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Tetrahydrobiopterin Prevents Nitration of Tyrosine Hydroxylase by Peroxynitrite and Nitrogen Dioxide

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ABSTRACT

Tyrosine hydroxylase (TH) is the initial and rate-limiting enzyme in the synthesis of the neurotransmitter dopamine. TH is inhibited and nitrated at tyrosine residues in vitro by the reactive nitrogen species peroxynitrite and nitrogen dioxide (NO_2) and in vivo by drugs that damage dopamine neurons. Tetrahydrobiopterin, which is the essential cofactor for TH and is concentrated in dopamine neurons, completely blocks nitration of tyrosine residues in TH caused by peroxynitrite or NO_2. Various tetrahydro- and dihydro- analogs of tetrahydrobiopterin, including 6,7-dimethyl-tetrahydropterin, 6-methyl-tetrahydropterin, 6-hydroxymethyl-tetrahydropterin, tetrahydropterin, 7,8-dihydrobiopterin, 7,8-dihydroxanthopterin, and sepiapterin, also prevent nitration of tyrosines caused by the reactive nitrogen species. Biopterin and pterin, the fully oxidized forms of the pterin molecule, fail to block peroxynitrite- or NO_2-induced nitration of TH. Reduced pterins prevent neither the inhibition of TH activity nor cysteine modification caused by peroxynitrite or NO_2, despite blocking tyrosine nitration. However, dithiothreitol prevents and reverses these effects on TH of tetrahydrobiopterin and reactive nitrogen species. Using an enhanced green fluorescent protein-TH fusion construct as a real-time reporter of intracellular tyrosine nitration, tetrahydrobiopterin was found to prevent NO_2-induced tyrosine nitration in intact cells but to leave TH activity inhibited. These results indicate that tetrahydrobiopterin prevents the tyrosine-nitrating properties of peroxynitrite and NO_2. Tetrahydrobiopterin-derived radical species formed by reaction with reactive nitrogen species may account for inhibition of TH via mechanisms that do not involve tyrosine nitration.

Peroxynitrite ($ONOO^-$) is formed by the near-diffusion limited reaction between nitric oxide and superoxide (Huie and Padmaja, 1993). $ONOO^-$ is a powerful oxidant that modifies proteins, DNA, membrane lipids, and mitochondria, and a combination of these effects can ultimately result in cytotoxicity and cell death (Beckman and Koppenol, 1996). Perhaps the best known property of $ONOO^-$ is its ability to create nitrates of free tyrosine or tyrosine residues in proteins. Measures of nitrotyrosine are used increasingly as an index of $ONOO^-$ action under conditions in which cells can be damaged and in a variety of disease states. For example, treatment of animals with methamphetamine (Imam et al., 1999) or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Ara et al., 1998; Ferrante et al., 1999) results in damage to dopamine neurons that has been attributed, at

least in part, to $ONOO^-$. Each of these drugs causes an increase in nitrotyrosine (free and in proteins) levels in brain, suggesting a link between this posttranslational modification and dopamine neuronal degeneration. Direct injection of free 3-nitrotyrosine into mouse brain also causes striatal neurodegeneration (Mihm et al., 2001). Tyrosine hydroxylase (TH; tyrosine 3-monooxygenase; EC 1.14.16.2), the initial and rate-limiting enzyme in dopamine (DA) biosynthesis, is nitrated and inactivated by $ONOO^-$ (Ara et al., 1998; Kuhn et al., 1999a, 2002; Blanchard-Fillion et al., 2001). Based on results in animal models of Parkinson's disease, and considering data showing that postmortem brain tissue from persons with Parkinson's disease contains proteins that are immunoreactive for nitrotyrosine (Good et al., 1998), $ONOO^-$ has emerged as a possible participant in DA neurodegeneration.

The role of $ONOO^-$ in cellular or neuronal toxicity has

ABBREVIATIONS: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; TH, tyrosine hydroxylase; DA, dopamine; BH_2, 7,8-dihydrobiopterin; $6MPH_4$, (6R,S)-6-methyl-5,6,7,8-tetrahydropterin; $6OH-MPH_4$, (6R,S)-6-hydroxymethyl-5,6,7,8-tetrahydropterin; BH_2, 7,8-dihydrobiopterin; BH_4, (6R)-5,6,7,8-tetrahydrobiopterin; $DMPH_4$, 6,7-dimethyl-5,6,7,8-tetrahydropterin; PH_4, 5,6,7,8-tetrahydropterin; DTT, dithiothreitol; DTPA, diethylenetriaminepenta-acetic acid; PMAB, polyethylene oxide maleimide activated biotin; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide; PAPA/NO, propylamine propylamine NONOate; eGFP, enhanced green fluorescent protein; ECL, enhanced chemiluminescence; PH_2, 7,8-dihydropterin; XH_2, 7,8-dihydroxanthopterin; PAGE, polyacrylamide gel electrophoresis; HEK, human embryonic kidney; PBS, phosphate-buffered saline.

become a matter of debate recently. Real-time measures of tyrosine nitration in intact cells indicate that $ONOO^-$ may not cross the plasma membrane in sufficient amounts to cause intracellular tyrosine nitration (Espey et al., 2002a,b). Arguing from the basis of chemical and kinetic reactivity, Pfeiffer and Mayer (1998) question whether $ONOO^-$ is at all effective as a tyrosine nitrating species in vivo. $ONOO^-$ is by no means the only possible nitrating species and a strong case can be made for nitrogen dioxide (NO_2) as a relevant nitrating reagent in intact cells (Espey et al., 2002b).

The tyrosine-nitrating properties of $ONOO^-$ and NO_2 are not often considered within the context of cellular phenotype, but this could be extremely important if specific cells contained endogenous substances that modified the tyrosine nitrating properties of reactive nitrogen species. DA neurons, obviously, are characterized by their high content of DA, and it has been shown that DA (and many other catechols) reacts with $ONOO^-$ to form the DA o-quinone (Kerry and Rice-Evans, 1998) at the expense of tyrosine nitration. In addition to DA, catecholamine neurons contain high levels of (6R)-5,6,7,8-tetrahydrobiopterin (BH_4) (Levine et al., 1981), the essential cofactor for TH, and one of numerous cofactors for nitric-oxide synthase. BH_4 is quite reactive with a number of radical species (Nakamura et al., 2001; Vasquez-Vivar et al., 2001; Patel et al., 2002) as well as with $ONOO^-$ (Milstien and Katusic, 1999; Kohnen et al., 2001) and NO_2 (Hyun et al., 1995).

In view of the possibility that tyrosine nitration of TH and other proteins within DA neurons is an early marker of neurodegeneration and may be a mechanism by which these neurons are damaged (Ara et al., 1998), we have investigated the influence of BH_4 on nitration of TH. We report herein that the tyrosine-nitrating effects of $ONOO^-$ and NO_2 on TH are prevented by BH_4 in vitro and in intact cells expressing an enhanced green fluorescent protein-TH fusion protein as a real-time reporter construct of tyrosine nitration (Espey et al., 2002b). These results suggest that endogenous factors within DA neurons, including BH_4, may shift the toxic actions of reactive nitrogen species toward pathways that do not involve tyrosine nitration.

Materials and Methods

Materials. The following pterins were obtained from Dr. B. Schircks Laboratories (Jona, Switzerland): (6R,S)-6-methyl-5,6,7,8-tetrahydropterin (6MPH$_4$); (6R,S)-6-hydroxymethyl-5,6,7,8-tetrahydropterin (6OH-MPH$_4$); 7,8-dihydrobiopterin (BH$_2$); 7,8-dihydropterin (PH$_2$); BH$_4$; 6,7-dimethyl-5,6,7,8-tetrahydropterin (DMPH$_4$), 5,6,7,8-tetrahydropterin (PH$_4$), sepiapterin, biopterin, and pterin. Dithiothreitol (DTT), ascorbic acid, diethylenetriamine penta acetic acid (DTPA), cysteine, glutathione, glutathione-agarose, and horseradish peroxidase were obtained from Sigma (St. Louis, MO). Polyethylene oxide maleimide-activated biotin (PMAB) and horseradish peroxidase-conjugated streptavidin were obtained from Pierce Endogen (Woburn, MA). Myeloperoxidase and 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (PTIO) were purchased from Calbiochem (La Jolla, CA), and catalase was a product of Roche Molecular Biochemicals (Indianapolis, IN). Thrombin, pGEX fusion protein vectors, and horseradish peroxidase-conjugated goat-anti-mouse IgGs were products of Amersham Biosciences (Piscataway, NJ). A monoclonal antibody against nitrotyrosine and PAPA/NO was purchased from Cayman Chemical Company (Ann Arbor, MI). The mammalian cell expression vector pEGFP-C3 and G-418 (Geneticin) were purchased from BD Biosciences Clontech (Palo Alto, CA). LipofectAMINE 2000 was a product of Invitrogen (Carlsbad, CA). Enhanced chemiluminescence (ECL) reagents were products of PerkinElmer Life Sciences (Boston, MA) and Bio-Max MR film was from Kodak (Rochester, NY). All other reagents were obtained from commercial sources in the highest possible purities.

Cloning and Assay of TH. TH was cloned by reverse-transcriptase polymerase chain reaction of rat brain RNA, and its sequence was verified by nucleotide sequencing. The full-length form of TH was ligated in-frame into pGEX-4T2 and expressed as a glutathione S-transferase-fusion protein. The recombinant protein was purified by glutathione-agarose affinity chromatography and the glutathione S-transferase fusion tag was removed by thrombin cleavage, resulting in a highly purified TH preparation (>95% pure) as described previously (Kuhn et al., 1999a,b). TH catalytic activity was assessed by the tritium-release method as described previously (Kuhn et al., 1999a,b). Protein was measured using the method of Bradford (1976).

Preparation of $ONOO^-$, NO_2, Pterins, and Treatment of TH. $ONOO^-$ was synthesized by the quenched-flow method of Beckman et al. (1994), and its concentration was determined using the extinction coefficient $\in_{302} = 1670$ M^{-1} cm^{-1}. The hydrogen peroxide contamination of $ONOO^-$ solutions was removed by manganese dioxide chromatography and filtration. $ONOO^-$ was added to TH with vigorous mixing in 50 mM potassium phosphate buffer, pH 7.4, containing 100 μM DTPA, and incubations were carried out for 15 min at 30°C, after which the enzyme was diluted with 10 volumes of 50 mM potassium phosphate buffer, pH 6, and assayed for catalytic activity or post-translational modification (see below). The volume of $ONOO^-$ added to the enzyme samples was always less than 1% (v/v) and did not influence pH. NO_2 was produced by reacting horseradish peroxidase or myeloperoxidase (specified below) with hydrogen peroxide (100 μM) in the presence of sodium nitrite (10–500 μM) as described by Espey et al. (2002b). TH was exposed to NO_2-generating conditions in the presence or absence of pterins for 60 min at 30°C, after which the enzyme was diluted with 10 volumes of 50 mM potassium phosphate, pH 6, and assayed for catalytic activity or post-translational modification as described for $ONOO^-$. All pterins except PH$_2$ and XH$_2$ were dissolved in water and added immediately before $ONOO^-$ or NO_2 when tested. PH$_2$ and XH$_2$ were prepared by oxidation of PH$_4$ in potassium phosphate buffer, pH 6.8, as described previously (Heales and Hyland, 1989).

SDS-PAGE and Western Blot Analysis of TH. After treatment with $ONOO^-$ or NO_2 with or without pterins, TH was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on 10% gels according to the method of Laemmli (1970). Proteins were transferred to nitrocellulose, blocked in Tris-buffered saline containing Tween 20 (0.1%, v/v) and nonfat dry milk (5%, w/v), and probed with a monoclonal antibody specific for nitrotyrosine. After overnight incubations with the primary antibody at a dilution of 1:2,000, blots were washed and incubated with goat anti-mouse secondary antibody conjugated with horseradish peroxidase (diluted 1:2,000), and immunoreactive protein bands were visualized with ECL.

Modification of Cysteine Residues in TH by $ONOO^-$ and NO_2. The effect of $ONOO^-$ and NO_2 with or without pterins on TH cysteine residues was determined with the use of the thiol-reactive biotinylation reagent PMAB as described previously (Kuhn et al., 2002). PMAB reacts selectively with reduced cysteines in proteins and does not react with cysteines that have been oxidized. Untreated TH or enzyme exposed to $ONOO^-$ or NO_2 with or without pterins as described above was diluted with 100 mM Tris HCl, pH 8.5, for subsequent labeling with PMAB (50 μM). Proteins were labeled for 60 min at room temperature in the dark, after which they were subjected to SDS-PAGE and blotting to nitrocellulose. PMAB reactivity was detected with horseradish peroxidase-conjugated streptavidin and visualized with ECL. The effect of dithiothreitol (DTT) on modification of TH catalytic function by $ONOO^-$ and NO_2 with or without BH_4 was also tested to demonstrate cysteine involvement. DTT (2 mM) was added immediately before exposure of TH to either

reactive nitrogen species and pterins to test for prevention of TH inhibition or it was added after treatment of TH to test for reversal. When DTT was added after treatment, samples were incubated at room temperature for an additional 30 min, after which TH catalytic assays were completed as described above.

Direct Real-Time Evaluation of Tyrosine Nitration with Enhanced Green Fluorescent Protein. A fusion protein comprised of enhanced green fluorescent protein (eGFP) and TH was constructed by cloning the full-length cDNA of TH into the vector pEGFP-3C at its *XhoI/Bam*HI restriction sites in the multiple cloning site. In this orientation, the eGFP fusion tag was upstream of the TH amino terminus, and the entire fusion protein complex had a molecular mass of 87 kDa. The pEGFP/TH fusion vector was stably transfected into HEK293 cells and selection was carried out in G-418 (300 μg/ml) and by observing eGFP fluorescence with a fluorescence microscope. Both elements of the eGFP/TH fusion protein retained their respective functionality (i.e., fluorescence and TH catalytic activity; see below). Cells were maintained in Dulbecco's modified Eagle's medium (high glucose) containing 10% fetal calf serum in an atmosphere of 5% CO_2. Real-time evaluation of eGFP/TH tyrosine nitration was carried out as described by Espey et al. (2002b). Intact cells (1×10^6) were washed into phosphate-buffered saline (PBS) pH 7.4 containing DTPA (100 μM) and exposed to NO_2 for 15 min via incubation with PTIO and PAPA/NO at 37°C in cells preloaded or not with BH$_4$ as described previously for DA (Park et al., 2002). The extent of intracellular tyrosine nitration caused by NO_2 was monitored through measures of reductions in eGFP/TH fluorescence, with emission at 488 nm and excitation at 512 nm (Espey et al., 2001, 2002b) in an Aminco-Bowman Series 2 fluorescence spectrometer. Immediately after measures of fluorescence, intact cells were placed on ice, washed three times with ice-cold PBS, and sonicated in 60 μl of potassium phosphate buffer, pH 6. TH activity was subsequently measured in the cell supernatant after sedimentation of membranes by centrifugation at 40,000*g* for 15 min at 4°C.

Results

Treatment of TH (10 μM) with ONOO$^-$ (100 μM) resulted in the nitration of tyrosine residues in the TH monomer (60 kDa) as shown in Fig. 1A. The addition of increasing concentrations of BH$_4$ to TH before ONOO$^-$ resulted in a progressive reduction in the extent of TH nitration. Concentrations of BH$_4$ between 1 and 5 μM seemed to have little influence on the nitrating effects of ONOO$^-$, whereas BH$_4$ concentrations between 10 and 100 μM completely prevented TH nitration. The generality of this effect of BH$_4$ was characterized by testing a series of related pterins for their effects on ONOO$^-$-mediated nitration of tyrosine residues in TH. The results in Fig. 1B show that equimolar concentrations (50 μM) of all reduced pterins tested blocked the effects of ONOO$^-$ on TH. The tetrahydro- (BH$_4$, 6MPH$_4$, DMPH$_4$, and 6OH-MPH$_4$) and dihydro- (BH$_2$ and sepiapterin) pterins were equally effective in preventing ONOO$^-$-induced nitration of TH. Only biopterin, the fully oxidized form of BH$_4$, was without effect on TH nitration (Fig. 1B, last lane).

The oxidation of BH$_4$ by ONOO$^-$ produces BH$_2$, PH$_2$, and XH$_2$ in roughly equal proportions (Milstien and Katusic, 1999). Therefore, PH$_2$ and XH$_2$ were synthesized from PH$_4$ by oxidation, and these pterins were tested for their effects on ONOO$^-$-induced nitration of TH. PH$_2$ is unstable without the addition of reducing agents and spontaneously rearranges to XH$_2$ (Heales and Hyland, 1989). Considering that reducing agents prevent ONOO$^-$-induced modification of TH (Kuhn et al., 1999a), we made no attempts to stabilize PH$_2$, testing a mixture of PH$_2$ and XH$_2$. The results in Fig. 1C

show that PH$_4$ and PH$_2$/XH$_2$ (50 μM each) were effective in preventing ONOO$^-$-induced nitration of tyrosine residues in TH, whereas pterin, the fully oxidized form of PH$_4$, had no effect. These results parallel those obtained with BH$_4$ and its reduced and oxidized analogs.

ONOO$^-$ (100 μM) inhibits TH catalytic activity by 50%, as shown in Fig. 2A, and increasing concentrations of BH$_4$ did not modify the effects of ONOO$^-$ on the enzyme. BH$_4$ did not alter TH activity if tested in the absence of ONOO$^-$ (data not shown). The pterins that were shown to block ONOO$^-$-induced nitration of TH (Fig. 1, B and C) were tested for their effects on TH activity and the results are included in Fig. 2, B and C. It can be seen that all pterins, regardless of their chemical status (i.e., tetrahydro-, dihydro-, or fully oxidized), were without influence on the inhibition of TH activity caused by ONOO$^-$. The reductions in TH activity caused by ONOO$^-$ with or without pterins were statistically significant ($p < 0.05$, Bonferroni's test), and none of the pterins significantly modified the magnitude of the ONOO$^-$ effect on TH.

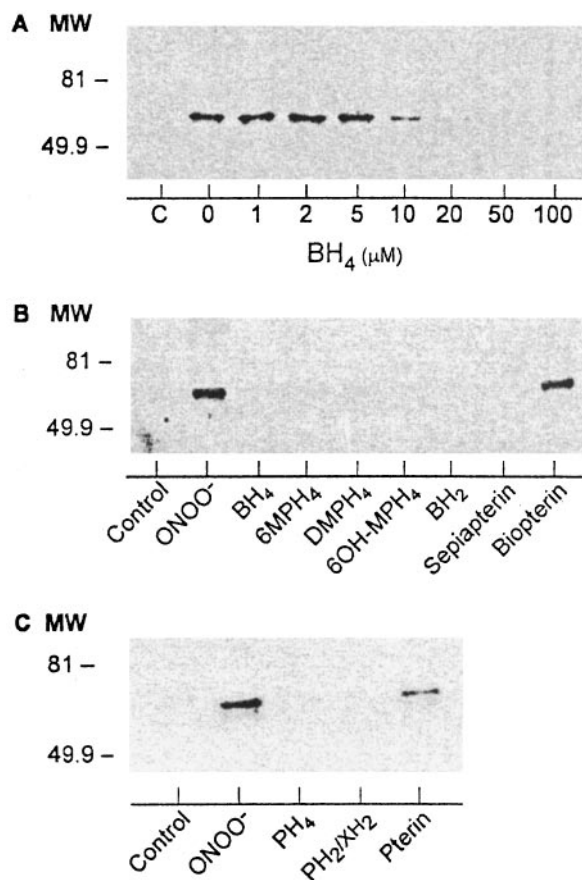NO_2 nitrated tyrosine residues in TH as described above for ONOO$^-$. Figure 3A shows that increasing concentrations

Fig. 1. Effects of BH$_4$ and related pterins on ONOO$^-$-induced nitration of tyrosine residues in TH. Purified TH (10 μM) was treated with ONOO$^-$ (100 μM) in 50 mM potassium phosphate buffer, pH 7.4, containing DTPA (100 μM) for 10 min at 30°C, and samples were exposed to SDS-PAGE and immunoblotting with a monoclonal antibody against nitrotyrosine. Immunoreactivity was visualized with ECL. A, TH was treated with control conditions (C) or with ONOO$^-$ (100 μM) in the presence of increasing concentrations of BH$_4$ (0–100 μM). B and C, TH was treated with control conditions (control), ONOO$^-$ (100 μM), or with ONOO$^-$ (100 μM) plus the indicated pterin (50 μM each). The molecular mass markers on the ordinate are in kilodaltons and are indicated by prestained protein standards.

of BH_4 caused a gradual reduction in NO_2-induced nitration of TH. Concentrations of BH_4 between 1 and 5 μM substantially reduced the nitrating effects of NO_2, and concentrations above 10 to 20 μM resulted in complete prevention of tyrosine nitration in the enzyme. The generality of pterin-induced prevention of tyrosine nitration by NO_2 was found to be very similar to that shown by $ONOO^-$. Figure 3B shows that all tetrahydro- and dihydropterins tested (50 μM each) were effective in blocking NO_2-induced nitration. Biopterin did not alter the nitrating effects of NO_2, as was the case with $ONOO^-$. PH_2 and XH_2 are not known to be products of NO_2

Fig. 2. Effects of BH_4 and related pterins on $ONOO^-$-induced inhibition of TH. Purified TH (10 μM) was exposed to $ONOO^-$ (100 μM) as described in the legend to Fig. 1. Where indicated, pterins were added just before $ONOO^-$. After incubation, samples were diluted 1:10 with potassium phosphate buffer, pH 6, and TH catalytic activity was assayed as described under *Materials and Methods*. Data are presented as percentage of control TH activity and represent means ± S.E.M. of five experiments run in duplicate. A, TH was treated with $ONOO^-$ alone or with concentrations of BH_4 varying from 0 to 100 μM. The effect of $ONOO^-$ alone on TH activity was statistically significant ($p < 0.05$, Bonferroni's test) and the addition of BH_4 did not modify the effect of $ONOO^-$ on TH. B and C, TH was treated with control conditions, $ONOO^-$, or $ONOO^-$ plus the indicated pterins (50 μM for each). The effect on TH activity of $ONOO^-$ alone, and in the presence of the pterins, was statistically significant (*, $p < 0.05$, Bonferroni's test). None of the pterins tested changed the effect of $ONOO^-$ on TH activity.

reaction with BH_4, so their effects on NO_2-induced tyrosine nitration were not tested. However, PH_4 was as effective as BH_4 in blocking NO_2-induced nitration of TH (data not shown).

The effect of BH_4 on NO_2-induced alterations in TH catalytic activity was investigated and the results are presented in Fig. 4A. NO_2, like $ONOO^-$, caused a significant reduction in TH activity. If any one of the components required for NO_2 production (i.e., horseradish peroxidase, hydrogen peroxide, sodium nitrate) was omitted from the reaction, TH activity was not inhibited, indicating that the inactivation was mediated by NO_2 (data not shown). Figure 4A also shows that BH_4 caused a slight protection of TH against NO_2-induced inhibition. Concentrations of BH_4 between 5 and 20 μM reduced the inhibition of TH caused by NO_2 from 50% to about 40%, and concentrations of 50 to 100 μM were slightly more protective (35% inhibition versus 50% inhibition in control subjects). Despite the partial protection against the inhibitory effects of NO_2 afforded by BH_4, TH activity remained significantly reduced ($p < 0.05$, ANOVA). Structure-activity analysis of the effects of pterins on NO_2-induced inactivation of TH established that tetrahydro- and dihydropterins partially protected TH against inhibition. $6MPH_4$, $DMPH_4$, $6OH-MPH_4$, and sepiapterin (all in concentrations of 50 μM), like BH_4, reduced the inhibition of TH caused by NO_2 from 45 to 50% to about 30%. BH_2 was slightly more effective than the other reduced pterins in this regard, allowing only 13% inhibition of TH by NO_2. With the exception of BH_2, TH remained significantly inhibited after exposure to NO_2, despite the partial protection afforded by the reduced
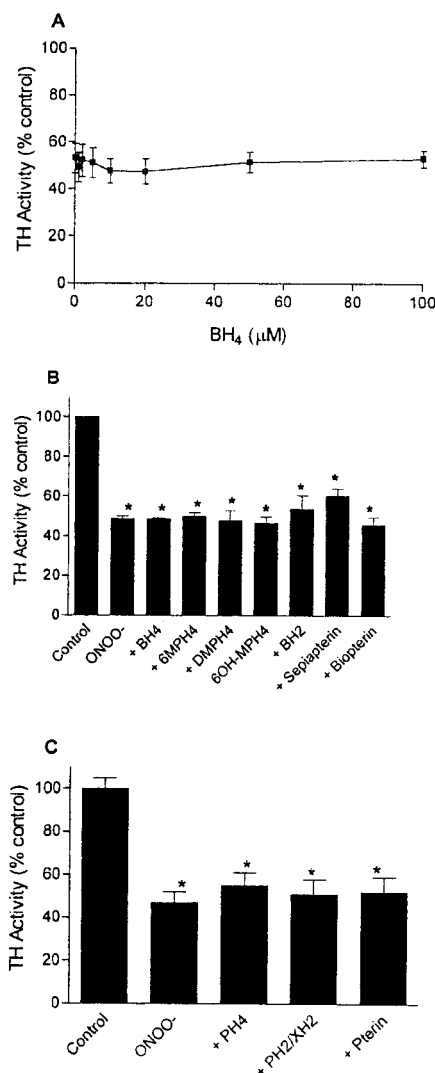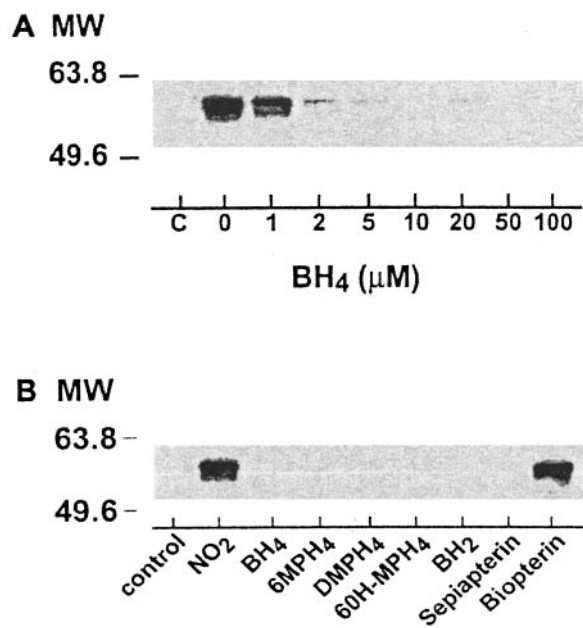

Fig. 3. Effects of BH_4 and related pterins on NO_2-induced nitration of tyrosine residues in TH. Purified TH (10 μM) was treated with control conditions, NO_2, or NO_2 plus the indicated pterins (50 μM each) for 60 min at 30°C, and samples were exposed to SDS-PAGE and immunoblotting with a monoclonal antibody against nitrotyrosine. Immunoreactivity was visualized with ECL. A, TH was treated with control conditions (C) or with NO_2 in the presence of increasing concentrations of BH_4 (0–100 μM). B, TH was treated with control conditions (control), NO_2, or NO_2 plus the indicated pterin (50 μM each). The molecular mass markers on the ordinate are in kilodaltons and are indicated by prestained protein standards.

pterins ($p < 0.05$, Bonferroni's test). Biopterin did not change the inhibitory effects of NO_2 on TH activity.

The mechanism by which TH is inhibited by at least $ONOO^-$ involves nitration of critical tyrosine residues (Ara et al., 1998; Blanchard-Fillion et al., 2001) or oxidation of cysteine residues (Kuhn et al., 1999a, 2002). In view of results showing that reduced pterins block nitration of TH by $ONOO^-$ and NO_2 without preventing the inactivation of catalytic function, the status of cysteine residues in TH was assessed through the use of PMAB after treatment of the enzyme. This analysis was restricted to BH_4 (50 and 100 μM) and biopterin. Figure 5 shows that both $ONOO^-$ and NO_2 caused substantial reductions in PMAB labeling of TH, indicative of cysteine modification. It can also be seen in Fig. 5 that PMAB labeling of TH was greater in the presence of BH_4 than in its absence when combined with either $ONOO^-$ or NO_2. A higher concentration of BH_4 (100 μM) was no more effective in preventing cysteine modification of TH cysteines, in agreement with results on catalytic activity (Fig. 2). Biopterin did not change the effects of $ONOO^-$ or NO_2 on TH cysteine labeling by PMAB. Densitometric scans of the data in Fig. 5 indicate that $ONOO^-$ and NO_2 reduced PMAB labeling to about 20% of control in the absence of BH_4. PMAB labeling was reduced to 58% of control after treatment of TH with $ONOO^-$ + BH_4 (50 μM) and to 62% of control by
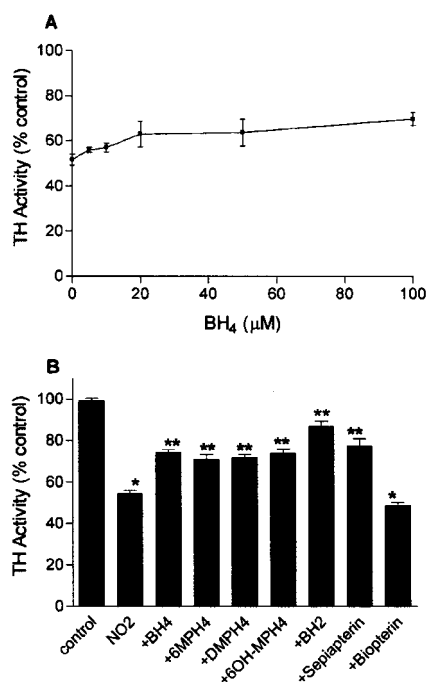
Fig. 4. Effects of BH_4 and related pterins on NO_2-induced inhibition of TH. Purified TH (10 μM) was exposed to NO_2 as described in the legend to Fig. 2. Where indicated, pterins were added just before NO_2. After incubation, samples were diluted 1:10 with potassium phosphate buffer, pH 6, and TH catalytic activity was assayed as described under *Materials and Methods*. Data are presented as percentage of control TH activity and represent means ± S.E.M. of five experiments run in duplicate. A, TH was treated with NO_2 alone or with NO_2 plus BH_4 concentrations varying from 0 to 100 μM. The effect of NO_2 alone on TH activity was statistically significant ($p < 0.05$, Bonferroni's test). The overall effect of BH_4 was significantly different from control conditions and from the effect of NO_2 alone ($p < 0.05$, ANOVA). B, TH was treated with control conditions, NO_2, or NO_2 plus the indicated pterins (50 μM for each). Significant differences from control (*, $p < 0.05$, Bonferroni's test) or from both control and NO_2 (**, $p < 0.05$ Bonferroni's test) are indicated over the respective bars.
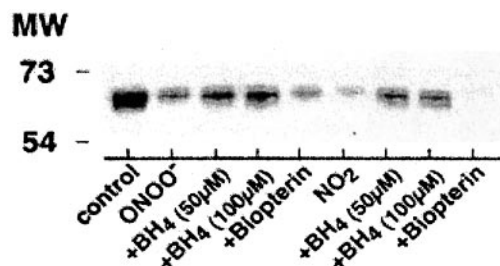
Fig. 5. Effects of BH_4 and biopterin on $ONOO^-$- and NO_2-mediated modification of cysteine residues in TH. TH (10 μM) was treated with $ONOO^-$ or NO_2 in the absence or presence of BH_4 (50 or 100 μM) or biopterin (50 μM) as described under *Materials and Methods*. Samples were subsequently labeled with PMAB (50 μM) in 100 mM Tris HCl, pH 8.5, for 60 min under conditions of reduced lighting. After labeling, samples were subjected to SDS-PAGE and blotting to nitrocellulose. PMAB reactivity was visualized by ECL using horseradish peroxidase-conjugated streptavidin. The molecular mass markers on the ordinate are in kilodaltons and are indicated by prestained protein standards.

$ONOO^-$ and BH_4 (100 μM). A similar situation existed when NO_2 was tested with BH_4. PMAB labeling was reduced to 50% of control after treatment with either concentration of BH_4 in the presence of NO_2. These results indicate that the $ONOO^-$ or NO_2-induced modification of cysteine residues in TH was partially mitigated by BH_4 and are consistent with the effects of reduced pterins on inhibition of TH by $ONOO^-$ and NO_2 (see above). Table 1 shows that inhibition of TH by $ONOO^-$ or NO_2, in the presence of BH_4, was prevented and reversed by DTT. In contrast, DTT does not reverse the effects of either nitrating species on TH activity in the absence of BH_4.

Espey et al. (2002b) recently introduced a method to measure intracellular tyrosine nitration directly and in real-time based on the sensitivity of eGFP to nitration-induced reductions in fluorescence. We created stable transformants expressing an eGFP/TH fusion protein in HEK293 cells. This cell line was chosen for use presently because of its low endogenous content of BH_4 (R. A. Levine, personal communication). Exposure of these cells to NO_2 via treatment with PTIO-PAPA/NO caused a significant reduction (40%) in eGFP fluorescence, as shown in Fig. 6. When cells were preloaded with BH_4 before exposure to NO_2, the nitration-

TABLE 1
Effects of DTT on inhibition of TH caused by $ONOO^-$ or NO_2 ± BH_4

TH was treated with reactive nitrogen species ± BH_4 as described under *Materials and Methods*. DTT (2 mM) was added immediately before treatment and remained in the samples throughout exposure conditions. In other experiments, DTT was added immediately after treatment and samples were incubated at room temperature for 30 min before assays for TH catalytic activity were carried out. Data are reported as percentage of control TH activity and represent mean ± S.E.M. of four separate experiments carried out in duplicate.

Treatment	No DTT	DTT Before Treatment	DTT After Treatment
	%	%	
Control	100	97 ± 5	94 ± 6
$ONOO^-$	54 ± 2[a]	97 ± 5[b]	56 ± 3[a]
$ONOO^-$ + BH_4 (50 μM)	56 ± 6[a]	78 ± 3[b]	78 ± 5[b]
$ONOO^-$ + BH_4 (100 μM)	58 ± 5[a]	81 ± 7[b]	76 ± 7[b]
NO_2	58 ± 4[a]	97 ± 6[b]	62 ± 5[a]
NO_2 + BH_4 (50 μM)	72 ± 4[a]	94 ± 3[b]	83 ± 4[b]
NO_2 + BH_4 (100 μM)	68 ± 4[a]	95 ± 4[b]	86 ± 6[b]

[a] Significantly different from control, $P < 0.05$ using Bonferroni's test.
[b] Significantly different from respective no-DTT group, $P < 0.05$ using Bonferroni's test.
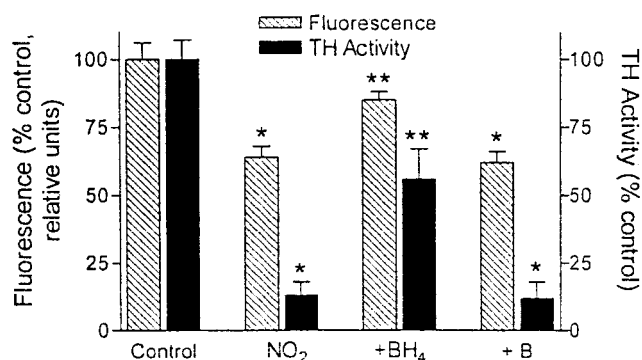
Fig. 6. Effects of BH_4 on NO_2-induced reductions in eGFP/TH fluorescence in intact cells. HEK293 cells (1×10^6) stably expressing an eGFP/TH fusion protein were treated with PTIO-PAPA/NO to generate NO_2 as described by Espey et al. (2002b). Cells were treated for 15 min at 37°C after which the fluorescence of eGFP/TH was measured by emission at 488 nM and excitation at 512 nm. Where indicated, cells were incubated with BH_4 or biopterin (50 μM each) for 15 min at 37°C before exposure to NO_2, and fluorescence was monitored in washed cells as described above. After fluorescence measures, cells were washed three times in ice-cold PBS and lysed for measures of soluble TH catalytic activity. Results are presented as percentage of control for each measure and are the mean ± S.E.M. of four to six experiments run in duplicate. Significant differences from control (*, $p < 0.05$ for fluorescence and TH activity, Bonferroni's test) or from both control and NO_2 (**, $p < 0.05$ for fluorescence and TH activity, Bonferroni's test) are indicated over the respective bars.

induced reduction in fluorescence was largely prevented (Fig. 6). Biopterin had no effect on the nitration-induced reduction in eGFP fluorescence. TH activity was inhibited by about 80% after exposure of intact cells to NO_2. It can also be seen in Fig. 6 that BH_4 provided partial protection against NO_2-induced inhibition of TH, whereas biopterin was without effect, as seen in in vitro studies (above). In agreement with Espey et al. (2002b), $ONOO^-$ in concentrations up to 1000 μM, added as a bolus or by slow decomposition of SIN-1, did not cause intracellular tyrosine nitration as measured by reductions in eGFP/TH fluorescence, nor did it inhibit TH activity (data not shown).

Discussion

$ONOO^-$ is a powerful oxidant that can damage cell organelles, compromise membrane integrity through lipid peroxidation, and modify DNA, properties that underlie its cytotoxicity (Beckman and Koppenol, 1996). $ONOO^-$ is perhaps best known for the ability to nitrate tyrosine residues in proteins (Ischiropoulos, 1998). Increases in tyrosine nitration seen after treatment of animals with MPTP (Ara et al., 1998; Ferrante et al., 1999) or methamphetamine (Imam et al., 1999) implicate $ONOO^-$ as a causative factor in DA neuronal damage caused by these drugs. The detection of nitrated proteins (Good et al., 1998) in postmortem brain from persons with Parkinson's disease has focused attention on nitrotyrosine as a marker for $ONOO^-$ action and as an early biochemical event in degeneration of DA neurons (Ara et al., 1998).

The ability of $ONOO^-$ to nitrate proteins in intact cells has become an issue of debate recently. Espey and colleagues monitored protein-tyrosine nitration in real-time and observed that $ONOO^-$ caused very little nitration, apparently as a result of poor penetration through the plasma membrane (Espey et al., 2002a,b). Pfeiffer and Mayer (1998) have

argued that $ONOO^-$ is a poor tyrosine nitrating reagent when its chemical reactivity and kinetics of decomposition are considered. $ONOO^-$ is not the only nitrating species, and a strong case can be made for other nitroxyl-derived species, including NO_2, as in vivo tyrosine-nitrating reagents (Espey et al., 2002a,b). Studies of tyrosine nitration do not often consider the possibility that cell phenotype could influence both the generation and effects of reactive nitrogen species. This is particularly important in the case of DA neurons. BH_4 is localized selectively in monoaminergic neurons (Levine et al., 1981) and its concentration in DA nerve endings is approximately 100 μM (Lovenberg et al., 1979). BH_4 is quite reactive with $ONOO^-$ (Milstien and Katusic, 1999; Kohnen et al., 2001) and NO_2 (Hyun et al., 1995). Considering the possibility that BH_4 could alter the reactivity of $ONOO^-$ and NO_2 toward tyrosine residues, it was very important to assess the effects of the pterins on tyrosine nitration in TH.

BH_4 prevented nitration of TH caused by $ONOO^-$ or NO_2. Structure-activity analysis revealed that reduced pterins (tetrahydro- and dihydro-forms) shared with BH_4 the ability to prevent nitration of TH. The fully oxidized species biopterin and pterin failed to block nitration. These findings are in agreement with Widner et al. (1998), who showed that a variety of oxidized pterins, including neopterin, biopterin, and pterin do not modify $ONOO^-$-induced nitration of free tyrosine at neutral pH. All of the tetrahydropterins tested (BH_4, $6MPH_4$, $DMPH_4$, PH_4, and $6OH-MPH_4$) are TH cofactors, whereas the dihydropterins tested (BH_2, XH_2, and sepiapterin) are not (Kato et al., 1980). Because cofactor activity was not an common property among those pterins that prevent $ONOO^-$-induced tyrosine nitration, it seems that a direct interaction of the pterins with $ONOO^-$ (Widner et al., 1998; Milstien and Katusic, 1999; Kohnen et al., 2001) or NO_2 (Hyun et al., 1995) prevents nitration, not an indirect interaction of the pterins with TH that makes it a poorer substrate for tyrosine nitration.

It has been suggested that $ONOO^-$ inhibits TH through nitration of tyrosine residues (Ara et al., 1998; Blanchard-Fillion et al., 2001). TH is also sensitive to inhibition via cysteine modification (Kuhn et al., 1999a,b; Borges et al., 2002), so the possibility that this was the basis for lowered TH catalytic function was examined. $ONOO^-$ and NO_2 each caused substantial reductions in PMAB labeling of TH, indicative of cysteine modification. These results agree well with previous studies showing cysteine modification in TH by at least $ONOO^-$ (Kuhn et al., 2002) and extend them to NO_2. Treatment of TH with $ONOO^-$ or NO_2 in the presence of BH_4 resulted in reduced PMAB labeling but to a lesser extent than that caused by the reactive nitrogen species alone. DTT prevented inhibition of TH caused by $ONOO^-$ and NO_2, in keeping with its ability to react directly with reactive nitrogen species. However, although DTT did not reverse the effects of $ONOO^-$ or NO_2 alone on TH, it did reverse inhibition of the enzyme caused by either reactive nitrogen species in the presence of BH_4. These results fall short of identifying the chemical species produced by the reaction of $ONOO^-$ or NO_2 with reduced pterins, but they do suggest that the reactant modifies cysteine residues in TH by a mechanism that is fundamentally different from the mechanism(s) by which $ONOO^-$ or NO_2 alone modify cysteines [e.g., oxidation beyond sulfenic acid (Radi et al., 1991)]. BH_4-derived radical species (Kohnen et al., 2001; Patel et al., 2002) formed by

reaction with nitrating species may account for inhibition of TH via mechanisms that do not involve tyrosine nitration.

Evidence for nitration of TH by $ONOO^-$ in intact cells has been difficult to obtain. Ara et al. (1998) found that $ONOO^-$ nitrated TH in PC12 cell lysates. We have not been able to establish that TH is nitrated after treatment of intact PC12 cells with $ONOO^-$. Several factors could account for this failure and led us to consider an alternative approach to the problem. First, it does not seem that $ONOO^-$ penetrates intact cells in sufficient concentrations to cause tyrosine nitration in cytoplasmic proteins (Espey et al., 2002b). Second, $ONOO^-$ is formed from the reaction of nitric oxide with superoxide, and high concentrations of these reactants must be maintained at or near 1:1 stoichiometry to avoid secondary reactions that form species incapable of tyrosine nitration (Pfeiffer and Mayer, 1998; Thomas et al., 2002). Third, PC12 cells contain high catecholamine and BH_4 concentrations (Anastasiadis et al., 1998), which could diminish $ONOO^-$-induced tyrosine nitration. Fourth, it is possible that immunoblotting is too insensitive to detect low levels of TH nitration. The method of Espey et al. (2002b) was used to monitor tyrosine nitration in intact cells through measures of fluorescence reductions in an eGFP/TH fusion protein stably expressed in HEK293 cells. We chose this cell line because of its extremely low endogenous BH_4 content. The use of fluorescence is also a far more sensitive measure of nitration than immunoblotting. It was observed that NO_2 caused a significant reduction in eGFP/TH fluorescence and TH catalytic activity in intact cells. The magnitude of the reduction in TH activity was greater than the reduction in eGFP fluorescence (about 50%) and stands in contrast to in vitro results in which TH activity was inhibited by 50% upon exposure to NO_2. The reasons for this difference are not immediately evident but could result from use of different methods of NO_2 production (i.e., chemical versus enzymatic) or an attack on cellular TH by nitric oxide generated through PAPA/NO decomposition. Nitric oxide would not alter eGFP fluorescence (Espey et al., 2002b) but could inhibit TH activity. BH_4 largely prevented the reduction in eGFP/TH fluorescence caused by NO_2. Although BH_4 provided partial protection against NO_2-induced inhibition of TH activity, the enzyme remained significantly inhibited. These results indicate that cellular BH_4 can modulate tyrosine nitration.

Nitrotyrosine immunoreactivity has been used as a marker for $ONOO^-$ production in vivo and could represent an early pathological event in neurodegenerative processes. However, the present results cast in a different light the use of nitrotyrosine as an early marker or mediator of cytotoxicity in DA neurons for two reasons. First, BH_4 prevents $ONOO^-$- and NO_2-induced tyrosine nitration in TH. Precursors of BH_4 and products of its reaction with at least $ONOO^-$ (i.e., BH_2, PH_2, and XH_2) are also effective in preventing nitration of tyrosine residues. BH_4 is not the only species found in DA neurons that can mitigate tyrosine nitration. DA is obviously concentrated in these neurons and is known to react with $ONOO^-$ and NO_2. This interaction prevents nitration of free tyrosine (Kerry and Rice-Evans, 1999) and tyrosine residues in at least TH (Park et al., 2003). Second, BH_4 and related pterins are actually cytoprotective. BH_4 mediates the preferential resistance of DA neurons to damage caused by glutathione depletion (Nakamura et al., 2000) and even protects against MPTP-induced neurotoxicity (Madsen et al., 2003). BH_4 also

lowers superoxide production by nitric-oxide synthase (Rosen et al., 2002) and scavenges superoxide in DA neurons (Nakamura et al., 2001).

In summary, the DA neuronal phenotype seems to have a significant impact on the chemical reactivity of $ONOO^-$ and NO_2 by preventing tyrosine nitration. Based on the very gradual loss of DA neurons in Parkinson's disease and considering that DA neurons retain substantial DA and BH_4 synthetic capacity throughout its progression (Lovenberg et al., 1979), it may be more accurate to consider that tyrosine nitration of proteins, as assessed in postmortem tissue, represents a late-occurring event that emerges with the loss of BH_4 and DA, not an early insult.

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