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Protein tyrosine nitration in cellular signal transduction pathways

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Abstract

How specificity and reversibility in tyrosine nitration are defined biologically in cellular systems is poorly understood. As more investigations identify proteins involved in cell regulatory pathways in which only a small fraction of that protein pool is modified by nitration to affect cell function, the mechanisms of biological specificity and reversal should come into focus. In this review experimental evidence has been summarized to suggest that tyrosine nitration is a highly selective modification and under certain physiological conditions fulfills the criteria of a physiologically relevant signal. It can be specific, reversible, occurs on a physiological time scale, and, depending on a target, can result in either activation or inhibition.

Keywords

Nitric oxide; tyrosine nitration; peroxynitrite; superoxide; uncoupling

Introduction

Reactive nitrogen species (RNS) as signaling molecules have been studied in terms of four primary mechanisms: (1) binding to metal centers; (2) nitrosylation of thiol and amine groups; (3) nitration of Tyr; and (4) oxidation of thiols (both Cys and Met). The most extensively studied is NO• binding to the heme group of soluble guanylate cyclase, stimulating cyclic guanosine monophosphate production and activation of protein kinase G. NO• also binds to the heme group of cytochrome c oxidase with consequential inhibition of cytochrome c oxidase activity. This is one proposed mechanism of mitochondrial electron transport regulation that minimizes mitochondrial reactive oxygen species (ROS) generation (1). More recent studies on RNS-dependent signal transduction mechanisms have focused on protein modifications. Two protein modifications, in particular, nitrosylation of thiols to produce S-nitrosothiol and nitration of Tyr residues to produce nitroTyr, are being intensely investigated in this context.

S-nitrosylation has been shown in numerous investigations to fulfill the criteria of a physiologically relevant signal in that it can be specific, reversible, occurs on physiological

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time scale, and depending on a target, can result in either activation or inhibition (2,3). Stamler and associates propose that S-nitrosylation of protein Cys residues is the prototypic redox-based post-translation mechanism comparable to protein phosphorylation (4). Transcription factors, caspases, receptor tyrosine kinases and protein tyrosine phosphatases as well as a number of other proteins are reversibly S-nitrosylated after the treatment of cells with NO• donors or activation of signal transduction pathways (5–8). Caspases when S-nitrosylated at their active site Cys are inhibited (6,7,9,10). S-nitrosylation of proteins such as protein tyro-sine phosphatases and their inhibition has been shown to intersect with other signal transduction pathways, e.g. receptor tyrosine kinases (5,11).

The biological signficance of Tyr nitration, on the other hand, remains ill-defined. However, accumulating data show a strong link between protein Tyr nitration and the mechanisms involved in formation of many pathological conditions and diseases: post-radiation response, acute and chronic inflammation, acute and chronic rejection of the allograft, chronic hypoxia, tumor vascularization and microenvironment, atherosclerosis, myocardial infarction, chronic obstructive pulmonary disease, diabetes, Parkinson's disease, Alzheimer's disease, and others (12–29). For this reason researchers are looking at new pharmacological strategies of inhibiting protein Tyr nitration as an additional type of treatment for many pathological conditions (30–39). As discussed below there is also accumulating experimental evidence that protein Tyr nitration can under certain conditions have the specificity necessary for normal physiological cellular signaling pathways. This review will focus its attention on this latter argument that specific protein Tyr nitration fulfills the criteria of a signal transducing mechanism.

In vitro analysis of protein Tyr nitration

In most cases the amount of Tyr nitration of an individual protein obtained under endogenous conditions is very low. Thus to examine sites of nitration, investigators use RNS donors and purified proteins to maximize yield of nitration. Interpretation of results from these *in vitro* experiments requires consideration of the type and concentration of RNS donor used and ultimately *in vivo* validation under appropriate environmental conditions.

Selectivity in which Tyrs are nitrated can depend on the type of RNS (or RNS donors). For example, glutamate dehydrogenase is nitrated by both Hemin–H_2O_2–NO_2^- and the peroxynitrite ($OONO^-$) donor, SIN-1 (40). Mass spectrometric analyses of the nitrated enzyme demonstrated completely different Tyr nitration patterns in the same protein: Tyr262 and Tyr471 after nitration by Hemin–H_2O_2–NO_2^- and Tyr401 and Tyr493 after nitration by SIN-1. Another example is seen in comparing $ONOO^-$ and tetranitromethane (TNM)-dependent Tyr nitration of cytochrome c (41). The main reaction products of cyto-chrome c obtained with $ONOO^-$ are two mono-nitrated protein molecules, one with nitroTyr97 and the other with nitroTyr74. Based on abundance, Tyr97 is preferentially nitrated by $ONOO^-$. TNM treatment of cyto-chrome c, on the other hand, generates three products: two mono-nitrated cytochrome c species (nitroTyr74 or nitroTyr67) and a single di-nitrated cytochrome c with both Tyr67 and Tyr74 nitrated. Other investigators have reported TNM-dependent nitration of cytochrome c at Tyr67 alone (42). Similar divergent results are found for glycogen phosphorylase b in comparing Tyr nitration by $ONOO^-$ and

TNM. Low doses of $ONOO^-$ (<50μM) nitrate glycogen phosphorylase b on Tyr613 with five more Tyrs (Tyr83, Tyr84, Tyr90, Tyr74, and Tyr75) nitrated at higher $ONOO^-$ concentrations (>75 μM) (43). In contrast, TNM nitrates glycogen phosphorylase b on Tyr75, Tyr203, Tyr262, Tyr280, Tyr403, Tyr552, and Tyr647 (44).

Lennon and co-investigators proposed one explanation for the differential nitration obtained with $ONOO^-$ and TNM based on their studies with prion protein isoforms (45). TNM, unlike $ONOO^-$, is soluble in organic solvents, and thus in principle more readily reacts with buried Tyr residues than $ONOO^-$. This may be true for the prion protein, however this does not account for the results comparing the nitration patterns of other proteins with these RNS donors (41,42,44).

The studies cited above not only reveal differences in nitration patterns depending on RNS donor but also on the concentration of donor. Other examples include mammalian aldolase A and the tumor suppressor protein p53. Peroxynitrite in the concentration range of 100–200 μM nitrates mammalian aldolase A at Tyr243, Tyr342, and Tyr363. Increasing the $ONOO^-$ concentration to 500 μM results in nitration of one additional Tyr residue, Tyr222 (46). Treatment of cells with the NO•-donor DETA NONOate at concentrations of 20–50 μM that mimic a chronically low inflammatory state nitrates p53 protein on Tyr327. However, at higher DETA NONOate concentrations (≥200 μM) additional Tyr(s) of p53 become nitrated (47). As will be discussed below, not only is donor concentration important in selectivity but also the exposure time of the substrate with donor (28).

In vivo validation is ultimately required to determine the physiological significance of the nitrative event. Some proteins are equivalently nitrated *in vitro* and *in vivo* although this is often not the case especially at extremes of RNS concentration and time. For example, Tyr nitration analysis of human nitric oxide synthase-2 (NOS2) *in vitro* (by $ONOO^-$) and in muscle samples from a subset of three patients with sepsis showed identical nitrated Tyrs (48). Mass spectrometry demonstrated nitration of only 4 Tyrs among the 31 Tyrs residues present in NOS2. Nitrated Tyr was located at positions 299 and 336 in the three patients examined. In addition, Tyr446 was nitrated in one patient, whereas Tyr698 was nitrated in another patient. Those 4 Tyrs (located at positions 299, 336, 446, and 698) were nitrated when NOS2 was incubated with $ONOO^-$ *in vitro*.

In previous work, we (49) showed that IκBα was equally nitrated on Tyr181 and Tyr305 *in vivo* (by ionizing radiation-dependent activation of constitutive NOS activity in intact cells) as well as *in vitro* with lysates from cells overexpressing IκB (by bolus $ONOO^-$). However, in a study on p53 nitration following treatment of cells with ionizing radiation or with low doses of an NO• donor mimicking the inflammatory microenvironment a clear difference was observed between these treatments and *in vitro* nitration with $ONOO^-$ or *in vivo* with excessively high doses of a NO• donor (47). p53 was selectively nitrated on Tyr327 in the tetramerization domain following the radiation exposure or at low NO• donor concentrations but treatment with $ONOO^-$ *in vitro* was less selective with at least two additional Tyrs nitrated including one in the DNA binding domain. Experiments using NO• donors at different concentrations or radiation and with cells expressing p53 molecules differing in

Tyr mutant status demonstrated that selective nitration of p53 had significant consequences in terms of gene expression profiles.

Aslan et al. (2003) compared Tyr nitration of actin in liver and kidney homogenates from sickle cell disease mouse with *in vitro* nitration by $ONOO^-$ (50). Three of 15 actin tyrosine residues were nitrated (Tyr91, Tyr198, and Tyr240) *in vivo*. *In vitro* treatment with $ONOO^-$ modified four residues (Tyr53, Tyr198, Tyr240, and Tyr362) with nitration of Tyr362 not consistently observed in some experiments.

In vivo, the skeletal muscle creatine kinase of untreated 34-month old rats is nitrated at both Tyr14 and Tyr20 but of none of the other seven Tyr residues at positions 39, 82, 125, 140, 173, 174, and 279 (51). In contrast, *in vitro* exposure of creatine kinase to $ONOO^-$ nitrates exclusively Tyr82. These investigators suggested that one or more of the following factors likely cause these distinctly different patterns. One mechanism may result from proteosome degradation of specific nitrated isoforms of creatine kinase. Nitrated proteins can be preferential targets of proteosome proteolysis (52). A nitrating agent may generate nitroTyr predominantly at one specific location in the protein and negligible yields of nitroTyr at other positions. Nevertheless, protein isoforms containing the nitroTyr at these other positions may accumulate with age if they are resistant to degradation, and at the same time, the protein isoforms containing nitroTyr at the predominant target Tyr residue are susceptible to degradation. Alternatively, $ONOO^-$ might not be the (only) nitrating agent *in vivo*. For example, Zeng et al. (2005) investigated myeloperoxidase (MPO)-mediated apoA-I nitration and chlorination *in vitro* and *in vivo* and attempted to relate the degree of site-specific modifications to loss of apoA-I lipid binding and cholesterol efflux functions (53). Of the seven Tyr residues in apoA-I, Tyr192, Tyr166, Tyr236, and Tyr29 were nitrated in MPO-mediated reactions. The favored modification site following exposure to MPO-generated oxidants was Tyr192. MPO-dependent nitration proceeded with Tyr166 as a secondary site and with Tyr236 and Tyr29 modified only minimally. A parallel study of apoA-I in human atherosclerotic tissue revealed its nitration at the MPO-preferred sites, Tyr192 and Tyr166. In contrast, *in vitro* nitration with 100 μM $ONOO^-$ generated nitroTyr at three Tyr residues: Tyr166, Tyr8, and Tyr236. The remaining Tyr residues were detected in these analyses as exclusively in the un-nitrated form.

There is also the potential for a time-dependent selectivity for *in vivo* Tyr nitration. Sharov et al. (2006) identified age-dependent post-translational modifications of skeletal muscle glycogen phosphorylase b isolated from F1 hybrids of Fisher 344 × Brown Norway rats (54). This study revealed the accumulation of nitroTyr on Tyr113, Tyr161, and Tyr573. Although nitration of Tyr113 was detected for both young and old rats, nitroTyr at positions 161 and 573 was identified only for enzyme isolated from aged 34-months old rats. The *in vitro* reaction of $ONOO^-$ with glycogen phosphorylase b resulted in the nitration of Tyrs, 51, 52, 113, 155, 185, 203, 262, 280, 404, 473, 731, and 732. Thus, the *in vitro* nitration conditions only mimic the nitration of a single Tyr residue observed *in vivo*, suggesting alternative mechanisms controlling the nitration and accumulation of nitroTyr *in vivo*.

In summary to this section, it is apparent that *in vitro* experiments with isolated proteins can provide some evidence for potential nitration sites but this evidence can also be misleading

both positively and negatively. The use of RNS donors with intact cells while under some circumstances an improvement can also be misleading since as discussed below it does not always recreate the subcellular RNS gradients that are generated *in vivo*. This may be especially important in identifying low abundance proteins involved in signal transduction pathways and whose activities may be subtly modulated by Tyr nitration. A clearer, more in depth understanding of those physiochemical and microenvironmental factors that determine specificity in Tyr nitration is needed.

Protein structure and Tyr nitration

There is selectivity in Tyr nitration, since as shown above not all proteins in a tissue under analysis are nitrated nor are all Tyrs of a nitrated protein nitrated. As discussed by several investigators (55–59), one would expect a relatively high degree of Tyr nitration given the moderate hydrophilic character of Tyr and its relatively high degree of surface exposure (only 15% of Tyr residues are at least 95% buried). However, that this is not the sole criterion is born out by studies of human serum albumin, the most abundant plasma protein. Only 2 of its 18 Tyrs are nitrated by $ONOO^-$ *in vitro*. In plasma samples from patients with acute respiratory distress syndrome 5 proteins not including serum albumin are nitrated. These results as do many of the studies cited above imply specificity especially *in vivo*.

No consensus primary sequence for Tyr nitration has been defined. A recent analysis of amino acid sequences of proteins nitrated both *in vitro* and *in vivo* (aged rat tissues) suggests three common features: (1) one or more acidic residues near the nitratable Tyr, (2) nearby turn-inducing amino acids, Pro or Gly, and (3) mostly exclusion of sulfur containing Met and Cys. An earlier analysis of 10 identified nitrated proteins suggested the following consensus sequence, H-X-[DE]-H-X(2,3)-H(2)-X(2,4)-Y, where H is any hydrophobic amino acid and X is any amino acid (59). The increased frequency of hydrophobic amino acids near the target Tyr is suggested to be consistent with the observation of apparent selective nitration of Tyrs in transmembrane domains of membrane proteins. A difficulty in defining a consensus sequence based on comparing linear peptide sequences containing nitrated Tyrs is that the sequences of non-nitrated Tyrs are not simultaneously compared. This is seen in another study on nitrated proteins from rat brains using mass spectrometry to identify and compare both nitrated and non-nitrated Tyr containing peptide sequences in terms of primary sequence and three dimensional structures near the nitrated Tyr for the proteins of known high-resolution structure (60). This analysis as did previous studies demonstrated an *in vivo* preference for nitration of surface exposed Tyrs in loops and also a modest preference for an Asp or Glu near the nitrated Tyr. However, in contrast to the earlier investigations, most all peptide sequences containing nitroTyr also contained a Cys or Met suggesting that these amino acids do not inhibit Tyr nitration *in vivo*. Furthermore, this analysis of *in vivo* nitrated proteins also revealed the overwhelming presence of a basic amino acid (Lys or Arg) in the 9 amino acid peptide window in nitrated versus non-nitrated Tyr peptides.

Souza et al. (58) made an interesting suggestion concerning specificity based on pulse radiolysis experiments with lysozyme and a two step model for Tyr nitration. The initial step in Tyr nitration is the oxidation of the phenolic ring of Tyr to yield the one-electron

oxidation product, Tyr•. The second step involves the termination reaction of one radical Tyr• with another, $•NO_2$. The diffusion limiting reaction of NO• with superoxide ($O_2•^-$) generates $ONOO^-$ that after protonation to peroxynitrous acid yields •OH and $•NO_2$. At physiological levels of CO_2, the nitrosoperoxocarbonate ($ONOOCO_2$) forms which homolyzes to •NO and $CO_3•^-$. The carbonate radical, $CO_3•^-$, is a much more effective one-electron oxidant than the hydroxyl radical, •OH, and thus it is observed that nitration yields are higher in the presence of CO_2. The pulse radiolysis experiments initially show oxidation of Trp yielding an indolyl radical and subsequently by intramolecular electron transfer resulted in the oxidation of Tyr to Tyr•. As suggested by Souza et al. (58), a stabilized Tyr• could be prone to reaction with $•NO_2$ far from the site of the initial oxidation step. Molecular interactions that stabilize the Tyr• or the nitrating species would presumably provide specificity to the final nitration step. A basic amino acid in close proximity suggests the possible involvement of electrostatic interactions stabilizing nitrating anions such as $ONOOCO_2^-$.

Specificity in the nitration process may not only be derived from close proximity as defined by the primary amino acid sequence but from higher protein structure levels. An example of this is seen in the consensus sequence for S-nitrosylation. Analysis of the primary sequences of S-nitrosylated proteins and the realization that acid-base catalysis alternatively promotes protein S-nitrosylation and denitrosylation suggest a degenerate consensus sequence, (G,S,T,C,Y,N,Q,)(K,R,H,D,E,) C(D,E) (61). The acid-base motif does not have to reside in the linear primary sequence but can be generated from the tertiary structure of the protein (62). For example, replacement of the acidic (Asp355) or basic (Arg357 and Arg363) amino acids located in the vicinity of Cys121 by Ser leads to a marked reduction in the ability of NO• to S-nitrosylate and inactivate hepatic methionine adenosyltransferase.

We have examined two proteins nitrated *in vivo* by mild oxidative/nitrosative stresses that may provide some clues as to long distance interactions that determine specificity in the nitration process or its consequences (47,49). IκBα, the inhibitory protein of the transcription factor nuclear factor-κB (NF-κB) is nitrated on 2 of 8 Tyrs, Tyr181, and Tyr305, after activation of the endogenous NOS. The crystallographic structure of the complex shows the fingers 3/4, 4/5, and 5/6 of IκBα contact the p50 subunit of NF-κB. Tyr181 and Asn182 extending from finger 3/4 have multiple contacts with p50. Tyr181 forms hydrogen bonds with p50 Lys252 and Arg258, π-stacks with Tyr351, and makes multiple van der Waals contacts with Ala260, Pro327, and Leu349 of p50. Using a software program to calculate on an atom-by-atom basis the enthalpic interactions of Tyr181 with all other atoms within 8 Å, an in silico nitration of Tyr181 induced signifi-cant destabilizing changes in these interactions of about 3 kcal/mol. In contrast, the “nitration” enhanced Tyr181 interactions with other IκBα residues within 8 Å consistent with a net stabilizing effect. Major contributions to this positive interaction are the acid/base and hydrogen bond interactions of the nitro-group with Arg140 of IκBα. The role of Tyr305 nitration is not clear since the available crystallographic structures of IκBα use a truncated version of the inhibitor protein. However, Tyr305 is in the PEST domain of IκBα and thus may be involved in modulation of IκBα stability *in vivo*.

Under similar mild oxidative/nitrosative conditions the tumor suppressor protein, p53, is nitrated *in vivo* on 1 of 9 Tyrs, Tyr327 in the tetramerization domain (47). The consequences of this nitrative event as demonstrated by combined functional, biochemical and molecular approaches is enhanced oligomerization of p53 and its transcriptional activation. A transcriptional analysis using p53 specific mRNA microarrays revealed that selective nitration of Tyr327 at low NO• donor concentrations resulted in an mRNA expression spectrum very different from that obtained with ionizing radiation or at very high NO•. An in silico structural analysis based on the tetrameric p53 structure demonstrated increased stability of the tetramer by interaction of the nitrated Tyr327 of one monomer with Arg333 of an adjacent monomer. Thus, this same Arg-Tyr/nitroTyr interaction appears to play an important role but with very different consequences compared to IκBα/NFκB—enhanced protein-protein interactions. The significance of this nitroTyr-Arg interaction in broader terms will depend on future studies relating protein structure and the nitrative process.

The above discussion is limited to non-metalloproteins. With metalloproteins containing a metal center or heme group different nitration mechanisms are involved. These are discussed in more detail by Souza et al. (47,49,58). With these proteins the metal center promotes nitration of an adjacent Tyr. For example with manganese super-oxide dismutase (Mn-SOD), one mechanism proposes that a Mn-O complex oxidizes Tyr34 to •Tyr34 which then reacts with $\bullet NO_2$ to form the nitrated Tyr.

Subcellular location of the target protein and sites of RNS generation

Specificity in nitration of specific proteins can also result from the subcellular locations of the NO• generators. As discussed in more detail below, the catalytic cycle of all three isoforms of NOS can become uncoupled with the result that NOS no longer generates NO• but superoxide anion, $\bullet O_2^-$. The subcellular locations of the different isoforms differ with NOS1 predominantly localized to the plasma membrane although some investigators propose a mitochondrial like NOS1; NOS2 is found in the cytosol, peroxidase-containing secretory granules and peroxisomes; and NOS3 associates with the Golgi, endoplasmic reticulum, and plasma membrane caveolae. There are also other less defined mechanisms for cellular NO• generation including the cytoplasmic enzyme, alde-hyde oxidase (63), and a nitrite reductase activity found in mitochondria (64). Because NO• is a relatively stable free radical and can diffuse substantial cellular distances, localized formation of nitrating RNS may also depend on the subcellular location of ROS generators. Especially prominent are the mitochondrial electron transport chain and plasma membrane caveolae NADPH oxidase as well as specialized organelles such as peroxisomes.

A careful high-resolution immunoelectron microscopy study evaluated the subcellular distribution of Tyr-nitrated proteins in different cells and tissues (65). This analysis revealed that endogenous nitrated proteins distributed differently depending on cell type but that for the most part localized in compartments in close proximity to the high NO• producing NOS2 and enzymes involved in ROS generation. Because this study only evaluates high abundance nitrated proteins, more subtle changes in protein nitration due to low levels of NO• generated by the constitutive NOS isoforms, NOS1 and NOS3, would not necessarily have been detected. This is important because, as described below, a number of low abundance

proteins involved in signal transduction pathways are nitrated under very mild oxidative/nitrosative conditions. For example, binding of caveolin-1 to NOS3 inhibits NOS3 activity in endothelial cells and disruption of this interaction leads to hyperactive NOS3 and increased protein Tyr nitration including protein kinase G (66). Nitration of protein kinase G inhibits its activity and in lungs this inhibition can result in pulmonary hypertension. Alternatively, nitration of a critical high abundance protein can be limited to a subcellular compartment minimizing cellular damage but permitting propagation of a signal throughout the cell. An example of this is seen with the mitochondrial enzyme, Mn-SOD, a protein nitrated in rejected human kidney allografts and in tissue culture cells after exposure to ionizing radiation (26,67). For rejected human allograft tissue, the chronic oxidative/nitrosative stress results in significant nitration and inhibition of mitochondrial Mn-SOD enzymatic activity. Inactivation of Mn-SOD increases the lifetime of $\bullet O_2^-$ and hence formation of $ONOO^-$ from NO• by a “positive feedback cycle” (67). Tyr nitration of Mn-SOD is also observed after exposing cells to ionizing radiation. However, inhibition in enzymatic activity is not detected, possibly because only a small fraction of a very abundant cellular protein is nitrated (67). Nonetheless, even if enzyme inhibition is limited to a single mitochondrion within a cell and not detectable by standard assay, significant biological effects can still accrue. This is because of intermitochondrial propagation of an oxidative signal from one mitochondrion to adjacent mitochondria via a reversible permeability transition, e.g. (67–70). Thus a potentially lethal oxidative event can be limited to a single mitochondrion amongst hundreds within a cell while at the same time initiating and amplifying reversible cellular signaling pathways.

Regulation of NOS activity by uncoupling of the NOS catalytic cycle

An important discovery about the enzyme activity of all NOS isoforms is that it can become uncoupled and as a consequence shift producing NO• to generating $ONOO^-$ or $O_2\bullet^-$. The catalytic cycle of all NOS isoforms involves the transfer of electrons coupled with the oxidation of arginine with the cofactor tetrahydrobiopterin (BH4) donating electrons to the ferrous-dioxygen complex to initiate oxidation. If BH4 is limiting which can be the case under chronic inflammatory conditions, coupling becomes less efficient and more $O_2\bullet^-$ is generated. Under these conditions NOS have been termed “peroxynitrite synthases” (71–73). This further amplifies the uncoupling with the generation of more powerful oxidants thus produced ($\bullet NO_2$, $\bullet CO_3^-$, •OH) further depleting BH4.

NOS activity can also be uncoupled with the generation of $\bullet O_2^-$ by depleting the substrate arginine (74). Physiologically this may not be relevant except under conditions of high arginase expression or elevated levels of endogenous inhibitors such as the asymmetric dimethlyarginines. Previous studies (75,76) have demonstrated that elevated cytoplasmic arginase expression uncouples NOS activity in endothelial cells contributing to endothelial dysfunction and redirects arginine metabolism to the formation of polyamines and l-proline critical for smooth muscle proliferation and collagen synthesis. Under hypoxic conditions, elevated arginase expression is critical for proliferation of human pulmonary microvascular endothelial cells. Increased arginase activity also sensitizes NOS activity to inhibition by asymmetric dimethyl arginines (ADMA) and l-NMMA (NG-monomethyl- l-arginine). These l-arginine analogues are produced from the proteolytic degradation of proteins

containing methylated residues(77). ADMA and l-NMMA are inhibitors of all three NOS. ADMA is degraded by DDAHs (dimethylarginine dimethylaminohydrolases), levels of ADMA/l-NMMA are critical factors in modulating angiogenesis.

Regardless of the mechanism of uncoupling, the consequences may represent a critical switching mechanism for cell growth. When fully coupled, the primary product of NOS is NO• and downstream signaling is dominated by NO• dependent pathways including soluble guanylate cyclase/protein kinase G and S-nitrosylation. Uncoupled NOS on the other hand produces potent oxidants such as $ONOO^-$ and •OH initiating different downstream signaling. Depending on their levels and cellular context, these latter oxidants are not necessarily cytotoxic but may actually stimulate cytoprotective mechanisms, e.g. NF-κB (49).

Reversibility of protein Tyr nitration

An important characteristic of all regulatory signal transduction mechanisms is reversibility. With respect to phosphorylation this is accomplished by apposing kinase and phosphatase activities. Proteolysis of post-translationally modified proteins is another mechanism that can irreversibly modulate a pathway and this is commonly seen in change in state signaling as in cell cycle transitions and proteolysis of cyclins. With respect to nitrated proteins there is evidence for both “denitrase” activity and enhanced proteolysis of nitrated proteins.

Early studies by Murad and associates identified a “denitrase” activity in lysates from spleen, liver and cultured macrophages(78,79). By the classical protein criteria of heat and trypsin sensitivities, the denitrase was a protein with substrate specificity. For example, the denitrase activity from activated macrophages denitrated histone H1.2 but not CuZn-SOD. The activity was inducible in macrophages by lipopolysaccharide (LPS). Subsequent studies by other investigators also demonstrated a denitrase activity in LPS-stimulated macrophages that was selective for nitrated calmodulin, glutamine synthetase and the human smooth muscle calcium channel, hCa(v)1.2b (78–82). Nitration and as a consequence inhibition of Src kinase phosphorylation of the calcium channel protein was reversed by treating the channel protein with the LPS-activated macrophage lysate. In the one study in which it was examined, no intermediate stage involving formation of amino-Tyr was observed in the apparent denitrase mechanism. All the above studies controlled for possible proteolysis with appropriate loading controls and, because conditions were used to yield maximal levels of nitrated protein, it is unlikely that proteolysis of a small amount of nitrated could explain the results.

Other studies in which time courses of nitration/ denitration of specific proteins were followed are also consistent with a denitration mechanism. Mn-SOD, NF-κB inhibitor protein, IκBα, the tumor suppressor protein, p53, and a number of undefined proteins are transiently nitrated after an exposure to a clinically relevant dose of radiation (47,49,67). With respect to IκBα and p53 that were studied in detail, western blot analysis with and without a proteosome inhibitor suggests that selective proteolysis of the nitrated proteins could not account for the apparent denitration. However, only about 5–10% of each protein was nitrated leaving open the possibility that the protein analysis was insufficiently sensitive to detect rapid turnover of the nitrated proteins. The frequency of transient nitration of IκBα

after radiation corresponded to that observed for tumor necrosis factor dependent IKK-dependent phosphorylation (83). Both nitration and phosphorylation of IκBα result in the dissociation of IκBα from NF-κB and transcriptional activation although the IKK-dependent transcriptional response was much more robust. One explanation for this difference in magnitude of the transcriptonial responses is probably that phosphorylation triggers ubiquitination and proteolysis of IκBα whereas the nitration mechanism did not.

Nitration and denitration of mitochondrial proteins have also been studied (46,84). With highly purified rat liver mitochondria, nitration of several mitochondrial proteins decreased within 5 min of hypoxia/anoxia and was completely blocked by 20 min with no apparent loss of proteins as detected by 2D gel electrophoresis and western blot. Moreover, nitration of these same proteins subsequently recovered within minutes of re-oxygenation. If mitochondria were incubated with l-arginine under oxygenated conditions, there was a progressive increase in the nitrated proteins. No increase or decrease was observed when the mitochondria were incubated with non-metabolizable d-arginine and d-arginine did not inhibit the recovery in nitrated proteins upon re-oxygenation. These results suggest that the mitochondria have a mechanism for NO• generation independent of arginine. A number of the proteins nitrated and denitrated were identified by mass spectroscopy and shown to be involved in electron transport and the TCA cycle. These proteins are nuclear encoded so that it is difficult to argue for a proteolysis/ resynthesis cycle to explain the results. As concluded by these investigators, the nitration/denitration cycling observed with these mitochondrial proteins has many of the characteristics of a signaling pathway including reversibility, responsiveness on a physiological time scale and selectivity.

Enhanced proteolysis and resynthesis of nitrated proteins represents an alternative mechanism for nitration reversibility (55,56,58,85). This mechanism is also seen with phosphorylation of some proteins, e.g. cyclins and IκB. Generally speaking, oxidative modification of proteins increases their susceptibility to proteolysis. Nitrated proteins appear to be more rapidly degraded by proteo-some activity but not by calpain or proteases sensitive to a serine protease inhibitor. Most of these studies used $ONOO^-$ to modify the target proteins and thus cannot rule out oxidation of other amino acids as contributing to enhanced protein degradation. One exception is the study with CuZn-SOD where only Tyr108 was nitrated without any other amino acid modification as validated by mass spectrometry. Nitrated CuZn-SOD was degraded by proteosomes at a 1.8-fold greater rate than the native protein (85).

In summary, an understanding of reversibility of protein Tyr nitration in the context of signal transduction mechanisms remains elusive. Although the evidence for denitrase activities in cells and mitochondria is compelling, conclusive proof will require identification of the denitrase proteins. The use of endogenously nitrated proteins with a defined nitrated Tyr or proteins “nitrated” by incorporation of nitroTyr during *in vitro* translation as substrates may be important in identifying denitrases as well as defining the physiological significance of selective proteolysis.

Conclusions

In this short review, we have attempted to provide experimental evidence suggesting that Tyr nitration under certain physiological conditions fulfills the criteria of a physiologically relevant signal in that it can be specific, reversible, occurs on a physiological time scale, and depending on a target, can result in either activation or inhibition. How specificity in nitration is defined biologically and mechanisms of reversibility in cellular systems are poorly understood. This is in part the result of how past studies have mostly focused on nitrated proteins that are either in high abundance, show a high degree of nitration or are nitrated under non-physiological conditions. As more investigations identify proteins involved in cell regulatory pathways in which nitration of only a small fraction of that protein pool is necessary to affect cell function, the underlying mechanisms of biological specificity and reversal of protein Tyr nitration should come into focus. This is becoming apparent in a number of recent studies demonstrating nitration of key regulatory proteins including p53 (47), IκBα (49), protein kinase Cϵ (86), protein phosphatase 2A (87), β-catenin (88), histone deacetylase II (89), smooth muscle l-type calcium channel (81), ERK1/2 (90), and Akt (90,91).

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