**Galla rhois** exerts its antiplatelet effect by suppressing ERK1/2 and PLCβ phosphorylation

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**Abstract**

*Galla rhois* and its components have various biological activities, including protective effects on liver cells as well as antimetastatic, antiplatelet, and antibacterial effects. In the present study, we identified the antiplatelet activity and possible mechanism of action of a *G. rhois* extract (GRE). We investigated the effect of GRE and its components on rabbit platelet activation, and their possible molecular mechanisms. The GRE inhibited collagen-, AA-, and thrombin-induced platelet aggregation as well as serotonin secretion, in a concentration-dependent manner. The GRE significantly inhibited the production of lipoygenase-mediated 12-hydroxyeicosatetraenoic acid. The GRE effectively suppressed thrombin-stimulated PLCβ3 phosphorylation and collagen-induced ERK1/2 phosphorylation, in addition, the GRE significantly restored the cAMP level, which had decreased due to collagen or thrombin. Among the components of GRE, methyl gallate inhibited the collagen-induced platelet activation through suppression of ERK phosphorylation, penta-O-galloyl-β-D-glucoside inhibited the thrombin-induced platelet activation through suppression of PLCβ phosphorylation.

These results indicate that the GRE including methyl gallate and penta-O-galloyl-β-D-glucoside suppressed platelet activation by inhibiting ERK1/2 and PLCβ3 phosphorylation.

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**1. Introduction**

Platelet activation plays an essential role in the prevention and treatment of cardiovascular and atherothrombotic diseases (Marcus and Safier, 1993; Ruggeri, 2002; Vilahur et al., 2004a). Accordingly, the mechanism of platelet activation and aggregation is considered a new avenue for the development of antithrombotic agents (Badimon, 2001; Vilahur et al., 2004b). Platelet activation can be initiated through various receptor pathways by agonists such as collagen, thrombin, and ADP, which induce platelet activation and trigger platelet signaling events arising from intracellular signaling molecules (Brash, 1985; Garcia-Arguinzonis et al., 2010; Molins et al., 2010). During platelet activation, intracellular calcium is mobilized by the phosphorylation of phospholipase C (PLC) isoforms (β and γ) by activating receptors (Nby et al., 1996; Shattil and Brass, 1987), resulting in the hydrolysis of arachidonic acid (AA) from phospholipid membranes (Brash, 1985). AA is converted to metabolites such as thromboxane (TXB) A2, prostaglandin (PG), and 12-hydroxyeicosatetraenoic acid (12-HETE) by cyclooxygenase (COX)-1 and 12-lipoxygenase (LOX) (Aharony et al., 1982; Brash, 1985).

ERK1/2 is activated through a thrombin-mediated pathway to stimulate cytosolic phospholipase A2 (cPLA2) (Borsch-Haubold et al., 1998; Kramer et al., 1995; Lin et al., 1993). Therefore, mitogen-activated protein kinases, including ERK1/2, induce granule secretion, platelet aggregation, and thrombosis (Li et al., 2001; Oury et al., 2002; Roger et al., 2004; Sakurai et al., 2004).

*Galla rhois* (*Rhus javanica*) is a traditional herbal medicine used to treat various diseases, including diarrhea, dysentery, hemorrhaging, thrombosis, and skin diseases (Kim et al., 2005; Shim et al., 2003; Song et al., 2002). Moreover, its components have various biological activities, including a protective effect on liver cells as well as antimetastatic, antiplatelet, and antibacterial effects (Ahn et al., 1998; Ata et al., 1996; Park et al., 2008; Song et al., 2002). Although Song et al. (2002) demonstrated the antithrombotic and antiplatelet activity of a *G. rhois* extract (GRE), its target mechanism has not been established. Accordingly, we identified the antiplatelet activity and possible mechanism of action of a GRE.

**2. Materials and methods**

**2.1. Materials**

TXB2, prostaglandin PGD2, and 12-HETE were obtained from Cayman Chemical (Ann Arbor, MI, USA). [3H] AA (250 μCi mmol−1) was purchased from New England Nuclear (Boston, MA, USA). Indomethacin, bovine serum albumin (BSA), ethylene...
glycol-bis-(β-aminoethy1 ether)-N,N,N′,N′-tetraacetic acid (EGTA), serotonin creatinine sulfate, o-phthalaldehyde, imipramine, acetylsalicylic acid (ASA, aspirin), and dimethyl sulfoxide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Collagen, AA, and thrombin were purchased from Chrono-Log Co. (Haverton, PA, USA). All other chemicals were of analytical grade. The standard compound of gallic acid was purchased from TCI (Tokyo, Japan) and methyl gallate, syringic acid and penta-O-galloyl-β-D-glucose were purchased from Sigma−Aldrich (St. Louis, MO, USA). The purity of marker compound determined using HPLC was higher than 98%. HPLC grade acetonitrile and methanol were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). Analytical grade acetic acid was obtained from Duksan. (Gyeonggi-do, Korea). The 3rd distilled water was filtered through a 0.45 µm membrane filter (ADVANTEC, Japan) after analysis.

2.2. Animals

New Zealand white rabbits (weight, 2.5−3 kg each) were purchased from Samtako Animal Co. (Osan, Korea) and acclimated for 1 week at 24 ± 1°C and 55 ± 5% humidity. The animals had free access to a commercial pellet diet obtained from Samyang Co. (Wonju, Korea) and drinking water. All animal studies were carried out in accordance with Korean Institute of Oriental Medicine Care Committee Guidelines.

2.3. GRE preparation

Galla rhois was obtained from Yeongcheon Oriental Herbal Market (Yeongcheon, Korea). All voucher specimens were deposited in the herbal bank at the Center for Herbal Medicine Improvement Research, Korean Institute of Oriental Medicine. To prepare the GRE, dried G. rhois (50.0 g) was placed in 1000 ml of distilled water and extracted by heating for 3 h at 115°C (Gyeongseo Extractor Cosmos-600, Inchon, Korea). After extraction, the solution was filtered using a standard test sieve (150 µm). (Retsch, Haan, Germany), freeze-dried, and maintained in desiccators at 4°C before use. One acquisition was 2.32 g; the yield was 4.64%.

2.4. Washed rabbit platelet preparation and platelet aggregation

Blood was withdrawn from the ear artery of male white rabbits and collected directly into 0.15% (v/v) of anticoagulant citrate dextrose solution containing 0.8% citric acid, 2.2% trisodium citrate, and 2% dextrose (w/v). Washed platelets were prepared as described previously (Lee et al., 2009). Briefly, platelet rich plasma (PRP) was obtained by centrifugation of rabbit blood at 230 × g for 10 min. Platelets were pelleted by centrifugation of the PRP at 800 × g for 15 min and washed with HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 3.8 mM HEPES, pH 6.5) containing 0.35% BSA and 0.4 mM EGTA. The washed platelets were suspended in HEPES buffer (pH 7.4) and adjusted to 4 × 10^10 cells/ml. Platelet aggregation was measured with an aggregometer (Chrono-Log Co.) according to the turbidimetric method of Born and Cross (1963). The washed platelet suspension was incubated at 37°C in the aggregometer with stirring at 1200 rpm, and various concentrations of the GRE or its components or their subcomponents were added. After 3 min of preincubation, platelet aggregation was induced by the addition of collagen (5 µg/ml), AA (100 µM), or thrombin (0.05 U/ml).

2.5. Serotonin secretion

Serotonin release was measured as described previously (Lee et al., 2009). In brief, to prevent the reuptake of secreted serotonin, imipramine (a serotonin reuptake inhibitor, 5 µM) was added to the PRP. Washed rabbit platelets were treated with various concentrations of the GRE at 37°C for 3 min prior to the addition of agonist (5 µg/ml collagen, 100 µM AA, or 0.05 U/ml thrombin) for 5 min. An aliquot (0.35 ml) of the washed rabbit platelets was mixed with 5 mM EDTA on ice and centrifuged at 12,000 × g for 2 min. The supernatant was mixed with 6 M trichloroacetic acid (TCA) and centrifuged at 12,000 × g for 2 min. An aliquot (0.3 ml) of the TCA supernatant was mixed with 1.2 ml of 0.5% o-phthalaldehyde diluted 1:10 in ethanol, 1 M NaCl, placed in a boiling water bath for 10 min, and cooled on ice. Excess lipid was extracted with chloroform, and the fluorophore was measured at excitation and emission wavelengths of 360 and 475 nm, respectively. Serotonin creatinine sulfate was used as the standard solution to calculate serotonin release.

2.6. Immunoblotting

To assess platelet proteins, platelets were preincubated with the GRE or its components at the indicated concentrations and then stimulated with collagen (10 µg/ml) or thrombin (0.05 U/ml) for 5 min. The reaction was terminated by the addition of Laemmli sample buffer, and the mixture was boiled for 5 min. Proteins were resolved by 7.5% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting was performed as described previously (Lee et al., 2010). All antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). The immunoreactivities of the proteins were detected and visualized using an enhanced chemiluminescence kit, and the detected protein quantities were normalized to those of β-actin or the respective total protein, respectively. The intensity of each band was quantified using the Scion-Image for Windows program (Scion Corp., Wellesley, MA, USA).

2.7. AA liberation

PRP was preincubated with [3H]-AA (1 µCi/ml) at 37°C for 1.5 h and then washed as described previously (Lee et al., 2009). Briefly, [3H]-AA−labeled platelets (4 × 10^10 cells/ml) were pretreated with 100 µM 3-amino-1-[m−(trifluoro-methyl)phenyl]-2-pyrazoline (BW755C), a potent inhibitor of COX and LOX, and the platelets were preincubated with various concentrations of the GRE or ASA (COX inhibitor) at 37°C for 3 min in the presence of 1 mM GaCl3 and then stimulated with collagen (50 µg/ml). The reaction was terminated by adding chloroform/methanol/HCl (200/200/1, v/v/v). Lipids were extracted and separated by thin-layer chromatography (TLC) on silica gel G plates with a petroleum ether−diethyl ether:acetic acid (40:40:1, v: v: v) development system. The area corresponding to each lipid was scraped off, and radioactivity was detected by liquid scintillation counting.

2.8. AA metabolites

AA metabolites such as TXB2, PGD2, and 12-HETE were measured as described previously (Lee et al., 2009). Briefly, washed rabbit platelets (4 × 10^10 cells/ml) were preincubated with various concentrations of the GRE or ASA at 37°C for 3 min and further incubated with a mixture of [3H]-AA (1 µCi/ml) and unlabeled AA (2 µM) for 5 min. The reaction was terminated by adding stop solution (2.6 mM EGTA and 130 µM BW755C). Lipids were extracted and separated by TLC on silica gel G plates with an ethyl acetate:isoctane:acetic acid:water (11:5:2:10, v:v:v:v) development system. The area corresponding to each lipid was scraped off, and radioactivity was detected by liquid scintillation counting.

2.9. Adenylyl cyclase activity

Washed rabbit platelets were preincubated with various concentrations of the GRE at 37°C for 3 min and then exposed to collagen (10 µg/ml) or thrombin (0.05 U/ml). The reaction was terminated by adding lysis buffer containing dodecyl-trimethylammonium bromide. Cyclic adenosine monophosphate (cAMP) levels were assayed using an enzyme-linked immunosorbent assay kit according to the manufacturer’s protocol.

2.10. Preparing of standard solution and GRE sample

The stock solutions of gallic acid (GA), methyl gallate (MG), syringic acid (SA) and penta-O-galloyl-β-D-glucose (TGGC) were prepared by dissolving 1 mg of each standard in 1 ml of pure methanol to yield a final concentration of 1000 µg/ml. These stock solutions were stored at −4°C before analysis. Each stock solution was diluted to the appropriate concentration range for the preparation of the calibration curves.
A 1 mg of GRE within 10 mL of 3rd distilled water was extracted by ultrasonication and filtered through a 0.2 mm syringe membrane filter (Whatman Ltd, Maidstone, UK) before injection into the HPLC system for analysis. All solutions were stored at -4 °C in a refrigerator.

2.11. HPLC-DAD analysis

In this study, the HPLC analysis were performed using the Waters HPLC 2695 system equipped with a pump, an auto sampler, a column oven, and a 996 photodiode array UV/VIS detector (Waters HPLC System, MA, USA), and the Empower software program was used for data acquisition and processing. The chromatographic column that was used in this experiment was commercially available and was obtained from Optimapak (C18, 4.6 × 250 mm, 5 μm). The column oven temperature was kept at 30 °C. The injection volume was 10 μL, and the flow rate of the mobile phase was 1.0 mL/min. The wavelength of the UV detector was set at 270 nm. The mobile phase was composed of water containing 0.25% acetic acid (A) and acetonitrile containing 0.25% acetic acid (B). The run time was 90 min and the mobile-phase program was the gradient elution, as follows: 5% (v/v) B at 0–20 min; 20–45% B at 20–45 min; 45–90% B at 45–90 min.

2.12. Statistical analysis

All results are expressed as means ± standard error and were analyzed using an analysis of variance. A p-value <0.05 was considered significant.

Fig. 2. Effect of a Galla rhois extract (GRE) on serotonin secretion. Washed rabbit platelets were preincubated with the indicated concentrations of the GRE for 3 min, and the effect of the GRE on serotonin level was measured in collagen- (5 μg/ml) (A), arachidonic acid- (AA, 100 μM) (B), and thrombin-stimulated (0.05 U/ml) (C) platelets. The results are the average of three similar and independent experiments. The values shown are means ± standard error. *p < 0.05; **p < 0.01.

Fig. 3. Effects of a Galla rhois extract (GRE) on phospholipase (PLC)γ2 and PLCβ3 phosphorylation and their downstream pathway. Platelets were preincubated with the GRE at the indicated concentrations and then stimulated with collagen (5 μg/ml) or thrombin (0.05 U/ml) for 5 min. The reaction was terminated by the addition of Laemmli sample buffer, and the mixture was boiled for 5 min. The gel images are representative blots of four similar independent experiments. The values shown are means ± standard error. *p < 0.05; **p < 0.01.
3. Results

3.1. Antiplatelet effect of the GRE

To confirm the antiplatelet activity of the GRE, the ability of the GRE to inhibit platelet aggregation was investigated using washed rabbit platelets in vitro. Fig. 1 shows that the GRE inhibited collagen-, AA-, and thrombin-induced rabbit platelet aggregation in a concentration-dependent manner, respectively. In addition, we confirmed that the antiplatelet effect of the GRE was not due to cellular cytotoxicity using a WST-1 assay (data not shown).

In addition, the GRE potently inhibited collagen-, AA-, and thrombin-induced serotonin secretion (Fig. 2), consistent with our platelet aggregation results. Serotonin is secreted from activated platelets during platelet aggregation (Nishihira et al., 2006). The GRE inhibited serotonin secretion in a concentration-dependent manner, with inhibition percentages of 2.5%, 42.2%, and 66.9% for collagen (Fig. 2A); 40.8%, 66.3%, and 69.2% for AA (Fig. 2B); and 12.7%, 52.7%, and 60.6% for thrombin (Fig. 2C) at 100, 300, and 500 µg/ml, respectively. In addition, the total platelet serotonin content was expressed as lysis in Fig. 2.

3.2. GRE suppresses PLCβ3, p47, and ERK1/2 phosphorylation

To examine the molecular basis of the observed antiplatelet activity, the GRE was added to platelets treated with collagen or...
thrombin, and PLCγ2 and PLCβ3 phosphorylation was assessed. In addition, to investigate the effects of the GRE on the downstream pathway of collagen- or thrombin-induced PLCγ2 and PLCβ3 activation, ERK1/2 and p47 phosphorylation was measured in collagen- and thrombin-stimulated platelets.

Fig. 3A shows that the GRE effectively reduced ERK1/2 and p47 phosphorylation stimulated by collagen, whereas PLCγ2 was unaffected. Moreover, the GRE markedly suppressed thrombin-induced PLCβ3, ERK1/2, and p47 phosphorylation (Fig. 3B). These results indicate that the GRE not only downregulated the activation of ERK1/2 and p47 phosphorylation for collagen-induced platelet activation but also suppressed p47 phosphorylation by downregulating PLCβ3 phosphorylation and ERK1/2 phosphorylation during thrombin-induced platelet activation.

3.3. The GRE did not affect PLCγ2 phosphorylation-mediated AA liberation

Collagen was added to platelets to confirm the selective involvement of the GRE in PLCγ2-mediated AA and DAG formation. Fig. 4 shows that the GRE had no effect. These results indicate that the GRE did not affect upstream signaling for AA and DAG liberation, consistent with our PLCγ2 phosphorylation result. ASA was used as a negative control, but did not affect AA or DAG liberation at 100 μg/ml.

3.4. The GRE significantly inhibited LOX-mediated 12-HETE conversion

The production of COX-mediated TXB2 and PGD2 and LOX-mediated 12-HETE was measured in AA-treated platelets to investigate the effects of the GRE on AA metabolism. The GRE significantly suppressed 12-HETE production at 500 μg/ml (Fig. 5C), whereas it did not affect TXB2 or PGD2 production (Fig. 5A and B).

3.5. GRE significantly activated adenylyl cyclase

To assess the effect of the GRE on adenylyl cyclase activation, cAMP was measured in collagen- and thrombin-treated platelets. Increases in cAMP are known to decrease the intracellular Ca2+ level in platelets due to the inhibition of Ca2+ mobilization into the dense tubular system through the suppression of PLC and protein kinase C (Walter et al., 1993). The GRE significantly restored the cAMP level decreased by collagen or thrombin at a concentration of 500 μg/ml, indicating that the GRE increased the cAMP level as much as that in the unstimulated control, consistent with the downregulation of PLC and p47 phosphorylation (Fig. 6).

3.6. HPLC analysis of standard and GRE

To find the optimum absorbance for each analyte, we obtained the UV/Vis spectra at the range of 190–400 nm (data not shown). Optimum absorbances for all analytes were as follows: at GA: 268 nm, MG: 269 nm, SA: 273 nm and PGG: 277 nm. Hence, GA, MG, SA and PGG were determined to be detected at 270 nm. The qualitative identification of four standards in GRE were achieved via RP-HPLC, and based on the comparisons of UV spectra with those of the standard compounds.

The four standards peaks were appeared at GA: 7.65 min, MG: 27.55 min, SA: 42.25 min and PGG: 77.51 min (Fig. 7A). This result shows that in the mixture of four standard compounds and the sample were successfully separated and simultaneously analyzed. The identification of the certified compounds of GRE was based on the comparisons of their retention times (tR) and chromatogram with those of the standard compounds in Fig. 7. The linearity of detection for each analyte was examined using four standards solutions: at GA: 0.1, 10, 100 and 400 μg/mL, MG, SA and PGG: 0.005, 0.5, 5 and 10 μg/mL. The calibration curve between the peak area and the concentration of four standards form the certified standard GRE sample shown excellent linearity in the range. The correlation coefficient was 0.9993–0.9999 (Table 1).

3.7. Determination of activity components

To determine the activity components of GRE, we investigated the effect of PGG, SA, GA and MG on platelet aggregation and the mechanism underlying the antiplatelet activity of GRE. On collagen-stimulated platelet aggregation, MG only showed the inhibition effect in a concentration-dependent manner. PGG, SA and GA weakly inhibited the thrombin-stimulated platelet aggregation, however, MG did not affect. In addition, MG concentration-dependently inhibited ERK phosphorylation induced by collagen, PGG significantly inhibited PLCβ3 phosphorylation induced by thrombin. These results indicated that the inhibition effect of GRE in collagen-induced platelet activation is due to suppression of ERK phosphorylation by MG, the inhibition effect of GRE in thrombin-induced platelet activation is due to suppression of PLCβ phosphorylation by PGG.

4. Discussion

As mentioned above, the antiplatelet effect of GRE has been already reported by Song et al. (2002), thus, we should demonstrate to involve additional scientific findings (Song et al., 2002). Because the findings of previous report just showed the activity of GRE
without the molecular biological evidence, the present study identified the antiplatelet activity and possible mechanism of action of GRE for establishing the target mechanism. The GRE produced in this study possessed antiplatelet activity involved in inhibiting serotonin secretion, and the molecular basis for the antiplatelet activity of GRE might be mediated by suppressing PLCβ3, p47, and ERK1/2 phosphorylation and inhibiting LOX-mediated AA metabolism.

Platelet aggregation and activation are major targets for thrombotic complications such as myocardial infarction, acute coronary syndrome, atherosclerosis, stroke, and ischemia [Brass, 2003; Majid et al., 2001; Ruggeri, 2002]. Thus, inhibiting platelet activation while treating thrombotic disease leads to suppressed thrombosis formation and progression and, therefore, is important for preventing complications after an acute coronary complication (Wijns et al., 2010). Platelet activation is mainly mediated through the adhesion of platelets to the site of injury and through the action of endogenous agonists such as collagen, ADP, and thrombin, followed by the release of TXA2 and serotonin, which act as amplifying factors during platelet aggregation (Farndale et al., 2004; Jackson et al., 2003). Stimulating platelets with agonists such as collagen, thrombin, and ADP causes PLC phosphorylation (Mangin et al., 2003). PLC is a key enzyme in platelet signal transduction. Among PLC isoforms, PLCβ2 and PLCβ3 are involved in collagen- and thrombin-dependent signaling in platelets, respectively (Bunney and Katan, 2006; Ragab et al., 2007).

In the present study, the GRE effectively reduced ERK1/2 and p47 phosphorylation stimulated by collagen, whereas PLCc2 was unaffected. Moreover, the GRE markedly suppressed thrombin-induced PLCβ3, ERK1/2, and p47 phosphorylation. These results indicate that the GRE not only downregulated ERK1/2 and p47 phosphorylation during collagen-induced platelet activation but also suppressed p47 phosphorylation by downregulating PLCβ3 and ERK1/2 phosphorylation during thrombin-induced platelet
activation. As a result, the GRE not only inhibited collagen-, AA-, and thrombin-induced rabbit platelet aggregation but also potently inhibited collagen-, AA-, and thrombin-induced serotonin secretion in a concentration-dependent manner, consistent with our platelet aggregation results. However, the GRE did not affect AA liberation via cPLA2 activation or AA metabolite production through COX and LOX, as a downstream signaling pathway of PLCγ2 activation (Singer et al., 1997), except 12-HETE production. The negative effect of the GRE on AA liberation is accordance with its lack of effect on PLCγ2 phosphorylation. This result suggests that the GRE may have inhibited LOX activity rather than COX-1 activity because 12-HETE was simultaneously produced from AA via the LOX pathway.

An elevated level of cAMP in plateletes decreases intracellular Ca2+ concentrations because the uptake of Ca2+ into the dense tubular system negatively affects the actions of PLC and PKC (Walter et al., 1993). In our results, the GRE restored the cAMP level compared with that in the stimulated control, suggesting that the GRE increased the cAMP level as much as that in the unstimulated control, consistent with the downregulation of PLC and p47 phospholipase. These results indicated that MG and PGC as the most significant constituent of GRE inhibit the collagen or thrombin-induced platelet activation. We can suggest that GRE extract has multi-target for the inhibition of platelet activation including the suppression of ERK and PLCγ3 phosphorylation by MG and PGC.

5. Conclusion

Consequently, we suggest that the GRE including MG and PGC inhibited platelet aggregation by means of an antiplatelet effect, possibly due to the suppression of ERK1/2 and PLCγ3 phosphorylation, which is involved in the inhibition of LOX activation and recovery of the cAMP level, and that these effects may be useful for treating cardiovascular diseases by modulating platelet activation.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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