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Effect of Cordycepin-Enriched WIB801C from *Cordyceps militaris* Suppressing Fibrinogen Binding to Glycoprotein IIb/IIIa

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Abstract

In this study, we investigated the effects of cordycepin-enriched (CE)-WIB801C, a n-butanol extract of *Cordyceps militaris*-hypha on collagen-stimulated platelet aggregation. CE-WIB801C dose dependently inhibited collagen-induced platelet aggregation, and had a synergistic effect together with cordycepin (W-cordycepin) from CE-WIB801C on the inhibition of collagen-induced platelet aggregation. CE-WIB801C and cordycepin stimulated the phosphorylation of VASP (Ser^{157}) and the dephosphorylation of PI3K and Akt, and inhibited the binding of fibrinogen to glycoprotein IIb/IIIa ($\alpha IIb/\beta_3$) and the release of ATP and serotonin in collagen-induced platelet aggregation. A-kinase inhibitor Rp-8-Br-cAMPS reduced CE-WIB801C-, and cordycepin-increased VASP (Ser^{157}) phosphorylation, and increased CE-WIB801C-, and cordycepin-inhibited the fibrinogen binding to $\alpha IIb/\beta_3$. Therefore, we demonstrate that CE-WIB801C-, and cordycepin-inhibited fibrinogen binding to $\alpha IIb/\beta_3$ are due to stimulation of cAMP-dependent phosphorylation of VASP (Ser^{157}), and inhibition of PI3K/Akt phosphorylation. These results strongly indicate that CE-WIB801C and cordycepin 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, Cordycepin, VASP (Ser^{157}), PI3K/Akt, Fibrinogen binding

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 ($\alpha IIb/\beta_3$), platelet membrane integrin, then subsequently cause "outside-in signaling" by binding fibrinogen to $\alpha IIb/\beta_3$ (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 $\alpha IIb/\beta_3$ to inhibit the final common pathway for platelet aggregation (Laurent *et al*., 1999; Sudo *et al*., 2003). $\alpha IIb/\beta_3$ activation is also stimulated by phosphatidylinositol 3-kinase (PI3K)/Akt phosphorylation (Morello *et al*., 2009). In special, the interaction between $\alpha IIb/\beta_3$ and plasma fibrinogen is known to involve in $[Ca^{2+}]_i$ mobilization, tyrosine phosphorylation of Src and Syk, activation of phosphatidylinositol 4,5-bisphosphate hydrolysis by phospholipase C-γ_2 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 (Ser^{157}) through cAMP- dependent protein kinase

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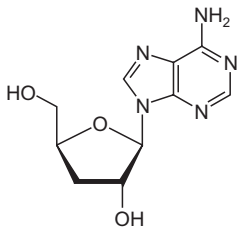

Cordycepin (3'-deoxyadenosine)

Fig. 1. Chemical structure of cordycepin (3'-deoxyadenosine).

(A-kinase) activation to inhibit $[Ca^{2+}]_i$ mobilization (Ok *et al*., 2012), and caffeic acid, a phenolic acid, is reported to phosphorylate VASP (Ser^{157}) through cAMP/A-kinase pathway to inhibit $[Ca^{2+}]_i$ mobilization (Lee *et al*., 2014a). In addition, Abciximab, etifibatide, tirofiban, and lamifiban are known as αIIb/β_3 antagonists (Lincoff *et al*., 2000; Sabatine and Jang, 2000).

In previous reports (Cho *et al*., 2006, 2007a, 2007b), we suggested that cordycepin (3'-deoxyadenosine, Fig. 1) inhibits its $[Ca^{2+}]_i$ mobilization in collagen-, TXA_2 analogue U46619-, and tumor promoter thapsigargin-activated human platelets. In addition, cordycepin strongly inhibited the phosphorylation of Ca^{2+}-dependent proteins (myosin light chain and pleckstrin) by suppressing collagen-, and U46619-elevated $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ mobilization *via* cAMP-dependent phosphorylation of inositol 1, 4, 5-trisphosphate receptor (IP_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/β_3.

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-cGMPS, 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-phosphor-VASP (Ser^{157}), anti-phosphor-VASP (Ser^{239}), anti-PI3K, anti-phosphor-PI3K, anti-Akt, anti-phosphor-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 (Piseataway, NJ., USA). Enhanced chemiluminesence 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 (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. 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 C_{18} 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_2PO_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 $NaHCO_3$, 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 (PIRB12-072).

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 (CE-WIB801C, authentic cordycepin, W-cordycepin,

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 (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 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 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 (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 (Ser^{157}), anti-phosphor-VASP (Ser^{239}), 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, 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 $\alpha IIb/\beta_3$

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) in the presence of Alexa Flour 488-human fibrinogen (30 μg/mL) for 5 min at 37°C. The reaction was stopped by the addition of 0.5% paraformaldehyde in phosphate-buffered saline, and the samples were placed in the dark. Alexa Fluor 488-fibrinogen binding to platelets was determined using flow cytometry (BD Biosciences, San Jose, CA., USA) and fibrinogen binding to $\alpha IIb/\beta_3$ was analyzed using cellQuest software.

Determination of ATP secretion

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 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 ATP secretion from dense body. ATP secretion was measured with a luminometer (BioTek Instruments, Winooski, VT., USA) using ATP assay kit.

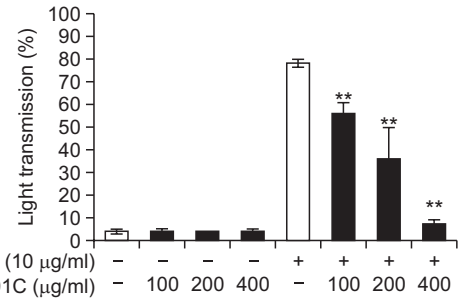

Fig. 2. Effects of CE-WIB801C on collagen-induced platelet aggregation. Measurement of platelet aggregation was carried out as described in "Materials and Methods." The data are expressed as the mean ± S.E.M. (n=4). ***p*<0.001 versus the collagen-stimulated platelets.

Determination of serotonin secretion

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 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, Winoosku, 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-

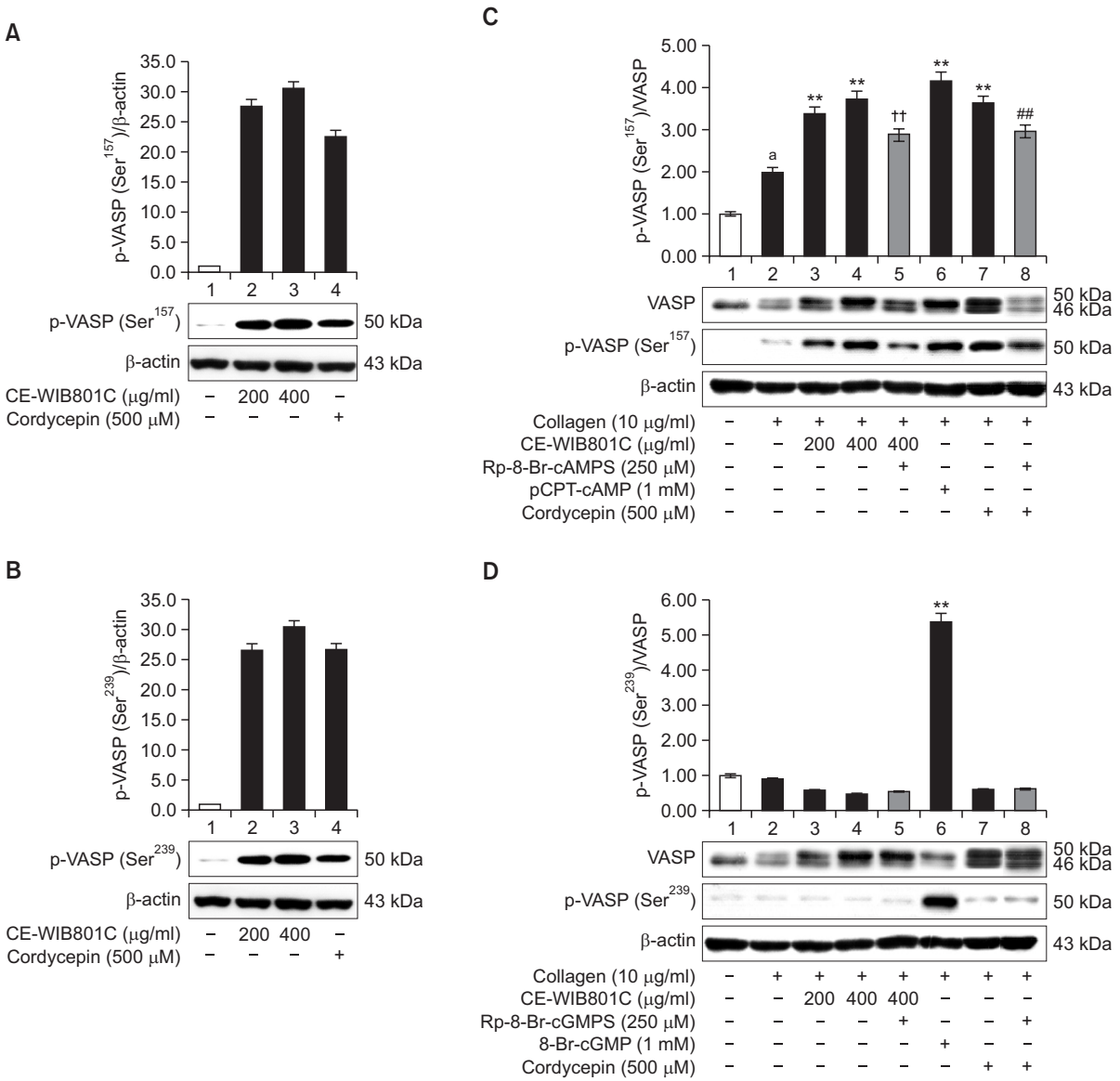

Fig. 3. Effects of CE-WIB801C and cordycepin on VASP phosphorylation. (A) Effect of CE-WIB801C and cordycepin on p-VASP (Ser^{157}). (B) Effect of CE-WIB801C and cordycepin on p-VASP (Ser^{239}). Lane 1, Intact platelets (base); Lane 2, CE-WIB801C (200 μg/mL); Lane 3, CE-WIB801C (400 μg/mL); Lane 4, Cordycepin (500 μM). (C) Effect of CE-WIB801C and cordycepin on p-VASP (Ser^{157}). 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)+pCPT-cAMP (1 mM); Lane 7, Collagen (10 μg/mL)+cordycepin (500 μM); Lane 8, Collagen (10 μg/mL)+cordycepin (500 μM)+Rp-8-Br-cAMPS (250 μM). (D) Effect of CE-WIB801C and cordycepin on p-VASP (Ser^{239}). 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-cGMPS (250 μM); Lane 6, Collagen (10 μg/mL)+8-Br-cGMP (1 mM); Lane 7, Collagen (10 μg/mL)+cordycepin (500 μM); Lane 8, Collagen (10 μg/mL)+cordycepin (500 μM)+Rp-8-Br-cGMPS (250 μM). Western blotting was performed as described in "Materials and Methods." The data are expressed as the mean ± S.E.M. (n=3). $^{**}p<0.001$ versus the collagen-stimulated platelets, $^{\dagger\dagger}p<0.001$ versus the collagen-stimulated platelets in the presence of CE-WIB801C (400 μg/mL), $^{\#\#}p<0.001$ versus the collagen-stimulated platelets in the presence of cordycepin (500 μM).

nist in this study. The light transmission in response to various concentrations of CE-WIB801C (100, 200, 400 μg/mL) in intact platelet was 4.3 ± 0.6% (at 100 μg/mL of CE-WIB801C), 4.0 ± 0.0% (at 200 μg/mL of CE-WIB801C), and 4.3 ± 0.6 (at 400 μg/mL of CE-WIB801C), respectively, which were not significantly different from that (4.0 ± 1.0%) in resting platelets. These mean that CE-WIB801C alone did not affect on 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 $CaCl_2$, 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).

Table 1. Changes of p-VASP (Ser[157])/VASP ratio and fibrinogen binding

	p-VASP (Ser[157]) /VASP	Δ (%)		Fibrinogen binding (%)	Δ (%)	
Collagen (10 μg/mL)	2.10 ± 0.20	0		77.2 ± 5.5	0	
CE-WIB801C (400 μg/mL)+Collagen (10 μg/mL)	3.74 ± 0.81	+78.1[1)]	0	8.3 ± 0.8	-89.2[2)]	0
CE-WIB801C (400 μg/mL) +Rp-8-Br-cAMPS (250 μM)+Collagen (10 μg/mL)	2.88 ± 0.57	-	-23.0[3)]	40.4 ± 1.1	-	+386.7[4)]
CE-WIB801C (400 μg/mL) +Rp-8-Br-cGMPS (250 μM)+Collagen (10 μg/mL)	3.84 ± 0.79	-	+2.7[5)]	8.8 ± 0.5	-	+6.0[6)]

Data were from Fig. 3C, 3D, 5B. [1)] to [2)] Δ (%)=[(CE-WIB801C+Collagen)-Collagen]/Collagen×100, [3)] to [4)] Δ (%)=[(CE-WIB801C+Rp-8-Br-cAMPS+Collagen)-(CE-WIB801C+Collagen)]/(CE-WIB801C+Collagen)×100, [5)] to [6)] Δ (%)=[(CE-WIB801C+Rp-8-Br-cGMPS+Collagen)-(CE-WIB801C+Collagen)]/(CE-WIB801C+Collagen)×100.

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 (Ser[157]) as compared with that (Fig. 3A lane 1) of intact platelets, control. Collagen increased weakly the phosphorylation of Ser[157] [p-VASP (Ser[157])] at 50 kDa of VASP (Fig. 3C lane 2) as compared with that of intact platelets (Fig. 3C lane 1), which involves in feedback inhibition by collagen (Gambaryan *et al*., 2010). A-kinase activator pCPT-cAMP stimulated VASP phosphorylation, indicating VASP phosphorylation (Ser[157]) is dependent on cAMP in collagen-induced platelet aggregation (Fig. 3C lane 6). CE-WIB801C (400 μg/mL), and cordycepin (500 μM) also increased p-VASP (Ser[157]) (Fig. 3C lane 4, 7), and CE-WIB801C (400 μg/mL) increased to 78.1% the ratio of p-VASP (Ser[157]) to VASP in collagen-induced platelet aggregation (Table 1). A-kinase inhibitor Rp-8-Br-cAMPS decreased to 23.0% CE-WIB801C-elevated p-VASP (Ser[157]) (Fig. 3C lane 5 and Table 1). With regard to the p-VASP (Ser[239]), G-kinase activator increased p-VASP (Ser[239]) (Fig. 3D lane 6). CE-WIB801C alone (200, 400 μg/mL), and cordycepin alone (500 μM) (Fig. 3B lane 2, 3, and 4) also potently increased p-VASP (Ser[239]) as compared with that (Fig. 3B lane 1) of intact platelets, control. However, CE-WIB801C (200, 400 μg/mL), and cordycepin (500 μM) did not almost increase the p-VASP (Ser[239]) (Fig. 3D lane 3, 4, and 7) as compared with that (Fig. 3D lane 2) by collagen. G-kinase inhibitor Rp-8-Br-cGMPS did not change p-VASP (Ser[239]) in the presence of CE-WIB801C (400 μg/mL) or cordycepin (500 μM) in collagen-induced platelet aggregation (Fig. 3D lane 5, 8).

Effects of CE-WIB801C and cordycepin on fibrinogen binding to αIIb/β3

Next, we investigated whether the VASP phosphorylation by CE-WIB801C involved in inhibition of fibrinogen binding to αIIb/β3. As shown in Fig. 4, collagen activated fibrinogen binding to αIIb/β3 (Fig. 4A-b), and increased the degree of fibrinogen binding to αIIb/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 αIIb/β3 (Fig. 4A-c, 4B), and its inhibitory degree was 89.2% as compared with that (77.2 ± 5.5%) by collagen (Table 1).

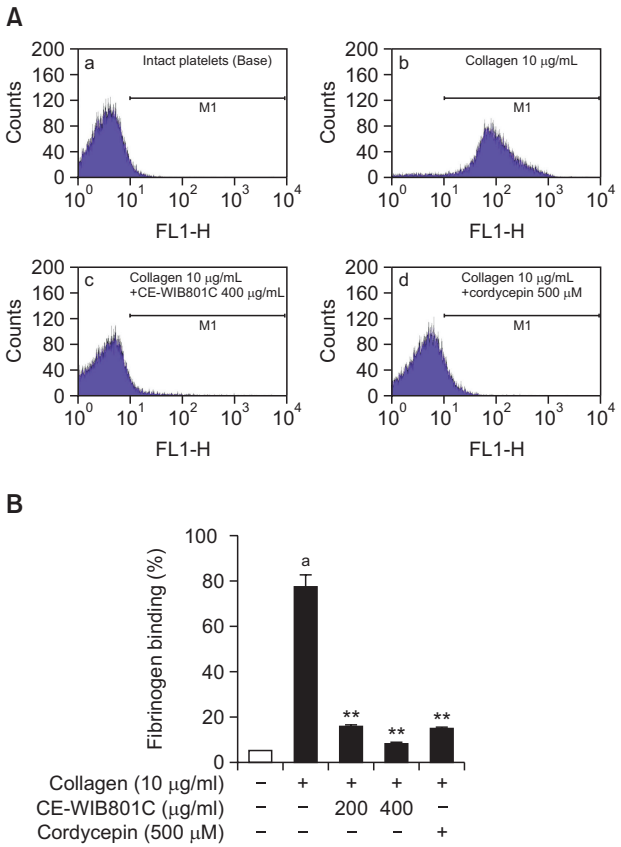

Fig. 4. Effects of CE-WIB801C and cordycepin on collagen-induced fibrinogen binding. (A) The flow cytometry histograms on fibrinogen binding. a, Intact platelets (base); b, Collagen (10 μg/mL); c, Collagen (10 μg/mL)+CE-WIB801C (400 μg/mL); d, Collagen (10 μg/mL)+cordycepin (500 μM). (B) Effects of CE-WIB801C and cordycepin on collagen-induced fibrinogen binding (%). Determination of fibrinogen binding to αIIb/β3 was carried out 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.

Cordycepin also potently inhibited collagen-activated fibrinogen binding to αIIb/β3 (Fig. 4A-d and 4B). Because the inhibition of αIIb/β3 is resulted from cAMP/A-kinase- and cGMP/G-kinase-mediated VASP phosphorylation, and it is known that

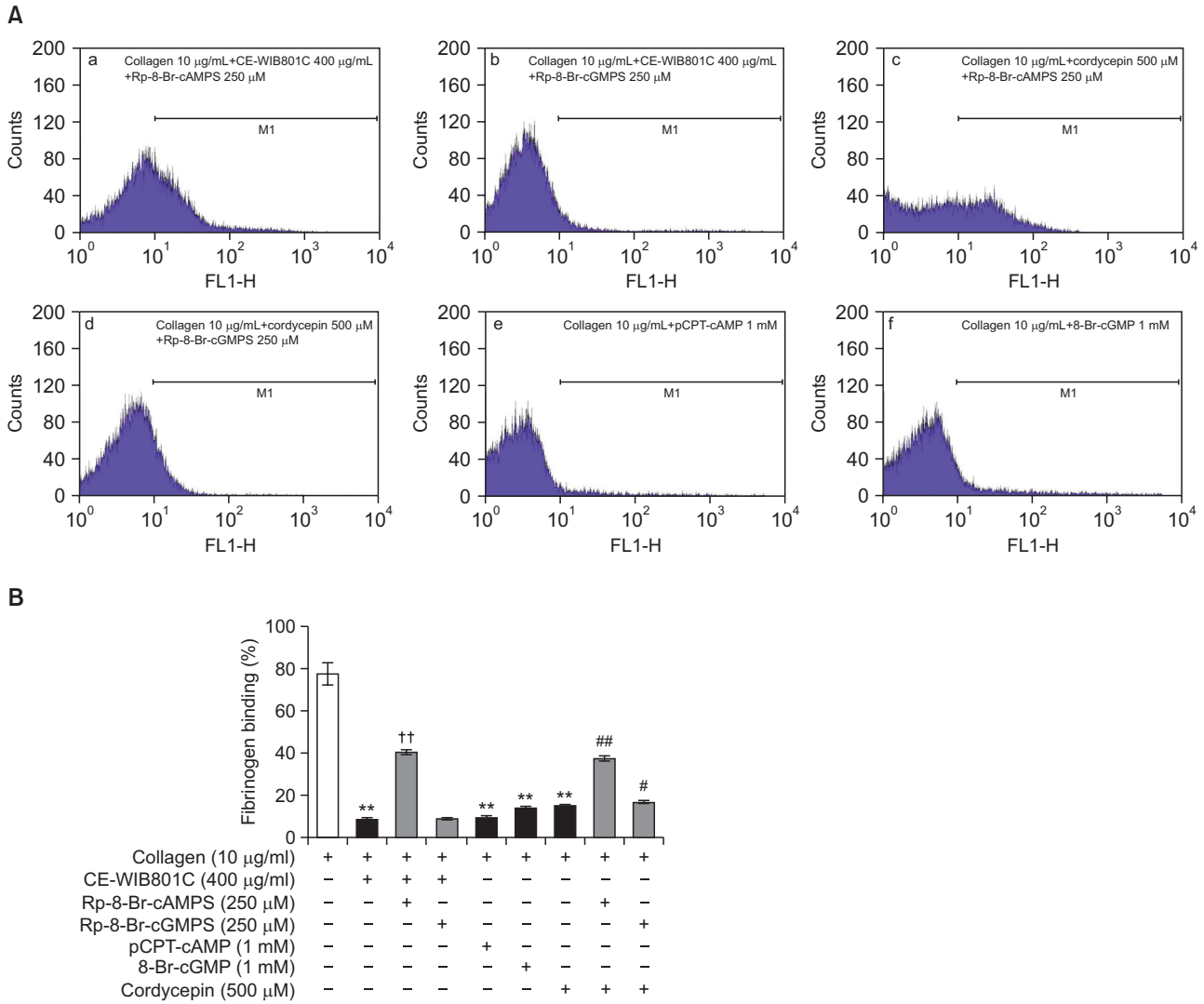

Fig. 5. Effects of CE-WIB801C and cordycepin on collagen-induced fibrinogen binding in the presence of A-kinase inhibitor (Rp-8-Br-cAMPS), or G-kinase inhibitor (Rp-8-Br-cGMPS). (A) The flow cytometry histograms on fibrinogen binding. a, Collagen (10 μg/mL)+CE-WIB801C (400 μg/mL)+Rp-8-Br-cAMPS (250 μM); b, Collagen (10 μg/mL)+CE-WIB801C (400 μg/mL)+Rp-8-Br-cGMPS (250 μM); c, Collagen (10 μg/mL)+cordycepin (500 μM)+Rp-8-Br-cAMPS (250 μM); d, Collagen (10 μg/mL)+cordycepin (500 μM)+ Rp-8-Br-cGMPS (250 μM); 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 $\alpha IIb/\beta_3$ was carried out as described in "Materials and Methods." The data are expressed as the mean ± S.E.M. (n=4). $^{**}p<0.001$ versus the collagen-stimulated platelets, $^{\dagger\dagger}p<0.001$ versus the collagen-stimulated platelets in the presence of CE-WIB801C (400 μg/mL). $^{\#}p<0.05$ versus the collagen-stimulated platelets in the presence of cordycepin (500 μM). $^{\#\#}p<0.001$ versus the collagen-stimulated platelets in the presence of cordycepin (500 μM).

cAMP- and cGMP-increasing compounds involve in inhibition of $\alpha IIb/\beta_3$ (Horstrup *et al*., 1994; Barragan *et al*., 2003), we investigated whether the inhibition of fibrinogen binding to $\alpha IIb/\beta_3$ by CE-WIB801C was contributed to which kinase of A-kinase and G-kinase. A-kinase activator pCPT-cAMP and G-kinase activator 8-Br-cGMP inhibited collagen-induced fibrinogen binding to $\alpha IIb/\beta_3$, respectively (Fig. 5A-e, f and Fig. 5B). It was confirmed that cAMP/A-kinase and cGMP/G-kinase pathway involve in inhibition of fibrinogen binding to $\alpha IIb/\beta_3$ in collagen-induced platelet aggregation. CE-WIB801C-inhibited fibrinogen binding to $\alpha IIb/\beta_3$ (Fig. 4A-c and Fig. 4B) was elevated by A-kinase inhibitor Rp-8-Br-cAMPS (Fig. 5A-a and Fig. 5B), and its stimulatory degree was 386.7% as compared with that (8.3%) by both CE-WIB801C and collagen (Table 1). However, CE-WIB801C-inhibited fibrinogen binding to $\alpha IIb/\beta_3$ (Fig. 4A-c and Fig. 4B) was not almost elevated by G-kinase inhibitor Rp-8-Br-cGMPS (Fig. 5A-b and Fig. 5B), and its stimulatory degree was 6.0% as compared with that (8.3%) by both CE-WIB801C and collagen (Table 1).

Effects of CE-WIB801C on PI3K/Akt phosphorylation

As apposed to the phosphorylated VASP, PI3K/Akt phosphorylation stimulates $\alpha IIb/\beta_3$ activation and fibrinogen binding (Zhang *et al*., 1996; Chen *et al*., 2004). Thus, we investigated

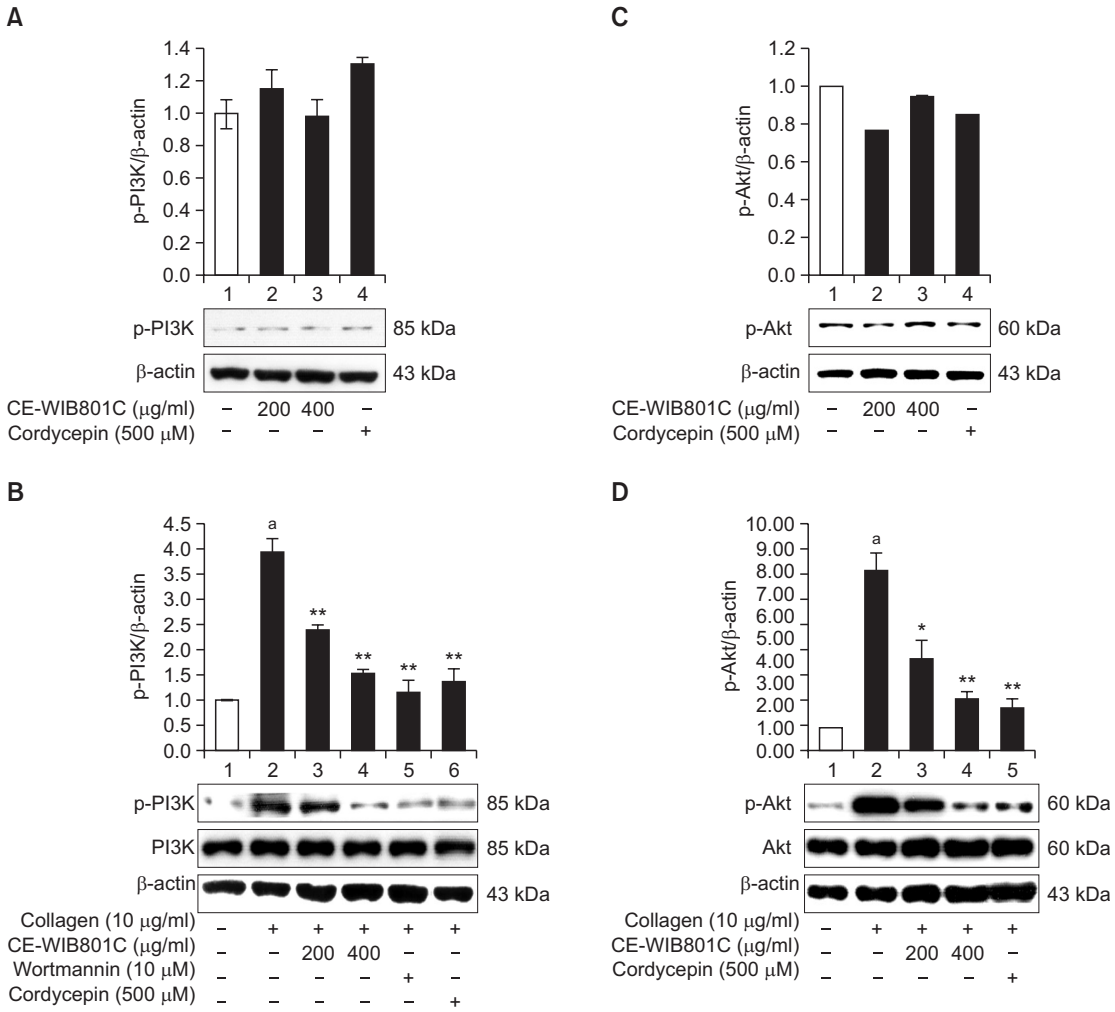

Fig. 6. Effects of CE-WIB801C and cordycepin on PI3K phosphorylation and Akt. (A) Effects of CE-WIB801C and cordycepin on PI3K phosphorylation in intact platelets. Lane 1, Intact platelets (base); Lane 2, CE-WIB801C (200 μg/mL); Lane 3, CE-WIB801C (400 μg/mL); Lane 4, Cordycepin (500 μM). (B) Effects of CE-WIB801C and cordycepin on PI3K 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)+Wortmannin (10 μM); Lane 6, Collagen (10 μg/mL)+Cordycepin (500 μM). (C) Effects of CE-WIB801C and cordycepin on Akt phosphorylation in intact platelets. Lane 1, Intact platelets (base); Lane 2, CE-WIB801C (200 μg/mL); Lane 3, CE-WIB801C (400 μg/mL); Lane 4, Cordycepin (500 μM). (D) Effects of CE-WIB801C and cordycepin on Akt 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)+cordycepin (500 μM). The data are expressed as the mean ± S.E.M. (n=3). [a]$p<0.05$ versus non-stimulated platelets, *$p<0.05$, **$p<0.001$ versus the collagen-stimulated platelets.

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 $\alpha IIb/\beta_3$ release ATP and serotonin out of dense bodies to aggregate platelets, which due to the $[Ca^{2+}]_i$ mobilization by fibrinogen binding to $\alpha IIb/\beta_3$ (Weiss *et al*., 1974; Michal and Motamed, 1976; Kamruzzaman *et al*., 2013). Because CE-WIB801C also inhibited $[Ca^{2+}]_i$ mobilization (Lee *et al*., 2014b), and fibrinogen binding to $\alpha IIb/\beta_3$ (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-

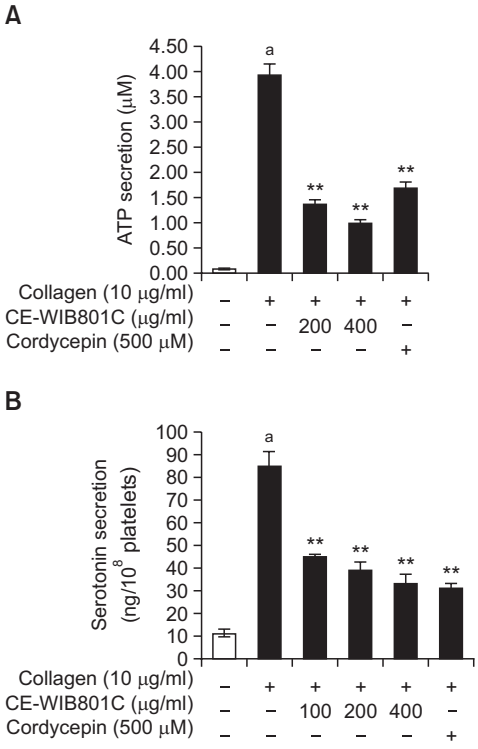

Fig. 7. Effects of CE-WIB801C and cordycepin on ATP and serotonin release. (A) Effect of CE-WIB801C and cordycepin on ATP release in collagen-induced platelets. (B) Effect of CE-WIB801C and cordycepin on serotonin release in collagen-induced platelets. ATP and serotonin secretion were measured as described 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.

leased to 3.91 ± 0.24 μM ATP from platelets, it's the stimulatory degree was 4,244% as compared with that (0.09 ± 0.02 μM) in intact platelets, control (Fig. 7A). However, CE-WIB801C, and cordycepin potently inhibited ATP release by collagen (Fig. 7A).

With regard to serotonin release, as shown in Fig. 7B, collagen potently released serotonin to 84.7 ± 6.5 ng/10^8 platelets, and its stimulatory degree was 636.5% as compared with that (11.5 ± 1.6 ng/10^8 platelets) in intact platelets, control (Fig. 7B). However, CE-WIB801C, and cordycepin inhibited collagen-elevated serotonin release (Fig. 7B).

Effects of cordycepin, W-cordycepin on collagen-induced platelet aggregation

CE-WIB801C (100, 200, 400 μg/mL) dose dependently inhibited collagen-induced platelet aggregation (Fig. 2). To investigate whether cordycepin (W-cordycepin) of CE-WIB801C involved in inhibition of collagen-induced platelet aggregation by CE-WIB801C, we purified cordycepin from CE-WIB801C with prep-HPLC (Lee *et al*., 2014c). The concentration of cordycepin (Mw. 251.14) was 81.98 ± 1.37 mg/g-CE-WIB801C (8.2%) (Lee *et al*., 2014b). The concentration of CE-WIB801C that inhibited collagen-induced platelet aggregation was at least 100, 200, 400 μg/mL (Fig. 2, Lee *et al*., 2014b). In this study, therefore, to investigate the synergistic effect of cordycepin, or W-cordycepin with CE-WIB801C on collagen-induced platelet 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. Furthermore, the inhibitory degree of collagen-induced platelet aggregation by CE-WIB801C together with cordycepin or W-cordycepin was also increased in a dose dependent manner. Therefore, it is thought that cordycepin or W-cordycepin with CE-WIB801C have a synergistic inhibitory effect on collagen-induced platelet aggregation, and the inhibition (Fig. 2) is resulted from antiplatelet function of W-cordycepin in CE-WIB801C.

DISCUSSION

A downstream pathway of both cAMP/A-kinase and cGMP/G-kinase involves in VASP phosphorylation to inhibit fibrinogen binding to αIIb/β_3. Ser^{157} at 50 kDa of VASP is phosphorylated by the cAMP/A-kinase pathway, whereas Ser^{239} 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 (Ser^{157}) 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 (Ser^{157}) 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 (Ser^{157}) phosphorylation *via* A-kinase activation in collagen-induced platelet aggregation. If not so, CE-WIB801C-, and cordycepin-induced VASP (Ser^{157}) phosphorylation would not be inhibited by A-kinase inhibitor Rp-

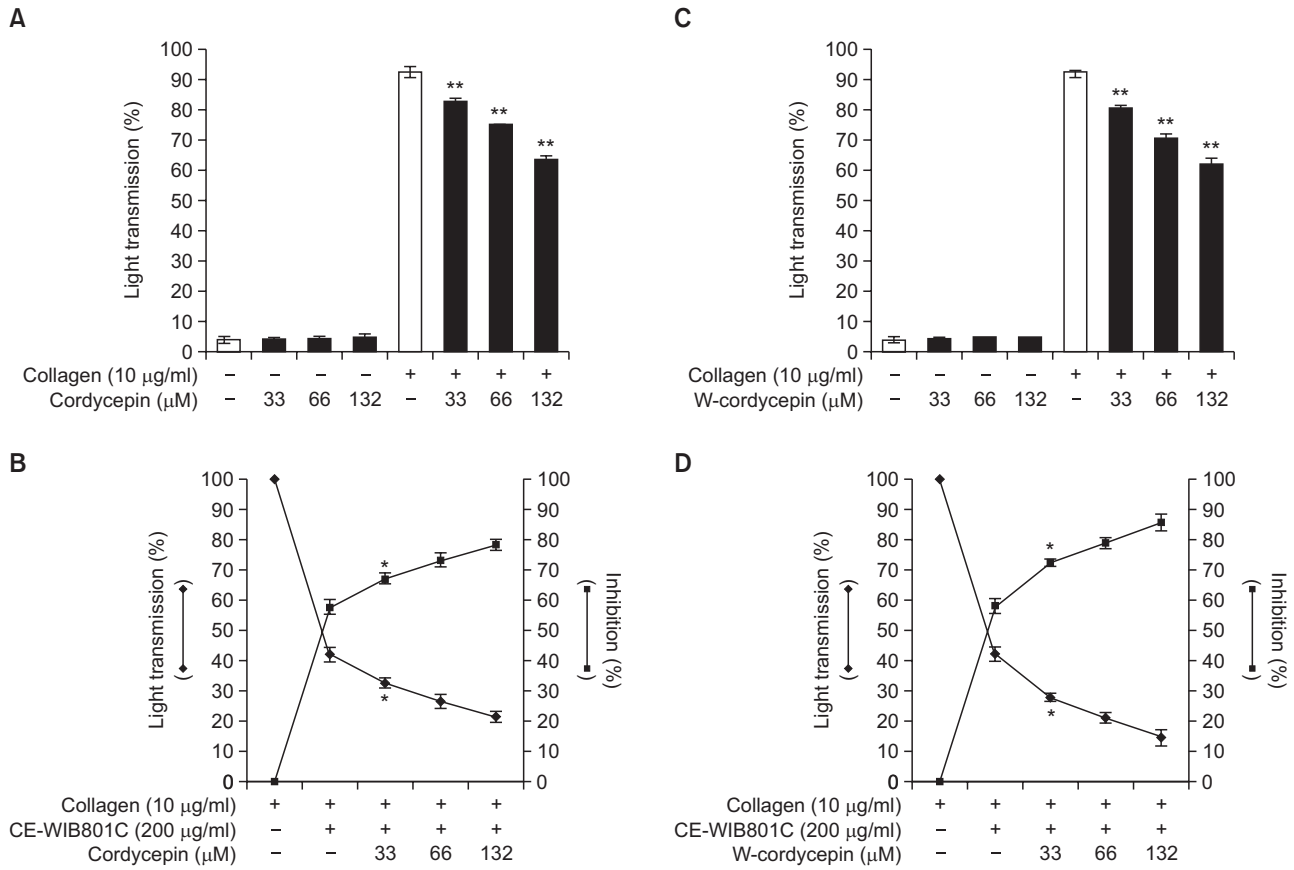

Fig. 8. Effects of cordycepin or W-cordycepin on collagen-induced platelet aggregation. (A) Effects of cordycepin on collagen-induced platelet aggregation. (B) The synergistic inhibitory effects of cordycepin with CE-WIB801C on collagen-induced platelet aggregation. (C) Effects of W-cordycepin on collagen-induced platelet aggregation. (B) The synergistic inhibitory effects of W-cordycepin with CE-WIB801C on collagen-induced platelet aggregation. Measurement of platelet aggregation 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 the collagen-stimulated platelets. The light transmission and inhibitory degree by cordycepin or W-cordycepin, and cordycepin or W-cordycepin plus CE-WIB801C were expressed as a percentage of that by collagen (100% : aggregation, 0% : inhibition).

8-Br-cAMPS in collagen-induced platelet aggregation. With regard to VASP (Ser^{239}) phosphorylation, CE-WIB801C and cordycepin phosphorylated G-kinase substrate VASP (Ser^{239}) in intact platelets, but did not phosphorylate that in collagen-induced platelet aggregation. Unfortunately, we could not explain the different phenomena of VASP (Ser^{239}) phosphorylation caused by CE-WIB801C and cordycepin in intact platelets and 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-indcued VASP (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 (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, cilostazole, 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 Willigen 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 (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 $[Ca^{2+}]_i$ to phosphorylate Ca^{2+}/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 $[Ca^{2+}]_i$ mobilization, which

also supports the facts that CE-WIB801C induced the cAMP-dependent phosphorylation of IP3R to inhibit $[Ca^{2+}]_i$ mobilization (Lee *et al*., 2014b). In our another study, we observed that cordycepin inhibits $[Ca^{2+}]_i$ mobilization by phosphorylating IP3R in collagen-induced platelet aggretion (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^{157}), and dephosphorylation of PI3K and Akt to inhibit fibrinogen binding to $\alpha IIb/\beta_3$.

Platelet aggregation is generated at site of vascular wall injury, and is involved in the formation of thrombus. 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 fibroblast, 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-κB activity. In addition, cordycepin is known to inhibit protein synthesis by attenuating Akt/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 antithrombotic-, and antiatherosclerotic-effects without inflammation and progression of atherosclerotic 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 $[Ca^{2+}]_i$ mobilization, and inhibit $\alpha IIb/\beta_3$ 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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REFERENCES

Barragan, P., Bouvier, J. L., Roquebert, P. O., Macaluso, G., Commeau, P., Comet, B., Lafont, A., Camoin, L., Walter, U. and Eigenthaler, M. (2003) Resistance to thienopyridines: clinical detection of coronary stent thrombosis by monitoring of vasodilator-stimulated phosphoprotein phosphorylation. *Catheter. Cardiovasc. Interv.* **59**, 295-302.

Castro-Malaspina, H., Rabellino, E. M., Yen, A., Nachman, R. L. and Moore, M. A. (1981) Human megakaryocyte stimulation of proliferation of bone marrow fibroblasts. *Blood* **57**, 781-787.

Chen, J., De, S., Damron, D. S., Chen, W. S., Hay, N. and Byzova, T. V. (2004) Impaired platelet responses to thrombin and collagen in AKT-1-deficient mice. *Blood* **104**, 1703-1710.

Cho, H. J., Cho, J. Y., Rhee, M. H., Lim, C. R., and Park, H. J. (2006) Cordycepin (3'-deoxyadenosine) inhibits human platelet aggregation induced by U46619, a TXA2 analogue. *J. Pharm. Pharmacol.* **58**, 1677-1682.

Cho, H. J., Cho, J. Y., Rhee, M. H. and Park, H. J. (2007a) Cordycepin (3'-deoxyadenosine) inhibits human platelet aggregation in a cyclic AMP- and cyclic GMP-dependent manner. *Eur. J. Pharmacol.* **558**, 43-51.

Cho, H. J., Cho J. Y., Rhee, M. H., Kim, H. S., Lee, H. S., and Park, H. J. (2007b) Inhibitory effects of cordycepin (3'-deoxyadenosine), a component of *Cordyceps militaris*, on human platelet aggregation induced by thapsigargin. *J. Microbiol. Biotechnol.* **17**, 1134-1138.

Clutton, P., Folts, J. D. and Freedman, J. E. (2001) Pharmacological control of platelet function. *Pharmacol. Res.* **44**, 255-264.

Eigenthaler, M., Nolte, C., Halbrügge, M. and Walter U. (1992) Concentration and regulation of cyclic nucleotides, cyclic-nucleotide-dependent protein kinases and one of their major substrates in human platelets. Estimating the rate of cAMP-regulated and cGMP-regulated protein phosphorylation in intact cells. *Eur. J. Biochem.* **205**, 471-481.

Gambaryan, S., Kobsar, A., Rukoyatkina, N., Herterich, S., Geiger, J., Smolenski, A., Lohmann, S. M. and Walter, U. (2010) Thrombin and collagen induce a feedback inhibitory signaling pathway in platelets involving dissociation of the catalytic subunit of protein kinase A from an NFkappaB-IkappaB complex. *J. Biol. Chem.* **285**, 18352-18363.

Hayashi, H. and Sudo, T. (2009) Effects of the cAMP-elevating agents cilostamide, cilostazol and forskolin on the phosphorylation of Akt and GSK-3beta in platelets. *Thromb. Haemost*. **102**, 327-335.

Holash, J., Maisonpierre, P. C., Compton, D., Boland, P., Alexander, C. R., Zagzag, D., Yancopoulos, G. D. and Wiegand, S. J. (1999) Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science* **284**, 1994-1998.

Horstrup, K., Jablonka, B., Hönig-Liedl, P., Just, M., Kochsiek, K. and Walter, U. (1994) Phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein at Ser^{157} in intact human platelets correlates with fibrinogen receptor inhibition. *Eur. J. Biochem.* **225**, 21-27.

Jennings, L. K. (2009) Role of platelets in atherothrombosis. *Am. J. Cardiol.* **103**, 4A-10A.

Kaibuchi, K., Sano, K., Hoshijima, M., Takai, Y. and Nishizuka, Y. (1982) Phosphatidylinositol turnover in platelet activation; calcium mobilization and protein phosphorylation. *Cell Calcium* **3**, 323-335.

Kamruzzaman, S. M., Yayeh, T., Ji, H. D., Park, J.Y., Kwon, Y. S., Lee, I. K., Kim, S., Oh, S. H., Kim, S. D., Roh, S. S., Yun, B. S. and Rhee, M. H. (2013) p-Terphenyl curtisian E inhibits in vitro platelet aggregation via cAMP elevation and VASP phosphorylation. *Vascul. Pharmacol.* **59**, 83-89.

Kim, H. G., Shrestha, B., Lim, S. Y., Yoon, D. H., Chang, W. C., Shin, D. J., Han, S. K., Park, S. M., Park, J. H., Park, H. I., Sung, J. M., Jang, Y., Chung, N., Hwang, K. C. and Kim, T. W. (2006) Cordycepin inhibits lipopolysaccharide-induced inflammation by the suppression of NF-kappaB through Akt and p38 inhibition in RAW 264.7 macrophage cells. *Eur. J. Pharmacol.* **545**, 192-199.

Laurent, V., Loisel, T. P., Harbeck, B., Wehman, A., Gröbe, L., Jockusch, B. M., Wehland, J., Gertler, F. B. and Carlier, M. (1999) Role of proteins of the Ena/VASP family in actin-based motility of Listeria monocytogenes. *J. Cell Biol.* **144**, 1245-1258.

Lee, D. H., Kim, H. H., Cho, H.J., Bae, J. S., Yu, Y. B. and Park, H. J. (2014a) Antiplatelet effects of caffeic acid due to Ca^{2+} mobilization-inhibition via cAMP-dependent inositol-1, 4, 5-trisphosphate receptor phosphorylation. *J. Atheroscler. Thromb.* **21**, 23-37.

Lee, D. H., Kim, H. H., Cho, H. J., Yu, Y. B., Kang, H. C., Kim, J. L., Lee, J. J. and Park, H. J. (2014b) 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. *Biomol. Ther.* **22**, 223-231.

Lee, D. H., Kwon, H. W., Kim, H. H., Lim, D. H., Nam, G. S., Shin, J. H., Kim, Y. Y., Kim, J. L., Lee, J. J., Kwon, H. K. and Park, H. J. (2014c) Cordycepin-enriched WIB801C from *Cordyceps militaris* inhibits ADP-induced $[Ca^{2+}]_i$ mobilization and fibrinogen binding via phosphorylation of IP3R and VASP. *Arch. Pharm. Res.* DOI 10.1007/s12272-014-0436-z. [Epub ahead of print]

Li, Z., Ajdic, J., Eigenthaler, M. and Du, X. (2003) A predominant role for cAMP-dependent protein kinase in the cGMP-induced phosphorylation of vasodilator-stimulated phosphoprotein and platelet inhibition in humans. *Blood* **101**, 4423-4429.

Lincoff, A. M., Califf, R. M. and Topol, E. J. (2000) Platelet glycoprotein IIb/IIIa receptor blockade in coronary artery disease. *J. Am. Coll. Cardiol.* **35**, 1103-1115.

Michal, F. and Motamed, M. (1976) Shape change and aggregation of blood platelets: interaction between the effects of adenosine and diphosphate, 5-hydroxytryptamine and adrenaline. *Br. J. Pharmacol.* **56**, 209-218.

Morello, F., Perino, A. and Hirsch, E. (2009) Phosphoinositide 3-kinase signalling in the vascular system. *Cardiovasc. Res.* **82**, 261-271.

Nagai, R., Suzuki, T., Aizawa, K., Shindo, T. and Manabe, I. (2005) Significance of the transcription factor KLF5 in cardiovascular remodeling. *J. Thromb. Haemost.* **3**, 1569-1576.

Nishikawa, M., Tanaka, T. and Hidaka, H. (1980) Ca2+-calmodulin-dependent phosphorylation and platelet secretion. *Nature* **287**, 863-865.

Ok, W. J., Cho, H. J., Kim, H. H., Lee, D. H., Kang, H. Y., Kwon, H. W., Rhee, M. H., Kim, M. and Park, H. J. (2012) Epigallocatechin-3-gallate has an anti-platelet effect in a cyclic AMP-dependent manner. *J. Atheroscler. Thromb.* **19**, 337-348.

Packham, M. A. and Mustard, J. F. (1986) The role of platelets in the development and complications of atherosclerosis. *Semin. Hematol.* **23**, 8-26.

Payrastre, B., Missy, K., Trumel, C., Bodin, S., Plantavid, M. and Chap, H. (2000) The integrin alpha IIb/beta 3 in human platelet signal transduction. *Biochem. Pharmacol.* **60**, 1069-1074.

Phillips, D. R., Nannizzi-Alaimo, L. and Prasad K.S. (2001) β3 tyrosine phosphorylation in αIIb/β_3 (platelet membrane GP IIb-IIIa) outside-in integrin signaling. *Thromb. Haemost.* **86**, 246-58.

Phillips, D. R., Conley, P. B., Sinha, U. and Andre, P. (2005) Therapeutic approaches in arterial thrombosis. *J. Thromb. Haemost.* **3**, 1577-1589.

Sabatine, M. S. and Jang, I. K. (2000) The use of glycoprotein IIb/IIIa inhibitors in patients with coronary artery disease. *Am. J. Med.* **109**, 224-237.

Schwartz, S. M. and Ross, R. (1984) Cellular proliferation in atherosclerosis and hypertension. *Prog. Cardiovasc. Dis.* **26**, 355-372.

Schwartz, S. M. and Reidy, M.A. (1987) Common mechanisms of proliferation of smooth muscle in atherosclerosis and hypertension. *Hum. Pathol.* **18**, 240-247.

Schwartz, S. M., Heinmark, R. L. and Majesky, M. W. (1990) Developmental mechanisms underlying pathology of arteries. *Physiol. Rev.* **70**, 1177-1209.

Seppä, H., Grotendorst, G., Seppä, S., Schiffmann, E. and Martin, G. R. (1982) Platelet-derived growth factor in chemotactic for fibroblasts. *J. Cell Biol.* **92**, 584-588.

Shattil, S. J. and Newman, P. J. (2004) Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood.* **104**, 1606-1615.

Smolenski, A., Bachmann, C., Reinhard, K., Hönig-Liedl, P., Jarchau, T., Hoschuetzky, H. and Walter U. (1998) Analysis and regulation of vasodilator-stimulated phosphoprotein serine 239 phosphorylation in vitro and in intact cells using a phosphospecific monoclonal antibody. *J. Biol. Chem.* **273**, 20029-20035.

Sudo, T., Ito, H. and Kimura, Y. (2003) Phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) by the anti-platelet drug, cilostazol, in platelets. *Platelets* **14**, 381-390.

van Willigen, G. and Akkerman, J. W. (1991) Protein kinase C and cyclic AMP regulate reversible exposure of binding sites for fibrinogen on the glycoprotein IIB-IIIA complex of human platelets. *Biochem. J.* **273**, 115-120.

Weiss, H. J., Tschopp, T. B., Rogers, J. and Brand, H. (1974) Studies of platelet 5-hydroxytryptamine (serotonin) in storage pool disease and albinism. *J. Clin. Invest.* **54**, 421-433.

Wong, Y. Y., Moon, A., Duffin, R., Barthet-Barateig, A., Meijer, H. A., Clemens, M. J. and de Moor, C. H. (2010) Cordycepin inhibits protein synthesis and cell adhesion through effects on signal transduction. *J. Biol. Chem.* **285**, 2610-2621.

Yue, G. G., Lau, C. B., Fung, K. P., Leung, P. C., and Ko, W. H. (2008) Effects of *Cordyceps sinensis*, *Cordyceps militaris* and their isolated compounds on ion transport in Calu-3 human airway epithelial cells. *J. Ethnopharmacol.* **117**, 92-101.

Zhang, J., Zhang, J., Shattil, S. J., Cunningham, M. C. and Rittenhouse, S. E. (1996) Phosphoinositide 3-kinase gamma and p85/phosphoinositide 3-kinase in platelets. Relative activation by thrombin receptor or beta-phorbol myristate acetate and roles in promoting the ligand-binding function of alphaIIbbeta3 integrin. *J. Biol. Chem.* **271**, 6265-6272.