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Protective effects of peony glycosides against corticosterone-induced cell death in PC12 cells through antioxidant action

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ABSTRACT

Aim of the study: Previous studies in our laboratory have shown that total glycosides of peony (TGP) produced antidepressant-like action in various mouse models of behavioral despair. However, the molecular mechanism by which TGP exerts antidepressant-like effect is not fully understood. This study examined the protective ffects of TGP against corticosterone-induced neurotoxicity in rat pheochromocytoma (PC12) cells and ts possible mechanisms.

Materials and methods: The direct antioxidant effect of TGP was investigated by using a 2,2′-azinobis-(3-ethylbenzothiazoline- 6-sulphonic acid) (ABTS) radical cation-scavenging assay in a cell-free system. PC12 cells were treated with 200 μM of corticosterone in the absence or presence of TGP in varying concentrations for 48 h. Cell viability, lactate dehydrogenase (LDH) activity, intracellular reactive oxygen species (ROS) level, malondialdehyde (MDA) content, glutathione (GSH) content, superoxide dismutase (SOD) activity, and catalase (CAT) activity were then determined.

Results: TGP displayed antioxidant properties in the cell-free system, and the IC50 value in the ABTS radical cation-scavenging assay was 9.9 mg/L. TGP treatment at increasing doses (1–10 mg/L) protected against corticosterone-induced cytotoxicity in PC12 cells in a dose-dependent manner. The cytoprotection afforded by TGP treatment was associated with decreases in the intracellular ROS and MDA levels, and increases in the GSH level, SOD activity, and CAT activity in corticosterone-treated PC12 cells.

Conclusion: The results suggest that TGP has a neuroprotective effect on corticosterone-induced neurotoxicity in PC12 cells, which may be related to its antioxidant action.

1. Introduction

Depression is a common psychiatric disorder, yet the pathogenesis of depression is not well understood. In recent years, a large number of experimental and clinical observations have supported the notion that hippocampal neuronal atrophy or destruction is involved in the pathogenesis of depression (Manji and Duman, 2001; Fuchs et al., 2004). Several putative mechanisms have been implicated in this damage. One is the hyperactivation of the hypothalamic–pituitary–adrenal (HPA) axis, which is characterized by elevated levels of circulating glucocorticoids in the blood (Sapolsky, 2000; Murray et al., 2008). It is well known that the HPA axis is activated in response to stress, which results in the increased concentration of glucocorticoid in the circulating blood. Under normal conditions, the blood glucocorticoid level is tightly regulated by a negative feedback mechanism. However, it has been reported that high concentrations of blood glucocorticoid are maintained in patients with depression due to the dysfunction of this feedback mechanism (Johnson et al., 2006). High glucocorticoid levels cause pathological damage to the hippocampal neurons both *in vitro* and *in vivo* (Sapolsky, 2000; Zhu et al., 2006a; Li et al., 2007; Murray et al., 2008), and can induce depression-like behavior in animals (Johnson et al., 2006; Murray et al., 2008). The PC12 cell line, which possesses typical neuron features and expresses a high level of glucocorticoid receptors, has been widely used in a variety of studies. It has been shown that high concentrations of glucocorticoid can induce cellular damage in PC12 cells (Li et al., 2003a,b, 2004; Zhu et al., 2006b), while different types of classical antidepressants have been demonstrated to protect against cytotoxicity induced by glucocorticoid in PC12 cells (Li et al., 2003b). This suggests that antidepressants may act by mitigating glucocorticoid-induced neurotoxicity.

The root of *Paeonia lactiflora* Pall. (Family: Ranunculaceae), commonly known as peony, is one of the most commonly used medicinal herbs in China, Korea, and Japan. Previous studies in our laboratory have demonstrated the antidepressant-like effect of ethanol extract of peony and total glycosides of peony (TGP) in mice using forced swim and tail suspension tests (Mao et al., 2008a,b). The antidepressive effect of TGP has also been observed in mice exposed to chronic mild stress (Mao et al., 2009a). However,

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the molecular mechanism by which TGP exerts its antidepressant-like effect is not fully understood. This study aimed to examine the neuroprotective effect of TGP on PC12 cells exposed to corticosterone, which is a principal glucocorticoid. Given that oxidative stress is considered to be a possible molecular mechanism involved in corticosterone-induced neurotoxicity (McIntosh et al., 1998a,b; Zafir and Banu, 2009) and is known to play an important role in the pathogenesis of depression (Bilici et al., 2001; Khanzode et al., 2003), this study also investigated whether the neuroprotective effects of TGP are related to its antioxidant properties using an ABTS radical cation-scavenging assay in a cell-free system and measuring the intracellular ROS level, MDA level, GSH level, SOD activity, and CAT activity in corticosterone-treated PC12 cells with and without TGP co-treatment.

2. Materials and methods

2.1. Drugs and reagents

TGP (light yellow brown powder) was supplied by Ningbo Liwah Pharmaceutical Co., Ltd. (Zhejiang, China). TGP contained 30% (w/w) of paeoniflorin and 10% (w/w) of albiflorin, as determined by high-performance liquid chromatography (Mao et al., 2008b, 2009a,b). A voucher sample (TGP071024) has been deposited in the School of Chinese Medicine for future reference. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) was obtained from Invitrogen (Carlsbad, CA, USA). 5,5-Dithiobis(2-nitrobenzoic acid), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), ABTS, corticosterone, Dulbecco's Modified Eagle Medium, and thiobarbituric acid were obtained from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum, heat-inactivated horse serum, penicillin, and streptomycin were purchased from Gibco (Grand Island, NY, USA). All other chemicals and reagents were of analytical grade.

2.2. ABTS radical cation-scavenging assay

The assay was performed according to the method of Ghanta et al. (2007) with minor modifications. The ABTS stock solution was prepared by reacting ABTS (7 mM) and potassium persulphate (2.45 mM) and allowing the mixture to stand for at least 16 h in the dark to generate ABTS radical cations. The working solution was prepared by diluting the stock solution with ethanol such that its absorbance reached 0.7 ± 0.02 at 734 nm ($A_{control}$). The reaction was performed in a 1 mL volume flask containing different concentrations of TGP in 10 μL volumes and 990 μL of ABTS working solution. Trolox was used as the standard. The absorbance (A_{sample}) at 734 nm was noted exactly 6 min after the reaction mixture was prepared. The percentage inhibition of radical formation due to the antioxidant properties of TGP was calculated using the formula: % inhibition = $[(A_{control} - A_{sample})/A_{control}] \times 100$. Three replicate tests were performed. The antioxidant activity of TGP was expressed as IC50, which was defined as the concentration of test material required to cause a 50% decrease in initial ABTS radical cation concentration.

2.3. Cell culture and treatment

PC12 cells obtained from the American Type Culture Collection (Rockville, MD, USA) were cultured as described elsewhere (Mao et al., 2009b). The experimental design contained five treatment groups: non-treated control, 200 μM of corticosterone, and 200 μM of corticosterone plus TGP (1, 5, or 10 mg/L). The concentration of corticosterone used was selected based on the results of Gao et al. (2008). The cells were seeded onto 6-well culture plates at a density of 2×10^6 cells/well, unless otherwise specified. The cells were stabilized at 37 °C for 48 h. They were then cultured in serum-free medium and incubated with the corresponding drugs for another 48 h.

2.4. Cell viability assay

Cell viability was measured by trypan blue exclusion assay according to the method of Cho et al. (2009). Briefly, at the end of the drug treatment, the cells were suspended. After centrifugation at $600 \times g$ for 6 min, the cells were resuspended in 200 μL of phosphate-buffered saline (PBS). The total cell suspension was mixed with 200 μL of 0.4% trypan blue staining solution for 5 min at room temperature. The cells were then loaded into a hemocytometer, and those exhibiting dye uptake were counted under a microscope. The percentage of stained cells was counted by scoring one hundred and fifty cells.

2.5. LDH activity assay

The release of LDH activity was measured as an *in vitro* marker for cellular toxicity. LDH activity was measured using a LDH diagnostic kit (STANBIO Laboratory, USA) according to the manufacturer's protocol. Briefly, PC12 cells were seeded in 24-well culture plates at a density of 1×10^5 cells/well. At the end of the drug treatment, the medium was collected. Subsequently, 100 μL of the medium was added to a polystyrene cuvette containing 1 mL of LDH reagent. The cuvette was placed immediately into a spectrophotometer and maintained at 30 °C. After stabilization for 1 min, the absorbance at 340 nm was recorded at 1 min intervals for 3 min. The change in absorbance was expressed in concentration units per liter. To determine the intra-cellular LDH activity, the cells were washed with D-Hanks solution and then scraped from the plates into 500 μL of ice-cold PBS (0.1 M, containing 0.05 mM of EDTA) and homogenized. The homogenate was centrifuged ($4000 \times g$) at 4 °C for 30 min. The resulting supernatant was collected for the LDH activity assay. LDH leakage was expressed as a percentage (%) of total LDH activity (LDH in the medium + LDH in the cell), according to the equation % LDH released = (LDH activity in the medium/total LDH activity) × 100.

2.6. Measurement of intracellular ROS level

The intracellular ROS level was measured using DCFH-DA (Yokozawa et al., 2007). DCFH-DA is a nonfluorescent compound that is enzymatically converted to the strongly fluorescent compound DCF in the presence of ROS. Briefly, PC12 cells were seeded onto a 96-well culture plate at a density of 2×10^4 cells/well. At the end of the drug treatment, the cells were washed with D-Hanks and incubated with DCFH-DA at a final concentration of 10 μM for 30 min at 37 °C in darkness. The cells were then washed twice with D-Hanks solution to remove the extracellular DCFH-DA, and the fluorescence intensity of the DCF was measured with a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

2.7. MDA, GSH, SOD, and CAT assay

PC12 cells were seeded onto 100-mm^2 dishes at a density of 5×10^6 cells/dish. At the end of the drug treatment, the cells were washed with D-Hanks, scraped from the plates into 1 mL of ice-cold PBS (0.1 M, containing 0.05 mM of EDTA), and homogenized. The homogenate was centrifuged at $4000 \times g$ for 30 min at 4 °C. The resulting supernatant was stored at −80 °C until required for analysis. The MDA content was measured as previously described (Okhawa et al., 1979). Briefly, 100 μL of the supernatant was mixed with 1.5 mL of acetic acid (20%, v/v, pH 3.5), 1.5 mL of thiobarbituric

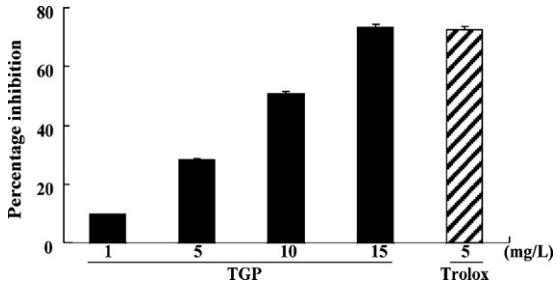

Fig. 1. Percentage inhibition of ABTS radical cations by TGP. The values given are the mean ± SD of three independent experiments.

acid (0.8%, w/v), and 200 μL of sodium dodecyl sulphate (8%, w/v). Each reaction mixture was heated for 60 min at 95 °C and cooled to room temperature. 5 mL of n-butanol was then added. After mixing and centrifugation at 3000 × *g* for 10 min, the organic layer was collected and the absorbance measured at 532 nm. The GSH level was measured according to the method of Gulati et al. (2007). Briefly, 100 μL of the supernatant was mixed with 200 μL of trichloroacetic acid (25%) and 200 μL of saline. The mixture was centrifuged at 3000 × *g* for 10 min at 4 °C, and then 200 μL of the resulting supernatant was mixed with 1 mL of phosphate buffer (100 mM, pH 8.0) and 50 μL of 5,5-dithiobis-2-nitrobenzoic acid (3 mM). The solution was maintained at room temperature for 5 min and its absorbance measured at 412 nm. The SOD activity was measured using a SOD activity assay kit (BioVision, Mountain View, CA) according to the manufacturer's protocol. Briefly, 20 μL of supernatant was added with 200 μL of water soluble tetrazolium working solution and 20 μL of enzyme working solution to a 96-well plate. After incubating the plate at 37 °C for 20 min, the absorbance at 450 nm was read using a microplate reader. The CAT activity was measured according to the method of Shen et al. (2009). Briefly, 50 μL of the supernatant was mixed with 1.95 mL of phosphate buffer (0.05 M, pH 7.0) and 1.0 mL of hydrogen peroxide (19 mM). The CAT activity was then estimated from the decrease in absorbency at 240 nm for 2 min. The protein content was measured using the Bradford method with bovine serum albumin as the standard (Bradford, 1976).

2.8. Statistical analysis

The data were expressed as the mean ± standard deviation (SD). Multiple group comparisons were performed using one-way analysis of variance (ANOVA) followed by Dunnett's test to detect any inter-group differences. Differences were considered to be statistically significant at $p < 0.05$.

3. Results

3.1. ABTS radical cation-scavenging capacity of TGP

Fig. 1 shows the ABTS radical cation-scavenging potential of TGP. TGP at different concentrations (1–15 mg/L) was found to effectively scavenge ABTS radical cations (10–73% inhibition), with an average IC50 value of 9.9 mg/L. Trolox (at 5 mg/L), which was used as the reference compound, showed a 72% inhibition.

3.2. Effect of TGP on corticosterone-induced cytotoxicity

According to the results of the trypan blue exclusion assay (Fig. 2A), treating PC12 cells with 200 μM of corticosterone for 48 h induced cytotoxicity, with cell viability being reduced to 30% of the control value. When the cells were treated with different TGP concentrations of 1, 5, and 10 mg/L in the presence of 200 μM of corticosterone for 48 h, the cell viability values increased to 38%, 45%,

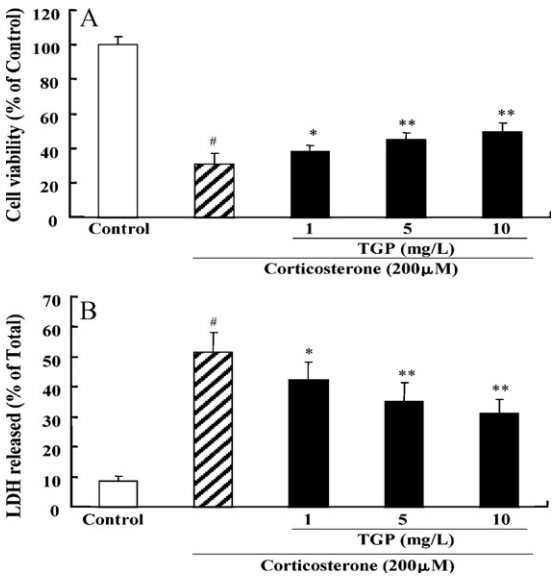

Fig. 2. Effect of TGP on the viability of corticosterone-treated PC12 cells. Cell viability was measured by trypan blue exclusion assay (A) and LDH assay (B). The values given are the mean ± SD of six independent experiments. $^{\#}p < 0.01$ compared with the control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the corticosterone group.

and 49%, respectively, of the control value. To further investigate the protective effects of TGP, a LDH assay was performed. As shown in Fig. 2B, when PC12 cells were incubated with 200 μM of corticosterone for 48 h, the percentage of LDH leakage increased from 8% (control) to 52%. In contrast, when the cells were treated with different concentrations of TGP (1, 5 and 10 mg/L) in the presence of 200 μM corticosterone for 48 h, the percentage of LDH leakage dropped to 42%, 35%, and 31%, respectively.

3.3. Effect of TGP on corticosterone-induced oxidative stress

As shown in Fig. 3, oxidative stress was assessed by measuring the intracellular ROS level, MDA level, GSH level, SOD activity, and CAT activity. After exposure to 200 μM corticosterone for 48 h, the intracellular ROS level and MDA level of the PC12 cells significantly increased to 195% and 214% of the control value, respectively, whereas the GSH level, SOD activity, and CAT activity significantly decreased to 42%, 40%, and 43% of the control value, respectively, suggesting that corticosterone may induce oxidative stress. When the PC12 cells were incubated with different concentrations of TGP (1, 5 and 10 mg/L) in the presence of 200 μM of corticosterone for 48 h, both the intracellular ROS level (160%, 148%, and 129% of the control value, respectively) and the MDA level (183%, 160%, and 145% of the control value, respectively) significantly decreased, whereas the GSH level (55%, 63%, and 71% of the control value, respectively) and SOD activity significantly increased (52%, 57%, and 60% of the control value, respectively) compared with the corticosterone group. Co-treatment with TGP (5 and 10 mg/L) also significantly increased the CAT activity (59% and 60% of the control value, respectively) in the corticosterone-treated PC12 cells.

4. Discussion

This study found that corticosterone caused a significant decrease in cell viability and an increase in LDH leakage in PC12 cells, confirming its neurotoxicity. In this *in vitro* model, TGP partly reversed the changes induced by corticosterone, supporting the antidepressant-like effect of TGP reported *in vivo*. Consistent with these findings, animal studies have demonstrated that

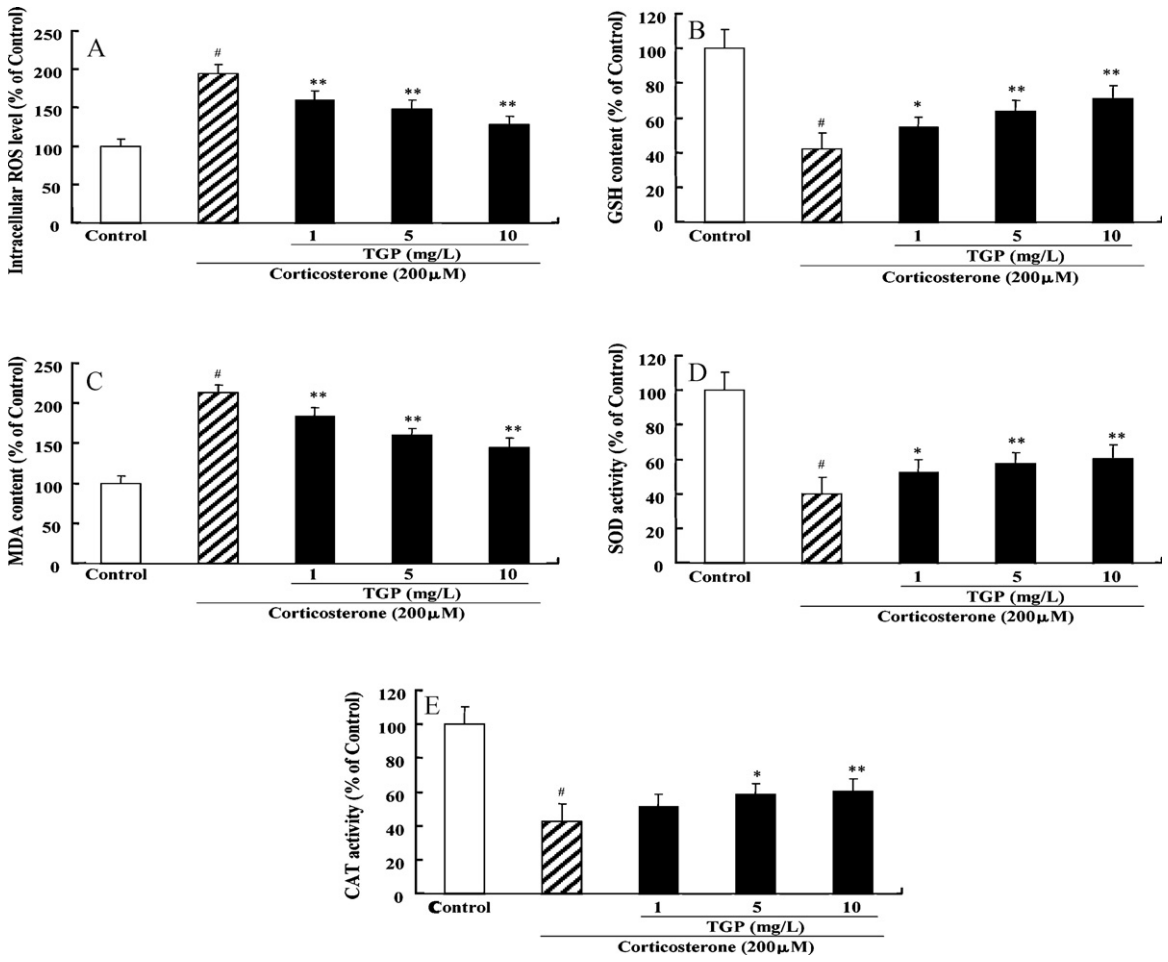

Fig. 3. Effect of TGP on corticosterone-induced oxidative stress in PC12 cells. Oxidative stress was assessed by measuring the intracellular ROS level (A), MDA level (B), GSH level (C), SOD activity (D), and CAT activity (E). The values given are the mean ± SD of six independent experiments. $^{\#}p < 0.01$ compared with the control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the corticosterone group.

abnormally high corticosteroid levels can induce depression-like behavior (Johnson et al., 2006; Murray et al., 2008) and many functional changes in the hippocampal neurons (Sapolsky, 2000; Murray et al., 2008). *In vitro* studies have also reported that high concentrations of corticosterone induce damage in primary cultured hippocampal neurons and cultured PC12 cells that can be reversed by antidepressants (Li et al., 2003a,b, 2004, 2007; Zhu et al., 2006a,b).

Oxidative stress, which is defined as a disturbance in the balance between the production of ROS and antioxidant defense systems, may contribute to neuronal injury induced by corticosterone (McIntosh et al., 1998a,b; Schmidt et al., 2002; Zafir and Banu, 2009). ROS, which are mainly composed of superoxide anions, hydrogen peroxide, and hydroxyl radicals, are produced when cells generate energy by reducing molecular oxygen to water (Wakamatsu et al., 2008). Previous *in vitro* and *in vivo* studies have shown that corticosterone treatment causes the level of ROS to significantly increase (Zafir and Banu, 2009). Excessive ROS levels are known to cause damage to major macromolecules in cells, including lipids, proteins, and nucleic acids (Niebrój-Dobosz et al., 2004; Zhao et al., 2008), culminating in neuronal dysfunction and depression. MDA, which is a by-product of lipid peroxidation, is produced under oxidative stress and reflects oxidative damage to the plasma membrane and the resultant production of thiobarbituric acid reactive substances that is proportional to lipid peroxidation and oxidant stress (Xiao et al., 2008). There are also antioxidant defense systems in cells for scavenging ROS to prevent cell damage. SOD and CAT are two important antioxidant enzymes. It has been shown that SOD has the ability to transform superoxide anions to hydrogen peroxide, which is subsequently scavenged by CAT (Xiao et al., 2008). GSH, which is the most abundant endogenous antioxidant, is also involved in antioxidant defense (Mueller et al., 2001). Elevated GSH level, SOD activity and CAT activity provide a repair mechanism for oxidized membrane components (Xiao et al., 2008). This study found that the treatment of PC12 cells with corticosterone caused a marked rise in oxidative stress as characterized by excessive ROS and MDA production and a reduction in GSH level and SOD and CAT activity. However, co-treatment with TGP attenuated these changes in the corticosterone-treated PC12 cells, suggesting that the neuroprotective effect of TGP may be related to its antioxidant ability. The ABTS cation radical-scavenging assay, which is one of the most widely used methods for screening the antioxidant activity of plant extracts (Ghanta et al., 2007), was employed to investigate the direct antioxidant effects of TGP in a cell-free system. The results clearly showed that TGP displays antioxidant properties.

In summary, TGP treatment may protect against corticosterone-induced cell death in PC12 cells in a dose-dependent manner. The cell viability of corticosterone-treated cells afforded by TGP was increased in parallel with the increases in the GSH level, SOD activity, and CAT activity, suggesting the protective effect of TGP was mediated by its antioxidant action.

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