Water Extract from Rice Bran Fermented with *Lactobacillus plantarum* Hong Inhibits Thromboxane A₂ Production Associated Microsomal Enzyme Activity in Human Platelets

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In this study, we investigated the effect of rice bran water extract fermented with *Lactobacillus plantarum* Hong (RBLw), on activities of cyclooxygenase-1 (COX-1) and thromboxane A₂ synthase (TXAS), thromboxane A₂ (TXA₂) production associated microsomal enzymes and evaluated its the antiplatelet effect. RBLw, containing 13.5 mg of ferulic acid, dose-dependently inhibited ADP-induced platelet aggregation, and inhibited the production of TXA₂, an aggregation molecule. In addition, RBLw directly inhibited COX-1 activity in a dose-dependent manner, but not TXAS activity in platelet microsomal fraction having cytochrome c reductase (an endoplasmic reticulum marker enzyme) activity and expressing COX-1 (72 kDa) and TXAS (60.5 kDa) proteins. These results suggest that RBLw selectively inhibited the activity of COX-1 rather than TXAS to attenuate TXA₂ production in ADP-activated platelets. Thus, we demonstrate that RBLw might have direct COX-1 antagonistic function for platelet aggregation-mediated diseases, such as thrombosis, myocardial infarction, atherosclerosis, and ischemic cerebrovascular disease.

Key Words: Rice bran fermentation, Thromboxane A₂, Microsomal fraction, Cyclooxygenase-1, Thromboxane A₂ synthase

INTRODUCTION

The aggregation-inducing molecule thromboxane A₂ (TXA₂) plays an important role in agonists (eg. collagen, thrombin, ADP)-induced platelet aggregation (Malmsten et al., 1975; Lewis and Watts, 1982; Li et al., 2010), which can

cause cardiovascular diseases such as thrombosis, atherosclerosis and myocardial infarction (Schwartz et al., 1990). When diverse agonists such as collagen, thrombin, and ADP activate platelets, membrane phospholipids (i.e. phosphatidylinositol 4,5-bisphosphate) are broken down, and subsequently produce TXA₂ from arachidonic acid (AA). This potently induces platelet activation and vasoconstriction in an autacoidal action (Hamberg et al., 1975; Samuelsson et al., 1978; Gesele et al., 1991). Agonists-produced TXA₂ interacts with its receptor in other platelets in an autacoidal reaction, and subsequently increases intracellular free Ca²⁺, a platelet aggregation-inducing molecule, via G_{αq} of G protein-coupled receptor (Jennings, 2009; Cattaneo et al., 1991; Moriyama et al., 1988). TXA₂ production associated enzymes

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are cyclooxygenase-1 (COX-1) and thromboxane A₂ synthase (TXAS), which are located in microsomes (Carey et al., 1982). COX-1 produces prostaglandin G₂ (PGG₂) from substrate AA, TXAS produces TXA₂ from prostaglandin H₂ (PGH₂) that is oxidized from PGG₂ by endoperoxidase. Therefore, inhibition of COX-1 or TXAS is very useful to evaluate an antiplatelet effect of any substance or compound. For instance, COX-1 inhibitor aspirin and TXAS inhibitor ozagrel are being used as anti-platelet agents (Patrono, 2001).

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 is known to have 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 (RB) has a neuroprotective effect in ischemic brain injury (Baek et al., 2014; Hong et al., 2015), and anti-platelet effect *via* cAMP-dependent phosphorylation of vasodilator stimulated phosphoprotein (VASP) (VASP-Ser¹⁵⁷) (Kim et al., 2014; Kim et al., 2015). In the present study, we investigated whether rice bran water extract fermented with *Lactobacillus plantarum* Hong (RBLw), a different water extract from RB, inhibits the specific activities of COX-1 and TXAS to suppress TXA₂ production, and found a novel function of RBLw in ADP-activated human platelets.

MATERIALS AND METHODS

Materials

ADP was purchased from Chrono-Log Co. (Havertown, PA, USA). COX-fluorescent activity kit, TXB₂ enzyme immunoassay (EIA) kit, and thromboxane A₂ synthase substrate prostaglandin H₂ were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). Anti-COX-1 and anti-TXAS were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 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 chemiluminescence (ECL) solution was purchased from GE Healthcare (Chalfont St,

Giles, Buckinghamshire, UK).

Preparation of rice bran water extract fermented with *Lactobacillus plantarum* Hong (KFCC 11556P) (RBLw)

RBLw was prepared according to the following method: *Lactobacillus plantarum* Hong (KFCC 11556P) was inoculated and cultured in MRS broth medium (Difco Laboratories, Detroit, MI, USA) at 35°C 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 (rice bran 100 g : 900 ml distilled water) was inoculated with the starter at a ratio of 5% and fermented at 35°C. Rice bran was fermented using the starter at 35°C, with shaking at 120 rpm for 36 hours. The culture broth was autoclaved at 121°C 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 (Fig. 1). The resultant concentrate was designated as RBLw and kept at 4°C until used. For experiments to investigate the effects on platelet aggregation, RBLw was dissolved in saline (0.9% NaCl).

Determination of total phenolic content of RBLw

Total phenolic content was determined by using a modification of Singleton's method (Singleton and Rossi, 1965). RBLw was dissolved in 50% methanol at a concentration of 0.1% (w/v). A 2.0 mL aliquot 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 RBLw.

Fig. 1. Preparation of rice bran water extract fermented with *Lactobacillus plantarum* Hong (KFCC 11556P) (RBLw)

Detection of phenolic compounds of RBLw by HPLC

Because it has been reported that rice bran contains phenolic compounds, we tested for the presence of phenol compounds in RBLw using high performance liquid chromatography (HPLC) (Goufo and Trindade, 2014). After initially dissolving the RBLw in distilled water (100 mg/ml), the pH of the dissolved sample was adjusted to pH 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 mm × 4.6 mm id, 5 µm, Tosoh, Japan) was used at a temperature of 40 °C. The mobile phase consisted of methanol (A) and 50 mM NaH₂PO₄, 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 human platelet-rich plasma (PRP) and washed 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 residual red blood cells, and then centrifuged for 10 min at 1,300 g to obtain platelet pellets. The platelet pellets were then resuspended in platelet-poor plasma to a final concentration of 5×10^8 /mL.

Human washed platelets were prepared from PRP to obtain microsomal fraction, a source of COX-1 and TXAS. PRP was centrifuged for 10 min at 125 g to remove residual red blood and white 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 NaHCO₃, 0.36 mM NaH₂PO₄, 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 NaHCO₃, 0.36 mM NaH₂PO₄, 0.49 mM MgCl₂, 5.5 mM glucose, 0.25% gelatin, pH 6.9) to a final concentration of 5×10^8 /mL. All of the above procedures were carried out at 25 °C 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

PRP (10^8 platelets/mL) were preincubated for 3 min at 37 °C with or without substances, then stimulated with ADP (20 µM) 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 platelet poor plasma was used as a reference (transmission 0%). RBLw was dissolved in saline (0.9% NaCl).

Measurement of thromboxane B₂

PRP (10⁸ platelets/mL) were preincubated for 3 min at 37°C with or without substances, then stimulated with ADP (20 µM) for 5 min. The reactions were terminated by the addition of ice-cold EDTA (5 mM) and indomethacin (0.2 mM). The amount of TXB₂, a stable metabolite of TXA₂, was determined with synergy HT multi-model microplate reader (BioTek Instruments, Winooski, VT., USA) using a TXB₂ EIA kit. RBLw was dissolved in saline (0.9% NaCl).

Isolation of microsomal fraction

Washed platelets (5 × 10⁸ platelets/mL) containing suspension buffer (pH 6.9) with 1% protease inhibitor were sonicated for 20 sec, 1 cycle, and 10 times on ice with a sonicator (Bandelin, HD2070, Germany) to obtain platelet lysates. Homogenates were centrifuged at 1,500 g for 15 min at 4°C, then the supernatant was ultracentrifuged at 105,000 g for 1 hr at 4°C to obtain the microsomal fraction (F1-cellular fraction) containing endoplasmic reticulum (ER) membrane (Carey et al., 1982). The supernatant was termed F2-cellular fraction. All of the separated fractions (homogenates, F1-, and F2-cellular fraction) were identified by cytochrome c reductase (a marker enzyme of ER membrane) (Lagarde et al., 1981) and used as enzyme sources in Western blot as described below.

Cytochrome c reductase activity assay

Cytochrome c reductase is a flavoprotein localized in the ER. Cytochrome c reductase activity of the fractions (homogenates, F1-, and F2-cellular fraction) was assayed by using cytochrome c reductase (NADPH) assay kit (Sigma Chemical Corp, St. Louis, MO, USA). The reaction was initiated by addition of NADPH and the reduction of cytochrome c is monitored by the increase of absorbance at 550 nm for 7 min with kinetic program.

Western blot analysis of cyclooxygenase-1 (COX-1) and TXA₂ synthase (TXAS)

Protein concentrations in the separated fractions were measured by using Bradford method. Proteins of each fraction (30 µg) were separated on 8% SDS-PAGE (1.5 mm gel). The separated proteins were electrophoretically transferred to PVDF membranes (GE Healthcare, New Jersey, USA). Immunoblot was performed by using primary antibody (COX-1, 1:200, TXAS, 1:100) and horseradish peroxidase (HRP) conjugated secondary antibody at a dilution of 1:5,000 from Santa Cruz Biotechnology Inc. (CA, USA). After secondary antibody (anti-mouse IgG-HRP or anti-goat IgG-HRP) treatment, detection of antibody-bound protein in the membrane was performed with enhanced chemiluminescence (ECL) solution (GE Healthcare, Buckinghamshire, UK).

Cyclooxygenase-1 (COX-1) activity assay

For the measurement of COX-1 activity, the F1-cellular fraction (microsomal fraction, 20 µg proteins) of platelets was pre-incubated with or without various concentrations of RBLw or aspirin (500 µM) at 37°C for 30 min. COX-1 activity of the treated F1-cellular fraction was assayed with COX-1 fluorescent assay kit (Cayman Chemical Co, Ann Arbor, MI, USA) according to the procedure described by manufacturer.

Thromboxane A₂ synthase (TXAS) activity

For the measurement of TXAS activity of the F1-cellular fraction (microsomal fraction, 20 µg proteins) was pre-incubated with ozagrel (11 nM, IC₅₀), a positive control as a TXAS inhibitor, and with or without various concentrations of RBLw at 37°C for 30 min. The reaction is initiated by the addition of PGH₂. Incubation was allowed to proceed for 1 min at 37°C. The reactions are terminated by the addition of 1 M citric acid. After neutralization with 1 N NaOH, the concentration of TXB₂, a stable metabolite of TXA₂, was determined by using TXB₂ EIA kit according to the procedure described by manufacturer.

Statistical analysis

The experimental results are expressed as the mean ±

Table 1. Total phenolic acid content of RBLw

	Total phenolic (TP) content (mg / 100 g RBLw)	Ferulic acid (FA) content (mg / 100 g RBLw)	FA/TP (%)
RBLw	36.4 ± 0.4	13.5	37.1

The content of total phenolic was determined as described in "Materials and Methods". Ferulic acid content was from Table 2. The data are expressed as the mean ± S.E.M. (n = 4).

Fig. 2. HPLC chromatograms of RBLw and ferulic acid. (A) The chromatogram of ferulic acid. (B) The chromatogram of RBLw. HPLC was performed as described in "Materials and Methods". F, ferulic acid

S.E.M. Data was 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* values of less than 0.05 were considered to be statistically significant.

RESULTS

The content of total phenolics and ferulic acid in RBLw

When the concentration of total phenolics was determined by using gallic acid as a standard, a concentration of total phenolics of 36.4 ± 0.4 mg in 100 g RBLw was obtained

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

		RT (min)	Calibration curve ^{a)}	r^2	Test range ($\mu\text{g/mL}$)	$x^{\text{b)}$ ($\mu\text{g/mL}$)	Content (mg/100 g-RBLw)
Authentic compound	Ferulic acid	13.89	$y=0.0002x+9.3815$	0.9993	12.5~500	–	–
RBLw	Peak F	13.66	–	–	–	13.5	13.5

^{a, b)} y, peak areas of analytes; x, concentrations of analytes in 100 mg/mL RBLw.

Fig. 3. Effect of RBLw on ADP-induced human platelet aggregation. (A) The concentration threshold of ADP on platelet aggregation. (B) Effect of RBLw pretreatment on ADP-induced platelet aggregation. Measurement of platelet aggregation was performed as described in the "Materials and Methods" section. Inhibition rate by RBLw was expressed as the percentage of the ADP-induced aggregation rate. The data are expressed as the mean $\pm$ S.E.M. ($n = 4$). ** $P < 0.001$ versus the ADP-stimulated platelets.

neuroprotective effect in rats (Baek et al., 2014), we analyzed the ferulic acid content in RBLw with HPLC. As shown in Fig. 2A, authentic ferulic acid was observed at a retention time of 13.89 min. As shown in Fig. 2B, the retention time (13.66 min) of peak F was almost identical with that of ferulic acid. Accordingly, we propose that peak F is a compound corresponding to ferulic acid. The concentration of peak F, as calculated from the calibration curve, was 13.5 mg/100 g RBLw (Table 2), which corresponded to 37.1% of the total phenolic content (Table 1).

Effect of RBLw on ADP-induced human platelet aggregation

The concentration of ADP that induced maximal platelet aggregation was approximately 20 μM (Fig. 3A). Therefore, 20 μM of ADP was used as the platelet agonist in this study. When PRP ($10^8/\text{mL}$) were activated with ADP (20 μM), the aggregation rate increased to $56.3 \pm 1.0\%$. However, various concentrations of RBLw (10 to 500 $\mu\text{g/mL}$) significantly inhibited ADP-stimulated platelet aggregation in a dose-dependent manner (Fig. 3B).

Effect of RBLw on TXA_2 production

The amount of TXA_2 (determined as TXB_2) in intact platelets was $4.2 \pm 0.2 \text{ ng}/10^8$ platelets, which was markedly increased to $24.0 \pm 3.4 \text{ ng}/10^8$ platelets by ADP (20 μM). RBLw (50~500 $\mu\text{g/mL}$), however, potently reduced the production of TXA_2 (Fig. 4, Table 3) in a dose-dependent manner. These results show that RBLw may inhibit the activity of COX-1 or TXAS in a dose-dependent manner to suppress the production of TXA_2 in ADP-induced platelet aggregation.

(Table 1). Because it has been reported that rice bran water extract supplemented with ferulic acid has a synergistic

Table 3. Effects of RBLw on TXB₂ production in ADP-induced platelet aggregation

	TXB ₂	
	ng/10 ⁸ platelets	Change (%)
Intact (control)	4.2 ± 0.2	–
ADP (20 µM)	24.0 ± 3.4	+ 471.4 ¹⁾
RBLw (50 µg/mL) + ADP (20 µM)	8.8 ± 1.2**	- 63.3 ²⁾
RBLw (100 µg/mL) + ADP (20 µM)	6.0 ± 0.6**	- 75.0 ³⁾
RBLw (150 µg/mL) + ADP (20 µM)	5.6 ± 0.2**	- 76.7 ⁴⁾
RBLw (250 µg/mL) + ADP (20 µM)	5.5 ± 0.5**	- 77.1 ⁵⁾
RBLw (500 µg/mL) + ADP (20 µM)	4.7 ± 0.1**	- 80.4 ⁶⁾

Data were from Fig. 4. ¹⁾Change (%) = (ADP-intact) / intact × 100; ^{2) to 6)}Change (%) = [ADP-(RBLw+ADP) / ADP] × 100. The data are expressed as the mean ± S.E.M. (n = 4). **P < 0.001 versus the ADP-stimulated platelets.

Fig. 4. Effects of RBLw on ADP-induced TXA₂ production. Measurement of TXB₂, a stable metabolite of TXA₂, was performed as described in "Materials and Methods". The data are expressed as the mean ± S.E.M. (n = 4). ^aP < 0.05 versus non-stimulated platelets, **P < 0.001 versus the ADP-stimulated platelets.

Determination of enzyme source of COX-1 and TXAS

To determine whether RBLw is involved in the inhibition of COX-1 and TXAS, enzyme sources having COX-1 or TXAS were needed. We separated F1-, and F2-cellular

Fig. 5. Determination of enzyme source of cyclooxygenase-1 (COX-1) and thromboxane A₂ synthase (TXAS). (A) Cytochrome c reductase activities in homogenates, F1-, and F2-cellular fraction. NADPH-cytochrome c reductase, the marker enzyme for dense tubular system in platelets (100 µg of total protein) was assayed as described in "Materials and Methods". (B) Western blot analysis of COX-1 and TXAS in homogenates, F1-, F2-cellular fraction. Western blotting was performed as described in "Materials and Methods". H: homogenates; F1: F1-cellular fraction (microsomal fraction); F2: F2-cellular fraction. **P < 0.001 compared with that of homogenates.

fractions from platelet lysates (homogenates), and measured the activity of cytochrome c reductase, an endoplasmic reticulum (microsomes) marker enzyme. As shown in Fig. 5A, we found that the F1-cellular fraction has the highest specific activity of cytochrome c reductase. Next, we determined which fraction from platelet lysates expresses both COX-1 and TXAS proteins. As the result, remarkably high expressions of COX-1 (72 kDa) and TXAS (60.5 kDa) were observed in F1-cellular fraction (Fig. 5B). Because these are in accordance with that F1-cellular fraction has the highest activity of cytochrome c reductase, the F1-cellular fraction is likely the microsomal fraction. This microsomal fraction was used to determine the activity of COX-1 and TXAS.

Fig. 6. Effects of RBLw on cyclooxygenase-1 (COX-1) and thromboxane A₂ synthase (TXAS) activities. (A) Effect of RBLw on COX-1 activity in F1-cellular fraction (microsomal fraction). COX-1 activity was performed as described in "Materials and Methods". (B) Effect of RBLw on TXAS activity in F1-cellular fraction (microsomal fraction). TXAS activity was performed as described in "Materials and Methods". The data are expressed as the mean $\pm$ S.E.M. (n = 4). * P <0.05, ** P <0.001 compared with that of non-treated F1-cellular fraction (microsomal fraction).

Effect of RBLw on COX-1 activity

To determine if the inhibitory effect of TXA₂ production by RBLw was due to the direct suppression of COX-1, a cell-free enzyme assay method was used. As shown in Fig. 6A, COX-1 activity in the absence of RBLw (control) was 18.3 ± 2.1 nmol/mg-protein/min. However, RBLw dose-dependently inhibited COX-1 activity. Aspirin (500 µM), a

positive control of COX-1, inhibited COX-1 activity to 13.7 ± 2.5 nmol/mg-protein/min (Fig. 6A), which was weak compared with that by RBLw (150 µg/mL).

Effect of RBLw on TXAS activity

To determine whether the inhibitory effect on TXA₂ production by RBLw was due to the direct suppression of TXAS, the aforementioned cell-free enzyme assay method was also used. As shown in Fig. 6B, TXAS activity by RBLw-untreated platelets was 655.4 ± 55.7 ng/mg-protein/min. However, RBLw (250 µg/mL) almost did not inhibit TXAS activity. In addition, ozagrel (11 nM), a positive control of TXAS, potentially inhibited TXAS activity (Fig. 6B).

DISCUSSION

TXA₂ is a strong agonist on resting platelets as a positive feedback promoter on activated platelets (Ruggeri, 2002; Jennings, 2009), and also acts as a vasoconstrictor and a bronchoconstrictor (FitzGerald, 1991) in an autacoidal action. Thus, a compound that can inhibit TXA₂ production-associated enzymes (COX-1 or TXAS), TXA₂ production or TXA₂ action could be applied as an anti-thrombotic agent (Clutton et al., 2001). Presently, we tried to explain the inhibitory mechanism of RBLw in TXA₂ production by determining the activities of COX-1 and TXAS. In this study, we established that RBLw inhibits COX-1 activity to attenuate TXA₂ production in ADP-induced platelet aggregation. Presently, it is unknown whether ferulic acid of RBLw is directly or indirectly involved in inhibition of COX-1 activity. In the current study, because COX-1 activity was determined with microsomal fraction prepared from washed platelets, and was inhibited by RBLw, it is suggested that RBLw is involved in direct inhibition of COX-1 activity. Accordingly, considering our previous report that authentic ferulic acid inhibited ADP-induced platelet aggregation (Kim et al., 2014), it is inferred that ferulic acid of RBLw may directly involve in inhibition of COX-1 activity to suppress the production of TXA₂, a potent stimulator in platelet aggregation.

RBLw inhibited potentially COX-1 activity rather than TXAS activity (Fig. 6A, B), which is in accord with reports

that the inhibition of agonists-induced platelet aggregation is dependent on inhibition of COX-1 rather than TXAS (Lewis and Watts, 1982; Jang et al., 2002). Because these results suggest that inhibition of TXA₂ by RBLw is responsible for the suppression of microsomal specific COX-1, it is shown that RBLw may be used as a COX-1 inhibitor. In addition, TXA₂ binds to its receptor in platelets, and subsequently mobilizes Ca²⁺ to activate platelets (Ohkubo et al., 1996; Cattaneo et al., 1991; Su et al., 1999). This means that TXA₂ and Ca²⁺ are involved in thrombogenesis by intensifying platelet aggregation. Therefore, the inhibitory effect of ADP-elevated TXA₂ production by RBLw could be related to the attenuation of Ca²⁺ to suppress the thrombogenesis. It is known that aspirin is involved in inhibition of COX-1 activity *via* acetylation of COX-1 protein (Roth et al., 1975; DeWitt et al., 1990). At present, it is unknown how RBLw inhibits COX-1 activity, and whether RBLw acetylates COX-1 protein in the same as aspirin. With regard to this, further studies are necessary.

In conclusion, RBLw that inhibits TXA₂ production *via* attenuation of COX-1 activity may have therapeutic potential for the treatment or prevention of platelet aggregation-mediated diseases, such as thrombosis, myocardial infarction, atherosclerosis, and ischemic cerebrovascular disease.

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Conflict of interest

The authors declare no conflict of interest.

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