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Current Topics

Nitration in Neurodegeneration: Deciphering the "Hows" "nYs"†

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ABSTRACT: Recent literature has ushered in a new awareness of the diverse post-translational events that can influence protein folding and function. Among these modifications, protein nitration is thought to play a critical role in the onset and progression of several neurodegenerative diseases. While previously considered a late-stage epiphenomenon, nitration of protein tyrosine residues appears to be an early event in the lesions of amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease. The advent of highly specific biochemical and immunological detection methods reveals that nitration occurs in vivo with biological selectively and site specificity. In fact, nitration of only a single Tyr residue is often sufficient to induce profound changes in the activity of catalytic proteins and the three-dimensional conformation of structural proteins. Presumably, nitration modifies protein function by altering the hydrophobicity, hydrogen bonding, and electrostatic properties within the targeted protein. Most importantly, however, nitrative injury may represent a unifying mechanism that explains how genetic and environmental causes of neurological disease manifest a singular phenotype. In this review and synthesis, we first examine the pathways of protein nitration in biological systems and the factors that influence site-directed nitration. Subsequently, we turn our attention to the structural implications of site-specific nitration and how it affects the function of several neurodegeneration-related proteins. These proteins include Mn superoxide dismutase and neurofilament light subunit in amyotrophic lateral sclerosis, α-synuclein and tyrosine hydroxylase in Parkinson's disease, and τ in Alzheimer's disease.

Pathways of Protein Nitration

Nitric oxide (NO),[1] a small, diffusible signaling ligand, was originally discovered to play a role in normal physiological processes, including neurotransmission and vascular dilation. Soon after this seminal discovery, it was postulated that •NO may also mediate oxidative toxicity by reacting with superoxide ($^{\bullet}O_2^-$) to form peroxynitrite ($ONOO^-$) (*1*). •NO-mediated injury most likely results from the formation of highly reactive secondary intermediates, such as nitrogen

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[1] Abbreviations: AA, arachidonic acid; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; $^{\bullet}CO_3^-$, carbonate radical; DA, dopamine; EPO, eosinophil peroxidase; L-DOPA, L-dihydroxyphenylalanine; mAb, monoclonal antibody; MN, motor neuron; M^nX, transition metal center; MPO, myeloperoxidase; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NF, neurofilament; NFT, neurofibrillary tangle; •NO, nitric oxide radical; NO_2^-, nitrite; $^{\bullet}NO_2$, nitrogen dioxide radical; NO_2^+, nitronium ion; NOS, nitric oxide synthase; 3-NT, 3-nitrotyrosine; $^{\bullet}O_2^-$, superoxide radical; •OH, hydroxyl radical; ONOOH, peroxynitrous acid; $ONOOCO_2^-$, nitrosoperoxycarbonate; PD, Parkinson's disease; SOD, superoxide dismutase; TH, tyrosine hydroxylase; •Tyr, tyrosyl radical.

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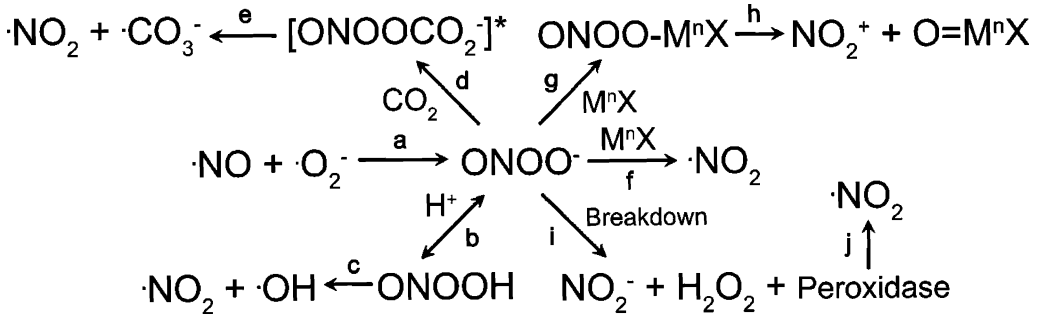

FIGURE 1: Pathways of $ONOO^-$-mediated protein nitration. $ONOO^-$ is generated from the near-diffusion-limited reaction of $^{\bullet}NO$ and $^{\bullet}O_2^-$ radicals (scheme a). Once formed, $ONOO^-$ can direct one- and two-electron oxidation reactions that result in the formation of reactive secondary intermediates (schemes b−j). In most cases, protein nitration proceeds through a free radical mechanism whereby a tyrosyl radical ($^{\bullet}$Tyr) recombines with $^{\bullet}NO_2$ to form 3-NT. However, a free radical-independent process has also been proposed whereby a transition metal (M^nX)−ONOO intermediate decomposes to form a nitronium ion (NO_2^+). The nitronium ion may then directly attack protein Tyr residues (schemes g and h). Please see the text for details.

dioxide ($^{\bullet}NO_2$) and hydroxyl ($^{\bullet}OH$) radicals, rather than from $^{\bullet}NO$ or $ONOO^-$ itself (*2*). While multiple mechanisms are thought to contribute to protein nitration in vivo, this work focuses on the most disease relevant and kinetically feasible pathways. Specifically, we will review the reactions of $ONOO^-$ with carbon dioxide (CO_2) and transition metal centers and determine the process by which they generate secondary nitrating species.

While the role of $ONOO^-$ as the primary in vivo nitrating agent has recently been called into question, it is clear that $ONOO^-$ directs one- and two-electron oxidation reactions that induce protein Tyr nitration. The reaction of $^{\bullet}NO$ with $^{\bullet}O_2^-$ to form $ONOO^-$ is extremely rapid, with a rate constant of at least 10^{10} M^{-1} s^{-1} (Figure 1, scheme a) (*3*). While $ONOO^-$ does not react directly with its protein targets, it can generate secondary intermediates, such as $^{\bullet}NO_2$, $^{\bullet}OH$, and carbonate ($^{\bullet}CO_3^-$) radicals, that participate in free radical biochemistry. For example, given that $ONOO^-$ has a pK_a of 6.8, ~20% of $ONOO^-$ will exist as its conjugate acid, peroxynitrous acid (ONOOH), at physiological pH (Figure 1, scheme b) (*4*). ONOOH is far more labile than $ONOO^-$ and, in the higher-energy trans conformation, may undergo spontaneous homolysis to yield $^{\bullet}NO_2$ and $^{\bullet}OH$ (Figure 1, scheme c) (*5*). These reactive free radicals can abstract a hydrogen atom from Tyr to form tyrosyl radicals ($^{\bullet}$Tyr). $^{\bullet}$Tyr radicals, in turn, recombine with $^{\bullet}NO_2$ in a near-diffusion-limited reaction to form 3-nitrotyrosine (3-NT).

Notably, proton-mediated $ONOO^-$ decomposition is a slow process. As such, it is far more likely that $ONOO^-$ first reacts with a strong Lewis acid (i.e., CO_2 or a redox-active metal ion) that will destabilize the ONO−O bond to generate reactive free radicals (*6*). For example, $ONOO^-$ reacts catalytically with CO_2 to form an intermediate adduct, nitrosoperoxycarbonate ($ONOOCO_2^-$), that decomposes into $^{\bullet}NO_2$ and $^{\bullet}CO_3^-$ radicals (Figure 1, schemes d and e) (*7*). These species are fully competent to nitrate protein Tyr residues. Therefore, CO_2 at physiological concentrations (~1.5 mM) may preferentially direct the activity of $ONOO^-$ toward protein nitration (*8*). The overall consequences of $ONOO^-$ formation in vivo will ultimately be decided by the reaction kinetics and concentration of substrates.

As mentioned above, $ONOO^-$ also reacts with transition metal centers (i.e., M^nX), such as those present within enzyme active sites, to catalyze protein nitration. Reaction of $ONOO^-$ with a redox-active metal ion polarizes the ONO−O bond, leading to homolytic cleavage and formation of the nitrating species, $^{\bullet}NO_2$ (Figure 1, scheme f). Alternatively, an electrophilic aromatic nitration mechanism has been proposed whereby $ONOO^-$ induces nitration without the generation of free radicals (*9*). The latter process, which is thought to occur in some, but not all, metal-catalyzed nitration reactions, proceeds via an ONOO−M^nX intermediate to yield a nitronium ion (NO_2^+). The NO_2^+ species may then directly attack protein Tyr residues (Figure 1, schemes g and h).

Under conditions of inflammatory stress, tissue leukocytes can activate heme peroxidases, such as myeloperoxidase (MPO) and eosinophil peroxidase (EPO), which may further contribute to oxidative damage. These enzymes catalyze the oxidation of nitrite (NO_2^-), a normal byproduct of $^{\bullet}NO$ metabolism, to $^{\bullet}NO_2$, which, in turn, may cause protein nitration (Figure 1, schemes i and j) (*10*).

Factors that Direct Site-Specific Nitration

Protein nitration occurs in vivo with biological selectivity and site specificity (Table 1) (reviewed in ref *11*). However, unlike site-directed kinases whose activity requires the recognition of a consensus sequence, a singular factor does not appear to govern the selectivity of most nitrating agents. Instead, a combination of factors may direct nitrating species toward their respective targets.

Perhaps most importantly, the local environment of Tyr residues within the secondary and tertiary protein structure assumes a pivotal role in directing nitrative events. For example, Tyr residues located within a carboxylic acid-rich environment (i.e., nearby Glu and Asp residues) are often targeted for nitration (*11*). A possible explanation for this selectivity is that the carboxyl group of Glu forms hydrogen bonds with the adjacent Tyr residue. These electrostatic forces could influence the local concentration of nitrating species near specific Tyr residues, thereby directing site-specific nitration. Alternatively, similar electrostatic interactions may stabilize highly reactive intermediates that are necessary for nitration.

Other factors that may regulate site-specific nitration include the absence of steric hindrances, the proximity to turn-inducing residues (i.e., Pro and Gly), and a paucity of nearby Cys and Met residues. Cys residues are capable of forming intra- and intermolecular disulfide bridges that can sterically hinder the accessibility of nitrating agents to Tyr.

Table 1: Examples of Site-Specific Nitration in Neurodegenerative Disease

Disease, Protein	Total no. of Tyr residues	Tyr Residues Nitrated[a]	Refs	Effects of Nitration[b]
ALS, Mn SOD	9	Y34 ≫ Y45 and Y193	*13*	Enzyme inactivation (Y34)
ALS, NF-L	20	Y17, Y138, Y177, and Y265	*46*	Inhibition of homologous and heterologous assembly in vitro
PD, α-synuclein	4	Y39, Y125, Y133, and Y136	*60*, *65*−*67*	Inhibits assembly in vitro (Y39, Y125, Y133, Y136); low concentrations of nitrated species facilitate assembly of nonmodified monomers; inhibits lipid binding and assumption of α-helical structure (Y39); inhibits proteolysis (Y39)
PD, TH	15	Y423 ≫ Y428 and Y432	*75*	Enzyme inactivation (Y423)
AD, τ	5	Y18 and Y29 ≫ Y197 and Y394	*83*−*85*, *93*	Inhibits assembly in vitro (Y18, Y394); alters assembly kinetics in vitro (Y18, Y29, Y197, Y394); alters filament morphology (Y18, Y29, Y197, Y394); induces formation of the Alz-50 epitope (Y197 ≫ Y18, Y29, Y394); inhibits microtubule binding and stability (Y18, Y29, Y197, Y394); induces deficits in filament nucleation and elongation (Y18); induces deficits in filament nucleation (Y29)

[a] The symbol ≫ denotes a hierarchical pattern of Tyr nitration. [b] Parentheses indicate the effects of nitration at a specific residue.

In addition, both Cys and Met serve as alternative targets for oxidative modifications. Several recent reports have shown that a hydrophobic environment may promote Tyr nitration by either concentrating $^{\bullet}NO$ within the hydrophobic protein core or extending the half-life of the $^{\bullet}NO_2$ moiety (*12*).

Proximity-facilitated selectivity may also contribute to the specific nature of Tyr nitration. In other words, if a nitrating agent is generated in the proximity of a Tyr residue, then nitration is more likely to occur at this position. A salient example of this process can be seen in the $ONOO^-$-mediated nitration of Mn superoxide dismutase. Under inflammatory conditions, $ONOO^-$ reacts with the redox-active metal center in Mn superoxide dismutase to generate reactive free radicals. The production of these species proximal to Tyr34 in the enzyme active site results in the exclusive nitration of this residue (*13*, *14*). It should also be noted that while the nature of the nitrating agent can direct both protein and site specificity, neither the intracellular protein concentration nor the molar fraction of Tyr residues within the protein influences nitration selectivity (*11*).

As mentioned above, multiple factors likely contribute to site-specific nitration in vivo. It is important to include, however, that only a select number of proteins are modified by nitrotyrosination. Moreover, site-specific nitration appears to correlate with changes in the function of both catalytic and structural proteins in neurological disease. In the following section, we will examine specific neurodegeneration-related proteins whose structure and/or function is altered by site-specific nitration.

Amyotrophic Lateral Sclerosis: The Role of Cu,Zn Superoxide Dismutase

Amyotrophic lateral sclerosis (ALS) is the most common adult motor neuron disease. The pathological hallmarks of ALS include the selective deterioration and death of neurons in both the upper and lower motor pathways. This process culminates in respiratory muscle paralysis, which typically leads to death within 5 years of onset. Microscopically, motor neuron loss is accompanied by intracellular accumulations of neurofilament (NF) protein within the cell body and proximal axonal swellings (*15*). Importantly, one-fifth of familial ALS cases are caused by dominant missense mutations in the antioxidant enzyme Cu,Zn superoxide dismutase (SOD1) (*16*). Despite the fact that nitration does not influence SOD1 activity, mutations in this enzyme lead to the SOD1-catalyzed nitration of other proteins. In addition, SOD1 shares many similarities with Mn superoxide dismutase (SOD2), which is nitrated and inactivated in disease states (see below). For these reasons, we will first examine the function of SOD1 under normal and pathological circumstances.

SOD1, a ubiquitously expressed homodimer, catalyzes the conversion of $^{\bullet}O_2^-$ anions into oxygen and hydrogen peroxide within the cell cytoplasm (*17*). During the two-step dismutation of $^{\bullet}O_2^-$, the Cu ion redox cycles between Cu(II) and Cu(I), and then back to Cu(II). Given the causal role of SOD1 mutations in ALS, it was suggested that SOD1 may serve a protective role by scavenging $^{\bullet}O_2^-$ before it reacts with $^{\bullet}NO$ to form $ONOO^-$ (*18*). However, ALS-associated SOD1 mutations do not appear to invoke a loss of protein function but instead may yield a toxic gain of function. In support of this notion, SOD1-deficient mice fail to manifest an ALS phenotype (*19*). In addition, SOD1 activity does not appear to correlate with ALS progression in humans and transgenic mouse models (*20*).

One hypothesis for how SOD1 mutations could elicit a toxic gain of function is that mutations alter the conformation of the enzyme active site, thereby allowing $ONOO^-$ greater access to the redox-active metal ions. This idea is supported by multiple lines of evidence. First, the majority of SOD1 mutations localize to the β-barrel structure of SOD1 at residues known to influence protein stability (*21*). Second, the Zn binding affinity of several ALS-associated SOD1 mutants is decreased up to 30-fold compared to that of wild-type SOD1 (*21*). The Zn ion assumes a key structural role in SOD1 by stabilizing two loop structures that comprise the active site (*22*). In addition, the Zn ion may confer stability to the reduced Cu(I) intermediate during the dismutation reaction. Third, Zn-deficient wild-type SOD1 catalyzes $ONOO^-$-mediated nitration with a 2-fold greater efficiency than Zn-replete SOD1 (*21*). Therefore, while wild-type SOD1 prohibits $ONOO^-$ from accessing the enzyme active site (*23*), it appears that Zn-deficient SOD1 allows $ONOO^-$ entry by relaxing the resides that comprise the active site channel (*24*).

Without a full complement of Zn ions, the SOD1 enzyme may operate in a "reverse" fashion to initiate a pathogenic

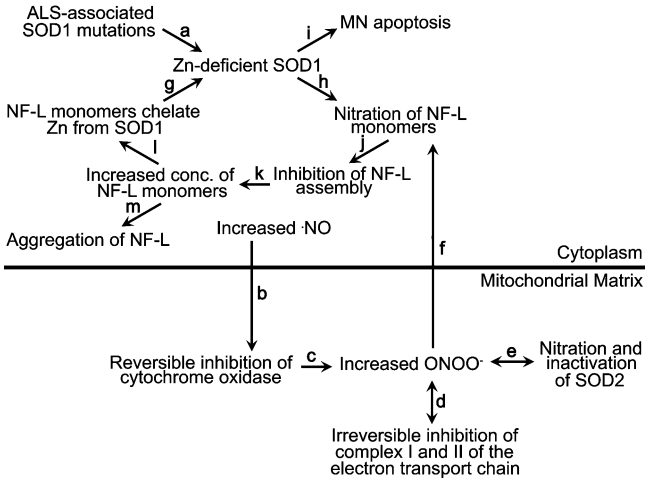

FIGURE 2: Hypothetical loss of function (SOD2) and gain of function (NF-L) cascades to explain the selective motor neuron (MN) vulnerability in ALS pathogenesis. Both ALS-associated SOD1 mutations and NF-L monomers promote the formation of Zn-deficient SOD1 (schemes a and g). Zn-deficient SOD1 catalyzes nitration much more efficiently than Zn-replete SOD1, resulting in site-directed nitration of NF-L monomers and motor neuron apoptosis (schemes h and i). Nitrated NF-L monomers are unable to polymerize into mature filaments (scheme j), leading to a greatly increased cytoplasmic concentration of NF-L monomers (scheme k). These nitrated NF-L proteins may assemble into insoluble aggregates via mass action (scheme m) or chelate additional Zn ions from native SOD1 proteins (scheme l). In addition, •NO, which is overproduced in ALS brains, may diffuse into the mitochondrial matrix (scheme b) and inhibit key enzymes involved in mitochondrial respiration. This process leads to high-output $ONOO^-$ production, irreversible inactivation of SOD2, and further intracellular oxidative injury (schemes c−f).

cascade (Figure 2, scheme a). While the reasons for this are not completely understood, it is thought that SOD1 mutations render the enzyme a more efficient oxidizing agent (*25*). As a result, the reduced Cu ion can be reoxidized by molecular oxygen to generate $^\bullet O_2^-$ at, or near, the enzyme active site (*26*). Given that $^\bullet O_2^-$ reacts with •NO at a 3-fold faster rate than with native SOD (*3*), nitric oxide can effectively outcompete SOD1 to generate $ONOO^-$. $ONOO^-$, in turn, may react catalytically with the active site Cu to form a nitronium-like intermediate (NO_2^+) which nitrates intra- and intermolecular Tyr residues (*27*). Because mutant SOD1 has a markedly reduced capacity to scavenge $^\bullet O_2^-$ (*28*), this process would necessarily increase steady-state $^\bullet O_2^-$ levels and, consequently, $ONOO^-$ concentration. It is noteworthy that SOD1 function is not altered by nitration of its single Tyr residue (Tyr108). Therefore, SOD1 may continually catalyze nitration, irrespective of its nitrative state. SOD1-catalyzed nitration is one of the fastest known reactions involving $ONOO^-$ (*29*). As such, this process may also lead to the site-directed nitration of the neurofilament light subunit (see below).

ALS: The Role of Mn Superoxide Dismutase

Mn superoxide dismutase (SOD2) is a metalloenzyme that scavenges $^\bullet O_2^-$ within the mitochondrial matrix. In humans, SOD2 consists of a homotetramer containing one Mn atom per subunit (*30*). Similar to SOD1, SOD2 catalyzes a one-electron redox reaction that results in $^\bullet O_2^-$ dismutation. Intriguingly, while SOD2 appears to be essential for survival, SOD1 does not. For example, SOD2-deficient mice survive to only 3 weeks of age and manifest numerous signs of oxidative toxicity (*31*). In contrast, the motor neurons of SOD1 null mice develop normally (*19*). Moreover, knockdown of SOD2 levels in transgenic mice expressing ALS-associated SOD1 mutations exacerbates disease progression (*32*). Taken together, these findings suggest that SOD2 assumes a critical role in mitigating nitrative and/or oxidative injury in ALS.

In a landmark paper by MacMillan-Crow and colleagues, SOD2 was identified as the first endogenous target of $ONOO^-$-mediated nitration (*33*). In this report, they also show that nitration induces the inactivation of SOD2 during chronic rejection of renal allografts (*33*). In fact, the concentration of $ONOO^-$ required to inactivate 50% of SOD2 was estimated to be 10 μM (*33*), a highly feasible concentration given that $^\bullet O_2^-$ and •NO are markedly upregulated during inflammatory conditions. Soon thereafter, another group showed that, following exposure to $ONOO^-$, only three of the nine total Tyr residues in SOD2 (Tyr34, Tyr45, and Tyr193) are modified (Table 1) (*13*). Most importantly, however, SOD2 inactivation correlates with the exclusive nitration of Tyr34 (*13*). While Tyr34 is less accessible to the bulk solvent than many other Tyr residues in SOD2, it lies in the proximity of the Mn active site (*34*).

A plausible explanation for the select nitration of Tyr34 is that the cationic residues lining the channel entrance guide $ONOO^-$ from the exterior of the protein into the active site by the same mechanism as $^\bullet O_2^-$ (*14*). $ONOO^-$ may then catalytically react with the Mn center to form reactive intermediates (i.e., either NO_2^+ or $^\bullet NO_2$ via heterolytic or homolytic cleavage, respectively) that nitrate the proximal Tyr34 residue. However, the current literature cannot rule out the possibility that, in the higher-energy trans conformation, $ONOO^-$ may directly access the Mn active site to spontaneously nitrate Tyr34.

A structural appreciation of the SOD2 active site may provide further clues about the functional basis of its inactivation. Substrate access to the redox-active Mn center is governed by a narrow, solvent-filled channel. The tightly packed side chains of four highly conserved "gateway" residues (i.e., Tyr34, His30, Trp169, and Glu170) shield the active site from the bulk solvent flow (*35*). As mentioned earlier, the basic amino acids lining the channel guide the substrate from the enzyme exterior to the active site by electrostatic forces. Tyr34, positioned at the vertex of the substrate channel, lies ~5 Å from the Mn atom in the enzyme active site (*36*). Therefore, under conditions of nitrative stress, the addition of a bulky nitro group onto Tyr34 would necessarily restrict access of the substrate to the enzyme active site and deter its physiological function. Furthermore, mutational analyses have shown that Tyr34 may participate in a hydrogen bonding chain that relays protons to enzymatic intermediates (*35*). Once the phenol ring is nitrated in the ortho position, however, its pK_a changes from 10 to 7.5. This event may also result in deprotonation of the hydroxyl group and disrupt the proton relay system necessary for redox cycling of the Mn atom.

Taken together, these studies suggest that site-specific nitration of SOD2 at Tyr34 may contribute to the oxidative toxicity manifest in ALS pathogenesis. Here, we propose one potential mechanism to explain how select nitration of SOD2 leads to the neuronal damage seen in ALS. The

inciting step may well occur at the level of •NO overproduction. For example, •NO, which is known to be upregulated in the brains of ALS patients (*37*), can diffuse into the mitochondrial matrix to reversibly inhibit cytochrome oxidase (Figure 2, scheme b). This event effectively interferes with mitochondrial respiration, thereby increasing $•O_2^-$ concentration and, consequently, $ONOO^-$ production (Figure 2, scheme c) (*38*). $ONOO^-$, in turn, irreversibly inhibits complexes I and II of the electron transport chain, leading to an amplification of $ONOO^-$ within the mitochondrial matrix (Figure 2, scheme d). While SOD2 may attempt to scavenge the excess $•O_2^-$, the reaction between $•O_2^-$ and •NO to form $ONOO^-$ is kinetically favorable (*3*). High levels of $ONOO^-$ may then lead to the self-catalyzed nitration of SOD2 at Tyr34, resulting in irreversible enzyme inactivation (Figure 2, scheme e). Given the primary protective role of SOD2 in neuronal mitochondria, the decreased concentration of SOD2 would lead to further oxidative toxicity. In addition, this feed-forward cycle of oxidative damage could precipitate the nitration of other disease-related proteins (i.e., neurofilament light subunit; Figure 2, scheme f) and, eventually, induce apoptotic cell death (*39*).

ALS: The Role of Neurofilament Light Subunit

Neurofilament (NF) aggregates within the cell soma and proximal axonal swellings are an early sign of motor neuron (MN) injury in ALS. NFs are the most abundant structural proteins within MNs, and polymerization of these proteins is critical for the integrity and diameter of MN axons. Structurally, each NF subunit contains a highly conserved rod and head domain. The rod domain is comprised of a rich network of α-helices that intertwine to form a superhelix of parallel coiled coils (*40*). The head domain is primarily responsible for maintaining lateral associations between the NF subunits (*41*). During NF assembly, mature filaments are formed by complex intersubunit associations.

While requisite for axonal function, NFs may also confer a selective vulnerability to MNs in ALS. The evidence supporting this notion is severalfold. First, in sporadic cases of ALS, selective deterioration occurs only in large-caliber MNs harboring high concentrations of NFs (*42*). Indeed, interference with NF assembly has previously been demonstrated to precipitate MN cell death in animal models (*43*). Second, mutations in highly conserved regions of NF subunits lead to a disease phenotype in sporadic ALS patients (*44*). This latter finding provides strong genetic evidence that NF dysfunction alone may be sufficient to cause ALS.

Several studies have demonstrated that 3-NT immunoreactivity colocalizes with NF-rich inclusions in both the upper and lower MNs of sporadic ALS cases (*45*). This observation strongly suggests that nitration occurs in deteriorating MNs. More importantly, NF light subunit (NF-L) is a select target of SOD1-catalyzed nitration in vitro, and nitrated NF-L monomers markedly inhibit the assembly of unmodified NF proteins (*46*). Using mass spectrometric techniques, Crow et al. (*46*) demonstrated that, of the 20 total Tyr residues in NF-L, Tyr17 in the head domain and Tyr138, Tyr177, and Tyr265 in the rod domain were specifically nitrated (Table 1). Notably, each of these Tyr residues lies within regions of the NF-L molecule known to be essential for NF assembly. For example, Tyr17, which resides in the head domain, is essential for the polymerization of NFs and other structurally related proteins (*47*). The other three Tyr residues targeted for nitration in NF-L are sequestered within the coiled coil structure of the rod domain. These Tyr residues are likely involved in intermolecular hydrophobic interactions that confer stability upon the NF polymer (*46*). Therefore, the addition of a nitro group onto the phenolic ring may alter both the hydrophobicity and three-dimensional conformation of the NF-L subunit. This mechanism could explain the disruption of NF polymerization following nitration. Together, these data reveal that site-specific nitration in the rod and head domains of NF-L may promote MN dysfunction in ALS.

Previously, it was shown that the high Zn binding capacity of NF-L can selectively remove Zn ions from both wild-type and mutant SOD1 (Figure 2, scheme g) (*21*). Therefore, given the high concentration of NFs within MNs, it is entirely possible that NF-L monomers can extract Zn ions from the enzyme active site. For reasons discussed above, Zn-deficient SOD1 functions in "reverse" to promote $ONOO^-$ formation and, thus, protein Tyr nitration. This event may induce both the site-specific nitration of NF-L monomers (scheme h) and MN apoptosis (scheme i) (*25*). The former process would inhibit native NF-L subunit assembly (scheme j), resulting in a markedly increased concentration of cytosolic NF-L monomers (scheme k). The NF-L monomers may further chelate Zn ions from SOD1 (scheme l), thereby perpetuating the cycle, or assemble into aggregates via mass action (scheme m). Furthermore, the increased levels of $ONOO^-$ may traverse the mitochondrial membrane to inactivate SOD2 (scheme e), which would further contribute to mitochondrial damage. This process may represent the toxic gain of function that is responsible for selective MN vulnerability in ALS.

Parkinson's Disease: The Role of α-Synuclein

Parkinson's disease (PD) is the second most common age-related neurodegenerative disorder. Clinically, PD is characterized by a constellation of signs and symptoms, including resting tremor, bradykinesia, muscular rigidity, and postural instability. These symptoms are attributed to the selective destruction of dopaminergic neurons within the nigrostriatal system. The pathological signatures of PD include Lewy bodies and Lewy neurites, which consist of intraneuronal aggregates of the α-synuclein protein.

α-Synuclein is a small, heat-stable protein that exhibits a natively unfolded, random coil structure in solution. Given its location within presynaptic nerve terminals, α-synuclein may regulate the trafficking of lipid secretory vesicles (*48*). In addition, α-synuclein has been shown to prevent the aggregation of other proteins in a chaperone-like fashion (*49*). Within the α-synuclein protein, four Tyr residues are located at positions 39, 125, 133, and 136 (Table 1). Three of these Tyr residues are located in the Asp- and Glu-rich carboxy terminus. This polar, highly flexible region has been proposed to facilitate protein−protein interactions (*50*). On the other hand, Tyr39 lies within the amino terminus of α-synuclein. This domain exhibits a tendency to form amphipathic α-helices that can associate with lipid membranes (*51*).

Several lines of evidence suggest that $ONOO^-$-mediated nitration may play a mechanistic role in PD pathogenesis

(*52*, *53*). Much of this evidence comes from studies using the Parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as a model of dopaminergic cell death. For example, mice with genetically induced increases in either the cytoplasmic or mitochondrial forms of SOD are resistant to MPTP-mediated neurotoxicity (*54*, *55*). In addition, transgenic mice with a targeted deletion in the SOD2 gene exhibit a marked sensitivity to MPTP toxicity (*56*). Not surprisingly, the neurotoxic effects of MPTP are abrogated by inhibition of neuronal nitric oxide synthase (nNOS) (*57*) and exacerbated by overexpression of the high-output glial NOS (inducible NOS, or iNOS) and nNOS (*58*). Taken together, these data provide strong genetic evidence that production of $ONOO^-$ within dopaminergic neurons may cause the selective cell death observed in PD.

In a cell culture model, $ONOO^-$ treatment of HEK293 cells stably transfected with α-synuclein results in the select nitration of α-synuclein (*59*). In addition, select nitration of α-synuclein occurs in the striatum and ventral midbrain following treatment of mice with MPTP (*59*). Despite this protein selectivity, however, nitration of α-synuclein does not exhibit site specificity in vitro (*60*) or in vivo (*52*). Presumably, this lack of site selectivity is due to the intrinsically unstructured nature of α-synuclein that allows nitrating species equal access to each Tyr residue.

Immunohistochemical studies have demonstrated that 3-NT cosegregates with α-synuclein lesions in diverse synucleinopathies (*53*). These reports, however, were conducted using a polyclonal antibody that recognizes 3-NT in a manner independent of protein context. As a result, the precise protein targets of nitration were not delineated. More recently, using monoclonal antibodies (mAbs) that recognize nitrated α-synuclein, another group showed that α-synuclein nitration occurs on all four Tyr residues in the hallmark lesions of PD, dementia with Lewy bodies, the Lewy body variant of Alzheimer's disease, and multiple-system atrophy (*52*). These antibodies also label the insoluble, but not the soluble, fraction of diseased synucleinopathy brains, suggesting that Tyr nitration may reduce the solubility of α-synuclein (*52*).

Under physiological conditions, α-synuclein exists in equilibrium between membrane-associated and soluble forms (Figure 3, scheme a). Soluble α-synuclein adopts a natively unfolded structure (*61*). The binding of protein chaperones to α-synuclein may further enhance its unstructured state. Once engaged at the lipid membrane, α-synuclein exhibits an α-helical structure that is less prone to filament assembly (*62*) and nitrative modification (scheme b) (*63*). In disease states, however, α-synuclein assumes a *β*-pleated sheet conformation and readily assembles into filaments (*64*). Therefore, nitrative modifications may influence the biochemical properties of α-synuclein in several important ways. First, nitration induces the formation of α-synuclein oligomers with an increased level of *β*-pleated sheet structure (scheme c) (*65*). When present at low concentrations, these nitrated proteins facilitate the rate-limiting nucleation step of α-synuclein assembly (scheme f) (*66*). At high concentrations, however, these species inhibit filament assembly (scheme d) (*67*), presumably by increasing the critical concentration. Second, nitration of Tyr39 has been shown to displace α-synuclein from lipid vesicles, thereby preventing the assumption of an α-helical conformation (scheme g) (*66*). Third, nitrated α-synuclein may also exhibit a prolonged half-life due to a reduced capacity for proteosomal degradation (scheme e) (*66*). Collectively, these events would increase the size of the intracellular pool of free α-synuclein and promote aggregate formation. It should also be noted that nitrated α-synuclein monomers, which resemble protofibrils, may exert a toxic gain of function by forming neurotoxic pores in the plasma membrane (*68*).

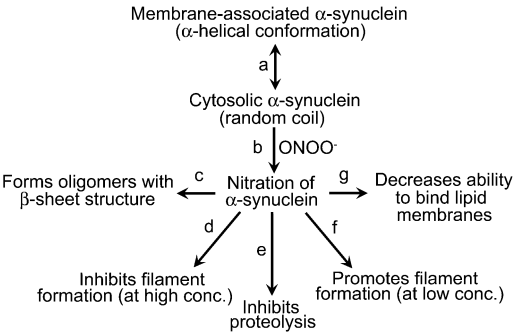

FIGURE 3: Hypothetical gain of function mechanism for explaining how nitration may influence the pathological self-assembly of α-synuclein. Under normal circumstances, α-synuclein exists in equilibrium between membrane-associated (α-helical conformation) and cytosolic forms (random coil conformation) (scheme a). Nitration of natively unfolded α-synuclein (scheme b) increases its tendency to form oligomers with *β*-sheet structure (scheme c), decreases its ability to associate with phospholipid membranes (scheme g), and reduces its capacity for proteolytic degradation (scheme e). At high concentrations, nitrated α-synuclein proteins inhibit filament assembly (scheme d). At substoichiometric levels, however, nitrated α-synuclein species facilitate polymerization (scheme f).

PD: The Role of Tyrosine Hydroxylase

Tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, catalyzes the conversion of Tyr to L-dihydroxyphenylalanine (L-DOPA). Following its synthesis in the cytosol, L-DOPA is converted to dopamine (DA) and sequestered within presynaptic secretory vesicles. Deficits in catecholamine synthesis are thought to play a critical role in PD pathogenesis. While the early decline in DA levels in PD was originally attributed to the loss of dopaminergic cells, it was later shown that the decrement in DA levels greatly exceeded neuronal death (*69*). Moreover, in idiopathic PD patients, TH activity is lost early in the disease process with a concomitant DA deficiency (*70*). Together, these data strongly suggest that inhibition of TH activity occurs prior to dopaminergic cell death.

A large body of evidence suggests that nitration occurs in the brains of sporadic PD patients (*52*, *53*). Intriguingly, administration of MPTP to mice results in the $ONOO^-$-mediated nitration and inactivation of TH (*71*). While the precise mechanism of MPTP-mediated toxicity has not been elucidated, MPTP can selectively accumulate within dopaminergic neurons where it is converted to its active metabolite, MPP^+ (*72*). Redox cycling of MPP^+ with intracellular enzymes may then form $ONOO^-$, which is capable of nitrating protein Tyr residues (*73*). Importantly, in the striatum of MPTP-treated mice, the early loss of TH activity correlates temporally with the decrease in DA levels, whereas the levels of TH protein remain constant (*71*). Moreover, mice overexpressing SOD1 were rescued from MPTP-induced neurotoxicity (*71*). Collectively, these findings

provide the first direct evidence that nitration may induce TH dysfunction in the early stages of PD.

To understand the functional basis of $ONOO^-$-mediated TH inactivation, a structural appreciation of the TH active site is required. The TH active site consists of a narrow cleft through which substrates must traverse. At the bottom of this cleft, three residues (His331, His336, and Glu376) chelate the redox-active ferrous ion required for catalysis (*74*). Additionally, two loop structures shield the active site entrance from the bulk solvent flow. Tyr423 and Tyr428 comprise the first and last residues of the initial loop (*74*). While the aromatic side chain of Tyr423 projects into the active site channel, the aromatic rings of Tyr428 and Tyr432 extend away from the channel interior. Accordingly, nitrative modification of Tyr423 would constrict the width of the active site channel from 15 to ~12.5 Å (*75*), thereby inhibiting access of the substrate to the enzyme active site.

As might be predicted from these structural analyses, $ONOO^-$-mediated TH nitration in vitro occurs with site specificity toward Tyr423 and, to a lesser extent, Tyr428 and Tyr432 (Table 1) (*75*). Mutational analyses reveal that Tyr423 is critical for TH function and that nitration of Tyr423 is both necessary and sufficient for TH inactivation (*75*). Moreover, $ONOO^-$ reacts with TH in vitro with a second-order rate constant (*75*). This latter finding is consistent with a transition metal-catalyzed reaction. Due to the fact that residues Tyr423, Tyr428, and Tyr432 lie in the proximity of the ferrous ion in the enzyme active site, transition metal catalysis most likely accounts for the site-specific nitration of TH.

Alzheimer's Disease: The Role of τ

Alzheimer's disease (AD) is a progressive amnestic dementia typified by the pathological misfolding and deposition of the microtubule-associated τ protein. Clinically, AD manifests as an insidious deterioration of cognitive, language, behavioral, and executive functions. Pathologically, the postmortem signatures of AD brain include the neurofibrillary tangle (NFT), neuritic plaques, and neuropil threads. The NFT, whose spatiotemporal distribution closely parallels cognitive decline in patients with AD, is largely comprised of the τ protein assembled into paired helical and straight filaments (*76*).

Similar to α-synuclein, τ is a natively unfolded protein in solution dominated by random coil structure. Abnormal modifications of τ, including phosphorylation, proteolysis, and conformational changes, are thought to play a critical role in the series of events leading to NFT formation. Previously, we showed that τ undergoes a dynamic series of conformational changes during tangle evolution (*77*). For example, in early-stage tangles, τ assumes a conformation whereby the amino terminus comes into close apposition with the third microtubule binding domain. This event is concomitant with early filamentous changes in τ and is detected by the Alz-50 antibody (*78*).

Five Tyr residues (Tyr18, Tyr29, Tyr197, Tyr310, and Tyr394) span the length of the τ protein. Tyr18 and Tyr29 reside within the amino terminus of τ, a region lacking significant secondary structure. Notably, removal of the amino terminus inhibits τ polymerization in vitro (*79*). This latter finding supports a facilitative role for the amino terminus in τ assembly, likely by its contribution to the Alz-50 conformation. On the other hand, Tyr197 is located within the Gly- and Pro-rich interior of τ that is thought to impart flexibility to the τ molecule (*80*). Tyr310 lies within a critical interaction motif (^{306}VQIVYK^{311}) in the third microtubule binding repeat that is essential for τ assembly in vitro (*81*). Finally, the carboxy terminus of τ harbors residue Tyr394. This region protects against τ filament formation by interacting with the microtubule binding domain to prevent formation of the Alz-50 epitope (*82*).

In recent years, our laboratory has made significant progress toward delineating the structural and functional consequences of τ nitration in vitro. Specifically, we have shown that $ONOO^-$ preferentially nitrates Tyr residues at the amino terminus (Tyr18 and Ty29), and, to a lesser extent, in the proline-rich (Tyr197) and carboxy-terminal (Tyr394) regions (Table 1) (*83*, *84*). This hierarchical pattern of nitration is also observed in preformed τ filaments (*85*) which are conformationally distinct from τ monomers (*78*). Select nitration of the amino terminus may be due to its lack of secondary structure, its high degree of solvent exposure, its carboxylic acid-rich nature, and/or its proximity to turn-inducing residues (*83*).

Previously, we demonstrated that arachidonic acid (AA)-induced in vitro assembly accurately models τ polymerization under near-physiological conditions (*86*). Using this model system, we showed that the overall effect of τ nitration is to inhibit assembly in vitro (*83*). This inhibitory effect appears to be specific for the 3-NT modification, as pseudophosphorylation at residues Tyr18, Tyr29, Tyr197, and Tyr394 does not influence τ assembly (*83*). The mechanism underlying this inhibition may be explored through a careful analysis of τ assembly in vitro. AA-induced τ polymerization proceeds through a ligand-mediated mechanism whereby filament nucleation is followed by elongation of the nascent polymer (*87*). The initial step in this process involves τ binding to the anionic surface of AA micelles (*88*). Therefore, it is entirely possible that the amino terminus, a region known to interact with numerous cellular constituents, facilitates the binding of τ to AA. Nitration of residues Tyr18 and Tyr29 may increase the net negative charge at the amino terminus and repel τ from the anionic surface of AA, thereby preventing τ assembly. In fact, given that τ filaments have been shown to arise from, or terminate within, plasma membranes (*89*), a similar mechanism may also hold true in vivo.

To delineate the effects of τ nitration at *individual* Tyr residues, we also generated τ mutants singly nitrated at Tyr18, Tyr29, Tyr197, and Tyr394. Using these reagents, we showed that site-specific Tyr nitration dramatically alters the rate and/or extent of τ polymerization in vitro (*84*). Select nitration of Tyr29 and Tyr197 increases the average length of τ filaments without affecting the steady-state polymer mass. In contrast, nitration at residues Tyr18 and Tyr394 decreases the average length and/or number of synthetic τ filaments. This translates into a marked reduction in overall filamentous mass and an increased critical concentration. Interestingly, the degree of nitration (Tyr29 and Tyr18 $\gg$ Tyr394 and Tyr197) does not correlate with the effect of each nitrated mutant on τ polymerization. Thus, it is the location, not the extent, of nitration that influences τ function (*84*). However, it should be noted that nitration of wild-type τ inhibits assembly to a greater extent than any singly nitrated

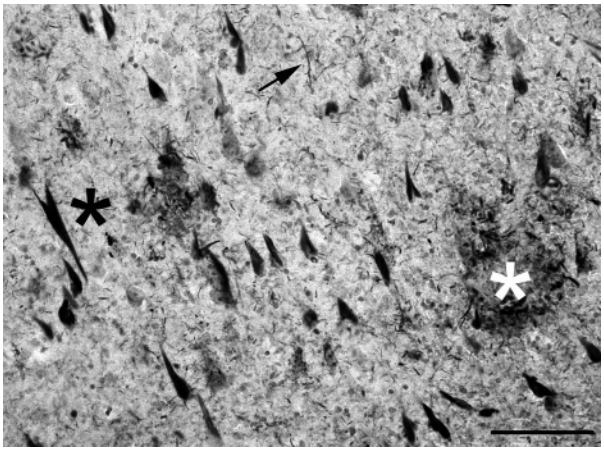

FIGURE 4: Tau-nY29, a novel, nitro-τ-specific antibody, labels the triad of fibrillar τ pathologies in Alzheimer's brain. A pathologically advanced case of AD hippocampus (CA1 region) was stained with the Tau-nY29 antibody at a concentration of 1 μg/mL (*93*). As shown, Tau-nY29 labels neurofibrillary tangles (black asterisk), neuritic plaques (white asterisk), and neuropil threads (arrow). The calibration bar represents 50 μm. Immunohistochemical staining courtesy of J. F. Reyes (Northwestern University).

τ mutant (*83*). This latter finding suggests that nitration of multiple Tyr residues may have a combinatorial effect on assembly inhibition.

In copolymerization assays, we demonstrated that site-specific nitration inhibits the ability of τ to promote tubulin assembly (*85*). In addition, affinity measurements reveal that, even in the face of decreased polymer mass, site-specific τ nitration promotes the pathological Alz-50 conformation (*85*). These findings, combined with evidence that Alz-50-positive τ filaments cannot stabilize microtubules (*90*), suggest that the Alz-50 epitope may be conformationally incompatible with microtubule stabilization. Therefore, even though nitration inhibits τ polymerization in vitro, it may also promote a toxic gain of function by inducing the Alz-50 epitope.

Immunohistochemical studies show that 3-NT cosegregates with NFTs in post-mortem AD brain (*91*). In fact, an immunological probe raised against nitrated α-synuclein that also recognizes nitrated τ decorates NFTs and other τ inclusions in AD-affected brains (*92*). Intriguingly, this antibody labels pretangles in early-stage AD pathology (Braak stage I−II) but stains far fewer fibrillar τ inclusions in advanced AD cases (Braak stage V−VI) (*92*). This latter finding implies that τ nitration may be an early-stage event during tangle evolution. In support of these data, we have recently raised a mAb that specifically detects τ when nitrated at residue Tyr29 (*93*). This reagent, termed Tau-nY29, robustly stains the fibrillar τ lesions in AD brain (Figure 4) and differentially labels the neuronal and glial τ pathology of diverse non-AD tauopathies (*93*). In double-label immunofluorescence studies, Tau-nY29 colocalizes to a remarkable degree with the early-stage marker of τ pathology, Alz-50 (*93*). Moreover, while Tau-nY29 detects paired helical filaments and soluble τ purified from severely affected AD brains (Braak stage V−VI), it does not label soluble τ from mildly affected AD brains (Braak stage III) (*93*). This observation supports the notion that τ nitration is a disease-specific event that occurs on τ monomers prior to filament formation.

Taken together, these data suggest potential pathogenic pathways of τ nitration (Figure 5). Under normal circumstances, the intracellular concentration of τ is approximately 2−4 μM (*94*), >99% of which is bound to the microtubule lattice (Figure 5, scheme a) (*87*). Aberrant post-translational modifications, including hyperphosphorylation, can displace τ from the microtubule, thereby increasing the concentration of free τ available for aggregation (*95*). On the basis of the evidence presented above, nitration of the τ protein (scheme b) would likely inhibit filament formation by increasing the critical concentration (scheme e), prevent τ−microtubule interactions (scheme c), and promote the pathological Alz-50 conformation (scheme d). While these events may appear to exert disparate effects on τ polymerization in vitro, it is possible that their cumulative effect in vivo would promote τ self-association and NFT formation (see Discussion).

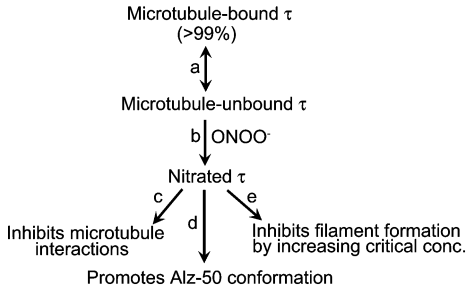

FIGURE 5: Hypothetical gain of function cascade to explain how nitration influences the pathological misfolding and deposition of the τ protein. Under physiological circumstances, τ exists in equilibrium between microtubule-bound (>99%) and cytosolic forms (scheme a). Nitration of the τ protein (scheme b) leads to microtubule instability (scheme c), assumption of the pathological Alz-50 conformation (scheme d), and inhibition of filament assembly (scheme e).

Discussion

In this review and synthesis, we illustrate the biologically relevant pathways of protein nitration, the factors that govern site-specific nitration, and the select proteins affected by nitration in neurodegenerative disease. Current findings reveal that site-specific nitration can profoundly alter the function of both catalytic and structural proteins through distinct mechanisms. For catalytic proteins, site-directed nitration of Tyr residues within the enzyme active site appears to directly interfere with enzyme−substrate docking. For structural proteins, nitration may alter the three-dimensional conformation of individual subunits, thereby modifying intersubunit associations.

For example, in the catalytic proteins SOD2 and TH, enzymatic activity is markedly inhibited by nitration of Tyr residues within, or near, the substrate access channel. The select nitration of Tyr residues proximal to the enzyme active site most likely occurs via a metal-catalyzed reaction. Presumably, for both SOD2 and TH, Tyr nitration sterically hinders access of the substrate to the active site, thereby inhibiting enzyme function. In general, to induce a loss of protein function, a large proportion of critical Tyr residues must be modified (*2*). Given the favorable kinetics of metal-catalyzed nitration (*29*), however, a large fraction of nitrated Tyr residues may well be possible.

In contrast, α-synuclein, NF-L, and τ are structural proteins that participate in homologous and/or heterologous polymerization. As previously characterized, the mechanism of α-synuclein and τ assembly involves a nucleation/elongation reaction (*67*, *87*). While the precise mechanism of NF-L

polymerization has not been established, this protein may also exhibit nucleation-dependent assembly. On the basis of the Oosawa model of nucleated polymerization (*96*), nucleation occurs by the sequential addition of monomeric units, each forming single contacts with one another. Polymer elongation, on the other hand, not only involves the apposition of adjacent monomers but also requires complex intermonomer associations. Nitration of structural proteins may induce conformational changes that interfere with the intra- and intersubunit associations necessary for polymer extension. In support of this notion, NF-L (*46*), α-synuclein (*65*), and τ (*84*) all form nucleated structures with a reduced capacity for elongation following treatment with $ONOO^-$. These studies suggest that nitrated monomers can assemble into oligomeric nucleation centers but are unable to interact with (or polymerize from) the nucleation core.

It is important to note that $ONOO^-$-mediated nitration occurs with high efficacy in vitro (*85*). As such, very few nonmodified monomers would be available for polymerization in these studies. On the other hand, the efficacy of protein nitration is much lower in vivo (*97*), and a large number of nonmodified subunits would be available for promotion of polymer extension from a nitrated, nucleation-competent nidus. This latter phenomenon may explain why nitrated α-synuclein monomers inhibit polymerization at high concentrations (*65*, *67*) but facilitate assembly at low concentrations (*66*). In vivo, nitration of neurologically relevant structural proteins may seed the assembly of insoluble protein aggregates, thereby contributing to the neurodegenerative cascade. This mechanism fits the criteria for a toxic gain of function, whereby a small fraction of modified proteins (i.e., nitrated monomers) produce a signal that is amplified into a biologically significant response (i.e., induction of nucleated polymerization) (*2*).

To validate Tyr nitration as a pathologically relevant modification, nitration should occur in the initial stages of disease pathology. While timing these events in post-mortem tissues is often difficult, highly specific immunological and proteomic detection methods have revealed that nitration occurs in the early stages of disease. For example, site-specific nitration of Tyr423 correlates temporally with the early loss of TH activity in patients with idiopathic PD (*70*). Further, in double-label immunofluorescence studies, novel mAbs that specifically recognize nitrated τ colocalize with markers of early-stage τ pathology (*93*). These data suggest that nitration is a disease-specific, early-stage event that contributes to the onset and progression of several neurological diseases. However, we cannot exclude the possibility that Tyr nitration is a temporally regulated event that occurs in multiple stages of the disease process.

In addition to its effects on protein function, Tyr nitration may also interfere with intracellular signaling cascades. Tyr phosphorylation has long been recognized as a transducer of cellular signaling. While Tyr phosphorylation is tightly regulated by Tyr kinases and phosphatases, enzymes that selectively remove nitro groups have not yet been identified (*11*). Given that nitration and phosphorylation are mutually exclusive events at a single Tyr residue, nitration may permanently interfere with phospho-Tyr signaling (*98*). For example, Tyr18 of τ can be phosphorylated by the src family Tyr kinase, fyn, whose expression is upregulated in a subset of AD neurons (*99*). Moreover, phosphorylation of Tyr18 occurs on τ filaments purified from AD-affected brains (*99*). While nitration of Tyr18 inhibits τ filament formation in vitro (*84*), phosphorylation at this residue is permissive toward τ polymerization (*83*). Therefore, it is feasible that nitration and phosphorylation compete for the modification of critical Tyr residues, thereby influencing protein function.

Another possible explanation for the select nitration of disease-related proteins is that 3-NT induces an immunogenic response that helps to remove insoluble aggregates. In fact, one report has shown that nitrated peptides can elicit an immune response in transgenic mice otherwise incapable of mounting an immune reaction (*100*). Therefore, nitrative modifications may represent a mechanism for removing dysfunctional proteins via immune-mediated or proteosomal pathways.

In conclusion, site-specific nitration may serve as a pathogenic mechanism for inactivating catalytic proteins and promoting the aberrant assembly of structural proteins in neurodegenerative disease. Moreover, a growing body of evidence suggests that nitration is an early-stage event that may play a mechanistic role in the onset and progression of several neurodegenerative diseases. While this work affords mechanistic insights into the structural and functional implications of protein nitration, further studies must be performed in vivo to precisely delineate the effects of nitration in human disease.

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