Inhibitory Effects of Cordycepin on Platelet Activation *via* Regulation of Cyclic Adenosine Monophosphate-downstream Pathway

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Platelet activation is essential at the sites of vascular injury, which leads to hemostasis through adhesion, aggregation, and secretion process. However, potent and continuous platelet activation may be an important reason of circulatory disorders. Therefore, proper regulation of platelet activation may be an effective treatment for vascular diseases. In this research, inhibitory effects of cordycepin (3'-deoxyadenosine) on platelet activation were determined. As the results, cordycepin increased cAMP and cGMP, which are intracellular Ca^{2+} -antagonists. In addition, cordycepin reduced collagen-elevated $[\text{Ca}^{2+}]_i$ mobilization, which was increased by a cAMP-dependent protein kinase (PKA) inhibitor (Rp-8-Br-cAMPS), but not a cGMP-protein kinase (PKG) inhibitor (Rp-8-Br-cGMPS). Furthermore, cordycepin increased IP_3RI (Ser¹⁷⁵⁶) phosphorylation, indicating inhibition of IP_3 -mediated Ca^{2+} release from internal store *via* the IP_3RI , which was strongly inhibited by Rp-8-Br-cAMPS, but was not so much inhibited by Rp-8-Br-cGMPS. These results suggest that the reduction of $[\text{Ca}^{2+}]_i$ mobilization is caused by the cAMP/A-kinase-dependent IP_3RI (Ser¹⁷⁵⁶) phosphorylation. In addition, cordycepin increased the phosphorylation of VASP (Ser¹⁵⁷) known as PKA substrate, but not VASP (Ser²³⁹) known as PKG substrate. Cordycepin-induced VASP (Ser¹⁵⁷) phosphorylation was inhibited by Rp-8-Br-cAMPS, but was not inhibited by Rp-8-Br-cGMPS, and cordycepin inhibited collagen-induced fibrinogen binding to $\alpha\text{IIb}/\beta_3$, which was increased by Rp-8-Br-cAMPS, but was not inhibited by Rp-8-Br-cGMPS. These results suggest that the inhibition of $\alpha\text{IIb}/\beta_3$ activation is caused by the cAMP/A-kinase-dependent VASP (Ser¹⁵⁷) phosphorylation. In conclusion, these results demonstrate that inhibitory effects of cordycepin on platelet activation were due to inhibition of $[\text{Ca}^{2+}]_i$ mobilization through cAMP-dependent IP_3RI (Ser¹⁷⁵⁶) phosphorylation and suppression of $\alpha\text{IIb}/\beta_3$ activation through cAMP-dependent VASP (Ser¹⁵⁷) phosphorylation. These results strongly indicated that cordycepin might have therapeutic or preventive potential for platelet activation-mediated disorders including thrombosis, atherosclerosis, myocardial infarction, or cardiovascular disease.

Key Words: Cordycepin, Ca^{2+} mobilization, cAMP, Inositol trisphosphate receptor, VASP, Fibrinogen binding

INTRODUCTION

Platelets are activated by several molecules (i.e., collagen, ADP and thrombin) at the sites of vascular injury, which is essential hemostatic process. However, it can also lead to

circulatory disorders, including thrombosis, atherosclerosis, or myocardial infarction (Schwartz et al., 1990). Therefore, inhibition of platelet activation might be an effective approach to prevent vascular diseases. When collagen is released from the vascular endothelial cell in vascular injury, it binds to the receptor glycoprotein VI (GP VI), and then

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platelets activate by tyrosine kinase-dependent mechanisms, which is related to phospholipase C- γ_2 (PLC- γ_2) phosphorylation (Wonerow et al., 2002). The phosphorylated PLC- γ_2 produces diacylglycerol (DG) and inositol 1, 4, 5-trisphosphate (IP₃) hydrolyzing phosphatidylinositol 4, 5-bisphosphate (PIP₂). IP₃ mobilizes cytosol free Ca²⁺ ([Ca²⁺]_i) from dense tubular system through IP₃ receptor type I (IP₃RI), which works as calcium ion channel (Quinton et al., 1992). The increased [Ca²⁺]_i phosphorylates myosin light chain (MLC) *via* activation of Ca²⁺/calmodulin-dependent protein kinase, which induces platelet aggregation and granule secretion (Nishikawa et al., 1980; Kaibuchi et al., 1982). On the other hand, cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP) has antiplatelet effects by decreasing the [Ca²⁺]_i mobilization (Menshikov et al., 1993; Schwarz et al., 2001). The cAMP or cGMP activate cAMP- or cGMP-dependent protein kinases (PKA or PKG), which phosphorylate substrate proteins, IP₃RI and vasodilator-stimulated phosphoprotein (VASP) (Halbrügge et al., 1990; Butt et al., 1994). IP₃RI (Ser¹⁷⁵⁶) phosphorylation results in inhibition of [Ca²⁺]_i mobilization (Quinton et al., 1992; Schwarz et al., 2001), and VASP (Ser¹⁵⁷ and Ser²³⁹) phosphorylation leads to inhibition of fibrinogen binding to glycoprotein IIb/IIIa (α IIb/ β_3), so that they consequentially inhibit platelet activation (Laurent et al., 1999; Sudo et al., 2003). Therefore, regulation of these cascades is good factor for evaluating antiplatelet effects of any compound or substance.

A species of *Cordyceps* known as ingredient of Chinese traditional medicine is prescribed for inflammatory and cancer diseases, and its major component is cordycepin (3'-deoxyadenosine). In previous report, cordycepin (3'-deoxyadenosine, Fig. 1) from *Cordyceps militaris* has antiplatelet effects in cAMP- and cGMP-dependent manner, which is involved in down-regulation of [Ca²⁺]_i and TXA₂ (Cho et al., 2007). However, its pathway remains uncertain and it is unknown how cordycepin regulates cAMP- or cGMP downstream pathway (i.e. phosphorylation of IP₃RI and VASP). In this study, we investigated how cordycepin regulates cAMP- or cGMP downstream pathway to inhibit platelets activation. These provide novel information and possibility of cordycepin as an antiplatelet drug.

Cordycepin (3'-deoxyadenosine)

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

MATERIALS AND METHODS

Materials

Cordycepin, Fura 2-AM, and other reagents were purchased from Sigma Chemical Corporation (St. Louis, MO., USA). Collagen was purchased from Chrono-Log Co. (Havertown, PA., USA). Lysis buffer and Antibodies for western blotting were purchased from Cell Signaling Co. (Beverly, MA., USA). Polyvinylidene difluoride (PVDF) membrane and enhanced chemiluminescence (ECL) solution were obtained from GE Healthcare (Chalfont St., Giles, Buckinghamshire, UK), and Fibrinogen Alexa Fluor 488 conjugate was purchased from Invitrogen Molecular Probes (Eugene, OR., USA).

Preparation of washed platelets

Human platelet-rich plasma (PRP) treated by ACD anti-coagulant (2.2% sodium citrate, 0.8% citric acid, 2.45% dextrose) was offered from Korean Red-Cross Blood Center (Changwon, Korea). PRP was centrifuged at 1,300 g for 10 min to obtain platelets removing a little red blood cell, and the platelets were washed twice with washing buffer (138 mM NaCl, 12 mM NaHCO₃, 5.5 mM glucose, 2.7 mM KCl, 1 mM EDTA, and 0.36 mM NaH₂PO₄, pH 6.5). The washed

platelets were suspended in suspending buffer (138 mM NaCl, 12 mM NaHCO₃, 5.5 mM glucose, 2.7 mM KCl, 0.49 mM MgCl₂, 0.36 mM NaH₂PO₄, and 0.25% gelatin, pH 6.9) to 5×10⁸/mL of final concentration. All this procedures were carried out avoiding platelet aggregation at 25 °C.

Measurement of cAMP and cGMP level

Washed platelets (10⁸/mL) were pre-incubated with the compound in 2 mM CaCl₂ at 37 °C for 3 min, and stimulated by 10 µg/mL collagen at 37 °C for 5 min. The reaction was terminated with 80% ice-cold ethanol, and cAMP and cGMP were determined by cAMP and cGMP EIA kits using synergy HT multi-model microplate reader.

Measurement of cytosolic-free Ca²⁺ mobilization

Fura 2-loaded platelets were prepared by incubating PRP in the presence of 5 µM Fura 2-AM at 37 °C for 60 min, and were pre-incubated with the compound in 2 mM CaCl₂ at 37 °C for 3 min, and stimulated with 10 µg/mL collagen at 37 °C for 5 min to determine cytosolic-free Ca²⁺ ([Ca²⁺]_i) mobilization. Fura 2 fluorescence was determined by a spectrofluorometer (SFM 25; Bio-Teck Instrument, Italy) in the excitation wavelength changing each 0.5 second from 340 to 380 nm and the emission wavelength at 510 nm. The [Ca²⁺]_i were calculated by the method of Schaeffer (Schaeffer et al., 1989).

Western blot for analysis of IP₃RI-, VASP-phosphorylations

Washed platelets (10⁸/mL) were pre-incubated with the compound in 2 mM CaCl₂ for 3 min, and stimulated with 10 µg/mL collagen at 37 °C for 5 min. The reactions were terminated with equal volume (250 µL) of lysis buffer (Cayman Chemical Co., MI., USA). Platelet lysates containing 15 µg protein were used for analysis. The effects of cordycepin on IP₃RI-, VASP-phosphorylation were analyzed by western blotting. 6~8% SDS-PAGE were used for electrophoresis, and PVDF membrane was used for gel transfer. The membranes were visualized with ECL, which was analyzed by the Quantity One, Ver. 4.5 (Hercules, CA., USA).

Measurement of fibrinogen binding to platelets

Washed platelets (10⁸/mL) were pre-incubated with the compound in 2 mM CaCl₂ for 3 min, and stimulated with 10 µg/mL collagen in the presence of 30 µg/mL Alexa Flour 488-human fibrinogen at room temperature for 5 min. The reaction was terminated with 0.5% paraformaldehyde in phosphate-buffered saline, which were placed in the dark. Alexa Fluor 488-fibrinogen binding to platelets was measured using flow cytometry (San Jose, CA., USA) and the data are analyzed by cellQuest software.

Statistical analyses

These data were expressed as the mean ± SEM accompanied with the number of observations. These data were assessed through analysis of variance (ANOVA). If the analysis indicated significant differences among the control group means, the each group was compared through the Newman-Keuls method. *P*<0.05 was considered to have statistically significant mean.

RESULTS & DISCUSSION

We examined whether cordycepin was concerned with elevation of cAMP or cGMP in collagen-stimulated platelet activation. As the result, collagen reduced intracellular cAMP production to 46.2% as compared with basal level (Table 1, Fig. 2A). However, when platelets were incubated in the presence of both cordycepin and collagen, the level of cAMP was increased to 8.3 ± 0.7 pmol/10⁹ platelets (Table 1, Fig. 2A), which increased collagen-decreased cAMP level to 196.4% (Table 1, Fig. 2A). On the contrary, collagen reduced intracellular cGMP production to 25.0% as compared with basal level (Table 1, Fig. 2B). However, when platelets were incubated in the presence of cordycepin and collagen, the cGMP level was increased to 46.7% as compared with the result (3.0 ± 0.4 pmol/10⁹ platelets) by collagen only (Table 1, Fig. 2B). The intracellular cAMP and cGMP levels are regulated by balance between cyclic nucleotide-producing enzymes such as adenylate/guanylate cyclase, and hydrolyzing enzymes such as cAMP/cGMP phosphodiesterases (PDEs). It is known that platelets have PDE₂, PDE₃, and

Table 1. Changes of cAMP and cGMP

	cAMP		cGMP		cAMP/cGMP	
	pmol/10 ⁹ platelets	Change (%)	pmol/10 ⁹ platelets	Change (%)	Ratio	Change (%)
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	- 25.0 ²⁾	0.9	- 30.7 ³⁾
Collagen (10 µg/mL) + cordycepin (500 µM)	8.3 ± 0.7 ^{**}	+ 196.4 ⁴⁾	4.4 ± 0.6 [*]	+ 46.7 ⁵⁾	1.9	+ 111.1 ⁶⁾

Data are from Fig. 2A, B. 1) to 3) Changes to base; 4) to 6) Changes to collagen

1) and 2) of inhibition (%) = (Base - Collagen)/Base × 100

4) and 5) of inhibition (%) = [(Collagen + cordycepin) - Collagen]/Collagen × 100

3) ratio decrease (%) = (Base - Collagen)/Base × 100

6) ratio increase (%) = [(Collagen + cordycepin) - Collagen]/Collagen × 100

These data were expressed as the mean ± SEM (n = 4). ^aP<0.05 compared with base, *P<0.05, **P<0.001 compared with the collagen-stimulated platelets.

Fig. 2. Effect of cordycepin on cAMP and cGMP production. (A) Effect of cordycepin on cAMP production in collagen-induced platelets. (B) Effect of cordycepin on cGMP production in collagen-induced platelets. The level of cAMP and cGMP was examined as described in "Methods". These data were expressed as the mean ± SEM (n = 4). These data were expressed as the mean ± S.E.M. (n = 4). *P<0.05 compared with the non-stimulated platelets, *P<0.05, **P<0.001 compared with the collagen-stimulated platelets.

PDE₅ (Schwarz et al., 2001; Walter et al., 2009). PDE₂ hydrolyzes both cAMP and cGMP as cGMP-stimulated PDE, PDE₃ hydrolyzes cAMP rather than cGMP as cGMP-inhibited PDE, and PDE₅ hydrolyzes cGMP only as cGMP-binding-cGMP specific PDE. Because cordycepin increased both cAMP and cGMP in collagen-induced platelet aggregation, although it is thought that cordycepin might involve inhibition of PDE₂ to increase both cAMP and cGMP, we

cannot exclude that cordycepin might involve activation of adenylate cyclase to produce cAMP from ATP, because cordycepin-elevated cAMP level was decreased by adenylate cyclase inhibitor SQ22536 (Cho et al., 2007). Nieswandt group provided an evidence for cross-talk between collagen receptor GPVI and G_i-coupled receptor during collagen-induced platelet aggregation (Nieswandt et al., 2001). If so, there is a possibility that collagen-reduced cAMP was medi-

Fig. 3. Effects of cordycepin on $[Ca^{2+}]_i$ mobilization. (A) Effects of cordycepin on collagen-stimulated $[Ca^{2+}]_i$ mobilization. (B) Effects of cordycepin on collagen-stimulated $[Ca^{2+}]_i$ mobilization in the presence of Rp-8-Br-cAMPS (PKA inhibitor), or Rp-8-Br-cGMPS (PKG inhibitor). The level of $[Ca^{2+}]_i$ was examined as described in "Methods". These data were expressed as the mean $\pm$ SEM ($n = 4$), and ^a $P < 0.05$ compared with the non-stimulated platelets, ^{**} $P < 0.001$ versus the collagen-stimulated platelets, ^{††} $P < 0.001$ compared with the collagen-stimulated platelets in the presence of 500 μ M cordycepin.

ated *via* G_i -coupled receptor, and by contrast, the elevation of cAMP level achieved by cordycepin was induced by G_s -coupled receptor through adenylate cyclase activation. This is supported from report (Johnston-Cox et al., 2011) adenosine and the analogues increase cAMP level *via* adenosine receptor A_{2A} , which are coupled to G_s -coupled receptor. Cordycepin is an analogue of adenosine as 3'-deoxyadenosine. cGMP is known to be produced *via* the activation of guanylate cyclase in the presence or absence of nitric oxide (NO) synthesized in platelets (Pasqui et al., 1991). Because cordycepin did not produce NO in platelets (Cho et al., 2007), the elevation of cGMP achieved by cordycepin is independent on NO/guanylate cyclase pathway.

In addition, collagen-elevated $[Ca^{2+}]_i$ was potentially decreased by 500 μ M of cordycepin, and PKA activator pCPT-cAMP or PKG activator 8-Br-cGMP reduced collagen-elevated $[Ca^{2+}]_i$ (Fig. 3A). Because $[Ca^{2+}]_i$ level could be reduced through cAMP/PKA or cGMP/PKG downstream pathway, next, we investigated whether cordycepin is concerned with cAMP/PKA or cGMP/PKG downstream pathway to decrease $[Ca^{2+}]_i$ level. As shown in Fig. 3B, $[Ca^{2+}]_i$ level in the presence of both collagen and cordycepin was

217.2 $\pm$ 3.6 nM, which was significantly elevated by PKA inhibitor Rp-8-Br-cAMPS to 283.9 $\pm$ 8.2 nM (Fig. 3B). On the other hand, the $[Ca^{2+}]_i$ level in the presence of both collagen and cordycepin was not elevated by PKG inhibitor Rp-8-Br-cGMPS (Fig. 3B). Because $[Ca^{2+}]_i$ reduction could be resulted from cAMP/PKA-, or cGMP/PKG-phosphorylated IP_3RI (Ser¹⁷⁵⁶), next we checked whether cordycepin induces IP_3RI (Ser¹⁷⁵⁶) phosphorylation. As shown in Fig. 4 lane 3, and the ratio of phospho- IP_3RI (Ser¹⁷⁵⁶) [p- IP_3RI] to β -actin strongly increased in the presence of collagen and cordycepin. However, Rp-8-Br-cAMPS potently decreased the ratio of p- IP_3RI increased by both collagen and cordycepin (Fig. 4 lane 4), but not Rp-8-Br-cGMPS (Fig. 4 lane 5). In addition, the ratio of p- IP_3RI were increased in the presence of both collagen and 1 mM pCPT-cAMP as PKA activator, or 1 mM 8-Br-cGMP as PKG activator (Fig. 4 lane 6 and 7). Accordingly, cAMP/PKA-dependent IP_3RI (Ser¹⁷⁵⁶) phosphorylation exclusively contributed to the inhibition of $[Ca^{2+}]_i$ mobilization by cordycepin in collagen-activated platelets.

Another downstream pathway of cAMP/PKA or cGMP/PKG is VASP phosphorylation, which is concerned with

Fig. 4. Effects of cordycepin on inositol 1,4,5-trisphosphate receptor type I (IP₃RI) (Ser¹⁷⁵⁶) phosphorylation. The phosphorylation was determined as described in "Materials and Methods". These data were expressed as the mean $\pm$ SEM (n = 4), and ^a*P* < 0.05 compared with the non-stimulated platelets, ^{**}*P* < 0.001 compared with the collagen-stimulated platelets, ^{††}*P* < 0.001 compared with the collagen-stimulated platelets in the presence of 500 μM cordycepin.

α Ib/ β ₃ inactivation inhibiting platelet aggregation. The cAMP/PKA pathway phosphorylates Ser¹⁵⁷ of VASP, whereas the cGMP/PKG pathway phosphorylates Ser²³⁹ of VASP (Horstrup et al., 1994; Smolenski et al., 1998). Therefore, examination of VASP phosphorylation is a useful indicator for monitoring cAMP/PKA- or cGMP/PKG-pathway. As the results, collagen increased weakly phosphorylation of Ser¹⁵⁷ at 50 kDa of VASP [p-VASP (Ser¹⁵⁷)] (Fig. 5 lane 2). It is known that thrombin or collagen is concerned with a feedback inhibition by increasing p-VASP (Ser¹⁵⁷) (Gambaryan et al., 2010). A PKA activator pCPT-cAMP increased p-VASP (Ser¹⁵⁷) (Fig. 5 lane 6), which means that cAMP/PKA pathway results in phosphorylation of VASP (Ser¹⁵⁷). In addition, cordycepin increased the ratio of p-VASP (Ser¹⁵⁷) in collagen-induced platelet activation (Fig. 5 lane 3), and

Rp-8-Br-cAMPS decreased cordycepin-elevated p-VASP (Ser¹⁵⁷) (Fig. 5 lane 4). On the other hand, even if PKG activator 8-Br-cGMP increased p-VASP (Ser²³⁹) indicating the activation of PKG *via* cGMP (Fig. 5 lane 7), the p-VASP (Ser²³⁹) was not observed in the presence of both collagen and cordycepin (Fig. 5 lane 3). In addition, p-VASP (Ser²³⁹) was not changed by Rp-8-Br-cGMPS in the presence of cordycepin (Fig. 5 lane 5). Accordingly, the elevation of p-VASP (Ser¹⁵⁷) by cordycepin was obviously resulted from cAMP/PKA pathway in collagen-activated platelets. As the result, cordycepin phosphorylated VASP (Ser¹⁵⁷), but VASP (Ser²³⁹) is not. These can be explained by the previous report that a small elevation in cAMP is enough to activate most PKA potently, whereas even several fold elevation of the cGMP level may stimulate only a small fraction of total

Fig. 5. Effects of cordycepin on VASP phosphorylation. The phosphorylation was determined as described in "Methods". These data were expressed as the mean $\pm$ SEM ($n = 4$), and ^a $P < 0.05$ compared with the non-stimulated platelets, ^{**} $P < 0.001$ compared with the collagen-stimulated platelets, ^{††} $P < 0.001$ compared with the collagen-stimulated platelets in the presence of 500 µM cordycepin.

PKG (Eigenthaler et al., 1992). This report reflects that a little cAMP level is enough to phosphorylate VASP (Ser¹⁵⁷), but more cGMP level is required to phosphorylate VASP (Ser²³⁹). In this study, cordycepin increased cAMP level 1.9 fold more than cGMP level (Table 1). Accordingly, it is thought that cordycepin-elevated cGMP level is not enough to phosphorylate VASP (Ser²³⁹), which is similar that natriuretic peptides weakly induce cGMP-dependent phosphorylation of VASP (Ser²³⁹) (Borgogone et al., 2013), and PKA plays predominately to inhibit platelet aggregation (Li et al., 2003).

Phosphorylated VASP is known to inhibit fibrinogen binding to α IIB/ β_3 complex, which are concerned with inhibition of platelet activation (Horstrup et al., 1994; Barragan et al., 2003). Therefore, we examined whether the phosphorylation of VASP by cordycepin are associated with inhibition of fibrinogen binding to α IIB/ β_3 . As shown in Fig. 6, collagen activated fibrinogen binding to α IIB/ β_3 (Fig. 6A-b) compared with intact cell, basal (Fig. 6A-a), and increased fibrinogen binding to α IIB/ β_3 by $83.2 \pm 7.3\%$ as compared with intact platelets, basal (Fig. 6B). However, cordycepin inhibited collagen-activated fibrinogen binding to α IIB/ β_3

Fig. 6. Effect of cordycepin on fibrinogen binding. (A) The flow cytometry histograms on collagen-induced fibrinogen binding. a, Intact platelets; b, Collagen (10 µg/mL); c, Collagen (10 µg/mL) + cordycepin (500 µM); d, Collagen (10 µg/mL) + cordycepin (500 µM) + Rp-8-Br-cAMPS (250 µM); e, Collagen (10 µg/mL) + cordycepin (500 µM) + Rp-8-Br-cGMPS (250 µM); f, Collagen (10 µg/mL) + pCPT-cAMP (1 mM); g, Collagen (10 µg/mL) + 8-Br-cGMP (1 mM). (B) Effect of cordycepin on collagen-induced fibrinogen binding (%). The reactions were determined as described in "Methods". These data were expressed as the mean $\pm$ SEM (n = 4), and ^a*P* < 0.05 compared with non-stimulated platelets, ^{**}*P* < 0.001 compared with the collagen-stimulated platelets, [†]*P* < 0.05, ^{††}*P* < 0.001 compared with the collagen-stimulated platelets in the presence of 500 µM cordycepin.

by $14.5 \pm 1.2\%$ (Fig. 6B). The inhibition of $\alpha\text{IIb}/\beta_3$ is resulted from cAMP/PKA- or cGMP/PKG-mediated VASP phosphorylation, and it is known that cAMP- or cGMP-increasing compounds is associated with inhibition of $\alpha\text{IIb}/\beta_3$ (Horstrup et al., 1994; Hauser et al., 1999; Barragan et al., 2003), so that we examined whether the inhibition of fibrinogen binding to $\alpha\text{IIb}/\beta_3$ by cordycepin was contributed to cyclic-nucleotide of cAMP or cGMP. PKA activator pCPT-

cAMP or PKG activator 8-Br-cGMP inhibited collagen-stimulated fibrinogen binding to $\alpha\text{IIb}/\beta_3$ (Fig. 6A-f, g). It was confirmed that cAMP/PKA and cGMP/PKG pathway involve the inhibition of fibrinogen binding to $\alpha\text{IIb}/\beta_3$ in collagen-induced platelet activation. Cordycepin-inhibited fibrinogen binding to $\alpha\text{IIb}/\beta_3$ (Fig. 6A-c) was elevated by PKA inhibitor Rp-8-Br-cAMPS (Fig. 6A-d). However, cordycepin-inhibited fibrinogen binding to $\alpha\text{IIb}/\beta_3$ was very

slightly elevated by PKG inhibitor Rp-8-Br-cGMPS than Rp-8-Br-cAMPS (Fig. 6A-e). These results are in accord with that the inhibition of p-VASP (Ser¹⁵⁷) by Rp-8-Br-cAMPS was stronger than Rp-8-Br-cGMPS (Fig. 5). Therefore, it is inferred that cordycepin-mediated α Ib/ β ₃ inhibition was resulted from cAMP dependent-VASP (Ser¹⁵⁷) phosphorylation.

In conclusion, cordycepin contributed to cAMP-dependent phosphorylation of IP₃RI (Ser¹⁷⁵⁶) and VASP (Ser¹⁵⁷) to inhibit both [Ca²⁺]_i mobilization and fibrinogen binding to α Ib/ β ₃, which accompanied the inhibition of platelet activation. Antiplatelet agents such as thienopyridine derivatives (i.e. clopidogrel, ticlopidine) have characteristics that inhibit [Ca²⁺]_i mobilization, phosphorylate VASP and inhibit α Ib/ β ₃ activation, which is mediated by cAMP- or cGMP-dependent manner (Barragan et al., 2003). Therefore, we suggest that cordycepin might represent a useful agent in prevention and therapy of vascular diseases (i.e., thrombosis, myocardial infarction, atherosclerosis, and ischemic cerebrovascular disease) associated with platelet activation.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

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