Damage to dopaminergic neurons is mediated by proliferating cell nuclear antigen through the p53 pathway under conditions of oxidative stress in a cell model of Parkinson's disease

DA-WEI LI[1,2], GUANG-REN LI[3], BEI-LIN ZHANG[2], JING-JING FENG[2] and HUA ZHAO[1,2]

[1]Neuroscience Research Center, The First Hospital of Jilin University; [2]Department of Physiology, College of Basic Medical Sciences, Jilin University; [3]Department of Neurology, The Third Hospital of Jilin University, Changchun, Jilin 130021, P.R. China

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Abstract. Oxidative stress is widely considered as a central event in the pathogenesis of Parkinson's disease (PD). The mechanisms underlying the oxidative damage-mediated loss of dopaminergic neurons in PD are not yet fully understood. Accumulating evidence has indicated that oxidative DNA damage plays a crucial role in programmed neuronal cell death, and is considered to be at least partly responsible for the degeneration of dopaminergic neurons in PD. This process involves a number of signaling cascades and molecular proteins. Proliferating cell nuclear antigen (PCNA) is a pleiotropic protein affecting a wide range of vital cellular processes, including chromatin remodelling, DNA repair and cell cycle control, by interacting with a number of enzymes and regulatory proteins. In the present study, the exposure of PC12 cells to 1-methyl-4-phenylpyridinium (MPP^+) led to the loss of cell viability and decreased the expression levels of PCNA in a dose- and time-dependent manner, indicating that this protein may be involved in the neurotoxic actions of MPP^+ in dopaminergic neuronal cells. In addition, a significant upregulation in p53 expression was also observed in this cellular model of PD. p53 is an upstream inducer of PCNA and it has been recognized as a key contributor responsible for dopaminergic neuronal cell death in mouse models of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD. This indicates that MPP^+-induced oxidative damage is mediated by the downregulation of PCNA through the p53 pathway in a cellular model of PD. Thus, our results may provide some novel insight into the molecular mechanisms responsible for the development of PD and provide new possible therapeutic targets for the treatment of PD.

Correspondence to: Professor Hua Zhao, Neuroscience Research Center, The First Hospital of Jilin University, 126 Xinmin Street, Changchun, Jilin 130021, P.R. China
E-mail: zhua@jlu.edu.cn

Abbreviations: PD, Parkinson's disease; ROS, reactive oxygen species; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PCNA, proliferating cell nuclear antigen; DCF, 2'7'-dichlorodihydrofluorescein

Key words: Parkinson's disease, 1-methyl-4-phenylpyridinium, reactive oxygen species, apoptosis, proliferating cell nuclear antigen, p53

Introduction

Parkinson's disease (PD) is a common and chronic neurodegenerative disorder caused by the selective and progressive loss of dopaminergic neurons in the substantia nigra, leading to a depletion of the dopamine neurotransmitter in the striatum (1). While the etiology remains unclear, environmental toxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; a lipophilic molecule that rapidly crosses the blood-brain barrier), have been suggested to be involved in the pathogenesis of PD (2-5). Having crossed the barrier, it is oxidized in the brain to its toxic metabolite, 1-methyl-4-phenylpyridinium (MPP^+), by monoamine oxidase type B (MAO-B) (6). MPP^+ then enters the dopaminergic neurons via the dopamine transporter and is transported to the mitochondria where it causes the inhibition of mitochondrial respiration and energy depletion, by interacting with the respiration complex I (7), leading to reactive oxygen species (ROS) production (8-11). ROS production is widely recognized as a major initiator triggering sequential events leading to the degeneration of dopaminergic neurons (12-15). Postmortem studies of the brains of patients with PD have shown increased levels of 4-hydroxy-2-nonenal (HNE), a by-product of lipid peroxidation (16,17) and carbonyl modifications of soluble proteins (18), supporting the involvement of oxidative damage in dopaminergic neuron degeneration. The well-known parkinsonism inducers, MPTP, rotenone and 6-hydroxydopamine (6-OHDA) have been shown to cause ROS production and the degeneration of dopaminergic neurons in animal models, further supporting the involvement of oxidative stress in the pathogenesis of PD (19-23). The molecular mechanisms responsible for the gradual loss of dopaminergic neurons under conditions of oxidative stress are not yet fully understood.

However, DNA damage-mediated cell death has been suggested to be associated with neuronal cell death in PD (24). This is supported by analyses showing selective increases in levels of DNA and RNA oxidation products, 8-hydroxy-

deoxyguanosine and 8-hydroxy-guanosine, in the substantia nigra during postmortem studies of brains affected by PD (25,26). Oxidative DNA damage has also been observed in the brains tissue of mice exposed to MPTP and other neuronal toxins that induce a PD-like pathology (27). Proliferating cell nuclear antigen (PCNA) is a well known determinant of DNA biological function, including DNA replication and repair, as well as cell cycle control (28,29), and thus plays a crucial role in maintaining in the integrity of the genome, as well as cell survival. Previous studies have shown that PCNA plays a role in the repair of DNA damage under conditions of oxidative stress (30,31). In this study, we examined the changes in the levels of this protein in MPP^+-stimulated PC12 cells, in order to identify potential causes of dopaminergic neuron degeneration and to elucidate the underlying molecular mechanisms. Our results demonstrated that MPP^+ induced the loss of cell viability and the apoptosis of dopaminergic neuronal cells, in a time- and dose-dependent manner. MPP^+ also decreased PCNA protein expression, and this was accompanied by the impairment of PC12 cells, suggesting a correlation between the levels of this protein and damage to PC12 cells under conditions of oxidative stress. Notably, MPP^+ induced the significant upregulation of p53 expression, which is an upstream modulator of PCNA and has been recognized as a key contributor responsible for dopaminergic neuronal cell death in mouse models of MPTP-induced PD (32,33). Overall, these findings indicate that PCNA may play a crucial role in oxidative stress-induced damage to dopaminergic neurons, thus, providing a therapeutic target for molecular-based strategies in the treatment of PD.

Materials and methods

Drugs and chemicals. All reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Cell culture. The rat adrenal pheochromocytoma cell line, PC12, was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 4 mM L-glutamine and 10% inactivated fetal serum, (Gibco, Grand Island, NY, USA). The cultures were maintained in a 5% CO_2 incubator at 37˚C.

Cell viability assay. The viability of the PC12 cells was evaluated using the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT is reduced by metabolically active cells to form blue formazan crystals. The PC12 cells were plated on 96-well plates at a density of 30,000 cells/cm^2 and incubated for 24 h. To assess the toxicity of MPP^+ to the PC12 cells, the cells were exposed to various concentrations of MPP^+ (0.5, 1 and 2 mM). MTT solution (5 mg/ml) was then added to each well and the cells were incubated for 4 h. The culture medium was removed, and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance of the colored solution was measured at 570 nm using a microplate reader (Epoch; BioTek, Winooski, VT, USA). The results are expressed as a percentage of the absorbance of the control culture wells (cells not exposed to MPP^+). The experiment was repeated 3 times.

Nuclear staining assay. Morphological changes in the cell nuclei induced by MPP^+ were evaluated using acridine orange/ethidium bromide (AO/EB) staining. The PC12 cells were plated on 6-well plates at a density of 30,000 cells/cm^2 and incubated in DMEM for 24 h. Following exposure to 1 mM MPP^+ for 48 h, the cells were washed and resuspended in phosphate-buffered saline (PBS) followed by the addition of AO/EB (final concentration 1 μg/ml). The cells were then examined under a fluorescence microscope (IX71; Olympus Corp., Tokyo, Japan). Living cells with intact structures were stained green, whereas early apoptotic cells exhibited condensed green nuclei, and late apoptotic cells exhibited condensed red-orange chromatin. At least 300 cells were randomly observed and the number of apoptotic cells is expressed as a percentage of the total number of cells counted.

DNA fragmentation assay. DNA denaturation in the apoptotic cells was determined by a single-stranded DNA (ssDNA) assay using an ssDNA apoptosis enzyme-linked immunosorbent assay (ELISA) kit (Chemicon International, Temecula, CA, USA), according to the manufacturer's instructions. This procedure is based on the ability of a monoclonal antibody to detect ssDNA in apoptotic cells but not in necrotic cells. The cells at a concentration of 30,000 cells/cm^2 were cultured for 24 h, followed by treatment with various concentrations (0.5, 1, 2 mM) of MPP^+. Following 24 h of incubation, the staining of ssDNA was performed, and ssDNA fragmentation was determined by measuring the absorbance at a wavelength of 405 nm using a microplate reader (Epoch; BioTek).

Measurement of oxidative stress. Oxidative stress was measured in the PC12 cells using 2'-7'-dichlorofluorescein diacetate (DCFH-DA) based on the ROS-dependent oxidation of DCFH-DA to fluorescent dichlorofluorescein (DCF). DCFH-DA easily crosses the membrane into cells and is converted into non-fluorescent dichlorofluorescein (DCFH) by intracellular esterase. DCFH is then oxidized into highly fluorescent DCF by intracellular ROS, thereby the density of fluorescence reflects an overall index of oxidative activity. Following exposure, the cells were incubated in bovine serum albumin (BSA)-free DMEM with DCFH-DA at a final concentration of 20 μM for 30 min at 37˚C. Thereafter, the cells in each group were analyzed by flow cytometry using the FL1 flow cytometer detection channels (BD Biosciences, San Jose, CA, USA). The excitation wavelength was 485 nm and the reading was performed at 530 nm.

Western blot analysis. Following exposure to MPP^+, the PC12 cells were harvested and lysed with cell lysis solution containing 4% sodium dodecyl sulfate (SDS), 2 mM EDTA, 50 mM Tris-HCl (pH 6.8). Equal amounts of protein were loaded onto a 12% SDS-polyacrylamide gel. Following electrophoretic separation, the gels were transferred onto PVDF membranes (Amersham Biosciences, Uppsala, Sweden). The membranes were subsequently incubated in Tris-buffered saline/Tween-20 (TBST) buffer supplemented with 5% fat-free milk for 1 h. The membranes were then blotted with mouse monoclonal anti-rat PCNA antibodies (Cat. no. 610664; BD Biosciences) and mouse monoclonal anti-rat p53 antibodies (Cat. no. 554157; BD Biosciences), and horseradish peroxidase-conjugated anti-mouse secondary antibodies (Cat. no. R-21455; Pierce Biotechnology, Inc.,

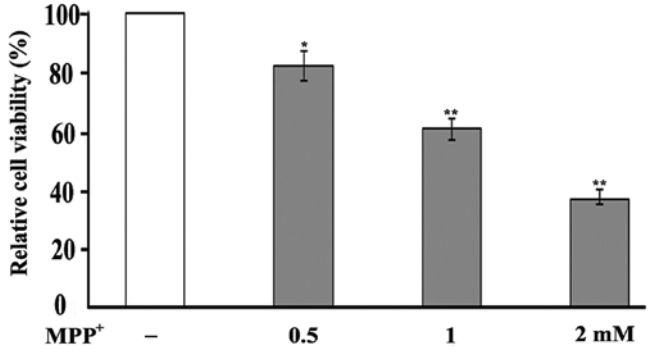

Figure 1. 1-Methyl-4-phenylpyridinium (MPP^+) reduces the viability of PC12 cells. PC12 cells were exposed to the indicated concentrations of MPP^+ for 48 h. Cell viability was determined by an MTT assay as described in the Materials and methods. Data are presented as the means ± SEM of 3 independent experiments performed in sixplicate. $^*P<0.05$ or $^{**}P<0.01$ vs. control (no exposure).

Rockford, IL, USA) were used as the secondary antidodies. β-actin was used as an internal control.

Statistic analysis. Data are expressed as the means ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) or a Student's t-test. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

MPP^+ induces the loss of the viability of PC12 cells. The oxidative damage induced by MPP^+ to the PC12 cells was examined by MTT assay, a colorimetric assay used for measuring the activity of mitochondrial dehydrogenases in metabolically active cells. The measurements revealed a decrease in cell viability following the exposure of PC12 cells to MPP^+ in a dose-dependent manner. Following 48 h of exposure to 0.5 mM MPP^+, cell viability was reduced to 82% of the control, while exposure to 1 and 2 mM MPP^+ decreased cell viability to 61 and 37% of the control, respectively (Fig. 1).

MPP^+ induces the apoptosis of dopaminergic neurons. To examine the MPP^+-induced apoptosis of the PC12 cells, an AO/EB staining assay and a DNA fragmentation assay were performed. Apoptosis is a process of programmed cell death characterized by a series of distinct nuclear morphological changes, which can be detected by AO/EB staining. Exposure to MPP^+ significantly increased the percentage of apoptotic cells in a concentration-dependent manner (Fig. 2). To further examine the toxic effects of MPP^+ on the PC12 cells, DNA fragmentation was investigated by an ssDNA assay. The results revealed an increase in DNA fragmentation following exposure to MPP^+ (Fig. 3), thus incating that MPP^+ is toxic to PC12 cells.

MPP^+ induces the production of ROS. To determine whether MPP^+-induced damage is mediated by oxidative damage in PC12 cells, the level of ROS production was evaluated by flow cytometry with DCFH-DA. DCFH-DA is a stable compound that easily diffuses into cells where it is converted into DCFH by intracellular esterase. DCFH is then trapped within cells and oxidized to highly fluorescent DCF by intracellular ROS; thereby, the intensity of the fluorescence produced by DCF may reflect an intracellular oxidative state. Exposure to MPP^+ induced a significant increase in DCFH oxidation in the PC12 cells (Fig. 4), which supports the hypothesis that oxidative damage is involved in the degeneration of dopaminergic neurons.

MPP^+ decreases PCNA expression in dopaminergic neuronal cells. To determine whether PCNA is involved in dopaminergic neuronal cell death under conditions of oxidative stress, the

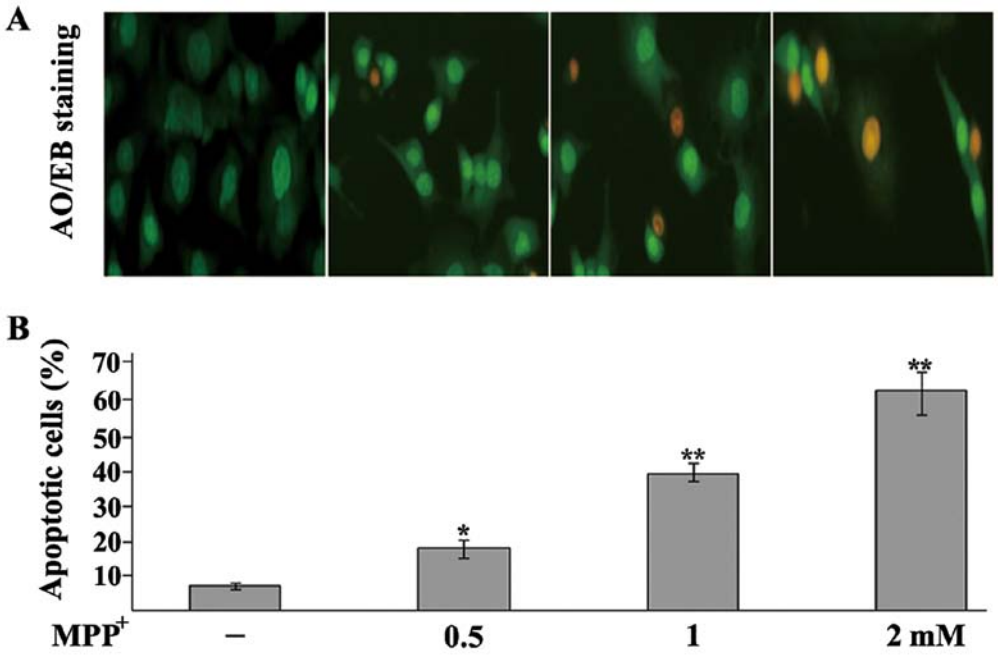

Figure 2. Effect of 1-methyl-4-phenylpyridinium (MPP^+) on neuronal cell apoptosis. (A) PC12 cells were exposed to the indicated concentrations of MPP^+ for 48 h, then apoptosis was analyzed using the acridine orange/ethidium bromide staining (AO/EB) assay. (B) The histograms show the percentage of apoptotic cells. Data are presented as the means ± SEM. n=3 experiments. $^*P<0.05$ or $^{**}P<0.01$ vs. control (no exposure).

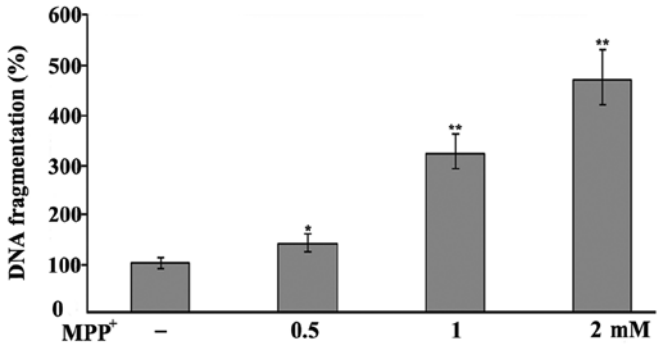

Figure 3. Determination of the effects of MPP+ on the apoptosis of PC12 cells using a single-stranded DNA assay. The histograms show the percentage of cells with DNA fragmentation. Data are presented as the means ± SEM. n=3 experiments. *P<0.05 or **P<0.01 vs. control (no exposure).

expression levels of PCNA were measured in a cellular model of MPP+-induced PD. Firstly, we examined whether MPP+ induced toxicity to the PC12 cells in a time-dependent manner. The PC12 cells were exposed to 1 mM MPP+, and then cell viability was determined after 12, 24 and 48 h by an MTT assay. The results revealed that exposure of the PC12 cells to 1 mM MPP+ for 12 h caused cell viability to decrease to 87% of the control, whereas exposure for 24 and 48 h decreased cell viability to 77 and 61% of the control, respectively (Fig. 5A). In addition, exposure to 1 mM MPP+ reduced PCNA protein expression in the PC12 cells. Consistent with the changes observed in cell viability, PCNA expression was decreased from 12 h and further decreased until 48 h following exposure to 1 mM MPP+ in the PC12 cells (Fig. 5B), thus indicating that PCNA is involved in the MPP+-induced degeneration of dopaminergic neurons.

MPP+ increases the epression of p53 in dopaminergic neurons. In order to elucidate the mechanisms through which MPP+ decreases PCNA expression in MPP+-exposed PC12 cells, we examined a well-known PCNA upstream regulator, p53. p53 has been suggested to play a pivotal role in dopaminergic neuronal cell death in a mouse model of MPTP-induced PD (33,34), and transcriptional activation is the principal mechanism through which PCNA expression is regulated. Our results revealed that p53 expression was upregulated following exposure to 0.5 mM MPP+ and further upregulated following exposure to 1 and 2 mM MPP+ in the PC12 cells (Fig. 6A). In contrast to p53 expression, PCNA expression was decreased in a dose-dependent manner following exposure to the indicated

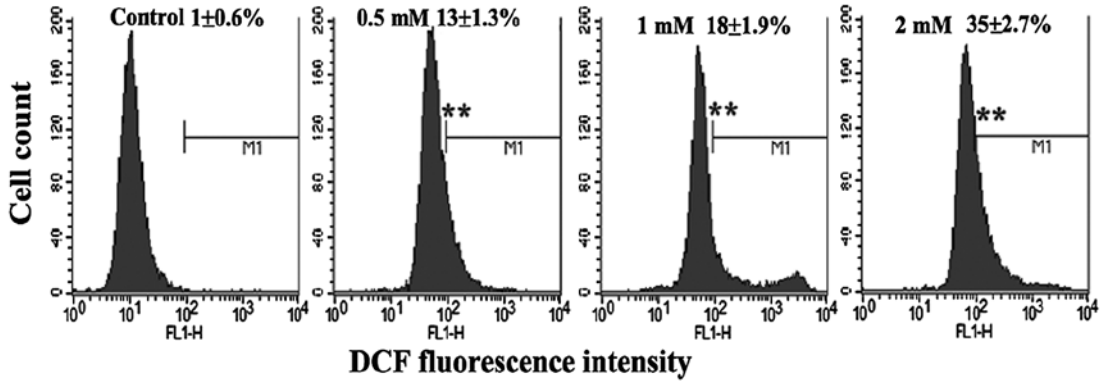

Figure 4. Effects of 1-methyl-4-phenylpyridinium (MPP+) on reactive oxygen species (ROS) production. PC12 cells were exposed to the indicated concentrations of MPP+ for 48 h. The production of ROS in the cells was measured by flow cytometry with DCFH-DA. The graphs display the relative levels of ROS production compared with the control (cells not exposed to MPP+). Data are presented as the means ± SEM. n=3 experiments. **P<0.01 vs. control.

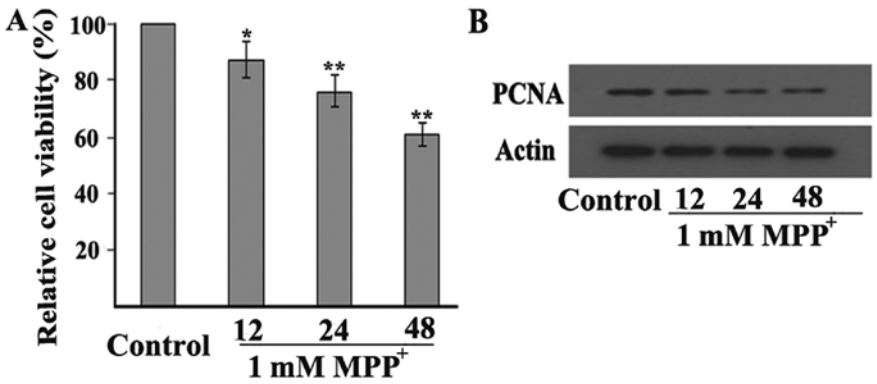

Figure 5. 1-Methyl-4-phenylpyridinium (MPP+) reduces cell viability and proliferating cell nuclear antigen (PCNA) expression. (A) PC12 cells were exposed to 1 mM MPP+, then the cell viability was determined after 12, 24 and 48 h using an MTT assay. Data are presented as the means ± SEM of 3 independent experiments performed in sixplicate. Error bars represent the means ± SEM (*P<0.05 and **P<0.01). (B) PCNA protein expression in PC12 cells exposed to 1 mM MPP+ for the indicated periods of time was determined by western blot analysis. Actin expression was used as a loading control.

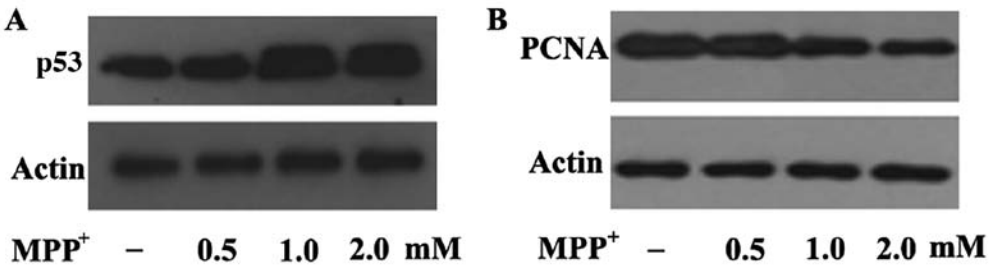

Figure 6. Expression levels of proliferating cell nuclear antigen (PCNA) and p53 in 1-methyl-4-phenylpyridinium (MPP^+)-exposed PC12 cells. PC12 cells were exposed to 1 mM MPP^+ for 48 h. The expression levels of (A) p53 and (B) PCNA were determined by western blot analysis, and actin expression was used as a loading control.

concentrations of MPP^+ (Fig. 6B), suggesting a negative correlation between p53 and PCNA expression under conditions of oxidative stress.This further indicates that MPP^+-induced oxidative damage is mediated by the downregulation of PCNA through the p53 pathway in a cellular model of PD.

Discussion

In this study, we demonsrated that the pleiotropic protein PCNA is involved in the damage to dopaminergic neurons in neurodegenerative conditions, and that the downregulated expression of this protein may be mediated by the p53 signaling pathway. PCNA is an essential protein involved in DNA replication and repair (35), and the dysregulation of its expression may aggravate oxidative stress-induced DNA damage, a central event involved in the neuronal cell death in PD (25). The downregulation of PCNA is at least partly responsible for the DNA damage-mediated death of dopaminergic neurons, thus providing a potential target for the molecular-based therapeutic management of PD.

PD is a common neurodegenerative movement disorder, clinically characterized by rigidity, resting tremor, bradykinesia and postural instability, caused by the degeneration and death of dopaminergic neurons in the pars compacta of the substantia nigra (36). Although the cellular and molecular mechanisms underlying the loss of dopaminergic neurons in PD remain unclear, accumulating evidence indicates that increased levels of oxidative stress play a crucial role in triggering a programmed cell death cascade, involved in the pathogenesis of PD (24,37). Oxidative damage is a pathological event responsible for a number of human diseases, including cardiovascular, metabolic, inflammatory and neurodegenerative diseases, as well as cancer (25,27,38-41). Dopaminergic neurons are more prone to oxidative damage due to high levels of lipids, iron as well as dopamine metabolism (42-51). Oxidative stress is mainly elicited by the excessive production of ROS, including hydrogen peroxide (H_2O_2), superoxide anion and hydroxyl radical (52-54). The overproduction of ROS damages nucleic acids, including DNA and RNA, finally causing cell death. This pathological mechanism is thought to be at least partly responsible for the death of dopaminergic neurons in PD. Postmortem studies on PD-affected brains and on brain tissue from mice exposed to MPTP and other neuronal toxins that induce a PD-like pathology have shown increased oxidative DNA damage, selectively targeting dopaminergic neurons of substantia nigra pars compacta (25,27), strongly implicating DNA damage-induced cell death as a causative factor of PD. DNA is the most important determinant of cell survival and death. Replication and repair are required for DNA integrity, since DNA is frequently subjected to damage by endogenous and environmental toxic agents (55). Under pathological conditions, numerous mechanisms are involved in DNA repair to protect against DNA damage (56,57). PCNA is an essential protein in DNA replication, and its function was originally described as the auxiliary protein of DNA polymerases (35). However, PCNA has also been shown to affect multiple vital cellular processes, including chromatin remodeling, DNA repair and cell cycle control (35,58). PCNA has no intrinsic enzymatic activity, and its complex role in cells depends on its capacity to regulate other proteins. PCNA interacts with a wide range of enzymes and regulatory proteins, such as cyclin-dependent kinases (CDKs) (59) or the CDK inhibitor p21/waf1 (60), which allows this protein to modulate a wide range of biological functions. In differentiated neutrophils, for example, it was found that cytoplasmic PCNA sequesters procaspases and prevents their activation, promoting the cell survival (61). Additionally, PCNA plays a crucial role in the repair of DNA damage under conditions of oxidative stress (30,31). In this study, we found that the neurotoxin, MPP^+, a well-established inducer of parkinsonism-like symptoms in humans and primates, induced an increase in ROS productino and in the number of apoptotic dopaminergic neurons, supporting the involvement of oxidative stress in the pathogenesis of PD. Importantly, exposure to MPP^+ also decreased the expression level of PCNA in a time- and dose-dependent manner, suggesting the involvement of PCNA in MPP^+-induced neuronal toxicity in PD. The downregulation of this protein may aggravate DNA damage under pathological conditions due to the crucial role of PCNA in maintaining DNA integrity against various insults including oxidative damage. However, the mechanisms responsible for this change in PCNA expression in a cellular model of MPP^+-induced PD remain unclear.

The transcription factor p53 modulates a set of target genes that are involved in a wide range of cellular processes, including cell cycle progression, DNA repair, apoptosis and cellular stress responses (62-65). p53-dependent apoptosis in neuronal cells is mainly mediated by DNA damage (66,67). The overproduction of ROS activates p53, leading to further DNA damage under conditions of oxidative stress. It is well known that p53 is an upstream inducer of PCNA. The interaction of p53 with the PCNA promoter, the specific sequence for the p53 binding site, regulates the production of this protein. Higher concentra-

tions of wild-type p53 inhibit the PCNA promoter and reduce PCNA expression (68,69). Evidence has indicated that p53 is upregulated and plays a pivotal role in dopaminergic neuronal cell death in mouse models of MPTP-induced PD (33,34). It has been demonstrated that p53 inhibitors are highly effective in reducing damage to dopaminergic neurons and in preserving motor function in a mouse model of PD (33). Consistent with these reports, our results demonstrated that MPP^+ significantly increased p53 expression in dopaminergic neuronal cells, supporting the involvement of p53 in the pathogenesis of PD. In addition, a decrease in PCNA expression was also observed in the cells exposed to MPP^+, and this expression pattern is in contrast to that of p53 expression, suggesting a negative correlation between p53 and PCNA expression under conditions of oxidative stress. Taken together, these findings suggest that a PCNA-dependent apoptotic pathway is a potential molecular mechanism that is involved in neuronal cell death in PD, and the p53 signaling pathway is also implicated in this process.

In the present study, we present evidence that MPP^+-induced oxidative damage is mediated by the downregulation of PCNA through the p53 pathway in a cellular model of PD. The cellular and molecular mechanisms responsible for the effects of PCNA on dopaminergic neurons require further elucidation, and may provide a potential and efficient therapeutic target for molecular-based strategies for the treatment of PD.

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