ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 7 & 8, 2005

Forum Original Research Communication

S-Thiolation of Tyrosine Hydroxylase by Reactive Nitrogen Species in the Presence of Cysteine or Glutathione

MAHDIEH SADIDI,[1] TIMOTHY J. GEDDES,[1,3] and DONALD M. KUHN[1–3]

ABSTRACT

Tyrosine hydroxylase (TH) is the initial and rate-limiting enzyme in the biosynthesis of the neurotransmitter dopamine. Peroxynitrite ($ONOO^-$) and nitrogen dioxide (NO_2) inhibit TH catalytic function and cause nitration of protein tyrosine residues. Exposure of TH to either $ONOO^-$ or NO_2 in the presence of cysteine (or glutathione) prevents tyrosine nitration and results in *S*-thiolation instead. TH catalytic activity is suppressed by *S*-thiolation. Dithiothreitol prevents and reverses the modification of TH by *S*-thiolation, and returns enzyme activity to control levels. *S*-Nitrosothiols, which are known to *S*-thiolate proteins, can be formed in the reaction of cysteine or glutathione with reactive nitrogen species. Therefore, *S*-nitrosoglutathione (GSNO) was tested for its ability to modify TH. Fresh solutions of GSNO did not modify TH, whereas decomposed GSNO resulted in extensive *S*-thiolation of the protein. Dimedone, a sulfenic acid trap, prevents *S*-thiolation of TH when included with GSNO during its decomposition. Taken together, these results show that TH is *S*-thiolated by $ONOO^-$ or NO_2 in the presence of cysteine. *S*-Thiolation occurs at the expense of tyrosine nitration. Glutathione disulfide *S*-oxide, which forms spontaneously in the decomposition of GSNO and which is found in tissue undergoing oxidative stress, may be the species that *S*-thiolates TH. *Antioxid. Redox Signal.* 7, 863–869.

INTRODUCTION

Dopamine (DA) plays numerous important roles as a neurotransmitter, ranging from regulation of motor and endocrine function to participating in high-level cognitive activities. DA neurons are particularly sensitive to damage caused by disease, drugs, and environmental contaminants, and it is under these conditions that one can appreciate the importance of maintaining equilibrium within these neurons. For instance, Parkinson's disease is a debilitating neurodegenerative condition that is characterized by the extensive loss of neurons within the nigrostriatal DA system. Drugs of abuse like methamphetamine can also cause long-term reductions in the function of DA nerve endings. The molecular mechanisms by which the DA neuronal system is damaged are not understood, but attention is increasingly focused on oxidative and nitrosative stress.

A common thread running among most conditions associated with toxicity to DA neurons is an alteration in cellular glutathione (GSH). GSH maintains the essential thiol status of cells and critical proteins by preventing oxidation and/or by scavenging free radicals. The recognition that GSH levels are significantly lowered in Parkinson's disease (13) and after methamphetamine intoxication (29) may indicate that DA neurons have been targeted by reactive oxygen and nitrogen species. However, a reduction in the cellular levels of GSH is not necessarily the same as a loss of GSH from cells, or an oxidation of GSH to GSSG. In fact, it is becoming apparent that, under conditions of cellular stress, GSH can form disulfide bonds with protein cysteine residues, a posttranslational modification referred to as *S*-thiolation (when disulfide forms between cysteine and protein) or *S*-glutathionylation (when disulfide forms between cysteine of GSH and protein).

Tyrosine hydroxylase (TH) is the initial and rate-limiting enzyme in the biosynthesis of DA, and the level of its activity is tightly regulated by various posttranslational modifications, including phosphorylation. More recently, it has become evident that TH function can be altered under conditions of oxidative and nitrosative stress through direct modification of the protein. Peroxynitrite ($ONOO^-$) inhibits TH

[1]Department of Psychiatry and Behavioral Neurosciences and [2]Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, and [3]John D. Dingell VA Medical Center, Detroit, MI 48201.

catalytic function via a mechanism that involves nitration of tyrosine residues (1, 4) or direct oxidation of cysteines (5, 31). We have proposed that DA neurons contain numerous factors, including DA (31), tetrahydrobiopterin (23), and reduced nicotinamide nucleotides (22), that block the tyrosine-nitrating effects of reactive nitrogen species on TH, lowering the possibility that nitrotyrosine formation is the posttranslational event that mediates reductions in DA neuronal function (26). Instead, the interaction of $ONOO^-$ with cellular components of DA neurons can lead to varying posttranslational modifications of cysteine residues in TH that have as much impact on its function as phosphorylation. One of these components could be GSH. TH can be *S*-glutathionylated *in vitro* as well as in intact PC12 cells under conditions of oxidative stress (5). In view of the importance of TH to normal DA neuronal function, and realizing that $ONOO^-$ could interact with cellular GSH, we considered the possibility that reactive nitrogen species could lead to *S*-thiolation of TH.

MATERIALS AND METHODS

Materials

Thrombin and pGEX-4T vectors were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). Cysteine, GSH, GSH-agarose, dithiothreitol (DTT), dimedone, diamide, diethylenetriaminepentaacetic acid (DTPA), *S*-nitrosoglutathione (GSNO), horseradish peroxidase (HRP), and myeloperoxidase were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.). HRP-conjugated streptavidin was a product of Pierce (Rockford, IL, U.S.A.). The water-soluble biotinylating reagent biotin-X-NHS was obtained from Calbiochem (San Diego, CA, U.S.A.). Western Lightning™ Chemiluminescence Reagent Plus was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA, U.S.A.). HRP-conjugated goat anti-mouse IgG was obtained from Cappel/Organon (West Chester, PA, U.S.A.). An anti-nitrotyrosine monoclonal antibody was purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.) and an anti-GSH monoclonal antibody was obtained from ViroGen (Watertown, MA, U.S.A.). All other reagents were of analytical grade from commercial sources.

Cloning and assay of TH

TH was expressed as a glutathione *S*-transferase-fusion protein. The recombinant protein was purified by GSH-agarose affinity chromatography, and the fusion tag was removed by thrombin cleavage, resulting in a highly purified TH preparation (>95% pure) as previously described (5, 25). Protein was measured using the method of Bradford (6).

Preparation of $ONOO^-$ and nitrogen dioxide (NO_2) and treatment of TH

$ONOO^-$ was synthesized by the quenched-flow method of Beckman *et al.* (3), and its concentration was determined by the extinction coefficient ϵ_{302} = 1,670 M^{-1} cm^{-1}. $ONOO^-$ was added to TH with vigorous mixing in 50 m*M* potassium phosphate buffer, pH 7.4, containing 100 μ*M* DTPA, and incubations were carried out for 15 min at 30°C. NO_2 was produced by reacting HRP with hydrogen peroxide (100 μ*M*) in the presence of sodium nitrite (200 μ*M*) as described by Espey and colleagues (12). TH was exposed to NO_2 for 60 min at 30°C. After incubations with $ONOO^-$ or NO_2, TH was diluted with 10 volumes of 50 m*M* potassium phosphate, pH 6, and assayed for catalytic activity or posttranslational modification.

Posttranslational modification of TH by S-thiolation (or S-glutathionylation) and tyrosine nitration

TH (5 μ*M*) was exposed to *S*-thiolating conditions as described by Eaton *et al.* (10). In brief, TH was incubated with varying concentrations of diamide, biotinylated cysteine (bCYS), or diamide + bCYS in 50 m*M* potassium phosphate buffer, pH 7.5, containing 100 μ*M* DTPA for 15 min at 30°C. GSH was used in place of bCYS in parallel reactions to extend the generality of TH modification beyond cysteine. Where indicated, diamide was replaced with $ONOO^-$ or NO_2. Attempts to prevent or reverse the effects of *S*-thiolation on TH were carried out by adding DTT before or after treatment, respectively. In some experiments, TH was exposed to fresh GSNO, prepared by reacting equimolar concentrations of GSH with acidified sodium nitrite. The effects of fresh GSNO on TH were compared with those of commercial GSNO preparations that had been stored at −20°C for 6 months (referred to as "aged" hereafter) or to GSNO that was allowed to decompose in the dark at room temperature for 60 h after synthesis. The concentration of all GSNO solutions was determined by the extinction coefficient ϵ_{330} = 767 M^{-1} cm^{-1} (28). TH was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotting to nitrocellulose, and blots were blocked in Tris-buffered saline containing Tween-20 (0.1%, vol/vol) and 5% nonfat dry milk. When TH was *S*-thiolated with bCYS, modified protein was detected by incubating blots with HRP-conjugated streptavidin (1:4,000 dilution, 30 min at room temperature), followed by enhanced chemiluminescence. When GSH or GSNO was used to modify TH, *S*-glutathionylation of the protein was detected by probing blots with a monoclonal antibody against protein-bound GSH (1:1,000 dilution, 16 h at 4°C). Nitration of TH tyrosine residues was detected with a monoclonal antibody specific for nitrotyrosine (1:2,000 dilution, 16 h at 4°C). After exposure to primary antibodies, blots were washed and incubated with HRP-conjugated streptavidin (1:4,000, 1 h at room temperature), followed by enhanced chemiluminescence.

RESULTS

TH is inhibited by ~60% upon exposure to diamide + bCYS (1 m*M* each) as shown in Fig. 1A. Neither diamide nor bCYS alone reduced TH activity (data not shown). If DTT was added before or after exposure of the enzyme to diamide + bCYS, inhibition of catalysis was prevented or reversed, respectively, suggesting that TH cysteine residues were modified. Figure 1B shows that diamide + bCYS caused extensive *S*-thiolation of TH, whereas diamide or bCYS alone did not.

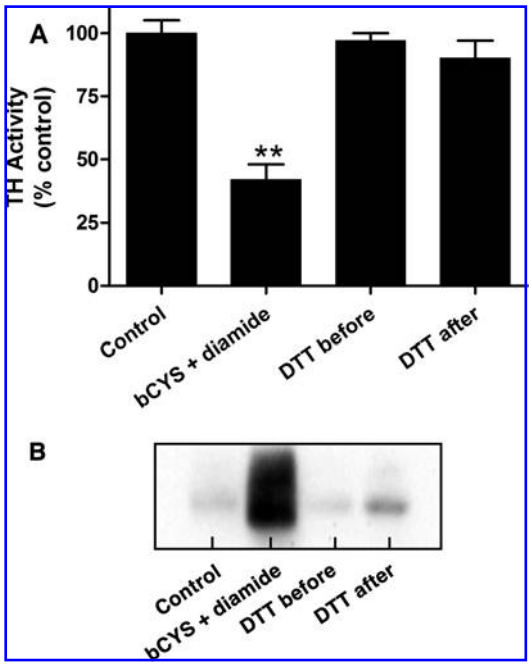

FIG. 1. Inhibition and *S*-thiolation of TH by diamide and bCYS. TH (5 μ*M*) was incubated with diamide (1 m*M*), bCYS (1 m*M*), or their combination, and the effects on enzyme activity **(A)** and posttranslational modification **(B)** were determined. Controls were wild-type enzyme incubated as described, but not exposed to diamide or bCYS. Where indicated, DTT (2 m*M*) was added before or after exposure of TH to *S*-thiolating conditions. Enzyme activity in A is reported as % control and represents the mean ± SEM of three or four experiments carried out in duplicate. *S*-Thiolation of TH in B was detected by probing nitrocellulose blots with HRP-conjugated streptavidin, followed by enhanced chemiluminescence. The effect of *S*-thiolation on TH catalytic activity was significantly different from control (**$p < 0.01$), Bonferroni's test.

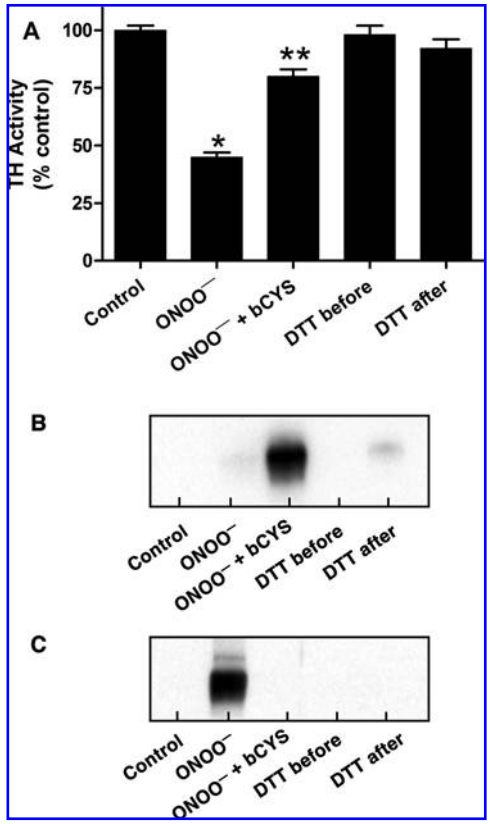

FIG. 2. $ONOO^-$ inhibits TH and causes protein *S*-thiolation in the presence of bCYS. TH (5 μ*M*) was incubated with $ONOO^-$ (100 μ*M*) or $ONOO^-$ + bCYS (1 m*M*) as indicated, and the effects on enzyme activity **(A)**, *S*-thiolation **(B)**, and tyrosine nitration **(C)** were determined. Controls were wild-type enzyme incubated as described, but not exposed to $ONOO^-$ or bCYS. Where indicated, DTT (2 m*M*) was added before or after exposure of TH to $ONOO^-$ + bCYS. Enzyme activity in A is reported as % control and represents the mean ± SEM of three or four experiments carried out in duplicate. *S*-Thiolation of TH (B) was detected as described in Fig. 1. Modification of TH by tyrosine nitration (C) was measured by probing blots with an anti-nitrotyrosine antibody. The effect of *S*-thiolation on TH catalytic activity was significantly different from control (*$p < 0.01$) and $ONOO^-$ (**$p < 0.05$), Bonferroni's test.

In agreement with data shown in Fig. 1A, DTT prevented and reversed the modification of TH caused by diamide + bCYS. These results suggest that TH is inhibited and modified by *S*-thiolation, in agreement with our previous studies (5).

$ONOO^-$ was tested for effects on TH because it can react with cysteine and GSH to form *S*-nitrosothiols (2, 35, 38) that can *S*-thiolate proteins (20, 30). Figure 2A shows that $ONOO^-$ significantly lowers TH activity. The effect of $ONOO^-$ on TH is partially prevented by bCYS, as expected (24), but TH activity remains significantly below control levels. DTT provides complete protection from the inhibition caused by $ONOO^-$ + bCYS, and it also reverses this effect. By contrast, the inhibition of TH caused by $ONOO^-$ alone is not reversed by DTT (24). Inhibition of TH by $ONOO^-$ + bCYS is accompanied by protein *S*-thiolation, an effect that is prevented and reversed by DTT (Fig. 2B). Figure 2C shows that $ONOO^-$ nitrates tyrosine residues in TH and establishes that bCYS completely prevents this effect. Thus, in the presence of bCYS, $ONOO^-$-mediated *S*-thiolation of TH occurs to the expense of tyrosine nitration.

$ONOO^-$ may have limited capacity to cause tyrosine nitration *in vivo* (12, 32) so NO_2 was also tested as an alternative reactive nitrogen species. Figure 3A shows that NO_2 significantly inhibits TH catalytic function. The extent of TH inhibition by NO_2 was mitigated by bCYS, but the enzyme remained significantly inhibited nonetheless. DTT prevented and reversed the effects of NO_2 + bCYS on TH activity, but did not reverse the inhibitory effects of NO_2 alone on TH activity (data not shown). Figure 3B shows that TH inhibition by NO_2 + bCYS is associated with extensive protein *S*-thiolation. DTT prevented NO_2-mediated *S*-thiolation of TH in the presence of bCYS and caused the chemical reduction of the bCYS-TH disulfide. Finally, Figure 3C shows that NO_2 alone causes extensive tyrosine nitration within TH. However, the NO_2-induced tyrosine nitration is completely blocked by bCYS and is replaced by *S*-thiolation instead.

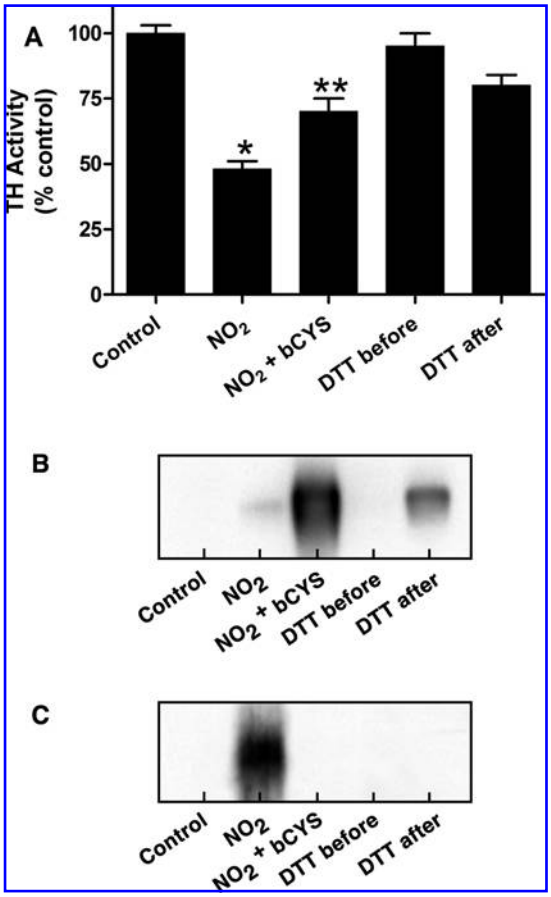

FIG. 3. NO_2 inhibits TH and causes protein *S*-thiolation in the presence of bCYS. TH (5 μ*M*) was incubated with NO_2 (100 μ*M*) or NO_2 (100 μ*M*) + bCYS (1 m*M*) as indicated, and the effects on enzyme activity **(A)**, *S*-thiolation **(B)**, and tyrosine nitration **(C)** were determined. Controls were wild-type enzyme incubated as described, but not exposed to NO_2 or bCYS. Where indicated, DTT (2 m*M*) was added before or after exposure of TH to NO_2 + bCYS. Enzyme activity in A is reported as % control and represents the mean ± SEM of three or four experiments carried out in duplicate. Modification of TH by *S*-thiolation (B) or tyrosine nitration (C) was detected as described in Fig. 2. The effect of *S*-thiolation on TH catalytic activity was significantly different from control (*$p < 0.01$) and NO_2 (**$p < 0.05$), Bonferroni's test.

Cysteine and GSH react with $ONOO^-$ to form *S*-nitrosothiols, such as *S*-nitrosocysteine and GSNO. Because *S*-nitrosothiols can *S*-thiolate proteins, we considered the possibility that an *S*-nitrosothiol was modifying TH during exposure to cysteine (or GSH) + $ONOO^-$. GSNO was used in these experiments because we found increased background labeling of TH when bCYS was used to synthesize *S*-nitrosocysteine. Freshly synthesized GSNO (up to 10 m*M*) did not result in *S*-glutathionylation of TH as shown in Fig. 4. However, it now appears that glutathione disulfide S-oxide [GS(O)SG)], a spontaneous degradation product of GSNO, may be the major thiolating species in solutions of GSNO (16, 27, 36). Aged commercial preparations of GSNO were tested for their effects on TH and were found to cause extensive *S*-glutathionylation of the protein as shown in Fig. 4. Similarly, when fresh GSNO was allowed to decompose in air for 60 h at room temperature, conditions that lead to formation of GS(O)SG (16, 36), TH was *S*-glutathionylated. The decomposition of GSNO to GS(O)SG involves a glutathione sulfenic acid intermediate, and this intermediate can be trapped by dimedone, a specific sulfenic acid scavenger (36). When freshly prepared GSNO was decomposed in the presence of excess dimedone (10 m*M*), *S*-glutathionylation of TH was prevented (Fig. 4). Similarly, dimedone prevents TH *S*-glutathionylation by aged commercial GSNO (data not shown). Freshly prepared GSNO caused only minor inhibition of TH catalytic activity (10% inhibition), whereas both preparations of decomposed GSNO inhibited the enzyme by ~30%. Dimedone itself does not modify TH catalytic activity, but the mild inhibitory effects of decomposed GSNO on TH activity were prevented by this sulfenic acid trap (data not shown).

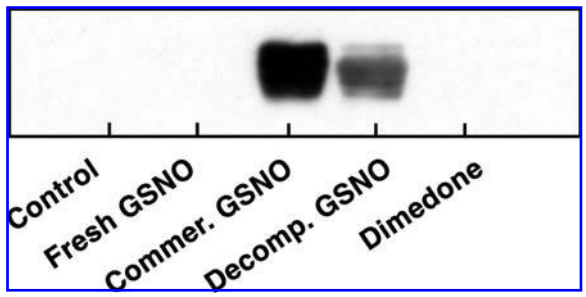

FIG. 4. *S*-Glutathionylation of TH by decomposed GSNO. TH (5 μ*M*) was exposed to freshly prepared GSNO (5 m*M*), or to equimolar concentrations of aged commercial GSNO or fresh GSNO that had been allowed to decompose for 60 h at room temperature, as indicated. In some experiments, decomposition of freshly prepared GSNO was carried out in the presence of a twofold molar excess of dimedone to scavenge sulfenic acid intermediates. TH *S*-glutathionylation was detected with the use of an anti-glutathione antibody and enhanced chemiluminescence as described in Materials and Methods.

DISCUSSION

Oxidation of protein thiols beyond sulfenic acid to sulfinic acid or sulfonic acid reflects modifications that are usually not reversible, and represents a potentially harmful consequence of oxidative stress. It is generally accepted that low-molecular-weight thiols like cysteine and GSH prevent oxidative modification of proteins as part of the cellular defense against stress. However, it is becoming increasingly apparent that cysteine or GSH can covalently modify proteins by forming mixed disulfides with them, especially under conditions of oxidative stress. The posttranslational modification of cysteine residues within proteins by the formation of a disulfide with cysteine or GSH is referred to as *S*-thiolation or *S*-glutathionylation, respectively. *S*-Thiolation is emerging as a dynamic regulatory mechanism that has many parallels with protein phosphorylation, including rapid onset, substrate specificity, reversibility (chemical and enzymatic), and tar-

get-dependent functional consequences. A growing number of proteins are now known to be modified by *S*-thiolation, including protein kinases (17, 40) and phosphatases (34), actin (8), and glyceraldehyde-3-phosphate dehydrogenase (11), to list but a few. The impact of *S*-thiolation on a target protein is hard to predict and can range from protection (*i.e.,* by masking of a critical cysteine against irreversible oxidation) to loss of function (21).

The aim of the present experimentation was to assess the effects of cysteine (and GSH) on modification of TH by reactive nitrogen species. $ONOO^-$ is perhaps best known for its ability to nitrate free tyrosine or tyrosine residues in proteins (19), and this posttranslational modification is often used as an indirect measure of $ONOO^-$ production in intact systems (18). $ONOO^-$ has been implicated as an etiological factor in the DA neuronal degeneration associated with Parkinson's disease and in other neurodegenerative and neurotoxic conditions (15, 37), largely through findings of increased tissue levels of nitrotyrosine. Exposure of TH to $ONOO^-$ inhibits its catalytic activity and results in extensive tyrosine nitration of the protein (1, 4). However, $ONOO^-$ is also a powerful oxidant, and we have attributed the inhibition of TH by $ONOO^-$ to its ability to modify cysteine residues (24, 25). Although the extent to which $ONOO^-$ causes tyrosine nitration in intact cells has been questioned on the basis of chemical and kinetic constraints (12, 32), it is clear that $ONOO^-$ could encounter cysteine or GSH within the intracellular environment. Interactions of $ONOO^-$ with intracellular thiols could readily alter its reactive properties and, in the process, create downstream reactants (*e.g., S*-nitrosothiols) that have equal potential to modify critical cellular proteins. Therefore, we hypothesized that $ONOO^-$ and NO_2 would react with cysteine (or GSH) and lead to *S*-thiolation of TH.

The present experiments were facilitated by the development of a new approach to the study of *S*-thiolation. Eaton *et al.* used bCYS to monitor cellular *S*-thiolation with streptavidin-labeled probes (10). This approach has now been used successfully to identify a number of cardiac proteins that are modified by *S*-thiolation during ischemia and reperfusion (9–11, 14), and it was extended to the present studies of TH as a substrate for *S*-thiolation. Treatment of TH with diamide, a specific cysteine oxidant, in the presence of bCYS, led to a significant inhibition of catalytic activity. Neither diamide nor bCYS alone lowered enzyme function. DTT prevented the inhibitory effects of diamide + bCYS on TH activity and restored catalytic function to TH when added after treatment. These effects of diamide + bCYS on enzyme activity were accompanied by *S*-thiolation of the protein. DTT prevented and reversed modification of TH by diamide + bCYS as well. The characteristics of the modification of TH by diamide + bCYS are entirely consistent with protein *S*-thiolation and confirm our previous studies showing that TH is inhibited by *S*-glutathionylation after treatment with diamide + GSH (5).

Having established that TH is *S*-thiolated with the use of bCYS, reactive nitrogen species were substituted for diamide to determine if they promoted *S*-thiolation as well. $ONOO^-$ inhibits TH activity and bCYS was found to provide some protection, in agreement with previous results (24). The inhibition caused by $ONOO^-$ + bCYS was prevented and reversed by DTT, whereas that associated with $ONOO^-$ alone was not reversible. TH is *S*-thiolated when treated with $ONOO^-$ in the presence of bCYS and this modification is prevented and reversed by DTT, in agreement with results seen on catalytic function of the enzyme. $ONOO^-$ alone causes tyrosine nitration in TH, an effect that is completely prevented by bCYS. NO_2 could be substituted for $ONOO^-$ and GSH could be substituted for cysteine without changing the pattern of effects on TH. These findings demonstrate the fundamental differences between $ONOO^-$-induced tyrosine nitration and $ONOO^-$-mediated *S*-thiolation of TH in the presence of bCYS. First, the inhibitory effects of $ONOO^-$ alone on TH activity cannot be reversed by DTT, whereas those of $ONOO^-$ + bCYS can. Second, $ONOO^-$-mediated nitration of tyrosine residues in TH is completely prevented by bCYS, and *S*-thiolation occurs instead.

The reaction of low-molecular-weight thiols with reactive nitrogen species can yield *S*-nitrosothiols like GSNO (2, 35, 38) that lead to protein *S*-glutathionylation (20, 30, 39). To investigate the possibility that *S*-nitrosothiols might be mediating the *S*-thiolating effects of bCYS and reactive nitrogen species on TH, GSNO was tested for its effects on TH. Freshly prepared GSNO was virtually devoid of effects on TH. It is now known that GSNO undergoes spontaneous decomposition, generating several products that mediate *S*-glutathionylation of proteins. The most potent glutathionylating species in decomposed solutions of GSNO has been identified as GS(O)SG (16). Therefore, solutions of freshly synthesized GSNO were allowed to decompose before testing for effects on TH. We observed that GS(O)SG (from decomposed GSNO) caused much greater levels of *S*-thiolation of TH than equimolar solutions of fresh GSNO. Similarly, decomposed GSNO caused more inhibition of TH activity than freshly prepared GSNO. These results are consistent with a growing body of evidence that decomposition products of GSNO, and *S*-nitrosocysteine as well (16), are potent protein *S*-thiolating species. This possibility is strengthened by the observation that dimedone, a sulfenic acid trap, prevents TH *S*-thiolation when added to GSNO prior to its decomposition. The formation of GS(O)SG is thought to proceed through sulfenic acid intermediates (36), explaining how dimedone prevents *S*-thiolation of TH by GSNO decomposition products.

The present data suggest that a product formed in the reaction of $ONOO^-$ or NO_2 with cysteine or GSH [*e.g.,* GS(O)SG] mediates *S*-thiolation of TH. However, protein *S*-thiolation could occur by other mechanisms while still involving the same three reactants used presently, *i.e.,* TH, cysteine (or GSH), and $ONOO^-$. These include: (a) thiol–disulfide exchange between TH cysteines and GSSG; (b) oxidation of TH cysteines to form thiyl radicals or sulfenic acids that interact with cysteine (or GSH) to produce a mixed disulfide; (c) nucleophilic attack of TH thiolate on GSNO, resulting in a mixed disulfide; (d) oxidation of GSH to GSH-sulfenic acid and subsequent formation of a mixed disulfide with a TH cysteine; and (e) nitrosation of TH cysteines, followed by interaction with cysteine (or GSH) to form mixed disulfides. These mechanisms have been delineated in several excellent reviews (7, 16, 21).

$ONOO^-$ has been implicated in the damage to DA neurons that occurs in neurodegenerative diseases or after ingestion of neurotoxic drugs of abuse. This conclusion is based, in large

part, on observation of elevated levels of nitrotyrosine in affected tissues. DA neurons are endowed with various factors that prevent the tyrosine nitrating effects of $ONOO^-$ and NO_2, including DA itself (31), the TH cofactor tetrahydrobiopterin (23), and reduced nicotinamide nucleotides (22), leading us to conclude that $ONOO^-$-induced nitrotyrosine formation most likely represents a late-occurring event in the process of degeneration of these neurons and not an early sign of distress or damage (26). The present results now add cysteine and GSH to the list of intraneuronal factors that prevent $ONOO^-$-induced tyrosine nitration and establish *S*-thiolation as a potential early response to stress. Reactive oxygen species can also interact with low-molecular-weight thiols to form *S*-thiolating species (33), so it is possible that cysteine and GSH channel reactive oxygen/nitrogen species toward *S*-thiolation and away from tyrosine nitration.

ACKNOWLEDGMENTS

These studies were supported by National Institutes of Health grants DA10756 and DA14392, and by a Department of Veterans Affairs Merit Award.

ABBREVIATIONS

bCYS, biotinylated cysteine; DA, dopamine; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; GSH, glutathione; GSNO, *S*-nitrosoglutathione; GS(O)SG, glutathione disulfide *S*-oxide; HRP, horseradish peroxidase; NO_2, nitrogen dioxide; $ONOO^-$, peroxynitrite; TH, tyrosine hydroxylase.

REFERENCES

1. Ara J, Przedborski S, Naini AB, Jackson-Lewis V, Trifiletti RR, Horwitz J, and Ischiropoulos H. Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Proc Natl Acad Sci U S A* 95: 7659–7663, 1998.
2. Balazy M, Kaminski PM, Mao K, Tan J, and Wolin MS. *S*-Nitroglutathione, a product of the reaction between peroxynitrite and glutathione that generates nitric oxide. *J Biol Chem* 273: 32009–32015, 1998.
3. Beckman JS, Chen J, Ischiropoulos H, and Crow JP. Oxidative chemistry of peroxynitrite. *Methods Enzymol* 233: 229–240, 1994.
4. Blanchard-Fillion B, Souza JM, Friel T, Jiang GCT, Vrana K, Sharov V, Barron L, Schoneich C, Quijano C, Alvarez B, Radi R, Przedborski S, Fernando GS, Horwitz J, and Ischiropoulos H. Nitration and inactivation of tyrosine hydroxylase by peroxynitrite. *J Biol Chem* 276: 46017–46023, 2001.
5. Borges CR, Geddes T, Watson JT, and Kuhn DM. Dopamine biosynthesis is regulated by *S*-glutathionylation. Potential mechanism of tyrosine hydroxylase inhibition during oxidative stress. *J Biol Chem* 277: 48295–48302, 2002.
6. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254, 1976.
7. Cotgreave IA and Gerdes RG. Recent trends in glutathione biochemistry—glutathione–protein interactions: a molecular link between oxidative stress and cell proliferation? *Biochem Biophys Res Commun* 242: 1–9, 1998.
8. Dalle-Donne I, Giustarini D, Rossi R, Colombo R, and Milzani A. Reversible *S*-glutathionylation of Cys 374 regulates actin filament formation by inducing structural changes in the actin molecule. *Free Radic Biol Med* 34: 23–32, 2003.
9. Eaton P and Shattock MJ. Purification of proteins susceptible to oxidation at cysteine residues: identification of malate dehydrogenase as a target for *S*-glutathiolation. *Ann N Y Acad Sci* 973: 529–532, 2002.
10. Eaton P, Byers HL, Leeds N, Ward MA, and Shattock MJ. Detection, quantitation, purification, and identification of cardiac proteins *S*-thiolated during ischemia and reperfusion. *J Biol Chem* 277: 9806–9811, 2002.
11. Eaton P, Wright N, Hearse DJ, and Shattock MJ. Glyceraldehyde phosphate dehydrogenase oxidation during cardiac ischemia and reperfusion. *J Mol Cell Cardiol* 34: 1549–1560, 2002.
12. Espey MG, Xavier S, Thomas DD, Miranda KM, and Wink DA. Direct real-time evaluation of nitration with green fluorescent protein in solution and within human cells reveals the impact of nitrogen dioxide vs. peroxynitrite mechanisms. *Proc Natl Acad Sci U S A* 99: 3481–3486, 2002.
13. Fitzmaurice PS, Ang L, Guttman M, Rajput AH, Furukawa Y, and Kish SJ. Nigral glutathione deficiency is not specific for idiopathic Parkinson's disease. *Mov Disord* 18: 969–976, 2003.
14. Fuller W, Parmar V, Eaton P, Bell JR, and Shattock MJ. Cardiac ischemia causes inhibition of the Na/K ATPase by a labile cytosolic compound whose production is linked to oxidant stress. *Cardiovasc Res* 57: 1044–1051, 2003.
15. Good PF, Hsu A, Werner P, Perl DP, and Olanow CW. Protein nitration in Parkinson's disease. *J Neuropathol Exp Neurol* 57: 338–342, 1998.
16. Huang KP and Huang FL. Glutathionylation of proteins by glutathione disulfide *S*-oxide. *Biochem Pharmacol* 64: 1049–1056, 2002.
17. Humphries KM, Juliano C, and Taylor SS. Regulation of cAMP-dependent protein kinase activity by glutathionylation. *J Biol Chem* 277: 43505–43511, 2002.
18. Ischiropoulos H. Biological tyrosine nitration: a pathophysiological function of nitric oxide and reactive oxygen species. *Arch Biochem Biophys* 356: 1–11, 1998.
19. Ischiropoulos H. Biological selectivity and functional aspects of protein tyrosine nitration. *Biochem Biophys Res Commun* 305: 776–783, 2003.
20. Ji Y, Akerboom TP, Sies H, and Thomas JA. *S*-Nitrosylation and *S*-glutathiolation of protein sulfhydryls by *S*-nitrosoglutathione. *Arch Biochem Biophys* 362: 67–78, 1999.
21. Klatt P and Lamas S. Regulation of protein function by *S*-glutathiolation in response to oxidative and nitrosative stress. *Eur J Biochem* 267: 4928–4944, 2000.

22. Kuhn DM and Geddes TJ. Reduced nicotinamide nucleotides prevent nitration of tyrosine hydroxylase by peroxynitrite. *Brain Res* 933: 85–89, 2002.
23. Kuhn DM and Geddes TJ. Tetrahydrobiopterin prevents nitration of tyrosine hydroxylase by peroxynitrite and nitrogen dioxide. *Mol Pharmacol* 64: 946–953, 2003.
24. Kuhn DM, Aretha CW, and Geddes TJ. Peroxynitrite inactivation of tyrosine hydroxylase: mediation by sulfhydryl oxidation, not tyrosine nitration. *J Neurosci* 19: 10289–10294, 1999.
25. Kuhn DM, Sadidi M, Liu X, Kreipke C, Geddes T, Borges C, and Watson JT. Peroxynitrite-induced nitration of tyrosine hydroxylase: identification of tyrosines 423, 428, and 432 as sites of modification by matrix-assisted laser desorption ionization time-of-flight mass spectrometry and tyrosine-scanning mutagenesis. *J Biol Chem* 277: 14336–14342, 2002.
26. Kuhn DM, Sakowski SA, Sadidi M, and Geddes TJ. Nitrotyrosine as a marker for peroxynitrite-induced neurotoxicity: the beginning or the end of the end of dopamine neurons? *J Neurochem* 89: 529–536, 2004.
27. Li J, Huang FL, and Huang KP. Glutathiolation of proteins by glutathione disulfide *S*-oxide derived from *S*-nitrosoglutathione. Modifications of rat brain neurogranin/RC3 and neuromodulin/GAP-43. *J Biol Chem* 276: 3098–3105, 2001.
28. Mathews WR and Kerr SW. Biological activity of *S*-nitrosothiols: the role of nitric oxide. *J Pharmacol Exp Ther* 267: 1529–1537, 1993.
29. Moszczynska A, Turenne S, and Kish SJ. Rat striatal levels of the antioxidant glutathione are decreased following binge administration of methamphetamine. *Neurosci Lett* 255: 49–52, 1998.
30. Padgett CM and Whorton AR. Cellular responses to nitric oxide: role of protein *S*-thiolation/dethiolation. *Arch Biochem Biophys* 358: 232–242, 1998.
31. Park S, Geddes TJ, Javitch JA, and Kuhn DM. Dopamine prevents nitration of tyrosine hydroxylase by peroxynitrite and nitrogen dioxide: is nitrotyrosine formation an early step in dopamine neuronal damage? *J Biol Chem* 278: 28736–28742, 2003.
32. Pfeiffer S and Mayer B. Lack of tyrosine nitration by peroxynitrite generated at physiological pH. *J Biol Chem* 273: 27280–27285, 1998.
33. Poole LB, Karplus PA, and Claiborne A. Protein sulfenic acids in redox signaling. *Annu Rev Pharmacol Toxicol* 44: 325–347, 2004.
34. Rao RK and Clayton LW. Regulation of protein phosphatase 2A by hydrogen peroxide and glutathionylation. *Biochem Biophys Res Commun* 293: 610–616, 2002.
35. Schrammel A, Gorren AC, Schmidt K, Pfeiffer S, and Mayer B. *S*-Nitrosation of glutathione by nitric oxide, peroxynitrite, and $^{\bullet}NO/O_2^{\bullet-}$. *Free Radic Biol Med* 34: 1078–1088, 2003.
36. Tao L and English AM. Protein *S*-glutathiolation triggered by decomposed *S*-nitrosoglutathione. *Biochemistry* 43: 4028–4038, 2004.
37. Torreilles F, Salman-Tabcheh S, Guerin M, and Torreilles J. Neurodegenerative disorders: the role of peroxynitrite. *Brain Res Brain Res Rev* 30: 153–163, 1999.
38. van der Vliet A, Eiserich JP, Halliwell B, and Cross CE. Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite. A potential additional mechanism of nitric oxide-dependent toxicity. *J Biol Chem* 272: 7617–7625, 1997.
39. Viner RI, Williams TD, and Schoneich C. Peroxynitrite modification of protein thiols: oxidation, nitrosylation, and *S*-glutathiolation of functionally important cysteine residue(s) in the sarcoplasmic reticulum Ca-ATPase. *Biochemistry* 38: 12408–12415, 1999.
40. Ward NE, Stewart JR, Ioannides CG, and O'Brian CA. Oxidant-induced *S*-glutathiolation inactivates protein kinase C-alpha (PKC-alpha): a potential mechanism of PKC isozyme regulation. *Biochemistry* 39: 10319–10329, 2000.

Address reprint requests to:
Donald M. Kuhn, Ph.D.
Department of Psychiatry and Behavioral Neurosciences
Wayne State University School of Medicine
2125 Scott Hall, 540 E. Canfield
Detroit, MI 48201

E-mail: donald.kuhn@wayne.edu

Received for publication October 5, 2004; accepted January 19, 2005.

This article has been cited by:

1. Mirko Zaffagnini , Mariette Bedhomme , Christophe H. Marchand , Samuel Morisse , Paolo Trost , Stéphane D. Lemaire . 2012. Redox Regulation in Photosynthetic Organisms: Focus on Glutathionylation. *Antioxidants & Redox Signaling* **16**:6, 567-586. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
2. Rodney P. Guttmann, Tamara J. Powell. 2012. Redox Regulation of Cysteine-Dependent Enzymes in Neurodegeneration. *International Journal of Cell Biology* **2012**, 1-8. [CrossRef]
3. Sergio Rosales-Corral , Russel J. Reiter , Dun-Xian Tan , Genaro G. Ortiz , Gabriela Lopez-Armas . 2010. Functional Aspects of Redox Control During Neuroinflammation. *Antioxidants & Redox Signaling* **13**:2, 193-247. [Abstract] [Full Text HTML] [Full Text PDF] [Full Text PDF with Links]
4. Peiran Liu, Malloree A. Tarnowski, Brian W. O'Mara, Wei Wu, Haiying Zhang, James K. Tamura, Michael S. Ackerman, Li Tao, Michael J. Grace, Reb J. Russell. 2009. Characterization of S-thiolation on secreted proteins from E. coli by mass spectrometry. *Rapid Communications in Mass Spectrometry* **23**:20, 3343-3349. [CrossRef]
5. U. Bardullas, J.H. Limón-Pacheco, M. Giordano, L. Carrizales, M.S. Mendoza-Trejo, V.M. Rodríguez. 2009. Chronic low-level arsenic exposure causes gender-specific alterations in locomotor activity, dopaminergic systems, and thioredoxin expression in mice. *Toxicology and Applied Pharmacology* **239**:2, 169-177. [CrossRef]
6. Xing-Huang Gao, Mariette Bedhomme, Laure Michelet, Mirko Zaffagnini, Stéphane D. LemaireChapter 12 Glutathionylation in Photosynthetic Organisms **52**, 363-403. [CrossRef]
7. X.-H. Gao, M. Bedhomme, D. Veyel, M. Zaffagnini, S. D. Lemaire. 2008. Methods for Analysis of Protein Glutathionylation and their Application to Photosynthetic Organisms. *Molecular Plant* **2**:2, 218-235. [CrossRef]
8. Steven R. Danielson, Julie K. Andersen. 2008. Oxidative and nitrative protein modifications in Parkinson's disease. *Free Radical Biology and Medicine* **44**:10, 1787-1794. [CrossRef]
9. Rebecca L. Charles, Philip Eaton. 2008. Redox signalling in cardiovascular disease. *PROTEOMICS – Clinical Applications* **2**:6, 823. [CrossRef]
10. David J. Eyerman, Bryan K. Yamamoto. 2007. A rapid oxidation and persistent decrease in the vesicular monoamine transporter 2 after methamphetamine. *Journal of Neurochemistry* **103**:3, 1219-1227. [CrossRef]
11. Ernst van Faassen, Anatoly F. VaninLow-molecular-weight S-nitrosothiols 173-199. [CrossRef]
12. Dr. Irfan Rahman , Se-Ran Yang , Saibal K. Biswas . 2006. Current Concepts of Redox Signaling in the Lungs. *Antioxidants & Redox Signaling* **8**:3-4, 681-689. [Abstract] [Full Text PDF] [Full Text PDF with Links]
13. Philip Eaton , Michael J. Shattock . 2005. Protein S-Thiolation: Emphasis on Cell Signaling and Gene Expression. *Antioxidants & Redox Signaling* **7**:7-8, 839-840. [Citation] [Full Text PDF] [Full Text PDF with Links]