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Short communication

Reduced nicotinamide nucleotides prevent nitration of tyrosine hydroxylase by peroxynitrite

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Abstract

Tyrosine hydroxylase (TH) is the initial and rate-limiting enzyme in the biosynthesis of the neurotransmitter dopamine (DA). TH activity is inhibited by peroxynitrite ($ONOO^-$) by a mechanism that involves nitration of tyrosine residues and oxidation of cysteine residues in the enzyme. Reduced forms of the nicotinamide adenine dinucleotide cofactors, NADH and NADPH, protect TH from inhibition by $ONOO^-$ and prevent nitration of tyrosine residues. NAD, the oxidized form of the cofactors, neither protects TH from $ONOO^-$-induced inhibition nor prevents the nitration of tyrosine residues in the enzyme. These results suggest that the redox status of the nicotinamide nucleotide cofactors could influence the ability of $ONOO^-$ to modify proteins that are important to the function of DA neurons.

Theme: Disorders of the nervous system

Topic: Degenerative disease: Parkinson's

Keywords: Tyrosine hydroxylase; NADH; NADPH; NAD; Peroxynitrite; Tyrosine nitration

Tyrosine hydroxylase (TH) is the initial and rate-limiting enzyme in the biosynthesis of the neurotransmitter dopamine (DA). TH is inhibited by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) by a mechanism that is thought to involve peroxynitrite ($ONOO^-$)-induced nitration of tyrosine residues in the enzyme [1]. These in vivo results with MPTP are paralleled by in vitro studies showing that TH is inactivated by $ONOO^-$ through modifications of tyrosine [1] or cysteine [20] residues. Methamphetamine, a drug of abuse that inhibits TH and causes damage to DA nerve endings [3,7], appears to exert at least some of its neurotoxic properties through the production of $ONOO^-$ as well [11–13,23]. Using nitration of tyrosine residues in proteins as a marker of $ONOO^-$ action [4,14], it has been suggested that the damage to or destruction of DA neurons that takes place after drugs like MPTP or methamphetamine, or in Parkinson's disease, is mediated by this powerful oxidant [9,10,30]. DA neurons obviously contain high concentrations of DA, selectively, and the natural cofactor for TH, tetrahydrobiopterin (BH4), is also found in very high concentrations in DA neurons [25,26,28]. Both DA [16,17] and BH4 [29] are identified targets for oxidation by $ONOO^-$ and their properties as a neurotransmitter and enzyme cofactor, respectively, are abolished by their reaction with $ONOO^-$. We have observed recently that both DA and BH4 prevent the $ONOO^-$-induced nitration of tyrosine residues in TH without preventing inhibition of the enzyme (unpublished observations). Therefore, it is clear that DA neurons contain species that can react with $ONOO^-$ to cause a shift in its chemical properties as well (i.e., reduction in tyrosine nitration).

The nicotinamide adenine dinucleotide cofactors are found in neurons, including those that use DA as a neurotransmitter. The reduced forms of these cofactors, NADH and NADPH, are required for the function of the enzymes that synthesize BH4 [15,31] including dihydropteridine reductase. NADH and NADPH are now known to

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react with $ONOO^-$ at rates that are faster than the reaction of glutathione and cysteine with $ONOO^-$ [6,18], suggesting the possibility that they could mediate the influence of $ONOO^-$ on proteins that are important for DA neuronal function, including TH. Therefore, the aim of the present study was to assess the ability of NADH and NADPH to modulate the effects of $ONOO^-$ on TH. The results suggest that the reduced forms of these cofactors, but not their oxidized form NAD, prevent both $ONOO^-$-induced inhibition of TH and nitration of tyrosine residues. NADH and NADPH can be added to the list of substances found in DA neurons that prevent the tyrosine nitrating properties of $ONOO^-$, suggesting the possibility that this post-translational modification is not an early event in the destructive process in DA neurons.

TH was cloned and expressed in *Escherichia coli* as a GST-fusion protein as previously described [20,21]. The GST-fusion tag was removed from TH by thrombin cleavage, resulting in a highly purified (>95% pure) preparation of enzyme. $ONOO^-$ was synthesized by the quenched-flow method of Beckman et al. [2] and its concentration was determined by the extinction coefficient $\epsilon_{302}=1670\ M^{-1}\ cm^{-1}$. The hydrogen peroxide contamination of $ONOO^-$ solutions was removed by manganese dioxide chromatography and filtration. $ONOO^-$ (100 μM) was added to TH (10 μM) 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. The effects of NAD, NADH, and NADPH (all from Sigma, St. Louis, MO, USA) on TH inhibition and nitration by $ONOO^-$ was assessed by adding the cofactors to enzyme preparations just prior to $ONOO^-$. Upon completion of incubations with $ONOO^-$ and other additions, enzyme samples were diluted with 10 volumes of 50 mM potassium phosphate, pH 6 and assayed for catalytic assay [24], or they were prepared for electrophoresis (below).

Following treatment with $ONOO^-$ ± the nicotinamide adenine dinucleotide cofactors, TH was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 10% gels [22]. Proteins (3–5 μg/lane) were transferred to nitrocellulose, blocked in Tris-buffered saline containing Tween-20 (0.1%, v/v) and non-fat dry milk (5%, w/v), and probed with a monoclonal antibody specific for nitrotyrosine (Cayman Chemical, Ann Arbor, MI, USA). After overnight incubations with the primary antibody at a dilution of 1:2000, blots were washed incubated with goat anti-mouse secondary antibody conjugated with horseradish peroxidase (Cappel, West Chester, PA, USA; diluted 1:2000), and immunoreactive protein bands were visualized with enhanced chemiluminescence (ECL) reagents (NEN Life Science Products, Boston, MA, USA).

TH activity was lowered to about 45% of control by 100 μM $ONOO^-$. Increasing concentrations of the reduced nicotinamide cofactors NADH and NADPH, added just prior to $ONOO^-$, protected the enzyme from inhibition. It can be seen in Fig. 1 that concentrations of NADH and NADPH between 5 and 20 μM decreased the $ONOO^-$-induced inhibition of TH from 45% of control to approximately 65–70% of control. Over this same concentration range, NADH provided more protection against inhibition of TH by $ONOO^-$ than did NADPH. Maximal protection of TH against the inhibitory effects of $ONOO^-$ was provided by concentrations of 50–100 μM NADH and NADPH where catalytic activity reached 80–85% control. The reduced cofactors did not alter TH activity when tested without $ONOO^-$ (data not shown). The inhibitory effect of $ONOO^-$ alone on TH activity was significant ($P<0.01$, Student's *t*-test). The overall effect of the cofactors on TH activity was significant ($P<0.001$, analysis of variance, ANOVA). The protective effects of NADH and NADPH were significantly different from $ONOO^-$ alone ($P<0.001$, Bonferroni's test) and from untreated controls ($P<0.001$, Bonferroni's test). Finally, NADH was significantly more protective than NADPH at concentrations of 5–20 μM ($P<0.05$, Bonferroni's test) but not at the higher concentrations. The oxidized cofactor NAD provided no protection against the inhibition of TH caused by $ONOO^-$ over the same concentration range at which NADH and NADPH were protective. These data are included in Fig. 1 as well.

Fig. 2 shows the effects of NADH, NADPH, and NAD

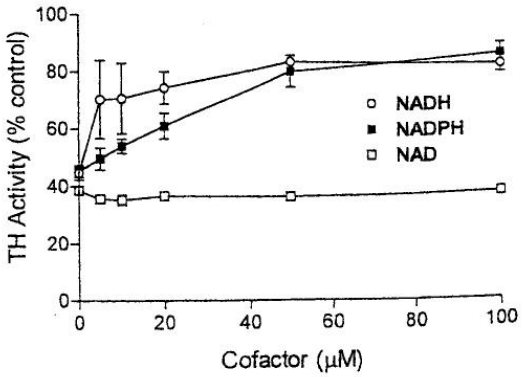

Fig. 1. Effects of NAD, NADH, and NADPH on the $ONOO^-$-induced inhibition of TH. Purified, recombinant TH (10 μM) was incubated with $ONOO^-$ (100 μM) alone or with the indicated concentrations of NAD, NADH, or NADPH in 50 mM potassium phosphate buffer, pH 7.4 containing 100 μM DTPA for 15 min at 30 °C. The cofactors were added just prior to $ONOO^-$. After treatment, samples were diluted with 10 volumes of buffer and remaining TH activity was assayed. Data are presented as TH activity (% control) and are the mean±S.E.M. of four experiments carried out in duplicate. The effect of $ONOO^-$ alone on TH activity was significant ($P<0.001$). The effects of NADH and NADPH were significantly different from untreated controls and from $ONOO^-$ alone ($P<0.001$ for each), but were not different from each other. The effect of NAD was not different from $ONOO^-$ alone.

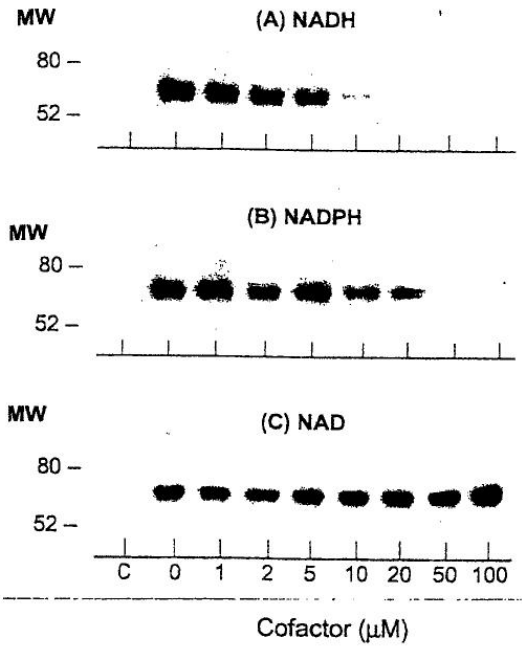

Fig. 2. Effects of NAD, NADH, and NADPH on the $ONOO^-$-induced nitration of tyrosine residues in TH. TH (10 μM) was treated with $ONOO^-$ (100 μM) alone or with the indicated concentrations of NADH (A), NADPH (B), or NAD (C) in 50 mM potassium phosphate buffer, pH 7.4 containing 100 μM DTPA for 15 min at 30 °C. The cofactors were added just prior to $ONOO^-$. After treatment, proteins were run on SDS–PAGE and blotted to nitrocellulose for probing with a monoclonal antibody against nitrotyrosine (diluted 1:2000). After incubation with an HRP-labeled secondary antibody (diluted 1:5000), nitrated TH was visualized with ECL. Molecular weight (MW) markers are shown to the left of each immunoblot and are in kDa. These experiments were repeated on four separate occasions with the same results.

on the $ONOO^-$-induced nitration of tyrosine residues in TH. It can be seen that the reduced cofactors NADH (Fig. 2A) and NADPH (Fig. 2B) prevented, in a concentration-dependent manner, the nitration of tyrosines in TH. The ability of NADH and NADPH to prevent tyrosine nitration caused by $ONOO^-$ paralleled their ability to protect TH from inhibition. Concentrations of each between 5 and 20 μM substantially reduced the extent of TH nitration, and this $ONOO^-$-induced posttranslational modification of TH was completely prevented if the concentration of NADH or NADPH exceeded 20–50 μM. It can be seen that NADH was more potent than NADPH in preventing nitration of TH, consistent with its higher potency in protecting TH from inhibition by $ONOO^-$ (see Fig. 1). By contrast, NAD did not have an effect on TH nitration caused by $ONOO^-$ at any concentration tested, as shown in Fig. 2C.

The reduced nicotinamide nucleotides play a variety of essential roles in neurons including participation in electron-transfer reactions, cellular redox regulation, and serving as cofactors for the enzymes that participate in the synthesis of BH4. These cofactors are also very important in the mitochondrial matrix [33]. NADH and NADPH react with $ONOO^-$ at rates that are faster than the reaction of $ONOO^-$ with GSH or cysteine [18]. Therefore, it is possible that NADH and NADPH could modulate the effects of $ONOO^-$ in DA neurons under conditions of oxidative and nitrosative stress. Considering that $ONOO^-$, as judged by the nitration of tyrosine residues in neuronal proteins, is thought to be a primary factor in the drug (i.e., MPTP and methamphetamine)- and disease-induced (i.e., Parkinson's disease) damage to DA neurons [9–11,30,32], it is important to determine if NADH and NADPH could modify the effects of $ONOO^-$ on TH. It was observed that the reduced forms of the cofactors, NADH and NADPH, were very effective at protecting against the $ONOO^-$-induced inactivation of TH and they also prevented the nitration of tyrosine residues. By comparison, NAD prevented neither the inactivation of TH nor the nitration of tyrosine residues caused by $ONOO^-$. We observed consistently that a ratio of NADH or NADPH to $ONOO^-$ of approximately 1:5 was sufficient to completely prevent the effects of $ONOO^-$ on TH. Similarly, the interaction of NADH with $ONOO^-$ prevents the nitration of proteins in submitochondrial particles [33]. Therefore, it appears possible that the reduced nicotinamide adenine dinucleotides could provide protection of at least TH from the damaging effects of $ONOO^-$. The chemical basis for the enhanced potency of NADH over NADPH in protecting TH from $ONOO^-$-induced inhibition and tyrosine nitration is not immediately clear. It was originally proposed that the reactivity of $ONOO^-$ with NADH and NADPH was identical [18]. However, re-examination of this reaction has revealed that NADH does not react directly with $ONOO^-$, but with its intermediates hydroxyl radical and nitrogen dioxide [8]. NADPH is relatively inert toward freely diffusing radicals [19] and this could help explain why it is less potent in protecting TH from $ONOO^-$.

The reaction between $ONOO^-$ and NADH or NADPH generates NAD^+ and H_2O_2 via the production of the superoxide radical [18]. TH is an oxidatively labile enzyme [20,21] and it is somewhat perplexing that H_2O_2 would not result in some degree of inhibition of TH. The prevention of TH inhibition by NADH and NADPH was significant, but not total, so it is possible that the residual inhibition of the enzyme seen in the presence of NADH and NADPH reflects an effect of the peroxide. This possibility does not appear to be that likely because the addition of catalase did not provide any more protection of TH from inhibition than NADH or NADPH (unpublished observations), and the yield of H_2O_2 from the reaction of NADH with $ONOO^-$ is only 10% of the amount of NADH consumed [18].

NADH and NADPH are not known to be concentrated in DA neurons, but DA and BH4 are, obviously. Both DA [16,17] and BH4 [29] interact with $ONOO^-$ and, in the

process, the ability of $ONOO^-$ to cause nitration of free tyrosine or tyrosine residues in cytoplasmic proteins such as TH is prevented. These observations suggest that the concentration of $ONOO^-$ would have to overcome the very high levels of endogenous DA, BH4, and NADH/NADPH in DA neurons before tyrosine nitration could occur. It has been proposed that the nitration and inhibition of TH that occurs after MPTP might be one of the early biochemical events in the degenerative process that characterizes Parkinson's disease [1]. Similarly, the DA nerve-ending damage that is seen after administration of methamphetamine is thought to be mediated by $ONOO^-$, and is accompanied by increases in the levels of nitrotyrosine [11]. Based on the ability of DA, BH4, and the reduced nicotinamide adenine dinucleotides to prevent $ONOO^-$-induced tyrosine nitration, we suggest that nitration of tyrosines by $ONOO^-$ is more likely to reflect a late-occurring event in DA neurons undergoing damage, made possible by prior depletion of DA, BH4, NADH, and NADPH. It is well known that both MPTP [27] and methamphetamine [5] cause a redistribution of DA from storage vesicles into the cytoplasm, and measures of tyrosine nitration after these drugs are usually taken after extensive depletion of endogenous DA has occurred. It is concluded from the present studies that DA neurons contain several endogenous factors, including NADH and NADPH, that interact with $ONOO^-$ and prevent its tyrosine-nitrating properties.

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