Reactive nitrogen species in cellular signaling

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Abstract

The transduction of cellular signals occurs through the modification of target molecules. Most of these modifications are transitory, thus the signal transduction pathways can be tightly regulated. Reactive nitrogen species are a group of compounds with different properties and reactivity. Some reactive nitrogen species are highly reactive and their interaction with macromolecules can lead to permanent modifications, which suggested they were lacking the specificity needed to participate in cell signaling events. However, the perception of reactive nitrogen species as oxidizers of macromolecules leading to general oxidative damage has recently evolved. The concept of redox signaling is now well established for a number of reactive oxygen and nitrogen species. In this context, the post-translational modifications introduced by reactive nitrogen species can be very specific and are active participants in signal transduction pathways. This review addresses the role of these oxidative modifications in the regulation of cell signaling events.

Keywords: Nitric oxide, nitrosylation, nitration, cell signaling

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Introduction

Transitory addition or removal of a prosthetic group allows a signal to be passed by inducing a change in the target molecule that alters its activity. Phosphorylation is the most widely discussed form of signal transduction and kinase cascades are very well characterized. Recently, reactive nitrogen species have been gaining attention as signal transduction mediators rather than damaging oxidizers of macromolecules. Oxidative modifications are difficult to study because the variety of targets makes it difficult to distinguish between relevant modification and collateral damage. In addition, when added exogenously, the concentrations of oxidants necessary to see consistent results are often damaging to the cell causing the concept of oxidation to become synonymous with cellular damage and senescence. However, the concept of oxidative signaling is now well established in the literature for a number of reactive oxygen and nitrogen species. The strongly oxidative nitrogen-based molecules and radical products of their decomposition are seen as too reactive to have the specific functions required for signal transduction. In addition, the modifications may be permanent causing them to be excluded from studies of signal mechanisms.[1] However, nitrated proteins are removed by degradation[2] and possible other mechanism yet to be fully characterized.[3–6] In addition, new technologies that allow the study of proteins with single residue modification in the absence of undesirable oxidative modification or damage to other cell components provide new opportunities for the investigation of oxidative signaling mediated by tyrosine nitration.[7–9]

Oxidative modifications are often unstable and prone to further oxidation or interactions with reducing agents. On the other hand, the conditions of analysis can produce oxidative modifications that were not there in the first place increasing the difficulty for the analysis of oxidative modification. However, the field of redox proteomics has developed a number of methodologies to deal with these limitations. Combinations of biotin-based techniques, alkylating agents, and indirect fluorescent labeling can be used to find and ''trap'' the redox modifications, which can be identified with improved antibodies or without gels by using liquid chromatography and mass spectrophotometry. The multidimensional approach of redox proteomics can help provide insight into the mechanisms of endogenous redox signaling, and help provide diagnostic tools as we move forward.[10–13]

Nitric oxide, nitrosation, and nitrosylation

Post-translational modifications involving reactive nitrogen species share a common progenitor: nitric oxide (NO). Nitric oxide is produced from L-arginine by three main isoforms of nitric oxide synthase (NOS): epithelial NOS (eNOS), related to vasodilation and vascular regulation; neuronal NOS (nNOS), which is linked to intracellular

signaling; and inducible NOS (iNOS), which has a variety of situational functions. While nitric oxide production by nNOS and eNOS is tightly regulated by calcium by a calmodulin-dependent mechanism,[14,15] iNOS is activated in response to various endotoxin or cytokine signals, which can lead to the rapid production of large fluxes of nitric oxide. iNOS expression is regulated by well characterized signal pathways including MAPK and JNK/STAT,[16,17] suggesting that the inducible production of nitric oxide must be tightly controlled. Several disease states have been linked to the deregulation of nitric oxide production, indicating that aberrant production of nitric oxide and its products can have deleterious consequences for the cells.[18–23] All products formed by nitric oxide reactions are collectively denominated reactive nitrogen species, which include a number of compounds with very different chemical properties and reactivity.

Nitric oxide is a very versatile molecule with multiple functions and mechanisms of action. Soon after the discovery of nitric oxide it became evident that it could have opposing effects. Nitric oxide was described as a diffusible radical that results in vasodilation and a key player in the circulatory system. The Nobel Prize winning work of Murad et al. identified nitric oxide as ligand of the soluble guanylyl cyclase, which stimulates the production of cGMP.[14,15,24] Nitric oxide-dependent production of cGMP has wide variety of targets, and plays a role in the regulation of several functions in the nervous system.[25–32] However, it soon became clear that not all activities of nitric oxide were mediated by production of cGMP. Oxidative products of nitric oxide were soon reported in macromolecules and a number of proteins.

Early on, nitrosylation of thiols in cysteine residues was accepted as a possible post-translational modification,[33] often linked to reactions with oxygen or glutathione.[1,34] Several mechanisms of nitrosylation have been described, including oxidative S-nitrosation, trans-nitrosylation by small molecular weight nitric oxide carriers like S-nitrosoglutathione, and metalloprotein-catalyzed S-nitrosylation.[35–37] Nitrosylation occurs in a functionally diverse group of proteins in diverse subcellular locations and in different conditions, regulating a variety of cellular processes.[36–38]

The first clearly defined role of nitrosylation in the nervous system was identified 20 years ago with the discovery that nitric oxide regulation of the NMDA receptor was mediated by S-nitrosylation.[39–41] Lipton et al. identified nitric oxide produced by nNOS as both a positive and negative regulator of the NMDA glutamate ionotropic receptor, depending on redox state. Direct S-nitrosation of the NMDA receptor suppresses its activity, but reaction with the spontaneously formed nitric oxide/superoxide product peroxynitrite activates the receptor.[40] Another early target of nitrosylation is p21ras, which plays a central role in this paradoxical activity. The nitric oxide-mediated S-nitrosylation status of the NMDA receptor helps to regulate p21ras[39] and it was suggested that Ras is one of the central steps in how redox signals transmit their messages.[41] Recent work showed that introduction of GSNO as a nitric oxide donor transiently nitrosylates Ras and Src kinase at the plasma membrane, and is reversed within 20–40 min. Src kinase induces the diacylglycerol pathway, causing translocation of RasGRP1 to the Golgi apparatus, activating Golgi-associated Ras. In contrast, eNOS-generated nitric oxide induced by EGF only activates Ras at the membrane. This suggests a way in which a cell can use the EGFR proliferation pathway in the absence of EGF,[42] which may have interesting ramifications in cancer.

Mannick et al. identified S-nitrosylation as a regulatory step in caspase activity.[43,44] Nitrosylation of a thiol in the active site leads to mitochondrial localization and inactivation of caspase-3 zymogens. Fas signaling activates caspase-3 by denitrosylation of the active site thiol and translocation to the cytoplasm during the initiation of apoptosis. Nitrosylation also participates in the regulation of the apoptotic signaling through the modification of glyceraldehyde-3-phosphate dehydrogenase (GADPH). Sen et al. reported that nitric oxide produced in response to a broad range of apoptotic stimuli nitrosylates GADPH, abolishing its normal metabolic function in glycolysis and enabling it to complex with an E-3 ubiquitin ligase. The complex GADPH/E3 ubiquitin ligase translocates to the nucleus where it stimulates apoptosis.[45] In addition, Talbott et al. reported S-nitrosation as one of the mechanism of regulation of death receptor-mediated apoptosis.[46] The FLICE-inhibitory protein is a regulator on the NF-κB/caspase-8 death pathway, and its cleavage and processing is dependent on nitrosylation in cysteine residues 254 and 259.

Oxidative modifications by peroxynitrite

Further nitric oxide mediated post-translational modifications occur though the formation of other reactive nitrogen species. Nitric oxide produces peroxynitrite through the diffusion-limited reaction with superoxide.[47] This molecule gained attention when, after studying the ability of superoxide dismutase to prevent injury in many disease states, Beckman et al identified peroxynitrite as the source of this damage.[48,49] In an aqueous environment, the protonated peroxynitrous acid forms and can rapidly decompose into nitrogen dioxide ($^{\bullet}NO_2$) and an extremely reactive hydroxyl radical ($^{\bullet}OH$)[24,26] with an efficiency of approximately 30%, though these values are controversial.[48,50,51] The hydroxyl radical is reactive enough to remove electrons from nearly any biological molecule, for instance the reaction with tyrosine creates a tyrosyl radical which can then react with nitrogen dioxide to form nitrotyrosine. In addition, peroxynitrite directly reacts with CO_2 yielding nitrosoperoxycarbonate ($ONOOCO_2-$), which decomposes into nitrogen dioxide and the carbonate radical ($^{\bullet}CO_3-$) that has a similar reactivity to the hydroxyl radical.[1] The production of these carbonate and hydroxyl radicals and peroxynitrite's ability to react strongly with metal centers[1,50,52] caused its presence to be associated with catastrophic cytotoxic circumstances (Figure 1). Peroxynitrite was seen as too reactive to participate in signaling events and its reaction with other molecules was considered to be damage rather than an oxidative modification.

The ability of peroxynitrite to modify proteins and other macromolecules has been harnessed by the phagocytes in

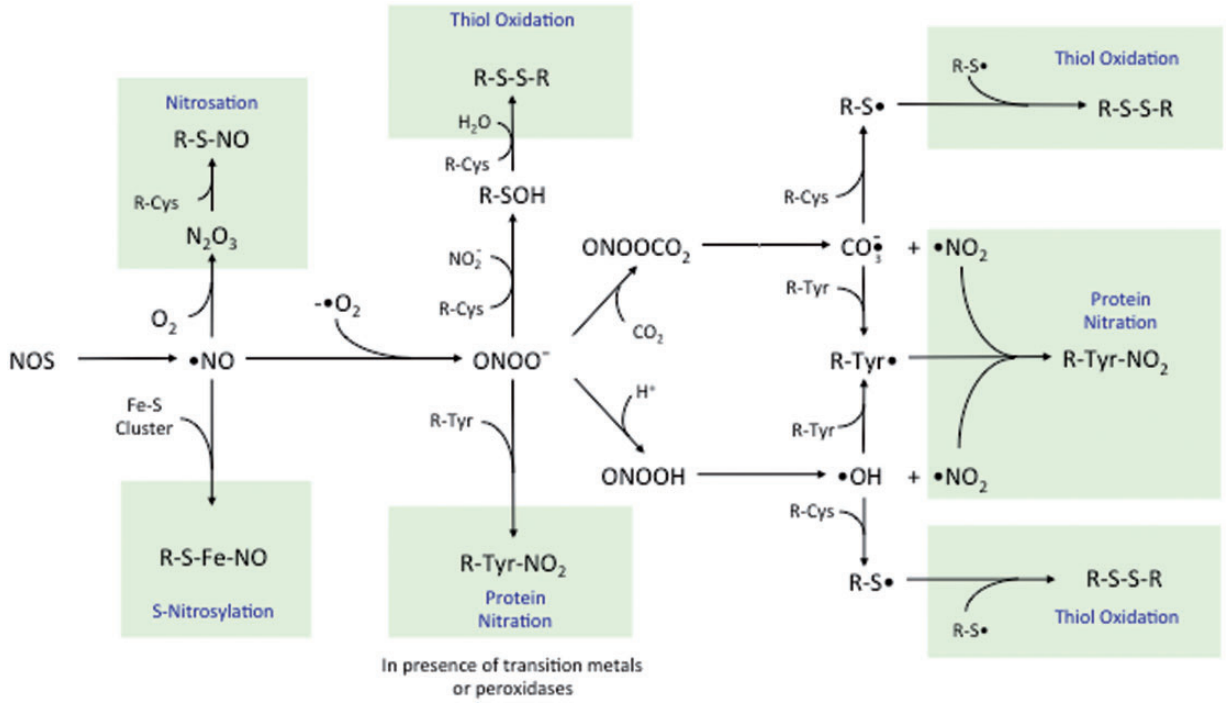

Figure 1 Nitric oxide reactions and protein post-translational modifications

the immune system. Both activated macrophages and neutrophils kill invading bacteria by engulfing them and releasing oxidative species in the ''respiratory burst''. After phagocytosis of antigenic particles, NADPH oxidase produces large amounts of superoxide and iNOS generates nitric oxide,[53–56] which combine to create peroxynitrite. It was found that the bacterial-killing function of activated macrophages was dependent on the presence of peroxynitrite, and that inhibiting either superoxide or nitric oxide formation inhibits this function.[55]

It is important to note that while peroxynitrite is used to protect the organism from pathogens, overproduction or dysregulation of these pathways is also damaging. The same respiratory burst used by immune cells is also responsible for chronic inflammatory responses. After observing rat models of traumatic brain injury, Hall et al. noted a six-fold increase in iNOS transcript. The subsequent increase in peroxynitrite formation switched the response from protective to neuropathogenic.[19] In sickle cell disease, characterized by multiple ischemia-reperfusion like events, peroxynitrite mediated damage occurs in the microvasculature epithelium when blood flow intermittently blocked by aberrant red blood cells is restored. The oxidative damage observed in reperfusion injury, and perhaps in sickle cell disease, has been recently shown to originate from the reversal of respiratory complex 1 in mitochondria causing excess superoxide production, which could then react with nitric oxide produced by eNOS and iNOS.[57–59]

Peroxynitrite overproduction results in tissue damage characterized by oxidation of proteins leading to loss of function. Peroxynitrite affects cellular metabolism by lipid peroxidation,[49] DNA damage,[60] and mitochondrial damage.[61] While peroxynitrite may have important cellular functions, if its levels become too high, it will cause catastrophic oxidative damage to cells and tissues. For this reason peroxynitrite has been implicated in a wide variety of disease states (Table 1).

Peroxynitrite can also oxidize thiols.[75] In fact, thiols act as scavengers of peroxynitrite because thiol oxidation is the fastest direct reaction of peroxynitrite. It is important to note that thiol oxidation and nitration/nitrosylation are not mechanistically exclusive and they are competing with each other in biological relevant conditions (Figure 1).

It had been shown that peroxynitrite leads to apoptosis and although the exact mechanism was unclear at the time, it was assumed that the oxidant caused general oxidative damage by randomly modifying macromolecules eventually leading to p38 activation and cell death.[76] Since then, evidence accumulated suggesting that peroxynitrite mediated cell death was not random death due to oxidative damage, but was a tightly regulated process.[77–79] The Akt and MLK/MAP kinase pathways were identified as directly involved in the regulation of peroxynitrite-induced apoptosis.[80] Due to the long-lasting nature of peroxynitrite modifications, peroxynitrite activation of a regulated pathway suggests a possible mechanism by which the cells could measure levels of oxidative stress. Theoretically, the accumulation of these modifications due to continuous oxidative damage could reach a threshold where the cell should commit to programmed cell death, activating p38 and leading to apoptosis. Considering this possibility creates an interesting question: If some of the peroxynitrite-induced disease states occur in a regulated pathway, could peroxynitrite function as a signaling intermediate in normal cellular processes?

Table 1 A short list of diseases and peroxynitrite's pathological role

Disease	Peroxynitrite mediated alterations	Target	Source
Amyotrophic lateral sclerosis (ALS)	Apoptosis of motor neurons	Tyrosine nitration	Zhu et al.[62]
	Cu,Zn-SOD damage	Tyrosine nitration	Kamisaki et al.[63]
Parkinson's disease	General mitochondrial damage	Iron/sulfur cluster reactions	Csermely et al.[64]
	Tyrosine hydroxylase impairment	Tyrosine nitration	Stebbins et al.[65]
	α-synuclein modifications	Tyrosine nitration	Wiech et al.[66]
Diabetes	DNA damage/binding	Guanine nitration	Franco et al.[67]
	Flow mediated dilation disruption	Tyrosine nitration	Cassuto et al.[23]
Rheumatoid arthritis	H2A histone modification, induction of antibody response	Tyrosine nitration	Franco and Estevez[68]
Lupus erythematosus	H1 histone modification, induction of antibody response	Tyrosine nitration	Franco and Estevez,[68] Yamakura et al.[69]
Vitiligo	Mitochondrial DNA changes, immunogenic response	Guanine nitration	Jin et al.[70]
Liver diseases	Broad spectrum protein nitration, Conversion of Hsp90 to toxic species	Tyrosine nitration	Blanchard-Fillion et al.[71]
Atherosclerosis	Prostaglandin synthase impairment	Tyrosine nitration	Deeb et al.[18]
	LDL oxidation and accumulation	Tyrosine nitration	Przedborski et al.[72]
Pulmonary hypertension	Hyperactive eNOS secondary to caveolin depletion impairs PKG activity	Tyrosine nitration	Zhao et al.[22]
	Inhibition of platelet activity	Cysteine nitrosation	Tripathi et al.[73]
Chronic heart disease	Low serum Ferroxidase I activity	Tyrosine nitration	Khan et al.[74]

Tyrosine nitration

To help answer that question, we must look at another type of nitric oxide based, peroxynitrite mediated post-translational modification—protein nitration, one of the best characterized peroxynitrite modifications. Originally identified as a footprint of peroxynitrite presence, nitration of tyrosine is the post-translational modification seen in many of the diseases in which peroxynitrite is produced (Table 1).[1] Nitrotyrosine is present in a relatively small number of proteins, and occurs in limited positions on these proteins.[1] Recently, protein nitration has been gaining traction as a signal transduction mechanism, as suggested many years ago by Ischiropoulos and Al-Mehdi.[81] We have previously shown that peroxynitrite-induced apoptosis can be prevented by tyrosine-containing peptides that scavenge the products of peroxynitrite decomposition, preventing nitration of proteins.[82] Traditionally, nitration is seen as an irreversible process, making it poorly suited for the dynamics of signal transduction.[83]

However, in some circumstances cells need long term signaling. Nitration is then a potential mechanism to engender lasting alterations to cell functionality, such as memory formation, cellular differentiation, or in major metabolic shifts. Growing evidence shows that post-translational modification of proteins by nitration can be used to modulate their activity in ways that other redox signals cannot. We have recently shown that nitrotyrosine levels are dynamically regulated during fetal heart development.[84] This temporal pattern does not correlate with apoptosis, but with myocyte differentiation. The permanent changes that happen in development are well suited to a signal transduction mechanism that is not readily reversible. In 2013, researchers reported that the activity of hydroxypyruvate reductase from Arabidopsis plants was regulated by site-specific tyrosine nitration.[85] This loss of function modification was reported in a normal, no-stress condition, and no effect was observed after exposure to hydrogen peroxide, suggesting nitration was the intended functional modification, not a by-product of other reactive species. Finding pathways such as this where nitration is used in normal conditions supports the hypothesis that nitration can be a controlled mechanism, and not only a sign of oxidative damage. In addition, nitration of proteins in liver caused by acetaminophen is catalyzed by superoxide dismutase (SOD).[86–88] Gene deletion of SOD prevents both nitration and damage in the liver produced by high doses of acetaminophen. These results suggest that in addition to the more accepted radical unregulated oxidative mechanisms, endogenous tyrosine nitration may be catalyzed, increasing the level of regulation.

Furthermore, Kamasaki et al. reported the ability of spleen and lung homogenates to reduce nitrotyrosine in a nitrated BSA sample.[89] They proposed the presence of a ''nitrotyrosine denitrase'', although were unable to isolate the source of this activity. Recently, Deeb et al. characterized this denitrase, showing that it rapidly reversed COX-1 heme-dependent nitration in a regulated and substrate-selective manner.[3] Using more modern techniques they were able to isolate, identify and quantify the denitrase in several tissue types and elucidate methods for regulation of its activity. These findings indicate that nitration is not as permanent as once believed, but a dynamic, well regulated process very suitable for signal transduction.

Nitration of heat shock protein 90

We identified heat shock protein 90 (Hsp90) as a target of post-translational nitration in PC12 cells after incubation with peroxynitrite and cultured motor neurons deprived of trophic factors using monoclonal antibodies specific only to the nitrated form of the protein.[69] Hsp90 has multiple cellular functions and is one of the most common proteins in the cell, making up 1–2% of the total protein profile in the cytoplasm. This chaperone has a very broad range of functions: protein folding, stabilization, translocation, activity modulation, and degradation.[90–92]

Further study revealed that nitration of a single tyrosine residue on Hsp90 (Y33 or Y56) was necessary and sufficient to cause a gain of function converting this pro-survival chaperone into a pro-apoptotic protein.[62] Nitrated Hsp90 has been identified in the spinal cords of patients suffering from sporadic ALS and in SOD mutant ALS model mice.[62,93] Interestingly, the amount of nitrated Hsp90 also correlates with disease progression, increasing as the disease progresses, suggesting that the gain of apoptotic function by nitrated Hsp90 participates in the progressive motor neuron death characteristic of this disease (Dennys, Trias, Ramdial and Estevez, unpublished observations).

Nitration has been reported in many disease states and a large body of research has been focused on preventing or reducing these reactions, but relatively little intellectual attention has been directed towards the endogenous signaling function of these reactions. If considered in this light, this oxidative post-translational modification can been seen as something other than a stressor for the cell, but critical cellular mechanisms for maintenance and normal function. Understanding the relative proportion of the different nitration mechanisms in normal conditions, as well as how nitration is regulated could result in a better understanding of the pathogenic mechanisms activated in many disease states. It may also shed some light on the general failure of antioxidant therapies.

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