Mini Review
Strategies for Profiling Native *S*-Nitrosylation

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ABSTRACT:

Cysteine is a uniquely reactive amino acid, capable of undergoing both nucleophlilic and oxidative post-translational modifications. One such oxidation reaction involves the covalent modification of cysteine via the gaseous second messenger nitric oxide (NO), termed S*-nitrosylation (SNO). This dynamic post-translational modification is involved in the redox regulation of proteins across all phylogenic kingdoms. In mammals, calcium-dependent activation of NO synthase triggers the local release of NO, which activates nearby guanylyl cyclases and cGMP-dependent pathways. In parallel, diffusible NO can locally modify redox active cellular thiols, functionally modulating many redox sensitive enzymes. Aberrant SNO is implicated in the pathology of many diseases, including neurodegeneration, inflammation, and stroke. In this review, we discuss current methods to label sites of SNO for biochemical analysis. The most popular method involves a series of biochemical steps to mask free thiols followed by selective nitrosothiol reduction and capture. Other emerging methods include mechanism-based phosphine probes and mercury enrichment chemistry. By bridging new enrichment approaches with high-resolution mass spectrometry, large-scale analysis of protein nitrosylation has highlighted new pathways of oxidative regulation.* © *2013 Wiley Periodicals, Inc. Biopolymers 101: 173–179, 2014.*

Keywords: *nitrosylation; post-translational modification; nitric oxide*

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INTRODUCTION

Sulfur is the lightest element that can produce stable exceptions to the octet rule because of the presence of "*d*" orbitals. Typical cysteine residues in proteins have a side chain pK_a values of 8.0,[1] and thus ~10% of cysteine thiols are in their reactive thiolate form at physiological pH. However, many redox active or catalytic cysteine residues have dramatically reduced pK_a values. Such thiolates have evolved to promote catalysis or redox regulation. For example, the pK_a of the catalytic cysteine in methionine sulfoxide reductase is reduced to 5.7 upon substrate binding.[2,3] Similarly the active site thiol of glutaredoxin has a low pK_a near 3.5.[4,5] Such altered acid-dissociation constants enhance thiol reactivity, which in turn promote reactions with electrophilic oxidants to produce distinct post-translational modifications.

In this review, we focus on the chemistry and dynamics of protein *S*-nitrosylation (SNO). This unique oxidative modification directly modulates the localization and activity of cellular proteins involved in cellular growth and regulation.[6,7] In neurons, stimulus-dependent depolarization leads to calcium influx, calmodulin activation, and stimulation of nitric oxide (NO) synthases.[8–10] Local NO release induces spatially restricted SNO of channels, phosphatases, and other redox

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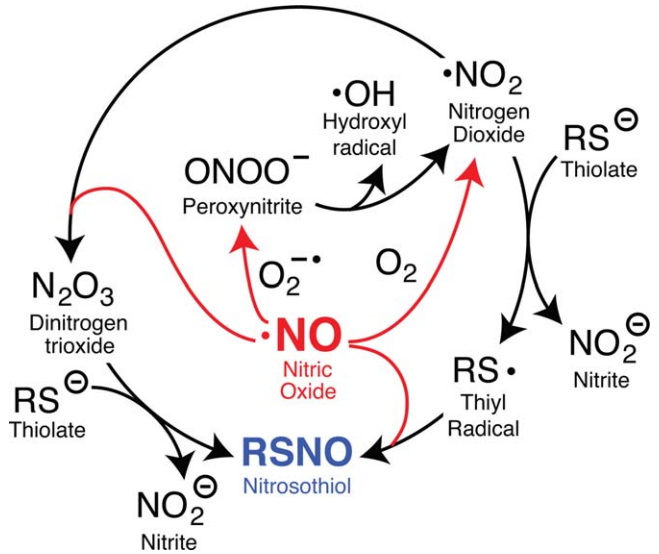

FIGURE 1 Formation of nitrosothiols from nitric oxide (NO) occurs through distinct oxidative pathways,[17–21] each involving two molecules of NO for each nitrosothiol formed. Superoxide radical = $O_2^{-\bullet}$. Oxygen = O_2.

active thiols.[6,11,12] Emerging proteomics studies implicate hundreds of endogenous sites of nitrosylation,[13–16] although the stoichiometry and functional consequences of these post-translational remains an active research area.

There are several mechanistic routes leading to protein SNO (Figure 1). Heme-dependent NO synthases generate NO via a two-step, five-electron oxidation of L-arginine.[22–25] This reaction uses two moles of molecular oxygen and 3/2 moles of NADPH per mole of NO formed.[26] NO itself is not especially reactive towards protonated cellular thiols, particularly under aerobic conditions.[27–29] To generate nitrosothiols, NO must first undergo secondary oxidation to nitrogen dioxide, which occurs via at least two distinct pathways.[30] In the first pathway, NO reacts with a superoxide radical to make peroxynitrite. Peroxynitrite (p*Ka* of 6.5[31]) converts to peroxynitrous acid at physiological pH, which undergoes hemolytic cleavage to form the hydroxyl and the nitrogen dioxide radicals. The nitrogen dioxide radical can in turn react with NO to form dinitrogen trioxide, which reacts with thiolates to form nitrosothiols.[29] In a second pathway, nitrogen dioxide reacts with a thiolate to generate nitrite and a thiyl radical.[17] The resulting thiyl radical is the only species able to react directly with NO to generate nitrosothiols. Additionally, metal-dependent formation of thiyl radicals[32] promotes SNO by one-electron oxidation of thiols to thiyl radicals, or through metal-nitrosyl complex intermediates.[33,34] Furthermore, iron and NO spontaneously react to form dinitrosyliron complexes (DNIC), which can be intermediates in nitrosothiol formation.[35–38] All of these routes generate diffusible reactive radicals with enhanced reactivity towards thiols with reduced pK_a values, such as catalytic or redox-active thiols.

SNO is reversible, either by NO release or by direct transfer to other cellular thiols.[39,40] Such trans-nitrosylation reactions mobilize the exchange of NO from one protein to another, relaying nitroso-oxidation through multiple carriers.[41–44] Trans-nitrosylation provides another route for the dynamic exchange of nitrosothiols. Millimolar glutathione levels maintain an intracellular reducing environment that protects proteins from oxidative modifications.[45] Abundant glutathione scavenges nitrosothiols by trans-nitrosylation, yielding a reduced protein thiol and nitrosoglutathione, which is reduced either by *S*-nitrosoglutathione reductase (GSNOR) or thioredoxin cascades. Such thiol exchange reactions are prevalent at physiological pH and predominate in comparison to hydrolysis reactions,[46] and are driven by the levels of reduced thiols in a given environment.[47] Several studies have demonstrated trans-nitrosylation cascades relay the nitroso adduct from one protein thiol to another, eventually nitrosylating and inactivating select enzymes, for example nuclear chromatin-modifying enzymes.[48] This model suggests stable SNO sites are protected from the cellular environment, masked inside proteins or membranes. These studies demonstrate the surprising resilience of certain nitrosylated proteins in face of millimolar glutathione, and hints at orchestrated pathways of nitrosothiol transfer in cellular regulation. Indeed, hydrophobicity does enhance the rate of reaction between NO and oxygen by several fold,[49] suggesting that thiols in hydrophobic environments, either in membranes or in hydrophobic protein domains, may be more prone to stable SNO.[6] Overall, protein SNO is modulated by thiol pK_a, vicinal hydrophobicity,[6] proximity to NOS enzymes and by activities of redox enzymes such as thioredoxin, GSNOR, and accessibility to reduced glutathione.

Protein nitrosylation functionally regulates protein activities by transiently occupying thiol residues. Functional cysteines often reside in the active sites of enzymes, such as phosphatases, proteases, acyl-transferases, and ubiquitin ligases.[50] These thiols reside in environments that promote thiolate formation by reducing the side-chain pK_a, leading to a more redox-active cysteine. Importantly, the active site of any enzyme is more likely to be protected from the environment, which likely prevents exchange with bulkier thiols. Accordingly, more stable SNO is inversely correlated with thiolate exposure, and stabilized in protected environments. Indeed, nitrosylation of cellular phosphatases potentiates kinase cascades to promote cell growth, or inactivates lipid phosphatases during ischemic stroke.[51–53]Because of the selective targeting of functional cysteines, nitrosylation may serve as a general redox switch important for the reversible inactivation of functional cysteine residues. For

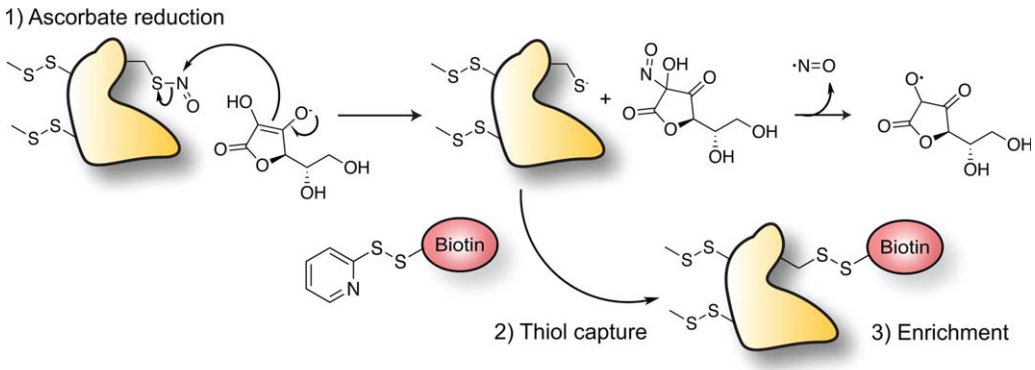

FIGURE 2 Biotin-switch technique for ascorbate-dependent reduction of nitrosothiols. Enriched proteins are either analyzed by SDS–PAGE or annotated by mass spectrometry.

example, nitrosylation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) recruits the ubiquitin ligase Siah1, and the GAPDH-Siah1 complex translocates to the nucleus where Siah1 targets nuclear proteins for degradation.[54–56] Similarly, argininosuccinate synthase, the enzyme that generates arginine from citrulline,[57] is inhibited by SNO at Cys132.[58] Elevated NO levels induce argininosuccinate synthase nitrosylation and inactivation, providing an autoregulatory loop that prevents excess oxidant production.

SNO can also directly compete other reversible cysteine post-translational modifications, such as protein palmitoylation. Upon synaptic stimulation, protein palmitoyl thioesterases remove the membrane anchoring lipids from the neuronal scaffolding protein PSD-95, promoting egress from the postsynaptic density.[59] Simultaneous activity-dependent calcium influx activates neuronal NO synthase (nNOS), which is stably associated with PSD-95 via PDZ-dependent interactions. This locally generated flux of NO directly nitrosylates the newly deacylated thiols, thus blocking further palmitoylation and membrane association. The mutually competitive modification of PSD-95 cysteines orchestrates synaptic release through an exchange of specific cysteine post-translational modifications.[59] Furthermore, SNO of the NMDA receptor leads to channel desensitization and channel closing, preventing excitotoxicity.[52] This cascade of post-translational events ensures proper membrane release of PSD-95, and ensures proper channel desensitization. This example raises the question if such mutually competitive modifications are unique for PSD-95, or if many proteins undergo dynamic exchange of cysteine PTMs. Furthermore, PSD-95 nitrosylation is channeled by protein–protein interactions, which provides selectivity for the deacylated thiols. The breadth of cysteine modifications competition is unknown. Interestingly, several palmitoylated proteins, including Ras and G-proteins, are rapidly depalmitoylated after receptor stimulation,[60–62] which coincides activation of NADPH oxidases and NO synthases.

ASCORBATE-DEPENDENT ENRICHMENT STRATEGIES

Sensitive and selective labeling tools are critical for the precise detection and annotation of SNO. Despite the acceptance of SNO as an important protein regulatory modification, there remains a lack of direct methods to study this reversible redox modification. Early approaches used chemiluminescent, colorimetric, or electrochemical methods to detect total NO liberated from nitrosylated thiols in a sample.[63] These methods measure bulk release, and do not distinguish between heme, metabolite, or protein sources. Furthermore, these approaches eliminate any information about the sites and dynamics of nitrosylation on select proteins.

The "biotin-switch" technique was a major advance in the study and annotation of *S*-nitrosylated proteins.[64] This widely adopted method involves a series of biochemical steps, beginning with addition of the alkylating agent 2-iodacetamide, or by methyl methanethiosulfonate (MMTS) to block all free thiols. After removing the thiol capture reagents, the sample is treated with ascorbate, which reduces nitrosothiols to generate free sulfhydryl groups. This approach is selective for SNO over other oxidative modifications, largely due to the unique mechanism of indirect reduction (Figure 2).[65,66] In the presence of nitrosothiols, ascorbate undergoes a trans-nitrosation reaction involving nitrosonium (NO^+) transfer to generate nitrosoascorbate, which decomposes to NO and the semidehydroascorbate radical. Therefore, ascorbate does not directly donate an electron for nitrosothiol reduction. This distinct mechanism is thought to provide chemical orthogonality to other oxidative modifications, making ascorbate an ideal nitrosothiol-selective reducing agent.[66] Following ascorbate-mediated reduction, the newly unmasked thiols groups are then captured using a pyridyldithiol-activated, sulfhydryl-reactive biotin-linked probes for affinity enrichment.[64] While these reagents are commonly available, several commercial kits are available that include ascorbate, metal-chelating buffers, MMTS, and

iodoacetamide detection reagents. Recent adaptations for quantitative proteomics labeling strategies are also commercially available, and have been used to quantify individual sites of SNO.[67] Thiol resin assisted capture (RAC) replaces biotin and streptavidin purification with direct disulfide capture to activated thiol resin immediately after ascorbate treatment.[68,69] This approach simplifies the procedure, and eliminates nonspecific enrichment of endogenous biotinylated carboxylases.

Ultimately, the biotin-switch purification method is indirect, and highly dependent on the complete alkylation of all free thiols. Protein nitrosylation is a low abundance modification, so even low levels of uncapped thiols can lead to a high false positive rate. Interestingly, a heating step is generally included during thiol capping, often to 50°C. *S*-nitrosothiols are known to undergo thermal decomposition via homolytic cleavage of the S—N bond to yield the corresponding disulfides and NO, the latter is then oxidized to nitrogen dioxide.[70,71] This step may introduce later complications, as the thermal stability of distinct nitrosothiols has not been thoroughly evaluated. Clearly, prolonged heating will promote NO release, and potentially suppress detection of labile nitrosothiols. Furthermore, after ascorbate reduction, newly free thiols are able to exchange with existing disulfide bonds, scrambling native sites of nitrosylation. RAC is likely to reduce the extent of scrambling by providing high excess of activated disulfides for immediate capture.[69] Another source of false-positives is sunlight driven disulfide reduction, which can be eliminated by performing all procedures in complete darkness.[72] This unfortunate restraint makes sample preparation more tedious, but is essential to eliminate nonspecific disulfide reduction. Overall, biotin-switch technique is the current standard for nitrosothiol labeling, enrichment, and analysis.

CAVEATS OF NITRIC OXIDE DONORS

Thousands of *S*-nitrosylated proteins have been reported from ascorbate-dependent enrichment using the biotin-switch method and mass spectrometry.[13] Unfortunately, nearly all reported proteomics data was collected from biological samples after the addition of exogenous or physiological nitrosylating agents. Upon donor release, gaseous NO is oxidized by molecular oxygen to form a peroxynitrite radical, which then reacts with a second NO molecule to generate two molecules of nitrogen dioxide. This reaction is limited by an apparent third-order rate law ($k = 2.5 \times 10^6\ M^{-2}s^{-1}$),[73–75] which means the reaction rate depends on the product of square of the NO concentration and molecular oxygen.[73] Therefore, at very low NO concentrations the reaction is very slow, but at high NO concentrations the reaction is extremely rapid. Thus, depending on the concentration of NO released, the half-life of the reaction can range from 0.5 s to 50 h.[73] When donors release NO at higher than physiological concentrations, it is likely that nonphysiological nitrosothiols are formed at less activated thiols. Therefore, it is difficult to interpret the results of proteomics experiments performed on donor treated samples.[76]

In a recent study, two NO donors (spermine NONOate and CysNO) were compared to understand their ability to form nitrosothiols.[77] Surprisingly, spermine NONOate ($t_{1/2}$ = 39 and 230 min) produces a high amount of DNIC and very low amount of nitrosothiols. Conversely, Cys-NO ($t_{1/2} \leq 2$ min)[77,78] efficiently produced nitrosothiols. Since, the majority of nitrosylation proteomics experiments rely on NO donor-treated samples, much of the current literature should be carefully interpreted. While NO donors are important tools for many experiments, it is important in the future to focus on detecting endogenous nitrosothiols.

EMERGING NITROSOTHIOL ENRICHMENT STRATEGIES

Given the limitations of the biotin-switch method, several alternative approaches have recently been reported (Figure 3A). In a series of innovative reports, Xian presents triaryl-substituted phosphines as a novel chemoselective reaction for conversion of nitrosothiols to a stable substituted thiobenzamide.[79,80] This reductive ligation reaction mechanism is similar to the Staudinger ligation, and is initiated by nitrosothiol reaction with the phosphine to form an azaylide, which then undergoes an intramolecular reaction and hydrolysis to yield the substituted thiobenzamide adduct. Additional variants of this reaction proceed by a similar azaylide intermediate, but undergo distinct rearrangements to yield varying products. The bis-ligation reaction uses phosphine-thioester probes to form disulfide-iminophosphorane products.[81] Importantly, the nitrogen originating from NO transforms to the iminophosphorane, providing an analytical linkage to both the originating NO and thiol. This methodology has been used to quantify the formation of *S*-nitrosoglutathione in activated macrophages by mass spectrometry, and allowed sensitive profiling of other nitrosylated metabolites in cell lysates.[82] The one-step disulfide method similarly uses a phosphine-thioester to first form the thiobenzamide adduct and thiolate, followed by intermolecular thioester exchange with the released thiolates to generate a disulfide linkage.[81] The reaction results in disulfide formation and elimination of the phosphine oxide. Finally, alkyl-aryl phosphines react with SNO generation of the azaylide, followed by reductive elimination to generate dehydroalanine.[83] Dehydrolalanine is an electrophilic Michael acceptor,

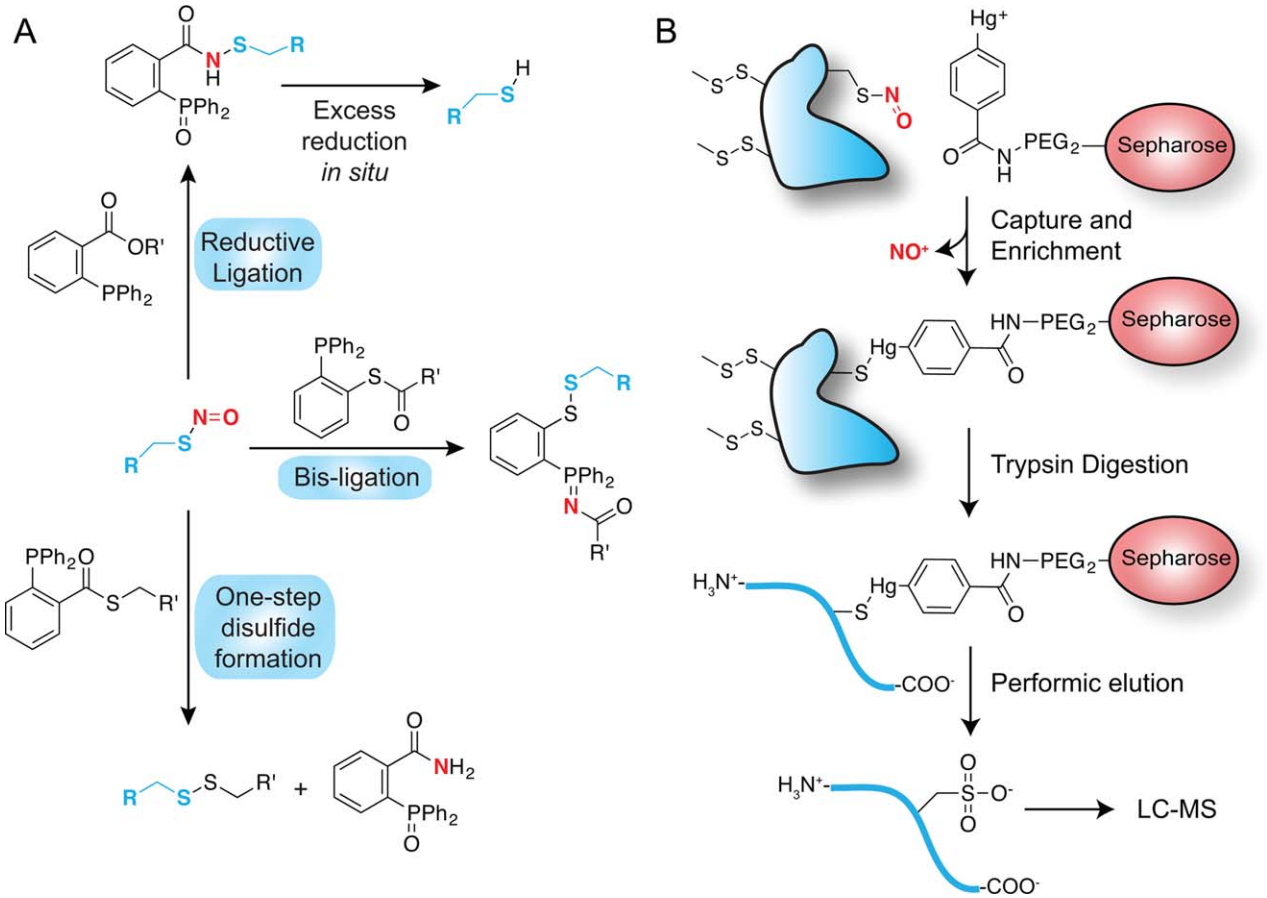

FIGURE 3 Chemoselective nitrosothiol labeling methods. In both approaches, free thiols are first blocked by addition of MMTS. (A) Triaryl-phosphine ligation methods. Three reactions are shown that describe recent reports of nitrosothiol-selective phosphine reactions. The reductive ligation approach was demonstrated on fixed cells, but led to over-reduction to the free thiol. The one-step disulfide formation reaction was demonstrated on cell lysates after nitric oxide donor treatment. (B) Phenyl-mercury enrichment of nitrosothiols for proteomic annotation. Sepharose beads or biotin are linked to phenyl-mercury for nitrosothiol enrichment, followed by trypsin digestion. Nitrosylated peptides are released from the resin by perfomic acid oxidation to the sulfonic acid for mass spectrometry annotation.

and can be linked to a variety of tagged nucleophiles for straightforward chemoselective enrichment.

In cell lysates, excess phosphine reagent led to complete reduction of nitrosothiols to free thiols,[79] suggesting these methods may require careful optimization. To avoid this pitfall, free thiols were first alkylated with NEM, followed by selective nitrosothiol reduction with triaryl-phosphines. The resulting free thiols were labeled using biotin or fluorophore-linked maleimide reagents for nitrosothiol detection. In gel-based experiments, phosphine reduction demonstrated superior selectivity to dithiothreitol, which nonselectively reduced disulfides and sulfenylated thiols.[79] This methodology was used in fixed cells to detect nitrosothiols after lipopolysaccharide stimulation in macrophages. Overall, these mechanistic phosphine probes show early promise as an alternative to ascorbate-dependent enrichment and proteomic analysis.

Organo-mercury enrichment methods have emerged as an alternative approach for direct nitrosothiol labeling and enrichment[84,85] (Figure 3B). Nitroso-cysteines react directly with phenylmercury to yield a stable thiol-mercury bond. This reaction is direct, selective, and highly efficient. By coupling the phenyl mercury to biotin or agarose beads, nitrosothiols can be directly labeled and enriched from tissue lysates. Bound peptides are then released from the resin by mild performic acid treatment, which oxidizes the thiol to the sulfonic acid for selective detection by mass spectrometry.[85] The fate of MMTS-capped thiols is not reported, although they are presumably simultaneously

oxidized. This suggests MMTS should be replaced with iodoacetamide reagent to block free thiols before analysis. Furthermore, methionine is similarly oxidized by perfomic acid to the sulfone, adding additional complexity to the proteomic analysis. Nonetheless, this method lead to the identification of nearly a thousand nitrosylation sites summed across mouse brain, heart, liver, kidney, lung, and thymus tissues. Furthermore, about half or more of these sites were absent in eNOS knockout mice, suggesting the majority of nitrosylation originates from NO synthases.[84] Interestingly, more than 70% of the nitrosylation sites in heart tissue were found on mitochondrial proteins.[84] Further analysis suggests widespread regulation of metabolic enzymes in involved in glycolysis, gluconeogenesis, pyruvate metabolism, the Kreb's cycle, oxidative phosphorylation, amino acid metabolism, ketone body formation, and fatty acid pathways. The simplicity of this method offers several advantages over ascorbate-dependent capture methods, particularly since it is a direct enrichment followed by a unique oxidation to sulfonate for direct proteomic annotation. Furthermore, this method is the first to report robust differences by comparative proteomic analysis of eNOS knockout mice. This data provides key evidence that NO synthase activation contributes to nitrosylation *in vivo*, and suggests supplementation with NO donors could have broader implications on cellular metabolism.

CONCLUSIONS

Protein SNO has emerged as an important oxidative post-translational modification. Several labeling methods have emerged that take advantage of the unique reactivity, either by reduction and capture, or by direct chemical labeling. Importantly, such methodologies have enabled proteomic analysis of SNO in cells and tissues. In conjunction with isotopic labeling methods, quantitative proteomic profiling of SNO will enable in-depth global profiling, independent of NO donors. With the development of selective methods for each distinct cysteine post-translational modification, multiplexed analysis has the potential to discover new cellular pathways orchestrated post-translational exchanges. Such analysis will integrate palmitoylation dynamics, and explore the mutual competition at distinct cysteine residues between NO and hydrogen peroxide. Importantly, each of these modifications are labile, and rapidly hydrolyzed in the presence of free thiols, such as glutathione. Therefore, we anticipate such tools will enable a greater understanding of the role of compartmentalization in thiol modification stability and function.

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