Neuroprotective effects of Total Saikosaponins of Bupleurum yinchowense on corticosterone-induced apoptosis in PC12 cells

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ABSTRACT

Ethnopharmacological relevance: The root of Bupleurum yinchowense Shan et Y. Li, a well-known medicinal plant in China, was originally documented in the “Shennong’s Herbal”, which is the oldest Chinese materia medica monographs. It has the action of soothing liver and relieving constraint for emotional instability such as depression, anxiety and phobia. The in vivo experiment of our previous study has showed an efficacy of Total Saikosaponins (TSS) from Bupleurum yinchowense in acute stress and chronic unpredictable mild stress models. Nevertheless, there are no studies on the cytoprotection and potential mechanisms of TSS on corticosterone-induced apoptosis in PC12 cells. The present study focuses on cytoprotection against corticosterone-induced neurotoxicity in PC12 cells and its underlying molecule mechanisms of the antidepressant-like effect of TSS.

Materials and methods: The PC12 cells were treated with 250 μM corticosterone in the absence or presence of different concentrations of TSS for 24 h, then the cell viability, lactate dehydrogenase (LDH) release, Hoechst 33342 and propidium iodide (PI) double staining and the DNA fragmentation of the apoptotic PC12 cells were determined. The mitochondrial permeability transition pore (mPTP), mitochondrial membrane potential (MMP), intracellular Ca²⁺ ([Ca²⁺]i) concentration and western blot analysis of caspase-3, glucose-regulated protein 78 (GRP78), growth arrest and DNA damage inducible proteins 153 (GADD-153), X-box DNA-binding protein-1 (XBP-1), Bax, Bcl-2 were investigated.

Results: Pretreatment of PC12 cells with TSS (3.125, 6.25, 12.5, 25 μg/ml) partly reversed corticosterone-induced neurotoxicity in a dose dependent manner. TSS (25 μg/ml) reversed the increase of dead cells in the Hoechst 33342 stain, the accumulation in LDH leakage and the number of TUNEL positive cells induced by corticosterone to PC12 cells. Moreover, the cytoprotection of TSS was proved to be associated with the homeostasis of intracellular Ca²⁺, the stabilization of ER stress via the down-regulation of GRP78, GADD-153, XBP-1, and the restoration of mitochondrial function, which included mPTP, MMP and caspase-3 activity. Furthermore, TSS (25 μg/ml) markedly ameliorated up-regulation of Bax and down-regulation of Bcl-2 in corticosterone-induced PC12 cells.

Conclusion: The result depicted that antidepressant-like effect of TSS in vivo may be associated with the cytoprotection of neuron, and the neuroprotective mechanisms were correlated with inhibiting the ER stress and the mitochondrial apoptotic pathways.

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1. Introduction

Major depression is a common but sometimes fatal disorder, which has been considered to be the second leading cause of ailments worldwide (Moussavi et al., 2007). Although major depression has multiple pathogenesis, most recently available articulations of antidepressants and molecular targets still focus on monoamine disorder hypothesis that the monoamine neurotransmitters are depleted in major depression. However, the antidepressants presently used caused a variety of side-effects such as cardiac toxicity, sexual dysfunction, body weight gain and sleep disorder; moreover, the cure rate of depression is still as low as 70% approximately by using monoamine-like drug (Bostwick, 2010; Murrough, 2012). Thus, a safer, better-tolerated drug with higher effect is still on the way. From this point of view, the therapeutic discovery in this field might have drawn more attention to Ethnodrugs.

Bupleurum yinchowense Shan et Y. Li, a well-known medicinal plant in China, was originally documented in the “Shennong’s Herbal”, which is the oldest Chinese materia medica monographs.
It was recorded that the root of *Bupleurum yinchowense* has the action of relieving fever, soothing liver and relieving constraint, raising yang *qi* in patterns of spleen or stomach deficiency, and is used for improving the symptoms of emotional instability such as depression, anxiety, phobia, as well as dizziness, vertigo, chest and flank pain and menstrual problems (Xiao, 2002; Liang et al., 2012). Our recent research showed that Total Saikosaponins (TSS) from *Bupleurum yinchowense* had antidepressant-like effect in acute and chronic unpredictable mild stress models (Sun et al., 2012). However, the molecular mechanisms that TSS exerted antidepressant-like effects have not yet been elucidated.

The pathogenesis of depression remains poorly understood, but in recent years, a large number of experimental and clinical observations showed that major depression was closely associated with reduced hippocampal volumes and a positive correlation exists between the hippocampus atrophy and the time course of the depression (Li et al., 2003; Saylam et al., 2006). Postmortem analyses also indicated a reduction of the neuronal cells in hippocampus of patients with major depression (Stockmeier et al., 2004). To date, the mechanism of neuronal degeneration in depression still remains to be uncovered. Among several putative mechanisms of antidepressants, the hyperactivation of hypothalamic–pituitary–adrenal (HPA) axis has achieved much more appreciation. It is well known that HPA axis is activated in response to stress. Under normal conditions, glucocorticoid level in blood is sensitively regulated by HPA axis via negative feedback; it will rise when chronically stressful situation or depression occurs. The persistently high concentration of blood glucocorticoids causes the dysfunction of HPA axis, exacerbates the lesion in the nervous system, and even aggravates the depression (Murphy, 1997; Sapolsky, 2000). It has been reported that high corticosterone level could induce depression-like behaviors in mice (Murray et al., 2008), and damage the normal hippocampal neurons both in vitro and in vivo (Zhu et al., 2006a; Li et al., 2007). The rat pheochromocytoma (PC12) cell line, which possesses typical neuron features and expresses high levels of glucocorticoid receptors, is one of the widely used neuronal cell lines in a variety of studies.

The present study aimed to examine the neuroprotective effects of TSS from *Bupleurum yinchowense* and further investigates its underlying mechanisms in corticosterone-induced PC12 cells. The current findings demonstrated that TSS partly reversed the apoptosis of corticosterone-induced PC12 cell via inhibiting the endoplasmic reticulum (ER) stress and the mitochondrial apoptotic pathways.

### 2. Materials and methods

#### 2.1. Preparation of Total Saikosaponins (TSS)

The roots of *Bupleurum yinchowense* were collected from Dingxi County, Gansu Province, China, August 2009, and authenticated by Professor Bengang Zhang of the Institute of Medicinal Plant, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China, where a voucher specimen (No. 20090815) has been deposited in Herbarium of the institute.

The powdered root of *Bupleurum yinchowense* (500 g) was extracted with 60% ethanol containing 0.5% ammonia aqua (three volumes, each 1 L) at room temperature for 6 h. The ethanol extracts were combined and evaporated in *vacuo*. The concentrate was diluted with distilled water, then subjected to a column containing D101 macroporous resin (1 kg) (Cang Bon Adsorber Technology Co., Ltd., China), and eluted successively with water and ethanol–water (9:1, v/v). The ethanol was evaporated in *vacuo* to yield a pale yellow residue (32 g), which is called Total Saikosaponins (TSS). Five main monomeric compounds in TSS were determined using HPLC and the content of Saikosaponins a, c, d, e and f was 10.12%, 2.84%, 14.13%, 1.52% and 2.14%, respectively. An HPLC profile is obtainable from the author.

#### 2.2. Drugs and reagents

Fetal bovine serum, heat-inactivated horse serum, penicillin and streptomycin were purchased from Gibco (Grand Island, NY, USA). Corticosterone, Dulbecco’s Modified Eagle Medium (DMEM) and thiobarbitalic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies to β-actin (1:2000), caspase-3 (1:200), bcl-2 (1:200) family, GRP78 (1:200), XBP-1 (1:200), GADD-153 (1:200), as well as the second antibodies labeled with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (1:1000) were purchased from Santa Cruz Biotechnol (Santa Cruz, CA, USA). All other chemicals and reagents are of analytical grade.

#### 2.3. Cell culture and treatment

PC12 cells were grown in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 5% (v/v) heat-inactivated horse serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured in a humidified 95% air and 5% CO2 atmosphere at 37 °C. For all experiments, cells in the exponential phase of growth were used. To study the neuroprotective effect of TSS, PC12 cells were divided into non-treated control, corticosterone (250 μM), TSS, corticosterone (250 μM) plus TSS groups in all experiments. TSS were applied 24 h prior to the treatment with corticosterone, and was also present in the medium during corticosterone incubation.

#### 2.4. Cell viability assay

Cell viability was evaluated with the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Kumamoto, Japan). In brief, PC12 cells cultured in 96-well plates were treated as indicated (Fig. 1), and then gently washed with PBS. After washing, 90 μl of culture medium and 10 μl of CCK-8 solution were added to each well. The plate was maintained at 37 °C for 2 h and the absorption was measured at 450 nm on a microplate reader (Spectrafluor, TECAN, Sunrise, Austria). Cell viability was expressed as a percentage of the control.

![Fig. 1. Effect of Total Saikosaponins on the cell viability by CCK-8 assay in corticosterone-treated PC12 cells. The results are expressed as mean ± SD (n=3). **P < 0.01 as compared with control group; *P < 0.05 and **P < 0.01 as compared with the corticosterone group. TSS: Total Saikosaponins; Cort: corticosterone.](image-url)
2.5. Assessment with Hoechst 33342 and propidium iodide (PI) double staining

To further investigate the protective effect of TSS, Hoechst 33342 and PI double-fluorescent staining was assayed. The Annexin V/propidium iodide (PI) assay kit was purchased from Invitrogen (Eugene, OR, USA). The PC12 cells were cultured on coverslips in 24-well plates for 24 h. After the indicated treatment, the cells were incubated with 5 μg/ml Hoechst 33342 for 15 min, washed twice with PBS, then incubated with 1 μg/ml PI working solution for another 15 min (Saravia et al., 2009), and then visualized by inverted fluorescence microscopy (Leica, Germany). The apoptotic nuclei were counted in at least 200 cells from five randomly selected fields in each treatment, and expressed as a percentage of the total number of nuclei counted.

2.6. LDH activity assay

LDH activity was measured using a LDH diagnostic kit (STAN-BIO Laboratory, USA) according to the manufacturer’s protocol. PC12 cells were cultured in 6-well plates. At the end of the drug treatment, the medium was collected, 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 intracellular 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 (4000g) at 4 °C for 30 min. The 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.

2.7. TUNEL staining

The DNA fragmentation of the apoptotic PC12 cells was detected using the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling TUNEL kit (Roche Diagnostics Corp., IN, USA). The cells were cultured on cover slips for 24 h. At the end of the drug treatment, the cells were fixed by incubation in a 10% neutral buffered formalin solution for 30 min at room temperature. Then the cells were incubated with a methanol solution containing 0.3% H2O2 for 30 min at room temperature, and then incubated with a permeabilizing solution (0.1% sodium citrate and 0.1% Triton X-100) for 2 min at 4 °C. The cells were incubated with the TUNEL reaction mixture for 60 min at 37 °C and visualized by inverted fluorescence microscopy (Leica, Germany). TUNEL-positive nuclei were counted in four nonoverlapping fields per cover slip, and then converted to percentage by comparing TUNEL-positive counts with the total cell nuclei as determined by Hoechst 33342 counterstaining.

2.8. Measurement of mitochondrial permeability transition pore (mPTP) opening

The opening of mPTP in corticosterone-treated PC12 cells was determined using the calcine–cobalt quenching method. The cells were cultured in 24-well plates. After treatments, PC12 cells were loaded with calcine dye and in the presence of cobalt chloride (CoCl2, 1 mmol/L) at 37 °C for 30 min. Images were acquired using an inverted fluorescence microscope (Leica, Germany) with excitation and emission wavelengths of 488 and 505 nm, respectively.

2.9. Measurement of mitochondrial membrane potential (MMP)

5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetrathiacycliazolyl-carboxyanine iodide (JC-1, Invitrogen, Eugene, OR, USA) was used to determine the changes on MMP in corticosterone-treated PC12 cells. In brief, the cells were suspended in warm medium at approximately 1 x 10⁶ cells/ml and then incubated with JC-1 (2 mM final concentration) for 30 min in the dark. After incubation, the cells were washed twice with PBS and visualized using an inverted fluorescence microscopy (Leica, Germany). Monomeric JC-1 green fluorescence emission and aggregate JC-1 red fluorescence emission were measured on a microplate reader. The MMP of PC12 cells in each treatment group was calculated as the ratio of red to green fluorescence.

2.10. Measurement of intracellular calcium level

After treatments, PC12 cells were collected and prepared to generate a 0.5 ml cell suspension for each sample. The cell suspension was added Fura-2/AM (final concentration 5 μM) and shaken at 37 °C for 1 h, and then centrifuged twice at 1000 rpm for 5 min. The cells were re-suspended in HEPES buffer solution (containing NaCl 132, KCl 3, glucose 10, HEPES 10 and CaCl₂ 2 mM, pH 7.4); finally, the fluorescence intensity was measured by a microplate reader (SpectraFluor; Tecan, Sunrise, Austria) with excitation and emission wavelengths of 340 and 500 nm, respectively.

2.11. Western blot analysis

At the end of treatments, the PC12 cells were harvested, washed once with PBS, then lysed with a cell lysis buffer containing 1% phenylmethylsulfonylfluoride. The whole cell lysates were centrifuged at 12,000 rpm for 5 min at 4 °C, and the supernatant was collected. Protein concentration was determined by bicinchoninic acid assay. Equal amounts of protein (10 μg) were separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels and transferred onto nitrocellulose membranes. These membranes were incubated with 5% (w/v) non-fat milk powder in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST) for 40 min to block nonspecific binding sites. The membranes were then incubated overnight at 4 °C with the primary antibodies. After washing with TBST, the membranes were incubated for 1 h at room temperature with the secondary antibodies. After rewashing with TBST, the bands were developed by enhanced chemiluminescence.

2.12. Statistical analysis

The results are presented as means ± standard deviation (SD). All statistical analysis were performed using the SPSS 17.0 software. Student’s t-test was performed, and differences were accepted as statistically significant at P < 0.05. All experiments were performed for a minimum of three times.

3. Results

3.1. Effect of TSS on corticosterone-induced apoptosis in PC12 cells by CCK-8

According to the results of CCK-8 assay (Fig. 1), the viability of PC12 cells, which was measured with the absorbance of formazan detected in each well, when exposed to corticosterone at a concentration of 250 μM for 24 h was significantly decreased as compared with the control group (P < 0.01) and the survival rate was 58.4% of control. When the cells were pretreated with TSS at different concentrations (3.125, 6.25, 12.5, 25 and 50 μg/ml) for
24 h, in the presence of 250 μM of corticosterone for 24 h, the cell viability values changed to 63.8%, 65.9%, 68.7%, 74.4% and 52.5%, respectively, of the control. The result revealed that 25 μg/ml of TSS possessed the best neuroprotective effects on the corticosterone-induced apoptosis. On the contrary, the cell apoptosis intensified when TSS was 50 μg/ml, and indicated the cytotoxicity of TSS. Therefore, 25 μg/ml of TSS was used in the subsequent research.

3.2. Effect of TSS on corticosterone-induced apoptosis of PC12 cells by Hoechst 33342 and PI double staining

As illustrated in the microphotographs and the histogram (Fig. 2), the nucleus of dead cells could be penetrated by propidium iodide (PI) which released red fluorescence, while the living cells could just be stained by the Hoechst 33342 and illustrated the blue fluorescence. After treatment with 250 μM of corticosterone for 24 h, the amount of apoptotic cells (red fluorescence) was significantly increased, the cell survival rate decreased to 55.60% as compared with the control group. By contrast, on pretreatment with TSS (25 μg/ml) in the presence of 250 μM corticosterone, the cell viability obviously elevated to 77.20% of the control, and TSS treatment alone did not change the apoptosis rate of the PC12 cells.

3.3. Effect of TSS on corticosterone-induced LDH leakage in PC12 cells

The effect of TSS on the LDH leakage in corticosterone-induced PC12 cells is shown in Fig. 3; the cell viability was based on the

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**Fig. 2.** Effects of Total Saikosaponins on the cell survival in corticosterone-treated PC12 cells by Hoechst 33342 and PI double staining method. (A) The photomicrographs in double-fluorescent staining with Hoechst 33342 and PI, representative images of PI-positive cells (red, apoptosis cells) and Hoechst counterstaining (blue, normal cells). (B) Cell survivals (%) were detected by manually counting the cells as in (A). Results were expressed as mean ± SD (n = 3). **P < 0.01 as compared with the control group; ##P < 0.01 as compared with corticosterone group. TSS: Total Saikosaponins; Cort: corticosterone. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)**
measurement of LDH activity released from damaged cells. After treatment of PC12 cells with 250 μM corticosterone for 24 h, the LDH leakage was significantly increased as compared with the control group \((P < 0.01)\) and the percentage of LDH leakage increased from 12.7% (control) to 56.8%. Pretreatment of PC12 cells with TSS (25 μg/ml) in the presence of 250 μM corticosterone for 24 h resulted in the decrease of LDH leakage \((P < 0.01)\) and the percentage of LDH leakage was 34.9%, meanwhile the percentage was 14.3% with the TSS (25 μg/ml) treatment alone.

3.4. Effect of TSS on internucleosomal DNA fragmentation in corticosterone-induced PC12 cells

The apoptosis in PC12 cells was further examined using TUNEL staining. During the apoptosis, the DNA strand breaks were detected by enzymatically labeling the free 3′-OH termini with modified nucleotides. These new DNA ends that were generated upon DNA fragmentation were typically localized in morphologically identifiable nuclei and apoptotic bodies. In contrast, normal nuclei, which have relatively insignificant numbers of DNA 3′–OH ends, usually do not stain with the kit. Fig. 4 shows PC12 cells treated with 250 μM corticosterone for 24 h alone; TUNEL-positive cells with green fluorescence significantly increased to 38.9% of total cells, while only 5.3% in control \((P < 0.01)\). In contrast, preincubation with TSS (25 μg/ml) resulted in a dramatic decrease in the TUNEL-positive cell count by almost 24.7% \((P < 0.01)\). TSS (25 μg/ml) treatment alone had no effect on the apoptosis rate of the PC12 cells.
3.5. Effect of TSS on the opening of mPTP in corticosterone-induced P12 cells

The influence of TSS on corticosterone-induced mPTP opening was assayed using the calcein–cobalt quenching method. Under the normal conditions, calcein could selectively gather in the mitochondria and illustrated the green fluorescence; when mPTP opened unusually, the profound release of calcein to the cytosol would be quenched by cobalt. As shown in Fig. 5, treatment with corticosterone (250 μM) caused a significant decrease in green fluorescence compared to control, consistent with mPTP opening. On pretreatment with TSS (25 μg/ml) in the presence of corticosterone, a comparatively intensive green fluorescence was observed, while treatment with TSS (25 μg/ml) alone did not influence green fluorescence. The results revealed that TSS markedly induced the closure of the mPTP.

3.6. Effect of TSS on corticosterone-induced MMP in PC12 cells

The effect of TSS on corticosterone-induced MMP was assayed by JC-1 staining. JC-1 exhibits potential-dependent accumulation in mitochondria. In normal cells, JC-1 accumulates and forms dimeric J-aggregates in the mitochondria, giving off a bright red fluorescence. However, when the potential is disturbed, the dye cannot access the transmembrane space and remains in the cytoplasm in its monomer form, giving off a bright green fluorescence. Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. As shown in Fig. 6, treatment of PC12 cells with corticosterone (250 μM) caused a significant decrease in the red/green fluorescence intensity ratio compared to the control group ($P < 0.01$). However, TSS (25 μg/ml) pretreatment prevented the effect of corticosterone on the red/green fluorescence intensity ratio. Thus, the results reflect the restoration of TSS on the MMP in corticosterone-induced PC12 cells.

3.7. Effect of TSS on corticosterone-induced intracellular Ca$^{2+}$ concentration in PC12 cells

As shown in Fig. 7, Fura-2 would transmit from the cytomembrane and combined with intracellular free calcium, and generated different intensity fluorescences. After treatment of PC12 cells with 250 μM corticosterone for 24 h, the concentration of [Ca$^{2+}$]i markedly increased as compared with the control group ($P < 0.01$) and the concentration of [Ca$^{2+}$]i was 289.4%, while for the control group it was 100%. By contrast, pretreatment with TSS (25 μg/ml) in the presence of 250 μM corticosterone for 24 h observably decreased as compared with the control group ($P < 0.01$) and the [Ca$^{2+}$]i concentration was 231.8%. However, the concentration of [Ca$^{2+}$]i was 109.8% in the TSS treated alone group.

3.8. Effect of TSS on caspase-3 activation in corticosterone-induced PC12 cells

To further discuss the mitochondrial apoptotic pathway in the neuroprotective activity of TSS against corticosterone-induced PC12 cell, the activation of caspase-3 was also detected. As shown in Fig. 8A, the caspase-3 activity up-regulated in the corticosterone-treated group compared with the control, whereas TSS (25 μg/ml) pretreatment caused a significant decrease in caspase-3 activity.

3.9. Effect of TSS on ER stress activation in corticosterone-induced PC12 cells

In order to explore whether corticosterone-induced apoptosis in PC12 cell is related to ER stress, the activation of ER biomarkers, GRP78, GADD-153 and XBP-1, was analyzed by western blot. As shown in Fig. 8B–D, the expression of GRP78, GADD-153 and XBP-1.

Fig. 5. Effect of Total Saikosaponins on the opening of mPTP in corticosterone-treated PC12 cells. The opening of the mPTP was determined using mitochondrial calcein fluorescence. Green fluorescence is representative of healthy mitochondria fluorescence. Treatment with corticosterone caused a decrease in green fluorescence compared to control; pretreatment with Total Saikosaponins in the presence of corticosterone results in intensity of green fluorescence; treatment with Total Saikosaponins alone did not influence fluorescence intensity. TSS: Total Saikosaponins; Cort: corticosterone. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
XBP-1 significantly increased in PC12 cells after 250 μM corticosterone treatment. However, the up-regulation of these three biomarkers were attenuated by pretreatment with TSS (25 μg/ml).

3.10. Effect of TSS on modulating Bcl-2 and Bax expression in corticosterone-induced PC12 cells

To delineate the mechanism of anti-apoptosis effect of TSS, the expression of apoptosis-related proteins in PC12 cells in response to corticosterone and TSS treatment was assessed. Fig. 9A and B shows that corticosterone treatment significantly up-regulated the expression of pro-apoptotic protein Bax, but down-regulated that of anti-apoptotic protein Bcl-2. However, pretreatment with TSS (25 μg/ml) blocked these effects.

4. Discussion

The PC12 cells treated with high concentration of glucocorticoid to induce the neuronal damage have been widely used as an in vitro experimental model of depression (Mao et al., 2011; Mao et al., 2012a, 2012b). In the present study, our results revealed that corticosterone markedly decreased the cell viability, obviously increased the number of dead cells in the Hoechst 33342 staining.
and accumulated LDH leakage in PC12 cells, confirming its neurotoxicity in PC12 cells. Moreover, the DNA fragmentation of the apoptotic PC12 cells detected by TUNEL kit also proved the neurotoxicity of corticosterone. Consistently, abnormally high corticosteroid levels could cause many functional changes in the cultured hippocampal neurons as well as PC12 cells (Li et al., 2004b; Mao et al., 2011a, 2011b). In this in vitro model, on pretreatment with TSS (25 μg/ml) in presence of 250 μM corticosterone, the changes of PC12 cells induced by corticosterone were partly reversed, supporting the antidepressant-like effect of TSS as reported by our previous in vivo study. The result implied that antidepressant-like effect of TSS may be associated with its
neuroprotective effects. In order to elucidate the mechanism by which TSS attenuated the physiological changes induced by corticosterone, we investigated the apoptotic pathways in which TSS involved, including Ca\textsuperscript{2+} homeostasis, ER stress, mitochondrial damage and Bcl-2 family expression.

As reported in literature, Ca\textsuperscript{2+} homeostasis participates positively in different forms of intracellular signaling which might determine whether cells live or die (Dong et al., 2006). In the case of corticosterone-induced cell apoptosis in PC12 cells, several studies have showed that the death of cells are related to Ca\textsuperscript{2+} overloading (Li et al., 2004a, 2004b; Zhu et al., 2006b). Our results demonstrated that the corticosterone-induced [Ca\textsuperscript{2+}]i was obviously elevated, while in presence of TSS (25 μg/ml), [Ca\textsuperscript{2+}]i overloading was partially attenuated which was consistent with the cell viability. The results suggested that the neuroprotective effects of TSS in corticosterone-induced PC12 cells might be mediated, at least partly, by inhibition of [Ca\textsuperscript{2+}]i overloading.

ER is the main Ca\textsuperscript{2+} store of the cell and the major intracellular Ca\textsuperscript{2+} signaling site. Upon ER stress, unrestricted release of Ca\textsuperscript{2+} from the ER followed by mitochondria uptake has a function in cell death (Zhu et al., 2000; Hajnoczky et al., 2003). In response to prolonged ER stress, GADD-153, a leucine zipper transcription factor, which is expressed at low levels in normal situation, is forcefully expressed in extreme stress (Ron and Habener, 1992). The increase of GADD-153 leads to enhanced sensitization of the cells to ER stress (Oyadomari and Mori, 2004). The ER stress induced by the GADD-153 is associated with the GRP78, which would dissociate from ER transmembrane receptor PRK-like ER kinase, inositol-requiring enzyme 1, and lead to their activation and increase further stress (Bertolotti et al., 2000; Shen et al., 2002). XBP-1 is a basic leucine zipper family transcription factor. Upon ER stress, the enrichment of XBP-1 expression affects cell fate decisions between cytoprotective and pro-apoptotic outcomes (Lee et al., 2003). As typical ER biomarkers, the expression of GRP78, GADD-153 and XBP-1 was significantly increased in PC12 cells after corticosterone exposure. In the meantime, their expression was attenuated when pretreated with TSS (25 μg/ml), suggesting the cytoprotection of TSS to the corticosterone-induced neurotoxicity partly via ER stress-mediated pathway.

As a main participant of ER stress pathway, mitochondria is involved in the regulation of signal transduction cascades, which is known as mitochondrial pathways of apoptosis (Hajnoczky et al., 2006). In extreme ER stress, the excessive accumulation of Ca\textsuperscript{2+} in mitochondria would lead to over capacity and impairment of organelles function because of a transient depolarization of the inner mitochondrial membrane (IMM) and thus the opening of the mitochondrial permeability transition (mPTP) (Bernardi, 1999; Hajnoczky et al., 2006). The uncontrolled opening of mPTP leads to collapse of MMP, whose disruption is considered the “point of no return” in the apoptosis cascade of events (Kroemer et al., 2007). The disintegration of mitochondria triggers the release of cytochrome C which increases the activity of caspase-3 (Kroemer and Reed, 2000). In the current study, corticosterone caused the pathological and abnormal opening of mPTP and the disruption of MMP in PC12 cells; by contrast, TSS (25 μg/ml) partly reversed the uncontrolled opening of mPTP and stabilized MMP. Therefore, it appears that TSS restored corticosterone-induced dysfunction of mitochondria by attenuating the impairment of IMM. Furthermore, in the cells pretreated with TSS (25 μg/ml), the expression of caspase-3 was partly decreased compared with corticosterone-injured PC12 cells. Such an investigation may provide an important evidence to suggest that the inhibition of mitochondrial pathways might directly contribute to the anti-apoptosis effects of TSS as the downstream process of the apoptosis, or as a result of upstream cascade reactions.

Additionally, the published research has identified that Bcl-2 family can simultaneously stimulate multiple mechanisms in order to achieve protection or damnification of the cells (Reed, 1997; Dispersyn et al., 1999; Pinton et al., 2001). The Bcl-2 family proteins can contribute several homologous proteins including anti-apoptotic proteins (Bcl-2, etc.) and pro-apoptotic proteins (Bax, etc.) (Kosten et al., 2008). The imbalance of pro- and anti-apoptotic proteins have been proved to influence the steady-state Ca\textsuperscript{2+} content of ER and the resisting force of cells for apoptosis (Pinton et al., 2001; Scorrano and Korsmeyer, 2003). In basal conditions, Bcl-2 can lower total ER [Ca\textsuperscript{2+}]i; thus there is less Ca\textsuperscript{2+} to be released, so Bcl-2 inhibits Ca\textsuperscript{2+} mobilization from ER to mitochondria (Szalai et al., 1999). Knocking out the pro-apoptotic Bax and Bak leads to a decrease of the steady-state Ca\textsuperscript{2+} concentration in ER, and renders the cells more resistant to apoptosis (Scorrano and Korsmeyer, 2003). The attenuation of Bcl-2 and enhancement of Bax were detected in the corticosterone-induced apoptosis of PC12 cells in the present study, consistent with the other studies (Quadrilatero and Hoffman-Goetz, 2005; Yoshimura et al., 2007). In contrast, the measurement of PC12 cells pretreated with TSS (25 μg/ml) counteracted the abnormal expression changes of Bcl-2 and Bax, which were induced by the corticosterone. Taken together, these results suggested that TSS reestablished the balance of Bcl-2 and Bax.

The conclusion that can be drawn from the results obtained in this \textit{in vitro} model of depression showed that TSS, which partly reverses the pathological changes induced by corticosterone, was beneficial in the cytoprotection of neurons, because it might contribute to the stabilization of ER and inhibition of the mitochondrial apoptosis pathway via stabilizing the Ca\textsuperscript{2+} homeostasis and the regulation of Bcl-2 family. The present study suggested that the cytoprotection of TSS may be one of available mechanisms on its antidepressant-like effects. Regarding the clinical and animal effects, as well as our results, it is worth of further study for TSS, which can be a potential candidate to develop antidepressants.

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