1-Methyl-4-phenylpyridinium (MPP⁺)-induced apoptosis and mitochondrial oxidant generation: role of transferrin-receptor-dependent iron and hydrogen peroxide

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1-Methyl-4-phenylpyridinium (MPP⁺) is a neurotoxin used in cellular models of Parkinson's Disease. Although intracellular iron plays a crucial role in MPP⁺-induced apoptosis, the molecular signalling mechanisms linking iron, reactive oxygen species (ROS) and apoptosis are still unknown. We investigated these aspects using cerebellar granule neurons (CGNs) and human SH-SY5Y neuroblastoma cells. MPP⁺ enhanced caspase 3 activity after 24 h with significant increases as early as 12 h after treatment of cells. Pre-treatment of CGNs and neuroblastoma cells with the metalloporphyrin antioxidant enzyme mimic, Fe(III)tetrakis(4-benzoic acid)porphyrin (FeTBAP), completely prevented the MPP⁺-induced caspase 3 activity as did overexpression of glutathione peroxidase (GPx1) and pre-treatment with a lipophilic, cell-permeable iron chelator [*N,N'*-bis-(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid, HBED]. MPP⁺ treatment increased the number of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labelling)-positive cells which was completely blocked by pre-treatment with FeTBAP. MPP⁺ treatment significantly decreased the

aconitase and mitochondrial complex I activities; pre-treatment with FeTBAP, HBED and GPx1 overexpression reversed this effect. MPP⁺ treatment increased the intracellular oxidative stress by 2–3-fold, as determined by oxidation of dichlorodihydrofluorescein and dihydroethidium (hydroethidine). These effects were reversed by pre-treatment of cells with FeTBAP and HBED and by GPx1 overexpression. MPP⁺-treatment enhanced the cell-surface transferrin receptor (TfR) expression, suggesting a role for TfR-induced iron uptake in MPP⁺ toxicity. Treatment of cells with anti-TfR antibody (IgA class) inhibited MPP⁺-induced caspase activation. Inhibition of nitric oxide synthase activity did not affect caspase 3 activity, apoptotic cell death or ROS generation by MPP⁺. Overall, these results suggest that MPP⁺-induced cell death in CGNs and neuroblastoma cells proceeds via apoptosis and involves mitochondrial release of ROS and TfR-dependent iron.

Key words: caspase 3, glutathione peroxidase, metalloporphyrin, nitric oxide synthase, oxidative stress, Parkinson's disease.

INTRODUCTION

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Figure 1) is a potent *in vivo* neurotoxin that produces degeneration of nigrostriatal neurons in primates and rodents and, therefore, has been used in many studies as a model of Parkinson's Disease [1–3]. MPTP conversion into 1-methyl-4-phenylpyridinium (MPP⁺; Figure 1) is required for toxicity, and this conversion is carried out by monoamine oxidase type B in the brain [4]. MPP⁺ is transported into dopaminergic neurons by the dopamine transporter and is concentrated into mitochondria, where it produces cessation of oxidative phosphorylation by blockade of the reoxidation of NADH dehydrogenase by coenzyme Q [5]. ATP depletion leading to the loss of plasma and mitochondrial membrane potential differences is postulated to contribute to cell death in dopaminergic neurons [6]. The former results in dis-

turbances in calcium homeostasis as well as opening of the mitochondrial transition pore. However, some studies have suggested that MPP⁺-induced cell death involves multiple mechanisms, and not complex I inhibition alone [7,8].

Several *in vivo* and *in vitro* studies find that MPP⁺ exerts oxidative stress on cells. MPTP treatment produces an increase in brain hydroxyl radicals in mice [9], and high concentrations of MPP⁺ have been shown to increase reactive oxygen species (ROS) in neuroblastoma cells [9], but lower, lethal concentrations do not produce ROS in dopaminergic neurons in primary culture [8]. In animals, overexpression of antioxidant enzymes protects against MPTP toxicity [10], and antioxidant molecules protect against MPP⁺ toxicity in neuronal cell lines [11] and dopaminergic neurons in primary culture [12]. MPP⁺ has been shown to induce superoxide production by isolated mitochondria via inhibition of complex I [13]. However,

Abbreviations used: carboxy-DCF, carboxy-2',7'-dichlorofluorescein; carboxy-DCFH, carboxy-2',7'-dichlorodihydrofluorescein; carboxy-H₂DCFDA, carboxy-2',7'-dichlorodihydrofluorescein diacetate; CGN, cerebellar granule neuron; CGN⁺GPx1, GPx1-overexpressing CGN; DFO, desferrioxamine (desferral); DHE, dihydroethidium (hydroethidine); DPBS, Dulbecco's PBS; FBS, foetal bovine serum; FeTBAP, Fe(III)tetrakis(4-benzoic acid)porphyrin; GFP, green fluorescent protein; GPx1, glutathione peroxidase; HBED, *N,N'*-bis-(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid; Hsp, heat-shock protein; IRP, iron-regulatory protein; LDH, lactate dehydrogenase; MnSOD, manganese superoxide dismutase; MPP⁺, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; L-NAME, L-nitroarginine-*N*-methyl ester; L-NMA, *N*^G-methyl-L-arginine; nNOS, neuronal NOS; NOS, nitric oxide synthase; ROS, reactive oxygen species; TfR, transferrin receptor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labelling.

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Figure 1 Structures of selected compounds used in the present study

other studies have implicated nitric oxide synthase (NOS) [14] and other mitochondrial enzymes [15] as a source of oxygen radicals.

Although the neurotoxic effects of MPTP are confined to dopaminergic neurons of the nigrostriatal pathway *in vivo*, MPP⁺ is toxic to other cell types *in vitro*. In particular, several studies have demonstrated that low concentrations of MPP⁺ produce cell death in glutamatergic cerebellar granule neurons (CGNs) *in vitro* [16,17]. CGNs also express a high level of neuronal NOS (nNOS) that is activated in response to increased intracellular calcium. There is clear evidence that MPP⁺-induced cell death in CGNs ultimately results from autocrine excitotoxicity resulting from massive glutamate release, as both the *N*-methyl-D-aspartate (NMDA) channel blocker MK801 and inhibitors of glutamate exocytosis are protective [17]. MPP⁺-induced cell death in CGNs also has characteristics of apoptosis [16,17]. Cell death is preceded by caspase activation and is prevented by inhibitors of caspase 3 but not caspase 1 [16].

The objective of the present study was to test the hypothesis that MPP⁺ induces apoptotic cell death through an intrinsic mechanism involving the mitochondrial pathway and that the ROS generated in the mitochondrial respiratory chain are responsible for MPP⁺-induced toxicity. In the present study, we used intracellular iron chelators [desferrioxamine (desferral or DFO) and *N,N'*-bis-(2-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid (HBED)], antioxidant enzyme mimic [Fe(III) tetrakis(4-benzoic acid)porphyrin (FeTBAP)], and overexpression of glutathione peroxidase (GPx1) to probe the mechanism of MPP⁺-induced cell death. The metalloporphyrin antioxidant, FeTBAP (Figure 1), is cell-permeable and decomposes intracellular superoxide and H₂O₂ [18]. Results indicate that MPP⁺-mediated mitochondrial generation of superoxide, H₂O₂ and cellular iron are responsible for apoptosis in dopaminergic and non-dopaminergic cellular systems. Implications for treating Parkinson's disease with cell-permeable iron chelators and antioxidant enzymes are discussed.

MATERIALS AND METHODS

Materials

Carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H₂DCFDA) and dihydroethidium (hydroethidine or DHE) were purchased from Molecular Probes Inc. (Eugene, OR, U.S.A.). FeTBAP was synthesized according to the published method [19]. DFO and HBED were a gift from Dr Cherakuri Muralikrishna (National Cancer Institute, Bethesda, MD,

U.S.A.). MPP⁺ iodide, NADH, ubiquinone-1 and L-nitro-arginine-*N*-methyl ester (L-NAME), as well as all salts and buffers, were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [³H]MPP⁺ (specific radioactivity, 78 Ci/mmol) was purchased from DuPont NEN Life Science Products Inc. (Boston, MA, U.S.A.). Cell-permeable caspase 3 inhibitor 1 (DEVD-CHO) and caspase inhibitor 1 (Z-VAD-FMK) were purchased from Calbiochem, CA, U.S.A. Lactate dehydrogenase (LDH) assay kit was purchased from Sigma. LIPOFECTAMINE™ Plus reagent was obtained from Gibco BRL (Gaithersburg, MD, U.S.A.). pcDNA3 containing the full-length manganese superoxide dismutase (MnSOD) gene and recombinant adenovirus Ad-CMV-GPx1 were gifts from Dr Larry Oberley (University of Iowa, Iowa City, IA, U.S.A.). Ad-CMV-GFP (recombinant adenovirus expressing green fluorescent protein) was obtained from the Adenoviral Core Facility (Medical College of Wisconsin, Milwaukee, WI, U.S.A.). Monoclonal antibody, 42/6, against human transferrin receptor (TfR) was a gift from Dr Ian Towbridge (Salk Institute, San Diego, CA, U.S.A.).

Culturing of rat CGNs

CGNs from cerebellum were prepared from 6–8-day-old pups of either sex exactly as described previously in [20], except that the cells were plated at a density of 1.4×10^6 cells/ml on to six-well dishes (Fisher Scientific, Pittsburgh, PA, U.S.A.) that had been coated with poly-D-lysine. More than 90% of the cells in the cultures were small neurons with considerable neuritic outgrowth and cell clustering, features typical of CGNs in primary culture. The remaining cells were astrocytes.

Culturing of human neuroblastoma cells

Human neuroblastoma cells (SH-SY5Y) were obtained from the American Type Cell Collection, transferred to 75-cm² filter vent flasks (Costar, Cambridge, MA, U.S.A.), grown in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal bovine serum (FBS), 4 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin, and incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. For experiments, cells were seeded in six-well dishes and grown to 70–80% confluence. At 12 h before the start of treatment, the medium was replaced with Dulbecco's modified Eagle's medium containing 2% (v/v) FBS. The above conditions were applied to all of the experiments performed in this study.

Cell treatments

Unless otherwise stated, all experiments were performed in CGNs cultured *in vitro* for at least 10 days. On day 10, CGNs were pre-treated for 2 h with one of the following: medium alone, FeTBAP (10 μ M) or L-NAME (100–1000 μ M), DFO (10 μ M), HBED (10 μ M) or caspase inhibitors (5 μ M), followed by the addition of MPP⁺ to a final concentration of 50 μ M. Following a 24 h incubation, the cell-culture medium was aspirated and cells were washed once with Dulbecco's PBS (DPBS) and collected by either trypsinization or gentle scraping. The cell pellets were washed twice with DPBS and used for subsequent assays. For the time-course experiments, CGNs were treated with 50 μ M MPP⁺ and cells were collected by gentle scraping at different time points. Neuroblastoma cells were treated when the cells were grown to 70–80% confluence. Cell-treatment conditions were the same as described for CGNs, except 5 mM MPP⁺ was used for treatment and the cells were incubated for 16 h.

Caspase 3 activity

Caspase 3 activity was determined in CGN and neuroblastoma cell lysates using the ApoAlert caspase 3 activity Kit (Clontech, Palo Alto, CA, U.S.A.). After collection, cells were suspended in 100 μ l of lysis buffer supplied in the kit and passed through a 24-gauge needle ten times to ensure complete lysis. The lysate was centrifuged at 4 °C for 10 min at 9300 *g* and 50 μ l of the clear supernatant was used for the activity assay according to the manufacturer's protocol. An increase in the absorbance at 405 nm was considered as an index of caspase 3 activity.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labelling (TUNEL) assay

CGNs were grown in four-well chamber slides at a density of 1×10^6 cells/ml. After treatment, the medium was aspirated and cells were washed twice in a Coplin jar containing DPBS, then were fixed in 4% (v/v) formaldehyde in DPBS at 4 °C for 25 min as described by the manufacturer of the ApoAlert[®] DNA Fragmentation Assay Kit. Fluorescence images were acquired using a Nikon microscope with illumination from a 150 W xenon arc lamp and processed and stored using Metamorph software (Universal Imaging Corporation, West Chester, PA, U.S.A.). Standard FITC filter settings (excitation 480/30 nm and emission 535/40 nm) were used for green fluorescence and rhodamine filter settings (excitation 540/25 nm and emission 605/55 nm) were used to detect propidium-iodide-stained cells (red fluorescence). The images were overlaid and the proportion of cells positive for both red and green fluorescence (yellow cells) was calculated in three different fields of view using Metamorph software.

Measurement of oxidative stress

The determination of intracellular oxidant production was based on the oxidation of carboxy-H₂DCFDA, resulting in the formation of the fluorescent compound carboxy-2',7'-dichloro-fluorescein (carboxy-DCF) [21]. Following treatment of cells with MPP⁺, the medium was aspirated, and cells were washed twice with DPBS and then placed in 1 ml of cell culture medium without FBS. Carboxy-H₂DCFDA was added to a final concentration of 10 μ M and cells were incubated for 20 min. The cells were again washed with DPBS and maintained in 1 ml of culture medium. Cellular fluorescence was monitored at wavelengths of 480/30 nm (excitation) and 535/40 nm (emission).

The fluorescence-intensity values from three different fields of view were calculated using the Metamorph software to give a mean value.

DHE staining

The redox-sensitive, cell-permeable fluorophore DHE was used to evaluate the cellular production of superoxide *in vivo*. DHE is oxidized by superoxide to a novel product which binds to DNA enhancing intracellular fluorescence [22]. Unlike lucigenin, DHE does not undergo redox-cycling to form superoxide artifactually [23]. Following treatment of CGNs or neuroblastoma cells, culture medium was aspirated and cells were washed once with DPBS and incubated in fresh culture medium without FBS. DHE (10 μ M) was added to the cells for 30 min, at which time fluorescence images were obtained at excitation and emission wavelengths of 540/25 nm and 605/55 nm respectively. The fluorescence-intensity values from three different fields of view were determined using Metamorph software and the mean values were calculated.

Complex I activity

The activity of mitochondrial complex I was measured as described in [24]. Cell pellets were frozen and thawed three times. The lysate was centrifuged at 800 *g* for 10 min to remove nuclei and non-lysed cells. A 50 μ l volume of the supernatant was added to 1 ml of potassium phosphate buffer (10 mM, pH 8.0) containing NADH (100 μ M) in a 1 ml cuvette at 37 °C. The difference in the rate of NADH oxidation ($\lambda_{\text{max}} = 340$ nm, $\epsilon = 6.22 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) in the presence (5 μ l) and absence of 50 μ M ubiquinone-1 was used as an index of complex I activity. The enzyme activity was also examined in the presence of 1 μ M rotenone.

Aconitase activity

CGNs were washed twice with cold DPBS and lysed with buffer containing 0.2% (v/v) Triton X-100, 100 μ M diethylene-triaminepenta-acetic acid and 5 mM citrate in DPBS. The activity of aconitase was measured in 100 mM Tris/HCl (pH 8.0) containing 20 mM D,L-trisodium isocitrate at 37 °C. An absorption coefficient for *cis*-aconitate of 3.6 mM at 240 nm was used [25].

Intracellular MPP⁺ uptake

Cells in six-well plates were washed and pre-incubated in transport buffer containing 25 mM Tris, pH 8.5, 280 mM mannitol, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgSO₄ and 5 mM glucose. After 5 min, the buffer was replaced and 50 μ M MPP⁺ and 5.0 mM [³H]MPP⁺ (200 nCi) were added for 30 min. Cells were pre-incubated with 10 μ M FeTBAP and 10 μ M HBED for 15 min before the addition of [³H]MPP⁺. After incubating for 30 min, the buffer was removed by aspiration and cells were washed twice with ice-cold transport buffer. The cells were then scraped into water and transferred to vials for measuring the radioactivity.

LDH-release assay

To assess the cytotoxicity of MPP⁺ under our experimental conditions, we measured the LDH release in treated cells. Cell culture medium (50 μ l) was used to analyse the LDH activity by measuring the oxidation of NADH at 340 nm as described in the manufacturer's protocol (Sigma Chemical Co.).

GPx1-activity assay

GPx1 activity was measured as described by Paglia and Valentine [26]. Cells from 6 cm culture dishes were homogenized in 50 mM Tris/HCl buffer (pH 7.6), containing 0.1 mM EDTA. After centrifugation (1000 *g* for 15 min at 4 °C), the supernatant was assayed for GPx1 activity. The reaction mixture (1.6 ml) consisted of 100 µg of cytosolic protein, 0.14 mM NADPH, 2 mM GSH and 1 unit/ml GSSG reductase in 50 mM Tris/HCl buffer containing 0.1 mM EDTA at 37 °C. The reaction was initiated by adding 20 µl of *tert*-butyl-hydroperoxide (6.6 mM) as a substrate. NADPH disappearance was monitored spectrophotometrically at 340 nm. GPx1 activity was expressed in units/mg of protein, where one unit is equal to 1 nmol of NADPH oxidized/min.

Overexpression of MnSOD in CGNs

On day 8, CGNs were transfected individually with 2 µg each of pcDNA3 containing the full-length MnSOD gene and pE-GFP-N1 (control plasmid) using LIPOFECTAMINETM Plus reagent according to the manufacturer's protocol. Following 4 h of incubation, one volume of complete medium was added to the cells to arrest transfection. After 24 h, the medium was replaced and the cells were allowed to recover for 24 h. On day 10, the transfection efficiency was monitored by assessing the expression of a GFP and by the increase in MnSOD activity as measured using the cytochrome *c* reduction method [27]. For experiments involving the detection of DHE fluorescence, CGNs were transfected with 2 µg of pcDNA3 containing the full-length MnSOD gene.

Overexpression of GPx1

On day 8, adenoviral infection with GPx1 was performed in a serum-free medium for 1 h at a multiplicity of infection (MOI) of 500 particles/cell [28], followed by the addition of an equal volume of fresh medium containing 20 % FBS and 50 nM sodium selenite. Incubation was continued for 24 h. The medium was replaced 24 h after infection with medium containing 10 % FBS and the cells were treated with MPP⁺ on day 10. These conditions produced nearly 100 % transfection with recombinant adenovirus, as assessed with Ad-CMV-GFP reporter-gene expression.

Determination of TfR levels

Following the termination of the experiment, CGNs and neuroblastoma cells were washed with ice-cold DPBS and resuspended in 100 µl of RIPA buffer [20 mM Tris/HCl, pH 7.4, 2.5 mM EDTA, 1 % (v/v) Triton X-100, 1 % (w/v) sodium deoxycholate, 1 % (w/v) SDS, 100 mM NaCl and 100 mM sodium fluoride]. To 10 ml of RIPA buffer, 1 mM sodium vanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 10 µg/ml pepstatin inhibitors were added. The cells were homogenized by passing the suspension through a 25 gauge needle (ten strokes). The lysate was centrifuged at 12000 *g* for 10 min and the supernatant was used for analysis. Protein was determined by the Lowry method, and 20 µg was used for the Western blot analysis. The proteins were resolved by SDS/PAGE (8 % gels) and blotted on to nitrocellulose membranes. Membranes were washed with Tris-buffered saline (140 mM NaCl, 50 mM Tris/HCl, pH 7.2) containing 0.1 % (v/v) Tween 20 and 5 % (w/v) skimmed milk to block the non-specific protein binding. Membranes were incubated with a mouse monoclonal antibody against human TfR [Zymed Laboratories Inc., San Francisco, CA, U.S.A.; 1 µg/ml antibody in Tris-buffered saline containing 0.1 % (v/v) Tween 20

for 2 h at room temperature (25 °C)], washed five times, and then incubated with horseradish-peroxidase-conjugated rabbit anti-mouse IgG (1:5000) for 1.5 h at room temperature. The TfR band was detected using the ECL[®] method (Amersham Biosciences, Little Chalfont, Buckinghamshire, U.K.). Statistical significance was obtained using Student's *t* test employing the Sigmaplot software.

RESULTS

MPP⁺-induced caspase 3 activation and apoptosis: effect of metalloporphyrin antioxidant

Treatment of CGNs with 50 µM MPP⁺ induced a time-dependent activation of caspase 3 activity (Figure 2A). The activity of caspase 3 was increased nearly 2-fold as early as 12 h following MPP⁺ treatment and reached a maximum at 48 h. Pre-treatment of CGNs with the antioxidant enzyme mimetic, FeTBAP (10 µM), completely prevented MPP⁺-induced caspase 3 activation (Figure 2B). Pre-treatment with 100–1000 µM L-NAME did not protect CGNs from MPP⁺-induced apoptosis (Figure 2B) or cell death (results not shown). The TUNEL assay also demonstrated significant apoptosis following 24 h incubation with 50 µM MPP⁺ (Figure 2C and Figure 2D). Pre-treatment of CGNs with FeTBAP before the addition of MPP⁺ reduced the number of TUNEL-positive cells to control values; however, 1 mM L-NAME did not inhibit MPP⁺-induced TUNEL-positive cells (Figure 2C and Figure 2D). Pre-treatment with HBED (10 µM) or GPx1 overexpression significantly decreased MPP⁺-induced TUNEL-positive staining (Figure 2C). The cellular uptake of [³H]MPP⁺ was not inhibited by FeTBAP or HBED (results not shown). No significant increase in LDH release from cells treated with MPP⁺ alone or in the presence of FeTBAP or HBED was noticeable (Figure 2E). These results indicate that ROS and redox-active intracellular iron, but not nitric oxide or reactive nitrogen species, are responsible for MPP⁺-induced apoptosis in CGNs.

MPP⁺-induced intracellular oxidative stress as measured by carboxy-DCF and DHE staining

We used carboxy-DCF staining to assess the production of the intracellular oxidants, H₂O₂ and peroxynitrite in response to MPP⁺. This assay is based on the oxidation of a non-fluorescent carboxy-2',7'-dichlorodihydrofluorescein (carboxy-DCFH) probe to the green fluorescent product carboxy-DCF. Previous results have shown that H₂O₂ alone does not oxidize carboxy-DCFH to carboxy-DCF and that the presence of haem or redox metal ion is required for carboxy-DCFH oxidation to carboxy-DCF [29]. Results indicate that there is nearly a 3-fold increase in the intensity of carboxy-DCF staining in CGNs treated with 50 µM MPP⁺ for 24 h (Figure 3A and Figure 3B). Pre-treatment of the cells with the antioxidant FeTBAP significantly decreased carboxy-DCF fluorescence as compared with cells that were treated with MPP⁺ alone (Figure 3A and Figure 3B). Pre-treatment with L-NAME (Figure 3A and Figure 3B) and N^G-methyl-L-arginine (L-NMA; results not shown) did not have a marked effect on carboxy-DCF staining in cells that were exposed to MPP⁺.

To investigate whether or not MPP⁺ induces superoxide generation in CGNs, we employed DHE staining. In this assay, superoxide anion oxidizes DHE to a novel product that binds to DNA, leading to enhancement in fluorescence [22]. Results indicate that there is nearly a 2–3-fold increase in superoxide-

Figure 2 MPP⁺ treatment results in caspase 3 activation and TUNEL-positive staining in CGNs

(A) CGNs were treated with 50 μ M MPP⁺ for different time intervals before determination of caspase 3 activity in the supernatant. (B) CGNs were treated with MPP⁺ in the presence and absence of FeTBAP and L-NAME, as indicated. After incubation, cells were collected by gentle scraping and were lysed. Caspase 3 activity was determined in the supernatant. (C) CGNs were treated with MPP⁺ in the presence and absence of FeTBAP, HBED, L-NAME, nitric oxide synthase inhibitor and CGNs⁺GPx1 for 24 h. Cells were then washed and fixed before a TUNEL assay was performed as indicated in the Materials and methods section. The overlaid images of FITC- and propidium-iodide-stained cells (yellow cells) indicate apoptotic nuclei. (D) Apoptotic nuclei as a percentage of total cells. Results are representative of three different fields of view. (E) CGNs and neuroblastoma cells were treated with 50 μ M and 5.0 mM MPP⁺ in the presence and absence of FeTBAP (10 μ M) and HBED (10 μ M) for 16 h. The cytotoxicity of these compounds was assessed by monitoring the LDH release. As a positive control, LDH release was measured from cells treated with 0.1% (v/v) Triton X-100. Results in (A), (B), (D) and (E) are means $\pm$ S.D. of three separate experiments. *, $P < 0.01$ as compared with the control; #, $P < 0.01$ as compared with the MPP⁺-treated group. pNA, *p*-nitroanilide.

dependent product formation in CGNs following a 24 h treatment with 50 μ M MPP⁺ (Figure 3C and Figure 3D). Pre-treatment of CGNs with FeTBAP inhibited DHE-derived superoxide-specific product staining to control values. Pre-

treatment with L-NAME did not affect MPP⁺-induced DHE-superoxide product staining.

We then investigated the effect of overexpressing MnSOD (to decompose superoxide anion to H₂O₂) and GPx1 (to

Figure 3 MPP⁺ treatment increases oxidative stress in CGNs

CGNs were treated with MPP⁺ in the presence and absence of FeTBAP and L-NAME for 24 h. Following incubation, cells were washed once with DPBS and incubated with 10 μ M carboxy-H₂DCFDA for 20 min as indicated in the Materials and methods section. (A) Fluorescence micrographs were captured with a Nikon fluorescence microscope equipped with FITC filter settings. (B) Mean fluorescence intensity values from three different fields of view obtained using Metamorph software. (C) CGNs were treated with MPP⁺ in the presence and absence of FeTBAP and L-NAME for 24 h. Following incubation, cells were washed once with DPBS and incubated with 10 μ M DHE for 30 min, before the medium was replaced with fresh DPBS. The fluorescence images were captured using a Nikon fluorescence microscope equipped with rhodamine filter settings. (D) The mean intensity of fluorescence from three different fields of view obtained using the Metamorph software. (E) Efficiency of plasmid transfection as shown by the expression of GFP in CGNs 48 h after co-transfection with 1 μ g each of pcDNA3 containing the MnSOD gene and pE-GFP-N1 plasmid. (F) MnSOD and GPx1 enzyme activities in untransfected, MnSOD-gene-transfected and GPx1-overexpressed CGNs (48 h following transfection). Enzyme activities were determined as described in the Materials and methods section. (G) Fluorescence micrographs showing the DHE staining in untransfected, MnSOD-gene-transfected and adenoviral-expressed-GPx1 in CGNs following treatment with 50 μ M MPP⁺ for 24 h. (H) Arbitrary fluorescence intensity values of DHE-stained CGNs from three different fields of view obtained using Metamorph software. Results shown in (B), (D), (F) and (H) are representative of two separate experiments. *, $P < 0.05$ as compared with the control; #, $P < 0.05$ as compared with the MPP⁺-treated group.

Figure 4 Effect of MPP⁺ on carboxy-DCF staining and caspase 3 activation in CGNs and CGNs⁺GPx1

(A) CGNs and CGNs⁺GPx1 were incubated with 50 μ M MPP⁺ for 48 h and the morphology of neurons was observed using phase-contrast microscopy. (B) Efficiency of adenoviral infection in CGNs as shown using a control vector containing the GFP reporter gene, Ad-CMV-GFP. (C) CGNs and CGNs⁺GPx1 were incubated with 50 μ M MPP⁺ for 24 h. Following incubation, cells were washed once with DPBS and incubated with 10 μ M carboxy-H₂DCFDA for 20 min as indicated in the Materials and methods section. Fluorescence micrographs were captured with a Nikon fluorescence microscope equipped with FITC filter settings. (D) Mean fluorescence intensity values from three different fields of view obtained using Metamorph software. (E) CGNs and CGNs⁺GPx1 were treated with 50 μ M MPP⁺ for 24 h. After incubation, cells were collected by gentle scraping and were lysed. Caspase 3 activity was determined in the supernatant. Results shown in (D) and (E) are the means $\pm$ S.D. of three separate experiments. *, $P < 0.05$ as compared with the control; #, $P < 0.05$ as compared with the MPP⁺-treated group. pNA, p -nitroanilide.

detoxify H₂O₂ to H₂O). CGNs were transfected using the LIPOFECTAMINETM Plus reagent, which resulted in a transfection efficiency of 30–40 %. The expression profile was quantitatively monitored by observing the GFP expression following co-transfection with the control plasmid pE-GFP-N1 (Figure 3E). An 8-fold increase in GPx1 and a 2-fold increase in MnSOD

activities were measured in overexpressed cells (Figure 3F). CGNs overexpressing MnSOD (CGNs⁺MnSOD) greatly decreased MPP⁺-induced DHE-superoxide product staining, while overexpression of GPx1 by CGNs (CGNs⁺GPx1) had no effect on MPP⁺-induced staining from DHE oxidation (Figure 3G and Figure 3H). This result is consistent with the interpretation that

Figure 5 MPP⁺ treatment inhibits complex I and aconitase activities

CGNs were treated with MPP⁺ in the presence and absence of FeTBAP and L-NAME for 24 h. At the end of incubation, cells were washed three times with DPBS, gently scraped and collected by brief centrifugation. Cell pellets were lysed by freeze-thawing three times. (A) The complex I activity assay was carried out as indicated in the Materials and methods section. (B) Aconitase activity was carried out by measuring the conversion of isocitrate into *cis*-aconitate at 240 nm as described in the Materials and methods section. Results shown in (A) and (B) are the means $\pm$ S.D. of three separate experiments. *, $P < 0.01$ as compared with control; #, $P < 0.01$ as compared with the MPP⁺-treated group.

oxidized DHE staining in MPP⁺-treated CGN is indicative of superoxide reaction. Results from these studies indicate that intracellular oxidative stress induced by MPP⁺ is dependent on O₂⁻ and H₂O₂. Although GPx1 overexpression did not affect superoxide generation, we tested the hypothesis that MPP⁺-induced toxicity and oxidative stress are inhibited in CGNs^{+GPx1}. In these studies, GPx1 was administered by adenovirus and the efficiency of adenoviral gene expression in CGNs is nearly 100%, as shown using a reporter gene Ad-CMV-GFP (Figure 4B). Figure 4(A) shows that, following a 48 h treatment with MPP⁺, a majority of the neurons were dead, as evidenced by the cell morphology; however CGNs^{+GPx1} were more resistant to MPP⁺

toxicity. MPP⁺-induced carboxy-DCF staining was also reduced to control values in CGNs^{+GPx1} (Figure 4C and Figure 4D), indicating that H₂O₂ generation was reduced by GPx1 overexpression. In addition, MPP⁺-induced caspase 3 activity was reduced to control values following GPx1 overexpression (Figure 4E).

MPP⁺-induced inhibition of mitochondrial complex I and aconitase activities

Previous studies have demonstrated that MPP⁺ inhibits mitochondrial complex I activity irreversibly and that this inhibition is blocked when ROS scavengers are present [30]. Consistent with these previous reports, CGNs treated with 50 μ M MPP⁺ for 24 h exhibited a 3-fold decrease in complex I activity of mitochondria that were isolated from the treated cells (Figure 5A), as did rotenone (results not shown). The cytosolic aconitase activity was also decreased by 50% following MPP⁺ treatment (Figure 5B). Pre-treatment of CGNs with FeTBAP restored the complex I and aconitase activities nearly to control values, while L-NAME did not alter the effect of MPP⁺ on either of these enzyme activities. These findings implicate a role for ROS in MPP⁺-induced inactivation of mitochondrial enzymes.

Effect of iron chelators on MPP⁺-induced carboxy-DCF staining and caspase 3 activation

As iron has been implicated in the pathogenesis of Parkinson's disease and in MPP⁺-induced toxicity [31,32], we examined the effect of cell-permeable iron chelators in CGNs treated with MPP⁺. Figure 6(A) shows that pre-treatment of CGNs with a cell-permeable iron chelator HBED (10 μ M) significantly inhibited carboxy-DCF staining caused by MPP⁺ to the control values (Figure 6A and Figure 6B), as did treatment with DFO. The iron chelators were also effective inhibitors of MPP⁺-induced caspase 3 activation (Figure 6C).

MPP⁺-induced apoptosis and oxidative stress in neuroblastoma cells: effects of metalloporphyrin, iron chelator and GPx1 overexpression

We have also investigated the effect of MPP⁺ in neuroblastoma cells. In agreement with an earlier study [33], we found that MPP⁺-induced toxicity in neuroblastoma cells requires higher concentrations than in CGNs. MPP⁺ induced a 5-fold enhancement of caspase 3, following a 16 h treatment with 5 mM MPP⁺ (Figure 7A). MPP⁺-induced caspase 3 activation was significantly diminished in cells that were pre-treated with FeTBAP and HBED, and in cells overexpressing GPx1 (Figure 7B). Treatment with L-NAME had no effect on MPP⁺-induced caspase 3 activation. There is nearly a 2-fold increase in carboxy-DCF and a 3-fold increase in DHE-superoxide product staining in neuroblastoma cells treated for 16 h with 5 mM MPP⁺ (Figure 7C–Figure 7F). Pre-treatment of neuroblastoma cells with either 10 μ M FeTBAP or 10 μ M HBED reduced the fluorophore staining to that of the control values, implicating a role for ROS in the toxicity. L-NAME had no effect on MPP⁺-induced carboxy-DCF or DHE staining, suggesting that NO[•] or NO⁻-derived oxidants do not play a major role in MPP⁺ toxicity in neuroblastoma cells.

MPP⁺-induced caspase activation: effect of anti-TfR antibody

We monitored the effect of MPP⁺ on the expression of cellular TfR levels by Western blotting analysis. Figure 8(A) and Figure

Figure 6 Iron chelators inhibit MPP⁺-induced carboxy-DCF staining and caspase 3 activation

CGNs were treated with MPP⁺ in the presence and absence of the iron chelators, DFO and HBED, for 24 h. Following incubation, cells were washed once with DPBS and incubated with 10 μ M carboxy-H₂DCFDA for 20 min as indicated in the Materials and methods section. **(A)** Fluorescence micrographs were captured with a Nikon fluorescence microscope equipped with FITC filter settings. **(B)** Mean fluorescence intensity values from three different fields of view obtained using Metamorph software. **(C)** Caspase 3 activity was determined as described in the Materials and methods section. Results shown in **(B)** and **(C)** are the means $\pm$ S.D. of three separate experiments. *, $P < 0.05$ as compared with the control; #, $P < 0.05$ as compared with the MPP⁺-treated group. pNA, *p*-nitroanilide.

8(B) show that MPP⁺ treatment produced an increase in TfR levels within 2–4 h in CGNs and within 1–2 h in neuroblastoma cells. Previously, it has been reported that mitochondrial oxidative stress and aconitase inactivation result in increased TfR levels and TfR-dependent iron transport [34]. The involvement of TfR in MPP⁺-induced apoptosis was confirmed further using the monoclonal (IgA) anti-TfR antibody (42/6) that specifically binds to the extracellular domain of the TfR and blocks receptor endocytosis [35]. This antibody recognizes both human and bovine TfR [36]. In the presence of 42/6, iron cannot enter the cell through TfR. Figure 8(C) shows that, in the presence of anti-TfR antibody, MPP⁺-induced caspase 3 activation was inhibited in CGNs and neuroblastoma cells. In control experiments, when CGNs and neuroblastoma cells were incubated with 12 μ g/ml IgG-class antibody that does not bind to the extracellular domain of TfR, MPP⁺-induced caspase 3 proteolytic activity was not inhibited (results not shown). The anti-TfR antibody treatment did not affect the uptake of MPP⁺ into cells (results not shown). These results strongly suggest a critical role of TfR in MPP⁺-induced apoptotic signalling.

DISCUSSION

In the present study, we have shown that the cellular oxidative stress is significantly enhanced in MPP⁺-treated dopaminergic

and non-dopaminergic cells, as demonstrated using both DHE- and carboxy-DCF-staining assays. Antioxidant strategies involving overexpression of GPx1 and metalloporphyrin antioxidants diminished MPP⁺-induced caspase 3 activation and protected the neuronal cells from oxidative cell death. Results implicate a role for TfR-dependent iron and hydrogen peroxide in the ultimate toxicity of MPP⁺ (Scheme 1).

MPP⁺-induced generation of superoxide anion in mitochondria: interaction with iron–sulphur centres

Results reported in Figure 5, as well as those in [30], clearly demonstrate that MPP⁺ causes an inhibition of mitochondrial complex I. As pre-treatment with antioxidants nullifies this inhibition, it is likely that this inhibition is mediated by free-radical damage. MPP⁺ has been shown to inhibit NADH dehydrogenase-linked oxidation, resulting in the loss of ATP and glutathione [5,37]. Blockade of electron leakage from mitochondrial NADH dehydrogenases leads to increased superoxide generation. Mitochondrial respiratory-chain inhibitors (e.g., rotenone, antimycin) have been reported to increase superoxide formation [38]. Patients with mitochondrial complex I deficiency have elevated levels of mitochondrial superoxide [39]. It has been shown that superoxide specifically reacts with 4Fe-4S centres

Figure 7 MPP⁺-induced apoptosis and oxidative stress in neuroblastoma cells: effects of antioxidant (enzyme) supplementation and iron chelators

(A) Caspase 3 activity in neuroblastoma cells following treatment with different concentrations of MPP⁺ for 16 h. (B) Neuroblastoma cells were treated with 5 mM MPP⁺ in the presence or absence of FeTBAP, HBED, L-NAME and cells overexpressing GPx1. Following incubation, caspase 3 activity was measured in the supernatant. Neuroblastoma cells were treated with 5 mM MPP⁺ in the presence and absence of FeTBAP, HBED and L-NAME for 16 h. Following incubation, cells were washed once with DPBS and incubated with 10 μ M carboxy-H₂DCFDA for 20 min as indicated in the Materials and methods section. (C) Fluorescence micrographs were captured with a Nikon fluorescence microscope equipped with FITC filter settings. (D) Mean fluorescence intensity values from three different fields of view obtained using Metamorph software. (E) Neuroblastoma cells were treated with 5 mM MPP⁺ in the presence and absence of FeTBAP, HBED and L-NAME for 16 h. Following incubation, cells were washed once with DPBS and incubated with 10 μ M DHE for 30 min, before the medium was replaced with fresh DPBS. The fluorescence images were captured using a Nikon fluorescence microscope equipped with rhodamine filter settings. (F) Mean fluorescence intensity values from three different fields of view obtained using Metamorph software. Results shown in (B), (D) and (F) are the means $\pm$ S.D. of three separate experiments. *, $P < 0.05$ as compared with the control; #, $P < 0.05$ as compared with the MPP⁺-treated group. pNA, *p*-nitroanilide.

present in mitochondrial complex I and II and aconitase [40,41]. Aconitase is rapidly inactivated by superoxide ($k = 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$) in the presence and absence of substrate, and is inactivated relatively slowly by peroxynitrite and nitric oxide [41]. During the reaction between the iron-sulphur clusters and super-

oxide, the [4Fe-4S]²⁺ is quantitatively oxidized to the [3Fe-S]¹⁺ cluster by superoxide, releasing Fe²⁺ and H₂O₂. This facilitates the formation of potent oxidants, hydroxyl radical or perferryl iron in mitochondria. Results from our laboratory indicate that, unlike paraquat, MPP⁺ is unable to catalyse superoxide form-

Figure 8 MPP⁺ induces TfR levels in CGNs and neuroblastoma cells: effect of TfR antibody on MPP⁺-induced caspase activation

(A) CGNs and (B) neuroblastoma cells were treated with 50 μ M and 5 mM MPP⁺ for different time periods. Following incubation, cells were lysed in RIPA buffer and TfR levels were determined by Western blot analysis using an anti-TfR antibody. Band intensity was measured by densitometric analysis using AlphaImagerTM software (Alpha Innotech Corp., San Leandro, CA, U.S.A.). (C) Cells were pre-incubated with TfR antibody for 1 h before the addition of 50 μ M MPP⁺ for CGNs and 5 mM for neuroblastoma cells. Caspase 3 activity in cell lysates was measured as described in the Materials and methods section. Results indicated for all histograms are the means $\pm$ S.D. of three separate experiments. *, $P < 0.05$ as compared with the control; #, $P < 0.05$ as compared with the MPP⁺-treated group. Ab, antibody; pNA, *p*-nitroanilide.

ation via redox-cycling (J. Vasquez-Vivar, C. J. Hillard and B. Kalyanaraman, unpublished work). Thus the enhanced formation of mitochondrial superoxide anion in the presence of MPP⁺ is not due to the redox-cycling of the MPP⁺ radical, the one-electron reduction intermediate of MPP⁺. Previously, it was shown that MPP⁺-induced ROS formation is not mitochondrial in origin, but results from dopamine oxidation [42]. However, this mechanism is unlikely to be operative in our system, as MPP⁺-induces apoptosis in both dopaminergic and non-dopaminergic cells via similar mechanisms and elicits similar effects in the presence of inhibitors.

MPP⁺-induced oxidation of carboxy-DCFH to carboxy-DCF

The assay based on carboxy-DCFH oxidation to carboxy-DCF has been used frequently to measure intracellular H₂O₂ or oxidative stress [30,31,39,40]. The cell-permeable non-fluorescent probe, carboxy-H₂DCFDA, is hydrolysed by intracellular esterases to form the active probe, carboxy-DCFH [21]. However, H₂O₂ does not convert carboxy-DCFH into carboxy-DCF, except in the presence of a catalyst (cytochromes, peroxidases or redox-active metal ions) [29]. In the present study, we show that MPP⁺ induces carboxy-DCFH oxidation to carboxy-DCF and

Scheme 1 Schematic representation of the inhibitory effects of GPx1, antioxidant enzyme mimetic and iron chelators on MPP⁺-induced apoptosis

In general, agents that detoxify H₂O₂ or chelate intracellular iron are proposed to inhibit the oxidant-induced TIR-dependent iron-signalling mechanism.

that the oxidation is inhibited both by GPx1 overexpression and by cell-permeable iron chelators (Figure 1). Based on these observations, we propose that a higher oxidant (hydroxyl radical or a perferryl iron) formed from the interaction between intracellular iron and hydrogen peroxide is responsible for carboxy-DCFH oxidation to carboxy-DCF. Although the actual source of iron still remains to be determined, it is conceivable that the iron released from mitochondrial iron-sulphur centres and/or TfR-mediated iron uptake is responsible for intracellular formation of a higher oxidant. The oxidant-induced inactivation of mitochondrial iron-sulphur proteins has been shown to stimulate the cellular iron-sensing mechanism through activation of iron-regulatory proteins (IRPs) [43].

The role of intracellular iron and H₂O₂ in MPP⁺ toxicity

Inhibition of MPP⁺-mediated apoptosis in cells either overexpressing GPx1 or supplemented with the cell-permeable iron chelator, HBED, strongly implies that oxidants (hydroxyl radical and/or a perferryl iron) are responsible for MPP⁺ toxicity in CGNs and neuroblastoma cells. This is consistent with the reports that iron chelators that are systemically administered to MPTP-treated rats prevent the progressive loss of nigrostriatal dopamine neurons [44]. HBED has a very high affinity for iron and forms complexes having 3:1, 2:1 and 1:1 chelator to iron ratios [Fe(III) stability constant, 10⁴⁰] [45]. This binding constant is much higher than that of the iron-DFO complex (stability constant, 10³¹). We attribute the protective effect of metalloporphyrin antioxidant, FeTBAP, to scavenging of H₂O₂ and/or superoxide anion [18,36,46]. Recently, we have shown that FeTBAP also induces heat-shock proteins (Hsp70 and Hsp32) in endothelial cells and myocytes [18]. Thus the anti-

apoptotic effect of FeTBAP in MPP⁺-treated cells could be due to ROS scavenging and/or Hsp70 induction.

MPP⁺-induced oxidant generated from the reaction between intracellular iron and H₂O₂ could have additional pathophysiological relevance with respect to protein aggregation. It has been recently reported that MPP⁺ promotes aggregation of α -synuclein, a brain presynaptic protein, in neuroblastoma cells [47]. Aggregated α -synuclein has been suggested as having a role in the pathogenesis of Parkinson's disease [48]. Iron levels are reportedly increased in brains of patients suffering from Parkinson's disease [32,33]. Iron and H₂O₂ also exacerbated α -synuclein aggregation *in vitro* [49]. Collectively, these findings imply that MPP⁺-induced α -synuclein aggregation in neuroblastoma cells might be due to oxidants formed from iron and H₂O₂.

Lack of involvement of NO[•] and reactive nitrogen species in MPP⁺ toxicity in CGNs and neuroblastoma cells

The findings of the present study show that NOS inhibitors (L-NAME, L-NMA or 7-nitroindazole; 0.1–1 mM) did not inhibit MPP⁺-induced carboxy-DCF staining, nor did they inhibit MPP⁺-induced apoptosis as measured by caspase 3 activation. The same concentrations of these agents inhibited endogenous NOS activity, as determined by nitrite measurements (results not shown). Although these results are consistent with earlier reports using CGNs [18,19], it has been shown that MPP⁺-induced degeneration of the substantia nigra was attenuated in mutant mice lacking the nNOS gene, but not in mice lacking the endothelial NOS gene [50]. The nNOS-mutant mice also showed a decrease in MPP⁺-induced nitrotyrosine levels in the striatum [50]. These results suggest that under *in vivo* conditions, NO[•] and

peroxynitrite are involved in MPP⁺-induced neurotoxicity. Thus there is a dichotomy between the cell-culture experiments reported here and the published *in vivo* experiments. Although these differences cannot at present be reconciled, many inflammatory factors (e.g. cytokine modulation) could influence MPP⁺ toxicity under *in vivo* conditions.

In conclusion, using dopaminergic and non-dopaminergic cell systems, we have demonstrated that MPP⁺-induced apoptosis is caused by hydrogen peroxide and intracellular iron (Scheme 1). Cell-permeable iron chelators, antioxidant enzyme mimetics or GPx1 overexpression readily afford protection against MPP⁺-induced apoptosis. These findings provide a mechanistic rationale for using antioxidant and iron-chelator therapy for treatment of Parkinsonism induced by environmental toxins.

Oxidative stress, TfR expression and neuronal apoptosis

The interplay between ROS and iron-induced redox signalling, leading to cell proliferation and apoptosis, is becoming increasingly evident [35,36]. One of the pathways by which neuronal cells acquire iron is via the TfR, which facilitates the uptake of iron-loaded transferrin. TfRs are expressed on the surface of cells that use iron. Under conditions when the [4Fe-4S] cluster in aconitase is disassembled under oxidant stress, the IRPs bind to the iron-responsive elements present on 3' and 5' untranslated regions of TfR and ferritin mRNAs respectively. The increased binding to TfR mRNA stabilizes the mRNA, resulting in increased mRNA translation and TfR synthesis. Cells that are exposed to hydroperoxides or drugs that induce intracellular ROS increase the expression of the TfR mRNA due to the induction of IRPs, which trigger a signal for increased iron uptake. The continued uptake of iron via the TfR contributes to intracellular hydroxyl radicals or other higher oxidants of iron, mitochondrial damage and apoptosis. Although, at first glance, this process seems counterintuitive (i.e. increased cellular uptake of iron in response to oxidative stress), a large proportion of a cell's iron requirement is for the assembly of iron clusters and haem synthesis (e.g. cytochromes) in mitochondria and, thus, the partial inactivation of mitochondrial iron-sulphur proteins (e.g. aconitase and complex I) in the presence of MPP⁺ is sufficient to stimulate cellular iron signalling.

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