INTRODUCTION

Platelet aggregation is caused by "inside-out signaling" and "outside-in signaling", which is absolutely essential for the formation of a hemostatic plug when normal blood vessels are injured. However, platelet aggregation can also cause circulatory disorders, such as thrombosis, atherosclerosis, and myocardial infarction (Schwartz et al., 1990). Various agonists (i.e., collagen, thrombin, ADP) induce "inside-out signaling" to bind fibrinogen to glycoprotein IIb/IIIa (αIIb/β3), platelet membrane integrin, and subsequently cause "outside-in signaling" by binding fibrinogen to αIIb/β3 (van Willigen and Akkerman, 1991; Payrastre et al., 2000; Phillips et al., 2001). Vasodilator-stimulated phosphoprotein (VASP) phosphorylation inhibits VASP affinity for contractile protein filamentous actin, and fibrinogen binding to αIIb/β3 to inhibit the final common pathway for platelet aggregation (Laurant et al., 1999; Sudo et al., 2003). αIIb/β3 activation is also stimulated by phosphatidylinositol 3-kinase (PI3K)/Akt phosphorylation (Morello et al., 2009). In special, the interaction between αIIb/β3 and plasma fibrinogen is known to involve in [Ca2+]i mobilization, tyrosine phosphorylation of Src and Syk, activation of phosphatidylinositol 4,5-bisphosphate hydrolysis by phospholipase C-γ2 phosphorylation, cytoskeleton reorganization and granule secretion (Clutton et al., 2001; Jennings, 2009). Therefore, the stimulation of VASP phosphorylation, and the inhibition of PI3K/Akt phosphorylation are very useful for evaluating the antiplatelet effect of substances or compounds. For instance, a major catechin analogue, (-)-epigallocatechin-3-gallate from green tea, is known to produce cAMP via adenylate cyclase activation and subsequently phosphorylates VASP (Ser157) through cAMP-dependent protein kinase.
In addition, cordycepin strongly inhibited the phosphorylation and tumor promoter thapsigargin-activated human platelets. In this study, we investigated the novel effects of cordycepin (3'-deoxyadenosine, Fig. 1) inhibiting collagen-induced [Ca\textsuperscript{2+}]\textsubscript{i} mobilization (Ok et al., 2014b). In previous reports (Cho et al., 2006, 2007a, 2007b), we suggested that cordycepin (3'-deoxyadenosine, Fig. 1) inhibits [Ca\textsuperscript{2+}]\textsubscript{i} mobilization in collagen-, TXA\textsubscript{2} analogue U46619-, and tumor promoter thapsigargin-activated human platelets. In addition, cordycepin strongly inhibited the phosphorylation of Ca\textsuperscript{2+}-dependent proteins (myosin light chain and plakstrin) by suppressing collagen-, and U46619-elevated [Ca\textsuperscript{2+}]\textsubscript{i} mobilization (Cho et al., 2006, 2007a). In recent, we prepared cordycepin-enriched WIB801C (Compound from 2008 First Project of Bioteam, Whanin Pharm. Co., Ltd., Suwon, Korea), a n-butanol extract from Cordyceps militaris-hypha (Lee et al., 2014b), and reported that cordycepin-enriched (CE) WIB801C has an antiplatelet effect by inhibiting collagen-induced [Ca\textsuperscript{2+}]\textsubscript{i} mobilization via cAMP-dependent phosphorylation of inositol 1, 4, 5-trisphosphate receptor (IP\textsubscript{3R}) in human platelet (Lee et al., 2014b). In this study, we investigated the novel effects of CE-WIB801C on the phosphorylation of VASP and dephosphorylation of PI3K and Akt affecting on fibrinogen binding to αIIb/β3.

**MATERIALS AND METHODS**

**Materials**

Collagen was purchased from Chrono-Log Co. (Havertown, PA., USA). ATP assay kit was purchased from Biomedical Research Service Center (Buffalo, NY, USA). Cordycepin, A-kinase inhibitor Rp-8-Br-cAMPS, cGMP-dependent protein kinase (G-kinase) inhibitor Rp-8-Br-cGMP, A-kinase activator pCPT-cAMP; and G-kinase activator 8-Br-cGMP were obtained from Sigma Chemical Corporation (St. Louis, MO., USA). Serotonin ELISA kit was purchased from Labor Diagnostika Nord GmbH & CO. (Nordhorn, Germany). Anti-VASP, anti-phospho-VASP (Ser\textsuperscript{157}), anti-phospho-VASP (Ser\textsuperscript{239}), anti-PI3K, anti-phospho-PI3K, anti-Akt, anti-phospho-Akt, and 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 (Pisateaway, N.J., USA). Enhanced chemiluminescence solution (ECL) was from GE Healthcare (Chalfont St., Giles, Buckinghamshire, UK). Fibrinogen Alexa Fluor 488 conjugate was obtained from Invitrogen Molecular Probes (Eugene, OR., USA).

**Preparation of CE-WIB801C and quantity of cordycepin**

The preparation of CE-WIB801C was performed according to the method of our previous report (Lee et al., 2014b). Culture-solution of Cordyceps militaris-hypha was concentrated with a rotary vacuum evaporator (Eylea 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 (Eylea N3000, Rikakikai Co. Ltd., Tokyo, Japan) under reduced pressure (40°C), and was lyophilized and stored at -20°C until used. The quantity of cordycepin in CE-WIB801C was determined with calibration curve of (authentic cordycepin : 50, 100, 200, and 400 μg/mL) by HPLC as described in our previous report (Lee et al., 2014b).

**Purification of cordycepin in CE-WIB801C with prep-HPLC**

The purification of cordycepin from CE-WIB801C was performed according to the Lee method (Lee et al., 2014c). The methanol (50%) extract from CE-WIB801C was dissolved with 50% methanol and then purified by prep-HPLC. An Agilent 1100 liquid chromatography system (Palo Alto, CA, USA), equipped with vacuum degasser, quaternary gradient pump, autosampler and DAD, connected to an Agilent ChemStation software. A Jupiter C18 column (250 mm×21.2 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\textsubscript{2}PO\textsubscript{4} (B) using the following program: 0-30 min, 15% B. The flow rate was at 25 mL/min and sample injection volume was 1.5 mL. The UV detection was operated at 254 nm. The purified cordycepin were freeze-dried using a freeze dryer (Clean-vac 24T, Biotron, Korea) to obtain powder, which were analyzed and calculated by analytic HPLC above condition. The cordycepin was dissolved in distilled water, and used to investigate the effects on platelet aggregation. In this study, the cordycepin from CE-WIB801C was called as W-cordycepin to differentiate from authentic cordycepin.

**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\textsubscript{3}, 0.36 mM Na\textsubscript{2}HPO\textsubscript{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\textsubscript{3}, 0.36 mM Na\textsubscript{2}HPO\textsubscript{4}, 0.49 mM MgCl\textsubscript{2}, 5.5 mM glucose, 0.25% gelatin, pH 6.9) to a final concentration of 5×10\textsuperscript{9}/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 (PIRB12-072).

**Measurement of platelet aggregation**

Washed platelets (10\textsuperscript{9}/mL) were preincubated for 3 min at 37°C in the presence of 2 mM CaCl\textsubscript{2} with or without substances (CE-WIB801C, authentic cordycepin, W-cordycepin, Lee et al. Inhibitory Effects of Fibrinogen Binding of CE-WIB801C
and so on), 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).

**Western blot for analysis of VASP-, PI3K-, and Akt-phosphorylations**

Washed platelets (10⁹/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 in an aggregometer (Chrono-Log, Corp., Havertown, PA., USA) at a constant stirring speed of 1,000 rpm. The reactions were terminated by adding an equal volume (250 µL) of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₃ EDTA, 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 phenylmethylsulfonyl fluoride, pH 7.5). Platelet lysates containing the same protein (20 µg) were used for the analysis. Protein concentrations were measured using a bicinchoninic acid protein assay kit (Pierce Biotechnology, IL., USA). The effects of substances on VASP-, PI3K-, and Akt-phosphorylation were analyzed using 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-VASP, anti-phosphor-VASP (Ser157), anti-PI3K, anti-phosphor-PI3K, anti-Akt, anti-phosphor-Akt, and anti-rabbit IgG-HRP were 1:1000, 1:1000, 1:1000, 1:1000, 1:1000, 1:1000, and 1:10000, respectively. The membranes were visualized using ECL. The blots were analyzed using the Quantity One, Ver. 4.5 (Bio-Rad, Hercules, CA., USA).

**Determination of fibrinogen binding to αIIb/β3**

Washed platelets (10⁹/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 at 37°C in an aggregometer (Chrono-Log, Corp., Havertown, PA., USA) at a constant stirring speed of 1,000 rpm. The reaction was terminated by the addition of ice-cold 2 mM EDTA, the samples were centrifuged and supernatants were used for the assay of serotonin secretion. Serotonin secretion was measured with a Synergy HT Multi-Model Microplate Reader (BioTek Instruments, Winooski, VT., USA) using serotonin ELISA kit (Labor Diagnostika Nord GmbH & CO., Nordhorn, Germany).

**Statistical analysis**

The experimental results are expressed as the mean ± S.E.M. accompanied by the number of observations. The data were assessed using an analysis of variance (ANOVA). If the analysis indicated significant differences between the group means, then each group was compared according to the Newman-Keuls method. *p<0.05 was considered to be statistically significant.

**RESULTS**

**Composition of cordycepin in CE-WIB801C**

The quantity of cordycepin in WIB801C was about 8.2% (81.98 ± 1.37 mg/g-WIB801C (Lee et al., 2014b). 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. The yield of cordycepin from CE-WIB801C with prep-HPLC was 80.7% (Lee et al., 2014c).

**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., 2014a). Therefore, collagen (10 µg/mL) was used as the platelet ago-
CE-WIB801C dose dependently inhibited collagen-induced platelet aggregation as compared with that in intact platelets without CE-WIB801C. When washed platelet platelets (10^8/mL) were activated with collagen (10 μg/mL) in the presence of 2mM CaCl₂, the aggregation was increased to 78.1 ± 1.7% (Fig. 2). However, various concentrations (100, 200, 400 μg/mL) of CE-WIB801C dose dependently inhibited collagen-induced platelet aggregation (Fig. 2).
Effects of CE-WIB801C and cordycepin on VASP phosphorylation

Because 200 and 400 μg/mL of CE-WIB801C (Fig. 2), and 500 μM cordycepin (Cho et al., 2007a) significantly inhibited collagen-induced platelet aggregation, we used 200 and 400 μg/mL of CE-WIB801C, and 500 μM cordycepin. CE-WIB801C alone (200, 400 μg/mL), and cordycepin alone (500 μM) (Fig. 3A lane 2, 3, and 4) potently increased p-VASP (Ser157) as compared with that (Fig. 3A lane 1) of intact platelets, control. Collagen increased weakly the phosphorylation of Ser157 [p-VASP (Ser157)] at 50 kDa of VASP (Fig. 3C lane 2) as compared with that (77.2 ± 5.5%) of intact platelets, basal (Table 1). However, CE-WIB801C (200, 400 μg/mL), and cordycepin (500 μM) in collagen-induced platelet aggregation (Fig. 3C lane 5 and 6) also potently increased p-VASP (Ser157) (Fig. 3C lane 5 and 6). CE-WIB801C (400 μg/mL), and cordycepin (500 μM) also increased p-VASP (Ser157) (Fig. 3C lane 4, 7), and CE-WIB801C (400 μg/mL) increased to 78.1% the ratio of p-VASP (Ser157) to VASP in collagen-induced platelet aggregation (Fig. 3C lane 6). CE-WIB801C (400 μg/mL), and cordycepin (500 μM) increased p-VASP (Ser157) as compared with that (Fig. 3B lane 1) of intact platelets, control.

Next, we investigated whether the VASP phosphorylation by CE-WIB801C involved in inhibition of fibrinogen binding to η2/β3. As shown in Fig. 4, collagen activated fibrinogen binding to η2/β3 (Fig. 4A-b), and increased the degree of fibrinogen binding to η2/β3 up to 77.2 ± 5.5% as compared with that (5.4 ± 0.2%) of intact platelets, basal (Table 1). However, CE-WIB801C inhibited collagen-activated fibrinogen binding to η2/β3 (Fig. 4A-c, 4B), and its inhibitory degree was 89.2% as compared with that (77.2 ± 5.5%) by collagen (Table 1). Cordycepin also potently inhibited collagen-activated fibrinogen binding to η2/β3 (Fig. 4A-d and 4B). Because the inhibition of η2/β3 is resulted from cAMP/A-kinase- and cGMP/G-kinase-mediated VASP phosphorylation, and it is known that

| Collagen (10 μg/mL) | p-VASP (Ser157) | Δ (%) | Fibrinogen binding (%) | Δ (%) |
|---------------------|-----------------|-------|------------------------|-------|
| CE-WIB801C (400 μg/mL)+Collagen (10 μg/mL) | 2.10 ± 0.20 | 0 | 77.2 ± 5.5 | 0 |
| CE-WIB801C (400 μg/mL) | 3.74 ± 0.81 | +78.1 1 | 8.3 ± 0.8 | -89.2 2 |
| +Rp-8-Br-cAMPS (250 μM)+Collagen (10 μg/mL) | 2.88 ± 0.57 | -23.0 3 | 40.4 ± 1.1 | 0.4 |
| CE-WIB801C (400 μg/mL) | 3.84 ± 0.79 | +2.7 5 | 8.8 ± 0.5 | 0.6 6 |

Data were from Fig. 3C, 3D, 5B. 1 to 6: Δ (%)=[(CE-WIB801C+Collagen)-Collagen]/Collagen×100.

Table 1. Changes of p-VASP (Ser157)/VASP ratio and fibrinogen binding

Fig. 4. Effects of CE-WIB801C and cordycepin on collagen-induced fibrinogen binding. (A) The flow cytometry histograms on fibrinogen binding. a, Intact platelets (base); b, Collagen (10 μg/mL); c, Collagen (10 μg/mL)+CE-WIB801C (400 μg/mL); d, Collagen (10 μg/mL)+cordycepin (500 μM). (B) Effects of CE-WIB801C and cordycepin on collagen-induced fibrinogen binding (%). Determination of fibrinogen binding to η2/β3 was carried out as described in “Materials and Methods.” The data are expressed as the mean ± S.E.M. (n=4). *p<0.05 versus non-stimulated platelets, **p<0.001 versus the collagen-stimulated platelets.
Determination of fibrinogen binding to lagen (10 μg/mL)+8-Br-cGMP (250 μM). A-kinase activator pCPT-cAMP and G-kinase inhibitor (Rp-8-Br-cAMPS) (250 μM). (B) Effects of CE-WIB801C and cordycepin on collagen-induced fibrinogen binding in the presence of A-kinase inhibitor (Rp-8-Br-cAMPS) or G-kinase inhibitor (Rp-8-Br-cGMPS). Determination of fibrinogen binding to αIIb/β3 was carried out as described in “Materials and Methods.” The data are expressed as the mean ± S.E.M. (n=4). **p<0.01 versus the collagen-stimulated platelets, *p<0.05 versus the collagen-stimulated platelets in the presence of cordycepin (500 μM). **p<0.01 versus the collagen-stimulated platelets in the presence of cordycepin (500 μM). (Collagen 10 μg/mL)+cordycepin (500 μM). (Collagen 10 μg/mL)+pCPT-cAMP (1 mM); f, Collagen (10 μg/mL)+8-Br-cGMP (1 mM), (B) Effects of CE-WIB801C and cordycepin on collagen-induced fibrinogen binding (%) in the presence of A-kinase inhibitor (Rp-8-Br-cAMPS) or G-kinase inhibitor (Rp-8-Br-cGMPS).

Fig. 5, Effects of CE-WIB801C and cordycepin on collagen-induced fibrinogen binding in the presence of A-kinase inhibitor (Rp-8-Br-cAMPS), or G-kinase inhibitor (Rp-8-Br-cGMPS). (A) The flow cytometry histograms on fibrinogen binding. a, Collagen (10 μg/mL)+CE-WIB801C (400 μg/mL); b, Collagen (10 μg/mL)+CE-WIB801C (400 μg/mL)+Rp-8-Br-cAMPS (250 μM); c, Collagen (10 μg/mL)+cordycepin (500 μM)+Rp-8-Br-cGMPS (250 μM); d, Collagen (10 μg/mL)+cordycepin (500 μM)+8-Br-cGMP (1 mM); e, Collagen (10 μg/mL)+pCPT-cAMP (1 mM); f, Collagen (10 μg/mL)+8-Br-cGMP (1 mM). (B) The flow cytometry histograms on fibrinogen binding. a, Collagen (10 μg/mL)+CE-WIB801C (400 μg/mL); b, Collagen (10 μg/mL)+CE-WIB801C (400 μg/mL)+Rp-8-Br-cAMPS (250 μM); c, Collagen (10 μg/mL)+cordycepin (500 μM)+Rp-8-Br-cGMPS (250 μM); d, Collagen (10 μg/mL)+cordycepin (500 μM)+8-Br-cGMP (1 mM); e, Collagen (10 μg/mL)+pCPT-cAMP (1 mM); f, Collagen (10 μg/mL)+8-Br-cGMP (1 mM). (B) Effects of CE-WIB801C and cordycepin on collagen-induced fibrinogen binding (%) in the presence of A-kinase inhibitor (Rp-8-Br-cAMPS) or G-kinase inhibitor (Rp-8-Br-cGMPS). Determination of fibrinogen binding to αIIb/β3, was carried out as described in “Materials and Methods.” The data are expressed as the mean ± S.E.M. (n=4). **p<0.01 versus the collagen-stimulated platelets, *p<0.05 versus the collagen-stimulated platelets in the presence of cordycepin (500 μM). **p<0.01 versus the collagen-stimulated platelets in the presence of cordycepin (500 μM).

Effects of CE-WIB801C on PI3K/Akt phosphorylation

As apposed to the phosphorylated VASP, PI3K/Akt phosphorylation stimulates αIIb/β3 activation and fibrinogen binding (Zhang et al., 1996; Chen et al., 2004). Thus, we investigated
the effect of CE-WIB801C, and cordycepin on phosphorylation of PI3K and its downstream molecule Akt. CE-WIB801C alone (200, 400 μg/mL), and cordycepin alone (500 μM) almost did not change p-PI3K (Fig. 6A lane 2, 3, and 4) as compared with that (Fig. 6A lane 1) of intact platelets, control. PI3K inhibitor wortmannin suppressed collagen-induced PI3K phosphorylation (Fig. 6B lane 5). Collagen potently phosphorylated PI3K (Fig. 6B lane 2) as compared with that (Fig. 6B lane 1) of intact platelets, however, CE-WIB801C (Fig. 6B lane 3, 4) and cordycepin (Fig. 6B lane 6) inhibited collagen-induced PI3K phosphorylation. CE-WIB801C alone (200, 400 μg/mL), and cordycepin alone (500 μM) almost did not change p-Akt (Fig. 6C lane 2, 3, and 4) as compared with that (Fig. 6C lane 1) of intact platelets, control. Collagen elevated the phosphorylation of PI3K target molecule Akt (Fig. 6D lane 2), however, CE-WIB801C (Fig. 6D lane 3, 4) and cordycepin (Fig. 6D lane 5) inhibited collagen-induced Akt phosphorylation.

Effects of CE-WIB801C on ATP and serotonin release
Collagen, ADP, and thrombin that activate αIIb/β3 release ATP and serotonin out of dense bodies to aggregate platelets, which due to the [Ca$^{2+}$] mobilization by fibrinogen binding to αIIb/β3 (Weiss et al., 1974; Michal and Motamed, 1976; Kamruzzaman et al., 2013). Because CE-WIB801C also inhibited [Ca$^{2+}$] mobilization (Lee et al., 2014b), and fibrinogen binding to αIIb/β3 (Fig. 4A-c), we investigated whether CE-WIB801C, and cordycepin have inhibitory effect on collagen-elevated ATP and serotonin release. As shown in Fig. 7A, collagen re-
aggregation, we used 33, 66, 132 μM of cordycepin, or W-cordycepin corresponding to cordycepin concentration that contains in CE-WIB801C (100, 200, 400 μg/mL). As the results, as shown in Fig. 8A and 8C, the light transmissions (4.3 ± 0.6–5.0 ± 1.0%) in response to various concentration (33, 66, 132 μM) of cordycepin, and W-cordycepin alone was not significantly different from that (4.0 ± 1.0%) in resting platelets. However, cordycepin and W-cordycepin inhibited collagen-induced platelet aggregation in a dose (33, 66, 132 μM) dependent manner. With regard to the synergistic effect of cordycepin or W-cordycepin with CE-WIB801C on collagen-induced platelet aggregation. As shown in Fig. 8B and 8D, when platelets were activated by collagen in the presence of both CE-WIB801C (200 μg/mL) and cordycepin (33, 66, 132 μM) (Fig. 8B) or W-cordycepin (33, 66, 132 μM) (Fig. 8D), the aggregation was dose dependently decreased as compared with that (42.1 ± 2.4%) by CE-WIB801C (200 μg/mL) alone. The aggregation (21.5 ± 1.8%) by both cordycepin (132 μM) and CE-WIB801C (200 μg/mL) was lower as compared with that (42.1 ± 2.4%) by CE-WIB801C (200 μg/mL) alone in collagen-induced platelet aggregation. In addition, the aggregation (14.4 ± 2.7%) by both W-cordycepin (132 μM) and CE-WIB801C (200 μg/mL) was lower as compared with that (42.1 ± 2.4%) by CE-WIB801C (200 μg/mL) alone in collagen-induced platelet aggregation.

### DISCUSSION

A downstream pathway of both cAMP/A-kinase and cGMP/G-kinase involves in VASP phosphorylation to inhibit fibrinogen binding to cllb/l5. Ser157 at 50 kDa of VASP is phosphorylated by the cAMP/A-kinase pathway, whereas Ser229 at 50 kDa of VASP is phosphorylated by the cGMP/G-kinase pathway (Horstrup et al., 1994; Smolenski et al., 1998). CE-WIB801C and cordycepin phosphorylated A-kinase substrate VASP (Ser229) in intact platelets, and collagen-activated platelets. It is unknown whether CE-WIB801C increases cAMP level in intact platelets. Cordycepin (500 μM) increased cAMP level about 100 fmol/10^9 platelets in intact platelets (Cho et al., 2007a). We could not explain how the cAMP level (100 fmol/10^9 platelets) that was increased by cordycepin (500 μM) involved in VASP (Ser229) phosphorylation in intact platelets. However, this phenomenon could be explained from the report (Eigenthaler et al., 1992) that a very small elevation in cAMP is enough to activate most A-kinase. CE-WIB801C elevated cAMP level to 18.1 ± 1.0 pmol/10^9 platelets (Lee et al., 2014b), and cordycepin (500 μM) increased cAMP level to 7.5 pmol/10^9 platelets in collagen-induced platelet aggregation (Cho et al., 2007a). Therefore, it is thought that CE-WIB801C and cordycepin increase the level of cAMP, and subsequently involve in VASP (Ser229) phosphorylation via A-kinase activation in collagen-induced platelet aggregation. If not so, CE-WIB801C, and cordycepin-induced VASP (Ser229) phosphorylation would not be inhibited by A-kinase inhibitor Rp.
8-Br-cAMPS in collagen-induced platelet aggregation. With regard to VASP \((\text{Ser}^{239})\) phosphorylation, CE-WIB801C and cordycepin phosphorylated G-kinase substrate VASP \((\text{Ser}^{239})\) in intact platelets, but did not phosphorylate that in collagen-activated platelets, which are necessary to study in the future. It is known that A-kinase plays predominately in the cGMP-phosphorylated VASP to inhibit platelet aggregation (Li et al., 2003), which reflects that CE-WIB801C- and cordycepin-phosphorylated VASP \((\text{Ser}^{157})\) phosphorylation was inhibited by A-kinase inhibitor Rp-8-Br-cAMPS, and was dependent on cAMP/A-kinase pathway.

A-kinase inhibitor Rp-8-Br-cAMPS increased CE-WIB801C- and cordycepin-inhibited fibrinogen binding to \(\alpha IIb/\beta 3\). This means that CE-WIB801C and cordycepin inhibit fibrinogen binding to \(\alpha IIb/\beta 3\) via cAMP/A-kinase pathway. In this report, we have established that the inhibitory effect by CE-WIB801C and cordycepin on collagen-induced \(\alpha IIb/\beta 3\) activation is due to cAMP/A-kinase-dependent VASP \((\text{Ser}^{157})\) phosphorylation. It is known that the phosphorylation of PI3K and Akt involves in \(\alpha IIb/\beta 3\) activation, an index of fibrinogen binding to \(\alpha IIb/\beta 3\), in collagen-, ADP-, thrombin-induced platelet activation (Morello et al., 2009). cAMP-elevating agents (i.e. cilostamide, cilostazol, and forskolin) are known to inhibit PI3K- and Akt-phosphorylation in collagen-induced platelet aggregation (Hayashi and Sudo, 2009) and cAMP/A-kinase pathway is known to involve in suppression of \(\alpha IIb/\beta 3\) activation (van Wijligen and Akkerman, 1991; Payrastre et al., 2000; Shattil and Newman, 2004). Our findings show that CE-WIB801C (Lee et al., 2014b), and cordycepin (Cho et al., 2007a) increases cAMP and subsequently stimulates the phosphorylation of VASP \((\text{Ser}^{157})\), on the contrary, inhibits the phosphorylation of both PI3K and Akt to inhibit fibrinogen binding to \(\alpha IIb/\beta 3\) on collagen-induced platelet aggregation.

A lot of agonists such as collagen, thrombin and ADP mobilize \([\text{Ca}^{2+}]\) to phosphorylate \(\text{Ca}^{2+}/\text{calmodulin-dependent myosin light chain (20 kDa, which involves in granule secretion such as ATP and serotonin (Nishikawa et al., 1980; Kaibuchi et al., 1982).

The inhibition of ATP and serotonin secretion by CE-WIB801C and cordycepin is associated with the elevation of cAMP level and the inhibition of \([\text{Ca}^{2+}]\) mobilization, which
also supports the facts that CE-WIB801C induced the cAMP-dependent phosphorylation of IP3R to inhibit [Ca\textsuperscript{2+}] mobilization (Lee et al., 2014b). In our another study, we observed that cordycepin inhibits [Ca\textsuperscript{2+}] mobilization by phosphorylating IP3R in collagen-induced platelet aggregetion (Data not shown). In addition, inhibition of 20kDa phosphorylation by cordycepin (Cho et al., 2006; 2007a) might be resulted in inhibition of ATP and serotonin release by cordycepin.

The purified W-cordycepin from CE-WIB801C, and cordycepin inhibited collagen-induced platelet aggregation. In addition, because W-cordycepin, and cordycepin had a synergistic inhibitory effect with CE-WIB801C on collagen-induced platelet aggregation, it is thought that the inhibition of collagen-induced platelet aggregation by CE-WIB801C might be resulted from the inhibitory effect by at least W-cordycepin. If so, this reflects the possibility that W-cordycepin in CE-WIB801C would directly or indirectly involve in phosphorylation of VASP (Ser\textsuperscript{157}), and dephosphorylation of PI3K and Akt to inhibit fibrinogen binding to \alpha\textsubscript{IIb}b\textbeta{3}.

Platelet aggregation is generated at site of vascular wall injury, and is involved in the formation of thrombi. During the formation of thrombus, platelets release cell growth proteins such as platelet-derived growthfactor (PDGF), and vascular endothelial growth factor (VEGF) (Holash et al., 1999; Castro-Malaspina et al., 1981). It is well established that PDGF- and VEGF induce the proliferation of fibrobist, vascular smooth cells, and epithelial cells, and subsequently enhance the rate of atherosclerosis lesion progression (Seppä et al., 1982; Schwartz and Ross, 1984; Packham and Mustad, 1986; Schwartz and Reidy, 1987; Nagai et al., 2005). The progression of atherosclerosis is strongly induced by inflammatory cell such as monocyte/macrophage, and neutrophil (Philips et al., 2005). Although CE-WIB801C and cordycepin have antiplatelet effects, if CE-WIB801C and cordycepin do not inhibit inflammation by leukocyte, the progression of atherosclerosis lesion would be generated at site of vascular wall injury, and a question for antiplatelet effects of CE-WIB801C or cordycepin might be raised. Kim et al. (2006) reported that cordycepin inhibits lipopolysaccharide-induced inflammation by inhibiting Akt phosphorylation and NF-\kappaB activity. In addition, cordycepin is known to inhibit protein synthesis by attenuating Akt/\textit{mTOR} signaling pathway in NIH3T3 fibroblast, which means that cordycepin has an inhibitory effect on proliferation of NIH3T3 fibroblast (Wong et al., 2010). Considering our results and these two previous reports that cordycepin inhibits platelet aggregation, inflammation, and fibroblast proliferation, it is thought that CE-WIB801C, and cordycepin may have anti-thrombotic-, and antiatherosclerotic-effects without inflammation and progression of atherosclerosis lesion at site of vascular wall injury. Therefore, CE-WIB801C and cordycepin is highlighted as an non-toxic antiplatelet compound.

Antiplatelet drugs such as thienopyridine derivatives (i.e. ticlopidine, clopidogrel) have characteristics that phosphorylate VASP, inhibit [Ca\textsuperscript{2+}] mobilization, and inhibit \alpha\textsubscript{IIb}b\textbeta{3} activation, which is mediated by cAMP or cGMP (Barragan et al., 2003). Therefore, it is thought that CE-WIB801C and cordycepin as well as thienopyridine derivatives may also represent a useful tool in the therapy and prevention of vascular diseases associated with platelet aggregation.

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