Research Article

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Berberine Protects against Hydrogen Peroxide-Induced Oxidative Damage in PC12 Cells through Activation of ERK1/2 Pathway

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Abstract

Oxidative stress is a much-recognized phenomenon linked with the progression of Neurodegenerative Diseases (NDs) due to imbalances in redox homeostasis. Increasing evidence indicates that excessive Reactive Oxygen Species (ROS), impairing the physiological functions of neurons via inducing cell apoptosis, is the main cause of NDs. The drug candidates are required that can effectively protect neurons from oxidative stress insult to slow down the process of neurodegenerative diseases. In present study, we investigated the protective effect and the underlying mechanisms of berberine (BBR, an isoquinoline alkaloid isolated from the herb Rhizoma coptidis, against oxidative damage in PC12 cells. It was found that BBR was able to suppress hydrogen peroxide (H_2O_2)-induced cell death in PC12 cells. Flow cytometry revealed that BBR significantly reduced the apoptosis of PC12 cells exposed to H_2O_2. Western blot analysis displayed that BBR stimulated the extracellular regulated ERK1/2 survival signaling, while application of PC12 cells with ERK1/2 pathway inhibitor PD98059 blocked the neuroprotective effect of BBR. These results together indicated that BBR is a potential protectant, and it protects PC12 cells against H_2O_2 toxicity through activated the ERK1/2 pathway.

Keywords: Berberine; Degenerative disease; PC12 cells; ERK1/2

Introduction

Neurodegenerative diseases (ND), such as Alzheimer's (AD) and Parkinson's disease (PD), are chronic degenerative pathologies of the Central Nervous System (CNS). NDs are characterized by progressive loss of specific neurons which further leads to a decline in brain functions [1,2]. Despite the fact that pathologies have different clinical features, there are some common hallmarks, such as synaptic dysfunctioning, oxidative stress, and inflammation [3]. The NDs are caused by an enhancement of ROS production via mitochondria and NADPH oxidase (NOX), which seems to account for tissue injury and neurodegeneration [4,5]. Nowadays, NDs are chronic and incurable conditions, and the disabling effects may continue for years or even decades representing an enormous disease load, regarding human suffering and economic cost [6,7]. In neuronal cells, the over production of ROS is considered as one of the risk factors for NDs [8,9]. Disruption of redox homeostasis is a key phenotype of many pathological conditions. Hydrogen peroxide (H_2O_2), the main source of ROS, can cause cell membrane injury as well as lipid peroxidation and DNA damage in variety cells. However, antioxidants were able to protect cells against H_2O_2-induced cell death via reducing ROS production [10].

Recently, a number of natural medicinal plants have been tested for their therapeutic properties, revealing that the raw extracts or isolated pure compounds from them had more effective properties than the whole plant itself for the treatment of ND and other disease [11,12]. In the last decade, more and more attention has been paid to the antioxidant activities of natural products and compounds isolated from plants which usually have higher efficacy and lower side effects. Berberine (BBR) is an isoquinoline alkaloid (5,6-

dihydrodibenzoquinolizinium derivative), which belongs to the structural class of protoberberines and is extracted from several medicinal herbs, particularly in the genus berberis [8]. Previous studies have shown that BBR has abundant pharmacological activities, including antioxidant, anticancer, anti-inflammation, antidepressant, neuroprotection, hepatoprotection, cerebroprotection and cardioprotection [13,14]. In recent years, BBR has been reported to exert beneficial effects in neurodegenerative and neuropsychiatric disorders because of its dual antioxidant and anti-apoptotic activities [15-18].

It has been found that BBR-mediated neuroprotection against neuronal apoptosis is regulated by several pathways including Akt/GSK3β, ERK1/2, AMPK, Nrf2/HO-1 survival/apoptotic signaling pathway as well as JNK and Caspase-3 activity inhibition [19,20]. Different studies have shown that BBR inhibited superoxide anions and had radical scavenging activity against the highly reactive hydroxyl radicals, while the mechanisms and signaling pathways involved in its antioxidant effects are still not very clear. The rat pheochromocytoma PC12 cells are useful neuronal models for the study of neuronal degenative disorders such as Alzheimer's disease, and also widely used to investigate free ROS biochemical pathways involved in cell death and neuroprotection [21]. Hence, this cell line is a suitable model for studyi--ng oxidative stress-induced neuronal injury.

In present study, we discovered that BBR was able to protect PC12 cells against H_2O_2-induced oxidative damage via inhibiting the apoptosis. We also displayed that the neuroprotective effect of BBR is mediated through the ERK1/2 pathway. These findings suggest that BBR is able to protect PC12 cells against H_2O_2 injury by the MAPK pathway and further support that the BBR administration might be a possible therapeutic approach for the treatment of AD.

Materials and Methods

Materials

Dimethyl Sulfoxide (DMSO), poly-L-lysine and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). Fetal Bovine Serum (FBS), Penicillin-Streptomycin (PS), DMEM and trypsin were obtained from Invitrogen (Carlsbad, USA). Methyl Thiazolyl Tetrazolium (MTT) was got from Molecular Probes (Eugene, OR, USA). Annexin V-FITC/PI Apoptosis Detection Kit was purchased from BD Biosciences (San Diego, CA, USA). Anti-β-actin and phospho-ERK1/2 antibodies were obtained from Cell Signaling Technology (Woburn, USA). ERK1/2 inhibitor PD98059 was obtained from Selleckchem.

Cell culture and treatment

The rat pheochromocytoma PC12 cell, NIH clone, was kindly provided by Dr. Gordon Guroff (National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD, USA). Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% heat-inactivated horse serum, 5% Fetal Bovine Serum (FBS) and antibiotics (100 U/mL penicillin; 100 µg/mL streptomycin) as previously described [22,23]. Cells were incubated at 37°C with 5% CO_2 humidified atmosphere. All experiments were carried out 24 h after the cells were seeded. For the protection assay, PC12 cells were pre-treated with BBR for 2 h, and then treated with H_2O_2 for 24 h in all experiments. In addition, BBR was removed from the cell culture medium before H_2O_2 exposure.

MTT assay

Mitochondrial activity, a measure of cell death, was measured by the MTT assay as previously described [24]. Briefly, PC12 cells were seeded in 96-well plates at a density of $\times 10^3$ cells/well. After serum starvation, the cultures were incubated with different reagents for 24 h. Thereafter, MTT (0.5 mg/mL) was added to each well for an additional 4 h. Subsequently, medium was removed, and DMSO (100 µl) was added to each well to solubilize the formazan salt. Absorbance was measured at 490 nm by Infinite M200 PRO Multimode Microplate (Tecan, Switzerland). The relative cell viability was presented as a percentage compared with the control group.

Apoptosis of PC12 cells measured by flow-cytometry

The PC12 cells after treatment were analyzed by flow cytometer. Briefly, the PC12 cells were collected by centrifugation (2000 rpm for 5 min) and washed with PBS for two times. Cells were suspended in 400 µl of 1X Binding Buffer. 5 µl Annexin V-EGFP mix was added in each sample followed by the addition of 10 µl Propidium Iodide; mixed and kept away from light at room temperature for 20 min. Data acquisition and analysis were performed using BD C Sample plus.

Western blot analysis

The western blotting in this study was carried out as described previously [25]. Briefly, cells after different treatment were washed once with ice-cold PBS, then lysed in RIPA buffer (10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 µg/ml leupeptin, 0.1% aprotinin, 1 mM iodoacetamide, 200 µg/ml bacitracin, 20 µg/ml soybean trypsin inhibitor, 10 mM NaCl and 0.25% Triton X-100) for 15 min on ice. Protein concentrations were measured by BCA protein assay kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions. For western blotting, samples (20 µg protein/lane) were separated by SDS-PAGE gel with a pre-stained protein ladder (5 µl) as a molecular weight marker, and then transferred to PVDF membranes. The p-ERK1/2 was determined by phospho-specific antibody while β-actin was used as a loading control. Immuno-reactive bands were visualized by ECL kit according to the manufacturer's instructions. The intensity of band was quantified using Image J software. The experiments were repeated for 3 times by using independent cultures.

Statistical analysis

Statistical analysis and data handling were performed using SPSS version 16.0. All experiments were repeated for 3 times. All the data for continuous variables were expressed as the mean ± standard deviation (X ± SD) along with ranges. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison. The null hypothesis was rejected at $P<0.05$.
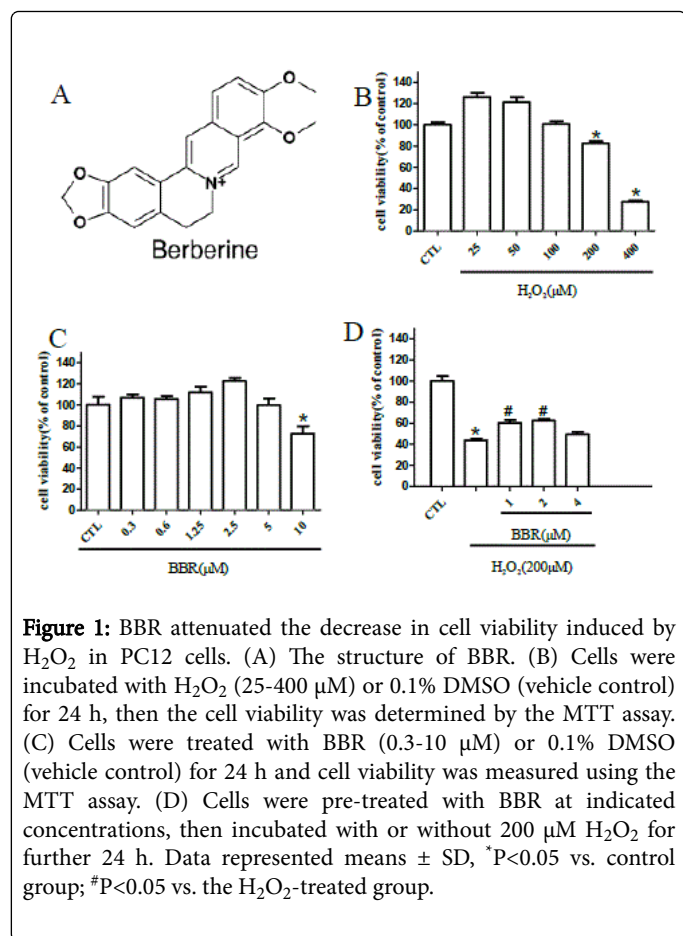
Results

BBR attenuated the decrease in cell viability induced by H_2O_2 in PC12 cells

To calculate the cytotoxicity of H_2O_2, PC12 cells were incubated with different concentrations of H_2O_2 for 24 h and the cell viability was measured by MTT assay. As shown in Figure 1, H_2O_2 treatment for 24 h significantly decreased the cell viability of PC12 cells in a concentration-dependent manner. H_2O_2 at 200 µM caused about 20% loss in cell viability, thus this concentration of H_2O_2 was used in the coming experiments to produce the cell insult and to measure neuroprotection. To investigate the cytotoxicity of BBR, PC12 cells were treated with different concentrations of BBR for 24 h, and the cytotoxicity was measured by MTT assay. As shown in Figure 2, BBR did not show any cytotoxicity between 0.315 and 5 µM, and these concentrations were used in further experiments. To examine the protective effects of BBR, PC12 cells were incubated with BBR for 2 h and then exposed to H_2O_2 for 24 h. The result form MTT assay revealed that pre-treatment with 1 or 2 µM of BBR significantly attenuated H_2O_2-induced cell viability loss in a concentration-dependent manner.

Protective effect of BBR on H_2O_2-induced apoptosis in the PC12 cells

It was reported that cell death caused by H_2O_2 was mainly mediated by apoptosis [26] and BBR was able to protect cells from apoptosis in various cell types [20,27]. We therefore investigated whether BBR could reduce the cell apoptosis caused by H_2O_2 in PC12 cells. Cell apoptosis was tested by flow cytometry, and the data indicated that H_2O_2 exposure markedly increased apoptosis in PC12 cells, while BBR pre-treatment significantly reduced the apoptosis caused by H_2O_2 (Figure 2).

Figure 1: BBR attenuated the decrease in cell viability induced by H_2O_2 in PC12 cells. (A) The structure of BBR. (B) Cells were incubated with H_2O_2 (25-400 μM) or 0.1% DMSO (vehicle control) for 24 h, then the cell viability was determined by the MTT assay. (C) Cells were treated with BBR (0.3-10 μM) or 0.1% DMSO (vehicle control) for 24 h and cell viability was measured using the MTT assay. (D) Cells were pre-treated with BBR at indicated concentrations, then incubated with or without 200 μM H_2O_2 for further 24 h. Data represented means ± SD, *P<0.05 vs. control group; #P<0.05 vs. the H_2O_2-treated group.

Figure 2: Protective effect of BBR on H_2O_2-induced apoptosis in the PC12 cells. (A) Photographs of representative cultures measured by flow cytometry. (B) Quantitative analysis of (A). The data were represented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. control group; #P<0.05 vs. the H_2O_2-treated group.

BBR stimulated the ERK1/2 signaling pathway in PC12 cells

ERK1/2 pathway phosphorylation is a key biochemical event responsible for cell survival and apoptosis. BBR was able to activate ERK1/2 signaling in mice as reported earlier [28]. We tested whether ERK1/2 pathway is involved in BBR-induced neuroprotective effects in PC12 cells. As shown in Figure 3, phosphorylation of ERK1/2 was gradually increased after the addition of BBR in a concentration-dependent fashion. This data suggested ERK1/2 pathway participated in the protective action of BBR.
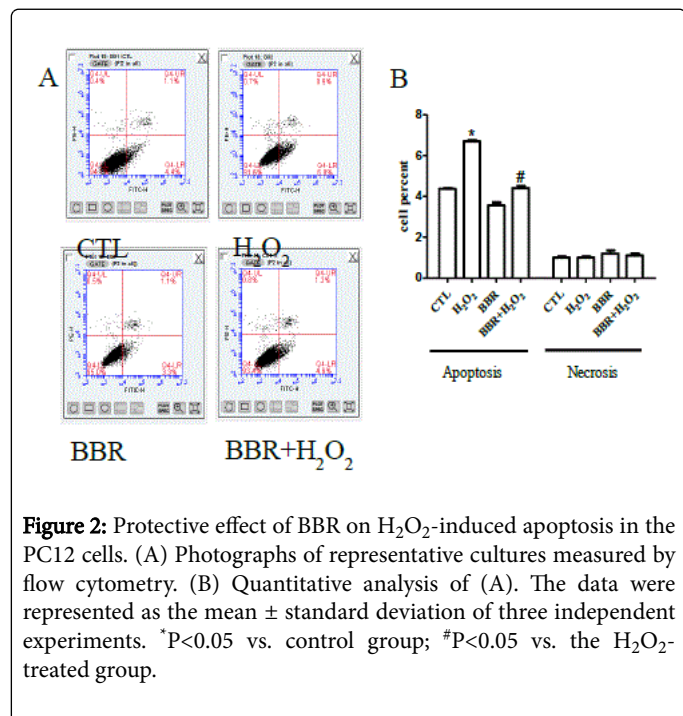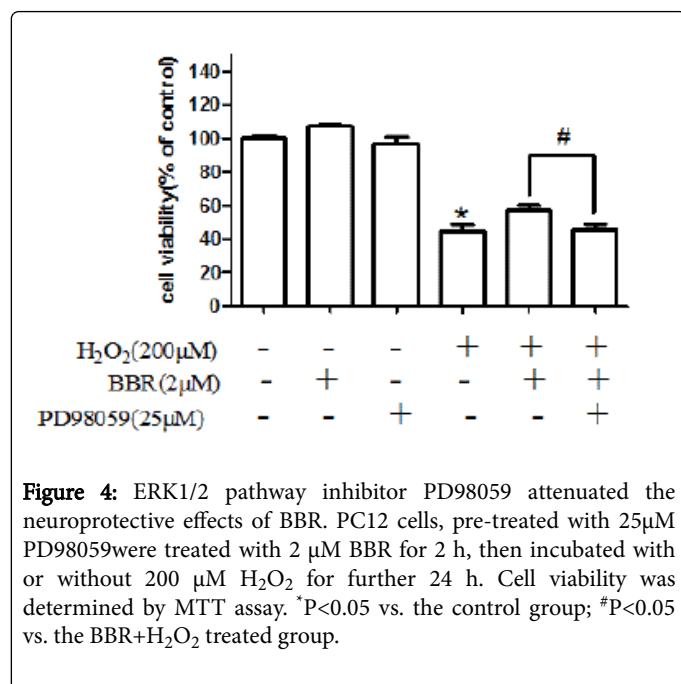

Figure 3: Involvement of ERK1/2 signaling in the neuroprotective effect of BBR. (A) PC12 cells were treated with BBR in different concentrations. The expression of phosphorylated ERK1/2 and β-actin were detected by western blotting with specific antibodies. (B) Quantification of representative protein band from western blotting. *P<0.05 vs. control group.
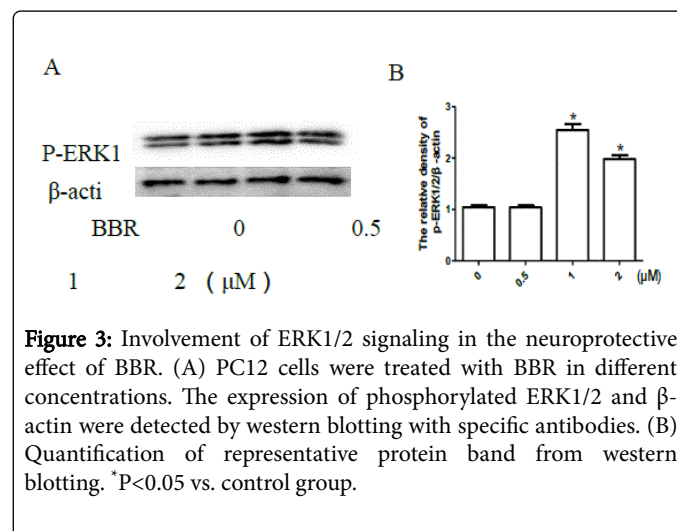
The ERK1/2 pathway inhibitor PD98059 attenuated the protective effects of BBR

To further support the role of ERK1/2 signaling pathway involved in the neuroprotective effect of BBR, PC12 cells were pre-incubated with 25 μM PD98059 (a specific inhibitor of MEK which is an upstream kinase of ERK1/2), and then the neuroprotective effect of BBR on H_2O_2-induced injury was investigated by MTT assay. As shown in Figure 4, pre-incubation with PD98059 markedly reduced the neuroprotective effect of BBR on H_2O_2-induced cell viability loss. These results indicated that the neuroprotective effect of BBR was mediated by ERK1/2 pathway.

Discussion

Numerous scientific reports emphasized that oxidative stress plays a crucial role in the pathophysiology of NDs [29-32]. ROS, caused by oxidative stress, lead to lipid peroxidation as well as protein oxidation, resulting in plasma membrane broken and cross-linking of cytoskeletal biomolecules [33-36]. Antioxidants can reduce or delay oxidation process though preventing the initiation or propagation of oxidizing chain reactions. In this study, neuroprotective effect of BBR on H_2O_2-induced oxidative damage was investigated. Our results showed that 200 μM H_2O_2 significantly increased the cell apoptosis in PC12 cells, while pre-treatment with BBR was able to significantly attenuate the cell viability loss induced by H_2O_2. Further study displayed that the neuroprotective effect of BBR was mediated by the ERK1/2 pathway.

Figure 4: ERK1/2 pathway inhibitor PD98059 attenuated the neuroprotective effects of BBR. PC12 cells, pre-treated with 25µM PD98059were treated with 2 µM BBR for 2 h, then incubated with or without 200 µM H_2O_2 for further 24 h. Cell viability was determined by MTT assay. *$P<0.05$ vs. the control group; #$P<0.05$ vs. the BBR+H_2O_2 treated group.

Excessive oxidative stress caused mitochondrial dysfunction and has been proposed to be associated with NDs and brain aging [37]. Therefore, reducing oxidative damage to neuronal cells could be a promising preventive and therapeutic approach [7]. Increasing evidences suggest that many phytochemicals can activate pathways that prevent or reverse oxidative injury. BBR is able to quench superoxide anions and exert radical scavenging activity [38]. In culture cells, BBR was able to inhibit ROS production and prevent generation of superoxide anions caused by NADPH oxidase in the LPS stimulated human monocyte-derived macrophages [39]. It has been reported that BBR inhibited iNOS expression to attenuate NO production. In addition, several reports have shown that BBR induces antioxidant defence by enhancement of the levels of non-enzymatic antioxidants [15,40].

Biologically, ERK1/2 pathway is a key signaling component that plays a crucial role in the regulation of most of cellular processes linked to stimulation such as cell proliferation, cell apoptosis and differentiation. Our results provided mechanistic evidence to support that BBR protected PC12 cells from H_2O_2-induced oxidative damage via ERK1/2 activation.

In summary, our results demonstrated that BBR is able to protect PC12 cells from H_2O_2-induced oxidative damage and this protective effect is mediated, at least in part by activated ERK1/2 signaling.

Acknowledgements

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