We investigated whether suppression of nitric oxide (NO) implicated in the protective effect of echinacoside (ECH), a phenylethanoid glycoside, on H$_2$O$_2$-induced injury to the rat pheochromocytoma cell line (PC12 cells). Data show that application of ECH to H$_2$O$_2$-injured PC12 cells (HIPCs) increased cell viability and decreased the necrotic ratio. Laser scanning confocal microscopic (LSCM) analysis suggested that ECH exerted an inhibitory effect on the formation of NO. In addition, RT-PCR analysis revealed that ECH down-regulated p65 and iNOS mRNA expressions in HIPCs. In summary, suppression of NO is related to the protective effect of ECH on HIPCs.

Keywords: Echinacoside, ECH, apoptosis, NO, iNOS, NF-κB.

Alzheimer’s and Parkinson’s diseases are neurodegenerative disorders characterized by apoptosis and neuron loss where the effects of oxidative stress are thought to play critical roles [1]. Because H$_2$O$_2$ has been used extensively to induce cell apoptosis, and PC12 cells show many properties just like primary cultured neuronal cells [2], the HIPCs model has been used widely to develop drugs for neurodegenerative diseases in vitro [3,4]. Studies have demonstrated that NO synthesized by iNOS promotes neurotoxicity and plays an important role in H$_2$O$_2$–mediated cell apoptosis [5]. NF-κB is an essential transcription factor mediating iNOS induction and can be inversely activated by NO [6]. Although the complicated interaction among NF-κB, iNOS and NO in the process of cell apoptosis remains unknown, it is a fact that inhibition of NO makes a major contribution to cytoprotection, and a compound suppressing the generation of NO could be a potential candidate for use in the intervention of neurodegenerative diseases.

ECH (Figure 1) was extracted from Echinacea angustifolia in 1950 and defined as a phenylethanoid glycoside. It exists also in several other plants such as Cistanche tubulosa (Schrenk) Wight. In China, glycosides isolated from the stems of C. tubulosa have been approved as a treatment for vascular dementia. This is a government-approved drug containing phenylethanoid glycosides which have shown antioxidant properties and a neuroprotective function with ECH as a quality control compound (content more than 25%) [7,8]. It has recently been demonstrated that ECH protects against hepatotoxicity and is a potent promoter of neuronal survival that is induced by TNF-α and MPTP [9,10]. However, these studies provide little information about the relationship between NO and the protective effect of ECH on oxidative stress-induced injury in vitro. In order to explore this subject, the cell survival, apoptosis and necrosis, and the change of NO, iNOS and p65 mRNA expressions were determined.

Cell viability determined by MTT reduction was markedly decreased and the necrosis ratio detected using flow cytometry was increased after PC12 cells were exposed to H$_2$O$_2$. However, when the cells were pre- and co-incubated with ECH, H$_2$O$_2$-induced cell
Table 1: Attenuation of H2O2-induced PC12 cell injury by ECH.

<table>
<thead>
<tr>
<th>Group</th>
<th>Survival(%)</th>
<th>Necrosis(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 5.1</td>
<td>1.67 ± 0.20</td>
</tr>
<tr>
<td>H2O2</td>
<td>27.9 ± 8.0 ***</td>
<td>4.83 ± 0.78 ***</td>
</tr>
<tr>
<td>H2O2+ECH 5μg/mL</td>
<td>35.5 ± 10.8</td>
<td>3.54 ± 0.50</td>
</tr>
<tr>
<td>H2O2+ECH 10μg/mL</td>
<td>51.0 ± 13.4 **</td>
<td>2.59 ± 0.68 **</td>
</tr>
<tr>
<td>H2O2+VE 10μg/mL</td>
<td>56.6±12.4***</td>
<td>3.22 ± 0.73 ***</td>
</tr>
</tbody>
</table>

Data are expressed as percentages of cell viability relative to the control, with mean ± SD (n = 5). ### P < 0.001 compared with control; ** P < 0.01, *** P < 0.001 compared with H2O2.

Toxicity was significantly attenuated (Table 1). The pictures of PC12 cells stained by Hoechst 33342 are shown in Figure 2. The apoptotic cells can be distinguished by their typical morphological appearance: chromatin condensation and nuclear fragmentation with nuclei being stained much lighter than normal cells.

Figure 2: H2O2 induced cell apoptosis stained by Hoechst 33342 and the effect of ECH. “→” shows the apoptotic cells. A: Control; B: H2O2; C: ECH 5μg/mL; D: ECH 10μg/mL; E: VE 10μg/mL.

To determine whether NO was involved in the protection of ECH on H2O2-induced PC12 cell injury, LSCM was utilized to monitor the change of NO level. After PC12 cells were treated with 200 μM of H2O2 for 30 min, NO increased rapidly during the first 28 min and then attained a peak. The treatment with ECH (5, 10 μg/mL) for 30 min prior to H2O2 treatment significantly suppressed the time course curve (Figure 3A). Moreover, ECH application caused a remarkable decrease in the peak value of NO by 33.1% (P < 0.05) and 43.3% (P < 0.01), (Figure 3B). The results suggest that ECH reduces the NO level elevated by H2O2.

Figure 3: Effect of ECH on the time course of NO and peak value of Ft/F0 (n = 20-25). Data are expressed as Ft/F0 and mean ± SD. ### P < 0.001 compared with control; ** P < 0.01 compared with H2O2.

We also measured the effects of ECH on H2O2-induced up-regulation of p65 and iNOS mRNA by RT-PCR. As shown in Figure 4, p65 and iNOS mRNA expressions in PC12 cells increased (P < 0.01) when cells were treated with 400 μM of H2O2. However, ECH significantly suppressed the up-regulated p65 and iNOS mRNA.

Figure 4: Effect of ECH on the expressions of p65 and iNOS mRNA in H2O2-induced PC12 cells. (A): Photographs of agarose gel electrophoresis of the p65 and iNOS and β-actin mRNA RT-PCR product. The level of mRNA was quantified by densitometric analysis and expressed as targeted mRNA / β-actin and mean ± SD. ### P < 0.001 compared with control; * P < 0.05, ** P < 0.01, *** P < 0.001 compared with H2O2.

NO, a free radical, is synthesized by three different types of NO synthase including the constitutive Ca2+/calmodulin-dependent neuronal and endothelial isoforms (nNOS and eNOS) and the inducible Ca2+/calmodulin-independent isoform (iNOS). NO is synthesized by different types of NO synthase, which shows divergent effects on neuronal cells. Unlike the protective effect of eNOS, NO produced by nNOS and iNOS has a neurotoxic action which derives in part from the reaction of NO with superoxide anions to form peroxynitrite to alter cell functions [11]. iNOS was assumed to have an important role during CNS injuries [12]. For example, it has been reported that cerebral...
ischemia-reperfusion injury in rats induced NF-κB and iNOS activation and taxifolin ameliorated the injury by modulating iNOS and NF-κB expressions [13]. The relationships among the NF-κB, iNOS and NO remain unclear in HIPCs. We propose that H2O2 can elicit a rapid rise in intracellular Ca2+, which could activate Ca2+-dependent nNOS, and NO produced by nNOS could stimulate NF-kB activity, leading to increased synthesis of iNOS, which would then promote greater NO release and further activation of NF-κB, resulting in a vicious cycle in which NO seems to have a critical role.

The results indicate that ECH protects PC12 cells against H2O2-induced apoptosis, necrosis and increases cell viability through a route of suppressing NO formation. Several mechanisms are proposed to explain this suppression. First, experiments showed that ECH decreased the level of NO by down-regulating the expressions of NF-kB and iNOS. Secondly, ECH reduced the concentration of Ca2+ in HIPCs with a lower Ca2+-dependent nNOS activity. Thirdly, a possible mechanism is suggested by ECH’s polyphenolic structure, since polyphenolic compounds are known to be potent antioxidants and free radical scavengers. Therefore, suppression of NO by ECH protects H2O2-induced PC12 cells injury.

**Experimental**

**Materials and cell culture:** ECH (purity >98%, HPLC) and vitamin E (V E, purity >98.8%, GC) were supplied by the National Institute for the Control of the Pharmaceutical and Biological Products (Nicpbp, China). PC12 cells were purchased from Shanghai Institute of Biochemistry and Cell Biology (Sibcb, China). Cells were maintained in DMEM (Gibco, USA) supplemented with 5% heat-inactivated horse serum, 10% fetal bovine serum (Gibco, USA), 100 U/mL penicillin, and 100 U/mL streptomycin. Culture flasks were kept in humidified 5% CO2/95% air at 37°C. The medium was changed every 3 days.

**Experimental protocols:** PC12 cells were grown to 80-90% confluence and then replanted at an appropriate density (according to the particular experiment) on either culture plates or dishes. To induce oxidative stress, fresh H2O2 was prepared from a 30% stock solution prior to each experiment. In all experiments, two doses (5, 10 μg/mL) of either ECH or 10 μg/mL of V E were pre-incubated with PC12 cells for 30 min, after which 400 μM of H2O2 was added (except in NO evaluation). Assays were performed 4 h after H2O2 was added. V E, a strong antioxidant that has anti-apoptosis properties [14], was used as a positive control.

**Measurement of cell viability:** PC12 cells were seeded in a 96-well plate at a density of 2 × 104 cells per well. After treatment with ECH and H2O2, as described above, cells were incubated with 0.5 mg/mL MTT solution (Sigma, USA) for 4 h at 37°C. Formazan crystals that formed in intact cells were dissolved with 200 μL of dimethylsulfoxide (DMSO), and the absorbance was measured at 570 nm using a microplate reader (Molecular Devices Co., Spectra MAX 340, USA). Cell viability measurements were calculated as a percentage of the absorbance of the control sample.

**Determination of apoptotic cells with Hoechst 33342 staining and necrotic cells with PI combined flow cytometry:** To further evaluate the effect of ECH on the H2O2-induced apoptosis of PC 12 cells, apoptotic nuclei staining in H2O2-administered PC12 cells was examined. After treatment with H2O2 for 4 h with or without ECH, the cells were rinsed with PBS and fixed with 4% paraformaldehyde for 5 mins. The fixed cells were washed twice with PBS and incubated with the DNA-binding dye Hoechst 33342 at 10 μg/mL. Nuclear morphology was examined by Olympus IX 70 fluorescence microscopy (Tokyo, Japan). Individual nuclei were visualized to distinguish the apoptotic cells by their typical morphological appearance: chromatin condensation, nuclear fragmentation with nuclei being stained more lightly or smaller than normal cells [15]. In addition, separate groups of cells were also harvested and stained with PI (propidium iodide, Caltag, USA) to evaluate the percentage of necrotic cells using flow cytometry (Becton-Dickinson, USA). Data were analyzed using CellQuest software (Becton-Dickinson).

**Measurement of NO change:** A LSCM (Zeiss 510, Germany) was used to evaluate the relative change of NO by detecting DAF-2 fluorescence (10 μM, Molecular probes, USA). Fluorescence was measured at an excitation of 488 nm and emission of 515 nm - 565 nm. Following the addition of 200 μM of H2O2 to the dish, laser scanning was used to obtain a series of images. Image frames were acquired every 30 secs and image acquisition was completed within 30 mins following H2O2 addition. Images were analyzed quantitatively using Zeiss LSM software to determine the change in fluorescence intensity within a cell (20-25 cells were analyzed in each group). The fluorescence at each time point (Ft) after H2O2 addition was divided by the fluorescence before H2O2 addition (F0) to obtain the ratio Ft/F0 [16]. ECH and V E were incubated with PC12 cells for 30 mins prior to H2O2 irritation.

**RT-PCR analysis of NF-κB p65 and iNOS mRNA:** After treatment, as mentioned in “Experimental protocols”, 5 × 105 cells were rinsed twice with cold
PBS and total RNA was extracted according to the Kit supplier’s instructions. For PCR amplification, the specific primers used were as follows: (1) iNOS sense primer: 5′-GCCTCGCTCTGGAAGAAGA-3′, antisense primer: 5′-TCCATGCAGACACCTT-3′; (2) p65: sense primer: 5′-AAGATCAATGGCTACACAGG-3′; anti-sense primer: 5′-CCTCAATGCTTCTTCTTGCG-3′; (3) β-actin: sense primer: 5′-GAGGGAAATCGTGCGTGAC-3′, anti-sense primer: 5′-TTGGCATAGTAGGTCTTTACG-3′. The primer sets yield PCR products of 477, 493 and 272 bp for iNOS, p65 and β-actin, respectively. The PCR procedure was performed at 42ºC for 30 min, and 94ºC for 3 min, followed by 35 cycles at 94ºC for 20 s, 60ºC for 1 min, and extension at 72ºC for 5 min. PCR products were examined by electrophoresis on 2% agarose ethidium bromide gel and visualized by an ultraviolet gel documentation system. The intensity of the PCR products was quantified by scanning densitometry using NIH Image 1.61 software. The level of gene mRNA expression was expressed as the ratio of the intensity of the gene PCR products to the corresponding β-actin PCR product.

Statistical analysis: Data are presented as mean ± SD. Differences between group mean values were calculated using analysis of variance, and comparisons between means were performed by the two-tailed Student’s t-test. Statistical significance was defined as P < 0.05.

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