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Original article

Neuroprotection against hydrogen peroxide-induced toxicity by *Dictyophora echinovolvata* polysaccharide via inhibiting the mitochondria-dependent apoptotic pathway

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ABSTRACT

Neuronal apoptosis caused by toxic stimuli such as oxidative stress is believed to be one of the major reasons in the pathologenesis of neurodegenerative diseases. In the current study, the neuroprotective effects of the crude polysaccharide fraction of edible *Dictyophora echinovolvata* (DEVP) against H_2O_2-induced cytotoxicity were investigated using PC12 cells. Following exposure of PC12 cells to 750 μM H_2O_2, a significant reduction in cell viability and the number of FDA-stained viable neurons as well as an increase in the number of PI-stained dead cells were observed. Furthermore, H_2O_2 treatment significantly upregulated the protein expression of Bax, cleaved caspases 3 and cytosolic cytochrome c, and down-regulated Bcl-2 levels. 2 h pre-treatment with VP reversed these changes caused by H_2O_2, including inhibiting neuronal loss and decreasing Bax, cleaved caspases 3 and cytosolic cytochrome c levels, as well as increasing Bcl-2 levels. These results taken together demonstrated that DEVP provided a substantial neuroprotection against H_2O_2-induced toxicity in PC12 cells, at least partly through inhibiting the mitochondrial apoptotic pathway. These findings suggested that DEVP might be a potential candidate for further preclinical study for preventing neurodegenerative diseases in which oxidative stress and apoptosis are involved.

1. Introduction

Neurodegenerative diseases are characterized by the progressive functional neuronal loss in various brain regions, such as the hippocampus, substantia nigra and striatum [1,2]. Though the exact causes of neurodegenerative diseases are still unknown, increasing lines of evidence indicate that oxidative stress plays an important role in the pathology of these devastating disorders [3].

Normally, hydrogen peroxide (H_2O_2) is formed as a natural byproduct of enzymatic oxidase actin and thereby acts as an endogeneous source of hydroxyl free radicals. However, excessive exogenous H_2O_2 could elevate oxidative stress, such as reactive oxygen species (ROS), beyond the protective capacity of endogenous antioxidant defenses and induce apoptotic neuronal death by initiating mitochondrial dysfunction [4,5], which is associated with the increase in Bax/Bcl-2 protein ratio, activation of caspase-3 and release of cytochrome-c. Thus, H_2O_2 has been widely used as an inducer of neuronal injury to probe the neuroprotective effects and underlying molecular mechanisms of novel pharmacotherapy [6–8].

Dictyophora indusiata is an edible fungus in China. The folkloric consumption of *Dictyophora indusiata* in ancient China began as early as 618 CE, which pointed mainly to the nutritional bioactivities, like benefits to eyes and tonics to cardiovascular systems; and partially to the medicinal effect like mental tranquilization, antitumor, and tonics, and so forth. This fungus

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has high nutritional value because it is rich in vitamins, polysaccharide and micro minerals. *Dictyophora echinovolvata* is one of the species of *Dictyophora indusiata*, and at present most of the market share of *Dictyophora indusiata* sold in China belongs to this species due to its easy cultivation. In recent years, anti-tumor [9,10], anti-proliferative [11,12] and anti-oxidative activities [13–15] of *Dictyophora indusiata* have been reported in different models. However, there is little about the neuroprotective effects of *Dictyophora echinovolvata*. In the current study, we investigated the neuroprotection and underlying mechanisms of *Dictyophora echinovolvata polysaccharide* (DEVP) in a H_2O_2–induced neurotoxicity cell model, and found for the first time that DEVP protected against H_2O_2–induced neuronal death in PC12 cells, possibly through the inhibition of the mitochondrial apoptotic pathway.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). Fluorescein diacetate (FDA), propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and H_2O_2 were obtained from Sigma (St Louis, MO, USA). Primary antibodies against Bax, Bcl-2, caspases-3, and β-actin were from Cell Signaling Technology (Beverly, MA, USA). Secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. DEVP preparation

The fruiting body of *Dictyophora echinovolvata*.was purchased from a commercial market in Shunchang, Fujian province of China. The homogenate of D. echinovolvata gelatine was boiled in distilled water for 2 h. After centrifugation to remove debris fragments, the supernatant was concentrated by rotary evaporation. Protein was removed with the use of Sevage method. The crude polysaccharide fraction of D. echinovolvata gelatine was obtained through precipitation with 4 vol of 95% ethanol, centrifugation and freeze-drying.

2.3. PC12 cell

PC12 cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin/streptomycin at 37 °C under an atmosphere of 95% air and 5% CO_2.

2.4. MTT assay

Cell viability was examined as reported previously [16]. Briefly, 24 h after plating, cells were incubated with different concentrations of H_2O_2 for 24 h (cell model establishment), or pre-treated with gradually increasing concentrations of VP for 2 h, then exposed to H_2O_2 for 24 h. After H_2O_2 challenge, cells were incubated with MTT solution for 2–4 h, and the resulted formazan was measured at a test wavelength of 570 nm.

2.5. Image analysis of FDA/PI viability staining

To differentiate viable cells from dead cells, a double staining procedure with the two specific fluorophores, FDA and PI, was carried out. Briefly, after H_2O_2 challenge, cells were incubated with 10 μg/ml FDA and 5 μg/ml PI for 5–10 min, then photographed using a fluorescence microscopy.

2.6. Intracellular ROS measurement

Intracellular ROS level was evaluated using the fluorescent probe DCFH-DA as reported previously [17]. Briefly, 4 h after H_2O_2 challenge, PC12 cells were incubated with 10 μM DCFH-DA in FBS-free medium at 37 °C for 30 min, then scanned with a plate reader at 485 nm excitation and 520 nm emission.

2.7. Western blotting

Mitochondria-dependent apoptotic signaling pathway was analyzed using Western blotting assay as previously described [17,18]. Briefly, after H_2O_2 challenge, cells were lysed and protein was extracted. Following concentration measurement using Bradford assay, protein (25–35 μg) was separated on SDS–polyacrylamide gel, and then transferred onto a polyvinyldifluoride membrane. Thereafter, membrane was incubated in blocking buffer (5% milk powder in Tris-Buffered Saline/Tween 20), probed with primary antibodies (Bax, Bcl-2, caspases-3, cytochrome c and β-actin) and secondary antibodies. The blots were developed using an enhanced chemiluminescence plus kit, then evaluated by densitometric analysis.

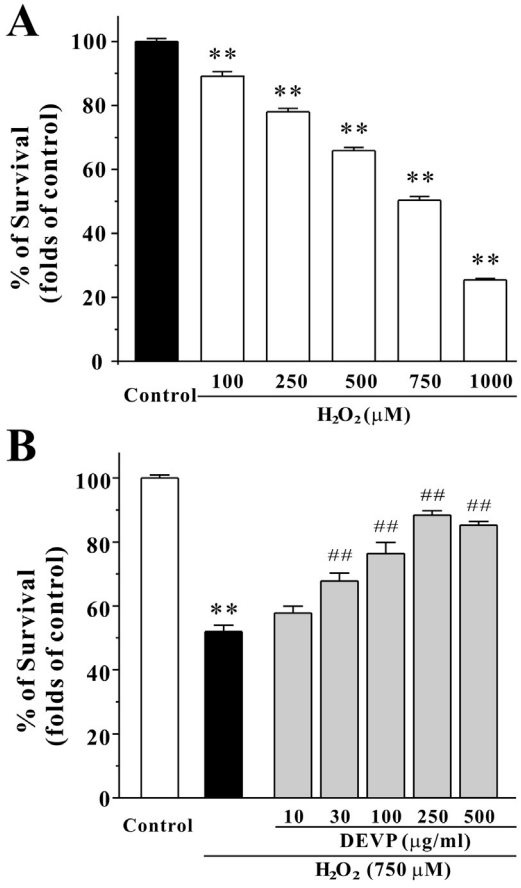

Fig. 1. DEVP substantially protected against H_2O_2–induced neuronal death in PC12 cells. (A), H_2O_2 markedly caused neuronal death in PC12 cells in a concentration-dependent manner. PC12 cells were treated with different concentrations of H_2O_2 (100–1000 μM) for 24 h, then subjected to MTT assay for measuring cell viability. **, $p < 0.01$, compared to control group. (B), DEVP prevented neuronal loss in PC12 cells caused by H_2O_2. PC12 cells were pre-treated with different concentrations of DEVP (10–500 μM) for 2 h, and then exposed to 750 μM H_2O_2 for 24 h, and finally subjected to MTT assay for measuring cell viability. **, $p < 0.01$, compared to control group; ##, $p < 0.01$, compared to H_2O_2 group.