Knockdown of cytosolic NADP⁺-dependent isocitrate dehydrogenase enhances MPP⁺-induced oxidative injury in PC12 cells

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INTRODUCTION

Parkinson's disease (PD) is a relatively common neurodegenerative disorder manifested by bradykinesia, rest tremor, rigidity, gait abnormalities, and postural disturbance (1). A variety of markers and indices in PD patients and animal models indicate the involvement of reactive oxygen species (ROS) and oxidative stress in the pathogenesis of PD. These include lipid peroxidation (2, 3), reduced glutathione (4), increased levels of iron, and the reduction of ferritin levels in the substantia nigra pars compacta of PD patients (5). Exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces PD-like symptoms as well as neurotoxicity in humans and animal species (6). MPTP is metabolized in the brain by monoamine oxidase-B to its toxic metabolite, 1-methyl-4-phenylpyridium ion (MPP⁺), which is then selectively taken up into dopaminergic neurons (7). MPTP and its toxic metabolite MPP⁺ induce rat lung in a manner similar to that of a structurally-related toxicant, paraquat (8). Therefore, ROS are implicated in the mechanism of MPP⁺-induced neurotoxicity in animals (9).

Antioxidant enzymes, which protect against oxidative damage induced by ROS, could be susceptible to the damaging effects of MPP⁺. It is implied that the inactivation of antioxidant enzymes by MPP⁺ may result in the disruption of cellular redox homeostasis and subsequently exacerbate the harmful effects of MPP⁺ as well as ROS. Cytosolic (IDPc) and mitochondrial NADP⁺-dependent isocitrate dehydrogenase (IDPm) catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate, thus generating NADPH (10). NADPH is an essential co-factor in the reduction of glutathione by glutathione reductase (11). In addition, NADPH is a reducing equivalent for the activity of the NADPH-dependent thioredoxin system, which also confers cellular protection against oxidative damage (12). It is also required to maintain the antioxidant enzyme catalase in its active form (13). Glucose 6-phosphate dehydrogenase (G6PDH), a key enzyme in the pentose phosphate pathway, has long been regarded as the major enzyme generating cytosolic NADPH. However, it has been reported that IDPc in rat liver has an approximately 20-fold higher specific activity than G6PDH (14). Recently, we demonstrated that IDPc protects cells against various types of oxidative stress (15-17). In this aspect, the attenuated activity of IDPc may result in the disruption of the antioxidant defense system and subsequently lead to a pro-oxidant condition.

In this report, we used PC12 cells transfected with IDPc small interfering RNA (siRNA) to assess the role of IDPc in cellular defense against MPP⁺-induced oxidative injury. The data suggest that IDPc plays an important role in protecting against oxidative damage induced by MPP⁺.

RESULTS AND DISCUSSION

Knockdown of PC12 IDPc by siRNA

Since MPP⁺ induces an increase in ROS and IDPc is susceptible to modification by ROS (18), modulation of IDPc activity in PC12 cells exposed to MPP⁺ was measured. When PC12 cells were treated with 1 mM MPP⁺ for 2 days, cell-derived IDPc activity was significantly attenuated, and this inhibition
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was not induced by fragmentation or degradation of the enzyme (Fig. 1A). In order to elucidate the effect of suppressed IDPc activity on MPP⁺-induced cytotoxicity in PC12 cells, siRNA methodology was employed. The silencing of gene expression by siRNA duplex is a powerful tool for the study of gene function in vivo (19). To examine whether or not the transient transfection of human IDPc siRNA can selectively knockdown IDPc gene expression, we assayed the activity and protein expression of IDPc in PC12 cells. Cells transfected with IDPc siRNA displayed significantly lower levels of IDPc protein and IDPc activity. When the IDPc siRNA-transfected PC12 cells were treated with 1 mM MPP⁺ for 2 days, decreased IDPc activity was significant (Fig. 1B). When cultured PC12 cells were treated with 1 mM MPP⁺ for 2 days, cells transfected with IDPc siRNA were significantly more sensitive than control cells transfected with scrambled siRNA. The antioxidant N-t-butyl hydroxyamine (NtBHA) (20) effectively protected against viability loss of MPP⁺-treated cells (Fig. 1C).

**Effects of IDPc siRNA on MPP⁺-induced oxidative damage in PC12 cells**

To obtain association between cell death and ROS formation in the control and IDPc siRNA-transfected cells exposed to MPP⁺, the levels of intracellular peroxides in PC12 cells were determined with xylenol orange. Fig. 2A illustrates that the intracellular level of peroxides in IDPc siRNA-transfected PC12 cells were significantly higher than those in control cells when cells were treated with 1 mM MPP⁺ for 2 days. The occurrence of protein oxidation and lipid peroxidation was evaluated as a marker indicative of oxidative damage to the cells. Oxidative stress is known to introduce carbonyl groups into the amino acid side chains of proteins, in particular lysine, arginine, proline, and threonine residues (21). Besides, it is well established that oxidative stress in various cells usually leads to the accumulation of potent, cytotoxic lipid peroxidation products such as malondialdehyde (MDA) and 4-hydroxynonenal (22). Both the levels of carbonyl groups in proteins and MDA of the IDPc siRNA-transfected cells were significantly higher than those of control cells. Taken together, these results indicate that the suppressed activity of IDPc by siRNA enhances MPP⁺-induced cellular oxidative damage.

**Effects of IDPc siRNA on apoptosis induced by MPP⁺**

Although ROS induce necrotic cell death at higher concentrations, lower concentrations of ROS induce apoptotic cell death (23). We hypothesized that the knockdown of IDPc expression in PC12 cells would decrease cellular reduction po-

![Fig. 1](https://bmbreports.org)
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Potential and increase ROS formation, thereby inducing apoptosis at lower concentrations of ROS. To evaluate the relationship between IDPc activity and MPP⁺-induced apoptotic cell death, the effect of transfection of IDPc siRNA on the cellular features of apoptosis was studied. As expected, the extent of DNA fragmentation following treatment with 0.2 mM MPP⁺ for 2 days was more apparent in the IDPc siRNA-transfected PC12 cells than in the control cells (Fig. 3A). To measure the possible perturbation of cell cycle regulation by IDPc siRNA, we analyzed the effects of IDPc siRNA on PC12 cells exposed to MPP⁺ by flow cytometry. When PC12 cells were exposed to 0.2 mM MPP⁺ for 2 days, a sub-G1 peak in the DNA histogram increased for the IDPc siRNA-transfected cells as compared to the control cells (Fig. 3B). Measurement of caspase-3 activity with a specific chromogenic substrate, Ac-DEVD-pNA, confirmed that the activation of caspase-3 by MPP⁺ was en-

Fig. 2. Effects of IDPc siRNA on cellular redox status and oxidative damage in PC12 cells exposed to MPP⁺. (A) The levels of peroxides in PC12 transfectant cells was determined by xylene orange. Data are presented as means ± S.D. of three separate experiments. *P < 0.01 versus control scrambled siRNA-transfected cells exposed to MPP⁺. (B) Protein carbonyl content was determined in cell-free extracts using DNPH. Data are presented as means ± S.D. of three separate experiments. **P < 0.05 versus control scrambled siRNA-transfected cells exposed to MPP⁺. (C) Lipid peroxidation of PC12 cells after exposure to MPP⁺. The levels of MDA accumulated in the cells were determined by a TBARS assay. Data are presented as means ± S.D. of three separate experiments. **P < 0.05 versus scrambled siRNA-transfected cells exposed to MPP⁺.

Fig. 3. MPP⁺-induced apoptosis in IDPc siRNA transfectant PC12 cells. (A) PC12 cells were treated with 0.2 mM MPP⁺ for 2 days. DNA fragmentation was measured by diphenylamine assay. Data are presented as means ± S.D. of three separate experiments. **P < 0.05 versus scrambled siRNA transfected cells exposed to MPP⁺. (B) Cell cycle analysis of cellular DNA content was performed by flow cytometry. The sub-G₁ region (presented as ‘M1’) includes cells undergoing apoptosis. The number of each panel refers to the percentage of apoptotic cells. (C) Activation of caspase-3 in PC12 transfectant cells unexposed or exposed to 0.2 mM MPP⁺ for 2 days. PC12 cells were lysed and centrifuged, after which the supernatant was added to Ac-DEVD-pNA and subjected to analysis of caspase colorimetric activity. The protease activity of caspase-3 was calculated by monitoring the absorbance at 405 nm. Data are presented as means ± S.D. of three separate experiments. *P < 0.01 versus scrambled siRNA transfected cells exposed to MPP⁺. (D) Immunoblot analysis of apoptotic marker proteins in PC12 transfectant cells exposed to 0.2 mM MPP⁺ for 2 days. Cell extracts were subjected to 10-12.5% SDS-PAGE and immunoblotted with antibodies against caspase-3, cleaved PARP, and lamin B. β-Actin was used as an internal control.
hanced in IDPc-depleted PC12 cells (Fig. 3C). As shown in Fig. 3D, immunoblotting also revealed that MPP⁺-induced cleavage of procaspase-3 was more pronounced in the IDPc siRNA-transfected cells. Furthermore, knockdown of IDPc increased the cleavage of the caspase substrates PARP and lamin B (Fig. 3D). Taken together, our results indicate that MPP⁺ induces cleavage of procaspase-3 to the active form of caspase-3, which then induces the degradation of PARP and lamin B.

In summary, the data presented herein indicate that the down-regulation of IDPc in PC12 cells results in pro-oxidant conditions, which consequently leads to an increase in oxidative injury and cell death upon exposure to MPP⁺. These results indicate that the attenuation of IDPc activity is probably one of the intermediary events in MPP⁺-induced cytotoxicity in PC12 cells.

**MATERIALS AND METHODS**

**Materials**

β-NAD⁺, isocitrate, NIBHA, 2,4-dinitrophenyl hydrazine (DNPH), diphenylamine, and MPP⁺ were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA) and Cell Signaling (Beverly, MA, USA). Recombinant mouse IDPc was used to prepare polyclonal anti-IDPc antibodies in rabbits.

**siRNA transfection**

IDPc siRNA and control (scrambled) siRNA were purchased from Samchully Pham (Seoul, Korea). The sequences of our IDPc and scrambled siRNA were as follows. For IDPc, sense and antisense IDPc siRNA were 5'-GGACUUGGCUGCUUCGC AUUdTdT-3' and 5'-'AUGAUGACCUGAGUGAAUGdTdT-3', respectively. For the scrambled control, sense and antisense siRNAs were 5'-CUGAUGACCUGAGUGAAUGdTdT-3' and 5'- CAUUCACUCAGGUCAUCAGdTdT-3', respectively. The PC12 cells were transfected with 40 nM oligonucleotides using Lipofectamine RNAi MAX (Invitrogen, Carlsbad, CA, USA).

**Enzyme assays**

Cytosolic fraction of the cells was added to 1 ml of 40 mM Tris buffer, pH 7.4, containing NADP⁺ (2 mM), MgCl₂ (2 mM), and isocitrate (5 mM). The activity of IDPc was determined by the production of NADPH at 340 nm at 25°C. For the caspase-3 activity assays, cells were washed three times with chilled PBS and then incubated with 75 μl of lysis buffer (50 mM Tris-Cl, pH 7.4, 1 mM EDTA, 10 mM EGTA, 10 μM digitonin, 0.5 mM PMSF) for 30 min at 37°C. The contents from three wells were pooled and centrifuged at 20,000 x g for 20 min at 4°C. The supernatant was mixed (1:1) with reaction buffer (100 mM HEPES, 1 mM EDTA, 10 mM DTT, 0.5 mM PMSF, 10% glycerol). Reactions were initiated by the addition of 5 μl (5 mg/ml) of the colorimetric agent Ac-DEVD-pNA (caspase-3 substrate), and the caspase activity was measured at an absorbance of 405 nm for 1 h after the incubation of the mixture at 37°C. Caspase activity was calculated as (absorbance/mg of protein in treated sample)/absorbance/mg of protein in control sample).

**Immunoblot analysis**

Cell extract proteins were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were subsequently subjected to immunoblot analysis using the appropriate antibodies. Immunoreactive antigen was then recognized using horseshadish peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

**Cellular redox status and oxidative damage**

The intracellular production of peroxides was assessed using the ferric sensitive dye xylene orange. The concentration of thiobarbituric acid-reactive substances (TBARS) in the cell lysate was determined as an indicative marker of lipid peroxidation. The cell extracts (500 μl) were mixed with 1 ml of TBA solution (0.375% thiobarbituric acid in 0.25 N HCl containing 15% (w/v) trichloroacetic acid) and heated at 100°C for 15 min. Then, the reaction was stopped on ice, and the absorbance was measured at 535 nm. The protein carbonyl content was determined spectrophotometrically using the DNPH-labeling procedure.

**Apoptosis analysis**

To measure apoptotic DNA fragmentation, cells were collected by centrifugation, resuspended in 250 μl of 10 mM Tris and 1 mM EDTA, pH 8.0 (TE buffer), and incubated with one additional volume of lysis buffer (5 mM Tris, 20 mM EDTA, and 0.5% Triton X-100, pH 8.0) for 30 min at 4°C. After lysis, the intact chromatin (pellet) was separated from the DNA fragments (supernatant) by centrifugation for 15 min at 13,000 x g. The pellets were resuspended in 500 μl of TE buffer, and samples were precipitated by the addition of 300 μl of 10% trichloroacetic acid at 4°C. The samples were pelleted at 4,000 x g for 10 min, and the supernatant was removed. After the addition of 300 μl of 5% trichloroacetic acid, samples were boiled for 15 min. The DNA content was quantitated using the diphenylamine reagent. The percentage of fragmented DNA was calculated as the ratio of the DNA content in the supernatant to that in the pellet. For FACS analysis, cells were collected at 2,000 x g for 5 min, washed once with cold PBS, and fixed in 70% ethanol. Then, ethanol was decanted by centrifugation and stained with 1 ml of solution containing 50 mg/ml of PI, 1 mg/ml of RNase A, and 1.5% Triton X-100 for at least 1 h in
REFERENCES