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Inhibitory Effect of Ginkgolide B on Platelet Aggregation in a cAMP- and cGMP-dependent Manner by Activated MMP-9

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Extracts from the leaves of the *Ginkgo biloba* are becoming increasingly popular as a treatment that is claimed to reduce atherosclerosis, coronary artery disease, and thrombosis. In this study, the effect of ginkgolide B (GB) from *Ginkgo biloba* leaves in collagen (10 μg/ml)-stimulated platelet aggregation was investigated. It has been known that human platelets release matrix metalloproteinase-9 (MMP-9), and that it significantly inhibited platelet aggregation stimulated by collagen. Zymographic analysis confirmed that pro-MMP-9 (92-kDa) was activated by GB to form an MMP-9 (86-kDa) on gelatinolytic activities. And then, activated MMP-9 by GB dose-dependently inhibited platelet aggregation, intracellular Ca^{2+} mobilization, and thromboxane A_2 (TXA_2) formation in collagen-stimulated platelets. Activated MMP-9 by GB directly affects down-regulations of cyclooxygenase-1 (COX-1) or TXA_2 synthase in a cell free system. In addition, activated MMP-9 significantly increased the formation of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), which have the anti-platelet function in resting and collagen-stimulated platelets. Therefore, we suggest that activated MMP-9 by GB may increase the intracellular cAMP and cGMP production, inhibit the intracellular Ca^{2+} mobilization and TXA_2 production, thereby leading to inhibition of platelet aggregation. These results strongly indicate that activated MMP-9 is a potent inhibitor of collagen-stimulated platelet aggregation. It may act a crucial role as a negative regulator during platelet activation.

Keywords: Cyclic nucleotide, Ginkgolide B, Intracellular Ca^{2+}, Matrix metalloproteinase-9, Platelet aggregation

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Introduction

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 (Direu and De Feudis, 2000; Van Beek, 2000). In numerous experimental models, a *ginkgo* extract was found to affect vascular and metabolic disturbances as well as neurological and behavioral activities, especially dementia (Chatterjee, 1984; Karcher *et al*., 1984; Tang and Eisenbrand, 1992). Among the constituents of *Ginkgo biloba*, terpene trilactones such as bilobalide, ginkgolide A (GA), ginkgolide B (GB), ginkgolide C (GC), along with flavonoides, have been identified as the active constituents of the *Ginkgo* extract for inhibition of the binding of PAF (Braquet, 1986; Hasler, 2000). In this report, we investigated the anti-platelet mechanism of GB in collagen-stimulated platelets.

Matrix metalloproteinases (MMPs) are a family of Zn^{2+}- and Ca^{2+}-dependent enzymes, which are important in the resorption of extracellular matrices. It is known that MMPs have been implicated in the tissue remodeling, which accompanies inflammation, bone resorption, wound healing, thrombosis, atherosclerosis, and the invasion of tumors (Ray and Stetler-Stevenson, 1994). Most MMPs are synthesized and secreted as inactive proenzymes (Lijene, 2001). 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 (Ray and Stetler-Stevenson, 1994; Brikedal-Hansen, 1995). MMP-9 is secreted as a 92-kDa proenzyme and can be activated to be 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 this may be associated with the process of

hemostasis and thrombosis. Sheu *et al*. (2004) 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 GB from *Ginkgo biloba* in collagen-stimulated platelets.

Thrombosis plays an important role in the pathogenesis of acute coronary syndromes, and vessel wall injury leads to the adherence of platelets and subsequent platelet activation. Platelet aggregation is absolutely 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 infraction (Schwartz *et al*., 1990). 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 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. An increase in $[Ca^{2+}]_i$ activates both the Ca^{2+}/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 (Kaibuchi *et al*., 1982; Nishikawa *et al*., 1980). In addition, diacylglycerol also can be hydrolyzed by diacylglycerol lipase to produce arachidonic acid (20 : 4), a precursor of TXA_2, which is a potent platelet aggregation agent generated from 20 : 4 liberated when PIP_2 is broken down by collagen, thrombin and ADP (Nishikawa *et al*., 1980; Kaibuchi *et al*., 1982; Mehshikove *et al*., 1993). Verapamil and theophylline have an anti-platelet function that elevates the cyclic adenosine monophosphate (cAMP) level, and then decreases the $[Ca^{2+}]_i$, 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 (Menshikove *et al*., 1993). 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 $[Ca^{2+}]_i$ (Pasqui *et al*., 1991) and has a role in inhibiting platelet activation (Rodomski *et al*., 1990).

In this study, we suggest that GB stimulates the activity of MMP-9, and strongly inhibits $[Ca^{2+}]_i$ elevation and TXA_2 formation, and simultaneously increases the intracellular levels of cAMP and cGMP, which have an inhibitory effect on cyclooxygense-1 (COX-1) or TXA_2 synthase in collagen-stimulated human platelet aggregation.

Materials and Methods

Materials. GB (Fig. 1) from *Ginkgo biloba* leaves was purchased from the Sigma-Aldrich Co., and collagen was obtained from the Chrono-Log Corporation. Protein molecular weight standards were obtained from Bio-Rad Laboratories. Fura 2-AM was obtained from Sigma Chemical Co. cAMP- and cGMP-enzymeimmunoassay (EIA) kits were purchased from R&D systems, Inc. and a TXB_2 EIA system were obtained from Amersham Bioscience.

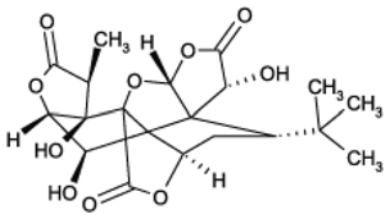

Fig. 1. Chemical structure of GB from *Ginkgo biloba* Leaves.

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 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 suspended in suspending 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 procedures above 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 of exogenous $CaCl_2$ with or without GB and then stimulated with 10 μg of collagen/ml for 5 min. Aggregation was monitored using an aggregometer (Chrono-Log, Corp.) at a constant stirring speed of 1,000 rpm. Each aggregation rate was evaluated as an increase in light transmission. The suspending buffer was used as reference. GB was dissolved in dimethylsulfoxide (DMSO) (0.001%), and subtracted the effect of DMSO from the results.

Zymography of MMP-9. Washed platelets (10^8/ml) were preincubated for 3 min at 37°C with various concentrations of GB in the presence of 2 mM $CaCl_2$ and then stimulated with 10 μg of collagen/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, pH 8.0, 0.5% Deoxycholic acid) that contained a protease inhibitor, 0.5 mM sodium orthovanadate, and 1 mM phenylmethylsulphonylfluoride (PMSF), and centrifuged at 14000 × *g* at 4°C for 30 min. Supernatant was used as the cytosol fraction for the detection of activated MMP-9. Gelatin zymography was performed to detect MMP-9 activity in the extracted samples from rat platelets. Proteins in a sample were separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels containing 1 mg/ml gelatin. Samples (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 $ZnCl_2$, pH7.5) 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 30% methanol and 10% acetic acid solution, until active bands become clear. The digested area appeared clear on a blue background indicating the location of gelatinase.

Measurement of cAMP and cGMP. Washed platelets (10^8/ml) were preincubated for 3 min at 37°C with various concentrations of GB in the presence of 2 mM $CaCl_2$, and then stimulated with 10 μg of collagen/ml for 5 min for platelet aggregation. The aggregation was terminated by adding 80% ice-cold ethanol. cAMP and cGMP were measured using the respective EIA kits of cAMP and cGMP. Because GB was dissolved in DMSO (0.001%), the levels of cAMP and cGMP were calculated by subtracting the effect of DMSO, respectively.

Determination of $[Ca^{2+}]_i$. Platelet-rich plasma was incubated with 5 μM fura 2-AM at 37°C for 60 min. Because fura 2-AM is light-sensitive, the tube containing the platelet-rich plasma was covered with aluminum foil during loading. The fura 2-loaded washed platelets were also 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 GB in the presence of 2 mM $CaCl_2$ and then stimulated with 10 μg of collagen/ml for 5 min for evaluation of $[Ca^{2+}]_i$. Fura 2 fluorescence was measured with a spectrofluorimeter (RF-5301 PC, SHIMADZU, JAPAN) with an excitation wavelength that changed every 0.5 sec from 340 nm to 380 nm; the emission wavelength was set at 510 nm. The $[Ca^{2+}]_i$ values were calculated using the method of Schaeffer (Schaeffer and Blaustein, 1989). Because GB was dissolved in DMSO (0.001%), the levels of cAMP and cGMP were calculated by subtracting the effect of DMSO, respectively.

Measurement of TXB_2. Washed platelets (10^8/ml) were then preincubated with or without GB for 3 min in the presence of 2 mM $CaCl_2$, and activated for 5 min with 10 μg/ml of collagen. The reactions were terminated by adding ice-cold 5 mM EDTA and 0.2 mM indomethacin. 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 GB on 20 : 4 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 GB for 5 min, and then 100 pmol of 20 : 4 was added to 200 μl of the lysate. The lysate mixtures were incubated further for 10 min, and the amount of TXB_2 was determined as described above.

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

Results and Discussion

Inhibitory effect of GB on platelet aggregation. In this study, we used GB (Fig. 1) from *Ginkgo biloba* leaves, a traditional Chinese medicine, to investigate the anti-platelet function. The concentration of collagen that induced maximal platelet aggregation was approximately 10 μg/ml (Cho *et al.*, 2004). Therefore, 10 μg of collagen/ml was used as a platelet agonist in this study. Since $[Ca^{2+}]_i$ is a critical regulator of platelet aggregation, it was used to examine the effects of GB in the presence of 2 mM $CaCl_2$. When washed platelets (10^8/ml) were activated with 10 μg of collagen/ml in the presence of 2 mM $CaCl_2$, various concentrations of GB (1, 10, 50, 100, and 500 μM) significantly reduced the collagen (10 μg/ml)-stimulated platelet aggregation in a dose-dependent manner (Fig. 2). From this result, we focused on the MMP-9 activity by GB, and investigated whether GB affects the MMP-9 activity in collagen-stimulated platelets.

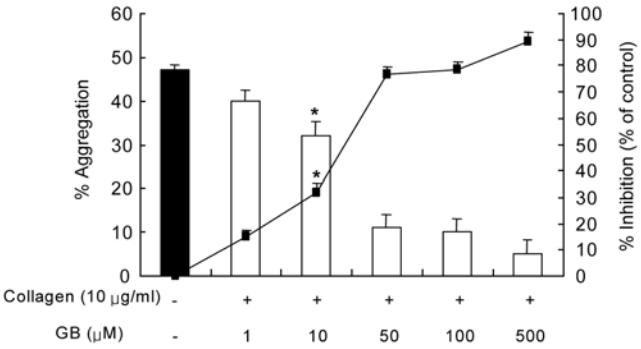

Fig. 2. Effects of GB pretreatment on collagen-induced platelet aggregation. Washed platelets (10^8/ml) were preincubated with various concentrations of GB (1 to 500 μM) in the presence of 2 mM $CaCl_2$ for 3 min at 37°C, and then stimulated with collagen (10 μg/ml) for 5 min. Platelet aggregation (%) was recorded as an increase in light transmission. Inhibition by GB was recorded as percentage of the collagen-induced aggregation rate. Data are expressed as means ± S.D. (n = 4). $*p < 0.05$ and $**p < 0.001$ compared with that of collagen alone.

Effect of GB on MMP-9 activity in washed platelets. 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 demonstrable with the use of various agonists, such as collagen, thrombin, ADP, U46619, and arachidonic acid. Nakamura *et al.* (1998) suggested that human plasma MMP-9 concentrations ranged from 34.2 ± 16.6 to 52.4 ± 26.6 ng/ml. Sheu *et al.* (2004) indicated that cytoplasm was the main storage compartment for MMP-9 in resting and collagen-stimulated platelets. Therefore, we used the cytoplasm fraction to detect the MMP-9 activity. To determine whether platelet activation might cause changes in MMP-9 activity in the cytoplasm, we used the cell lysates treated with or without GB in the collagen-stimulated platelets. When platelets were preincubated with GB, GB 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 GB (50 and 100 μM) to form an

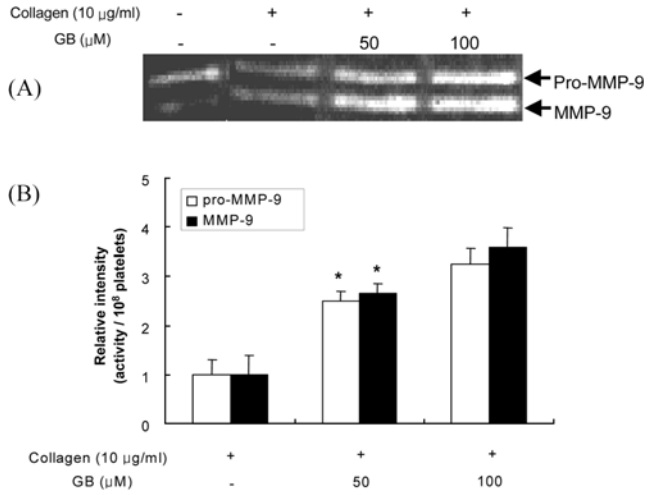

Fig. 3. The effects of GB-induced MMP-9 activity in collagen-stimulated platelets. (A) Analysis of MMP-9 activity in collagen-stimulated platelets by zymography. (B) The quantitative analysis of MMP-9 activation in collagen-stimulated platelets. Washed platelets (10^8/ml) were preincubated with various concentrations of GB in the presence of 2 mM $CaCl_2$ for 3 min at 37°C, 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, pH 8.0, 0.5% deoxycholic acid) 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".

activated MMP-9 (86-kDa), as shown by gelatinolytic activities. These results suggest that the activated MMP-9 by GB may inhibit up-stream of platelet aggregation when platelets were stimulated by collagen (10 μg/ml). Therefore, this partly infers that activated MMP-9 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 (Sheu *et al*., 2004). Thus, we measured the cAMP and cGMP production by GB in resting and collagen-stimulated platelets.

Effects of GB 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 (Jang *et al*., 2002). We next investigated whether GB up-regulated the cellular level of cAMP/cGMP, endogenous negative regulators of platelet aggregation (Qi *et al*., 1996; Homer and Wanstall, 2002; Park *et al*., 2004). As shown in Fig. 4A, 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. When the platelets, however, were incubated in the presence of both GB and collagen, GB (10 to 500 μM) significantly increased the cAMP level in a dose-dependent manner. On the other hand, GB (10 to 500 μM) alone progressively increased the cAMP level from 9.4 ± 0.5 to 14.5 ± 1.0 pmol/10^8 platelets in comparison with the control levels (4.9 ± 0.5 pmol/10^8 platelets) in resting platelets (Fig. 4A). It is interesting to note that GB modulated the production of cAMP in resting and in collagen-stimulated platelets.

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

Above results suggest that GB 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 (Sudo *et al*., 2003), IP_3 receptor (Komalvials and Lincoln, 1994), and TXA_2 receptor (Kinsella *et al*., 1994). Therefore, GB might block the platelet aggregation *via* enhanced levels of cAMP and cGMP and its linked PKA/PKG activity. In other words, the activated MMP-9 by GB (Fig. 3) increased the intracellular cAMP and cGMP levels to inhibit the collagen-stimulated platelet aggregation (Fig. 4).

Effects of GB on the regulation of aggregation-inducing molecules, $[Ca^{2+}]_i$ and TXA_2. Of several aggregation-inducing molecules, Ca^{2+} and TXA_2 are known to be essential for platelet aggregation (Charo *et al*., 1997). 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. 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, and 100 μM) of GB in a dose-dependent manner (87% inhibition at 100 μM), suggesting that the inhibitory activity of GB on collagen-stimulated platelet aggregation was due to lowering of the level of $[Ca^{2+}]_i$. Therefore, GB significantly blocked Ca^{2+} release seemed to be critical to the GB-mediated inhibition of platelet aggregation (Fig. 5A).

TXA_2 is a potent stimulus of platelet aggregation, and its receptor G-protein (Gq)-PLC-IP_3 signaling pathway is activated by collagen treatment (Wang *et al*., 1998). Therefore, we next examined whether GB blocked the production of TXA_2 under collagen exposure. The TXA_2 (determined as TXB_2) level in intact platelets was 1.5 ± 0.3 pg/10^5 platelets, and this was

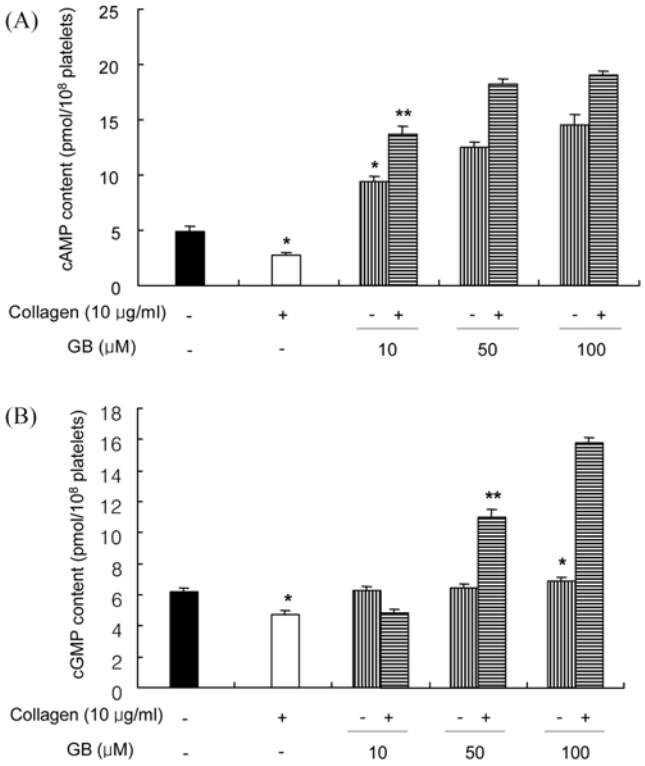

Fig. 4. Effects of GB on cAMP and cGMP production in resting and collagen-stimulated platelets. Washed platelets (10^8/ml) were preincubated with or without GB for 3 min in the presence of 2 mM $CaCl_2$ and then stimulated with collagen (10 μg/ml) for 5 min at 37°C. The reactions were terminated by adding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

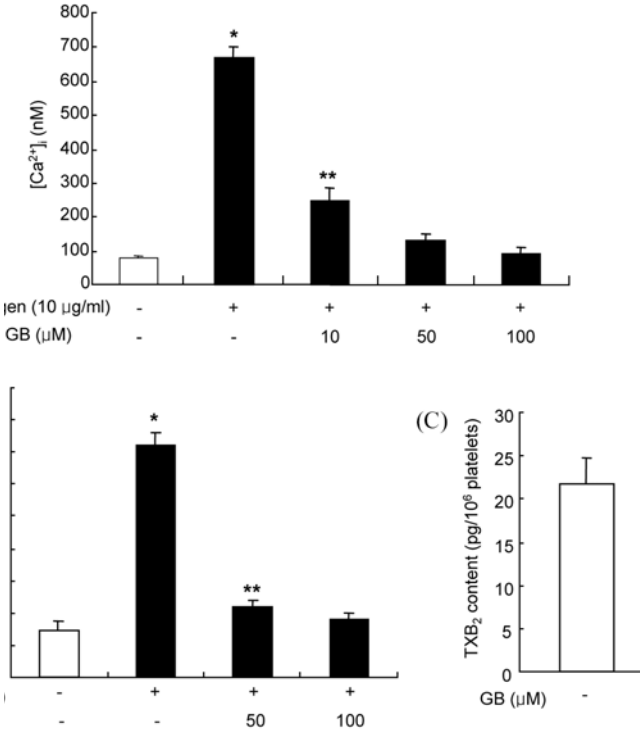

Fig. 5. Effects of GB on collagen-stimulated Ca^{2+} mobilization and TXB_2 production. (A) Effects of GB on collagen-induced $[Ca^{2+}]_i$ mobilization. Fura 2-loaded platelets (10^8/ml) were preincubated with various concentrations of GB in the presence of 2 mM $CaCl_2$ for 3 min at 37°C, and then collagen (10 μg/ml) was added. $[Ca^{2+}]_i$ was determined as described in "Materials and Methods". (B) Effects of GB on the production of TXB_2 stimulated by collagen. Washed platelets (10^8/ml) were preincubated with GB for 3 min in the presence of 2 mM $CaCl_2$, and then stimulated with collagen (10 μg/ml). (C) Effects of GB 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 ± S.D. (n = 4). *$p < 0.001$ compared with basal level. **$p < 0.001$ compared with that of collagen-induced platelets.

markedly increased to 7.2 ± 0.4 pg/10^5 platelets in the collagen-stimulated platelets (Fig. 5B). However, GB significantly reduced the production of TXA_2 in a dose-dependent manner (75% inhibition at 100 μM). To determine if the inhibitory effect on TXA_2 release of GB was due to the direct suppression of COX-1 or TXA_2 synthase, cell-free enzyme assay method was used. When platelet lysates were incubated with or without GB (100 μM) for 5 min at 37°C in the presence of AA (20 : 4), a substrate of COX-1, GB treatment almost inhibited the TXA_2 production (99.5% inhibition at 100 μM) as compared with intact platelets (Fig. 5C), suggesting that the decrease in TXA_2 production by GB possibly is directly related to inhibition of its metabolic enzyme, COX-1 or TXA_2 synthase.

In conclusion, the most important thing of this study suggested that GB significantly inhibit the collagen-stimulated platelet aggregation. GB increases both intracellular cAMP and cGMP level and MMP-9 activity, thereby leading to inhibition of the TXA_2 production and intracellular Ca^{2+} mobilization. Moreover, GB directly diminished the COX-1 or TXA_2 synthase activity, resulting in decrease TXA_2 formation, an aggregation-inducing molecule, ultimately leading to inhibition of intracellular TXA_2-mediated Ca^{2+} mobilization and platelet aggregation. Therefore, GB may inhibit the vascular disease associated with platelet aggregation, such as atherosclerosis, myocardial infarction, coronary artery disease, and thrombosis. These results suggest that GB may be a physiologically effective negative feedback regulator during the platelet aggregation.

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