International Journal of Biological Macromolecules 48 (2011) 726–735

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Chitooligosaccharide-mediated neuroprotection is associated with modulation of Hsps expression and reduction of MAPK phosphorylation

Golsa Joodi, Niloufar Ansari, Fariba Khodagholi*

Neuroscience Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Article history:
Received 18 December 2010
Received in revised form 14 February 2011
Accepted 21 February 2011
Available online 26 February 2011

Keywords:
Chitooligosaccharides
Hsp
MAPK
Oxidative stress
PC12 cells

ABSTRACT

There is mounting evidence implicating the role of oxidative stress induced by reactive oxygen species (ROS) in neurodegenerative disease, including Alzheimer's disease. In this study we aimed to investigate the possible protective effect of chitooligosaccharide (COS), an antioxidant oligosaccharide, on hydrogen peroxide induced apoptosis in NGF-differentitated rat pheochromocytoma (PC12) cells. COS treatment reversed the decrease of cell viability induced by H_2O_2 and this was associated with diminished intracellular ROS and decreased level of cytosolic Ca^{2+}. Additionally, COS contributed to up-regulation of Bcl-2, down regulation of Bax protein and reduction of cleaved Caspase-3 protein. COS treatment stabilized Nrf2 in nucleus and increased the Hsp70 level within cell while down-regulated Hsp90 expression. Moreover, COS could inhibit the phosphorylation of different mitogen activated protein kinases (MAPKs), whose aberrant phosphorylation has been implicated in AD. Our findings suggest that heat shock response and MAPK cascades are both involved in cell survival, and by concomitantly regulating both pathways, COS can be a promising agent in treating neurodegenerative diseases.

1. Introduction

Oxidative stress is the major culprit in neuronal death observed in neurodegenerative diseases, such as Alzheimer's disease (AD) [1]. This imbalance between prooxidant and antioxidant factors in favor of prooxidants can contribute to accumulation of intracellular reactive oxygen species (ROS). ROS are normal byproducts of aerobic respiration and their level is strictly controlled by various cellular antioxidant compounds and enzymes [2]. While at lower levels ROS play physiological roles, their overproduction is associated with cytotoxic reactions leading to apoptosis [3]. Accordingly, antioxidant therapy has been adopted to prevent the oxidative stress induced neuronal cell death. The use of drug substances derived from plants has a long tradition in medicine. Together with their derivatives, and synthetic compounds deduced from natural product precursors, they represent a major part of today's pharmaceutical market. Although neuroprotective effects of various antioxidant compounds have been observed, the underlying molecular mechanism needs to be further elucidated.

Chitooligosaccharides (COS) are depolymerized products of chitosan, a natural polysaccharide with various biological activities [4] including antitumor [5,6], antimicrobial [7–9], immune-stimulant [10], and anti-apoptotic effects [11,12]. Yang et al. have reported the effects of COS on neuronal differentiation and neurite outgrowth of PC12 cells [13]. Recently, the antioxidative properties of chitosan and its derivatives have remarked the most attention [14,15]. Pour solubility in water and high viscosity of the solution of chitosan limit the use of chitosan in food and biomedical applications. Unlike chitosan, COS have good solubility in water and its solution has low viscosity which make it attractive in different applications.

In a previous study, we showed that COS can decrease neuronal cell death by activating the NF-E2-related factor2 (Nrf2)-antioxidant response element (ARE) signaling pathway [16]. This pathway controls the expression of genes which are involved in the detoxification and elimination of reactive oxidants [17]. In this study we aimed to determine the possible effect of COS on cellular responses essential for sensing environmental changes and adapting to them.

One such response is called heat shock response (HSR). The heat shock proteins (Hsps), also known as molecular chaperones, are an important class of prosurvival proteins that are induced by various stimuli and play essential roles in cell survival, stress response, and protein management. To the best of our current knowledge, the chaperone system in eukaryotic cells revolves around the ATPase activities of Hsp70 and Hsp90, the two primary chaperone scaffolds. Other chaperones and co-chaperones act through manipulating the activities of Hsp70 and Hsp90, thereby facilitating either folding of the client or its degradation [18].

Mitogen-activated protein kinase (MAPK) cascades are another major signaling pathway involved in cell proliferation, differenti-

* Corresponding author. Tel.: +98 21 22429768; fax: +98 21 22432047.
E-mail address: khodagholi@sbmu.ac.ir (F. Khodagholi).

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doi:10.1016/j.ijbiomac.2011.02.011

ation and adaptation [19]. Evidence shows that MAPK family may play a critical role in neuronal apoptosis [20] and its members are emerging as novel targets in treating neurodegenerative diseases.

Given the significance of these two cellular components in regulating cell fate, their interplay can be exploited to obtain more effective protection under stressful conditions. In the present study, we assessed the effect of COS pretreatment on Hsp expression and MAPK activation after H_2O_2 exposure in NGF-differentiated PC12 cells.

2. Materials and methods

2.1. Materials

Antibodies directed against Hsp70, Hsp90, phospho-p38 MAPK (Thr180/Tyr182), phospho-SAPK/JNK (Thr183/Tyr185), p38 MAPK, SAPK/JNK, Bax, Bcl2 and β-actin were obtained from Cell Signaling Technology. Phospho-ERK1/2 and ERK1/2 antibodies were obtained from ABCAM. Nrf2 and Lamin B2 antibodies were obtained from Santa Cruz Biotechnology. All the other reagents, unless otherwise stated, were from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture and differentiation

Rat pheochromocytoma (PC12) cells obtained from Pasteur Institute (Tehran, Iran) were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, Aldrich), supplemented with 10% horse serum, 5% fetal bovine serum and 1% antibiotic mixture comprising penicillin–streptomycin, in a humidified atmosphere at 37 °C with 5% CO_2. Growth medium was changed three times a week. PC12 cells were differentiated by treating with nerve growth factor (NGF) (50 ng/ml) every other day for 6 days.

2.3. Treatment conditions

Soluble COS was prepared by enzymatic hydrolysis of chitosan with chitosanase [21].

Briefly, 500 mg of chitosan was dissolved in 100 ml of acetate buffer (0.1 M). Enzyme solution (3.48 μg/ml, pH 5.7) was added to initiate reaction. The reaction mixture was incubated and shaked at 37 °C for 96 h. The mixture was centrifugated for 20 min at 1000 × *g*. The precipitated chitosan was removed and the supernatant containing COS was collected.

Differentiated PC12 cells were pretreated with different concentrations (50, 100 and 150 μg/ml) of COS for 1 h. The cells were then treated with H_2O_2 (150 μM) for 24 h.

2.4. Measurement of cell viability

Cell viability was determined by the conventional MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) reduction assay. The dark blue formazan crystals formed in intact cells were solubilized in dimethyl sulphoxide and the absorbance was measured at 550 nm. Results were expressed as the percentages of reduced MTT, assuming the absorbance of control cells as 100%.

2.5. Acridine orange/ethidium bromide (AO/EB) double staining

Apoptosis was determined morphologically after staining the cells with AO/EB followed by fluorescence microscopy inspection. Briefly, PC12 cells were seeded in a 6-well plate and were treated with different concentrations (50, 100 and 150 μg/ml) of COS followed by adding H_2O_2 (150 μM). After 24 h, the cells were harvested and washed three times with phosphate buffered saline (PBS) and were adjusted to a density of 1×10^6 cells/ml of PBS. AO/EB solution (1:1, v/v) was added to the cell suspension in a final concentration of 100 μg/ml. Cellular morphology was evaluated by fluorescence microscope (Zeiss, Germany).

2.6. Measurement of intracellular ROS

The fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCF-DA) was used to monitor intracellular accumulation of ROS. For this purpose, DCFH-DA solution (10 μM) was added to the suspension of the cells (1×10^6/ml) and the mixture was incubated at 37 °C for 1 h. Cells were then washed twice with PBS and the fluorescence intensity was measured by the Varian Cary Eclipse spectrofluorometer with excitation and emission wavelengths of 485 nm and 530 nm, respectively.

2.7. Measurement of intracellular calcium level

PC12 cells were collected and Fura-2/AM (at the final concentration of 5 μM) was added to the cell suspension. The suspension was 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 132 mM NaCl, 3 mM KCl, 10 mM glucose, 10 mM HEPES and 2 mM $CaCl_2$ (pH 7.4). The fluorescence intensity was measured by the Varian Cary Eclipse spectrofluorometer with excitation and emission wavelengths of 340 nm and 500 nm, respectively.

2.8. Western blot analysis

After treatment of PC12 cells with different concentrations of COS for 1 h followed by H_2O_2 exposure for 24 h, the cells were harvested and lysed using lysis buffer containing 1% Triton X-100, 1% SDS, 10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM sodium pyrophosphate, 2 mM Na_3VO_4, 1 mM NaF, 0.5% sodium deoxycholate, 10% glycerol, 1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin and 60 μg/ml aprotinin. Protein concentrations were determined according to Bradford's method [22]. Standard plot was generated by using bovine serum albumin. Nuclear and cytoplasmic proteins were isolated as described by Kutuk and Basaga [23]. Total proteins were electrophoresed in 12% SDS–PAGE gel, transferred to polyvinylidene fluoride membranes and probed with specific antibodies. Immunoreactive polypeptides were detected by chemiluminescence using enhanced electrochemiluminescence (ECL) reagents (Amersham Bioscience, USA) and subsequent autoradiography. Quantification of results was performed by densitometric scan of films. Data analysis was done by Image.J.

2.9. Measurement of glutathione levels

The concentration of glutathione (GSH) was determined in whole cell lysate using dithionitrobenzoic acid (DTNB) method at 412 nm [24] and GSH concentrations were expressed as μmol/mg protein.

2.10. Superoxide dismutase activity assay

Superoxide dismutase (SOD) activity was measured based on the extent inhibition of amino blue tetrazolium formazan formation in the mixture of nicotinamide adenine dinucleotide, phenazine methosulphate and nitroblue tetrazolium (NADH–PMS–NBT), according to the method of kakkar et al. [25]. Assay mixture contained 0.1 ml of cell lysate, 1.2 ml of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 ml of phenazine methosulphate (186 μM), 0.3 ml of NBT (300 μM) and 0.2 ml of