Tetrahydrobiopterin scavenges superoxide in dopaminergic neurons

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SUMMARY
Increased oxidative stresses are implicated in the pathogenesis of Parkinson's disease (PD), and dopaminergic neurons may be intrinsically susceptible to oxidative damage. However, the selective presence of tetrahydrobiopterin (BH4) makes dopaminergic neurons more resistant to oxidative stress caused by glutathione depletion. To further investigate the mechanisms of BH4 protection, we examined the effects of BH4 on superoxide levels in individual living mesencephalic neurons. Dopaminergic neurons have intrinsically lower levels of superoxide than nondopaminergic neurons. In addition, inhibiting BH4 synthesis increased superoxide in dopaminergic neurons, while BH4 supplementation decreased superoxide in nondopaminergic cells. BH4 is also a cofactor in catecholamine and NO production. In order to exclude the possibility that the antioxidant effects of BH4 are mediated by dopamine and NO, we used fibroblasts in which neither catecholamine nor NO production occurs. In fibroblasts, BH4 decreased baseline reactive oxygen species (ROS), and attenuated ROS increase by rotenone and antimycin A. Physiologic concentrations of BH4 directly scavenged superoxide generated by potassium superoxide in vitro. We hypothesize that BH4 protects dopaminergic neurons from ordinary oxidative stresses generated by dopamine and its metabolites and that environmental insults or genetic defects may disrupt this intrinsic capacity of dopaminergic neurons and contribute to their degeneration in PD.

Key words: superoxide, glutathione, buthionine-(S,R)-sulfoximine, Parkinson’s disease, sepiapterin, hydroethidine, 2,7-dichlorodihydrofluorescein; spin trapping; DEPMPO
INTRODUCTION

Parkinson’s Disease (PD) is characterized by the selective degeneration of dopaminergic neurons in the substantia nigra (SN) pars compacta. While the etiology and pathogenesis of this cell death in most cases of PD remain unknown, it has been widely hypothesized that increased oxidative stress and mitochondrial dysfunction contribute to dopaminergic neuronal degeneration. Autopsy studies have noted numerous abnormalities indicative of increased oxidative damage in the SN (1-5). In particular, decreased glutathione in the SN has been found in autopsy brains from individuals with incidental Lewy bodies, presumed to represent presymptomatic PD (6). In addition, mitochondrial complex I activity is also decreased in the SN of PD patients (7,8), and complex I inhibition can increase the formation of reactive oxygen species (9,10).

Although these findings suggest that dopaminergic neurons are susceptible to oxidative stress, studies in vivo have not observed dopaminergic neuronal death following the oxidative stress generated by glutathione depletion (11-13). Furthermore, mesencephalic dopaminergic neurons in primary monolayer (14) and reaggregate (15) cultures are less susceptible to the toxicity of glutathione depletion than nondopaminergic neurons.

To explain this unanticipated resistance of dopaminergic neurons to oxidative stress, we hypothesized that dopaminergic neurons may contain additional antioxidants for the metabolism of ROS. One candidate is tetrahydrobiopterin (BH$_4$), which is present abundantly in dopaminergic neurons where it functions as a cofactor for tyrosine hydroxylase in catecholamine synthesis. Recently, BH$_4$ has also been found to decrease superoxide formed by xanthine/xanthine oxidase (16), a macrophage/phorbol myristate acetate reaction system (17),
and by NOS (18,19), and to protect against a variety of oxidative stressors (20-23). BH₄ also mediates the marked preferential resistance of dopaminergic neurons to glutathione depletion (15). Therefore, BH₄ may provide dopaminergic neurons with an additional pathway for the metabolism of reactive oxygen species (ROS).

In this paper, we delineate a mechanism underlying the increased resistance of dopaminergic neurons to oxidative stress. First, using microfluorimetry of individual living neurons, we show that dopaminergic neurons have lower levels of superoxide than nondopaminergic neurons. We then show that the presence of BH₄ is both sufficient and necessary to maintain low superoxide levels in dopaminergic neurons, and in fibroblasts that have been engineered to synthesize BH₄ but do not produce dopamine or NO. Lastly, we show that BH₄ decreases superoxide in vitro through a direct scavenging mechanism.

EXPERIMENTAL PROCEDURES

Mesencephalic Cultures

Cultures were prepared as described previously (15). Briefly, timed pregnant Sprague-Dawley rats were purchased from Harlan Sprague Dawley (Madison, WI, U.S.A.). Pieces of ventral mesencephalon were dissected from embryonic gestation day 14 (E14) rat embryos (day of conception defined as day 0), and then incubated for 20 min at 37 °C in 0.4% trypsin in calcium- and magnesium-free Hanks’ balanced salt solution (HBSS). Pieces were then triturated in 0.015% DNAse using flame-polished Pasteur pipettes. Cells were plated onto poly-L-lysine coated glass coverslips at a density of 200,000 viable cells/cm². Cells were grown in high glucose
Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. After 48 h, the medium was changed, and 10 µg/ml 5-fluoro-2-deoxyuridine was added to inhibit glial growth. Cells were grown an additional 4-6 d prior to study. Glia represented less than 15% of the total cells in cultures, based on the number of cells that lacked immunoreactivity to neurofilament protein 200 (NFP) or neuron-specific nuclear protein (NeuN) antibodies.

Measurement of ROS

Relative superoxide levels were determined by measuring the rate of increase in ethidium (Et) fluorescence generated by the oxidation of hydroethidine (HEt) by superoxide (24). All measurements were made with a Nikon Diaphot epifluorescence microscope, and illumination with a 150 W Xe arc (attenuated by a neutral density 1.5, ultraviolet-grade filter; Omega Optical, Brattleboro, VT). Et imaging used standard rhodamine optics (excitation 510-560 nm; dichroic mirror 580 nm; emission >590 nm) (Nikon, Melville, NY). Images were formed using a 40X Fluor NA 0.85 objective (Nikon) and collected on a Hamamatsu ICCD (sensitivity set at 7.0). Images were 8-bit (256 intensity levels), and 16 frames were averaged every 10 seconds. Background subtraction was made using the first image obtained when HEt solution was added. Data acquisition was controlled by MetaFluor or MetaMorph software (Universal Imaging Corp.), and average intensity over identified regions of interest were logged to hard disk and displayed in real time. The relative superoxide level for each cell was determined from the slope of the increase in ethidium fluorescence over time, fit by linear regression. HEt, a dye freely permeable to cells (25,26), was freshly prepared and used at a final concentration of 3.2 µM.

We also measured the oxidation of 2,7-dichlorodihydrofluorescein (DCHF) to 2,7-dichlorofluorescein (DCF) which can be caused by various ROS and reactive nitrogen species.
(27). Images were obtained in the presence of 33 μM DCHF at 5 sec intervals for 15 min. Images were corrected by subtracting an autofluorescence image obtained before dye addition. Optics utilized excitation at 460-490, dichroic mirror 500, emission >515nm. Rate of fluorescence increase was determined by fitting a line to the final 5 min by linear regression.

To quantify fluorescence, cell somata were circled, and the average cytosolic fluorescence computed. Because systematic differences in somal volumes between dopaminergic and nondopaminergic neurons would alter fluorescence intensities, any volume difference could confound our interpretation of ROS differences. Accordingly, mean cross sectional somal areas (and hence cell volume) were compared between dopaminergic and nondopaminergic neurons and were found to be comparable (data not shown).

Immunohistochemistry and post hoc identification of dopaminergic neurons

Neurons in which fluorescence measurements were made were identified as either dopaminergic or nondopaminergic based on post-hoc TH-immunohistochemistry. After each study, the location of the field of cells was marked on the slide. Cells were then washed with 0.1 M PBS, and fixed in 4% paraformaldehyde/0.1 M phosphate buffer for 15 minutes. Cells were exposed to 0.6% H₂O₂ in PBS for 15 minutes to remove endogenous peroxidase activity, and preincubated with 0.1 M PBS containing 1% BSA and 0.2% Triton X-100 for 30 minutes. Cells were incubated at room temperature overnight in primary polyclonal anti-TH antiserum (1:1000). Cultures were next incubated for 1 hr with anti-rabbit biotinylated secondary antibody at 1:200 dilution, and an additional 1 hr with an avidin-biotin conjugate of peroxidase (Vectastain ABC kit). Dopaminergic neurons were visualized as a black reaction product, using diaminobenzidine.
tetrahydrochloride (DAB) in the presence of cobalt chloride (0.02%) and nickel ammonium sulfate (0.02%).

Following immunohistochemistry, the original field of imaged cells field was found and re-imaged. The orientation and location of stained cells were matched, and neurons were identified as dopaminergic or nondopaminergic based on immunostaining.

Western blot analysis

Fibroblasts were grown to confluence in T162 flasks prior to harvest. Cells were resuspended in 200 µl PBS, sonicated and centrifuged at 16,000 g for 2 minutes. The supernatant was frozen at -20 °C.

A total of 25 µl of supernatant from each sample were electrophoresed on 10% SDS-PAGE gels, and transferred to a polyvinylidene difluoride (PVDF) membrane. Following transfer, membranes were incubated overnight in 5% nonfat milk, and then incubated 1 h with primary antibodies against endothelial NOS (eNOS) at 1:500, inducible NOS (iNOS) at 1:1000, or constitutive NOS (cNOS, neuronal or brain NOS) at 1:175 in 3% milk. Membranes were then incubated for 1 h with the appropriate secondary (anti-rabbit horseradish peroxidase or antimouse horseradish peroxidase, 1:2000) in 3% milk, and then developed using ECL.

In Vitro Superoxide Measurements

Saturated potassium superoxide (KO$_2$) solution was made by mixing KO$_2$ in dimethyl sulfoxide, centrifuging at 1500 rpm for 5 min, and then decanting the supernatant (24). This procedure yields about 3.6 mM solution (28). The stock solution of KO$_2$ was then added to buffer
containing 10 μM HEt (for a final concentration of 150 μM), and Et fluorescence was monitored every 20s for 160s, using a fluorometer. The superoxide level was determined from the slope of the increase in Et fluorescence over time, fit by linear regression. When indicated, BH₄ was added immediately prior to adding KO₂, at final concentrations ranging from 20 nM to 200 μM. Cu/Zn-SOD (9 U/mL) was used as a control.

The effects of BH₄ on KO₂-generated superoxide levels were also measured by electron paramagnetic resonance (EPR) spectrometry using a spin trapping agent 5-diethoxy-phosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO) (19,29). DEPMPO was prepared as a 40 mM solution in 50 mM sodium phosphate buffer, pH 7.4, treated with an iron chelator, diethlenetriaminepentaacetic acid (DTPA; N,N-bis[2-(bis[carboxymethyl]amino)ethyl]glycine) (1 mM). Catalase (300 U/ml) was also added to prevent other oxygen radicals from contaminating the EPR spectra. Spectra were recorded within 1 min after mixing, with a Varian E-12 EPR spectrometer equipped with a century series bridge (E-102) and a TM011 cavity mounted horizontally in the magnet. EPR spectra were recorded at X-band (9.45 GHz), using 30 mW of power, a time constant of 0.1 s and modulation amplitude of 0.5 G. Spectra were acquired in 30 s. The mean and standard error of the signal amplitude was calculated as the signal height of the 5th line of the DEPMPO spectrum taken from nine or ten measurements from each of 3 separate experiments.

**Statistical analysis**

Statistical analyses were performed using the GB-STAT statistical package (Dynamic Microsystems, Silver Spring, MD). Standard errors were calculated for each mean, and
statistical differences between groups were determined by ANOVA followed by Newman-Keuls post hoc test as indicated.

Materials

Dulbecco’s modified Eagle's medium (DMEM) and calcium- and magnesium-free Hanks’ balanced salt solution (HBSS) were purchased from Gibco (Grand Island, NY); and fetal bovine serum from Intergen (Purchase, NY). Trypsin was purchased from Worthington Biochemical Corporation (Freehold, NJ); 15 mm diameter glass coverslips from Carolina Biological (Burlington, NC); 2,4-diamino-6-hydroxypyrimidine (DAHP), N-acetylseryotonin (NAS), DNase, L-buthionine-[S,R]-sulfoximine (BSO), N-nitro-L-arginine methyl ester (L-NAME), DPTA and potassium superoxide from Sigma (St. Louis, MO), DEMPPO from OXIS Health Products (Portland, OR). Antibodies against tyrosine hydroxylase (TH) antiserum were obtained from Pel-Freez (Rogers, AR); antibodies against eNOS, iNOS and cNOS from Biomol (Plymouth Meeting, PA); Vectastain ABC kit from Vector Laboratories (Burlingame, CA); mouse macrophage RAW 264.7 and bovine pulmonary artery endothelium cell lines were obtained from ATCC (Manassas, VA); and whole rat brain extract from Biomol (Plymouth Meeting, PA). Hydroethidine (HEt) and 2,7-dichlorodihydrofluorescein (DCHF) were from Molecular Probes (Eugene, OR, USA), sepiapterin from RBI (Natick, MA) and tetrahydrobiopterin (BH₄) from Schircks (Jona, Switzerland). SDS-PAGE and PVDF membranes were from Novex (Carlsbad, CA).

RESULTS

BH₄ decreases superoxide in primary mesencephalic neurons
We first examined the effects of BH₄ on superoxide levels in individual mesencephalic dopaminergic and nondopaminergic neurons using Et fluorescence. Dopaminergic (n = 56) neurons had significantly lower baseline levels of superoxide than nondopaminergic neurons (n = 924), consistent with the endogenous presence of BH₄ within dopaminergic neurons (Fig. 1). Sepiapterin (20 µM) is converted to BH₄ intracellularly (30), and increases BH₄ levels in total mesencephalic neurons comparable to those in dopaminergic neurons (31). Sepiapterin significantly decreased superoxide in nondopaminergic neurons to levels comparable to those in dopaminergic neurons, without further decreasing superoxide in dopaminergic neurons (n = 246 and 14 respectively) (Fig. 1). To decrease BH₄ levels, we inhibited BH₄ synthesis using DAHP (1 mM) and NAS (0.1 mM). Each of these compounds decreases BH₄ to less than 5% of control levels in cell lines (30). In mesencephalic neurons, DAHP and NAS in combination decrease BH₄ levels to about 16% of control (15). BH₄ depletion increased superoxide in dopaminergic neurons without affecting superoxide in nondopaminergic neurons (n = 20 and 316 respectively). Furthermore, BH₄ depletion increased superoxide levels in dopaminergic neurons beyond the levels of nondopaminergic neurons. The observation that DAHP and NAS failed to alter BH₄ levels in nondopaminergic neurons is consistent with the absence of endogenous BH₄ in these cells. Therefore, superoxide levels are inversely related to BH₄ levels in mesencephalic neurons.

**BH₄ decreases ROS independent of catecholamines and NOS**

BH₄ is employed as a required cofactor by catecholamine synthesis enzymes and by nitric oxide synthase, both of which are expressed in our cultures of mesencephalic neurons. Because products of both these enzyme systems can modulate superoxide production, altering BH₄ levels in mesencephalic cultures could indirectly modulate superoxide by affecting levels of catecholamines or NO. To understand BH₄ effects on superoxide levels in the absence of BH₄-
dependent enzymes, we examined superoxide levels in primary cultures of fibroblasts (PF) and cultures of a fibroblast line genetically engineered to express GTP cyclohydrolase (PFG), the rate-limiting enzyme in BH₄ synthesis. Neither of these cell types synthesizes catecholamines. PFG produce BH₄ but not L-DOPA or dopamine (32).

PFG produced BH₄ and PF did not (Table 1). PFG also had much lower levels of superoxide compared with PF (Fig 2K). Addition of sepiapterin (20 µM) to PF markedly increased BH₄ levels (Table 1) and also decreased Et fluorescence dramatically compared with controls (Fig. 2K). The decreased superoxide level in sepiapterin-treated PF was comparable to superoxide levels in PFG (Fig. 2K). Inhibition of BH₄ synthesis in PFG by DAHP (1 mM) and NAS (1 mM) decreased BH₄ PFG to undetectable levels (Table 1), and increased superoxide levels. These data indicate that BH₄ is sufficient to decrease superoxide, independent of its role as a cofactor for dopamine synthesis.

To determine if BH₄ lowers ROS through its effect on NOS, we examined whether fibroblasts express NOS. NOS has been shown to produce superoxide instead of NO in the absence of BH₄ and NO can also scavenge superoxide (33). Western blot analyses did not detect eNOS, iNOS or cNOS expression in either PF or PFG (Fig. 3). To provide further evidence for the absence of NOS effect on superoxide levels in our system, we examined the effect of L-NAME on superoxide levels. Treatment of fibroblasts with L-NAME (1 mM) for 2 h had no significant effect on basal superoxide levels or following treatment with sepiapterin (20 µM) (Table 2). These data indicate that BH₄ decreases superoxide levels in fibroblasts independent of NOS.
To further investigate the effects of BH₄ on ROS, we employed DCF fluorescence. Oxidation of DCFH to DCF by various ROS and reactive nitrogen species has been noted (27). Sepiapterin (20 µM) decreased DCF fluorescence in PF (Fig. 2L), and PFG had lower levels of DCF fluorescence than PF (Fig. 2L). In addition, inhibition of BH₄ synthesis in PFG by DAHP (1 mM) and NAS (1 mM) increased DCF fluorescence. These DCF data were consistent with our Et fluorescence data. Taken together, our data using Et and DCF fluorescence suggest that BH₄ decreases both superoxide and downstream superoxide metabolites such as peroxides and other ROS.

**BH₄ decreases mitochondrial ROS**

In order to assess the effects of BH₄ on mitochondrial production of superoxide, rotenone and antimycin A were used to block electron transport distal to the primary sites of superoxide formation in complexes I and III, respectively (10,34-36). Both rotenone (20 nM) and antimycin A (1 µM) significantly increased superoxide levels in control fibroblasts. In both cases, this increase in superoxide was significantly attenuated in cells that either expressed BH₄ or were pre-treated with sepiapterin (Fig. 4). These data indicate that BH₄ either interferes with mitochondrial ROS generation, directly scavenges ROS formed within mitochondria, or both.

Mitochondrial depolarization-induced release of ethidium and subsequent fluorescence enhancement following binding to nucleic acids can complicate the interpretation of ethidium-based methods of superoxide measurement (37). To test for this complication in our system, we used myxothiazole to depolarize mitochondria without increasing superoxide production. Treatment of fibroblasts with myxothiazole (2 µM) plus oligomycin (2 µg/ml) inhibited the slow Et fluorescence increase observed under baseline conditions (data not shown). In addition,
subsequent treatment with FCCP (1 µM) did not produce an increase in Et fluorescence, demonstrating the lack of artifactual increase in Et fluorescence under our experimental conditions.

**BH₄ directly scavenges ROS**

To investigate whether BH₄ can scavenge superoxide directly, we used an *in vitro* model of KO₂-generated superoxide (24,38). Consistent with previous observations, KO₂ increased superoxide as indicated by an increased rate of HEt conversion into Et (Fig. 5). However, this KO₂-induced increase in superoxide levels was significantly attenuated by pre-incubation with BH₄ at concentrations as low as 2 µM. The effect of 200 µM BH₄ on scavenging superoxide was equivalent to that of 9 U/mL SOD. BH₄ (20 µM) alone did not alter the fluorescence of 100 nM Et *in vitro* (data not shown), excluding the possibility that BH₄ directly interferes with Et fluorescence.

Although KO₂ generates primarily superoxide, other ROS may be subsequently generated and scavenged by BH₄. Therefore, to provide more direct evidence that BH₄ scavenges superoxide, we also used EPR with the spin trapping agent DEPMPO. DEPMPO is more sensitive and specific for superoxide detection than other spin traps, due to the formation of a more stable adduct (19,29). The mean signal height of the 5th line of the DEPMPO spectrum corresponding to superoxide was 275.9 ± 7.5 (SEM, an arbitrary unit, n=27) in the KO₂ (50 µM) group. This value was attenuated to 127.7 ± 2.7 (n=30, p< 0.01 by Fisher’s LSD protected t-Test) when 200 µM BH₄ was added to the mixture (Fig. 6). There was no detectable superoxide signal when SOD (100 U/ml) was added as a positive control to scavenge superoxide (Fig. 6C). Taken
together, these data strongly suggest that BH$_4$ decreases ROS through a direct scavenging mechanism.

**DISCUSSION**

This study provides the first evidence for intrinsic differences in antioxidant capacity between dopaminergic and nondopaminergic neurons. Dopaminergic neurons have intrinsically lower levels of superoxide than nondopaminergic neurons, consistent with their increased resistance to oxidative stresses such as glutathione depletion (14,15,31). In addition, our data indicate that physiologic levels of BH$_4$ within dopaminergic neurons are necessary and sufficient for maintaining lower ROS, since the inhibition of BH$_4$ synthesis selectively increased ROS within dopaminergic neurons and providing exogenous BH$_4$ selectively decreased ROS in nondopaminergic neurons. Our data further demonstrate that BH$_4$ decreases ROS generated from mitochondria and does so independent of any effect on dopamine synthesis or NOS. Finally, our in vitro experiments show that BH$_4$ scavenges superoxide directly.

The lower ROS level in dopaminergic neurons is surprising given the overwhelming evidence for oxidative stress in PD and the selective degeneration of dopaminergic neurons in PD. One possible explanation is that the balance of oxidative stress to antioxidant defense in cultured rodent fetal mesencephalic neurons may be different from adult human dopaminergic neurons *in vivo*. In addition, the intrinsic oxidative capacity of dopaminergic neurons in the normal state may be different from that in disease states. Dopaminergic neurons are also likely to face a higher baseline level of oxidative stress, due to ROS formed from the degradation of dopamine. Thus, monoamine oxidase metabolizes dopamine to H$_2$O$_2$, which may be detoxified primarily by the glutathione/glutathione peroxidase system. In addition, ROS may be generated
by TH through partial uncoupling of the hydroxylation reaction (39). Therefore, in order for normal dopaminergic neurons to survive, they may require an intrinsically increased antioxidant capacity.

Our results suggest that BH₄ provides an intrinsic antioxidant capacity unique to dopaminergic neurons. Thus, BH₄ protects against BSO (15), H₂O₂, UV light (22), and paraquat toxicity (40). Compared with nondopaminergic neurons, dopaminergic neurons are also more resistant to NO toxicity, but equally vulnerable to peroxynitrite toxicity (41,42). This observation, together with the fact that superoxide reacts rapidly with NO to form toxic peroxynitrite anions, led Sawada et al. (41,42) to hypothesize that normal dopaminergic neurons have low superoxide levels. The protective effect of decreased superoxide against NO toxicity has also been inferred from data showing that high levels of MnSOD are protective (43). Our data provide direct evidence supporting the hypothesis that dopaminergic neurons have low superoxide levels and demonstrate that lower superoxide levels in dopaminergic neurons is due to BH₄.

BH₄ could lower superoxide levels by interfering with the production of superoxide or by scavenging it. In most cell types, mitochondria are the major site of superoxide generation (44,45), and their metabolic products such as H₂O₂, peroxynitrite anions and hydroxyl radicals constitute the majority of intracellular ROS. BH₄ attenuated the superoxide levels generated by mitochondrial inhibitors such as rotenone and antimycin A (Fig. 4). Once superoxide is formed its two most characterized fates are SOD-catalyzed dismutation to H₂O₂ and reaction with NO to form toxic peroxynitrite anions. BH₄ functions as an essential cofactor for NOS (46), and NOS can be uncoupled to produce superoxide when concentrations of either BH₄ or the substrate
arginine are suboptimal (19,33). Conversely, NOS can also decrease superoxide levels, since NO formed by NOS reacts rapidly with superoxide to form peroxynitrite (47). Therefore, BH₄ could lower superoxide levels both by decreasing production by NOS (48,49), and by increasing superoxide scavenging by NO. To examine whether there are direct effects of BH₄ on superoxide independent of NOS, we used fibroblasts that do not express NOS (Fig. 3). These fibroblasts had lower superoxide levels than control fibroblasts when genetically modified to produce BH₄ or provided with the BH₄ precursor sepiapterin (Fig. 2K). Thus, the superoxide lowering effect of BH₄ is not mediated through NO.

We provide the first direct evidence that BH₄ scavenges superoxide, by showing its effect on superoxide generated nonenzymatically by KO₂ (24) using both Et fluorescence and EPR measurements. Although BH₄ has previously been hypothesized to scavenge superoxide directly, these studies generated superoxide by xanthine/xanthine oxidase or a macrophage/phorbol myristate acetate reaction system (16,17,19,22,40), and hence BH₄ could have inhibited these superoxide generating enzymes. We saw a BH₄-mediated decrease in superoxide levels at a range of concentrations (2 – 200 µM) similar to the estimated concentrations of BH₄ in nigrostriatal dopaminergic neurons (50). Furthermore, the observation that BH₄ significantly attenuates basal superoxide levels within dopaminergic neurons in culture demonstrates the physiological relevance of its effect.

A potential relationship between the role of BH₄ as an antioxidant and the pathogenesis of PD is a tantalizing idea. Dopaminergic neurons in the SN have lower levels of GTP cyclohydrolase 1 mRNA than dopaminergic neurons in the VTA (51), and lower GTP cyclohydrolase protein than norepinephrine neurons in the locus ceruleus or serotonergic neurons
in the dorsal raphe nucleus (52), and are therefore presumed to have comparatively low levels of BH₄. Low levels of BH₄ could contribute to the greater susceptibility of dopaminergic neurons in the SN versus other catecholaminergic neurons in PD. In addition, the strongest known risk factor for PD is age, and BH₄ levels are known to decrease significantly with increasing age in mice (53) and humans (54). In particular, Chen et al. (55) reported that the number of GTPCH-immunoreactive nigral neurons were decreased more than TH-immunoreactive neurons in aged monkeys.

In conclusion, BH₄ directly scavenges superoxide, and thereby provides dopaminergic neurons with an enhanced antioxidant capacity. We propose that this intrinsic resistance of dopaminergic neurons to oxidative stress is critical to their survival, and disturbances in this capacity through genetic mutations or exposure to exogenous toxins could underlie their demise in PD. Understanding these processes may lead to therapeutic interventions to protect both dopaminergic and nondopaminergic neurons from a variety of oxidative stressors.
References

1. Sian, J., Dexter, D. T., Lees, A. J., Daniel, S., Agid, Y., Javoy-Agid, F., Jenner, P., and Marsden, C. D. (1994) *Ann Neurol* **36**, 348-355

2. Yositaka, A., Hattori, N., Uchida, K., Tanaka, M., Stadtman, E. R., and Mizuno, Y. (1996) *Proc Natl Acad Sci USA* **93**, 2696-2701

3. Alam, Z. I., Daniel, S. E., Lees, A. J., Marsden, D. C., Jenner, P., and Halliwell, B. (1997) *J Neurochem.* **69**, 1326-1329

4. Alam, Z. I., Jenner, A., Daniel, S. E., Lees, A. J., Cairns, N., Marsden, C. D., Jenner, P., and Halliwell, B. (1997) *J Neurochem.* **69**, 1196-1203

5. Jenner, P., and Olanow, C. W. (1998) *Ann Neurol.* **44**, S72-S84

6. Dexter, D. T., Sian, J., Rose, S., Hindmarsh, J. G., Mann, V. M., Cooper, J. M., Wells, F. R., Daniel, S. E., Lees, A. J., Schapira, A. H. V., Jenner, P., and Marsden, C. D. (1994) *Ann Neurol* **35**, 38-44

7. Schapira, A. H. V., Mann, V. M., Cooper, J. M., Krige, D., Jenner, P. J., and Marsden, C. D. (1992) *Ann Neurology* **32**, S116-S124

8. Mann, V. M., Cooper, J. M., Krige, D., Daniel, S. E., Schapira, A. H. V., and Marsden, C. D. (1992) *Brain* **115**, 333-342

9. Kwong, L. K., and Sohal, R. S. (1998) *Arch Biochem Biophys.* **350**, 118-126

10. Turrens, J. F., and Boveris, A. (1980) *Biochem J* **191**, 421-427

11. Toffa, S., Kunikowska, G. M., Zeng, B. Y., Jenner, P., and Marsden, C. D. (1997) *J Neural Transm.* **104**, 67-75

12. Pileblad, E., Magnusson, T., and Fornstedt, B. (1989) *J Neurochem.* **52**, 978-980

13. Wullner, U., Loschmann, P., Schulz, J., Schmid, A., Dringen, R., Eblen, F., Turski, L., and Klockgether, T. (1996) *Neuroreport* **7**, 921-923
14. Nakamura, K., Wang, W., and Kang, U. J. (1997) *J Neurochem* **69**, 1850-1858

15. Nakamura, K., Won, L., Heller, A., and Kang, U. J. (2000) *Brain Res* **873**, 203-211

16. Wever, R. M. F., van Dam, T., nan Rijn, H. J. M., de Groot, F., and Rabelink, T. J. (1997) *Biochem. Biophys. Res Commun.* **237**, 340-344

17. Kojima, S., Icho, T., Kajiwara, Y., and Kubota, K. (1992) *FEBS Lett* **304**, 163-166

18. Stroes, E., Hijmering, M., Zandvoort, M., Wever, R., Rabelink, T. J., and Faassen, E. E. (1998) *FEBS Lett.* **438**, 161-164

19. Vasquez-Vivar, J., Kalyanaraman, B., Martasek, P., Hogg, N., Masters, B. S. S., Karoui, H., Tordo, P., and Pritchard, K. A., Jr. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 9220-9225

20. Heales, S. J. R., Blair, J. A., Meinschad, C., and Ziegler, I. (1988) *Cell Biochem Function* **6**, 191-195

21. Koshimura, K., Murakami, Y., Tanaka, J., and Kato, Y. (1998) *J Neurosci Res* **54**, 664-672

22. Shen, R., and Zhang, Y. (1993) *Adv Exp Med Biol* **338:351-354**, 351-354

23. Ishii, M., Shimizu, S., Momose, K., and Yamamoto, T. (1999) *J Cardiovasc Pharmacol.* **33**, 295-300

24. Bindokas, V., Jordan, J., Lee, C., and Miller, R. (1996) *J Neurosci.* **16(4)**, 1324-1336

25. Gallop, P. M., Paz, M. A., Henson, E., and Latt, S. A. (1984) *Biotechniques* **2**, 32-36

26. Carter, W. O., Narayanan, P. K., and Robinson, J. P. (1994) *J Leukoc Biol* **55**, 253-8.

27. Ischiropoulos, H., Gow, A., Thom, S. R., Kooy, N. W., Royall, J. A., and Crow, J. P. (1999) *Methods Enzymol.* **301**, 367-373

28. Reiter, C. D., Teng, R. J., and Beckman, J. S. (2000) *J Biol Chem* **275**, 32460-32466.

29. Roubaud, V., Sankarapandi, S., Kuppusamy, P., Tordo, P., and Zweier, J. L. (1997) *Anal Biochem* **247**, 404-411
30. Smith, G. K., Duch, D. S., Edelstein, M. P., and Bigham, E. C. (1992) *J. Biol. Chem.* **267**, 5599-5607

31. Nakamura, K., Wright, D. A., Wiatr, T., Kowlessur, D., Milstien, S., Lei, X. G., and Kang, U. J. (2000) *J Neurochem* **74**, 2305-2315

32. Bencsics, C., Wachtel, S. R., Milstien, S., Hatakeyama, K., Becker, J. B., and Kang, U. J. (1996) *J. Neurosci.* **16**, 4449-4456

33. Pou, S., Pou, W. S., Bredt, D. S., Snyder, S. H., and Rosen, G. M. (1992) *J Biol Chem* **267**, 24173-24176

34. Herrero, A., and Barja, G. (1997) *J Bioenerg Biomembr.* **29**, 241-249

35. Pitkanen, S., and Robinson, B. H. (1996) *J Clin Invest.* **98**, 345-351

36. Hensley, K., Pye, Q. N., Maidt, M. L., Stewart, C. A., Robinson, K. A., Jaffrey, F., and Floyd, R. A. (1998) *J Neurochem* **71**, 2549-2557

37. Budd, S. L., Castilho, R. F., and Nicholls, D. G. (1997) *FEBS Lett* **415**, 21-24

38. Lokesh, B. R., and Cunningham, M. L. (1986) *Toxicol Lett* **34**, 75-84.

39. Haavik, J., Almas, B., and Flatmark, T. (1997) *J. Neurochem* **68**, 328-332

40. Kojima, S., Ona, S., Iizuka, I., Arai, T., Mori, H., and Kubota, K. (1995) *Free Radic. Res.* **23**, 419-430

41. Sawada, H., Kawamura, T., Shimohama, S., Akaike, A., and Kimura, J. (1996) *J Neurosci Res* **43**, 503-510

42. Sawada, H., Shimohama, S., Kawamura, T., Akaike, A., Kitamura, Y., Taniguchi, T., and Kimura, J. (1996) *J Neurosci Res* **46**, 509-518

43. Gonzalez-Zuleta, M., Ensz, L. M., Mukhina, G., Lebovitz, R. M., Zwacka, R. M., Enmgelhardt, J. F., Oberley, L. W., Dawson, V. L., and Dawson, T. M. (1998) *J. Neurosci.* **18**, 2040-2055
44. Skulachev, V. P. (1996) Q.Rev.Biophys. 29, 169-202

45. Turrens, J. F. (1997) Biosci Rep 17, 3-8.

46. Mayer, B., and Werner, E. R. (1995) Naunyn-Schmiedeberg's Arch.Pharmacol. 351, 453-463

47. Schmidt, H. H., Hofmann, H., Schindler, U., Shutenko, Z. S., Cunningham, D. D., and Feelisch, M. (1996) Proc Natl Acad Sci U S A 93, 14492-14497

48. Xia, Y., Tsai, A. L., Berka, V., and Zweier, J. L. (1998) J Biol Chem 273, 25804-25808

49. Vasquez-Vivar, J., Hogg, N., Martasek, P., Karoui, H., Pritchard, K. A., Jr, and Kalyanaraman, B. (1999) J Biol Chem 274, 26736-26742

50. Levine, R. A., Miller, L. P., and Lovenberg, W. (1981) Science 214, 919-921

51. Lentz, S. I., and Kapatos, G. (1996) Neurochem.Int. 28, 569-582

52. Hirayama, K., and Kapatos, G. (1998) J.Neurochem. 70, 164-170

53. Yoshida, Y., Eda, S., and Masada, M. (2000) Brain Dev 22 Suppl 1, 45-49

54. Furukawa, Y., and Kish, S. J. (1998) Neurology 51, 632-634

55. Chen, E. Y., Kallwitz, E., Leff, S. E., Cochran, E. J., Mufson, E. J., Kordower, J. H., and Mandel, R. J. (2000) J Comp Neurol 426, 534-548
Figure Legends

Fig. 1  Superoxide levels of individual mesencephalic neurons

Superoxide levels were estimated in individual living mesencephalic neurons using HEt microfluorimetry as described previously (24). Cells were then processed for TH-immunohistochemistry, and the original field of cells was relocated to identify dopaminergic neurons. Sepiapterin (20 µM) (S) was given to provide BH4 intracellularly while DAHP (1 mM) and NAS (0.1 mM) (N + D) were used to block BH4 synthesis. Superoxide levels were normalized to the mean superoxide in control nondopaminergic neurons. Data are expressed as means ± SEM (bars).  

\[ \text{a } p < 0.01 \text{ versus nondopaminergic neurons in the same treatment; } b \ p < 0.01 \text{ versus respective dopaminergic and nondopaminergic control groups by Newman-Keuls post hoc test.} \]

Fig. 2  BH4 effect on ROS levels in fibroblasts

A-E show differential interference contrast images of fibroblasts and F-J show Et microfluorimetry. A, F show control fibroblasts (PF); B, G are PF incubated with sepiapterin (20 µM) (S); C, H are PF incubated with DAHP (1 mM) and NAS (1 mM) (N + D). D, I show BH4-expressing fibroblasts (PFG) and E, J show PFG incubated with N + D. K shows quantitation of the Et fluorescence values normalized to the mean levels in untreated PF. L shows quantitation of DCF fluorescence. The data are expressed as means ± SEM.  

\[ \text{a } p < 0.05, \ b \ p < 0.01 \text{ versus untreated PF; } c \ p < 0.01 \text{ versus untreated PFG, by Newman-Keuls post hoc test, n = 23-56 cells per group for Et; n = 37-61 for DCF microfluorimetry.} \]

Fig. 3  NOS expression in fibroblasts
NOS expression was examined in PF and PFG by Western blot analysis using antibodies against cNOS, eNOS and iNOS. Positive controls were obtained from extracts of mouse macrophage RAW 264.7 cells treated for 16 hr with LPS (30 µg/ml) prior to harvest for iNOS (iN), bovine pulmonary artery endothelial cells for eNOS (eN), and whole brain extract from rat (10 µg) for cNOS (cN). St, standard; B, blank. The expected molecular weight of each NOS protein is indicated.

Fig. 4  BH₄ effect on mitochondrial sources of ROS

Data shows the acute effects of rotenone (20 nM) and antimycin A (1 µM) on superoxide levels measured by Et fluorescence in control fibroblasts (PF), PF incubated with sepiapterin (20 µM) (PF/S), and BH₄-expressing fibroblasts (PFG). Et fluorescence values were first normalized to the mean level in untreated PF. The increase in superoxide level was then calculated by subtracting the respective PF, PF/S and PFG control groups. Data show mean ± SEM. a p < 0.05 versus PF in the same treatment, b p < 0.01 versus PF in the same treatment; c p < 0.01 versus respective PF, PF/S and PFG control groups by Newman-Keuls post hoc test; n = 11-53 cells per group.

Fig. 5  BH₄ effect on superoxide in vitro measured by ethidium fluorescence
The effect of KO\(_2\) (O) on Et fluorescence was examined in the presence of HEt (H, 10 µM) and increasing concentrations of BH\(_4\) (B), as described in methods. SOD (9 U/mL) was used as a control. Data are expressed as means ± SEM. \(^a p< 0.05, \ ^b p < 0.01\) versus HO, by Newman-Keuls post hoc test, \(n = 3\) per group.

**Fig. 6**  BH\(_4\) effect on superoxide in vitro measured by electron paramagnetic resonance of a spin trap agent

The EPR spectra of DEPMPO-OOH spin trap adduct obtained from (A) KO\(_2\) in buffer solution; (B) KO\(_2\) in the presence of 200 µM of BH\(_4\) and (C) KO\(_2\) and SOD (100 U/ml). The concentration of DEPMPO was 40 mM. The X-band spectrometer settings were: 30 mW of power, sweep range 200G, time const. 0.1 sec, scan time 30 s, mod. ampl. 0.63 G.
### Table 1. BH₄ levels of genetically modified fibroblasts

|         | baseline | DAHP /NAS | Sepiapterin |
|---------|----------|-----------|-------------|
| PF      | 0 ± 0    | 0 ± 0     | 946 ± 214   |
| PFG     | 212 ± 29.7° | 0 ± 0°    | ND          |

Control fibroblasts (PF) and fibroblasts expressing BH₄ (PFG) were cultured in the presence of DAHP (1 mM) and NAS (1 mM) or sepiapterin (20 µM). After 2 d, cultures were harvested and BH₄ levels (pmol/mg protein) were measured. Data show mean ± SEM. °p < 0.01 versus PF; °p < 0.01 versus respective untreated controls by Bonferroni t-tests. n = 5-7 separate cultures per group with 12 fields counted in each culture. ND: not done.
Table 2 - Effect of NOS inhibitor on superoxide levels

|             | baseline     | L-NAME (1mM)  |
|-------------|--------------|---------------|
| PF          | 100 ± 8.18   | 109 ± 7.39    |
| PF+S        | 27.0 ± 5.38<sup>a</sup> | 28.2 ± 3.94<sup>a</sup> |

Fibroblasts (PF) were treated with or without L-NAME (1mM) for 2 h in the presence or absence of sepiapterin (20 µM) (PF/S) and superoxide levels were measured by the Et fluorescence method. Et fluorescence values are normalized to the mean level in untreated PF. Data are expressed as mean ± SEM. <sup>a</sup> p < 0.01 versus PF and PF/L-NAME by Newman-Keuls <i>post hoc</i> test, n = 21-25 cells per group.
**Figure 1**

A bar graph showing the superoxide level (%) relative to non-DA control for Control, S, and D+N groups. The graph compares Dopaminergic and Nondopaminergic conditions. Bars with different letters (a, b, ab) indicate significant differences between groups.
Figure 2
Figure 3
Figure 4

Increase in Et Fluorescence Over Respective Controls

Rotenone

Antimycin

0 200 400 600 800

PFG
PF/S
PF

bc
ac
b

bc
bc
bc
Figure 5

The figure shows a bar graph with the y-axis labeled as "Et fluorescence" and the x-axis labeled with different treatments and concentrations of HO/B200nM, HO/B2uM, HO/B20uM, and HO/SOD. The treatments are represented as bars with error bars indicating standard deviation. The graph compares the effects of these treatments on Et fluorescence.
Figure 6

A

B

C
