Research Article

Inhibitory effects of thromboxane A₂ generation by ginsenoside Ro due to attenuation of cytosolic phospholipase A₂ phosphorylation and arachidonic acid release

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Background: Thromboxane A₂ (TXA₂) induces platelet aggregation and promotes thrombus formation. Although ginsenoside Ro (G-Ro) from Panax ginseng is known to exhibit a Ca²⁺-antagonistic antiplatelet effect, whether it inhibits Ca²⁺-dependent cytosolic phospholipase A₂ (cPLA₂) activity to prevent the release of arachidonic acid (AA), a TXA₂ precursor, is unknown. In this study, we attempted to identify the mechanism underlying G-Ro-mediated TXA₂ inhibition.

Methods: We investigated whether G-Ro attenuates TXA₂ production and its associated molecules, such as cyclooxygenase-1 (COX-1), TXA₂ synthase (TXAS), cPLA₂, mitogen-activated protein kinases, and AA. To assay COX-1 and TXAS, we used microsomal fraction of platelets.

Results: G-Ro reduced TXA₂ production by inhibiting AA release. It acted by decreasing the phosphorylation of cPLA₂p, p38-mitogen-activated protein kinase, and c-Jun N-terminal kinase1, rather than by inhibiting COX-1 and TXAS in thrombin-activated human platelets.

Conclusion: G-Ro inhibits AA release to attenuate TXA₂ production, which may counteract TXA₂-associated thrombosis.

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1. Introduction

Platelets are activated via breakdown of phosphatidylinositol 4,5-bisphosphate in the plasma membrane (PM) to inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DG) by phospholipase C (PLC) [1]. IP₃ mobilizes Ca²⁺ from the endoplasmic reticulum to the cytoplasm, and Ca²⁺/calmodulin-dependent protein kinase [2]. Apart from phosphorylating pleckstrin by binding to protein kinase C, DG acts as a donor of arachidonic acid (AA) [3], a precursor of thromboxane A₂ (TXA₂) [4]. TXA₂ is an autacoid produced from AA by the actions of cyclooxygenase-1 (COX-1) and TXA₂ synthase (TXAS) and initiates thrombogenesis [5–7]. Antithrombotic drugs, such as aspirin, imidazole, and indomethacin, block TXA₂ production by inhibiting COX-1 or TXAS activity [8].

Mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38-MAPK, are phosphorylated in thrombin-activated human platelets [9–11]. Phosphorylated p38-MAPK and ERK2 induce TXA₂ production [12–14]. Moreover, the phosphorylation of p38-MAPK is essential for the activation of cytosolic phospholipase A₂ (cPLA₂), leading to AA release [14]. Thrombin elevates the intracellular Ca²⁺ level, leading to the translocation of cPLA₂ from the cytosol to the PM. Subsequently, p38-MAPK activates cPLA₂ by phosphorylating it at Ser⁵⁰⁵ [15]. Therefore, it may be beneficial to evaluate the antplatelet potential

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of a compound on TXA₂ production in relation to phosphorylation of MAPKs.

Roots of *Panax* (*P. ginseng*) are used in traditional Oriental medicine. In a previous study, we reported that total saponin from Korean Red Ginseng inhibits both COX-1 and TXAS to reduce the production of TXA₂ [16]: however, its individual components have not yet been evaluated. Therefore, we evaluated the effects of ginsenoside Ro (G-Ro), an oleanane-type saponin (Fig. 1) in *P. ginseng*, on the production of TXA₂ along with its associated enzymes and signaling molecules.

2. Materials and methods

2.1. Materials

G-Ro was obtained from Ambo Institute (Daejon, Korea). Thrombin was obtained from Chrono-Log Corporation (Havertown, PA, USA); Fura 2-AM was obtained from Invitrogen Molecular Probes (Eugene, OR, USA). Aspirin was obtained from Sigma Chemical Corporation (St. Louis, MO, USA). Thromboxane B₂ (TXB₂) enzyme immunoassay (EIA) kit, COX-1 fluorescence activity assay kit, oza-grel, and prostaglandin H₂ were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Anti-phospho-cPLA₂ (Ser505), anti-phospho-p38-MAPK, anti-phospho-JNK (1/2), and cPLA₂ were purchased from Cell Signaling Technology (Beverly, MA, USA). PD98059, SB203580, and anti-β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinylidene difluoride membrane and enhanced chemiluminescence solution were purchased from GE Healthcare (Piscataway, NJ, USA). Human AA EIA kit was obtained from Cusabio (Wuhan, Hubei, China).

2.2. Preparation of washed human platelets

Human platelet-rich plasma with acid–citrate–dextrose solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose) was purchased from Korean Red Cross Blood Center (Changwon, Korea). It was centrifuged for 10 min at 1,300 × g to obtain the platelet pellets. The platelets were washed twice using a washing buffer (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 a suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.36 mM NaH₂PO₄, 0.49 mM MgCl₂, 5.5 mM glucose, and 0.25% gelatin, pH 6.9) to a final concentration of 5 × 10⁸ cells/mL. All the aforementioned procedures were performed at 25°C to preserve platelet activity. These experiments were approved (PIRB12-072) by the National Institute for Bioethics Policy Public Institutional Review Board (Seoul, Korea).

2.3. Determination of platelet aggregation

Platelets (10⁸ cells/mL) were preincubated, with or without G-Ro, in a CaCl₂ (2 mM) solution for 3 min at 37°C. They were stimulated with thrombin (0.05 U/mL) and allowed to aggregate for 5 min in an aggregometer (Chrono-Log Corporation). Platelet aggregation rate was determined as an increase in light transmission. G-Ro was dissolved in the platelet suspension buffer (pH 6.9), and MAPK inhibitors were dissolved in 0.1% dimethyl sulfoxide.

2.4. Western blot analysis of COX-1 and TXAS, and phosphorylation of p38-MAPK, JNK1/2, and cPLA₂α

Platelet aggregation was terminated by adding an equal volume (250 μL) of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM ATPase, 1 mM Na₃VO₄, 1 μg/mL leupeptin, and 1 mM phenylmethanesulfonyl fluoride, pH 7.5). Protein content in the platelet lysate was measured using a bicinchoninic acid protein assay kit (Pierce Biotechnology, IL, USA). COX-1 and TXAS were analyzed by Western blotting after separating equal amounts of total protein (30 μg) in the lysate, microsomal, and cytosol fractions of platelets via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (8%, 1.5 mm). Phosphorylation of p38-MAPK, JNK1/2, and cPLA₂α was evaluated by Western blotting after separating 15 μg of total protein by SDS-PAGE (6%, 1.5 mm). A Polyvinylidene difluoride membrane was used for protein transfer. The primary and secondary antibodies were diluted 1:1,000 and 1:10,000, respectively. The membranes were visualized using an enhanced chemiluminescence solution. The degrees of phosphorylation were analyzed using the Quantity One 1-D analysis software, Version. 4.5 (Bio-Rad, Hercules, CA, USA).

2.5. Measurement of TXB₂

Because TXA₂ is unstable and gets converted spontaneously to TXB₂, it was quantified by determining the TXB₂ content [4]. After platelet aggregation, the reaction was terminated by adding ice-cold EDTA (5 mM) and indomethacin (0.2 mM) to prevent the metabolism of AA to TXA₂. The amount of TXB₂, a stable metabolite of TXA₂, was determined using a TXB₂ EIA kit according to the procedure described by the manufacturer.

2.6. Isolation of microsomal fraction

Washed platelets (10⁸ cells/mL), suspended in a buffer (pH 7.4) with 1% protease inhibitor, were sonicated 10 times at 100% sensitivity for 20 s on ice (Bandelin, HD2070, Germany) to obtain the platelet lysate. The microsomal fraction, containing endoplasmic reticulum membrane, was obtained by ultracentrifugation at 105,000 × g for 1 h at 4°C [16].

2.7. AA release

The reaction was terminated after platelet aggregation, and the aggregates were centrifuged at 200 × g at 4°C for 10 min. AA in the supernatant was quantified using an AA EIA kit (Cusabio), and the absorbance was measured at 450 nm using a Synergy HT multimode microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

2.8. COX-1 activity assay

The microsomal fraction of platelets was preincubated with aspirin (500 μM), a positive control, or with various concentrations of G-Ro and other reagents at 37°C for 30 min. COX-1 activity was assayed with a COX-1 fluorescence assay kit (Cayman Chemical Co).

2.9. TXAS activity assay

The microsomal fraction of platelets was preincubated with ozagrel (11 nM, IC₅₀), a positive control, or with various concentrations of G-Ro and other reagents at 37°C for 5 min. The reaction was initiated by adding prostaglandin H₂, and the samples were incubated at 37°C for 1 min; the reaction was terminated by adding citric acid (1 M). After neutralization with 1 N NaOH, the amount of TXB₂ was determined using a TXB₂ EIA kit according to the procedure described by the manufacturer.
2.10. Statistical analyses

All experimental results are indicated as the mean ± standard deviation accompanied by the number of trials. Significant differences were determined by analysis of variance followed by the Newman-Keuls multiple comparisons method. All statistical analyses were conducted using the SPSS 21.0.0.0 software (SPSS, Chicago, IL, USA). A p value < 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of G-Ro on platelet aggregation

We used thrombin at a dose of 0.05 U/mL, which induces maximum human platelet aggregation [17] to stimulate the platelets in this study. Thrombin increased platelet aggregation up to 92.5 ± 1.2%. However, G-Ro reduced the thrombin-induced platelet aggregation in a dose-dependent manner (Fig. 1B).

3.2. Effects of G-Ro on TXA2 production

We determined whether G-Ro reduced platelet aggregation by inhibiting TXA2 production (by measuring the TXB2 level). As shown in Fig. 1C, thrombin increased TXB2 level (49.2 ± 1.6 ng/10^8 platelets), whereas G-Ro dose-dependently (50–300 μM) reduced the TXB2 level that was induced by thrombin; G-Ro (300 μM) inhibited the thrombin-mediated elevation in TXB2 level by 94.9%.

3.3. Effects of G-Ro on activities of COX-1 and TXAS

We evaluated the activities of COX-1 (70 kDa) and TXAS (58 kDa) in the microsomal fraction to investigate whether they contributed to the reduction in TXB2 by G-Ro (Fig. 2A, lane 2). COX-1 activity in the absence of G-Ro (negative control) was 2.3 ± 0.1 nmol/mg protein. However, G-Ro dose-dependently (50–300 μM) reduced its activity (Fig. 2B); at 300 μM, COX-1 activity was reduced by 26.4% of that of the negative control. TXAS activity in the absence of G-Ro (negative control) was 220.8 ± 1.8 ng/mg protein/min. However, G-Ro dose-dependently (50–300 μM) reduced its activity (Fig. 2C); at 300 μM, TXAS activity was reduced by 22.9% of that of the negative control. We observed that G-Ro (300 μM) reduced COX-1 (26.4%) and TXAS (22.9%) activities to similar extents.

3.4. Effects of G-Ro on cPLA2α phosphorylation and AA release

The inhibitory effect of G-Ro (300 μM) on TXB2 production (94.9%, Fig. 1C) was significantly higher than those on COX-1 (26.4%, Fig. 2B) and TXAS (22.9%, Fig. 2C) activities. This suggested that G-Ro might also inhibit AA release, a precursor of TXA2, from PM phospholipids to reduce TXA2 production in thrombin-activated platelets.

Because Ca^{2+}-dependent cPLA2α is activated by phosphorylation [18] and releases AA from PM phospholipids in thrombin-activated human platelets [10], we investigated the effect of G-Ro on the phosphorylation of cPLA2α. As shown in Fig. 3A, G-Ro inhibited the thrombin-mediated phosphorylation of cPLA2α (Ser505) in a dose-dependent manner as it is reported that cPLA2α is activated by phosphorylation [18,19]. At 300 μM, G-Ro inhibited the thrombin-induced cPLA2α (Ser505) phosphorylation by 96.5% (Fig. 3A). Moreover, it reduced the thrombin-induced AA release in a dose-dependent manner (Fig. 3B); at 300 μM, it inhibited AA release by 61.1% of that induced by thrombin (2159.2 ± 29.0 ng/10^8 platelets).

3.5. Effects of G-Ro on the phosphorylation of MAPKs

Platelets contain MAPKs, such as ERK, JNK, and p38-MAPK [20], that phosphorylate Ser505 of cPLA2α [10,14,18,19,21–23].

![Fig. 1. Effects of G-Ro on thrombin-induced human platelet aggregation and thromboxane B2 production. (A) Structure of G-Ro. (B) Effect of G-Ro on thrombin-induced human platelet aggregation. (C) Effect of G-Ro on thromboxane B2 production. Platelet aggregation and thromboxane B2 production were carried out as described in "Materials and methods" section. The data are expressed as the mean ± standard deviation (n = 4). *p < 0.05 versus the thrombin-stimulated human platelets, **p < 0.01 versus the thrombin-stimulated human platelets. TXB2, thromboxane B2.](image)

![Fig. 2. Effects of G-Ro on COX-1 and TXAS activities. (A) Determination of the effects of the enzyme sources on COX-1 and TXAS activities. (B) Determination of the effects of G-Ro on COX-1. (C) Determination of the effects of G-Ro on TXAS activities. Western blot analysis and COX-1 and TXAS activities were determined as described in "Materials and methods" section. The data are expressed as the mean ± standard deviation (n = 4). *p < 0.05 versus the thrombin-stimulated human platelets. COX-1, cyclooxygenase-1; TXAS, thromboxane A2 synthase.](image)
Therefore, we investigated whether G-Ro inhibited the phosphorylation of cPLA2α (Ser505) in thrombin-activated human platelets. Thrombin-mediated p38-MAPK phosphorylation (Fig. 4A, lane 2) was dose-dependently (50–300 μM) inhibited by G-Ro (Fig. 4A, lanes 3–6). Furthermore, the p38-MAPK inhibitor, SB203580, attenuated the thrombin-induced phosphorylation of p38-MAPK (Fig. 4A, lane 7).

Thrombin phosphorylated JNK1 (46 kDa), but not JNK2 (54 kDa), as shown (Fig. 4B, lane 2). G-Ro attenuated the thrombin-induced phosphorylation of JNK1 in a dose-dependent manner (Fig. 4B, lanes 3–6). The inhibitor of JNK, SP600125, inhibited the phosphorylation of both JNK1 and JNK2 in thrombin-activated human platelets (Fig. 4B, lane 7).

3.6. Effects of MAPK inhibitors on cPLA2α phosphorylation, AA release, and TXA2 production

Furthermore, we investigated whether MAPK inhibitors inhibited the phosphorylation of cPLA2α. Thrombin extensively phosphorylated cPLA2α; however, it was inhibited by SB203580 (40 μM). Nevertheless, PD98059 (40 μM) and SP600125 (40 μM) did not influence the thrombin-induced cPLA2α phosphorylation (Fig. 5A).

Among the MAPK inhibitors, only SB203580 (40 μM), a p38-MAPK inhibitor, strongly inhibited the thrombin-mediated cPLA2α phosphorylation. This suggested that p38-MAPK induces cPLA2α phosphorylation and may stimulate TXA2 production by promoting AA release. Therefore, we tested this hypothesis using SB203580.

We observed that it inhibited the thrombin-induced AA release and TXA2 production by 75.2% and 91.6%, respectively (Figs. 5B, 5C).

4. Discussion

The autacoid TXA2, produced in platelets, constricts blood vessels and initiates thrombogenesis [7,24,25]. P. ginseng compounds, such as ginsenoside Rp1 [26], panaxadiol, and panaxatriol saponins [27–29], inhibit TXA2 production and attenuate platelet aggregation. In this study, we evaluated whether G-Ro inhibits thrombin-induced platelet aggregation by decreasing TXA2 production and investigated the mechanisms underlying the attenuation of AA release. We sought to identify the TXA2 antagonistic potential of G-Ro for development into an antiplatelet agent.

G-Ro inhibited TXA2 production to abolish thrombin-induced platelet aggregation. We determined the activities of COX-1 (70 kDa) and TXAS (58 kDa) in the microsomal fraction, which has the highest activity of cytochrome c reductase (an endoplasmic reticulum marker enzyme) to justify this inhibitory effect [16]. G-Ro reduced the production of TXA2 more than it reduced the activities of COX-1 and TXAS, suggesting that it may also inhibit AA release by cPLA2α and AA utilization by COX-1 and TXAS in thrombin-activated platelets. As expected, G-Ro strongly inhibited both thrombin-
induced Ca\textsuperscript{2+}-dependent cPLA\textsubscript{2} \((\text{Ser}^{505})\) phosphorylation and AA release. These results verify that the reduction in intracellular Ca\textsuperscript{2+} level by G-Ro \cite{30} prevents the binding of cPLA\textsubscript{2} to its PM substrates, such as phosphatidylcholine (PC), phosphatidyserine (PS), and phosphatidyethanolamine (PE). Accordingly, the Ca\textsuperscript{2+} antagonist effects of G-Ro \cite{30} reduce AA release from cPLA\textsubscript{2} substrates (PC, PS, and PE) to decrease TXA\textsubscript{2} production. Moreover, thrombin-elevated intracellular Ca\textsuperscript{2+} hydrolyzes the AA bond at position 2 of PS, PC, and PE in the PM of human platelets \cite{31}, indicating that the AA, bound at position 2 of glycerophospholipids, is attacked by Ca\textsuperscript{2+}-dependent cPLA\textsubscript{2}. Thrombin also activates Ca\textsuperscript{2+}-dependent PLC\textsubscript{b} to produce DG and IP\textsubscript{3} from phosphatidylinositol 4,5-bisphosphate in the PM. DG is hydrolyzed to AA and glycerol via the DG\textsuperscript{–}-monooacylglycerol–lipase pathway \cite{1}. Accordingly, we cannot rule out G-Ro-mediated inhibition of the PLC\textsubscript{b}/DG\textsuperscript{–}lipase/monoacylglycerol–lipase pathway to reduce AA release in thrombin-activated platelets.

In the present study, G-Ro inhibited the activities of both the AA release enzyme (cPLA\textsubscript{2}) and AA utilization enzymes (COX-1 and TXAS) to decrease the thrombin-induced TXA\textsubscript{2} production. These enzymes are known to be activated by phosphorylated MAPKs \cite{12,14,19,20,21,22,23,24,25,26,27,28,29}. Therefore, we used MAPK inhibitors to investigate whether G-Ro requires inhibition of thrombin-phosphorylated MAPKs for attenuating TXA\textsubscript{2} production. SB203580 (a p38-MAPK inhibitor) inhibited the thrombin-induced p38-MAPK phosphorylation, cPLA\textsubscript{2}a phosphorylation, AA release, and TXA\textsubscript{2} production. These results confirm that thrombin-phosphorylated p38-MAPK increases AA release and TXA\textsubscript{2} production by promoting cPLA\textsubscript{2}a phosphorylation.

Similar to SB203580, G-Ro attenuated thrombin-induced p38-MAPK phosphorylation, cPLA\textsubscript{2}a phosphorylation, AA release, and TXA\textsubscript{2} production. Therefore, we can assume that G-Ro inhibits thrombin-induced AA release and TXA\textsubscript{2} production by preventing the phosphorylation of both p38-MAPK and cPLA\textsubscript{2}a. Moreover, G-Ro is reported to inhibit the thrombin-mediated phosphorylation of ERK2 \cite{30} and JNK1. However, G-Ro failed to inhibit AA release through suppression of ERK2 and JNK1. Instead, it induced cPLA\textsubscript{2}a phosphorylation in thrombin-activated platelets. Furthermore, both PD98059 (ERK inhibitor) and SP600125 (JNK inhibitor) did not inhibit thrombin-induced cPLA\textsubscript{2}a phosphorylation. Therefore, other platelet-activating mechanisms, such as Ca\textsuperscript{2+} influx \cite{9,20,42,43} and COX-1 activation by ERK2 \cite{30} and serotonin release by JNK1 \cite{20}, might have led to the suppression of ERK2 and JNK1 by G-Ro. Many compounds of ginseng, such as G-Ro, G-Rp4, Rg3-enriched red ginseng extract, and G-Rp1, inhibit the phosphorylation of MAPKs to attenuate Ca\textsuperscript{2+} influx and serotonin release in platelets \cite{26,42,43}.

We previously showed that G-Ro inhibits thrombin-induced Ca\textsuperscript{2+}-dependent platelet-activating reactions, including granule secretion, fibrinogen binding, and fibrin clot retraction, by upregulating the cyclic adenosine monophosphate (cAMP)-dependent phosphorylation of IP\textsubscript{3} \cite{32} and vasodilator-stimulated phosphoprotein \cite{33}. In this study, we observed that G-Ro attenuated thrombin-induced TXA\textsubscript{2} production by inhibiting AA release, and this effect was due to the inhibition of Ca\textsuperscript{2+}-dependent cPLA\textsubscript{2a} phosphorylation by p38-MAPK. In addition, G-Ro abolishes Ca\textsuperscript{2+}-dependent p-selectin expression in thrombin-activated platelets \cite{30}. Because its expression in activated platelets causes leukocytic inflammatory atherosclerosis, G-Ro may counteract inflammation and atherosclerosis \cite{44,45}. The in vitro and in vivo antiinflammatory activities of G-Ro and Korean Red Ginseng are reported \cite{46,47,48}.

In conclusion, G-Ro attenuates TXA\textsubscript{2} production by inhibiting p38-MAPK-mediated cPLA\textsubscript{2}a phosphorylation and AA release. It also reduced the activities of microsomal COX-1 and TXAS in thrombin-activated human platelets. Combined with previous reports \cite{30,44,48,49}, G-Ro holds significant antplatelet potential.

Conflicts of interest

The authors declare no conflict of interest.

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