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Review

NO Means No and Yes: Regulation of Cell Signaling by Protein Nitrosylation

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Protein nitrosylation is emerging as a key mechanism by which nitric oxide regulates cell signaling. Nitrosylation is the binding of a NO group to a metal or thiol (−SH) on a peptide or protein. Like phosphorylation, nitrosylation is a precisely targeted and rapidly reversible posttranslational modification that allows cells to flexibly and specifically respond to changes in their environment. An increasing number of proteins have been identified whose activity is regulated by intracellular nitrosylation. This review focuses on proteins regulated by endogenous nitrosylation, the chemistry underlying nitrosylation, the specificity and reversibility of nitrosylation reactions, methods to detect protein nitrosylation, and the role of coordinated protein nitrosylation/denitrosylation in cell signaling.

INTRODUCTION

Nitric oxide (NO) is a free radical molecule produced by the intracellular enzyme nitric oxide synthase (NOS) that regulates a wide range of biologic functions including vasodilation, neurotransmission, inflammation and cell death.[1] A key mechanism by which NO regulates these diverse physiologic processes is via posttranslational modification of proteins. NO can oxidize, nitrate or nitrosylate proteins.[2] Nitration refers to the attachment of an NO_2 group to a tyrosine or less commonly a tryptophan residue. Nitrosylation refers to the attachment of an NO group to a transition metal or a thiol, usually a cysteine residue (S-nitrosylation). Whereas nitration is an irreversible modification that may be responsible for some of the toxic effects of NO,[3–7] nitrosylation is a reversible modification involved in cell signaling.[8] Although the functions of many proteins have been modified by protein nitrosylation in cell free systems, it has been much more difficult to identify proteins whose function is regulated by endogenous nitrosylation in cells. However, in recent years nitrosylation has been shown to regulate the function of an increasing number of intracellular proteins.[8] These data raise the possibility that nitrosylation is a form of signal transduction regulation comparable to phosphorylation.[9,10] Both modifications are rapidly reversible and precisely targeted, allowing cells to respond quickly, accurately and flexibly to physiologic stimuli.

METAL NITROSYLATION

Guanylate cyclase is the classic example of a protein whose function is regulated by metal nitrosylation.[11] NO binding to the heme iron of guanylate cyclase induces a conformational change that activates the enzyme and increases 3′5′-guanosine monophosphate (cGMP) production.[12,13] Nitrosylation of guanylate cyclase is a mechanism underlying many biological activities of NO including vasodilation. More recently, several other proteins have been identified whose function is regulated by intracellular metal nitrosylation. As will be discussed in more detail below, NO binds to the heme iron of

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cytochrome *c* during apoptosis, stimulating the pro-apoptotic activity of cytochrome *c*.[14] In contrast, the activity of cytochrome oxidase, the terminal electron acceptor of the mitochondrial electron transport chain, is inhibited by metal nitrosylation. NO binds in competition with oxygen to the iron/copper binuclear center of cytochrome oxidase leading to an inhibition of oxygen consumption.[15–17] Non-heme iron nitrosylation of the iron–sulfur cluster protein aconitase leads to the loss of an iron atom from the iron–sulfur cluster and inhibition of aconitase activity during episodes of cardiac allografts rejection.[18] Several other proteins including catalase[19] are regulated by metal nitrosylation *in vitro*, but it remains to be determined if they are nitrosylated intracellularly by endogenous sources of NO.

S-NITROSYLATION

The function of an increasing number of proteins has also been shown to be regulated by S-nitrosylation. Like metal nitrosylation, S-nitrosylation can either inhibit or stimulate protein function. The activities of the caspase family of cysteine proteases, the *N*-methyl-D-aspartate (NMDA) class of glutamate receptor in the brain, the skeletal muscle ryanodine receptor and methionine adenosyl transferase (MAT) are inhibited by endogenous intracellular S-nitrosylation of a single critical cysteine residue on each protein. Caspase activity is inhibited by S-nitrosylation of the caspase catalytic site cysteine.[20–24] NMDA receptor activity is inhibited by S-nitrosylation of a critical cysteine on the NR2a regulatory subunit.[25,26] Similarly, the activity of MAT, the enzyme that synthesizes the methyl donor S-adenosyl methionine, is inhibited by S-nitrosylation of cysteine 121.[27,28] In contrast, the activity of the Ras family of G proteins and thioredoxin are stimulated by endogenous S-nitrosylation. S-nitrosylation of a critical cysteine on Ras family members enhances guanine-nucleotide exchange activity, resulting in an increase in active GTP-bound protein.[29,30] The redox regulatory and anti-apoptotic activity of thioredoxin is stimulated by S-nitrosylation of cysteine 69.[31] Again, the function of many additional proteins is modified by S-nitrosylation in cell free systems, but the physiologic relevance of these findings remains to be determined.

CHEMISTRY OF NITROSYLATION

Whereas most classical posttranslational modifications like phosphorylation are enzyme-dependent, nitrosylation is, at least in part, an enzyme-independent modification. Instead nitrosylation is mediated by intracellular redox chemistry. Although the chemistry underlying nitrosylation is incompletely understood, in general, NO binds to transition metals or thiyl radicals whereas NO^+ or an equivalent (such as NO_2 or N_2O_3) binds to –SH groups.[9] Therefore protein nitrosylation will be dependent on the relative formation and colocalization of reduced thiols, thiyl radicals, NO and NO^+ equivalents within a cell. For instance, reactions between NO and O_2 are accelerated 300-fold in lipid membranes leading to increased production of the NO^+ equivalents NO_2 and N_2O_3.[32] Therefore, proteins inserted in membranes are likely targets of NO_2 and N_2O_3-mediated S-nitrosylation. Protein nitrosylation also results from transnitrosation reactions in which NO groups are directly transferred from one thiol to another.[33]

SPECIFICITY OF NITROSYLATION

Despite the fact that virtually all proteins contain cysteine residues, many proteins contain metals, and most if not all cells produce NO, only a precisely defined subset of protein targets appear to be nitrosylated intracellularly. For instance, only 1 of 50 free thiols on the ryanodine receptor is targeted for nitrosylation.[34,35] The remarkable specificity of nitrosylation reactions is conferred both by colocalization of NOS with nitrosylation targets and, in the case of S-nitrosylation, by consensus motifs that target specific cysteine residues for nitrosylation. Specificity of phosphorylation reactions is achieved by analogous mechanisms. Kinases colocalize intracellularly with protein targets and consensus motifs targets specific serine, threonine or tyrosine residues for phosphorylation.

Colocalization of NOS with nitrosylation targets is well exemplified by the NMDA receptor. The neuronal isoform of NOS (nNOS) associates with the NMDA receptor complex. Stimulation of the NMDA receptor results in an influx of calcium that activates nNOS. Local production of NO and related species by nNOS leads to the specific nitrosylation of three components of the NMDA receptor complex: Dexras, guanylate cyclase, and the Nr2a subunit of the receptor.[36–38] Nitrosylation stimulates the guanine-nucleotide exchange activity of Dexras, increases cGMP production by guanylate cyclase and inhibits NR2a leading to a downregulation of NMDA receptor activity.[26,30,38,39] Thus colocalization of NOS with 3 nitrosylation targets within the NMDA receptor complex modulates downstream signaling from the receptor.

Specificity of nitrosylation reactions is also conferred by consensus motifs. Two consensus motifs for S-nitrosylation reactions have been identified. The first is an acid/base consensus

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sequence in which the target cysteine is located between basic and acidic amino acids either in the primary amino acid sequence or the tertiary structure of the protein.[40–42] The acid/base consensus motif is well exemplified in MAT. Although each subunit of MAT has 10 free cysteines, only cysteine 121 is targeted for nitrosylation, leading to an inhibition in enzyme activity.[27,28] Analysis of the tertiary structure of MAT reveals that cysteine 121 is flanked by an acidic and basic amino acid. Site-directed mutagenesis of the flanking acidic or basic residues inhibits S-nitrosylation and abrogates the NO responsiveness of MAT, supporting the importance of the consensus motif for targeting the nitrosylation reaction.[28]

Hydrophobic cores of proteins represent a second kind of S-nitrosylation "consensus motif". NO and O_2 are concentrated within hydrophobic cores, leading to increased production of nitrosylating species such as NO_2 and N_2O_3.[35] Therefore cysteine residues within hydrophobic cores are targeted for S-nitrosylation. Allosteric changes in protein conformation that create localized hydrophobic pockets may allow a protein to catalyze its own nitrosylation. For instance, the single cysteine residue that is targeted for nitrosylation in the ryanodine receptor is located within a hydrophobic pocket that is generated during a redox-driven conformational change in the protein.[34,35,42]

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DENITROSYLATION

Just as phosphorylation is reversed by dephosphorylation, nitrosylation is reversed by denitrosylation. However, unlike dephosphorylation, denitrosylation can be accomplished non-enzymatically due to the redox-sensitivity of S–NO and metal–NO bonds.[9] A variety of factors including transition metals, reducing agents, pH and pO_2 shifts and UV light can lead to the loss of S–NO and/or metal–NO bonds.[40,43] Thus, denitrosylation may simply require altering the redox environment of a protein. For instance, treatment of endothelial cells with TNFα or oxidized lipoproteins leads to the denitrosylation of multiple proteins perhaps due to intracellular redox shifts triggered by these agents.[31] Denitrosylation may also result from translocation of a protein from a subcellular compartment that allows stable S–NO or metal–NO bond formation to a subcellular compartment whose redox environment favors denitrosylation. For example, although caspase-3 zymogens are located both in the cytoplasm and the mitochondrial intermembrane space, it is predominantly the mitochondrial subpopulation that is S-nitrosylated in resting cells.[44] During apoptosis, caspase-3 zymogens translocate from the mitochondria into the cytoplasm and are denitrosylated. These data suggest that the mitochondrial intermembrane space may be a privileged site allowing stable protein nitrosylation whereas the reducing environment of the cytoplasm may promote denitrosylation except under conditions of oxidative stress or in hydrophobic microenvironments. Denitrosylation has also been reported to be enzyme-mediated. Specifically, formaldehyde dehydrogenase has been shown to denitrosylate the S-nitrosylated peptide S-nitrosoglutathione (GSNO), an NO donor in transnitrosation reactions. Deletion of the gene in yeast or mice leads to increased intracellular levels of GSNO and S-nitrosylated proteins.[45]

DETECTION OF NITROSYLATED PROTEINS

Although nitrosylation is emerging as a novel mechanism of signal transduction regulation, the nitrosylation field is still in its infancy. It remains to be determined if nitrosylation is a ubiquitous regulator of cell signaling. One of the difficulties facing the nitrosylation field is a lack of easy, sensitive and specific methods to identify S-nitrosylated proteins in cells. The levels of endogenously nitrosylated intracellular proteins are at the limits of detection of all currently available techniques, each method has potential drawbacks, and none can be considered the gold standard. Therefore, nitrosylation measurements should be confirmed using at least two different methods to validate results. Since S–NO and metal–NO bonds are redox-sensitive, great care must be taken during sample preparation to avoid artifactual loss and/or gain of S–NO or metal–NO bonds. The conditions favoring S–NO or metal–NO bond stability vary from protein to protein. For instance, some S–NO bonds are most stable at low pH whereas others are most stable at neutral pH. Consequently, the best method for detecting protein nitrosylation will vary between proteins. Ideally control experiments should be performed for each protein of interest to determine the optimal method and conditions for nitrosylation detection.

Currently, the most commonly used techniques to detect endogenous intracellular protein nitrosylation are chemical reduction/chemiluminescence, the biotin switch assay, 2,3-Diamino-napthalene (DAN) or 4,5-diaminofluorescein (DAF-2) assays, and immunofluorescent or immunohistochemical staining with an anti-SNO antibody. In chemical reduction/chemiluminescence assays, NO groups are released from nitrosylated proteins using reductants and the released NO is measured by chemiluminescence.[24,46–48] This assay is very sensitive but requires a nitric oxide analyzer that is not available in many laboratories. In addition, most

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chemical reduction/chemiluminescence methods are better suited for detecting S–NO than metal–NO bonds. In the DAN or DAF-2 assays, S-nitrosylated proteins are treated with mercuric chloride to release NO from S–NO bonds. The released NO reacts with DAN or DAF-2 causing the compounds to fluoresce.[31,49,50] The extent of DAN or DAF-2 fluorescence is proportional to the amount of NO released from each sample. In our hands, this assay is less sensitive than chemical reduction/chemiluminescence but has the advantage of not requiring a nitric oxide analyzer. In the biotin switch assay, S-nitrosylated proteins are selectively labeled with biotin and then are purified on immobilized streptavidin or are analyzed on anti-biotin immunoblots.[51] This assay is relatively sensitive and specific and does not require specialized pieces of equipment. The anti-SNO antibody is a rabbit antiserum generated against S-nitrosocysteine that detects S-nitrosylated proteins by immunofluorescent and immunohistochemical staining.[35,52] However, the antiserum has been reported to detect some unmodified thiols, so appropriate controls must be performed to assure that the staining is S-NO specific.[35] Metal nitrosylation can be assessed by analyzing the UV–visible spectra (or EPR spectra if sufficient protein is available) of immunoprecipitated metal-containing proteins.

REGULATION OF FAS-INDUCED APOPTOSIS BY PROTEIN NITROSYLATION

Regulation of cell signaling by protein nitrosylation is well exemplified in the Fas signaling pathway. Fas is a cell surface receptor that induces apoptotic cell death.[53–55] Fas ligation leads to the activation of a family of cysteine proteases called caspases. Caspases are expressed as relatively inactive zymogens that are cleaved to form fully active enzymes.[56] A group of initiator caspases including caspase-8 and -9 cleave and activate a group of executioner caspases including caspase-3. The executioner caspases cleave specific cellular targets leading to cell death.[57–61] In some cells, apoptotic cascades are amplified after Fas ligation due to the release of proteins such as cytochrome *c* and caspases from the mitochondrial intermembrane space into the cytoplasm.[62–64] In the cytoplasm, cytochrome *c* forms a multiprotein complex with the adaptor protein Apaf1 and caspase-9 called the apoptosome.[65–69] The apoptosome further cleaves and activates executioner caspases leading to enhanced apoptotic cell death.

In resting cells caspase-3 zymogens in mitochondria are kept inactive via S-nitrosylation of their catalytic site cysteine.[21,24,44] Caspase-3 may be stably S-nitrosylated in mitochondria due to an association between S-nitrosylated caspase-3 and NOS[70] (Fig. 1a). Moreover S-nitrosylated but not denitrosylated caspase-3 associates with acid sphingomyelinase (ASM) in mitochondria.[70] The association of S-nitrosylated caspase-3 with ASM provides another level of apoptosis regulation by inhibiting capase-3 cleavage and activation by initiator caspases.[70] When cells are stimulated by Fas ligand, caspase-3 becomes denitrosylated. Denitrosylation stimulates caspase-3 activity by two mechanisms. First, denitrosylation allows the catalytic site of caspase-3 to function.[24] In addition, denitrosylated caspase-3 presumably dissociates from ASM, allowing initiator caspases to cleave caspase-3 to its fully active form (Fig. 1b).

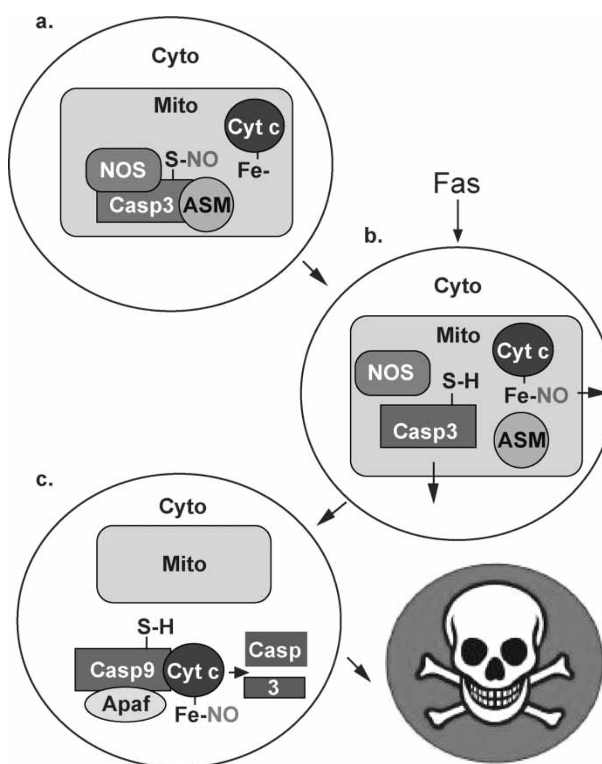

FIGURE 1 *Regulation of Fas-induced apoptosis by protein nitrosylation*. (**a**) In resting cells, caspase-3 zymogens (Casp3) in mitochondria (Mito) are S-nitrosylated due to an association with NOS. S-nitrosylation inhibits caspase-3 activity by two mechanisms. First, S-nitrosylation of the catalytic site cysteine directly inhibits the protease activity of caspase-3. Secondly, acid sphingomyelinase (ASM) specifically associates with S-nitrosylated caspase-3 in mitochondria. The association with ASM prevents caspase-3 cleavage to its fully active tetrameric form by initiator caspases. (**b**) After Fas stimulation, mitochondrial caspase-3 is denitrosylated and released into the cytoplasm (Cyto). Denitrosylation frees the catalytic site of caspase-3 and leads to a dissociation of ASM, allowing caspase-3 to be cleaved to its active form. Concurrently, cytochrome *c* (Cyt c) is nitrosylated on its heme iron and is released from mitochondria into the cytoplasm. (**c**) In the cytoplasm, nitrosylation of cytochrome *c* in combination with denitrosylation of caspase-3 increases caspase-3 cleavage and activation by the apoptosome (a complex containing cytochrome *c*, Apaf1 and caspase-9). Increased caspase-3 activation promotes apoptotic cell death.

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Thus S-nitrosylation/denitrosylation serves as an off/on switch for caspase-3 function during apoptosis.

Cytochrome *c* activity is also regulated by nitrosylation during Fas-induced apoptosis. However, in contrast to caspase-3, cytochrome *c* is not nitrosylated in resting cells. Instead, when cells receive an apoptotic stimulus, cytochrome *c* is nitrosylated on its heme iron in mitochondria and then is rapidly released into the cytoplasm.[14] In the cytoplasm, heme nitrosylated cytochrome *c* stimulates caspase-3 cleavage by the apoptosome[14] (Fig. 1c). Thus coordinated denitrosylation of caspase-3 and heme nitrosylation of cytochrome *c* serve to enhance caspase activation and Fas-induced apoptosis. It remains to be determined if denitrosylation of caspase-3 is directly linked to nitrosylation of cytochrome *c* in mitochondria via a direct transfer of a NO group from the catalytic site cysteine of caspase-3 to the heme iron of cytochrome *c*.

CONCLUSION

Nitrosylation is emerging as a redox-sensitive posttranslational modification that is a key mechanism underlying many of the physiologic effects of NO. However, protein nitrosylation is a relatively new scientific field and much remains to be learned about its role in cell signaling. Technical advances that facilitate the detection and quantification of endogenously nitrosylated proteins are needed to determine if nitrosylation is a ubiquitous regulator of signal transduction. Moreover, future studies may reveal that nitrosylation is only one of multiple redox-mediated posttranslational modifications, such as sulfenic acid and mixed disulfide formation,[2] that play critical roles in the regulation of cell signaling.

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