Inhibitory Effect of Ethanol Extract of *Piper longum* L. on Rabbit Platelet Aggregation through Antagonizing Thromboxane A₂ Receptor

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Injury to the blood vessel wall triggers rapid platelet activation and platelet plug formation, followed by the occurrence of blood coagulation and the formation of fibrin-containing thrombi that occlude the site of injury. These effects limit vital blood loss at the site of injured tissue, but may also block narrow or diseased vessels, leading to ischemia and/or tearing in vital organs. In addition, platelet aggregation and subsequent thrombus formation in coronary and cerebral arteries may cause myocardial infarction and stroke, respectively. Platelets play important roles in thrombus formation in injured blood vessels. Platelet aggregation is regulated by several physiological agonists such as 5-hydroxytryptamine (5-HT), epinephrine, adenosine 5'-diphosphate (ADP), and thromboxane A₂ (TXA₂) in a concentration-dependent manner. TXA₂, a metabolite of arachidonic acid with a chemical half-life of about 30 s, is released from activated platelets, binds to TXA₂ receptors and causes platelet shape change and aggregation as a positive feedback mediator. TXA₂ receptor, one of the G protein-coupled receptors, interacts with heterotrimeric G proteins G₁₁₁, G₁₂₀, and G₁₂₁. It is known that stimulation of the TXA₂ receptor results in platelet shape change mainly through the G₁₂₁ pathway and subsequent aggregation through the G₁₁₁ pathway. The activation of phospholipase C-β (PLC-β) via G₁₂₁ produces inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), as second messengers, through hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂). IP₃ increases intracellular Ca²⁺ concentration by releasing Ca²⁺ from storage sites and DAG activates protein kinase C (PKC).

TXA₂-induced platelet aggregation is important for thrombus formation in vascular beds, and because of this non-steroidal anti-inflammatory drugs (NSAIDs) and TXA₂ synthase inhibitor such as aspirin, indomethacin, and oazagrel are clinically used for reducing TXA₂-mediated platelet aggregation by decreasing TXA₂ synthesis in platelets.

Fingeridol, which has been used as a spice, contains various constituents such as piperine, piperlongumine, and β-(3,4,5-trimethoxyphenyl)propionic acid (TMPPA). *Piper longum* L. contains a constituent(s) that inhibits platelet aggregation as a non-competitive thromboxane A₂ receptor antagonist.

**Key words** *Piper longum* L.; platelet aggregation; thromboxane A₂ receptor

**MATERIALS AND METHODS**

**Materials** 9,11-Dideoxy-9α,11α-epoxymethano-prostaglandin F₂α (U46619) and [15-[1α,2α(Z),3α,4α]]-7-[2-[(phenylamino)carbonyl]hydrazine[methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-hepentoic acid (SQ29548) were obtained from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). [3H]Inositol and [3H]SQ29548 were from Perkin Elmer Life Science (Boston, MA, U.S.A.). Thrombin was from Wako Pure Chemicals (Osaka, Japan). All other chemicals used were of reagent grade or the highest quality available.

**Preparation of *Piper longum* L. Fractions** *Piper longum* L. (200 g) without crushing was extracted with 21 of hot water (90 °C) for 1 h, and the extract was dried by a rotary evaporator under reduced pressure followed by lyophilization (extract-A). In the same way, crushed *Piper longum* L. (200 g) was extracted with 21 of cold water (extract-B), 21 of hot water (90 °C) (extract-C), 21 of 30% ethanol (extract-D), 21 of 70% ethanol (extract-E), 21 of 100% ethanol (extract-
F), or 21 of 100% butanol (extract-G), and the extract was evaporated under reduced pressure followed by lyophilization. The obtained extracts were 20.8 g for extract-A, 19.8 g for extract-B, 28.1 g for extract-C, 19.0 g for extract-D, 17.5 g for extract-E, 14.4 g for extract-F, and 13.9 g for extract-G. The extracts were dissolved in 100% dimethylsulfoxide (DMSO) to make a concentration of 100 mg/ml, and were used with a concentration of DMSO <1%.

**Determination of Components in Piper longum L. Extracts** For the determination of piperine, Piper longum L. extracts were dissolved in 99% ethanol and analyzed by reversed-phase HPLC (Column ODS, Wakosil-II5c-18HG, 4.6×250 mm at 40 °C, UV determined at 345 nm). The flow rate was 1.0 ml/min, and the solvents used for separation were mixtures of acetonitrile/tetrachloroacetic acid (TFA) and 0.1% TFA/methanol, increasing 0.1% TFA/methanol (50—95% linear gradient).

**Preparation of Washed Platelets** Fresh blood was obtained from male rabbits (Japanese white rabbits weighing about 2.5—3.5 kg), collected into plastic tubes containing acid citrate dextrose solution (1/6 volume of blood), composed of citric acid (65 mM), trisodium citrate (85 mM), and dextrose (2%). Then, the blood was centrifuged at 250×g for 12 min to obtain platelet-rich plasma. The platelet-rich plasma was centrifuged at 90×g for 12 min at room temperature (20—25 °C). The pellet was washed twice with Tyrode/HEPES solution (NaCl 138.3 mM, KCl 2.68 mM, MgCl₂·6H₂O 1.0 mM, NaHCO₃ 4.0 mM, HEPES 10 mM, glucose 0.1%, and bovine serum albumin 0.35% at pH 6.35). The resultant pellet was suspended in the second wash buffer (Tris–HCl 10 mM, NaCl 140 mM, pH 7.4) and filtrated with a glass fiber filter (GC50, Advantec, Tokyo) under reduced pressure. After two additional washes with the washing buffer, radioactivity in the filter was counted by liquid scintillation spectrophotometer (Beckman, LS 6000 IC, Fullerton, CA, U.S.A.).

**Receptor Binding Assay** The platelets (1×10⁸ platelets) were incubated with [³H]SQ29548 (final 7.5 nM) and the Piper longum L. extract in 200 μl of Tyrode/HEPES solution (pH 7.35) at 37 °C for 5 min. The reactions were terminated by addition of 4 ml ice-cold washing buffer (Tris–HCl 10 mM, NaCl 140 mM, pH 7.4) and filtrated with a glass fiber filter (GC50, Advantec, Tokyo) under reduced pressure. After two additional washes with the washing buffer, radioactivity in the filter was counted by liquid scintillation spectrophotometer. Specific binding was obtained by subtracting nonspecific binding in the presence of 10 μM SQ29548.¹⁷

**Data Analysis** Data are expressed as means±S.E.M. Significant differences were determined by Tukey’s–Kramer or Scheffe test.

**RESULTS**

**Effects of Piper longum L. Extracts on U46619-Induced Platelet Aggregation** First, we examined the effects of several extracts of Piper longum L. on U46619-induced platelet aggregation (Fig. 1). As the results, hot water extract without crushed Piper longum L. (extract-A), cold water extract with crushed Piper longum L. (extract-B), and hot water extract (extract-C) at a concentration of 100 μg/ml slightly inhibited U46619 (3 μM)-induced platelet aggregation. On the other hand, 30% ethanol extract (extract-D) and 70% ethanol extract (extract-E) at a concentration of 100 μg/ml partially inhibited U46619-induced platelet aggregation. Moreover, 100% ethanol extract (extract-F) and 100% butanol extract (extract-G) at a concentration of 100 μg/ml with indicated concentrations of the Piper longum L. extract in the presence of LiCl (10 mM) for 5 min. Then, platelets were stimulated with U46619 for 15 min. The reactions were terminated by addition of an equal volume of ice-cold 10% trichloroacetic acid (TCA). The TCA extracts were washed three times with diethyl ether to remove TCA, and the diethyl ether was removed by keeping the samples at 47 °C for 30 min. [³H]inositol phosphates were separated by anion exchange column (Bio-Rad AG 1X-8, 100—200 mesh, formate form), as previously described.¹⁶ Radioactivity of the eluate was counted by liquid scintillation spectrophotometer (Beckman, LS 6000 IC, Fullerton, CA, U.S.A.).

**Measurement of Inositol Phosphates** Washed platelets in albumin-free Tyrode/HEPES solution (pH 7.35) were labeled with 25 μCi/ml [³H]inositol at 37 °C for 1 h. Then, platelets were washed with albumin-containing Tyrode/HEPES solution (pH 7.35), and suspended at 3×10⁸ platelets/ml. After preincubation for 10 min, platelets were treated with indicated concentrations of the Piper longum L. extract in the presence of LiCl (10 mM) for 5 min. Then, platelets were stimulated with U46619 for 15 min. The reactions were terminated by addition of an equal volume of ice-cold 10% trichloroacetic acid (TCA). The TCA extracts were washed three times with diethyl ether to remove TCA, and the diethyl ether was removed by keeping the samples at 47 °C for 30 min. [³H]inositol phosphates were separated by anion exchange column (Bio-Rad AG 1X-8, 100—200 mesh, formate form), as previously described.¹⁶ Radioactivity of the eluate was counted by liquid scintillation spectrophotometer (Beckman, LS 6000 IC, Fullerton, CA, U.S.A.).

**Determination of Platelet Aggregation** Platelet aggregation was determined by a standard turbidimetric method using an aggregometer (PAM-6C, Merbanix, Tokyo, Japan).⁴ Platelet aggregation was expressed as an increase in light transmission. The levels of light transmission were calibrated as 0% for a platelet suspension and 100% for the Tyrode/HEPES solution (pH 7.35). Platelet suspension (3×10⁸ platelets/ml, 0.3 ml) in a cuvette was preincubated at 37 °C for 3 min under continuous stirring at 1000 rpm then CaCl₂ was added at a final concentration of 1 mM for 3 min. After the preincubation of the Piper longum L. extract for 5 min, platelet aggregation was monitored for 10 min after the addition of U46619 or thrombin.
strongly inhibited U46619-induced platelet aggregation.

**Piperine, Piperlongumine, and TMPPA Contents in *Piper longum* L. Extracts** The above extracts of *Piper longum* L. contained piperine, piperlongumine, and TMPPA at different levels (Table 1). Contents of piperine and piperlongumine were high in relatively hydrophobic extract solutions. On the other hand, the content of TMPPA was similar in the extract solutions employed in the present study. The major constituent in 70% ethanol extract, 100% ethanol extract, and 100% butanol extract was piperine, and that in hot water extract without crushing, cold water extract, hot water extract, and 30% ethanol extract was TMPPA. Since 100% ethanol extract and 100% butanol extract had potent inhibitory activity on U46619-induced platelet aggregation, 100% ethanol extract was selected and used further to analyze the detailed mechanism.

**Effect of 100% Ethanol Extract on U46619- or Thrombin-Induced Platelet Aggregation** To examine the detailed mechanism of inhibitory activity, we compared the effect of 100% ethanol extract on U46619- and thrombin-induced platelet aggregations (Fig. 2A). Although the extract (200 μg/ml) inhibited U46619 (3 μM)-induced platelet aggregation, it did not inhibit that induced by thrombin (0.05 U/ml). Figure 2B shows the concentration–response curves for the 100% ethanol extract in inhibition of U46619-induced platelet aggregation. The extract inhibited U46619-induced platelet aggregation in a concentration-dependent manner with an IC_{50} value of about 70 μg/ml. On the other hand, the extract only slightly inhibited thrombin-induced platelet aggregation (Fig. 2B). In addition, the extract also slightly inhibited ADP- and 5-HT-induced platelet aggregation (data not shown), suggesting that the 100% ethanol extract selectively inhibited U46619-induced platelet aggregation.

**Inhibitory Mode of U46619-Induced Platelet Aggregation by 100% Ethanol Extract** Because the 100% ethanol extract inhibited U46619-induced platelet aggregation, we examined the inhibitory mode of U46619-induced platelet aggregation by the 100% ethanol extract. U46619 caused platelet aggregation in a concentration-dependent manner, and the maximum response to U46619 was reduced by the 100% ethanol extract in a concentration-dependent manner from 50 to 200 μg/ml (Fig. 3). Therefore the inhibitory mode of U46619-induced platelet aggregation by the 100% ethanol extract was considered non-competitive.

**Effect of 100% Ethanol Extract on U46619-Induced Platelet Aggregation**

Because the 100% ethanol extract selectively inhibited U46619-induced platelet aggregation, we examined the effect of the 100% ethanol extract on U46619-induced phosphoinositide hydrol-

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**Table 1. Contents of Piperine, Piperlongumine, and TMPPA in Several Extracts of *Piper longum* L.**

<table>
<thead>
<tr>
<th>Extract</th>
<th>Piperine (% w/w)</th>
<th>Piperlongumine (% w/w)</th>
<th>TMPPA (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract-A</td>
<td>0.2</td>
<td>0.1</td>
<td>3.0</td>
</tr>
<tr>
<td>Extract-B</td>
<td>0.2</td>
<td>0.0</td>
<td>2.8</td>
</tr>
<tr>
<td>Extract-C</td>
<td>0.5</td>
<td>0.3</td>
<td>3.4</td>
</tr>
<tr>
<td>Extract-D</td>
<td>2.5</td>
<td>0.5</td>
<td>4.9</td>
</tr>
<tr>
<td>Extract-E</td>
<td>15.6</td>
<td>1.2</td>
<td>3.2</td>
</tr>
<tr>
<td>Extract-F</td>
<td>15.9</td>
<td>2.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Extract-G</td>
<td>22.0</td>
<td>3.4</td>
<td>3.8</td>
</tr>
</tbody>
</table>

Extract-A, hot water extract without crushing of *Piper longum* L.; extract-B, cold water extract; extract-C, hot water extract; extract-D, 30% ethanol extract; extract-E, 70% ethanol extract; extract-F, 100% ethanol extract; extract-G, 100% butanol extract.

**Phosphoinositide Hydrolysis** It is known that stimulation of the TXA2 receptor causes activation of PLC-β through G_{q,11}, resulting in phosphoinositide hydrolysis. Because the 100% ethanol extract selectively inhibited U46619-induced platelet aggregation, we examined the effect of the 100% ethanol extract on U46619-induced phosphoinositide hydrol-
In the present study, we demonstrated that 100% ethanol extract (extract-F) and 100% butanol extract (extract-G) of *Piper longum* L. potently inhibited rabbit platelet aggregation in response to TXA₂ receptor agonist U46619 *in vitro* (Fig. 1). The 100% ethanol extract inhibited U46619-induced platelet aggregation in a concentration-dependent manner, while it only weakly inhibited thrombin-induced platelet aggregation (Fig. 2). Since the 100% ethanol extract also weakly inhibited ADP- and 5-HT-induced platelet aggregation, it seems that the extract selectively inhibits TXA₂ receptor-mediated platelet aggregation. It is known that U46619 causes platelet aggregation mediated mainly through TXA₂ receptor followed by G₉/₁₁, which activates PLC-β, resulting in the production of IP₃ and DAG as second messengers from PIP₂. Figure 4 shows that the 100% ethanol extract inhibited U46619-induced phosphoinositide hydrolysis and [³H]SQ29548 binding to TXA₂ receptor in a concentration-dependent manner. We obtained similar IC₅₀ values of the 100% ethanol extract of *Piper longum* L. to the TXA₂ receptor-mediated events; 70 µg/ml for U46619-induced platelet aggregation, 64 µg/ml for U46619-induced phosphoinositide hydrolysis, and 75 µg/ml for [³H]SQ29548 binding to TXA₂ receptor. Because the IC₅₀ value of the 100% ethanol extract of *Piper longum* L. on U46619-induced platelet aggregation was correspondent with the IC₅₀ on U46619-induced phosphoinositide hydrolysis and [³H]SQ29548 binding to TXA₂ receptor, the site of action of *Piper longum* L. ethanol extract is assumed the TXA₂ receptor.

On the other hand, the 100% ethanol extract only weakly inhibited aggregation induced by thrombin, ADP, or 5-HT. TXA₂ is released as a positive feedback mediator when platelets are activated by physiological agonists such as thrombin, ADP, and 5-HT. It has been shown that a TXA₂ receptor antagonist partially inhibits platelet aggregation induced by thrombin or ADP. Therefore it is assumed that the 100% ethanol extract can inhibit platelet aggregation partially induced by other stimulants except a TXA₂ receptor agonist.

HPLC analysis revealed that the 100% ethanol extract contained 15.9% piperine, 2.2% piperlongumine, and 0.9% TPMPA. When we used 200 µg/ml of the 100% ethanol extract, the concentration of piperine was 32 µg/ml (110 µM), piperlongumine was 4.4 µg/ml (14 µM), and TPMPA was 1.8 µg/ml (7.5 µM). To determine the active substance(s), we examined the effect of piperine (110 µM), piperlongumine (14 µM), or TPMPA (7.5 µM) on U46619-induced platelet aggregation, but none of these substances inhibited U46619-induced platelet aggregation (Iwashita and Nakahata, unpublished observation). These results suggest that *Piper longum* L. extracts contain active constituent(s) other than piperine, piperlongumine, and TPMPA.

TXA₂ is active in various conditions and diseases such as thrombogenesis, hypertension, bronchial asthma, and poor peripheral blood circulation mediated via its pharmacological actions of platelet aggregation and contraction of smooth muscle cells. The COX inhibitors aspirin and indomethacin, TXA₂ synthesis inhibitor ozagrel, and TXA₂ receptor antagonist seratrodast have been used for the treatment of these diseases. In the present study, we showed for the first time that the extract from *Piper longum* L. had an inhibitory effect on platelet aggregation as a TXA₂ receptor antagonist. These
results suggest that the extract from *Piper longum* L. contains some active substance useful for the treatment of the above-mentioned diseases through inhibition of TXA₂ receptor-mediated events.

In conclusion, the 100% ethanol extract of *Piper longum* L. inhibited U46619-induced platelet aggregation, phosphoinositide hydrolysis, and [³H]SQ29548 binding to TXA₂ receptor in a concentration-dependent manner with similar inhibitory potency. Therefore the ethanol extract of *Piper longum* L. contains a constituent(s) that inhibits platelet aggregation as a TXA₂ receptor antagonist.

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**REFERENCES**