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Research article

The inhibitory mechanism of crude saponin fraction from Korean Red Ginseng in collagen-induced platelet aggregation

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ABSTRACT

Background: Korean Red Ginseng has been used as a traditional oriental medicine to treat illness and to promote health for several thousand years in Eastern Asia. It is widely accepted that ginseng saponins, ginsenosides, are the major active ingredients responsible for Korean Red Ginseng's therapeutic activity against many kinds of illness. Although the crude saponin fraction (CSF) displayed antiplatelet activity, the molecular mechanism of its action remains to be elucidated.

Methods: The platelet aggregation was induced by collagen, the ligand of integrin $\alpha_{II}\beta_I$ and glycoprotein VI. The crude saponin's effects on granule secretion [e.g., calcium ion mobilization and adenosine triphosphate (ATP) release] were determined. The activation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated protein kinase 1/2 (ERK1/2), c-Jun N-terminal kinases (JNKs), and p38 MAPK, and phosphoinositide 3-kinase (PI3K)/Akt was analyzed by immunoblotting. In addition, the activation of integrin $\alpha_{II}b\beta_{III}$ was examined by fluorocytometry.

Results: CSF strongly inhibited collagen-induced platelet aggregation and ATP release in a concentration-dependent manner. It also markedly suppressed $[Ca^{2+}]_i$ mobilization in collagen-stimulated platelets. Immunoblotting assay revealed that CSF significantly suppressed ERK1/2, p38, JNK, PI3K, Akt, and mitogen-activated protein kinase kinase 1/2 phosphorylation. In addition, our fraction strongly inhibited the fibrinogen binding to integrin $\alpha_{IIb}\beta_3$.

Conclusion: Our present data suggest that CSF may have a strong antiplatelet property and it can be considered as a candidate with therapeutic potential for the treatment of cardiovascular disorders involving abnormal platelet function.

1. Introduction

Platelet aggregation is essential for the normal hemostatic process when blood vessels are injured. However, aberrant platelet activation causes cardiovascular diseases such as thrombosis, atherosclerosis, and myocardial infarction [1]. Several agonists [e.g., collagen, thrombin, and adenosine diphosphate (ADP)] can bind to glycoprotein receptors or G-protein coupled receptors, and lead to subsequent activation of intracellular downstream signaling molecules. The activation of these receptors can stimulate phospholipase C, which decomposes phosphatidylinositol 4,5-bisphosphate into two second messengers, namely, inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol [2,3]. IP_3 binds to IP_3 receptor of calcium storage pool (i.e., dense tubular system) and releases Ca^{2+} into the cytoplasm. Thus, the intracellular calcium ion ($[Ca^{2+}]_i$) concentration can be increased by more than 10-fold. $[Ca^{2+}]_i$ can bind to the calcium-binding protein calmodulin and activate myosin light-chain kinase. This kinase causes the phosphorylation of the regulatory light chain of myosin,

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which in turn causes the activation of platelet [4,5]. As the result, platelet aggregation occurs and the activation of platelet that goes beyond this range causes cardiovascular diseases such as thrombosis, atherosclerosis, and myocardial infarction. Therefore, the suppression of platelet function by natural products represents a promising approach to the prevention of thrombosis.

To suppress or prevent the atherosclerosis and thrombosis, many antiplatelet drugs have been developed [6]. However, these antiplatelet drugs have had various side effects in some patients, such as gastrointestinal side effects, hemorrhage, and reduction of platelets [7–10]. As a result, increasing attention has been paid to dietary supplements, prevention of cardiovascular diseases for the safety of drug use, and development of oriental medicines for treatment [11].

Korean Red Ginseng has been used as a traditional oriental medicine to treat illness and promote health for several thousand years. Moreover, it is reported that Korean Red Ginseng is an effective folk medicine against stress, heart failure, hypertension, diabetes, and so on [12,13]. Korean Red Ginseng contains many active ingredients such as polysaccharides, peptides, fatty acids, mineral oils, and ginsenosides. Among these components, ginseng saponin (i.e., ginsenoside) is one of the well-known bioactive ingredients in ginseng, and thus far more than 30 ginsenosides have been isolated and characterized. Ginsenosides are classified into protopanaxadiol (PPD) and protopanaxatriol (PPT) saponins. PPD includes ginsenoside Rb1 (G-Rb1), G-Rb2, G-Rc, and G-Rd, whereas PPT contains G-Rg1, G-Re, G-Rf, and G-Rg2.

Although the antiplatelet activities of saponin fraction and several ginsenosides have been reported [14–17], the molecular mechanism of its action is not yet well understood.

In this study, we have demonstrated that the crude saponin fraction (CSF) has strong inhibitory activities on collagen-induced platelet aggregation. In addition, we determined the molecular mechanism underlying the antiplatelet activity of CSF.

2. Materials and Methods

2.1. Materials

CSF was obtained from KGC Research Institute (Daejeon, Korea). CSF was analyzed by HPLC and the composition of ginsenosides is as follows: 134 mg/g of G-Rg1, 106 mg/g of G-Re, 52 mg/g of G-Rf, 181 mg/g of G-Rb1, 165 mg/g of G-Rc, 108 mg/g of G-Rb2, 18 mg/g of Rb3, 72 mg/g of Rd, 44 mg/g of F2, 28 mg/g of Rg3. Collagen was obtained from Chrono-Log Co. (Havertown, PA, USA). Fura-2-acetoxymethyl ester (Fura-2/AM) and dimethyl sulfoxide (DMSO) were from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies to phospho-p44/42 [p-extracellular signal-regulated protein kinase 1/2 (p-ERK1/2)], p44/42 (T-ERK1/2), phospho-p38 (p-p38), p38 (T-p38), phospho-SAPK (phospho-stress-activated protein kinase)/c-Jun N-terminal kinase (p-JNK), SAPK/JNK (T-JNK), phospho-phosphoinositide 3-kinase (phospho-PI3K; p-p85), PI3K (T-p38), phospho-mitogen-activated protein kinase kinase (p-MEK1/2), MEK1/2 (T-MEK1/2), phospho-Akt (p-Akt), and Akt (T-Akt) were acquired from Cell Signaling Technology (Beverly, MA, USA). Adenosine triphosphate (ATP) assay kits were purchased from Biomedical Research Service Center (Buffalo, NY, USA). Fibrinogen Alexa Fluor 488 conjugate was obtained from Molecular Probes (Eugene, OR, USA). All other chemicals were of reagent grade.

2.2. Experimental animals

Male Sprague Dawley rats (300–350 g) were obtained from Orient Co. (Seoul, Korea). The animals were acclimated for 1 wk before the experiments, and housed in an air-conditioned animal room with a 12/12-h light/dark cycle at a temperature of $22 \pm 1^{\circ}C$ and humidity of 50% ± 10%. All animals were provided with a laboratory diet and water *ad libitum*. All experimental protocols involving the use of animals were conducted in accordance with the National Institutes of Health guidelines and approved by the Committee on Animal Care at the Kyungpook National University.

2.3. Washed rat platelet preparation

Blood was withdrawn from the abdominal vein of rats and collected directly into an anticoagulant citrate dextrose solution that contained 0.8% citric acid, 2.2% trisodium citrate, and 2% dextrose (w/v). Washed platelets were prepared as previously described. In brief, platelet-rich plasma (PRP) was obtained by centrifuging rat blood samples at 230*g* for 10 min. Platelets were precipitated by centrifugation of the PRP at 800*g* for 15 min and washed with HEPES buffer (137mM NaCl, 2.7mM KCl, 1mM $MgCl_2$, 5.6mM glucose, and 3.8mM HEPES; pH 6.5) containing 0.35% bovine serum albumin and 0.4mM ethylene glycol tetraacetic acid (EGTA). The washed platelets were resuspended in HEPES buffer (pH 7.4) and the cell dilution was adjusted to 4×10^8 cells/mL.

2.4. Platelet aggregation

Platelet aggregation was evaluated as previously described [18]. Aggregation was monitored by measuring light transmission with an aggregometer (Chrono-Log Co.) at constant stirring speed (1,200 rpm). The washed platelets (3×10^8/mL) were preincubated at 37°C for 2 min with either CSF or vehicle and then stimulated with 2.5 mg/mL collagen. The mixture was further incubated for 5 min with stirring at 170*g*. The vehicle concentration was less than 0.1% to minimize the effect of this reagent.

2.5. $[Ca^{2+}]_i$ measurement

The intracellular calcium ion concentration ($[Ca^{2+}]_i$) was measured with Fura-2/AM as previously described [19]. In brief, the platelets were incubated with 5μM of Fura-2/AM for 30 min at 37°C and washed. The Fura-2-loaded platelets (3×10^8/mL) were then preincubated with MAE for 2 min at 37°C in the presence of 1mM $CaCl_2$, and subsequently stimulated with collagen for 5 min. Fluorescent signals were recorded using a Hitachi F-2500 fluorescence spectrofluorometer (Hitachi, Japan). Light emission was measured at 510 nm, with simultaneous excitation at 340 and 380 nm that changed every 0.5 seconds. Fura-2 fluorescence in the cytosol measured with the spectrofluorometer was calculated as previously described by Schaeffer and Blaustein [20] using the following formula: $[Ca^{2+}]_i$ 224nM $\times (F - F_{min})/(F_{max} - F)$, where 224nM is the dissociation constant of the Fura-2–Ca^{2+} complex, and F_{min} and F_{max} represent the fluorescence intensity levels at very low and very high Ca^{2+} concentrations, respectively. In our experiment, F_{max} was the fluorescence intensity of the Fura-2–Ca^{2+} complex at 510 nm after the platelet suspension containing 1mM of $CaCl_2$ had been solubilized with Triton X-100 (0.1%). F_{min} was the fluorescence intensity of the Fura-2–Ca^{2+} complex at 510 nm after the platelet suspension containing 20mM Tris/3mM of EGTA had been solubilized with Triton X-100 (0.1%). *F* represented the fluorescence intensity of the Fura-2 complex at 510 nm after the platelet suspension was stimulated with collagen with or without crude saponin fraction in the presence of 1mM $CaCl_2$.

2.6. ATP release assay

Washed platelets (3×10^8/mL) were preincubated for 2 min at 37°C with various concentrations of CSF and then stimulated with 2.5 μg/mL collagen. After the reaction was terminated, the cells were