Protective effects of DL-3-n-butylphthalide in the lipopolysaccharide-induced mouse model of Parkinson's disease

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Abstract. DL-3-n-butylphthalide (NBP) is extracted from rapeseed and exhibits multiple neuroprotective effects, exerted by inhibiting the inflammatory process, including reducing oxidative stress, improving mitochondrial function and reducing neuronal apoptosis. The present study aimed to investigate the neuroprotective effects of NBP in a lipopolysaccharide (LPS)-induced mouse model of Parkinson's disease (PD). The behavior of mice was assessed using the rotarod test and open-field test, the amount of tyrosine hydroxylase in the substantia nigra pars compacta was evaluated by immunohistochemistry, and the levels of phosphorylated c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase 14 (p38) and extracellular signal-regulated kinase 1 were determined by western blotting. It was demonstrated that the LPS-induced behavioral deficits were significantly improved. LPS-induced dopaminergic neurodegeneration was relieved following treatment with NBP, as determined from tyrosine hydroxylase-positive cells. Phosphorylation of JNK and p38 was significantly inhibited following treatment with NBP. Therefore in the present study, a role for NBP has been established in the treatment of a PD murine model, laying the experimental basis for the treatment of PD with this agent.

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Abbreviations: NBP, DL-3-n-butylphthalide; PD, Parkinson's disease; TH, tyrosine hydroxylase; SNpc, substantia nigra pars compacta; MAPK, mitogen-activated protein kinase; LPS, lipopolysaccharide; MPTP, methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDA, 6-hydroxydopamine

Key words: DL-3-n-butylphthalide, lipopolysaccharide, mouse model, Parkinson's disease, tyrosine hydroxylase

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder, which is caused by degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (1). Despite a number of studies, the accurate pathogenesis of PD remains unclear and increasing evidence has demonstrated that activation of the microglia, with the attendant oxidative stress and neuroinflammation, may be crucial events in the pathogenesis of PD (2,3). Additionally, it has been demonstrated that the mitogen-activated protein kinase (MAPK) pathway serves an important role in the pathogenesis of PD (4). Modulating the MAPK pathway represents a promising approach for the prevention and treatment of PD.

In the past few years, several neurotoxic molecules, including 6-hydroxydopamine (6-OHDA) and methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), have been utilized to establish PD models, by causing an inflammatory response; however, it is difficult to confirm whether neuroinflammation is the consequence or cause in injured dopaminergic neurons (5). However, a PD model induced by lipopolysaccharide (LPS), as an effective glial cell activator, has demonstrated a gradual and selective reduction in dopaminergic neurons in the SNpc (6). Several clinical and basic studies have indicated that LPS-induced PD models demonstrate the basic characteristics of PD pathology, including anti-inflammatory actions (similar to nonsteroidal anti-inflammatory drugs, cyclo-oxgenase-2 inhibitors, inducible nitric oxide synthase inhibitors) and anti-oxidative properties, particularly NADPH inhibitors (7-9).

In south China, DL-3n-butylphthalide (NBP) is extracted from rapeseed and was approved for clinical use by the China Food and Drug Administration in 2002 (Beijing, China). NBP has been demonstrated to possess multiple neuroprotective effects exerted by inhibiting the inflammatory process including reducing oxidative stress, improving mitochondrial function and reducing neuronal apoptosis (10,11). NBP also suppresses the generation of peroxynitrite, superoxide and nitric oxide (12). Previously, NBP has been reported to be able to prevent oxidative damage and reduce mitochondrial dysfunction in a 1-methyl-4-phenylpyridinium^{+}-induced cellular model of PD (13).

Therefore, in the present study, the neuroprotective effects of NBP on LPS-induced inflammatory nerve damage in a PD mouse model were examined and the possible mechanisms were investigated.

Materials and methods

Reagents. Anti-c-Jun N-terminal kinase (JNK), MAPK 14 (p38) and extracellular signal-regulated kinase (ERK), phosphorylated (p)-p38, p-JNK, and p-ERK rabbit antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA; cat. nos. 9252, 9212, 4695, 9211, 9251 and 9101, respectively). Anti-β-actin rabbit polyclonal antibody was purchased from Beyotime Institute of Biotechnology (Haimen, China; cat. no. AA128). Protease inhibitor cocktail and extraction buffer [radioimmunoprecipitation assay (RIPA) lysis buffer; cat. no. P0013B] were purchased from the Beyotime Institute of Biotechnology. Enhanced chemiluminescence (ECL) reagents were purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China). LPS was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and NBP was obtained from CSPC Pharmaceutical Co., Ltd. (Shijiazhuang, China).

Animals and treatment. Adult male C57BL/6 mice (n=30) weighing 18-20 g were purchased from the Comparative Medicine Center at Yangzhou University [Yangzhou, China; cat. no. scxk(Su) 2012-004] and housed under pathogen-free conditions with a 12-h light/dark cycle, 60-70% relative humidity and temperature of 20-22˚C, and received food and water *ad libitum* in their cages. All animals were ~10 weeks of age at the start of administration. Prior to the experiment, the animals were allowed 10 days to adapt to the new environment. All mice were randomly separated into 3 groups (n=10/group) as follows: i) The control group, which was treated with saline; ii) the LPS-treatment group, which received a single intraperitoneal injection of LPS at 5 mg/kg to generate the PD model; and iii) the NBP-treatment group, which received gastric perfusions of NBP (120 mg/kg) dissolved in soybean oil once a day for 30 days, following injection with LPS (similar to the LPS-treatment group). All experiments commenced following 7 months of treatment. The present study was approved by the ethics committee of Bengbu Medical College (Bengbu, China).

Rotarod test. The rotarod test is widely used to assess motor and coordination abilities, particularly bradykinesia in mice (14). In the present study, a rotarod (3-cm diameter) was utilized at a fixed speed of 20 rpm. The retention time for each mouse resting on the revolving rod was recorded. The rotarod test was repeated 3 times for each mouse, with 30-min rest periods in between the tests. The retention time of each mouse was determined and used for comparison.

Open-field test. Following treatment, motor behavior was analyzed in an open-field test. The apparatus consisted of a square (40x40 cm) with a surrounding wall (height, 30 cm). The square floor was divided into 16 (4x4) sub-squares using transverse and longitudinal segments. Mice were placed in the center of the structure and then the behavior of the mice was recorded for 5 min. When each mouse repeated the test 3 times, their spontaneous activity was analyzed for 30 min. The line crossings, grooming and rearing of each mouse were recorded and analyzed.

Immunohistochemistry. Following treatment, 3 mice were randomly selected from each group for the subsequent study. Mice were deeply anesthetized with 10% chloral hydrate (3 ml/kg; Sigma-Aldrich) at room temperature and perfused through the ventriculus sinister with 100 ml saline, followed by 4% paraformaldehyde for 20 min. The midbrains were collected and fixed with 4% paraformaldehyde at 4˚C for 24 h. Subsequently, the midbrains were embedded in paraffin and cut (35-μm-thick) and processed for immunohistochemistry. The sections were blocked with 5% bovine serum albumin (cat. no. P0007; Beyotime Institute of Biotechnology) dissolved in PBS at room temperature for 1 h.

The sections were incubated with primary monoclonal antibodies at 37˚C overnight [rabbit anti-tyrosine hydroxylase (TH); 1:1,000; cat. no. 2791; Cell Signaling Technology, Inc.]. Then, the sections were rinsed with PBS, incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 50 min (goat anti-rabbit Immunoglobulin G, 1:800 (cat. no. GB23303; Goodbio Technology Co., Ltd., Wuhan, China). Sections were subsequently incubated for 30 min in peroxidase substrate mixing liquid and then washed and detected with a DAB staining kit (cat. no. K5007; Dako; Agilent Technologies, Santa Clara, CA, USA) at room temperature for 3-10 min, terminating the coloration when brown granules (positive cells) were observed.

Finally, the sections were counterstained with hematoxylin at room temperature for 3 min, dehydrated with deionized water, dried and sealed. The immunopositive cells in the stained sections were analyzed under an optical microscope (Nikon Corporation, Tokyo, Japan). The number of TH-positive cells (magnification, x40) in every sixth section was counted by two researchers blinded to the treatment.

Western blot analysis. RIPA lysis buffer was used to extract proteins from the ventral mesencephalon. Tissues were lyzed in RIPA buffer supplemented with protease inhibitor cocktail. Then, the lysates were centrifuged (8,000 x g for 10 min at 4˚C) and the protein concentration in the extracts was determined using the Bradford assay.

Equal amounts (30 μg) of protein samples were electrophoresed on 12% denaturing polyacrylamide gels and then transferred onto nitrocellulose membranes (EMD Millipore, Billerica, MD, USA) using a semi-dry blotting apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 2 h at 4˚C. The membrane was then blocked with 5% bovine serum albumin (cat. no. P0007; Beyotime Institute of Biotechnology) dissolved in TBS containing Tween [TBST; 10 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% Tween-20] at room temperature for 1 h. Following washing with TBST buffer 3 times, membranes were incubated with primary antibodies against JNK, ERK, p38, p-JNK, p-ERK and p-p38 (all 1:1,000) at 4˚C overnight. Then, the membranes were washed 3 times with TBST for 10 min and incubated with the HRP-conjugated secondary antibodies (cat. no. GB23303; Goodbio Technology Co., Ltd.) at room temperature for 1 h (1:5,000). An anti-β-actin antibody (1:1,000) was used as an internal control.

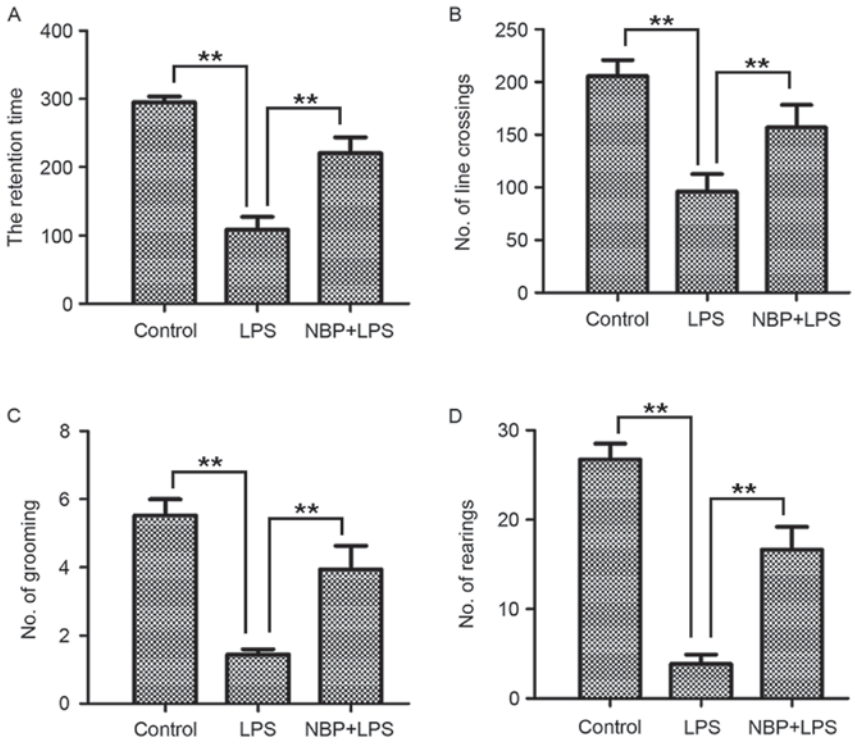

Figure 1. NBP attenuates behavioral impairment in intranasal LPS-induced Parkinson's disease model mice, as assessed by the rotarod test and open field test. (A) Retention times during which each mouse rested on the revolving rod in the control group, LPS-treatment group and NBP-treatment group. Number of (B) line crossing, (C) grooming and (D) rearing actions of each mouse, in the control group, LPS-treatment group and NBP-treatment group. Data are representative of three independent experiments. The values are expressed as the mean ± standard deviation. **P<0.01. NBP, DL-3-n-butylphthalide; PD, Parkinson's disease; LPS, lipopolysaccharide.

Band intensities were detected with ECL reagents (cat. no. KGP1123; Nanjing KeyGen Biotech Co., Ltd.) and measured with Quantity one Software version 25.0.0 (Bio-Rad Laboratories, Inc.).

Statistical analysis. All values were expressed as the mean ± standard deviation. Results were analyzed using one-way analysis of variance followed by Tukey post-hoc tests using SPSS software version 18.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

NBP attenuates behavioral impairment in intraperitoneal LPS-induced PD mice. To investigate whether NBP protects mice from LPS-induced neurotoxicity, the behavioral tests were conducted, including the rotarod and the open-field test.

Rotarod test results demonstrated that the retention time of the control group was 298.61±2.94 sec (Fig. 1A). Compared with the control group, the LPS-treatment group (103.78±46.01 sec) had a significantly reduced retention time (P<0.01). However, the NBP-treatment group, who demonstrated marked improvement in retention time (Fig. 1A), had significantly enhanced retention times (221.83±53.88 sec) compared with mice in the group treated with LPS (P<0.01).

As presented in Fig. 1B-D, the open field test, which assessed the degree of locomotory capacity demonstrated that the number of line crossing, rearing and grooming actions in the control group was 205.56±26.82, 27.06±3.59 and 5.50±0.84, respectively. In the LPS-treatment group, the line crossing (96.00±28.65), rearing (3.83±1.87) and grooming (1.44±0.27) activities were decreased significantly (all P<0.01; Fig. 1B-D). In the NBP treatment group, the line crossing (156.67±37.23), rearing (16.78±4.64) and grooming (3.94±1.22) behaviors were significantly increased compared with the LPS-treatment group (all P<0.01; Fig. 1B-D). Therefore, the mice treated with NBP demonstrated a significant improvement in behavioral performance in the open field test compared with the LPS-treatment group.

These results demonstrated that NBP could protect mice from LPS-induced behavioral impairment.

NBP inhibits LPS-induced dopaminergic neuronal loss. TH-positive cells are an important pathological indicator for assessing whether a drug exhibits protective effects on dopaminergic neurons (15). Immunoblotting was also used to analyze the number of TH-positive cells in the SNpc (Fig. 2). In the LPS-treatment group, the number of TH-positive cells in the SNpc (122.33±30.29) was significantly reduced compared with the control group (242.33±19.14; P<0.01). Compared with the LPS-treatment group, the number of TH-positive cells in the NBP treatment group (191.67±23.12) were significantly increased (P<0.01), indicating that NBP could inhibit the loss of TH.

NBP inhibits LPS-induced inflammatory signaling pathways involving JNK and p38. A previous study demonstrated that the MAPK signaling pathway serves an important role in the pathogenesis of PD (4). To evaluate whether the MAPK

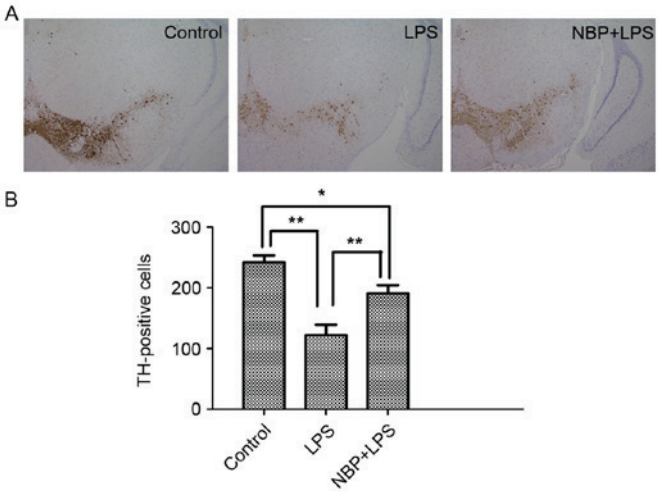

Figure 2. NBP inhibits LPS-induced dopaminergic neuronal loss. TH was detected by immunohistochemical staining in the SNpc of Parkinson's disease model mice. (A) TH-positive cells distribution in the control group, LPS-treatment group and NBP-treatment group. Magnification, x100. (B) Quantification of TH-positive cells in the SNpc for each group. Data are representative of three independent experiments. The values are expressed as mean ± standard deviation *P<0.05, **P<0.01. TH, tyrosine hydroxylase; SNpc, substantia nigra pars compacta; NBP, DL-3-n-butylphthalide; LPS, lipopolysaccharide; PD, Parkinson's disease.

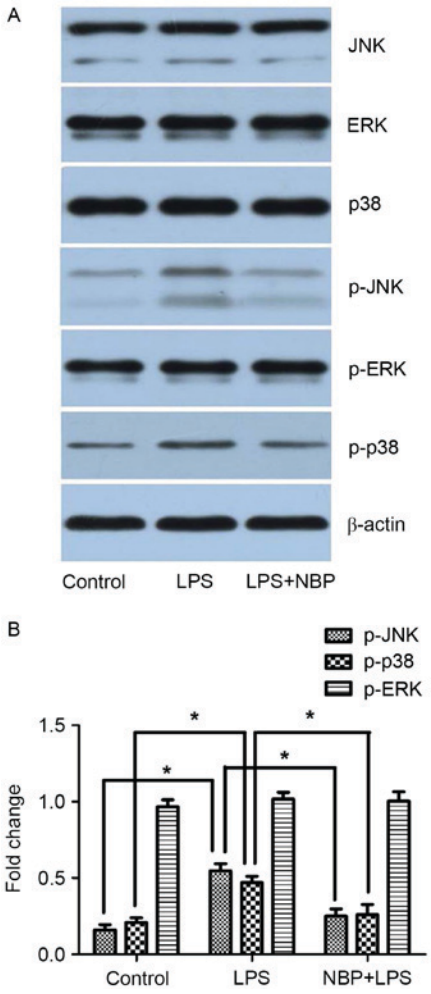

Figure 3. NBP inhibits LPS-induced inflammatory signaling pathways involving JNK and p38. (A) Levels of p-JNK, p38 p-p38 and p-ERK were determined by western blotting. β-actin was used as an internal control. (B) Band intensities of p-JNK, p-p38 and p-ERK were quantified using densitometry. The values are expressed as mean ± standard deviation. *P<0.05. Data are representative of three independent experiments. p-, phosphorylated; JNK, c-Jun N-terminal kinase; p38, mitogen-activated protein kinase 14; ERK, extracellular signal-regulated kinase; NBP, DL-3-n-butylphthalide; LPS, lipopolysaccharide.

pathway is involved in the protective effect exerted by NBP, the alterations in JNK, p38 and ERK in mice treated with LPS and NBP were investigated.

As demonstrated in Fig. 3, the levels of p-JNK, p-p38 and p-ERK in the control group were 0.23±0.08, 0.28±0.08 and 0.99±0.24, respectively. The levels of p-JNK, p-p38 and p-ERK in the LPS-treatment group were 0.56±0.13, 0.52±0.17, and 1.0±0.27, respectively, which indicated phosphorylation of JNK, p38, and ERK, although no significant difference was observed with ERK. The levels of p-JNK, p-p38 and p-ERK in the NBP-treatment group were 0.31±0.09, 0.34±0.11, and 0.85±0.21, respectively. Compared with the LPS-treatment group, treatment with NBP significantly inhibited phosphorylation of JNK and p38 (P<0.05), but had little effect on ERK. Compared with the control group, treatment with NBP had no significant effect on the phosphorylation of JNK and p38 (P>0.05).

Therefore, these results indicated that inhibition of the JNK and p38 pathway is involved in the protective effect of NBP against LPS-induced neurotoxicity.

Discussion

PD is a common age-associated neurodegenerative disorder, characterized by dopaminergic neuron loss in the SNpc. In the later stages of human PD, there are clear signs of microglial activation and inflammation that may contribute to the progression of the disease (16,17).

The LPS model has been widely used in studies of the biochemical, neurochemical and inflammatory markers, and functional behaviors (18). It has previously been reported that administration of LPS induced symptoms of PD, dopaminergic neuron death and microglial activation (19,20).

NBP has been reported to possess a range of pharmacological effects. It has been demonstrated that NBP protects the brain from ischemia, inhibits platelet aggregation, reduces the area of cerebral infarcts and decreases neuronal apoptosis and oxidative brain damage (10, 21-26). Additionally, NBP has also been demonstrated to serve a potential therapeutic role in Alzheimer's and can decrease the cytotoxicity of MPTP in a cellular model of PD (27).

Therefore, in the present study, a purely inflammation-driven (LPS-induced) mouse model of PD was employed. It was also examined whether NBP can suppress inflammatory nerve damage induced by LPS in a mouse model and further investigated the possible mechanisms.

Behavioral tests, including the open-field test and the rotarod test, can be used to observe movement disorders in the mouse. TH is the restricting enzyme in dopamine synthesis; immunohistochemical analysis of TH allows assessment of the number and function of dopaminergic neurons in the SN (28). Behavioral tests are crucial to evaluate whether a drug exhibits protective effects on dopaminergic neurons, while the number of TH-positive cells is an important pathological indicator. In the present study, the mice treated with LPS exhibited the basic characteristics of PD.

The mice exhibited movement disorder. The retention times of mice treated with LPS was markedly reduced and the numbers of line crossing, rearing, and grooming actions of mice treated with LPS were decreased. These mice demonstrated a decrease in TH-positive cells. However, the group that received NBP treatment demonstrated an improvement in behavior and increase in TH-positive cells. These results suggested that NBP markedly improved behavior performance and pathological indicator test results in the LPS-induced mouse model of PD, but the exact underlying mechanism remains unclear.

The MAPK superfamily comprises 3 major branches, including JNK, p38 and ERK (29). These subfamilies serve key roles in a variety of cellular responses, including immunity, apoptosis, autophagy, cell survival, cell growth and cell division (29, 30-32).

JNK is markedly activated in models of PD induced by LPS, 6-OHDA and MPTP (33,34). A previous study demonstrated that activation of the JNK pathway is associated with neural cell apoptosis in models of PD (34). The inhibitor of JNK, SR-3306, can reduce the loss of neurons in a rat model of PD, suggesting that JNK inhibitors possess a protective effect on neurons in PD (35). p38 is also activated in neurotoxic PD models (36). Furthermore, inhibitors of p38 are effective in the treatment of PD (4). ERK is involved in regulating cell proliferation, development and apoptosis. In a cell model of PD, chronic treatment with MPTP can decrease mitochondrial oxidative phosphorylation in dopaminergic neural cells, and inhibiting ERK activation by adding the inhibitor U0126 reversed the mitochondrial morphological alterations and the loss of mitochondrial proteins observed in cells chronically stressed with MPTP (37). A study has also revealed that the ERK signaling pathway may be an important target for the treatment of PD (38).

In the present study, the expression of p-JNK, p-p38 and p-ERK in mice treated with LPS and NBP was observed. The results of the present study revealed that mice treated with NBP demonstrated inhibited LPS-induced phosphorylation of JNK and p38, but had no effect on ERK. These results indicated that neuroprotection mediated by NBP involves the JNK and p38 pathways.

In conclusion, the present study demonstrated that NBP may serve a valuable neuroprotective role, due to its ability to regulate MAPK pathways in models of PD. These results on the effect of NBP may lay the basis for the development of novel clinical drugs for treating PD.

Acknowledgements

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