1-Methyl-4-phenyl-pyridinium time-dependently alters expressions of oxoguanine glycosylase 1 and xeroderma pigmentosum group F protein in PC12 cells

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Abstract: Objective To determine if DNA excision repair enzymes oxoguanine glycosylase 1 (OGG1) and xeroderma pigmentosum group F protein (XPF) are involved in the pathogenesis of Parkinson's disease (PD) in a cell model. **Methods** PC12 cells were treated with 1-Methyl-4-phenylpyridine ion (MPP⁺) for various periods of time to induce oxidative DNA damage. MTT assay was used to determine cell viability. Immunocytochemistry with antibody against 8-hydroxy-2'-deoxyguanosine (8-oxodG) was used to evaluate oxidative DNA damage. Immunoblotting was used to detect the protein levels of OGG1 and XPF. **Results** MPP⁺ treatment (1 mmol/L) for 18 h and 24 h reduced cell viability to 78.6% and 70.3% of the control, respectively, in a time-dependent way. MPP⁺ increased the immunoreactivity of 8-oxodG in the cytoplasm at 3 h and in the nucleus at 24 h of treatment. With the treatment of MPP⁺, the expression of OGG1 was significantly increased at 1 h, reaching a peak at 3 h, and then it was decreased at 24 h, as compared to that with vehicle treatment. The same effect was exerted on XPF level, except that the XPF level reached a peak at 18 h of MPP⁺ treatment. Moreover, the maximally-increased protein level of OGG1 by MPP⁺ was approximately 2-fold higher than that of XPF. **Conclusion** MPP⁺ treatment could time-dependently induce increases in OGG1 and XPF expressions in PC12 cells. Also, this study indicates that the base and nucleotide excision repair pathways may be compensatorily activated in the early stage of pathogenesis in the cells after MPP⁺ treatment.

Keywords: 1-Methyl-4-phenyl-pyridinium; oxoguanine glycosylase 1; xeroderma pigmentosum group F protein; PC12 cells

1 Introduction

Parkinson's disease (PD), one of the most common progressive neurodegenerative disorders, is neuropathologically characterized by a selective and profound loss of dopaminergic neurons in the substantia nigra^[1]. Although the pri-

mary cause of PD remains unclear, there has been increasing evidence suggesting the involvement of oxidative stress in PD pathogenesis^[2-6]. The postmortem studies demonstrate that 8-hydroxy-2'-deoxyguanosine (8-oxodG), a biomarker of oxidative DNA damage, accumulates in substantia nigra neurons^[7-10].

Generally, oxidative DNA damage is repaired by base excision repair (BER)^[11]. An intact process of BER involves several steps that rely on many enzymes, among which oxoguanine glycosylase 1 (OGG1) is the most important. OGG1

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Article ID: 1673-7067(2010)01-0001-07
Received date: 2009-09-22; Accepted date: 2009-11-19

participates in the base recognition step and limits the rate of BER. It has been shown that OGG1 activity is inversely related to the level of 8-oxodG in brain regions^[12]. Alternatively, oxidative DNA damage can be repaired by nucleotide excision repair (NER)^[13-18], which is a major DNA repair pathway in removing a wide range of DNA damage including DNA double helix distortion^[19]. Approximately 20-30 proteins are involved in NER, participating in successive steps of damage recognition, local opening of the DNA double helix around the injury, incision of the damaged strand on either side of the lesion, and nascent DNA synthesis/ligation^[19]. XPF, a protein whose functional defect is closely related to xeroderma pigmentosum (XP) in human, functions principally as a structure-specific endonuclease in a form of heterodimer with ERCC1 in NER.

The involvement of DNA repair system in pathology of PD is supported by accumulating data. Postmortem investigations have shown higher expression levels of MutT homolog and human MutY homolog in the substantia nigra in PD brains^[9,20]. And both of these 2 enzymes are associated with error-avoiding mechanisms against oxidative DNA damage. For the more important BER pathway, the expression of OGG1 is greatly increased in early stage but not in advanced stage of PD^[21]. Furthermore, in a mouse model of PD, the activity of OGG1 is increased during 12-48 h after 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) injection in substantia nigra, but returns to base level at 72 h^[22]. These data indicate that BER level is elevated in the beginning and falls to base level at later stage after oxidative stress. However, it is not clear whether this fall implicates a decline of DNA damage or is just a discompensation of the repair system. To address this question, 1-Methyl-4-phenylpyridinium (MPP⁺) was added into cultured PC12 cells to induce oxidative damage. Besides, whether and how OGG1 and XPF would be altered in cultured PC12 cells after MPP⁺ treatment were also analyzed.

2 Materials and methods

2.1 Reagents and chemicals MPP⁺ was obtained from RBI (Sonoma, CA, USA). Dulbecco's Modified Eagle's Medium (DMEM), horse serum (HS), fetal bovine serum (FBS) and 3-

(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT) were all purchased from GIBCO Life Technologies (Gaithersburg, MD, USA). Poly-L-lysine, 3,3'-diaminobenzidine tetrachloride (DAB) and trypsin were from Sigma (St Louis, MO, USA). Polyvinylidene difluoride (PVDF) microporous membrane was obtained from Bio-Rad (Hercules, CA, USA). The monoclonal antibody against OGG1 was from Novus Biologicals (Littleton, CO, USA). The polyclonal antibody against XPF was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal antibody against 8-oxodG was purchased from Chemicon (Temecula, CA, USA).

2.2 PC12 cell culture and treatment PC12 cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in high-glucose DMEM supplemented with 10% FBS, 5% HS, 100 units/mL penicillin, 100 µg/mL streptomycin and 3.7 g/L NaHCO₃. Cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂ and subcultured every 2-3 d at approximately 70%-80% confluence. Throughout the experiments, cells were cultured in poly-L-lysine-coated plates, dishes or coverslips, and exposed to 1 mmol/L MPP⁺ for different hours. For MTT assay, cells were plated at a density of 2×10⁴ cells/well in 96-well dishes and treated with MPP⁺ for 1 h, 18 h, and 24 h, respectively. For 8-oxodG immunocytochemical analysis, cells were exposed to MPP⁺ for 3 h, 18 h, and 24 h, respectively, on coverslips in dishes at a density of 5×10⁵ cells/dish. For western blot analysis, cells were harvested at 1 h, 2 h, 3 h, 6 h, 12 h, 18 h and 24 h of MPP⁺ treatment in 6-well plates at a density of 5×10⁵ cells/well. Untreated cells were used as control.

2.3 Cell viability assay Cell viability was determined by MTT assay^[23], which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenase. Briefly, MTT was added to each well at a final concentration of 0.5 mg/mL, and the plates were further incubated for 4 h at 37 °C until the final detection. At the end point, the culture medium was removed and the purple formazan crystals were dissolved in 150 µL DMSO. The absorbance was read at 570 nm with a microplate reader (Tecan Sunrise TC, Männedorf, Switzerland), and the viability was normalized to the percentage of control viability.

2.4 8-oxodG immunocytochemistry After MPP⁺ exposure

for indicated hours, the medium was removed and the cells were rinsed immediately with 1×PBS at room temperature. After being fixed in 70% ethanol at -20 °C for 10 min on ice, PC12 cells were incubated with 100 µg/mL RNase A prepared in TEN buffer (10 mmol/L Tris-HCl, pH 7.4; 1 mmol/L EDTA, pH 7.6; and 400 µmol/L NaCl) for 60 min at 37 °C. Then, cells were incubated with 2 N HCl at room temperature for 10 min to denature DNA. After that, cells were neutralized with 50 mmol/L Tris-base for an additional 5 min. After being blocked with 10% FBS for 30 min at 37 °C, the cells were incubated with mouse anti-8-oxodG monoclonal antibody (1:300) for 1 h at 37 °C followed by overnight incubation at 4 °C. After that, the cells on the coverslips were exposed to 0.3% H₂O₂ for 30 min to exclude the endogenous peroxidase activity. The cells were then washed and incubated with biotinylated anti-mouse IgM (1:200) for 1 h. Subsequently, they were incubated with avidin-biotin peroxidase (1:200) for 1 h, and stained with 0.05% DAB in the presence of 0.03% H₂O₂. Cells in negative control group received the same treatment except that they were not exposed to the primary antibody. The 8-oxodG immunopositive cells were observed using optical scanning laser microscopy (TCS NT, Leica, Germany).

2.5 Western blot analysis After 1 mmol/L MPP⁺ treatment, PC12 cells were washed with ice-cold 1×PBS and lysed in RIPA buffer containing 1×PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L sodium orthovanadate and a protease inhibitor cocktail on ice for 30 min. And then protein lysates were centrifugated at 12 000 g for 20 min to remove the pellets. The supernatant was carefully transferred and measured using the BCA protein assay reagent. Subsequently, 50 µg protein of each sample was separated on 12% SDS-PAGE and electrophoretically transferred onto a PVDF microporous membrane. The membranes were then blocked with 10% skim milk in 10 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 1% Nonidet P-40 at room temperature (25 °C) for 1 h, and incubated with goat anti-XPF (1:500) polyclonal antibody and mouse anti-OGG1 (1:1 000) monoclonal antibody at 4 °C overnight. After being washed, membranes were incubated with horseradish peroxidase-conjugated IgG (1:3 000), and immunoreactivity was visualized by ECL system.

2.6 Statistical analysis Data were expressed as mean±SEM. Statistical analysis was determined using one-way analysis of variance (ANOVA) followed by student's *t*-test. *P* < 0.05 was considered to be statistically significant.

3 Results

3.1 MPP⁺ reduced cell viability and induced oxidative DNA damage in cultured PC12 cells In the present study, MTT assay was used to reveal the changes of cell viability. As shown in Fig. 1, MPP⁺ treatment time-dependently reduced the cell viability. The cell viability percentage was dramatically decreased to 78.6% and 70.3% of the control level at 18 h and 24 h of MPP⁺ treatment, respectively (*P* < 0.05).

To investigate the effect of MPP⁺ on cellular oxidative DNA damage, the formation of 8-oxodG, a biomarker of oxidative DNA stress, was detected by immunostaining in PC12 cells following MPP⁺ exposure for 3 h, 18 h and 24 h, respectively. As shown in Fig. 2, in the control group, few 8-oxodG immunopositive cells were observed and the immunoreactivity was mainly located in cytoplasm (Fig. 2A, E), indicating that DNA damage might occur in the mitochondria. Then, the 8-oxodG immunoreactivity (8-oxodG⁺) gradually increased in a time-dependent way. At 3 h of MPP⁺ treatment, the 8-oxodG⁺ signals increased and were limited to the cytoplasm (Fig. 2B, F). At 18 h, the 8-oxodG⁺ signals further in-

Fig. 1 Assessment of cell viability in PC12 cells following MPP⁺ exposure. MPP⁺ treatment caused the decrease of cell viability in PC12 cells in a time-dependent manner, compared to the vehicle group. Data were expressed as mean±SEM (*n*=4) and analyzed by one-way ANOVA. **P* < 0.05 vs control.

Fig. 2 8-oxodG immunostaining in cytoplasm and nuclei of the PC12 cells following MPP⁺ treatment for different hours. MPP⁺ time-dependently induced increases of 8-oxodG immunostaining in cytoplasm and nucleus in PC12 cells. The 8-oxodG immunoreactivity was first limited in the cytoplasm in the control (A, E) and the 3-h MPP⁺ treatment groups (B, F). With the increase of MPP⁺ exposure time, the 8-oxodG signals were transferred from cytoplasm to nucleus. The 8-oxodG immunopositive signals appeared in both the cytoplasm and the nucleus at 18 h (C, G) and only in the nucleus at 24 h (D, H) of MPP⁺ treatment. Photographs E-H were the magnifications of photographs A-D, correspondingly. Scale bar: 50 µm (A-D), scale bar: 25 µm (E-H).

creased and started to transfer to the nuclei from cytoplasm (Fig. 2C, G). Subsequently, at 24 h of MPP⁺ treatment, most of the surviving cells were positive for 8-oxodG, and the signals were located mainly in nucleus (Fig. 2D, H). These results demonstrate that MPP⁺ could induce oxidative DNA damage in a time-dependent manner, and this damage was transferred from cytoplasm to nuclei.

3.2 MPP⁺ time-dependently induced the expressions of OGG1 and XPF in cultured PC12 cells To investigate the effect of MPP⁺ on DNA excision repair, the expression levels of BER and NER rate limiting factors OGG1 and XPF, were determined, respectively, by Western blot analysis. Western blot (Fig. 3A) and the corresponding densitometric analysis (Fig. 3B) demonstrated that OGG1 expression was altered by MPP⁺ treatment. Compared with that of the control, OGG1 protein level in MPP⁺ group increased significantly by 5.0-fold ($P < 0.05$) at 1 h, by 5.4-fold at 2 h ($P < 0.05$), and by 6.3-fold at 3 h of the treatment ($P < 0.05$). Besides, OGG1 protein level began to decrease from 6 h, and at 24 h of treatment, it was below the control level.

Meanwhile, the level of NER factor XPF in the PC12 cells was also time-dependently changed by MPP⁺. As shown in Fig. 4, the XPF level significantly increased by 2.2-fold ($P < 0.05$)

at 1 h of treatment, reaching a peak of 3.9-fold increase at 18 h ($P < 0.05$) of MPP⁺ treatment. At 24 h, it was still higher than the control level, but with no significant difference (Fig. 4B).

Fig. 3 MPP⁺ treatment induced a significant increase in OGG1 protein level in PC12 cells in a time-dependent way. A: OGG1 levels were detected by western blot analysis. Cells were treated with vehicle (24 h) or MPP⁺ for different hours. B: Densitometric analysis showed that OGG1 expression level increased at 1 h, reaching a peak at 3 h, and then declined gradually. Data were from 4 separate measurements, and analyzed by one-way ANOVA followed by Student's *t*-test. * $P < 0.05$ vs the control.

Fig. 4 The time-dependent increases of XPF protein level in PC12 cells following MPP⁺ treatment. **A:** OGG1 expression in the cytoplasm of PC12 cells was detected by western blot analysis. Cells were treated with vehicle (24 h) or MPP⁺ for different hours as indicated in the obsica of the figure. **B:** Densitometric analysis showed that XPF protein level increased at 1 h, reaching a peak at 18 h of MPP⁺ treatment, and then it declined. Data were from 4 separate measurements, and analyzed by one-way ANOVA followed by Student's *t*-test. **P* < 0.05 vs the control.

4 Discussion

In the present study, we find that oxidative DNA damage and reduction of cell viability could be detected at 18 h of MPP⁺ treatment. Besides, increases in OGG1 and XPF expressions were detected at as early as 1 h of MPP⁺ treatment, and lasted at least 18 h. Moreover, up-regulation of OGG1 and XPF occurred earlier than DNA oxidative damage in MPP⁺-treated cells. Taken together, the present results suggest that both BER and NER DNA repair pathways are involved in the pathological process of neurodegeneration in this *in vitro* PD model established by MPP⁺ treatment. Comparing the time-course of cell viability changes with the expressions of DNA repair factors, we suggest that OGG1 and XPF may play compensatory roles in the early stage of degenerative process in the cells following MPP⁺ treatment.

It is well documented that oxidative stress contributes to neurodegeneration in the substantia nigra in PD patients, mechanisms of which are very complex^[2-6, 24-26]. In the present study, we established a cell model of PD using MPP⁺

treatment, according to previous descriptions^[27]. Using this model, we confirm that oxidative damage and cell death are both present in PD conditions, as indicated by accumulation of 8-oxodG⁺ signal, a biomarker of oxidative DNA damage^[7-10], and reduction of cell viability. Consistent with our previous studies^[27,28], MPP⁺/MPTP could induce oxidative DNA damage in the cytoplasm at early stage, which was then translocated into the nuclei. It is also worth mention that cell viability was reduced at 18 h of MPP⁺ treatment when 8-oxodG⁺ signal was translocated to the nuclei, suggesting that the presence of nuclei oxidative DNA damage may indicate the destination of cell death.

It is commonly known that DNA oxidative damage is repaired via activation of both BER and NER pathways, based on the severity of DNA damage^[13-16,29]. Theoretically, DNA repair function should be activated once DNA damage occurs in the cells. Here we used MPP⁺ treatment to induce oxidative damage as indicated by 8-oxodG staining (Fig. 2). Under this experimental condition, we propose that both BER and NER pathways are involved in the neuropathogenesis of PD, since MPP⁺ treatment could time-dependently up-regulate the expressions of OGG1 and XPF in PC12 cells (Fig. 3, 4). More interestingly, the up-regulation of both OGG1 and XPF expressions occurred when oxidative DNA damage appeared in the cytoplasm of the cells after MPP⁺ treatment, and disappeared when oxidative DNA damage existed in the nuclei of the cells. This phenomenon is consistent with a previous *in vivo* study^[22], which reports that MPTP injection induces OGG1 overexpression in the substantia nigra of rat brain at early periods (12 h and 48 h) but not at late time (72 h)^[22]. The balance between DNA damage and repair activation could determine the cell fate. Once the amount of damaged DNA exceeds the capacity of DNA repair system, the cells will die. This may help explain our present findings that the contents of OGG1 and XPF started to be back down when oxidative DNA damage became more severe and cell viability was reduced at later period of MPP⁺ treatment. Therefore, we speculate that the up-regulation of DNA repair enzymes in the early stage of MPP⁺ treatment may act as a cellular compensatory mechanism to counteract DNA damage.

In summary, the present results suggest that DNA exci-

sion repair levels could be regulated with accumulation of oxidative DNA damage after MPP⁺ treatment in PC12 cells. This study provides novel insights that changes of DNA excision repair function may be involved in the pathophysiological process of neuron degeneration following oxidative stress. Better understanding of the neuronal DNA repair mechanisms will help to develop new strategies to protect against neurodegenerative diseases.

Acknowledgement: This work was supported by the National Natural Science Foundation of China (No. 30770660, J0730860).

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1- 甲基 -4- 苯基吡啶时程依赖性改变 PC12 细胞内氧基 - 鸟嘌呤 DNA 糖苷酶和着色性干皮病蛋白 F 的表达

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摘要: **目的** 探讨离体条件下 DNA 剪切修复相关蛋白酶氧基 - 鸟嘌呤 DNA 糖苷酶 (OGG1) 和着色性干皮病蛋白 F (XPF) 是否参与帕金森病 (PD) 的病理过程。 **方法** 用 1 mmol/L 1-甲基-4-苯基吡啶 (MPP⁺) 处理 PC12 细胞不同时间, 诱导细胞产生 DNA 氧化损伤, 建立 PD 细胞模型。在此模型上, 采用 MTT 法检测细胞活力; 采用 8-氧基 -7,8-双氢脱氧鸟嘌呤 (8-oxodG) 免疫细胞化学技术检测细胞 DNA 氧化损伤; 用 Western blot 技术检测细胞内 OGG1 和 XPF 的蛋白表达水平。 **结果** MPP⁺ 时程依赖性地降低细胞活力, 18 h 和 24 h 后细胞活力分别降至对照组的 78.6% 和 70.3%。MPP⁺ 显著增加了 8-oxodG 免疫阳性反应, 在 3 h 时 8-oxodG 主要分布于细胞质, 在 24 h 时其主要位于细胞核内。此外, MPP⁺ 显著改变了 OGG1 和 XPF 的蛋白表达水平。在 MPP⁺ 处理 1 h 后, OGG1 和 XPF 蛋白表达水平均显著上调, 分别于 3 h 和 18 h 达到顶峰, 随后依次下降, 且 OGG1 的峰值几乎是 XPF 的两倍。 **结论** MPP⁺ 时程依赖性地增加了 PC12 细胞内 XPF 和 OGG1 的表达水平。本研究提示, 在 MPP⁺ 致 PD 的病理模型早期, 碱基切除修复和核苷酸剪切修复可能被代偿性地激活。

关键词: 1- 甲基 -4- 苯基吡啶; 氧基 - 鸟嘌呤 DNA 糖苷酶; 着色性干皮病蛋白 F; PC12 细胞