The inhibitory activity of ginsenoside Rp4 in adenosine diphosphate-induced platelet aggregation

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A B S T R A C T
Background: Korean ginseng, Panax ginseng Meyer, has been used as a traditional oriental medicine to treat illness and promote health for several thousand years. Ginsenosides are the main constituents for the pharmacological effects of P. ginseng. Since several ginsenosides, including ginsenoside (G)-Rg3 and G-Rp1, have reported antiplatelet activity, here we investigate the ability of G-Rp4 to modulate adenosine diphosphate (ADP)-induced platelet aggregation. The ginsenoside Rp4, a similar chemical structure of G-Rp1, was prepared from G-Rg1 by chemical modification.

Methods: To examine the effects of G-Rp4 on platelet activation, we performed several experiments, including antiplatelet ability, the modulation of intracellular calcium concentration, and P-selectin expression. In addition, we examined the activation of integrin αIIbβ3 and the phosphorylation of signaling molecules using fibrinogen binding assay and immunoblotting in rat washed platelets.

Results: G-Rp4 inhibited ADP-induced platelet aggregation in a dose-dependent manner. We found that G-Rp4 decreased calcium mobilization and P-selectin expression in ADP-activated platelets. Moreover, fibrinogen binding to integrin αIIbβ3 by ADP was attenuated in G-Rp4-treated platelets. G-Rp4 significantly attenuated phosphorylation of extracellular signal-regulated protein kinases 1 and 2, p38, and c-Jun N-terminal kinase, as well as protein kinase B, phosphatidylinositol 3-kinase, and phospholipase C-γ phosphorylations.

Conclusion: G-Rp4 significantly inhibited ADP-induced platelet aggregation and this is mediated via modulating the intracellular signaling molecules. These results indicate that G-Rp4 could be a potential candidate as a therapeutic agent against platelet-related cardiovascular diseases.

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1. Introduction

Cardiovascular diseases (CVDs) are the highest cause of death in Western and Asian countries due to epidemiological transition, lifestyle changes, nutrition transition, and economic consequences in the modern world [1,2]. Recent studies have suggested that CVDs are mainly caused by abnormal platelet activation and aggregation. Atherosclerosis, one of the major CVDs, is a chronic inflammatory disease in the arterial vessel wall, and thus, causing plaque and thrombus formation [3,4].

Platelets, derived from megakaryocytes of bone marrow, are important factors relating to the progression of atherosclerosis. Platelets play an essential role in hemostasis, thrombosis, inflammatory processes, atherosclerosis, and tumor metastasis [5–7]. When there is vascular injury, platelets exposed to subendothelial matrix components including von Willebrand factor and collagen trigger adhesion and activation [8]. Platelet activation leads to the secretion of platelet granules including insulin-like growth factor 1, platelet-derived growth factor, transforming growth factor-beta, platelet factor 4, adenosine diphosphate (ADP), adenosine triphosphate, and serotonin, which contain other platelet agonists such as ADP, collagen, thrombin, and the generation of lipid-based agonists such as thromboxane A2, resulting in an increase in intracellular calcium ion ([Ca²⁺]) and platelet shape change. These
secondary agonists amplify the signals leading to fully platelet-cascade activation [9].
ADP plays an essential role in the process of hemostasis by signaling through G-protein coupled receptors (GPCR), P2Y1 and P2Y12 receptors. The Gq-coupled P2Y1 receptor plays a role in ADP-induced platelet shape change. P2Y1 receptor signaling is important for GPCR-mediated integrin activation, platelet granule release, and consequently platelet aggregation [10]. In addition, Gi-coupled-P2Y12 receptor induces platelet activation in the presence of the GPCR agonist ADP. The role of P2Y12 receptors in promoting platelet aggregation is consistent with inhibitory effects on cAMP, which relieves the inhibitory effect of cAMP-dependent protein kinase on platelet activation.

Korean ginseng (Panax ginseng Meyer) has been used as a traditional Korean medicine. This folk medicine has been used to treat illness and promote health for several thousand years [11]. The therapeutic potential of Korean ginseng has been discussed in a multitude of in vitro and in vivo studies [12]. Recently ginseng's marvelous effects have been studied in a wide range of pathological conditions, such as CVDs, immune diseases, ischemic heart disease, stroke, nervous disorders, and cancer [12]. Moreover, the ginseng-mediated protective mechanism is reported to be due to antioxidant, anti-inflammatory, antiaging, and antiapoptotic activity. In addition, the antiplatelet activities by ginseng extracts and some ginsenosides have also been determined [13,14]. However, information on the effect of ginsenoside (G)-Rp4 on platelet activation is limited. G-Rp4, a derivative from G-Rg1, is verified to be chemically stable in comparison to G-Rg1.

In this study, the antiplatelet properties of G-Rp4 were investigated using ADP-stimulated platelet aggregation. We further determined whether G-Rp1 affected intracellular downstream signal transductions (i.e., intracellular calcium ion concentration, granule secretions, signaling molecule phosphorylation, and fibrinogen binding to active integrin αIIbβ3).

2. Materials and methods

2.1. Materials

G-Rp4 with 99% purity was obtained from Ambo Institute (Seoul, Korea). ADP was purchased from Chronolog (Haverton, PA, USA). Fura-2-acetoxyethyl ester (Fura-2/AM) was procured from Sigma–Aldrich Co. (St. Louis, MO, USA). Mouse monoclonal to CD62P antibody and goat polyclonal to mouse immunoglobulin (Ig) G-H&L (fluorescein isothyocianate) antibodies were gained from Abcam (Cambridge, UK). Alexa Fluor488 fibrinogen conjugate was from Molecular Probes (Eugene, OR, USA). All other chemicals were of reagent grade. Antibodies of phospho-extracellular signal-regulated kinase-2 (ERK2; p44/p42), total-ERK2 (p44/42), phospho-p38, total-p38, phospho-c-Jun N-terminal kinase (JNK), total JNK, phospho-protein kinase B (Akt), total Akt, phospho-phosphatidylinositol 3-kinase (PI3K; p85/p55), and PI3K (p85), phospho-phospholipase C (PLC)-γ, total PLCγ, were purchased from Cell Signaling (Beverly, MA, USA).

2.2. Washed rat platelet preparation

Male Sprague Dawley 8–10-wk old rats (240–250g) were acquired from Orient Co. (Seoul, Republic of Korea). The rats were maintained in a standard laboratory animal facility with free access to food and water and they were acclimatized to these conditions for at least 1 wk before use. Whole blood from the rats was collected using a 23G needle inserted into the left ventricle of the heart and then transferred to a 14-mL round test tube including 1 mL of acid citrate dextrose solution (85mM trisodium citrate, 83mM dextrose, and 21mM citric acid) as an anticoagulant. Platelet-rich plasma (PRP) was prepared by centrifugation of the whole blood samples at 350 g for 7 min twice at room temperature, and the PRP was washed with washing buffer (Tyrode buffer 4). Washed platelets were then tenderly resuspended in Tyrode buffer (137mM NaCl, 12mM NaHCO3, 5.5mM glucose, 2mM KCl, 1mM MgCl2, 0.3mM NaHPO4, pH 7.4) to a final concentration of 3 × 10^8 platelets/mL [15].

2.3. Platelet aggregation in vitro assay

Platelet aggregation was performed as previously described [16,17]. PRP (3 × 10^8 platelets/mL) was preincubated for 2 min at 37°C in the presence of 1mM exogenous CaCl2 with various concentrations (6.25–50μM) of G-Rp4 and then platelet aggregation was encouraged by ADP (10μM). The reaction mixture was further incubated for 5 min with stirring at 250 g. The concentration of the vehicle was held at < 0.05%. The aggregation was monitored using an aggregometer (Chronolog, Haverton, PA, USA) at a constant stirring of 250 g and aggregation rates were measured as the light transmission changes were recorded for 9 min.

2.4. [Ca^{2+}]i measurement

The [Ca^{2+}]i was measured with Fura-2/AM as previously described [15]. Briefly, the washed platelets were incubated with Fura-2/AM (5μM) for 30 min at 37°C and platelet aggregation assay was done using the washed platelets. The Fura-2-loaded platelets (3 × 10^8/mL) were then preincubated with G-Rp4 for 2 min at 37°C in the presence of 1mM CaCl2, and subsequently stimulated with ADP for 5 min. Fluorescent signals were recorded using a F-2500 fluorescence spectrofluorometer (Hitachi, Ltd., Tokyo, Japan). Light emission was measured at 510 nm with simultaneous excitation at 340 nm and 380 nm that changed every 0.5 s. Fura-2 fluorescence in the cytosol measured with the spectrofluorometer was calculated as previously described [18,19].

2.5. Determination of P-selectin expression

Washed platelets, treated with various concentrations (6.25–50μM) of G-Rp4 or vehicle were stimulated with ADP (10μM) and incubated at 37°C for 5 min. The reaction was discontinued, and platelets were centrifuged consecutively and resuspended in cold phosphate-buffered saline (PBS), containing 10% fetal bovine serum and 1% sodium azide. Platelets were then incubated with CD62P primary antibody in 3% bovine serum albumin/PBS for 30 min at 4°C in the dark. Next, platelets were fixed using 0.5% paraformaldehyde and washed three times by centrifugation at 2,000 ×g for 5 min and resuspended in cool PBS one after another. Fluorescein isothiocyanate-conjugated secondary antibody was incubated in 3% bovine serum albumin/PBS for 30 min at 4°C in the dark. Flow-cytometry analysis was performed on a FACSArray II flow cytometer using CellQuest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) [11].

2.6. Determination of fibrinogen binding to integrin αIIbβ3

Washed platelets were at first treated with G-Rp4 or vehicle and incubated for 5 min at room temperature. Then, 10μM ADP was added together with 200 μg/mL Alexa Fluor 488-human fibrinogen (Molecular Probes, Eugene, OR, USA), and the sample was incubated at 37°C for 15 min. Alexa Fluor 488-fibrinogen binding to platelets was determined by flow cytometry using FACSArray III flow cytometer (Becton Dickinson Immunocytometry Systems), and
2.7. Immunoblotting

Platelets (5 × 10^8/mL) were activated with ADP for 5 min in the presence of 1mM CaCl2 with G-Rp4 (6.25–50μM) and instantly dissolved in sample buffer (0.125M Tris-HCl at pH 8.6, 2% fetal bovine serum, 2% β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue in the presence of 1mM phenethylsulfonylfluoride, 2 μg/mL aprotnin, 1 μg/mL leukemia, and 1 μg/mL pepstatin). Protein concentration was measured using bicinchoninic acid assay (PRO-MEASURE, iNtRON Biotechnology, Kyungki-Do, Korea) on ice. After boiling for 5 min, the proteins were resolved by electrophoresis in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membranes in a transfer buffer [25mM Tris (pH 8.5) and 20% methanol]. The membrane was blocked with 5% skim milk, washed, and subjected to immunoblotting with ERK1/2, p38, JNK, Akt, PI3K, PLCγ antibodies. The immunoblots were again incubated with horseradish peroxidase secondary antibody and the membranes were visualized using enhanced chemiluminescence (iNtRON Biotechnology).

2.8. Statistical analysis

Data were analyzed by one-way analysis of variance, using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA) tool, followed by a post hoc Dunnett’s test in order to determine the statistical significance of the differences between treatment groups. All data are presented as means ± standard deviation, and p < 0.05 were considered to statistically significant.

3. Results

3.1. Inhibitory effect of G-Rp4 on ADP-induced platelet aggregation

The concentration of ADP (10μM) could induce full activation and aggregation in rat washed platelets. Therefore, 10μM of ADP was used as the ligand to induce platelet aggregation in the further studies. While washed platelets were strongly activated by 10μM of ADP in the presence of 1mM CaCl2, G-Rp4 significantly reduced ADP-induced platelet aggregation in a concentration-dependent manner with an IC50 of 30.86 ± 0.12μM (Fig. 1B). However, G-Rg1 has not shown any antiplatelet activity in ligand-induced platelet aggregation (Fig. 1A).

3.2. G-Rp4 reduces ADP-induced [Ca^{2+}] mobilization

The release of granule contents boosts platelet activation signals and is considered to be an important indicator of the platelet-activating cascade. [Ca^{2+}] mobilization plays an essential role in platelet aggregation such as platelet activation and thrombus formation. Thus, we investigated the effects of G-Rp4 on ADP-induced [Ca^{2+}] mobilization. Pretreatment of the washed platelets with the various concentration of G-Rp4 (6.25–25μM) remarkably prevented the rise in ADP-stimulated [Ca^{2+}] (Fig. 2).

3.3. G-Rp4 inhibits P-selectin expression in ADP-activated platelets

Since granule secretions are important markers of platelet activation in the earlier phase of platelet aggregation, we determined whether G-Rp4 preincubation affected the granule release in
ADP-stimulated platelets. ADP-stimulated P-selectin secretion was remarkable and concentration-dependently reduced in G-Rp4 treated platelets (Figs. 3A and 3B). It showed that platelet α-granule secretory activity was significantly and dose-dependently impaired by G-Rp4 pretreatment.

3.4. G-Rp4 reduces fibrinogen binding to activated integrin-αIIbβ3

The ligation of integrin-αIIbβ3 function is the principal consequence of activation and adhesion in platelets and then aggregation as a result of the adhesive substrates bound to the membranes of activated platelets. Therefore, we determined the function of G-Rp4 on the response mechanism in integrin-αIIbβ3 activation. ADP-stimulated fibrinogen binding to its receptor was concentration-dependently inhibited in various G-Rp4-treatmented platelets (Fig. 4). As expected, ethylene glycol tetra-acetic acid (10μM) significantly blocked fibrinogen binding to active integrin-αIIbβ3 [20]. This result suggested that G-Rp4 may impair integrin-αIIbβ3 conformational changes for high correspondence fibrinogen binding site exposure (inside-out signaling) that arises as a result of prior platelet agonist interactions [21–23].

The next step of outside-in signaling implies a number of intracellular effectors, which is essentially associated with the integrin β3 subunit, PLCγ and PI3K [24–26].

3.5. G-Rp4 suppresses ADP-stimulated platelet mitogen-activated protein kinases phosphorylations

The antiplatelet effect is known to inhibit phosphorylation of mitogen-activated protein kinases (MAPKs; p38 MAPK, ERK, and

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**Fig. 3.** Effect of ginsenoside (G)-Rp4 on adenosine diphosphate (ADP)-activated P-selectin expression. Washed platelets (3 × 10^9/mL) were incubated with or without a G-Rp4 (6.25–25μM) in the presence of 1mM CaCl2 at 37°C for 2 min. The platelets were stimulated with adenosine diphosphate (10μM) for 3 min at 37°C. (A) Flow cytometry analysis was performed on a FACSAria III flow cytometer using CellQuest software. (B) The data are presented as mean ± standard deviation of three independent experiments. *p < 0.01. **p < 0.001 versus ADP-activated control. FITC, fluorescein isothiocyanate.
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4. Discussion

Ginseng contains saponins, proteins, amino acids, nucleic acids, fatty acid components, essential oils, polyacetylenes, phenolic compounds, terpenoids, and various other components. Among them, ginsenosides are the pharmacologically active components found in ginseng that are classified into 20(S)-propanaxatriolts such as Re and Rg1 and 20(S)-propanaxadiolts like Rg2 and Rh1, according to a site of glycoside [32].

Until now, research on ginseng has been carried out in order to investigate its pharmacological efficacy and various other medical and biological aspects. Ginsenosides are known to exhibit various effects by acting on physical functions such as central nervous, endocrine, immune, and metabolic systems [11,33—35]. Among these ginsenosides, Rg1 was reported to be efficacious for antifatigue and memory progression. Besides, G-Rp1 derived from G-Rg3 was reported to be potent in antiplatelet activity, but information on the effect of the stable compound G-Rp4 derived from G-Rg1 on platelet activation is limited. Therefore, we first examined whether G-Rp4 affected ADP-induced platelet aggregation, and then investigated its molecular mechanism of antiplatelet activity by analyzing downstream signal transduction (e.g., [Ca\textsuperscript{2+}]), mobilization, P-selectin secretion, MAPK and PI3K/Akt phosphorylation, and fibrinogen binding to active integrin αIIbβ3).

Previous reports showed that G-Rp1 has potent antiplatelet activity through the mechanisms of PI3K, PLC\textgamma, MAPKs, and phosphorylation of vasodilator-stimulated phosphoprotein by increasing cAMP [11]. In the present study, we have shown that G-Rp4 mediates its antiplatelet activity by phosphorylation of PLC\textgamma, PI3K, Akt, and MAPKs through signaling of GPCR P2Y12 binding to ADP.

Platelets, also called thrombocytes, are small, irregular-shaped blood cells derived from megakaryocytes that serve diverse roles in different pathological or physiological pathways in hemostasis and inflammation [13]. Platelet activation and aggregation occurs via many biochemical and biophysical pathways. Platelet agonists, such as collagen, ADP, thrombin, and thromboxane A2, activate platelets through GPCR pathways [36]. Platelets are usually present in the inactive state, but activation with agonists like collagen, ADP, thrombin, and thromboxane A2 turn on the active state of platelets [37]. Platelet aggregation occurs when membrane receptors bind to

Fig. 4. Ginsenoside (G)-Rp4 reduces fibrinogen binding to activated integrin-αIIbβ3. The inhibitory effect of G-Rp4 in fibrinogen binding to integrin-αIIbβ3 in adenosine diphosphate (ADP)-stimulated platelets was examined using flow cytometry analysis. Washed platelets (3 × 10\textsuperscript{5}/mL) were pretreated CaCl\textsubscript{2} 1mM with or without G-Rp4 at 37°C for 2 min and then the platelets were added to 10μM ADP with Alexa Fluor 488-human fibrinogen (200 μg/mL), and the sample was incubated at 37°C for 15 min. The bar graphs show the mean ± standard deviation of three independent experiments. *p < 0.05. **p < 0.001 versus ADP-activated control. EGTA, ethylene glycol tetra-acetic acid.

3.6. G-Rp4 inhibits the phosphorylation of PLC\textgamma and PI3K/AKT proteins involved in platelet glycoprotein signaling

PI3K plays a remarkable role in platelet—ADP receptor signaling pathway. Platelet glycoproteins and the activation of PLC\textgamma is significant for the most part of functional reactions to ADP, containing aggregation and alpha-granule secretion, so here we demonstrated the effect of G-Rp4 on the expression levels of the indicated proteins [31]. G-Rp4 suppressed ADP-stimulated PLC\textgamma and PI3K/AKT phosphorylation in a dose-dependent manner (Fig. 6).

Fig. 5. Effects of ginsenoside (G)-Rp4 on adenosine diphosphate (ADP)-stimulated phosphorylation of mitogen-activated protein kinases (MAPKs). Washed platelets (3 × 10\textsuperscript{5}/mL) were pretreated CaCl\textsubscript{2} 1mM with or without G-Rp4 at 10μM ADP for 5 min at 37°C. After completing the responses, total platelet proteins were extracted. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. (A) The membranes were then investigated with antibodies against total and phospho-extracellular-signal-regulated kinases 1 and 2 (ERK1/2), p38, stress-activated protein kinases/jun amino-terminal kinases (JNK). Antibody binding was visualized with chemiluminescence. All immunoblots are representative of three or four independent experiments. *p < 0.05. **p < 0.001 versus control.
Effects of ginsenoside (G)-Rp4 on adenosine diphosphate (ADP)-stimulated phosphorylation of phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), and phospholipase Cγ (PLCy). Washed platelets (3 x 10^6/mL) were preincubated at 37°C for 2 min with vehicle or various concentration of G-Rp4. The platelets were then stimulated with 10μM ADP at 37°C for 5 min. After completing the reactions, total platelet proteins were extracted. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. (A) The membranes were then incubated with antibodies against phospho-PLCy, phospho-PI3K, and phospho-Akt. Antibody binding was visualized with chemiluminescence. (B) The bar graph shows the mean ± standard deviation of three or four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle control.

In conclusion (Fig. 7), this study indicates that the inhibitory effect of G-Rp4 occurs in a concentration-dependent manner in ADP-induced platelet aggregation. In addition, G-Rp4 markedly suppressed intracellular downstream signal transduction (i.e., intracellular calcium mobilization and P-selectin expression). Furthermore, we found that G-Rp4 notably inhibited the phosphorylation of all three MAPKs (i.e., ERK1/2, JNK, and p38 MAPK) and PI3K/Akt phosphorylation. Finally, integrin-αIIbβ3 activation, the final step of full platelet activation, was remarkably suppressed by G-Rp4 pretreatment. Based on our present results, we suggest that G-Rp4 showed antiplatelet activity via the modulation of intracellular downstream signaling pathways.

Fig. 6. Effects of ginsenoside (G)-Rp4 on adenosine diphosphate (ADP)-induced platelet aggregation. Akt, protein kinase B; G, ginsenoside; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase.

Fig. 7. The schematic diagram for possible inhibitory activity of ginsenoside Rp-4 on adenosine diphosphate (ADP)-induced platelet aggregation. Akt, protein kinase B; G, ginsenoside; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase.
signaling components. Therefore, C-RP4 can be regarded as a potential therapeutic agent for preventing CVDs, such as thrombosis and ischemia.

**Conflicts of interest**

The authors declare that they have no competing interests.

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