Biomedical Science Letters 2015, 21(2): 103~114
http://dx.doi.org/10.15616/BSL.2015.21.2.103
eISSN : 2288-7415

Original Article

Inhibitory Effects of Rice Bran Water Extract Fermented *Lactobacillus plantarum* due to cAMP-dependent Phosphorylation of VASP (Ser^{157}) on human Platelet Aggregation

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In this study, we investigated the effect of rice bran water extract fermented with *Lactobacillus plantarum* KCCM-12116 (RBLp) on ADP (20 μM)-, collagen (10 μg/mL)-, and thrombin (0.2 U/mL)-stimulated platelet aggregation. RBLp dose-dependently inhibited ADP-, collagen-, and thrombin-induced platelet aggregation, with IC_{50} values of 501.1, 637.2, and > 2,000 μg/mL, respectively. The platelet aggregation induced by ADP plus RBLp (750 μg/mL) was increased by the adenylate cyclase inhibitor, SQ22536, and the cAMP-dependent protein kinase (A-kinase) inhibitor, Rp-8-Br-cAMPS. Treatment with RBLp increased the phosphorylation of VASP (Ser^{157}), an A-kinase substrate, which was also inhibited by SQ22536 and Rp-8-Br-cAMPS. It is thought that the RBLp-induced increases in cAMP contributed to the phosphorylation of VASP (Ser^{157}), which in turn resulted in an inhibition of ADP-induced platelet aggregation, thereby indicating that RBLp has an antiplatelet effect *via* cAMP-dependent phosphorylation of VASP (Ser^{157}). Thus, RBLp may have therapeutic potential for the treatment (or prevention) of platelet aggregation-mediated diseases, such as thrombosis, myocardial infarction, atherosclerosis, and ischemic cerebrovascular disease.

Key Words: cAMP, cAMP-dependent protein kinase, Platelet aggregation, Rice bran fermentation, Vasodilator-stimulated phosphoprotein-Ser^{157} phosphorylation

INTRODUCTION

Platelet aggregation is absolutely essential for the formation of a hemostatic plug when normal blood vessels are injured. However, it can also cause cardiovascular diseases, such as thrombosis, atherosclerosis and myocardial infarction (Schwartz et al., 1990). When platelets are stimulated by agonists, such as adenosine diphosphate (ADP), thrombin and TXA_2, phosphatidylinositol 4,5-bisphosphate is hydrolyzed by phospholipase C-β *via* a G-protein-coupled receptor to generate inositol 1,4,5-trisphosphate and diacylglycerol (Guidetti et al., 2008; Berridge et al., 1984; Jennings, 2009). It is particularly well-known that ADP binds to the G_q-coupled $P2Y_1$ receptor, which mediates PLC-β, and the G_i-coupled $P2Y_{12}$ receptor, which mediates inhibition of

*Received: April 22, 2015 / Revised: June 15, 2015
Accepted: June 22, 2015
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adenylate cyclase, thereby amplifying platelet aggregation (Cattaneo, 2005).

Intracellular cyclic adenosine monophosphate (cAMP), as an antiplatelet regulator, decreases $[Ca^{2+}]_i$ mobilization (Menshikov et al., 1993; Schwarz et al., 2001). The antiplatelet effects of cAMP are mediated *via* cAMP-dependent protein kinases (A-kinases), which phosphorylate substrate proteins, such as vasodilator-stimulated phosphoprotein (VASP) (Halbrügge et al., 1989; Halbrügge et al., 1990; Butt et al., 1994). VASP (Ser^{157} and Ser^{239}) phosphorylation involves in inhibition of VASP's affinity for the contractile protein, filamentous actin, as well as an inhibition of fibrinogen binding to glycoprotein IIb/IIIa ($\alpha IIb/\beta_3$), which inhibits platelet aggregation (Laurent et al., 1999; Sudo et al., 2003). Therefore, measuring increases in the levels of cAMP or phosphorylated VASP is a very useful method for evaluating the antiplatelet effects of substances or compounds. For instance, a major catechin analogue, (−)-epigallocatechin-3-gallate (EGCG) from green tea, is known to produce cAMP *via* adenylate cyclase activation, which subsequently phosphorylates VASP-Ser^{157} through A-kinase activation to inhibit platelet aggregation (Ok et al., 2012). Furthermore, verapamil and theophylline exert antiplatelet functions by elevating cAMP levels (Gasser and Betteridge, 1991), and are therefore currently used as antiplatelet agents to prevent and/or treat cardiovascular diseases (Menshikov et al., 1993).

Rice bran is produced as a by-product in the rice milling process by a method in which the outer layer of the rice grain is removed. Rice bran has various biological effects, including anti-inflammatory, cholesterol-lowering, antioxidant and anti-diabetic activities (Qureshi et al., 2002; Jun et al., 2012; Hou et al., 2010). Recently, it has been reported that a water extract from rice bran has a neuroprotective effect in ischemic brain injury (Baek et al., 2014). In the present study, we investigated the effect of RBLp on VASP phosphorylation in ADP-induced platelet aggregation and evaluated its anti-platelet effects.

MATERIALS AND METHODS

Materials

ADP, collagen and thrombin were purchased from Chrono-Log Co. (Havertown, PA, USA). cAMP enzyme immunoassay (EIA) kit and LDH cytotoxicity assay kit were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). SQ22536, Rp-8-Br-cAMPS and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Anti-phosphor-VASP (Ser^{157}), anti-rabbit IgG-horseradish peroxidase conjugate (HRP), and lysis buffer were obtained from Cell Signaling (Beverly, MA, USA). Polyvinylidene difluoride (PVDF) membranes were purchased from GE Healthcare (Piscataway, NJ, USA). Enhanced chemiluminesence (ECL) solution was purchased from GE Healthcare (Chalfont St, Giles, Buckinghamshire, UK).

Preparation of rice bran water extract fermented with *Lactobacillus plantarum* (RBLp)

RBLp was prepared according to the following method: *Lactobacillus plantarum* KCCM-12116 was inoculated and cultured in MRS medium at 35℃ with shaking at 120 rpm; a 12 hour incubated culture broth was used as a starter. Rice bran was obtained from the Gimhae Rice Processing Complex just after milling the rice cultivar of Samkwang (Gimhae, Korea). Rice bran was pretreated with autoclaving immediately upon receipt. After cooling to room temperature, the rice bran material was vacuum-packed and stored in the refrigerator until further use. Fermentation was performed as follows: a rice bran suspension (1:9) was inoculated with the starter at a ratio of 5% and fermented at 35℃. Rice bran was fermented using the starter under the conditions of 35℃, with shaking at 120 rpm for 36 hours. The culture broth was autoclaved at 121.5℃ for 15 min to inactivate the bacteria and then centrifuged at 8,000 ×g for 10 min. The supernatant was filtered and concentrated to 54 Brix by vacuum evaporation. The resultant concentrate was designated as RBLp and kept in a refrigerator (4℃) until further use. For experiments to investigate the effects on platelet aggregation, RBLp was dissolved in saline (0.9% NaCl).

Determination of total phenolic content of RBLp

Total phenolic content was determined by using a modification of Singleton's method (Singleton and Rossi, 1965). RBLp was dissolved in 50% MeOH at a concentration of 0.1% (w/v). An aliquot of 0.2 mL of the dissolved sample

was reacted with 1.0 mL of 10% Folin-Ciocalteu reagent for 4 min at room temperature, and then 0.8 mL of a saturated sodium carbonate solution (approximately 75 g/L) was added to the reaction mixture. After incubation at room temperature for 30 min, the mixture was centrifuged at 3,000 rpm and the supernatant was removed. The absorbance readings of the supernatants were taken at 765 nm. Gallic acid was used as a reference standard, and the results are expressed as milligrams of gallic acid equivalents (mg gallic acid)/100 g RBLp.

Detection of phenolic compounds of RBLp by HPLC

Because it has been reported that rice bran contains phenolic compounds, we tested for the presence of phenol compounds in RBLp using high performance liquid chromatography (HPLC) (Goufo and Trindade, 2014). After initially dissolving the RBLp in distilled water (100 mg/mL), the pH of the dissolved sample was adjusted to 2~3 with 2 N HCl. The sample was extracted three times with 0.5 mL ethylacetate, concentrated by vacuum rotary evaporation until dry, dissolved with 0.5 mL methanol, and then analyzed by HPLC. An Agilent 1100 liquid chromatography system (Palo Alto, CA, USA) equipped with a vacuum degasser, a quaternary gradient pump, an autosampler and a diode array detector (DAD) was used, which was controlled by Agilent ChemStation software. A TSKgel ODS-100V column (150 × 4.6 mm id, 5 μm, Tosoh, Japan) was used at a temperature of 40℃. The mobile phase consisted of methanol (A) and 50 mM NaH_2PO_4, pH 2.5 (adjusted with phosphoric acid) (B). The following program was used: 0~20 min, 30% A and 70% B, with a flow rate of 1.0 mL/min and a sample injection volume of 5 μL. UV detection was operated at 310 nm. A calibration curve was constructed by injecting seven concentrations (12.5, 25, 50, 62.5, 125, 250, 500 μg/mL) of authentic ferulic acid in duplicate, and then plotting the peak areas against the concentrations of each analyte.

Preparation of washed human platelets

Human platelet-rich plasma (PRP) anti-coagulated with an acid-citrate-dextrose solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose) were obtained from the Korean Red Cross Blood Center (Changwon, Korea). PRP was centrifuged for 10 min at 125 ×g to remove red blood cells, and then 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℃ to avoid platelet aggregation resulting from any effect of low temperatures. The Korea National Institute for Bioethics Policy Public Institutional Review Board (Seoul, Korea) approved these experiments (PIRB12-071).

Measurement of platelet aggregation

Washed platelets (10^8/mL) were preincubated for 3 min at 37℃ in the presence of 2 mM $CaCl_2$ with or without substances, then stimulated with ADP (20 μM) or collagen (10 μg/mL) or thrombin (0.2 U/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 a reference (transmission 0%). RBLp was dissolved in saline (0.9% NaCl).

Lactate dehydrogenase (LDH) cytotoxicity assay

To assess whether RBLp has cytotoxicity, we examined the effect of RBLp on LDH release *in vitro*, a stable enzyme normally found in the cytosol of cells, but releases into the supernatant upon damage of cell membrane. Washed platelets (10^8/mL) were incubated 5 min at 37℃ with RBLp (500, 750 μg/mL), and then supernatant was measured with a synergy HT multi-model microplate reader (BioTek Instruments, Winooski, VT, USA) using a LDH cytotoxicity assay kit.

Measurement of cAMP

Washed platelets (10^8/mL) were preincubated for 3 min at 37℃ with or without substances in the presence of 2 mM $CaCl_2$, and then stimulated with ADP (20 μM) for 5 min to

induce platelet aggregation. The aggregation was terminated by the addition of 80% ice-cold ethanol. cAMP was measured with a synergy HT multi-model microplate reader (BioTek Instruments, Winooski, VT, USA) after using a cAMP EIA kit.

Western blot analysis for VASP-phosphorylation

Washed platelets (10^8/mL) were preincubated with or without substances in the presence of 2 mM $CaCl_2$ for 3 min and then stimulated with ADP (20 μM) for 5 min at 37℃. 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, pH 7.5), supplemented with 2.5 mM sodium pyrophosphate, 1 mM of the serine/threonine phosphatase inhibitor, β-glycerophosphate, 1 mM ATPase, alkaline and acid phosphatase, the protein phosphotyrosine phosphatase inhibitor, Na_3VO_4, 1 μg/mL of the serine and cysteine protease inhibitor, leupeptin, and 1 mM of the serine protease and acetylcholinesterase inhibitor, phenylmethanesulfonyl fluoride. Platelet lysates containing the same protein (15 μg) were used for analysis. Protein concentrations were measured by using the bicinchoninic acid protein assay kit (Pierce Biotechnology, USA). The effects of substances on VASP phosphorylation were analyzed by Western blotting. An 8~10% SDS-PAGE gel was used for electrophoresis and a

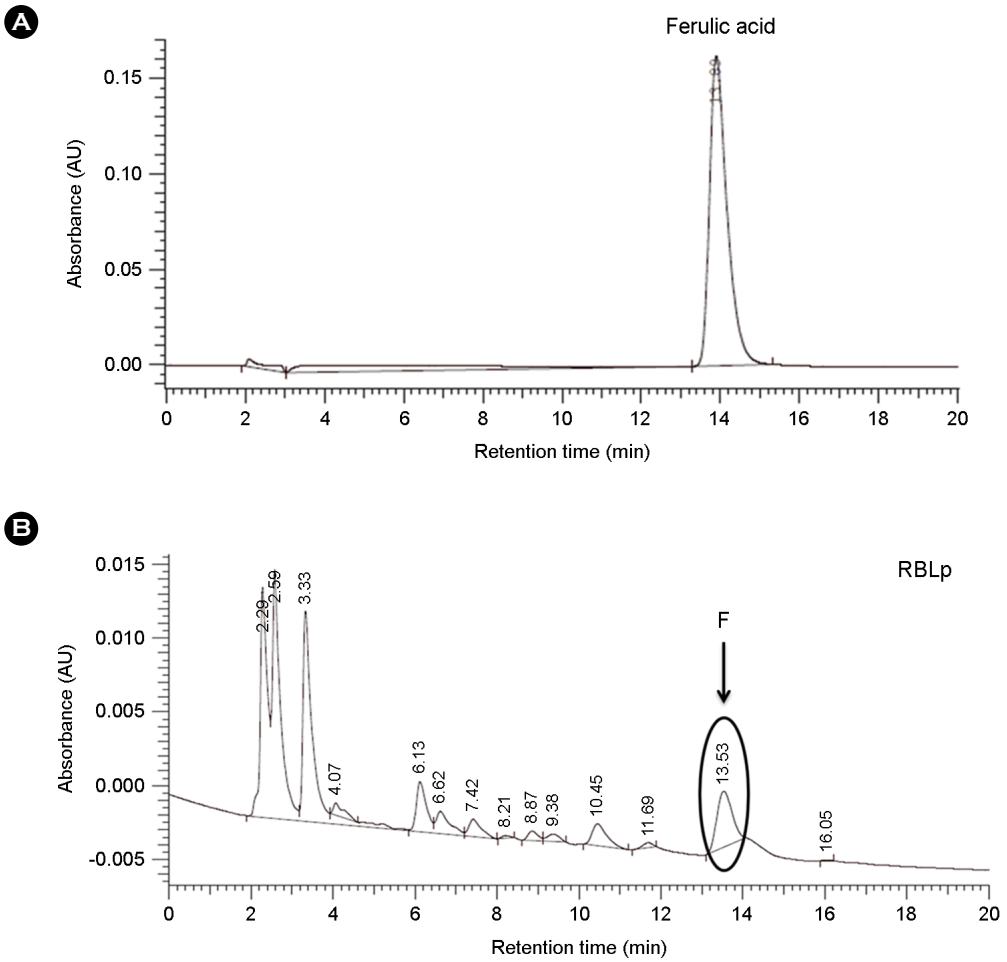

Fig. 1. HPLC chromatograms of RBLp and ferulic acid. (A) The chromatogram of ferulic acid. (B) The chromatogram of RBLp. HPLC was performed as described in "Materials and Methods".

Table 1. Total phenolic acid content of RBLp

	Total phenolic (TP) content (mg/100 g RBLp)	Ferulic acid (FA) content (mg/100 g RBLp)	FA/TP (%)
RBLp	37.5 ± 0.6	18.4	49.1

Table 2. Calibration curve and content of ferulic acid in RBLp

		RT (min)	Calibration curve[a)]	r^2	Test range (μg/mL)	x[b)] (μg/mL)	Content (mg/100 g-RBLp)
Authentic compound	Ferulic acid	13.89	$y = 0.0002x + 9.3815$	0.9993	12.5~500	−	−
RBLp	Peak F	13.53	−	−	−	18.4	18.4

[a, b)] y, peak areas of analytes ; x, concentrations of analytes in 100 mg/mL RBLp.

PVDF membrane was used for protein transfer from the gel. The dilutions for anti-phosphor-VASP (Ser^{157}) and anti-rabbit IgG-HRP were 1:1,000 and 1:10,000, respectively. The membranes were visualized using ECL. Blots were analyzed with the software, Quantity One, Ver. 4.5 (Bio-Rad, Hercules, CA, USA).

Statistical analysis

The experimental results are expressed as the mean ± S.E.M., accompanied by the number of observations. Data were assessed by analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared by the Newman-Keuls method. $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSIONS

The content of total phenolics and ferulic acid in RBLp

When the concentration of total phenolics was determined by using gallic acid as a standard, a concentration of total phenolics of 37.5 ± 0.6 mg in 100 g RBLp was obtained, as shown in Table 1. Because it has been reported that rice bran water extract supplemented with ferulic acid has a synergistic neuroprotective effect in rats (Baek et al., 2014), we analyzed the ferulic acid content in RBLp with HPLC. As shown in Fig. 1A, authentic ferulic acid was observed at a retention time of 13.89 min. As shown in Fig. 1B, the retention time (13.53 min) of peak F was almost identical with that of ferulic acid. Accordingly, it is thought that peak F is a compound corresponding to ferulic acid. The concentration of peak F, as calculated from the calibration curve, was 18.4 mg/100 g RBLp (Table 2), which corresponded to 49.1% of the total phenolic content (Table 1).

Effect of RBLp on cytotoxicity

To determine whether RBLp has cytotoxicity, LDH release assay was examined. As the results, RBLp (500, 750 μg/mL) do not have cytotoxicity as compared with that of control in platelets (Fig. 3). Therefore, the anti-platelet effects of RBLp did not be affected by its toxic effect.

Effect of RBLp on ADP-, collagen-, and thrombin-induced platelet aggregation

The concentration of ADP that induced maximal platelet aggregation was approximately 20 μM (Fig. 2A). Therefore, 20 μM of ADP was used as the platelet agonist in this study. When washed platelets (10^8/mL) were activated with ADP (20 μM) in the presence of 2 mM $CaCl_2$, the aggregation rate was increased to 56.3 ± 1.0%. However, various concentrations of RBLp (100 to 1,000 μg/mL) significantly inhibited ADP-stimulated platelet aggregation in a dose-dependent manner (Fig. 2B), with a half-maximal inhibitory concentration (IC_{50}) of approximately 501.1 μg/mL (Fig. 2C).

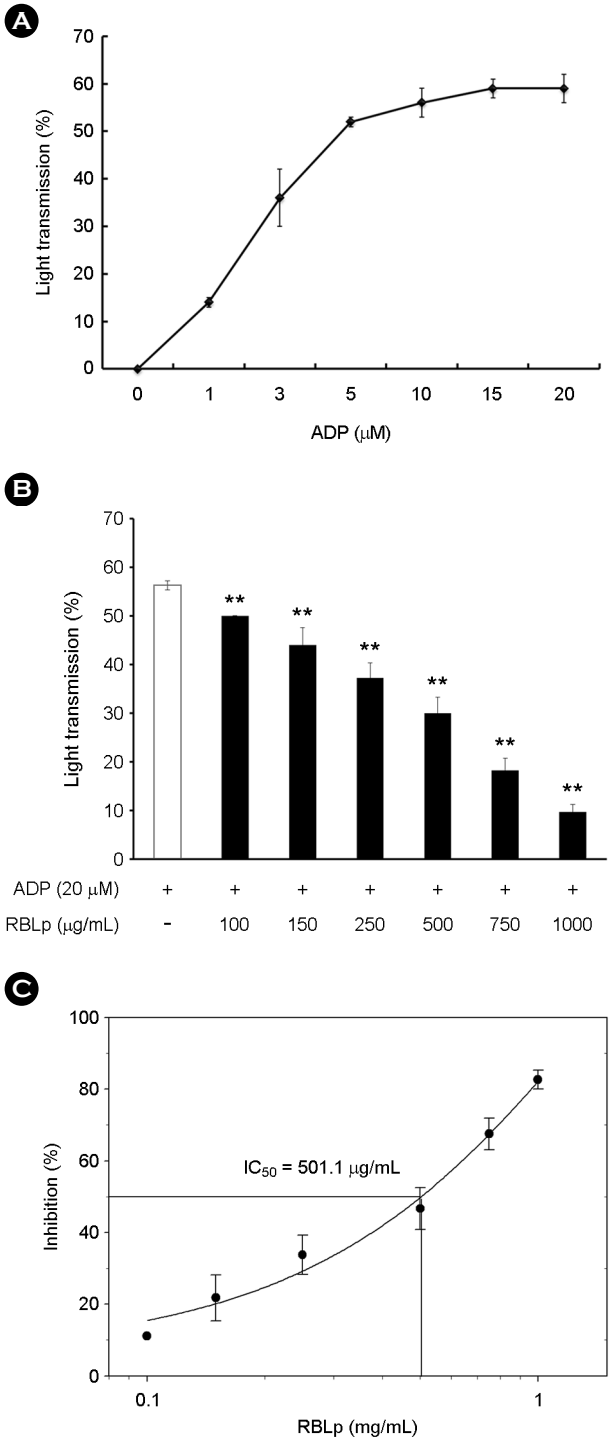

Fig. 2. Effect of RBLp on ADP-induced platelet aggregation. (A) The concentration threshold of ADP on platelet aggregation. (B) Effect of RBLp pretreatment on ADP-induced platelet aggregation. (C) IC_{50} value of RBLp on ADP-induced platelet aggregation. Meas-urement of platelet aggregation was performed as described in the "Materials and Methods" section. Inhibition rate by RBLp was expressed as the percentage of the ADP-induced aggregation rate. The IC_{50} value of RBLp was calculated by using a 4-parameter log fit method. The data are expressed as the mean ± S.E.M. (n = 4). $^{**}P < 0.001$ versus the ADP-stimulated platelets.

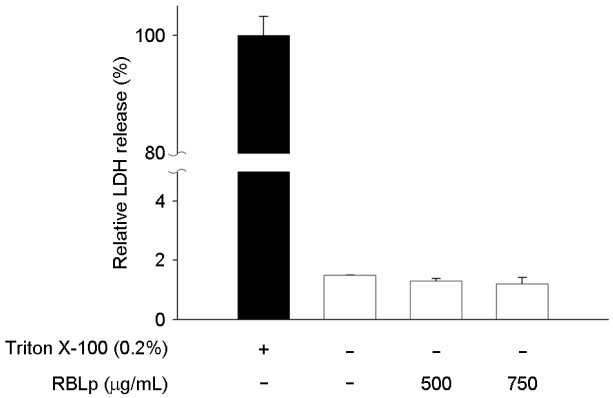

Fig. 3. Effect of RBLp on LDH release in washed platelets. Measurement of LDH release was performed as described in the "Materials and Methods" section. The data are expressed as the mean ± S.E.M. (n = 3).

Similarly, when washed platelets (10^8/mL) were activated with collagen (10 μg/mL) in the presence of 2 mM $CaCl_2$, the aggregation rate was increased to 66.7 ± 4.0%. However, various concentrations of RBLp (100 to 1,000 μg/mL) significantly inhibited collagen-stimulated platelet aggregation in a dose-dependent manner (Fig. 4A), with an IC_{50} of approximately 637.2 μg/mL (Fig. 4B). Thrombin-induced platelet aggregation was increased to 68.3 ± 2.1%. However, RBLp inhibited thrombin-stimulated platelet aggregation in a dose-dependent manner (100 to 2,000 μg/mL, Fig. 5A), but its IC_{50} cannot be calculated (over 2,000 μg/mL, Fig. 5B). Because RBLp had the strongest inhibitory effect on ADP-induced platelet aggregation with the lowest IC_{50} value (Figs. 2B, C), we next evaluated the antiplatelet effect of RBLp by assaying antiplatelet molecules (i.e., cAMP, VASP phosphorylation) on ADP-induced platelet aggregation. We evaluated 500 and 750 μg/mL of RBLp, which inhibited approximately 47 and 67% of ADP-induced platelet aggregation.

Effect of RBLp on cAMP production

Intracellular cAMP, an antiplatelet regulator, is produced by adenylate cyclase from ATP. cAMP inhibits platelet aggregation *via* the cAMP/A-kinase pathway. If a substance enhances the production of cAMP, the substance would be expected to have anti-platelet effects *via* the cAMP/A-kinase

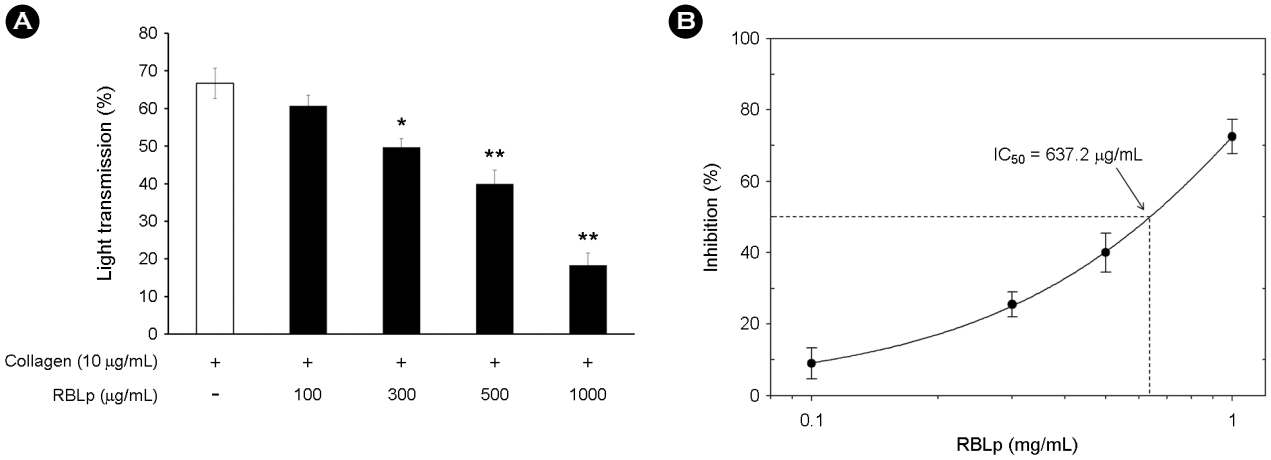

Fig. 4. Effect of RBLp on collagen-induced platelet aggregation. (A) Effect of RBLp pretreatment on collagen-induced platelet aggregation. (B) IC_{50} value of RBLp on collagen-induced platelet aggregation. Measurement of platelet aggregation was performed as described in the "Materials and Methods" section. Inhibition rate by RBLp was expressed as the percentage of the collagen-induced aggregation rate. The IC_{50} value of RBLp was calculated by using a 4-parameter log fit method. The data are expressed as the mean ± S.E.M. (n = 4). $^{*}P < 0.05$, $^{**}P < 0.001$ versus the collagen-stimulated platelets.

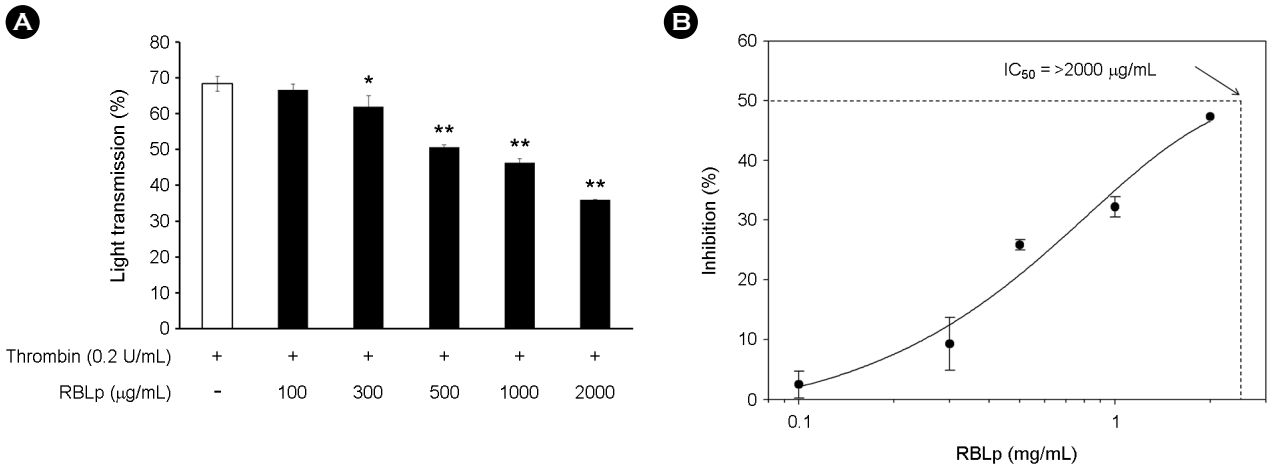

Fig. 5. Effect of RBLp on thrombin-induced platelet aggregation. (A) Effect of RBLp pretreatment on thrombin-induced platelet aggregation. (B) The IC_{50} value of RBLp on thrombin-induced platelet aggregation. Measurement of platelet aggregation was performed as described in the "Materials and Methods" section. Inhibition rate by RBLp was expressed as the percentage of the thrombin-induced aggregation rate. The IC_{50} value of RBLp was calculated by using a 4-parameter log fit method. The data are expressed as the mean ± S.E.M. (n = 4). $^{*}P < 0.05$, $^{**}P < 0.001$ versus the thrombin-stimulated platelets.

pathway. As shown in Fig. 6, ADP decreased intracellular cAMP levels from 2.09 ± 0.17 pmoL/10^9 platelets (basal level) to 0.99 ± 0.04 pmoL/10^9 platelets. When platelets, however, were stimulated in the presence of both RBLp (1,000 μg/mL) and ADP, the level of cAMP was increased to 2.35 ± 0.13 pmoL/10^9 platelets (Fig. 6). ADP induces platelet aggregation by decreasing cAMP levels. Therefore, the RBLp-induced increase in cAMP levels (Fig. 6) could explain the inhibition of ADP-induced platelet aggregation by RBLp (Fig. 2B).

Effect of RBLp on ADP-induced platelet aggregation in the presence of an adenylate cyclase inhibitor or a cAMP-dependent protein kinase (A-kinase) inhibitor

If RBLp increased cAMP levels by activating adenylate cyclase to inhibit ADP-induced platelet aggregation (Fig. 2B), then ADP-induced platelet aggregation would increase in the presence of an adenylate cyclase inhibitor that inhibits the generation of cAMP. Here, to observe an apparent change by these inhibitors, we used 750 μg/mL of RBLp that potently more inhibited than that by IC_{50} value (about 500 μg/mL) in ADP-induced platelet aggregation. As shown in Fig. 7A, platelet aggregation (18.5 ± 0.7%) induced by RBLp (750 μg/mL) plus ADP (20 μM) was increased by 29.0 ± 1.0% in the presence of the adenylate cyclase inhibitor, SQ22536 (50 μM). This result indicates that RBLp may elevate cAMP levels *via* activation of adenylate cyclase, thereby resulting in an inhibition of ADP-induced platelet aggregation. Otherwise, platelet aggregation (18.5 ± 0.7%) in the presence of RBLp plus ADP would not be increased to 56.8% in the presence of an adenylate cyclase inhibitor (Table 3). On the other hand, SQ22536 alone did not significantly affect ADP-induced platelet aggregation (Fig. 7A).

The inhibition of platelet aggregation by cAMP is caused *via* activation of cAMP-dependent protein kinase (A-kinase). Therefore, platelet aggregation would be expected to be increased in the presence of the A-kinase inhibitor, similar to the effect seen with the adenylate cyclase inhibitor, SQ22536. Accordingly, we investigated whether RBLp-inhibited platelet aggregation is increased by the A-kinase inhibitor, Rp-8-Br-cAMPS. As shown in Fig. 7B, platelet aggregation upon treatment with RBLp (750 μg/mL) plus ADP (20 μM) was

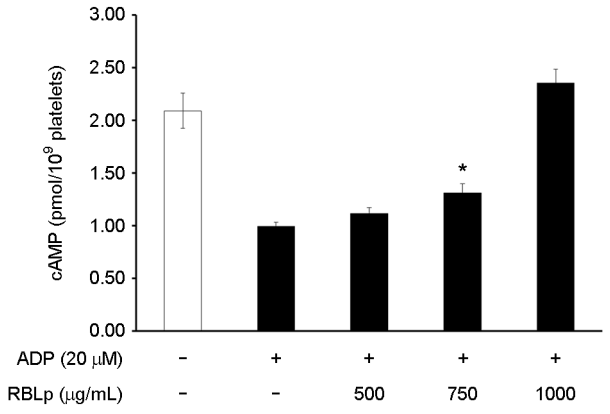

Fig. 6. Effect of RBLp on cAMP production. Measurement of cAMP was performed as described in the "Materials and Methods" section. The data are expressed as the mean ± S.E.M. (n = 4). $^{*}P < 0.05$ versus the ADP-stimulated platelets.

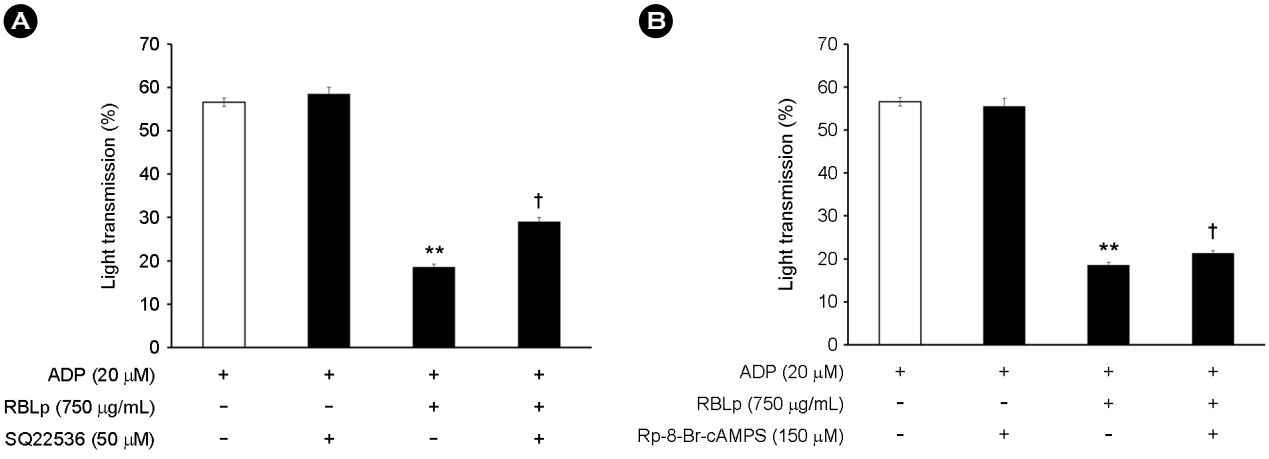

Fig. 7. Effects of RBLp in the presence of an adenylate cyclase inhibitor or an A-kinase inhibitor on ADP-induced platelet aggregation. (A) Effect of RBLp in the presence of the adenylate cyclase inhibitor, SQ22536. (B) Effect of RBLp in the presence of the A-kinase inhibitor, Rp-8-Br-cAMPS. Measurement of platelet aggregation was performed as described in the "Materials and Methods" section. The data are expressed as the mean ± S.E.M. (n = 4). $^{**}P < 0.001$ versus the ADP-stimulated platelets; $^{\dagger}P < 0.05$ versus the ADP-stimulated platelets in the presence of RBLp (750 μg/mL).

increased by Rp-8-Br-cAMPS (150 μM). These results suggest that the inhibitory mechanism of ADP-induced platelet aggregation by RBLp is dependent on the cAMP/A-kinase pathway. Otherwise, platelet aggregation (18.5 ± 0.7%) in the presence of RBLp and ADP would not be increased to 16.4% in the presence of the A-kinase inhibitor (Table 3). On the other hand, Rp-8-Br-cAMPS alone had no significant effect on ADP-induced platelet aggregation (Fig. 7B).

Effect of RBLp on VASP phosphorylation

The downstream pathway of cAMP/A-kinase involves in the phosphorylation of VASP, which results in the inhibition of platelet aggregation. Ser^{157} at 50 kDa of VASP is phosphorylated by the cAMP/A-kinase pathway (Horstrup et al., 1994; Smolenski et al., 1998). Therefore, the phosphorylation

Table 3. Changes of platelet aggregation in the presence of SQ22536 or Rp-8-Br-cAMPS

	ADP (20 μM) + RBLp (750 μg/mL)	ADP (20 μM) + RBLp (750 μg/mL) + SQ22536 (50 μM)	ADP (20 μM) + RBLp (750 μg/mL) + Rp-8-Br-cAMPS (150 μM)
Platelet aggregation (%)	18.3 ± 2.5①	29.0 ± 1.0②	21.3 ± 0.6③
Δ (%)	0	+ 56.8④	+ 16.4⑤

Platelet aggregations are from Fig. 7. Δ (%) ④; ②-①/①×100. Δ (%) ⑤; ③-①/①×100.

Table 4. Changes of p-VASP (Ser^{157})/β-actin ratio in the presence of SQ22536 or Rp-8-Br-cAMPS

	ADP (20 μM) + RBLp (750 μg/mL)	ADP (20 μM) + RBLp (750 μg/mL) + SQ22536 (50 μM)	ADP (20 μM) + RBLp (750 μg/mL) + Rp-8-Br-cAMPS (150 μM)
p-VASP (Ser^{157})/β-actin	6.2 ± 0.3①	3.1 ± 0.2②	2.8 ± 0.1③
Δ (%)	0	- 50.0④	- 54.8⑤

p-VASP (Ser^{157})/β-actin ratio are from Fig. 8. Δ (%) ④; ②-①/①×100. Δ (%) ⑤; ③-①/①×100.

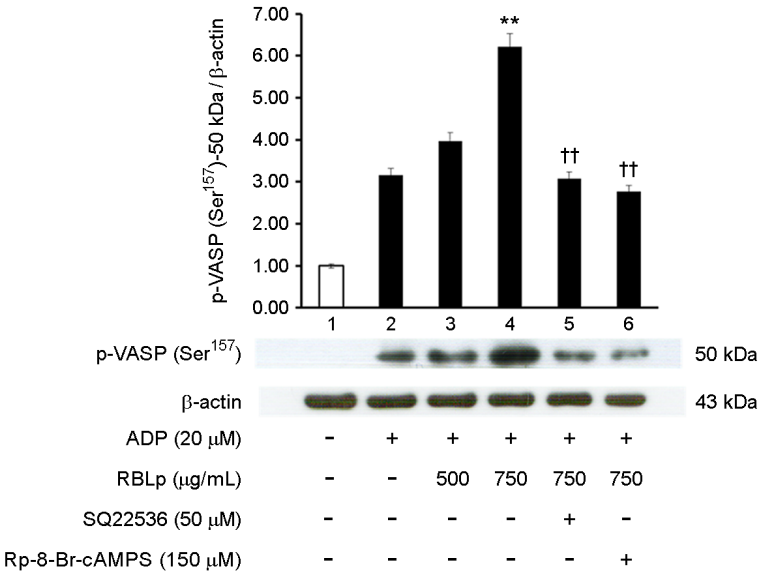

Fig. 8. Effect of RBLp on VASP phosphorylation. Lane 1, Unstimulated platelets (base); Lane 2, ADP (20 μM); Lane 3, ADP (20 μM) + RBLp (500 μg/mL); Lane 4, ADP (20 μM) + RBLp (750 μg/mL); Lane 5, ADP (20 μM) + RBLp (750 μg/mL) + SQ22536 (50 μM); Lane 6, ADP (20 μM) + RBLp (750 μg/mL) + Rp-8-Br-cAMPS (150 μM). Western blotting was performed as described in "Materials and Methods". The data are expressed as the mean ± S.E.M. (n = 4). $^{**}P < 0.001$ versus the ADP-stimulated platelets; $^{††}P < 0.001$ versus the ADP-stimulated platelets in the presence of RBLp (750 μg/mL).

of Ser^{157} at 50 kDa of VASP can function as a useful indicator for monitoring the cAMP/A-kinase pathway. ADP slightly increased the phosphorylation of 50 kDa VASP (Ser^{157}) [p-VASP (Ser^{157})] and thus the ratio of p-VASP (Ser^{157}) to β-actin (Fig. 8, lane 2), suggesting that ADP is involved in feedback inhibition by elevating p-VASP (Ser^{157} and Ser^{239}) (Gambaryan et al., 2010). The ratio of p-VASP (Ser^{157}) to β-actin was dose-dependently increased in the presence of both ADP and RBLp (500 and 750 μg/mL, respectively) (Fig. 8, lanes 3, 4). As shown in Fig. 8 (lane 5) and Table 4, treatment of platelets with both ADP (20 μM) and RBLp (750 μg/mL) resulted in the phosphorylation of 50 kDa VASP (Ser^{157}), which was inhibited by the adenylate cyclase inhibitor, SQ22536 (50 μM). In addition, the phosphorylation of VASP (Ser^{157}) by both ADP and RBLp also was decreased in the presence of Rp-8-Br-cAMPS (Fig. 8, lane 6 and Table 4). These results indicate that RBLp induces the phosphorylation of VASP (Ser^{157}) through adenylate cyclase activation, cAMP elevation, and A-kinase activation. Otherwise, the ratio of phosphorylated VASP (Ser^{157}) to β-actin following treatment with both RBLp (750 μg/mL) and ADP (20 μM) would not be decreased in the presence of the adenylate cyclase or A-kinase inhibitor (Fig. 8, Table 4). It is well established that cAMP/A-kinase-dependent VASP (Ser^{157}) phosphorylation is involved in inhibition of ADP-induced platelet aggregation (Halbrügge et al., 1989; Halbrügge et al., 1990; Butt et al., 1994). In addition, Kim et al. (2014) demonstrated that both SQ22536 and Rp-8-Br-cAMPS inhibited ADP-induced VASP (Ser^{157}) phosphorylation. These results suggest that SQ22536 and Rp-8-Br-cAMPS inhibited cAMP/A-kinase-dependent VASP (Ser^{157}) phosphorylation by inhibiting cAMP production and A-kinase activity.

With regards to the regulatory effects of VASP (Ser^{157}) phosphorylation induced by phenolic compounds on platelet aggregation, epigallocatechin-3-gallate (Ok et al., 2012) and caffeic acid (Lee et al., 2014) have been found to also elevate cAMP levels and induce phosphorylation of VASP *via* a cAMP/A-kinase pathway, leading to an inhibition of platelet aggregation. RBLp contains ferulic acid, a phenolic compound, and appears to involve cAMP-dependent phosphorylation of VASP (Ser^{157}), resulting in an inhibition of platelet aggregation. In the present study, however, it is unknown whether the ferulic acid present in RBLp was directly involved in cAMP-dependent phosphorylation of VASP (Ser^{157}). This possible mechanism should be investigated in future studies. There are reports that ferulic acid, along with its derivatives, possess antiplatelet effects (Yasuda et al., 2003; Wang and Ou-Yang, 2005), however, their mechanism of action remains unknown. Therefore, because in RBLp, 49.1% of the total phenolics found is composed of ferulic acid (Table 1), the inhibition of ADP-, collagen-, and thrombin-induced platelet aggregation by RBLp (Figs. 2B, 4A, 5A) may result from the action of the ferulic acid that is present in RBLp.

It is inferred that ferulic acid in RBLp (Fig. 1B) may have inhibitory effect on ADP-induced platelet aggregation. In our previous report (Kim et al., 2014), we investigated whether authentic ferulic acid (FA) has an antiplatelet activity on ADP-induced platelet aggregation. To investigate the antiplatelet activity of FA, we used 0.15, 0.24, 0.49 μM of sodium ferulate (MW. 216.17). As shown in our previous report (Kim et al., 2014), ferulic acid (FA) dose dependently inhibited ADP-induced platelet aggregation. These results suggest that antiplatelet effect of RBLp may be resulted from ferulic acid (Fig. 1B) in RBLp.

Antiplatelet drugs, such as thienopyridine derivatives (i.e., ticlopidine, clopidogrel), induce the phosphorylation of VASP, which is mediated by cAMP or cGMP (Barragan et al., 2003). Therefore, RBLp, along with these thienopyridine derivatives, may represent useful therapeutic tools in the treatment or prevention of vascular diseases associated with platelet aggregation.

Acknowledgements

This study was supported by a grant from the High Value-added Food Technology Development Program [112074-3], iPET (Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries).

Conflict of interest

The authors declare no conflict of interest.

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