Inhibitory Effects of Water Extract from Rice Bran Due to cAMP-dependent Phosphorylation of VASP (Ser\textsuperscript{157}) on ADP-induced Platelet Aggregation

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In this study, we investigated the effect of water extract from rice bran (RB) on ADP (20 μM)-stimulated platelet aggregation. RB dose-dependently inhibited ADP-induced platelet aggregation, and its IC\textsubscript{50} value was 224.0 μg/mL, which was increased by adenylate cyclase inhibitor SQ22536 and cAMP-dependent protein kinase (A-kinase) inhibitor Rp-8-Br-cAMPS. RB elevated the phosphorylation of VASP (Ser\textsuperscript{157}) which was also inhibited by SQ22536 and Rp-8-Br-cAMPS. It is thought that RB-elevated cAMP contributed to the phosphorylation of VASP (Ser\textsuperscript{157}) to inhibit ADP-induced platelet aggregation. Therefore, we demonstrate that RB has an antiplatelet effect via cAMP-dependent phosphorylation of VASP (Ser\textsuperscript{157}), and RB may have preventive or therapeutic potential for platelet aggregation-mediated diseases, such as thrombosis, myocardial infarction, atherosclerosis, and ischemic cerebrovascular disease.

Key Words: Rice bran, Platelet aggregation, cAMP-dependent protein kinase, cAMP, Vasodilator-stimulated phosphoprotein- Ser\textsuperscript{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\textsubscript{2}, phosphatidylinositol 4, 5-bisphosphate is hydrolyzed by phospholipase C-β via G-protein coupled receptor to inositol 1, 4, 5-trisphosphate and diacylglycerol (Guidetti et al., 2008; Berridge et al., 1984; Jennings et al., 2009). In special, it is known that ADP binds to the G\textsubscript{q}-coupled P2Y\textsubscript{1} receptor, which mediates PLC-β, and the G\textsubscript{i}-coupled P2Y\textsubscript{12} receptor, which mediates inhibition of adenylylate cyclase and amplifies platelet aggregation (Cattaneo, 2005).

Intracellular cyclic adenosine monophosphate (cAMP) as an antiplatelet regulator decrease the [Ca\textsuperscript{2+}] mobilization (Menshikov et al., 1993; Schwarz et al., 2001). The antiplatelet effects of cAMP are mediated via cAMP-dependent protein kinases (A-kinase), which phosphorylates substrate protein, vasodilator stimulated phosphoprotein (VASP) (Halbrügge et al., 1989; Halbrügge et al., 1990; Butt et al., 1994). VASP (Ser\textsuperscript{157} and Ser\textsuperscript{239}) phosphorylation involves in inhibition of VASP affinity for contractile protein filamentous actin, and fibrinogen binding to glycoprotein IIb/IIIa (αIIb/β\textsubscript{3}) to inhibit platelet aggregation (Laurent et al.,
Therefore, elevating cAMP and phosphorylating VASP are very useful for evaluating the antiplatelet effect of substances or compounds. For instance, a major catechin analogue, (-)-epigallocatechin-3-gallate (EGCG) from green tea, is known to produce cAMP via adenylyl cyclase activation and subsequently phosphorylates VASP-Ser157 through A-kinase activation to inhibit platelet aggregation (Ok et al., 2012). Furthermore, verapamil and theophylline have antiplatelet function that by elevating the cAMP level (Gasser et al., 1991), and are currently used to prevent and/or treat cardiovascular diseases as antiplatelet agents (Menshikov et al., 1993).

Rice bran is produced as a by-product in the rice milling process, a method in which the outer layer of the rice grain is removed. Rice bran has various biological effects like anti-inflammatory, cholesterol-lowering, antioxidant and anti-diabetic activities (Qureshi et al., 2002; Jun et al., 2012; Hou et al., 2010). In recent, it is reported that water extract (RB) from rice bran has a neuroprotective effect on ischemic brain injury (Baek et al., 2014).

In the present study, we investigated the effect of RB on VASP phosphorylation in ADP-induced platelet aggregation, and evaluated the anti-platelet effect.

**MATERIALS AND METHODS**

**Materials**

ADP was purchased from Chrono-Log Co. (Havertown, PA., USA). Sodium ferulate was purchased from AK Scientific Inc. (Union City, CA., USA). cAMP enzyme immunoassay (EIA) kit was 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 (Ser157), 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 (Piseatway, NJ., USA). Enhanced chemiluminesence solution (ECL) was from GE Healthcare (Chalfont St, Giles, Buckinghamshire, UK).

**Preparation of rice bran water-extract (RB)**

Rice bran was obtained from Gimhae Rice Processing Complex just after milling rice cultivar of Samkwang (Gimhae, Korea). To inactivate enzymes, rice bran was autoclaved at 121°C for 30 min. After cooling to room temperature, rice bran was vacuum-packed and stored in the freezer until use.

RB was prepared according to the following methods; 100 g of rice bran was mixed with 900 mL distilled water followed by hot water extraction at 121.5°C for 15 min. After cooling to room temperature, the mixture was centrifuged at 8,000 × g for 10 min. The supernatant was filtrated and concentrated to 54 brix by vacuum evaporation. The yield of water extract from RB was 2.6%. The resultant concentrate was designated as RB and kept in a refrigerator (4°C) until use. RB was dissolved in saline (0.9% NaCl) to investigate the effects on platelet aggregation.

**Determination of total phenolic content of RB**

Total phenolic content was determined by modified Singleton's method (Singleton et al., 1965). RB was dissolved 50% MeOH at the concentration of 0.1% (w/v). 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 saturated sodium carbonate solution (about 75 g/L) was added into the reaction mixture. After incubation at room temperature for 30 min, the mixture was centrifuged 3,000 rpm, and the supernatant was taken. The absorbance readings of the supernatant were taken at 765 nm. Gallic acid was used as a reference standard, and the results were expressed as milligram gallic acid equivalent (mg gallic acid)/100 g RB.

**Detection of phenolic compounds of RB with HPLC**

Because it is reported that rice bran contains phenolic compounds, we detected phenol compounds in RB with high performance liquid chromatography (HPLC) (Goufo and Trindade, 2014). RB was dissolved in distilled water (100 mg/mL), for the first time, and the pH of dissolved sample was set to 2~3 with 2N HCl, the sample was extracted three times with 0.5 mL ethylacetate, and was
concentrated by vacuum rotary and vacuum-dried, and then was dissolved with 0.5 mL methanol, and then it was analyzed by HPLC. An Agilent 1100 liquid chromatography system (Palo Alto, CA., USA), equipped with vacuum degasser, quaternary gradient pump, autosampler and diode array detector (DAD), connected to an Agilent ChemStation software. A TSKgel ODS-100V column (150 mm × 4.6 mm id, 5 μm, Tosoh, Japan) was used at a column temperature of 40°C. The mobile phase consisted of methanol (A) and 50 mM NaH2PO4 (B), pH 2.5 with phosphoric acid using the following program: 0~20 min, 30% A and 70% B. The flow rate was at 1.0 mL/min and sample injection volume was 5 μL. The UV detection was operated at 310 nm.

Preparation of washed human platelets

Human platelet-rich plasma (PRP) anti-coagulated with acid-citrate-dextrose solution (0.8% citric acid, 2.2% sodium citrate, 2.45% glucose) were obtained from Korean Red Cross Blood Center (Changwon, Korea). PRP was centrifuged for 10 min at 125 × g to remove a little red blood cells, and was centrifuged for 10 min at 1,300 × g to obtain the platelet pellets. The platelets were washed twice with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM 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 × 10⁸/mL. All of the above procedures were carried out at 25°C to avoid platelet aggregation from any effect of low temperatures. The Korea National Institute for Bioethics Policy Public Institutional Review Board (Seoul, Korea) approved these experiments.

Measurement of cAMP

Washed platelets (10⁹/mL) were preincubated for 3 min at 37°C with or without substances in the presence of 2 mM CaCl₂, and then stimulated with ADP (20 μM) for 5 min for platelet aggregation. The aggregation was terminated by the addition of 80% ice-cold ethanol. cAMP was measured with synergy HT multi-model microplate reader (BioTek Instruments, Winooski, VT., USA) using cAMP EIA kit.

Western blot for analysis of VASP-phosphorylation

Washed platelets (10⁹/mL) were preincubated with or without substances in the presence of 2 mM CaCl₂ for 3 min and then stimulated with ADP (20 μM) for 5 min at 37°C. The reactions were terminated by adding an equal volume (250 μL) of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₂EDTA, 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 Na₃VO₄, 1 μg/mL serine and cysteine protease inhibitor leupeptin, and 1 mM serine protease and acetylcholinesterase inhibitor phenylmethanesulfonyl fluoride, pH 7.5). Platelet lysates containing the same protein (15 μg) were used for analysis. Protein concentrations were measured by using bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology, USA). The effects of substances on VASP-phosphorylation were analyzed by western blotting. A 8~10% SDS-PAGE was used for electrophoresis and a PVDF membrane was used for protein transfer from the gel. The dilutions for anti-phosphor-VASP (Ser157), 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 (Bio-Rad, Hercules, CA., USA).
**Statistical analyses**

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 DISCUSSION**

**The contents of total phenolics and ferulic acid in RB**

When total phenolics was determined by using gallic acid as a standard, the contents of total phenolics in RB was contained, as shown in Table 1, 24.0 ± 1.0 mg in 100 g RB. Because it is reported that RB supplemented with ferulic acid has a synergistic neuroprotective effect in rat (Baek et al., 2014), we analyzed ferulic acid content in RB with HPLC. As shown in Fig. 1A, authentic ferulic acid was observed at 13.89 min, retention time, in HPLC chromatogram. As shown in Fig. 1B, the retention time (13.69 min) of peak F was almost in accord with that of ferulic acid. Accordingly, it is thought that peak F is a compound

<table>
<thead>
<tr>
<th>Total phenolic (TP) content (mg/100g RB)</th>
<th>Ferulic acid (FA) content (mg/100g RB)</th>
<th>FA/TP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB</td>
<td>24.0 ± 1.0</td>
<td>21.1</td>
</tr>
</tbody>
</table>

$\text{Table 1. Total phenolic content of RB}$

![Fig. 1. HPLC chromatograms of RB and ferulic acid. (A) The chromatogram of ferulic acid. (B) The chromatogram of RB. HPLC was performed as described in "Materials and Methods."}
corresponding to ferulic acid. The content of ferulic acid calculated from calibration curve, the content of peak F corresponding to ferulic acid was 21.1 mg/100 g-RB (Table 2), which was corresponded to 87.9% of total phenolic contents (Table 1).

**Effect of RB on ADP-induced platelet aggregation**

The concentration of ADP-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 (10⁷/mL) were activated with ADP (20 μM) in the presence of 2 mM CaCl₂, the aggregation rate was increased up to 64.5 ± 3.0%. However, various concentrations of RB (50 to 1,000 μg/mL) significantly inhibited ADP-stimulated platelet aggregation in a dose-dependent manner (Fig. 2B), and its the half-maximal inhibitory concentration (IC₅₀) was approximately 224.0 μg/mL (Fig. 2C).

**Effect of RB on cAMP production**

Intracellular cAMP is known as antiplatelet regulators. cAMP is produced by adenylate cyclase from ATP. cAMP

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**Table 2. Calibration curve and content of ferulic acid in RB**

<table>
<thead>
<tr>
<th></th>
<th>RT (min)</th>
<th>Calibration curve ⁴</th>
<th>r²</th>
<th>Test range (μg/mL)</th>
<th>Contents (mg/100g-RB)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Authentic compound</td>
<td>Ferulic acid</td>
<td>13.8</td>
<td>y=0.0002x+9.3815</td>
<td>0.9993</td>
<td>12.5~500</td>
</tr>
<tr>
<td>RB</td>
<td>Peak F</td>
<td>13.7</td>
<td>-</td>
<td>-</td>
<td>21.1</td>
</tr>
</tbody>
</table>

⁴, ⁵⁶ y, peak areas of analytes; x, concentrations of analytes in 100 mg/mL RB (μg/mL).

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**Fig. 2.** Effect of RB on ADP-induced platelet aggregation. (A) The concentration threshold of ADP on platelet aggregation. (B) Effect of RB pretreatment on ADP-induced platelet aggregation. (B) IC₅₀ value of RB on ADP-induced platelet aggregation. Measurement of platelet aggregation was carried out as described in "Materials & Methods" section. Inhibition rate by RB was recorded as percentage of the ADP-induced aggregation rate. IC₅₀ value of RB was calculated by 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 ADP-stimulated platelets.
inhibits platelet aggregation via cAMP/A-kinase pathway. If some substance enhances the production of cAMP, the substance could have anti-platelet effects via cAMP/A-kinase pathway. As shown in Fig. 3, ADP decreased intracellular cAMP level from 2.09 ± 0.17 pmoL/10⁹ platelets (basal level) to 0.99 ± 0.28 pmoL/10⁹ platelets. When platelets, however, were stimulated in the presence of both RB and ADP, the level of cAMP was increased to 2.38 ± 0.19 pmoL/10⁹ platelets by RB (250 µg/mL) (Fig. 3). ADP induces platelet aggregation by decreasing cAMP level. Therefore, the increase of cAMP by RB (Fig. 3) would contribute to the inhibition of ADP-induced platelet aggregation by RB (Fig. 2B).

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

If RB increased cAMP level by activating adenylate cyclase to inhibit ADP-induced platelet aggregation (Fig. 2B), then ADP-induced platelet aggregation would be increased in the presence of an adenylate cyclase inhibitor that inhibits the generation of cAMP. As shown in Fig. 4A, surprisingly, the platelet aggregation (28.7 ± 1.5%) by RB (250 µg/mL) plus ADP (20 µM) was increased by 51.7 ± 1.5% in the presence of adenylate cyclase inhibitor SQ22536 (50 µM). This result means that RB may elevate the cAMP level via activation of adenylate cyclase to inhibit ADP-induced platelet aggregation. Otherwise, the platelet aggregation (28.7 ± 1.5%) by RB (250 µg/mL) plus ADP (20 µM) would not be increased to 80.1% in the presence of adenylate cyclase inhibitor SQ22536 (50 µM) (Table 3). On the other hand, adenylate cyclase inhibitor SQ22536 did not significantly affect on ADP-induced platelet aggregation (Fig. 4B).

The inhibition of platelet aggregation by cAMP is caused via activation of cAMP-dependent protein kinase (A-kinase). Therefore, platelet aggregation would be increased in the presence of A-kinase inhibitor, and would be accord with that by cAMP inhibitor SQ22536. Accordingly, we investigated whether RB-inhibited platelet aggregation is increased by A-kinase inhibitor, Rp-8-Br-cAMPS. As shown in Fig. 4B, the platelet aggregation by RB (250 µg/mL) plus ADP (20 µM) was increased by A-kinase inhibitor, Rp-8-Br-cAMPS (150 µM). These results suggest that the inhibitory mode of ADP-induced platelet aggregation by RB is dependent on cAMP/A-kinase pathway. Otherwise, the platelet aggregation (28.7 ± 1.5%) in the presence of RB (250 µg/mL) and ADP (20 µM) would not be increased to 62.7% in the presence of A-kinase inhibitor Rp-8-Br-cAMPS (150 µM) (Table 3). On the other hand, A-kinase inhibitor Rp-8-Br-cAMPS did not significantly affect on ADP-induced platelet aggregation (Fig. 4B).

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

<table>
<thead>
<tr>
<th></th>
<th>ADP (20 µM) + RB (250 µg/mL)</th>
<th>ADP (20 µM) + RB (250 µg/mL) + SQ22536 (50 µM)</th>
<th>ADP (20 µM) + RB (250 µg/mL) + Rp-8-Br-cAMPS (150 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet aggregation (%)</td>
<td>28.7 ± 1.5</td>
<td>51.7 ± 1.5</td>
<td>46.7 ± 1.2</td>
</tr>
<tr>
<td>Δ (%)</td>
<td>0</td>
<td>+ 80.1</td>
<td>+ 62.7</td>
</tr>
</tbody>
</table>

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

Fig. 3. Effect of RB on cAMP production. Measurement of cAMP was carried out as described in "Materials & Methods" section. The data are expressed as the mean ± S.E.M. (n = 4). *P<0.05 versus the ADP-stimulated platelets.
Effect of RB on VASP phosphorylation

Downstream pathway of cAMP/A-kinase involves in phosphorylating VASP to inhibit 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, phosphorylation of Ser^{157} at 50 kDa of VASP is a useful indicator for monitoring cAMP/A-kinase pathway. ADP increased weakly the phosphorylation of VASP (Ser^{157}) [p-VASP (Ser^{157})] at 50 kDa and the ratio of p-VASP (Ser^{157}) to β-actin (Fig. 5A lane 2). It reflects that ADP involves in a 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 RB (150 and 250 μg/mL) (Fig. 5A lane 3, 4). As shown in Fig. 5A lane 5, both ADP (20 μM) and RB (250 μg/mL)-phosphorylated VASP (Ser^{157}) at 50 kDa [p-VASP (Ser^{157})] were inhibited by adenylate cyclase inhibitor SQ22536 (50 μM). In addition, the phosphorylation of VASP (Ser^{157}) by both ADP and RB
was also decreased in the presence of A-kinase inhibitor Rp-8-Br-cAMPS (Fig. 5A lane 6). These results indicate that RB phosphorylates VASP (Ser^{157}) through adenylate cyclase activation, cAMP elevation, and A-kinase activation. Otherwise, the ratio of VASP (Ser^{157}) phosphorylation to β-actin by both RB (250 μg/mL) and ADP (20 μM) would not be decreased in the presence of SQ22536 (50 μM), adenylate cyclase inhibitor, or Rp-8-Br-cAMPS (150 μM), A-kinase inhibitor, which were returned to the basal level, intact platelets (Fig. 5B). It is established that cAMP/A-kinase/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, both adenylate cyclase inhibitor SQ22536 and A-kinase inhibitor 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 regard to the regulatory effects of VASP (Ser^{157}) phosphorylation by phenolic compounds on platelet aggregation, epigallocatechin-3-gallate (Ok et al., 2012) and caffeic acid (Lee et al., 2014) also elevated cAMP level and phosphorylated VASP via cAMP/A-kinase pathway to inhibit platelet aggregation. RB contains ferulic acid, a phenolics, and seems to involve in cAMP-dependent phosphorylation of VASP (Ser^{157}) to inhibit platelet aggregation.

In the present study, however, it is unknown whether ferulic acid in RB directly involved in cAMP-dependent phosphorylation of VASP (Ser^{157}). These should be study in the future. There are reports that ferulic acid, and its derivatives has an antiplatelet effects (Yasuda et al., 2003; Wang and Ou-Yang, 2005), but its antiplatelet mechanism is unknown. Therefore, because RB contains 87.9% of ferulic acid among total phenolics (Table 1), the inhibition of ADP-induced platelet aggregation by RB (Fig. 2B) may be resulted from the action of ferulic acid in RB.

**Effect of ferulic acid on ADP-induced platelet aggregation**

It is inferred that ferulic acid in RB (Fig. 1B) may have inhibitory effect on ADP-induced platelet aggregation. Therefore, 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) corresponding to ferulic acid concentration that...
contains in RB (150, 250, 500 μg/ml). As shown in Fig. 6, ferulic acid (FA) dose dependently inhibited ADP-induced platelet aggregation. These concentration of FA that inhibited ADP-induced platelet aggregation is very low as compared with that by caffeic acid, an analogue of FA (Lee et al., 2014). These results suggest that antiplatelet effect of RB may be resulted from ferulic acid (Fig. 1B) in RB.

Antiplatelet drugs such as thienopyridine derivatives (i.e. ticlopidine, clopidogrel) have characteristics that phosphorylate VASP, which is mediated by cAMP or cGMP (Barragan et al., 2003). Therefore, it is thought that RB as well as thienopyridine derivatives may represent a useful tool in the therapy and prevention of vascular diseases associated with platelet aggregation.

Acknowledgements
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REFERENCES

Fig. 6. Effect of ferulic acid on ADP-induced platelet aggregation. Measurement of platelet aggregation was carried out as described in “Materials & Methods” section. The data are expressed as the mean ± S.E.M. (n = 4). * P<0.05 versus the ADP-stimulated platelets.
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