Neuroscience Letters 516 (2012) 247–252

Contents lists available at SciVerse ScienceDirect

Neuroscience Letters

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

DL-3-n-Butylphthalide protects rat bone marrow stem cells against hydrogen peroxide-induced cell death through antioxidation and activation of PI3K-Akt pathway

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ARTICLE INFO

Article history:
Received 12 February 2012
Received in revised form 28 March 2012
Accepted 1 April 2012

Keywords:
DL-3-n-Butylphthalide
Oxidative stress
Bone marrow stem cells
Antioxidant
PI3K/Akt

ABSTRACT

Bone marrow stem cells (BMSCs) have been one of the most important cell sources for cell replacement therapy (CRT) in cerebral infarction. However, long-lasting oxidative stress during stroke, which plays an important role in neuron damage, deteriorates the microenvironment for cell survival, differentiation and removal. Thus the outcome of CRT in ischemic diseases was poor. DL-3-n-Butylphthalide (NBP) has protective effects on ischemic brain tissue through multiple mechanisms and has been used for stroke treatment in China for several years. In this study, hydrogen peroxide (H_2O_2) was used to induce oxidative stress injury to rat bone marrow stem cells (rBMSCs), imitating the microenvironment surrounding transplanted cells in the ischemic brain in vitro. The protective effects of NBP on rBMSCs against apoptosis induced by oxidative stress were investigated. Our results indicated that NBP could protect rBMSCs against apoptosis due to antioxidative properties and modulation of PI3K/Akt pathway. NBP could be used in combination with BMSCs for the treatment of cerebral infarction by improving the oxidative stress microenvironments and cell survival, however, further studies remain warranted.

1. Introduction

Ischemia stroke is one the most frequent causes of mortality in China. Cell replacement therapy (CRT) has recently emerged as an attractive and promising therapeutic strategy for the treatment of various ischemia-related disorders, including cardiovascular disease and stroke, in experimental studies [1,28]. However, the clinical exploitation of CRT in stroke was hampered by the fact that transplanted stem cells did not survive efficiently within the injured brain [9]. In recent studies, hypoxia and serum deprivation (hypoxia/SD), both components of ischemia [9,21], induce stem cells apoptosis and evidence [18] has pointed out oxidative stress long exists after stroke. These findings indicate that the ischemic microenvironment of the infarcted brain may not be conducive to transplanted cells survival. Therefore, protection of transplanted cells from apoptosis and enhancing their viability and survival in oxidative stress conditions by antioxidant and apoptosis-inhibitor may be crucial for the successful utilization in CRT of stroke.

Among several kinds of cell donor sources for CRT, bone marrow stem cells (BMSCs) are self-renewing and able to differentiate into neurons [11], osteoblasts [25], chondrocytes [25], skeletal muscle cells [7] and cardiomyocytes both in vitro [20] and in vivo [30]. The ease of isolation and ex vivo expansion in culture, combined with their multipotency and lack of ethical obstacles, make BMSCs a promising source of stem cells for neural regeneration [28].

DL-3-n-Butylphthalide (NBP) is developed from L-3-n-butylphthalide which is extracted as a pure component from seeds of Apium and approved by the State Food and Drug Administration (SFDA) of China for clinical use in stroke patients in 2002. The beneficial effects of NBP have been well established

Abbreviations: ANOVA, analysis of variance; (r)BMSCs, (rat)bone marrow stem cells; Bax, Bcl-2-associated x protein; Bcl-2, B cell lymphoma-2; Caspase, cysteinyl aspartate-specific protease; CRT, cell replacement therapy; DCFH-DA, fluorescent probe 2′,7′-dichlorofluorescein diacetate; DMSO, dimethylsulfoxide; FBS, fetal bovine serum; FCM, flow cytometry; H_2O_2, hydrogen peroxide; Hypoxia/SD, hypoxia and serum deprivation; L-DMEM, low glucose Dulbecco's modified Eagle's medium; MDA, malondialdehyde; NBP, DL-3-n-butylphthalide; NT-3, neural nutrients-3; PBS, phosphate buffer saline; PI, phosphatidyl inositide; PI3K-Akt, phosphatidylinositol 3 kinase-protein kinase B; PS, phosphatidylserine; ROS, reactive oxygen species; SFDA, State Food and Drug Administration; SOD, superoxide dismutase.

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http://dx.doi.org/10.1016/j.neulet.2012.04.003

in a variety of in vivo and in vitro models of stroke [17,14]. NBP exerts protective effect on ischemic brain tissue through multiple mechanisms such as improving energy metabolism [6], decreasing oxidative damage [4], and reducing neuronal apoptosis [2].

In this study, we demonstrated for the first time that NBP might protect rBMSCs against H_2O_2-induced apoptosis through PI3K-Akt pathway and enhance rBMSCs' resistance to oxidative stress, which suggests that NBP may possess the ability of protecting rBMSCs from oxidative injury. The present results suggest that CRT in combination with NBP administration could be a potential useful strategy to increase the cell survival in CRT for stroke.

2. Materials and methods

2.1. Materials

Male Sprague–Dawley rats weighing 110–150 g were used in this study (Shanghai Laboratory Animal Center, Chinese Academy Sciences). All procedures were performed according to the Nanjing Medical University animal care guidelines, which conform to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised, 1996). NBP (Shi Jia Zhuang Pharmacy, the product catalog number: 050298) was dissolved in DMSO before dilution with the culture medium. The final concentration of DMSO per well was 0.1%. The final concentrations of NBP in the NBP-pretreated groups were 0.1, 1, 10 and 100 μmol/L. The chemical structure of NBP is shown in Fig. 1C. Low glucose Dulbecco's modified Eagle's medium (L-DMEM), fetal bovine serum (FBS) and trypsin were from Hyclone. The annexin V-FITC Apoptosis Detection Kit was purchased from the BIOSEA (BIOSEA, Beijing, China). Polyclonal rabbit anti-phospho-antibody to Akt (Ser473) and Akt were purchased from Cell Signaling Technology (Danvers, MA, USA). LY294002, rabbit anti-Bcl-2, Bax, Caspase-3, β-actin and secondary antibody to rabbit were obtained from SantaCruz Biotechnology, Inc. (Delaware, CA, USA). MTT (3-[4,5-dimethylthiazolo-2]-2,5-diphenyltetrazolium bromide), SOD (Superoxide Dismutase) kits were from Booker Biotechnology Co. Ltd. (Booker, Nanjing, China), whereas lipid peroxidation MDA (Malondialdehyde, MDA) Assay Kit was purchased from Cell Biolabs (San Diego, CA, USA). Fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was from Sigma (St. Louis. MO, USA). All other chemicals were commercial products with analytic purity.

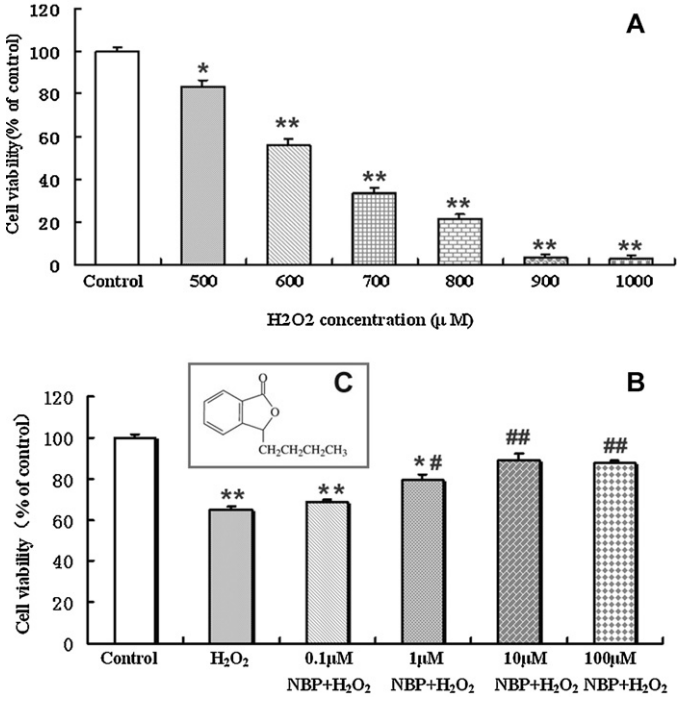

Fig. 1. (A) MTT analysis of cell viability in H_2O_2 treated rBMSCs. The viability of cells treated with 500, 600, 700, 800, 900 and 1000 μmol/L H_2O_2 reduced dose-dependently. (B) MTT analysis of cell viability in 600 μmol/L H_2O_2 treated rBMSCs with different doses of NBP pretreatment. The cell viabilities increased dose-dependently when pretreated with 1, 10, and 100 μmol/L NBP for 24 h before 600 μmol/L H_2O_2 treatment. (C) The chemical structure of NBP. (*P < 0.05 and **P < 0.01, compared to the control. #P < 0.05 and ##P < 0.01, compared to H_2O_2 group. Data were presented as mean ± S.E.M. n = 4 in each group.)

2.2. Cell culture

Culture of rBMSCs was performed as described previously [15]. All cells used in the assay were of passages 4–6. The quality of rBMSCs was ensured by flow cytometry as previously reported [15]. To study the protective effects of NBP, cells were pretreated with NBP or vehicle (DMSO) for 24 h and then incubated with H_2O_2 (600 μmol/L) for 4 h. To determine the effects of LY294002, cells were pretreated with LY294002 (final concentration: 25 μmol/L) for 1 h, followed by the treatments of NBP and H_2O_2.

2.3. Cell viability assay

Cell viability was assessed using the MTT assay [22]. After treatment with H_2O_2 and the different concentrations of NBP, cultured cells were incubated with 50 μl of 5 mg/ml MTT solution for 4 h at 37 °C and centrifuged at 400 × g for 5 min. DMSO (150 μl) was added to each well to dissolve the formazan and the absorbance was measured at 550 nm. Rat BMSCs without treatment were considered as controls, and treated groups were expressed as a percentage of control. The results were obtained from three independent experiments performed in quadruplicate.

2.4. Analysis of cell apoptosis

Apoptosis was determined by detecting phosphatidylserine (PS) exposure on cell plasma membrane with the fluorescent dye Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's protocols. In brief, cells were harvested and washed in ice-cold PBS, resuspended in 200 μl of binding buffer and incubated with 10 μl of Annexin V-FITC solution for 30 min at 4 °C in the dark. This was followed by a further incubation with 5 μl of propidium iodide (PI) for 5 min and then immediately analyzed by bivariate flow cytometry using a FACScan-LSR equipped with CellQuest (BD) software. Approximately 5–6 × 10^5 cells were analyzed in each of the samples.

2.5. Detection of MDA and SOD

For detection of cellular MDA and SOD concentrations, cells were rinsed twice with ice-cold PBS and then lysed in ice-cold lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1%TritonX-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 mM PMSF, and 10 μg/ml each of Leupeptin, Aprotinin, and Pepstatin] for 30 min. Cell lysates were centrifuged at 13,000 × g for 10 min at 4 °C and the protein concentration was determined by the BCA assay. The content of MDA was measured with a modified thiobarbituric acid method [24] using a UV-2550 spectrophotometer at 532 nm. MDA concentrations in the samples were calculated by a standard calibration curve of 1,1,3,3-tetraethoxypropone prepared in the same manner. SOD activity was measured using WST [33]. The results were expressed as a percentage of the control.