Ginkgolide C Inhibits Platelet Aggregation in cAMP- and cGMP-Dependent Manner by Activating MMP-9

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In this report, we investigated the effect of ginkgolide C (GC) from Ginkgo biloba leaves in collagen (10 μg/ml)-stimulated platelet aggregation. It has been known that matrix metalloproteinase-9 (MMP-9) is released from human platelets, and that it significantly inhibited platelet aggregation stimulated by collagen. Zymographic analysis confirmed that pro-MMP-9 (92-kDa) was activated by GC to form an activated MMP-9 (86-kDa) on gelatinolytic activities. And then, GC dose-dependently inhibited platelet aggregation, intracellular Ca²⁺ mobilization, and thromboxane A₂ (TXA₂) formation in collagen-stimulated platelets. In addition, GC significantly 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. Therefore, we demonstrate that the inhibitory effect of GC on platelet aggregation might be involved into the following pathways. GC may increase intracellular cAMP and cGMP production and MMP-9 activity, inhibit intracellular Ca²⁺ mobilization and TXA₂ production, thereby leading to inhibition of platelet aggregation. These results strongly indicate that GC is a potent inhibitor of collagen-stimulated platelet aggregation. It may be a suitable tool for a negative regulator during platelet activation.

Key words ginkgolide C; matrix metalloproteinase-9; cyclic nucleotide; intracellular Ca²⁺; platelet aggregation

Ginkgo biloba is one of the most ancient trees, and extracts from its leaves have been used in traditional medicine for several hundred years.¹–² There are numerous studies describing the beneficial effects of Ginkgo biloba extracts on patients with disturbances in vigilance, memory, and cognitive functions associated with aging and senility, and on those with all types of dementias, mood changes, and the inability to cope with daily stressors.³–⁴ In addition, it is of great interest because its leaves possess pharmacological properties that include radical scavenging, blood flow improvement, vasoprotection, and anti-platelet activating factor (PAF) activity.⁵–⁶ Among the constituents of Ginkgo biloba, terpene trilactones such as bilobalide, ginkgolide A (GA), ginkgolide B (GB), ginkgolide C (GC), along with flavonoids, have been identified as the active constituents of the Ginkgo extract for the inhibition of the binding of PAF.⁷–⁸ The ability of GC to inhibit collagen-stimulated platelet aggregation has not been clarified up until now.

Blood platelets are absolutely required for hemostatic plug formation when normal vessels are injured. However, the interactions between platelets and collagen can also cause circulatory disorders, such as thrombosis, atherosclerosis, and myocardial infarction.⁹ Inhibition of the platelet-collagen interaction might be a promising approach to the prevention of thrombosis. An important role in the mechanism by which collagen induces platelet aggregation is played by thromboxane A₂ (TXA₂) formation, which also contributes to an increase in cytosolic free Ca²⁺ level ([Ca²⁺]ᵢ) in collagen-activated platelets. An increase in [Ca²⁺]ᵢ activates both the Ca²⁺/calmodulin-dependent phosphorylation of myosin light chain (20-kDa) and the diacylglycerol-dependent phosphorylation of cytosolic protein (40- or 47-kDa) to induce platelet aggregation.¹⁰ In addition, diacylglycerol also can be hydrolyzed by diacylglycerol lipase to produce arachidonic acid, a precursor of TXA₂, which is a potent platelet aggregation agent generated from arachidonic acid liberated when PIP₂ is broken down by stimulation with collagen, thrombin and ADP.¹¹–₁₃ Verapamil and theophylline have an antiplatelet function that elevates the cyclic adenosine monophosphate (cAMP) level, and then decreases the [Ca²⁺]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.¹⁴ It is believed that cGMP is produced via the activation of guanylate cyclase in the presence or absence of nitric oxide (NO). NO, synthesized in platelets, decreases agonist-elevated [Ca²⁺]ᵢ, and has a role in inhibiting platelet activation.¹⁵

Matrix metalloproteinases (MMPs) belong to a family of zinc- and calcium-dependent proteases and are capable of degrading basement membrane as well as extracellular matrix surrounding cells. It is known that MMPs have been implicated in the tissue remodeling, which accompanies inflammation, bone resorption, wound healing, thrombosis, atherosclerosis, and tumor invasion.¹⁶ Most MMPs are synthesized and secreted as inactive proenzymes.¹⁷ MMP-9, also known as gelatinase B, has a broad range of substrate specificity for different native collagens (types IV, VI, VII, and X) as well as denatured collagens (gelatine) and elastin.¹⁸ MMP-9 is secreted as a 92-kDa proenzyme and can be activated to be an 86-kDa active form.¹⁹ Vascular smooth muscle and endothelial cells are known to synthesize and release MMPs including MMP-9,¹⁶,²⁰ suggesting that this may be associated with the process of hemostasis and thrombosis. Sheu et al. demonstrated that human platelets release MMP-9, and that activated MMP-9 significantly inhibited platelet aggregation stimulated by collagen. We therefore investigated the effect of MMP-9 on platelets, and found out the anti-platelet mechanism of ginkgolide C (GC) from Ginkgo biloba in collagen-stimulated platelets.

In the present report, we indicate that GC increases the ac-
tivity of MMP-9, strongly inhibits [Ca$^{2+}$], elevation and TXA$_2$ production, and simultaneously increases the intracellular levels of cAMP and cGMP in collagen-stimulated human platelet aggregation.

MATERIALS AND METHODS

Materials  GC (Fig. 1) from Ginkgo biloba leaves was purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.), and collagen was obtained from Chrono-Log Corporation (Havertown, PA, U.S.A.). Protein molecular weight standards were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Fura 2-AM was obtained from Sigma Chemical Co. cAMP- and cGMP-enzymeimmunoassay (EIA) kits were purchased from R&D systems, Inc. (Minneapolis, MN, U.S.A.), and a TXB$_2$ EIA system was obtained from Ame- sham Bioscience (Buckinghamshire, U.K.).

Preparation of Washed Rat Platelets  Blood was collected from Sprague-Dawley rats (6—7 weeks, male), and anti-coagulated with ACD solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose). Platelet-rich plasma was centrifuged at 1250×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$_2$PO$_4$, 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$_2$PO$_4$, 0.49 mM MgCl$_2$, 5.5 mM glucose, 0.25% gelatin, pH 7.4) to a final concentration of 5×10$^9$/ml. All of the above procedures were carried out at 25°C to avoid platelet aggregation on cooling.

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 GC and then stimulated with collagen (10$^8$/ml) for 5 min. Aggregation was monitored using an aggregometer (Chrono-Log Corp., Havertown, PA, U.S.A.) at a constant stirring speed of 1000 rpm. Each aggregation rate was evaluated as an increase in light transmission. The suspension buffer was used as the reference. GC was dissolved in dimethylsulfoxide (DMSO) (0.001%), 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 GC 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 phenylmethylsulphonylfluoride (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 (SDS-PAGE) using 10% gels containing 1 mg/ml gelatin. Samples containing 25 µg protein 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 µM 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.

Measurement of cAMP and cGMP  Washed platelets (10$^7$/ml) were preincubated for 3 min at 37°C with various concentrations of GC in the presence of 2 mM CaCl$_2$, and 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. Since GC was dissolved in DMSO (0.001%), the levels of cAMP and cGMP were calculated by subtracting the effect of DMSO.

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, so the tube containing the 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$^9$/ml) were preincubated for 3 min at 37°C with various concentrations of GC 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 spectrofluorometer (RF-5301 PC, Shimadzu, Japan) with an excitation wavelength that changed every 0.5 s 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.$^{21}$ Since GC was dissolved in DMSO (0.001%), the levels of cAMP and cGMP were calculated by subtracting the effect of DMSO.

Measurement of TXB$_2$  Washed platelets (10$^9$/ml) were preincubated with or without GC 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 GC 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 GC for 5 min, and then 100 pmol of arachidonic acid was added to 200 µl lystate. The lystate mixtures were incubated for a further 10 min, and the amount of TXB$_2$ was determined as described above.

Statistical Analysis  All data are shown as mean±S.D. Student’s t-test was used for data analysis and paired or unpaired comparison was used where necessary.

RESULTS

Inhibitory Effect of GC on Platelet Aggregation  The concentration of collagen that induced maximal platelet ag-
Aggregation was approximately 10 μg/ml. Therefore, 10 μg of collagen/ml was used as a platelet agonist in this study. Since [Ca^{2+}], is a critical regulator of platelet aggregation, it was used to examine the effects of GC in the presence of 2 mM CaCl₂. When washed platelets (10⁸/ml) were activated with 10 μg of collagen/ml in the presence of 2 mM CaCl₂, various concentrations of GC (1, 10, 50, 100, 500 μM) significantly reduced the collagen (10 μg/ml)-stimulated platelet aggregation in a dose-dependent manner (Fig. 2).

**Effect of GC 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 GC in the collagen-stimulated platelets. When platelets were preincubated with GC, GC concentration-dependently increased the activity of MMP-9 in collagen-stimulated platelets (Fig. 3). Zymographic analysis confirmed that pro-MMP-9 (92-kDa) was activated by GC (50, 100 μM) to form an activated MMP-9 (86-kDa), as shown by gelatinolytic activities. These results suggest that the GC increases the MMP-9 activity to inhibit the platelet aggregation in collagen-stimulated platelets. We therefore investigated the effect of intracellular cAMP and cGMP levels by GC in collagen-stimulated platelets.

**Effects of GC on the Formation of cAMP and cGMP**

The elevation of the platelet activating reagent-induced aggregation is known to be lowered by either the production of cGMP or cAMP. We next investigated whether GC up-regulated the cellular level of cAMP and cGMP. As shown in Fig. 4A, collagen decreased intracellular cAMP level from 4.9 ± 0.5 pmol/10⁸ platelets (basal level), to 2.7 ± 0.3 pmol/10⁸ platelets in the washed platelets. When the platelets, however, were incubated in the presence of both GC and collagen, GC (10 to 500 μM) significantly increased...
the cAMP level in a dose-dependent manner. On the other hand, GC (10 to 500 μM) alone progressively increased the cAMP level from 7.0 ± 0.6 to 12.7 ± 0.8 pmol/10^8 platelets in comparison with the control levels (4.9 ± 0.5 pmol/10^8 platelets) in resting platelets (Fig. 4B). It is interesting to note that GC modulated the production of cAMP in resting and in collagen-stimulated platelets.

As shown in Fig. 4C, 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 in the washed platelets. When the platelets, however, were incubated in the presence of both GC and collagen, GC (10 to 500 μM) significantly increased the cGMP level in a dose-dependent manner. With the change of cAMP level in platelets, GC alone also increased the level of cGMP in resting platelets (Fig. 4D). These results indicate that GC regulates the production of cGMP in resting and collagen-stimulated platelets.

**Effects of GC on the Regulation of Aggregation-Inducing Molecules, [Ca^{2+}], and TXA2.** As shown in Fig. 5A, 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. However, this was significantly reduced by various concentrations (10, 50, 100 μM) of GC in a dose-dependent manner (67% inhibition at 100 μM), suggesting that the inhibitory activity of GC on collagen-stimulated platelet aggregation was due to lowering of the level of [Ca^{2+}]_i.

TXA2 is a potent stimulator of platelet aggregation, and its receptor G-protein (Gq)-PLC-IP3 signaling pathway is activated by collagen treatment. Therefore, we next examined whether GC blocked the production of TXA2 under collagen exposure. The TXA2 (determined as TXB2) level in intact platelets was 1.5 ± 0.3 pg/10^8 platelets, and this was markedly increased to 7.2 ± 0.4 pg/10^8 platelets in the collagen-stimulated platelets (Fig. 5B). However, GC significantly reduced the production of TXA2 in a dose-dependent manner (73% inhibition at 100 μM). To determine if the inhibitory effect on TXA2 release of GC was due to the direct suppression of COX-1 or TXA2 synthase, cell-free enzyme assay method was used. When platelet lysates were incubated with or without GC for 5 min at 37°C in the presence of AA (20:4), a substrate of COX-1, GC (100 μM) possibly is not directly related to inhibition of its metabolic enzyme, COX-1 or TXA2 synthetase (Fig. 5C).

**DISCUSSION**

In this report, we used GC (Fig. 1) from *Ginkgo biloba* leaves, a traditional Chinese medicine, to investigate the antiplatelet effect. When platelets (10^8/ml) were preincubated with various concentrations of GC (1 to 500 μM), GC significantly inhibited the collagen-stimulated platelet aggregation (Fig. 2).

Sheu et al. suggested that pro-MMP-9/activated MMP-9 is present in human platelets, and the inhibition of activated MMP-9 was demonstrable with the use of various agonists, such as collagen, thrombin, ADP, U46619, and arachidonic acid. Nakamura et al. suggested that human plasma MMP-9 concentrations ranged from 34.2 ± 16.6 to 52.4 ± 26.6 ng/ml. Sheu et al. indicated that cytoplasm was the main storage compartment for MMP-9 in resting and collagen-stimulated platelets. Therefore, we used the cytoplasmic fraction to detect the MMP-9 activity. As shown in Fig. 3, when platelets were preincubated with GC (50, 100 μM) in collagen-stimulated platelets, pro-MMP-9 was activated by GC. These results indicate that the platelet aggregation by collagen stimulation affects the MMP-9 activity, and GC increased the MMP-9 activity to inhibit the platelet aggregation in collagen-stimulated platelet.

GC may affect the cAMP or cGMP regulation, and this is in accord with the concept that intracellular cAMP and cGMP level are responsible for platelet aggregation. Thus, we measured the cAMP and cGMP production by GC in resting and collagen-stimulated platelets. As the results, GC acts as a strong intracellular inducer of cAMP/cGMP, endogenous negative regulators of platelet aggregation, in the presence of collagen (Figs. 4A, C). On the other hand, GC alone interestingly increased the cAMP and cGMP production in intact platelets. These results suggest that GC might directly affect the activity of adenylate cyclase and cAMP-dependent PDE as well as guanylate cyclase and cGMP-dependent PDE. The increased cAMP and cGMP levels participate in activating PKA and PKG and consequently these enzymes phosphorylate their substrate proteins, resulting in negative regulation of platelet aggregation. The negatively regulated substrate proteins include vasodilator-stimulated phosphoprotein, a regulator of actin dynamics and TXA2 receptor. Therefore, GC might block the platelet aggregation *via* enhanced levels of cAMP and...
cGMP and their linked PKA andPKG activity. In brief, GC (Fig. 3) increased the intracellular cAMP and cGMP levels to inhibit the collagen-stimulated platelet aggregation.

Among several aggregation-inducing molecules, Ca\(^{2+}\) and TXA\(_2\) are known to be essential for platelet aggregation.\(^{32}\) Collagen-activated platelets require an adequate concentration of intracellular Ca\(^{2+}\) for aggregation, because the formation of platelet is accompanied by the migration of platelets and their adhesion. However, GC significantly blocked Ca\(^{2+}\) release which seems to be critical to the GC-mediated inhibition of platelet aggregation (Fig. 5). In the case of TXA\(_2\), GC suppressed collagen-induced TXA\(_2\) production by 73% at 100 \(\mu\)M, and by contrast, it blocked Ca\(^{2+}\) release by 67% at 100 \(\mu\)M. On the other hand, GC blocked TXA\(_2\) formation from arachidonic acid without directly affecting the COX-1 or TXA\(_2\) synthase. From these results, we suggest that collagen-induced TXA\(_2\) formation and Ca\(^{2+}\) mobilization were markedly inhibited by GC, and that GC doesn’t directly affect the COX-1 or TXA\(_2\) synthase associated with TXA\(_2\) production from arachidonic acid (Fig. 5).

In conclusion, the most important result of this study is that GC significantly inhibited the collagen-stimulated platelet aggregation. This inhibitory effect may involve the following mechanisms. (1) GC may increase the MMP-9 activity and intracellular cAMP and cGMP levels, thereby leading to inhibition of the TXA\(_2\) production and intracellular Ca\(^{2+}\) mobilization. (2) Moreover, GC diminishes TXA\(_2\) formation, an aggregation-inducing molecule, ultimately leading to inhibition of intracellular TXA\(_2\)-mediated Ca\(^{2+}\) mobilization and platelet aggregation. Therefore, these results suggest that GC may be a physiologically effective negative feedback regulator during platelet aggregation.

Acknowledgments This work was supported by the Medical Science and Engineering Research Centers (MRC) program from Ministry of Science and Technology (MOST)/Korea Science and Engineering (KOSEF) (R13-2005-013-01003-0), Korea.

REFERENCES