Passive Cutaneous Anaphylaxis-Inhibitory Action of Tectorigenin, a Metabolite of Tectoridin by Intestinal Microflora

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Tectoridin isolated from the flowers of *Pueraria thunbergiana* (Leguminosae) are metabolized to tectorigenin by human intestinal microflora. When tectoridin was orally administered to rats, tectorigenin, but not tectoridin, was detected in urine after β-glucuronidase hydrolysis. The main metabolite tectorigenin potently inhibited the passive cutaneous anaphylaxis reaction and inhibited *in vitro* the release of β-hexosaminidase from RBL-2H3 cells induced by IgE. These results suggest that tectoridin is a prodrug, which can be transformed into the active agent tectorigenin by human intestinal bacteria and can be a candidate for antiallergic agent.

Key words tectoridin; tectorigenin; passive cutaneous anaphylaxis; intestinal bacteria; allergy

From the flowers of *Pueraria thunbergiana* (Leguminosae), which have been used in Chinese medicine,[1] Kurihara *et al.* and Kubo *et al.* isolated a series of isoflavonoids.[2—5] Recently, Park *et al.* have isolated glycitin, tectoridin, 6″-*O*-xylosyltectoridin, and 6″-*O*-xylosylglycitin from the flowers of *P. thunbergiana*.[6]

Most traditional medicines are administered orally, and components of these medicines inevitably come into contact with intestinal microflora in the alimentary tract. Most components are transformed by intestinal bacteria before being absorbed from the gastrointestinal tract.[7,8] The metabolism of the main components 6″-*O*-xylosyltectoridin and tectoridin isolated from the flowers of *P. thunbergiana* was studied. Tectoridin was transformed into tectorigenin by human intestinal bacteria (Fig. 1), and the transformant tectorigenin showed more potent cytotoxicity against tumor cells.[9] However, studies on metabolites of tectoridin in urine and blood were not performed. Therefore, we isolated the main metabolite in urine after tectoridin was orally administered to rats and investigated the inhibitory effect of tectoridin and its main metabolite tectorigenin on the passive cutaneous anaphylaxis (PCA) reaction.

MATERIALS AND METHODS

Materials and Cells RBL-2H3 rat basophil cells and RAW 264.7 murine macrophage cells were purchased from the Korean Cell Line Bank. Egg albumin, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, anti-dinitrophenyl (DNP)-IgE, DNP-human serum albumin (HSA), Evans blue, and disodium cromoglycate (DSCG) were purchased from Sigma Chemical Co. (U.S.A.). Griess reagent was purchased from Promega Co. (U.S.A.). Tectoridin was isolated from dried flowers (4.8 kg) of *P. thunbergiana* according to our previous method.[10]

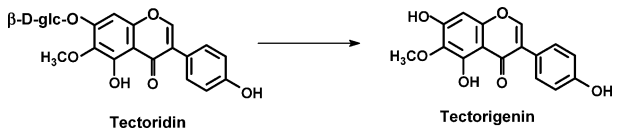

Fig. 1. Proposed Metabolic Pathway of Tectoridin by Human Intestinal Microflora

Tectoridin (Purity, >95%): Colorless needles (MeOH), mp >300 °C. $[\alpha]_D^{20}$ −46.0° [*c*=0.25, dimethyl sulfoxide (DMSO)]. UV λ_{max}^{MeOH} (log ε): 209.4 (4.39), 266.2 (4.46). IR ν_{max}^{Kbr} cm^{-1}: 3472 (OH), 1635 (α,β-unsaturated ketone), 1517, 1498, 1469, 1433 (aromatic C=C), 1100—1000 (glycoside). FAB-MS: 463 $[M+H]^+$.

TLC TLC of tectoridin and its metabolite tectorigenin was performed on silica gel plates (Merck, Silica-gel 60F254) with $CHCl_3$/MeOH (4 : 1). The chromatograms of these compounds were quantitatively assayed with a Shimadzu CS-9301 TLC scanner.

Animals Male Sprague–Dawley (SD) rats and Institute of Cancer Research (ICR) mice were supplied from Daehan Experimental Animal Breeding Center. All animals were housed in wire cages at 20—22 °C and 50±10% humidity, fed standard laboratory chow (Samyang Feed Production Co.), and allowed water *ad libitum*. All procedures relating to animals and their care conformed with the international guidelines Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) and Guiding Principles for the Care and Use of Laboratory Animals of Kyung Hee University, Korea.

Isolation of Metabolites To obtain the metabolites of tectoridin by human intestinal bacteria, a reaction mixture was prepared containing tectoridin 2.0 g and 5 g of fresh human feces in a final volume of 500 ml of anaerobic dilution medium in an anaerobic glove box (Coy Laboratory Products Inc., MI, U.S.A.). The mixture was incubated at 37 °C for 24 h and the reaction mixture was extracted three times with ethyl acetate. The EtOAc-soluble portion of the reaction mixture was dried on a rotary evaporator under reduced pressure and subjected to silica gel column chromatography (2.5×15 cm) with $CHCl_3$/MeOH (10 : 1—10 : 2). Tectoridin and tectorigenin (0.19 g) were obtained from these fractions incubated with tectoridin. Tectoridin and tectorigenin were identified according to the previously reported method.[6]

Tectorigenin (Purity, >97%): Yellowish amorphous solid, mp 230—233 °C. UV, λ_{max}^{MeOH} 264 nm (log ε 4.11), 237 nm (sh, log ε 3.18). IR, ν_{max}^{Kbr} 3447, 2921, 1648 and 1023 cm^{-1}.

Assay of Antiallergic Activity in RBL-2H3 Cells The

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inhibitory activity of test agents against the release of β-hexosaminidase from RBL-2H3 cells was evaluated according to the method of Choi *et al.*[11] RBL-2H3 cells were grown in DMEM supplemented with 10% fetal bovine serum and L-glutamine. Before experiments, the cells were dispensed into 24-well plates at the concentration of 5×10^5 cells/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 sensitization. The cells were washed with 500 μl of siraganian buffer [119 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 25 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), and 40 mM NaOH at pH 7.2] and incubated in 160 μl of siraganian buffer containing 5.6 mM glucose, 1 mM $CaCl_2$, and 0.1% BSA for additional 10 min at 37 °C. Then cells were exposed to 40 μl of test agents for 20 min, followed by the treatment with 20 μl of antigen (DNP-HSA, 1 μg/ml) for 10 min at 37 °C to activate cells to evoke allergic reactions (degranulation). The reaction was stopped by cooling in an ice bath for 10 min. The reaction mixture was centrifuged at 2000 rpm for 10 min 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 200 μl of 0.1 M Na_2CO_3/$NaHCO_3$ and the absorbance measured by ELISA reader at 405 nm.

PCA Reaction An IgE-dependent PCA reaction was measured according to the previous method of Katayama *et al.*[12] The male ICR mice (22—25 g) were injected intradermally with 10 μg of anti-DNP IgE into each of two 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 3% Evans blue PBS containing 200 μg of DNP-HSA *via* the tail vein. Test compounds were administered 1 h prior to the DNP-HSA injection. Thirty minutes after the DNP-HSA injection, the mice were killed and their dorsal skins removed for measurement of the pigmented area. After extraction with 1 ml of 1.0 N KOH and 4 ml of a mixture of acetone and 0.6 N phosphoric acid (13 : 5), the amount of dye was determined colorimetrically (absorbance at 620 nm).

Assay of Antioxidative Activity 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, superoxide anion radical generation, and xanthine oxidase (XO)-inhibitory activities of compounds isolated were measured according to the method of Xiong *et al.*[13]

Nitric Oxide Analysis Nitric oxide (NO) was determined by measuring the amount of nitrite from cell culture supernatant using the Griess reagent according to the manufacturer's protocol.[14] RAW 264.7 cells were stimulated with lipopolysaccharide (LPS) (1 μg/ml) and test compounds for 24 h. Cells were briefly centrifuged, and 150 μl of cell culture supernatant was mixed with 150 μl of Griess reagent and incubated 10 min at room temperature (light protected). The absorbance was measured using an ELISA reader at 540 nm against a calibration curve with sodium nitrate as a standard.

Pharmacokinetic Study Tectoridin (40 mg/rat) was orally administered to three male SD rats (200—220 g), and urine was collected continuously at 4, 8, 12, 24, 36, 48, and 60 h postdosing. The urine samples were treated according to the previously reported method.[15] The urine (3 ml) was incubated at 37 °C for 20 h with β-glucuronidase (10000 units, Sigma Co. MO, U.S.A.) in 1 ml of 1 M sodium acetate buffer (pH 4.5), and then applied on a preconditioned C_{18} solid-phase column (Sep-Pak, Waters Co., U.S.A.) preconditioned with 6 ml of 95% ethanol and 10 ml of distilled water. The column was washed with 4 ml of 10% methanol in distilled water and then eluted with 2 ml of methanol.

The eluted sample was analyzed by HPLC (Waters System: column, μ-Bondapak C_{18} (3.9×300 mm); elution solvent, methanol/water/glacial acetic acid (55 : 42.5 : 2.5); elution rate, 1.0 ml/min; detection wavelength, 280 nm) (Fig. 2). The retention times of metabolites were as follows: tectoridin, 6.41 min; tectorigenin, 11.45 min; and ponciretin (internal standard), 18.36 min.

Statistical Analysis All the data from the *in vivo* experiments are expressed as mean±standard deviation, and the statistical significance of differences was determined using Duncan's multiple-range test.

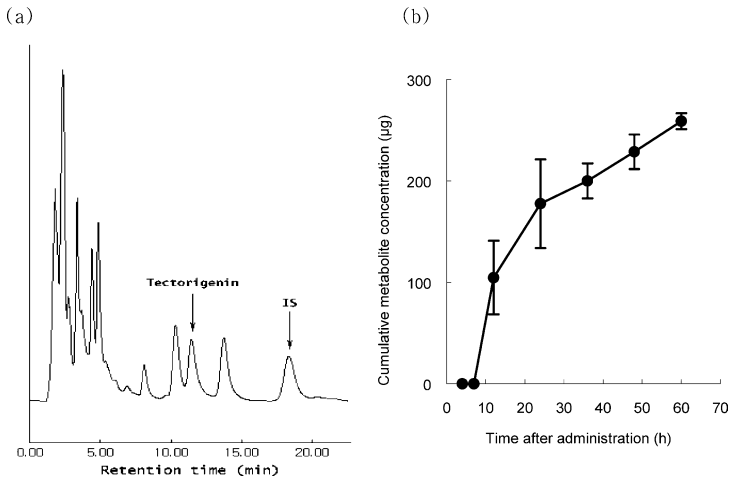

Fig. 2. HPLC Chromatogram (a) and Time Course of Tectorigenin Concentration (b) in the Urine of SD Rats

Tectoridin was administered to rats (200 mg/kg), and total urine was collected in metabolic cages (at 4, 8, 12, 24, 36, 48, 60 h). The urine was incubated for 24 h at 37 °C with β-glucuronidase. The level of tectorigenin was measured using HPLC (Waters system: column, μ-Bondapak C_{18} (3.9×300 nm); elution solvent, methanol/water/glacial acetic acid (55 : 42.5 : 2.5); elution rate, 1.0 ml/min; detection wavelength, 280 nm). The retention times of metabolites were as follows: tectoridin, 6.41 min; tectorigenin, 11.45 min; and ponciretin (internal standard), 18.36 min.

RESULTS

Metabolism of Tectoridin by Human Fecal Bacteria When tectoridin was incubated with human feces, it was metabolized to tectorigenin, as previously reported.[9] To understand the biotransformation of tectoridin in the entire body, tectoridin (250 mg/kg) was orally administered to rats and the compounds in urine were periodically analyzed by HPLC after β-glucuronidase hydrolysis. As shown in Fig. 1, the main compound detected in urine was tectorigenin. The total excreted was 0.8—1% of the orally administered tectoridin dose within 24 h. However, tectoridin was not detected.

Protective Effect of Tectoridin against the PCA Reaction To isolate antiallergic compounds from natural products, isoflavones from the flowers of *P. thungergiana* were orally administered to mice and their inhibitory activities against the *in vivo* PCA reaction were measured. Tectoridin showed the most potent inhibition.[4] Therefore we measured the inhibitory activity on β-hexosaminidase release from RBL-2H3 cells induced by IgE (Table 1). Tectoridin showed weak inhibitory activity. However, tectorigenin exhibited potent inhibition, with an IC_{50} value of 0.193 mM. Therefore we measured the PCA-inhibitory activity of tectorigenin (Table 2). Intraperitoneally administered tectorigenin showed potent inhibitory activity and significantly inhibited the PCA reaction at doses of 50 mg/kg with inhibitory activity of 98±14.4%. However, intraperitoneally administered tectoridin had weak inhibitory activity.

Inhibition of NO Production and Active Oxygen Radical-Scavenging Activity of Tectoridin and Tectorigenin When the effects of tectoridin and tectorigenin on the activity of hyaluronidase and their active oxygen radical-scavenging activity were investigated, these substances (0.2 mM) showed almost no inhibition of the activation of hyaluronidase and scavenging of active oxygens (Table 3).

The stimulation of RAW 264.7 cells with LPS also induced NO production. Tectoridin and tectorigenin weakly inhibited NO production in LPS-stimulated RAW 264.7 cells, although L-NMMA, which is a representative inhibitor, potently inhibited the enzyme activity (Table 3).

Table 1. Inhibitory Effects of Tectoridin and Tectorigenin on the Release of β-Hexosaminidase from RBL-2H3 Cells Induced by IgE

Agent	IC_{50} (mM)
Tectoridin	0.478
Tectorigenin	0.193
Dexamethasone	0.027
Azelastine	0.023

Table 2. Inhibitory Effects of Tectoridin, Tectorigenin, Disodium Cromoglycate (DSCG), and Azelastine on Passive Cutaneous Anaphylaxis in Mice

Agent	Dose (mg/kg)	Inhibition (%)	
		p.o.	i.p.
Tectoridin	25	14±6.4[b]	—
	50	54±5.1[d]	62±16.7[d,e]
Tectorigenin	25	—[a]	56±17.3[c,d]
	50	—	98±14.4[e,f]
Azelastine	10	81±4.3[e,f]	80±5.6[e,f]
	25	93±9.3[e,f]	—
DSCG	100	37±0.2[c,d]	—

All agents were administered *p.o.* or i.p. prior to challege with antigen. Values are expressed as mean±S.D. (n=5). *a*) Not determined. *b, c, d, e, f*) Those with the same letter are not significantly different ($p<0.05$).

Table 3. Antioxidative Effect and Inhibition of NO Production by Tectoridin and Tectorigenin

Agent	IC_{50} (μM)			
	DPPH	Superoxide anion	XO	NO production
Tectoridin	>1000	>1500	>100	0.53
Tectorigenin	989	134	76	0.17
Caffeic acid	0.4	0.4	—	—
Allopurinol	—[a]	—	0.04	—
L-NMMA	—	—	—	0.04

a) Not determined. DPPH, 1,1-diphenyl-2-picrylhydrazyl; XO, xanthine oxidase; NO, nitric oxide.

DISCUSSION

Allergic diseases such as asthma and atopic dermatitis are based on IgE-mediated pharmacologic processes of a variety of cell populations such as mast cell and basophils.[16] Degranulation of mast cells and basophils with antigen-crosslinked IgE causes anaphylaxis.[17] The present study evaluated the inhibitory activity of isoflavones, which are the main components in the flower of *P. thunbergiana*, against the PCA reaction. Orally administered tectoridin potently inhibited the PCA reaction. However, when their glycosides were administered intraperitoneally, PCA-inhibitory activity did not increase. Tectorigenin potently inhibited the PCA reaction. The inhibitory activity was more potent than that of other isoflavones, such as daidzein, which was also reported to be a PCA inhibitor.[18] Tectorigenin exhibited the most potent inhibitory activity. Although its potency is weaker than that of azelastine (10 mg/kg i.p.), it potently inhibited the PCA reaction. These results confirmed that tectorigenin exhibits potent inhibitory activity against β-hexosaminidase release from RBL-2H3 cells induced by IgE, but its glycoside tectoridin showed weak inhibitory activity. Tectorigenin not only weakly inhibited NO production, but also scavenged *in vitro* active oxygens, as in the previous reports.[10,19] Tectoridin and tectorigenin did not inhibit the activation of hyaluronidase at a concentration of 5 mM. These results suggest that the PCA-inhibitory mechanism of tectorigenin may be due to membrane stabilization and free radical-scavenging activity.

We found that tectoridin was quickly metabolized to tectorigenin when incubated with human intestinal bacteria in a previous study.[9] In the present study, we also found that when tectoridin was orally administered to rats, tectorigenin was detected in urine after β-glucuronidase hydrolysis. These results suggest that the isoflavone glycoside tectoridin can be easily transformed into its aglycone by intestinal microflora and the transformed aglycone may inhibit the PCA reaction. Therefore we believe that tectoridin is a prodrug that has antiallergic properties and that tectorigenin is a candidate the therapeutic agent for the treatment of IgE-induced atopic allergy.

REFERENCES

1) Kim T. J., "Korean Resources Plants," Vol. II, Seoul National University Press, Seoul, 1986, p. 322.
2) Kurihara T., Kikuchi M., *Yakugaku Zasshi*, **95**, 1283—1285 (1975).
3) Kurihara T., Kikuchi M., *Yakugaku Zasshi*, **93**, 1201—1205 (1973).
4) Kurihara T., Kikuchi M., *Yakugaku Zasshi*, **96**, 1486—1488 (1976).
5) Kubo M., Sasaki M., Namba K., Naruto S., Nishimura H., *Chem. Pharm. Bull.*, **23**, 2449—2457 (1975).
6) Park H. J., Park J. H., Moon J. O., Lee K. J., Jung W. T., Lee H. K., Oh S. R., *Phytochemistry*, **51**, 147—151 (1999).
7) Kobashi K., Akao T., *Biosci. Microflora*, **16**, 1—7 (1997).
8) Kim D.-H., Yu K.-W., Bae E.-A., Park H.-J., Choi J.-W., *Biol. Pharm. Bull.*, **21**, 360—365 (1998).
9) Bae E.-A., Han M. J., Lee K. T. , Choi J.-W., Park H.-J., Kim D.-H., *Biol. Pharm. Bull.*, **22**, 1314—1318 (1999).
10) Lee H.-W., Choo M.-K., Bae E.-A., Kim D.-H., *Liver Int.*, **23**, 221—226 (2003).
11) Choi O. H., Kim J. H., Kinet J. P., *Nature* (London), **380**, 634—635 (1996).
12) Katayama S., Shionoya H., Ohtake S., *Microbiol. Immunol.*, **22**, 89—101 (1978).
13) Xiong Q., Kadota S., Tani T., Namba T., *Chem. Pharm Bull.*, **19**, 1580—1595 (1996).
14) Choo M.-K., Park E.-K., Han M. J., Kim D.-H., *Planta Med.*, **69**, 518—522 (2003).
15) Han Y.-O., Han M. J., Park S.-H., Kim D.-H., *J. Pharmacol. Sci.*, **93**, 331—336 (2003).
16) Stevens R. L., Austen K. F., *Immunol. Today*, **10**, 381—386 (1989).
17) Ohmori H., Hideki M., Takaki T., *Eur. J. Immunol.*, **20**, 2499—2503 (1990).
18) Choo M.-K., Park E.-K., Yoon H.-K., Kim D.-H., *Biol. Pharm. Bull.*, **25**, 1328—1332 (2002).
19) Kim H. K., Cheon B. S., Kim Y. H., Kim S. Y., Kim H. P., *Biochem. Pharmacol.*, **58**, 759—765 (1999).