Antiplatelet Effects of Cordycepin-Enriched WIB-801CE from *Cordyceps militaris*: Involvement of Thromboxane A₂, Serotonin, Cyclooxygenase-1, Thromboxane A₂ Synthase, Cytosolic Phospholipase A₂

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A species of the fungal genus *Cordyceps* has been used as an ingredient of traditional Chinese medicine. In this study, we prepared cordycepin-enriched WIB-801CE, an ethanol extract from culture solution of *Cordyceps militaris*-hypha, and evaluated its antiplatelet effects on human platelet aggregation. WIB-801CE dose-dependently inhibited ADP-, collagen-, and thrombin-induced platelet aggregation. These antiplatelet effects by WIB-801CE were associated with the attenuation of thromboxane A₂ (TXA₂) production and serotonin release by ADP, collagen, and thrombin. The inhibition of TXA₂ production by WIB-801CE was due to the inhibition of cyclooxygenase-1, TXA₂ synthase, and cytosolic phospholipase A₂ activity. Therefore, these data suggest that WIB-801CE may be a beneficial component against protection from platelet aggregation-mediated thrombotic disease.

Key Words: Cordycepin, WIB-801CE, TXA₂, Serotonin, TXAS, COX-1, cPLA₂

INTRODUCTION

Platelet aggregation by various agonists (i.e., collagen, thrombin, ADP, adrenaline) is absolutely required for the formation of a hemostatic plug when normal blood vessels are injured. However, this physiological reaction can also cause cardiovascular diseases such as thrombosis, atheroscle-

rosis and myocardial infarction (Schwartz et al., 1990). When various platelet agonists bind to their receptors (i.e., P₂Y, glycoprotein VI, protease-activated receptors) and activate platelets, membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) is hydrolyzed by phospholipase C_{γ2} and C_β to produce inositol 1,4,5-trisphosphate (IP₃), and diacylglycerol (DG) (Samuelsson et al., 1978; Berridge et al., 1984; Guidetti et al., 2008; Jennings, 2009). IP₃ binds to its receptor type I

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(IP₃RI) which then mobilizes cytosolic free Ca²⁺ ([Ca²⁺]_i) from the dense tubular systems (DTS). Increased [Ca²⁺]_i activates both the Ca²⁺/calmodulin-dependent phosphorylation of myosin light chain (20 kDa) and the DG-dependent phosphorylation of pleckstrin (47 kDa) to induce granule secretion (i.e., serotonin, ATP, ADP) and platelet aggregation (Nishikawa et al., 1980; Kaibuchi et al., 1982). DG is hydrolyzed by DG- and monoacylglycerol-lipase to produce arachidonic acid (AA), which is metabolized to TXA₂ via cyclooxygenase-1 (COX-1) and thromboxane A₂ synthase (TXAS). TXA₂ is known to intensify platelet aggregation and the formation of thrombus by binding to its receptors in resting platelets, and induces vasoconstriction as an autacoidal action (Hamberg et al., 1975; Samuelsson et al., 1978; Gesele et al., 1991; He and Yang, 1999). An important role in the agonist-induced platelet aggregation is played by aggregation-inducing molecules TXA₂ and granule secretion (i.e., serotonin, ATP) (Malmsten et al., 1975; Lewis and Watts, 1982; Li et al., 2010). Therefore, inhibition of TXA₂ production and granule secretion is very useful to evaluate an antiplatelet effect of any substance or compound. For instance, COX-1 inhibitor aspirin that inhibits TXA₂ production has used as antiplatelet drug (Patrono, 2001), which has a characteristics that secondarily prevents cardiovascular events, such as myocardial infarction, stroke and cardiovascular death (Tendera and Wojakowski, 2003).

A species of the fungal genus *Cordyceps* is an ingredient in the traditional Chinese medicine and is known for its anti-inflammatory and anti-cancerous properties (Cunningham et al., 1951; Ng and Wang, 2005). It is well established that cordycepin (3'-deoxyadenosine), a major component of *Cordyceps militaris*, attenuates [Ca²⁺]_i and TXA₂ production in collagen-induced human platelet aggregation (Cho et al., 2007) (Fig. 1). Recently, we reported that cordycepin-enriched n-butanol extract (WIB801C) from *Cordyceps militaris* has antiplatelet effects by inhibiting TXA₂ production and [Ca²⁺]_i mobilization in collagen-, and ADP-induced human platelet aggregation (Lee et al., 2014; Lee et al., 2015).

In this study, we prepared WIB-801CE (Compound from 2008 First Project of Biotechnology, Whanin Pharm. Co., Ltd., Suwon, Korea), an ethanol extract from culture solution

of *Cordyceps militaris*-hypha, and analyzed the composition of cordycepin with high performance liquid chromatography (HPLC). In present study, we observed that WIB-801CE contains enough cordycepin, and investigated whether WIB-801CE involves in attenuation of TXA₂ production and its associated COX-1 and TXAS activities, Ca²⁺-dependent cytosolic phospholipase A₂ (cPLA₂) activity, and serotonin release.

MATERIALS AND METHODS

Materials

WIB-801CE was provided from Whanin Pharmaceutical Corporation (Suwon, Korea). Thrombin, collagen and ADP were obtained from Chrono-Log Corporation (Havertown, PA., USA). Serotonin ELISA kit was purchased from Labor Diagnostika Nord GmbH & Corporation (Nordhorn, Germany). Cordycepin, protease inhibitor cocktail, and 9,11-dideoxy-9 α ,11 α -methanoepoxyprostaglandin F₂ α (U46619) were obtained from Sigma Chemical Corporation (St. Louis, MO., USA). TXB₂ enzyme immunoassay (EIA) kit, COX-1 fluorescent activity assay kit, and prostaglandin H₂ for TXAS assay were purchased from Cayman Chemical (Ann Arbor, MI., USA). Anti-phosphor-cPLA₂ 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 (Piscataway, NJ., USA). Enhanced chemiluminescence solution (ECL) was from GE Healthcare (Chalfont St., Giles, Buckinghamshire, UK).

Preparation of WIB-801CE

Cordyceps militaris was cultivated in Whanin Pharmaceutical Corporation (Suwon, Korea) and culture-solution of *Cordyceps militaris*-hypha was concentrated up to 50° Brix with a rotary vacuum evaporator (Eyela N3000, Rikakikai Co., Ltd., Tokyo, Japan) at 60°C. The Brix was measured with refractometer (Atago Co., Ltd., Tokyo, Japan). The concentrate was extracted by extraction-shaker (Cosmos 660, Kyungseo Co., Ltd., Seoul, Korea) for 4 h at 40°C one time with distilled water/95% ethanol (1 : 3.5, v/v), which was filtered one time 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 (60°C), and was lyophilized and stored at -20°C until used. This was named as cordycepin-enriched WIB-801CE (Compound from 2008 First Project of Biotechnology, Whanin Pharm. Co., Ltd., Suwon, Korea).

Analysis of cordycepin in WIB-801CE with HPLC

WIB-801CE was dissolved with 75% methanol, then analyzed by high performance liquid chromatography (HPLC). An Alliance 2695 liquid chromatography system (Milford, MA, USA), equipped with vacuum degasser, quaternary gradient pump, autosampler and photodiode array detector, was connected to Empower software. A hydrosphere C₁₈ column (250 mm×4.6 mm id, 5 µm, YMC) was used at a column temperature of 30°C. The applied-mobile phase gradient program was 0.01 M KH₂PO₄/methanol (95 : 5, v/v) at 0 min and held for 5 min; 0.01 M KH₂PO₄/methanol (70 : 30, v/v) at 20 min and held for 6 min; 0.01M KH₂PO₄/methanol (95 : 5, v/v) at 27 min and held 6 min for chromatographic balance. In this step, 99.8% of methanol was used. The flow rate was at 1.0 mL/min and sample injection volume was 10 µL. The UV detection was operated at 254 nm.

Preparation of human platelets

Human platelet-rich plasma (PRP) with acid-citrate-dextrose solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose) was supplied from Korean Red Cross Blood Center (Changwon, Korea). PRP was centrifuged for 10 min at 125 ×g to remove red blood cells and white cells, and was centrifuged for 10 min at 1,300 ×g to obtain platelet pellets. The platelets were washed twice with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.36 mM NaH₂PO₄, 5.5 mM glucose, and 1 mM Na₂EDTA, pH 6.5). The washed platelets were then resuspended in suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.36 mM NaH₂PO₄, 0.49 mM MgCl₂, 5.5 mM glucose, 0.25% gelatin, pH 6.9) to a final concentration of 5×10⁸/mL. Washed platelets were used to investigate the platelet aggregation by collagen and thrombin, PRP (5×10⁸/mL) was used to investigate the platelet aggregation by ADP and

U46619. 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/PIRB12-072-01) approved these experiments.

Measurement of platelet aggregation

To investigate the effect on human platelet aggregation, human washed platelets (10⁸/mL) were pre-incubated for 3 min at 37°C with or without substances in the presence of 2 mM CaCl₂, then stimulated with thrombin (0.025 U/mL) and collagen (5 µg/mL). ADP (20 µM)- and U46619 (10 µM)-induced platelet aggregation was measured with PRP, removed red blood cells and white cells, in the absence of 2 mM CaCl₂. After adding agonists, each aggregation was performed for 5 min using an aggregometer (Chrono-Log Corporation, Havertown, PA, USA) at a constant stirring speed of 1,000 rpm. Each aggregation rate was determined as an increase in light transmission. The suspension buffer and PPP were used as the reference (transmission 0). WIB-801CE was dissolved in platelet suspension buffer (pH 6.9). U46619 was dissolved in 0.01% dimethyl sulfoxide (DMSO). 0.01% DMSO did not affect the platelet aggregation (0.3 ± 0.6%).

Measurement of TXB₂

To investigate the effect of WIB-801CE on autacoidal aggregation-inducing molecule, TXA₂ production, the aggregation was terminated by adding ice-cold 5 mM EDTA and 0.2 mM indomethacin to inhibit subsequent conversion of AA to TXA₂. The amounts of TXB₂, a stable metabolite of TXA₂, were determined using a TXB₂ EIA kit according to the procedure described by the manufacturer.

Determination of serotonin release

To investigate the effect of WIB-801CE on autacoidal aggregation-inducing molecule, serotonin release, the aggregation was centrifuged at 4°C for 10 min at 200 ×g. The supernatants were used for the assay of serotonin release. Serotonin release was measured with a Synergy HT multi-Model Microplate Reader (BioTek Instruments, Winooski, VT, USA) using serotonin ELISA kit.

Preparation of platelet lysates

We prepared platelet lysates (homogenates) for measurement of COX-1 and TXAS. Non-stimulated platelets (10^8 /mL), and ADP-, collagen-, and thrombin-stimulated platelets (10^8 /mL) in presence of 1% protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO., USA) were sonicated 10 times at sensitivity 100% for 20 sec on ice with a model HD 2070 sonicator (Bandelin Electronic, Bandelin, Germany) to obtain platelet lysates. Next, the platelet lysates were centrifuged at $12,000 \times g$ for 15 min at 4°C to remove cell debris. The supernatant was used to measure COX-1 and TXAS activity.

Measurement of COX-1 activity

For measure COX-1 activity, platelet lysates containing 10 μg of protein were used. COX-1 activities were measured with Synergy HT multi-model microplate reader (BioTek Instruments, Winooski, VT., USA) using COX fluorescent activity assay kit according to the procedure described by manufacturer.

Measurement of TXAS activity

For measure TXAS activity, platelet lysates containing 20 μg of protein were used. The reaction for assay of TXAS activity was initiated by the addition of TXAS substrate prostaglandin H_2 (PGH_2) and allowed to proceed for 1 min at 37°C . The reaction was terminated by the addition of 1 M citric acid, then was neutralized with 1 N NaOH. The concentration of TXA_2 was determined as thromboxane B_2 (TXB_2), a stable metabolite of TXA_2 , which was measured with Synergy HT multi-model microplate reader (BioTek Instruments, Winooski, VT., USA) using TXB_2 EIA kit.

Western blot for analysis of cPLA₂ phosphorylation

The aggregation was 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 $\mu\text{g}/\text{mL}$ serine and cysteine protease

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

inhibitor leupeptin, and 1 mM serine protease and acetylcholinesterase inhibitor phenylmethanesulfonyl fluoride, pH 7.5). Platelet lysates were suspended in their equal volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer (62.5 mM Tris-HCl, 10% glycerol, 1% SDS, 1% β -mercaptoethanol, 0.01% bromphenol blue, pH 6.8), then were boiled to completely denature the proteins for 5 min. Aliquots containing 15 μg of protein from each sample tube were subjected to SDS-PAGE (8%, 1.5 mm gel).

Proteins in the gel were transferred to PVDF membrane in the presence of transfer buffer (25 mM Tris-HCl, 192 mM glycine, 20% methanol, pH 8.3). PVDF membrane was washed one time for 5 min with Tris-buffered saline with tween 20 (25 mM Tris-HCl, 140 mM NaCl, 2.7 mM KCl, 0.1% tween 20, pH 7.4), then was blocked with blocking buffer (25 mM Tris-HCl, 140 mM NaCl, 2.7 mM KCl, 0.1% tween 20, 5% skim milk, pH 7.4) for 1 h at room temperature, and subsequently was washed three times for 5 min. The protein phosphorylation was observed by using Western blotting. The dilutions for anti-phosphor-cPLA₂ and anti-rabbit IgG-HRP were 1:1,000 and 1:10,000, respectively. The membranes were visualized using ECL. Blots were analyzed by using the Quantity One, Ver. 4.5 (BioRad, Hercules, CA, USA).

Statistical analyses

The experimental results are indicated as the mean $\pm$ standard deviation accompanied by the number of observations. Data were determined by analysis of variance (ANOVA). If this analysis showed significant differences among the group means, then each group was compared by the Newman-Keuls method. Statistical analysis was carried out according to the SPSS 21.0.0.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

RESULTS

Composition of cordycepin in WIB-801CE

Cordyceps militaris, source of WIB-801CE, contains

cordycepin as detected by HPLC in our experiment (Fig. 1) (Huang et al., 2003). As shown in Fig. 2, peak 1 was observed at 19.988 min of the retention time (Fig. 2A), which was almost in accord with the retention time (19.980 min) of authentic cordycepin (Fig. 2B). The concentration of peak 1 in WIB-801CE corresponding to cordycepin was 69.30 ± 0.2 mg/g-WIB-801CE (approximately $6.93 \pm 0.02\%$, Table 1). Whole fruiting body myelia of *Cordyceps militaris* is known to contain 0.16% of cordycepin, and whole fruiting body, stroma, and larva of *Cordyceps sinensis* does not contain cordycepin (Yue et al., 2008). Therefore, the cordycepin content in WIB-801CE that we used in this study was much higher than those in whole fruiting body myelia of *Cordyceps militaris*, and in whole fruiting body, stroma, and larva of *Cordyceps sinensis*.

Fig. 2. HPLC chromatograms of WIB-801CE and authentic cordycepin. (A) The chromatogram of WIB-801CE. (B) The chromatogram of authentic cordycepin. HPLC was performed as described in "Materials and Methods".

Table 1. Content of cordycepin in WIB-801CE

	Retention time (min)	Area (mAU × s)	Concentration of sample (µg/mL)	Contents (mg/g-WIB-801CE)
Authentic cordycepin	19.980	2,949.35	100.3	–
WIB-801CE				
Peak 1	19.988	2,278.34	1,006	69.30 ± 0.20

The content of cordycepin in mg/g-WIB-801CE was expressed using the following equation : mg/g-WIB-801CE = (area of peak 1 / area of authentic cordycepin) × (concentration of authentic cordycepin / concentration of WIB-801CE) × (% of purity of authentic cordycepin / 100%) × (1,000 mg / 1 g). Purity of authentic cordycepin was 98% by manufactured. The data are given as the mean ± standard deviation (n=3).

Fig. 3. Effects of WIB-801CE on various agonists-induced platelet aggregation. (A) The concentration threshold of ADP on human platelet aggregation (inset). Effects of WIB-801CE on ADP-induced human platelet aggregation. (B) The concentration threshold of collagen on human platelet aggregation (inset). Effects of WIB-801CE on collagen-induced human platelet aggregation. (C) The concentration threshold of thrombin on human platelet aggregation (inset). Effects of WIB-801CE on thrombin-induced human platelet aggregation. The data are expressed as the mean ± standard deviation (n=4). * $P < 0.05$, ** $P < 0.001$ versus the each agonist-stimulated platelets. IC₅₀, half maximal inhibitory concentration.

Effects of WIB-801CE on ADP-, collagen-, and thrombin-induced human platelet aggregation

The concentration of ADP which induced maximal human platelet aggregation was approximately 10 µM (Fig. 3A, inset). But, as cordycepin and WIB801C, an analogue substance of WIB-801CE, inhibited 20 µM of ADP-induced

platelet aggregation (Lee et al., 2015) in this study 20 µM of ADP was used as a platelet agonist. When PRP (10⁸/mL) were activated with ADP (20 µM), the aggregation rate was increased to 67.7 ± 1.5%. However, various concentrations of WIB-801CE (50 to 400 µg/mL) significantly suppressed ADP-induced human platelet aggregation in a dose-dependent manner (Fig. 3A).

Fig. 4. Effects of WIB-801CE on TXB₂ production and U46619-induced platelet aggregation. (A) Effects of WIB-801CE on ADP-induced TXB₂ production. (B) Effects of WIB-801CE on collagen-induced TXB₂ production. (C) Effects of WIB-801CE on thrombin-induced TXB₂ production. (D) Effects of WIB-801CE on U46619-induced platelet aggregation. Measurement of TXB₂ production and platelet aggregation were carried out as described in "Materials and Methods". The data are expressed as the mean $\pm$ standard deviation (n=4). ^a*P*<0.05 versus the unstimulated platelets, **P*<0.05, ***P*<0.001 versus the each agonist-stimulated platelets. NS, not significant versus the unstimulated platelets. Δ (%) = [(WIB-801CE + agonist) - agonist] / agonist $\times$ 100.

The concentration of collagen-induced maximal human platelet aggregation was approximately 5 μ g/mL, and its degree was $83.3 \pm 3.1\%$ (Fig. 3B, inset). Therefore, 5 μ g/mL of collagen was used as a platelet agonist in this study. As shown in Fig. 3B, when washed human platelets (10^8 /mL) were activated with collagen (5 μ g/mL) in the presence of 2 mM CaCl₂, the aggregation rate was increased to $83.0 \pm 1.0\%$. However, WIB-801CE (50 to 300 μ g/mL)-dose dependently inhibited collagen-induced human platelet aggregation (Fig. 3B).

The concentration of thrombin-induced maximal human platelet aggregation was approximately 0.025 U/mL, and its degree was $91.7 \pm 3.2\%$ (Fig. 3C, inset). Therefore, 0.025 U/mL of thrombin was used as a platelet agonist in this study.

As shown in Fig. 3C, when washed human platelets (10^8 /mL) were stimulated with thrombin (0.025 U/mL) in the presence of 2 mM CaCl₂, the aggregation rate was increased to $92.3 \pm 1.0\%$. However, WIB-801CE (50 to 400 μ g/mL)-dose dependently attenuated thrombin-induced human platelet aggregation (Fig. 3C).

Effects of WIB-801CE on TXA₂ production, and its analogue U46619-induced platelet aggregation

We, next, investigated whether the inhibition of ADP-, collagen-, and thrombin-induced human platelet aggregation by WIB-801CE resulted from the reduction of TXA₂ production. The TXA₂ level (determined as TXB₂) in resting platelets was 1.4 ± 0.2 ng/10⁸ platelets, and ADP (20 μ M)

potently increased TXA₂ level to 24.5 ± 1.6 ng/10⁸ platelets (Fig. 4A). This suggests that ADP increased TXA₂ production to 1,650% as compared with that (1.4 ± 0.2 ng/10⁸ platelets) by resting platelets. But, WIB-801CE alone (300, 400 µg/mL) did not affect the TXA₂ production (Fig. 4A) in resting platelets. WIB-801CE dose (100 to 400 µg/mL)-dependently reduced TXA₂ production in ADP (20 µM)-activated platelets (Fig. 4A). 150 µg/mL of WIB-801CE, IC₅₀ to ADP-induced platelet aggregation (Fig. 3A), attenuated ADP-elevated TXA₂ level (24.5 ± 1.6 ng/10⁸ platelets) to 14.9 ± 1.2 ng/10⁸ platelets (39.2%) (Fig. 4A).

Collagen (5 µg/mL) strongly increased TXA₂ level to 120.9 ± 7.1 ng/10⁸ platelets (Fig. 4B). This suggest that collagen increased TXA₂ production to 8,537.7% as compared with that (1.4 ± 0.2 ng/10⁸ platelets) by resting platelets. WIB-801CE alone (100, 150 µg/mL) did not affect the TXA₂ production in resting platelets, but WIB-801CE dose (50 to 150 µg/mL)-dependently inhibited TXA₂ production in collagen (5 µg/mL)-activated platelets (Fig. 4B). 100 µg/mL of WIB-801CE, IC₅₀ to collagen-induced platelet aggregation (Fig. 3B), reduced collagen-elevated TXA₂ level (120.9 ± 7.1 ng/10⁸ platelets) to 63.2 ± 3.4 ng/10⁸ platelets (47.7%) (Fig. 4B).

Thrombin (0.025 U/mL) potently elevated TXA₂ level to 47.5 ± 0.8 ng/10⁸ platelets (Fig. 4C). This suggest that thrombin increased TXA₂ production to 3,292.8% as compared with that (1.4 ± 0.2 ng/10⁸ platelets) by resting platelets. WIB-801CE alone (300, 400 µg/mL) did not affect the TXA₂ production in resting platelets, however, WIB-801CE attenuated TXA₂ level in thrombin (0.025 U/mL)-activated platelets in a dose (100 to 400 µg/mL)-dependent manner (Fig. 4C). 200 µg/mL of WIB-801CE, IC₅₀ to thrombin-induced platelet aggregation (Fig. 3C), decreased thrombin-increased TXA₂ level (47.5 ± 0.8 ng/10⁸ platelets) to 41.1 ± 1.7 ng/10⁸ platelets (13.5%) (Fig. 4C).

TXA₂ is produced by agonist-activated platelets and subsequently acts as a positive feedback promotor on resting platelets, which is connected to the intensification of thrombus (Halushka, 1995). This means that TXA₂ is a strong agonist against resting platelets and TXA₂ antagonistic substance or compound may be used beneficially as an anti-thrombotic agent. Therefore, we investigated whether WIB-

Fig. 5. Effects of WIB-801CE on COX-1 activity. (A) Effects of WIB-801CE on ADP-induced COX-1 activity. (B) Effects of WIB-801CE on collagen-induced COX-1 activity. (C) Effects of WIB-801CE on thrombin-induced COX-1 activity. Measurements of COX-1 activity was carried out as described in "Materials and Methods". The data are expressed as the mean $\pm$ standard deviation (n=4). ^aP<0.05 versus the basal. NS, not significant versus each agonist-stimulated human platelets, *P<0.05 versus each agonist-stimulated human platelets. Δ (%) = [(ADP-WIB-801CE) / (ADP-basal)] $\times$ 100.

801CE, inhibiting TXA₂ production, inhibits TXA₂ mimetic compound U46619-induced platelet aggregation. As shown in Fig. 4D, the concentration of U46619-induced maximal

platelet aggregation was 10 μ M (Fig. 4D, inset). WIB-801CE dose (12.5 to 300 μ g/mL)-dependently inhibited U46619 (10 μ M)-induced human platelet aggregation (Fig. 4D), and its inhibitory dose is very low as compared with doses (50 to 400 μ g/mL) that attenuated ADP-, collagen-, and thrombin-induced human platelet aggregation (Fig. 3A-C).

Effects of WIB-801CE on COX-1 activity

We investigated whether the inhibition of TXA₂ production by WIB-801CE resulted from the inhibition of COX-1 activity. ADP (20 μ M) increased COX-1 activity from basal activity (0.24 ± 0.02 nmoL/protein-mg/min) to 0.36 ± 0.04 nmoL/protein-mg/min (Fig. 5A). In contrast, WIB-801CE dose (100 to 400 μ g/mL)-dependently inhibited ADP-induced COX-1 activity (Fig. 5A). 150 μ g/mL of WIB-801CE, IC₅₀ to ADP-induced platelet aggregation (Fig. 3A), attenuated ADP-induced COX-1 activity (100%) to 33.3% (Fig. 5A). 400 μ g/mL of WIB-801CE decreased ADP-induced COX-1 activity (0.36 ± 0.04 nmoL/protein-mg/min) to 0.24 ± 0.04 nmoL/protein-mg/min (100%) (Fig. 5A).

Collagen (5 μ g/mL) and thrombin (0.025 U/mL) increased COX-1 activity from basal activity (0.24 ± 0.02 nmoL/protein-mg/min) to 0.71 ± 0.02 nmoL/protein-mg/min, and 0.80 ± 0.06 nmoL/protein-mg/min, respectively (Fig. 5B, C). However, WIB-801CE did not inhibit collagen-, and thrombin-induced COX-1 activity (Fig. 5B, C). These results suggest that WIB-801CE inhibited ADP-induced COX-1 activity to reduce TXA₂ production but did not attenuate collagen- and thrombin-induced COX-1 activity.

Effects of WIB-801CE on TXAS activity

We investigated whether the inhibition of TXA₂ production by WIB-801CE was due to the attenuation of TXAS activity. Collagen (5 μ g/mL) elevated TXAS activity from basal activity (264.90 ± 1.88 ng/protein-mg/min) to 309.80 ± 5.01 ng/protein-mg/min (Fig. 6A). However, WIB-801CE dose (50 to 150 μ g/mL)-dependently attenuated collagen-induced TXAS activity (Fig. 6A). 100 μ g/mL of WIB-801CE, IC₅₀ to collagen-induced platelet aggregation (Fig. 3B), decreased collagen-elevated TXAS activity (309.80 ± 5.01 ng/protein-mg/min) to 278.3 ± 5.5 ng/protein-mg/

Fig. 6. Effects of WIB-801CE on TXAS activity. (A) Effects of WIB-801CE on collagen-induced TXAS activity. (B) Effects of WIB-801CE on ADP-induced TXAS activity. (C) Effects of WIB-801CE on thrombin-induced TXAS activity. Measurements of TXAS activity was carried out as described in "Materials and Methods". The data are expressed as the mean $\pm$ standard deviation (n=4). ^a P <0.05 versus the basal. NS, not significant versus each agonist-stimulated human platelets, ^{*} P <0.05 versus each agonist-stimulated human platelets. Δ (%) = [(collagen-WIB-801CE) / (collagen-basal)] $\times$ 100.

min (70.2%) (Fig. 6A).

ADP (20 μ M) increased TXAS activity from basal activity

Fig. 7. Effects of WIB-801CE on serotonin release. (A) Effects of WIB-801CE on ADP-induced serotonin release. (B) Effects of WIB-801CE on collagen-induced serotonin release. (C) Effects of WIB-801CE on thrombin-induced serotonin release. The data are expressed as the mean $\pm$ standard deviation ($n=4$). ^a $P<0.05$ versus the unstimulated platelets, NS, not significant versus the unstimulated platelets. * $P<0.05$, ** $P<0.001$ versus the each agonist-stimulated platelets. Δ (%) = [Agonist- (agonist + WIB-801CE)] / agonist $\times$ 100.

(264.90 $\pm$ 1.88 ng/protein-mg/min) to 297.40 $\pm$ 2.54 ng/protein-mg/min (Fig. 6B), which suggests that ADP (20 μ M)

elevated TXAS activity to 12.3%. But WIB-801CE did not inhibit ADP-induced TXAS activity (Fig. 6B). In addition, thrombin (0.025 U/mL) increased very weakly TXAS activity from basal (264.90 $\pm$ 1.88 ng/protein-mg/min) to 275.30 $\pm$ 0.30 ng/protein-mg/min, which was not inhibited by WIB-801CE (Fig. 6C). The above results suggest that WIB-801CE attenuated collagen-induced TXAS activity to inhibit TXA₂ production, but did not inhibit ADP- and thrombin-induced TXAS activity.

Effects of WIB-801CE on serotonin release

Platelets do not synthesize neurotransmitter serotonin, which is incorporated by platelets and stored in dense body of platelets (Fraer and Kilic, 2015). Serotonin is released out of dense body when platelets are aggregated by various agonists (i.e., ADP, collagen, thrombin), and subsequently intensify platelet aggregation to generate the thrombosis (Moerland et al., 2011; Fraer and Kilic, 2015). Therefore, we investigate the effect of WIB-801CE on agonist-released serotonin. WIB-801CE alone (100 to 400 μ g/mL) did not release serotonin out of resting platelets (Fig. 7A-C). This means that WIB-801CE alone did not affect serotonin release, a marker of platelet activation, out of resting platelets. ADP (20 μ M), collagen (5 μ g/mL), and thrombin (0.025 U/mL) released serotonin 207.6 $\pm$ 8.1 ng/10⁸ platelets (Fig. 7A), 154.9 $\pm$ 9.5 ng/10⁸ platelets (Fig. 7B), and 384.4 $\pm$ 4.9 ng/10⁸ platelets (Fig. 7C), respectively. However, 150 μ g/mL of WIB-801CE, IC₅₀ to ADP-induced platelet aggregation (Fig. 3A), attenuated ADP-induced serotonin release (207.6 $\pm$ 8.1 ng/10⁸ platelets) to 100.9 $\pm$ 1.4 ng/10⁸ platelets (51.4%) (Fig. 7A). 100 μ g/mL of WIB-801CE, IC₅₀ to collagen-induced platelet aggregation (Fig. 3B), reduced collagen-elevated serotonin release (154.9 $\pm$ 9.5 ng/10⁸ platelets) to 63.6 $\pm$ 9.5 ng/10⁸ platelets (58.9%) (Fig. 7B). 200 μ g/mL of WIB-801CE, IC₅₀ to thrombin-induced platelet aggregation (Fig. 3C), diminished thrombin-elevated serotonin release (384.4 $\pm$ 4.9 ng/10⁸ platelets) to 275.0 $\pm$ 1.3 ng/10⁸ platelets (28.5%) (Fig. 7C).

Effects of WIB-801CE on cPLA₂ activity

cPLA₂ is Ca²⁺-dependently activated, and subsequently hydrolyzed membrane phospholipids (i.e., phosphatidyl-

Fig. 8. Effects of WIB-801CE on cPLA₂ phosphorylation. (A) Effects of WIB-801CE on ADP-induced cPLA₂ phosphorylation. (B) Effects of WIB-801CE on collagen-induced cPLA₂ phosphorylation. (C) Effects of WIB-801CE on thrombin-induced cPLA₂ phosphorylation. cPLA₂ phosphorylation was determined as described in "Materials and Methods" section. The data are expressed as the mean $\pm$ standard deviation (n=4). * P <0.05, ** P <0.001 versus the each agonist-stimulated platelets.

choline) to release AA, precursor of TXA₂. Therefore, we investigated whether WIB-801CE inhibits cPLA₂ activity to attenuate the production of TXA₂ (Fig. 4). cPLA₂ activity is determined by its phosphorylation status. ADP (20 μM), collagen (5 μg/mL), and thrombin (0.025 U/mL) potently stimulated cPLA₂ activity, respectively (Fig. 8). WIB-801CE, however, inhibited these agonist-induced cPLA₂ activities in a dose-dependent manner (Fig. 8). Especially, WIB-801CE without having inhibitory effect on COX-1 (Fig. 5C) and TXAS (Fig. 6C) activities attenuated cPLA₂ activity in thrombin-induced platelet aggregation (Fig. 8C).

DISCUSSION

Platelet aggregation is a marker of platelet activation which is controlled by various agonists (i.e., ADP, collagen, thrombin)-produced TXA₂. Therefore, to observe the inhibition of agonist-induced TXA₂ production it is important to evaluate the antiplatelet effect of any substance or compound (Schwartz et al., 1990; Duguid, 1946; Cahill and Newland, 1993; Grau et al., 1998). WIB-801CE potently attenuated TXA₂ production to inhibit ADP-, collagen- and thrombin-induced platelet aggregation. Especially, ADP is an autacoidal platelet agonist that is released by thrombin- and collagen-stimulation. Since WIB-801CE inhibits ADP-induced platelet aggregation and TXA₂ production it may be considered as a beneficial antiplatelet agent. TXA₂ precursor AA is produced by phospholipase C (PLC) or cPLA₂, and subsequently metabolized to TXA₂ by COX-1 and TXAS (Hamberg et al., 1975; Samuelsson et al., 1978; Gresle et al., 1991). COX-1 produces PGG₂ from AA, which is oxidized to PGH₂ by endoperoxidase. TXAS produces finally TXA₂ from PGH₂. Accordingly, it is considered that WIB-801CE inhibited TXA₂ production by protecting the use of AA by COX-1 in ADP-activated platelets, and PGH₂ by TXAS in collagen-activated platelets. In addition, there is the possibility that WIB-801CE diminishes Ca²⁺-dependent cPLA₂ activity to reduce AA supply in ADP-, collagen- and thrombin-activated platelets. Accordingly, WIB-801CE seems to inhibit the activities of COX-1, TXAS, and cPLA₂ to attenuate TXA₂ production in ADP- and collagen-activated platelets. Although WIB-801CE did not inhibit COX-1 and TXAS

activities, it attenuated TXA₂ production and cPLA₂ activity in thrombin-activated platelets. These results mean that WIB-801CE attenuated the production of TXA₂ by inhibiting the supply of AA *via* inhibition of cPLA₂ activity in thrombin-activated platelets.

With regard to PLC activity, it has been shown by our previous report that cordycepin does not inhibit PLC γ ₂ activity, and IP₃ production by PLC γ ₂ in collagen-induced human platelet aggregation (Cho et al., 2007). In this study, because we did not measure PLC activity, it is unknown whether WIB-801CE inhibits PLC activity to attenuate the supply of AA. This should be investigated in the future. Various agonist-elevated [Ca²⁺]_i also involves in activation of Ca²⁺/calmodulin-dependent myosin light chain kinase (MLCK) and integrin glycoprotein IIb/IIIa (α IIb/ β ₃) to activate platelets. The release of granule compounds is an index of platelet activation, and is generated by MLCK-phosphorylated MLC (20 kDa) (Nishikawa et al., 1980; Kaibuchi et al., 1982). It is well established that cordycepin in WIB-801CE attenuates [Ca²⁺]_i, and subsequently inhibits the phosphorylation of 20 kDa by Ca²⁺-dependent MLCK in collagen-, and U46619-activated human platelets (Cho et al., 2006; Cho et al., 2007). WIB-801CE also inhibited ADP-, collagen- and thrombin-elevated [Ca²⁺]_i (data not shown). Therefore, the decrease of [Ca²⁺]_i by WIB-801CE and cordycepin is resulted in the inhibition of ADP-, collagen-, and thrombin-induced serotonin release.

Because agonist-produced TXA₂ itself binds to its receptor as autacoidal agonist, and subsequently elevates [Ca²⁺]_i to stimulate the granule secretion (Halushka et al, 1995), the inhibition of U46619-induced human platelet aggregation by WIB-801CE might be also involved in attenuation of serotonin and [Ca²⁺]_i as evidenced that cordycepin in WIB-801CE attenuated U46619-elevated [Ca²⁺]_i and Ca²⁺-dependent 20 kDa phosphorylation (Cho et al., 2006). Because WIB-801CE contains cordycepin as well as other unknown compounds, it is unknown whether cordycepin is the key ingredient in WIB-801CE that contributes to the inhibition of platelet aggregation. Recently, we showed that cordycepin purified from butanol extracts (BE) of *Cordyceps militaris* has antiplatelet effect (Lee et al., 2015a; Lee et al., 2015b), and it has a synergistic inhibitory effect with BE. As shown

in Table 1, because WIB-801CE contains cordycepin, it is thought cordycepin in WIB-801CE might contribute to the antiplatelet effect of WIB-801CE.

In conclusion, cordycepin-enriched WIB-801CE, an ethanol extract from *Cordyceps militaris*, inhibited thrombosis-generation molecules (i.e. TXA₂, serotonin) increased by platelet aggregation. Therefore, we suggest that WIB-801CE and cordycepin might be considered a beneficial and effective agent for the treatment or protection from thrombosis, atherosclerosis, and myocardial infarction *via* inhibition of platelet aggregation.

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Conflict of interest

The authors declare no conflict of interest.

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