Neuroscience Letters 475 (2010) 89–94

Contents lists available at ScienceDirect

Neuroscience Letters

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

DL-3-n-Butylphthalide prevents oxidative damage and reduces mitochondrial dysfunction in an MPP+-induced cellular model of Parkinson's disease

Jin-zhong Huang[a,c,1], Ying-zhu Chen[c,1], Min Su[a], Hui-fen Zheng[a], Ya-ping Yang[a,b], Jing Chen[a], Chun-Feng Liu[a,b,*]

[a] Department of Neurology, Second Affiliated Hospital of Soochow University, Suzhou 215004, China
[b] Laboratory of Aging and Nervous Diseases, Institute of Neuroscience, Soochow University, Suzhou 215004, China
[c] First Clinical Medical School of Yangzhou University, Yangzhou 225001, China

ARTICLE INFO

Article history:
Received 11 January 2010
Received in revised form 3 March 2010
Accepted 22 March 2010

Keywords:
DL-3-n-Butylphthalide
Parkinson's disease
Oxidative stress
Mitochondrial membrane potential

ABSTRACT

The aim of the present study was to explore the neuroprotective effects and mechanisms of action of DL-3-n-butylphthalide (NBP) in a 1-methyl-4-phenylpyridiniumion (MPP^+)-induced cellular model of Parkinson's disease (PD). NBP was extracted from seeds of *Apium graveolens* Linn. (Chinese celery). MPP^+ treatment of PC12 cells caused reduced viability, formation of reactive oxygen, and disruption of mitochondrial membrane potential. Our results indicated that NBP reduced the cytotoxicity of MPP^+ by suppressing the mitochondrial permeability transition, reducing oxidative stress, and increasing the cellular GSH content. NBP also reduced the accumulation of α-synuclein, the main component of Lewy bodies. Given that NBP is safe and currently used in clinical trials for stroke patients, NBP will likely be a promising chemical for the treatment of PD.

Parkinson's disease (PD) is a neurodegenerative disease characterized by the progressive loss of dopaminergic neurons in the substantia nigra. Recent studies have implicated mitochondrial dysfunction and increased oxidative stress in the dopaminergic cell degeneration, although the etiological factors remain unclear [15]. Oxidative and nitrative stresses produced by mitochondrial dysfunction are regarded as important causes of protein accumulation in the pathogenic process [21]. Increased oxidative radicals result in covalent modification of α-synuclein (α-syn), leading to conformational changes and accumulation of the protein [1], a critical step in the development of PD [13,18]. Oxidative stress such as NO and its free radical metabolites also inhibit the function of the mitochondrial respiratory chain [1]. At the same time, overproduction of aggregated α-syn causes mitochondrial dysfunction and increased levels of reactive oxygen species (ROS) [14], which results in positive feedback during PD pathology. Currently, most PD therapeutic studies focus on preventing or blocking this vicious cycle.

DL-*3n-butylphthalide* (NBP) was extracted from the seeds of *Apium graveolens* Linn. (Chinese celery). NBP has been synthesized and received approval by the State Food and Drug Administration (SFDA) of China for clinical use in stroke patients in 2002. It has been shown to have multiple neuroprotective effects by reducing oxidative stress [8], improving mitochondrial function [19], blocking inflammatory reactions, and reducing neuronal apoptosis [5]. NBP also suppresses the production of peroxynitrite, superoxide, and nitric oxide [19]. Therefore, in the present study, we examined the neuroprotective effects of NBP on MPP^+-induced toxicity in PC12 cells, a cellular model of PD.

NBP (generously provided by Shijiazhuang Pharmaceutical Group NBP Pharma Co., Ltd.) was dissolved in dimethyl sulfoxide (DMSO). MPP^+, vitamin E, and H_2O_2 were from Sigma–Aldrich Co. (St Louis, MO, USA). Anti-syn and actin antibodies were from Santa Cruz Biotechnology, CA.

PC12 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated horse serum (Gibco, Langley, OK, USA). Cells were seeded in 6- or 96-well plates and grown to a confluence of 60–70%. Cells were then treated with 1 mM MPP^+ for 24 h followed by different concentrations of NBP (0.01, 0.1, 1.0, 10 or 100 μM) for 4 h.

Cell viability was assessed using the 3-[4,5-dimethylthiazolo-2]-2,5diphenyltetrazolium bromide (MTT) assay [23]. After treatment with MPP^+, H_2O_2, vitamin E, and the different concentrations of NBP, cultured cells were incubated with 10 μl of 10 mg/ml MTT solution for 2 h at 37 °C and centrifuged at 400 × *g* for 10 min. DMSO (100 μl) was added to each well to dissolve the formazan and the absorbance was measured at 570 nm. PC12 cells without treatment were considered as controls, and treated groups were expressed as a percentage of control.

* Corresponding author at: Department of Neurology, Second Affiliated Hospital of Soochow University, 1055 Sanxiang Road, Suzhou 215004, China.
Tel.: +86 512 67783307; fax: +86 512 68284303.
E-mail address: liucf@suda.edu.cn (C.-F. Liu).
[1] These authors contributed equally to this work.

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doi:10.1016/j.neulet.2010.03.053

The total glutathione (GSH) and oxidative glutathione (GSSG) were determined by colorimetric microplate assay kits (Beyotime Institute of Biotechnology, China). After treatment, PC12 cells were collected and centrifuged at 10,000 × *g* for 10 min at 4 °C. The cells were re-suspended in 20 μl cell medium. Ten microliters of cells was mixed with 30 μl 5% metaphosphoric acid, then frozen and thawed twice using liquid nitrogen and 37 °C water. The samples were centrifuged and the supernatant was used for GSH and GSSG assays. The total GSH level was measured by the DTNB-GSSG recycling assay [2]. The GSSG level was quantified by the same method as for total GSH after the supernatant was treated with 1 mol/L 2-vinylpyridine solution to remove the reduced GSH. The amount of reduced GSH was obtained by subtracting the amount of GSSG from that of total GSH.

Production of the nitric oxide derivative nitrite was determined with a nitrite detection kit (Beyotime Institute of Biotechnology, China). Briefly, PC12 cells were treated with MPP^+ and different concentrations of NBP. After 24 h incubation, 100 μl of cell medium or $NaNO_2$ standard samples were mixed with 100 μl of Griess reagent. After 15 min incubation at room temperature, optical density was read by a microplate reader at 540 nm. The standard curve produced by $NaNO_2$ was generated for quantification.

Intracellular ROS was measured using the non-fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA). PC12 cell were plated at a density of 1×10^5/well in 96-well plates. One day after plating, the cells were treated with MPP^+ for 24 h and NBP (0, 0.1, 1.0, 10 or 100 μM) for 4 h. DCFH-DA, diluted to a final concentration of 10 μM with DMEM/F12, was added to the cultures and incubated for 20 min at 37 °C. After DCFH-DA treatment, the PC12 cells were washed with cold PBS, collected, and subjected to flow cytometry (Becton Dickinson FACSCalibur) for ROS assay. The fluorescence was expressed as a percentage of total area.

The JC-1 probe was used to measure mitochondrial depolarization in PC12 cells as described previously [6]. Briefly, PC12 cells were treated with MPP^+ alone or with NBP, and then were incubated with an equal volume of JC-1 staining solution (5 μg/ml) at 37 °C for 20 min and rinsed twice with PBS. Mitochondrial membrane potential was monitored with a flow cytometer under Argon-ion 488 nm laser excitation. The ratio of green/red fluorescence intensity was calculated as an indicator of the loss of mitochondrial transmembrane potential.

Cells were harvested in Mammalian Protein Extraction Reagent buffer (Pierce, IL) containing 1× protease inhibitor cocktail (Pierce, IL), NaF (5 mmol/L), and Na_3VO_4 (200 μmol/L). Lysates were centrifuged at 4 °C for 2 min at 14,000 × *g* and resolved on 12% gels. Proteins were transferred to nitrocellulose membranes (Bio-Rad, CA). The membrane blots were blocked with 5% milk in TBST (50 mmol/L Tris, pH 7.6, 150 mmol/L NaCl, 0.1% Tween 20) and incubated with anti-α-syn or anti-actin antibody overnight at 4 °C. Immunolabeling was detected using the TMB Stabilized substrate for HRP (Promega). Membranes probed with antibodies and actin was used as loading controls. The images were captured and densitometric analysis was performed using Image J software (NIH, Bethesda, MD, USA).

Cells were seeded on coverslips placed in 24-well dishes. After treatment with MPP^+ for 24 h and/or NBP for 4 h, cells were fixed in 4% paraformaldehyde for 15 min, and then permeabilized with 0.1% Triton-X-100 (Sigma) for 15 min. Anti-α-syn antibody was incubated with the cells for 2 h at room temperature and Cy3-conjugated anti-goat antibodies (1:500; Beyotime Institute

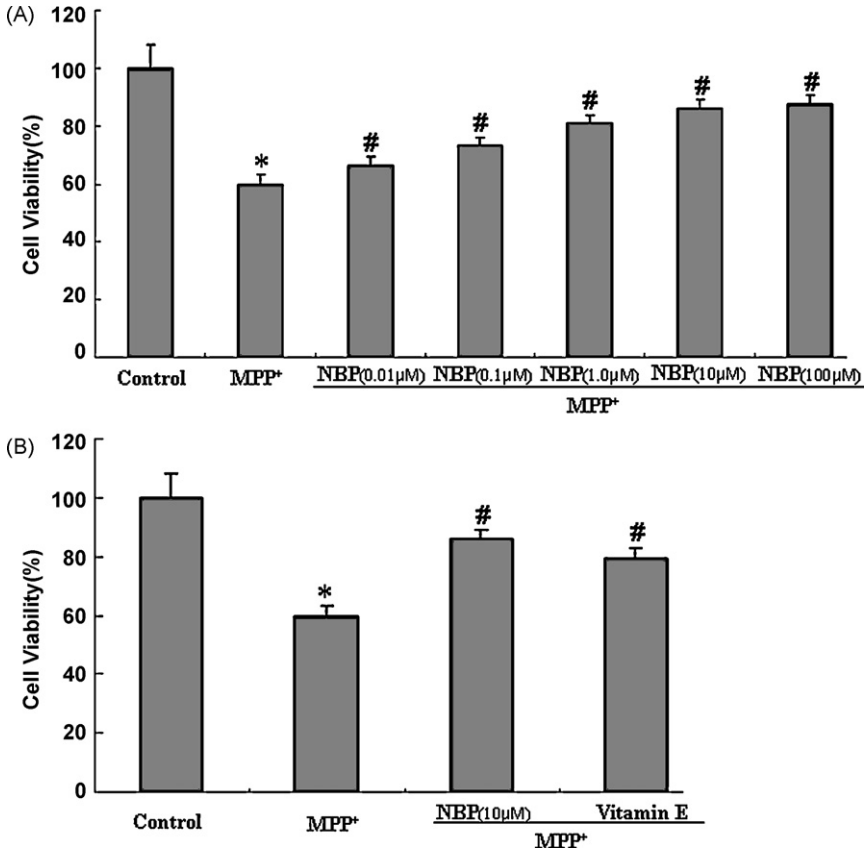

Fig. 1. Protective effect of NBP on cytotoxicity in PC12 cells induced by MPP^+. (A) PC12 cells were treated with NBP (0.01, 0.1, 1.0, 10, or 100 μM) for 4 h following 24 h of 1 mM MPP^+, and cell viability was determined by MTT assay. NBP significantly reduced MPP^+-induced cell death in a dose-dependent manner. (B) PC12 cells treated with 1 mM MPP^+ for 24 h and NBP (10 μM) or vitamin E (50 μM) for 4 h. Viability of PC12 cells was determined by MTT assay. $^*P < 0.05$ versus control; $^\#P < 0.05$ versus MPP^+ alone; $n = 4$ in each group.

of Biotechnology) were used for 1 h. Finally, after the cells were incubated with DAPI (3 μg/ml, Sigma) for 10 min, the slides were mounted in Citifluor (Citifluor Ltd.). The cells were visualized under a Leica TCS ST2 confocal microscope.

All data are expressed as mean ± SD. The significance of intergroup differences was evaluated by one-way analysis of variance (ANOVA: Duncan's test for post hoc comparisons). A probability less than 0.05 was considered statistically significant.

PC12 cells treated with 1 mM MPP^+ for 24 h, compared with the control group, showed a survival rate of 60 ± 3.2%. When the cells were treated with 1 mM MPP^+ for 24 h and then treated with different concentrations (0.01, 0.1, 1.0, 10 and 100 μM) of NBP for 4 h, the survival rates were 66.2 ± 3.1%, 73.2 ± 3.0%, 81.2 ± 2.7%, 86.1 ± 2.9% and 87.4 ± 3.2%, respectively. The protective effects of NBP on MPP^+-induced cell death displayed dose-dependence (Fig. 1A). Compared to the group treated with MPP^+ alone, NBP (0.01 μmol/L) reduced MPP^+-induced cytotoxicity in PC12 cells ($P = 0.04$, Fig. 1A). At the doses of 0.1 and 10 μmol/L, NBP prevented MPP^+-induced cytotoxicity ($P < 0.01$, Fig. 1A). However, the protection by NBP at 100 μmol/L was not significantly different from that by 10 μmol/L ($P = 0.31$, Fig. 1A), suggesting that 10 μmol/L NBP is the maximal protective dose. Therefore, we chose three concentrations (0.1, 1.0, and 10 μM) for the subsequent tests. Because previous studies indicated that NBP is an antioxidant, we compared the protective effect of NBP with that of vitamin E, a known antioxidant, on MPP^+-induced cell death. Our results demonstrated that 10 μM NBP and 50 μM vitamin E had similar protective effects ($P > 0.05$, Fig. 1B).

Reduction of cellular GSH increases the sensitivity of neurons to toxic insults and mitochondrial disfunction [12]. Therefore, we determined whether the protective effect of NBP on MPP^+-toxicity was related to the inhibitory effect on GSH depletion. The GSH content in control PC12 cells was 5.34 ± 0.06 nmol/mg protein. Treatment with 1 mM MPP^+ for 24 h reduced GSH content by 53% compared with the control group. This depletion was inhibited by the addition of NBP ($P < 0.05$, Fig. 2A). In addition, the GSH content in cells treated with 10 μM of NBP alone was not significantly different from control cells (data not shown), suggesting that NBP does not increase GSH synthesis in physiological conditions.

Excess NO contributes to the formation of free radicals that are involved in the damage to dopaminergic neurons and leads to the development of PD symptoms [7,20]. In PC12 cells, MPP^+ treatment increased the NO level, while NBP blocked the increase of NO induced by MPP^+. These data demonstrated that NBP protects a cellular model of PD though inhibition of NO stress (Fig. 2B).

In addition, NBP reduced MPP^+-induced accumulation of ROS in PC12 cells measured by flow cytometry. A robust production of ROS was found in the MPP^+ group, but NBP inhibited the MPP^+-induced increase of ROS at all three concentrations ($P < 0.01$, Fig. 3A).

To further confirm the anti-oxidative effects of NBP, we treated PC12 cells with H_2O_2 (200 μM) for 16 h and NBP (1.0 μM) for 4 h and measured the cell viability. H_2O_2 caused cell death and decreased cell viability; however, NBP treatment significantly reduced the impaired cell viability induced by H_2O_2, suggesting that NBP is an effective antioxidant in H_2O_2-induced toxicity ($P < 0.01$, Fig. 3C).

We assessed the effect of MPP^+ on the mitochondrial transmembrane potential. PC12 cells treated with 1 mM MPP^+ showed decreased red fluorescence intensity and increased green fluorescence intensity, which indicated a decrease in mitochondrial transmembrane potential. NBP reduced this MPP^+-induced decrease of mitochondrial transmembrane potential ($P < 0.01$, Fig. 3B).

Oxidation is associated with α-syn accumulation; after treatment with MPP^+ (1 mM) for 24 h, an increased signal of α-syn accumulation at 17 kDa was found in PC12 cells. Different concentrations of NBP (0.1, 1.0, and 10 μM) decreased the α-syn

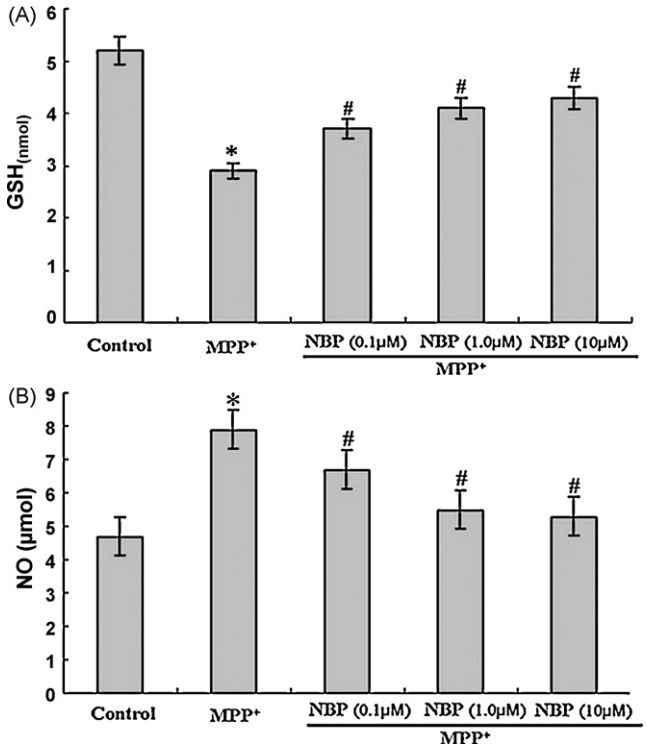

Fig. 2. Effects of NBP on GSH and NO content. After treatment with 1 mM MPP^+ and different concentrations of NBP, GSH content was determined. GSH depletion was significantly inhibited by NBP (A). PC12 cells were treated with MPP^+ and different concentrations of NBP and then NO production was measured. The data show that NBP protects a cellular model of PD though inhibition of NO stress (B). $^*P < 0.05$ versus control; $^\#P < 0.05$ versus MPP^+ alone; $n = 4$ in each group.

accumulation compared with the MPP^+ group ($P < 0.05$, Fig. 4A). Quantification of the α-syn level confirmed its reduction by NBP treatment ($P < 0.05$, Fig. 4B). We also detected cellular α-syn accumulation in PC12 cells by fluorescent immunostaining. PC12 cells were treated with MPP^+ or/and NBP (1.0 μM) and stained with anti-α-syn antibody. MPP^+ treatment resulted in shrunken and dead cells, and increased α-syn accumulation. NBP reversed the morphological changes and reduced the cellular α-syn level (Fig. 4C).

Previous studies have shown that NBP has neuroprotective effects in stroke patients, and it was approved for clinical use in stroke patients by the State Food and Drug Administration of China in 2002. This is the first study to demonstrate that NBP has significant protective effects in a cellular model of PD by reducing oxidative stress, increasing mitochondrial transmembrane potential, and raising GSH levels.

MPP^+ is the active metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridin (MPTP) and has been widely used as a dopaminergic neurotoxin because it causes a severe PD-like syndrome with loss of dopaminergic cells in humans and rodents [22]. It is proposed that MPP^+ causes mitochondrial dysfunction, oxidative stress, energy failure, and activation of genetic programs leading to cell death [24]. PC12 cells have been widely used to generate cell models of PD induced by MPP^+ to explore the potential molecular mechanisms underlying PD because this cell line has many of the features of mid-brain dopamine neurons, such as synthesizing and storing dopamine and norepinephrine [3].

MPP^+ caused cell death in PC12 cells (Fig. 1) and this was significantly reduced by NBP. At the same time, our results showed that MPP^+-induced cell loss by decreasing mitochondrial membrane potential (Fig. 4). Previous studies have indicated that MPP^+ also causes the release of cytochrome c into the cytosol, which leads to the activation of caspase-3 and the execution of apoptosis [17].

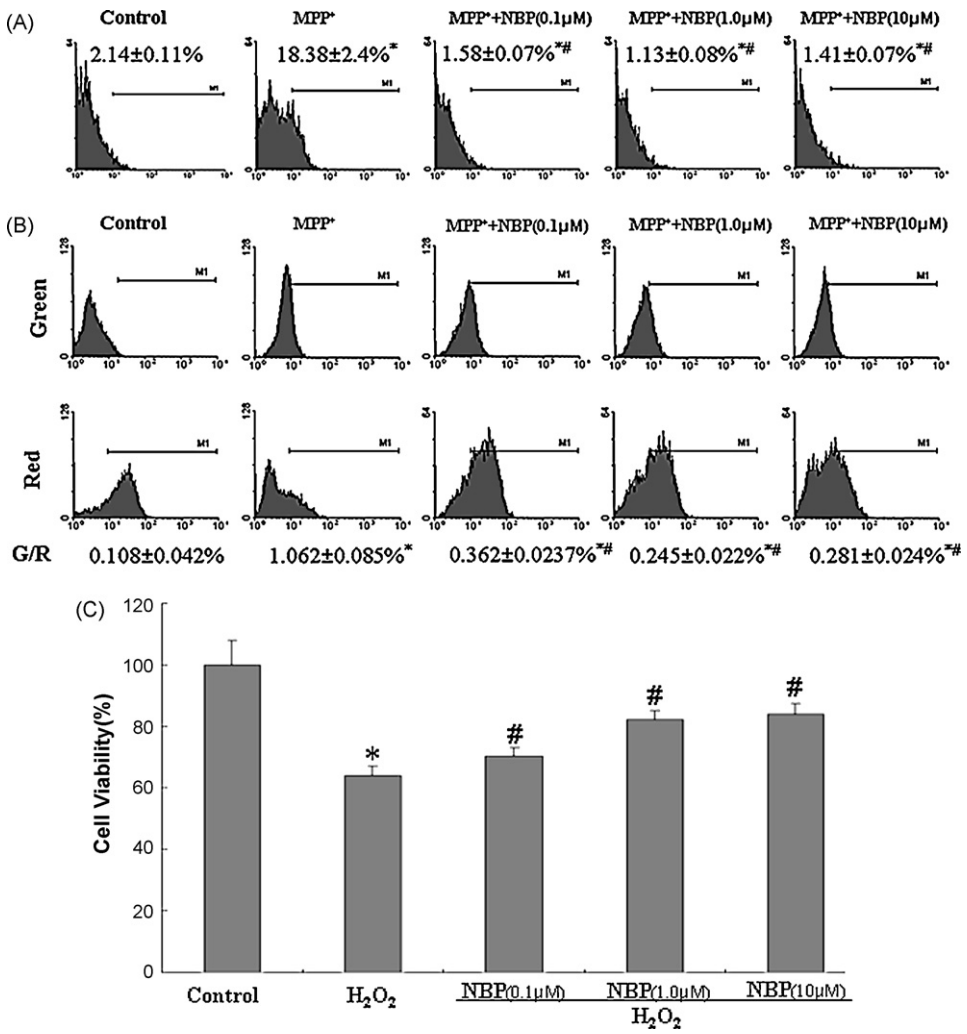

Fig. 3. Effects of NBP on ROS and the mitochondrial transmembrane potential in PC12 cells treated with MPP^+. (A) After cells were exposed to 1 mM MPP^+ alone for 24 h and/or various concentrations of NBP (0.1, 1.0, or 10 μM) for 4 h, the fluorescence intensity of DCF was measured by flow cytometry. (B) Effect of NBP on PC12 cells treated with 1 mM MPP^+, NBP, and JC-1; red (R) and green (G) fluorescence intensity were quantified by flow cytometry. The ratio of green/red fluorescence intensity (G/R) was calculated and plotted. $^*P < 0.05$ versus control; $^{\#}P < 0.05$ versus MPP^+ alone; $n = 4$ in each group. (C) Viability of PC12 cells treated with H_2O_2 (200 μM) for 16 h and NBP (10 μM) for 4 h.

This suggested that MPP^+ can cause cell toxicity through multiple pathways. After NBP treatment, MPP^+-induced cell death was prevented and mitochondrial membrane potential was increased significantly in the dose range of 0.1–10 μM. The anti-oxidative activity of 10 μM NBP had same protection as 50 μM of the known antioxidant vitamin E. These data demonstrated the highly potent protective effects of NBP on MPP^+-induced toxicity in PC12 cells.

Glutathione, the major antioxidant within cells that acts as a free radical scavenger is markedly decreased in the substantia nigra of PD patients [9]. Treatment with 1 mM MPP^+ for 24 h depleted the GSH content by 53%, and this depletion was significantly inhibited by NBP. NBP treatment alone did not increase cellular GSH levels. The increase of cellular GSH by NBP in MPP^+ treated cells may have been due to the inhibition of free radical production.

Generation of ROS is an inevitable outcome of oxygen-dependent respiration. However, when the production of free radicals increases abnormally or the defense mechanisms such as decreased GSH are damaged, free radicals result in lipid peroxidation and cellular dysfunction by attacking the polyunsaturated sites in biological membranes [26]. NO has emerged as a key endogenous modulator of neuronal function and is recognized as a signaling molecule for vasodilation and neurotransmission [4], but it can react with other ROS to form the highly toxic peroxynitrite. Peroxynitrite can then convert to highly toxic intermediates, such as nitrogen dioxide, carbonate, and hydroxyl radicals [4,25]. The excess NO may contribute to the formation of free radicals that are involved in the damage of dopaminergic neurons, leading to the development of PD symptoms. It is known that mice null for iNOS are resistant to neurodegeneration caused by MPTP, and iNOS inhibitors protect against the damage of dopaminergic neurons in MPTP-treated mice [7,20].

In our study, MPP^+ caused mitochondrial dysfunction (decreased mitochondrial transmembrane potential) and resulted in increased production of ROS and NO. Oxidative stress destroyed the defense mechanisms such as depleted cellular GSH after MPP^+ treatment. NBP inhibited the production of ROS and the decrease of mitochondrial transmembrane potential induced by MPP^+. The protection by NBP in H_2O_2-induced toxicity further supported the hypothesis that NBP is an antioxidant (Fig. 3C).

Lewy bodies and Lewy neurites are major pathological hallmarks of PD. Importantly, α-syn is the major component of these proteinaceous inclusions. ROS are known to cause protein misfolding and accumulation [11]; early studies showed that oxidative damage to proteins forms toxic aggregates and ultimately induces

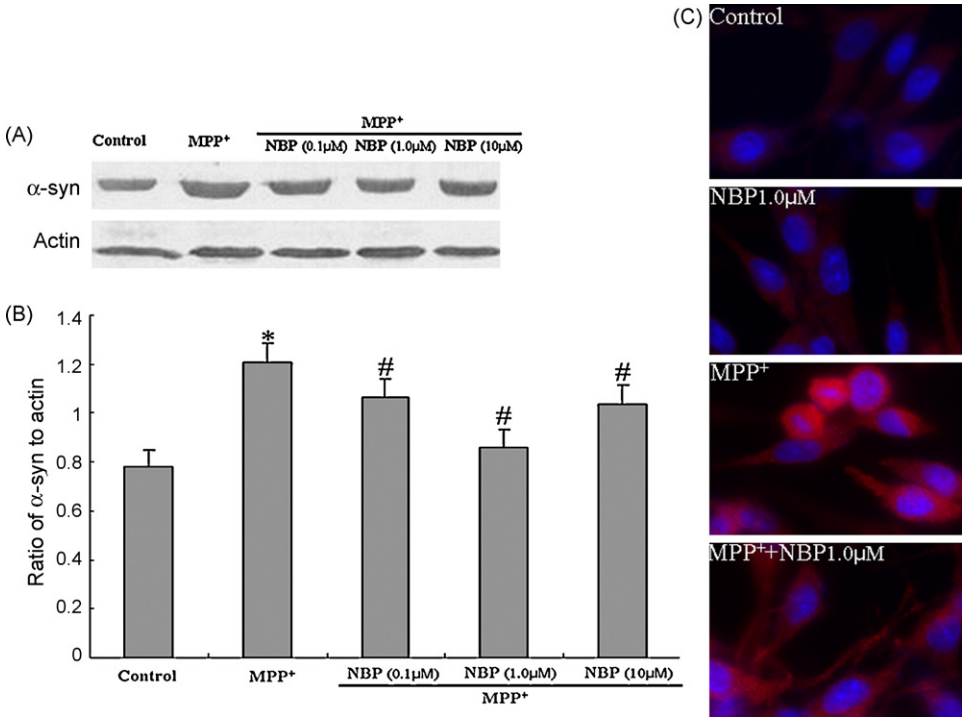

Fig. 4. Effect of NBP on α-syn expression after MPP^+ treatment. PC12 cells were treated with MPP^+ alone (1 mM) for 24 h and NBP for 4 h; the α-syn levels were determined by Western blotting analysis. (A) Representative gels of α-syn levels. (B) Densitometry of α-syn bands was analyzed. Actin was used as loading control. $^*P < 0.05$ versus control; $^\#P < 0.05$ versus MPP^+ alone; $n = 4$ in each group. (C) Micrographs of PC12 cells treated with MPP^+ or/and NBP (1.0 μM) and stained with anti-α-syn antibody, followed by Cy3-labeled secondary antibodies and DAPI (200×).

neurodegeneration in PD [16]. Antioxidants efficiently decrease α-syn oligomer content and prevent protein carbonylate formation [10], suggesting that oxidative stress is involved in the mechanisms underlying α-syn accumulation in the pathogenic process.

In the present study, we found that MPP^+ toxicity induced the production of ROS and NO and caused a significant increase in α-syn accumulation. NBP treatment reduced the production of ROS and NO, and also decreased the α-syn accumulation, as demonstrated by western blot analysis and immunostaining. Our results showed that NBP ameliorated MPP^+-induced reactive oxygen species production, attenuated the mitochondrial transmembrane potential loss, prevented the depletion of GSH, and reduced the accumulation of α-syn. NBP affords significant neuroprotection against MPP^+-induced injury in PC12 cells. These findings support the theory that NBP-mediated cytoprotection in a cellular model of PD is due to inhibition of oxidative stress.

Acknowledgements

This work was supported by the Suzhou Social Progress Foundation (SS0701), and the Jiangsu Province Foundation of China (07KJB320109).

References

[1] A. Abeliovich, Y. Schmitz, I. Farinas, D. Choi-Lundberg, W.H. Ho, P.E. Castillo, N. Shinsky, J.M. Verdugo, M. Armanini, A. Ryan, M. Hynes, H. Phillips, D. Sulzer, A. Rosenthal, Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system, Neuron 25 (2000) 239–252.
[2] M.A. Baker, G.J. Cerniglia, A. Zaman, Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples, Anal. Biochem. 190 (1990) 360–365.
[3] M.F. Beal, Experimental models of Parkinson's disease, Nat. Rev. Neurosci. 2 (2001) 325–334.
[4] D.S. Bredt, S.H. Snyder, Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme, Proc. Natl. Acad. Sci. U.S.A. 87 (1990) 682–685.
[5] Q Chang, X.L. Wang, Effects of chiral 3-n-butylphthalide on apoptosis induced by transient focal cerebral ischemia in rats, Acta Pharmacol. Sin. 24 (2003) 796–804.
[6] J. Chen, S.Y. Wu, Z.G. Ou-Yang, Y.S. Zhen, Synergy of gemcitabine and lidamycin associated with NF-kappaB downregulation in pancreatic carcinoma cells, Acta Pharmacol. Sin. 29 (2008) 614–619.
[7] T. Dehmer, J. Lindenau, S. Haid, J. Dichgans, J.B. Schulz, Deficiency of inducible nitric oxide synthase protects against MPTP toxicity in vivo, J. Neurochem. 74 (2000) 2213–2216.
[8] G.X. Dong, Y.P. Feng, Effects of NBP on ATPase and anti-oxidant enzymes activities and lipid peroxidation in transient focal cerebral ischemic rats, Zhongguo Yi Xue Ke Xue Yuan Xue Bao 24 (2002) 93–97.
[9] R. Dringen, Glutathione metabolism and oxidative stress in neurodegeneration, Eur. J. Biochem. 267 (2000) 4903.
[10] A.R. Esteves, D.M. Arduino, R.H. Swerdlow, C.R. Oliveira, S.M. Cardoso, Oxidative stress involvement in alpha-synuclein oligomerization in Parkinson's disease cybrids, Antioxid. Redox Signal. 11 (2009) 439–448.
[11] B.I. Giasson, J.E. Duda, I.V. Murray, Q. Chen, J.M. Souza, H.I. Hurtig, H. Ischiropoulos, J.Q. Trojanowski, V.M. Lee, Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions, Science 290 (2000) 985–989.
[12] A.G. Hall, Review: the role of glutathione in the regulation of apoptosis, Eur. J. Clin. Invest. 29 (1999) 238–245.
[13] S.J. Heales, J.P. Bolanos, V.C. Stewart, P.S. Brookes, J.M. Land, J.B. Clark, Nitric oxide, mitochondria and neurological disease, Biochim. Biophys. Acta 1410 (1999) 215–228.
[14] L.J. Hsu, Y. Sagara, A. Arroyo, E. Rockenstein, A. Sisk, M. Mallory, J. Wong, T. Takenouchi, M. Hashimoto, E. Masliah, Alpha-synuclein promotes mitochondrial deficit and oxidative stress, Am. J. Pathol. 157 (2000) 401–410.
[15] P. Jenner, Oxidative stress in Parkinson's disease, Ann. Neurol. 53 (Suppl. 3) (2003) S26–S36, discussion: S36–S38.
[16] P. Jenner, D.T. Dexter, J. Sian, A.H. Schapira, C.D. Marsden, Oxidative stress as a cause of nigral cell death in Parkinson's disease and incidental Lewy body disease. The Royal Kings and Queens Parkinson's Disease Research Group, Ann. Neurol. 32 (Suppl.) (1992) S82–S87.
[17] Y.J. Kim, H.H. Ko, E.S. Han, C.S. Lee, Lamotrigine inhibition of rotenone- or 1-methyl-4-phenylpyridinium-induced mitochondrial damage and cell death, Brain Res. Bull. 71 (2007) 633–640.
[18] V.M Lee, J.Q. Trojanowski, Mechanisms of Parkinson's disease linked to pathological alpha-synuclein: new targets for drug discovery, Neuron 52 (2006) 33–38.
[19] L. Li, B. Zhang, Y. Tao, Y. Wang, H. Wei, J. Zhao, R. Huang, Z. Pei, DL-3-n-butylphthalide protects endothelial cells against oxidative/nitrosative stress, mitochondrial damage and subsequent cell death after oxygen glucose deprivation in vitro, Brain Res. 1290 (2009) 91–101.

[20] G.T. Liberatore, V. Jackson-Lewis, S. Vukosavic, A.S. Mandir, M. Vila, W.G. McAuliffe, V.L. Dawson, T.M. Dawson, S. Przedborski, Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease, Nat. Med. 5 (1999) 1403–1409.
[21] S.A. Lipton, Z. Gu, T. Nakamura, Inflammatory mediators leading to protein misfolding and uncompetitive/fast off-rate drug therapy for neurodegenerative disorders, Int. Rev. Neurobiol. 82 (2007) 1–27.
[22] J. Lotharius, K.L. O'Malley, The parkinsonism-inducing drug 1-methyl-4-phenylpyridinium triggers intracellular dopamine oxidation. A novel mechanism of toxicity, J. Biol. Chem. 275 (2000) 38581–38588.
[23] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[24] S. Przedborski, M. Vila, MPTP: a review of its mechanisms of neurotoxicity, Clin. Neurosci. Res. 1 (2001) 407–418.
[25] C Szabo, H. Ischiropoulos, R. Radi, Peroxynitrite: biochemistry, pathophysiology and development of therapeutics, Nat. Rev. Drug Discov. 6 (2007) 662–680.
[26] P. Ying, S.F. Xu, G.Q. Chen, L. Wang, Y.P. Feng, X.L. Wang, L-3-n-Butylphthalide improves cognitive impairment induced by chronic cerebral, J. Pharmacol. Exp. Therap. 321 (2007) 902–910.