Tetrahydrobiopterin Prevents Nitrination 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 (NO2) 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 NO2. 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. Bipterin and pterin, the fully oxidized forms of the pterin molecule, fail to block peroxynitrite- or NO2-induced nitration of TH. Reduced pterins prevent neither the inhibition of TH activity nor cysteine modification caused by peroxynitrite or NO2, 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 NO2-induced tyrosine nitrination in intact cells but to leave TH activity inhibited. These results indicate that tetrahydrobiopterin prevents the tyrosine-nitrating properties of peroxynitrite and NO2. 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 have 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. Direct injection of free 3-nitrotyrosine into mouse brain also causes striatal neurodegeneration. Direct injection of free 3-nitrotyrosine into mouse brain also causes striatal neurodegeneration (Mihm et al., 2001). Tyrosine hydroxylation (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...
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, Pfieffer 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 (NO2) as a relevant nitrating reagent in intact cells (Espey et al., 2002b).

The tyrosine-nitrating properties of ONOO− and NO2 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 catechol)s react with ONOO− to form the DAquinone (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 (BH4) (Levine et al., 1981), the essential cofactor for TH, and one of numerous cofactors for nitric-oxide synthase. BH4 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 NO2 (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 BH4 on nitration of TH. We report herein that the tyrosine-nitrating effects of ONOO− and NO2 on TH are prevented by BH4 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 BH4, 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 (6MPH4); (6R,S)-6-hydroxymethyl-5,6,7,8-tetrahydropterin (6OH-MPH4); 7,8-dihydrobiopterin (BH2); 7,8-dihydrotetraphosphorin (PH2); BH4; 6,7-dimethyl-5,6,7,8-tetrahydropterin (DMPH4), 5,6,7,8-tetrahydropterin (PH4), sepiapterin, biopterin, and pterin.

**Preparation of ONOO−, NO2, 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 ε302 = 1670 M−1 cm−1. The hydrogen peroxide contamination of ONOO− solutions was removed by manganese dioxide chromatography and filtration. NO2 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. NO2 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 NO2-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 PH2 and XH2 were dissolved in water and added immediately before ONOO− or NO2 when tested. PH2 and XH2 were prepared by oxidation of PH4 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 NO2 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 NO2.** The effect of ONOO− and NO2 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., 1999). 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 NO2 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 dithioreitol (DTT) on modification of TH catalytic function by ONOO− and NO2 with or without BH4 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 if 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 Xhol/BamHI 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% CO₂. Real-time evaluation of eGFP/TH tyrosine nitration was carried out as described by Espey et al. (2002b). Intact cells (1 x 10⁶) were washed into phosphate-buffered saline (PBS) pH 7.4 containing DTPA (100 µM) and exposed to NO₂ for 15 min via incubation with PTIO and PAPA/NO at 37°C in cells preloaded or not with BH₄ as described previously for DA (Park et al., 2002). The extent of intracellular tyrosine nitration caused by NO₂ 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 Amino-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 BH₄ to TH before ONOO⁻ resulted in a progressive reduction in the extent of TH nitration. Concentrations of BH₄ between 1 and 5 µM seemed to have little influence on the nitrating effects of ONOO⁻, whereas BH₄ concentrations between 10 and 100 µM completely prevented TH nitration. The generality of this effect of BH₄ 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- (BH₄, 6MPH₄, DMPH₄, and 6OH-MPH₄) and dihydro- (BH₂ and sepiapterin) pterins were equally effective in preventing ONOO⁻-induced nitration of TH. Only bioppterin, the fully oxidized form of BH₄, was without effect on TH nitration (Fig. 1B, last lane).

The oxidation of BH₄ by ONOO⁻ produces BH₂, PH₂, and XH₂ in roughly equal proportions (Milstein and Katusic, 1999). Therefore, PH₂ and XH₂ were synthesized from PH₄ by oxidation, and these pterins were tested for their effects on ONOO⁻-induced nitration of TH. PH₂ is unstable without the addition of reducing agents and spontaneously rearranges to XH₂ (Heales and Hyland, 1989). Considering that reducing agents prevent ONOO⁻-induced modification of TH (Kuhn et al., 1999a), we made no attempts to stabilize PH₂, testing a mixture of PH₂ and XH₂. The results in Fig. 1C show that PH₄ and PH₂/XH₂ (50 µM each) were effective in preventing ONOO⁻-induced nitration of tyrosine residues in TH, whereas pterin, the fully oxidized form of PH₄, had no effect. These results parallel those obtained with BH₄ and its reduced and oxidized analogs.

ONOO⁻ (100 µM) inhibits TH catalytic activity by 50%, as shown in Fig. 2A, and increasing concentrations of BH₄ did not modify the effects of ONOO⁻ on the enzyme. BH₄ 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.

NO₂ nitrated tyrosine residues in TH as described above for ONOO⁻. Figure 3A shows that increasing concentrations...
of BH₄ caused a gradual reduction in NO₂-induced nitrination of TH. Concentrations of BH₄ between 1 and 5 μM substantially reduced the nitrating effects of NO₂, 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 NO₂ 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 NO₂-induced nitration. Biopterin did not alter the nitrating effects of NO₂, as was the case with ONOO⁻. PH₂ and XH₂ are not known to be products of NO₂ reaction with BH₄, so their effects on NO₂-induced tyrosine nitration were not tested. However, PH₄ was as effective as BH₄ in blocking NO₂-induced nitration of TH (data not shown).

The effect of BH₄ on NO₂-induced alterations in TH catalytic activity was investigated and the results are presented in Fig. 4A. NO₂, like ONOO⁻, caused a significant reduction in TH activity. If any one of the components required for NO₂ 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 NO₂ (data not shown). Figure 4A also shows that BH₄ caused a slight protection of TH against NO₂-induced inhibition. Concentrations of BH₄ between 5 and 20 μM reduced the inhibition of TH caused by NO₂ 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 NO₂ afforded by BH₄, TH activity remained significantly reduced (p < 0.05, ANOVA). Structure-activity analysis of the effects of pterins on NO₂-induced inactivation of TH established that tetrahydro- and dihydropterins partially protected TH against inhibition. 6MPH₄, DMPH₄, 6OH-MPH₄, and sepiapterin (all in concentrations of 50 μM), like BH₄, reduced the inhibition of TH caused by NO₂ from 45 to 50% to about 30%. BH₂ was slightly more effective than the other reduced pterins in this regard, allowing only 13% inhibition of TH by NO₂. With the exception of BH₂, TH remained significantly inhibited after exposure to NO₂, despite the partial protection afforded by the reduced

Fig. 2. Effects of BH₄ 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 BH₄ 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 BH₄ did not modify the effect of ONOO⁻ on TH. B and C, TH was treated with control conditions, ONOO⁻, or ONOO⁻ plus the indicated pterin (50 μM 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.

Fig. 3. Effects of BH₄ and related pterins on NO₂-induced nitration of tyrosine residues in TH. Purified TH (10 μM) was treated with control conditions, NO₂, or NO₂ plus the indicated pterin (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 (control), NO₂, or NO₂ plus the indicated pterin (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. B, TH was treated with control conditions (control), NO₂, or NO₂ 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 M) 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 M) 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 M) and to 62% of control by ONOO$^-$ and BH$_4$ (100 M). 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 transfectants 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

![Fig. 5. Effects of BH$_4$ and biopterin on ONOO$^-$ and NO$_2$-mediated modification of cysteine residues in TH. TH (10 M) was treated with ONOO$^-$ or NO$_2$ in the absence or presence of BH$_4$ (50 or 100 M) or biopterin (50 M) as described under Materials and Methods. Samples were subsequently labeled with PMAB (50 M) 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.](Image)

**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.

<table>
<thead>
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<th>Treatment</th>
<th>No DTT</th>
<th>Before Treatment</th>
<th>After Treatment</th>
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<tr>
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<td>94 ± 6</td>
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<tr>
<td>ONOO$^-$</td>
<td>54 ± 2$^a$</td>
<td>97 ± 5$^b$</td>
<td>56 ± 3$^b$</td>
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<tr>
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<td>56 ± 6$^a$</td>
<td>78 ± 3$^b$</td>
<td>78 ± 5$^b$</td>
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<tr>
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<td>81 ± 7$^b$</td>
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<tr>
<td>NO$_2$</td>
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<td>97 ± 6$^b$</td>
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<td>95 ± 4$^b$</td>
<td>86 ± 6$^b$</td>
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* Significantly different from control, $P < 0.05$ using Bonferroni’s test.

* Significantly different from respective no-DTT group, $P < 0.05$ using Bonferroni’s test.

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**Fig. 4.** Effects of BH$_4$ and related pterins on NO$_2$-induced inhibition of TH. Purified TH (10 M) 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 M. 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 M 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. 6. Effects of BH4 on NO2-induced reductions in eGFP/TH fluorescence in intact cells. HEK293 cells (1 × 10⁶) stably expressing an eGFP/TH fusion protein were treated with PTIO-PAPA/NO to generate NO2 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 BH4 or biopterin (50 μM each) for 15 min at 37°C before exposure to NO2, 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 NO2 (**, 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 NO2. It can also be seen in Fig. 6 that BH4 provided partial protection against NO2-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 (Ishchiropoulos, 1998). Increases in tyrosine nitration are seen after treatment of animals with MPTP (Ara et al., 1998; Ferrante et al., 1999) or methamphetamine (Imam et al., 2001). TH is also sensitive to inhibition via reactive nitrogen species. However, although DTT did not reverse the effects of ONOO− or NO2 alone on TH, it did reverse inhibition of the enzyme caused by either reactive nitrogen species in the presence of BH4. These results fall short of identifying the chemical species produced by the reaction of ONOO− or NO2 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 NO2 alone modify cysteines [e.g., oxidation beyond sulfenic acid (Radi et al., 1991)]. BH4-derived radical species (Kohnen et al., 2001; Patel et al., 2002) formed by 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 nitrosoyl-derived species, including NO2•, 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. BH4 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). BH4 is quite reactive with ONOO− (Milstien and Katusic, 1999; Kohnen et al., 2001) and NO2 (Hyun et al., 1995). Considering the possibility that BH4 could alter the reactivity of ONOO− and NO2 toward tyrosine residues, it was very important to assess the effects of the pterins on tyrosine nitration in TH.

BH4 prevented nitration of TH caused by ONOO− or NO2. Structure-activity analysis revealed that reduced pterins (tetrahydro- and dihydro-forms) shared with BH4 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 (BH4, 6MPH4, DMPH4, PH4, and 6OH-MPH4) are TH cofactors, whereas the dihydropterins tested (BH2, XH2, and sepiapterin) are not (Kato et al., 1980). Because cofactor activity was not a 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 NO2 (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., 2001), so the possibility that this was the basis for lowered TH catalytic function was examined. ONOO− and NO2 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 NO2. Treatment of TH with ONOO− or NO2 in the presence of BH4 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 NO2, in keeping with its ability to react directly with reactive nitrogen species. However, although DTT did not reverse the effects of ONOO− or NO2 alone on TH, it did reverse inhibition of the enzyme caused by either reactive nitrogen species in the presence of BH4. These results fall short of identifying the chemical species produced by the reaction of ONOO− or NO2 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 NO2 alone modify cysteines [e.g., oxidation beyond sulfenic acid (Radi et al., 1991)]. BH4-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$^-$/H$_2$O_2 in intact cells has been difficult to obtain. Ara et al. (1998) found that ONOO$^-$/H$_2$O_2-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 can 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$_3$, PH$_2$, and XV$_3$) 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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References


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