

Inhibitory Effects of Total Saponin Korean Red Ginseng on Thromboxane A₂ Production and P-Selectin Expression *via* Suppressing Mitogen-Activated Protein Kinases

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Ginseng has been widely used for traditional medicine in eastern Asia and is known to have inhibitory effects on cardiovascular disease (CVD) such as thrombosis, atherosclerosis, and myocardial infarction. Because, platelet is a crucial mediator of CVD, many studies are focusing on inhibitory mechanism of platelet functions. Among platelet activating molecules, thromboxane A₂ (TXA₂) and P-selectin play a central role in CVD. TXA₂ leads to intracellular signaling cascades and P-selectin plays an important role in platelet-neutrophil and platelet-monocyte interactions leading to the inflammatory response. In this study, we investigated the inhibitory mechanisms of total saponin fraction from Korean red ginseng (KRG-TS) on TXA₂ production and P-selectin expression. Thrombin-elevated TXA₂ production and arachidonic acid release were decreased by KRG-TS dose (25 to 150 µg/mL)-dependently *via* down regulation of microsomal cyclooxygenase-1 (COX-1), TXA₂ synthase (TXAS) activity and dephosphorylation of cytosolic phospholipase A₂ (cPLA₂). In addition, KRG-TS suppressed thrombin-activated P-selectin expression, an indicator of granule release *via* dephosphorylation of mitogen-activated protein kinases (MAPK). Taken together, we revealed that KRG-TS is a beneficial novel compound inhibiting TXA₂ production and P-selectin expression, which may prevent platelet aggregation-mediated thrombotic disease.

Key Words: Total saponin fraction, MAPKs, Thromboxane A₂, Arachidonic acid, P-selectin

INTRODUCTION

Platelets are activated at the sites of vascular injury. Upon activation, inositol-1,4,5-trisphosphate (IP₃) is released from

plasma membrane (Berridge and Irvine, 1984) and released IP₃ mobilizes Ca²⁺ from endoplasmic reticulum into cytoplasm. Intracellular Ca²⁺ stimulates translocation of cytosolic phospholipase A₂ (cPLA₂) from cytosol to membranes and cPLA₂ is phosphorylated at Ser⁵⁰⁵ by p38 mitogen-activated

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protein kinase (p38 MAPK) for full catalytic activity. The activated cPLA₂ hydrolyzes the sn-2 acyl bond of phospholipids and releases arachidonic acid (AA) (Kramer et al., 1996; McNicol and Shibou, 1998). The AA is a precursor of thromboxane A₂ (TXA₂) (Hamberg et al., 1975) and the TXA₂ biosynthesis is triggered by cyclooxygenase-1 (COX-1) and TXA₂ synthase (TXAS) (Needleman et al., 1976; Patrignani et al., 1999). TXA₂ is released from platelets and interacts with membrane receptor of other platelets in an autacoidal reaction, which acts as a positive promoter on activated platelets and resting platelets simultaneously (Jennings, 2009). It is well known that aspirin and ozagrel have anti-thrombotic effects by inhibiting the TXA₂ production. The mechanisms are concerned with COX-1 and TXAS (Patrono, 1994).

Selectins are expressed by activation or inflammatory response on various vascular cells, including platelets, leukocytes and endothelial cells (Kansas, 1996; Ley, 2003). L-selectin is present on leukocytes and E-selectin is present on endothelial cells and P-selectin is expressed on endothelial cells and platelets. The P-selectin in platelets is released by agonists from α -granule, and is re-expressed to the surface (Zarbock et al., 2007). P-selectin plays an important role in interactions with immune cells (von Hundelshausen and Weber, 2007).

Ginseng, the root of *Panax ginseng* Meyer, is known to have various pharmacological activities (Ernst, 2010; Kim and Park, 2011). Recently, it is reported that KRG-TS has an effect on CVD through reduction of blood pressure (Chung, 2010), anti-coagulation effects (Wee et al., 2010), endothelium relaxation (Jung et al., 2011), and inhibition of hypercholesterolemia-induced platelet aggregation (Hwang et al., 2008). In our previous report, we demonstrated that KRG-TS was involved in increase of cAMP level and subsequent reduction of $[Ca^{2+}]_i$ mobilization in thrombin-induced rat platelet aggregation (Lee et al., 2013). However, the inhibitory mechanism by KRG-TS is not fully understood. Thus, we demonstrated that the modulatory mechanism of TXA₂ production and P-selectin expression by KRG-TS on human platelets for prevention of CVD.

MATERIALS AND METHODS

Materials

KRG-TS was obtained from Korea Ginseng Corporation (Daejeon, Korea). Thrombin was purchased from Chrono-Log Corporation (Havertown, PA, USA). Aspirin was purchased from Sigma Chemical Corporation (St. Louis, MO, USA). TXB₂ EIA kit, COX-1 fluorescent activity assay kit, ozagrel, and Prostaglandin H₂ were purchased from Cayman Chemical (Ann Arbor, MI, USA). Anti-phosphor-cPLA₂ (Ser⁵⁰⁵), anti-phosphor-p38, anti-p38, anti-phosphor-ERK1/2, anti-ERK1/2, anti-phosphor-JNK1, and anti-rabbit IgG-horseradish peroxidase conjugate (HRP) and lysis buffer were purchased from Cell Signaling (Beverly, MA, USA). Anti- β -actin, anti-COX-1 and anti-TXAS were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PVDF membrane and ECL solution were purchased from GE Healthcare (Piscataway, NJ, USA). Human arachidonic release kit was purchased from (Cusabio, Wuhan, Hubei, China). CD62P (P-selectin) antibody was purchased from Biolegend (San Diego, CA, USA).

Preparation of washed human platelets

Human platelet-rich plasma (PRP) with ACD solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose) was supplied from Korean Red Cross Blood Center (Changwon, Korea). To remove red blood cells, it was centrifuged for 10 min at 250 g, then centrifuged for 10 min at 1,300 g. The platelets were washed using 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), then resuspended in suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.36 mM NaH₂PO₄, 0.49 mM MgCl₂, 5.5 mM glucose, 0.25% gelatin, pH 6.9). The human platelets in suspending buffer was adjusted to a final concentration of 5×10^8 /mL and aforementioned procedures were performed at 25 °C to maintain platelet activity. The approval (PIRB12-072) for these experiments was received from National Institute for Bioethics Policy Public Institutional Review Board (Seoul, Korea).

Determination of platelet aggregation

Human Platelets ($10^8/\text{mL}$) were preincubated with or without KRG-TS in the presence of 2 mM of CaCl_2 for 2 min at 37°C , then, stimulated with thrombin (0.05 U/mL). The platelet aggregation rate was determined as an increase in light transmission for 5 min using an aggregometer (Chrono-Log Corporation, Havertown, PA, USA).

Measurement of TXB_2

TXA_2 is unstable and quickly converted to thromboxane B_2 (TXB_2) in platelets. Thus, the amounts of TXA_2 were evaluated by measuring TXB_2 concentration (Hamberg et al., 1975). Thrombin-induced platelet aggregation was terminated by adding both ice-cold 5 mM EDTA and 0.2 mM indomethacin to inhibit subsequent metabolism of arachidonic acid to TXA_2 . The amounts of TXB_2 were determined using a TXB_2 EIA kit according to the procedure described by the manufacturer (Cayman Chemical Co, Ann Arbor, MI, USA).

Isolation of microsomal fraction

Human platelets ($10^8/\text{mL}$) containing suspending buffer (pH 7.4) with 1% protease inhibitor was sonicated at sensitivity 100% for 20 sec, 1 cycles, and 10 times on ice to obtain platelet homogenates using a sonicator (Bandelin, HD2070, Germany). And then, homogenates were ultracentrifuged at 105,000 g for 1 h at 4°C to obtain microsomal fraction containing endoplasmic reticulum (ER) membrane (Lagarde et al., 1981).

COX-1 activity assay

For the measurement of COX-1 activity, the microsomal fraction of platelets was pre-incubated with a positive control, aspirin (500 μM), and with various concentrations of KRG-TS at 37°C for 30 min. COX-1 activity was assayed with COX-1 fluorescent assay kit (Cayman Chemical Co, Ann Arbor, MI, USA).

Thromboxane A synthase activity

For the measurement of TXAS, microsomal fraction was preincubated with a positive control, ozagrel (11 nM, IC_{50}),

and with various concentrations of KRG-TS at 37°C for 5 min. The reaction is initiated by adding prostaglandin H_2 , and incubated at 37°C for 1 min. The reactions are terminated by the addition of 1 M citric acid and neutralized by 1 N NaOH, the amount of TXB_2 was determined by using TXB_2 EIA kit according to the procedure described by manufacturer (Cayman Chemical Co, Ann Arbor, MI, USA).

Arachidonic release

Thrombin-induced platelet aggregation was terminated, and centrifuged with 200 g at 4°C for 10 min, and supernatants were used for the assay of AA release EIA kit (Cusabio, Wuhan, Hubei, China). AA release was measured at 450 nm using a Synergy HT multi-Model Microplate Reader (BioTek Instruments, Winooski, VT, USA).

Western blot for analysis of COX-1, TXAS, cPLA₂, and MAPKs-phosphorylation

The platelet aggregation was terminated by adding a lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA , 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM ATPase, 1 mM Na_3VO_4 , 1 $\mu\text{g}/\text{mL}$ leupeptin, and 1 mM phenylmethanesulfonyl fluoride) then, platelet protein and microsomal fraction were measured using a BCA protein assay kit (Pierce Biotechnology, IL, USA). Protein (15 μg) and microsomal fraction (30 μg) were separated by SDS-PAGE (6%, 1.5 mm), then PVDF membrane was used for protein transfer. The dilutions for 1st antibody and 2nd antibody were 1:1,000 and 1:10,000, respectively. The membranes were visualized using ECL solution. The degrees of phosphorylation were analyzed using the Quantity One, Ver. 4.5 (BioRad, Hercules, CA, USA).

Determination of P-selectin release

After platelet aggregation, the platelets were resuspended by ice-cold PBS (pH 7.4) and cells were incubated with Alexa Fluor 488 anti-human CD62P for 60 min at 4°C under the dark condition. Next, platelets were washed three times by ice-cold PBS to reduce unbounded antibody and fixed using 0.5% paraformaldehyde. Alexa Fluor 488 anti-human CD62P binding to platelets were determined using

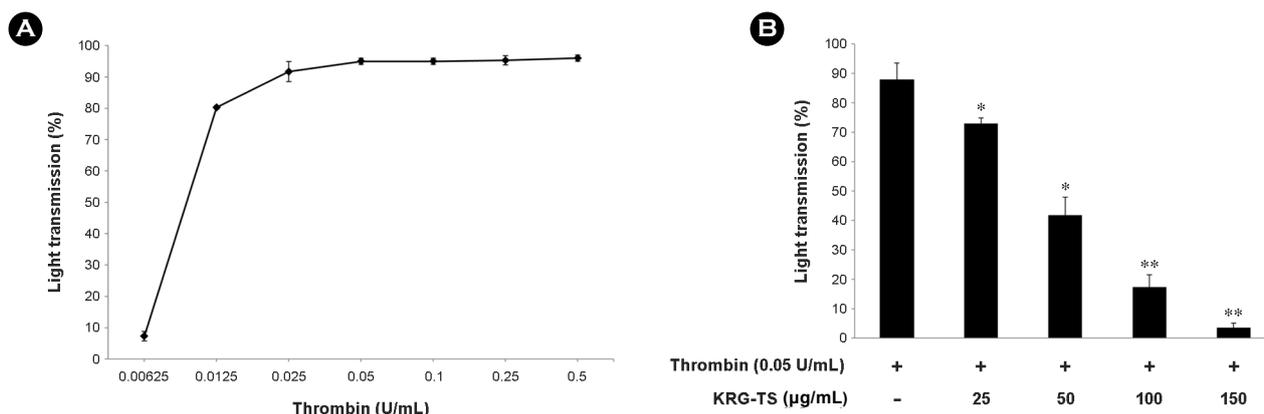


Fig. 1. Effects of KRG-TS on thrombin-induced human platelet aggregation. (A) The concentration threshold of thrombin on human platelet aggregation. (B) The effects of KRG-TS on thrombin-induced human platelet aggregation. Platelet aggregation was carried out as described in "Materials and Methods" section. The data are expressed as the mean \pm standard deviation (n=4). * P <0.05 versus the thrombin-stimulated human platelets, ** P <0.01 versus the thrombin-stimulated human platelets.

flow cytometry (BD Biosciences, San Diego, CA, USA) and data were analyzed using CellQuest software.

Statistical analyses

The experimental results are presented as the mean \pm standard deviation accompanied by the number of observations. Data were determined by analysis of variance (ANOVA). If this analysis showed significant differences among the group means, then each group was compared by the Newman-Keuls method. Statistical analysis was carried out according to the SPSS 21.0.0.0 (SPSS, Chicago, IL, USA). P <0.05 was considered to be statistically significant.

RESULTS

Effects of KRG-TS on thrombin-induced human platelet aggregation

The concentration of thrombin-elevated maximal human platelet aggregation was approximately 0.05 U/mL (Fig. 1A). Thus, thrombin (0.05 U/mL) was used as an agonist in this study. Thrombin increased human platelet aggregation rate was $87.8 \pm 5.7\%$. However, KRG-TS strongly reduced thrombin-induced platelet aggregation dose-dependently (Fig. 1B). In our previous report showed that various concentration of KRG-TS (20, 50, 100, 150 $\mu\text{g/mL}$) did not influence on unstimulated human platelet (Kwon et al., 2016).

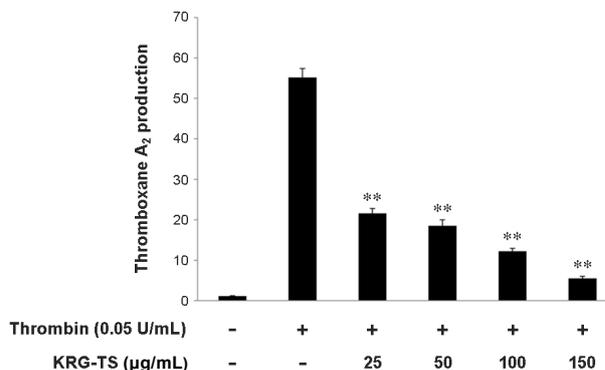


Fig. 2. Effects of KRG-TS on TXA₂ production. Effects of KRG-TS on TXA₂ production. Determination of TXA₂ production was carried out as described in "Materials and Methods" section. The data are expressed as the mean \pm standard deviation (n=3). ** P <0.01 versus the thrombin-stimulated human platelets.

Effects of KRG-TS on TXA₂ production and COX-1, TXAS activity

Next, we investigated TXA₂ production. The amount of TXA₂ was markedly increased by thrombin from 1.1 ± 0.1 , resting platelets, to $55.2 \pm 2.2 \text{ ng}/10^8$ platelets. However, KRG-TS reduced the TXA₂ production (Fig. 2). To determine whether KRG-TS involves in inhibition of COX-1 and TXAS, we needed enzyme sources having COX-1 or TXAS. We determined which fraction in platelets expresses COX-1 and TXAS in homogenates fraction, microsomal fraction,

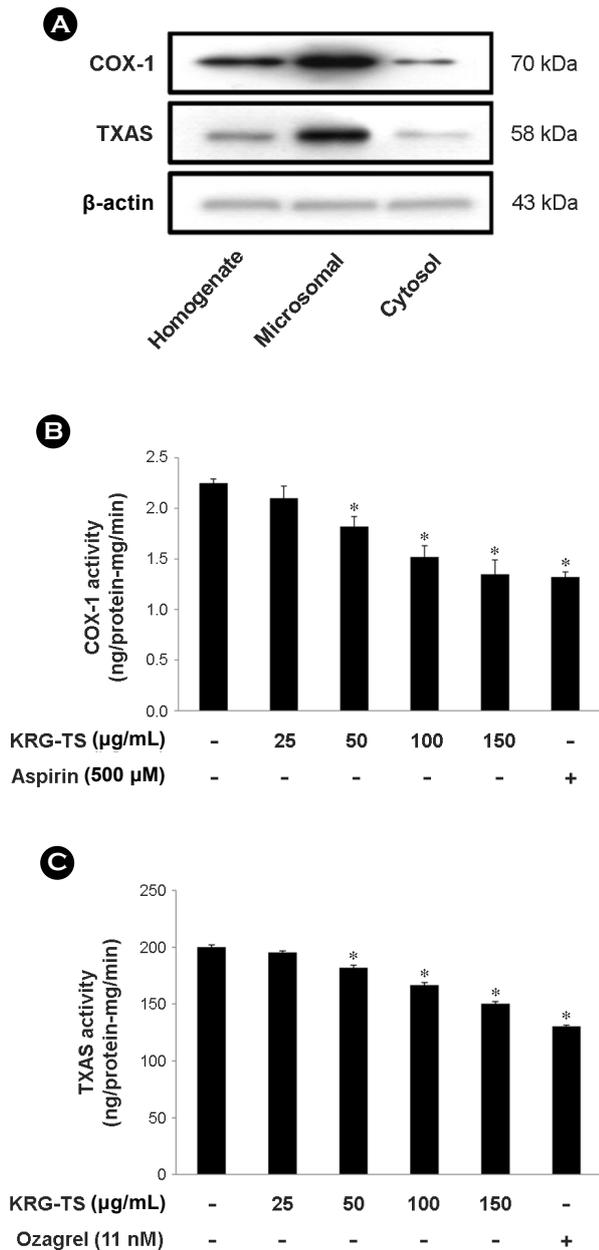


Fig. 3. Effects of KRG-TS on COX-1, and TXAS activity. (A) Determination of the effects of the enzyme sources on COX-1 and TXAS activities. (B) Effects of KRG-TS on COX-1 activity. (C) Effects of KRG-TS on TXAS activity. Western blot and COX-1 and TXAS activities were determined as described in "Materials and Methods" section. The data are expressed as the mean \pm standard deviation (n=4). * P <0.05 versus the thrombin-stimulated human platelets.

and cytosols fraction. As the result, high expressed COX-1 (70 kDa) and TXAS (58 kDa) were observed in microsomal fraction (Fig. 3A). Thus, we used to determine the activity

of COX-1 and TXAS using microsomal fraction. As shown in Fig. 3B, COX-1 activity of microsomal fraction in the absence of KRG-TS (control) was 2.3 ± 0.04 nmol/protein-mg/min. However, 50, 100, and 150 μ g/mL of KRG-TS inhibited COX-1 activity to 1.8 ± 0.10 , 1.5 ± 0.11 , and 1.4 ± 0.14 ng/protein-mg/min respectively. 500 μ M of aspirin, a positive control, inhibited COX-1 activity to 1.3 ± 0.05 nmol/protein-mg/min (Fig. 3B).

To determine whether KRG-TS is involved in TXAS, cell-free enzyme assay method with microsomal fraction of platelets was also used. In microsomal fraction (control), TXAS activity was 200.1 ± 1.8 ng/protein-mg/min (Fig. 3C). However, 50, 100, and 150 μ g/mL of KRG-TS inhibited TXAS activity to 182.0 ± 2.2 , 166.6 ± 2.1 , and 150.2 ± 1.8 ng/protein-mg/min respectively. In addition, 11 nM of ozagrel as a positive control was used, which inhibited TXAS activity to 130.2 ± 1.1 ng/protein-mg/min (Fig. 3C).

Effects of KRG-TS on Arachidonic acid release and cPLA₂ phosphorylation

In order to verify the inhibitory mechanism of KRG-TS on TXA₂ production, we investigated AA release. Thrombin-induced AA release increased to 1925.5 ± 22.2 , but KRG-TS dose (25, 50, 100, 150)-dependently inhibited the AA release (Fig. 4A). Moreover, because cPLA₂ acts as a key mediator to regulate the AA release in human platelets, we investigated whether KRG-TS inhibited the phosphorylation of cPLA₂. As shown in Fig. 4B, the cPLA₂ was strongly phosphorylated by thrombin (Fig. 4B, lane 2), but KRG-TS inhibited the cPLA₂-phosphorylation dose-dependently (Fig. 4B, lanes 3 to 5). These results indicated that KRG-TS inhibited TXA₂ production was due to the down regulation of cPLA₂/AA release pathway.

Effects of KRG-TS on MAPKs phosphorylation

The MAPKs are divided into three subgroups: extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK. It is reported that ERK2, JNK1 and p38 MAPK detected in human platelet and phosphorylated by thrombin (Bugaud et al., 1990; Kramer et al., 1995; Fadal-Wollbold et al., 2002). It is well known that the enzyme activity of cPLA₂ is achieved by its phosphory-

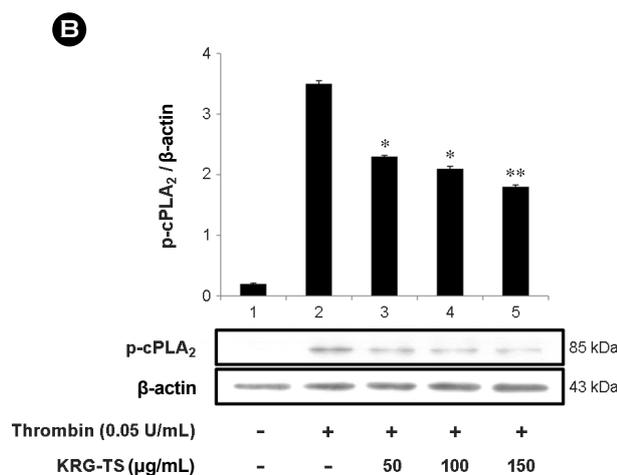
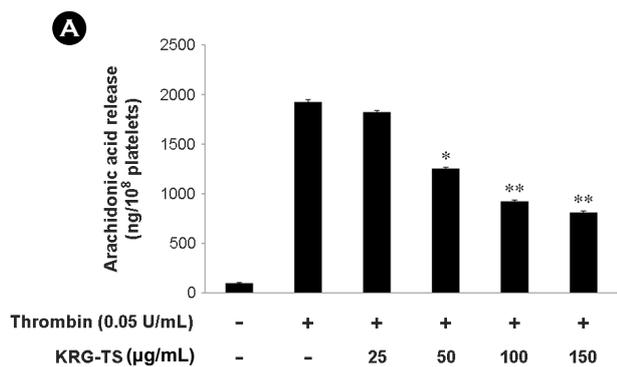


Fig. 4. Effects of KRG-TS on AA release and cPLA₂-phosphorylation. (A) Effects of KRG-TS on AA release. (B) Effects of KRG-TS on cPLA₂-phosphorylation. AA release assay and Western blot were determined as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n=3). **P*<0.05 versus the thrombin-stimulated human platelets, ***P*<0.01 versus the thrombin-stimulated human platelets.

lation and p38 MAPK phosphorylates cPLA₂ (Ser⁵⁰⁵). The p38 MAPK is also activated by its phosphorylation. Thus, we evaluated that KRG-TS has inhibitory effect on p38 MAPK phosphorylation. As shown in Fig. 5A, KRG-TS inhibited thrombin-elevated the phosphorylation of p38 MAPK dose (50 to 150 µg/mL)-dependently.

It has been revealed that ERK2 is involved in the TXA₂ production (Yacoub et al., 2006; Garcia et al., 2007). The specific inhibitors of ERK1/2 and p38 MAPK, PD98059 and SB203580, blocked COX-1 directly, and SB203580 inhibited the conversion of PGH₂ to TXA₂ (Börsch-Haubold et al., 1998), which means that MAPKs may influence on endo-

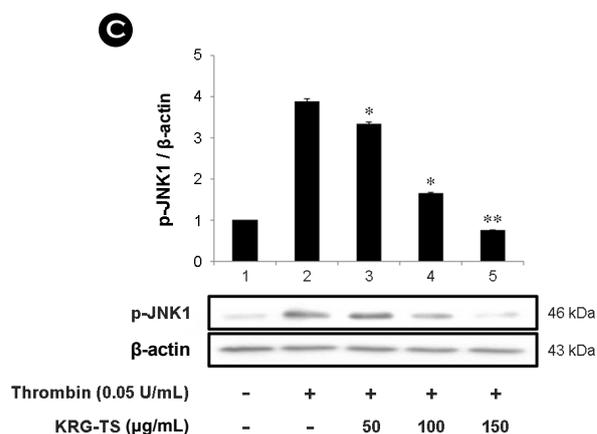
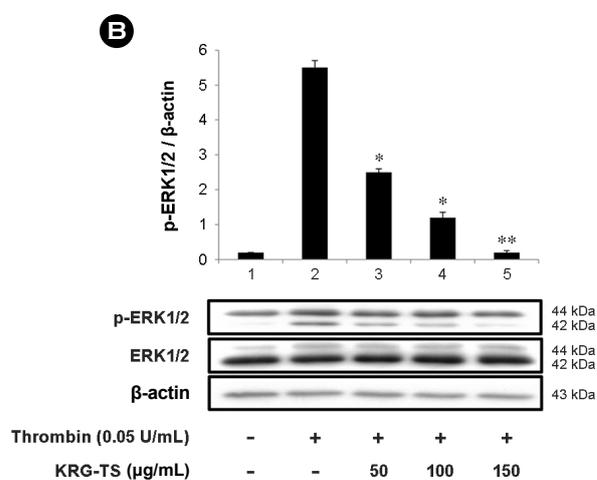
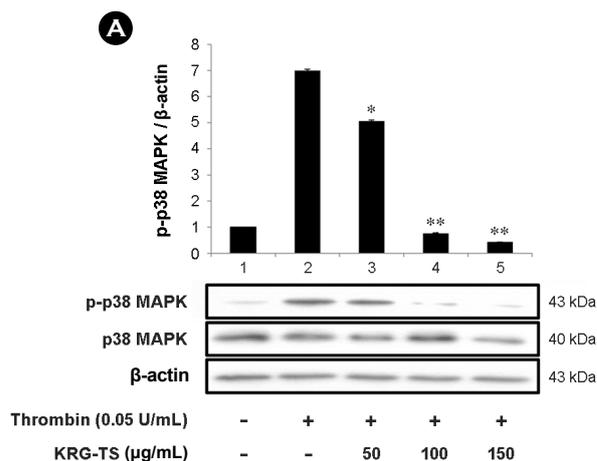


Fig. 5. Effects of KRG-TS on MAPKs-phosphorylation. (A) Effects of KRG-TS on p38 MAPK phosphorylation. (B) Effects of KRG-TS on ERK1/2 phosphorylation. (C) Effects of KRG-TS on JNK1 phosphorylation. Western blot was determined as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n=3). **P*<0.05 versus the thrombin-stimulated human platelets, ***P*<0.01 versus the thrombin-stimulated human platelets.

genous enzyme activity leading to TXA₂ production.

It is also known that phosphorylation of ERK is involved in extracellular Ca²⁺ influx (Rosado and Sage, 2001; Rosado and Sage, 2002) and the study in JNK1-deficient mice showed that JNK1 is involved in granule secretion and TXA₂ production (Adam et al., 2010). Thus, we investigated the

effect of KRG-TS on dephosphorylation of ERK2 and JNK1. As shown in Fig. 5B, thrombin potently phosphorylated ERK2 (42 kDa) of ERK1 (44 kDa) and ERK2 (42 kDa) (Fig. 5B, lane 2) as compared with those by control, unstimulated platelets (Fig. 5B, lane 1), and phosphorylated JNK1 (46 kDa) (Fig. 5C, lane 2) as compared with those by control

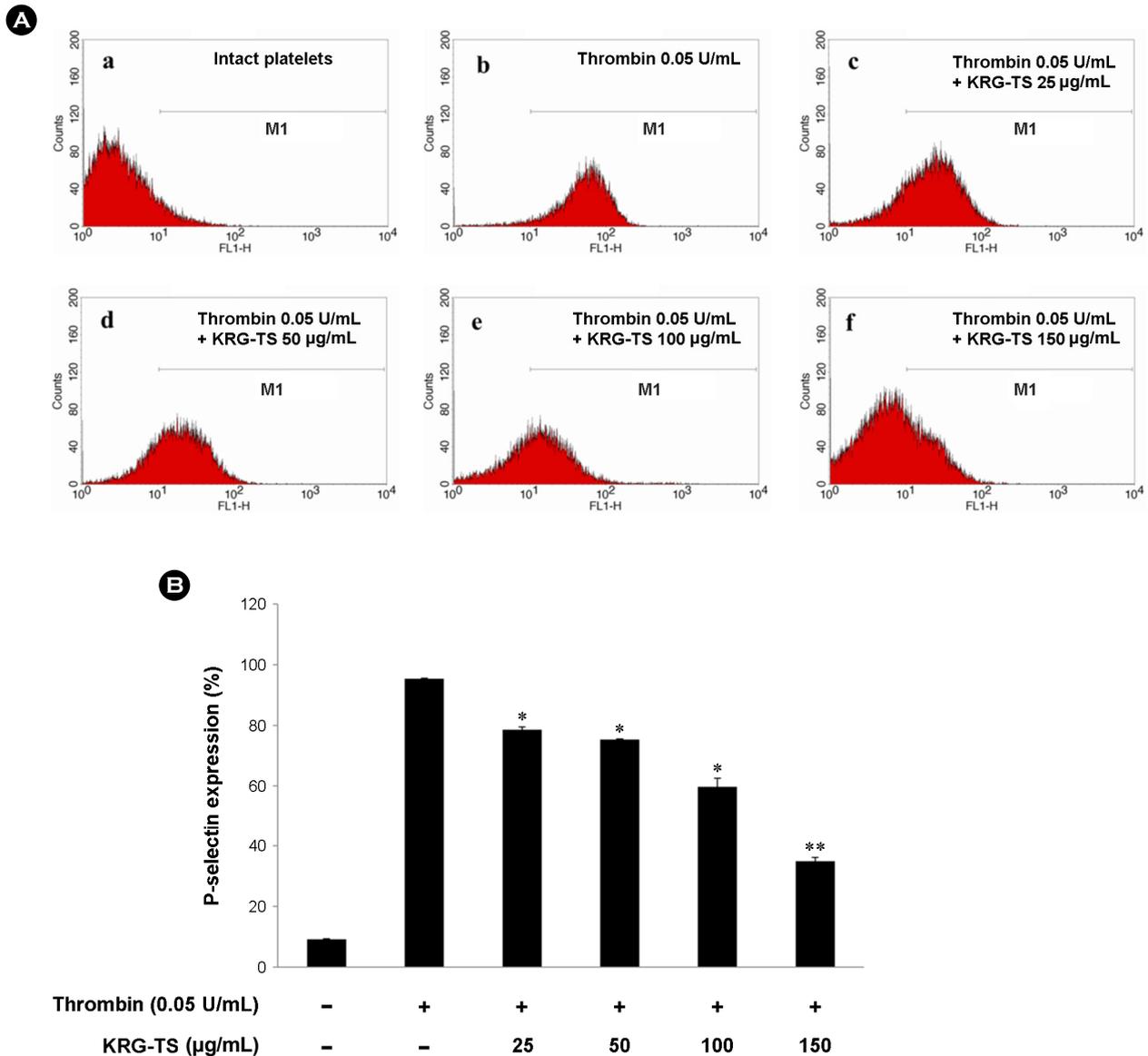


Fig. 6. Effects of KRG-TS on P-selectin expression. (A) The flow cytometry histograms on P-selectin expression. a, Intact platelets (base); b, Thrombin (0.05 U/mL); c, Thrombin (0.05 U/mL) + KRG-TS (25 µg/mL); d, Thrombin (0.05 U/mL) + KRG-TS (50 µg/mL); e, Thrombin (0.05 U/mL) + KRG-TS (100 µg/mL); f, Thrombin (0.05 U/mL) + KRG-TS (150 µg/mL). (B) Effects of KRG-TS on thrombin-induced P-selectin expression (%). Determination of P-selectin expression was carried out as described in "Materials and Methods" section. The data are expressed as the mean \pm standard deviation (n=3). **P*<0.05 versus the thrombin-stimulated human platelets, ***P*<0.01 versus the thrombin-stimulated human platelets.

(Fig. 5C, lane 1). However, KRG-TS dose (50 to 150 $\mu\text{g}/\text{mL}$)-dependently inhibited thrombin-induced the phosphorylation of ERK2 and JNK1 (Fig. 5B, lanes 3 to 5), (Fig. 5C lanes 3 to 5).

Effects of KRG-TS on P-selectin expression

Compounds in α -granule of platelets are known to involve in inflammation, coagulation and angiogenesis (Broos et al., 2011), these are also Ca^{2+} -dependently released by various platelet agonists. In special, P-selectin is involved in inflammation by binding to the P-selectin glycoprotein ligand-1 receptor on monocyte (von Hundelshausen and Weber, 2007; Zarbock et al., 2007). Therefore, we investigated the effect of KRG-TS on P-selectin expression on human platelet surface. Thrombin elevated the expression of P-selectin (Fig. 6A-B) as compared with that by unstimulated platelets (Fig. 6A-a). However, KRG-TS dose (25 to 150 $\mu\text{g}/\text{mL}$)-dependently inhibited thrombin-induced the expression of P-selectin (Fig. 6A-c~f, 6B). The expression of P-selectin is achieved by Ca^{2+} dependent kinase (Nishikawa et al., 1980), and ERK is involved in increase of intracellular Ca^{2+} concentration when platelets are activated (Rosado and Sage, 2002). In addition, JNK1 knockout mouse showed decreased granule release (Adam et al., 2010). Thus, it is thought that KRG-TS suppressed the P-selectin expression is achieved by dephosphorylation of ERK2 and JNK1.

DISCUSSION

Aspirin has a clear inhibition of TXA_2 production in platelets through the inactivation of COX-1. Several clinical trials have shown that inhibition of platelet COX-1 activity by aspirin leads the prevention of myocardial infarction and ischemic stroke. Aspirin reduces the risk of serious vascular events, but aspirin has a bleeding problem (Trialists'Collaboration, 2002). Therefore, a compound that can inhibit TXA_2 production has a potential for application as an anti-thrombotic agent.

KRG-TS decreased thrombin-elevated platelet aggregation dose-dependently (Fig. 1B), which is accordance with that KRG-TS inhibited thrombin-induced TXA_2 production (Fig. 2). However, it is not insufficient to understand an inhibitory

action of KRG-TS on TXA_2 production. Thus, we tried to explain its inhibitory mechanism by KRG-TS on TXA_2 production by assaying the activities of TXA_2 production-associated COX-1, TXAS, cPLA₂ and p38 MAPK. Because COX-1 and TXAS are localized in endoplasmic reticulum (Patrignani et al., 1999; Needleman et al., 1976), we isolated microsomes from cytosol in platelets, and confirmed the abundant expression of COX-1 (70 kDa) and TXAS (58 kDa) in microsomal fraction (Fig. 3A). We determined effects of KRG-TS on the activities of both enzymes by using microsomal fraction. As the results, KRG-TS inhibited COX-1 and TXAS activity in the presence of KRG-TS (50, 100, 150 $\mu\text{g}/\text{mL}$) directly like aspirin and ozagrel (Fig. 3B, 3C). Next, we focused on thrombin-induced AA release, upstream signaling molecule of TXA_2 and as shown in Fig. 4A, thrombin-induced AA release was decreased by KRG-TS. The AA release-associated signaling molecule, cPLA₂ and p38 MAPK are also diminished by KRG-TS (Fig. 4B, 4C). Comparing the inhibitory ratio of TXA_2 production and its associated enzymes (COX-1, TXAS) by KRG-TS, the inhibitory degree of TXA_2 production by KRG-TS (150 $\mu\text{g}/\text{mL}$) is very high as compared with inhibitory degrees of COX-1 and TXAS by KRG-TS (150 $\mu\text{g}/\text{mL}$). These results mean that the strong inhibition of TXA_2 production by KRG-TS is due to the various inhibitory effects such as inhibition of COX-1, TXAS, AA release and dephosphorylation of cPLA₂ and p38 MAPK.

The progression of atherosclerosis is triggered by inflammatory cell such as monocytes, neutrophils, and macrophages (Phillips et al., 2005). Thus, P-selectin is an important molecule for inflammatory response leading atherosclerosis. Although KRG-TS has antiplatelet effects through inhibition of TXA_2 production, if KRG-TS does not inhibit inflammation, atherosclerosis lesion would be generated at injury site of vascular wall. ATP and serotonin released from dense body are known to involve in amplification of platelet aggregation (Mustard and Packham, 1970; Holmsen and Day, 1970) and P-selectin released from α -granule is known to involve in causing of inflammation (von Hundelshausen and Weber, 2007; Zarbock et al., 2007). Because KRG-TS inhibited the P-selectin expression, it is thought that KRG-TS may inhibit aggregation-amplification and inflammation.

In real, many studies about anti-inflammatory activity by KRG-TS have been reported (Byeon et al., 2009; Park and Cho, 2009; Lee et al., 2014).

About dietary of a ginseng extract, It has reported that long-term (4 to 5 years) intake of red ginseng products (i.e. water extract, tea, drink) inhibit platelet aggregation, blood coagulation and hyperlipidemia, an index of atherosclerosis, and their effects were well also sustained in the subjects who have obesity, hyperlipidemia, and hypertension (Lee and Park, 1998; Park et al., 2000). Moreover, it has reported that dietary water-extract of Korean red ginseng (KRG-WE) suppressed rabbit platelet aggregation under hypercholesterolemia (Hwang et al., 2008) and oral administration (250 to 500 µg/kg-BW) of KRG-WE significantly inhibited platelet aggregation *ex vivo*, and KRG-WE (300, 500 µg/mL) inhibited washed rabbit platelet aggregation *in vitro* (Yang et al., 2015). With regard to the effects of ginsenosides on platelet aggregation, it is reported that ginsenoside Rg3 has inhibitory effect on collagen-induced blood platelet aggregation (Matsuda et al., 1985) and thromboxane A₂ production, ATP release and [Ca²⁺]_i mobilization (Lee et al., 1997). Furthermore, ginsenoside Rg3-enriched red ginseng extract showed inhibitory effects on ERK 1/2, JNK and p38 MAPK (Jeong et al., 2017). In our previous study, we also checked the inhibitory effects of G-Rg3 (20S, 20R) in thrombin induced human platelet (Shin et al., 2015). Moreover, we revealed the inhibitory effects of ginsenoside Ro on vasodilator-stimulated phosphoprotein and clot retraction (Shin et al., 2016). These reports suggest that inhibitory effects of KRG-TS are achieved by ginsenoside Rg3 and ginsenoside Ro. However, the inhibitory mechanism of ginsenosides is not fully understood.

In conclusion, we have revealed that anti-platelet effects of KRG-TS on TXA₂ production and P-selectin expression. KRG-TS suppressed the phosphorylation of cPLA₂, and p38 MAPK leading TXA₂ production. Simultaneously, KRG-TS decreased the phosphorylation of ERK2 and JNK1 leading P-selectin expression. These results show that the anti-platelet effects by KRG-TS are achieved through down regulation of MAPKs phosphorylation, which is useful to understand the anti-platelet effects of ginseng saponin we eat. Therefore, it is thought that KRG-TS would be a great potentiality

as a functional food in the therapy and prevention of CVD associated with platelet aggregation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

- Adam F, Kauskot A, Nurden P, Sulpice E, Hoylaerts MF, Davis RJ, Bryckaert M. Platelet JNK1 is involved in secretion and thrombus formation. *Blood*. 2010. 115: 4083-4092.
- Berridge MJ, Irvine RF. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature*. 1984. 312: 315-321.
- Börsch-Haubold AG, Pasquet S, Watson SP. Direct inhibition of cyclooxygenase-1 and-2 by the kinase inhibitors SB 203580 and PD 98059 SB 203580 also inhibits thromboxane synthase. *Journal of Biological Chemistry*. 1998. 273: 28766-28772.
- Broos K, Feys HB, De Meyer SF, Vanhoorelbeke K, Deckmyn H. Platelets at work in primary hemostasis. *Blood Reviews*. 2011. 25: 155-167.
- Bugaud F, Nadal-Wollbold F, Lévy-Toledano S, Rosa JP, Bryckaert M. Regulation of c-jun-NH2 terminal kinase and extracellular-signal regulated kinase in human platelets. *Blood*. 1990. 94: 3800-3805.
- Byeon SE, Choi WS, Hong EK, Lee J, Rhee MH, Park HJ, Cho JY. Inhibitory effect of saponin fraction from *Codonopsis lanceolata* on immune cell-mediated inflammatory responses. *Archives of Pharmacal Research*. 2009. 32: 813-822.
- Chung IM, Lim JW, Pyun WB, Kim H. Korean red ginseng improves vascular stiffness in patients with coronary artery disease. *Journal of Ginseng Research*. 2010. 34: 212-218.
- Ernst E. Panax ginseng: an overview of the clinical evidence. *Journal of Ginseng Research*. 2010. 34: 259-263.
- Fadal-Wollbold F, Pawlowski M, Lévy-Toledano S, Berrou E, Rosa JP, Bryckaert M. Platelet ERK2 activation by thrombin is dependent on calcium and conventional protein kinases C but not Raf-1 or B-Raf. *FEBS Letters*. 2002. 531: 475-482.
- Garcia A, Shankar H, Murugappan S, Kim S, Kunapuli SP. Regu-

- lation and functional consequences of ADP receptor-mediated ERK2 activation in platelets. *Biochemical Journal*. 2007. 404: 299-308.
- Hamberg M, Svensson J, Samuelsson B. Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proceedings of the National Academy of Sciences of the United States of America*. 1975. 72: 2994-2998.
- Holmsen H, Day HJ. The selectivity of the thrombin-induced platelet release reaction: subcellular localization of released and retained constituents. *The Journal of Laboratory and Clinical Medicine*. 1970. 75: 840-855.
- Hwang SY, Son DJ, Kim IW, Kim DM, Sohn SH, Lee JJ, Kim SK. Korean red ginseng attenuates hypercholesterolemia-enhanced platelet aggregation through suppression of diacylglycerol liberation in high-cholesterol-diet-fed rabbits. *Phytotherapy Research*. 2008. 6: 778-783.
- Jennings LK. Role of platelets in atherothrombosis. *The American Journal of Cardiology*. 2009. 103: 4A-10A.
- Jeong D, Irfan M, Kim SD, Kim S, Oh JH, Rhee MH. Ginsenoside Rg3-enriched red ginseng extract inhibits platelet activation and *in vivo* thrombus formation. *Journal of Ginseng Research*. 2017. 41: 548-555.
- Jung YH, Park KY, Jeon JH, Kwak YS, Song YB, Wee JJ, Rhee MH, Kim TW. Red ginseng saponin fraction A isolated from Korean red ginseng by ultrafiltration on the porcine coronary artery. *Journal of Ginseng Research*. 2011. 35: 325-330.
- Kansas GS. Selectins and their ligands: current concepts and controversies. *Blood*. 1996. 88: 3259-3287.
- Kim SK, Park JH. Trends in ginseng research in 2010. *Journal of Ginseng Research*. 2011. 35: 389-398.
- Kramer RM, Roberts EF, Striffler BA, Johnstone EM. Thrombin induces activation of p38 MAP kinase in human platelets. *Journal of Biological Chemistry*. 1995. 270: 27395-27398.
- Kramer RM, Roberts EF, Um SL, Börsch-Haubold AG, Watson SP, Fisher MJ, Jakubowski JA. p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A₂ (cPLA₂) in thrombin-stimulated platelets. evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA₂. *Journal of Biological Chemistry*. 1996. 271: 27723-27729.
- Kwon HW, Shin JH, Cho HJ, Rhee MH, Park HJ. Total saponin from Korean red ginseng inhibits binding of adhesive proteins to glycoprotein IIb/IIIa via phosphorylation of VASP (Ser 157) and dephosphorylation of PI3K and Akt. *Journal of Ginseng Research*. 2016. 40: 76-85.
- Lagarde M, Menashi S, Crawford N. Localisation of phospholipase A₂ and diglyceride lipase activities in human platelet intracellular membranes. *FEBS Letter*. 1981. 124: 23-26.
- Lee DH, Cho HJ, Kim HH, Rhee MH, Ryu JH, Park HJ. Inhibitory effects of total saponin from Korean red ginseng *via* vasodilator-stimulated phosphoprotein-Ser157 phosphorylation on thrombin-induced platelet aggregation. *Journal of Ginseng Research*. 2013. 37: 176-186.
- Lee JH, Park HJ. Effects of intaking of red ginseng products on human platelet aggregation and blood lipids. *Journal of Ginseng Research*. 1998. 22: 173-180.
- Lee SR, Park JH, Kim ND, Choi KJ. Inhibitory effects of ginsenoside Rg3 on platelet aggregation and its mechanism of action. *Journal of Ginseng Research*. 1997. 21: 132-140.
- Lee YJ, Han JY, Lee CG, Heo K, Park SI, Park YS, Kim JS, Yang KM, Lee KJ, Rhee MH, Kim SD. Korean red ginseng saponin fraction modulates radiation effects on lipopolysaccharide-stimulated nitric oxide production in RAW264. 7 macrophage cells. *Journal of Ginseng Research*. 2014. 38: 208-214.
- Ley K. The role of selectins in inflammation and disease. *Trends in Molecular Medicine*. 2003. 9: 263-268.
- Matsuda H, Kubo M, Tani T, Arichi S, Kitagawa I. Pharmacological study on panax ginseng CA MEYER V.: effects of red ginseng on the experimental disseminated intravascular coagulation (4). on ginsenoside-Rg3, Rh1 and Rh2. *The Japanese Society of Pharmacognosy*. 1985. 39: 123-125.
- McNicol A, Shibou TS. Translocation and phosphorylation of cytosolic phospholipase A₂ in activated platelets. *Thrombosis Research*. 1998. 92: 19-26.
- Mustard JF, Packham MA. Factors influencing platelet function: adhesion, release, and aggregation. *Pharmacological Reviews*. 1970. 22: 97-187.
- Needleman P, Moncada S, Bunting S, Vane JR, Hamberg M, Samuelsson B. Identification of an enzyme in platelet microsomes which generates thromboxane A₂ from prostaglandin endoperoxides. *Nature*. 1976. 261: 558-560.
- Nishikawa M, Tanaka T, Hidaka H. Ca²⁺-calmodulin-dependent phosphorylation and platelet secretion. *Nature*. 1980. 287: 863-865.
- Park HJ, Lee JH, Lee SJ, Ham HS, Cho HJ, Lim CR, Park KH. Effects of intaking of red ginseng products on relationship between obesity and blood lipids. *Korean Journal of Clinical Laboratory Science*. 2000. 6: 253-260.
- Park JS, Cho JY. Anti-inflammatory effects of ginsenosides from

- panax ginseng and their structural analogs. *African Journal of Biotechnology*. 2009. 8: 3682-3690.
- Patrignani P, Sciulli MG, Manarini S, Santini G, Cerletti C, Evangelista V. COX-2 is not involved in thromboxane biosynthesis by activated human platelets. *Journal of Physiology and Pharmacology*. 1999. 50: 661-667.
- Needleman P, Moncada S, Bunting S, Vane JR, Hamberg M, Samuelsson B. Identification of an enzyme in platelet microsomes which generates thromboxane A₂ from prostaglandin endoperoxides. *Nature*. 1976. 261: 558-560.
- Patrono C. Aspirin as an antiplatelet drug. *New England Journal of Medicine*. 1994. 330: 1287-1294.
- Phillips DR, Conley PB, Sinha U, Andre P. Therapeutic approaches in arterial thrombosis. *Journal of Thrombosis and Haemostasis*. 2005. 3: 1577-1589.
- Rosado JA, Sage SO. Role of the ERK pathway in the activation of store-mediated calcium entry in human platelets. *Journal of Biological Chemistry*. 2001. 276: 15659-15665.
- Rosado JA, Sage SO. The ERK cascade, a new pathway involved in the activation of store-mediated calcium entry in human platelets. *Trends in Cardiovascular Medicine*. 2002. 12: 229-234.
- Shin JH, Kwon HW, Cho HJ, Rhee MH, Park HJ. Inhibitory effects of total saponin from Korean red ginseng on [Ca²⁺]_i mobilization through phosphorylation of cyclic adenosine monophosphate-dependent protein kinase catalytic subunit and inositol 1, 4, 5-trisphosphate receptor type I in human platelets. *Journal of Ginseng Research*. 2015. 39: 354-364.
- Shin JH, Kwon HW, Cho HJ, Rhee MH, Park HJ. Vasodilator-stimulated phosphoprotein-phosphorylation by ginsenoside Ro inhibits fibrinogen binding to αIIb/β3 in thrombin-induced human platelets. *Journal of Ginseng Research*. 2016. 40: 359-365.
- Trialists/Collaboration A. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *British Medical Journal*. 2002. 324: 71-86.
- von Hundelshausen P, Weber C. Platelets as immune cells. *Circulation Research*. 2007. 100: 27-40.
- Wee JJ, Kim YS, Kyung JS, Song YB, Do JH, Kim DC, Lee SD. Identification of anticoagulant components in korean red ginseng. *Journal of Ginseng Research*. 2010. 34: 355-362.
- Yacoub D, Théorêt JF, Villeneuve L, Abou-Saleh H, Mourad W, Allen BG, Merhi Y. Essential role of protein kinase Cδ in platelet signaling, αIIbβ3 activation, and thromboxane A₂ release. *Journal of Biological Chemistry*. 2006. 281: 30024-30035.
- Yang Y, Lee J, Rhee MH, Yu T, Baek KS, Sung NY, Cho JY. Molecular mechanism of protopanaxadiol saponin fraction-mediated anti-inflammatory actions. *Journal of Ginseng Research*. 2015. 39: 61-68.
- Zarbock A, Polanowska-Grabowska RK, Ley K. Platelet-neutrophil interactions: linking hemostasis and inflammation. *Blood Reviews*. 2007. 21: 99-111.

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