Influence of Geijibokryunghwan on Platelet Aggregation, Cyclic AMP, Cyclic GMP, TXA2, Ca^{2+} Mobilization, Tyrosine Phosphorylation of PLC-γ2 and IP3 in Activated Platelets

Han Geu Kim, Jong Gu Kim, Seog Ha Kim, Eun-Ho Sa, Jae-Woo Kim, Jin Young Moon[2], Sun Dong Park[3], Dall Yeong Choi[4], Cherl Ho Kim[1], Won Hwan Park*

Departtment of Diagnostics, 1:Department of Biochemistry, 2:Depattmnet of AM-Pointology, 3:Department of Prescription, 4:Department of Pathology, College of Oriental Medicne, Dongguk University

Geijibokryunghwan has a wide range of therapeutic applications, and some reports have indicated that it has protective activity against atherosclerosis, and more specifically stroke and myocardial infarction. A recent report showed that atherosclerotic plaque volume can be reduced by supplying Geijibokryunghwan extracts for several years. In this study, we used a component of Geijibokryunghwan, which has been used for the prevention of atherosclerosis in Korea for several years, and has proven to be useful in lowering the occurrence of cerebral infarction. In a preliminary study, we found that Geijibokryunghwan potently suppressed platelet aggregation induced by various agonists. In this study, we sought to explore the mechanism by which Geijibokryunghwan inhibits platelet aggregations.

Key words : Geijibokryunghwan, atherosclerosis, activity, platelet, aggregations, Ca^{2+} mobilization

Introduction

Geijibokryunghwan has been used in herbal medicine for thousands of years, and it appears to contain a number of active compounds for medicinal use. Recently, antioxidative phenolic compounds of Geijibokryunghwan have been identified[1]. These phenolic compounds appear to be responsible for the beneficial effects of Geijibokryunghwan intake. Geijibokryunghwan has a wide range of therapeutic applications, and some reports have indicated that it has protective activity against atherosclerosis, and more specifically stroke and myocardial infarction[2]. Some investigations have shown that Geijibokryunghwan prevents both the development and progression of atherosclerosis[3]. Dietary Geijibokryunghwan increases fibrinolytic activity[2]. Geijibokryunghwan extracts can decrease the amount of low-density lipoprotein, serum triglyceride, and cholesterol and can prevent the oxidation of low-density lipoprotein[4]. Furthermore, a recent report showed that atherosclerotic plaque volume can be reduced by supplying Geijibokryunghwan extracts for several years[5]. With regard to platelet function, an animal in vivo experiment has shown that rabbits fed with Geijibokryunghwan extracts were well protected against thrombus formation induced by a lethal dose of collagen or arachidonic acid[6]. Geijibokryunghwan can also inhibit the responses of platelets and reduce TXB2 formation[7]. However, studies of Geijibokryunghwan on the precise mechanism of inhibition have been few.

In this study, we used a component of Geijibokryunghwan, which has been used for the prevention of atherosclerosis in Korea for several years, and has proven to be useful in lowering the occurrence of cerebral infarction[4,8,9]. In a preliminary study, we found that Geijibokryunghwan potently suppressed platelet aggregation induced by various agonists[1,6,7]. In this study, we sought to explore the mechanism by which Geijibokryunghwan inhibits platelet aggregations.

* To whom correspondence should be addressed at : Won Hwan Park, Department of Diagnostics, College of Oriental Medicne, Dongguk University, Sukjang-Dong 707, Kyung-Ju 780-714, Korea

· E-mail : diapwh@mail.dongguk.ac.kr, · Tel : 054-770-2373

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Materials and methods

1. Materials

The following materials were obtained from the indicated suppliers: PGI2 (Funakoshi); BSA (Sigma Chemical Co.); anti-PLC-γ2 pAb (Santa Cruz Biotechnology); anti-phosphotyrosine mAb (PY20) (Transduction Laboratories); anti-phosphotyrosine mAb (4G10) (Upstate Biotechnology, Inc.); D-myo-IP3 assay kit, TXB2 EIA system, cyclic AMP and cyclic

GMP EIA systems (Amersham Life Science); and fura 2-AM (Dojin Laboratories). CRP was a gift from Dept. of Biochemistry, College of Oriental Medicne, Dongguk University. Geijibokryunghwan was obtained from Dongguk oriental hospital, Kyungju, Korea (Scheme 1).

Scheme 1. Composition of Geijibokryunghwan (GBH, 桂枝茯苓丸)[8]

Cinnamomi Ramulus (桂枝)	1.33 g
Poria Cocos (茯苓)	1.33 g
Moutan Cortex Radicis (牧丹皮)	1.33 g
Paeoniae Radix (芍藥)	1.33 g
Persicae Semen (桃仁)	1.33 g
總量	6.65 g

2. Platelet separation

Platelets were obtained on the day of the experiment from volunteers who had taken no medication in the previous 2 weeks. The blood was centrifuged at 160 g for 15 min to obtain platelet-rich plasma. The platelet-rich plasma was centrifuged at 600 g for 15 min and resuspended in HEPES-Tyrode's buffer (138 mM NaCl, 3.3 mM NaH_2PO_4, 2.9 mM KCl, 1 mM $MgCl_2$, 1 ㎎/㎖ of glucose, and 20 mM HEPES, pH 7.4).

3. Platelet aggregation and Ca^{2+} measurement

Fura 2-AM at a final concentration of 3 μM was added to platelet-rich plasma, and the mixture was incubated for 30 min. After washing, fura 2-loaded platelets were resuspended in HEPES-Tyrode's buffer at a concentration of 1×10^8 cells/㎖. First, we used Geijibokryunghwan at a concentration of 10 ㎍/㎖ and evaluated the effects of Geijibokryunghwan on platelet aggregation at various time points including 30 sec and 1, 2, 5, and 10 min; there appeared to be no significant difference in its potency.

Therefore, we set the incubation time to be 5 min throughout the study. Washed platelets were incubated with various concentrations of Geijibokryunghwan for 5 min; then, the agonist was added to the suspension for evaluation of Ca^{2+} mobilization or platelet aggregation. Fura-2 fluorescence was measured with a Hitachi F 2000 fluorescence spectrophotometer with an excitation wavelength alternating every 0.5 sec from 340 to 380 nm; the emission wavelength was set at 510 nm. The $[Ca^{2+}]i$ values were determined from the ratio of fura-2 fluorescence intensity at 340 and 380 nm excitation. Platelet aggregation was measured with an AA-100 platelet aggregation analyzer (Kowa).

4. Measurement of cyclic AMP and cyclic GMP

Washed platelets were suspended in HEPES-Tyrode's buffer at a concentration of 3×10^8 cells/㎖. Platelets were incubated with various concentrations of Geijibokryunghwan for 5 min, and then the samples were lysed by adding a lysis reagent, which was obtained from the supplier of the cyclic AMP and cyclic GMP EIA kits. The amount of cyclic AMP or cyclic GMP in supernatants was measured with a cyclic AMP or cyclic GMP EIA system.

5. Measurement of TXB2

Washed platelets were suspended in HEPES-Tyrode's buffer at a concentration of 2×10^8 cells/㎖. The platelets were incubated with Geijibokryunghwan or an equal volume of PBS as vehicle for 5 min and then activated by 0.1 U/㎖ of thrombin or 0.5 ㎍/㎖ of CRP. Reactions were terminated after 1 min by adding 10 mM EDTA and 2 mM aspirin, and the samples were diluted 150-fold for TXB2 measurement. The amount of TXB2 was determined by using a TXB2 EIA kit.

To determine the direct effects of Geijibokryunghwan on arachidonic acid metabolism, the cells were first sonicated to obtain cell lysates. The cell lysates were incubated with various concentrations of Geijibokryunghwan for 5 min, and then 2 ㎕ of 10 μM arachidonic acid was added to 0.2 ㎖ of the lysate. The mixture was incubated further for 10 min, and the amount of TXB2 was determined as described above.

6. Immunoprecipitation of PLC-γ2

Washed platelets were adjusted to a concentration of 1×10^9 cells/㎖, and platelets pretreated with various concentrations of Geijibokryunghwan or with vehicle were stimulated with 0.5 g/㎖ of CRP. Reactions were terminated by adding an equal volume of ice-cold lysis buffer [2% Triton X-100, 100 mM Tris-HCl (pH 7.2), 2 mM EGTA, 2 mM vanadate, 1 mM PMSF, and 100 ㎍/㎖ of leupeptin]. The lysates were sonicated and centrifuged at 16,000 g for 5 min. The soluble fraction was precleared with protein A-Sepharose beads for 30 min. The supernatant was incubated with a polyclonal anti-PLC-γ2 antibody for 1 hr, and the immune complex was precipitated with protein A. Sepharose beads. After the mixture was rotated for 1 hr at 4℃, the Sepharose beads were washed three times with lysis buffer and once with 10 mM HEPES buffer. Finally, 50 ㎕ of HEPES buffer and 25 ㎕ of Laemmli buffer were added to the beads, and proteins were eluted by boiling for 3 min. Proteins were separated by SDS-PAGE(8%) under reducing conditions and transferred onto a nitrocellulose membrane. Tyrosine phosphorylation of PLC-γ2 was detected by western blotting, using an anti-phosphotyrosine mAb, 4G10.

7. Measurement of IP3

Platelets were suspended in HEPES-Tyrode's buffer at a concentration of 3×10^9 cells/㎖. After platelets were activated by 2 U/㎖ of thrombin or 2 ㎍/㎖ of CRP, an equal volume

of 15% TCA was added to the platelet suspension to terminate reactions, and the mixtures were kept on ice for 30 min. The mixtures were centrifuged at 2000 g for 15 min at 4℃, and the resultant supernatant was treated five times with 5 ㎖ of water-saturated diethyl ether to extract TCA. Residual ether was removed further in vacuo for 1 hr. The samples were neutralized by titration with 0.2 N NaOH, and the amount of IP3 was measured with an IP3 assay kit.

Results

1. Effects of Geijibokryunghwan on platelet aggregation induced by thrombin or CRP

Platelets were activated by thrombin or CRP throughout this study. CRP is known to be a potent activator of GPVI, a receptor for collagen. Washed platelets were incubated with various concentrations of Geijibokryunghwan for 5 min, and then 0.1 U/㎖ of thrombin or 0.5 ㎍/㎖ of CRP was added to a platelet suspension. The magnitude of platelet aggregation was assessed by changes in the optical density of platelet suspensions. Geijibokryunghwan potently inhibited platelet aggregation induced by thrombin or CRP in a concentration-dependent manner. Geijibokryunghwan at a concentration of 20 ㎍/㎖ completely blocked CRP-induced platelet aggregation (Fig. 1B) and at 40 ㎍/㎖ fully suppressed thrombin-induced platelet aggregation (Fig. 1A). The 50 values were 15 ㎍/㎖ for thrombin and 7.5 ㎍/㎖ for CRP. Similarly, Geijibokryunghwan also blocked ADP.

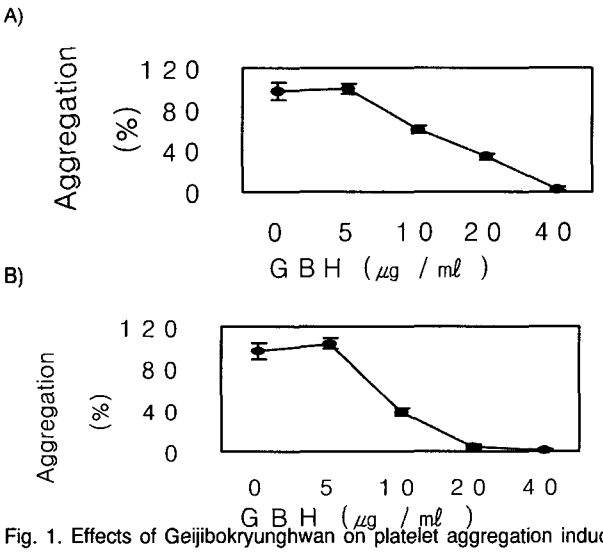

Fig. 1. Effects of Geijibokryunghwan on platelet aggregation induced by thrombin or CRP. Washed platelets were incubated with various concentrations of Geijibokryunghwan for 5 min, and then 0.1 U/㎖ of thrombin or 0.5 ㎍/㎖ of CRP was added to the platelet suspensions to activate platelets. (1A) Thrombin-induced platelet aggregation. (1B) CRP-induced platelet aggregation. The changes in optical density induced by 0.1 U/㎖ of thrombin were in the range of 60-70%, and with 0.5 ㎍/㎖ of CRP, 70-79%. The data are the means ± SD of five experiments.

2. Effects of Geijibokryunghwan on cyclic AMP and cyclic GMP

Cyclic AMP and cyclic GMP are important negative regulators of platelet functions, and many agents exert their inhibitory effects by increasing the intracellular concentration of cyclic AMP or cyclic GMP. They appear to suppress the early process of platelet activation[12,13]. To determine whether Geijibokryunghwan inhibits platelet activation by increasing the intracellular level of cyclic AMP or cyclic GMP, we examined the generation of intracellular cyclic AMP or cyclic GMP in resting platelets treated with Geijibokryunghwan. Platelets were incubated with various concentrations of Geijibokryunghwan for 5 min, and then the production of intracellular cyclic AMP or cyclic GMP was measured by using cyclic AMP or cyclic GMP EIA systems. As the positive controls, we simultaneously evaluated the effect of 0.1 μM PGE1 on cyclic AMP generation or cyclic GMP generation in resting platelets.

There was no significant increase in the level of intracellular cyclic AMP or cyclic GMP in platelets treated with Geijibokryunghwan up to the concentration of 20 ㎍/㎖. Although there was a slight increase in the level of cyclic AMP and cyclic GMP induced by 40 ㎍/㎖ of Geijibokryunghwan, it was far below the levels of intracellular cyclic AMP or cyclic GMP induced by PGE1 (Fig. 2A, 2B).

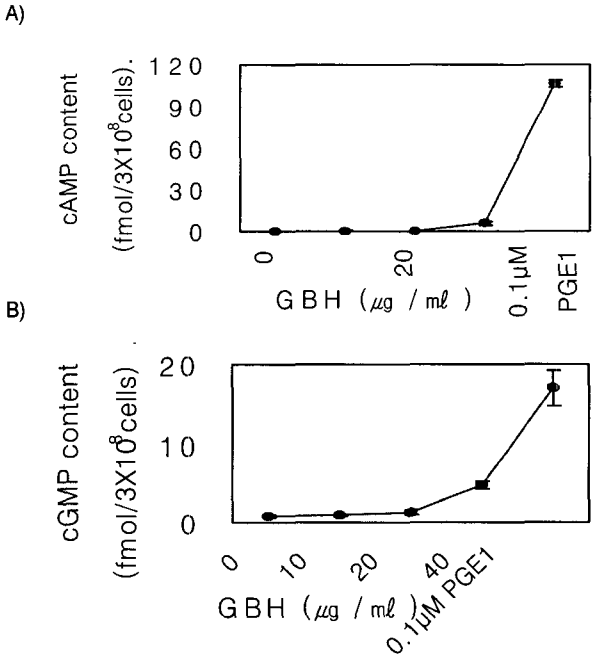

Fig. 2. Effects of Geijibokryunghwan on the production of cyclic AMP or cyclic GMP in resting platelets. Platelets were treated with Geijibokryunghwan or vehicle for 5 min, and then reactions were terminated by adding lysis reagent. The amount of cyclic AMP or cyclic GMP was measured by using a cyclic AMP or cyclic GMP EIA assay kit. (2A) Cyclic AMP. (2B) Cyclic GMP. The data represent the means of two experiments.

In a preliminary experiment, we evaluated the effects of various concentrations of PGE1 and SNP on the production of cyclic AMP or cyclic GMP in platelets, respectively. The level of cyclic GMP production induced by 40 ㎍/㎖ of Geijibokryunghwan was equal to that induced by 0.1 to 0.3 μM SNP, and the level of cyclic AMP production induced by 30 ㎍/㎖ of Geijibokryunghwan was equal to that induced by 2 nM PGE1. At these concentrations, SNP and PGE1 had no inhibitory effects on platelet aggregation induced by thrombin. These findings suggest that Geijibokryunghwan inhibits platelet aggregation independently of the production of cyclic AMP or cyclic GMP.

3. Effects of Geijibokryunghwan on TXB2 production induced by thrombin and CRP

We measured the formation of TXB2, the final and stable metabolite of TXA2, induced by thrombin or CRP, by using a TXB2 EIA system. Platelets pretreated with Geijibokryunghwan or with vehicle were stimulated by 0.1 U/㎖ of thrombin or 0.5 ㎍/㎖ of CRP. Thrombin or CRP induced the TXB2 production of 7.5 pg/10^5 cells, and 23 pg/10^4 cells in platelets, respectively. Geijibokryunghwan inhibited the formation of TXB2 induced by thrombin or CRP in a concentration-dependent manner. At the concentration of 40 ㎍/㎖, Geijibokryunghwan almost completely inhibited TXB2 formation induced by thrombin or CRP. The 50 values were approximately 20 ㎍/㎖ for both thrombin and CRP (Fig. 3A, 3B).

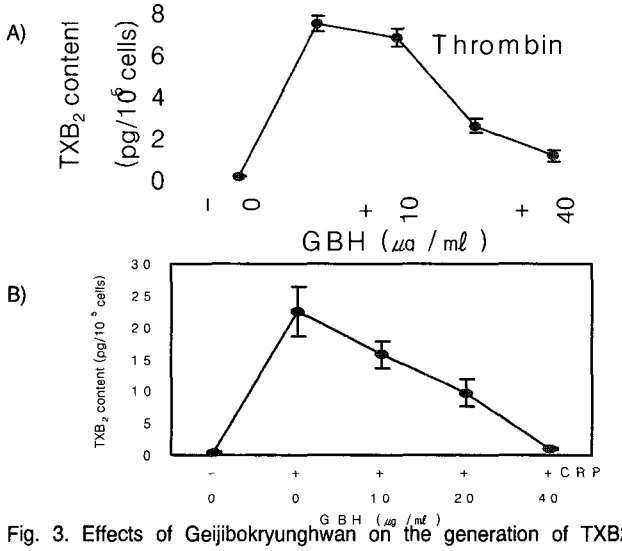

Fig. 3. Effects of Geijibokryunghwan on the generation of TXB2 induced by thrombin or CRP. Platelets were incubated with various concentrations of Geijibokryunghwan or with vehicle for 5 min, and then thrombin or CRP was added to the platelet suspension. Reactions were terminated by adding 10 mM EDTA and 2 mM aspirin. The amount of TXB2 was measured by using a TXB2 EIA kit. (3A) Thrombin-induced formation of TXB2. (3B) CRP-induced formation of TXB2. The data represent the means of two experiments.

4. Effects of Geijibokryunghwan on TXB2 production induced by arachidonic acid

To determine whether Geijibokryunghwan has a direct inhibitory effect on TXB2 production (cyclooxygenase or TXA2 synthase), platelets at a concentration of 3 × 10^8 cells/㎖ were first lysed by sonication and incubated with various concentrations of Geijibokryunghwan for 5 min. Then, 100 pmol arachidonic acid was added to 0.2 ㎖ of the cell lysate. After 10 min of incubation, the reaction was terminated by adding 10 mM EGTA and 2 mM aspirin (final concentrations); then the samples were diluted 150-fold with HEPES-Tyrode's buffer. TXB2 production was measured with a TXB2 EIA assay kit. Geijibokryunghwan up to the concentration of 20 ㎍/㎖ had no significant effect on TXB2 production in this system (Fig. 4). TXB2 production appeared to be slightly enhanced at 40 ㎍/㎖. However, the difference was not statistically significant ($P<0.05$). These findings taken together suggest that Geijibokryunghwan exerts its inhibitory effect on TXB2 production by suppressing the signal transduction pathway leading to TXB2 formation, but not by directly inhibiting cyclooxygenase or TXB2 synthetase.

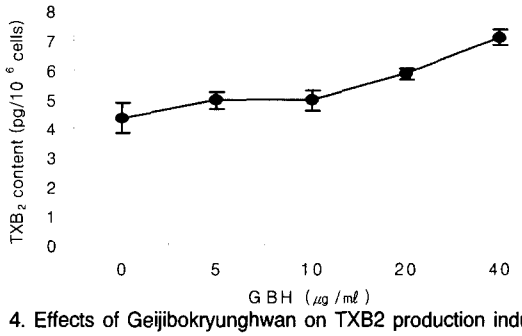

Fig. 4. Effects of Geijibokryunghwan on TXB2 production induced by arachidonic acid in a cell-free system. Washed platelets were sonicated three times for 5 sec, and Geijibokryunghwan was added to the lysates and incubated for 5 min. Then 100 pmol arachidonic acid was added to 0.2 ml of cell suspensions. After the mixture was incubated for 10 min, reactions were terminated by adding 10 mM EGTA and 2 mM aspirin. The samples were diluted 150-fold, and the amount of TXB2 was measured by using an EIA system. The data represent the means ± SD of three experiments.

5. Effects of Geijibokryunghwan on Ca^{2+} mobilization induced by thrombin or CRP

Ca^{2+} mobilization is a critical step in various aspects of platelet activation. Thus, we investigated the effects of Geijibokryunghwan on Ca^{2+} mobilization induced by thrombin or CRP. To exclude the secondary effect of TXA2 on Ca^{2+} mobilization, fura 2-loaded platelets were pretreated with 1 mM aspirin for 30 min. Platelets were incubated with various concentrations of Geijibokryunghwan for 5 min, and then thrombin or CRP was added to the suspension to induce Ca^{2+} mobilization. $[Ca^{2+}]i$ measurement was performed in the

presence of 200 μM EGTA or 1 mM Ca^{2+}, and the peak $[Ca^{2+}]i$ increase was used for evaluating the effects of Geijibokryunghwan. In both cases, Geijibokryunghwan suppressed Ca^{2+} mobilization induced by thrombin or CRP, suggesting that Ca^{2+} mobilization from intracellular Ca^{2+} stores and Ca^{2+} influx from the extracellular fluid were suppressed. In the absence of extracellular Ca^{2+}, Geijibokryunghwan at 20 ㎍/㎖ inhibited Ca^{2+} mobilization almost completely.

The IC_{50} values of Geijibokryunghwan were 10.2 ㎍/㎖ for thrombin and 17.1 ㎍/㎖ for CRP, which were similar to those required for blocking platelet aggregation (Fig. 5A, 5B). To characterize the effects of Geijibokryunghwan on Ca^{2+} mobilization, we employed thapsigargin, an inhibitor of Ca^{2+}-ATPase of Ca^{2+} stores, which induces Ca^{2+} influx by emptying internal Ca^{2+} stores. Geijibokryunghwan did not inhibit thapsigargin-induced Ca^{2+} influx, implying that Geijibokryunghwan had no direct effect on Ca^{2+} store-regulated Ca^{2+} entry.

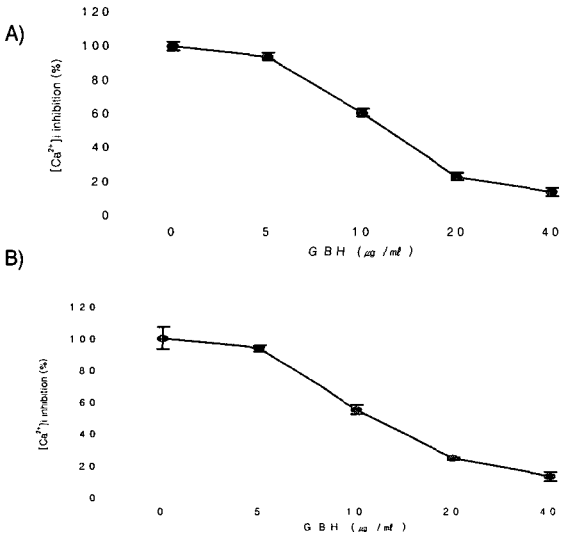

Fig. 5. Effects of Geijibokryunghwan on Ca^{2+} mobilization induced by thrombin or CRP. Fura 2-loaded platelets treated with aspirin were incubated with various concentrations of Geijibokryunghwan for 5 min in the presence of 200 μM EGTA, and then 0.1 U/ml of thrombin or 0.5 ㎍/ml of CRP was added to the suspension. (5A) Thrombin-induced Ca^{2+} mobilization. (5B) CRP-induced Ca^{2+} mobilization. The peak intracellular calcium concentrations induced by 0.1 U/ml of thrombin were in the range of 350-710 nM, and with 0.5 ㎍/ml of CRP, 280-460 nM. The data are the means ± SD of five experiments.

6. Effects of Geijibokryunghwan on tyrosine phosphorylation of PLC-γ2 induced by CRP

Although Geijibokryunghwan potently inhibited Ca^{2+} mobilization, the findings suggest that it does not exert its inhibitory effect by elevating the intracellular level of cyclic AMP and cyclic GMP, or by directly suppressing TXA2 production. We next sought to evaluate the signal transduction pathway leading to Ca^{2+} mobilization. Ca^{2+} mobilization from internal Ca^{2+} stores is mediated by IP3, which is produced by PLC. PLC-γ is activated in thrombin stimulation, and PLC-γ2 is activated in CRP-mediated platelet activation. Whereas the activation of PLC-γ2 can be assessed only by the level of IP3 production, PLC-γ2 activation also can be evaluated by the level of its tyrosine phosphorylation. Therefore, we sought to determine whether Geijibokryunghwan modified PLC-γ2 tyrosine phosphorylation induced by CRP. First, we evaluated the time course of tyrosine phosphorylation of PLC-γ2 induced by CRP. Thirty seconds after stimulation with CRP, PLC-γ2 appeared to be tyrosine-phosphorylated, with the maximum level of tyrosine phosphorylation occurring 1 min after platelet activation (Fig. 6A). Thus, the effect of Geijibokryunghwan on tyrosine phosphorylation of PLC-γ2 was determined 1 min after CRP stimulation. Even at the highest concentration of 40 ㎍/mL, which almost completely inhibits Ca^{2+} mobilization or platelet aggregation, Geijibokryunghwan had no inhibitory effect on tyrosine phosphorylation of PLC-γ2 induced by CRP (Fig. 6B).

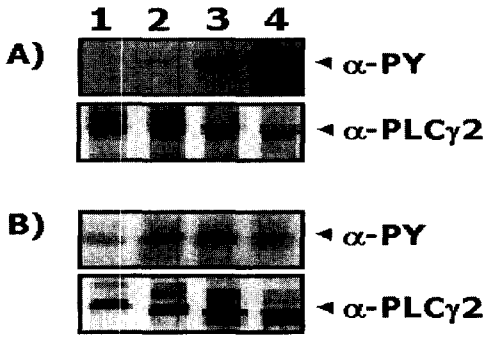

Fig. 6. Effects of Geijibokryunghwan on tyrosine phosphorylation of PLC-γ2 induced by CRP. Platelets were incubated with various concentrations of Geijibokryunghwan for 5 min, and then 0.5 ㎍/mL of CRP was added to the platelet suspensions. Reactions were terminated by adding lysis buffer. After immunoprecipitation with anti-PLC-γ2 pAb, the samples were subjected to western blotting with anti-phosphotyrosine mAb or anti-PLC-γ2 pAb. (6A) Time course of tyrosine phosphorylation of PLC-γ2 induced by CRP. The upper bands represent western blotting with anti-phosphotyrosine mAb; the lower bands represent western blotting with anti-PLC-γ2 pAb to confirm the recovery of PLC-2γ. Lane 1, 0 sec; lane 2, 30 sec; lane 3, 60 sec; lan 4, 180 sec. (6B) Effects of Geijibokryunghwan on tyrosine phosphorylation of PLC-γ2 induced by CRP. The upper bands represent western blotting with anti-phosphotyrosine mAb; the lower bands represent western blotting with anti-PLC-2γ pAb to confirm the recovery of PLC-γ2. The data are representative of three experiments. Lane 1, drug 0 ㎍/ml; lane 2, 10 ㎍/ml; lane 3, 20 ㎍/ml; lane 4, 40 ㎍/ml.

7. Effects of Geijibokryunghwan on IP3 formation induced by CRP

The results with PLC-γ2 suggest that Geijibokryunghwan does not affect PLC-γ2 activation induced by CRP, whereas it inhibits Ca^{2+} mobilization induced by CRP. We therefore examined the effects of Geijibokryunghwan on the production of IP3 induced by CRP and by thrombin. First, we determined the time course of IP3 production induced by CRP. Fifteen

seconds after stimulation with 2 ㎍/㎖ of CRP, IP3 formation could be detected, with the maximum level of IP3 generated at 30 sec. After 5 min of stimulation, IP3 formation decreased by approximately 70% of the peak value (Fig. 7A). Next, we evaluated the effect of Geijibokryunghwan on IP3 production induced by CRP. Platelets were pretreated with various concentrations of Geijibokryunghwan , and then stimulated with 2 ㎍/㎖ of CRP for 30 sec. Geijibokryunghwan up to the concentration of 30 ㎍/㎖ had no inhibitory effects on IP3 formation (Fig. 7B).

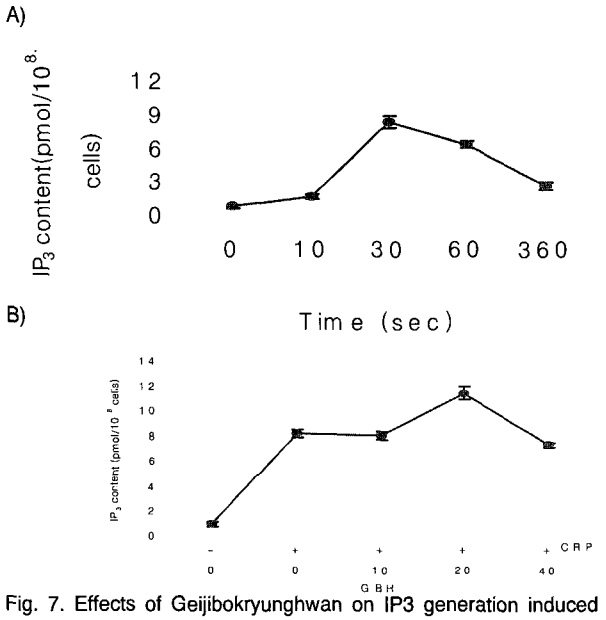

Fig. 7. Effects of Geijibokryunghwan on IP3 generation induced by CRP. Platelets were incubated with various concentrations of Geijibokryunghwan for 5 min, and then 2 ㎍/㎖ of CRP was added to the platelet suspensions. Reactions were terminated by adding an equal volume of ice-cold 15% TCA. IP3 was measured by using an Amersham IP3 assay kit (7A) Time course of IP3 production induced by CRP. (7B) Effects of various concentrations of Geijibokryunghwan on IP3 production induced by CRP. The data are the means ± SD of three experiments.

8. Effects of Geijibokryunghwan on IP3 formation induced by thrombin

Similar observations were obtained with thrombin-induced production of IP3 (Fig. 8). These findings suggest that Geijibokryunghwan at concentrations that potently block Ca^{2+} mobilization does not affect IP3 formation.

9. Effects of Geijibokryunghwan on IP3 binding to its receptor

Then, we asked whether Geijibokryunghwan modifies the binding between IP3 and the IP3 receptor. Various concentrations of Geijibokryunghwan were added to the membrane fractions of rat cerebellum, which contains abundant IP3 receptors (preparations contained in an Amersham IP3 assay kit), and incubated for 15 min; then, the IP3 binding to the IP3 receptor was determined, using the IP3 assay kit. Geijibokryunghwan at 30 ㎍/㎖ inhibited IP3 binding to its receptor by approximately 57%, whereas Ca^{2+} mobilization was almost completely suppressed at the same concentration of Geijibokryunghwan. Even at the concentration of 100 ㎍/㎖, Geijibokryunghwan failed to completely block the association between IP3 and its receptor (Fig. 9).

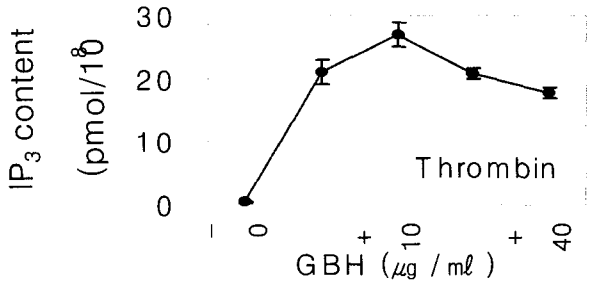

Fig. 8. Effects of Geijibokryunghwan on IP3 generation induced by thrombin. Platelets were incubated with various concentrations of Geijibokryunghwan for 5 min, and then 2 U/㎖ of thrombin was added to the platelet suspensions. Reactions were terminated 5 sec after stimulation by adding 15% TCA. IP3 was measured by using an Amersham IP3 assay kit. The data are the means ± SD of three experiments.

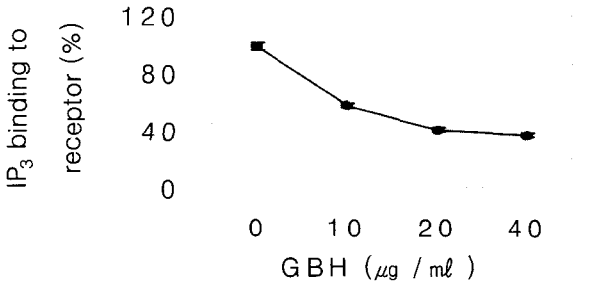

Fig. 9. Effects of Geijibokryunghwan on IP3 binding to its receptor. Various concentrations of Geijibokryunghwan incubated first with membrane fractions of rat cerebellum, and then the IP3 binding to the IP3 receptors was determined. The IP3 binding to its receptor was evaluated by the IP3 tracer and the tubes containing the membrane preparations provided by the supplier of the kit. The tracer bound to the membranes in the test tubes was in the range of 2123-2489 cpm/tube. The data are the means ± SD of four experiments.

Discussion and Conclusion

A number of studies have reported on the inhibitory effects of garlic on platelet aggregation, release response, and metabolism of arachidonic acid in vitro[10,11,12,13]. Although different preparations of garlic have been tested for their anti-platelet effects, there have been few studies using purified components of Geijibokryunghwan.

In this study, we sought to probe the mechanism by which Geijibokryunghwan inhibits platelet activation. First, we examined the effects of Geijibokryunghwan on platelet aggregation induced by various agonists. Geijibokryunghwan potently inhibited platelet aggregation induced by thrombin, CRP, U46619 (a TXA2 mimetic), ADP, or SFLL (a thrombin receptor agonist peptide). These findings suggest that

Geijibokryunghwan acts at a certain step of the signal transduction pathway common to these agonists.

Cyclic AMP and cyclic GMP are potent endogenous inhibitors of platelet function. A number of reports have shown that agents that increase the intracellular level of cyclic AMP or cyclic GMP suppress platelet aggregation as well as Ca^{2+} mobilization[14,15]. Therefore, we asked whether Geijibokryunghwan inhibits platelet aggregation or Ca^{2+} mobilization by increasing the intracellular level of cyclic AMP or cyclic GMP. In this study, platelets pretreated with various concentrations of Geijibokryunghwan failed to elevate intracellular cyclic AMP or cyclic GMP to a level that could suppress platelet function. Based on these results, we suggest that Geijibokryunghwan inhibits platelet aggregation and Ca^{2+} mobilization, independently of the cyclic AMP or cyclic GMP pathway[16].

TXA2 is an endogenous product of arachidonic acid that potently activates platelets. Many agents are known to inhibit platelet aggregation by blocking the synthesis of TXA2. Since several studies have reported that garlic extracts can block the generation of TXA2 by inhibiting cyclooxygenase or TXA2 synthetase[1,11,17], we examined whether Geijibokryunghwan modifies arachidonic acid metabolism in human platelets. Geijibokryunghwan effectively inhibited the generation of TXB2, a stable metabolite of TXA2, concentration-dependently in platelets activated by thrombin or CRP. However, since DT had no effect on TXB2 generation in a cell-free system, Geijibokryunghwan appears to have no direct effect on cyclooxygenase or TXA2 synthetase. The inhibitory effect of Geijibokryunghwan on TXA2 production in activated platelets suggests that Geijibokryunghwan acts at a certain step of the signal transduction pathway, leading to the release of arachidonic acid.

We then evaluated the effects of Geijibokryunghwan on Ca^{2+} mobilization induced by thrombin or CRP. Since we found that Geijibokryunghwan suppresses TXA2 production, platelets were first incubated with aspirin to exclude the secondary effects of TXA2. Geijibokryunghwan suppressed Ca^{2+} influx as well as Ca^{2+} mobilization from internal Ca^{2+} stores induced by thrombin or CRP. The Ca^{2+} mobilization induced by these agonists requires the production of IP3, which is an important second messenger for Ca^{2+} mobilization.

We first checked whether Geijibokryunghwan might have a direct inhibitory effect on Ca^{2+} influx. Thapsigargin, a Ca^{2+} -ATPase inhibitor, induces Ca^{2+} influx by depleting internal Ca^{2+} stores[18,19,20]. Ca^{2+} mobilization induced by thapsigargin is regulated by store-regulated Ca^{2+} entry, which is distinct from IP3-induced Ca^{2+} mobilization. Whereas Geijibokryunghwan inhibited IP3-mediated Ca^{2+} mobilization, it had no effect on thapsigargin-induced Ca^{2+} entry. These findings suggest that the inhibitory effects of Geijibokryunghwan on Ca^{2+} mobilization are specific for the IP3-mediated signal pathway.

Thrombin-induced Ca^{2+} release from intracellular Ca^{2+} stores involves PLC with resultant formation of IP[3][18,21,22]. Since we have no suitable method to assay the activity of PLC except for the measurement of IP3, we first assessed the production of IP3 induced by thrombin. We found that Geijibokryunghwan had no effect on the formation of IP3, whereas Geijibokryunghwan potently inhibited Ca^{2+} mobilization in platelets activated by thrombin. To further clarify the site of Geijibokryunghwan action, we investigated the signal transduction pathways involved in CRP-induced platelet activation.

Recent findings suggest that, upon the binding of CRP with the collagen receptor, Syk, a tyrosine kinase, is activated and undergoes tyrosine phosphorylation. Syk lies upstream of PLC-γ2, which catalyzes the production of IP3[19,20,23]. We first checked for Syk tyrosine phosphorylation and found that Geijibokryunghwan had no effect on tyrosine phosphorylation of Syk (results not shown). We next examined tyrosine phosphorylation of PLC-γ2 and IP3 production, the indices of PLC-γ2 activation, induced by CRP. Geijibokryunghwan, even at a high concentration of 40 ㎍/㎖, had no significant effects on tyrosine phosphorylation of PLC-γ2 or IP3 production induced by CRP.

Finally, we evaluated the binding of IP3 to the IP3 receptor in the presence or absence of Geijibokryunghwan by using rat cerebellum membranes, which are rich in IP3 receptors. Geijibokryunghwan showed a partial inhibition of the binding of IP3 to its receptor, even at a concentration of 30 ㎍/㎖, which almost completely suppressed Ca^{2+} mobilization.

Thus, the inhibitory effect of Geijibokryunghwan on Ca^{2+} mobilization may be partially attributed to the step of IP3 binding to its receptor. However, this incomplete effect points to the presence of an additional mechanism by which Geijibokryunghwan inhibits Ca^{2+} mobilization in human platelets. We suggest that Geijibokryunghwan also inhibits Ca^{2+} release at a site distal to IP3 binding to its receptor. Whereas the sulfur-containing structure of Geijibokryunghwan may react with the IP3 receptor, the precise biochemical property that renders Geijibokryunghwan reactive with the IP3 receptor remains elusive.

Our findings taken together suggest that Geijibokryunghwan is a unique, membrane-permeable inhibitor of Ca^{2+} mobilization, which acts on the IP3 receptor. It is of interest that an antibody against the IP3 receptor also inhibits

IP3-induced Ca^{2+} release, but does not interfere with the binding of IP3 to its receptor[24]. Since this is an antibody, it should be administered to cells whose membranes have been permeabilized by detergents or electroporation. The inhibitory mechanism of Geijibokryunghwan appears to be similar to those of xestospongin[25,26] and 2-aminoethoxydiphenyl borate [27], which are membrane-permeable blockers of IP3-induced Ca^{2+} release. They also do not block the binding of IP3 to the IP3 receptor. However, it should be noted that xestospongin at high concentrations also inhibits thapsigargin-induced Ca^{2+} release. Geijibokryunghwan, 2-aminoethoxydiphenyl borate, and xestospongin constitute a family of membrane-permeable agents that should be useful for investigating IP3-induced Ca^{2+} release.

We found that Geijibokryunghwan at 30 ㎍/㎖ slightly elevated the level of tyrosine phosphorylation of Syk and PLC-γ2 in the resting state (data not shown). However, as described in Results, Geijibokryunghwan even at the highest concentration tested had no effects on PLC-γ2 tyrosine phosphorylation in the activated platelets. Hence, we assume that the effect of Geijibokryunghwan is unrelated to the activity of tyrosine phosphatases.

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