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Elevated reactive oxygen species and antioxidant enzyme activities in animal and cellular models of Parkinson's disease

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Abstract

The dopaminergic neurotoxin *N*-methyl,4-phenyl-1,2,3,6 tetrahydropyridine (MPTP) causes a syndrome in primates and humans which mimics Parkinson's disease (PD) in clinical, pathological, and biochemical findings, including diminished activity of complex I in the mitochondrial electron transport chain. Reduced complex I activity is found in sporadic PD and can be transferred through mitochondrial DNA, suggesting a mitochondrial genetic etiology. We now show that MPTP treatment of mice and *N*-methylpyridinium (MPP^+) exposure of human SH-SY5Y neuroblastoma cells increases oxygen free radical production and antioxidant enzyme activities. Cybrid cells created by transfer of PD mitochondria exhibit similar characteristics; however, PD cybrids' antioxidant enzyme activities are not further increased by MPP^+ exposure, as are the activities in control cybrids. PD mitochondrial cybrids are subject to metabolic and oxidative stresses similar to MPTP parkinsonism and provide a model to determine mechanisms of oxidative damage and cell death in PD. © 1997 Elsevier Science B.V.

Keywords: Parkinson's disease; Mitochondria; MPTP; Reactive oxygen species; Antioxidant enzymes

1. Introduction

Parkinson's disease (PD) arises from the loss of dopaminergic neurons in the substantia nigra (SN), and can be produced experimentally following exposure to the neurotoxin *N*-methyl,4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [1,2]. *N*-methylpyridinium ion (MPP^+), the active toxin and two electron oxidation product of MPTP, is preferentially taken up by dopamine neurons and concentrated into mitochondria down the electrochemical gradient [3], where it selectively and potently inhibits complex I of the electron transport chain (ETC) [4]. This inhibition of

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complex I leads to impaired ATP production [5], loss of the mitochondrial membrane potential [6], and the formation of reactive oxygen species (ROS) [3–7]. Nigrostriatal MPTP neurotoxicity is also dependent on nitric-oxide synthase (NOS) [8–10], which forms nitric oxide ($NO^{\cdot}$), a potential precursor to the toxic peroxynitrite anion ($ONOO^{-}$) and hydroxyl free radical ($^{\cdot}OH$) [11,12]. These bioenergetic and oxidative stresses likely contribute to the death of vulnerable neurons [9,10,13,14]. Similar processes may occur in PD, as mitochondria from multiple PD tissues including platelets [15], fibroblasts [16], muscle [17,18], substantia nigra [19] and nonnigral brain [20,21] exhibit significantly reduced complex I activity, suggesting a systemic mitochondrial defect.

Parkinson's SN contains markers of oxidative damage to lipids [22], decreased total glutathione content [23], and increased levels of iron [24], which can catalyze the conversion of hydrogen peroxide (H_2O_2) into the highly reactive $^{\cdot}OH$. These findings support the theory that ROS may contribute to neuronal injury and eventual cell death in PD [9,10,13,14,20].

Mechanisms of protection from oxidative free radical damage include scavenging enzyme systems which detoxify ROS such as superoxide, $O_2^{\cdot -}$ (superoxide dismutase, SOD) or H_2O_2 (catalase, CAT and glutathione peroxidase, GPX). Intracellular glutathione functions as both a general free radical scavenger and a substrate for GPX when in its reduced form, which is continuously regenerated by glutathione reductase (GRD). We measured ROS production and the activities of these major antioxidant enzymes, and found that they are all increased in brains of mice treated with MPTP, in SH-SY5Y cells exposed to MPP^{+}, and in PD cybrid cells carrying the genetically transmitted complex I defect found in PD mitochondria.

2. Materials and methods

2.1. MPTP treatment of mice

We monitored the time course and brain regional distribution of production of $^{\cdot}OH$ and changes in antioxidant enzyme activities in C57BL/6 black mice treated systemically with MPTP. We treated male C57BL/6 mice with MPTP intraperitoneally (i.p.) 24 mg/kg b.i.d. for up to 48 h (maximum MPTP dose was 96 mg/kg).

2.2. Antioxidant enzymes in MPTP-treated mice

6 mice were sacrificed at each time-point (0, 2, 8, 24, 32, 48, 72, and 96 h) by decapitation and immediately put on ice and the brains dissected and placed on dry ice. The frontal cortex, striatum, ventral midbrain (which includes the SN), and cerebellum were isolated and placed in ice-cold PBS (20X volumes). The tissues were kept at −80°C until ready. Within 2 weeks, the tissues were thawed, 0.013% sodium cholate was added to lyse subcellular organelles, they were sonicated with an ultrasonic tip (Ultrasonics, Inc, Farmington, N.Y.) for one minute, and centrifuged at 12,000X *g* for 10 min at 4°C to pellet out insoluble materials. The supernatants were then kept on ice until the biochemical assays were performed. Neither the freeze/thawing of tissues nor the addition of this concentration of sodium cholate had discernible adverse effects on antioxidant enzyme activities.

2.3. Hydroxyl radical measurements in MPTP-treated mice

For the hydroxyl radical measurements, 4 or 5 mice were sacrificed at each time-point (0, 8, 24, 48, 72, and 168 h), after having received 100 mg/kg salicylic acid i.p one hour prior to decapitation. The isolated regions were frozen on dry ice, weighed, sonicated in 20 volumes of ice-cold 0.1 M HCL/0.1% sodium metabisulfite and centrifuged. Brain regional $^{\cdot}OH$ was estimated in supernatants by HPLC assay of tissue 2,3-dihydroxbenzoic acid (2,3-DHB), the stable and specific adduct between $^{\cdot}OH$ and salicylate [25].

2.4. Antioxidant enzyme activity measurements

Measurements of enzyme activities were performed according to standard methods. The SOD assay was done according to Nagi [26], via inhibition of the reduction of NBT by superoxide radical (pro-

duced from oxidation of glucose by glucose oxidase), using 2 mM KCN to inhibit the CuZn form and measure the activity of the Mn form, and using Sigma SOD as a reference (1U = 45 ng) to generate a standard curve. The CuZn SOD activity was determined by subtracting the Mn SOD from total SOD activity. GRD activity was measured according to Carlberg and Mannervik [27] by monitoring the oxidation of NADPH at 340 nm in the presence of GSSG. Selenium-dependent GPX activity was measured according to Carmagnol et al. [28] via coupling to the oxidation of NADPH by excess GRD monitored at 340 nm. The CAT assay was performed according to the spectrophotometric method of Aebi [29], monitoring the degradation of H_2O_2 at 240 nm.

2.5. Treatment of SY5Y neuroblastoma cells for enzyme assays

We measured antioxidant enzyme activities in control and SH-SY5Y cells exposed to 50 μM MPP^+. This concentration was chosen as one which should inhibit complex I, and was found to have an effect on the antioxidant enzymes, and yet produces negligible cell death in serum-containing media. We also measured antioxidant enzyme activities in SY5Y cells exposed to 96 h of 100 μM Se with or without 48 h MPP^+ treatment, to determine whether this would further upregulate GPX activities. The SY5Y cells were grown in media of Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Gaithersburg, MD) supplemented with 10% nondialyzed fetal bovine serum (Intergen, Purchase, NY) and 1X penicillin/streptomycin. The cells were grown to confluence, and 60–70 million cells were harvested for each sample with 5% trypsin–EDTA, washed twice with and suspended in 1 ml PBS/40 million cells. Cells were frozen at −80°C until assays, then were homogenized in sodium cholate (0.013%) and sonicated for 6 min at 50% duty cycle, then centrifuged for 10 min at 12,000X *g* at 4°C to isolate the supernatants. The cells were kept frozen no longer than 2 weeks, and neither the freezing and thawing nor this concentration of sodium cholate had any discernable adverse effects on enzyme activities. The supernatants were then assayed, with all samples being kept on ice, and the procedure repeated at least thrice for each time point.

2.6. Measurements of ROS in SY5Y cells

Intracellular reactive oxygen species were estimated with 2,7-dichlorofluorescin diacetate (DCFA) fluorescence combined with flow cytometry. DCFA is a cell-permeant nonfluorescent compound which is cleaved and retained intracellularly, and is converted by peroxides into fluorescent dichlorofluorescein (DCF) [30]. SY5Y cells were incubated for 45 min with 10 μM DCFA (Molecular Probes, Eugene OR) in serum-free DMEM medium containing a final [DMSO] of 0.05%. Cells were harvested with 5% trypsin, resuspended in DMEM and assayed on a Beckman-Dickinson FACS Station, using 488 nm excitation and 530/30 bp filter for detection. For each assay of MPP^+ effects, an equal number of events from cells not exposed to MPP^+ were also assayed. Data are expressed as average ratios of mean fluorescence of MPP^+ treated cells/mean fluorescence of control cells. Each [MPP^+] was assayed in three independent experiments.

2.7. Creation and treatment of PD cybrid cell lines

A clonal stock of human SH-SY5Y neuroblastoma cells lacking mtDNA (rho^0 cells, MitoKor Corporation, San Diego, CA) was created by prolonged exposure of these cells to low concentrations of ethidium bromide, which selectively depletes mtDNA [31]. Fusion of platelets from patients with sporadic PD with these mtDNA-deficient clonal SH-SY5Y cells was performed as previously described [32], resulting in transfer of mitochondria and mtDNA. These cytoplasmic hybrid cells (or ''cybrids'') were grown and harvested in the same manner as the SY5Y cells, except that their media was supplemented with 50 μg/ml uridine. Each line was grown and harvested separately at least twice, and assayed blinded at least once.

2.8. Statistical analyses

One way ANOVAs were performed on all enzyme activity time courses to determine normality, and post-hoc analyses were performed to determine significance of individual time-points. Tukey's test was performed on parametric data sets and Dunnett's test

on nonparametric data sets for each time-point compared to controls. T-tests were used to compare enzyme activities between Se-treated and control SY5Ys and between cybrids and controls. SigmaStat software (Jandel Scientific) was used for all statistical tests.

3. Results

3.1. ROS production and antioxidant enzyme activities in MPTP-treated mouse brains

We found evidence of increased ROS production in the brains of MPTP-treated mice and concomitant alterations in antioxidant enzyme activities in these animals. As shown in Fig. 1(a), tissue levels of 2,3-DHB, the specific adduct between $^{\cdot}OH$ and salicylate [25], increased significantly in the striatum and ventral midbrain. We observed the greatest relative increase in 2,3-DHB in the ventral midbrain, where the cell bodies of the dopaminergic SN cells (whose striatal terminals concentrate MPP^+) reside. We observed much smaller changes in cerebellar levels of 2,3-DHB after MPTP treatment.

As shown in Fig. 1(b), we found regionally specific increases in antioxidant enzyme activities in several brain regions. Overall, the greatest increases in activities were found in striatum, where GPX rose most significantly, by nearly 500%, and ventral midbrain, where total SOD, CAT, and GPX rose most significantly, each to about 300% of baseline. Less dramatic increases were found in the frontal cortex and cerebellum. These results are consistent with the selective nigrostriatal neurotoxicity of MPTP. Thiffault et al. [33] also recently showed that treatment of mice with a different MPTP regime acutely upregulated the activities of CAT in the SN and both isoforms of SOD in the striatum; however, they found decreased Mn SOD in the SN, failed to find increases in GPX, and did not measure GRD activity.

3.2. ROS and antioxidant enzyme activities in MPP $^+$-treated SY5Y cells

We also detected significant increases in ROS and scavenging enzyme activities in SY5Y neuroblastoma cells treated with MPTP's toxic metabolite, MPP^+.

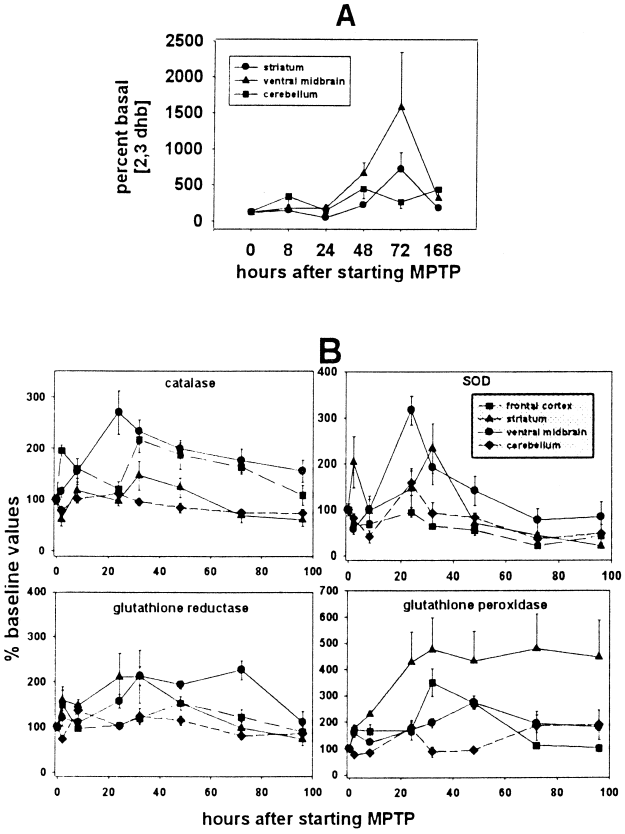

Fig. 1. Time course of changes in (A) hydroxyl radical production and (B) antioxidant enzyme activities in the brains of C57/BL6 mice treated with MPTP. Results for $^{\cdot}OH$ measurements are the means of data from 4–5 mice at each time point as measured by 2,3-DHB trapping. Results for enzyme activities in regions of the brains of MPTP-treated mice are means of data from 6 mice at each time point and are expressed as percentage of baseline control activity. CAT activity is measured in micromoles H_2O_2 consumed/min/mg protein, SOD in units SOD/mg protein, GRD and GPX as nanomoles NADPH consumed/min/mg protein. The following CAT enzyme time courses and time points were significant ($p < 0.05$) relative to controls: frontal cortex, and at 2, 8, 32, 48, 72, and 96 h; striatum; ventral midbrain and at 8, 24, 32, 48, 72, and 96 h; cerebellum at 72 h. The following total SOD enzyme time courses and time points were significant ($p < 0.05$) relative to controls: frontal cortex and at 48, 72, and 96 h; striatum; ventral midbrain and at 24, 32 h; cerebellum and at 72 h. The following GRD enzyme time courses and time points were significant ($p < 0.05$) relative to controls: striatum; ventral midbrain, and at 32 and 72 h; cerebellum at 72 h. The following GPX enzyme time courses and time points were significant ($p < 0.05$) relative to controls: frontal cortex and at 2, 8, 32, and 48 h; striatum and at 2, 8, 32, 48, 72, and 96 h; ventral midbrain and at 2, 8, 24, 48, 72, and 96 h.

Intracellular ROS, monitored by appearance of DCF fluorescence, increased after incubation with 50 μM

MPP^+ and peaked after 6–12 h, then declined to near baseline by 48 h exposure (Fig. 2(a)). This rise and fall in ROS detected in vitro with DCF fluorescence may be analogous to our observed trend in brain $^{\cdot}OH$ production after MPTP treatment of mice. Fig. 2(b) shows that increasing concentration of MPP^+ induced parallel increases in ROS levels in SH-SY5Y cells. As it is known that MPP^+ inhibits complex I in a dose-dependent manner [34], these results suggest a correlation between ROS production and degree of ETC inhibition. At concentrations at or above about 500 μM MPP^+, cell death began to occur (data not shown). As shown in Fig. 3, all the antioxidant enzyme activities increased in the SH-SY5Ys, with the Mn–SOD (mitochondrial isoform) and CAT activities rising to the greatest degree (approximately 400% and 300%, respectively) after exposure to MPP^+, similar to what we observed in vivo in mice treated with MPTP.

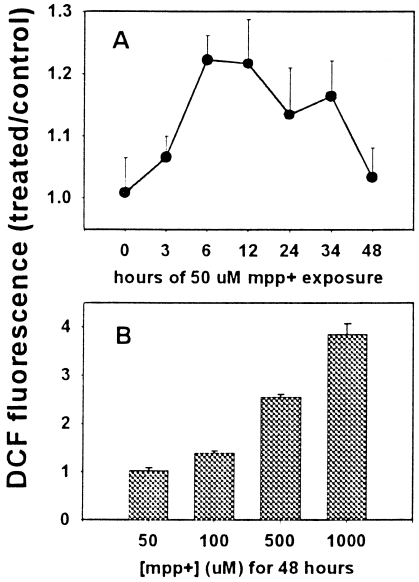

Fig. 2. Time course of changes in DCFA fluorescence measured with flow cytometry in SH-SY5Y cells exposed to 50 μM MPP^+. Data are means ± sem from three independent experiments and are expressed as fluorescence ratios for MPP^+-treated cells compared to simultaneously analyzed control (untreated) cells. The 6 h timepoint was significant ($p = 0.034$, unpaired t-test). (B). DCF fluorescence signal in SH-SY5Y cells exposed for 48 h to increasing [MPP^+]. Data are results from three independent experiments. The results were significant ($p < 0.001$, unpaired t-test) at 100, 500, and 1000 μM MPP^+.

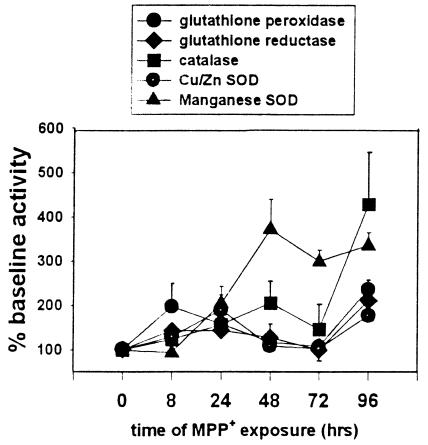

Fig. 3. Increased antioxidant enzyme activities in SY5Y cells treated with MPP^+. All activities are expressed as in Fig. 1. Data are from 3–4 independent experiments for each time point. The following enzyme time courses and timepoints were significant ($p < 0.05$): CAT, and at 8, 24, 48, 72, and 96 h; CuZn SOD, and 24 h; Mn SOD, 24, 48, 72, and 96 h; GRD, and 96 h; GPX, 8, 48, and 96 h.

3.3. Antioxidant enzyme activities in selenium-treated SY5Y cells

As recent reports in the literature have suggested that standard serum-supplemented media do not contain sufficient Se to allow maximal expression of Se–GPX activities [35], we determined the effects of added Se on GPX and GRD activities (Table 1). We treated cells for 96 h with 100 μM Se, a time period and Se-concentration which were found to be effective in significantly upregulating GPX activity in the SY5Y cells. This regimen produced significant elevations in both GPX and GRD, which presumably rose in compensation for increased GSSG formed by elevated GPX activity. The coincubation of Se with 50 μM MPP^+ further augmented the activities of both GPX and GRD significantly compared to treatment with MPP^+ alone. In contrast, the addition of MPP^+ to Se-treated cells did not further increase GPX activity, although it did significantly affect GRD activity, compared to Se alone (Table 1).

3.4. Antioxidant enzyme activities in PD cybrids

The cybrid cells employed in this study all share the same nuclear genes, and differ only in their

Table 1
MPP^+ and Se-supplementation augment the activities of GPX and GRD in SY5Y cells

Condition	Mean activity	Sem	P_1	P_2
GPX activity				
Control	40.2	3.57		
MPP^+	83.7	7.33	0.006	0.031
Se	106.7	2.60	< 0.001	0.53
Se + MPP^+	109.3	2.85	< 0.001	
GRD activity				
Control	59.0	4.72		
MPP^+	78.0	4.89	0.049	0.025
Se	88.3	3.22	0.007	0.058
Se + MPP^+	110.9	8.00	0.005	

The SY5Y cells were treated and harvested and GPX and GRD activities measured as in the methods. P_1 is the p-value calculated from an unpaired t-test of the indicated condition compared to control (untreated). P_2 is the p-value compared to the cells treated with MPP^+ and Se together. GRD and GPX activities are measured in nanomoles NADPH consumed/min/mg protein.

mtDNA. Persisting alterations in cellular functions after multiple cycles of cellular and mitochondrial replication and division must, therefore, derive from the source of mtDNA, not nuclear or environmental factors. Our previous study of PD mitochondrial cybrid cells revealed that they exhibit retention of the selective complex I defect, increased oxygen radical production, and increased sensitivity to MPP^+-induced apoptotic death [32]. In order to determine whether these SH-SY5Y cybrid cells compensate for their increased ROS burdens, we assayed cell lines derived from PD and age-matched control patients for antioxidant enzyme activities. All activities in PD cybrids were increased up to three-fold compared to control cybrids (Fig. 4). A 24 h incubation with 50 μM MPP^+ increased control cybrids' protective enzyme activities significantly to about the same levels as found in PD cybrids, but did not further significantly increase PD cybrid scavenging capacities. The one exception to this observation was the cytoplasmic CuZn SOD isoform, the activity of which decreased in both PD and control cells after MPP^+ exposure. This may derive from the known inhibition of CuZn SOD by H_2O_2 and $ONOO^-$ [36–38], although we

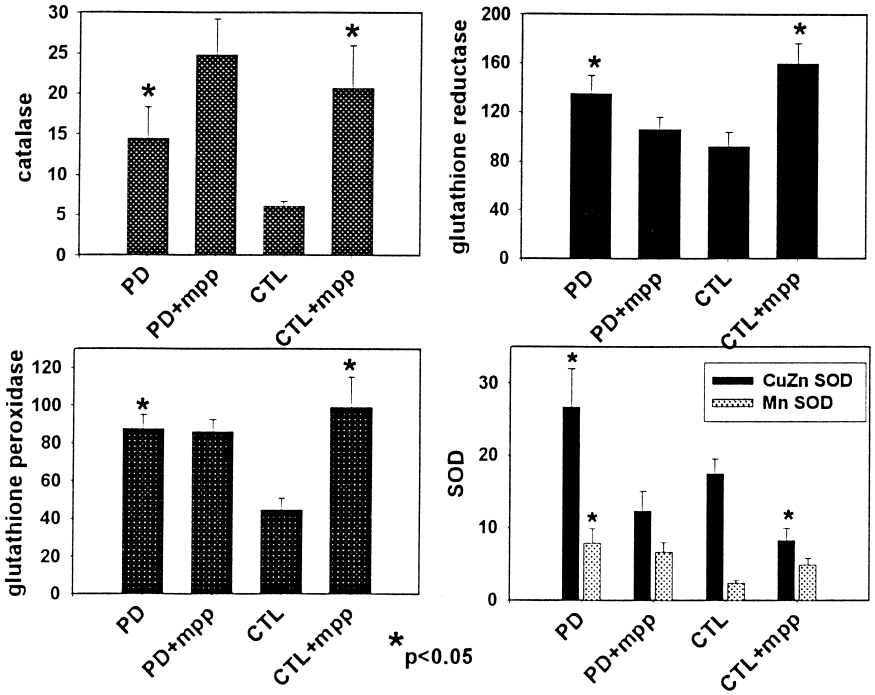

Fig. 4. Parkinson's disease cybrids show increased activities of the major antioxidant enzymes compared to controls, and do not respond significantly to MPP^+. Each enzyme is significantly elevated in the PD cybrids relative to controls ($p < 0.05$). Each of the control cybrids' enzymes rose significantly after 50 μ MPP^+ treatment ($p < 0.05$), while none of the PD cybrids' rose significantly. The ages of the PD patients ($n = 6$) and control patients ($n = 6$) were not significantly different ($p = 0.48$, unpaired t-test).

observed this decrease in activity only in cybrids, not in native host SH-SY5Y.

4. Discussion

We have shown that following our schedule of MPTP treatment of black mice, which results in loss of about one-third of nigral dopamine neurons [39], nigrostriatal production of $^{\cdot}OH$ increases with the greatest relative changes in the ventral midbrain. Temporally associated with the rise in this potentially damaging radical species are increases in the nigrostriatal activities of the major ROS-scavenging enzymes, which were also increased in both SY5Y cells after MPP^+ exposure and spontaneously in PD cybrids. Interestingly, the only predominantly mitochondrially-localized antioxidant enzyme, Mn SOD, exhibited some of the greatest increases relative to controls in both MPP^+-treated SY5Y cells and in PD cybrids (greater than 3-fold in both cases).

Although the regulation of the nuclear-encoded antioxidant enzymes is complex and not well-characterized, they are known to be affected by various oxidative stresses [40–46]. Therefore, it is possible that their upregulation shown here is due to the oxidative stress induced by complex I inhibition, although other factors related to complex I dysfunction such as decreased ATP-production [5,14], excitotoxicity [14], or impaired calcium-homeostasis [47] may be involved. That we have observed similar elevations in antioxidant enzyme activities in both in vivo and in vitro MPTP/MPP^+ models of PD and in PD cybrids relative to controls, argues that despite the obvious differences between the model systems, similar stressors might be operating in each case. Because the only difference between PD and control cybrids is the source of mitochondria and mtDNA, PD cybrids appear to be responding to stresses derived from their genetically transmitted complex I defect.

The elevation of antioxidant enzyme activities in control cybrids and lack of further significant increase in PD cybrids after MPP^+ exposure suggests that the mechanisms for their upregulation are maximally utilized in the PD cells, at least in the SH-SY5Y host cells used to make cybrids, and in response to this regimen of MPP^+. Yet, these defenses are inadequate, as evidenced by increased baseline ROS in PD cybrids and enhanced apoptotic cell death after MPP^+ exposure [32].

That scavenging capacities for several different ROS were increased in both experimental MPP^+ toxicity and in PD cybrids suggests that in both situations multiple ROS may be generated, most likely due to impaired complex I activity. H_2O_2, O_2^-, and $^{\cdot}OH$ have all been detected following complex I inhibition in vitro [7] and in vivo [48]. O_2^- can combine with $NO^{\cdot}$ to yield $ONOO^-$, which may directly oxidize proteins, lipids, and DNA [9,10], and has been found to degrade non-enzymatically to the highly reactive $^{\cdot}OH$ [11,12]. Formation of $^{\cdot}OH$ can also occur via H_2O_2 degradation catalyzed by Fe^{2+} (which is abundant in the SN [24]) in the Fenton reaction, providing at least three routes for the generation of this most destructive of ROS. These toxic ROS are capable of irreversibly damaging essential cellular constituents, and most likely contribute to the nigral cell death characteristic of PD [9,10,13,14,20].

It is interesting to note that the peak in $^{\cdot}OH$ production in vivo seems to be delayed with respect to the induction of enzyme activities, compared to the results in vitro, where ROS levels increased and peaked 24–36 h before maximal increases in antioxidant enzyme activities. There are several possible explanations for these observations. First, the time course of exposure to MPP^+ is substantially different in vivo compared to in vitro. With the in vivo MPTP injections, which occur four times over 48 h, tissue MPP^+ levels would be expected to rise rapidly after each injection, then fall as most MPP^+ is not retained. MPP^+ transport into dopamine terminals and thence into mitochondria in dopamine terminals would likely occur in a phasic manner following each MPTP injection. This is in contrast to the in vitro situation where MPP^+ is present at a constant low level. Steady state levels of intracellular and mitochondrial [MPP^+] would be expected to be reached within a short time.

Because the intracellular stimuli for increased expression of antioxidant enzymes remain to be fully determined, it is reasonable to propose a dissociation between the increases in free radical generation and increased antioxidant enzyme activities. It is tempting to assume that oxygen free radical generation is primarily responsible for stimulating the increase in

scavenging enzyme activities, but this may not be the only factor. Our speculation is that the final mitochondrial [MPP^+] achieved in vivo is higher than that in vitro, but that the time course of increase in mitochondrial [MPP^+] in vivo is slower than that in vitro. For this speculation to be tenable and explain our results, mitochondrial [MPP^+] would have to be related to the level of oxygen free radical production, and antioxidant enzyme expression would have to be stimulated either by low levels of oxygen radicals or some other factor(s).

Finally, we must consider the differences between the in vivo and in vitro detection of oxygen species. The salicylate trapping technique measures only 2,3-DHB, believed to reflect exclusively the result of hydroxyl radical attack on salicylic acid [25]. In contrast, the DCF technique used for the in vitro studies detects multiple reactive oxygen species, including hydrogen peroxide, hydroxyl radical, and nitric oxide [30]. These other oxygen species may have totally different time courses of appearance during MPP^+ toxicity compared to hydroxyl radical alone.

The elevation of Se–GPX activities by Se-supplementation is consistent with reports that standard tissue culture is Se-deficient with respect to Se–GPX requirements [35], and with reports that Se increases the stability and/or translation of GPX mRNA species [44,49–51]. The upregulation of GPX protein levels in response to Se suggests that cells in traditional tissue culture may be deficient in GPX protein, and hence subject to excess oxidative stress. The fact that the addition of MPP^+ to the Se-treated cells did not further significantly raise GPX activity suggests that GPX production was maximal with this [Se]. That GRD activities did further increase with combined MPP^+/Se suggests that the toxin may have increased the fraction of GPX protein which was maximally active, further increasing GSSG production, or that MPP^+ led to oxidation of GSH via ROS-mediated mechanisms. These results suggest that treatment of oxidatively-stressed PD tissues [22–24] or cybrids [32] with low Se concentrations would augment their radical-defenses and perhaps impede cell death. This proposal is consistent with reports of Se providing protection from the neurotoxic effects of MPTP in rat striatum [52] and the protective role of GPX in the PD brain [53].

Others have found in postmortem PD brains increased SOD [54–56] (although it is controversial whether only Mn SOD or both isoforms are higher) and decreased or unchanged CAT [57] and GPX [58] activities, an imbalance which may lead to excess H_2O_2 and hence hydroxyl radical production. CuZn SOD immunoreactivity has also been demonstrated in the Lewy bodies found in PD brains [59], suggesting a correlation between this neuropathological hallmark of PD and free radical scavenging. Damier et al. [53] demonstrated that GPX immunoreactivity is higher in glial cells surrounding surviving SN dopaminergic neurons in PD brains, whereas it is lowest in control brains in the areas most susceptible to PD. This suggests that surviving neurons elicit an adaptive response in glial GPX to the processes involved in PD neurodegeneration. Some reports also have shown that the activities of GPX and SOD are elevated in PD patients' serum [60], but not erythrocytes [61], which lack mitochondria, supporting the notion that the disease is mitochondrial and systemic. Overall, these studies suggest that antioxidant enzymatic defenses may be crucial for the prevention of cell death due to ROS-mediated neuronal damage in PD, and that impairment or limitation of these defenses may contribute to the pathogenesis of the disease.

If this schema is correct, then further scavenging of free radicals and inhibition of NOS would provide relief from the increased ROS burden associated with PD mitochondria. Involvement of $NO^{\cdot}$ in experimental MPTP parkinsonism [8–10] suggests an etiological role and potential therapeutic target for this gaseous, diffusible neurotransmitter free radical, whose normal function in the nervous system is still being defined [62,63]. Further upregulation of scavenging enzyme capacities may be accomplished with neurotrophins [64,65], whose expression can be induced in vitro by free radicals [66]. This neurotrophin-induced increase in scavenging enzymes may provide a mechanism for their ability to ameliorate MPP^+ neurotoxicity in vitro [67].

The creation of cybrids with mtDNA from Parkinson's patients offers a novel experimental paradigm for examining the effects of PD mtDNA on cellular physiology and pathophysiology [32]. We have shown that PD cybrids are characterized by a pattern of oxidative stress and increased antioxidant enzyme activities remarkably similar to those found in both in

vitro and in vivo MPP^+/MPTP models of PD. These results are clearly compatible with and strongly support the hypothesis that mitochondrial dysfunction, due to mutations in the mitochondrial genes for complex I, may underlie sporadic PD [15,32,68]. The resulting ETC impairments and increased ROS production may cause the prominent oxidative stress characteristic of PD, eventually leading to the death of susceptible neurons [9,13,14,20]. Both deletions and point mutations have already been found in sporadic PD patients' mitochondrial DNA [68–70]. Ultimately, the molecular genetic basis of the complex I defect in PD will be defined following sequencing of the mitochondrial genome, as has recently been reported for the selective complex IV defect in sporadic Alzheimer's disease [71].

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