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Anthraquinone derivative exerted hormetic effect on the apoptosis in oxygen-glucose deprivation-induced PC12 cells via ERK and Akt activated Nrf2/HO-1 signaling pathway

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ABSTRACT

There were accumulated evidences that agents may attenuate neurological disorders through a hormetic effect. This study was designed to investigate hormetic effect of BME on the oxygen-glucose deprivation (OGD)-induced mitochondrial apoptosis in NGF-differentiated PC12 cells. The effect of BME on the intracellular reactive oxygen species (iROS) formation and pro-survival signals mediated by ERK and Akt as well as transcription factor nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) pathways was also determined. The present results showed that, at low concentrations, pretreatment with BME triggered stress response by causing ROS production, then, activated survival-promoting signals via ERK and Akt activated Nrf2/HO-1 signaling pathway, resulting in decrease in cytotoxicity induced by the OGD. It may be accepted that mild pretreatment with BME stimulated transient and moderate ROS production, but activated hormetic signals and induced stress responsive genes. In contrast, high concentrations of BME displayed toxic action due to massive ROS production. These results suggested that the effect of BME on the OGD-induced PC12 cells may be hormetic mechanism including induction of oxidative stress and subsequent activation of stress response gene expression.

1. Introduction

Oxidative stress is implicated in neurodegenerative disorders including stroke and Alzheimer's disease (AD). The promotion of tolerance in neurons against ischemia reperfusion injury is considered as a current therapeutic strategy for oxidative injury [1–3]. Hormesis is defined as responses of cells or organisms to an exogenous or intrinsic factor including nitric oxide, carbon monoxide and glutamate, or exogenous stimuli, such as ischemic preconditioning and moderate-intensity excitatory stimulation [4–9], in which the factor induces beneficial effects at low doses and adverse effects at high doses. Hormesis exerts important protective effect in many diseases. For instance, accumulation of Polybrominated diphenyl ethers (PBDEs) in human tissues will consequently cause hepatotoxicity. But low concentration of PBDEs could cause hormesis effect in the human hepatoma HepG2 cell and DNA-PKcs/Akt pathway may be involved in proliferation increase and apoptosis depression [10]. Low dose radiation, which is the result of radiation hormesis, leads to reduction of ovarian cancer risk [11]. Therefore, the development of interventions that activate hormetic signaling pathways in neurons may be a promising approach for the prevention or treatment of neurological diseases. Among multiple hormetic mechanisms, the nuclear factor E2-related factor (Nrf2)/Heme oxygenase-1 (HO-1) pathway seems to be the pivotal antioxidant signaling mechanism [12,13].

Our previous report showed that, at lower concentrations (5, 10, 20 μM), anthraquinone derivative BME (N^1, N^3-di-*t*-butyloxycarboryl-N^1-(3-(1, 3, 8-tri-*O*-methylemodin-6-ylmethylamino) propyl) propane-1, 3-diamine) moderately elevated intracellular reactive oxygen species (iROS) and did not cause any cell damage in doxorubicin-resistant human myelogenous leukemia (K562/DOX) cells, accompanying with an increase in ERK1/2 activity which has been considered to be related to cell survival or growth [14] Whether this property of the agent is beneficial for the intervention of neurodegenerative disorders mentioned above may be worthy to determine. Recently, rat pheochromocytoma (PC12) cells under oxygen glucose deprivation (OGD) were introduced as an in vitro model of ischemic stroke to evaluate the pharmacological

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potential of various compounds [15–17]. Therefore, in the present report, the protective effect of BME at low concentrations on the OGD-induced mitochondrial apoptosis in PC12 cells was investigated and its action on the HO-1 induction as well as interactions with transcriptional factor Nrf2 and signaling molecules including PI3K/Akt and ERK were also observed.

2. Materials and methods

2.1. Materials

PC12 cells were purchased from Shanghai Institutes of Biological Sciense, Chinese Academy of Sciences. BME (with purity of 99.6) was synthesized [18] in Key Laboratory of Natural Medicine and Immune Engineering, Henan University, China. *N*-acetyl-L-cysteine (NAC), 3-(4, 5-Dinethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), and Rhodamine123 (Rh123) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and pancreatin were purchased from Gibco (NY, USA). Fetal bovine serum (FBS) was purchased from Sijiqing Biological Engineering Materials Co, Ltd (Hangzhou, China). Annexin V-FITC/PI Apoptosis Detection Kit was purchased from Key GEN Biological Engineering Materials Co, Ltd (Nanjing, China). ROS Assay Kit, Nuclear and Cytoplasmic Protein Extraction Kit, Cell lysis buffer for Western/IF, and PD98059 were purchased from Beytime Insititute of Biotecnology (Nantong, China). Primary antibodies against caspase-9 (1:250), caspase-3 (1:250), Bax (1:250), Bcl-2 (1:250), HO-1 (1:250), Nrf2 (1:250), catalase (1:250), ERK1/2 (1:250), Akt (1:250), β-actin (1:400), Lamin B (1:500), and secondary antibodies were purchased from Boster Bio-Engineering Limited Company (Wuhan, China). *Anti*-phospho-ERK1/2(Thr202 + Tyr204) (1:200) was purchased from Beijing Biosynthesis Biotechnology (Beijing, China). *Anti*-phospho-Akt (S473) (1:300) and LY294002 was purchased from Cell Signaling Technology, Inc (Boston, MA, USA). Triton X-100, Bovine serum albumin (BSA) and 4′, 6-diamidino-2-phenylindole (DAPI) were purchased from Beijing Solarbio Science &Technology Co.Ltd (Beijing, China).

2.2. Cell culture and treatment

PC12 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and cultured at 37 °C in a humidified atmosphere of 5% CO_2. The medium was changed every 48 h, and cells were plated at an appropriate density according to each experimental demand. BME was freshly prepared and diluted with phosphate-buffered saline (PBS) before the experiment. Cells were pretreated with various concentrations of BME or other agents for 6 h, then, exposed to OGD treatment for 6 h for experiments. Control group was treated with same volume of phosphate-buffered saline (PBS) as other drug-treatment group.

2.3. Oxygen-glucose deprivation

OGD was performed in PC12 cells according to the described methods [15,19]. Briefly, The cultures medium was replaced with glucose-free Earle's balanced salt solution (NaCl 143 mM, KCl 5.4 mM, $CaCl_2$ 1.8 mM, $MgSO_4{\cdot}7H_2O$ 0.8 mM, $NaH_2PO_4{\cdot}2H_2O$ 2.6 mM, $NaHCO_3$ 26.2 mM, HEPES 20.1 mM, pH adjusted to 7.4) and subsequently transferred into a gas-tight incubation chamber and flushed with gas (5% CO_2/95% N_2). After 30 min, the inlet and outlet valves of the chamber were closed. The chamber was placed in a humidified incubator at 37 °C for 6 h. During OGD, oxygen concentrations in the medium (<2.22 ± 0.10 ppm) were monitored with an oxygen electrode (East China University of Science and Technology, Shanghai, China).

2.4. MTT assay

PC12 cells were cultured at a density of 1×10^5/mL in a 96-well plate and allowed to attach overnight. After incubation with various chemicals, the cells were treated with MTT solution (5 mg/mL) for 4 h at 37 °C. The dark purple formazan crystals formed inside the intact mitochondria were solubilized with DMSO, and the absorbance was measured at 570 nm using a microplate reader (Multiskan Spectrum, Thermo Scientific).

2.5. Intracellular ROS (iROS) analysis

Flow Cytometry was used to analyze intracellular ROS with incubation of 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), which passively diffuses into the cells and is cleaved and oxidized to 2′, 7′-dichlorodihydrofluorescein. PC12 cells were seeded on 6-well plates at a density of 2×10^5/mL and allowed to attach overnight. After incubation with various chemicals, cells were collected and incubated with 500 μL H_2DCFDA (10 μM) at 37 °C for 20 min. Then cells were rinsed once with PBS and re-suspended in 500 μL PBS. After filtration, the suspension was analyzed by Flow Cytometry (BD FACSVerse).

2.6. Determination of glutathion, superoxide dismutase and catalase

After the cell treatment, the medium was removed and the cells were washed thrice with PBS. Cells were collected and dissociated by cell lysis buffer, cell lysis was carried out at 4 °C by vigorous shaking for 45 min. After centrifugation at 12000 rpm for 10 min, supernatant was separated and stored at −70 °C until use. The supernatant was used to measure the glutathion (GSH) content, superoxide dismutase (SOD) and catalase (CAT) activities using assay kit based on the specified manufacturer's instructions (Jiancheng Institute of Biotechnology, Nanjing, China).

2.7. Determination of mitochondrial transmembrane potential ($\triangle\Psi m$)

Rh123 was used to evaluate perturbations in mitochondrial transmembrane potential. PC12 cells were seeded in 6-well plates at a density of 2 × 105/mL and allowed to attach overnight. After treatments, cells were collected and incubated with 500 μL Rh123 (10 μM) for 20 min in the dark. Then cells were rinsed once with PBS and re-suspended in 500 μL PBS. The suspension was analyzed after filtration by Flow Cytometry (BD FACSVerse).

2.8. Determination of apoptotic rate

The apoptotic rate of PC12 cells was detected by Annexin V-FITC/PI double labeling method. Briefly, PC12 cells were seeded on 6-well plates at a density of 2 × 105/mL and allowed to attach overnight. After incubation with various chemicals, cells were harvested, washed with pre-chilled PBS (4 °C). The cell suspension was centrifuged and re-suspended in 195 μL Annexin V-FITC binding buffer and incubated with 5 μL Annexin V-FITC in the dark at ambient temperature for 10 min. Cells were then centrifuged, and the pellet was re-suspended in 195 μL binding buffer. Cells were then incubated with 10 μL PI solution on an ice bath in the dark and filtered with 75 μm nylon mesh. The suspension of each group was analyzed by Flow Cytometry (BD FACSVerse).

2.9. Western blot analysis

Following treatment, cells were collected and washed with PBS. After centrifugation, cell lysis was carried out at 4 °C by vigorous