Dl-butylphthalide inhibits rotenone-induced oxidative stress in microglia via regulation of the Keap1/Nrf2/HO-1 signaling pathway

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Abstract. Activated microglia are a source of superoxide which often increases oxidative stress in the brain microenvironment, increase production of reactive oxygen species (ROS) and directly or indirectly lead to dopaminergic neuronal death in the substantia nigra. Thus superoxide contributes to the pathogenesis of Parkinson's disease (PD). Evidence suggests that mitochondria are the main source of ROS, which cause oxidative stress in cells. Levels of ROS are thus associated with the function of the mitochondrial complex. Therefore, protecting the mitochondrial function of microglia is important for the treatment of PD. Dl-butylphthalide (NBP), a compound isolated from Chinese celery seeds, has been approved by the China Food and Drug Administration for the treatment of acute ischemic stroke. Recently, NBP demonstrated therapeutic potential for PD. However, the mechanism underlying its neuroprotective effect remains unclear. The present study aimed to investigate the effect of NBP on rotenone-induced oxidative stress in microglia and its underlying mechanisms. The results demonstrated that NBP treatment significantly increased mitochondrial membrane potential and decreased ROS level in rotenone-induced microglia. Western blot analysis showed that NBP treatment promoted entry of nuclear respiratory factor-2 (Nrf2) into the nucleus, increased heme oxygenase-1 (HO-1) expression and decreased the level of the Nrf2 inhibitory protein, Kelch-like ECH-associated protein 1. Overall, the findings indicated that NBP inhibited rotenone-induced microglial oxidative stress via the Keap1/Nrf2/HO-1 pathway, suggesting that NBP may serve as a novel agent for the treatment of PD.

Introduction

Parkinson's disease (PD), the second-most common neurodegenerative disorder that affects 2-3% of the population ≥65 years of age in the world, is characterized by quiescent tremor, motor retardation, myotonia and postural balance disorder (1). However, this disease has two main pathological characteristics: Massive degeneration and loss of dopaminergic neurons in substantia nigra and formation of Lewis bodies (1,2). Currently, the pathogenesis of PD is still unclear but mitochondrial damage leading to overproduction of reactive oxygen species (ROS) is an important cause of the activation of microglia and the loss of dopaminergic neurons (3,4). In addition, overwhelming evidence indicates that mitochondrial disorder and oxidative stress serve important roles in the development of PD (5,6). Furthermore, activated microglia, which can lead to oxidative stress, increase the level of ROS and directly or indirectly leading to the death of dopaminergic neurons in the substantia nigra, which is one of the pathological characteristics of PD (5). Activated microglia may thus be a key target for the treatment of PD.

Rotenone, an inhibitor of mitochondrial complex I, was first used in PD research in the 1980s (7). Research on the application of rotenone to treat Parkinson's disease has continued to increase (8). In terms of molecular mechanism, rotenone induces mitochondrial dysfunction and increases ROS production, which are implicated in the degeneration of dopaminergic neurons (9). Previous studies have reported that rotenone activates microglia cells by causing mitochondrial dysfunction and oxidative stress via inhibiting oxidative respiratory chain complex and increasing the production of ROS. Therefore rotenone treatment of cells can also be used as an *in vitro* model of PD (10,11).

Dl-butylphthalide (NBP), a compound isolated from Chinese celery, was approved by the China Food and Drug Administration for the treatment of acute ischemic stroke (12). Therapy using NBP has been recommended by Chinese

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guidelines for cerebral collateral circulation in ischemic stroke (13). The mechanisms of NBP in ischemic stroke treatment may be mediated through different processes including anti-oxidant activity, protection of mitochondria, anti-inflammation, anti-thrombosis and anti-apoptosis (14). A recent review indicated that the therapeutic effect of NBP is not limited to cerebrovascular diseases but also treats neuro-degeneration diseases (15). Our previous study reviewed the neuroprotective mechanism of NBP and found that reducing oxidative stress is one of the most important mechanisms (16). However, the mechanism through which NBP inhibits the microglial oxidative stress is not completely understood. Much less is known about its neuroprotection and regulation of the underlying signaling pathways.

Therefore, the present study aimed to investigate whether NBP exerts neuroprotective effects on rotenone-induced mitochondrial dysfunction and oxidative stress in BV2 cells. To elucidate the effects of the drug *in vitro* more fully, rotenone was used to induce damage to mitochondria in BV2 cells. Then, the effects of NBP on rotenone-induced morphological changes in microglia, mitochondrial dysfunction and ROS production and the underlying signaling pathways were examined. The results may aid in the development of novel treatment strategies for Parkinson's disease.

Materials and methods

Reagents. Rotenone was purchased from Sigma-Aldrich (Merck KGaA) and NBP was provided by China Shijiazhuang Pharmaceutical company. The Mitochondrial Membrane Potential Assay kit (with JC-1) was purchased from Elabscience Biotechnology. Dichlorodihydrofluorescein diacetate (DCFH-DA) and Cell Counting Kit-8 (CCK-8) were obtained from Beyotime Institute of Biotechnology. The antibodies against Kelch-like ECH-associated protein 1 (Keap1), nuclear respiratory factor-2 (Nrf2) and heme oxygenase-1 (HO-1) were obtained from Wanleibio and antibodies against β-tubulin and lamin B were purchased from Cell Signaling Technology, Inc. The NE-PER nuclear and cytoplasmic extraction reagent kit was obtained from Thermo Fisher Scientific, Inc.

Cell culture. BV2 microglial cells were provided by the Cell Culture Center of the Chinese Academy of Medical Sciences. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone; Cytiva) high glucose, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biological Industries) in a 5% CO_2 incubator set at 37°C. On reaching approximately 80% confluency, the cells were passaged at 1:3 ratio using trypsin (HyClone; Cytiva) solution (0.25% trypsin without EDTA).

Determination of cell viability. BV2 cell viability under different concentrations of rotenone and NBP was assessed using the CCK-8 assay. Briefly, BV2 cells were seeded in 96-well plates (Mettler-Toledo Rainin, LLC) at a density of 3,000 cells/well for 24 h. The medium was then replaced with DMEM with 10% FBS containing different doses of rotenone or NBP (3 replicate wells for each treatment). After 24 h, 10 μl CCK-8 reagent was added to each well. The absorbance was then recorded within 4 h at 450 nm using the Model 550

microplate reader (Bio-Rad Laboratories, Inc.). Data were collected from three independent experiments.

Detection of mitochondrial membrane potential. BV2 cells were seeded in 6-well plates at a density of $4x10^5$ cells/well for 24 h, followed by treatment with rotenone (0.05 μM) with or without NBP (200 μM) for 24 h. Next, the cells were harvested by using trypsin (0.25% trypsin without EDTA), resuspended in phosphate buffered saline (PBS), immediately stained with JC-1 (1 mg/ml in DMSO) and incubated at 37°C for 30 min in darkness. After washing twice in ice-cold PBS, the cells were analyzed using the CytoFLEX flow Cytometer (Beckman Coulter, Inc.). Data were collected from three independent experiments.

Measurement of intracellular ROS level. Intracellular ROS generation was examined using the DCFH-DA method. BV2 cells were seeded at a density of $4x10^5$ cells/well in 6-wells plates (Mettler-Toledo Rainin, LLC) for 24 h, followed by treatment with rotenone (0.05 μM) with or without NBP (200 μM)for 24 h. The cells were then incubated with 10 μM DCFH-DA for 30 min at 37°C in 5% CO_2. Next, the cells were washed thrice with PBS and visualized under a fluorescence microscope (Leica, Germany). Images were captured through band pass filters of 505-530 nm (10X). Finally, the fluorescence intensity of each group was analyzed by ImageJ 1.8 software (National Institutes of Health).

Preparation of nuclear extract. BV2 cells were seeded at a density of $4x10^5$ cells/well in 6-well plates for 24 h, followed by treatment with rotenone (0.05 μM) with or without NBP (200 μM) for another 24 h. The cells were then used for nuclear extract preparation using the NE-PER nuclear and cytoplasmic extraction reagent kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The concentration of the extracted protein was evaluated using a bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology).

Western blotting. In each group, proteins (20 μg for cytoplasmic protein and 5 μg for nuclear protein) were separated on 10% SDS-polyacrylamide gels (Beyotime Institute of Biotechnology) and then transferred onto polyvinylidene difluoride membranes (EMD Millipore). Subsequently, the membranes were blocked with Tris buffered saline with 5% Tween-20 containing 5% non-fat milk for 1 h at room temperature. The levels of Keap1 (cat. no. WL03285; Wanleibio), Nrf2 (cat. no. WL02135; Wanleibio) and HO-1 (cat. no. WL02400; Wanleibio) were detected by immunoblotting using specific primary and secondary antibodies. β-tubulin (cat. no. 2128S; Cell Signaling Technology, Inc.) and Lamin B (cat. no. 13435S; Cell Signaling Technology, Inc.) were used as controls to ensure equal loading of cell lysates. All primary antibodies were rabbit antibodies and were used at a concentration of 1:1,000. The secondary antibody (cat. no. IH-0011; Beijing Dingguo Changsheng Biotechnology Co., Ltd.) used was an anti-rabbit antibody and was used at a concentration of 1:5,000. The primary antibody was incubated at 4°C for 20 h and the secondary antibody was incubated at room temperature for 1 h. ECL (cat. no. 32106; Thermo Fisher Scientific) was used
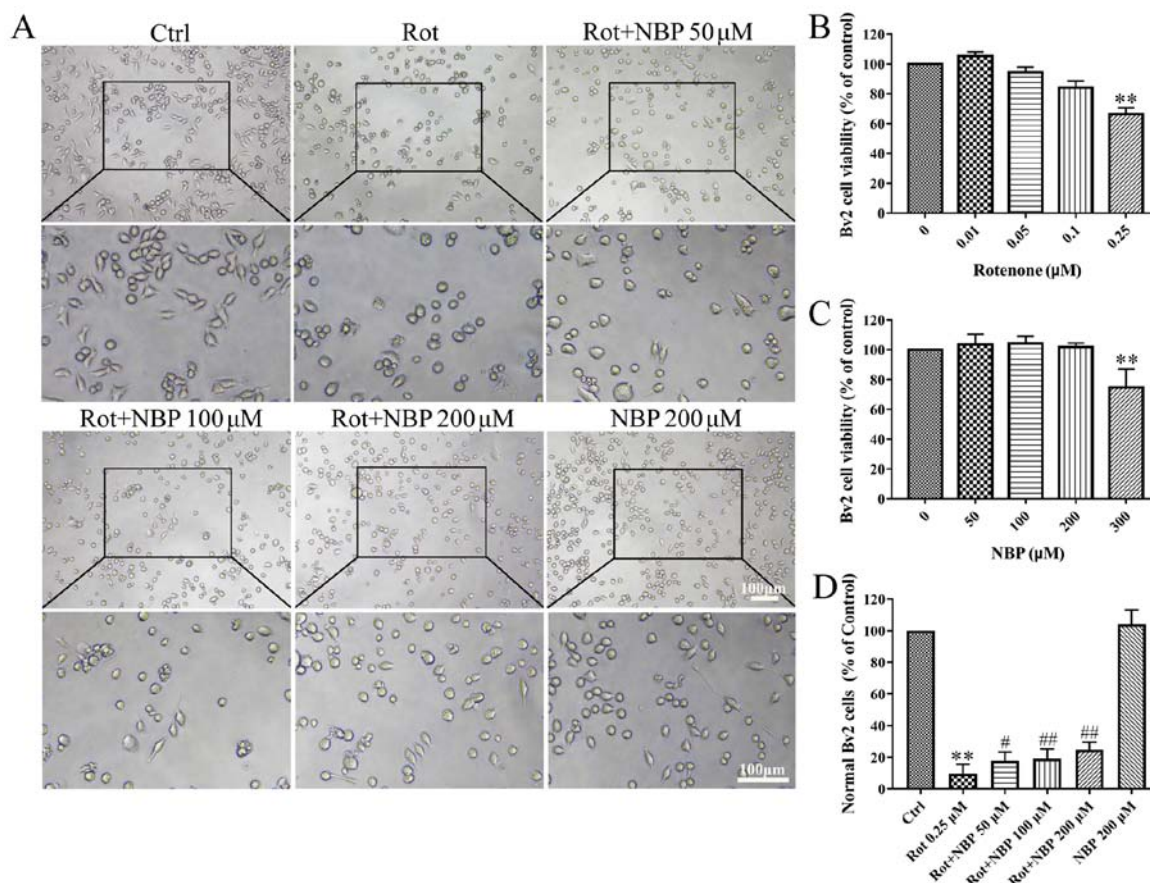
Figure 1. Effect of NBP on cell viability and form in rotenone treated BV2 microglia. (A) Cell morphology as observed under microscope following treatment with rotenone and NBP at different concentrations. (B) Cell viability of BV2 microglial cells as determined by CCK-8 assay following treatment of rotenone at different concentrations (0-0.25 μM) for 24 h. (C) Cell viability of BV2 microglial cells as determined by CCK-8 assay following treatment of NBP at different concentrations (0-300 μM) for 24 h. (D) The number of normal cells increased following addition of NBP to rotenone-treated cells. The morphology of BV2 cells was analyzed by ImageJ and the data were analyzed by one-way analysis of variance. All data were expressed as mean ± standard deviation for three independent experiments [**]P<0.01 vs. the control group; [#]P<0.05, [##]P<0.01 vs. the Rot (0.25 μM) group. NBP, dl-butylphthalide; Ctrl, control; rot, rotenone.

to develop the bands. The band intensity was analyzed using ImageJ 1.8 software. Data were analyzed by one-way analysis of variance (ANOVA).

Statistical analysis. All images were analyzed with ImageJ 1.8 software. Statistical analysis was performed by Dunnett test following one-way ANOVA using GraphPad Prism 8 software (GraphPad Software Inc.). All data were expressed as mean ± standard deviation from three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of different concentrations of NBP and rotenone in BV2 cells. First, the cytotoxicity of NBP and rotenone in BV2 cells was determined. The concentration of rotenone chosen was 0.25 μM with or without different dose of NBP for determination of the neuroprotective effect of NBP on BV2 cells. The results showed that NBP significantly protected microglia cells from rotenone-induced morphological changes. The degree of protection was dependent on the concentration of NBP as shown in Fig. 1A and D. Cell viability of the control was set at 100% as shown in Fig. 1B and C. Therefore, Rotenone

significantly diminished survival rates of BV2 cells while NBP rescued that in a dose-dependent manner.

NBP protects mitochondrial function. The mitochondrial membrane potential (MMP) controls ATP synthesis and the generation of ROS. A decrease in MMP represents a decrease in mitochondrial function (2). When stained with JC-1 dye, phycoerythrin A fluorescence of mitochondria indicates the formation of J-aggregates at high negative MMP and fluorescein isothiocyanate A fluorescence of mitochondria indicates the formation of JC-1 monomers at low MMP. The results of the present study showed that treatment of cells with rotenone (0.05 μM) for 24 h caused the MMP reduction and NBP significantly inhibited the formation of JC-1 monomers in rotenone-treated BV2 cells (Fig. 2).

NBP reduces intracellular ROS in rotenone-treated BV2 cells. The present study attempted to verify whether NBP reduced intracellular ROS generation in rotenone-treated BV2 cells. As shown in Fig. 3, the intracellular ROS of BV2 cells increased following treatment with rotenone for 24 h. However, BV2 cells treated with rotenone and NBP for 24 h showed a clear drop in ROS levels when compared with cells treated with rotenone alone. Treatment of cells with NBP
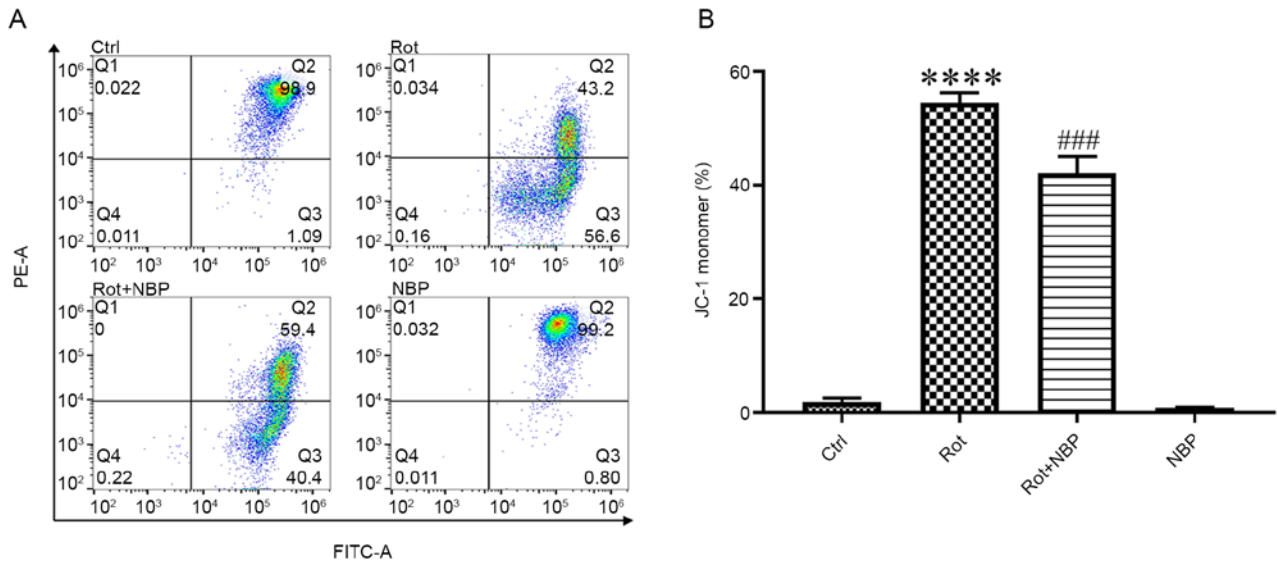
Figure 2. NBP inhibited rotenone-induced MMP reduction in microglia. (A) Scatter diagram of PE-A/FITC-A gating from control; Rot (0.05 μM), NBP (200 μM) + Rot (0.05 μM) and NBP (200 μM) groups. (B) Data analysis showed that Rot significantly increased the proportion of JC-1 monomers, while the addition of NBP reduced this change. All data are expressed as the mean ± standard deviation for three independent experiments. ****P<0.0001 vs. the control group; ###P<0.001 vs. the Rot group. NBP, dl-butylphthalide; MMP, mitochondrial membrane potential; PE, phycoerythrin; FITC, fluorescein isothiocyanate; Ctrl, control; Rot, rotenone.
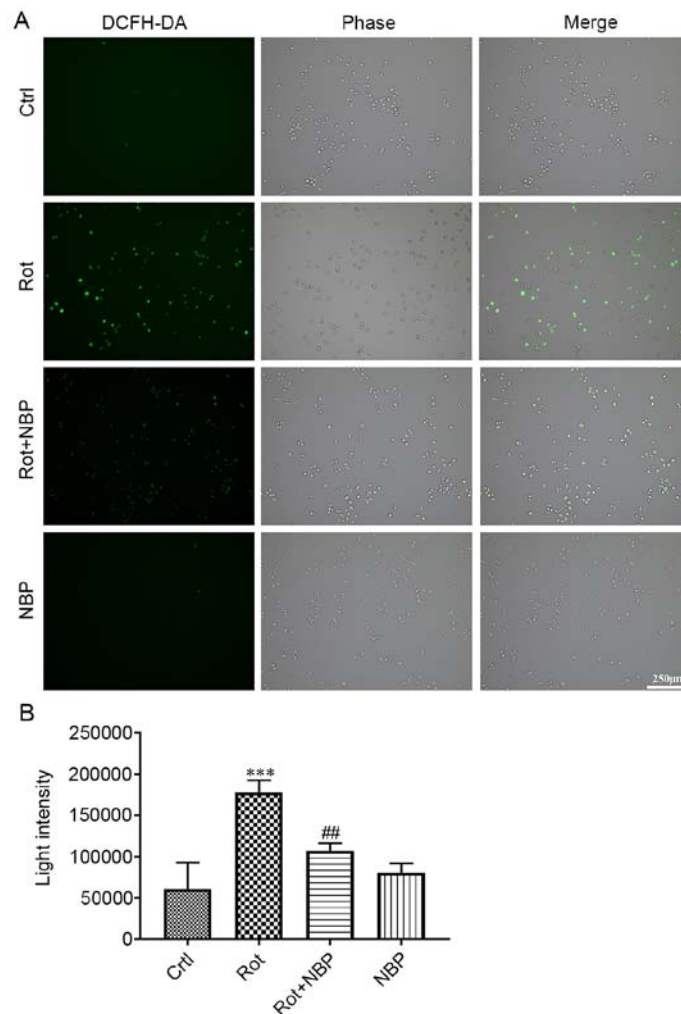

Figure 3. Effect of NBP on the generation of ROS in rotenone-induced BV2 microglia. (A) Following treatment with rotenone for 24 h, the intracellular ROS of BV2 cells significantly increased. (B) Inclusion of NBP in rotenone treated cells significantly decreased intracellular ROS. All data were expressed as mean ± standard deviation for three independent experiments ***P<0.001 vs. the control group; ##P<0.01 vs. the Rotenone group. NBP, dl-butylphthalide; ROS, reactive oxygen species; DCFH-DA, dichlorodihydrofluorescein diacetate; Ctrl, control; rot, rotenone.
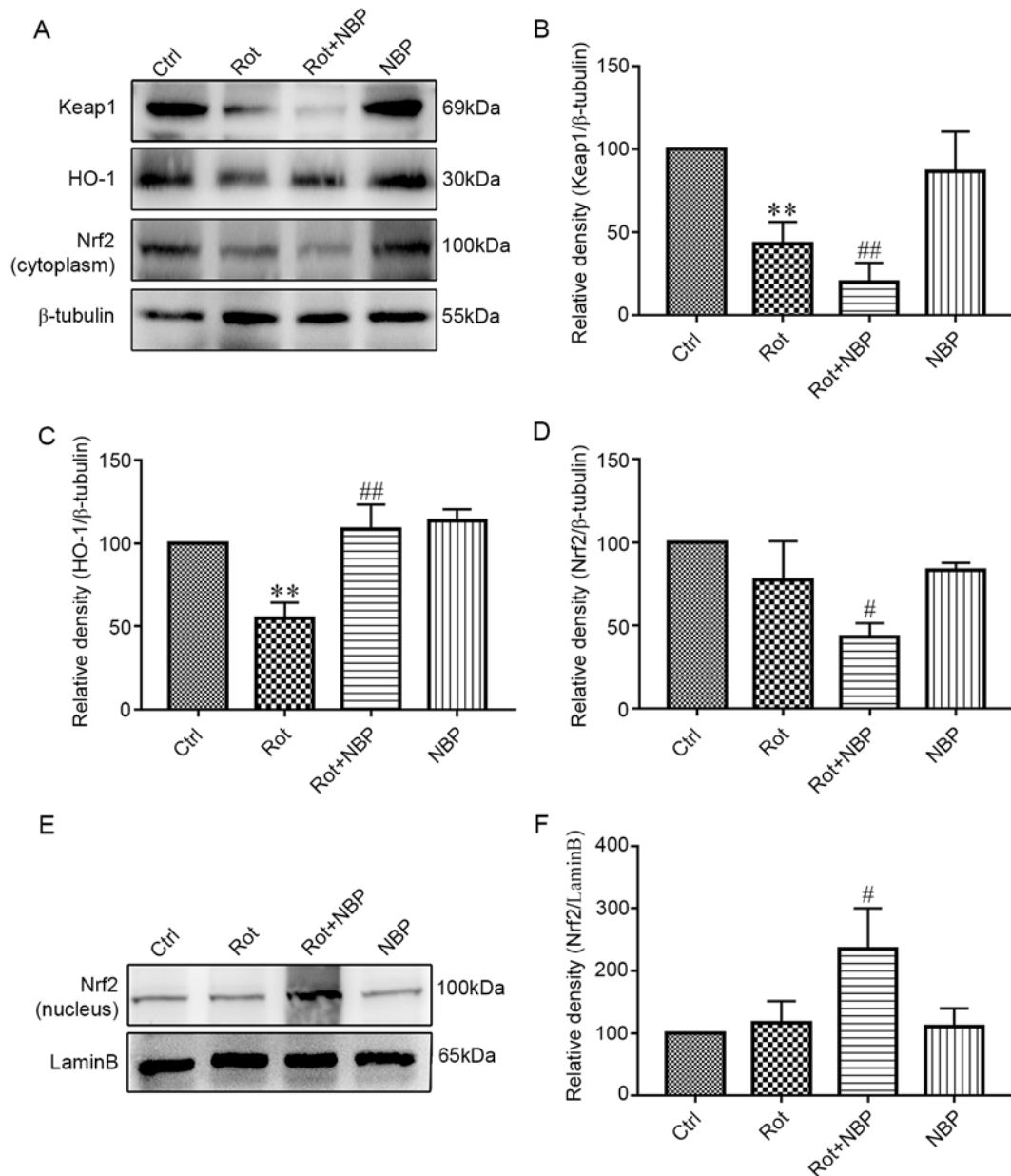
Figure 4. Effect of NBP on Keap1/Nrf2/HO-1 signaling pathway in rotenone-induced BV2 microglia. (A) The results of cytoplasmic protein expression of Keap1/Nrf2/HO-1 signaling pathway. (B) Inhibition of the levels of Keap1 in the cytoplasm by rotenone. Adding NBP significantly enhanced this inhibition but NBP alone had no effect on Keap1 expression. (C) Rotenone inhibited the levels of HO-1 but adding NBP eliminated the inhibition. When used alone, NBP had no effect on HO-1 expression. (D) Rotenone inhibited the levels of Nrf2 and adding NBP significantly enhanced this effect. However, NBP alone had no effect on Nrf2 expression. (E) The results of nuclear protein expression indicated that Nrf2 was promoted into the nucleus. (F) Rotenone increased the levels of Nrf2 in the nuclear and adding NBP significantly enhanced this effect. However, NBP alone had no effect on Nrf2 expression. The density of bands was analyzed by ImageJ and the data was analyzed by one-way analysis of variance. All data were expressed as mean ± standard deviation for three independent experiments [**]P<0.01 vs. the control group; [#]P<0.05, [##]P<0.01 vs. the Rotenone group. NBP, dl-butylphthalide; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear respiratory factor-2; HO-1, heme oxygenase-1; Ctrl, control; rot, rotenone.

alone had no effect on ROS levels when compared with the control group.

NBP activates Keap1/Nrf2/HO-1 signaling pathway. The Keap1/Nrf2/HO-1 signaling pathway was examined by western blotting. The expression of this pathway in the cytoplasm s shown in Fig. 4A. Following treatment with rotenone, the level of Keap1 in BV2 cells decreased significantly compared with the control group as shown in Fig. 4B. However, on inclusion of NBP in rotenone treated cells, the level of Keap1 decreased significantly (Fig. 4B). By contrast,

following rotenone treatment, the level of HO-1 in BV2 cells decreased significantly compared with control group, whereas on addition of NBP, the level of HO-1 increased significantly compared with cells treated with rotenone alone (Fig. 4C). Treatment of cells with rotenone had no significant effect on the level of Nrf2 in cytoplasm compared with control group. However, addition of NBP to rotenone treated cells significantly decreased the level of Nrf2 in cytoplasm compared with cells treated with rotenone alone (Fig. 4D). Additionally, treatment of cells with rotenone, significantly increased the level of Nrf2 in nucleus compared with control group, but addition of
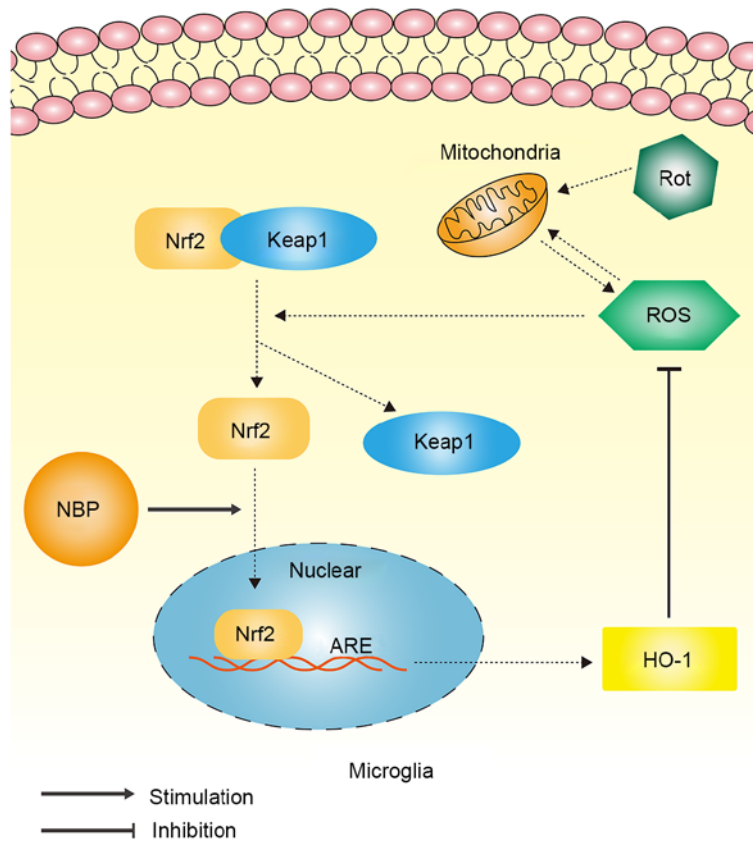
Figure 5. Mechanism of inhibition of rotenone-induced oxidative stress in microglia by NBP. Treatment with NBP decreased the level of ROS and protected mitochondrial function of BV2 cells, which may be through activating Keap1/Nrf2/HO-1 signaling pathway. NBP, dl-butylphthalide; ROS, reactive oxygen species; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear respiratory factor-2; HO-1, heme oxygenase-1; rot, rotenone; ROS, reactive oxygen species; ARE, antioxidant response element.

NBP to rotenone treated cells significantly increased the level of Nrf2 in nucleus compared with cells treated with rotenone alone (Fig. 4E and F).

Discussion

In recent years, NBP has been shown to have powerful effects against oxidative stress in different models. For instance, NBP inhibits oxidative stress in K141N-induced SH-SY5Y cells and in LPS-induced rats through activation of the Keap1/Nrf2/antioxidant response element (ARE) signaling pathway (17,18). Similarly, NBP reduces oxidative damage to provide neuroprotection in mice following tumor brain injury and in rats following carbon monoxide poisoning (19,20). In addition, NBP protects brain tissue against cerebral ischemia-reperfusion injury by its antioxidant activity via ERK signaling (21). NBP also works against H_2O_2-induced injury in neural stem cells by activation of the PI3K/Akt pathway (22). NBP increases the superoxide dismutase and catalase activity and reduces malondialdehyde activity in experimental auto-immune myositis model (23). Despite the above findings, it is unclear how NBP regulates microglia *in vitro* and the mechanisms involved remain to be elucidated. Additionally, little is known about its effect on the oxidative stress model of Parkinson's disease.

The pathogenesis of PD is closely associated with mito-chondrial dysfunction and oxidative stress (24). Mitochondria are the production factory for ATP in cells. When the mito-chondrion is damaged, there is a decrease of ATP, which causes dysfunction of the sodium potassium pump. This results in electrolyte and water balance disorder in the cell, causing difficulties in maintaining normal cell morphology (25). Thus, microglia are unable maintain the original spindle shape and become rounded following rotenone treatment (Fig. 1A). Following NBP treatment, the number of normal BV2 cells increased significantly, which suggested that NBP might protect mitochondrial function.

Mitochondria are surrounded by two layers of membrane, the inner and the outer membrane. The inner mitochondrial membrane (IMM) is implicated in mitochondrial energy conver-sion whereas the outer mitochondrial membrane is the principal platform for mitochondrial signaling (26). In the process of respi-ratory oxidation, the energy produced by mitochondria is stored in the IMM as electrochemical potential energy. This results in asymmetric distribution of protons and other ions on both sides of the inner membrane, which forms MMP, maintaining MMP is essential for ATP production (27). Therefore, mitochondrial function can be assessed indirectly by detecting MMP (28).

Mitochondrial dysfunction leads to excessive increase in intracellular ROS and activates microglia (10). ROS generated by mitochondria can induce rapid depolarization of MMP which further stimulates ROS generation resulting in an amplified ROS signal leading to further mitochondrial dysfunction (3). Thus, the level of MMP and ROS indicate the mitochondrial

function to an extent. Therefore, in the present study, the analyses of MMP and ROS indicated that the mitochondrial function of BV2 cells was preserved by treatment with NBP.

Nrf2 is a transcription factor responsible for reducing oxidative stress (29). In normal conditions, Nrf2 is sequestered in cytoplasm by Keap1, which keeps it in a resting state (30). This explains why the level of Nrf2 in the nucleus did not change when NBP was used alone. Under stressful condition, especially under oxidative stress, Nrf2 separates from Keap1 and transfers to the nucleus where it activates ARE and increases transcription of Nrf2-regulated genes (31). This increases the expression of HO-1 which, in turn, downregulates the level of intracellular ROS (32). Thus, Keap1/Nrf2/ARE signaling pathway is an important target for reducing oxidative stress (33).

The present study investigated whether NBP reduced intracellular ROS levels in BV2 cells via the Keap1/Nrf2/HO-1 signaling pathway. When rotenone was used alone, the level of HO-1 decreased, which may give a false impression. This is because the early rise of ROS consumed the original HO-1 in the cells and the decrease in intracellular expression level of Keap1 and Nrf2 was not statistically significant. This suggests that the pathway may be at the beginning of activation and so the new HO-1 had yet to be produced. The cells treated with rotenone and NBP (Rot+NBP) showed that NBP treatment promoted Nrf2 entry into the nucleus and increased HO-1 expression in rotenone treated BV2 cells. In addition, the data demonstrated that NBP treatment reduced the level of the Nrf2 inhibitory protein, Keap1. The overall mechanism by which NBP inhibited rotenone-induced oxidative stress in microglia is illustrated in Fig. 5.

In conclusion, the present study demonstrated that NBP inhibits rotenone-induced oxidative stress in microglia via Keap1/Nrf2/HO-1 signaling pathway. Therefore, the results supported the notion that NBP treatment could decrease oxidative stress and might have considerable value as a therapeutic agent against PD.

Acknowledgements

Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

RL and LZ were the major contributors in writing the manuscript and designing of the experiments. ZZ and RZ were responsible for designing and conducting experiments. JZ and SX were responsible for cell culturing and sample extractions. LZ and WB confirmed the authenticity of all the raw data. WB played a major role in the data analysis and experimental co-ordination. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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