Dopamine Prevents Nitrination of Tyrosine Hydroxylase by Peroxynitrite and Nitrogen Dioxide

IS NITROTYROSINE FORMATION AN EARLY STEP IN DOPAMINE NEURONAL DAMAGE?*

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Peroxynitrite and nitrogen dioxide (NO2) are reactive nitrogen species that have been implicated as causal factors in neurodegenerative conditions. Peroxynitrite-induced nitrination of tyrosine residues in tyrosine hydroxylase (TH) may even be one of the earliest biochemical events associated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced damage to dopamine neurons. Exposure of TH to peroxynitrite or NO2 results in nitrination of tyrosine residues and modification of cysteines in the enzyme as well as inactivation of catalytic activity. Dopamine (DA), its precursor 3,4-dihydroxyphenylalanine, and metabolite 3,4-dihydroxyphenylacetic acid completely block the nitrating effects of peroxynitrite and NO2 on TH but do not relieve the enzyme from inhibition. o-Quinones formed in the reaction of catechols with either peroxynitrite or NO2 react with cysteine residues in TH and inhibit catalytic function. Using direct, real-time evaluation of tyrosine nitration with a green fluorescent protein-TH fusion protein stably expressed in intact cells (also stably expressing the human DA transporter), DA was also found to prevent NO2-induced nitration while leaving TH activity inhibited. These results show that peroxynitrite and NO2 react with DA to form quinones at the expense of tyrosine nitration. Endogenous DA may therefore play an important role in determining how DA neurons are affected by reactive nitrogen species by shifting the balance of their effects away from tyrosine nitration and toward o-quinone formation.

Peroxynitrite (ONOO−)1 is formed in the chemical reaction between nitric oxide and superoxide (1). ONOO− is a powerful oxidant that can modify proteins and cell organelles, damage DNA, and cause lipid peroxidation, properties that are thought to underlie its cytotoxicity (2, 3). ONOO− nitrates free tyrosine and tyrosine residues in proteins, modifications that are used as markers of ONOO− action under conditions of cellular damage and in numerous diseases (4, 5). ONOO− has been implicated as a causal factor in dopamine (DA) neuronal damage that occurs after exposure to the neurotoxic amphetamines (6–8) and MPTP (9, 10). It is also suspected to play a role in the etiology of idiopathic Parkinson’s disease (11, 12).

The participation of ONOO− in cellular or neuronal toxicity is an evolving and complicated issue. Real-time measures of intracellular nitration indicate that ONOO− may not cross cell membranes in sufficient amounts to cause intracellular tyrosine nitration (13). This may be a reflection of preferential nitration of hydrophobic, transmembrane tyrosines by ONOO− as compared with tyrosines in the aqueous phase (14). Mayer and colleagues (15) have argued on chemical and kinetic grounds that ONOO− is altogether ineffective as a tyrosine nitrating species in vivo. ONOO− is by no means the only nitrating species and a strong case can be made for nitrogen dioxide (NO2) as a more relevant nitrating reagent (13, 16).

The tyrosine-nitrating properties of ONOO− and NO2 are not often considered within a context of cellular phenotype, but this could be extremely important in the case of DA neurons. ONOO− and NO2 react with DA to form o-quinones and various nitro-catechols (17–21). DA-mediated neurotoxicity is associated with increased formation of catechol-quinones, and quinones are known to modify cysteine residues in proteins (22–24), including TH (25). Catechol-quinones have also been implicated in Parkinson’s disease (26–30).

The interaction of DA with reactive nitrogen species could have important consequences in DA neurons by determining the pathway of toxicity, yet the influence of DA on the protein-nitrating properties of ONOO− and NO2 has not been considered. Given that tyrosine nitration of TH may be an early biochemical event in the DA neurodegeneration associated with Parkinson’s disease (9), we have studied the effects of DA on nitration of TH. It is found presently that catechols prevent nitrination of TH by ONOO− and NO2. Using intact cells expressing the human DA transporter (31, 32) along with a green fluorescent protein-TH fusion protein as a reporter of real-time nitration (13), we also observe that intact cellular tyrosine nitration is prevented by DA. These findings suggest that nitrotyrosine formation may be suppressed in DA neurons as long as

hydroxylase; ANOVA, analysis of variance; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PAPA, propylamine propylamine.
catechol synthesis and storage are intact and point to catechol-quinones as early participants in DA neuronal damage.

**EXPERIMENTAL PROCEDURES**

**Materials—**Tetrahydrobiopterin was obtained from Dr. B. Schircks Laboratories (Zurich, Switzerland). Dihydrodeminopentamethylenetriamine tetraacetate (DTTA), glutathione-agarose, DA, DOPA, DOPAC, sodium nitrite, sodium periodate, myeloperoxidase, and horseradish peroxidase were obtained from Sigma. Calbiochem was the source of 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3,3-dioxide (PTIO). Catalase and a monoclonal antibody against TH were products of Roche Applied Science. Thrombin and pGCTh, protein vectors were obtained from American Bio-science. The mammalian cell expression vector pEGFP-C3 was purchased from Clontech. Zeocin, pCMV/Zeo, and LipofectAMINE 2000 were products of Invitrogen. Nitro blue tetrazolium (NBT) was purchased from Aldrich Chemical Co. A monoclonal antibody against nitrotyrosine and PAPA/NO was purchased from Cayman Chemical Co. (Ann Arbor, MI), and horseradish peroxidase-linked goat-anti-mouse IgGs were products of Cappel. Polyethylene oxide-maleimide-activated biotin (PMAB) and horseradish peroxidase-linked streptavidin were purchased from Pierce. Enhanced chemiluminescence (ECL) reagents were products of PerkinElmer Life Sciences, and Bio-Max MR film was from Kodak. All other reagents were obtained from commercial sources in the highest possible purities.

**Cloning and Assay of TH—**TH was 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 the highest possible purities.

**Preparation of ONOO− and NO2− and Treatment of TH—**ONOO− was synthesized by the quenched-flow method of Beckman et al. (37), and its concentration was determined by the extinction coefficient ε290 = 1870 M−1 cm−1 (H2O2). Peroxide contamination of ONOO− solutions was removed by manganese dioxide chromatography and filtration (37). The typical concentration of stock ONOO− solutions ranged between 300 and 400 mM. 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. The volume of ONOO− added to the enzyme samples was always less than 1% (v/v) and did not influence pH. When tested, catechols were added immediately prior to ONOO− when tested. Upon completion of incubation with ONOO− and other additions, enzyme samples were diluted with 10 volumes of 50 mM potassium phosphate, pH 6, and assayed for catalytic activity or post-translational modification (nitration or quinolation) after SDS-PAGE and transfer to nitrocellulose (see below). 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. (13). TH was exposed to NO2−-generating conditions with or without catechols 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 above for ONOO−.

**Post-translation Modification of TH by Reactive Nitrogen Species and Catechol-quinones—**Following treatment with ONOO− or NO2− with or without catechols, TH was subjected to SDS-PAGE (62). Proteins were transferred to nitrocellulose, blocked in Tris-buffered saline containing 0.1% Tween (0.05% v/v) and 5% nonfat dry milk, and probed with a monoclonal antibody specific for nitrotyrosine (1:2000 dilution). After incubations with primary antibodies, blots were incubated with a goat anti-mouse secondary antibody conjugated with horseradish peroxidase, and immunoreactive protein bands were visualized with enhanced chemiluminescence. Catechol-quinone modification of TH was assessed in separate experiments by staining blots with NBT in the presence of 2 μM potassium glutamate buffer pH 10 as described previously (38). The effects of ONOO− and NO2− on TH activity were statistically significant (p < 0.05, ANOVA), but the effect of DA on ONOO−-induced inhibition of TH was not. Additional catechols were tested for their influence on the ONOO−-induced inactivation of TH. Fig. 1B shows that equimolar concentrations (20 μM) of DOPA, and DOPAC, in the presence of ONOO−, caused varying degrees of inhibition of TH. The effect of DOPAC was similar to that of DA in that it did not change the extent of inhibition caused by ONOO− alone. The effects of DOPA were significantly different from control and ONOO− alone (p < 0.05, Bonferroni’s test).

In view of the fact that catechols prevent the nitration of free tyrosine caused by ONOO− (20, 40), we tested their effects on ONOO−-induced nitration of tyrosine residues in TH. Fig. 2A shows that ONOO− (100 μM) caused extensive nitration of tyrosine residues in TH as measured by immunoblotting with a monoclonal antibody against nitrotyrosine. Catechol compounds were tested at a concentration of 20 μM, and each completely prevented the ONOO−-induced nitration of TH. Although a careful titration of the concentration effects of the catechols on ONOO−-induced nitration was not carried out, we have observed that molar ratios of about 1:5 (DA:ONOO−) are sufficient to block the TH-nitrating properties of ONOO−. The

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**RESULTS**

ONOO− caused a concentration-dependent inactivation of TH activity as previously reported (33, 34), and, at a concentration of 100 μM, TH activity was reduced to 50% of control. Fig. 1A presents data showing the effects on TH activity of adding DA in the presence of ONOO−. It can be seen that DA had little effect on the ONOO−-induced inhibition of TH. Concentrations up to 100 μM DA plus ONOO− did not change the effect on TH catalytic activity caused by ONOO− alone. The effect of ONOO− on TH activity was statistically significant (p < 0.05, ANOVA), but the effect of DA on ONOO−-induced inhibition of TH was not. Additional catechols were tested for their influence on the ONOO−-induced inactivation of TH. Fig. 1B shows that equimolar concentrations (20 μM) of DOPA, and DOPAC, in the presence of ONOO−, caused varying degrees of inhibition of TH. The effect of DOPAC was similar to that of DA in that it did not change the extent of inhibition caused by ONOO− alone. The effects of DOPA were significantly different from control and ONOO− alone (p < 0.05, Bonferroni’s test).

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Dopamine Prevents Nitration of Tyrosine Hydroxylase

**Fig. 1. Effect of DA and other catechols on the ONOO\(^{-}\)-induced inhibition of TH catalytic activity.** TH (10 \(\mu\)M) was incubated with ONOO\(^{-}\) (100 \(\mu\)M) alone or with the indicated additions in the presence of 100 \(\mu\)M DTPA for 15 min at 30 \(^\circ\)C. Reactions were diluted with 10 volumes of 50 mM potassium phosphate buffer at pH 7.4, and the remaining TH activity was assayed immediately. Results are expressed as percent control TH activity and are the mean \(\pm\) S.E. of five or six experiments carried out in duplicate. A, results with increasing concentrations of DA; B, results with equimolar concentrations of the indicated catechols (20 \(\mu\)M). The effects of DOPA were significantly different from both control (untreated) and ONOO\(^{-}\) in B (*, \(p < 0.05\), Bonferroni’s test).

**Fig. 2. Post-translational modification of TH after treatment with ONOO\(^{-}\) in the presence of DA and other catechols.** A, TH (10 \(\mu\)M) was treated with ONOO\(^{-}\) (100 \(\mu\)M) in the presence of 100 \(\mu\)M DTPA. The indicated catechols (20 \(\mu\)M each) were added just prior to ONOO\(^{-}\), and after a 15-min incubation at 30 \(^\circ\)C, samples were prepared for SDS-PAGE and immunoblotting with a monoclonal antibody against nitro-tyrosine (diluted 1:2000). Immunoreactivity was visualized by ECL. B, TH treated under the conditions described in A was analyzed by redox cycling staining after SDS-PAGE and electroblotting to nitrocellulose. Gels in A and B contained 10 \(\mu\)g of TH protein per lane.

The indicated catechols (20 \(\mu\)M) prevented tyrosine nitration in TH. When a blot similar to the one in Fig. 2A was exposed to redox cycling staining, it was observed that the catechol compounds in the presence of ONOO\(^{-}\) converted TH to a redox cycling protein. Fig. 2B shows that DA and DOPAC produced the strongest redox cycling staining in TH after exposure to ONOO\(^{-}\). DOPA was somewhat less potent than DA and DOPAC in this regard. These results with ONOO\(^{-}\)-DA interactions agree with previous studies showing that chemical or enzymatic conversion of DA to its aminochrome or \(o\)-quinone, respectively, modifies TH, converting the enzyme to a redox cycling quinoprotein (25).

Because the effects on TH of tyrosine nitrating species other than ONOO\(^{-}\) have not been investigated, we used increasing concentrations of sodium nitrite to generate a range of concentrations of NO\(_2\) in the presence of constant levels of horseradish peroxidase (25 units) and hydrogen peroxide (100 \(\mu\)M). Fig. 3A shows that TH was quite sensitive to inhibition by NO\(_2\). TH was inhibited by 40–50\% at a nitrite concentration of 200 \(\mu\)M; when the nitrite concentration reached 500 \(\mu\)M, TH activity was inactivated by 60–70\%. Omission of any one or two of the components needed to generate NO\(_2\) prevented inhibition of TH, indicating that the enzyme was not inhibited by the peroxidase, nitrite, or hydrogen peroxide. Substitution of myeloperoxidase for horseradish peroxidase produced the same inhibition of TH catalytic activity (data not shown). The effects of NO\(_2\) on TH activity were statistically significant (\(p < 0.01\), ANOVA). The effects of DA on the NO\(_2\)-induced inhibition of TH are presented in Fig. 3B. Low concentrations of DA (5–20 \(\mu\)M) slightly enhanced the inhibition of TH activity caused by NO\(_2\). For example, TH activity was reduced to about 50\% of control by NO\(_2\) in the absence of DA, whereas 20 \(\mu\)M DA increased the inhibitory effects of NO\(_2\) on TH to 40\% of control. Higher concentrations of DA (50–100 \(\mu\)M) did not further alter the NO\(_2\)-induced reduction in TH activity. The overall effect of DA on the inhibition of TH by NO\(_2\) was statistically significant (\(p < 0.05\), ANOVA). Equimolar concentrations of DOPA and DOPAC were also tested for effects on NO\(_2\)-induced inhibition of TH, and the results are included in Fig. 3C. The inhibitory effects of NO\(_2\) were not altered by DA or DOPA but were significantly increased by DOPAC. Whereas DA and DOPA increased the inhibition of TH by NO\(_2\) from 50\% to about 65\%, DOPAC plus NO\(_2\) resulted in a near-total inactivation of TH (i.e. 95\% inhibition).

The effect of catechols on nitrating activity of TH by NO\(_2\) was tested, and the results are presented in Fig. 4A. The NO\(_2\)-generating conditions that caused inhibition of TH catalytic activity (Fig. 3 above) resulted in the nitration of tyrosine residues in the TH monomer (60 kDa). Omission of any one of the components needed to produce NO\(_2\) prevented tyrosine nitration in TH (data not shown). It can be seen in Fig. 4A that equimolar concentrations (20 \(\mu\)M) of DA, DOPA, and DOPAC prevented NO\(_2\)-induced nitration of tyrosine residues in TH. In agreement with results using ONOO\(^{-}\) as the nitrating species (Fig. 2), exposure of TH to NO\(_2\) in the presence of any of the catechols converted the enzyme to a redox cycling quinoprotein, as shown in Fig. 4B.

In view of results showing that catechol prevention of TH
Nitration by ONOO\(^{-}\) or NO\(_2\) did not relieve the enzyme of inhibition, we tested the effects of these treatments on the status of cysteine residues in TH. In agreement with previous studies (33), ONOO\(^{-}\) lowered PMAB labeling of TH as shown in Fig. 5 (middle lane of each nitration condition), an indication of cysteine modification. NO\(_2\) caused similar reductions in PMAB labeling as seen with ONOO\(^{-}\). When ONOO\(^{-}\) or NO\(_2\) was combined with DA, the reduction in PMAB labeling was still observed. DOPA and DOPAC produced the same effects on PMAB labeling as seen with DA in combination with ONOO\(^{-}\) or NO\(_2\) (data not shown). Digital scans of the data in Fig. 5 indicate that the nitrating species reduced PMAB labeling by \(\sim 95\%\) and the DA-quinones also reduced labeling to roughly the same extent.

Espy et al. (13) 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/EM4 cells bearing the

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**Fig. 3.** The effect of DA and other catechols on the inhibition of TH by NO\(_2\). A, TH (10 \(\mu\)M) was incubated with varying concentrations of NaNO\(_2\) in the presence of constant concentration of horseradish peroxidase and hydrogen peroxide (as described under “Experimental Procedures”) to generate increasing amounts of NO\(_2\). B, NaNO\(_2\) was added to TH at a concentration of 200 \(\mu\)M with horseradish peroxidase and hydrogen peroxide, and the concentration of DA was varied as indicated. C, NO\(_2\) generating conditions used in B were combined with equimolar concentrations (20 \(\mu\)M) DA, DOPA, or DOPAC. All treatments were carried out for 60 min at 30 °C after which TH was diluted and assayed for remaining activity. Results in all panels are expressed as percent control TH activity and are the mean \(\pm\) S.E. four to five experiments carried out in duplicate. The overall effect of NaNO\(_2\) in A was statistically significant (\(p < 0.05\), ANOVA) as was the effect of DA in B. The effects of DA and DOPA on TH activity were significantly different from control (\(*\, p < 0.05\), Bonferroni’s test) but did not differ from those of NO\(_2\) alone. The effect of DOPAC was significantly different from control and all other treatment conditions (\(**\, p < 0.05\), Bonferroni’s test).

**Fig. 4.** Post-translational modification of TH after treatment with NO\(_2\) in the presence of DA and other catechols. A, TH (10 \(\mu\)M) was treated with NO\(_2\)-generating conditions as described in the legend to Fig. 3. The indicated catechols (20 \(\mu\)M each) were added just prior to NO\(_2\), and, after a 60-min incubation at 30 °C, samples were prepared for SDS-PAGE and immunoblotting with a monoclonal antibody against nitro-tyrosine (diluted 1:2000). Immunoreactivity was visualized by ECL. B, TH treated under the conditions described in A was analyzed by redox cycling staining after SDS-PAGE and electroblotting to nitrocellulose. Gels in A and B contained 10 \(\mu\)g of TH protein per lane.

**Fig. 5.** Effects of ONOO\(^{-}\) and NO\(_2\) on cysteine residues in TH. TH (10 \(\mu\)M) was treated with ONOO\(^{-}\) (100 \(\mu\)M) or NO\(_2\) (5 units of horseradish peroxidase, 100 \(\mu\)M hydrogen peroxide, and 200 \(\mu\)M sodium nitrite) in the absence or presence of DA (50 \(\mu\)M) as indicated. Samples were diluted with 100 mM Tris-HCl, pH 8.5, and labeled with PMAB (50 \(\mu\)M) 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 first lane under ONOO\(^{-}\) or NO\(_2\) treatment conditions show untreated TH and represent maximal PMAB labeling of cysteines. The second lanes show effects of nitration alone on labeling, and the last lane under each nitrating condition include DA plus either ONOO\(^{-}\) or NO\(_2\).
The quinone of DOPAC caused the greatest amount of redox cycling, whereas ONOO\(^{-}\) caused little if any intracellular nitration whereas NO\(_2\) did (13). The effect of NO\(_2\) on the functional aspects of TH or DA neurons has not been investigated.

TH is quite sensitive to inhibition by ONOO\(^{-}\) and NO\(_2\). Both reactive nitrogen species cause concentration-dependent reductions in TH catalytic function. The enzyme is also modified post-translationally by ONOO\(^{-}\) and NO\(_2\) as evidenced by tyrosine nitration and cysteine oxidation. Although it has been suggested that the cytotoxicity associated with ONOO\(^{-}\) and NO\(_2\) can be mediated by tyrosine nitration, the effects of these reactive nitrogen species are not often considered within the context of cellular phenotype. One case where this is particularly important is the DA neuron. These neurons are characterized, obviously, by their selective and high content of DA.

Catechols react with ONOO\(^{-}\) and NO\(_2\) to form o-quinones and other radical species (17) and, in the process, inhibit the nitration of free tyrosine (20, 40). As an initial step in assessing the influence of the DA phenotype on protein nitration, it was important to determine if catechols could modify the nitration of TH caused by reactive nitrogen species. We observed that DA, its precursor DOPA, and its metabolite DOPAC prevented ONOO\(^{-}\) and NO\(_2\)-induced nitration of tyrosine residues in TH. Despite prevention of tyrosine nitration in TH, the catechols did not relieve the enzyme of inhibition. Considering that ONOO\(^{-}\)-induced nitration of TH has been linked specifically to the loss of TH activity (9, 48), it is interesting that TH could be inhibited in the presence of ONOO\(^{-}\) or NO\(_2\), despite the prevention of tyrosine nitration. In view of results showing that catechols prevent tyrosine nitration caused by ONOO\(^{-}\) or NO\(_2\) without relieving the enzyme from inhibition, the possibility that another post-translational modification was mediating TH inhibition was investigated.

Catechol-quinones are known to attack protein cysteinyls (22, 30) and form redox cycling sites after binding (38, 49). TH (25), tryptophan hydroxylase (50, 51), and the dopamine transporter (32, 53) are examples of proteins that can be modified by DA-quinones and aminochromes. Quinone modification of each of these important proteins has the added effect of reducing their functional activity. Either ONOO\(^{-}\) or NO\(_2\), when combined with DA, DOPA, or DOPAC, modified TH to a redox cycling quinoprotein. However, subtle differences were noted between ONOO\(^{-}\) and NO\(_2\)-generated quinones and their impact on TH. For instance, DA did not alter the ONOO\(^{-}\)-induced inhibition of TH but slightly increased the inhibition caused by NO\(_2\). DOPA provided some protection against ONOO\(^{-}\)-induced inhibition of TH but was without effect on NO\(_2\). Finally, DOPAC did not change TH inhibition by ONOO\(^{-}\) but significantly increased the effects of NO\(_2\) on TH activity. These varying effects probably reflect differences in the chemical interactions between ONOO\(^{-}\) or NO\(_2\) and individual catechols. Any such differences in this regard do not mitigate the importance of the common property shared by catechols, the ability to prevent nitration of tyrosine residues in proteins while causing quinolization of cysteines.

The quinone of DOPAC caused the greatest amount of redox cycling in TH, especially when generated by ONOO\(^{-}\), and the DOPA quinone resulted in the lowest amount. The relationship between enzyme inhibition and redox cycling does not appear
to be directly correlated. Redox cycling by substituted quinones is a very complex chemical process and is difficult to use as a direct index of cysteine modification in TH. The o-quinones of DA (and other catechols as well) are extremely volatile, and the reactivity of any particular catechol-quinone will be determined by its access to sulfhydryls and by its electrophilicity (54, 55). The redox potential of substituted quinones is also very difficult to predict from their structures (49, 56), and, as an illustration, it has been shown that the redox cycling potential of DOPA-quinone is lower than that of many other protein-bound quinones (38). Thus, it appears that the total number of cysteines that are modified by catechol-quinones is a better predictor of the extent of TH inhibition than is the extent of redox cycling caused by a bound quinone.

We have argued recently that the ONOO$^-$/H$_2$O$_2$-induced inhibition of tryptophan hydroxylase (57) and TH (33, 34) is mediated by cysteine modification, not tyrosine nitration. The effects of NO$_2$ on TH have not been investigated, so we tested it along with ONOO$^-$ for effects on cysteine residues in TH using PMAB labeling. This sulfhydryl-specific reactive label reduced cysteine residues in proteins and its reactivity is diminished by treatment of proteins, including TH, with cysteine oxidants like ONOO$^-$ (33). In agreement with the effects of ONOO$^-$, NO$_2$ also reduced PMAB labeling of TH, indicative of cysteine modification. What is more, treatment of TH with ONOO$^-$ or NO$_2$, in the presence of DA (conditions preventing tyrosine nitration), resulted in reduced PMAB labeling as well. These data reinforce results with redox cycling and establish that catechol-quinones derived from ONOO$^-$ or NO$_2$ attack cysteine residues in TH. This post-translational modification appears to be the mechanism by which TH is inhibited when tyrosine nitration has been prevented by the catechols.

Evidence for nitration of TH by ONOO$^-$ in intact cells has been difficult to obtain. Ara et al. (9) treated PC12 cell lysates, not intact cells, with ONOO$^-$ and showed that TH was nitrated at selected tyrosine residues. We have not been able to establish that TH is nitrated after treatment of intact PC12 cells with ONOO$^-$, although this could account for the failure and led us to consider an alternative approach to the problem. First, it does not appear that ONOO$^-$ penetrates the plasma membrane of intact cells in sufficient concentrations to cause tyrosine nitration in cytoplasmic proteins (13). Second, ONOO$^-$ is formed de novo from the reaction of nitric oxide with superoxide and high concentrations of these reagents must be maintained at or near a 1:1 stoichiometry to avoid secondary reactions that form species incapable of tyrosine nitration. An imbalance in this stoichiometry can lead to a quenching of nitration and oxidation reactions or may even lead to the formation of nitrosating species (15, 58). Third, intact PC12 cells contain very high catecholamine concentrations that could also quench ONOO$^-$-induced tyrosine nitration. Fourth, it is possible that only a small number of the TH molecules in PC12 cells are nitrated, and immunoprecipitation and immunoblotting are too insensitive to detect TH nitration. We used the method of Espey et al. (13) to monitor tyrosine nitration in intact cells by NO$_2$ through measures of fluorescence reductions in an eGFP/TH fusion protein stably expressed in hDAT-bearing HEK293/EM4 cells. We chose this cell line because of its extremely low endogenous DA content and because the intracellular content of DA could be increased substantially via the hDAT. The use of fluorescence is also a far more sensitive way of measuring nitration than immunoblotting. It was observed that NO$_2$ caused a significant reduction in eGFP/TH fluorescence and TH catalytic activity in intact cells. It does not appear that ONOO$^-$ plays a role, possibly as a downstream by-product of NO$_2$ generation, because the ONOO$^-$ scavenger methionine did not prevent reductions in eGFP fluorescence or TH activity. The magnitude of the reduction in TH activity (near-total) was greater than the reduction in eGFP fluorescence (about 50%) and also stands in contrast to in vitro results where 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 (13) but could inhibit TH activity in vitro as well as in intact cells. The possibility that TH is modified by nitric oxide is currently under investigation. Pre-loading of cells with DA largely prevented the reduction in eGFP/TH fluorescence caused by NO$_2$. Although DA provided partial protection against NO$_2$-induced inhibition of TH activity, the enzyme remained inhibited. These results indicate that cellular DA can modulate tyrosine nitration. Furthermore, the loss of TH catalytic activity after exposure of intact cells to PTIO-PAPA/NO establishes that both elements of the eGFP/TH fusion protein had been modified by NO$_2$ (i.e. eGFP fluorescence and TH activity, respectively).

The present results provoke a re-consideration of ONOO$^-$-mediated tyrosine nitration as an early event in the neurotoxic process in DA neurons. It appears that DA, its precursor DOPA, and its metabolite DOPAC shift the balance of influence of ONOO$^-$ and NO$_2$ toward the formation of o-quinones at the expense of tyrosine nitration. Although most intracellular DA is sequestered within synaptic vesicles, where it might be protected from attack by ONOO$^-$ or NO$_2$, TH is a cytoplasmic enzyme, and newly synthesized DA appears in the cytoplasm before it is transported into vesicles. The cytoplasmic availability of DA is not determined solely by TH, particularly under conditions thought to cause the production of ONOO$^-$ in vitro. For example, methamphetamine (59) and MPTP (60, 61) cause extensive redistribution of DA from storage vesicles into the cytoplasm and extracellular space. Thus, ONOO$^-$ or NO$_2$-induced tyrosine nitration in DA neurons could be suppressed by endogenous catechols, reducing the likelihood that this post-translational modification is an early occurring event in DA neurodegeneration

Huang and colleagues have shown that intrastriatal injections of DA (22, 23) or systemic injections of methamphetamine (8), both of which result in damage to DA nerve endings, cause substantial increases in the levels of cysteinyl-DA adducts in proteins. Postmortem Parkinson’s tissue contains elevated levels of cysteinyl-catechol species (29, 52), and cerebrospinal fluid from individuals with Parkinson disease contain antibodies that recognize quinone-modified proteins (28). We have shown that TH that has been modified by DA-quinones acquires the ability to cause redox cycling of iron (25), reinforcing the cytotoxic potential of catechol-quinones that was established by the influential studies of Graham (52, 54, 55). Taken together, evidence is mounting that DA, and its catechol precursors and metabolites, in the form of their o-quinones, may play an early and influential role in determining the viability of DA neurons under conditions of oxidative or nitrosative stress.

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Dopamine Prevents Nitration of Tyrosine Hydroxylase by Peroxynitrite and Nitrogen Dioxide: IS NITROTYROSINE FORMATION AN EARLY STEP IN DOPAMINE NEURONAL DAMAGE?

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