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.

**Keywords** cyclic adenosine monophosphate · cyclic guanosine monophosphate · cyclooxygenase-1 · ginkgolide A · intracellular Ca\(^{2+}\) · platelet aggregation · thromboxane A\(_2\)

**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\(^{2+}\)]) in collagen-activated platelets. Increase in [Ca\(^{2+}\)], 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 TXA2; 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 [Ca2+], 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 [Ca2+], (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 possess 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 triacetones, 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 [Ca2+]i, elevation and TXA2 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 TXB2 ELA kits were bought from Cayman Chemical (USA). Prothrombin time (PT), activated partial thromboplastin time (aPTT) reagents, 0.25 mM CaCl2 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 NaHCO3, 0.36 mM Na2HPO4, 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 NaHCO3, 0.36 mM Na2HPO4, 0.49 mM MgCl2, 5.5 mM glucose, 0.25% gelatin; pH 7.4) to a final concentration of 5×108/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 (108/mL) were preincubated for 3 min at 37°C in the presence of 2 mM exogenous CaCl2 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 (108/mL) were preincubated for 3 min at 37°C with various concentrations of GA in the presence of 2 mM CaCl2 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 for 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 CaCl2, 0.02% NaN3, 1 mM ZnCl2; 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 [Ca2+]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 (108/mL) were preincubated for 3 min...