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A selenium polysaccharide from *Platycodon grandiflorum* rescues PC12 cell death caused by H_2O_2 via inhibiting oxidative stress

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ABSTRACT

In this paper, a selenium polysaccharide (PGP1) was isolated from the radix of *Platycodon grandiflorum*. We investigated the protective capacity of PGP1 against the hydrogen peroxide (H_2O_2)-induced oxidative damage in cultured rat pheochromocytoma (PC12) cells. Cells were pretreated with various doses of PGP1 (50, 100 and 200 μg/mL) for 24 h before exposure to 0.5 mM H_2O_2 for 12 h. Cell viability, LDH release, apoptotic rates, malondialdehyde (MDA) content, antioxidant enzyme superoxide dismutase (SOD) activity and intracellular accumulation of reactive oxygen species (ROS) were determined. The results showed pretreatment of PC12 cells with PGP1 prior to H_2O_2 exposure inhibited the decrease of cell viability, decreased the apoptotic rates, prevented membrane damage (LDH release) and attenuated intracellular ROS formation in PC12 cells injured by H_2O_2. Meanwhile, PGP1 increased SOD activity, while it decreased the level of MDA and the production of lipid peroxidation, in PC12 cells after H_2O_2 exposure. These findings suggested that PGP1 may be considered as a potential useful antioxidant agent in reducing neuronal oxidative damage via inhibiting oxidative stress.

1. Introduction

A number of studies indicate that oxidative stress-induced cell damage has been implicated in a variety of neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) [1–3]. Oxidative stress from reactive oxygen species (ROS) has been widely considered as a major cause of cell damage for the above neurodegenerative disorders [4,5], and hydrogen peroxide (H_2O_2) has been experimentally used to stimulate ROS production to form oxidative stress in nerve cells [6]. Under normal conditions, natural antioxidant systems in the human body keep the balance between oxidants and antioxidants, but during neurodegenerative disease conditions, there are a lager amount of ROS produced in the body. As is stated above, a widespread view is that an effective strategy for treating the central nervous system degeneration would be protecting neurons from oxidative injury, which is likely to be one of the most effective means. The essential trace element selenium has received much attention in the past decade for human health and acts as a critically important antioxidant [7,8]. A variety of selenium-enriched biological products has higher bioavailability and appears a safer choice as a dietary supplement than inorganic Se species [9,10]. Natural selenium polysaccharides compounds are explored as a novel selenium source in dietary supplements and it is also reported that Se contributes to increasing antioxidant activity of polysaccharides in many different cell types [11]. Therefore, identification and discovery of new antioxidants may aid in the development of drugs for treatment of various neurodegenerative diseases.

The radix of *Platycodon grandiflorum* is named as Jiegeng in China and has been widely used for its pharmacological activities in traditional oriental medicine for thousands of years, which include hepatoprotective, expectorant, and enhanced insulin sensitivity effects [12–15]. Most studies on the polysaccharides of *P. grandiflorum* have concentrated on the pharmacological aspects of immunostimulatory and anti-tumor effects [16–18]. Hence, *P. grandiflorum* polysaccharides are of great interest to researchers. Although some studies on the pharmacological effects of the polysaccharides of *P. grandiflorum* have been reported, little is known about the protective effect of *P. grandiflorum* polysaccharides on nervous system, especially at the cellular level. In this study, we used H_2O_2 induced injury model of PC12 cells to explore the protective effects of a polysaccahride from *P. grandiflorum* on H_2O_2-induced cytotoxicity and its mechanism of action in PC12 cells.

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2. Materials and methods

2.1. Materials and chemicals

Platycodon grandiflorum were purchased from local market in Jilin (China) and identified according to Chinese Pharmacopoeia. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). Hydrogen peroxide (H_2O_2), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), propidium iodide, and 2′,7′-dichlorofluorescein diacetate (DCFH2-DA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Kits for SOD, lactate dehydrogenase (LDH) and MDA were all purchased from Jiancheng Bioengineering Institute (Nanjing, China). Other chemicals were of the highest quality available.

2.2. Isolation and purification of polysaccharide from the radix of P. grandiflorum

The powdered *P. grandiflorum* radix (532.4 g) was extracted with ether using a Soxhlet apparatus, followed by maceration in ethanol overnight in order to remove some colored materials, oligosaccharides, and some small molecule materials. The residue was dried in air and then extracted with boiling water for three times under stirring (6 h for each). After cooling to the room temperature, the supernatant was collected by centrifugation, concentrated, and precipitated by adding four volumes of 95% ethanol (v/v). This precipitate dissolved in 200 mL distilled water was mixed with the same volume of organic working solvent (chloroform: *n*-butanol = 3:1) on a rotary-shaking apparatus to remove the protein by the method of Sevag [19]. The protein-free solution was collected by centrifugation, concentrated, and subjected to dialysis in a cellulose membrane (molecular weight cut-off 3500) against tap water for 24 h, and further against distilled water at room temperature for three successive days. After centrifugation, the supernatant was added with 3 volumes of 95% EtOH to precipitate crude polysaccharides (yield: 24.32*g*, 4.57% of the starting material). Crude polysaccharide CPGP dissolved in distilled water was fractionated on a DEAE-cellulose column (2.5 cm × 40 cm) coupled to an AKTA FPLC system, and eluted with a step-wise gradient of 0, 0.2, 0.4, and 1.0 M NaCl at a flow rate of 1 mL/min. The eluate was assayed for carbohydrate content by the phenol–sulfuric acid method [20]. The distilled water eluate was concentrated, dialyzed, lyophilized, and further purified by gel permeation chromatography on a Sephacryl S-400/HR column (2.6 cm × 80 cm) using 0.1 mol/L NaCl solution as an eluent. The major polysaccharide fraction was pooled, freeze-dried and named as PGP1. The yield rate of PGP1 was 0.18% (0.95 g) for the starting material. This process was repeated until the necessary quantities of PGP1 for future experiments were obtained.

2.3. General analysis and monosaccharide analysis

The carbohydrate content was determined by the PhOH–H_2SO_4 method using glucose as a standard [20]. The total uronic acid content was measured by the m-hydroxydiphenyl assay using galacturonic acid as a standard [21]. The protein content was estimated according to the Bradford method using bovine serum albumin (BSA) as the standard [22]. Se in SPC was quantified using the method of Cheng [23].

Gas chromatography (GC) was used for the identification and quantification of the monosaccharides. Briefly, the polysaccharides (5 mg) were hydrolyzed with 1 M TFA at 100 °C for 8 h, followed by evaporation to dryness and successive reduction with $NaBH_4$ and acetylation with Ac_2O–NaOAc at 120 °C for 1 h. The Ac_2O was destroyed with ice-water, and the resulting alditol acetates extracted with $CHCl_3$ [24] and analyzed by GC.

2.4. Cell culture and treatment

PC12 cell line was obtained from Shanghai Institute of Cell Biology (Shanghai, China) and was cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 5% heat inactivated horse serum, penicillin (100 IU/mL), and streptomycin (100 IU/mL) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. When the PC12 cells reached sub-confluence, they were treated with 0.25% trypsin in 0.02% EDTA solution, after which they were seeded into 96-well plate at a density of 5×10^4 cells/well for 48 h. H_2O_2 was freshly prepared from 30% stock solution prior to each experiment and added to the cells for the indicated times. To study the protective effect of PGP1, different concentration of PGP1 was added before exposure to 0.5 mM H_2O_2 for 6 h.

2.5. Analysis of cell viability by MTT assay

For the evaluation of the PGP1 or H_2O_2 on the proliferation of PC12 cells, cells (1×10^6 cells/mL) were treated with PGP1 at different concentrations (50, 100 and 200 μg/mL) or 0.5 mM H_2O_2 for indicated times at 37 °C. To assess the cytoprotection of PGP1 on H_2O_2-induced cell injury, PC12 cells were pretreated with PGP1 before 0.5 mM H_2O_2 was added, after which they were divided into 5 groups randomly: DMEM complete medium group, H_2O_2 injured group, groups treated with PGP1 at the concentrations of 100, 200 and 400 μg/mL, respectively. Finally, the cells' viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. After plate was maintained at 37 °C for 4 h, supernatant was removed and the cells were lysed in DMSO (200 μL), and the plate was shaken for 10 min The Absorbance were read by a spectrophotometer Bio-Rad 550 (Bio Rad, Milano, Italy) using a detective wavelength of 570 nm, with a reference wavelength of 650 nm. The following formula was used: Cell viability (%) = absorption value experimental group/absorption value of control group × 100%. Cell viability in the control cells treated with vehicle was expressed as 100%.

2.6. Measurement of apoptotic rates

The extent of apoptosis in PC12 cells was quantified by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (PI) [25]. After drug treatments, cells were collected and washed with ice-cold PBS and fixed with 70% ice-cold ethanol for 1 h. Then the fixed cells (1×10^5) were harvested by centrifugation at 1000*g* for 5 min, suspended in a solution consisting of 50 μg/mL RNAse A, 20 μg/mL PI, 0.1% Triton X-100 and 0.1 mM EDTA (pH 7.4), and incubated at 37 °C for 30 min in the dark. The apoptotic rates were measured with a FACScan flow cytometer (Becton Dickinson).

2.7. Morphological assay

The PC12 cells were harvested by centrifugation (1000 rpm for 5 min) and observed with a phase-contrast microscope (Nikon) to reveal cell configuration and possible changes as described previously [26].

2.8. Measurement of intracellular ROS level

Intracellular ROS levels were measured using the H_2O_2-sensitive DCFH-DA fluorescence [27]. After drug treatments, PC12 cells were washed with PBS and stained with DCFH-DA (40 μM) for 30 min in the dark. All the samples were centrifuged and the