination of fibrinogen binding to αIIb/β3
The platelet aggregation was conducted in the presence of Alexa Flour 488-human fibrinogen (30 μg/mL) for 5 min at 37°C. The reaction was stopped by the addition of 0.5% paraformaldehyde in cold PBS, 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).

Assay of platelet-mediated fibrin clot retraction
Human PRP 250 μL were preincubated with or without G-Ro (300 μM) for 10 min at 37°C, and incubated with thrombin (0.05 U/mL) for 20 min at 37°C. Photographs of fibrin clots were taken by a digital camera, and its area (at 20 min) was measured by NIH Image J Software (v1.46, National Institutes of Health, Bethesda, MD, USA). Percentage of clot retraction was calculated as follows:

\[ \text{Retraction (\%)} \text{ by thrombin} = \frac{\text{Control area} - \text{Thrombin area}}{\text{Control area}} \times 100 \]

Measurement of PT and APTT
To investigate whether G-Ro shows anticoagulant characteristics, we measured prothrombin time (PT) and activated partial thromboplastin time (APTT), markers of blood coagulation. The platelet poor plasma (PPP) (100 μL) was preincubated in a two-channel coagulator (Behnk Elektronik GmbH & Co., KG, Norderstedt, Germany) with gentle stirring for 1 min at 37°C. PT was determined as the time interval between the addition of PT reagent (100 μL) to the PPP and the formation of a fibrin clot. After preincubation of PPP for APTT measurement, 100 μL of APTT reagent was added to the PPP (100 μL) and incubated for 3 min at 37°C. Following incubation, 100 μL of 25 mM CaCl₂ was immediately added to the PPP containing APTT reagent. APTT was determined as the time required to form a fibrin clot.

Statistical analyses
The experimental results are reported as the mean± standard deviation accompanied by the number of observations. The data were compared via analysis of variance (ANOVA). Significant differences among the group means were compared using the Tukey-Kramer method. Statistical analysis was performed using SPSS 21.0.0.0 (SPSS, Chicago, IL, USA). P<0.05 was considered to be statistically significant.

RESULTS

Effects of G-Ro on thrombin-induced human platelet aggregation
Based on a previous report, 0.05 U/mL of thrombin was used as it maximally aggregates human platelets (16). The thrombin-induced aggregation rate was 95.8±1.7%, however, G-Ro dose-dependently reduced (50, 100, 200, and 300 μM) the light transmission. PI3K/Akt inhibitors, wortmannin (10 μM) and miltefosine (10 μM) also suppressed thrombin-induced light transmission, the aggregation rates were 45.8±3.6% and 35.5±1.3%, respectively. Moreover, PI3K/Akt inhibitors with G-Ro (300 μM) showed synergistic inhibitory effects (Fig. 2).

Effects of G-Ro on thrombin-induced PI3K- and Akt-phosphorylation
We could not directly confirm the activity of the regulatory subunit (p50, p55, and p85) and a catalytic subunit

![Fig. 2](image-url) Effects of ginsenoside Ro (G-Ro) and phosphoinositide 3-kinase (PI3K)/Akt inhibitors on thrombin-induced human platelet aggregation. Measurement of platelet aggregation was carried out as described in "MATERIALS AND METHODS" section. The rate of inhibition was expressed as the percentage of the thrombin-induced aggregation rate. The data are expressed as the mean± standard deviation (n=4). *P<0.05 versus the thrombin-stimulated human platelets.
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Fig. 3. Effects of ginsenoside Ro (G-Ro) on thrombin-induced phosphoinositide 3-kinase (PI3K)- and Akt-phosphorylation. (A) Effects of G-Ro on thrombin-induced PI3K (Tyr<sup>458</sup>) phosphorylation. (B) Effects of G-Ro on thrombin-induced Akt (Ser<sup>473</sup>) phosphorylation. Western blotting was performed as described in "MATERIALS AND METHODS" section.

(p110α, β, and δ) of PI3K. Thus, we investigated that G-Ro affects the PI3K signaling through phosphorylation and dephosphorylation. Because the stimulation of thrombin in human platelets has been shown to increase the phosphorylation of p85 (7,17), the experiment focused on the inhibitory effect of G-Ro on p85 phosphorylation. Thrombin accelerated PI3K-phosphorylation (Fig. 3A) compared to unstimulated platelets. However, G-Ro dose-dependently dephosphorylated (100 to 300 μM) thrombin-phosphorylated PI3K (Fig. 3A). In addition, thrombin-induced the phosphorylation of Akt (Fig. 3B), a downstream molecule, was dephosphorylated in a dose-dependent manner of G-Ro (100 to 300 μM) (Fig. 3B). Each positive control, wortmannin (10 μM) and miltefosine (10 μM) decreased PI3K and Akt phosphorylation (Fig. 3).

Effects of G-Ro on fibronectin adhesion

Next, we investigated fibronectin adhesion to α<sub>IIb</sub>/β<sub>3</sub>, which is an important reaction in outside-in signaling. Thrombin strongly stimulated the fibronectin adhesion to α<sub>IIb</sub>/β<sub>3</sub> as compared with unstimulated platelets, but not BSA, a negative control against fibronectin (Fig. 4A). G-Ro decreased thrombin-increased the adherence of fibronectin in a dose (50 to 300 μM)-dependent manner (Fig. 4A). The positive controls of α<sub>IIb</sub>/β<sub>3</sub>, eptifibatide, GR 144053, suppressed the adhesion, and those inhibitory degrees were equal to that by G-Ro (300 μM) (Fig. 4A). Thrombin-induced fibronectin adhesion was also inhibited by wortmannin (10 μM) and miltefosine (10 μM) (Fig. 4A), and showed synergistic inhibitory effects with G-Ro (300 μM) on fibronectin adhesion (Fig. 4B).

Effects of G-Ro on fibrinogen binding to α<sub>IIb</sub>/β<sub>3

Because G-Ro decreased fibronectin adhesion, G-Ro may influence fibrinogen binding to α<sub>IIb</sub>/β<sub>3</sub>. Thus, we investigated inhibitory effects of fibrinogen binding to α<sub>IIb</sub>/β<sub>3</sub> by G-Ro (300 μM), and PI3K/Akt inhibitors. Thrombin elevated fibrinogen binding to α<sub>IIb</sub>/β<sub>3</sub> (Fig. 5A-b and 5B), and its degree was 95.7±0.5. However, G-Ro (300 μM)
strongly attenuated the fibrinogen binding to αIIb/β3 (Fig. 5A-c), and its inhibitory degree was 88.1%. The fibrinogen binding to αIIb/β3 was also inhibited by both inhibitors, wortmannin (10 μM) and miltefosine (10 μM) (Fig. 5A-d and -e) and G-Ro (300 μM) plus wortmannin (10 μM) or miltefosine (10 μM) showed strong synergistic effects on fibrinogen binding to αIIb/β3 (Fig. 5A-f, -g, and 5B).

Effects of G-Ro on retraction of fibrin clot
The binding of fibronectin and fibrinogen is known to stimulate clot retraction for thrombus formation (18,19). Thus, we investigated whether G-Ro inhibits clot retraction. Thrombin accelerated the clot retraction (Fig. 6A), and its degree (at 20 min) was decreased 95% compared to (55.4±1.3 mm²) no thrombin, control (Fig. 6B). However, G-Ro very potently inhibited clot retraction that occurred within thrombin (Fig. 6A and 6B), which was attenuated up to 225% against that (2.7±0.2 mm²) occurred by thrombin (Fig. 6B). PI3K/Akt inhibitors, wortmannin (10 μM) and miltefosine (10 μM) also strongly inhibited clot retraction (Fig. 6A and 6B), which were attenuated up to 270% and 248% compared with thrombin (2.7±0.2 mm²) (Fig. 6B). In addition, G-Ro (300 μM) plus wortmannin (10 μM) or miltefosine (10 μM) had a synergistic effects on clot retraction, which were attenuated up to 507% and 510%, respectively compared with thrombin plus G-Ro (300 μM) (8.8±0.2 mm²) (Fig. 6C).

Effects of G-Ro on blood coagulation
Blood coagulation is connected to platelet aggregation and thrombosis (20,21). Accordingly, we investigated the
Clot Retraction Inhibition by Ginsenoside Ro

Fig. 6. Effects of ginsenoside Ro (G-Ro) and phosphoinositide 3-kinase (PI3K)/Akt inhibitors on fibrin clot retraction. (A) Photographs of fibrin clot and (B) effects of G-Ro on thrombin-retracted fibrin clot (%). (C) Synergistic effects of G-Ro with PI3K/Akt inhibitors on thrombin-retracted fibrin clot (%). Quantification of fibrin clot retraction was performed as describe in "MATERIALS AND METHODS" section. 1)[base−thrombin]/base×100. 2)[thrombin−(thrombin+G-Ro or PI3K/Akt inhibitors)]/thrombin×100. 3)[(thrombin+G-Ro)−(thrombin+G-Ro+inhibitor)]/(thrombin+G-Ro)×100. The data are expressed as the mean±standard deviation (n=4). *P<0.05 versus the thrombin-stimulated human platelets and †P<0.05 versus the thrombin-stimulated human platelets in the presence of G-Ro (300 μM).

Table 1. Effects of ginsenoside Ro (G-Ro) on blood coagulation (unit: s)

|          | PT    | APTT  |
|----------|-------|-------|
| PPP      | 12.8±0.1 | 29.9±0.1 |
| G-Ro 50 μM | 12.7±0.7   | 30.1±0.3  |
| G-Ro 100 μM | 13.1±0.2   | 30.7±0.5  |
| G-Ro 200 μM | 13.1±0.3  | 30.0±0.4  |
| G-Ro 300 μM | 13.2±0.1NS | 31.2±0.5NS |

The results were expressed as the mean±standard deviation (n=4).
PT, prothrombin time; APTT, activated partial thromboplastin time; PPP, platelet poor plasma.
NS, not significant.

The effects of G-Ro on blood coagulation time (PT and APTT) as an index of bleeding. As shown in Table 1, both PT and APTT were not significantly prolonged by G-Ro (50 to 300 μM) compared with those (PT, 12.8±0.1 s; APTT, 29.9±0.1 s) by PPP. These results suggest that G-Ro does not function as an anticoagulant.

DISCUSSION

It has been reported that Korean red ginseng has anti-coagulation effects by PT and APTT in vitro (22). Thus, we focused on the anti-coagulation effect by G-Ro, however, G-Ro did not prolong PT and APTT in vitro (Table 1), which shows that G-Ro has no anti-coagulant characteristics and may not influence fibrin production. The clot retraction is the most crucial step in repair and healing on damaged portions of blood vessels. The clot formation that is produced through coagulation factors and platelet aggregation is composed of fibrin and platelets. The coagulation phase, extrinsic and intrinsic pathways, generates thrombin that converts circulating fibrinogen in plasma into the fibrin. At the same time, the activated platelets are accumulated in the damaged blood vessel and build up a fibrin-platelet meshwork. Additional circulatory platelets and blood cells are trapped in the meshwork, which accelerate the extension of clot formation. The clot formation that seals off the damaged vessel begins to undergo retraction over a period of 30 to 60 min and pulls the cut edges together. Thus, this is clear evidence that the inhibitory effect of G-Ro on clot retraction is due to the downregulation of αIIb/β3 activity.
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 (23). The αIIb/β3 continuously intensifies the platelet aggregation (24) and the formation of thrombus via outside-in signaling pathway and influence the progression of atherosclerosis (25,26).

Activation of αIIb/β3 is connected to inflammation, a cause of atherosclerosis, and its associated proteins (platelet-derived growth factor, vascular endothelial growth factor, p-selectin, and interleukin 1β) are secreted from α-granules (18,27). Even though G-Ro inhibits fibronectin- and fibrinogen-binding to αIIb/β3 and fibrin clot retraction, if G-Ro could not influence inflammation, anti-platelet effects by G-Ro would be doubtful. In our previous study, we showed that G-Ro significantly inhibited the expression of p-selectin (28), which means that G-Ro may be involved in the inhibition of inflammation by suppressing thrombin-induced p-selectin expression. Moreover, some experiments showed that G-Ro had anti-inflammatory activity in vivo and in vitro (29,30). Therefore, our study suggests that G-Ro may influence thrombosis and atherosclerosis.

With regard to the synergistic effect of G-Ro with PI3K/Akt inhibitors on thrombin-induced human platelet function, as shown in Fig. 2, platelet aggregation was strongly inhibited by G-Ro (300 μM) or miletefosine (10 μM) (29). As shown in Fig. 4B, the inhibitory effects of fibronectin adhesion by G-Ro (300 μM) plus wortmannin (10 μM) or miletefosine (10 μM) was higher compared with G-Ro (300 μM) alone. In addition, the fibrinogen binding inhibition by G-Ro (300 μM) plus wortmannin (10 μM) (29) was similar inhibitory activity on phosphatidylinositol 3-kinase.

Furthermore, thrombin-induced clot retraction is inhibited by G-Ro (300 μM), wortmannin (10 μM), or miletefosine (10 μM) (29). Therefore, it is thought that G-Ro (300 μM), wortmannin (10 μM), and miletefosine (10 μM) showed similar inhibitory effect in thrombin-induced human platelets and two similar substances interacted with each other and showed a strong synergistic effect (Fig. 6C). These results suggest that G-Ro inhibits platelet function by inhibiting PI3K/Akt signaling from the thrombin-mediated G protein-coupled receptor.

Our results demonstrate that G-Ro inhibits fibronectin- and fibrinogen-binding to αIIb/β3, via dephosphorylation of PI3K/Akt. The downregulation of αIIb/β3 activity by G-Ro suppressed the thrombin-induced clot retraction. Therefore, our study elucidated the αIIb/β3 inhibitory mechanism by G-Ro. Thus, G-Ro may be used as a therapeutic agent for prevention of thrombosis and other platelet-mediated cardiovascular diseases.

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AUTHOR DISCLOSURE STATEMENT

The author declares no conflict of interest.

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