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Cytoprotective effect of tectorigenin, a metabolite formed by transformation of tectoridin by intestinal microflora, on oxidative stress induced by hydrogen peroxide

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Abstract

In the present study, the antioxidative properties of tectorigenin, a metabolite formed by transformation of tectoridin by intestinal microflora, were investigated. Tectorigenin was found to scavenge intracellular reactive oxygen species, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, and thus prevented lipid peroxidation. The radical scavenging activity of tectorigenin protected the viability of Chinese hamster lung fibroblast (V79-4) cells exposed to hydrogen peroxide (H_2O_2) via activation of extracellular signal regulated kinase (ERK) pathway. Furthermore, tectorigenin reduced the apoptotic cells formation and cell cycle arrest at G_2/M phase induced by H_2O_2. Tectorigenin increased the activities of cellular antioxidant enzymes like superoxide dismutase, catalase, glutathione peroxidase, and also increased their protein level. Taken together, these findings suggest that tectorigenin protected V79-4 cells against H_2O_2 damage, by enhancing the antioxidative activity and by activating ERK pathway.

Keywords: Tectorigenin; Reactive oxygen species; Antioxidant enzyme

1. Introduction

Isoflavones are biologically active compounds, which occur naturally in a variety of plants, with relatively high levels in soybean. Recently, Park et al (1999) have isolated a series of isoflavonoids; glycitin, tectoridin, 6-*O*-xylosyltectoridin, and 6-*O*-xylosylglycitin from the flowers of *Pueraria thunbergiana* (Leguminosae), which have been used in Chinese medicine. As most of the traditional medicines are administered orally, their components inevitably come into contact with intestinal microflora in the alimentary tract. Finally, most of the components are transformed by intestinal bacteria before being absorbed from gastrointestinal tract (Akao et al., 1994; Kim et al., 1998a). Tectoridin, a biologically active component, isolated from the flowers of *P. thunbergiana* was transformed into tectorigenin by human intestinal bacteria (Bae et al., 1999). The transformed tectorigenin showed more potent hypoglycemic activity, cytotoxicity against tumor cells, and anaphylaxis inhibitory activity than tectoridin (Bae et al., 1999; Park et al., 2004). These results suggested that tectoridin is a prodrug, which can be transformed into the active compounds by human intestinal bacteria.

Reactive oxygen species are known to cause oxidative modification of DNA, proteins, lipids and small cellular molecules and are associated with tissue damage and are the contributing factors for inflammation, aging, cancer, arteriosclerosis, hypertension and diabetes (Cooke et al., 1997;

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Darley-Usmar and Halliwell, 1996; Farinati et al., 1998; Laurindo et al., 1991; Nakazono et al., 1991; Palinski et al., 1995; Parthasarathy et al., 1992). Cells have developed a variety of antioxidant defense mechanisms, for protecting themselves against the detrimental effects of reactive oxygen species. Enzymatic defense mechanisms involve superoxide dismutase, which catalyzes dismutation of superoxide anion to hydrogen peroxide; catalase, which converts hydrogen peroxide into molecular oxygen and water, and glutathione peroxidase, which destroys toxic peroxides.

In the present study, we have investigated the protective effect of tectorigenin on cell damage induced by hydrogen peroxide and its protective mechanism.

2. Materials and methods

2.1. Preparation of tectoridin and tectorigenin

Tectoridin was isolated from dried flowers of *P. thunbergiana* according to previous method (Lee et al., 2003). To obtain the metabolites of tectoridin by human intestinal bacteria, a reaction mixture was prepared containing 2 g of tectoridin and *Bacteriodes spercoris* HJ-15, a human intestinal bacterium, in a final volume of 500 ml of anaerobic dilution medium in an anaerobic glove box (Coy Laboratory Products Inc., MI, USA). The reaction mixture was incubated at 37 °C for 24 h and was extracted three times with ethyl acetate. The ethyl acetate-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) as a mobile phase. Both tectoridin and tectorigenin (0.19 g) were obtained from these fractions (Fig. 1) and were identified according to the previously reported method (Park et al., 1999). The purity of tectorigenin (>98%) was analyzed by HPLC system (μ-Bondapak C18 (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). The retention time of tectorigenin was 11.45 min.

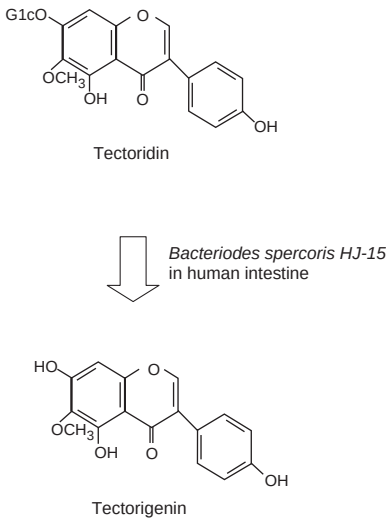

Fig. 1. Metabolic formation of tectorigenin. Tectorigenin is formed from the transformation of tectoridin by *Bacteriodes spercoris* HJ-15, a human intestinal bacterium.

The characteristics of tectorigenin are: yellow amorphous powder; mp 230–233 °C; UV $(MeOH)_{max}$ 264 nm (log ε 4.11), 237 nm (log ε 3.18); IR $(KBr)_{max}$ 3447, 2921, 1648, 1023 cm^{-1}

2.2. Reagents

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), and Hoechst 33342 were purchased from Sigma Chemical Company, St. Louis, USA, and thiobarbituric acid from BDH Laboratories, England. The other chemicals and reagents were of analytical grade. Primary rabbit polyclonal anti-ERK1/2 (44 kDa/42 kDa ERK) and -phospho-ERK1/2 (phosphorylated 44 kDa/42 kDa ERK) (Thr 202/Tyr 204) antibodies were purchased from Cell Signaling Technology (Beverly, USA). Primary sheep monoclonal superoxide dismutase, and catalase antibodies were purchased from Biodesign International Company (Maine, USA).

2.3. Cell culture

The Chinese hamster lung fibroblasts (V79-4) were obtained from the American Type Culture Collection. The V79-4 cells were maintained at 37 °C in an incubator, with a humidified atmosphere of 5% CO_2, and cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, streptomycin (100 μg/ml) and penicillin (100 units/ml).

2.4. Intracellular reactive oxygen species measurement

The DCF-DA method was used to detect the levels of intracellular reactive oxygen species (Rosenkranz et al., 1992). DCF-DA diffuses into cells, where it is hydrolyzed by intracellular esterase to polar 2′,7′-dichlorodihydrofluorescein. This non-fluorescent fluorescein analog gets trapped inside the cells and is oxidized by intracellular oxidants to a highly fluorescent, 2′,7′-dichlorofluorescein. The V79-4 cells were seeded in a 96-well plate at 1×10^5 cells/ml. Sixteen hours after plating, the cells were treated with various concentrations of tectoridin and tectorigenin and 30 min later, 1 mM H_2O_2 was added to the plate. The cells were incubated for an additional 30 min at 37 °C. The fluorescence of 2′,7′-dichlorofluorescein was detected at 485 nm excitation and at 535 nm emission, using a PerkinElmer LS-5B spectrofluorometer.

2.5. DPPH radical scavenging activity

Various concentrations of tectoridin and tectorigenin were added to a 1×10^{-4} M solution of DPPH in methanol, and the reaction mixture was shaken vigorously. After 5 h, the amount of remaining DPPH was determined at 520 nm using a spectrophotometer (Lo et al., 2004).

2.6. Lipid peroxidation inhibitory activity

Lipid peroxidation was assayed by thiobarbituric acid reaction (Ohkawa et al., 1979). V79-4 cells were seeded in a culture dish at 1×10^5 cells/ml. Sixteen hours after plating, the cells were treated with various concentrations of tectorigenin. One hour later, 1 mM H_2O_2 was added to the plate, and was incubated for

further 1 h. The cells were then washed with cold phosphate-buffered saline (PBS), scraped and homogenized in ice-cold 1.15% KCl. One hundred microliters of the cell lysates was mixed with 0.2 ml of 8.1% sodium dodecylsulfate, 1.5 ml of 20% acetic acid (adjusted to pH 3.5) and 1.5 ml of 0.8% thiobarbituric acid. The mixture was made up to a final volume of 4 ml with distilled water and heated to 95 °C for 2 h. After cooling to room temperature, 5 ml of *n*-butanol and pyridine mixture (15:1, v/v) was added to each sample, and the mixture was shaken well. After centrifugation at 1000×*g* for 10 min, the supernatant fraction was isolated, and the absorbance was measured spectrophotometrically at 532 nm.

2.7. Cell viability

The effect of tectorigenin on the viability of the V79-4 cells was determined using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide (MTT) assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase in the viable cells (Carmichael et al., 1987). To determine the cytoprotective effect of tectorigenin in H_2O_2 treated V79-4 cells, cells were seeded in a 96-well plate at 1×10^5 cells/ml. Sixteen hours after plating, the cells were treated with various concentrations of tectorigenin. One hour later, 1 mM H_2O_2 was added to the plate and incubated at 37 °C for an additional 24 h. Fifty microliters of the MTT stock solution (2 mg/ml) was then added into each well to attain a total reaction volume of 200 μl. After incubating for 4 h, the plate was centrifuged at 800×*g* for 5 min and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μl of dimethylsulfoxide and A_{540} was read on a scanning multi-well spectrophotometer. To determine the effect of tectorigenin on the viability of V79-4 cells during serum starvation, cells in 10% fetal calf serum were seeded in a 96-well plate at 1×10^5 cells/ml. Sixteen hours after plating, cells were serum starved (0.1% fetal calf serum), and then treated with various concentrations of tectorigenin. The plate was incubated at 37 °C for 24 h and the cell viability was measured using MTT test.

2.8. Flow cytometry analysis

Flow cytometry was performed to determine the content of apoptotic sub-G_1 hypo-diploid cells and cell distributions at each phase of a cell cycle (Nicoletti et al., 1991). The V79-4 cells were placed in a 6-well plate at 1×10^5 cells/ml. Sixteen hours after plating, the cells were treated with 10 μg/ml of tectorigenin. After a further incubation for 1 h, 1 mM H_2O_2 was added to the culture. After 24 h, the cells were harvested, and fixed in 1 ml of 70% ethanol for 30 min at 4 °C. The cells were washed twice with PBS, and then incubated for 30 min in dark at 37 °C in 1 ml of PBS containing 100 μg propidium iodide and 100 μg RNase A. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, USA). The proportion of sub-G_1 hypo-diploid cells and cell distributions at each phase of cell cycle was assessed by the histograms generated using the computer program, Cell Quest and Mod-Fit.

2.9. Nuclear staining with Hoechst 33342

The V79-4 cells were placed in a 24-well plate at 1×10^5 cells/ml. Sixteen hours after plating, the cells were treated with 10 μg/ml of tectorigenin and after further incubation for 1 h, 1 mM H_2O_2 was added to the culture. After 24 h, 1.5 μl of Hoechst 33342 (stock 10 mg/ml), a DNA specific fluorescent dye, was added to each well (1.5 ml) and incubated for 10 min at 37 °C. The stained cells were then observed under a fluorescent microscope, which was equipped with a CoolSNAP-Pro color digital camera, in order to examine the degree of nuclear condensation.

2.10. Superoxide dismutase activity

The V79-4 cells were seeded at 1×10^5 cells/ml, and 16 h after plating, the cells were treated with various concentrations of tectorigenin for 1 h. The harvested cells were suspended in 10 mM phosphate buffer (pH 7.5) and then lysed on ice by sonicating twice for 15 s. 1% of Triton X-100 was then added to the lysates and incubated for 10 min on ice. The lysates were separated, by centrifugation at 5000×*g* for 30 min at 4 °C in order to remove the cellular debris. The protein content of the supernatant was determined by the Bradford method (Bradford, 1976), with bovine serum albumin as the standard. The superoxide dismutase activity was used to detect the level of epinephrine auto-oxidation inhibition (Misra and Fridovich, 1972). Fifty micrograms of the protein was added to 500 mM of the phosphate buffer (pH 10.2) containing 0.1 mM EDTA and

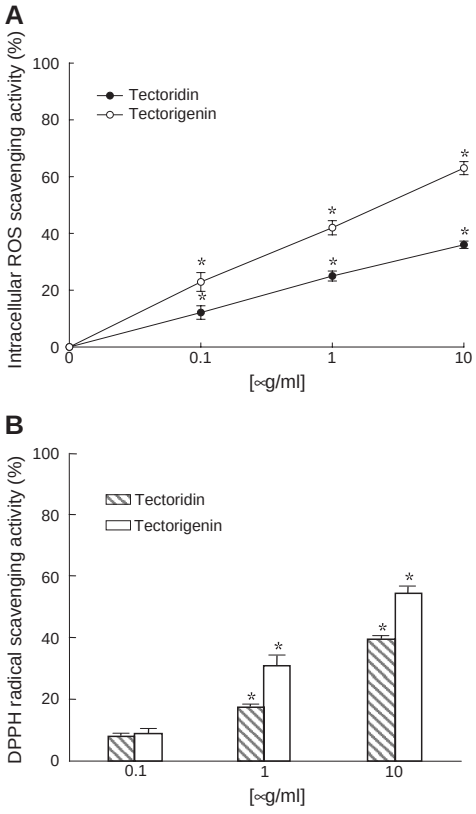

Fig. 2. Effect of tectoridin and tectorigenin on scavenging intracellular reactive oxygen species and DPPH radicals. (A) The intracellular reactive oxygen species generated was detected by DCF-DA method. (B) The amount of DPPH radicals was determined spectrophotometrically at 520 nm. The measurements were made in triplicate ($n=3$/group) and values are expressed as means±S.E.M. *Significantly different from control ($p<0.05$).

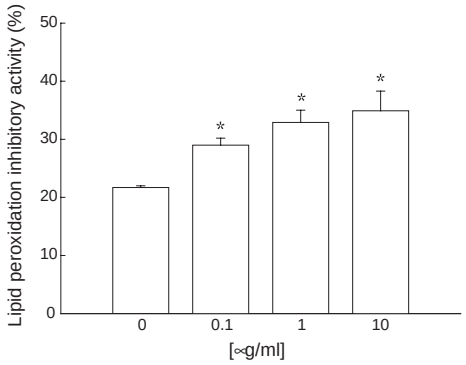

Fig. 3. Effect of tectorigenin on inhibition of lipid peroxidation. Lipid peroxidation was assayed by measuring the amount of malondialdehyde formation. The measurements were made in triplicate ($n=3$/group) and values are expressed as means±S.E.M. *Significantly different from control ($p<0.05$).

0.4 mM epinephrine. Epinephrine rapidly undergoes auto-oxidation at pH 10 to produce adrenochrome, which is a pink-colored product, measured at 480 nm using a UV/VIS spectrophotometer in kinetic mode. Superoxide dismutase inhibits the auto-oxidation of epinephrine. The rate of inhibition was monitored at 480 nm and one unit of enzyme activity was defined as the amount of enzyme required to produce 50% inhibition of enzyme activity. The superoxide dismutase activity is expressed as units/mg protein.

2.11. Catalase activity

Fifty micrograms of protein was added to 50 mM of phosphate buffer (pH 7) and 100 mM of H_2O_2 (v/v). This reaction mixture was incubated for 2 min at 37 °C and the absorbance was monitored at 240 nm for 5 min. The change in absorbance is proportional to the breakdown of H_2O_2 (Carrillo et al., 1991). The catalase activity is expressed as units/mg protein.

2.12. Glutathione peroxidase activity

Fifty micrograms of the protein was added to 25 mM of the phosphate buffer (pH 7.5), 1 mM EDTA, 1 mM NaN_3, 1 mM glutathione, 0.25 unit of glutathione reductase, and 0.1 mM NADPH. After incubation for 10 min at 37 °C, H_2O_2 was added to the reaction mixture at a final concentration of 1 mM. The absorbance was monitored at 340 nm for 5 min. The glutathione peroixdase activity was measured as the rate of NADPH oxidation at 340 nm (Paglia and Valentine, 1967). The glutathione peroixdase activity is expressed as units/mg protein.

2.13. Western blot

The V79-4 cells were placed in a plate at 1×10^5 cells/ml. Sixteen hours after plating, the cells were treated with 10 μg/ml of tectorigenin. The cells were harvested at the indicated times, and washed twice with PBS. The harvested cells were then lysed on ice for 30 min in 100 μl of a lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP 40] and centrifuged at 13,000×g for 15 min. Supernatants were collected from the lysates and protein concentrations were determined. Aliquots of the lysates (40 μg of protein) were boiled for 5 min and electrophoresed in 10% sodium dodecysulfate-polyacrylamide gel. Blots in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, USA), which were then incubated with primary rabbit monoclonal-ERK1/2, -phospho ERK1/2, primary sheep monoclonal superoxide dismutase, and -catalase antibodies. The membranes were further incubated with goat anti-rabbit or rabbit anti-sheep immunoglobulin G–horseradish peroxidase conjugates (Pierce, Rockland, USA), and then exposed to X-ray film. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (Amersham, Little Chalfont, USA).

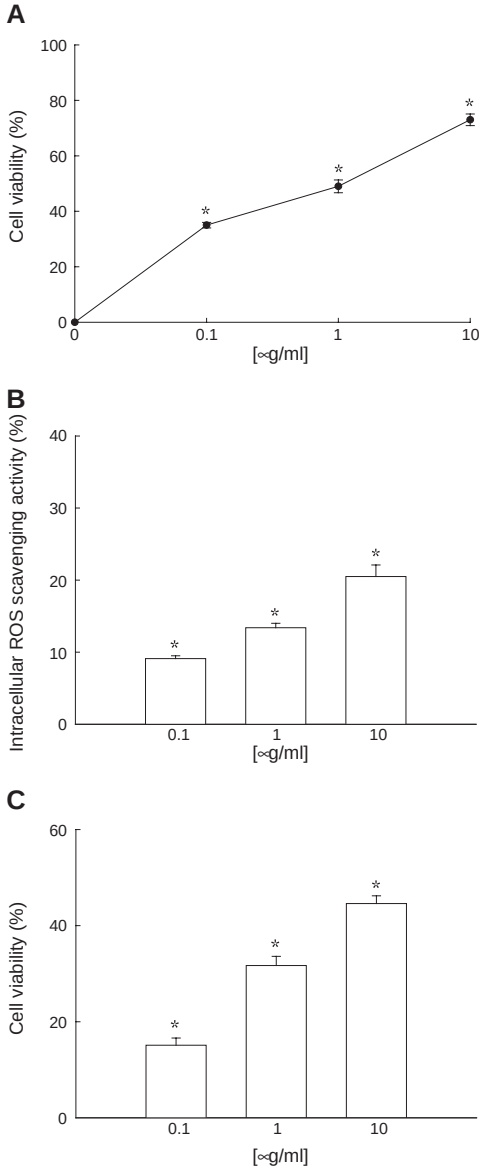

Fig. 4. Protective effect of tectorigenin on H_2O_2 or serum starvation induced oxidative damage of V79-4 cells. (A) The viability of V79-4 cells on H_2O_2 treatment was determined by MTT assay. (B) The intracellular reactive oxygen species generated by serum starvation was detected by DCF-DA method. (C) The viability of V79-4 cells on serum starvation was determined by MTT assay. The measurements were made in triplicate ($n=3$/group) and values are expressed as means±S.E.M. *Significantly different from control ($p<0.05$).

2.14. Nuclear extract preparation and electrophoretic mobility shift assay

The V79-4 cells were placed in plate at 1×10^5 cells/ml. Sixteen hours after plating, the cells were treated with 10 μg/ml of tectorigenin. The cells were harvested at the indicated times, and were then lysed on ice with 1 ml of lysis buffer (10 mM Tris–HCl, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, and 1% NP-40) for 4 min. After 10 min of centrifugation at 3000×*g*, the pellets were resuspended in 50 μl of extraction buffer (20 mM HEPES, pH 7.9, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, and 1 mM PMSF), incubated on ice for 30 min, and centrifuged at 13,000×*g* for 5 min. The supernatant was then harvested as nuclear protein extracts and stored at −70 °C after determination of protein concentration. Oligonucleotides containing transcription factor activator protein-1 (AP-1) consensus sequence (5′-CGC TTG ATG ACT CAG CCG GAA-3′) were annealed, labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase, and used as probes. The probes (50,000 cpm) were incubated with 6 μg of the nuclear extracts at 4 °C for 30 min in a final volume of 20 μl containing 12.5% glycerol, 12.5 mM HEPES (pH 7.9), 4 mM Tris–HCl (pH 7.9), 60 mM KCl, 1 mM EDTA, and 1 mM DTT with 1 μg of poly(dI–dC). Binding products were resolved on 5% polyacrylamide gel and the bands were visualized by autoradiography (Kim et al., 1998b).

2.15. Statistical analysis

All the measurements were made in triplicate and all values were represented as means±S.E.M. The results were subjected to an analysis of variance (ANOVA) using the Tukey test to analyze the difference. $p<0.05$ were considered as significant.

3. Results

3.1. Radical scavenging activity of tectoridin and tectorigenin

The radical scavenging effect of tectoridin and tectorigenin on the intracellular reactive oxygen species and DPPH free radical scavenging activities was compared. The intracellular reactive oxygen species scavenging activity of tectoridin was 12.1±2.3% at 0.1 μg/ml, 25.1±1.7% at 1 μg/ml, and 36.2±1.3% at 10 μg/ml (n=3/group). In case of tectorigenin, it was 22.9±3.3% at 0.1 μg/ml, 42.3±2.5% at 1 μg/ml, and 63.2±2.3% at 10 μg/ml (n=3/

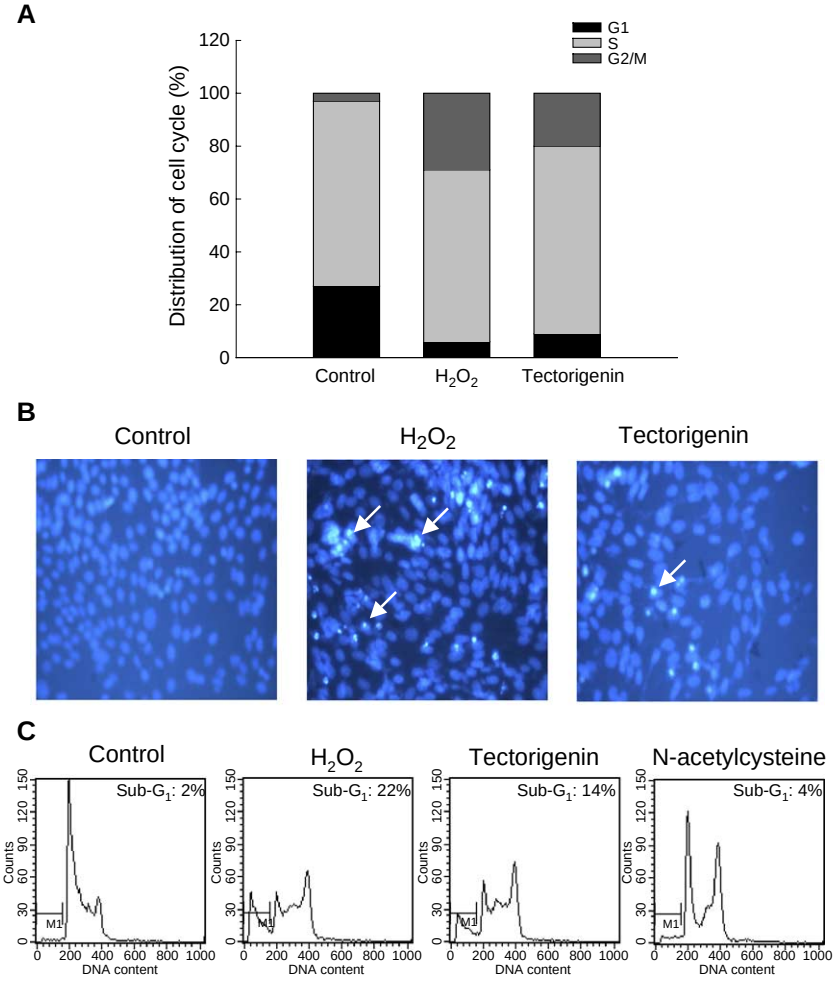

Fig. 5. Effect of tectorigenin on cell cycle arrest and apoptosis induced by H_2O_2 (A) The cell distribution at each phase of cell cycle was determined by flow cytometry. (B) Apoptotic body formation was observed under a fluorescent microscope after Hoechst 33342 staining and (C) apoptotic sub-G_1 DNA content was detected by flow cytometry after propidium iodide staining. Apoptotic bodies are indicated by arrows.

group) (Fig. 2A). *N*-acetylcysteine, which was used as a positive control, showed 83% inhibition of reactive oxygen species at 2 mM (data not shown). The reactive oxygen species scavenging activity of both the compounds was consistent with its DPPH radical scavenging activity (Fig. 2B). The DPPH radical scavenging activity of tectoridin showed 8.1±0.8% at 0.1 μg/ml, 17.3±1.1% at 1 μg/ml, and 39.3±1.3% at 10 μg/ml (n=3/group), and for tectorigenin, it was 9.0±1.2% at 0.1 μg/ml, 31.5±3.4% at 1 μg/ml, 54.3±2.3% at 10 μg/ml (n=3/group), when compared with 90% of *N*-acetylcysteine. However, the radical scavenging effect of tectorigenin in both the experiments was more effective when compared to tectoridin. From these results, we selected tectorigenin as active compound for further studies on radical scavenging effect.

3.2. Effect of tectorigenin on lipid peroxidation

The ability of tectorigenin to inhibit lipid peroxidation in H_2O_2 treated V79-4 cells was also tested. The generation of malondialdehyde, and related substances that react with thiobarbituric acid, was inhibited by tectorigenin. The activities of tectorigenin were 28.9±1.2% at 0.1 μg/ml, 32.9±2.1% at 1 μg/ml, and 34.9±3.4% at 10 μg/ml as compared to 21.7±0.3% in untreated group (n=3/group) (Fig. 3). However, inhibitory effect of *N*-acetylcysteine (31%) on lipid peroxidation was not so effective, when compared to its scavenging activity against H_2O_2 and DPPH radical.

3.3. Effect of tectorigenin on cell damage induced by H_2O_2 and serum starvation

The protective effect of tectorigenin on cell survival in H_2O_2 treated V79-4 cells was also measured. Cells were treated with tectorigenin at various concentrations for 1 h, prior to the addition to H_2O_2. The cell survival was determined 24 h later by the MTT assay. As shown in Fig. 4A, treatment with tectorigenin induced a dose-dependent increase in the cell survival; 34.8±1.1% at 0.1 μg/ml, 49.3±2.3% at 1 μg/ml, and 73.7±2.1% at 10 μg/ml (n=3/group). It is reported that serum starvation produces a marked accumulation of reactive oxygen species and results in cell death (Kang et al., 2003). We also examined whether tectorigenin showed the ROS scavenging effect and the protective effect on serum starved cells. The ROS scavenging effect by tectorigenin was determined after 6 h of serum starvation. As shown in Fig. 4B, tectorigenin showed the ROS scavenging activity of 9.1±0.4% at 0.1 μg/ml, 13.4±0.6% at 1 μg/ml, and 20.5±1.6% at 10 μg/ml (n=3/group). The cell survival was determined after 24 h of serum starvation. As shown in Fig. 4C, tectorigenin increased the cell survival: 15.1±1.5% at 0.1 μg/ml, 31.7±1.9% at 1 μg/ml, and 44.6±1.6% at 10 μg/ml (n=3/group). These results suggest that tectorigenin protects the cell damage induced by oxidative stress.

3.4. Effect of tectorigenin on cell cycle arrest and apoptosis induced by H_2O_2

The protective effect of tectorigenin was confirmed as cell distribution at cell cycle using flow cytometry. As shown in Fig. 5A, the cells exposed to H_2O_2 for 24 h induced cell cycle arrest at the G_2/M phase in 29%, when compared with 3% of control. The cells pretreated with tectorigenin prior to H_2O_2 treatment reduced the number of cells at the G_2/M phase to 20%. Cells pretreated with tectorigenin prior to H_2O_2 treatment recovered the cell population of S phase to 71%, when compared with 65% of the cells exposed only to H_2O_2. In order to study the cytoprotective effect of tectorigenin on apoptosis induced by H_2O_2, nuclei of V79-4 cells were stained with Hoechst 33342 for microscopy and with propidium iodide for flow cytometric analysis. The microscopic pictures in Fig. 5B show the control cells with intact nuclei, and the H_2O_2 treated cells showed significant nuclear fragmentation, a characteristic of apoptosis. However, when the cells were treated with tectorigenin for 1 h prior to H_2O_2 treatment, a dramatic decrease in nuclear fragmentation was observed. In addition to the morphological evaluation, the protective effect of tectorigenin against apoptosis was also confirmed by flow cytometry. As shown in Fig. 5C, an analysis of the DNA content in the H_2O_2 treated cells revealed an increase of 22% of apoptotic sub-G_1 DNA content. Treatment with 10 μg/ml of tectorgenin decreased the apoptotic sub-G_1 DNA content to 14%, showing 4% of the apoptotic sub-G_1 DNA content in *N*-acetylcysteine treated cells. These results suggest that tectorigenin protects cell viability by inhibiting H_2O_2 induced apoptosis.

3.5. Activation of ERK and AP-1 by tectorigenin

To better understand the protective mechanism of tectorigenin on V79-4 cells, the activation of ERK by Western blot analysis with phospho-ERK specific antibody was examined. As shown in Fig. 6A, tectorigenin activated phosphorylated ERK dramatically, within 3 h. However, there was no change in the total ERK protein level. Subsequently, we examined the effect of tectorigenin on DNA binding activity of AP-1, which is the downstream target of phospho-ERK pathway (Karin et al., 1997; Mukhopadhyay et al., 2001). As shown in Fig. 6B, AP-1 activity in tectorigenin treated cells was significantly increased. The

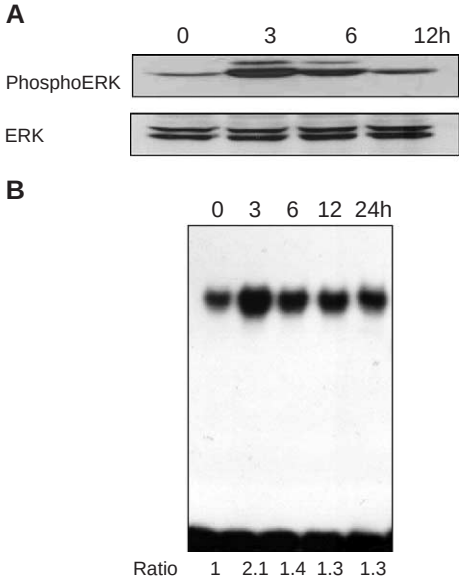

Fig. 6. Effect of tectorigenin on ERK and AP-1 activity (A) Cell lysates were electrophoresed and proteins of ERK1/2 and phospho-ERK1/2 were detected by their respective specific antibodies. (B) AP-1 specific oligonucleotide–protein complexes were detected by electrophoresis mobility shift assays. The values represent the normalized intensities of the AP-1 band against intensities of control band measured by bio-image analyzer system.

pattern of AP-1 activity was consistent with the pattern of activated ERK.

3.6. Effect of tectorigenin on the intracellular antioxidant systems

In order to investigate whether the radical scavenging activity of tectorigenin was mediated by the activities of antioxidant enzymes, the activities of superoxide dismutase, catalase and glutathione peroxidase in tectorigenin treated V79-4 cells were measured. Tectorigenin increased the activities of these three enzymes (Fig. 7A); in the superoxide dismutase activity, 23.5±1.2 U/mg protein at 0.1 μg/ml, 24.5±2.1 U/mg protein at 1 μg/ml, and 26.9±3.4 U/mg protein at 10 μg/ml, as compared to 13.5±0.3 U/mg protein of the control (n=3/group); in the catalase activity, 14.6±1.2 U/mg protein at 0.1 μg/ml, 17.3±1.1 U/mg protein at 1 μg/ml, and 24.7±3.1 U/mg protein at 10 μg/ml, as compared to 7.5±0.4 U/mg protein of the control (n=3/group); in the glutathione peroxidase activity, 12.5±1.1 U/mg protein at 0.1 μg/ml, 15.4±2.6 U/mg protein at 1 μg/ml, and 19.6±0.9 U/mg protein at 10 μg/ml, as compared to 11.7±0.5 U/mg protein of the control (n=3/group). To confirm the activation of superoxide dismutase and catalase by tectorigenin in terms of protein expression, the Western blot analysis was performed. As shown in Fig. 7B, the protein expressions of superoxide dismutase and catalase by tectorigenin were found to increase in a dose-dependent manner. The protein levels of the enzymes, superoxide dismutase (16 kDa) and catalase (42 kDa), induced by tectorigenin were consistent with the enzyme activity. The results show that the enhancement in antioxidant enzyme activities by tectorigenin may be associated with the inhibition of the production of reactive oxygen species.

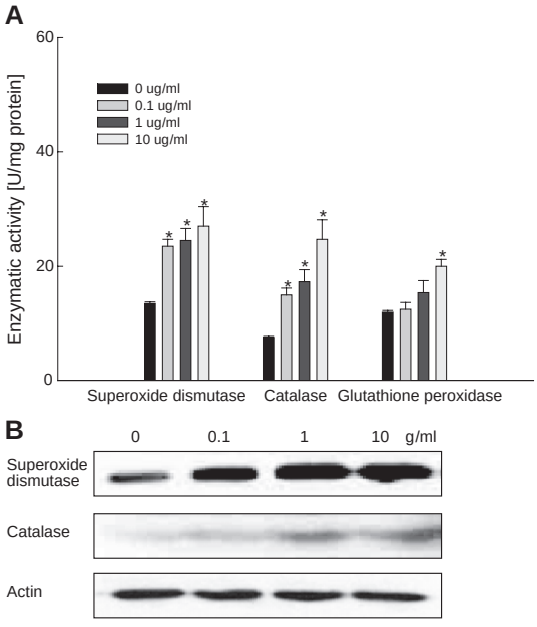

Fig. 7. Effect of tectorigenin on activity of antioxidant enzymes and its protein expression. (A) The detection methods for activities of antioxidant enzymes are described in Materials and methods. (B) Cell lysates were electrophoresed and proteins of superoxide dismutase and catalase were detected by their respective specific antibodies. The measurements were made in triplicate (n=3/group) and values are expressed as means±S.E.M. *Significantly different from control (p<0.05).

4. Discussion

In general, hydrophilic compounds with sugar moieties are not so easily permeable to the cellular membrane (Li et al., 1993), and hence glycosides are not easily absorbed form the gastrointestinal tract. In this study, tectorigenin (aglycon), a metabolite formed from the transformation of tectoridin (glycoside) by intestinal microflora, showed more potent antioxidant effect than tectoridin, suggesting that aglycon form may show active biological activity. Although some reports suggest that tectorigenin exhibit the antioxidant effect on free radical (Lee et al., 1999, 2000; Park et al., 2002), there is lack of evidence to show whether tectorigenin inhibits formation of reactive oxygen species on H_2O_2 induced cells. In our study, tectorigenin was found to decrease intracellular generation of reactive oxygen species and DPPH radical level, suggesting the enhanced viability of V79-4 cells in the presence of tectorigenin upon exposure to H_2O_2 and on serum starvation. This shows that tectorigenin scavenges intracellularly generated reactive oxygen species. The cells exposed to H_2O_2 exhibited distinct morphological features of apoptosis, such as nuclear fragmentation and an increase in 22% of sub-G_1 hypodiploid cells. However, cells that were pretreated with tectorigenin had significantly reduced percentage of apoptotic cells, as shown by morphology and 14% reduction in sub-G_1 DNA content. Our results are also consistent with the antioxidant activity of *N*-acetylcysteine, which also prevents H_2O_2 induced apoptosis, indicating that the inhibition of formation of reactive oxygen species may be important for cells to protect themselves against oxidative damage. The activation of ERK pathway induces cell proliferation, which leads to the induction of AP-1 activity, one of the transcription factors (Pages et al., 1991; Karin et al., 1997). Tectorigenin was found to significantly protect V79-4 cells against H_2O_2 induced damage, and the level of phosphorylated ERK and AP-1 activity was elevated in time-dependent manner, suggesting that the protective effect of tectorigenin on cells may be involved in activation of ERK pathway. Antioxidant enzymes like superoxide dismutase, catalase, and glutathione peroxidase play significant roles in effective augmentation of antioxidant defenses in cells. Superoxide dismutase converts superoxide radicals to hydrogen peroxide and subsequently to water by catalase and glutathione peroxidase. Tectorigenin was found to increase superoxide dismutase, catalase, and glutathione peroxidase activities and their protein levels, suggesting that the scavenging of reactive oxygen species may be related to the increased antioxidant activity. Therefore, the effects of tectorigenin on cell viability might involve dual actions: direct action on oxygen radical scavenging, as shown by DPPH radical scavenging, and indirect action through the induction of antioxidative enzymes.

In conclusion, tectorigenin, a metabolite of tectoridin, exerted intracellular reactive oxygen species and DPPH radical scavenging activity, promoted cell viability via

activation of ERK pathway, inhibited H_2O_2 induced apoptosis, and enhanced the effects of antioxidant enzymes.

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