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Echinacoside rescues the SHSY5Y neuronal cells from TNFα-induced apoptosis

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Abstract

We investigated the neuroprotective effect of echinacoside, one of the phenylethanoids isolated from the stems of *Cistanches salsa*, a Chinese herbal medicine, on tumor necrosis factor-alpha (TNFα)-induced apoptosis in human neuroblastoma (SHSY5Y) cells. Treatment of cultured SHSY5Y cells with TNFα 100 ng ml^{-1} for 36 h stimulated apoptosis, as demonstrated by typical morphological changes, cell viability, DNA laddering, annexin-V binding, intracellular reactive oxygen species, mitochondrial membrane potential and caspase-3 activity. However, simultaneous treatment with echinacoside (1, 10 or 100 μg ml^{-1}) attenuated the TNFα-mediated apoptosis. The antiapoptotic action of echinacoside was partially dependent on antioxidative stress effects, maintenance of mitochondria function, inhibition of caspase-3 activity and was also associated with increasing the expression of the antiapoptotic protein Bcl2. Thus, echinacoside has the neuroprotective capacity to antagonize TNFα-induced apoptosis in SHSY5Y cells and may be useful in treating some neurodegenerative diseases.

Keywords: Echinacoside; Apoptosis; TNFα; Mitochondrial membrane potential; Reactive oxygen species; Caspase-3

1. Introduction

Apoptosis is a genetically determined mechanism of programmed cell death that can be triggered by various internal and external stimuli. During nervous system development, apoptosis is normal, but its reemergence appears to contribute to neurodegenerative disorders such as Parkinson and Alzheimer diseases and delayed encephalopathy after acute carbon monoxide poisoning (Honig and Ronsenberg, 2000; Martin, 2001; Claude et al., 1997). Several lines of evidence have strongly suggested that oxidative stress, a cellular imbalance between the production and elimination of reactive oxygen species, leads to neuronal apoptosis and necrosis (Kelso et al., 2001; Brown et al., 1997; Freyer, 1998). Therefore, it is valuable to identify compounds that can antagonize the deleterious action of reactive oxygen species and act as an antioxidant to protect neurons from apoptosis.

Echinacoside is one of the phenylethanoids isolated from the stems of *Cistanches salsa*, a Chinese herbal medicine, which is an important crude drug used both as an antisenium and antifatigue agent (Xiong et al., 1999). Several phenylethanoids have been shown to possess free radical scavenging properties and protect oxidative-stress-induced toxic injuries (Xiong et al., 1996, 2000; Gao et al., 1999). Tumor necrosis factor-alpha (TNFα) is a toxic-interfering agent because of its important role in neurodegenerative diseases and being a well-characterized model of programmed cell death. Thus, we studied whether echinacoside can protect against TNFα-induced apoptosis in cultured SHSY5Y neuronal cells.

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2. Materials and methods

2.1. Reagents

Modified Eagle's medium (MEM) and fetal bovine serum (FBS) were purchased from GIBCO/BRL. Chromatin dye bisbenzimide (Hoechst 33342), poly-L-lysine, 3-(4,5-dimethylthiazal-z-yl)-2,5-diphenylterazolium (MTT), TNFα and 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) were obtained from Sigma (St. Louis, MD, USA). Primary mouse monoclonal antibodies to human Bcl-2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The annexin-V fluorescein isothcocyanate (FITC) apoptosis detection kit was purchased from Boehringer Mannheim (Indianapolis, IN, Germany). 5,5′ ,6,6′ -tetrachloral-1,1′ ,3,3′ -tetraethylbenzimidazol-carbocyanine iodide (JC-1) was purchased from Molecular Probes (Leiden, The Netherlands). Caspase-3 assay and DNA extraction kits were obtained from Promega (USA). Echinacoside from *C. salsa* was kindly supplied by Dr. Peng Fei Tu (Peking University Modern Research Center for Traditional Chinese Medicine). The purity of the compounds was shown to be more than 98% on high-performance liquid chromatography (HPLC). All other reagents or drugs were of analytical grade.

2.2. Cell culture

Neuronal SHSY5Y cells obtained from ATCC were maintained in MEM supplemented with 10% fetal bovine serum, 2 MM L-glutamine, 100 U ml^{-1} benzylpenicillin and 100 μg ml^{-1} streptomycin in a poly-L-lysine-coated culture flask in a humidified incubator with 5% CO_2 at 37 °C. The cells were passaged, and the culture medium was renewed every 2 to 3 d. Cells were treated with various concentrations of echinacoside or 100 ng ml^{-1} TNFα for 36 h before analysis.

2.3. Cell viability assay

Cell viability was determined by use of an MTT assay (Sladouski et al., 1993). Cells were seeded in 96-well plates at 1×10^4 cells per well and grown to 70% confluence in culture medium. The medium was replaced by medium containing various concentrations of echinacoside (1, 10 or 100 μg ml^{-1}) and/or 100 ng ml^{-1} TNFα. A total of 5 mg ml^{-1} MTT was added to each well after 36 h, and the culture continued to incubate for another 4 h at 37 °C. After the medium had been removed, cells and dye crystals were solubilized with 200 μl dimethylsulfoxide (DMSO), and optical density was measured at 570 nm by use of a model ELX-800 microplate assay reader (One Lambda).

2.4. Hoechst 33342 staining

After treatment with echinacoside and/or TNFα for 36 h, cells were harvested and fixed with 4% paraformaldyhyde for 30 min at room temperature then washed with prechilled phosphate-buffered saline (PBS) three times and exposed to 10 μg ml^{-1} Hoechst 33342 at room temperature in the dark for 10 min. Samples were observed under a fluorescence microscope (Isabella et al., 1997).

2.5. Analysis of DNA fragmentation

DNA was extracted by use of a DNA extraction kit (Promega) according to manufacturer's instructions after treated with echinacoside and/or TNFα for 36 h. Briefly, a 10-μl DNA sample was loaded onto 1.5% horizontal agarose gels containing ethidium bromide. Gels were run at 60 V for 1 h submerged in Tris acetic acid–EDTA (TAE) buffer, and DNA fragments were visualized using ultraviolet (UV) illumination (Moore and Matlashewski, 1994).

2.6. Determination of intracellular reactive oxygen species levels

Intracellular reactive oxygen species levels were measured with use of the fluorescent dye 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA; Sigma) staining method (LeBel et al., 1992). H_2DCFDA is a nonpolar compound that is converted into a nonfluorescent polar derivative (H_2DCF) by cellular esterases after incorporation into cells. H_2DCF is membrane-impermeable and rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) in the presence of intracellular reactive oxygen species (Sauer et al., 2001). After cells cultured on glass coverslips in every group were treated with echinacoside and/or TNFα for 36 h, they were incubated for 30 min at 37 °C with 20 μmol L^{-1} H_2DCFDA dissolved in PBS. Coverslips were then washed three times with PBS and analyzed under a confocal laser microscope (Leica, Germany). DCF was excited at 488 nm, and the emission filter was a 510-nm barrier filter. The fluorescent intensities and confocal laser microscopic images were held constant to allow for comparison of relative fluorescence intensities between control and experimental cells.

2.7. Mitochondrial membrane potential assay

The mitochondrial membrane potential of cells was measured with use of the probe JC-1(Cossarizza et al., 1995). JC-1 is able to enter mitochondria selectively, which appear green at low concentrations or at low membrane potential as a monomer. However, at high concentrations, mitochondria show as red fluorescent aggregates. JC-1 is sensitive to mitochondrial membrane potential, and the changes in the ratio between green and red fluorescence can provide information regarding the mitochondrial membrane potential. After the treated cells were loaded with 1 μmol L^{-1} JC-1 for 10 min at 37 °C,

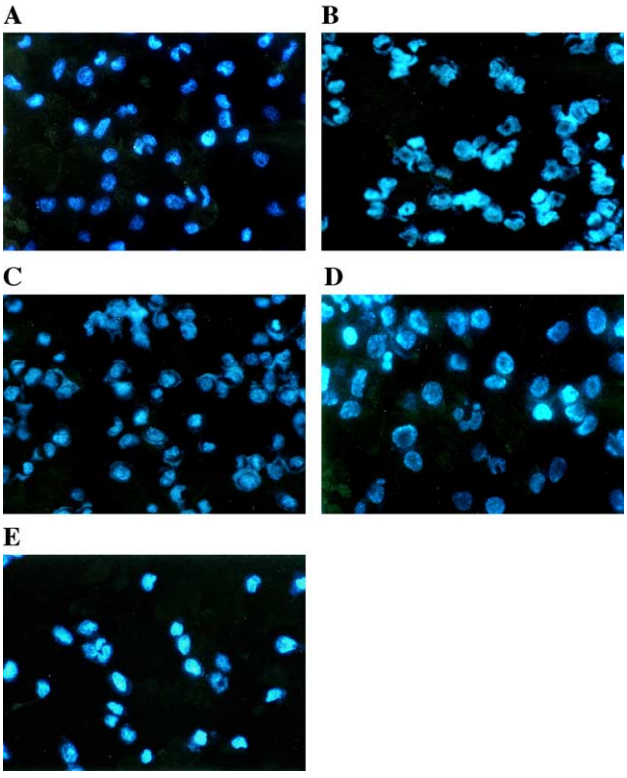

Fig. 1. Fluorescence photomicrographs of SHSY5Y cells after Hoechst 33242 staining (400×). (A) Control cells, (B) treatment with 100 ng ml^{-1} TNFα, (C) treatment with 100 ng ml^{-1} TNFα and 1 μg ml^{-1} echinacoside, (D) treatment with 100 ng ml^{-1} TNFα and 10 μg ml^{-1} echinacoside and (E) treatment with 100 ng ml^{-1} TNFα and 100 μg ml^{-1} echinacoside.

the fluorescent dye was excited at 490 nm, and the fluorescence intensities of both monomer and aggregated molecules were recorded at 590 nm under a confocal scanning laser microscope.

2.8. Flow cytometry analysis

We used annexin-V FITC apoptosis detection kit (Boehringer Mannheim) to bind annexin-V, which has a strong affinity for phosphatidylserine and can probe for apoptosis (Liu et al., 2002). In brief, cells were harvested and suspended in binding buffer at a final cell concentration of 10^6 cells/ml. Approximately 10^5 cells were incubated in the dark with annexin-V and propidium iodide for 15 min. Then, the suspension was analyzed with use of a FACS scan flow cytometer (Becton Dickinson, Heidelberg, Germany). Annexin-V FITC and propidium iodide-related fluorescence was recorded on FL1-H (525 nm) and FL2-H (575 nm) filters, respectively.

2.9. Caspase-3 activity assay

Caspase-3 activity was detected by use of the Apo-ONE™ Homogeneous Caspase-3 assay kit (Promega) according to the manufacturer's instructions. Briefly, cells were seeded into 96-well plates at 10^4 cells/well. After being exposed to various concentrations of echinacoside and/or 100 ng ml^{-1} TNFα for 36 h, cells were washed with ice-cold PBS. Then, 1 μl Z-DEVD-R110 and 99 μl caspase buffer were mixed to make the homogeneous caspase-3 reagent. A total of 100 μl homogeneous caspase-3 reagent was added to each well. The contents were gently mixed at 300–500 rpm for at least 30 s and incubated for 4 h at room temperature in the dark. The intensity of the fluorescence of the Z-DEVD-R110 substrate was measured at an excitation wavelength of 498 nm and an emission wavelength of 521 nm with use of a microplate spectrofluorometer (Wallac Victor^2™ 1420 Multilabel Counter, USA).

2.10. Western blotting

Cell samples were harvested, and lysis buffer was added. The lysate was centrifuged at 10,000 ×g for 15 min at 4 °C, the supernatants were collected, and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. The separated blots were electrophoretically transferred to nitrocellulose membrane and blocked in 5% nonfat milk for 2 h at 4 °C. Blots were then incubated with Bcl-2 (1:500) antibody overnight at 4 °C. The membrane was washed in PBS and incubated for 1 h at room temperature with peroxidase-conjugated goat antimouse antibody (1:5000). After further washing with PBS, the membrane was analyzed by use of the ECL method. Relative protein levels were quantified by scanning densitometry.

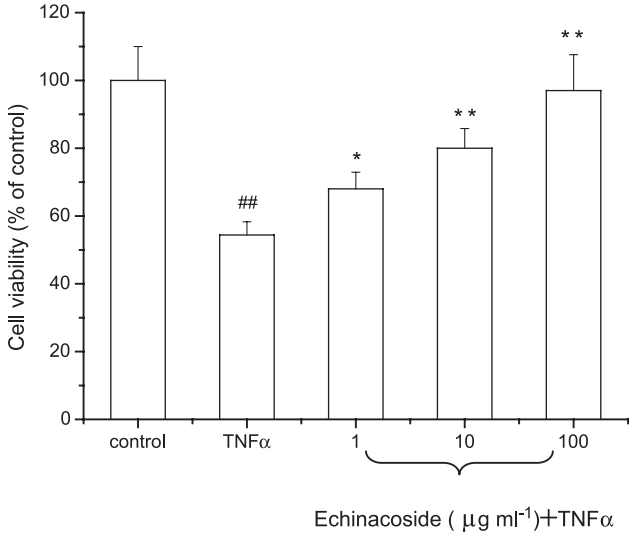

Fig. 2. Effect of echinacoside on TNFα-induced decrease in SHSY5Y cell viability. Cell viability was detected by MTT method as described in Materials and methods. n=8. Data are mean±S.E.M. $^{\#\#}p<0.01$ as compared to control cells, maintained in the absence of any stimuli; $^{*}p<0.05$, $^{**}p<0.01$ as compared to TNFα.

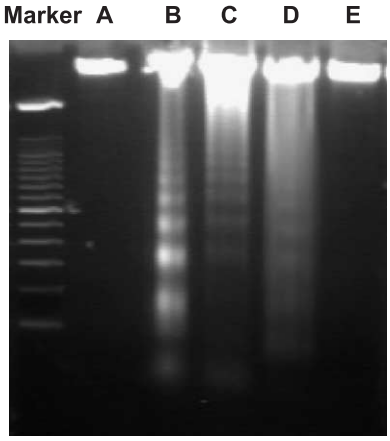

Fig. 3. Effect of echinacoside on TNFα-induced DNA fragmentation in SHSY5Y cells. (A) Control, (B) treatment with 100 ng ml^{-1} TNFα, (C) treatment with 100 ng ml^{-1} TNFα and 1 μg ml^{-1} echinacoside, (D) treatment with 100 ng ml^{-1} TNFα and 10 μg ml^{-1} echinacoside and (E) treatment with 100 ng ml^{-1} TNFα and 100 μg ml^{-1} echinacoside.

2.11. Statistical analysis

Results are expressed as the mean±S.E.M of triplicate values for each experiment. Statistical comparisons involved use of the Student's *t* test. Values at $p<0.05$ were considered to be statistically significant.

3. Results

3.1. Effect of echinacoside on cell nuclear morphology

The Hoechst 33342 staining that is sensitive to DNA was used to assess changes in nuclear morphology following echinacoside and TNFα treatment. The nuclei in normal cells were larger and exhibited diffused staining of the chromatin (Fig. 1A). However, after exposure to 100 ng ml^{-1} TNFα for 36 h, SHSY5Y cells underwent morphologic changes of apoptosis (Fig. 1B). A marked decrease was observed in the cells treated with echinacoside (Fig. 1C–E).

3.2. Effect of echinacoside on cell viability

After incubation with TNFα, approximately 42.9% of the cells underwent apoptotic death. Treatment with echinacoside (1, 10 or 100 μg ml^{-1}) decreased the cell death rate in a

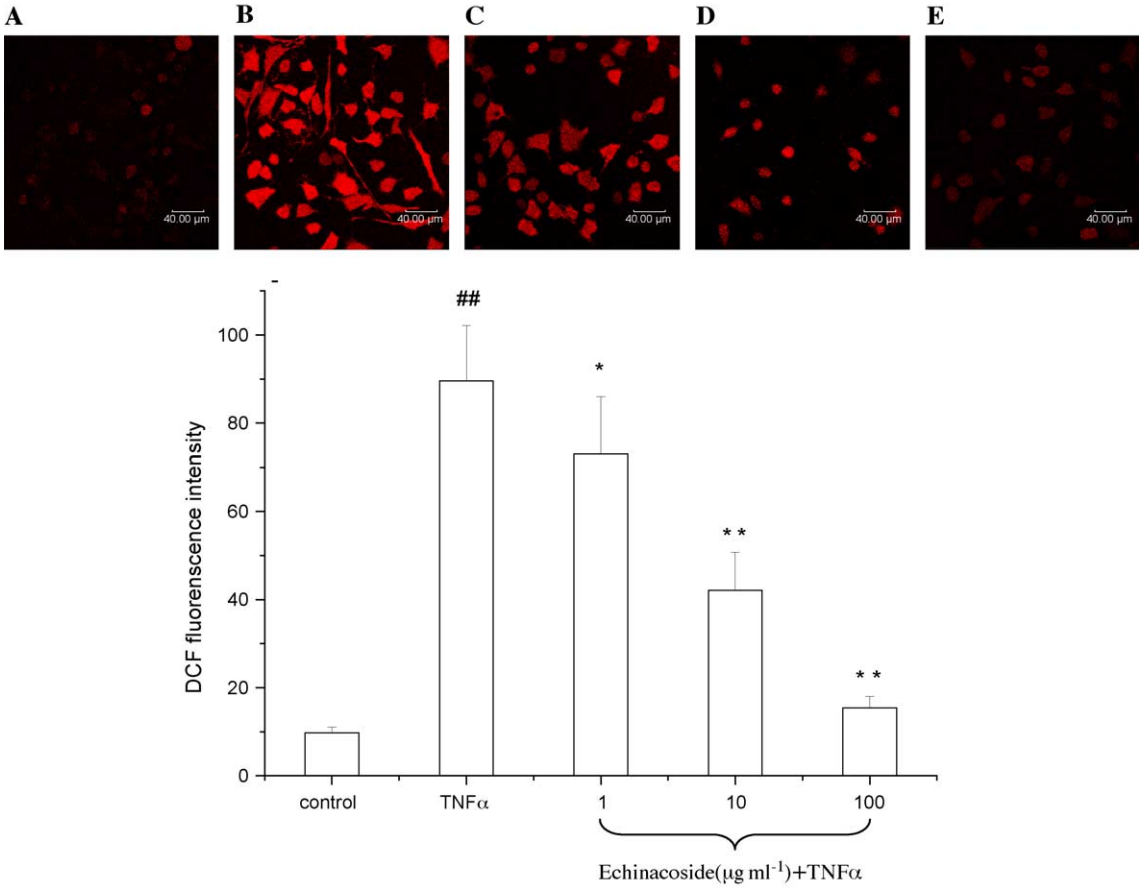

Fig. 4. Effect of echinacoside on level of intracellular reactive oxygen species in TNFα-induced SHSY5Y cells. (A) Laser confocal scanning microscope image of DCF fluorescence in control cells, (B) treatment with 100 ng ml^{-1} TNFα, (C) treatment with 100 ng ml^{-1} TNFα and 1 μg ml^{-1} echinacoside, (D) treatment with 100 ng ml^{-1} TNFα and 10 μg ml^{-1} echinacoside and (E) treatment with 100 ng ml^{-1} TNFα and 100 μg ml^{-1} echinacoside. $n=8$. Data are mean±S.E.M. $^{\#\#}p<0.01$ as compared to control cells; $^{*}p<0.05$, $^{**}p<0.01$ as compared to TNFα.

dose-dependant manner (cell death rate was 32%, 20% and 3%, respectively; Fig. 2); but echinacoside alone did not cause any apparent cytotoxicity (data not shown). Thus, echinacoside has a good neuroprotective effect on TNFα-induced cell death.

3.3. Effect of echinacoside on DNA fragmentation

After exposure of SHSY5Y cells to TNFα for 36 h, typically pronounced DNA laddering was observed. However, treatment cells with echinacoside inhibited TNFα-mediated DNA laddering, especially at the concentration of 100 μg ml^{-1}, which completely inhibits DNA fragmentation (Fig. 3).

3.4. Effect of echinacoside on reactive oxygen species formation

The level of reactive oxygen species generated in TNFα-induced cells increased markedly compared with controls (Fig. 4). However, the fluorescent intensity in echinacoside-treated cells decreased by 18%, 53% and 82% with 1, 10 or 100 μg ml^{-1} echinacoside treatment, respectively. Thus, echinacoside can inhibit the generation of reactive oxygen species in SHSY5Y cells induced by TNFα and has a neuroprotective effect.

3.5. Effect of echinacoside on mitochondrial membrane potential

Control cells exhibited numerous brightly staining mitochondria that emitted red-orange fluorescence and red/green ratio is 5.97±0.21, which was indicative of normal high membrane potential (Fig. 5A). TNFα treatment induced a transition in mitochondria permeability and a significant loss of membrane potential (red/green ratio is 0.35±0.02) (Fig. 5B). However, echinacoside treatment inhibited the collapse of mitochondrial membrane potential induced by TNFα in SHSY5Y cells with increasing dosage. Echinacoside gradually resumed the mitochondrial membrane potential with increasing concentrations, as indicated via the reappearance of red mitochondrial staining (Fig. 5C–E).

3.6. Effect of echinacoside on phospholipid phosphatidylserine exposure

The antiapoptotic action of echinacoside was also confirmed by measuring the presence of phosphatidylserine on the outer cell membrane. Fig. 6 shows the results of annexin-V binding experiments to measure the presence of phosphatidylserine on the outer cell membrane revealed approximately 37% of cells with apoptosis after TNFα treatment (Fig. 6B), but the proportion decreased to 25.9%, 18.3% and 8.2% with 1, 10 or 100 μg ml^{-1} echinacoside treatment, respectively (Fig. 6C–E).

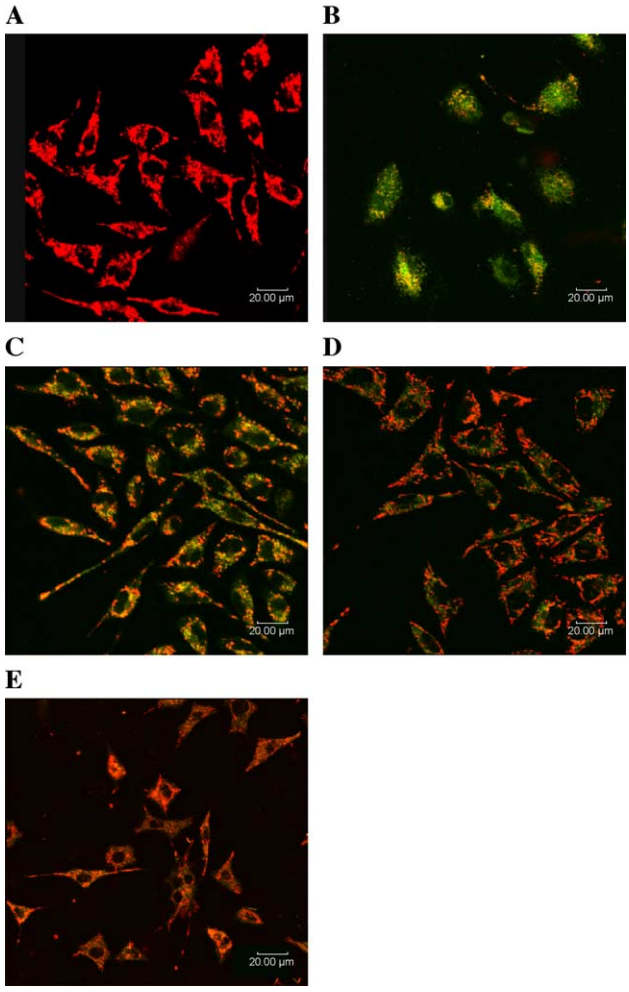

Fig. 5. Confocal laser scanning images of JC-1 fluorescence in SHSY5Y cells after 36-h exposure to TNFα in the absence or presence of echinacoside. (A) Control cells, (B) treatment with 100 ng ml^{-1} TNFα, (C) treatment with 100 ng ml^{-1} TNFα and 1 μg ml^{-1} echinacoside, (D) treatment with 100 ng ml^{-1} TNFα and 10 μg ml^{-1} echinacoside and (E) treatment with 100 ng ml^{-1} TNFα and 100 μg ml^{-1} echinacoside.

3.7. Effect of echinacoside on caspase-3 activity

Caspase-3 activity was increased by fivefold compared with controls after TNFα exposure (Fig. 7). In contrast, SHSY5Y cells which were simultaneously treated with (1, 10 or 100 μg ml^{-1}) echinacoside showed a significant decrease in caspase-3 activity compared with TNFα-treated cells at the same time point (Fig. 7). The data showed that treatment with echinacoside resulted in the inhibition of TNFα-induced activation of caspase-3.

3.8. Effect of echinacoside on expression of Bcl-2

Bcl-2 has been considered as an antiapoptosis agonist. Western blotting revealed an increased expression of Bcl-2 in the presence of TNFα. Echinacoside treatment enhanced the expression in a dose-dependent manner. This increased expression should be a compensatory and protective response

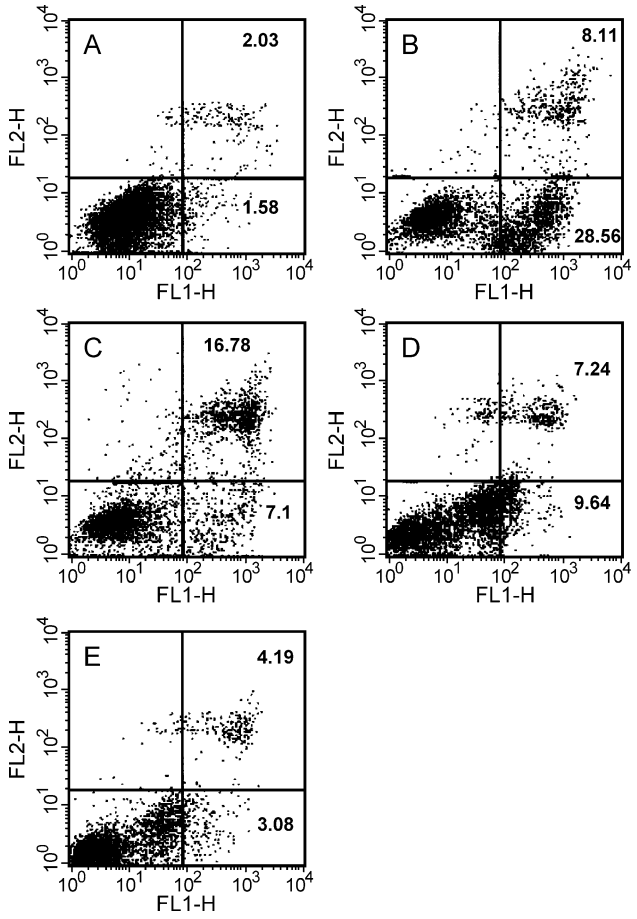

Fig. 6. Flow cytometric histograms of control SHSY5Y cells and cells exposed to TNFα alone or with echinacoside. (A) Control, (B) treatment with 100 ng ml^{-1} TNFα, (C) treatment with 100 ng ml^{-1} TNFα and 1 μg ml^{-1} echinacoside, (D) treatment with 100 ng ml^{-1} TNFα and 10 μg ml^{-1} echinacoside and (E) treatment with 100 ng ml^{-1} TNFα and 100 μg ml^{-1} echinacoside. Healthy cells are in the bottom left quadrant, apoptotic cells are in the bottom right quadrant, and necrotic and late apoptotic cells are in the upper right. Numbers in each quadrant are percentage of cells they contain.

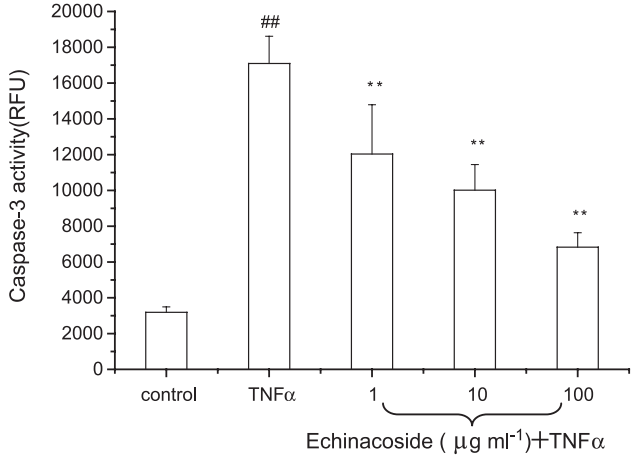

Fig. 7. Effect of echinacoside on TNFα-induced increase of caspase-3 activity. n=8. Data are mean±S.E.M. $^{\#\#}p<0.01$ as compared to control cells; $^{**}p<0.01$ as compared to TNFα.

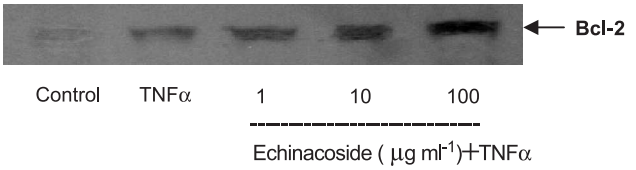

Fig. 8. The expression of Bcl-2 protein in SHSY5Y cells after 36-h exposure to TNFα in the absence or presence of echinacoside.

of the cells to TNFα-induced neurotoxicity, and echinacoside could markedly strengthen this potential (Fig. 8).

4. Discussion

C. salsa (C.A. Mey) G. Beck, one species of *Cistanches* which belongs to Orobanchaceae family, is a parasitic plant native in the northwest of China. The stem of this plant is an important traditional Chinese medicine and used for kidney deficiency, female infertility, morbid leucorrhea, neurasthenia and senile constipation due to colonic inertia. The major active constituents of this herb are phenylethanoid glycosides (Lei et al., 2001). Several phenylethanoids have been shown to possess free radical scavenging properties and protect oxidative-stress-induced toxic injuries (Xiong et al., 1996, 2000; Gao et al., 1999). However, the cellular and molecular mechanisms that underlie the actions are not fully understood. Echinacoside is one of the phenylethanoids isolated from the stems of *C. salsa*, which pharmacological and biological activities are not reported. The present study demonstrates for the first time that echinacoside has significant neuroprotective effects on TNFα-induced apoptosis in SHSY5Y neuronal cells by maintaining mitochondrial function, decreasing the generation of reactive oxygen species, increasing the expression of the antiapoptotic protein Bcl-2 and inhibiting caspase-3 activity through an antioxidation mechanism. These mechanisms may be through the individual neuroprotective effect of echinacoside or through its interaction with other factors and may lead to a decreased ratio of apoptosis in cells.

Previous studies have shown that oxidants or prooxidants are important regulators of apoptosis and can induce apoptosis (Gil et al., 2003; Coyle and Puttfarken, 1993; Olanow, 1993). Oxidative stress is a common element of apoptosis induced by various stimuli such as TNFα, serum deprivation and environmental toxin exposure, which usually do not exert a direct oxidant action. Gil et al. have reported that substances with antioxidative properties can inhibit apoptosis by decreasing the generation of reactive oxygen species and protecting oxidative-stress-induced toxic injuries (Gil et al., 2003). Inasmuch as we found that echinacoside treatment decreased the level of reactive oxygen species compared with TNFα treatment, it might protect neurons against apoptosis by directly scavenging intracellular reactive oxygen species.

Much evidence suggests that major alterations in mitochondrial function are critically involved in the apoptotic process. Several biochemical events, including reduced mitochondrial membrane potential, calcium ion influx and hyperproduction of reactive oxygen species and cytochrome *C* release, have been proposed to be necessary for apoptosis (Dallaporta et al., 1998; Heiden et al., 1997; John and Orrenius, 2002). These biochemical changes may result from alternations in the function of mitochondria (Krajewski et al., 1993). In addition, Bcl-2 is considered an antiapoptotic protein located mainly on the outer membrane of mitochondria (Hochenbery et al., 1993) and can inhibit apoptosis by suppressing the formation of reactive oxygen species or its effect (Kluck et al., 1997), thus preventing the release of cytochrome *C* from mitochondria and regulating intracellular calcium (Marcin et al., 1996). The Bcl-2 level has been demonstrated to be up-regulated in brains of patients with degenerative diseases. Such up-regulation might imply a compensatory response of the remaining neurons to protect against apoptosis. Oxidant-induced damage, including TNFα, has been shown to stimulate the endogenous antioxidant system (Wong and Goeddel, 1988; Briehl et al., 1997). It has been recently demonstrated that TNFα increased neuronal Bcl-2 expression in a nuclear factor kB (NF-kB)-dependent manner (Tamatani et al., 1999). In the present study, the Bcl-2 protein expression increased and mitochondrial membrane potential markedly decreased after exposure to TNFα, which indicate that the induced Bcl-2 protein expression may be part of protective response but that it is ineffective in blocking apoptosis. Following treatment with echinacoside, the Bcl-2 level was further enhanced, and the collapse of the mitochondrial membrane potential was rescued. Thus, echinacoside could promote the expression of Bcl-2 protein in maintaining mitochondrial membrane potential at a high level, suppress the dysfunction of mitochondria and counteract the toxicity of TNFα.

Outer stimuli can initiate apoptosis through the above mechanisms and may converge on the caspase pathway to execute the final phase of the apoptotic process. The caspase family of proteases consists of at least 14 mammalian members that are constitutively expressed in almost all cell types as inactive proenzymes (zymogens) that become processed and activated in response to a variety of proapoptotic stimuli (Kohler et al., 2002). Caspase-3 is a downstream member of the caspase cascade and acts as a central effector in the execution phase. When caspase-3 precursor protein CPP32 is activated by upstream signals such as the release of mitochondrial cytochrome *C*, the active caspase-3 cleaves specific aspartate residues in proteins with various structural, housekeeping and regulatory functions (Thornberry and Lazebnik, 1998; Dodel et al., 1999). These proteolytic events can lead to cell apoptosis and contribute to DNA fragmentation and nuclear morphologic changes. Stefanis et al. (1999) demonstrated that caspase inhibitors rescue cortical neurons from rapid apoptotic death after exposure to the DNA damaging agent camptothecin. Thus, substances that can inhibit the activity of caspase-3 might protect cells from apoptosis. Inasmuch as echinacoside markedly inhibited the caspase-3 activity in the TNFα-treated cells, it has the neuroprotective capacity.

In conclusion, echinacoside has a multifunctionally protective effect on damaged neurons. Because of its powerful antiapoptosis activity, it might be therapeutic for neurodegenerative and neurologic disabilities involving neuron apoptosis.

Acknowledgements

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