Protective effect of erythropoietin against 1-methyl-4-phenylpyridinium-induced neurodegeneration in PC12 cells

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Abstract: Objective The neuroprotective effect of erythropoietin (EPO) against 1-methyl-4-phenylpyridinium (MPP⁺)-induced oxidative stress in cultured PC12 cells, as well as the underlying mechanism, were investigated. **Methods** PC12 cells impaired by MPP⁺ were used as the cell model of Parkinson's disease. Methyl thiazolyl tetrazolium (MTT) was used to assay the viability of the PC12 cells exposed to gradient concentrations of EPO, and the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay was used to analyze the apoptosis ratio of PC12 cells. The expression of Bcl-2 and Bax in PC12 cells were examined by Western blot, and the reactive oxygen species (ROS), the mitochondrial transmembrane potential and the activity of caspase-3 in each group were detected by spectrofluorometer. **Results** Treatment of PC12 cells with MPP⁺ caused the loss of cell viability, which may be associated with the elevation in apoptotic rate, the formation of ROS and the disruption of mitochondrial transmembrane potential. It was also shown that MPP⁺ significantly induced the upregulation of Bax/Bcl-2 ratio and the activation of caspase-3. In contrast, EPO significantly reversed these responses and had the maximum protective effect at 1 U/mL. **Conclusion** The inhibitive effect of EPO on the MPP⁺-induced cytotoxicity may be ascribed to its anti-oxidative property and anti-apoptotic activity, and EPO may provide a useful therapeutic strategy for treatment of neurodegenerative diseases such as Parkinson's disease.

Keywords: 1-methyl-4-phenylpyridinium; PC12 cells; erythropoietin; oxidative stress; apoptosis

1 Introduction

Among the chemicals of interest to study neural degeneration, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been widely used as a dopaminergic neurotoxin because it causes a severe parkinsonian-like syndrome with loss of dopaminergic cells in animals and human beings^[1]. MPTP produces reactive oxygen species (ROS) *in vivo* via the active metabolite 1-methyl-4-phenyl pyridinium ion (MPP⁺), which stimulates the superoxide radical production *in vitro* and induces cell death in PC12 cells^[2]. Moreover, MPTP generates hydroxyl radicals to cause

membrane lipid peroxidation or DNA damage, which is thought to be a mechanism of dopaminergic neuron degeneration^[3]. Hence, chemicals that reduce the oxidative stress induced by MPP⁺ are supposed to be neuroprotective in Parkinson's disease.

Erythropoietin (EPO) is a cytokine that acts in erythroid progenitor proliferation and differentiation. Previous studies found that EPO could ameliorate the oxidative stress in several quite different disorders^[4-6]. It has been also reported that EPO displays efficient neuroprotective properties in a spectrum of different animal models, including ischemia/hypoxia, excitotoxic paradigms, traumatic brain and spinal cord injury, and retina/optic nerve damage in inflammatory/auto-immunological diseases^[7]. Recent studies have revealed a possible protective role of EPO in Parkinson's disease^[8-10].

However, few data exist regarding the protective value

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of EPO on MPP⁺ induced oxidative stress and the subsequent apoptosis in PC12 cells. In the present study, we tried to clarify whether EPO could substantially reduce MPP⁺-induced cellular death in PC12 cells via its anti-oxidant and anti-apoptosis characteristics.

2 Materials and methods

2.1 Materials Recombinant human EPO injection was obtained from Shenyang Sunshine Pharmaceutical Co. Ltd (China). MPP⁺, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorofluorescein diacetate (DCF-DA) and rhodamine 123 were from Sigma-Aldrich Inc. (St. Louis, MO, USA). Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit was from Biosea BCL (Beijing, China). The *in situ* cell death detection kit was from the Boehringer Mannheim, Co. (Mannheim, Germany). Bax and Bcl-2 antibodies were obtained from Santa Cruz (Santa Cruz, CA, USA). The Caspase-3 Fluorescent Assay kit was from R&D systems (Minneapolis, MN, USA). Dulbecco's modified Eagle's medium (DMEM), heat-inactivated calf serum, fetal bovine serum (FBS) were purchased from Gibco-BRL-Life Technologies (Gaithersburg, MD, USA). Bicinchoninic acid (BCA) kit and enhanced chemiluminescence (ECL) were purchased from Pierce Chemical Company (Rockford, IL, USA).

2.2 Cell culture Rat PC12 cells (adrenal gland; pheochromocytoma) were obtained from Chinese Type Culture Collection, China. PC12 cells were maintained in the DMEM supplemented with 10% heat-inactivated calf serum, 5% FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin in a water-saturated atmosphere at 37 °C with 5% CO₂. The culture medium was changed every three days and cells were subcultured about once a week. The medium was changed to serum-deprived medium or medium supplemented with 1% FBS 24 h before experiments and replanted in 96 and 24 well plates. The next day MPP⁺ and/or EPO were added to the naive PC12 cells. All experiments were carried out 24 h after the reagents were added.

2.3 Measurement of cell viability PC12 cells were seeded in 96-well plates at a density of 4×10^4 cells per well and treated with various concentrations of EPO (0.1, 0.3, 1, 3 or 10 U/mL) and/or 500 µmol/L of MPP⁺ at 37 °C. After incubation for up to 24 h, the medium was incubated with 10 µL of 5 mg/mL MTT solution for 4 h. Then the culture medium with MTT was removed and 200 µL DMSO was added to each well to dissolve the formazan. Absorbance was mea-

sured at 570 nm (540 nm as a reference) with a model 550-microplate reader. Cell viability was expressed as a percentage of the value in control cultures.

2.4 Assessment of apoptosis Flow cytometry was used to assess the membrane and nuclear events during apoptosis. Briefly, after the treatment period (24 h), the harvested cells were resuspended in 1 mL binding buffer. The aliquot (100 µL) was incubated with 5 µL Annexin V-FITC and 10 µL propidium iodide (PI) for 15 min in dark at room temperature and 400 µL PBS was added to each sample. The FITC and PI fluorescence were measured by FL-1 filter (530 nm) and FL-2 filter (585 nm), respectively, on BD-LSR flow cytometer using Cell Quest software; and 10 000 events were acquired.

2.5 TUNEL assay for apoptotic DNA fragmentation The DNA fragmentation of apoptotic cells were identified by the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) method which permits the specific labelling of the 3'-OH end of DNA breaks with modified nucleotides by TdT. Briefly, PC12 cells were seeded at a density of 1×10^5 cells/cm² on coverslips coated with poly-L-lysine. After washing with PBS, the cultures were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature. Endogenous peroxidase was quenched by incubation with 0.3% (V/V) hydrogen peroxide in methanol for 30 min at room temperature, and then the cells further permeabilized with 0.1% Triton X-100 in 0.1% sodium acetate for 5 min at 4 °C. Thereafter, the cells were labeled by incubation with the TUNEL reaction mixture for 60 min at 37 °C followed by labeled with peroxidase-conjugated anti-fluorescein anti-goat antibody (Fab fragment) for an additional 30 min. Subsequently, cells were incubated with diaminobenzidine substrate (DAB) to produce a dark brown precipitate. Slides were counterstained with hematoxylin stain. For each experiment, all treatments were performed in triplicate wells. The positive staining was identified as dark brown granules under the light microscope.

2.6 Measurement of intracellular reactive oxygen species formation Intracellular ROS was monitored by using the fluorescent probe DCF-DA, which could be oxidized to highly fluorescent compound dichlorofluorescein (DCF) by intracellular H₂O₂ or low-molecular-weight peroxides. PC12 cells (1×10^5 cells/mL) were treated with MPP⁺ and/or EPO for 24 h at 37 °C, then incubated with 1 mmol/L of DCF-DA for 1 h at 37 °C. Wash the reaction system three times with PBS. After centrifugation at 1 000 g for 5 min, the superna-

tants were removed and the pellets were resolved with 1% Triton X-100. Fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 540 nm using a fluorescence microplate reader.

2.7 Measurement of mitochondrial transmembrane potential Mitochondrial transmembrane potential was measured using the dye rhodamine 123, since the uptake of rhodamine 123 into mitochondria is shown to be a function of mitochondrial transmembrane potential. PC12 cells (4×10^4) were incubated with MPP⁺ for 4 h, followed by 10 μ mol/L rhodamine 123 (in DMEM) for 20 min at 37 °C. Cell suspension was centrifuged at 412 g for 10 min. Medium was removed and cells were dissolved with 1% Triton X-100. Fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 510 nm using a fluorescence microplate reader.

2.8 Western blot analysis After exposure to EPO and/or MPP⁺ for the indicated dosages and times, 5×10^6 cells were rinsed twice with cold PBS 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% Triton X-100, 0.1% SDS, 50 mmol/L sodium fluoride, and 1 mmol/L sodium vanadate) containing a protease inhibitor cocktail to obtain whole cell protein. Lysates were cleared by centrifugation. The protein concentration was determined by the BCA kit. Equal amounts of proteins were fractionated by SDS-polyacrylamide gel electrophoresis, and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% defatted milk in TBS-Tween (TBS-T) (50 mmol/L Tris, pH 7.6, 150 mmol/L NaCl, and 0.1% Tween-20) and incubated with the primary rabbit polyclonal antibody against Bcl-2 (1:500) or the mouse monoclonal antibody against Bax (1:500) overnight at 4 °C. The signals were detected by goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody and ECL. Finally gels were exposed to X-ray films (Fuji, Japan).

2.9 Measurement of caspase-3 activity The activity of caspase-3 was detected with the caspase-3 fluorometric assay kit (RnD Systems, Minneapolis, USA) according to the instruction manual. This kit uses synthetic tetrapeptide DEVD-AFC as the substrate. In the presence of active caspase-3, the substrate is cleaved between DEVD and AFC, releasing the fluorogenic AFC, which is then detected by the spectrofluorometry. The fluorogenic AFC reflects the activity of caspase-3. PC12 cells were incubated in medium containing serum-free medium with MPP⁺ and/or EPO.

Caspase activities in PC12 cells were measured after MPP⁺ and/or EPO treatment. After the incubation, cells were collected and lysed in a lysis buffer on ice for 10 min. The protein concentrations of the supernatant fluids were ascertained with the BCA kit. Samples containing 200 μ g of protein were mixed with the reaction buffer and DEVD-AFC substrate, and incubated for 2 h at 37 °C. The fluorescence was measured at an excitation wavelength of 400 nm and emission wavelength of 505 nm with fluorometric reader. A caspase-3-like activity was expressed as the relative content against that in the cells incubated in the serum medium without MPP⁺.

2.10 Statistical analysis Data were expressed as mean $\pm$ SD. The significance of inter-group difference was evaluated by one-way analyses of variance (ANOVA: Duncan's test for *post hoc* comparisons). $P < 0.05$ was considered statistically significant.

3 Results

3.1 Effect of EPO on MPP⁺-induced cell viability loss in PC12 cells The data demonstrated that EPO can promote PC12 cell proliferation even when the cells were cultured without FBS (Fig. 1A) or in the presence of small amounts of FBS (Fig. 1B).

Compared with the vehicle controls, exposure to 500 μ mol/L of MPP⁺ for 24 h resulted in 52.5% of the PC12 cells loss. The neurotoxic effect of MPP⁺ were significantly attenuated by EPO, when the cells were co-incubated with MPP⁺ and different concentrations (0, 0.1, 0.3, 1, 3, or 10 U/mL) of EPO for 24 h. The survival rate of PC12 cells was $(76.8 \pm 4.7)\%$, $(98.7 \pm 9.3)\%$, $(112.5 \pm 12.5)\%$, $(92.9 \pm 7.0)\%$, and $(64.6 \pm 5.1)\%$, respectively, when the cells were treated with the indicated concentrations of EPO for 24 h (Fig. 2). EPO exhibited neuroprotective effect on the MPP⁺-induced cytotoxicity in a dose-dependent manner in the PC12 cells, and maximal rescue occurred at a concentration of 1 U/mL (Fig. 1).

3.2 Effect of EPO against MPP⁺-induced apoptosis in PC12 cells In the control (Fig. 3A), 87.2% of cells were negative for Annexin V-FITC and PI binding. These are viable cells. PC12 cells exposed to 500 μ mol/L MPP⁺ for 24 h revealed that among the Annexin V positive cells (32.2%), 7.6% were PI negative (lower right quadrants) and 24.6% were PI positive (upper right quadrant), which indicated the background early apoptosis and the late apoptosis/necrosis, respectively (Fig. 3B). Simultaneous incubation with 0.1, 0.3, 1, 3 or 10 U/mL EPO significantly reduces the

Fig. 1 Proliferative effect of EPO on cultured PC12 cells. A: Cells treated with EPO for 24 h in the medium deprived serum; B: cells treated with EPO for 24 h in the medium supplemented with 1% FBS. Cell viability was assessed by MTT method as described in *Materials and methods*. Data are expressed as percent of values in untreated control cultures, and represent 3 replicate values in 4 separate experiments (means $\pm$ SD). * $P < 0.05$ vs control.

Fig. 2 Effect of EPO on the MPP⁺-induced decrease in PC12 cells viability. Cells were treated with 500 μ mol/L MPP⁺ for 24 h in absence or presence of EPO. Data are expressed as means $\pm$ SD of 3 replicate values in 4 separate experiments. * $P < 0.05$, ** $P < 0.01$ vs the group treated by MPP⁺ alone; ### $P < 0.01$ vs control (untreated group).

number of Annexin-V-labeled cells. The percentage of apoptosis cells was decreased to (16.7 $\pm$ 3.0)%, (11.3 $\pm$ 2.2)%, (10.2 $\pm$ 1.5)%, (14.4 $\pm$ 2.6)%, and (19.1 $\pm$ 2.5)% in 0.1, 0.3, 1, 3 and 10 U/mL EPO groups, respectively (Fig. 3C-G).

Consistent with the flow cytometric data, the results of TUNEL assay further confirmed that EPO-treated group could protect MPP⁺-induced apoptosis in PC12 cells. Apoptosis in the PC12 cells treated with 500 μ mol/L of MPP⁺ was determined by TUNEL staining, which could detect DNA fragmentation *in situ*. In this histochemical analysis, the appearance of intensely stained nuclei is indicative of apoptotic nuclei. EPO, at 1 U/mL, lowered the proportion of TUNEL positive cells (Fig. 4).

3.3 Effect of EPO on MPP⁺-induced ROS accumulation

To examine whether the inhibitory effect of EPO on the toxicity of MPP⁺ is mediated by the antioxidant ability, PC12

Fig. 3 Flow cytometric histograms of control PC12 cells and PC12 cells after 24 h exposure to 500 μ mol/L MPP⁺ alone or associated with EPO (0.1, 0.3, 1, 3 and 10 U/mL). After incubation, cells were harvested and labeled with a combination of Annexin V-FITC and propidium iodide. A: Control; B: 500 μ mol/L MPP⁺ treated alone; C: 500 μ mol/L MPP⁺ + 0.1 U/mL EPO; D: 500 μ mol/L MPP⁺ + 0.3 U/mL EPO; E: 500 μ mol/L MPP⁺ + 1 U/mL EPO; F: 500 μ mol/L MPP⁺ + 3 U/mL EPO; G: 500 μ mol/L MPP⁺ + 10 U/mL EPO. Data are means $\pm$ SD of 3 replicate values in 4 separate experiments. ** $P < 0.01$ vs MPP⁺ alone; ## $P < 0.01$ vs control.

Fig. 4 PC12 cells stained with TUNEL. PC12 cells were treated for 24 h exposure to 500 $\mu\text{mol/L}$ MPP⁺ alone or associated with EPO (1 U/mL). Apoptotic nuclei are those with dark brown precipitate. A: Control; B: 500 $\mu\text{mol/L}$ MPP⁺ treated alone; C: 500 $\mu\text{mol/L}$ MPP⁺ with 1 U/mL EPO; D: 1 U/mL EPO treated alone. The figures are representative for three different experiments. Scale bar, 10 μm .

cells were treated with 500 $\mu\text{mol/L}$ of MPP⁺ for 24 h, and the level of ROS was measured by DCF fluorescence. As shown in Fig. 5, when PC12 cells were exposed to MPP⁺, the intracellular ROS level was significantly increased from (100.0 $\pm$ 7.9)% (control) to (227.9 $\pm$ 21.5)% ($P < 0.01$), revealing that MPP⁺ enhanced ROS concentration in PC12 cells. However, simultaneous treatment with EPO and MPP⁺ effectively reduced ROS generation, and the suppressing effect was strengthened with the increase of EPO concentration. EPO inhibited the MPP⁺-induced ROS increase in DCF fluorescence, and at 1 U/mL, EPO showed the maximum inhibition.

3.4 Effect of EPO on the mitochondrial transmembrane potential loss caused by MPP⁺ Change in the mitochondrial transmembrane potential in PC12 cells treated with MPP⁺ was quantified by measuring the cellular retention of rhodamine 123. When PC12 cells were treated with 500 $\mu\text{mol/L}$ of MPP⁺ for 24 h at 37 $^{\circ}\text{C}$, the retention of rhodamine 123 decreased. EPO prevented the MPP⁺-induced decrease in the retention of rhodamine 123 (Fig. 6).

3.5 Effect of EPO on MPP⁺-induced downregulation of the Bax/Bcl-2 ratio In this study, we investigated whether EPO has any effect on the expressions of Bax and Bcl-2 in MPP⁺-treated cells by using Western blot analysis. As shown in Fig. 7A, Bax protein increased significantly in 500 $\mu\text{mol/L}$ MPP⁺-treated group compared with the control. However, EPO at 1 U/mL could decrease the Bax expression level almost to the normal value. In contrast, the level

Fig. 5 EPO reduced MPP⁺-induced accumulation of ROS. Data are expressed as means $\pm$ SD of 3 replicate values in 4 separate experiments. * $P < 0.05$, ** $P < 0.01$ vs MPP⁺ alone; ## $P < 0.01$ vs control.

Fig. 6 EPO blocked MPP⁺-induced mitochondrial membrane potential loss. Data are means $\pm$ SD of 3 replicate values in 4 separate experiments. ** $P < 0.01$ vs MPP⁺ alone; ## $P < 0.01$ vs control.

Fig. 7 Effect of EPO on the expression of Bax and Bcl-2 in PC12 cells. Cells were treated with MPP⁺ (500 μ mol/L) and/or EPO (1 U/mL) for 24 h. The levels of Bax and Bcl-2 were quantified by the densitometric analysis (A) and the Bax/Bcl-2 ratio was determined (B). These results are representative of three independent experiments. Data are expressed as means $\pm$ SD. * $P < 0.05$ vs MPP⁺ alone; [#] $P < 0.05$ vs control.

of Bcl-2 in the MPP⁺-treated group was significantly decreased compared with the control group. Also, expression of Bcl-2 was recovered with EPO treatment. The Bax/Bcl-2 ratio increased to 1.8-fold of control upon treatment with MPP⁺, while EPO prevented the MPP⁺-induced increase of Bax/Bcl-2 ratio (Fig. 7B).

3.6 Effect of EPO on MPP⁺-induced activation of caspase-3 Following 24 h treatment of PC12 cells with 500 μ mol/L of MPP⁺, we detected a (240.0 $\pm$ 14.6)% increase of caspase-3-like activity compared with the control. In contrast, PC12 cells simultaneously treated with EPO (0.1, 0.3, 1, 3, 10 U/mL) showed a significant decrease in caspase-3 activity compared with the MPP⁺-treated cells [(210.0 $\pm$ 20.0)%, (150.0 $\pm$ 12.5)%, (130.0 $\pm$ 14.4)%, (170.0 $\pm$ 13.1)%, and (220.0 $\pm$ 20.5)%, respectively, in different concentration groups] (Fig. 8). EPO alone did not show significant effect on the caspase-3 activity in PC12 cells. The results indicate that treatment with EPO resulted in inhibition of MPP⁺-induced activation of caspase-3.

Fig. 8 EPO inhibited MPP⁺-induced increase in caspase-3 activity. Values represent 3 replicate values in 4 separate experiments (means $\pm$ SD). * $P < 0.05$, ** $P < 0.01$ vs MPP⁺ alone; ^{##} $P < 0.01$ vs control.

4 Discussion

Researches during the past years have clearly demonstrated that EPO is a potent promoter of neuronal survival. Studies *in vitro* provided informations related to the molecular pathways involved in EPO action. These data showed that EPO may have a direct protective role against a variety of neurotoxic insults, such as hypoxic conditions^[11], glutamate toxicity^[12], free-radical injury^[13], and exposure to neurotoxicants^[14]. In addition, EPO receptor is abundantly expressed in adult dopaminergic neurons^[15], suggesting a direct effect of EPO on neurons. EPO can also protect dopaminergic neurons against the neurotoxicities of MPTP and 6-hydroxydopamine, and significantly reverse the corresponding behavioral deficits in mice^[8,10]. The protection of EPO in the central nervous system is mediated by a series of cellular pathways that maintain intricate links between one another. However, up to date, the mechanisms underlain the inhibitive effect of EPO on the neurotoxicity of MPP⁺ have not been fully understood. In order to explore this subject, we observed the effect of MPP⁺ on the PC12 cells retaining dopaminergic characteristics.

The present study showed that EPO exerted the neuroprotective effect in a concentration dependent manner against the cell viability loss and apoptosis induced by MPP⁺ in the PC12 cells. The neuroprotective effect of EPO reached maximum at 1 U/mL, while obviously decreased at larger doses. In accordance with others found in various cell culture models of neuronal cell death^[13,16], the survival-promoting action of EPO followed a bell-shaped dose-response curve in our *in vitro* paradigm. Increasing EPO concentration in the cell culture medium above an optimal

dose resulted in the decline of the cell rescue rate. In our experiments, EPO at concentrations higher than 1 U/mL (e.g. 10 U/mL) did not show stronger protective effects. Several studies have confirmed this particular dose-response behavior of EPO *in vivo*^[17,18], which might have relevance to the design of the upcoming clinical trials. As the recent human stroke study has demonstrated, EPO has beneficial effect on neurons in the context of cerebral ischemia^[19]. The clinical study about EPO could be further improved by optimizing the dose regimen in the future.

The subsequent experiments were designed to explore the neuroprotective mechanisms of EPO on MPP⁺-induced cell death in PC12 cells. A body of work has been generated to support the premise that MPP⁺ can directly lead to ROS formation and mitochondrial dysfunction^[20,21]. The antioxidants that could modulate ROS level have been shown to reduce apoptotic cell death in the dopaminergic cell lines and the PC12 cells treated with MPP⁺^[22]. EPO has been shown to afford the neuroprotection by its antioxidant effects^[6,23,24], which is further confirmed by our data that EPO is highly effective on inhibiting the MPP⁺-induced ROS formation and mitochondrial membrane potential loss in PC12 cells. The present results suggest that EPO attenuates the mitochondrial damage and cell death caused by oxidative stress in PC12 cells. Based on these findings, we postulated that the anti-oxidative properties of EPO may contribute to protecting PC12 cells from the damage of MPP⁺.

Some other mechanisms could also be pertinent to the protective effect of EPO. The PI3K/Akt pathway could be activated by EPO, and the survival promotion effect of EPO could be abolished by inhibiting this signaling pathway^[16]. Recently, PI3K and its downstream effector, the serine/threonine kinase (Akt), were reported to play roles in neuronal survival^[25,26]. For example, the overexpression of activated PI3K and Akt promoted the survival of superior cervical neurons in culture^[25], and activated Akt stimulated Bcl-2 and Bax expressions and showed the anti-apoptotic effect in several cell types, including hippocampal neurons and PC12 cells^[27,28]. Bcl-2 family has been known to play a key role in the mitochondrial apoptotic pathway^[29]. Bcl-2 has the anti-apoptotic property and is associated with the outer mitochondrial membrane^[30], where it maintains the integrity of mitochondrial membrane and inhibits the pro-apoptotic proteins being released from mitochondria^[31,32]. Because Bcl-2 is localized in the mitochondria^[30] (the active sources of ROS), it could prevent the depolarization of mi-

tochondrial membrane potential and the production of ROS by mitochondria, therefore prevent apoptosis^[33]. It was suggested that Bcl-2 overexpression may be involved in the inhibitive effect of EPO on the increased ROS concentration induced by MPP⁺ in the present study. In contrast, Bax is demonstrated to increase the ROS production in mitochondria^[34]. Cell survival in the early phases of apoptotic cascade depends mostly on the balance between the Bax and Bcl-2^[29]. EPO may exert its neuroprotective effect via differential regulating the expression of Bax and Bcl-2 to keep the balance between these two molecules^[35]. In neurons, Bcl-xL is shown to be strongly expressed after EPO is administrated^[36]. In addition, more recent work in cerebral microvascular endothelial cells (ECs) illustrates that upregulation of Bcl-xL by EPO may be necessary for preventing apoptosis^[37,38]. Our present study showed that MPP⁺ significantly increased the ratio of the pro-apoptotic Bax to the anti-apoptotic Bcl-2, which consists with the previous studies^[22,39]. However, EPO reduced the Bax expression and increased the Bcl-2 expression, thereby ameliorated the MPP⁺-induced Bax/Bcl-2 ratio elevation in PC12 cells. The results presented in this study further support the anti-oxidative effect of EPO on MPP⁺-induced apoptosis maybe, at least partly, mediated by regulating the expression of Bax and Bcl-2.

The increasing findings have shown that caspase-3 is activated during ROS exposure in MPP⁺-induced neurodegeneration^[22], which is consistent with our results. However, EPO effectively suppresses MPP⁺-induced activation of caspase-3, suggesting that EPO may act upstream of caspase-3 to block apoptosis. It is hypothesized that a compensatory induction of Bcl-2 was unable to counteract the apoptotic action of Bax, and thus resulted in caspase-3 activation and cell death^[40], which was closely associated to the pathogenesis of Parkinson's disease. From our own observation, a decrease in caspase-3 activity correlates well with a decrease in the Bax/Bcl-2 ratio. As pro-apoptotic Bax is thought to be upstream of caspase in the mitochondria-mediated apoptotic death pathway^[29], the mechanisms by which EPO inhibits MPP⁺-triggered activation of caspase-3 might include both its anti-oxidative activity and its regulatory function for Bcl-2 family.

In this study, we demonstrated that EPO afforded significant neuroprotection against MPP⁺-induced apoptosis in PC12 cells. EPO ameliorates MPP⁺-induced ROS production, attenuates the mitochondrial transmembrane

potential loss, downregulates the Bax/Bcl-2 ratio, and prevents the activation of caspase-3. These findings, taken together, support the theory that cytoprotection mediated by EPO is partly due to inhibition of the oxidative stress resulting from the mitochondrial apoptotic pathway.

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促红细胞生成素对 1- 甲基 -4- 苯基吡啶损伤的 PC12 细胞的保护作用

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摘要: 目的 探讨促红细胞生成素(erythropoietin, EPO)对 1-甲基-4-苯基吡啶离子(MPP⁺)诱导的 PC12 细胞变性损伤的保护作用及机制。方法 用 MPP⁺ 处理 PC12 细胞制作帕金森病细胞模型, 采用四甲基偶氮唑蓝法检测暴露于不同浓度 EPO 后细胞的活性; 流式细胞术与 DNA 断端原位标记法 (terminal deoxynucleotidyl transferase dUTP nick end labeling, TUNEL) 检测各组的细胞凋亡率; 免疫印迹法检测不同处理组 PC12 细胞 Bcl-2 和 Bax 的表达, 并采用荧光法观察不同处理组 PC12 细胞活性氧 (reactive oxygen species, ROS) 与线粒体膜电位水平以及 caspase-3 活性的变化。结果 MPP⁺ 可以使 PC12 细胞存活率下降, 凋亡率增高; 同时 PC12 细胞内 ROS 增多, 线粒体膜电位下降。MPP⁺ 还可以明显地提高 Bax/Bcl-2 比值并激活 caspase-3。而 EPO 可以抑制这些由 MPP⁺ 引发的改变, 并在 1 U/mL 时发挥最大保护作用。结论 EPO 可抑制 MPP⁺ 诱导的 PC12 细胞死亡, 其作用机制可能与其自身抗氧化和抗凋亡的特性有关。

关键词: 1- 甲基 -4- 苯基 - 吡啶; PC12 细胞; 促红细胞生成素; 氧化应激; 凋亡