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Research Paper

Berberine protects against 6-OHDA-induced neurotoxicity in PC12 cells and zebrafish through hormetic mechanisms involving PI3K/AKT/Bcl-2 and Nrf2/HO-1 pathways

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ABSTRACT

Berberine (BBR) is a renowned natural compound that exhibits potent neuroprotective activities. However, the cellular and molecular mechanisms are still unclear. Hormesis is an adaptive mechanism generally activated by mild oxidative stress to protect the cells from further damage. Many phytochemicals have been shown to induce hormesis. This study aims to investigate whether the neuroprotective activity of BBR is mediated by hormesis and the related signaling pathways in 6-OHDA-induced PC12 cells and zebrafish neurotoxic models. Our results demonstrated that BBR induced a typical hormetic response in PC12 cells, i.e. low dose BBR significantly increased the cell viability, while high dose BBR inhibited the cell viability. Moreover, low dose BBR protected the PC12 cells from 6-OHDA-induced cytotoxicity and apoptosis, whereas relatively high dose BBR did not show neuroprotective activity. The hormetic and neuroprotective effects of BBR were confirmed to be mediated by up-regulated PI3K/AKT/Bcl-2 cell survival and Nrf2/HO-1 antioxidative signaling pathways. In addition, low dose BBR markedly mitigated the 6-OHDA-induced dopaminergic neuron loss and behavior movement deficiency in zebrafish, while high dose BBR only slightly exhibited neuroprotective activities. These results strongly suggested that the neuroprotection of BBR were attributable to the hormetic mechanisms via activating cell survival and antioxidative signaling pathways.

1. Introduction

Hormesis is defined as a biphasic dose-response phenomenon that is characterized by a low-dose beneficial or stimulatory response and a high-dose toxic or inhibitory response, which has been observed in a broad range of biological and physiological systems [1–3]. According to the principles of hormesis, exposure to low concentrations of drugs, toxins or natural substances may protect against damage from a subsequent stressor, while at higher dose the toxic effect prevails [1,4]. Nowadays, hormesis becomes an essential concept to the biomedical sciences due to its important implications in therapeutics, in the clinical trial, in drug discovery and risk assessment for chemicals, radiation and pharmaceutical agents [2,5]. Hormesis has the potential to improve the quality of life during the normal lifespan, such as memory enhancement, bone strengthening, anxiety reduction, and protection against agonists inducing neuronal diseases [4,6]. Recently, the hormesis concept has been receiving increasing attention in the field of neuroscience, including the areas of neuroprotection, and drugs for Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), as well as in the areas of behavioral

Abbreviations: BBR, berberine; PD, Parkinson's disease; AD, Alzheimer's disease; HD, Huntington's disease; 6-OHDA, 6-hydroxydopamine; DMSO, dimethyl sulfoxide; Nom, nomifensine; MTT, thiazolyl blue tetrazolium bromide; PBS, phosphate buffered saline; PS, penicillin-streptomycin; FBS, fetal bovine serum; HS, horse serum; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; Nrf2, nuclear factor-E2-related factor 2; HO-1, heme oxygenase-1; FITC, fluorescein isothiocyanate; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; ZnPP, zinc protoporphyrin IX; FCM, flow cytometry; dpf, day post fertilization; TH, tyrosine hydroxylase; DA, dopaminergic; NDD, neurodegenerative disorders; MAPK, mitogen-activated protein kinases; JNK, c-Jun N-terminal kinase; sMaf, small Maf

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pharmacology [1,3,4].

A diverse range of chemicals (phytochemicals, endogenous agonists, synthetic drugs and neurotoxins) have been reported to display a hormetic responses in neuronal models [7,8]. Interestingly, some anti-AD drugs exhibit hormetic responses in improving memory [9]. In fact, neuroprotective agents seem to generally express their neuroprotective properties in a manner consistent with the hormetic response [7,10]. In addition, various phytochemicals in moderate amounts exert neuroprotective effects by enhancing neural cell viability and activating signaling pathways involving cell survival, anti-apoptosis and anti-oxidation defense [11,12]. However, substantial evidences are required to extensively elucidate the biomedical significance and the underlying mechanisms of hormesis.

Berberine (BBR), an isoquinoline alkaloid with PI3K-activating activity, occurs as an active constituent in the root, rhizome and stem bark of numerous medicinal plants with abundant pharmacological activities, including antioxidant, anticancer, anti-inflammation, antidepressant, neuroprotection, hepatoprotection, cerebroprotection, cardioprotection, nephroprotection, etc. [13]. In recent years, BBR has been demonstrated its beneficial effect in neurodegenerative and neuropsychiatric disorders because of its dual antioxidant and anti-apoptotic activities [14–16].

6-hydroxydopamine (6-OHDA) is widely used as a neurotoxin to generate experimental cell and animal models of PD [17]. PC12 cell line, derived from pheochromocytoma of rat adrenal medulla, has an embryonic origin from the neural crest and thus shows a variety of neuronal properties, including neurotransmitter release, neurite growth, response to nerve growth factor, etc. PC12 cell line has been widely used as a cellular model of neuronal development and neurological diseases [18]. Zebrafish is a well-established model in studies on development and drug discovery for neurological disorders. The high-fecundity and short generation time of zebrafish allow fast screening large numbers of drug candidates at low cost and the transparency of the zebrafish allows visualization of biochemical, physiological and anatomic states in a living vertebrate organism [19]. Moreover, the zebrafish shares genetic, physiologic and anatomic homology to higher order vertebrates, especially, the structure and function of the zebrafish brain are very similar to those of other vertebrates, and 6-OHDA is known to induce dopaminergic (DA) neuronal death and Parkinson's pattern in zebrafish.

Previous studies found that BBR exert neuroprotective effects against 6-OHDA and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine induced neurotoxicity in PC12 cells and a rat model of neurodegenerative diseases [20,21], whereas the underlying cellular and molecular mechanisms are still unclear. We have recently reported that BBR could enhance the survival of cancer cells at low doses via a hormetic mechanisms [22]. We speculate that the neuroprotective activity of BBR might be attributable to the hormetic mechanisms activated by relatively low dose BBR. To address this question, we characterized the hormetic and neuroprotective activities of BBR in 6-OHDA-induced neurotoxicity in PC12 cells and zebrafish, and investigated the involved signaling pathways in the current study.

2. Materials and methods

2.1. Chemicals and reagents

BBR with the purity greater than 99%, 6-OHDA, dimethyl sulfoxide (DMSO), nomifensine (Nom), and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich Co (St. Louis, MO, USA). F-12K medium, phosphate buffered saline (PBS), penicillin-streptomycin (PS) were purchased from Gibco (Maryland, USA). Fetal bovine serum (FBS) and horse serum (HS) were obtained from Invitrogen (Carlsbad, CA, USA). Antibodies against phosphor-phosphoinositide 3-kinase (p-PI3K), PI3K, Bcl-2, phosphor-protein kinase B (p-AKT), AKT, nuclear factor-E2-related factor 2 (Nrf2), heme oxygenase-1 (HO-1) and GAPDH, and secondary antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA) or Proteintech (Chicago, IL, USA). Secondary antibody fluorescein isothiocyanate (FITC) was obtained from Abcam Inc (Cambridge, MA, USA). Hoechst 33342 staining kit, LY294002, Annexin V-FITC/propidium iodide (PI) apoptosis detection kit, caspase-3 activity assay kit, cell cycle and apoptosis analysis kit, and the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) cell apoptosis detection kit were obtained from Beyotime Institute of Biotechnology (Nanjing, Jiangsu, China). Zinc protoporphyrin IX (ZnPP) was obtained from Enzo Life Sciences (Farmingdale, NY, USA).

2.2. Cell culture and drug treatments

The PC12 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in ATCC-formulated F-12K medium containing 15% (v/v) heat-inactivated HS, 2.5% (v/v) FBS, and 1% (v/v) PS, and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. For the following *in vitro* experiments, the working solutions of BBR were freshly dissolved and diluted in the basal medium.

2.3. Cell viability assay

Cell viability was measured using MTT method [23]. Briefly, PC12 cells (6×10^3 cells/well) were treated with increasing concentrations of BBR for 24 h in 96-well plates. To test the neuroprotective effect of low dose BBR against 6-OHDA-induced cell death, PC12 cells were pretreated with indicated concentrations of BBR for 24 h prior to the treatment of 250 μM 6-OHDA for a further 24 h. Then, the treated cells were incubated in 0.50 mg/mL MTT solution for 4 h at 37 °C. The supernatants were replaced with DMSO to dissolve the formazan crystals. The absorbance was determined using a microplate reader (BioTek, Winooski, VT, USA) with a test wavelength at 570 nm. The relative viability of treated cells was calculated comparing with OD value of control group.

2.4. TUNEL

TUNEL method was performed to label 3′-end of fragmented DNA of the apoptotic PC12 cells. The cells were treated as mentioned in cell viability assay, and fixed with 4% paraformaldehyde phosphate buffer saline for 45 min at room temperature, rinsed with PBS, and permeabilized by 0.1% Triton X-100 for 2 min on ice followed by TUNEL for 60 min at 37 °C in a humidified atmosphere in the dark. The TUNEL-positive cells were imaged using the InCell 2000 confocal microscope (GE Biosciences, Piscataway, NJ, USA). Quantitative analysis of apoptotic cells using the software modules supplied with the InCell 2000.

2.5. Annexin V-FITC/PI staining

The cells treated as indicated were washed with PBS and resuspended in the binding buffer containing Annexin V-FITC and PI for 15 min in the dark. Then the cells were detected by flow cytometry (FCM, FACS Canto™, BD, CA, USA). The percentage of apoptotic cells was counted using FlowJo software version 7.6.1 (Ashland, OR, USA).

2.6. FACS analysis for sub-G1 DNA determination

DNA fragmentation during apoptosis causes considerable loss of DNA content, resulting in an obvious sub-G1 peak [24]. For sub-G1 DNA content analysis, the treated cells were harvested and fixed in ice-cold 70% ethanol at −20 °C overnight, and then stained with PI for 15 min. Cells were maintained in the dark and analyzed by FCM. The percentage of cells in the sub-G1 phase of the cell cycle was calculated

using FlowJo software version 7.6.1.

2.7. Measurement of caspase-3 activity

Caspase-3 activity was carried out using the C1116 Caspase-3 Activity Assay kit according to the manufacturer's instructions. In brief, the treated cells were harvested and washed by ice-cold PBS, resuspended in lysis buffer on ice for 15 min, and centrifuged for 15 min at 16000*g*. Then the supernatant of each sample was incubated with Ac-DEVD-pNA/Ac-LEHD-pNA and detecting buffer at 37 °C for 120 min. The OD value was measured at 405 nm with a microplate reader.

2.8. Nrf2 immunofluorescence

The nuclear translocation of Nrf2 was determined by immunostaining as previously described [25]. Briefly, PC12 cells were treated with BBR (1 μM). Then, the cells were washed with PBS and fixed in 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, blocked with 1% BSA and then incubated with anti-Nrf2 antibody. The bound antibody was detected by FITC-IgG secondary antibody. DAPI was used for counterstaining of nuclei. The stained cells were washed with PBS and visualized using the InCell 2000 confocal microscope.

2.9. Western blotting

The collected cells were lysed with RIPA lysis buffer containing 1% phenylmethylsulfonyl fluoride and 1% protease inhibitor cocktail (Thermo, Rockford, IL, USA). The sample protein concentrations were determined using a BCA protein assay kit (Thermo, Rockford, IL, USA). Equivalent amounts of proteins from each group were separated by SDS-polyacrylamide gel electrophoresis, followed by transferring onto methanol activated PVDF membranes (Bio-Rad, Philadelphia, PA, USA). After being blocked with 5% skim milk with PBST buffer, the membranes were probed overnight at 4 °C with the indicated primary antibodies (1:1000) and incubated with the corresponding secondary antibodies (1:5000). Protein bands were detected by Bio-Rad ChemiDoc™ (Hercules, CA, USA).

2.10. Inhibitor Treatment

To clarify the roles of signaling pathways in low dose BBR-induced hormetic effect in PC12 cells, the cells were pretreated with or without 10 μM ZnPP (HO-1 inhibitor) or 10 μM LY294002 (PI3K/AKT pathway inhibitor) for 1 h and then treated with 1 μM BBR for 24 h. The cells were then subjected to the determination of cell viability of PC12 cells by MTT assay and hormesis-related protein levels by Western blotting. To analyze the roles of signaling pathways in low dose BBR-triggered neuroprotective effect in PC12 cells, the cells were treated with BBR (1 μM) for 24 h and then incubated with ZnPP for 1 h; PC12 cells were pre-incubated with LY294002 for 1 h and then treated with BBR (1 μM) for 24 h. Drug-treated cells were further treated with 250 μM 6-OHDA for 24 h. Then the cells were subjected to the measurement of cell viability by MTT assay and apoptosis by caspase-3 activity assay, sub-G1 DNA content analysis, and Annexin V-FITC/PI staining.

2.11. Anti-tyrosine hydroxylase (TH) whole-mount immunostaining

The AB strain of wild-type zebrafish was used in this study and all animal experiments were conducted following ethical guidelines of Institute of Chinese Medical Sciences, University of Macau. Zebrafish maintenance were performed as described previously [26]. Zebrafish embryos at 1 day post fertilization (dpf) were treated for 2 days with indicated concentrations of BBR in the presence or in the absence of 6-OHDA. Then zebrafish larvae were fixed in 4% paraformaldehyde in PBS for 30 min, rinsed, and stored at −20 °C in 100% MtOH. Whole-mount immunostaining and semi-quantification of TH^+ cells were performed as previously described [26]. Results are expressed as percentage of area of TH^+ cells of that of the untreated normal control group.

2.12. Locomotion behavioral test of zebrafish

Zebrafish larvae at 3 dpf were co-incubated with 250 μM 6-OHDA and Nom or BBR at the indicated concentrations for 4 days. Zebrafish at 7 dpf were transferred into 96-well plate (1 fish/well). Zebrafish behavior was analyzed using an automated video tracking system (Viewpoint, ZebraLab, LifeSciences). The swimming pattern and total distance traveled of each fish were recorded for 10 min. The larvae were allowed to accommodate to the new environment for 30 min

2.13. Statistical analysis

All the data were expressed as means ± SD of three independent experiments. One-way ANOVA followed with Tukey's multiple comparison are used in the GraphPad Prism statistical software (GraphPad Software, San Diego, CA, USA). The value of statistical significance is set at $P < 0.05$.

3. Results

3.1. Low dose BBR induced hormetic effect and attenuated 6-OHDA-induced cell damage in PC12 cells

To investigate the hormetic effect of BBR, PC12 cells were treated with BBR at concentrations ranging from 0.1 to 64 μM for 24 h. The cell viability of BBR was assessed by MTT assay. As shown in Fig. 1A, BBR at a concentration of 1 μM increased cell viability by 33.3% and did not show cytotoxicity up to the concentration of 8 μM. In contrast, treatment with BBR at a concentration of 64 μM significantly reduced cell viability by 47.3%. This biphasic dose-response phenomenon was in agreement with the typical character of hormesis [27,28].

We hypothesized that the hormetic effect of low dose BBR could attenuate 6-OHDA-induced cell damage. To test this hypothesis, PC12 cells were treated with 0.12–16 μM BBR for 24 h and then incubated with or without 250 μM 6-OHDA for a further 24 h. As shown in Fig. 1B, low dose BBR significantly protected PC12 cells from 6-OHDA-induced cell death. Particularly, 1 μM BBR exhibited the highest protective activity of 22.0% as compared to 6-OHDA alone. However, co-treatment of high dose BBR (16 μM) did not show any protective effect. Next, we determined whether low dose BBR could protect PC12 cells against 6-OHDA-induced apoptosis. The results from TUNEL staining showed that the apoptotic rates was decreased from 50.9% to 31.7% in the group of 6-OHDA (250 μM) used alone comparing to 6-OHDA plus BBR (1 μM) (Fig. 1C and D). Meanwhile, our results revealed that the induction of apoptosis by treatment of 6-OHDA was further evidenced by increased expression of cleaved caspase-3, decreased expression of Bcl-2, and increased activity of caspase-3. Interestingly, addition of low dose BBR significantly suppressed the level of cleaved caspase-3 expression, increased the levels of total caspase-3 and Bcl-2 expressions, and decreased the activity of caspase-3 in PC12 cells (Figs. 1E and 4G). These results demonstrated that the hormetic effect of low dose BBR significantly suppressed the *in vitro* 6-OHDA-induced neurotoxicity. The degree of suppression was dependent on the concentration of treatment. Our data also provided important information to discern the biomedical significance of hormesis.

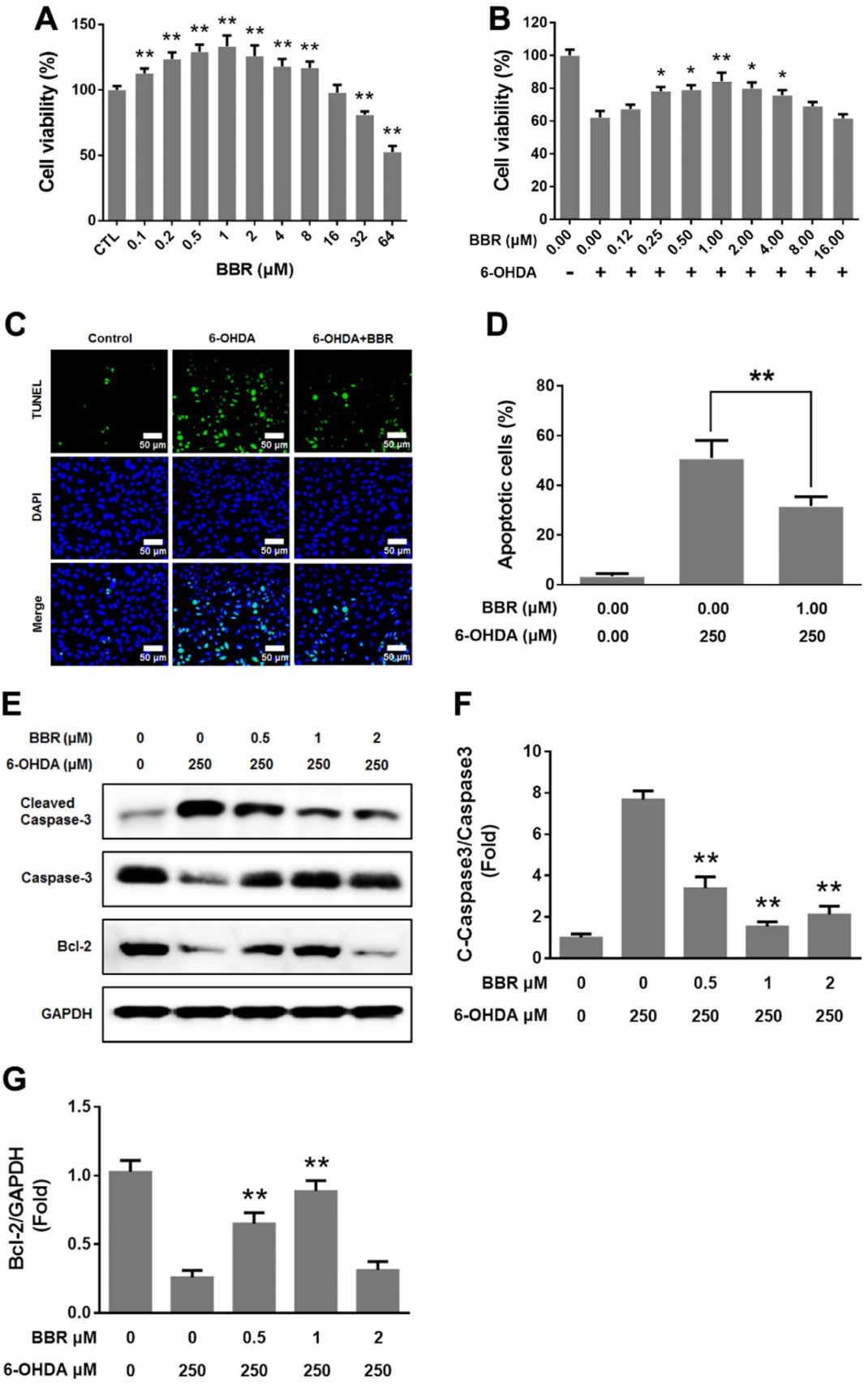

Fig. 1. BBR induced hormetic effect in PC12 cells and protective effects against 6-OHDA-induced cell damage. PC12 cells were treated with increasing concentrations of BBR for 24 h (A), and then incubated with or without 250 μM 6-OHDA for a further 24 h (B). The cell viability was measured using MTT method. (C) PC12 cells were pretreated with 1 μM BBR for 24 h and then treated with or without 250 μM 6-OHDA for 24 h were analyzed for apoptosis using Hoechst 33342 and TUNEL staining, and visualized by InCell Analyzer 2000 (20X objective). Scale bars represent 50 μm. (D) Quantification of apoptotic cells (C). PC12 cells were treated with indicated concentrations of BBR for 24 h, 6-OHDA was then added to the cells for a further 24 h and the protein levels of cleaved caspase-3, caspase-3 and Bcl-2 were detected by Western blot (E). (F) and (G) were densitometric analysis of (E) from three experiments. Values represent the mean ± SD (n = 3). $^{**}P < 0.01$, versus control group in (A); $^{*}P < 0.05$, $^{**}P < 0.01$ versus 6-OHDA-treated alone groups in (B), (D), (F) and (G).

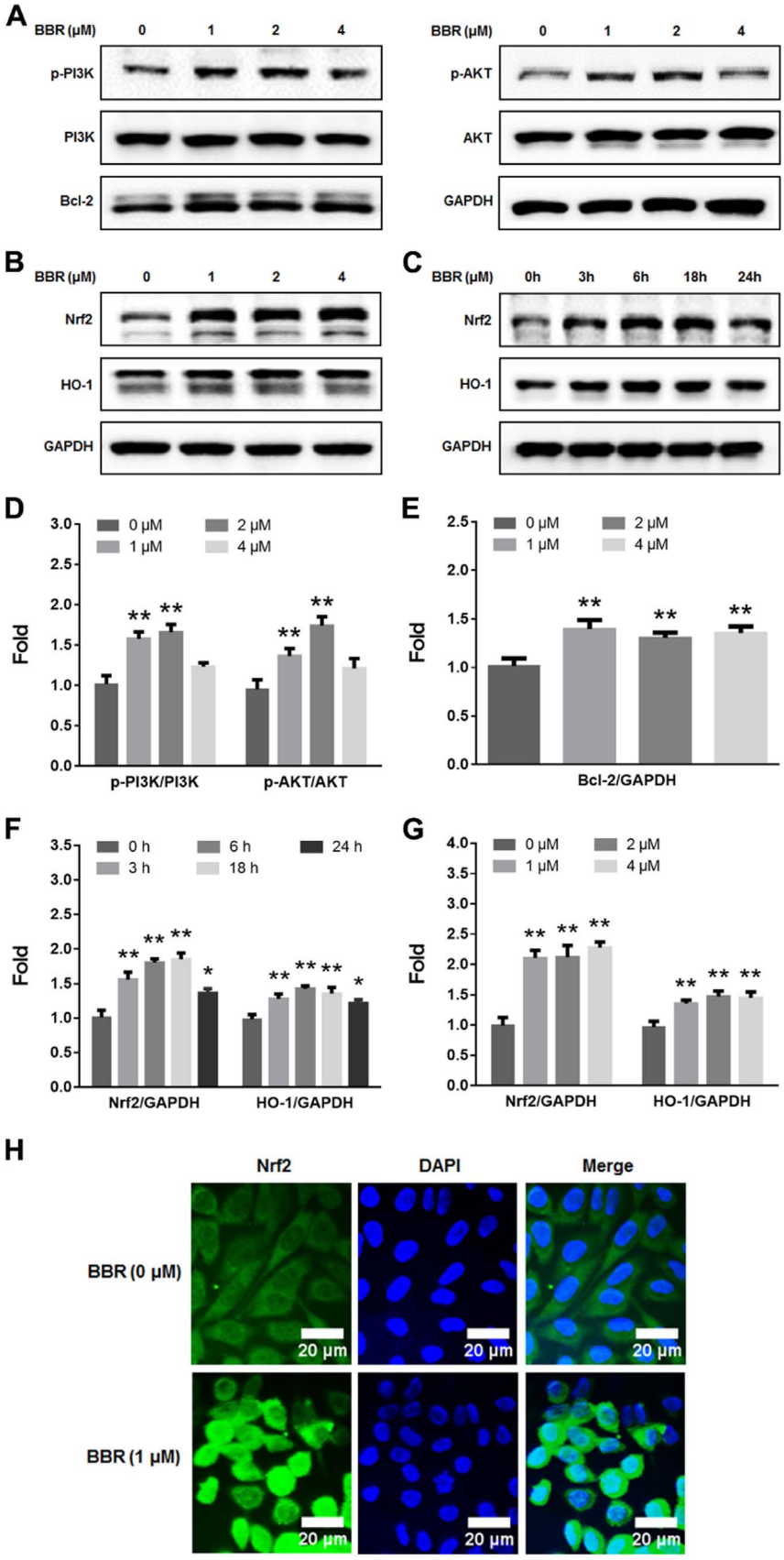

Fig. 2. Effects of BBR on the expression levels of components of PI3K/AKT/Bcl-2 and Nrf2/HO-1 signaling pathways. PC12 cells were treated with varying concentrations of BBR for 24 h (A, B), or the cells were treated with 1 μM BBR for different time points (C). Levels of total and phosphorylated proteins were determined by Western blot. (D), (E), (F) and (G) were densitometric analysis of (A), (B) and (C) from three experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, versus control. (H) Nuclear translocation of Nrf2 was detected using immunofluorescence assay. Analysis was performed by InCell 2000 confocal microscope (60X objective). Blue and green fluorescences indicate localization of nucleus (DAPI) and Nrf2 respectively. Scale bars represent 20 μm.

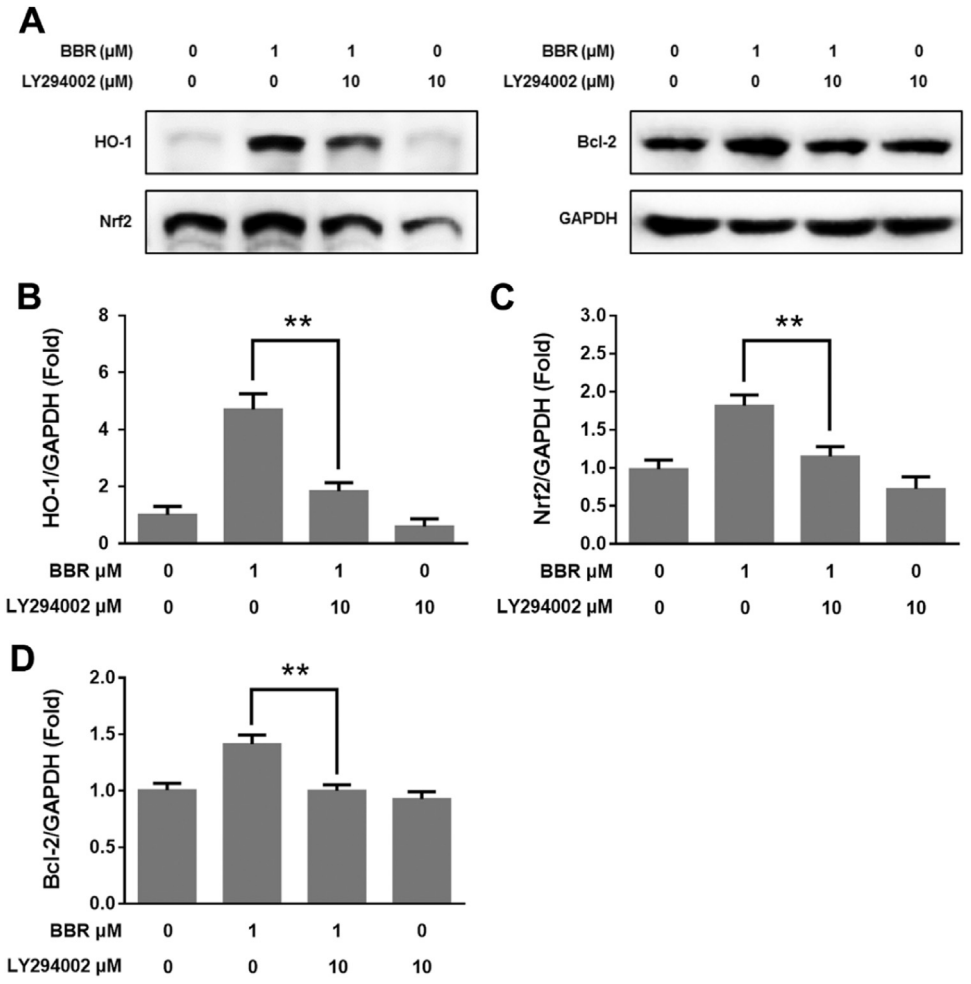

Fig. 3. The role of PI3K in BBR-induced hormetic effect in PC12 cells. PC12 cells were exposed to 1 μM BBR for 24 h with or without pretreatment of 10 μM LY294002 for 1 h. (A) The protein levels of HO-1, Nrf2 and Bcl-2 were detected by Western blot. (B), (C) and (D) were densitometric analysis of (A) from three experiments. Values represent the mean ± SD (n = 3). $^{**}P < 0.01$, compared to BBR-treated alone groups.

3.2. Low doses of BBR up-regulated PI3K/AKT/Bcl-2 and Nrf2/HO-1 pathways

Since PI3K/AKT/Bcl-2 pathway and Nrf2/HO-1 pathway play pivotal roles in cell growth/survival [29–31] and adaptive oxidative response [32–34], we hypothesized that PI3K/AKT/Bcl-2 and Nrf2/HO-1 signaling pathways were involved in the hormetic effect induced by low dose BBR. We examined the phosphorylated and total protein levels of PI3K, AKT, Bcl-2, HO-1 and Nrf2 treated with low dose BBR in PC12 cells by Western blotting assay. Our data showed that low doses of BBR upregulated the levels of p-PI3K and p-AKT, and the expression levels of Bcl-2, HO-1 and Nrf2 proteins in PC12 cells (Fig. 2A to C). To determine low dose BBR-mediated nuclear translocation of Nrf2, nuclear import of Nrf2 in control and treated cells was monitored by immunofluorescence. As shown in Fig. 2H, Nrf2 was mainly located in the cytoplasm of PC12 cells in the control group, and the fluorescence intensity of the nuclear Nrf2 was more significantly increased after BBR treatment (1 μM), indicating that BBR facilitates the Nrf2 accumulation in the nucleus. Moreover, LY294002 significantly reversed the increased protein levels of Bcl-2, HO-1 and Nrf2 (Fig. 3A). These results indicated that the hormetic effect of low dose BBR on PC12 cells were through activating PI3K/AKT/Bcl-2 and Nrf2/HO-1 signaling pathways.

3.3. Inhibition of PI3K/AKT/Bcl-2 and Nrf2/HO-1 pathways attenuated the hormetic and neuroprotective effects of low dose BBR

To further confirm the roles of PI3K/AKT/Bcl-2 cell growth/survival pathway and Nrf2/HO-1 antioxidant pathway in the hormetic effect of BBR, we tested whether the pathway inhibitors could affect the hormetic effect of BBR in PC12 cells. The MTT colorimetric assay revealed that low dose BBR increased viability of PC12 cells by about 30% (dose of 1 μM), which was consistent with previous result in Fig. 1A. However, co-treatment of BBR and 10 μM PI3K/AKT pathway inhibitor LY294002 (Fig. 4A), or 10 μM HO-1 inhibitor ZnPP (Fig. 4B), partially abolished the viability enhancement by low dose BBR in PC12 cells, indicating that PI3K/AKT/Bcl-2 and Nrf2/HO-1 pathways are involved, at least partially, in the hormetic effect of low dose BBR on PC12 cells.

We further investigated whether PI3K/AKT/Bcl-2 and Nrf2/HO-1 pathways participate in the neuroprotective effect of BBR at low doses in PC12 cells. As shown in Fig. 4A and B, the MTT colorimetric assay revealed that 1 μM BBR displayed about 30% neuroprotective activity comparing to the group of 6-OHDA alone treatment, which was similar to the result shown in Fig. 1B. However, addition of LY294002 (Fig. 4A) or ZnPP (Fig. 4B), the neuroprotective effect of BBR at low doses against 6-OHDA-induced cell death was markedly abolished comparing to the co-treatment of BBR and 6-OHDA group. We further assessed whether the neuroprotective effect of low dose BBR against 6-OHDA-induced apoptosis was affected by these inhibitors in PC12

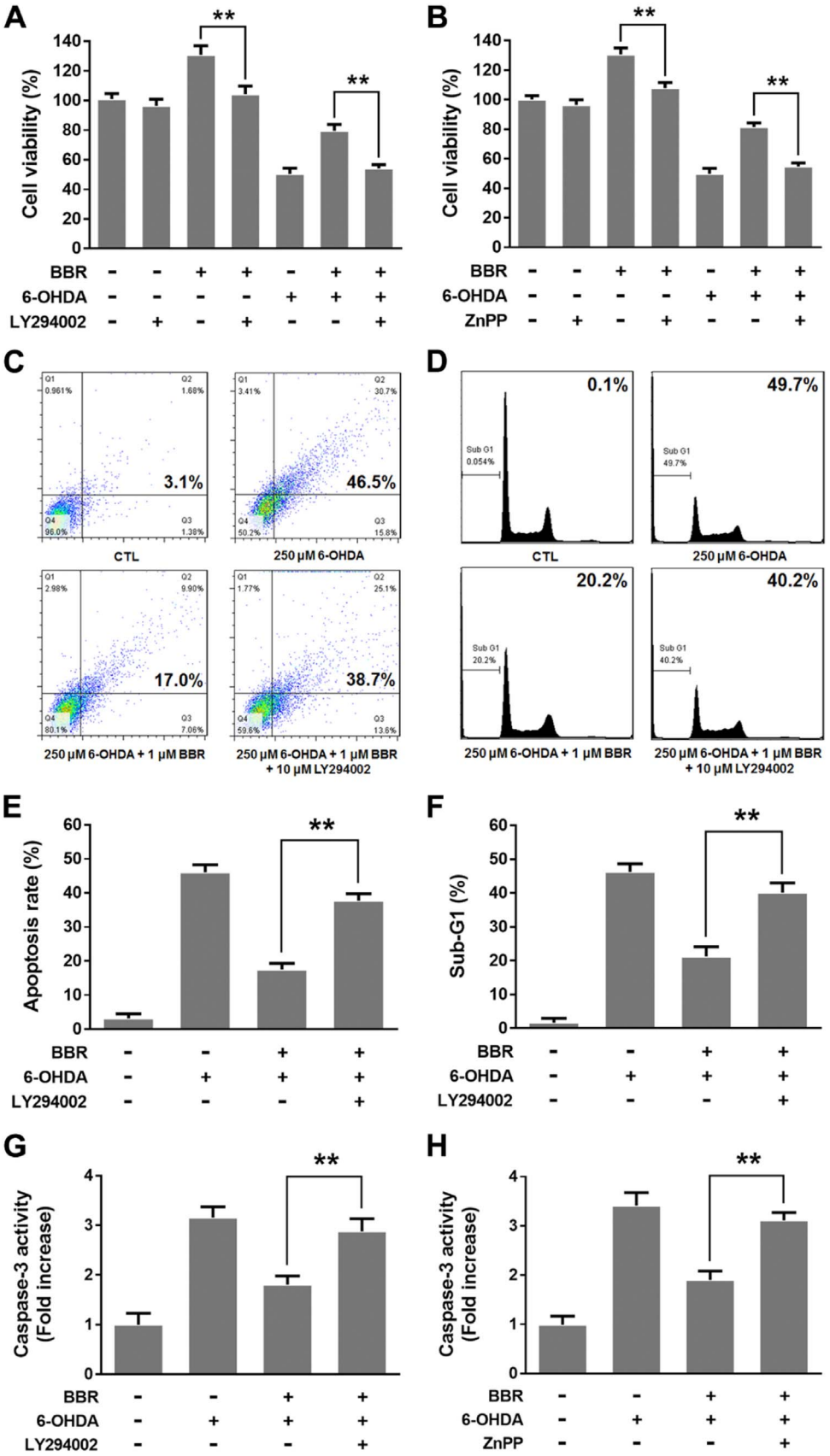

Fig. 4. PI3K and HO-1 inhibitors attenuated BBR-induced hormesis and neuroprotection in PC12 cells. (A) PC12 cells were pre-incubated with or without 10 μM LY294002 for 1 h and then treated with 1 μM BBR for 24 h; (B) PC12 cells were treated with 1 μM BBR for 24 h and then incubated with or without 10 μM ZnPP for 1 h. BBR and inhibitor-treated cells were further treated with or without 250 μM 6-OHDA for 24 h. Cell viability was detected by MTT assay (A) and (B). The effect of PI3K inhibitor on the neuroprotective activity of BBR against 6-OHDA-induced apoptosis was determined using Annexin V/PI staining (C) and subG1 peak analysis (D) by flow cytometry, and also was evaluated by determination of the activity of caspase-3 by ELISA reader (G and H). (E) and (F) were quantified results of (C) and (D), respectively. Values represent the mean ± SD (n = 3). $^{**}P < 0.01$, compared to BBR-treated alone groups or BBR + 6-OHDA-treated groups in (A) and (B). $^{**}P < 0.01$ versus BBR + 6-OHDA-treated groups in (E), (F), (G) and (H).

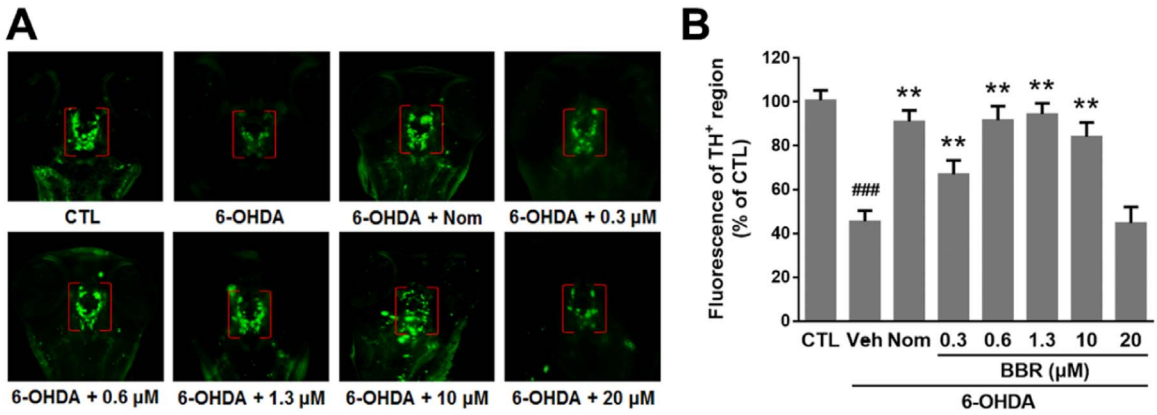

Fig. 5. The effect of BBR on 6-OHDA-induced dopaminergic (DA) neuron loss in zebrafish. Zebrafish embryos at 1 day post fertilization were exposed to different concentrations of BBR or nomifensine (Nom, used as a positive control), and then treated with or without 250 μM 6-OHDA for 48 h. Then zebrafish larvae were fixed for whole-mount immunostaining. (A) Representative morphology of DA neurons in the zebrafish brain indicated by immunostaining with antibody against tyrosine hydroxylase (TH). (B) Statistical analysis of TH^+ neuron in each group, 10 fish/group were used. Values represent the mean ± SD (n = 3). Data is expressed as a percentage of the control group. $^{\#\#\#}P < 0.01$ versus control (CTL) group, $^{**}P < 0.01$ versus vehicle (Veh) + 6-OHDA-treated group.

cells. Results from Annexin V/PI double staining assay (Fig. 4C), sub-G1 phase detection (Fig. 4D) and caspase-3 activity detection (Fig. 4G and H) indicated that the apoptotic rates were lower in PC12 cells co-treated with BBR and 6-OHDA comparing to the groups of 6-OHDA treatment alone. However, LY294002 and ZnPP attenuated the neuroprotective effects of low dose BBR against 6-OHDA-induced apoptosis in PC12 cells (Fig. 4C to H). These results demonstrated that PI3K/AKT/Bcl-2 and Nrf2/HO-1 pathways were involved, at least partially, in the neuroprotective effect of BBR at low doses on PC12 cells.

3.4. Low doses of BBR prevented 6-OHDA-induced dopaminergic (DA) neuronal loss in in vivo models of PD

To confirm the neuroprotective effect of low dose BBR *in vivo*, we evaluated the DA neuron system of zebrafish larvae by immunofluorescent staining with a specific antibody against anti-TH. As shown in Fig. 5, treatment with 6-OHDA decreased the number of DA neurons markedly in the diencephalon of zebrafish (indicated by the red brackets). Low dose BBR (0.3, 0.6 and 1.3 μM) significantly alleviated the loss of DA neurons in a dose-dependent manner, suggesting that low dose BBR exhibits protective effect against 6-OHDA-induced DA neuron death in zebrafish. However, high dose of BBR (20 μM) did not show protective effect against 6-OHDA-induced DA neuron loss.

3.5. Low doses of BBR rescued the deficit of locomotor activity in 6-OHDA-treated zebrafish

In zebrafish larvae, loss of dopamine stimulation results in deficits of locomotive behavior. As shown in Fig. 6, treatment with 6-OHDA markedly altered the swimming behavior and reduced the total swimming distance of zebrafish larvae (from 200 to 97 mm), whereas Nom reduced this deficit (172 mm). Under the same conditions, 0.3, 0.6 and 1.3 μM of BBR suppressed 6-OHDA-induced reduction in total movement distance in a concentration-dependent manner (126, 154 and 183 mm, respectively). However, high dose BBR (20 μM) only exerted slightly protective effect against 6-OHDA-induced movement decreases (125 mm).

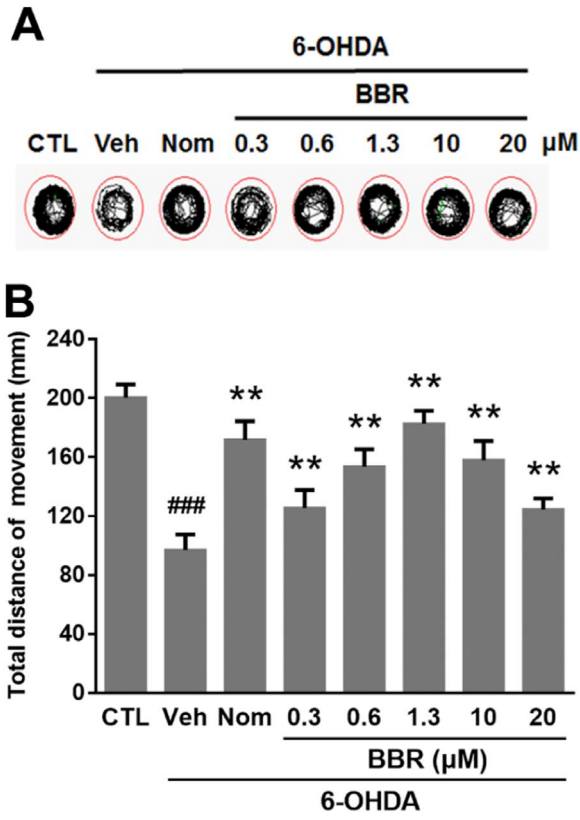

Fig. 6. The effect of BBR on 6-OHDA-induced deficits in the locomotor behavior of zebrafish. Zebrafish larvae at 3 day post fertilization were exposed to indicated concentrations of BBR with or without 250 μM 6-OHDA for 4 days. Then larvae were collected to perform locomotion behavior test using the Viewpoint Zebrabox system; the total distance traveled in 10 min was calculated. (A) Typical swimming patterns of control and different treatment groups. (B) Statistical analysis of total distance moved of control and different treatment groups, eight fish larvae per group were used and experiments were repeated three times. Nom used as a positive control. $^{\#\#\#}P < 0.01$ versus CTL group, $^{**}P < 0.01$ versus Veh + 6-OHDA-treated group.

4. Discussion

In recent years, phytochemicals have been proposed to generally induce hormetic response in neural system, or so called neurohormesis [12,35]. Since hormesis is an adaptive response which could lead to robust cellular protection against further damage in cells or organisms, we speculate that phytochemicals may generally induce hormetic response and activation of related cell growth/survival and anti-oxidation pathways to protect neural cell damage. In the current study, we investigated whether BBR could induce hormetic response and neuroprotective effect and their relationship in PC12 cells and zebrafish. We found that BBR induced a typical hormetic effect in PC12 cells, i.e. low dose BBR increased cell viability while relatively high dose BBR

decreased cell viability (Fig. 1A). Meanwhile, low dose BBR markedly protect PC12 cells from 6-OHDA-induced cytotoxicity and apoptosis, whereas relatively high dose BBR did not show obvious protective activity (Figs. 1B-G, 4G and H), indicating that the neuroprotective effect of BBR is in a hormetic dose response manner. These results are in line with our previous reports that low dose camptothecin exhibited hormetic and neuroprotective effects in PC12 cells [22], and low dose BBR attenuated the anticancer activity of chemotherapeutic drugs in B16-F10 melanoma cells [36]. Several studies have shown that BBR can recognize and directly bind specific DNA sequences by intercalation and form DNA triplexes or G-quadruplexes, thereby triggering DNA repair process and oxidative stress [37], which could be responsible for the induction of hormetic response by BBR [38]. Our data strongly suggested that the hormetic effect of BBR may be underlying its neuroprotective activity in PC12 cells. Furthermore, we tested and characterized the neuroprotective activity of BBR in 6-OHDA-induced zebrafish PD model, which is a powerful model for discovery of anti-PD neuroprotective agents [39,40], since the structure and function of the zebrafish brain are highly similar to those of other vertebrates [41] and 6-OHDA is generally used to cause DA neuronal death and Parkinson's pattern in animals [42]. We demonstrated that low dose BBR markedly attenuated the 6-OHDA-induced DA neuron loss and behavior movement deficiency in zebrafish. However, relatively high dose BBR exerted no or slightly neuroprotective effect, being indicative of a hormetic dose response (Figs. 5 and 6). These data further confirmed the role of hormetic response in the neuroprotective effect of low dose BBR. Evoking nonharmful endoplasmic reticulum stress and protective autophagy, which were thought to be hormetic mechanisms [43], could prevent neuronal cell death in animal PD models [1,44]. These were in accordance with the hypothesis that hormetic mechanisms could be novel targets for therapeutic intervention in neurodegenerative disorders (NDD) [6,45].

Hormesis is terminologically equivalent to adaptive response [27], which involves a broad range of stress response proteins and pathways [46]. Accumulating studies indicated that various phytochemicals could ameliorate neurological disorders through activating pro-survival and antioxidant pathways [12,47]. However, different stimuli may overwhelmingly activate one or more hormetic mechanisms in certain biological systems. In the present study, we found that low dose BBR treatment markedly increased the levels of p-PI3K and p-AKT, and the expression level of Bcl-2 (Fig. 2A). Our data also showed that the induction of Bcl-2 expression by low dose BBR could be attenuated by PI3K/AKT pathway inhibitor LY294002 (Fig. 3A), and co-treatment of LY294002 abolished the viability-enhancing effect and protective effect of BBR against 6-OHDA-induced cytotoxicity (Fig. 4A) and apoptosis (Fig. 4C, D and G) in PC12 cells. PI3K/AKT pathway is critical for neuronal survival by promoting cell survival and inhibiting apoptosis, and is also one of the major signaling pathways participating in hormesis [48]. AKT, a serine/threonine kinase, is the key mediator of PI3K-initiated signaling and can promote neuronal survival by upregulating the expression of Bcl-2 through cAMP-response element-binding protein [49]. Bcl-2 has been recognized for its pro-survival, antioxidant, antiapoptotic and cytoprotective functions, and plays a pivotal role in hormetic response [50]. Our results suggest that PI3K/AKT/Bcl-2 pathway plays a key role in BBR-elicited hormetic response and protective effect against 6-OHDA-caused apoptosis in PC12 cells. Previous studies have shown that PI3K/AKT pathway could be activated in response to oxidative stress [51,52]. We speculate that the activation of PI3K/AKT/Bcl-2 is attributable to the mild oxidative stress induced by low dose BBR.

Excessive oxidative stress caused mitochondrial dysfunction has been proposed to be associated with NDD [53] and brain aging [54]. Therefore, reducing oxidative damage to neuronal cells could be a promising preventive and therapeutic approach. Increasing evidence suggests that many phytochemicals can activate pathways that prevent or reverse oxidative injury [55]. The Nrf2/HO-1 signaling pathway is crucial for neuroprotection against oxidative stress [32–34] and involved in the process of hormesis [36,56–58]. Nrf2 is a stress-responsive transcriptional factor and an important effector protecting cells from oxidative injury, particularly neurodegenerative diseases. Under oxidative stress, Nrf2 releases from Keap1 and translocates into the nucleus and recruits the small Maf (sMaf) protein. The Nrf2-sMaf heterodimer then binds to the antioxidant response element (ARE) to promote the expression of many antioxidant enzymes, such as HO-1, NAD(P)H-quinone oxidoreductase, superoxide dismutase 3, glucuronosyltransferase-1a6 and glutathione S-transferase. These enzymes exert antioxidant and cytoprotective effects in response to oxidative stress [59,60]. Among the ARE-regulated phase II detoxifying enzymes and antioxidants, HO-1 is the major enzyme regulated by Nrf2 since it has the most strong AREs on its promoter, making it a potent therapeutic target for neurodegenerative diseases [61]. Numerous phytochemicals (e.g., curcuminoids, isothiocynates, Z-ligustilide, catechins, indoles, terpenes, diallyl sulfides) have been shown to activate one or more signaling pathways upstream of Nrf2/HO-1 axis, thus protecting neurons against oxidative stress [12,47]. In fact, the Nrf2 can be a downstream effector of several signal pathways, including MAPK, PI3K/AKT and protein kinase C to coordinate mammalian defense systems against electrophiles and oxidative stresses [62]. In the present study, low dose BBR significantly increased the levels of Nrf2 and HO-1 in PC12 cells (Fig. 2B and C), and promoted Nrf2 nuclear translocation (Fig. 2H). Inhibition of the Nrf2/HO-1 pathway with ZnPP (a HO-1 inhibitor) reversed the viability-enhancing (Fig. 4B) and neuroprotective effects of BBR (Fig. 4B and H) in PC12 cells. These results demonstrated that Nrf2/HO-1 pathway was also responsible for the hormetic and neuroprotective effects of BBR. In addition, LY294002 could reduce the levels of Nrf2 and HO-1 in the presence of BBR (Fig. 3A), suggesting that low dose BBR protected PC12 cells against 6-OHDA-induced cytotoxicity through enhancing the activity of Nrf2/HO-1 triggered by PI3K/AKT signaling pathway. This is in line with previous reports [47,63].

In summary, we demonstrated that relatively low dose BBR exhibited strong neuroprotective activity against 6-OHDA-induced neurotoxicity in PC12 cells and zebrafish through activating hormetic mechanisms involving up-regulated PI3K/AKT/Bcl-2 cell survival and Nrf2/HO-1 antioxidant pathways (as depicted in Fig. 7). This study provides new insight into the mechanisms for the neuroprotective effect of BBR. BBR has been approved for clinical trials in the treatment of hyperglycemia and hyperlipidemia [13]. However, the

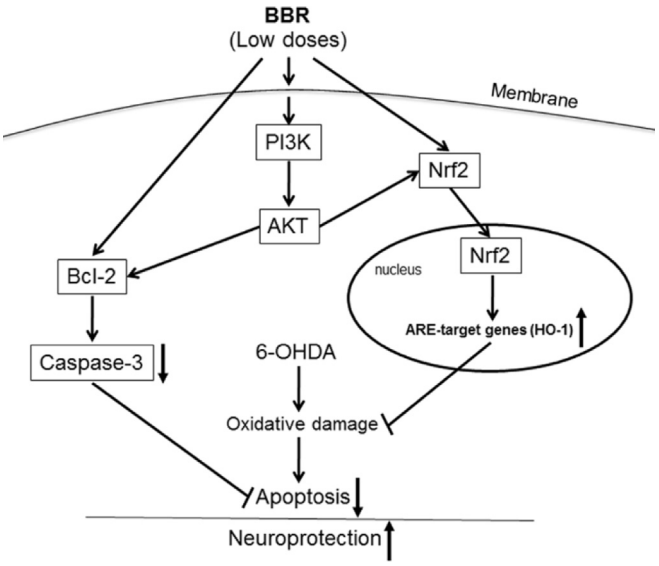

Fig. 7. A schematic model of upregulated PI3K/AKT/Bcl-2 cell survival pathway and Nrf2/HO-1 antioxidant pathway by low doses of BBR in PC12 cells.

preclinical and clinical studies of neuroprotective activities of BBR have not been documented to date. Our data and others strongly suggest that BBR is a potential candidate agent for the prevention and treatment of neurodegenerative diseases. In addition, we presented direct experimental evidences to confirm that induction of hormetic responses in neuronal cells could not only be a general mechanism for the neuroprotective activity of many phytochemicals with multiple targets [11,12], but also a new approach for the management of neurodegenerative disorders [6,45].

Author contributions

C.Z., K.L. and C.H. designed the research; C.Z., C.L., S.C., Z.L., X.J., K.W., J.B., Y.L. and X.W. performed the experiments; M.C., P.L., H.S., S.M.Y.L. and J.B.W. provided reagents and technical support; C.Z., C.L., K.L. and C.H. analyzed the data; C.Z. and C.H. wrote the paper.

Conflict of interests

The authors declare no conflict of interest.

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