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Total Saponin from Korean Red Ginseng Inhibits Thromboxane A_2 Production Associated Microsomal Enzyme Activity in Platelets

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Ginseng, the root of *Panax ginseng* Meyer, has been used frequently in traditional oriental medicine and is popular globally. Ginsenosides, which are the saponins in ginseng, are the major components having pharmacological and biological activities, including anti-diabetic and anti-tumor activities. In this study, we investigated the effects of total saponin from Korean red ginseng (TSKRG) on thrombin-produced thromboxane A_2 (TXA_2), an aggregating thrombogenic molecule, and its associated microsomal enzymes cyclooxygenase (COX)-1 and TXA_2 synthase (TXAS). Thrombin (0.5 U/mL) increased TXA_2 production up to 169 ng/10^8 platelets as compared with control (0.2 ng/10^8 platelets). However, TSKRG inhibited potently TXA_2 production to the control level in a dose-dependent manner, which was associated with the strong inhibition of COX-1 and TXAS activities in platelet microsomes having cytochrome c reductase activity. The results demonstrate TSKRG is a beneficial traditional oriental medicine in platelet-mediated thrombotic diseases via suppression of COX-1 and TXAS to inhibit production of TXA_2.

Keywords: Total saponin from Korean red ginseng, Cyclooxygenase 1, Thromboxane A_2 synthase

INTRODUCTION

Platelet aggregation is essential for normal hemostatic process when blood vessels are injured. However, it can also cause cardiovascular diseases such as thrombosis, atherosclerosis and myocardial infarction [1]. Hence, inhibition of platelet aggregation might be a promising target to the development of anti-thrombotic drugs and an approach for the prevention of cardiovascular disease. When platelets are activated by agonists such as collagen, thrombin and adenosine diphosphate (ADP), phosphatidylinositol 4, 5-bisphosphate (PIP_2) is broken down by phospholipase C which is activated through G-protein coupled receptor (GPCR) or glycoprotein VI. Diacylglycerol (DG) and inositol-1,4,5-trisphosphate (IP_3) are generated from PIP_2 [2,3]. IP_3 mobilizes Ca^{2+} from the dense tubular system into the cytoplasm. Ca^{2+} calmodulin complex activates myosin light chain kinase, which in turn phosphorylates the myosin light chain [4,5]. Moreover, DG phosphorylates pleckstrin through protein kinase C (PKC) and it is also used as a donor of arachidonic acid (AA) [6]. AA is a precursor of prostaglandin G_2/prostaglandin H_2 (PGH_2) and thromboxane A_2 (TXA_2) [7]. TXA_2 interacts with membrane receptors of other platelets in a autacoidal reaction, which results in elevation of intracellular free Ca^{2+}, a platelet aggregation-

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inducing molecule, via $G_{\alpha q}$ of GPCR [3,8,9]. Inhibition of TXA_2 production leads to anti-thrombosis.

Anti-thrombotic drugs such as aspirin, imidazole and indomethacin have anti-thrombotic effects by inhibiting the generation of TXA_2. The mechanisms are concerned with cyclooxygenase (COX)-1 and TXA_2 synthase (TXAS), which convert AA to TXA_2 [10,11]. Therefore, the inhibition of the medicated COX-1 and TXAS is very important to prevent platelet-induced thrombosis. For instance, aspirin, COX-1 inhibitor, and ozagrel, TXAS inhibitor are being used as anti-thrombotic agents [12,13].

It has been reported that nonsaponin fraction [14-16], aqueous fraction [17], polyacetylene compound [18], acidic polysaccharide [19], panaxatriol and panaxadiol [20-22], ginsenoside Rg_1 [17,23], and dihydroginsenoside Rg_3 [24] from *Panax ginseng* have anti-platelet effects on agonist-induced platelet aggregation. However, there are no reports concerning the involvement of ginseng compounds in the inhibition of COX-1 or TXAS to suppress platelet aggregation. In this study, we investigated whether total saponin from Korean red ginseng (TSKRG) inhibits the specific activities of COX-1 and TXAS to suppress TXA_2 production, and this provide new information of TSKRG-mediated anti-platelet activity.

MATERIALS AND METHODS

Materials

Total saponin from Korean red ginseng was obtained from KT&G Central Research Institute (Daejeon, Korea). Thrombin was purchased from Chrono-Log Corporation (Havertown, PA, USA). TXB_2 enzyme immunoassay kits were purchased from GE Healthcare (Buckinghamshire, UK). COX-fluorescent activity assay kit, ozagrel and prostaglandin H_2 were purchased from Cayman Chemical (Ann Arbor, MI, USA). Cytochrome c reductase (NADPH) assay kit and other reagents were obtained Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents used in the present study were of analytical grade.

Preparation of washed rat platelets

Whole blood was obtained from the abdominal vein of normal Sprague-Dawley rats (150-200 g), anti-coagulated with 0.38% sodium citrate solution and centrifuged at 220 ×g for 10 min to obtain the platelet-rich plasma. After removing the red blood cells, the preparation was centrifuged again at 1650 × g for 10 min to obtain platelet pellets. The platelets were washed twice with a washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.5 mM glucose, 1 mM EDTA, pH 6.5). Washed platelets were suspended in a buffer consisting of 138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.5 mM glucose, 0.49 mM $MgCl_2$ and 0.25% gelatin, pH 7.4, and adjusted to a final concentration of 5×10^8 platelets/mL. All of the procedures were carried out at room temperature (25°C) to avoid platelet aggregation from cooling [25]. Animals were maintained as per the principles and guidelines of the Ethics Committee of the Animal Care of Inje University.

Measurement of platelet aggregation

The washed platelets (10^8/mL) were pre-incubated at 37°C in the presence of 2 mM $CaCl_2$ with or without TSKRG. After 3 min, platelets were further stimulated with thrombin (0.5 U/mL) for 5 min. The aggregation was monitored using an aggregometer (Chrono-Log Corporation) with gentle stirring. Each aggregation rate was calculated as a percent of light transmission. The suspending buffer was used as a reference (transmission 0). TSKRG was dissolved in water; this had no effect on the results.

Measurement of thromboxane B_2

Because TXA_2 is unstable and quickly converted to thromboxane B_2 (TXB_2), the amounts of TXA_2 were evaluated by determining TXB_2 [7]. Washed platelets (10^8/mL) were pre-incubated at 37°C in the presence of 2 mM $CaCl_2$ with or without TSKRG or other reagents. After 3 min, platelets were further stimulated with thrombin (0.5 U/mL) for 5 min. The reaction was terminated by adding ice-cold 5 mM EDTA and 0.2 mM indomethacin to inhibit subsequent metabolism of AA to TXA_2. The amounts of TXB_2, a stable metabolite of TXA_2, were determined using a TXB_2 EIA kit according to the procedure described by the manufacturer. The assay is based on the competition between unlabelled TXB_2 and a fixed quantity of peroxidase-labelled TXB_2 for a limited number of binding sites on a TXB_2 specific antibody.

Isolation of microsomal fraction

Washed platelets (10^8 platelets/mL) in suspending buffer (pH 7.4) containing 1% protease inhibitor were sonicated once at a sensitivity of 100% for 20 s and 10 times on ice with a model HD2070 sonicator (Bandelin Electronic, Bandelin, Germany) to obtain platelet lysates. Each homogenate was ultracentrifuged at 105,000 ×g for 1 h at 4°C to obtain a microsomal fraction containing endoplasmic reticulum (ER) membrane. The supernatant was considered as a cytosolic fraction. All of the separat-

ed fractions were identified by cytochrome c reductase (an ER membrane marker enzyme) [26] and used as enzyme source in Western blots as described below.

Cytochrome c reductase activity assay

Cytochrome c reductase is a flavoprotein localized in the ER. Cytochrome c reductase activity of the fractions (homogenates, cytosols and microsomes of platelets) was assayed using a cytochrome c reductase (NADPH) assay kit (Sigma-Aldrich). The reaction was initiated by addition of NADH and the reduction of cytochrome c was monitored by the increase of absorbance at 550 nm for 7 min with a kinetic program.

Western blot analysis

Protein concentrations were measured using the Bradford method. Protein samples (30 μg) from each treatment platelets were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were electrophoretically transferred to a polyvinylidene fluoride membrane (GE Healthcare, Franklin Lakes, NJ, USA). An immunoblot was prepared using primary antibody (COX-1, 1:200 dilution; TXAS, 1:1000 dilution) and alkaline phosphatease-conjugated secondary antibody at a dilution of 1:10,000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After treatment with secondary antibody (anti-mouse of anti-goat IgG-horseradish peroxidase conjugate), detection of antibody-bound protein in the membrane was performed by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK).

Cyclooxygenase-1 activity assay

For the measurement of COX-1 activity, the microsomal fraction of platelets was pre-incubated with aspirin (500 μM), a positive control as a COX inhibitor, with or without various concentrations of TSKRG at 37°C for 30 min. COX-1 activity of the treated microsomal fraction was assayed with COX-1 fluorescent assay kit (Cayman Chemical).

Thromboxane A_2 synthase activity

For the measurement of TXAS activity, the microsomal fraction was preincubated with ozagrel (11 nM, IC_{50}), a positive control as a TXAS inhibitor, with or without various concentrations of TSKRG at 37°C for 5 min. The reaction was initiated by the addition of PGH_2. After incubation for 1 min at 37°C, the reaction was terminated by the addition of 1 M citric acid. After neutralization with 1 N NaOH, the amount of TXB_2, a stable metabolite of TXA_2, was determined using a TXB_2 enzyme immunoassay kit according to the procedure described by the manufacturer.

Statistical analyses

The experimental results are expressed as the mean± SEM. accompanied by the number of observations. Data were assessed by analysis of variance. If this analysis indicated significant differences among the group means, then each group was compared by the Newman-Keuls method. A p-value <0.05 was considered statistically significant.

RESULTS

Threshold concentration of thrombin causing platelet aggregation in a maximum

When washed platelets (10^8 platelets/mL) were incubated with various concentrations of thrombin, light transmission (%) indicating platelet aggregation was maximally increased by 0.25 U/mL of thrombin (Fig. 1). However, we used the 0.5 U/mL of thrombin.

Effect of total saponin from Korean red ginseng on thromboxane A_2 production

The amount of TXA_2 (determined as TXB_2) in intact platelets was 0.2 ng/10^8 platelets, which was markedly increased to 33.93±1.22 ng/10^8 platelets when platelets were stimulated with thrombin (0.5 U/mL). TSKRG (50-150 μg/mL), however, powerfully reduced the production of TXA_2 (Fig. 2). These results show that TSKRG may inhibit the activity of COX-1 or TXAS in a dose-dependent manner to suppress the production of TXA_2 in thrombin-induced platelet aggregation.

Determination of enzyme source of cyclooxygenase-1 and thromboxane A_2 synthase

To determine whether TSKRG is involved in the inhibition of COX-1 and TXAS, enzyme sources having COX-1 or TXAS were needed. We measured the activity of cytochrome c reductase, an ER marker enzyme, in homogenates, microsomes and cytosols of platelets. As shown in Fig. 3A, the microsomal fraction had the highest specific activity of cytochrome c reductase. Next, we determined the fraction from platelets that expressed COX-1 and TXAS. Remarkably high expressions of COX-1 (70 kDa) and TXAS (58 kDa) were observed in the microsomal fraction (Fig. 3B). These observations were consistent with the view that the microsomal fraction from platelets had the highest activity of cytochrome

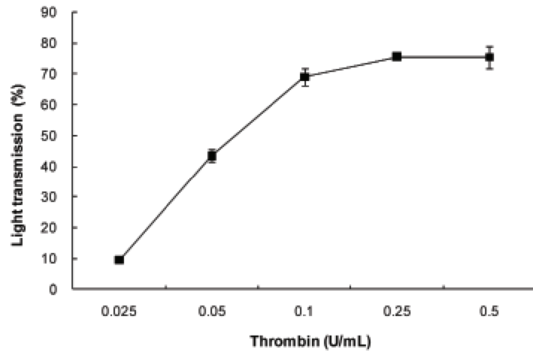

Fig. 1. The concentration threshold of thrombin on platelet aggregation. Washed platelets (10^8/mL) were stimulated with various doses of thrombin for 5 min in the presence of 2 mM $CaCl_2$ at 37°C. Platelet aggregation (%) was recorded as an increase in light transmission. Data are expressed as mean±SD (*n*=4).

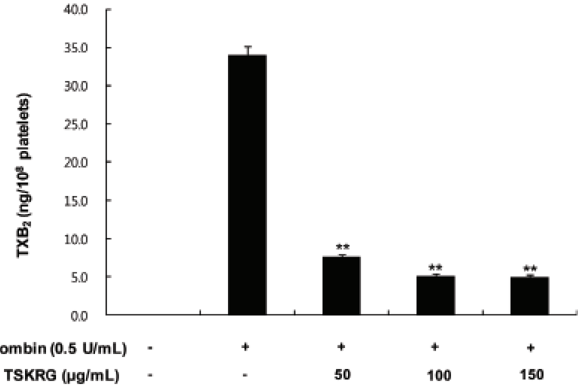

Fig. 2. Effect of total saponin from Korean red ginseng (TSKRG) on thrombin-induced thromboxane B_2 (TXB_2) generation. Washed platelets (10^8/mL) were preincubated for 3 min at 37°C in the presence of 2 mM $CaCl_2$ with or without TSKRG, and then thrombin (0.5 U/mL) was added. The reactions were terminated by adding ice-cold 5 mM EDTA and 0.2 mM indomethacin. The amounts of TXB_2 were determined using a TXB_2 enzyme immunoassay kit. Data are expressed as mean±SD (*n*=4). $^{**}p<0.001$ compared with that of thrombin-induced platelets without TSKRG.

c reductase. Thus, microsomal fractions were used to determine the activities of COX-1 and TXAS.

Effect of total saponin from Korean red ginseng on cyclooxygenase-1 activity

COX-1 converts AA to PGH_2. To determine if the inhibitory effect of TSKRG on TXA_2 production was due to the direct suppression of COX-1, a cell-free enzyme assay method with the microsomal fraction of platelets was used. As shown in Fig. 4A, COX-1 activity in the absence of TSKRG (control) was 900.7±40.7 pmol/min/mg protein. However, TSKRG produced dose-dependent inhibition of COX-1 activity; at 150 μg/mL, COX-1 activity was reduced to 397.0±11.5 pmol/min/mg protein. As a positive control, 500 μM of aspirin was used, which inhibited COX-1 activity to 340.1±31.1 nmol/min/mg protein (Fig. 4A). The 56% inhibition by 150 μg/mL TSKRG as compared with that of control (900.7±40.07 pmol/min/mg protein) was almost equal to the 62% inhibition elicited by 500 μM of aspirin.

Effect of total saponin from Korean red ginseng on thromboxane A_2 synthase activity

To determine whether the inhibitory effect on TXA_2 production of TSKRG was due to the direct suppression of TXAS, the aforementioned cell-free enzyme assay

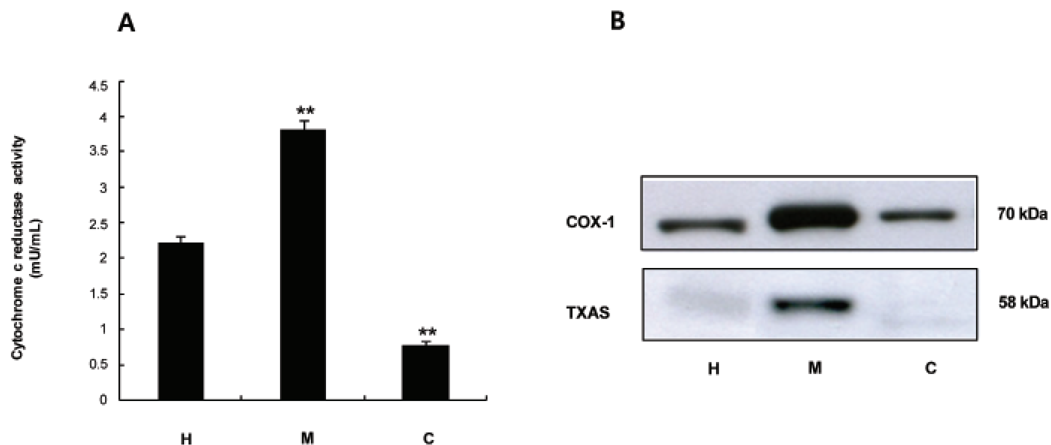

Fig. 3. Determination of enzyme source on cyclooxygenase (COX)-1 and thromboxane A_2 synthase (TXAS) activity. (A) Detection of cytochrome c reductase activities in homogenates, microsomes, and cytosols of platelets. NADPH-cytochrome c reductase, the marker enzyme for microsomes in platelets, was assayed by using a NADHP cytochrome c reductase assay kit. One hundred micrograms of protein were used. (B) Western blot analysis of COX-1 and TXAS in homogenates, microsomes, and cytosols of platelets. The 30 μg (TXAS, COX-1) of total protein on homogenates, microsomes, and cytosols of platelets was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the resolved proteins were transferred to polyvinylidene fluoride membranes and detected by enhanced chemiluminescence. H, homogenates; M, microsomes; C, cytosols. $^{**}p<0.001$ compared with that of total saponin from Korean red ginseng-non-treated microsomes.

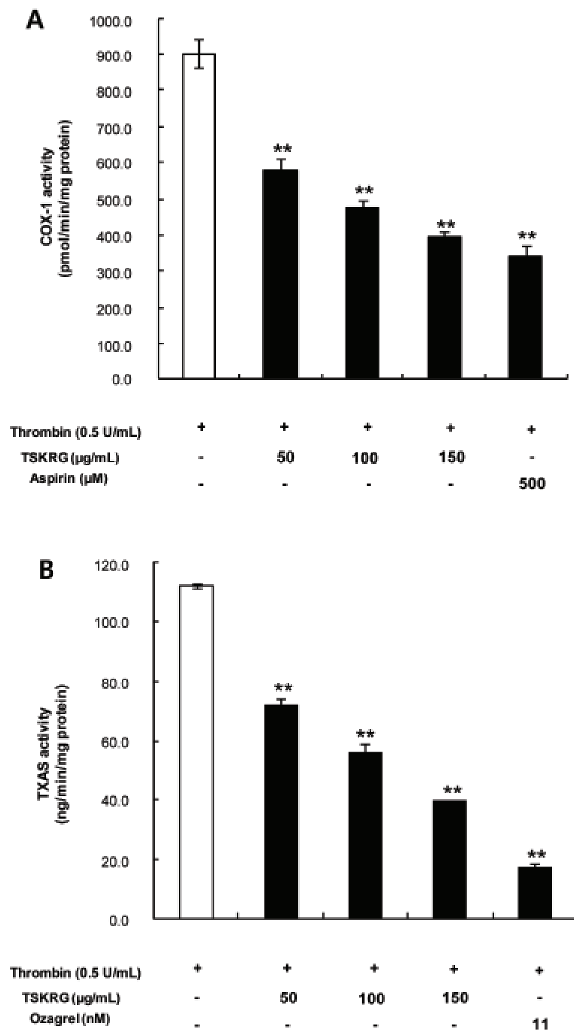

Fig. 4. Inhibitory effect of total saponin from Korean red ginseng (TSKRG) on cyclooxygenase (COX)-1 and thromboxane A_2 synthase (TXAS). (A) Effect of TSKRG on COX-1 activity in microsomes of platelets. The microsomal fraction (10 μg of total protein) of platelets was preincubated with or without various concentrations of TSKRG for 30 min at 37°C. The TSKRG-induced COX-1 activity was assayed with COX-1 fluorescent assay kit. (B) Effect of TSKRG on TXAS activity in platelet microsomes. The microsomal fraction (20 μg total protein) of platelets was preincubated for 5 min at 37°C with or without various concentrations of TSKRG. The reaction was initiated by adding 5 μM prostaglandin H_2 (PGH_2) and terminated by adding 1 M citric acid. The activity was measured as the production of PGH_2-mediated thromboxane B_2 (TXB_2) by TXB_2 EIA kit. The data are given as the mean±SD (*n*=4). $^{**}p<0.001$ compared with that of TSKRG-non-treated microsomal fraction of platelets.

method with the microsomal fraction of platelets was also used. In the TSKRG-untreated control, TXAS activity was 112.1±0.7 ng/min/mg protein. However, TSKRG inhibited TXAS activity in a dose-dependent fashion, to 39.6±0.3 ng/min/mg protein at 150 μg/mL. In addition, 11 nM ozagrel as a positive control was used; TXAS activity was 17.5±0.9 ng/min/mg protein (Fig. 4B). The 64.7% inhibition by 150 μg/mL TSKRG as compared with 112.1±0.7 ng/min/mg protein of the control (approximately 77%) to that (84.4%) by 11 nM of ozagrel.

DISCUSSION

TXA_2 acts as a positive feedback promoter on activated platelets and is a strong agonist on resting platelets [3,27]. TXA_2 is also a vasoconstrictor and a bronchoconstrictor [28]. Thus, a compound that can inhibit TXA_2 action or production has potential application as an antithrombotic agent [29,30]. Because ginsenosides from *P. ginseng* do not inhibit TXA_2 production in collagen-, ADP-, and AA-activated platelets [31], we investigated if TSKRG containing ginsenosides lacked inhibitory activity on agonist-produced TXA_2. TSKRG decreased thrombin-elevated TXA_2 level in a dose-dependent manner (Fig. 2), which is consistent with the report that panaxadial and panaxatriol inhibitor of thrombin- or adrenaline-induced TXA_2 production [21,22].

Although these results indicate that TSKRG inhibits a pathway involved in TXA_2 production, it is insufficient for a complete understanding of the inhibitory action of ginseng compounds on platelet aggregation. Presently, we tried to explain the inhibitory mechanism of TSKRG on TXA_2-production by assaying the activities of the TXA_2 production-associated microsomal enzymes, COX-1 and TXAS. Because COX-1 and TXAS are localized in the endoplasmic reticulum [32,33], we isolated microsomes from platelets, determined its marker enzyme, cytochrome c reductase, and observed the expression of COX-1 and TXAS. The activity of cytochrome c reductase was highest in the microsomal fraction rather than the cytosolic fraction or homogenates (Fig. 3A). The results mean that the microsomal fractions from platelet lysates contain COX-1 and TXAS.

As shown in Fig. 3B, COX-1 (70 kDa) and TXAS (50 kDa) were potently expressed in the microsomal fraction than in other cellular fractions. We determined the effects of TSKRG on the activities of COX-1 and TXAS in the microsomal fraction. TSKRG inhibited COX-1 and TXAS activities. In the TSKRG-untreated control, COX-1 activity was 900.7±40.7 pmol/min/mg protein. But, activity was decreased in the presence of TSKRG to 397.0±11.5 pmol/min/mg protein. As a positive control, 500 μM aspirin also inhibited COX-1 activity to 340.1±31.1 pmol/min/mg protein (Fig. 4A). These results suggest that TSKRG may be used as a COX-1 inhibitor with aspirin. In addition, TXAS activity in the absence of TSKRG was 112.1±0.7 ng/min/mg protein (Fig. 4B),

while TSKRG inhibited TXAS activity to 39.6±0.3 ng/min/mg protein. As a positive control, 11 nM ozagrel also inhibited TXAS activity to 17.5±0.9 ng/min/mg protein. TSKRG may be used as a TXAS inhibitor, similar to ozagrel. TSKRG that we used contained ginsenoside (G)-Rg_1, G-Re, G-Rb_1, G-Rc, G-Rg_2, G-Rb_2, G-Rd, and G-Rg_3, which were analyzed with HPLC-ELSD method (Zorbax ODS C_{18} column [250×4.6 mm id, 5 μm], Zorbax ODS C_{18} guard column [12.5×4.6 mm id, 5 μm]). Unfortunately, in the present study, we don't know which ginsenoside of TSKRG has inhibitory effect on thrombin-stimulated COX-1 and TXAS activities, these should be investigated in the future.

Thrombin is a strong stimulator of platelet aggregation, and activates phospholipase C_β via protease-activated receptors (PARs)-1 and PAR-4 of GPCR, which supplies TXA_2 precursor AA from phosphoinositides such as PIP_2, phosphatidylinositol 4-monophosphate (PIP), and phosphatidylinositol (PI) [3,34]. Accordingly, it cannot not excluded that TSKRG could inhibit phospholipase C activity to suppress the thrombin-mediated hydrolysis of phosphoinositides such as PI, PIP, and PIP_2. It can be inferred that panaxatriol from *P. ginseng* inhibited the thrombin-stimulated hydrolysis of PIP_2, PIP, and PI [20]. At present, although ginsenoside Rg_1, ginsenoside Rg_3, and dihydroginsenoside Rg_3 are known to have an anti-platelet effect [17,23,24,35], we are unable to discern which TSKRG ginsenoside is involved in the inhibition of COX-1 or TXAS activities to suppress TXA_2 production. Hepoxilin cyclopropane, which inhibits TXA_2 production by suppressing TXAS activity, inhibits the binding of TXA_2 to the TXA_2 receptor (TP) [36]. TXA_2 is a platelet agonist that binds to TP linked to both $G_{\alpha q}$ and G_{12}/G_{13} of GPCR [3]. G_i-stimulated phospholipase C_β hydrolyses PIP_2, PIP, and PI, which produces IP_3 that in turn mobilizes Ca^{2+} and DG to activate PKC. These signaling pathways are involved in facilitating platelet aggregation. G_{12}/G_{13} mediates the small GTPase Rho/Rho-kinase, which stimulates phosphorylation of myosin light chain to fully activate platelet aggregation and degranulation [37]. Considering previous reports [3,36,37], TXAS synthase activity inhibition by TSKRG may be involve in the down-regulation of G_i- and G_{12}/G_{13}-mediated platelet aggregation pathway. In addition, spinach saponin and hepoxilin cyclopropane that inhibit TXA_2 production increase cyclic adenosine monophosphate (cAMP) [36,38], which suppresses cytosolic free Ca^{2+}, an aggregation-inducing molecule. Because TSKRG inhibits TXA_2 production as well as spinach saponin and hepoxilin cyclopropane, TSKRG may be involved in increasing the production of cAMP. In conclusion, TSKRG has a beneficial anti-platelet effect within thrombotic diseases via the inhibition of COX-1 and TXAS activities.

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