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

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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 $[Ca^{2+}]_i$ mobilization and thromboxane A_2 (TXA_2) production. cAMP-dependent protein kinase (A-kinase) inhibitor Rp-8-Br-cAMPS increased the CE-WIB801C-downregulated $[Ca^{2+}]_i$ level in a dose dependent manner, and strongly inhibited CE-WIB801C-induced inositol 1, 4, 5-trisphosphate receptor (IP_3R) phosphorylation. These results suggest that the inhibition of $[Ca^{2+}]_i$ mobilization by CE-WIB801C is resulted from the cAMP/A-kinase-dependent phosphorylation of IP_3R. 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 resulted from the direct inhibition of COX-1 and TXAS. In this study, we demonstrate that CE-WIB801C with cAMP-dependent 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_3R

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-r_2 (PLC-r_2) *via* collagen receptor glycoprotein (GP) VI (Wonerow *et al*., 2002). Phosphorylated PLC-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+} ($[Ca^{2+}]_i$) from the endoplasmic reticulum *via* IP_3 receptor (IP_3R). An increase in the level of $[Ca^{2+}]_i$ activates both the Ca^{2+}/calmodulin-dependent phosphorylation of myosin light chain and the DG-dependent

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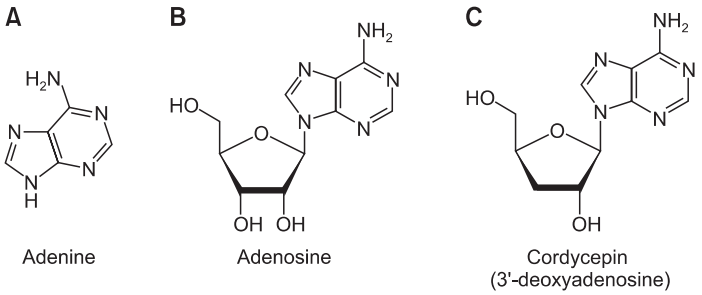

Fig. 1. Chemical structures of adenine, adenosine, and cordycepin (3'-deoxyadenosine). (A) The structure of adenine. (B) The structure of adenosine. (C) The structure of cordycepin (3'-deoxyadenosine).

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 A_2 (TXA_2), 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 $[Ca^{2+}]_i$ 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 IP_3R (Halbrügge and Walter, 1989; Halbrügge *et al*., 1990; Butt *et al*., 1994). IP_3R phosphorylation involves in inhibition of $[Ca^{2+}]_i$ 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. $[Ca^{2+}]_i$ and TXA_2) 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 $[Ca^{2+}]_i$. However, it is unknown how cordycepin involves in cAMP- and cGMP-downstream pathway (i.e. phosphorylation of IP_3R and VASP) by cAMP/A-kinase or cGMP/G-kinase. In this present study, we prepared WIB801C (*C*ompound from 20*08 First* Project of *B*ioteam, *W*han*i*n Pharm. Co., Ltd., Suwon, Korea), a n-butanol extract from *Cordyceps militaris*-hypha, and analyzed the composition of cordycepin in WIB801C with high performance liquid chromatography (HPLC). *Cordyceps* is known to contain nucleoside analogue such as cordycepin (3'-deoxyadenosine), adenine, and adenosine (Ng and Wang, 2005). Of these nucleoside analogue, we reported that cordycepin has an antiplatelet effect (Cho *et al*., 2007). Therefore, we investigated whether WIB801C contains cordycepin, adenine, and adenosine, and which, if any, adenine analogue has an antiplatelet effect. In this study, we found that WIB801C contains enough cordycepin, and investigated its antiplatelet effects to evaluate the efficacy that prevents or treats thrombotic disease.

In addition, we investigated the effects of cordycepin-enriched (CE)-WIB801C on upregulation of aggregation-inhibiting molecules (i.e. cAMP, cGMP), and downregulation of aggregation-inducing molecules (Ca^{2+}, TXA_2). In special, we set out to investigate in this study whether CE-WIB801C has inhibitory effect on collagen-induced $[Ca^{2+}]_i$ mobilization, and which, if any, cAMP and cGMP is responsible for the IP_3R phosphorylation to exert Ca^{2+}-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). TXB_2, 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-phosphor-IP_3-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 (Piseataway, 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 (*C*ompound from 20*08 First* Project of *B*ioteam, *W*han*i*n Pharm. Co., Ltd., Suwon, Korea). CE-WIB801C was dissolved with distilled water to investigate the effects on platelet aggregation.

Table 1. Calibration curves and contents of 2 peaks in CE-WIB801C

	RT (min)	Area (mAU×s)	Calibration curve[a)]	r^2	Test range (μg/ml)	χ[b)] (μg/ml)	Contents (mg/g-CE-WIB801C)
Authetic compound							
Adenine	6.6	-	y=43.600χ+7.959	0.9996	10-200	-	-
Cordycepin	14.8	-	y=27.618χ-64.657	0.9996	50-400	-	-
CE-WIB801C							
Peak 1	6.6	714 ± 11	-	-	-	16.21 ± 0.25	16.21 ± 0.25
Peak 2	14.8	2210 ± 38	-	-	-	81.98 ± 1.37	81.98 ± 1.37

[a,b)]y, peak areas of analytes; *x*, concentrations of analytes in 1 mg/ml CE-WIB801C (μg/ml).

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) C_{18} column (250 mm×4.6 mm id, 5 μm) and a Zorbax ODS C_{18} 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 KH_2PO_4 (B) using the following program: 0-30 min, 15% B. The flow rate was at 1.0 ml/min and sample injection volume was 10 μL. The UV detection was operated at 254 nm. To detect and analyze the nucleoside analogue, we used various concentrations (cordycepin: 50, 100, 200, and 400 μg/ml; adenosine: 4, 20, 100, and 200 μg/ml; adenine: 10, 20, 50, 100, 200 μg/ml) of each authentic compounds (cordycepin, adenosine, and adenine) in duplicate with HPLC, then the calibration curves were constructed by plotting the peak area against the concentration of each analyte with regression analysis, and we calculated linear equation from the calibration curve (Table 1).

Preparation of washed human platelets

Human platelet-rich plasma (PRP) anti-coagulated with acid-citrate-dextrose solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose) were obtained from Korean Red Cross Blood Center (Changwon, Korea). PRP was centrifuged for 10 min at 125×g to remove a little red blood cells, and was centrifuged for 10 min at 1,300 ×g to obtain the platelet pellets. 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 6.5). The washed platelets were then resuspended in suspension 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 6.9) to a final concentration of 5×10^8/ml. All of the above procedures were carried out at 25°C to avoid platelet aggregation from any effect of low temperature. The Korea National Institute for Bioethics Policy Public Institutional Review Board (Seoul, Korea) approved these experiments.

Measurement of platelet aggregation

Washed platelets (10^8/ml) were preincubated for 3 min at 37°C in the presence of 2 mM $CaCl_2$ with or without substances, then stimulated with collagen (10 μg/ml) for 5 min. Aggregation was monitored using an aggregometer (Chrono-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 (10^8/ml) were preincubated for 3 min at 37°C with or without substances in the presence of 2 mM $CaCl_2$, and then stimulated with collagen (10 μg/ml) for 5 min for platelet aggregation. The aggregation was terminated by the addition of 80% ice-cold ethanol. cAMP and cGMP were measured with synergy HT multi-model microplate reader (BioTek Instruments, Winooski, VT., USA) using cAMP and cGMP EIA kits.

Determination of cytosolic-free Ca^{2+} ($[Ca^{2+}]_i$)

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 10^8 platelets/ml were preincubated for 3 min at 37°C with or without substances in the presence of 2 mM $CaCl_2$, then stimulated with collagen (10 μg/ml) for 5 min for evaluation of $[Ca^{2+}]_i$. 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 $[Ca^{2+}]_i$ values were calculated using the method of Schaeffer (Schaeffer and Blaustein, 1989).

Western blot for analysis of IP_3R phosphorylation

Washed platelets (10^8/ml) were preincubated with or without substances in the presence of 2 mM $CaCl_2$ 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 Na_2EDTA, 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 Na_3VO_4, 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 IP_3R phosphorylation

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-phosphor-IP_3R 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 (10^8/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 μg/ml). The reactions were terminated by the addition of ice-cold EDTA (5 mM) and indomethacin (0.2 mM). The amount of TXB_2, a stable metabolite of TXA_2, was determined 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 (10^8/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 (10^8/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, Winoosku, 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 were observed. The retention time of peak 1 was 6.6 min, and peak 2 was 14.8 min (Fig. 2A, Table 1). We detected cordycepin and analyzed its composition. As shown in Fig. 2B, the retention times of authentic compounds, cordycepin, adenosine, and adenine, were 14.8, 11.7, and 6.6 min in order. The retention times of peak 1, and peak 2 were in accord with those of authentic adenine and cordycepin, but a certain peak corresponding authentic adenosine was not almost observed in WIB801C. Calibration curve were linear over the range of 50 to 400 μg/ml for cordycepin, and 10 to 200 μg/ml for adenine with $r^2>0.9996$ (Table 1). With regard to contents of cordycepin and adenine calculated from calibration curve, as shown in Table 1, the content of peak 2 corresponding to cordycepin was 81.98 ± 1.37 mg/g-WIB801C (about 8.2%), and the content of peak 1 corresponding to adenine was 16.21 ± 0.25 mg/g-WIB801C (about 1.62%).

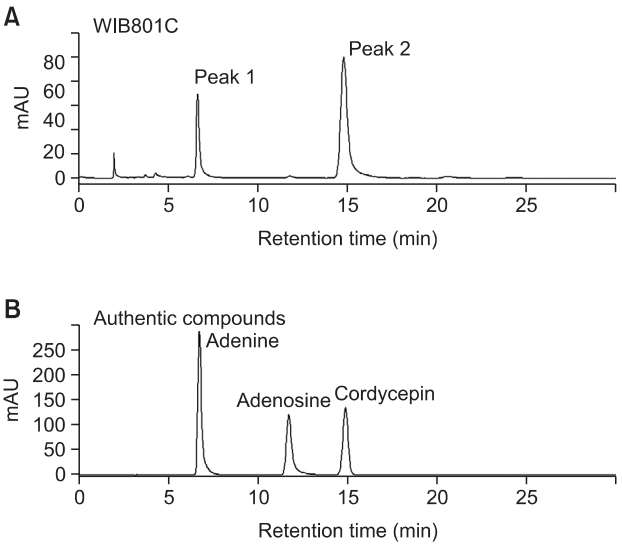

Fig. 2. HPLC chromatograms of CE-WIB801C and authentic compounds (adenine, adenosine, and cordycepin). (A) The chromatogram of CE-WIB801C. (B) The chromatogram of authentic compounds (adenine, adenosine, and cordycepin). HPLC was performed on a Zorbax ODS C_{18} column (250 mm×4.6 mm id, 5 μm) and a Zorbax ODS C_{18} guard column (12.5 mm×4.6 mm id, 5 μm) were used at 25°C. The mobile phase consisted of water (a) and methanol with 0.01M KH_2PO_4 (b) using the following program: 0-30 min, 15% b. The flow rate was at 1.0 ml/min and sample injection volume was 10 μl. The UV detection was operated at 254 nm.

It is known that whole fruiting body myelia of *Cordyceps militaris* contains 0.16% of cordycepin, and whole fruiting body, stroma, and larva of *Cordyceps sinensis* does not contain cordycepin (Yue *et al*., 2008). Accordingly, the cordycepin level in WIB801C that we used in this study is very higher than those in whole fruiting body myelia of *Cordyceps militaris*, or in whole fruiting body, stroma, and larva of *Cordyceps sinensis*. Thus, WIB801C is named as cordycepin-enriched WIB801C (CE-WIB801C) in this report.

Effects of CE-WIB801C on collagen-induced platelet aggregation

The concentration of collagen-induced maximal platelet aggregation was approximately 10 μg/ml (Lee *et al*., 2014). Therefore, collagen (10 μg/ml) was used as the platelet ago-

nist in this study. When washed platelets (10^8/ml) were activated with collagen (10 μg/ml) in the presence of 2 mM $CaCl_2$, 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 (IC_{50}) 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/10^9 platelets (basal level) to 2.8 ± 0.5 pmoL/10^9 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/10^9 platelets to 18.1 ± 1.0 pmoL/10^9 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/10^9 platelets (basal level) to 3.0 ± 0.4 pmoL/10^9 platelets (Table 2). Collagen reduced basal cGMP level to 25.0% to aggregate 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/10^9 platelets) achieved by collagen (10 μg/ml) alone (Table 2).

Effect of CE-WIB801C on $[Ca^{2+}]_i$ mobilization

As shown in Fig. 4A, collagen increased $[Ca^{2+}]_i$ level from 106.6 ± 2.1 nM (basal level) to 536.6 ± 45.0 nM. However, CE-WIB801C (400 μg/ml) decreased collagen-elevated $[Ca^{2+}]_i$ (536.6 ± 45.0 nM) to 124.9 ± 2.6 nM (Fig. 4A). This suggests that CE-WIB801C decreased collagen-elevated $[Ca^{2+}]_i$ level to 76.7% (Fig. 4A). The level of $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ reduction is resulted from cAMP/A-kinase-phosphorylated IP_3R, we next investigated whether CE-WIB801C involves in phosphorylation of IP_3R.

Effect of CE-WIB801C on IP_3R phosphorylation

The phosphorylation (p-IP_3R) of IP_3R and the ratio of p-IP_3R to β-actin were increased in the presence of A-kinase activator pCPT-cAMP (1 mM) (Fig. 5 lane 7), and G-kinase activator 8-Br-cGMP (1 mM) (Fig. 5 lane 8) as compared with collagen

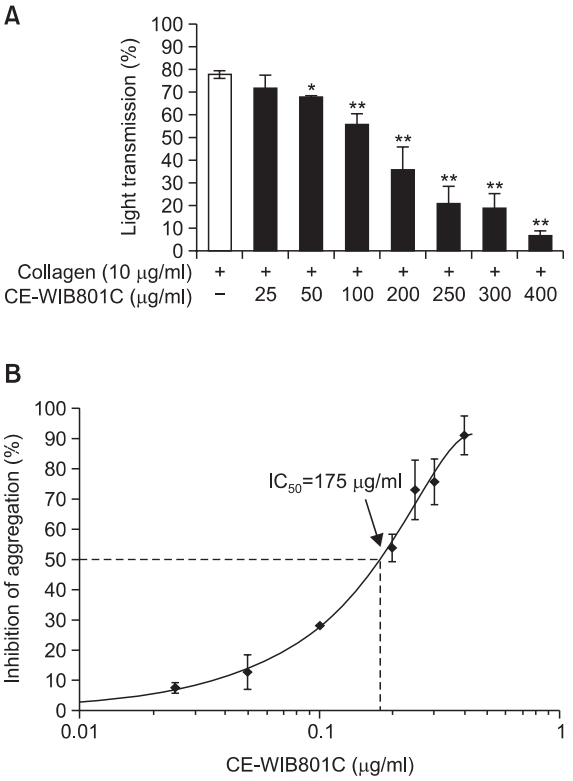

Fig. 3. Effects of CE-WIB801C on collagen-induced platelet aggregation. (A) Effects of CE-WIB801C pretreatment on collagen-induced platelet aggregation. (B) IC_{50} value of CE-WIB801C on collagen-induced platelet aggregation. Washed platelets (10^8/ml) were preincubated with or without various concentrations of CE-WIB801C (25 to 400 μg/ml) in the presence of 2 mM $CaCl_2$ for 3 min at 37°C, then stimulated with collagen (10 μg/ml) for 5 min in aggregometer. Platelet aggregation (%) was recorded as an increase in light transmission. Inhibition rate by CE-WIB801C was recorded as percentage of the collagen-induced aggregation rate. IC_{50} value of CE-WIB801C was calculated by 4-parameter log fit method. The data are expressed as the mean ± S.E.M. (n=4). *p<0.05, **p<0.001 versus the collagen-stimulated platelets.

Table 2. Changes of cAMP and cGMP

	cAMP		cGMP		cAMP/cGMP	
	pmoL/10^9 platelets	Δ (%)	pmoL/10^9 platelets	Δ (%)	Ratio	Δ (%)
Base	5.2 ± 0.4	-	4.0 ± 0.3	-	1.3	-
Collagen (10 μg/ml)	2.8 ± 0.5[a]	- 46.2[1)]	3.0 ± 0.4[a]	- 25[2)]	0.9	- 30.7[3)]
CE-WIB801C (400 μg/ml) + Collagen (10 μg/ml)	18.1 ± 1.0**	+ 546.4[4)]	4.6 ± 0.7*	+ 53.3[5)]	3.9	+ 333[6)]

1) to 3) Δ (%)=(Collagen-Base)/Base×100, 4) to 6) Δ (%)=[(CE-WIB801C+Collagen)-Collagen]/Collagen×100. The data are expressed as the mean ± S.E.M. (n=4). [a]p<0.05 versus non-stimulated platelets, *p<0.05, **p<0.001 versus the collagen-stimulated platelets.

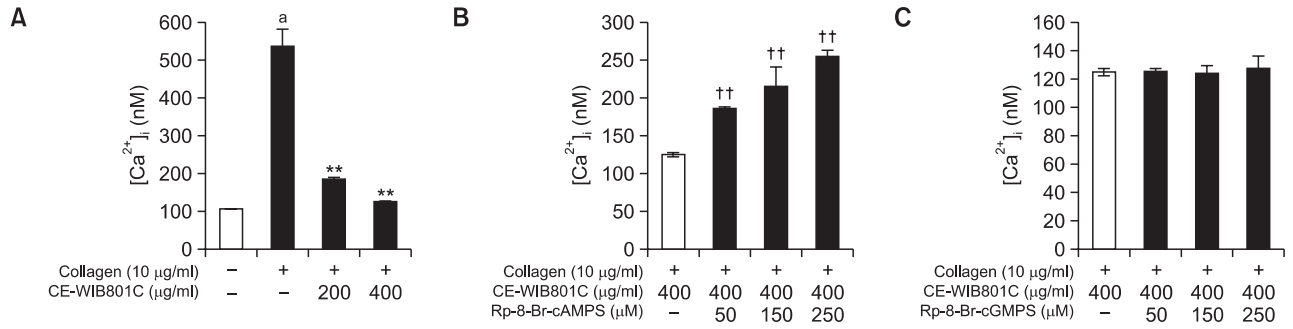

Fig. 4. Effects of CE-WIB801C on collagen-induced $[Ca^{2+}]_i$ mobilization, and in the presence of A-kinase inhibitor (Rp-8-Br-cAMPS) or G-kinase inhibitor (Rp-8-Br-cGMPS). (A) Effect of CE-WIB801C on collagen-induced $[Ca^{2+}]_i$ mobilization. (B) Effect of CE-WIB801C on collagen-induced $[Ca^{2+}]_i$ mobilization in the presence of A-kinase inhibitor (Rp-8-Br-cAMPS). (C) Effect of CE-WIB801C on collagen-induced $[Ca^{2+}]_i$ mobilization in the presence of G-kinase inhibitor (Rp-8-Br-cGMPS). Fura 2-loaded washed platelets (10^8/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. $[Ca^{2+}]_i$ was determined as described in "Materials and Methods". The data are expressed as the mean ± S.E.M. (n=4). $^a p<0.05$ versus non-stimulated platelets, $^{**}p<0.001$ versus the collagen-stimulated platelets, $^{††}p<0.001$ versus each control: the collagen-stimulated platelets in the presence of CE-WIB801C (400 μg/ml).

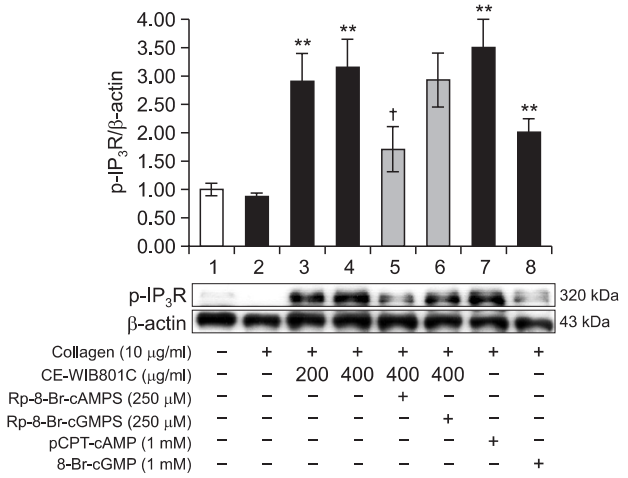

Fig. 5. Effects of CE-WIB801C on inositol 1,4,5-trisphosphate receptor (IP_3R) phosphorylation. Lane 1, Intact platelets (base); Lane 2, Collagen (10 μg/ml); Lane 3, Collagen (10 μg/ml)+CE-WIB801C (200 μg/ml); Lane 4, Collagen (10 μg/ml)+CE-WIB801C (400 μg/ml); Lane 5, Collagen (10 μg/ml)+CE-WIB801C (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 (10^8/ml) were preincubatedwith 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 the collagen-stimulated platelets in the presence of CE-WIB801C (400 μg/ml).

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

	p-IP_3R/β-actin	Δ (%)	
Collagen (10 μg/ml)	0.87 ± 0.07	0	
CE-WIB801C (400 μg/ml)	3.15 ± 0.50	+ 262.1[1)]	0
+ Collagen (10 μg/ml)			
CE-WIB801C (400 μg/ml)	1.71 ± 0.40	-	-45.7[2)]
+ Rp-8-Br-cAMPS (250 μM)			
+ Collagen (10 μg/ml)			
CE-WIB801C (400 μg/ml)	2.93 ± 0.48	-	-7.0[3)]
+ Rp-8-Br-cGMPS (250 μM)			
+ Collagen (10 μg/ml)			

Data were from Fig. 5. 1) Δ (%)=[(CE-WIB801C+Collagen)–Collagen]/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.

alone. These mean that cAMP/A-kinase and cGMP/G-kinase involve in IP_3R phosphorylation. As shown in Fig. 5 lane 3 and 4, p-IP_3R and the ratio of p-IP_3R to β-actin were dose dependently increased in the presence of both collagen and CE-WIB801C (200 and 400 μg/ml). However, the ratio (3.15) of p-IP_3R to β-actin by both collagen and CE-WIB801C (400 μg/ml) was decreased to 1.71 (45.7%) in the presence of A-kinase inhibitor Rp-8-Br-cAMPS (Fig. 5 lane 5, Table 3). On the other hand, the ratio (3.15, Fig. 5 lane 4) of p-IP_3R to β-actin by both collagen and CE-WIB801C (400 μg/ml) was not almost decreased in the presence of G-kinase inhibitor Rp-8-Br-cGMPS (Fig. 5 lane 6, Table 3). Accordingly, cAMP/A-kinase-dependent IP_3R phosphorylation exclusively contributed to the inhibitory effect of $[Ca^{2+}]_i$ mobilization achieved by CE-WIB801C on collagen-activated platelets.

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). This suggests that collagen increased TXA_2 production to 9,917% (Fig. 6A). However, CE-WIB801C potently reduced TXA_2 production to 5.5 ± 0.5 ng/10^8 platelets (90.8% inhibi-

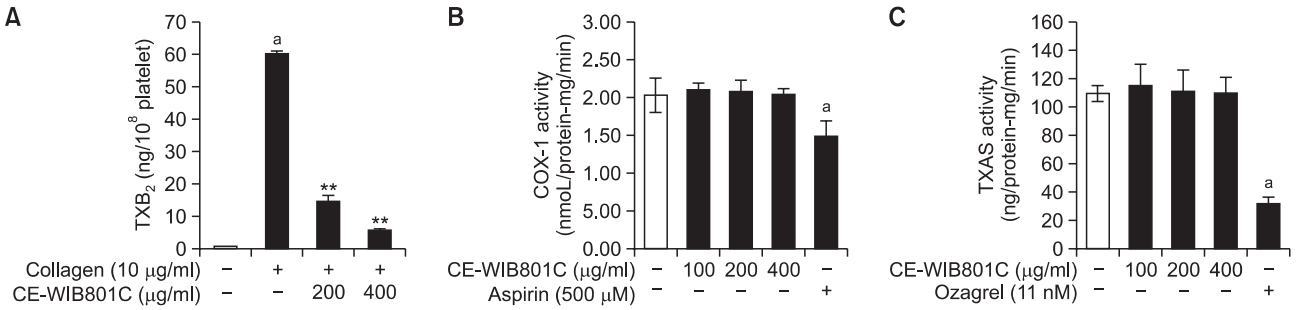

Fig. 6. Effects of CE-WIB801C on collagen-induced TXA_2 production, and TXA_2-associated enzymes (COX-1 and TXAS) activities. (A) Effects of CE-WIB801C on TXB_2 production. Washed platelets (10^8/ml) were preincubated with or without CE-WIB801C in the presence of 2 mM $CaCl_2$ for 3 min at 37°C, then stimulated with collagen (10 μg/ml) for 5 min in an aggregometer. TXA_2 was determined with the content of TXB_2 using TXB_2 EIA kit as described in "Materials and Methods". (B) Effects of CE-WIB801C on COX-1 activity in platelet lysates. (C) Effects of CE-WIB801C on TXAS activity in platelet lysates. COX-1 and TXAS activity was determined with COX fluorescent activity assay and TXB_2 EIA kit as described in "Materials and Methods". The data are expressed as the mean ± S.E.M. (n=4). $^a p<0.05$ versus non-stimulated platelets, $^{**}p<0.001$ versus the collagen-stimulated platelets.

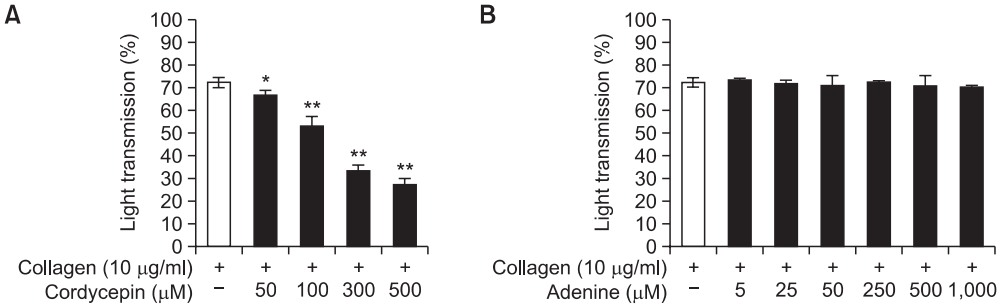

Fig. 7. Effects of cordycepin and adenine on collagen-induced platelet aggregation. (A) Effects of cordycepin pretreatment on collagen-induced platelet aggregation. (B) Effects of adenine pretreatment on collagen-induced platelet aggregation. Washed platelets (10^8/ml) were preincubated with or without various concentrations of cordycepin and adenine in the presence of 2 mM $CaCl_2$ for 3 min at 37°C, then stimulated with collagen (10 μg/ml) for 5 min. Platelet aggregation (%) was recorded as an increase in light transmission. The data are expressed as the mean ± S.E.M. (n=4). $^*p<0.05$, $^{**}p<0.001$ versus the collagen-stimulated platelets.

tion at 400 μg/ml) (Fig. 6A). TXA_2 production is concerned with COX-1 and TXAS, which convert 20:4 to TXA_2 (Patrono, 1994; Cipollone *et al*., 1997). Therefore, we investigated whether CE-WIB801C inhibited COX-1 and TXAS activities to inhibit TXA_2 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 TXA_2 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 $[Ca^{2+}]_i$ mobilization and TXA_2 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, TXA_2 is produced from 20:4 *via* cyclooxygenase-1 (COX-1) and TXA_2 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 TXA_2 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 TXA_2 production in collagen-induced platelet aggregation.

In real, TXA_2 precursor 20:4 is generated from DG/monoacylglycerol (MG) *via* DG-/MG-lipase pathway, and from phosphatidylinositol or phosphatidic acid *via* phospholipase A_2 (PLA_2), and these enzymes are activated by Ca^{2+} (Bell *et al*., 1979; Mauco *et al*., 1984; Moriyama *et al*., 1994). In the present study, although CE-WIB801C inhibited collagen-induced Ca^{2+} mobilization, it is unknown whether CE-WIB801C may involve in the indirect inhibition of DG-/MG-lipase or PLA_2 to at-

tenuate the supply of TXA_2 precursor 20:4, which is remained elusive, and should be studied in the future.

CE-WIB801C more increased exclusively cAMP than cGMP on collagen-induced 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 $[Ca^{2+}]_i$ mobilization in collagen-activated platelets. The levels of intracellular cAMP and cGMP are regulated by the balance between cyclic nucleotide-producing enzymes, adenylate/guanylate cyclases, and hydrolyzing enzymes, cAMP/cGMP phosphodiesterases (PDEs). It is known that platelets have PDE_2, PDE_3, and PDE_5 (Schwarz *et al*., 2001; Walter and Gambaryan, 2009). PDE_2 hydrolyzes cAMP than cGMP as cGMP-stimulated PDE, PDE_3 hydrolyzes cAMP rather than cGMP as cGMP-inhibited PDE, and PDE_5 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 PDE_3 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 Ca^{2+}-antagonistic reaction by cAMP or cGMP is mediated by A-kinase/IP_3R phosphorylation or G-kinase/IP_3R phosphorylation. CE-WIB801C elevated IP_3R phosphorylation, and this was inhibited by A-kinase inhibitor Rp-8-Br-cAMPS only, not G-kinase inhibitor Rp-8-Br-cGMPS. In addition, CE-WIB801C-decreased $[Ca^{2+}]_i$ was increased by A-kinase inhibitor Rp-8-Br-cAMPS in collagen-activated platelets. Accordingly, it is an evidence that the inhibition of $[Ca^{2+}]_i$ mobilization by CE-WIB801C is obviously due to cAMP/A-kinase-dependent IP_3R phosphorylation. Otherwise, CE-WIB801C-reduced $[Ca^{2+}]_i$ would not be increased by A-kinase inhibitor Rp-8-Br-cAMPS, and CE-WIB801C-elevated IP_3R 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 $[Ca^{2+}]_i$ 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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