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Effect of Tectorigenin Obtained from *Pueraria thunbergiana* Flowers on Phase I and -II Enzyme Activities in the Streptozotocin-induced Diabetic Rat

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Abstract – Tectorigenin has an apoptosis-inducing ability and immunosuppressive activity. We investigated the effect of tectorigenin on Phase I and II enzyme activities to elucidate the pharmacological action of the immunosuppressive tectorigenin in the diabetic rat. This compound was obtained from the hydrolysis of tectoridin isolated from the flower of *Pueraria thumbergiana* (Leguminosae). This crude drug (Puerariae Flos) has been used as a therapeutic for diabetes mellitus in traditional Korean medicine. Tecotrigenin inhibited the formation of malondialdehyde (MDA) and hydroxy radicals in serum and liver but promoted superoxide dismutase (SOD) activity. Low MDA contents and low xanthine oxidase and aldehyde oxidase activities were observed in the tectorigenin-treated rats, suggesting that such Phase I enzyme activities are the major source of lipid peroxidation. However, tectorigenin increased Phase II enzyme activities such as SOD, glutathione peroxidase and catalase, suggesting the activation of free radical-scavenging enzymes. The activities of tectorigenin were comparable to those of glibenclamide, which was employed as a positive control. These results suggest that tectorigenin may share some biological properties with glibenclamide in insulin-dependent-diabetes mellitus (IDDM).
Keywords: tectorigenin, *Pueraria thunergiana*, Phase I and II, tissue factor, reactive oxygen species, diabetes mellitus

Introduction

The flowers of *Pueraria thunbergiana* has been used as an anti-thirst drug for the treatment of diabetes mellitus and lingering intoxication (Kim, 1986). Recently, it seems that the traditional medicinal term, anti-thirst drug, mainly implies a crude antidiabetic drug in Korea. Tectorigenin shown in Fig. 1 is a component, which is present mainly in a glycosidic form in this plant rather than as an aglycone (Park *et al.*, 1999). Tectorigenin was obtained in a large amount by the hydrolysis of tectoridin (tectorigenin 7-O-glucoside), which was easily isolated from the flowers. The flowers of *P. thunbergiana* also contain isoflavone glycosides (tectoridin, glycitin, 6-O-tectoridin, and 6-O-glycitin) (Park *et al.*, 1999) and the saponins (soyasaponins, and kaikasaponin III) which have been found in the Leguminosae (Kinjo *et al.*, 1988).

After the large-scale isolation of tectorigenin from the flowers, we carried out various biological activity tests. It has been reported that the tectorgenin obtained has many biological activities, including hypoglycemic (Lee *et al.*, 2000), antimutagenic (Park *et al.*, 2002) and *in vitro* antioxidant effects *in vitro* (Lee *et al.*, 1999). In addition, we previously reported that human intestinal bacteria metabolize tectorigenin glycosides into tectorigenin (Bae *et al.*, 1999), and elucidated the apoptosis-inducing effect of tectorigenin and its associated signal transduction (Lee *et al.*, 2001). It was also reported that tectorigenin has an *in vitro* anti-inflammatory effect, in a study of the inhibitory effect of nitric oxide, prostaglandin E_2 and cyclooxygenase in lipopoly-saccharide-induced macrophage cells (Shin *et al.*, 1999, You *et al.*, 1999).

Previous biological assay results indicate that tectorigenin decreases immune-enhancing mediators in macrophage cells, which is manifested in various ways. Based on the above findings, we undertook, for the first time, to elucidate the *in vivo* antioxidant and blood circulatory effect of tectorigenin in the streptozotocin(STZ)-induced diabetic rat by investigating the activities of Phase I and II enzymes. Interestingly, Lee *et al.* (1999) reported that glibenclamide used as a therapeutic for hypoglycemic has an apoptotic capability similar to that of tectorigenin. Therefore, we also compared the effects of tectorigenin and glibenclamide on Phase I and -II enzyme activities in the STZ-induced

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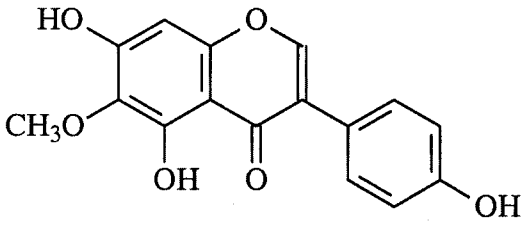

Fig. 1. Structure of tectorigenin obtained from *P. thunbergiana*.

diabetic rat.

Experimental

Chemicals – Tectorigenin was obtained by hydrolyzing of tectoridin easily, which was easily isolated by the crystallization of the n-BuOH fraction of the flower extract of *P. thunbergiana*, as previously reported (Lee *et al.*, 2000). NADPH, UDP-glucuronic acid (UDPGA), sodium xanthine, cytochrome C, N-methylnicotinamide and bovine serum albumin were from Sigma Co. (USA), malondialdehyde and 2-pyridone from Aldrich (USA), reduced- and oxidized glutathione from Fluka (USA), p-nitrophenol, 2,4-dinitrochlorobenzene, and thiobarbituric acid from Katayama (Japan).

Animals – Four week-old Sprague-Dawley male rats were purchased from Dae Han Biolink at Eumsung, Chungbuk, Korea. They were adapted to constant conditions (temperature: 20±2°C, humidity: 40-60%, light/dark cycle: 12 hr) for at least two weeks. The animals were fasted for twenty-four hours before the experiment, but supplied with water *ad libitum*. Because of dural enzyme activity variations, the animals were sacrificed at a fixed time (10:00 A.M.-12:00 A.M.).

Diabetic rats and tectorigenin treatment – Hyperglycemic rats were induced by injecting STZ (50 mg/kg) dissolved in 0.01 M citrate buffer into the tail vein. One week later, rats showing blood glucose concentrations of more than 300 mg/ml were considered hyperglycemic. Tectorigenin was administered intraperitoneally to hyperglycemic rats at 5 mg/kg or 10 mg/kg, respectively for a week. Likewise, glibenclamide was treated at 5 mg/kg (i.p.). Final blood samples were obtained after anesthetizing with CO_2 and were collected from the abdomianl aorta. After the blood glucose levels were measured, the sera were submitted for the assays as described in the following.

Measurement of lipid peroxide, hydroxy radical and SOD activity in serum-1) Lipid peroxide in serum: This content was measured according to the method of Yagi *et al.* (1987). After preincubating serum with 1/12N H_2SO_4 and 10% phosphotungsic acid for 5 min, it was centrifuged and the protein pellet obtained was resuspended in 1/12N H_2SO_4- and 10% phosphotungstic acid again the procedure repeated. The protein pellet was then resuspended in 1 ml distilled water, 0.67% thiobarbituric acid and 50% acetic acid at 95°C for 50 min, 5 ml of n-BuOH was added and the whole allowed to stand at room temperature. The red n-BuOH layer obtained cetrifuging for 10 min was analyzed by measuring the absorbance using spectrophotometer (Ex: 515 nm, Em 553 nm). Malondialdehyde (MDA; unit, nmole/ml of serum) was obtained from a standard curve.

2) Hydroxy radical: This was measured according to the method of Tateishi *et al.* (1987). Briefly, 333.3 μl of solution composed of 34.8 μl serum, 0.54 M NaCl. 0.1M potassium phosphate buffer (pH 7.4), 10 mM NaN_3, 7 mM deoxyrebose, 5 mM ferrous ammonium sulfate and distilled water was vortexed and stood at 37°C for 15 min. Sixty seven μl of this mixture was added to a solution of 75 μl 8.1%-sodium dodecyl sulfate, 67 μl of 20% acetic acid, and distilled water and then 222 μl of 1.2% thiobarbituric acid was added to this solution. The final solution was boiled for 30 min and cooled to room temperature before being centrifuged at 700×g for 5 min. The absorbance of the supernatant was determined at 532 nm using a spectrophotometer and concentration of hydroxy radical (unit: nmole/mg protein) present was calculated from a standard curve.

3) SOD activity: This was determined using the method of Oyanagui *et al.* (1984). Serum was 100-fold diluted with potassium phosphate buffer and 100 μl of the diluted solution was poured into a test tube and again the solutions of 200 μl of A (3 mM hydroxylamine/3 mM hypoxanthine) and 200 μl of B (7.5 mU/ml xanthine oxidase with 0.1 mM EDTA-2Na) and 500 μl distilled water added to the test tube was vortexed and then stood in 37°C water for 40 min. Two ml of solution C (300 mg of sulfanilic acid/5.0 mg N-1-naphtyl-ethylenediamine in 500 ml of 16.7% acetic acid) was then added to the final solution and the mix allowed to stand at room temperature for 20 min. The absorbance of the test solution was measured at 550 nm and the SOD activity in serum was calculated from a standard curve.

Measurement of hepatic lipid peroxide – The thiobarbituric acid (TBA) reactivity in the liver was measured as a marker of lipid peroxidation using the method of Ohkawa *et al.* (1979). An aliquot (0.4 ml) of 10% liver homogenate containing 0.9% NaCl was added to 1.5 ml of 8.1% SDS, 1.5ml of 20% acetate buffer (pH 3.5) and 1.5 ml of 0.8% TBA solution. The mixture was heated at 95°C for 1 h. After cooling, 5.0 ml of n-butanol:pyridine (15:1, v/v) was added for extraction, and the absorbance of the n-

butanol:pyridine layer was measured at 532 nm for the determination of TBA reactivity.

Measurement of hepatic xanthine oxidase (XO) and aldehyde oxidase (AO) (Phase I enzymes) activities – 1) XO Activity: The enzyme activity of xanthine oxidase was determined according to the Stripe and Dellas method (1969) (16). In brief, a mixture of 3.0 ml of 0.1M potassium phosphate buffer (pH 7.5) and 0.1 ml of 60 μM sodium xanthine (substrate) was reacted at 37°C. After the reaction, the protein of the mixture was removed by adding 20% trichloroacetic acid. The absorbance of the supernatant was measured at 292 nm, and the XO activity level was calculated using a standard calibration curve. Enzyme activity was expressed as nmoles of uric acid produced from 1 mg protein per minute. 2) AO Activity: The enzyme activity of aldehyde oxidase was determined according to the Rajagopalans method (1968). A mixture of 0.1 M potassium phosphate buffer (pH 7.5), N-methylnicotinamide (substrate) and enzyme solution was reacted. The absorbance of the produced 2-pyridone was recorded at 300 nm wavelenth. Enzyme activity was calculated using a standard calibration curve, and enzyme activity was expressed as nmoles of 2-pyridone produced of 1 mg of protein per minute.

Measurement of hepatic SOD, catalase, glutathione peroxidase (GPx) (Phase II enzymes) activities – 1) SOD Activity: The enzyme activity of superoxide dismutase was measured according to Marklunds method (1974). A solution of cytochrome C in 1.0 ml of 0.2 M potassium phosphate buffer (pH 8.6) containing 100 μM EDTA was allowed to stand on an ice bath for 20 min. A volume of 0.5 ml of alkaline DMSO or non-alkaline DMSO was added to the test or the blank solution, respectively, and the solution incubated at 37°C for 30 min. The absorbance of reducing cytochrome C was measured at 550 nm. Enzyme activity was defined as 1 unit for a 50% inhibition of the alkaline-mediated reduction of cytochrome C. 2) Catalase Activity: Catalase activity was determined according to Aebis method (1974). In brief, the reduction of 10 mM H_2O_2 (substrate) in 50 mM potassium phosphate buffer (pH 7.0) was estimated by the absorbance at 240 nm. Catalase activity was calculated using a molar absorption coefficient. The unit of enzyme activity was defined as the number of nmoles of hydrogen peroxide dissipated of 1 mg protein per min.; 3) GPx Activity: The enzyme activity of GPx was determined according to Paglia and Valentines method (1967). Enzyme solution was added to a mixture containing hydrogen peroxide and glutathione in 0.1mM of Tris buffer (pH 7.2) and followed by measuring the absorbance at 340 nm. Enzyme activity was calculated using a calibration curve. The unit of enzyme activity was expressed in nmoles of NADPH produced of 1 mg protein per min.

Results and Discussion

The effects of tectorigenin and glibenclamide on the lipid peroxide, the hydroxy radical and on SOD in the STZ-induced rat are shown in Table 1. The MDA content of the untreated group was observed to be 23.2±1.18 nmol/ml of protein whereas the value in the control was 48.9±3.20 nmol/ml of serum, which was approximately two-fold higher than normal. Tectorigenin significantly decreased the lipid peroxide level in the serum. The level of hydroxy radical (5.12±0.13 nmol/mg protein) in the control was also found to be twice as higher as that in the untreated rat (2.46±0.15 nmol/mg protein). Tectorigenin treatment significantly decreased the hydroxy radical of the control as well. STZ treatment decreased the value (3.06± 0.17 unit/mg protein) of the untreated group by 2.01±0.25 unit/mg protein whereas 5 and 10 mg/kg dose of tectorigenin significantly increased. Glibenclamide was found to have these effects as tectorigenin.

It has been reported that tectorigenin has anti-inflammatory, hepatoprotective and anti-diabetic effects, which may be associated with a reduction in reactive oxygen species *in vivo* and with its known *in vitro* immunosuppressive effect (Shin *et al.*, 1999, You *et al.*, 1999). Following on from our previous study upon the hypoglycemic effect of tectorigenin in STZ-induced diabetic rats (Lee *et al.*, 2000), we

Table 1. Effect of tectorigenin on the serum lipid peroxide, hydroxy radical content and superoxide dismutase activity in STZ-induced rat

Treatment	Dose (mg/kg)	LPO	Hydroxy radical[1)]	SOD[2)]
Normal		23.2 ± 1.18[3)]	2.46 ± 0.15	3.06 ± 0.17
STZ	50	48.9 ± 3.20a	5.12 ± 0.13	2.01 ± 0.25
Tectorigenin	5	38.6 ± 1.93*	3.72 ± 0.12**	2.47 ± 0.21
	10	34.2 ± 2.43*	3.52 ± 0.20**	2.74 ± 0.19*
Glibenclamide	5	26.4 ± 2.46**	2.37 ± 0.13***	2.97 ± 0.29**

[1)]Serum lipid peroxide content: MDA nmole/ml.
[2)]Serum hydroxy radical content: hydroxy radical/mg protein.
[3)]Values represent mean ± S.D. (n=6); Students *t* test compared with STZ: * $p<0.05$, ** $p<0.01$, *** $p<0.001$..

investigated the effects of tectorigenin on Phase I and II enzyme activities. The hypoglycemic and hypolipidemic effects of tectorigenin were confirmed in this experiment by measuring blood glucose and lipoprotein cholesterols (data not shown). In these studies, we found tectorigenin improves almost all biochemical parameters associated with the diabetic syndrome in this model.

By measuring the level of hydroxy radical and the activities of superoxide dismutase in the diabetic model by STZ treatment, we found tectorigenin decreases hydroxy radical levels and increases SOD activity. SOD converts the superoxide anions produced by the import of xenobiotics to hydrogen peroxide (Free *et al.*, 1980). Moreover, lipid peroxides released into the blood may cause vessel wall injury but are removed by the glutathione peroxidase-glutathione reductase system.

We also investigated the effects of tectorigenin on hepatic and serum Phase I and II enzymes, which are responsible for lipid peroxidation. Table 2 and 3 shows the effects of tectorigenin on lipid peroxide and Phase I and -II enzymes in the STZ-treated rat. The MDA of the control was 2.4-fold higher than in the untreated rats. Tectorigenin administration significantly decreased this lipid peroxidation. Table 3 shows the effects of tectorigenin on the activity of Phase II enzymes such as SOD, glutathione peroxidase and catalase in the STZ-treated rat. Tectorigenin treatment significantly increased those enzyme activities in the diabetic rat. Glibenclamide was found to have the same effects as tectorigenin.

In general, the detoxifying reactions in the liver mainly consists of Phase I reactions by the enzymes of the smooth endoplasmic reticulum, cytosol and mitochondria, and of Phase II reactions responsible for the conjugation. The cytosolic enzymes, xanthine oxidase and aldehyde oxidase, generate the superoxide anion radical, since these enzymes use oxygen as an electron acceptor. It is accepted that the superoxide anion radical is converted to hydrogen peroxide and finally cleaved to mainly toxic hydroxy radical. It appears that treatment with tectorigenin effectively decreased the level of hydroxy radical in the diabetic rat. Therefore, we suggest that these effects partly depend on the activity of the two hepatic cytosolic enzymes involved in the Phase I reactions. On the other hand, Phase II reactions are responsible for the final xenobiotic detoxification. Notable reactive oxygen species include the superoxide anion, the hydroxy radical and hydrogen peroxide (Batteli *et al.*, 1973). SOD plays a role in the metabolic conversion of the superoxide anion radical to hydrogen peroxide (Free *et al.*, 1973). Catalases highly expressed in the liver cleave hydrogen peroxide to harmless water and oxygen, and therefore the enzyme is an important free radical scavenging enzyme. The ubiquitously expressed glutathione peroxidase is also involved in such detoxification by converting peroxides to water (Lawrence and Burk, 1976), by using celluar antitoxidant glutathione as a substrate. Tectorigenin inhibited the decreases in SOD, catalase, and glutathione

Table 2. Effect of tectorigenin on the hepatic lipid peroxide, phase I enzyme activities in STZ-induced rat

Treatment	Dose (mg/kg)	LPO[1)]	XO[2)]	AO[3)]
Normal		20.4 ± 0.98[4)]	2.20 ± 0.39	1.56 ± 0.10
STZ	50	48.3 ± 2.47	5.51 ± 0.45	4.33 ± 0.14
Tectorigenin	5	33.7 ± 2.11*	4.48 ± 0.37*	3.97 ± 0.17*
	10	26.3 ± 1.99**	4.17 ± 0.33*	2.36 ± 0.16**
Glibenclamide	5	24.2 ± 1.82***	3.46 ± 0.37**	2.08 ± 0.19***

[1)]Serum lipid peroxide content: MDA nmole/g of tissue.
[2)]Xanthine oxidase: uric acid nmole/mg protein/min.
[3)]Aldehyde oxidase: 2-pyridone nmole/mg protein/min.
[4)]Values represent mean ± S.D. (n=6); Students *t* test compared with STZ: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Table 3. Effect of tectorigenin on the phase II enzyme activities

Treatment	Dose (mg/kg)	SOD[1)]	GPx[2)]	Catalase[3)]
Normal		9.45 ± 0.36[4)]	266.3± 16.3	2.65 ± 0.33
STZ	50	4.27 ± 0.21	156.1 ± 20.1	1.74 ± 0.18
Tectorigenin	5	5.00 ± 0.20*	193.4 ± 10.7*	2.00 ± 0.15
	10	6.98 ± 0.41**	220.5 ± 19.47*	2.35 ± 0.16*
Glibenclamide	5	7.34 ± 0.26**	241.5 ± 18.3**	2.44 ± 0.22*

[1)]Serum superoxide dismutase activity: unit/mg protein/min.
[2)]Glutathione peroxidase: oxidized NADPH nmole/mg protein/min.
[3)]Decrease H_2O_2 nmole/mg protein/min.
[4)]Values represent mean ± S.D. (n=6); Students *t* test compared with STZ: * $p<0.05$, ** $p<0.01$.

peroxidase caused by STZ treatment. These findings suggest that the inhibitory effect of tectorigenin on lipid peroxidation in the rat is attributable to a decrease in Phase I enzyme activity and an increase of Phase II activity, which may be associated with the inhibitory effect of tectorigenin on the formation of signal mediators by macrophages (Shin *et al.*, 1999, You *et al.*, 1999).

Therefore, tectorigenin in the flowers of *P. thunbergiana* may treat or prevent *diabetes mellitus* by the regulating the hepatic Phase I and II enzymes associated with the disease. Taking our results and those of others, it appears that these effects may be associated with the known immunosuppressive action of tectorigenin. The similarities between the biological actions of tectorigenin and glibenclamide in *diabetes mellitus* suggest that their modes of action are closely related.

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