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Antithrombotic and Antiallergic Activities of Rhaponticin from Rhei Rhizoma Are Activated by Human Intestinal Bacteria

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To evaluate the antithrombotic and antiallergic properties of rhaponticin extracted from Rhei Rhizoma, the *in vitro* and *ex vivo* inhibitory activities of rhaponticin and its metabolite, rhapontigenin, were measured. These compounds inhibited *in vitro* ADP- and collagen-induced platelet aggregation. Rhapontigenin was more potent, with IC_{50} values of 4 and 70 μg/ml, respectively. In *ex vivo* ADP- and collagen-induced rat platelet aggregation, these compounds also exhibited a potent inhibitory effect. The antiplatelet aggregation effects of rhaponticin and rhapontigenin were more potent than those of aspirin. Rhapontigenin showed significant protection from death due to pulmonary thrombosis in mice. Rhapontigenin also showed the strongest inhibitory activity against β-hexosaminidase release induced by DNP-BSA. These compounds inhibited PCA reaction in mice. Rhapontigenin intraperitoneally administered showed the strongest inhibitory activity and significantly inhibited PCA at doses of 25 and 50 mg/kg, with inhibitory activities of 48 and 85%, respectively. The inhibitory activity of orally administered rhaponticin was stronger than that of intraperitoneally administered rhaponticin. These results suggest that rhaponticin, in the rhizome of Rhei Rhizoma, is a prodrug that has extensive antiallergic and antithrombotic properties.

Key words: Antithrombosis, Antiallergy, Rhaponticin, Rhapontigenin, Rhei Rhizoma, Intestinal bacteria

INTRODUCTION

Because of its purgative, analgesic, antistroke and antiallergic activities, the rhizome of *Rheum undulatum* and *Rheum palmatum* (Family Polygonaceae) is frequently used in traditional herbal clinic as an ingredient in polyprescriptions, such as Chungpesagan-tang (Lee, 1996). Some anthraquinone and stilbene derivatives, sennoside A-F, chrysophanol, piceatannol and rhaponticin, were isolated from Rhei Rhizoma ad the main components (Okabe *et al.*, 1973; Kashiwata *et al.*, 1984; Ko *et al.*, 1995, 2000). Sennoside A-E have purgative effects, and stilbene derivatives possess antiallergic and anticoagulative properties (Matsuda *et al.*, 2001; Oshino *et al.*, 1978; Kim *et al.*, 2000).

Most herbal medicines are orally administered and their components are inevitably brought into contact with the intestinal microflora in the alimentary tract. Most of their components are metabolized by the intestinal bacteria before absorption from the gastrointestinal tract (Kobashi and Akao, 1997; Kim *et al.*, 2000). Sennoside A-E have purgative effects, and stilbene derivatives possess antiallergic and anticoagulative properties(Matsuda et al., 2001; Oshino *et al.*, 1978; Kim *et al.*, 2000)

Most herbal medicines are orally administered and their components are inevitably brought into contact with the intestinal bacteria before absorption. With regard to the metabolism of rhaponticin, we also reported that rhaponticin is transformed into rhapontigenin by the human intestinal microflora, and rhapontigenin may express antiallergic activity. Based on these findings, we believed that rhaponticin could be metabolized to its aglycone by intestinal bacteria, before being absorbed into the blood and being able to express its pharmacological effects. However, the relationship between its pharmacological properties, such as antithrombosis and antiallergy, and the metabolism of these compounds by human intestinal

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bacteria is not completely understood.

Therefore, we investigated the antithrombotic and antiallergic activities of Rhei Rhizoma, rhaponticin, and rhapontigenin, which is a metabolite of rhaponticin by human intestinal bacteria.

MATERIALS AND METHODS

Materials

Adenosine 5'-diphosphate (ADP), epinephrine, collagen, bovine serum albumin (BSA), prothrombin, thromboplastin, thrombin, hyaluronidase from bovine testis, *p*-nitrophenyl N-acetyl-β-D-glucosaminide, anti-dinitrophenol (DNP)-IgE, DNP-BSA, DNP-human serum albumin (HSA), Evans blue, hyaluronic acid potassium salts and disodium cromoglycate (DSCG) were purchased from Sigma Chemical Co. (USA). Rhaponticin was isolated from the rhizome of Rhei Rhizoma according to our previously reported method and rhapontigenin was prepared by the biotransformation of rhaponticin (Kim *et al.*, 2000). The other chemicals were all of analytical reagent grade.

Animals

Sprague-Dawley rats (male, 180-220 g) and ICR mice (male, 20-24 g) were purchased from Sam Yook Animal Co. (Korea) and acclimatized for 1 week at a temperature of 22 ± 1°C and a humidity of 55 ± 5% with free access to a commercial pellet diet (Samyang Co., Korea) and drinking water prior to the experiments. Animal experiments were carried out in accordance with international guidelines.

Preparation of platelets

Blood from rats was collected by cardiac puncture into a plastic flask containing 2.2% sodium citrate (1:9 v/v). Platelet rich plasma (PRP) was prepared by centrifugation of the blood at 120xg for 15min and further centrifuged at 850 xg for 10 min to prepare platelet poor plasma (PPP) (Aronson and Davidson, 1967). The supernatant was pooled and centrifuged at 600xg for 15min at room temperature. The platelet pellets were washed with modified Tyrode-HEPES buffer (129 mM NaCl, 2.8 mM KCl 3.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 2 mM EGTA, 5.6 mM glucose, 10 mM HEPES, 0.35% BSA, pH 7.4) and centrifuged at 600 x g for 15 min. The platelet pellets were then gently resuspended in Tyrode-HEPES buffer before being used in the experiment.

Assay of *in vitro* antiplatelet aggregation

Platelet aggregation was measured by turbidometry using a dual channel Whole Lumini-Ionized Calcium Aggregometer (Chrono-Log Co., Ltd, Havertown, PA, USA) according to the method of Born and Cross (1963). Briefly, rat PRP (300 μl) was incubated at 37°C for 2 min in the aggregometer, with stirring at 1200 rpm, and then stimulated with ADP and collagen. The samples and the aspirin reference agent were incubated with PRP for 3 min, followed by the addition of the aggregation agents. Changes in light transmission were recorded for 10 min after stimulation with these agents. Each inhibition rate was obtained from the maximal aggregation induced by respective agonist at several concentrations, and then the IC_{50} values were calculated from the data.

In vitro coagulation parameters

The plasma clotting times, activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT) were measured according to the manufactuers protocol. The plasma was incubated with the samples for 7 min at 37°C, and the coagulation was started by adding 100 μl of 20 mM $CaCl_2$, 100 μl of thromboplastin, and 100 μl of bovine thrombin into 100 μl of incubated plasma for the APTT, PT and TT assays, respectively

Assay of *ex vivo* antiplatelet aggregation

SD rats were used after overnight fasting. Rats were orally or intraperitoneally administered the samples (25 or 50 mg/kg). Blood was collected 3 h after sample treatment, PRP was isolated and platelet aggregation was measured. Platelet aggregation was induced by 80 μg/ml of collagen or 8 μM of ADP. The antiplatelet activities of the samples were investigated according to the method of Kimmura *et al.* (1985).

Assay of *in vivo* antithrombotic activity

The antithrombotic effects of the samples were investigated by the mouse thromboembolism test according to the method of DiMinno and Silver (1983). Male ICR mice were used after overnight fasting. The samples (25 and 50 mg/kg) and 0.5% CMC solution were administered orally. A mixture solution of collagen (110 μg) and epinephrine (13 μg) was injected into the mouse tail vein 90 min after sample treatment. The number of dead or paralyzed mice was recorded up to 15 min and the percentage of protection was calculated as follows: [1-(dead+paralyzed)/total] × 100

Assay of antiallergic activity in RBL-2H3 cell lines

The inhibitory activity of the samples against the release of β-hexosamindase from RBL-2H3 cells was evaluated according to Choi *et al.*(1996). RBL-2H3 cells were grown

in DMEM supplemented with 10% fetal bovine serum and L-glutamine. Before the experiment, cells were dispensed into 24 well plates at a concentration of 5×10^5 cells per well, using the medium containing 0.5 μg/ml of mouse monoclonal IgE, and were incubated overnight at 37°C in 5% CO_2 for the sensitization of the cells. The cells were washed with 500 μl of siraganian buffer (pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM PIPES, 40 mM NaOH) and incubated in 160 μl of siraganian buffer (5.6 mM glucose, 1 mM $CaCl_2$, 0.1% BSA were added) for an additional 10 min at 37°C. Then, the cells were exposed to 40 μl of the test materials for 20 min, followed by treatment with 20 μl of antigen (DNP-BSA, 1 μg/ml) for 10 min at 37°C, to cause the cells to evoke allergic reactions (degranulations). The reaction was stopped by cooling in an ice bath for 10 min. The reaction mixture was centrifuged and 25 μl aliquots of supernatant were transferred to 96 well plates and incubated with 25 μl of substrate (1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide) for 1 h at 37°C. The reaction was stopped by adding 0.2 ml of Na_2CO_3/$NaHCO_3$. The absorbance was measured by an ELISA reader at 405 nm.

PCA reaction

An IgE-dependent cutaneous reaction was measured according to the previous method of Katayama *et al.* (1978). The male ICR mice (25 - 30 g) were injected intradermally with 10 μg of anti-DNP IgE into each of four dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. Forty-eight hours later each mouse received an injection of 200 μl of 0.25% Evans blue PBS containing 100 μg of DNP-HSA *via* the tail vein. Test samples were administered 1 h prior to the DNP-HSA injection. Thirty minutes after the DNP-HSA injection, the mice were sacrificed and their dorsal skins were removed for measurement of the pigment area. After extraction with 1 ml of 1.0 N KOH and 9 ml of a mixture of acetone and phosphoric acid (5:13), the amount of dye was determined colorimetrically (absorbance at 620 nm).

RESULTS

Antithrombotic activities of Rhei Rhizoma, Rhaponticin and Rhapontigenin

When rhaponticin was incubated with human intestinal microflora, the main metabolite was rhapontigenin. Most human intestinal bacteria producing β-glucosidase potently transformed rhaponticin to rhapontigenin. Therefore, to evaluate the antithrombotic activity of rhaponticin, we measured the *in vitro* inhibitory effects of Rhei Rhizoma, rhaponticin and rhapontigenin on ADP- and collagen-induced platelet aggregation (Table 1). Rhei Rhizoma and these compounds inhibited ADP- and collagen-induced platelet aggregation. Rhapontigenin was the most potent, with IC_{50} values of 4 and 70 μg/ml, respectively. On *ex vivo* ADP- and collagen-induced rat platelet aggregation, these compounds also exhibited a potent inhibitory effects (Table 2). The antiaggregation activity of orally administered rhaponticin was weaker than that of intraperitoneally administered rhapontigenin. Rhei Rhizoma also inhibited *ex vivo* ADP- and collagen-induced platelet aggregation, however it only weakly inhibited *in vitro* ADP- and collagen-induced platelet aggregations. Aspirin, a reference drug, which is widely used as an antiplatelet drug in clinical practice, potently inhibited *in vitro* and *ex vivo* platelet aggregation. The antiplatelet aggregation effects of rhaponticin and rhapontigenin were more potent than those of aspirin.

When the *in vitro* inhibitory activity of these compounds against APTT, PT and TT-induced human plasma coagulation was measured, these compounds did not show inhibitory activity (Data not shown). The *in vivo* antithrombotic activities of these compounds by collagen and

Table 1. Effect of Rhei Rhizoma, Raphonticin and Raphontigenin on *in Vitro* Platelet Aggregation

Sample	IC_{50}[a] (mg/ml)	
	ADP[b]	Collagen[c]
Rhei Rhizoma	0.5	0.5
Rhaponticin	0.15	0.51
Rhapontigenin	0.004	0.07
Aspirin	0.03	0.16

[a]50% inhibitory concentration (IC_{50}) was calculated as follows: (control aggregation (%)-herbal medicine-treated aggregation (%)) / control aggregation (%) × 100 = inhibition (%)
[b]Final concentration was 0.008 mM.
[c]Final concentration was 0.08 mg/ml.

Table 2. Effect of Rhei Rhizoma, Rhaponticin and Rhapontigenin on *ex vivo* antiplatelet aggregation

Sample[a]	Dose(mg/kg)	Route	Platelet aggregation(%)	
			ADP[a]	Collagen[b]
Control	vehicle	-	58.0 ± 2.8	66.5 ± 2.1
Rhei Rhizoma	1000	*p.o*	47.0 ± 2.8	45.5 ± 4.9
Raphonticin	25	*p.o*	41.5 ± 21.9	-[c]
	50	*p.o*	16.5 ± 6.4	-
Rhapontigenin	5	*i.p*	44.0 ± 5.7	48.0 ± 2.8
Aspirin	50	*p.o*	47.5 ± 3.5	40.5 ± 3.4

[a] inal concentration was 0.008mM.
[b]final concentration was 0.08 mg/ml.
[c]not determined.
Samples were orally administered 3 h days before the test.
The results were expressed as mean SD (n=5).

Table 3. Anti-thrombosis activity of Rhei Rhizoma, Raphonticin and Rapontigenin

Sample	Dose(mg/kg)	Route	Protection(%)
Control	vehicle	-	20
Rhei Rhizoma	25	*p.o*	20
	50	*p.o*	40
Rhaponticin	25	*p.o*	60
	50	*p.o*	60
Rhapontigenin	25	*i.p.*	60
Aspirin	25	*p.o*	30
	50	*p.o*	60

The samples were orally administered 90 min before tail vein injection of epinephrine and collagen.

Table 4. Inhibitory Effect of Rhei Rhizoma, Raphonticin and Rapontigenin on the β-hexosamindase release from RBL 2H3 cells

Agent	IC_{50} (mM)
Rhei Rhizoma	0.32 mg/ml
Rhaponticin	>0.5
Rhapontigenin	0.03
DSCG	0.5

All values are means ± S.D. (n=3).

epinephrine were measured (Table 3). Rhaponticin and rhapontigenin showed significant protection from death due to pulmonary thrombosis in mice. The protective activity of rhaponticin was comparable to that of aspirin.

Antiallergic action of Rhaponticin and Rhapontigenin

The antiallergic activities of rhaponticin and rhapontigenin was measured by examining their inhibitory effects on the release of β-hexosaminidase from RBL 2H3 cells (Table 4). Rhapontigenin demonstrated the strongest inhibitory activity, on the β-hexosaminidase release induced by DNP-BSA, among the tested compounds. Its inhibitory activity was stronger than that of DSCG. However, rhaponticin did not show any inhibitory activity. To determine their inhibitory effects on PCA reaction in mice, these compounds were administered orally or intraperitoneally, 60 min prior to the challenge with antigen (Table 5). These compounds inhibited the PCA reaction in mice. Among the substances tested, rhapontigenin intraperitoneally administered showed the strongest inhibitory activity and significantly inhibited PCA at doses of 25 and 50 mg/kg, with an inhibitory activity of 48 and 85%, respectively. The inhibitory activity of orally treated rhaponticin was stronger than that of intraperitoneally treated rhaponticin.

When the inhibitory effects of rhaponticin and rhapontigenin on hyaluronidase activation, and their antioxidative activities were examined, rhapontigenin not only inhibited hyaluronidase, but also showed antioxidant activity on DPPH and free radical scavenging activities.

Table 5. Inhibitory Effect of Rhei Rhizoma, Raphonticin and Raphontigenin on PCA Reaction

	Dose (mg/kg)	Inhibition(%)	
		p.o.	*i.p.*
Rhei Rhizoma	250	31 ± 3	-[a]
Rhaponticin	100	53 ± 12	18 ± 3
Rhapontigenin	25	-	48 ± 9
	50	-	85 ± 14
DSCG	100	38 ± 12	-

[a]not determined.
All agents were administered *p.o.* or *i.p.* prior to challenge with antigen. Each experiment consisted of 5 observations.

Table 6. Hyaluronidase-Inhibitory and Antioxidant Activity of Rhaponticin and Rhapontigenin

Agent	IC_{50}(μM)			
	Hyaluronidase	DPPH	Superoxide anion	XOD
Rhei Rhizoma	>2000	-[a]	-	-
Rhaponticin	>2000	760	47	90
Rhapontigenin	140	280	8	10
Disodium cromoglycate	15200	-	-	-
Caffeic acid	-	14	2	-
Allopurinol	-	-	-	0.4

[a] not determined.

DISCUSSION

Rhei Rhizoma, as an ingredient in many polyprescriptions, has been used for its purgative, analgesic, antistroke and antiallergy properties. Its main components are sennosides and stilbenes. Rhaponticin, one of the stilbenes, is metabolized to rhapontigenin by human intestinal bacteria. Therefore, to evaluate the antithrombotic and antiallergic activities of rhaponticin and Rhei Rhizoma, we measured these activities for Rhei Rhizoma, rhaponticin and rhapontigenin.

Firstly, if blood vessels are damaged, platelet aggregation occurs rapidly to form haemostatic plugs or arterial thrombi at the sites of vessel injury or in the regions where the blood flow is disturbed. These thrombi are the source of the thromboembolic complications of arteriosclerosis, heart attacks, stroke, and peripheral vascular disease (Packham, 1994; Stein and Fuster, 1989; MacMahon and Sharpe, 1991). Therefore, the inhibition of the platelet function represents a promising approach for the prevention of thrombosis. In this study, rhaponticin and rhapontigenin exhibited an inhibitory

effect toward platelet aggregation *in vitro* and *ex vivo* and protected the mice from thromboembolism. Among these compounds, rhapontigenin, which is a metabolite of rhaponticin, transformed by human intestinal microflora prior to absorption in the intestine, was the more potent. Rhei Rhizoma also exhibited *in vitro* and *ex vivo* antiplatelet aggregation activity and protection against thromboembolism. Based on these findings, we can conclude that Rhei Rhizoma, and in particular rhapontigenin, in the case where rhaponticin is metabolized to rhapontigenin by human intestinal bacteria, could prevent the development of thrombosis or its recurrence.

Secondly, DSCG is an anti-allergic drug (Tasaka *et al.*, 1986). This drug inhibited the release of β-hexosaminidase from RBL-2H3 cells by DNP-BSA, which is an essential step in the pathological process of type I allergies (Choi *et al.*, 1996). These facts suggest that as powerful β-hexosaminidase release inhibitory substances, they can play a preeminent role in the development of new antiallergic drugs. In the present study, we investigated the inhibitory effects of rhaponticin and rhapontigenin on β-hexosaminidase release from RBL - 2H3 cells. These compounds inhibited β-hexosaminidase release. Among them, rhapontigenin, which is a metabolite of rhaponicin by human intestinal bacteria, was the more potent. Rhapontigenin de-monstrated potent antioxidant activity. These results support previous reports that antioxidants are useful for treating allergic diseases. Rhapontigenin also de-monstrated the most potent inhibitory effect on the PCA reaction. These results suggest that, when rhaponticin was administered orally, it was easily metabolized to rhapontigenin in the intestine, and the metabolite rhapontigenin was absorbed into the blood and was responsible for the inhibitory effect on the PCA reaction.

In this study, rhapontigenin, the metabolite of rhaponticin by human intestinal bacteria, demonstrated a potent inhibitory effect on thrombosis and allergy. Finally, we believe that rhaponticin in Rhei Rhizoma is a prodrug that has extensive antiallergic and antithrombotic properties.

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