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Mechanisms of MPP^+-induced PC12 Cell Apoptosis via Reactive Oxygen Species

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Summary: Apoptosis of dopaminergic neurons in the nigrostriatal projection plays a crucial role in the pathogenesis of Parkinson's disease (PD). Although the detailed mechanisms responsible for dopaminergic neuron loss are still under investigation, oxidative stress is identified as a major contributor for neuronal apoptosis. In the current study, we studied the effects of MPP^+, a substrate that mimics oxidative stress, on neuron-like PC12 cells and the underlying mechanisms. PC12 cells were cultured and treated by 100 μmol/L MPP^+ for 4, 8, 16, 24 and 48 h, respectively. For drug pretreatment, the PC12 cells were incubated with N-acetyl-l-cysteine (NAC, 5 mmol/L), an antioxidant, SP600125 (20 μmol/L) or PD98059 (100 μmol/L), two pharmacological inhibitors of JNK and ERK1/2, for 1 h before addition of MPP^+. Cell apoptosis was measured by flow cytometry. The mRNA expression of Cu^{2+}/Zn^{2+}-SOD, GSH-Px, Bcl-2 and Bax was detected by RT-PCR. The protein expression of p-ERK1/2 and p-JNK was determined by Western blotting. Our results showed that MPP^+ exposure could induce substantial PC12 cell apoptosis. The pretreatment of SP600125 or PD98059 could effectively reduce the apoptosis rate by reducing the ratio of Bax/Bcl-2 mRNA levels. MPP^+ exposure also induced high level of reactive oxygen species (ROS), marked by dramatic increase of Cu^{2+}/Zn^{2+}-SOD and GSH-Px mRNA levels. The elevated ROS was strongly associated with the activation of JNK and ERK1/2 signal pathways after MPP^+ exposure, since the pretreatment of NAC significantly reduced the upregulation of p-JNK and p-ERK1/2. Finally, the pretreatment of SP600125, but not PD98059, alleviated the increase of Cu^{2+}/Zn^{2+}-SOD and GSH-Px mRNAs induced by MPP^+, suggesting that the activation of the JNK signal pathway, but not the ERK1/2 signal pathway, could, in some degree, antagonize the generation of ROS induced by oxidative stress. In conclusion, our results suggest that JNK and ERK1/2 signal pathways, which are activated via ROS, play a crucial role in neuronal apoptosis induced by oxidative stress.
Key words: MPP^+; apoptosis; reactive oxygen species; JNK; ERK1/2; Cu^{2+}/Zn^{2+}-SOD; GSH-Px

Parkinson's disease (PD), one of the most common neurological degenerative disorders, is characterized by a selective degeneration of dopaminergic neurons in the nigrostriatal projection[1]. Morphological studies have shown that mesencephalic dopaminergic neurons ultimately undergo apoptosis in patients with PD[1, 2]. Although the detailed mechanisms responsible for dopaminergic neuronal death are still under investigation, one crucial factor is shown to be oxidative stress. For example, in patients with PD, the substantia nigra exhibits increased reactive oxygen species (ROS) and lipid peroxidation, reduced mitochondrial complex Ⅰ activity, and increased superoxide dismutase activity, all of which are consequences of oxidative stress[1–4].

Many reports implicated that, besides damaging cell structures through oxidative stress, ROS also act as second messengers to modulate various cellular signals associated with proliferation, cell cycle, and cell death[5–7]. Among the cellular responses associated with ROS, the mitogen-activated protein kinase (MAPK) pathways are well recognized. Members of each major MAPK subfamily—the extracellular signal-regulated protein kinase (ERK1/2), c-Jun N-terminal protein kinase (JNK), and p38MAPK have been shown to be activated in response to proinflammatory and other stress signals[8, 9].

In this study, we treated the neuron-like PC12 cells with MPP^+ to mimic the mesencephalic dopaminergic neurons that are under oxidative stress in PD. We investigated the role of candidate signal pathways that are redox-sensitive (ERK1/2 and JNK signal pathways) in MPP^+-induced apoptosis and examined their relationships with ROS.

1 MATERIALS AND METHODS

1.1 Cell Culture and Drug Treatment

PC12 cells were obtained from the Chinese Type Culture Collection in Wuhan University, China. They were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 5% horse serum (Gibco, USA), 100 μg/mL streptomycin, and 100 U/mL penicillin in a water-saturated atmosphere of 5% CO_2 at 37°C. One week before drug treatment, nerve growth

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factor (NGF) was added to the culture at a final concentration of 0.1 μg/mL to induce neuronal differentiation.

MPP^+ (final concentration: 100 μmol/L) was added to the dishes 24 h after PC12 cell plating (1×10^6/mL, 2.5 mL per well in 6-well plates). PC12 cells were then harvested after incubation with MPP^+ for 4, 8, 16, 24 and 48 h, respectively. For drug pretreatment, the PC12 cells were incubated with antioxidant N-acetyl-l-cysteine (NAC, 5 mmol/L), or two pharmacological inhibitors of JNK and ERK1/2, SP600125 (20 μmol/L) and PD98059 (100 μmol /L) for 1 h before addition of MPP^+.

1.2 Assessment of Apoptosis by Flow Cytometry

The Annexin Ⅴ/PI apoptosis detection kit (Sigma, USA) was used to assess membrane and nuclear events that occurred during apoptosis. After treatment, PC12 cells were harvested and washed with ice-cold PBS twice, suspended at a density of 1×10^6/mL. One hundred microliter suspension was then taken and incubated with 5 μL Annexin Ⅴ-FITC and 10 μL PI (20 μg/mL) for 15 min in the dark at room temperature. The FITC and PI signals were measured with a BD-LSR flow cytometer using the CellQuest software.

1.3 RT-PCR

Total RNA was extracted from PC12 cells using Trizol kit (Invitrogen, USA). The single-stranded cDNA was synthesized from total RNA using OligodT as primer. The primers used to detect Cu^{2+}/Zn^{2+}-SOD, GSH-Px, Bcl-2, Bax and β-actin were designed according to GenBank accession number (table 1). The cDNAs were amplified by PCR using the following conditions: 94°C for 5 min, then 30 cycles of 94°C for 30 s，59°C for 1 min and 72°C for 1 min, after the last cycle, 72°C for 5 min.

Table 1 The primer sequences of target genes

Target genes	Primer pairs	Product size (bp)
Cu^{2+}/Zn^{2+}-SOD	5'-TTCGAGCAGAAGGCAAGCGGTGAA-3' 5'-AATCCCAATCACACCACAAGCCAA-3'	389
GSH-Px	5'-GGGGCCTGGTGGTGCTCGGCT-3' 5'-CAATGGTCTGGAAGCGGCGGC-3'	354
Bcl-2	5'-CTGGTGGACAACATCGCTCTG-3' 5'-GGTCTGCTGACCTCACTTGTG-3'	228
Bax	5'-AAGCTGAGCGAGTGTCTCCGGCG-3' 5'-GCCACAAAGATGGTCACTGTCTGCC-3'	284
β-actin	5'-GAGCACCCTGTGCTGCTCACCGAGG-3' 5'-GTGGTGGTGAAGCTGTAGCCACGCT-3'	300

1.4 Western Blot Analysis

Cells were harvested and lysed in buffer (50 mmol/L Tris-HCl, pH 8.0, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 1% TritonX-100, 0.1% SDS, 50 mmol/L sodium fluoride, and 1 mmol/L sodium vanadate). Protein concentration was determined using the BCA kit (Pierce, USA). Equal amounts of protein were separated on an SDS-PAGE gel and electrophoretically transferred to a PVDF membrane. Membranes were blocked in TBS-T containing 5% nonfat dry milk and incubated overnight at 4°C with the primary antibody (anti-p-ERK1/2, 1:100; anti-p-JNK, 1:100; anti-GAPDH, 1:100; all anti-mouse antibodies from Santa Cruz Biotechnology, USA). After two 10-min rinses in TBS-T and one 10-min rinse in TBS, membranes were incubated with horseradish peroxidase-conjugated anti-mouse antibody (adjusted to 1:5000, Santa Cruz Biotechnology, USA) in TBS-T for 1 h at room temperature. The membrane was washed three times for 10 min each in TBS. Bands were visualized by ECL and quantified by HIPAS-1000 detection system.

1.5 Statistical Analysis

Quantitative data was expressed as $\bar{x}\pm s$. Data was analyzed by student's *t*-test for comparison. Statistical calculations were performed using a computer statistical package SPSS13.0. Significant difference was considered when a *P* value was <0.05.

2 RESULTS

2.1 MPP^+ Treatment Activated ERK1/2 and JNK Signal Pathways in PC12 Cells

The activation of ERK1/2 or JNK signal pathway was marked by the increased levels of phosphorylated ERK1/2 or JNK (p-ERK1/2 or p-JNK), respectively. Compared to the low level of p-ERK1/2 and p-JNK in normal PC12 cells, the exposure to MPP^+ resulted in a significant increase of both p-ERK1/2 and p-JNK levels, when normalized to GAPDH level that was unaffected (fig. 1, $P<0.05$). Such increase started at 4 h after treatment and lasted for at least 48 h in both cases.

2.2 MPP^+-induced PC12 Cells Apoptosis was Associated with JNK and ERK1/2 Pathways

Flow cytometric analysis demonstrated that the predominant cell death of PC12 cells after exposure to 100 μmol/L MPP^+ occurred via apoptosis. Starting from 8 h after MPP^+ incubation, the apoptosis rate increased significantly when compared to the control. Such elevation of apoptosis was in a time-dependent manner from 8 to 48 h (table 2).

On the molecular level, the expression of Bax mRNA increased significantly 4 h after MPP^+ exposure ($P<0.05$), which lasted for at least 48 h ($P<0.01$) (fig. 2). In contrast, the Bcl-2 mRNA level started to decrease at 8 h ($P<0.01$) and reached the lowest point at 48 h ($P<0.01$) (fig. 2). These results further confirmed that MPP^+ induced PC12 cell apoptosis.

To further investigate the role of the activated ERK 1/2 and JNK signal pathways in MPP^+-induced apoptosis, we incubated the PC12 cells with SP600125 (a JNK inhibitor) and PD98059 (an ERK1/2 inhibitor), respectively, for 1 h before addtion of 100 μmol/L MPP^+. We found that, starting from 16 h after MPP^+ application, the pretreatment with SP600125 (SP600125+MPP^+ group) or PD98059 (PD98059+MPP^+ group) significantly reduced the apoptosis rate when compared with the MPP^+ only group (table 2). These results indicated that ERK1/2 and JNK signal pathways play an important role in MPP^+

induced cell apoptosis.

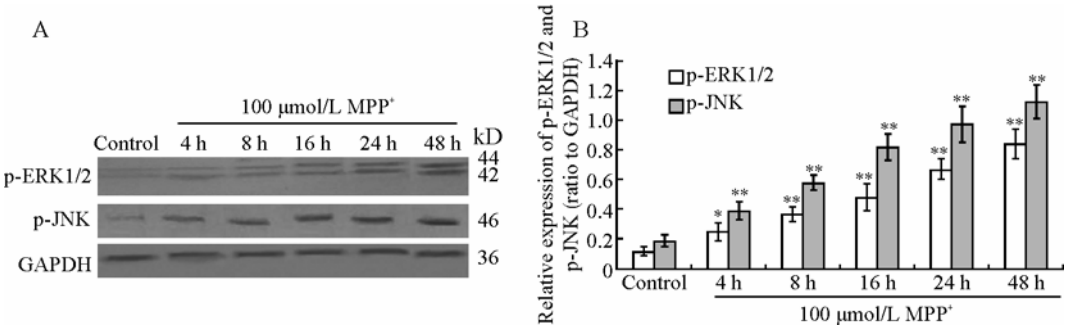

Fig. 1 Western blot analysis of p-ERK1/2 and p-JNK in MPP^+-treated PC12 cells

A: Western blotting showing expression levels of pERK1/2, pJNK and GAPDH (a loading control) in PC12 cells after incubation with 100 μmol/L MPP^+; B: Band intensities of p-ERK1/2 and p-JNK normalized to GAPDH. The expression levels of both p-ERK1/2 and p-JNK increased significantly in a time-dependent manner from 4 to 48 h. $^*P$<0.05, $^{**}P$<0.01 compared with control

Table 2 PC12 cell apoptosis rate in 100 μmol/L MPP^+ only, SP600125- and PD98059-pretreated groups (%, $\bar{x}\pm s$, n=4)

Groups	Control (0 h)	4 h	8 h	16 h	24 h	48 h
MPP^+	2.54±0.11	7.18±1.63	18.34±2.67**	29.14±2.22**	37.46±2.35**	45.11±4.35**
SP600125+MPP^+	2.54±0.11	3.89±1.35	10.83±1.09*	14.01±1.88$^{**▲▲}$	21.66±2.72$^{**▲▲}$	32.65±3.38$^{**▲▲}$
PD98059+MPP^+	2.54±0.11	4.66±1.53	13.21±1.75**	16.63±2.05$^{**▲}$	22.07±1.89$^{**▲▲}$	31.02±4.05$^{**▲▲}$

$^*P$<0.05, $^{**}P$<0.01 compared with control; $^▲P$<0.05, $^{▲▲}P$<0.01 compared with MPP^+ group

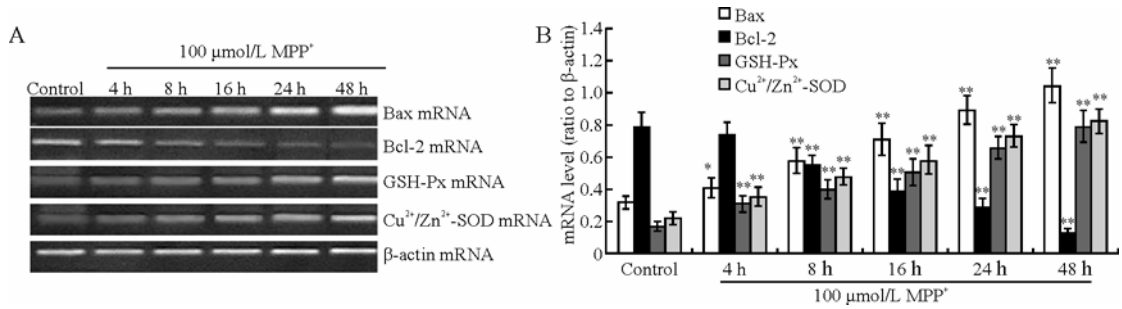

Fig. 2 RT-PCR analysis of Bax, Bcl-2, Cu^{2+}/Zn^{2+}-SOD and GSH-Px in MPP^+-treated PC12 cells

A: RT-PCR showing the mRNA expressions of Bax, Bcl-2, Cu^{2+}/Zn^{2+}-SOD and GSH-Px; B: Band intensities of Bax, Bcl-2, Cu^{2+}/Zn^{2+}-SOD and GSH-Px normalized to that of β-actin. After treated with100 μmol/L MPP^+, the Bax mRNA level in PC12 cells increased significantly at 4 h and lasted at least until 48 h. In contrast, the Bcl-2 mRNA level decreased at 8 h and reached the lowest point at 48 h. Starting from 4 h after MPP^+ incubation, both Cu^{2+}/Zn^{2+}-SOD and GSH-Px mRNA levels increased significantly in a time-dependent manner. $^*P$<0.05, $^{**}P$<0.01 *vs.* control group

2.3 ROS were Involved in the Activation of both ERK1/2 and JNK Signal Pathways in PC12 Cells after MPP^+ Exposure

To test that MPP^+ exposure results in dramatic elevation of ROS, we measured the mRNA levels of Cu^{2+}/Zn^{2+}-SOD and GSH-Px, two crucial antioxidant enzymes, at different time points. It turned out that, in both cases, they started to rise significantly at 4 h (P<0.01) in a time-dependent manner (fig. 2), serving to neutralize large amounts of ROS that were generated by MPP^+ exposure.

We further found that addition of NAC could significantly decrease the levels of ROS in PC12 cells induced by MPP^+ (data not shown). To test whether ROS play a role in the activation of ERK1/2 and JNK pathways, we incubated NAC for 1 h before addition of MPP^+. It was found that, although the level of p-ERK1/2 and p-JNK still increased in a time dependent manner (fig. 3), the increment for both genes was significantly reduced when compared to MPP^+ exposure only group (fig. 4A and 4B), with a more remarkable decrease in p-JNK (fig. 4B). These results indicated that ROS are involved in the activation of both ERK1/2 and JNK signal pathways in PC12 cells after MPP^+ exposure.

2.4 ERK1/2 and JNK Signal Pathways Modulated Gene Expression of Cu^{2+}/Zn^{2+}-SOD, GSH-Px, Bax and Bcl-2 after MPP^+ Treatment

To test how ERK1/2 and JNK signal pathways contributed to MPP^+-induced apoptosis and their relationships with ROS, we pharmacologically inhibited ERK1/2 and JNK respectively. When PC12 cells were incubated with SP600125 (a JNK inhibitor), the increase of Bax mRNA expression was delayed [8 h *vs*. 4 h as a significant increase relative to control groups (without MPP^+), P<0.01] and severely compromised (P<0.01 for 8, 16, 24, and 48 h between SP600125+MPP^+ and MPP^+ only groups, fig. 5). The down-regulation of Bcl2 mRNA, however, was unaffected (fig. 5). The increase of Cu^{2+}/Zn^{2+}-SOD and GSH-Px mRNA levels was also delayed (8 h *vs.* 4 h for Cu^{2+}/Zn^{2+}-SOD; 16 h *vs.* 4 h for

GSH-Px) and significantly disrupted by SP6000125 incubation (Cu^{2+}/Zn^{2+}-SOD: $P<0.01$ at 8, 16, 24, and 48 h; GSH-Px, $P<0.05$ at 16 h, and $P<0.01$ at 24 and 48 h between SP600125+MPP^+ and MPP^+ only groups, fig. 5).

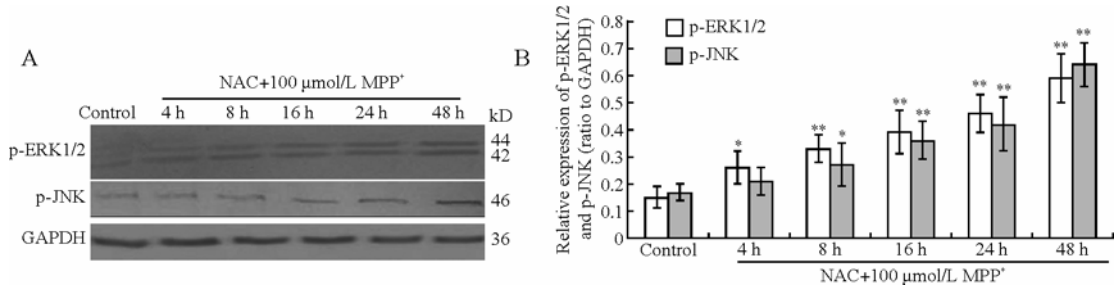

Fig. 3 Western blot analysis of p-ERK1/2 and p-JNK in P12 cells treated with NAC and MPP^+

A: Western blotting showing expression levels of p-ERK1/2, p-JNK and GAPDH (a loading control); B: Band intensities of p-ERK1/2 and p-JNK normalized to that of GAPDH. Levels of pERK1/2 or pJNK started to increase significantly when compared to the control at 4 h or 8 h, respectively, although in both cases the increments were much smaller than those induced by MPP^+ exposure only (seen in fig. 1). $^*P<0.05$, $^{**}P<0.01$ *vs.* control group

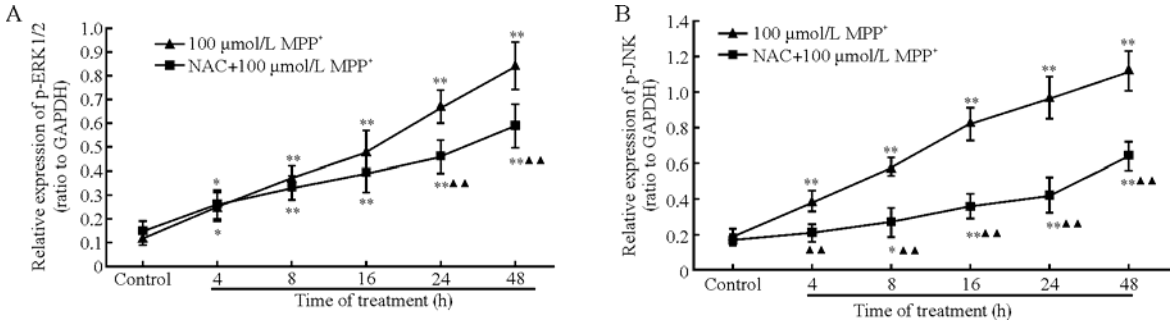

Fig. 4 Comparison of the protein expression of p-ERK1/2 (A) and p-JNK (B) between NAC+MPP^+ group and MPP^+ only group

Compared to the MPP^+ only group (PC12 cells only treated with 100 μmol/L MPP^+), both p-ERK1/2 and p-JNK levels in the NAC+100 μmol/L MPP^+ group were lower at the same time point. The level of p-JNK decreased more rapidly and remarkably. $^*P<0.05$, $^{**}P<0.01$ *vs.* control; $^{▲▲}P<0.01$ *vs.* MPP^+ only group

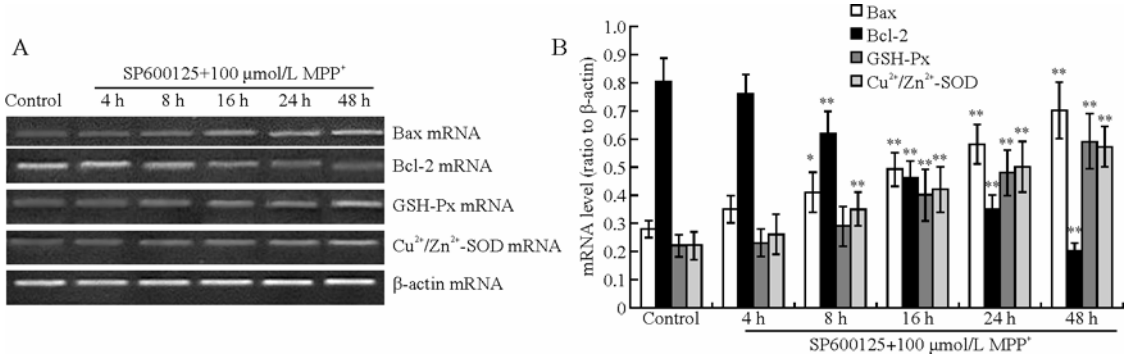

Fig. 5 RT-PCR analysis of Bax, Bcl-2, Cu^{2+}/Zn^{2+}-SOD and GSH-Px in PC12 cells treated by SP600125 and MPP^+

A: RT-PCR showing the mRNA expressions of Bax, Bcl-2, Cu^{2+}/Zn^{2+}-SOD and GSH-Px; B: Band intensities of Bax, Bcl-2, Cu^{2+}/Zn^{2+}-SOD and GSH-Px normalized to that of β-actin. When PC12 cells were incubated with SP600125 for 1 h before the addition of MPP^+, both Bax and Cu^{2+}/Zn^{2+}-SOD mRNA levels increased significantly after 8 h, and the GSH-Px mRNA level rose obviously after 16 h. The Bcl-2 mRNA level decreased obviously after 8 h in a time-dependent manner till 48 h. $^*P<0.05$, $^{**}P<0.01$ *vs.* control group

The incubation of PD98059, an ERK1/2 inhibitor, significantly reversed the expression changes of Bax and Bcl-2 induced by MPP^+ in PC12 cells (fig. 6). When compared to the MPP^+ only group, the expression of Bax mRNA relatively decreased and that of Bcl-2 mRNA relatively increased (Bax: $P<0.05$ at 8, 16 h, and $P<0.01$ at 24 and 48 h; Bc-2: $P<0.05$ at 8 h, and $P<0.01$ at 16, 24 and 48 h between PD98059+MPP^+ and MPP^+ only groups). In contrast, adding PD98059 had no overt effects on the increase of Cu^{2+}/Zn^{2+}-SOD and GSH-Px mRNA levels induced by MPP^+ (fig. 6).

These results indicated that both JNK and ERK1/2 play important roles in MPP^+-induced apoptosis. JNK signal pathway, but not ERK1/2 signal pathway, could potentially buffer the generation of ROS induced by MPP^+.

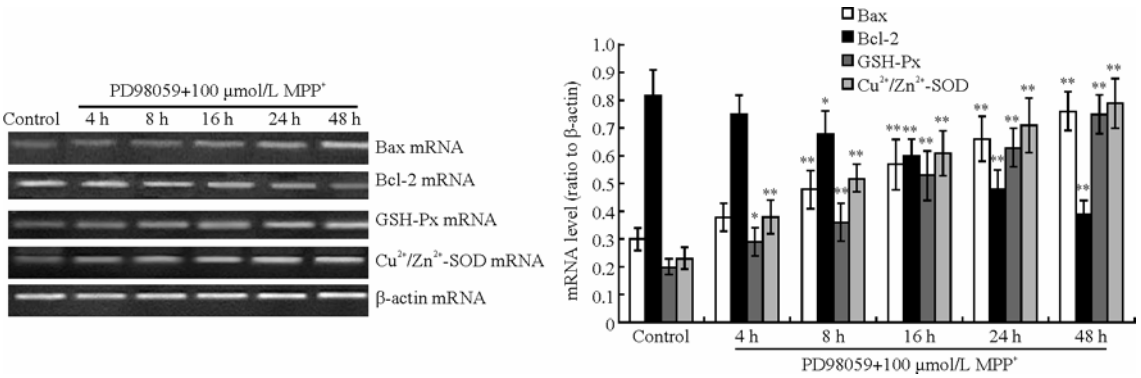

Fig. 6 RT-PCR analysis of Bax, Bcl-2, Cu^{2+}/Zn^{2+}-SOD and GSH-Px in PC12 cells treated by PD98059 and MPP^+

When PC12 cells were incubated with PD98059 for 1 h before the addition of MPP^+, GSH-Px and Cu^{2+}/Zn^{2+}-SOD mRNAs both increased significantly after 4 h, and the expression of Bax mRNA rose obviously after 8 h. The Bcl-2 mRNA level decreased obviously after 8 h in a time-dependent manner till 48 h. $^*P<0.05$, $^{**}P<0.01$ *vs.* control group

3 DISCUSSION

Oxidative stress is implicated in the neuronal cell death that is associated with multiple neurodegenerative disorders, such as Alzheimer disease, PD, Huntington disease and amyotrophic lateral sclerosis[10–13]. Studies have demonstrated that under oxidative stress, high levels of ROS, including free radicals such as super oxide (O^{2-}), hydroxyl radical (HO^-) and hydrogen peroxide (H_2O_2), may modify protein, lipid and DNA structures and consequently alter their functions, which cause severe cell damages and eventually cell death[14–18].

Emerging evidence suggests that under pathological conditions, excessive amounts of ROS induced by oxidative stress can activate certain signaling pathways by serving as second messengers. Activation of JNK, ERK1/2 and/or p38MAPK by oxidative stress-induced ROS has been described in various cell types[9, 19–21]. JNK, ERK1/2 and p38MAPK are three distinct groups of MAPKs. MAPK signaling cascades comprise highly conserved serine/threonine kinases connecting cell surface receptors to regulatory targets in response to various stimuli, and are known to be involved in the induction of cell death, as well as maintenance of cell survival[15, 22]. JNK is generally regarded as a mediator of apoptotic cell death in many cell types[23]. In contrast to JNK, ERK is regarded generally as an anti-apoptotic kinase. ERK1/2 usually functions as the cytoprotective machinery against apoptosis triggered by oxidative stress, tumor necrosis factor-alpha, growth factor deprivation, and apoptosis-inducing drugs[8, 24]. However, some reports also showed the pro-apoptotic role of ERK1/2 in Fas- and asbestos-initiated apoptosis. Therefore, the effect of ERK1/2 on apoptosis seems to be dependent on different cellular scenarios and cell types[25].

In this study, we chose PC12 cell lines as neuronal model system to investigate if MPP^+ treatment, which induces high level of ROS, would activate MAPKs signal pathways. Under normal culture conditions, PC12 cells have properties similar to those of undifferentiated neuron-like cells. When grown in the presence of nerve growth factor (NGF), they undergo differentiation and extend neurites, becoming neuronal phenotype[26]. We found that when PC12 cells were treated with MPP^+, ERK1/2 and JNK were significantly activated. However, such activation can be effectively blocked by the incubation of NAC, an antioxidant and ROS scavenger. These results indicated that MPP^+ treatment activated JNK and ERK1/2 signal pathways via ROS elevation.

We also found that by using SP600125, a specific inhibitor of JNK, the cell apoptosis rate decreased compared to that of the MPP^+-treated PC12 cells. In a similar manner, a specific ERK kinase inhibitor, PD98059, also reduced PC12 cell apoptosis rate. Therefore, the MPP^+-induced apoptosis was, at least, partially mediated by the activation of the JNK and ERK1/2 signal pathways. To further investigate the possible mechanisms by which the two signal pathways involved in MPP^+-induced apoptosis, we examined the gene expression of two apoptosis-related genes, Bax and Bcl-2.

It is now well established that Bcl is a multiple gene family, which consists of antiapoptotic proteins such as Bcl-2 and Bcl-xL, and proapoptotic proteins such as Bax, Bcl-xs and Bad[27]. Our study indicated that the JNK inhibitor SP600125 significantly inhibited the expression of Bax mRNA but had no effect on Bcl-2 mRNA. In contrast, the ERK1/2 inhibitor PD98059 relatively increased the expression of Bcl-2 mRNA and meanwhile substantially blocked Bax mRNA expression. These results revealed that JNK and ERK1/2 signal pathways could both promote apoptosis of PC12 cells through increasing Bax/Bcl-2 ratio.

To maintain cellular homoeostasis, intracellular levels of ROS are tightly controlled by antioxidant factors, including enzymes such as GSH-Px, Cu^{2+}/Zn^{2+}-SOD, catalase, and peroxiredoxins, as well as other redox-active proteins, such as thioredoxin and glutaredoxin, and small redox-active molecules, such as glutathione and NADPH[2, 4]. In our study, we found that SP600125 treatment could also decrease Cu^{2+}/Zn^{2+}-SOD and GSH-Px mRNAs at the same time. However, PD98059 had no effects on the expression of Cu^{2+}/Zn^{2+}-SOD and GSH-Px mRNAs. These results indicate that except for its role in inducing PC12 cells apoptosis, MPP^+ also promotes the expression of some antioxidant factors mediated by JNK signal pathway. Such phenomenon could be viewed as a protective compensatory mechanism, although it might not be robust enough to prevent the apoptosis.

In conclusion, our study indicated that MPP^+ induced PC12 cell apoptosis by activating JNK and

ERK1/2 signal pathways via ROS. Further studies on how JNK and/or ERK1/2 signal pathways interact with elevated ROS will definitely shed light on the research of mechanisms of oxidative stress-induced neuronal apoptosis that plays a crucial role in PD pathogenesis.

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