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ARTICLE

Anti-platelet Effect of Ginkgolide A from *Ginkgo biloba*

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Abstract Effects of ginkgolide A (GA) from *Ginkgo biloba* leaves in collagen (10 μg/mL)-stimulated platelet aggregation were investigated. Zymographic analysis confirmed that pro-matrix metalloproteinase-9 (MMP-9) (92 kDa) was activated by GA to form an activated MMP-9 (86-kDa) on gelatinolytic activities. GA concentration-dependently inhibited platelet aggregation, intracellular Ca^{2+} mobilization, and thromboxane A_2 (TXA_2) formation by inhibiting the cyclooxygenase-1 (COX-1) activity in collagen-stimulated platelets. In addition, GA increased the formation of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), which have an anti-platelet function in both resting and collagen-stimulated platelets. On the other hand, GA did not prolong prothrombin time (PT) and activated partial thromboplastin time (aPTT) associated with the extrinsic and intrinsic coagulation pathways on human plasma, respectively. Therefore, we suggest that the inhibitory effect of GA on platelet aggregation might involve the following pathway. GA may increase the MMP-9 activity and intracellular cAMP and cGMP production, inhibit intracellular Ca^{2+} mobilization, and decrease TXA_2 production by down-regulating the COX-1, thereby leading to inhibition of platelet aggregation without cytotoxicity. These results strongly indicate that GA is a potent inhibitor of collagen-stimulated platelet aggregation. It may play an important role as a negative regulator during platelet activation.

Jin-Hyeob Ryu and Ju-Ye Ro contributed equally to this work.

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Introduction

Platelet aggregation is essential to the formation of a hemostatic plug when normal blood vessels are injured. However, the interactions between platelets and collagen can also cause circulatory disorders, such as thrombosis, atherosclerosis, and myocardial infarction (Schwartz et al., 1990). Inhibition of the platelet-collagen interaction provides a promising approach to the prevention of thrombosis. It has been known that matrix metalloproteinase-9 (MMP-9) is released from human platelets, and that it significantly inhibits platelet aggregation stimulated by collagen (Ray and Stetler-Stevenson, 1994). MMP-9 is secreted as a 92-kDa proenzyme and can be activated into an 86-kDa active form (Brikedal-Hansen, 1995). Vascular smooth muscle and endothelial cells are known to synthesize and release MMPs including MMP-9 (Ray and Stetler-Stevenson, 1994; Sawicki et al., 1997), suggesting that they may be associated with the process of hemostasis and thrombosis.

An important role in the mechanism by which collagen induces platelet aggregation is played by thromboxane A_2 (TXA_2) formation (Cattaneo et al., 1991), which also contributes to an increase in cytosolic-free Ca^{2+} level ($[Ca^{2+}]_i$) in collagen-activated platelets. Increase in $[Ca^{2+}]_i$ activates both Ca^{2+}/calmodulin-dependent phosphorylation of myosin light chain (20 kDa) and diacylglycerol-dependent phosphorylation of cytosolic protein (40 or 47 kDa) inducing platelet aggregation (Nishikawa et al., 1980; Kaibuchi et al., 1982). In addition, diacylglycerol can also be hydrolyzed by diacylglycerol lipase to produce the precursor of TXA_2, arachidonic acid (20:4). TXA_2 is a potent platelet aggregation agent that is generated by cyclooxygenase-1 (COX-1) and TXA_2 synthase (TXAS) from arachidonic acid liberated when phospha-

tidylinositol-4,5-bisphosphate is broken down by collagen, thrombin, and ADP (Nishikawa et al., 1980; Kaibuchi et al., 1982; Menshikov et al., 1993). So, the agent which can be down-regulated the TXA_2 by decreasing the COX-1 or TXAS is available as anti-platelet drug.

Verapamil and theophylline have an antiplatelet function that elevates the level of cyclic adenosine monophosphate (cAMP), and then decreases $[Ca^{2+}]_i$, an essential factor for platelet aggregation. Vasodilators (such as molsidomine and nitroprusside) and cyclic guanosine monophosphate (cGMP) phosphodiesterase (PDE) inhibitors (such as zaprinast and erythro-9-[2-hydroxy-3-nonyl]adenine) elevate cGMP levels in platelets (Menshikov et al., 1993). cGMP is believed to be produced *via* the activation of guanylate cyclase in the presence or absence of nitric oxide (NO). NO, synthesized in platelets, decreases agonist-elevated $[Ca^{2+}]_i$ (Pasqui et al., 1991) and has a role in inhibiting platelet activation (Rodomski et al., 1990). Therefore, cAMP and cGMP are anti-platelet second messengers in platelet aggregation.

Ginkgo biloba, a Chinese herb, has been used in traditional Chinese medicine for thousands of years (Kleijnen and Knipschild, 1992). It is of great interest, because its leaves posses pharmacological properties that include radical scavenging, blood flow improvement, vasoprotection, and anti- platelet aggregating factor (PAF) activity (Van, 2000a; Direu and De, 2000). In numerous experimental models, *ginkgo* extract has been found to have positive effects on vascular and metabolic disturbances and has also been shown to have neurological and behavioral effects, especially in dementia (Agnoli et al., 1984; Karcher et al., 1984; Tang and Eisenbrand, 1992). The active constituents of *Ginkgo* extract that can inhibit the binding of PAF include terpene trilactones, such as bilobalide, ginkgolide A (GA), ginkgolide B (GB), ginkgolide C (GC), and flavonoids (Van, 2000b; Braquet, 1986). The ability of GA to inhibit collagen-stimulated platelet aggregation has not been clarified until now. In the present study, we report that in collagen-stimulated platelet aggregation, GA strongly inhibits $[Ca^{2+}]_i$ elevation and TXA_2 production via down-regulation of COX-1, and simultaneously increases the intracellular levels of cAMP and cGMP without cytotoxicity.

Materials and Methods

Materials. GA (Fig. 1) from *Ginkgo biloba* leaves was purchased from Sigma-Aldrich Co. (USA), and collagen was obtained from Chrono-Log Corporation (USA). Fura 2-AM was obtained from Sigma Chemical Co. cAMP- and cGMP enzyme-immuno assay kits were purchased from R&D systems, Inc. (USA), and lactate dehydrogenase (LDH) cytotoxicity assay, COX-1 activity assay, and TXB_2 EIA kits were bought from Cayman Chemical (USA). Prothrombin time (PT), activated partial thromboplastin time (aPTT) reagents, 0.25 mM $CaCl_2$ and protein molecular weight standards were bought from Thermo Scientific Inc. (USA). All other chemicals and reagents used in this study were purchased from Sigma-Aldrich Co. (USA).

Preparation of washed rat platelets. Blood was drawn from the antecubital veins of rats and anticoagulated with ACD solution (0.8% citric acid, 2.2% sodium citrate, and 2.45% glucose). Platelet-rich plasma was centrifuged at 125×*g* for 10 min to remove the red blood cells, and the platelets were washed twice with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.5 mM glucose, and 1 mM EDTA, pH 7.4). The washed platelets were then resuspended in suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 0.49 mM $MgCl_2$, 5.5 mM glucose, 0.25% gelatin; pH 7.4) to a final concentration of 5×10^8 /mL. All of the above procedures were carried out at 25°C to avoid platelet aggregation upon cooling. The Ethical Committees for Animal Experiments of Konyang University (Korea) approved this study.

Measurement of platelet aggregation. Washed platelets (10^8 /mL) were preincubated for 3 min at 37°C in the presence of 2 mM exogenous $CaCl_2$ with or without GA and then stimulated with collagen (10 μg/mL) for 5 min. Aggregation was monitored using an aggregometer (Chrono-Log, Corp., USA) at a constant stirring speed of 1,000 rpm. Each aggregation rate was evaluated as an increase in light transmission. The suspension buffer was used as the reference. GA was dissolved in dimethylsulfoxide (DMSO, 0.5%), and the effect of DMSO was subtracted from the results.

Gelatin-based zymography of MMP-9. Washed platelets (10^8 /mL) were preincubated for 3 min at 37°C with various concentrations of GA in the presence of 2 mM $CaCl_2$ and then stimulated with collagen (10 μg/mL) for 5 min for zymography. The platelets were lysed on ice for 1 h in RIPA buffer (150 mM NaCl, 1% NP40, 0.1% SDS, 50 mM Tris, 0.5% deoxycholic acid; pH 8.0) containing the protease inhibitors, sodium orthovanadate (0.5 mM), and phenylmethylsulphonyl fluoride (PMSF; 1 mM), and centrifuged at 14000×*g* at 4°C for 30 min. The supernatant was used for the detection of activated MMP-9 in the cytosolic fraction by gelatin zymography. The proteins in the samples were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis using 10% gels containing 1 mg/mL gelatin. Samples containing 25 μg proteins were electrophoresed at 120 V for 90 min. The gels were washed with 2.5% Triton X-100 for 1 h, and then incubated with developing buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 0.02% NaN_3, 1 mM $ZnCl_2$; pH 7.5) at 37°C for 24 h. The gels were stained with 2.5% Coomassie Blue R-250 in 50% methanol and 10% acetic acid for 20 min, and destained in a solution of 30% methanol and 10% acetic acid, until the active bands became clear. The digested area appeared clear on a blue background indicating the location of gelatinase.

Determination of $[Ca^{2+}]_i$. Platelet-rich plasma was incubated with 5 μM fura 2-AM at 37°C for 60 min. Fura 2-AM is light-sensitive, thus the tube containing platelet-rich plasma was covered with aluminum foil during loading. The fura 2-loaded washed platelets were prepared using the procedure described above. Fura 2-loaded washed platelets (10^8 /mL) were preincubated for 3 min

at 37°C with various concentrations of GA in the presence of 2 mM $CaCl_2$ and then stimulated with collagen (10 μg/mL) for 5 min for evaluation of $[Ca^{2+}]_i$. Fura 2 fluorescence was measured with a spectrofluorimeter (SHIMADZU, RF-5301 PC, Japan) with an excitation wavelength that was changed every 0.5 sec from 340 to 380 nm; the emission wavelength was set at 510 nm. The $[Ca^{2+}]_i$ values were calculated using the method of Schaeffer and Blaustein (1989). Because GA was dissolved in DMSO (0.5%), the levels of cAMP and cGMP were calculated by subtracting the effect of DMSO.

Measurement of TXB_2. Washed platelets (10^8 /mL) were preincubated with or without GA for 3 min in the presence of 2 mM $CaCl_2$, and activated for 5 min with collagen (10 μg/mL). The reactions were terminated by the addition of ice-cold EDTA (5 mM) and indomethacin (0.2 mM). The amount of TXB_2, a stable metabolite of TXA_2, was determined using a TXB_2 EIA kit. To determine the direct effects of GA on arachidonic acid metabolism, the cells were first sonicated with a sonicator (Bandelin, HD2070, Germany) to obtain platelet lysates. The platelet lysates were incubated with various concentrations of GA for 5 min, and then 100 pmol of arachidonic acid was added to 200 μL lysate. The lysate mixtures were incubated for further 10 min, and the amount of TXB_2 was determined as described above. GA was dissolved in DMSO (0.5%), and the effect of DMSO was subtracted from the results.

COX-1 activity assay. Platelets in suspending buffer containing 1% protease inhibitor were sonicated with a sonicator (Vibra-Cell, VCX 130, USA). The platelet lysates (10 μg-protein) were incubated with SC-560 (330 nM), a positive control as a COX-1 inhibitor, for 5 min at 25°C, with or without GA (0.5%). COX-1 activity was assayed with COX-1 activity assay kit (Cayman Chemical, USA) according to the manufacturer's recommendations. GA was dissolved in DMSO (0.5%), and the effect of DMSO was subtracted from the results.

TXA_2 synthase activity assay. Platelet lysates (10 μg-protein) were preincubated with ozagrel (11 nM), a positive control as a TXAS inhibitor, with or without GA (0.5%) at 37°C for 5 min. The reactions were 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, and neutralized with 1 N NaOH. The amount of TXB_2, a stable metabolite of TXA_2, was determined using a TXB_2 EIA kit according to the manufacturer's recommendations. GA was dissolved in DMSO (0.5%), and the effect of DMSO was subtracted from the results.

Measurement of cAMP and cGMP. Washed platelets (10^8 /mL) were preincubated for 3 min at 37°C with various concentrations of GA in the presence of 2 mM $CaCl_2$, and were then stimulated with collagen (10 μg/mL) for 5 min for platelet aggregation. The aggregation was terminated by the addition of 80% ice-cold ethanol. cAMP and cGMP were measured using cAMP and cGMP EIA kits. Because GA was dissolved in DMSO (0.5%), the levels of cAMP and cGMP were calculated by subtracting the effect of DMSO.

Assessment of PT and aPTT analyses. To investigate the effects of GA on coagulation pathway, we assessed PT and aPTT analyses. Citrated whole blood from adult, healthy volunteer donor was prepared by centrifuging at 125×g for 10 min. Platelet-poor plasma (PPP) with or without varying concentrations of GA was preincubated in coagulation analyzer (TS 4000, MD pacific, China) with gentle stirring for incubation at 37°C. PT was determined as the addition of PT reagent (0.2 mL) to preincubated PPP for 3 min, and then the formation time of a fibrin clot was detected. After preincubation of PPP with aPTT reagent, 0.1 mL of 0.25 mM $CaCl_2$ was added for determination of the fibrin clot time.

LDH assay. To assess whether GA has cytotoxicity on platelet aggregation reaction, we examined the effect of GA on LDH release, which is a stable enzyme normally found in the cytosol of cells, but rapidly releases into the supernatant upon damage of cell membrane. Washed platelets (10^8 /mL) were incubated for 5 min at 37°C with various concentrations of GA, and then the supernatant was measured by an LDH assay kit (Cayman Chemical) according to the manufacturer's recommendations. Because GA was dissolved in DMSO (0.5%), the levels of cAMP and cGMP were calculated by subtracting the effect of DMSO.

Statistical analysis. The experimental results are expressed as the means ± SEM and are accompanied by the number of observations. Data were with analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared by the Newman-Keuls method. p value less than 0.05 was considered statistically significant.

Results

Inhibitory effect of GA on platelet aggregation. The concentration of collagen that induces maximal platelet aggregation has been shown to be 10 μg/mL (Cho et al., 2004). Therefore, this concentration of collagen was used as a platelet agonist in our study. Since $[Ca^{2+}]_i$ is a critical regulator of platelet aggregation, GA (Fig. 1A) were examined in the presence of 2 mM $CaCl_2$. When washed platelets (10^8 /mL) were activated with 10 μg/mL collagen in the presence of 2 mM $CaCl_2$, GA (1, 10, 50, 100, and 500 μM) significantly reduced the collagen-stimulated platelet aggregation in a dose-dependent manner (Fig. 1B).

Effect of GA on MMP-9 activity in washed platelets. To determine whether platelet activation might cause changes in MMP-9 activity in the cytoplasm, we used the cell lysates treated with or without GA in the collagen-stimulated platelets. When platelets were preincubated with GA, GA concentration-dependently increased the activity of MMP-9 in collagen-stimulated platelets (Fig. 1C). Zymographic analysis confirmed that pro-MMP-9 (92 kDa) was activated by GA (50 and 100 μM) to form an activated MMP-9 (86-kDa), as shown by gelatinolytic activities. These results suggest that the activated MMP-9 by GA may inhibit up-stream of platelet aggregation when stimulated by collagen (10 μg/mL).

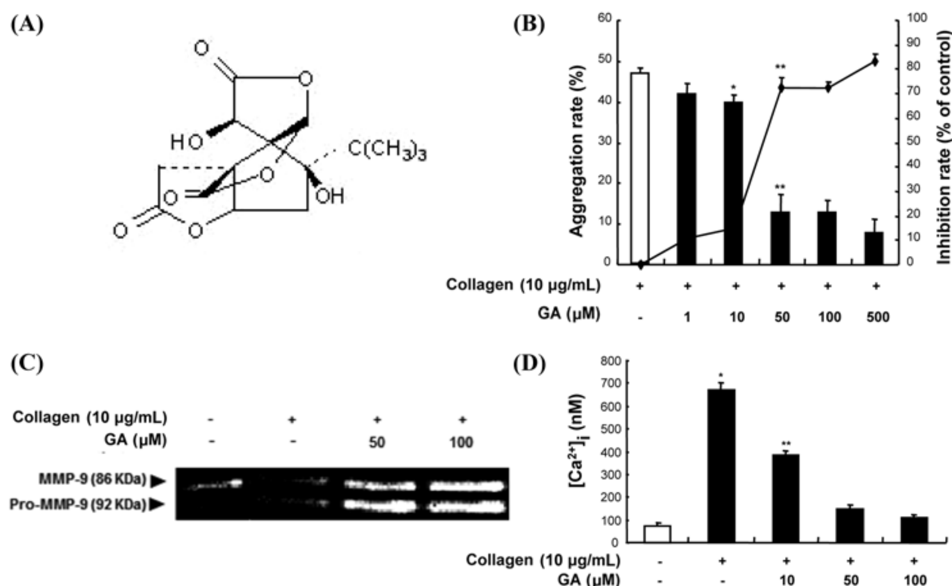

Fig. 1 Effects of GA in collagen-induced platelet aggregation, MMP-9 activity, and intracellular Ca^{2+} mobilization. (A) Chemical structure of GA from *Ginkgo biloba* leaves. (B) Effects of GA pretreatment on collagen-induced platelet aggregation. Washed platelets (10^8 /mL) were preincubated with varying concentrations of GA (1 to 500 μM) in the presence of 2 mM $CaCl_2$ for 3 min at 37ºC, and then stimulated with collagen (10 μg/mL) for 5 min. Platelet aggregation (%) was recorded as an increase in light transmission. Inhibition by GA was recorded as a percentage of the collagen-induced aggregation rate. (C) Effects of GA-induced MMP-9 activity in collagen-stimulated platelets. Washed platelets (10^8 /mL) were preincubated with various concentrations of GA in the presence of 2 mM $CaCl_2$ for 3 min at 37ºC, and then stimulated with collagen (10 μg/mL) for 5 min. Gelatin zymography was performed to detect MMP-9 activity from lysed sample with RIPA buffer (150 mM NaCl, 1% NP40, 0.1% SDS, 50 mM Tris, 0.5% deoxycholic acid; pH 8.0) that contained a protease inhibitor, 0.5 mM sodium orthovanadate, and 1 mM PMSF. MMP-9 activity was determined as described in “Materials and Methods”. (D) Effects of GA on collagen-induced $[Ca^{2+}]_i$ mobilization. Fura 2-loaded platelets (10^8 /mL) were preincubated with various concentrations of GA in the presence of 2 mM $CaCl_2$ for 3 min at 37ºC, and then collagen (10 μg/mL) was added. $[Ca^{2+}]_i$ was determined as described in “Materials and Methods”. Data are expressed as mean ± SEM (n = 4). **p* <0.001 was compared with that of basal level. ***p* <0.001 was compared with that of collagen-induced platelets.

Effects of GA on the regulation of aggregation-inducing molecules, $[Ca^{2+}]_i$ and TXA_2. When washed platelets (10^8 /mL) were stimulated by collagen (10 μg/mL), the level of $[Ca^{2+}]_i$ increased from 75 to 672 nM (Fig. 1D). However, this was significantly reduced by GA (10, 50, and 100 μM) in a dose-dependent manner (83% inhibition at 100 μM), suggesting that the inhibitory activity of GA on collagen-stimulated platelet aggregation was due to lowering of the level of $[Ca^{2+}]_i$.

TXA_2 is a potent stimulator of platelet aggregation, and its receptor G-protein (Gq)-PLC-IP_3 signaling pathway is activated by collagen treatment (Jang et al, 2002). Therefore, we next examined whether GA blocked the production of TXA_2 (determined as TXB_2) in collagen-treated platelets. The TXB_2 level in intact platelets was 0.4±0.1 ng/10^8 platelets, and this was markedly increased to 20.3±2.1 ng/10^8 platelets in the collagen-stimulated platelets (Fig. 2A). However, GA significantly reduced the production of TXB_2 in a dose-dependent manner (37.4% inhibition at 100 μM). In Fig. 2A, acetylsalicylic acid, TXA_2 down-regulator acting as a COX-1 inhibitor, is used as the positive control. To determine if the inhibitory effect on TXB_2 release of GA was due to the direct suppression of COX-1 or TXA_2 synthetase, a cell-free enzyme assay method was used. When platelet lysates were incubated with or without GA (100 μM) for 5 min at 37ºC in the presence of arachidonic acid, a substrate of COX-1, GA treatment inhibited TXB_2 production (45% inhibition at 100 μM) when compared with that of intact platelets (Fig. 2B), suggesting that the decrease in TXB_2 production by GA could be directly related to inhibition of its metabolic enzyme, COX-1 or TXA_2 synthetase. Subsequently, we determined the effects of GA on COX-1 and TXA_2 synthase (TXAS) activities. GA decreased the COX-1 activity in a dose-dependent manner, and 330 nM of SC-560, a selective COX-1 inhibitor, was used as a positive control (Fig. 2C). On the other hand, after the addition of PGH_2 (5 μM) for 1 min at 37ºC, platelet lysate with GA (100, 500 μM) did not affect the TXAS activity (Fig. 2D). When ozagrel (11 nM), a TXAS inhibitor, was used as a positive control, it decreased TXA_2 level to 211.1±10.7 ng/min/mg-protein as compared with that of control. These results indicate GA inhibited the COX-1 activity but not TXAS activity. Based on the results, we suggest that GA inhibits the collagen-induced platelet aggregation by decreasing $[Ca^{2+}]_i$ and TXA_2 production *via* inhibition of COX-1 activity.

Effects of GA on the formation of cAMP and cGMP. Increased platelet activating reagent-induced aggregation is known to be lowered by the production of either cGMP or cAMP (Wang et al, 1998). We next investigated whether GA up-regulated the cellular

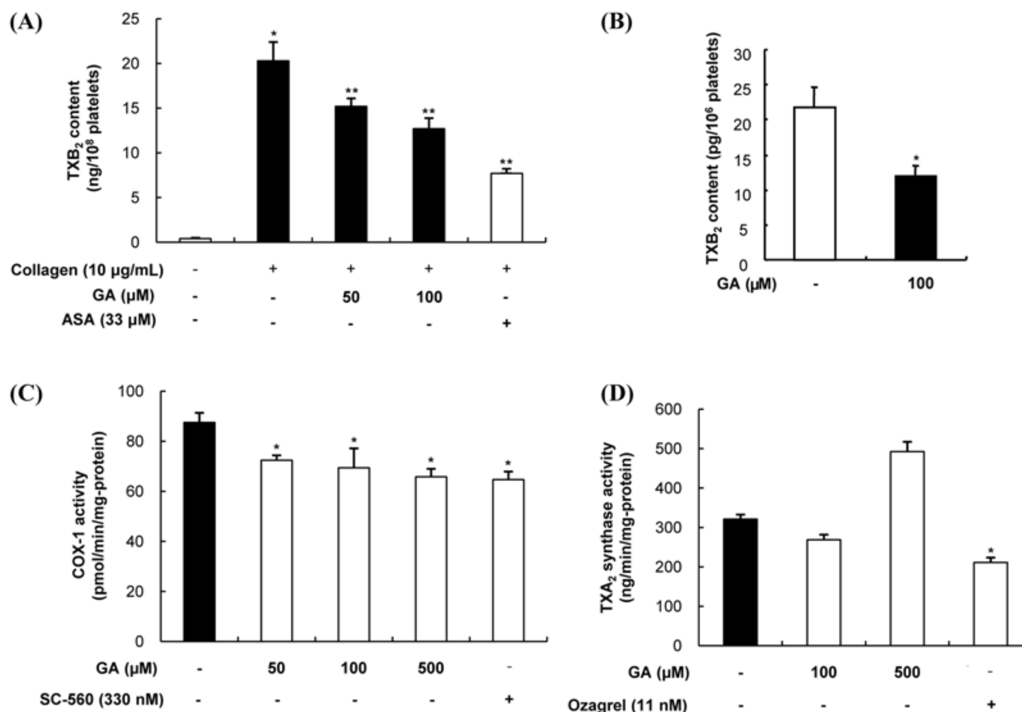

Fig. 2 The effects of GA in TXA_2 regulation. (A) Effects of GA on the production of TXB_2 stimulated by collagen. Washed platelets (10^8 /mL) were preincubated with GA for 3 min in the presence of 2 mM $CaCl_2$, and then stimulated with collagen (10 μg/mL). *p <0.001 was with that of basal level. **p <0.001 was compared with that of collagen-induced platelets. ASA, acetylsalicylic acid. (B) Effects of GA on the production of TXB_2 in intact platelets. TXB_2 was experimented as described in "Materials and Methods". The content of TXB_2 was measured using a TXB_2 EIA kit. Data are expressed as means ± SEM (n = 4). *p <0.001 was with that of control. (C) Effects of GA on COX-1 activity. Platelet lysate was incubated with or without GA or SC-560 (330 nM) as known as selective COX-1 inhibitor at 37°C for 30 min. COX-1 activity was determined by measuring peroxidase activity of COX. The peroxidase activity is assayed at 590 nm. Data represent means ± SEM (n = 3). *p <0.05 was compared with that of control. (D) Effect of GA on TXA_2 synthase activity. Washed platelets lysated by sonicator was pre-incubated with GA or ozagrel (11 nM), a TXA_2 synthase inhibitor, at 37°C for 30 min. Then PGH_2 solution (5 μM) was added as a substrate for TXA_2 synthase. TXA_2 synthase activity was determined by measuring TXA_2 formation. Data represent means ± SEM (n = 3). *p <0.05 was compared with that of control.

levels of cAMP and cGMP. Collagen decreased intracellular cAMP level from 4.9±0.5 pmol/10^8 platelets (basal level), to 2.7±0.3 pmol/10^8 platelets in the washed platelets (Fig. 3A). When the platelets were incubated in the presence of both GA and collagen, GA (10 to 100 μM) significantly increased cAMP levels in a dose-dependent manner. GA alone (10 to 100 μM) progressively increased cAMP level from 12.0±0.8 to 22.4±1.1 pmol/10^8 platelets in comparison to the control level (4.9±0.5 pmol/10^8 platelets) in the resting platelets (Fig. 3B). It is interesting to note that GA modulated the production of cAMP in both resting and collagen-stimulated platelets. As shown in Fig. 3C, collagen decreased intracellular cGMP level from 6.2±0.2 pmol/10^8 platelets (basal level), to 4.7±0.3 pmol/10^8 platelets. When the platelets were incubated in the presence of both GA and collagen, GA (10 to 100 μM) significantly increased the cGMP level in a dose-dependent manner. Similar to the cAMP results, GA alone also increased the level of cGMP in resting platelets (Fig. 3D). These results indicate that GA regulates the production of cGMP in resting and collagen-stimulated platelets. These results indicate GA has the anti-platelet effect by up-regulating the intracellular cAMP and cGMP production in resting and collagen-induced platelets, respectively.

Effects of GA on PT and aPTT on human plasma. To assess the effect of GA on blood coagulation, we performed PT and aPTT test on human plasma. PT and aPTT values were 13.8±0.4 sec and 32.4±0.2 sec on plasma only, respectively. GA (10 to 500 μM)-treated plasma was similar with those of control (Fig. 4A and B). These results suggest that GA does not affect extrinsic (determined as PT) and intrinsic (determined as aPTT) pathway on blood coagulation system.

Effects of GA on cytotoxicity. To assess whether GA has cell toxicity, washed platelets (10^8 /mL) were incubated with various concentrations of GA (1 to 500 μM) for 5 min as the same method of platelet aggregation reaction, and then LDH level was assayed. As the results, GA (1 to 500 μM) do not have toxicity as compared with that of control in platelets (Fig. 5). From these results, we demonstrate that GA is available of novel agent as anti-platelet drug within these concentrations.

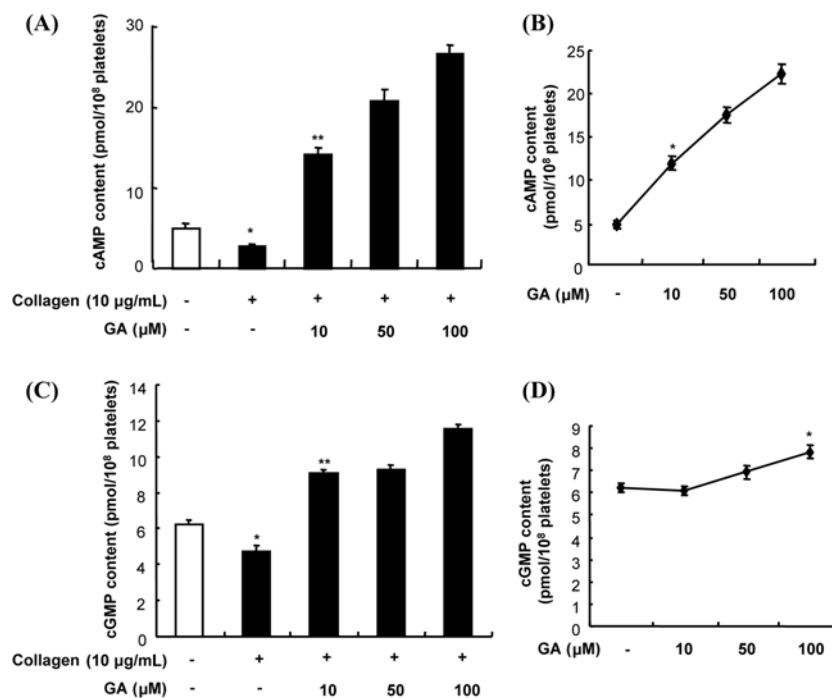

Fig. 3 The effects of GA on cAMP and cGMP production in resting and collagen-stimulated platelets. Washed platelets (10^8 /mL) were preincubated with or without GA for 3 min in the presence of 2 mM $CaCl_2$ and then stimulated with collagen (10 μg/mL) for 5 min at 37°C. The reactions were terminated by adding 80% ice-cold ethanol. cAMP and cGMP contents were measured using EIA kits. (A) Effects of GA on cAMP production in collagen-stimulated platelets. (B) Effects of GA on cAMP production in resting platelets. (C) Effects of GA on cGMP production in collagen-stimulated platelets. (D) Effects of GA on cGMP production in resting platelets. Data are expressed as mean ± SEM (n = 4). *p <0.05 was compared with that of basal level. **p <0.001 was compared with that of collagen-stimulated platelets.

Discussion

We used GA (Fig. 1A) from *Ginkgo biloba* leaves, a traditional Chinese medicine, to investigate anti-platelet function. When platelets (10^8 /mL) were preincubated with various concentrations of GA (1 to 500 mM), GA significantly inhibited the collagen-stimulated platelet aggregation (Fig. 1B). As shown in Fig. 3, the cAMP and cGMP production by GA in resting and collagen-stimulated platelets were measured. The results showed that, in the presence of collagen, GA acts as a strong intracellular inducer of platelets cAMP and cGMP, endogenous negative regulators of platelet aggregation (Qi et al., 1996; Homer and Wanstall, 2002; Park et al., 2004) (Fig. 3A and C). In addition, GA alone also increased the cAMP and cGMP production (cAMP >> cGMP) in the intact platelets (Fig. 3B and D). These results suggest that GA might directly affect the activity of adenylate cyclase or cAMP-dependent PDE as well as guanylate cyclase or cGMP-dependent PDE. The increased cAMP and cGMP levels participate in activating PKA and PKG. Consequently, these enzymes phosphorylate their substrate proteins, resulting in negative regulation of platelet aggregation. Such negatively regulated substrate proteins include vasodilator-stimulated phosphoprotein, a regulator of actin dynamics (Sudo et al., 2003), IP_3 receptor (Komalavilas and Lincoln, 1994), and TXA_2 receptor (Kinsella et al., 1994). Therefore, GA could block the platelet aggregation *via* enhanced levels of cAMP and cGMP and their linked PKA and PKG activities. In brief, GA increased the intracellular cAMP and cGMP levels to inhibit the collagen-stimulated platelet aggregation (Fig. 3).

Sheu et al. (2004) suggested that pro-MMP-9/activated MMP-9 is present in human platelets, and the inhibition of activated MMP-9 was demonstrated with the use of various agonists, such as collagen, thrombin, ADP, U46619, and arachidonic acid. We therefore investigated the effect of MMP-9 on platelets, and found the anti-platelet mechanism of GA from *Ginkgo biloba* in collagen-stimulated platelets. When platelets were preincubated with GA (50 and 100 mM) in collagen-stimulated platelets, pro-MMP-9 was activated by GA (Fig. 1C). These results indicate that the platelet aggregation by collagen stimulation affects the MMP-9 activity, and GA increased the MMP-9 activity to inhibit the platelet aggregation in collagen-stimulated platelet.

Among several aggregation-inducing molecules, Ca^{2+} and TXA_2 are known to be essential for platelet aggregation (Charo et al., 1977). Collagen-activated platelets require an adequate concentration of intracellular Ca^{2+} for aggregation, because the formation of platelets is accompanied by their migration and adhesion. However, GA significantly blocked Ca^{2+} release, which seems to be critical for the GA-mediated inhibition of platelet

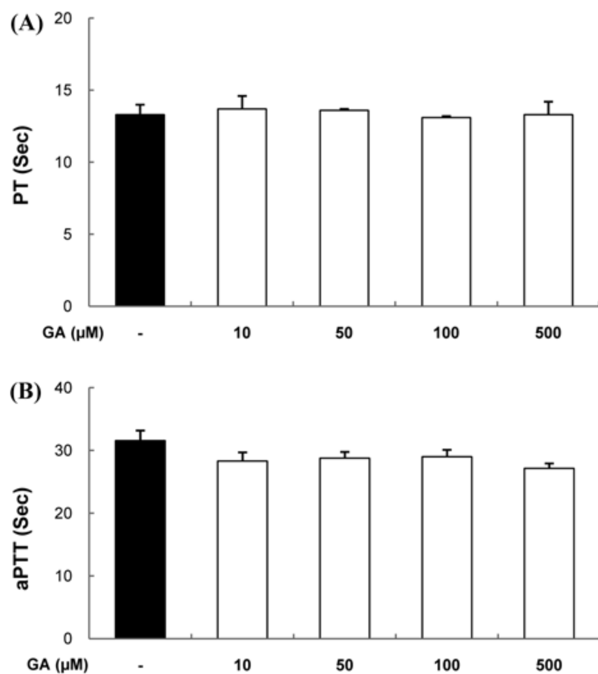

Fig. 4 The effect of GA on blood coagulation. (A) Effects of GA on PT in human plasma. Human plasma was pre-incubated with or without GA for 2 min at 37°C, and then treated with PT reagent for measurement. Data are expressed as means ± SEM (n = 3). (B) Effects of GA on aPTT in human plasma. After aPTT reagent was added, human plasma was pre-incubated with or without GA for 3 min at 37°C, and then treated with $CaCl_2$ for measurement. Data are expressed as means ± SEM (n = 3).

aggregation (Fig. 1C). GA at 100 μM suppressed collagen-induced TXB_2 production by 37.4% (Fig. 2A), whereas the same dose blocked Ca^{2+} release by 83%. Moreover, GA blocked TXB_2 formation from arachidonic acid *via* down-regulation of COX-1 activity in a cell-free system by 45% at 100 mM. Based on these results, we suggest that collagen-induced TXA_2 formation and Ca^{2+} mobilization were markedly inhibited by GA without cytotoxicity (Fig. 5), and that GA directly down-regulated COX-1 associated TXA_2 production from arachidonic acid. In the previous study, we suggested that GB and GC increase the MMP-9 activity and cyclic nucleotides (cAMP and cGMP), and decrease the $[Ca^{2+}]_i$ and TXA_2 production to inhibit the collagen-induced platelet aggregation (Cho and Nam, 2007a; Cho et al., 2007b). In the present study, we demonstrate that GA has the same mode of action to inhibit platelet aggregation such as GA and GB.

On the other hand, formation of occlusive thrombus is a powerful process, which requires coordinated series of events involving the blood coagulation system and platelet activation (Rivera et al., 2009). Blood coagulation systems have intrinsic (determined as aPTT) and extrinsic (determined as PT) systems. Normal value of PT and aPTT on human plasma were determined as 12.0–13.0 and 30.5–45.0 sec (Kratz and Lewandrowsko, 1998; Pagana and Pagana, 2010), respectively. At various concentrations of GA, GA did not delay PT and aPTT values (Fig. 4). In addition,

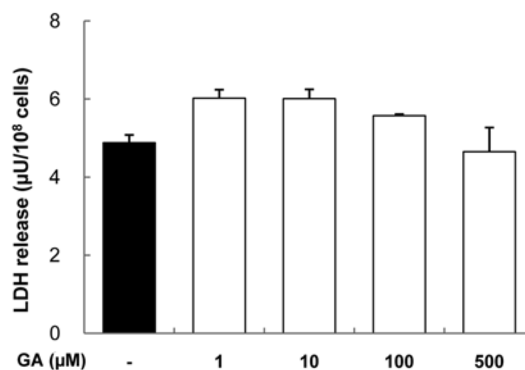

Fig. 5 The effect of GA on the LDH release in washed platelets. After 3 min incubation of washed platelets (10^8 /mL) with various concentrations of GA, LDH release was measured using LDH assay kit. Data are expressed as means ± SEM (n = 3).

GA has no cytotoxicity in the *in vitro* system (Fig. 5).

In conclusion, the most important result of this study is that GA significantly inhibited collagen-stimulated platelet aggregation without cytotoxicity (Fig. 5). This inhibitory effect may be due to the following mechanisms: GA increased the MMP-9 activity and the intracellular cAMP and cGMP levels, thereby leading to inhibition of TXA_2 production and intracellular Ca^{2+} mobilization. In addition, GA diminished the COX-1 activity, resulting in a decrease in the production of TXA_2, an aggregation-inducing molecule. This ultimately leads to inhibition of intracellular TXA_2-mediated Ca^{2+} mobilization and platelet aggregation. Therefore, GA could inhibit vascular diseases associated with platelet aggregation, such as atherosclerosis, myocardial infarction, and thrombosis. These results suggest that GA may be a physiologically effective negative feedback regulator during platelet aggregation induced by collagen.

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References

Agnoli A, Rapin JR, Scapagnini V, and Weitbrecht WV (1984) In *Effects of Ginkgo biloba Extract on Oraganic Cerebral Impairement*, (4th ed), John Libbey & Co Ltd, London.

Braquet P (1986) Proofs of involvement of PAF-acether in various immune disorders using BN 52021 (ginkgolide B): a powerful PAF-acether antagonist isolated from *Ginkgo biloba* L. *Adv Prost Res* **26**, 179–98.

Brikedal-Hansen H (1995) Proteolytic remodeling of extracellular matrix. *Curr Opin Cell Biol* **7**, 728–35.

Cattaneo M, Tenconi PM, Lecchi A, and Mannucci PM (1991) In vitro effects of picotamide on human platelet aggregation, the release reaction and thromboxane B_2 production. *Thromb Res* **62**, 717–24.

Charo IF, Feinman RD, and Detwiler TC (1977) Interrelations of platelet aggregation and secretion. *J Clin Invest* **60**, 866–73.

Cho HJ and Nam KS (2007a) Inhibitory effect of ginkgolide B on platelet aggregation in a cAMP-and cGMP-dependent manner by activated MMP-9. *J Biochem Mol Biol* **40**, 678–83.

Cho HJ, Ham HS, Lee TK, Jung YJ, Choi SA, Kang HC et al. (2004) Inhibitory effect of cordycepin on human platelet aggregation. *J Biomed Lab Sci* **10**, 1–8.

Cho HJ, Shon YH, and Nam KS (2007b) Ginkgolide C inhibits platelet aggregation in cAMP- and cGMP-dependent manner by activating MMP-9. *Biol Pharm Bull* **30**, 2340–4.

Direu K and De Feudis FV (2000) In *Ginkgo biloba: Chap 17*, (1st ed), Harwood Academic Publishers, Netherlands.

Homer KL and Wanstall JC (2002) Inhibition of rat platelet aggregation by the diazeniumdiolate nitric oxide donor MAHMA NONOate. *Br J Pharmacol* **137**, 1071–81.

Jang EK, Azzam JE, Dickinson NT, Davidson MM, and Haslam RJ (2002) Roles for both cyclic GMP and cyclic AMP in the inhibition of collagen-induced platelet aggregation by nitroprusside. *Br J Haematol* **117**, 664–75.

Kaibuchi K, Sano K, Hoshijima M, Takai Y, and Nishizuka Y (1982) Phosphatidylinositol turnover in platelet activation; calcium mobilization and protein phosphorylation. *Cell Calcium* **3**, 323–35.

Karcher L, Zagermann P, and Krieglstein J (1984) Effect of an extract of *Ginkgo biloba* on rat brain energy metabolism in hypoxia. *Naunyn Schmiedebergs Arch Pharmacol* **27**, 31–5.

Kinsella BT, O'Mahony DJ, and FitzGerald GA (1994) Phosphorylation and regulated expression of the human thromboxane A_2 receptor. *J Biol Chem* **269**, 29914–9.

Kleijnen J and Knipschild P (1992) *Ginkgo biloba*. *Lancet* **340**, 1136–9.

Komalavilas P and Lincoln TM (1994) Phosphorylation of the inositol 1,4,5-trisphosphate receptor by cyclic GMP-dependent protein kinase. *J Biol Chem* **269**, 8701–7.

Kratz A and Lewandrowski KB (1998) Case records of the Massachusetts General Hospital. Weekly clinicopathological exercises. Normal reference laboratory values. *N. Engl. J. Med* **339**, 1063–72.

Menshikov MYu, Ivanova K, Schaefer M, Drummer C, and Gerzer R (1993) Influence of the cGMP analog 8-PCPT-cGMP on agonist-induced increases in cytosolic ionized Ca^{2+} and on aggregation of human platelets. *Eur J Pharmacol* **245**, 281–4.

Nishikawa M, Tanaka T, and Hidaka H (1980) Ca^{2+}-calmodulin-dependent phosphorylation and platelet secretion. *Nature* **287**, 863–5.

Pagana KD and Pagana PJ (2010) *Mosby's manual of diagnostic and laboratory tests*. Mosby Elsevier, St. Louis, 396-397, 448–9.

Park WH, Kim HK, Nam KS, Shon YH, Jeon BH, Moon SK et al. (2004) Inhibitory effect of GBH on platelet aggregation through inhibition of intracellular Ca^{2+} mobilization in activated human platelets. *Life Sci* **75**, 3063–76.

Pasqui AL, Capecci PL, Ceccatelli L, Mazza S, Gistri A, and Laghi Pasini F (1991) Nitroprusside in vitro inhibits platelet aggregation and intracellular calcium translocation. Effect of hemoglobin. *Thromb Res* **61**, 113–22.

Qi R, Ozaki Y, Satoh K, Yang LB, Asazuma N, and Yatomi Y (1996) Intracellular levels of cyclic AMP and cyclic GMP differentially modify platelet aggregate size in human platelets activated with epinephrine or ADP. *J Cardiovasc Pharmacol* **28**, 215–25.

Ray JM and Stetler-Stevenson WG (1994) The role of matrix metalloproteases and their inhibitors in tumour invasion, metastasis and angiogenesis. *Eur Respir J* **7**, 2062–72.

Rivera J, Lozano ML, Navarro-Nunez L, and Vicente V (2009) Platelet receptors and signaling in the dynamics of thrombus formation. *Haematologica* **94**, 700–11.

Rodomski MW, Palmer RMJ, and Monacade S (1990) An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proceedings Proc Natl Acad Sci USA* **87**, 5193–7.

Sawicki G, Salas E, Murat J, Miszta-Lane H, and Radomski MW (1997) Release of gelatinase A during platelet activation mediates aggregation. *Nature* **386**, 616–8.

Schaeffer J and Blaustein MP (1989) Platelet free calcium concentrations measured with fura-2 are influenced by the transmembrane sodium gradient. *Cell Calcium* **10**, 101–3.

Schwartz SM, Heinmark RL, and Majesky MW (1990) Developmental mechanisms underlying pathology of arteries. *Physiol Rev* **70**, 1177–209.

Sheu JR, Fong TH, Liu CM, Shen MY, and Chen TL (2004) Expression of matrix metalloproteinase-9 in human platelets: regulation of platelet activation in in vitro and in vivo studies. *Br J Pharmacol* **143**, 193–201.

Sudo T, Ito H, and Kimura Y (2003) Phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) by the anti-platelet drug, cilostazol, in platelets. *Platelets* **14**, 381–90.

Tang W and Eisenbrand G (1992) In *Chinese Drugs of Plant Origin*, (1st ed), Springer Verlag, Germany.

Van Beek TA (2000a) In *Ginkgo biloba: Chap 1*, (1st ed), Harwood Academic Publishers, Netherlands.

Van Beek TA (2000b) In *Ginkgo biloba: Chap 7*, (1st ed), Harwood Academic Publishers, Netherlands.

Wang RG, Zhu Y, Halushka PV, Lincoln TM, and Mendelsohn ME (1998) Mechanism of platelet inhibition by nitric oxide: In vivo phosphorylation of thromboxane receptor by cyclic GMP-dependent protein kinase. *Proc Natl Acad Sci USA* **95**, 4888–93.