Cordycepin-Enriched WIB801C from Cordyceps militaris Inhibits Collagen-Induced $[\text{Ca}^{2+}]_i$, Mobilization via cAMP-Dependent Phosphorylation of Inositol 1, 4, 5-Trisphosphate Receptor in Human Platelets

Dong-Ha Lee$^{1,1,*}$, Hyun-Hong Kim$^{1,1}$, Hyun-Jeong Cho$^2$, Young-Bin Yu$^2$, Hyo-Chan Kang$^3$, Jong-Lae Kim$^4$, Jong-Jin Lee$^4$ and Hwa-Jin Park$^{1,*}$

1Department of Biomedical Laboratory Science, College of Biomedical Science and Engineering, Inje University, Gimhae 621-749,
2Department of Biomedical Laboratory Science, College of Medical Science, Konyang University, Daejeon 302-718,
3Department of Medical Laboratory Science, Dong-Eui Institute of Technology, Busan 614-715,
4Bioscience & Biotechnology Team, Central Research Center, Whanin Pharm. Co., Ltd., Suwon 443-766, Republic of Korea

Abstract
In this study, we prepared cordycepin-enriched (CE)-WIB801C, a n-butanol extract of Cordyceps militaris-hypha, and investigated the effect of CE-WIB801C on collagen-induced human platelet aggregation. CE-WIB801C dose-dependently inhibited collagen-induced platelet aggregation, and its IC$_{50}$ value was 175 µg/ml. CE-WIB801C increased cAMP level more than cGMP level, but inhibited collagen-elevated $[\text{Ca}^{2+}]_i$ mobilization and thromboxane A$_2$ (TXA$_2$) production. cAMP-dependent protein kinase (A-kinase) inhibitor Rp-8-Br-cAMPS increased the CE-WIB801C-downregulated $[\text{Ca}^{2+}]_i$ level in a dose dependent manner, and strongly inhibited CE-WIB801C-induced inositol 1, 4, 5-trisphosphate receptor (IP$_3$R) phosphorylation. These results suggest that the inhibition of $[\text{Ca}^{2+}]_i$, mobilization by CE-WIB801C is from the cAMP/A-kinase-dependent phosphorylation of IP$_3$R. CE-WIB801C suppressed TXA$_2$ production, but did not inhibit the activities of cyclooxygenase-1 (COX-1) and TXA$_2$ synthase (TXAS). These results suggest that the inhibition of TXA$_2$ production by WIB801C is not from the direct inhibition of COX-1 and TXAS.

In this study, we demonstrate that CE-WIB801C with cAMP-dependent $\text{Ca}^{2+}$-antagonistic antiplatelet effects may have preventive or therapeutic potential for platelet aggregation-mediated diseases, such as thrombosis, myocardial infarction, atherosclerosis, and ischemic cerebrovascular disease.

Key Words: CE-WIB801C, cAMP, TXA$_2$, Ca$^{2+}$, IP$_3$R

INTRODUCTION
Platelet aggregation is absolutely essential for 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). Accordingly, inhibition of the platelet-collagen interaction might be a promising approach for the prevention of thrombosis. It is known that collagen and its related peptide-induced stimulation of platelets activates tyrosine kinase-dependent mechanisms that involve the tyrosine phosphorylation of Syk and phospholipase C$_{c_r_2}$ (PLC$_{c_r_2}$) via collagen receptor glycoprotein (GP) VI (Wonerow et al., 2002). Phosphorylated PLC$_{c_r_2}$ hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP$_2$) to inositol 1, 4, 5-trisphosphate (IP$_3$) and diacylglycerol (DG). Moreover, IP$_3$ mobilizes cytosol free Ca$^{2+}$ ($[\text{Ca}^{2+}]_i$) from the endoplasmic reticulum via IP$_3$ receptor (IP$_3$R). An increase in the level of $[\text{Ca}^{2+}]_i$ activates both the Ca$^{2+}$/calmodulin-dependent phosphorylation of myosin light chain and the DG-dependent...
phosphorylation of pleckstrin to induce platelet aggregation (Nishikawa et al., 1980; Kaibuchi et al., 1982). In addition, DG can be hydrolyzed by DG- and monoacylglycerol-lipase to produce arachidonic acid (20:4), a precursor of thromboxane A₂ (TXA₂), which is a potent platelet aggregation agent. On the other hand, both intracellular cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) as antiplatelet regulators decrease the [Ca²⁺]i mobilization (Menshikov et al., 1993; Schwarz et al., 2001). The antiplatelet effects of cAMP and cGMP are mediated via cAMP- and cGMP-dependent protein kinases (A-kinase, G-kinase), which phosphorylate substrate protein IP₃R (Halbrügge and Walter, 1989; Halbrügge et al., 1990; Butt et al., 1994). IP₃R phosphorylation involves in inhibition of [Ca²⁺]i mobilization (Quinton and Dean, 1992; Cavallini et al., 1996; Schwarz et al., 2001) to inhibit platelet aggregation. Therefore, inhibiting the level of platelet aggregation-inducing molecules (i.e. [Ca²⁺]i, and TXA₂) or elevating the level of platelet aggregation-inhibiting molecules (i.e. cAMP and cGMP) is very useful for evaluating the antiplatelet effect of substances or compounds.

A species of the fungal genus Cordyceps is an ingredient of traditional Chinese medicine and is prescribed for inflammatory and cancer diseases (Cunningham et al., 1951; Ng and Wang, 2005). With regard to antiplatelet activity, cordycepin (3’-deoxyadenosine) is known to inhibit adenylate cyclase activity in platelets (Londos and Wolff, 1977; Haslam et al., 1978), and thus the elevation of cAMP would not be expected. A cordycepin analogue, 2’, 5’-dideoxyadenosine, does not affect on the inhibition of platelet aggregation, and the production of cGMP or cAMP is not altered by this analogue during collagen-induced platelet aggregation (Jang et al., 2002). In our previous report (Cho et al., 2007), we suggested that cordycepin (3’-deoxyadenosine, Fig. 1C) from Cordyceps militaris has an antiplatelet effect in a cAMP- and cGMP-dependent manner, which is associated with the down-regulation of [Ca²⁺]. However, it is unknown how cordycepin involves in cAMP- and cGMP-downstream pathway (i.e. phosphorylation of IP₃R and VASP) by cAMP/A-kinase or cGMP/G-kinase. In special, we set out to investigate in this study whether CE-WIB801C has inhibitory effect on collagen-induced [Ca²⁺] mobilization, and which, if any, cAMP and cGMP is responsible for the IP₃R phosphorylation to exert Ca²⁺-antagonistic effect.

**MATERIALS AND METHODS**

**Materials**

Collagen was purchased from Chrono-Log Co. (Havertown, PA., USA). Fura-2-AM, and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO., USA). TXB₂, cAMP and cGMP enzyme immunoassay (EIA) kit, and cyclooxygenase (COX) fluorescent activity assay kit were purchased from Cayman Chemical Co. (Ann Arbor, MI., USA). Anti-phospho-IP₃-receptor, anti-rabbit IgG-horseradish peroxidase conjugate (HRP), and lysis buffer were obtained from Cell Signaling (Beverly, MA., USA). Polyvinylidene difluoride (PVDF) membrane was from GE Healthcare (Piscataway, NJ., USA). Enhanced chemiluminesence solution (ECL) was from GE Healthcare (Chalfont St, Giles, Buckinghamshire, UK).

**Preparation of WIB801C**

Culture-solution of Cordyceps militaris-hypha was concentrated with a rotary vacuum evaporator (Eyela N3000, Rikakikai Co., Ltd., Tokyo, Japan) at 60°C. The concentrate was extracted by extraction-shaker (Cosmos 660, Kyungseo Co. Ltd., Seoul, Korea) at 60°C two times with n-butanol, which was filtered two times using a filter paper (Advantec No.2). The filtrate was completely concentrated by an evaporator (Eyela N3000, Rikakikai Co. Ltd., Tokyo, Japan) under reduced pressure (40°C), and was lyophilized and stored at -20°C until used. This was named as cordycepin-enriched (CE)-WIB801C (Compound from 2008 First Project of Bioteam, Whanin Pharm. Co., Ltd., Suwon, Korea). CE-WIB801C was dissolved with distilled water to investigate the effects on platelet aggregation.
Detection of cordycepin in WIB801C with HPLC

WIB801C was dissolved in 50% methanol, for the first time, and then it was analyzed by high performance liquid chromatography (HPLC). An Agilent 1100 liquid chromatography system (Palo Alto, CA., USA), equipped with vacuum degasser, quaternary gradient pump, autosampler and diode array detector, connected to an Agilent ChemStation software. A Zorbax octadecylsilane (ODS) C18 column (250 mm×4.6 mm id, 5 μm) and a Zorbax ODS C18 guard column (12.5 mm×4.6 mm id, 5 μm) were used at a column temperature of 25°C. The mobile phase consisted of water (A) and methanol with 0.01M KH2PO4, 5 mM MgCl2, 5.5 mM glucose, 0.25% gelatin, pH 6.9) to a final concentration of 5×108/ml. All of the above procedures were carried out substances in the presence of 2 mM CaCl2, then stimulated with collagen (10 μg/ml) for 5 min. The aggregation was terminated by adding an equal volume (250 μl) of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM serine/threonine phosphatase inhibitor β-glycerophosphate, 1 mM ATPase, alkaline and acid phosphatase, and protein phosphotyrosine phosphatase inhibitor Na3VO4, 1 μg/ml serine and cysteine protease inhibitor leupeptin, and 1 mM serine protease and acetylcholinesterase inhibitor phenylmethanesulfonyl fluoride, pH 7.5). Platelet lysates containing the same protein (15 μg) were used for analysis. Protein concentrations were measured by using bicinchoninic acid protein assay kit (Pierce Biotechnology, USA). The effects of substances on IP3 phosphorylation

Log Corporation, Havertown, PA., USA) at a constant stirring speed of 1,000 rpm. Each aggregation rate was calculated as an increase in light transmission. The suspension buffer was used as the reference (transmission 0).

Measurement of cAMP and cGMP

Washed platelets (10^9/ml) were preincubated for 3 min at 37°C with or without substances in the presence of 2 mM CaCl2, 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 with synergy HT multi-model microplate reader (BioTek Instruments, Winooski, VT., USA) using cAMP and cGMP EIA kits.

Determination of cytosolic-free Ca2+ ([Ca2+]i)

PRP 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 PRP was covered with aluminum foil during loading. The Fura 2-loaded washed platelets were prepared using the procedure described above and 10^9 platelets/ml were preincubated for 3 min at 37°C with or without substances in the presence of 2 mM CaCl2, then stimulated with collagen (10 μg/ml) for 5 min for evaluation of [Ca2+]i. Fura 2 fluorescence was measured with a spectrofluorometer (SFM 25; Bio-Teck Instrument, Italy) 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 [Ca2+]i values were calculated using the method of Schaeffer (Schaeffer and Blaustein, 1989).

Western blot for analysis of IP3R phosphorylation

Washed platelets (10^9/ml) were preincubated with or without substances in the presence of 2 mM CaCl2, for 3 min and then stimulated with collagen (10 μg/ml) for 5 min at 37°C. The reactions were terminated by adding an equal volume (250 μl) of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM serine/threonine phosphatase inhibitor β-glycerophosphate, 1 mM ATPase, alkaline and acid phosphatase, and protein phosphotyrosine phosphatase inhibitor Na3VO4, 1 μg/ml serine and cysteine protease inhibitor leupeptin, and 1 mM serine protease and acetylcholinesterase inhibitor phenylmethanesulfonyl fluoride, pH 7.5). Platelet lysates containing the same protein (15 μg) were used for analysis. Protein concentrations were measured by using bicinchoninic acid protein assay kit (Pierce Biotechnology, USA). The effects of substances on IP3R phosphorylation

Measurement of platelet aggregation

Washed platelets (10^9/ml) were preincubated for 3 min at 37°C in the presence of 2 mM CaCl2, with or without substances, then stimulated with collagen (10 μg/ml) for 5 min. Aggregation was monitored using an aggregometer (Chrono-
were analyzed by western blotting. A 6-8% SDS-PAGE was used for electrophoresis and a PVDF membrane was used for protein transfer from the gel. The dilutions for anti-phospho-IP$_3$R and anti-rabbit IgG-HRP were 1:1000 and 1:10000, respectively. The membranes were visualized using ECL. Blots were analyzed by using the Quantity One, Ver. 4.5 (Bio-Rad, Hercules, CA., USA).

Measurement of TXB$_2$
Washed platelets (10$^9$/ml) were preincubated with or without substances for 3 min in the presence of 2 mM CaCl$_2$ and activated for 5 min with collagen (10 $\mu$g/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 with synergy HT multi-model microplate reader (BioTek Instruments, Winoosku, VT., USA) using a TXB$_2$ EIA kit.

Measurement of cyclooxygenase-1 (COX-1) Activity
Washed platelets (10$^9$/ml) with 1% protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO., USA) were sonicated 10 times at sensitivity 100% for 20 seconds on ice with a model HD2070 sonicator (Bandelin Electronic, Bandelin, Germany) to obtain platelet lysates. The homogenates were centrifuged at 12,000×g for 15 min at 4°C to remove cell debris. The supernatant was used to measure COX-1 activity. The platelet lysates were pre-incubated with or without substances at 37°C for 30 min. COX-1 activity was measured with synergy HT multi-model microplate reader (BioTek Instruments, Winooski, VT., USA) using COX fluorescent activity assay kit.

Measurement of thromboxane A$_2$ Synthase (TXAS) Activity
Washed platelets (10$^9$/ml) with 1% protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO., USA) were sonicated 10 times at sensitivity 100% for 20 seconds on ice with a model HD2070 sonicator (Bandelin Electronic, Bandelin, Germany) to obtain platelet lysates. Next, the homogenates were centrifuged at 12,000×g for 15 min at 4°C to remove cell debris. The platelet lysates were pre-incubated with or without substances at 37°C for 30 min. The reaction was initiated by the addition of prostaglandin H$_2$ (PGH$_2$) and allowed to proceed for 1 min at 37°C. The reaction was then terminated by the addition of 1M citric acid. After neutralization with 1N NaOH, the concentration of thromboxane B$_2$ (TXB$_2$), a stable metabolite of TXA$_2$, was determined with synergy HT multi-model microplate reader (BioTek Instruments, Winooski, VT., USA) using TXB$_2$ EIA kit.

Statistical analyses
The experimental results are expressed as the mean ± S.E.M. accompanied by the number of observations. Data were assessed by analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared by the Newman-Keuls method. $p$<0.05 was considered to be statistically significant.

RESULTS
Composition of cordycepin in WIB801C
We analyzed the composition of cordycepin in WIB801C with HPLC, as shown in Fig. 2A, two peaks (peak 1, 2) mainly corresponding to adenine was 16.21 ± 0.25 mg/g-WIB801C (about 8.2%), and the content of peak 2 corresponding to cordycepin was 81.98 ± 1.37 mg/g-WIB801C (about 8.2%), and the content of peak 2 corresponding to cordycepin was 81.98 ± 1.37 mg/g-WIB801C (about 8.2%), and the content of peak 2 corresponding to cordycepin was 81.98 ± 1.37 mg/g-WIB801C (about 8.2%), and the content of peak 2 corresponding to cordycepin was 81.98 ± 1.37 mg/g-WIB801C (about 8.2%), and the content of peak 2 corresponding to cordycepin was 81.98 ± 1.37 mg/g-WIB801C (about 8.2%), and the content of peak 2 corresponding to cordycepin was 81.98 ± 1.37 mg/g-WIB801C (about 8.2%), and the content of peak 2 corresponding to cordycepin was 81.98 ± 1.37 mg/g-WIB801C (about 8.2%), and the content of peak 2 corresponding to cordycepin was 81.98 ± 1.37 mg/g-WIB801C (about 8.2%), and the content of peak 2 corresponding to cordycepin was 81.98 ± 1.37 mg/g-WIB801C (about 8.2%), and the content of peak 2 corresponding to cordycepin was 81.98 ± 1.37 mg/g-WIB801C (about 8.2%), and the content of peak 2 corresponding to cordycepin was 81.98 ± 1.37 mg/g-WIB801C (about 8.2%).

Effects of CE-WIB801C on collagen-induced platelet aggregation
The concentration of collagen-induced maximal platelet aggregation was approximately 10 $\mu$g/ml (Lee et al., 2014). Therefore, collagen (10 $\mu$g/ml) was used as the platelet ago-
ists in this study. When washed platelets (10⁸/ml) were activated with collagen (10 μg/ml) in the presence of 2 mM CaCl₂, the aggregation rate was increased up to 78.0 ± 1.7%. However, various concentrations of CE-WIB801C (25 to 400 μg/ml) significantly reduced collagen-stimulated platelet aggregation in a dose-dependent manner (Fig. 3A), and the half-maximal inhibitory concentration (IC₅₀) value was approximately 175 μg/ml (Fig. 3B).

Effects of CE-WIB801C on cAMP and cGMP production
As shown in Table 2, collagen decreased intracellular cAMP level from 5.2 ± 0.4 pmol/10⁹ platelets (basal level) to 2.8 ± 0.5 pmol/10⁹ platelets, which was reduced to 46.2% as compared with that of basal level (Table 2). When platelets, however, were incubated in the presence of both CE-WIB801C and collagen, 400 μg/ml of CE-WIB801C increased cAMP level from 2.8 ± 0.5 pmol/10⁹ platelets to 18.1 ± 1.0 pmol/10⁹ platelets (Table 2). This result suggests that CE-WIB801C (400 μg/ml) increased collagen-decreased cAMP level to 546.4% (Table 2). On the other hand, collagen decreased intracellular cGMP level from 4.0 ± 0.3 pmol/10⁹ platelets to 3.0 ± 0.4 pmol/10⁹ platelets (Table 2). When platelets, however, were incubated in the presence of both CE-WIB801C (400 μg/ml) and collagen (10 μg/ml), the cGMP level was increased to 53.3% as compared with that (3.0 ± 0.4 pmol/10⁹ platelets) achieved by collagen (10 μg/ml) alone (Table 2).

Effect of CE-WIB801C on [Ca²⁺] mobilization
As shown in Fig. 4A, collagen increased [Ca²⁺] level from 106.6 ± 2.1 nM (basal level) to 536.6 ± 45.0 nM. However, CE-WIB801C (400 μg/ml) decreased collagen-elevated [Ca²⁺] (536.6 ± 45.0 nM) to 124.9 ± 2.6 nM (Fig. 4A). This suggests that CE-WIB801C decreased collagen-elevated [Ca²⁺] level to 76.7% (Fig. 4A). The level of [Ca²⁺], in the presence of both collagen and CE-WIB801C was 124.9 ± 2.6 nM, however, which level was dose dependently increased by A-kinase inhibitor Rp-8-Br-cAMPS (50 to 250 μM) and was increased to 254.9 ± 9.4 nM (104.1%) (Fig. 4B). On the other hand, the level of [Ca²⁺] in the presence of both collagen and CE-WIB801C was not increased by G-kinase inhibitor Rp-8-Br-cGMPS (50 to 250 μM) (Fig. 4C). Because [Ca²⁺] reduction is resulted from cAMP/A-kinase-phosphorylated IP₃R, we next investigated whether CE-WIB801C involves in phosphorylation of IP₃R.

![Image](https://www.biomolther.org)

**Table 2. Changes of cAMP and cGMP**

|                | cAMP | cGMP | cAMP/cGMP |
|----------------|------|------|-----------|
|                | pmol/10⁹ platelets Δ (%) | pmol/10⁹ platelets Δ (%) | Ratio Δ (%) |
| Base           | 5.2 ± 0.4 | - | 4.0 ± 0.3 | - |
| Collagen (10 μg/ml) | 2.8 ± 0.5<sup>1) - 46.2<sup>2) | 3.0 ± 0.4<sup>4) - 25<sup>5) | 0.9 - 30.7<sup>3) |
| CE-WIB801C (400 μg/ml) + Collagen (10 μg/ml) | 18.1 ± 1.0<sup>2) + 546.4<sup>4) | 4.6 ± 0.7<sup>4) + 53.3<sup>3) | 3.9 + 333<sup>3) |

1) to 3) Δ (%) = (Collagen-Base)/Base x 100, 4) to 6) Δ (%) = [(CE-WIB801C+Collagen)-Collagen]/Collagen x 100. The data are expressed as the mean ± S.E.M. (n=4). *p<0.05, **p<0.001 versus the collagen-stimulated platelets.
Fig. 4. Effects of CE-WIB801C on collagen-induced [Ca^{2+}] mobilization, and in the presence of A-kinase inhibitor (Rp-8-Br-cAMP) or G-kinase inhibitor (Rp-8-Br-cGMPS). (A) Effect of CE-WIB801C on collagen-induced [Ca^{2+}] mobilization in the presence of A-kinase inhibitor (Rp-8-Br-cAMPS). (B) Effect of CE-WIB801C on collagen-induced [Ca^{2+}] mobilization in the presence of G-kinase inhibitor (Rp-8-Br-cGMPS). Fura 2-loaded washed platelets (10^7/ml) were preincubated with or without CE-WIB801C, the A-kinase inhibitor Rp-8-Br-cAMPS or the G-kinase inhibitor Rp-8-Br-cGMPS in the presence of 2 mM CaCl_2 for 3 min at 37°C, and then collagen (10 μg/ml) was added. [Ca^{2+}] was determined as described in "Materials and Methods". The mean ± S.E.M. (n=3). **p<0.001 versus each control: the collagen-stimulated platelets in the presence of CE-WIB801C (400 μg/ml).

Fig. 5. Effects of CE-WIB801C on inositol 1,4,5-trisphosphate receptor (IP_3R) phosphorylation. Lane 1, Intact platelets (base); Lane 2, Collagen (10 μg/ml); Lane 3, Collagen (10 μg/ml)+CE-WIB801C (200 μg/ml); Lane 4, Collagen (10 μg/ml)+CE-WIB801C (400 μg/ml); Lane 5, Collagen (10 μg/ml)+CE-WIB801C (400 μg/ml)+Rp-8-Br-cAMPS (250 μM); Lane 6, Collagen (10 μg/ml)+CE-WIB801C (400 μg/ml)+Rp-8-Br-cGMPS (250 μM); Lane 7, Collagen (10 μg/ml)+pCPT-cAMP (1 mM); Lane 8, Collagen (10 μg/ml)+8-Br-cGMP (1 mM). Washed platelets (10^7/ml) were preincubated with or without W-cordycepin, the A-kinase inhibitor Rp-8-Br-cAMPS or the G-kinase inhibitor Rp-8-Br-cGMPS, and A-kinase activator pCPT-cAMP or the G-kinase activator 8-Br-cGMP in the presence of 2 mM CaCl_2 for 3 min at 37°C, then stimulated with collagen (10 μg/ml) for 5 min at 37°C in an aggregometer. The reactions were terminated by adding an equal volume (250 μl) of lysis buffer. Proteins were separated by SDS-PAGE, transferred to PVDF, and immunoblotted with the indicated corresponding antibodies. The data are expressed as the mean ± S.E.M. (n=3). **p<0.001 versus the collagen-stimulated platelets, †p<0.05 versus non-stimulated platelets, **p<0.001 versus each control: the collagen-stimulated platelets in the presence of CE-WIB801C (400 μg/ml).

Table 3. Changes of p-IP_3R/b-actin ratio

|       | p-IP_3R/b-actin Δ (%) |       |       |       |       |       |       |
|-------|----------------------|-------|-------|-------|-------|-------|-------|
|       |                      |       |       |       |       |       |       |
|       |                      |       |       |       |       |       |       |
| Collagen (10 μg/ml) | 0.87 ± 0.07 | 0     |       |       |       |       |       |
| CE-WIB801C (400 μg/ml) | 3.15 ± 0.50 | + 262.1 | 0     |       |       |       |       |
| + Collagen (10 μg/ml) | 1.71 ± 0.40 | -       | -45.7 |       |       |       |       |
| CE-WIB801C (400 μg/ml) | 2.93 ± 0.48 | -       | -7.0  |       |       |       |       |
| + Rp-8-Br-cGMPS (250 μM) | 2.93 ± 0.48 | -       | -7.0  |       |       |       |       |
| + Collagen (10 μg/ml) | 2.93 ± 0.48 | -       | -7.0  |       |       |       |       |

Data were from Fig. 5. 1) Δ (%)=[(CE-WIB801C+Collagen)–Col-|lagen]/Collagen×100, 2) Δ (%)=[(CE-WIB801C+Rp-8-Br-cAMPS +Collagen)–(CE-WIB801C+Collagen)]/(CE-WIB801C+Collagen)×100, 3) Δ (%)=[(CE-WIB801C+Rp-8-Br-cGMPS+Collagen)–(CE-WIB801C+Collagen)]/(CE-WIB801C+Collagen)×100.

Table 3. Changes of p-IP_3R/b-actin ratio

|       | p-IP_3R/b-actin Δ (%) |       |       |       |       |       |       |
|-------|----------------------|-------|-------|-------|-------|-------|-------|
|       |                      |       |       |       |       |       |       |
|       |                      |       |       |       |       |       |       |
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| + Collagen (10 μg/ml) | 1.71 ± 0.40 | -       | -45.7 |       |       |       |       |
| CE-WIB801C (400 μg/ml) | 2.93 ± 0.48 | -       | -7.0  |       |       |       |       |
| + Rp-8-Br-cGMPS (250 μM) | 2.93 ± 0.48 | -       | -7.0  |       |       |       |       |
| + Collagen (10 μg/ml) | 2.93 ± 0.48 | -       | -7.0  |       |       |       |       |

Data were from Fig. 5. 1) Δ (%)=[(CE-WIB801C+Collagen)–Col-|lagen]/Collagen×100, 2) Δ (%)=[(CE-WIB801C+Rp-8-Br-cAMPS +Collagen)–(CE-WIB801C+Collagen)]/(CE-WIB801C+Collagen)×100, 3) Δ (%)=[(CE-WIB801C+Rp-8-Br-cGMPS+Collagen)–(CE-WIB801C+Collagen)]/(CE-WIB801C+Collagen)×100.

Effect of CE-WIB801C on TXA_2 production, and it-associated enzymes (COX-1 and TXAS) activities

The TXA_2 (determined as TXB_2) level in intact platelets was 0.6 ± 0.1 ng/10^8 platelets, and collagen (10 μg/ml) markedly increased TXA_2 level to 60.1 ± 1.0 ng/10^8 platelets (Fig. 6A). However, CE-WIB801C potently reduced TXA_2 production to 5.5 ± 0.5 ng/10^8 platelets (90.8% inhibi-
tion at 400 μg/ml) (Fig. 6A). TXA₂ production is concerned with COX-1 and TXAS, which convert 20:4 to TXA₂ (Patrono, 1994; Cipollone et al., 1997). Therefore, we investigated whether CE-WIB801C inhibited COX-1 and TXAS activities to inhibit TXA₂ production. As shown in Fig. 6B, aspirin (500 μM), a COX-1 inhibitor, significantly inhibited COX-1 activity from 2.03 ± 0.23 nmol/protein-mg/min (basal level) to 1.49 ± 0.20 nmol/protein-mg/min, however, CE-WIB801C did not inhibit COX-1 activity (Fig. 6B). In addition, ozagrel (11 nM), a TXAS inhibitor, significantly inhibited TXAS activity from 109.3 ± 5.6 ng/protein-mg/min (basal level) to 31.4 ± 4.8 ng/protein-mg/min, however, CE-WIB801C did not inhibit TXAS activity (Fig. 6C). These results mean that CE-WIB801C-reduced TXA₂ was not resulted from inhibition of COX-1 and TXAS activities.

**DISCUSSION**

CE-WIB801C contained mainly adenine (Fig. 1A) and cordycepin (Fig. 1C), and inhibited collagen-induced platelet aggregation, which is thought by cordycepin in WIB801C because authentic cordycepin inhibited collagen-induced platelet aggregation in a dose dependent manner (Fig. 7A), but authentic adenine did not inhibited collagen-induced platelet aggregation (Fig. 7B). It is established that cordycepin inhibits collagen-induced platelet aggregation in our previous report (Cho et al., 2007). CE-WIB801C significantly blocked both [Ca²⁺] mobilization and TXA₂ production, and increased the production of cAMP and cGMP in collagen-induced platelet aggregation, which are related with the inhibitory effect of collagen-induced platelet aggregation by CE-WIB801C. In collagen-activated platelets, TXA₂ is produced from 20:4 via cyclooxygenase-1 (COX-1) and TXA₂ synthase (TXAS) pathway. Therefore, even though it is thought that CE-WIB801C might involve in inhibition of COX-1 or TXAS to suppress collagen-produced TXA₂ level, because CE-WIB801C did not inhibit COX-1 and TXAS activities in a cell free system, it is inferred that CE-WIB801C would not directly involve in inhibition of COX-1 and TXAS activities to reduce TXA₂ production in collagen-induced platelet aggregation.

In real, TXA₂ precursor 20:4 is generated from DG/monoaac-glycerol (MG) via DG/αMG-lipase pathway, and from phosphatidylinositol or phosphatidic acid via phospholipase A₂ (PLA₂), and these enzymes are activated by Ca²⁺ (Bell et al., 1979; Maucou et al., 1984; Moriyama et al., 1994). In the present study, although CE-WIB801C inhibited collagen-induced Ca²⁺ mobilization, it is unknown whether CE-WIB801C may involve in the indirect inhibition of DG/αMG-lipase or PLA₂ to at-
tenuous the supply of TXA₂ precursor 20:4, which is remained elusive, and should be studied in the future.

CE-WIB801C more increased exclusively cAMP than cGMP on collagen-activated platelet aggregation, which trend is as well as those by phenolic compounds such as epigallocatechin-3-gallate (Ok et al., 2012), chlorogenic acid (Cho et al., 2012), and caffeic acid (Lee et al., 2014) that cAMP-dependently inhibited [Ca²⁺]i mobilization in collagen-activated platelets. The levels of intracellular cAMP and cGMP are regulated by the balance between cyclic nucleotide-producing enzymes, adenylyl/guanylyl cyclases, and hydrolyzing enzymes, cAMP/cGMP phosphodiesterases (PDEs). It is known that platelets have PDE₁, PDE₄, and PDE₅ (Schwarz et al., 2001; Walter and Gambaryan, 2009). PDE₂ hydrolyzes cAMP more than cGMP as cGMP-stimulated PDE, PDE₅ hydrolyzes cAMP rather than cGMP as cGMP-inhibited PDE, and PDE₆ hydrolyzes cGMP only as cGMP-binding-cGMP specific PDE. Because CE-WIB801C increased cAMP than cGMP in collagen-induced platelet aggregation, it is thought that CE-WIB801C might involve in inhibition of PDE₅ to increase both cAMP and cGMP. However, we have no obvious evidence how CE-WIB801C regulated the level of cAMP and cGMP. Further investigation along this line is underway.

The Ca²⁺-antagonistic reaction by cAMP or cGMP is mediated by A-kinase/IP₃ phosphorylation or G-kinase/IP₃ phosphorylation. CE-WIB801C elevated IP₃ phosphorylation, and this was inhibited by A-kinase inhibitor Rp-8-Br-cAMPS, not G-kinase inhibitor Rp-8-Br-cGMPS. In addition, CE-WIB801C-decreased [Ca²⁺]i was increased by A-kinase inhibitor Rp-8-Br-cAMPS, and CE-WIB801C-elevated IP₃ phosphorylation would not be decreased by A-kinase inhibitor Rp-8-Br-cAMPS.

Antiplatelet drugs such as thienopyridine derivatives (i.e. ticlopidine, clopidogrel) have characteristics that inhibit [Ca²⁺]i mobilization, which is mediated by cAMP or cGMP (Barragan et al., 2003). Therefore, it is thought that CE-WIB801C also may represent a useful tool in the therapy or prevention of vascular diseases (i.e. thrombosis, myocardial infarction, ischemic cerebrovascular disease, and atherosclerosis) associated with platelet aggregation.

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