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Inhibition of NO Synthesis Attenuates BH4-induced Toxicity in Nigrostriatal Dopaminergic System

Yeon Joo Jang[1], Yong Jin Lee[2] and Onyou Hwang[1]*

[1]Department of Biochemistry and Molecular Biology and [2]Asan Institute for Life Science, University of Ulsan College of Medicine, Seoul 138-736, Korea

ABSTRACT

Parkinson's disease (PD) is a movement disorder resulting from degeneration of the dopaminergic (DArgic) nigrostriatal pathway. Although the mechanism rendering this system more vulnerable is not completely understood, oxidative stress is believed to contribute to the pathogenesis. Lines of evidence indicate that nitric oxide (NO), via nitrosylation, may participate in the death of DArgic neurons in PD. We have previously observed that tetrahydrobiopterin (BH4), an obligatory cofactor for NO synthesis, causes DArgic cell death both in vivo and in vitro. In the present study we determined whether the production of NO after intrastriatal BH4 injection might contribute to degeneration of the nigrostriatal DArgic system. We observed that intrastriatal BH4 injection caused degeneration of DArgic fibers, which was accompanied by infiltration of microglial cells to the lesion site and increased NO synthesis activity. Co-injection of the NO synthesis inhibitor L-NAME attenuated the NO synthesis and nigrostriatal degeneration of DArgic system and partly reversed the motor deficit caused by injection with BH4 alone. Taken together, the present results suggest that increased NO production, probably by activated microglia, plays an important, albeit secondary, role in degeneration of nigrostriatal DArgic system following BH4 exposure.

Key words: tetrahydrobiopterin, nitric oxide, Parkinson's disease

INTRODUCTION

Dopaminergic (DArgic) neurons in the substantia nigra are known to be especially vulnerable to oxidative stress (Jenner and Olanow, 1998; Sian et al., 1994; Sofic et al, 1988). This selective vulnerability of the DArgic system is thought to contribute to the pathogenesis of Parkinson's disease (PD), a neurodegenerative disorder characterized by a selective loss of DArgic neurons in the substantia nigra pars compacta.

Nitric oxide (NO) is implicated in the pathophysiology of a variety of neurodegenerative diseases. It can form other reactive intermediates such as nitrate, nitrite, peroxynitrite and 3-nitrotyrosine, which lead to modification of important biomolecules including proteins and nucleic acids, ultimately causing cell death. There is considerable evidence that NO could be pivotal to the pathogenesis of PD. The concentration of nitrites is increased in PD cerebrospinal fluid (Qureshi et al., 1995), and 3-nitrotyrosine, an index of protein nitrosylation induced

*To whom correspondence should be addressed.
TEL: 82-2-3010-4279, FAX: 82-2-3010-4248
e-mail: oyhwang@amc.seoul.kr
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by the NO-derived molecule peroxynitrite, has been detected in substantia nigra (Good et al., 1998). Alpha-synuclein in Lewy bodies was found to contain nitrotyrosine produced from tyrosine by oxidative modification with peroxynitrite, a reactive nitrogen species (Paxinou et al., 2001).

NO is produced within cells by the actions of a group of enzymes called nitric oxide synthases (NOS): neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) forms. Of the three NOS, iNOS induced in activated microglia and macrophages are believed to be the major perpetrator of the NO-induced toxicity.

All forms of NOS require tetrahydrobiopterin (BH4) as an obligatory cofactor (Kwon et al., 1989). BH4 is thought to be required in the formation of NOS dimmer (Rafferty et al., 1999), the catalytically active form of the enzyme. Thus, the availability of BH4 is crucial in NOS activity and is known to regulate NOS activity (Werner-Felmayer et al., 1993; Canevari et al., 1999). The amount of BH4 seems to be limiting in the case of iNOS (Rosenkranz-Weiss et al., 1993), and expression of GTP cyclohydrolase I, the rate-limiting enzyme for BH4 synthesis, is co-regulated with iNOS (Hukkanen et al, 2003; Kumei et al., 2003).

We have previously reported that administration of BH4 into animals produces the typical characteristics observed in PD such as selective degeneration of the nigrostriatal system, loss of DA, apoptotic cell death, and motor deficit (Kim et al., 2003a; 2004). In vitro, BH4 exerts preferential toxicity on DA-producing cells over non-DArgic cells (Choi et al., 2000; 2003a) and leads to generation of oxidative stress, lipid peroxidation, protein modification and apoptotic death in DArgic cells (Choi et al., 2003a; 2003c). While this phenomenon observed in vitro with DArgic cells alone is thought to be mediated by increased oxidative stress derived from autooxidation of BH4 and DA, it is also possible that in vivo, where a variety of other cells including the glial cells are present, NO and activated microglia might also exacerbate the cell death process and contribute to chronic degeneration. This is supported by our finding that the degeneration continues even one month after BH4 injection into the striatum (Kim et al., 2003).

In the present study we therefore sought to determine whether NO also participates in the generation of the nigrostriatal DArgic system exposed to BH4. Specifically, in order to assess the contribution of NO in the BH4-induced nigrostriatal degeneration, we asked whether inhibition of NO synthesis might attenuate this degeneration.

MATERIALS AND METHODS

Chemicals

BH4 was obtained from RBI (Natick, MA). NG-nitro-L-arginine methyl ester (L-NAME), NADPH, nitroblue tetrazolium, ketamine, xylazine, ascorbate, sodium octylsulfate, triethylamine, and bovine serum albumin were purchased from Sigma Chemical (St. Louis, MO). Tissue-Tek O.C.T. compound was obtained from Sakura Finetek (Torrance, CA). Rabbit polyclonal antibody to tyrosine hydroxylase (TH) was obtained from Protos (New York, NY) and mouse anti-rat OX-42 antibody from Serotec (Raleigh, NC). Vectastain ABC kit and biotinylated secondary antibodies were from Vector Laboratories (Burlingame, CA) and Permount was from Fisher Scientific (Pittsburgh, PA). All other chemicals were reagent grades from Sigma or Merck (Rahway, NJ).

Animals

All procedures were performed in compliance with the guidelines set forth by the *Laboratory Animal Manual* of the Asan Institute for Life Sciences. Female Sprague-Dawley rats were bred and maintained at the animal facility of the Asan Institute for Life Sciences. They were housed in groups of three per cage in a temperature- and humidity-controlled room with a 12 h light-dark cycle. Food and water were available ad libitum.

Stereotaxic injections

Animals weighing 200～250 g were anesthetized with a mixture of ketamine (80 mg/kg) and xylazine (4 mg/kg i.p.). An injection cannula (26.5 gauge) was placed stereotaxically into the right striatum (AP, +0.7 mm from bregma; ML, 2.8 mm; DV, 4.6 mm below dura) according to the atlas (Paxinos and Watson, 1986). Injections of BH4 (200μg) and/or L-NAME (100μg) were made in 5μl containing 0.9% NaCl and 0.1% ascorbic acid, the vehicle shown to cause no significant changes in

monoamine levels and TH immunoreactivity (Ichitani et al., 1994). The solutions were freshly prepared just prior to use and were kept on ice before (in a lightproof container) and during (in cannula) the injection. The infusion was done at a rate of 0.5μl/min using a microinfusion pump (model 22, Harvard Apparatus). The cannula was left in place for 5 min before slowly withdrawing it in order to avoid reflux along the injection track. The wound was closed with suture and the animals were allowed to recover before they were returned to their cage. The entire procedure was well-tolerated by the animals.

Immunocytochemistry

Animals were deeply anesthetized (80 mg/kg ketamine and 20 mg/kg xylazine, i.p.) and transcardially perfused with normal saline containing heparin (100 USP units/ml) followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. Immunocytochemistry was performed as described previously (Hwang et al., 1998), using antisera against TH (1 : 1,000x), or OX-42 (1,000x), Vectastain ABC kit and biotinylated secondary antibodies.

NADPH-Diaphorase histochemistry

The histochemical staining was performed according to the method described by (Vincent and Kimura 1992). Tissue sections (postfixed for 2 h in 4% paraformaldehyde) were incubated for 1 h at 37°C with a solution containing 1 mg/ml NADPH, 0.25 mg/ml nitroblue tetrazolium, and 0.3% Triton X-100 in 0.1 M PB. The reaction was terminated by the addition of cold 0.1 M PB.

Forepaw adjusting steps

Motor deficit after the BH4 lesion was assessed by the animal s ability to adjust forepaw steps as described previously (Kim et al., 2003). Briefly, rats were held by one hand of the experimenter at the torso so that their hindlimbs were lifted. The right forepaw was held with the other hand and the left forepaw was allowed to step along on the surface of a treadmill (M.S.D., Seoul, Korea) moving at a rate of 7.5 cm/sec. The number of forepaw stepping during one round (90 cm) was counted. Each stepping test consisted of five trials and the average of the five trials was used for analysis. The test was performed by an experimenter blind to the treatment.

Quantitative morphological analyses. Up to five striatal sections, each separated by 120μm near the injection site, were selected. The area of lesion was determined to be the 2 mm^2 area (a diameter of 1.6 mm) around the injection site. Intensities of immunoreactivity and hischemical staining around the lesion were quantified by computer-assisted image analysis (BioRad Quantity One (version 4.2.1)). THimmunopositive cells in the substantia nigra were counted by the method of (Burke et al., 1992). Briefly, every third nigral sections, based on the atlas (Paxinos and Waston, 1986), were scanned under light microscopy, and TH-immunopositive substantia nigra pars compacta cells were counted manually. The number of neurons was expressed as the average of the counts obtained from the representative sections.

Data analyses

Comparisons were made using ANOVA and Newman-Keuls multiple comparisons test. $p < 0.05$ was considered statistically significant for all analyses.

RESULTS

We have previously reported that injection of BH4 into striatal tissue causes decreased TH immunoreactivity and DA accompanied by motor deficit (Kim et al., 2003). In the present study we first determined whether microglia cells and NO produced by these cells might participate in this degeneration. Animals were injected with BH4 in the right striatum and sacrificed after 4 weeks and the striatal tissues were subjected to various histochemical staining. The results showed that the area surrounding the injection site exhibited a loss of TH immunoreactivity, demonstrating damages to the striatal DArgic terminals (Fig. 1A). Consistently reproducible lesions were produced in all animals that received BH4. On the other hand, the animals injected with the vehicle alone maintained the dense TH immunoreactivity and only a very thin scar was visible along the needle track.

The adjacent sections were subjected to OX-42

staining in order to determine whether microglial infiltration has occurred. As shown in Fig. 1B, whereas the contralateral side showed little OX-42 immunoreactivity just above the corpus callusum, a dramatic increase in OX-42 immunoreactivity was observed around the lesion in the striatum created by the BH4 injection, indicating that microglia, and potentially peripheral leukocytes, have infiltrated the lesion site. To determine whether these immune cells might be activated and thus are producing NO, histochemical staining for NADPH-diaphorase was carried out adjacent sections, because NOS catalytic activity has been shown to account for the staining (Dawson et al., 1991; Hope et al., 1991). The results showed that NADPH-diaphorase staining was dramatically increased in the ipsilateral (Fig. 1D), but in the contralateral (Fig. 1C), side of BH4 injection. Therefore, we concluded that the BH4-induced degeneration of striatal tissue accompanied infiltration of microglia/leukocytes and increased NOS activity.

In order to determine the role of NO in BH4-induced DArgic degeneration, we asked whether cotreatment with the NOS inhibitor L-NAME might attenuate the degeneration. For this, L-NAME was co-injected with BH4 into the striatum of animals and the tissue was processed as the above. As shown in Fig. 2A, quantitative analysis showed that BH4 caused the intensity of TH immunostaining to decrease to about 45% of the vehicle-injected control ($p<0.05$). When L-NAME was injected alone with BH4, the intensity of TH immunostaining was slightly but significantly higher (58%; $p<0.05$), suggesting that DArgic degeneration has been attenuated by the NO synthesis inhibitor. However, it did not reach the intensity of immunoreactivity of the vehicle-treated control. As shown in Fig. 2B, the intensity of OX-42 immunoreactivity in the BH4 injected tissues increased by 5.8 fold. L-NAME/BH4 injected tissues tended to be lower (4.9 fold of vehicle-treated control) than those injected with BH4 alone, but this difference was not statistically significant ($p>0.05$). NADPH diaphorase histochemIstry (Fig. 2C) revealed lower staining in the L-NAME/BH4- treated striatum (2.4 fold of vehicle-treated control) compared to BH4 injected striatum (5.5 fold) ($p<0.05$), confirming that the NO forming activity has been decreased by the L-NAME treatment. Taken together, the results suggested that exposure to L-NAME leads to inhibition of NO production and attenuation of DArgic degeneration.

We have previously reported that striatal lesion caused by BH4 ultimately induces retrograde degeneration of the nigrostriatal pathway, leading to a loss of the DArgic neuronal cell bodies in the nigra (Kim et al., 2003a). In the present study we also asked whether the nigral DArgic neurons are protected in the presence of the NO synthesis

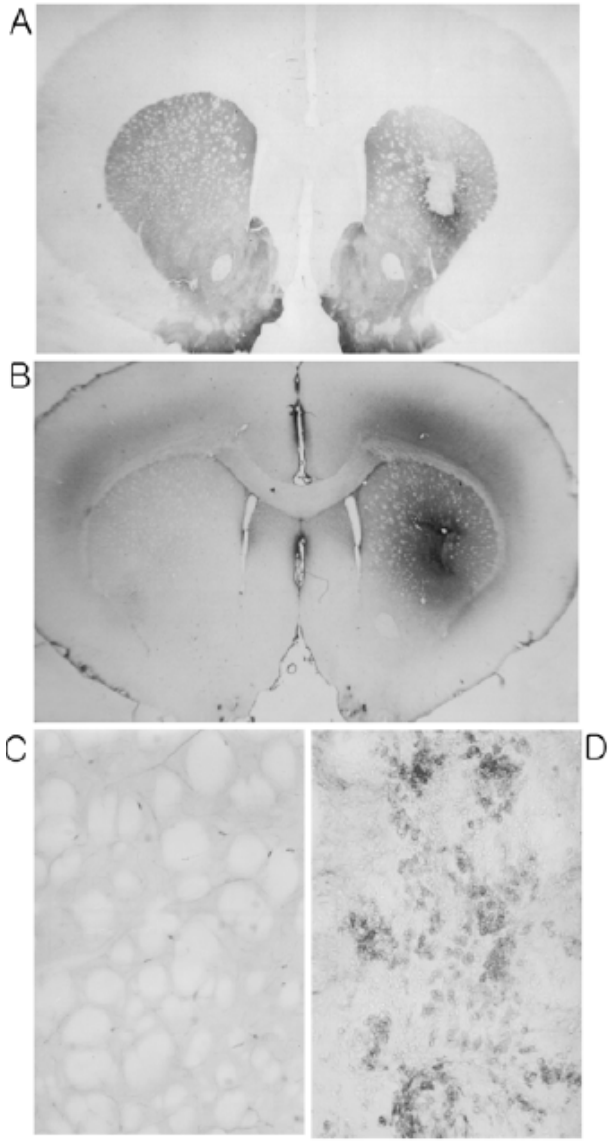

Fig. 1. Typical photomicrographs of TH (A) and OX-42 (B) immunostaining and NADPH diaphorase histochemical staining (C, D) 4 weeks after intrastriatal injection of BH4. C) contralateral and D) ipsilateral to the injection site. Scale bar=1.5 mm for A and B; 120μm for C and D.

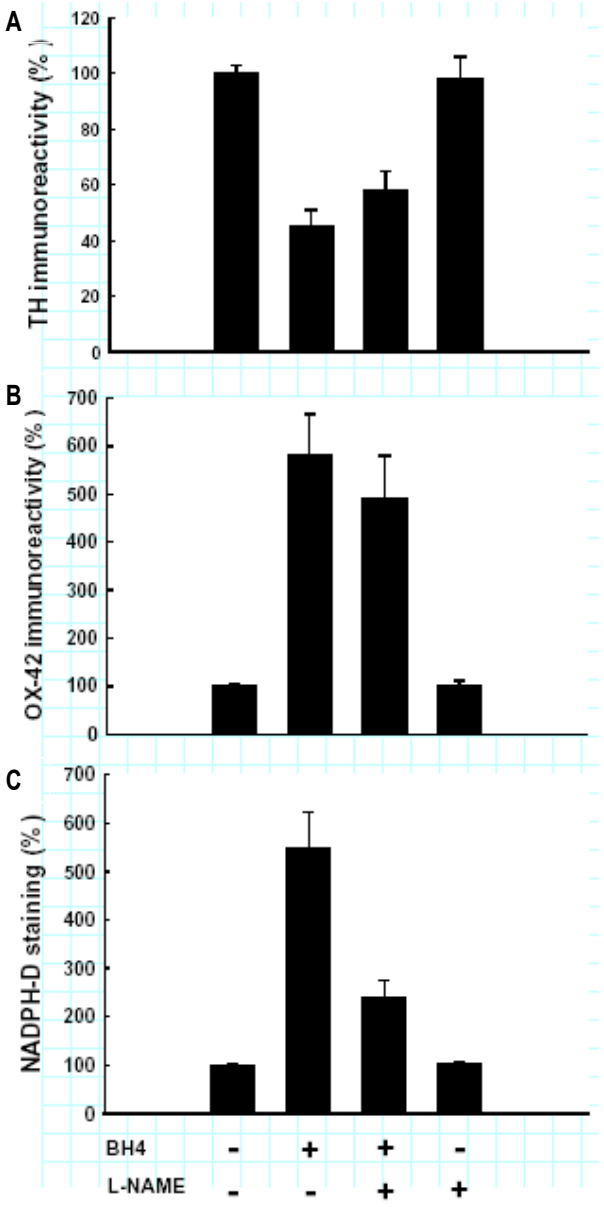

Fig. 2. Quantitative analysis of the striatal (A) TH immunoreactivity; (B) OX-42 immunoreactivity, and (C) NADPH diaphorase histochemical staining 4 weeks after intrastriatal injection of vehicle, BH4, BH4/L-NAME, or L-NAME. Data are means±SEM expressed as percentage of vehicle-injected control n=7.

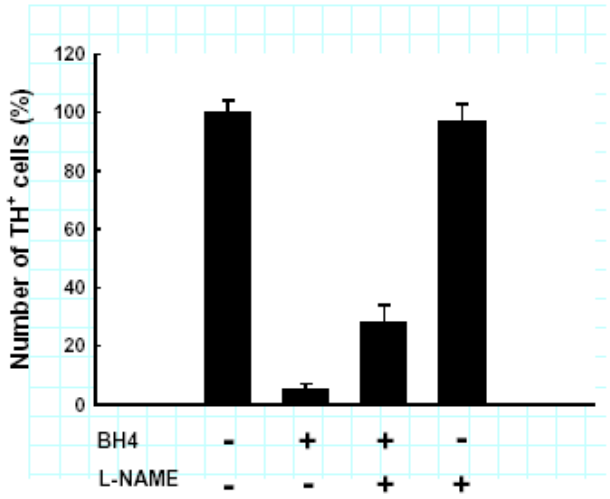

Fig. 3. Quantitative analysis of the number of TH-positive cell bodies in the ipsilateral substantia nigra 4 weeks after intrastriatal injection of vehicle, BH4, BH4/L-NAME, or L-NAME. Data are means±SEM expressed as percentage of vehicle-injected control n=7.

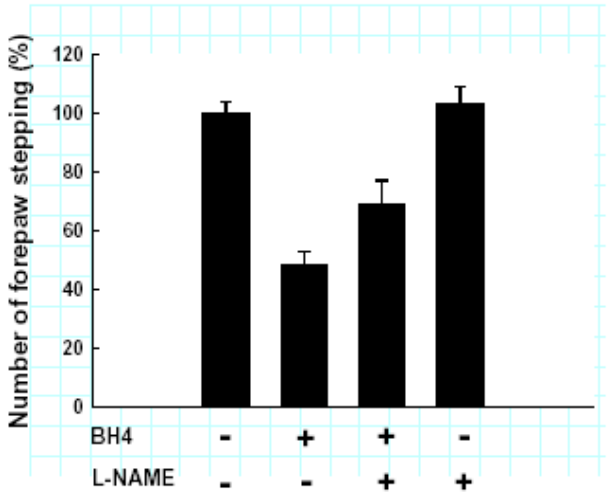

Fig. 4. Ability to adjust forepaw step. Animals were injected with vehicle, BH4, BH4/L-NAME, or L-NAME into the right striatum and the number of adjusting steps by the left forepaw was determined after 25 d. Data are means±SEM expressed as percentage of vehicle-injected control n=7.

inhibitor. For this, serial sections of the substantia nigra were taken and subjected to TH immunostaining and changes in the parent cell bodies were evaluated. TH immunoreactivity (space after TH) in the sections corresponding to bregma -4.4 to -4.8 was abolished only on the side ipsilateral to the injection. The DA cells of the ventral tegmental area were unaffected, demonstrating that this absence of nigral neurons was specifically caused by retrograde degeneration subsequent to the striatal damage (not shown). Quantitative analysis (Fig. 3) showed almost complete abolishment of the TH-immunopositive (space after TH) cells in the anterior part of the substantia nigra with only 5% of total cells remaining between the planes -4.4

and -4.6. In comparison, in L-NAME coinjected animals, 28% of total TH positive neurons remained. Therefore, L- NAME was able to protect the nigral DA cell bodies from retrograde degeneration after intrastriatal injection with BH4.

Nigrostriatal lesions by neurotoxins have been shown to accompany motor deficits (Chang et al., 1999; Przedborski et al., 1995; Schwarting and Huston, 1996). Deficits in forepaw adjusting steps provide a simple and consistent behavior phenomenologically similar to akinesia in early to moderate stages of PD (Olsson et al., 1995; Chang et al., 1999) and we have shown that BH4 injection causes deficit in forepaw adjusting steps (Kim et al., 2003a). Using this model, whether L-NAME co-injection could result in functional improvement of the BH4-induced motor deficit was tested 25 d post-injection. As shown in Fig. 4, the ipsilateral forepaw of the BH4-injected animals showed adjusting steps that were not significantly different from the vehicle- injected animals. On the other hand, the affected forepaw, i.e., the side contralateral to the lesioned striatum, showed dramatic reduction in the stepping ability (48% of the control). In the L-NAME/BH4- co-injected animals, the forepaw adjusting step abilities were significantly improved to 69% of control ($p < 0.05$ vs. BH4-injected). Thus, the BH4- induced damage to the nigrostriatal pathway accompanied a motor deficit related to DA depletion and inhibition of NO synthesis partially reversed this deficit.

DISCUSSION

We have previously reported that BH4 directly injected in the striatum causes selective degeneration of the nigrostriatal DArgic system and that this is accompanied by movement deficit (Kim et al., 2003a) that is phenomenologically similar to akinesia in early to moderate stages of PD (Olsson et al., 1995; Winkler et al., 1996; Chang et al., 1999). In the present study we demonstrate that NO synthesis and microglial infiltration increase around the lesion caused by BH4 and that inhibition of NO synthesis leads to protection of nigrostriatal DArgic system from BH4-induced degeneration and to improvement from the motor deficit.

In vitro experiments using a pure population of DArgic cells have demonstrated that the BH4 toxicity is found to primarily derive from oxidative stress generated by BH4 and DA (Choi et al., 2000; 2003a). BH4 can be autooxidized to superoxide and hydrogen peroxide (Davis and Kaufman, 1993; Davis et al., 1998), which can in turn react with DA to elicit formation of the reactive DA quinone (Berman and Hastings, 1999). Because BH4 is an obligatory cofactor for DA synthesis (Katz et al., 1976) and limiting for TH activity (Miwa et al., 1985), increased BH4 also leads to elevation of intracellular level of DA, contributing to the vulnerability of these cells.

In vivo situations where DArgic neurons, non-DArgic neurons, and glial cells coexist, however, involve another important and potentially toxic molecule: NO. Because BH4 is required for enzymatic activity of all forms of NOS (Kwon et al., 1989), the local increase in BH4 level would lead to an increase in NO synthesis. In addition, the damaged cells, initially by the oxidative stress generated by the BH4/DA interaction, might cause infiltration and activation of microglial cells, resulting in upregulation of iNOS. The increased local concentration of NO would in turn cause additional cytotoxic effect. Therefore, the primary damage seems to occur more readily within one day after BH4 exposure (Kim et al, 2003a), while NO might secondarily and more chronically contribute to the damage. Indeed, even long after the injection of BH4 (2 to 4 weeks post-injection), the striatal lesion continued to increase in size (Kim et al., 2003a), suggesting that secondary degeneration was indeed in progress. The fact that the NOS inhibitor could not completely protect against nigrostriatal degeneration or motor deficit induced by BH4 also supports the idea that NO is not the primary or the principal culprit. Therefore, roles of BH4 in vivo may be two fold: First, BH4 itself causes selective toxicity to DArgic cells by generation of oxidative stress via interaction with DA. Secondarily, this causes immunostimulation of microglial cells, which subsequently produces NO, whose presence exacerbates the degenerative process.

Because NO leads to cellular damage via inducing nitrotyrosylation of proteins, it was possible that in vivo, the NO produced nearby may contribute to the cellular damage. The increased num-

ber of microglial cells around the lesion area observed in the present study supports this view. In addition, the nNOS expressing neurons are in close contact with DA terminals in the striatum (Hwang et al., 1998; Fujiyama and Masuko, 1996), and BH4 can be taken up by these cells and utilized in NO synthesis (Choi et al., 2003b). In vitro, DA producing CATH.a cells have been shown to be susceptible to NO (Smith et al., 1998). Thus, it is possible to speculate that in addition to the microglia-derived NO, excessive extracellular BH4, whether released from the DA producing terminals by physiological effectors (Hwang et al., 1998) or experimentally supplied into the striatum, may stimulate NO synthesis, which in turn might exacerbate the BH4 toxicity. In addition, we have previously observed that NO can induce DA synthesis by upregulating TH gene expression (Kim et al., 2003b). Thus, increased NO may raise DA, which would further contribute to generation of oxidative stress.

Taken together, the present results suggest that the striatal damage following exposure to excessive BH4 might be exacerbated by subsequent NO production in activated microglia and that NO thus produced might contribute, at least in part, to degeneration of the nigrostriatal DArgic system.

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