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SNOs Differ
Methodological and Biological Implications
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Cellular signaling by endogenously generated nitric oxide (NO) was initially characterized as dependent on the ability of NO to bind heme, and in particular the heme of guanylate cyclase, thereby enhancing production of cGMP. However, it is now recognized that protein S-nitrosylation, the oxidative modification by NO of the thiol side chain of cysteine (Cys) to generate an S-nitrosothiol (SNO; first described in 1992) conveys the large part of the cellular influence of NO. The critical distinction between earlier and contemporary models of NO biology is the novel and unanticipated redox chemistry of NO that underlies a ubiquitous redox-based mechanism for both allosteric and active-site regulation of protein function (note that NO binding to heme in guanylate cyclase does not entail redox chemistry). S-nitrosylation operates ubiquitously across phylogeny from plants to microbes to man, and in all cell types, to regulate the function of proteins in most or all classes, and provides by far the most sophisticated and broadly operative system for redox regulation and signaling. Furthermore, dysregulated S-nitrosylation has been implicated in an increasingly broad spectrum of human pathophysiologies, including prominently cardiovascular disorders. Notably, SNOs in proteins are represented by several different forms, with both biological and methodological implications.

The discrepancy in the populations of SNO-proteins are blocked by a disulfide-forming reagent, the NO group is cleaved from S-nitrosylated Cys by treatment with ascorbate, and the newly available Cys thiols are coupled by a disulfide to biotin for subsequent analysis. Variants of this method have proliferated. Alternative blockers of free thiols including alkylating agents, alternative reducers of the S–NO bond, and multiple alternative reagents for labeling nascent thiols after SNO-specific reduction have been described. In addition, phenylmercury has been used to label SNOs without previous reduction, and phosphine-based approaches show great promise. Recent advances in redox proteomics and the use of isobaric reagents in conjunction with mass spectrometry have led to not only the identification of an increasing number of SNO-proteins but has also permitted relative quantification across various samples, allowing for greater insight into the regulation of signaling by protein S-nitrosylation.

Unexpectedly, proteomic analyses including the new study of Chung et al that have used alternative labels for nascent thiols have generated large disparities between identified sets of SNO-proteins. Rigorous controls, combined with accumulating evidence that alternative redox-based modifications target nonoverlapping sets of thiols, indicate that specificity (or lack thereof) is not the cause of disparity. Rather, as described below, these disparities probably highlight the fact that all biological SNOs are not alike, and in particular that the SNO linkage can vary substantially in chemical character. In addition, the free thiols generated from the reduction of SNOs are also not identical in terms of reactivity.

Chung et al performed a modified biotin switch on mice hearts and S-nitrosoglutathione (GSNO)–treated human embryonic kidney cells using 2 different thiol-reactive reagents to label thiols generated by ascorbate. Although the isobaric reagents used, cysteine-reactive tandem mass tag (cysTMT) and iodoacetyl tandem mass tag, differed only in their cysteine-reactive groups, and the number of SNO-proteins identified by the 2 methods was similar, the SNO-proteomes identified overlapped by only ≈30%. In combination, Chung et al identified 1008 SNO-sites (371 sites by both reagents) in GSNO-treated human embryonic kidney 293 cell lysates, as well as 315 endogenous SNO-sites (98 sites by both reagents) in untreated samples from mouse hearts. Many previously undescribed targets of S-nitrosylation were found in cardiac samples, including SERCA2A, α-actinin-2, and isoform 3 of LIM domain-binding protein 3. Their analysis suggests that studies identifying SNO-proteomes using only a single label will detect incomplete sets of SNO-proteins.

The discrepancy in the populations of SNO-proteins identified by different methods can be ascribed to 2 aspects of SNOs: first, all SNOs are not identical because they vary...
in their structure (chemical nature) and second, the reactivity and local environment of all S-nitrosylated cysteines residues are not identical. With regard to structural differences between SNOs, the chemical structure of the SNO group is determined by several factors, which reflect its reactivity. The R–S–NO bond dihedral angle may be present in cis- or trans-configuration. Furthermore, SNOs can have varying degrees of double-bond character (\(R–S–N=O\) to \(R–S+=N–O\)–) or might carry an unpaired electron (RSNO or RSNOH). SNOs can also interact with another thiol to form a nitroxyl disulfide [(RS)_2NO−]. Moieties in the near vicinity of SNOs including the aromatic amino acid tyrosine can mediate π–π interactions, and hemes can also influence the character of the SNO (ie, heme/thiol/NO redox coupling). SNOs of various forms cannot be differentiated from each other with current detection methods. Indeed, as far as is known, the sensitivity and specificity of current methods has only been determined for the simple cis-dihedral R–S–N=O. It should be noted that Chung et al11 used somewhat dissimilar conditions to reduce SNOs before labeling: 1 mmol/L ascorbate/1 mmol/L copper for cysTMT and 5 mmol/L ascorbate for iodoacetyl tandem mass tag. Because inclusion of copper changes the chemical mechanism by which NO is liberated from S–NO,19 it seems likely that the reactivities and chemistries of varied SNO-forms would differ under these disparate conditions. Moreover, the thermodynamic grounds for specificity of the biotin switch for SNO versus alternative oxidative modifications of thiols (sulfenic acid and mixed disulfides)14 does not hold in the presence of copper and so caution is warranted.

Another recent study used 2 different methods to enrich for isoproterenol-stimulated S-nitrosylation of calcium handling proteins in the heart.20 Although the phenylmercury and biotin switch methods identified similar numbers of SNO-proteins under resting conditions, the proportion of those that showed increases following isoproterenol was greater with cysTMT and 5 mmol/L ascorbate for iodoacetyl tandem mass tag. Because inclusion of copper changes the chemical mechanism by which NO is liberated from S–NO,19 it seems likely that the reactivities and chemistries of varied SNO-forms would differ under these disparate conditions. Another recent study used 2 different methods to enrich for isoproterenol-stimulated S-nitrosylation of calcium handling proteins in the heart.20 Although the phenylmercury and biotin switch methods identified similar numbers of SNO-proteins under resting conditions, the proportion of those that showed increases following isoproterenol was greater with phenylmercury. Again, the disparity in targets identified by the 2 methods can most likely be attributed to the existence of multiple SNO-populations.

Although not completely understood, the factors that determine the target specificity of S-nitrosylation include the local environment of the cysteine residue and the nature of the NO donor/source.21,22 Transnitrosylation from an SNO-protein or from low-molecular weight SNOs including GSNO or S-nitroso-coenzyme A is an important mode of S-nitrosylation. Distinct sets of proteins are S-nitrosylated by GSNO or S-nitroso-coenzyme A21,23 demonstrating differences in accessibility/reactivity of cysteines. By the same rationale, it seems likely that labeling and subsequent identification of SNO-sites reflect the nature of the thiol generated by ascorbate-dependent reduction, even when proteins are denatured (see below). In this regard, it is of interest that, compared with iodoacetyl tandem mass tag–labeled cysteines, cysTMT-labeled cysteines tended to be surrounded by more positively charged residues and cysTMT-labeled SNO-proteins also had a higher aliphatic index.14 In addition, enhanced labeling of samples from GSNO reductase knockout (GSNOR−/−) mice, which would preferentially promote SNO-sites that are regulated by GSNO levels, was preferentially observed with cysTMT.

Cysteine labeling reagents used for proteomics fall mainly into 2 classes: pyridyl-dithiol-based reagents that reversibly label thiols via a disulfide bond (including cysTMT, biotin HPDP, and thiopropyl sepharose) and iodoacetyl-based reagents that irreversibly alkylate cysteines by nucleophilic substitution of iodine with the sulfur atom (including iodoacetyl tandem mass tag and isotope-coded affinity tag). Other methods circumvent the reduction step and use reagents that directly react with SNO-cysteine. One such approach uses the reaction of phenylmercury compounds with SNO-cysteines (after blocking of free thiols) to form a tight bond with mercury to capture SNO-proteins.15 Direct reaction of SNOs with triarylpiphosphine has also been used to quantify GSNO in cells14 and early reports from the Tannenbaum group indicate that this reagent may provide unique molecular signatures for SNO-proteins as well. Considering the diverse chemistries involved in the labeling of cysteines using different reagents, it is easy to envision that the same level of sensitivity of detection will not be achieved with all reagents across all subpopulations of SNO-proteins. Thus, the use of >1 label, preferably involving different chemistries, would optimize capture of the SNO-proteome, a large proportion of which may otherwise be missed (Figure).

Some of the differences among SNO-proteins that might be expected to influence detectability will be negated by the fact that the detection assays are carried out under denaturing conditions (and certain SNO populations that are stabilized by secondary, tertiary or quaternary structure would be lost). However, commonly used denaturing detergents (sodium dodecyl sulfate) are highly charged and might themselves influence the efficacy of reducing and labeling reactions. Also, it is possible that denaturation of proteins might be incomplete. Several examples of sodium dodecyl sulfate–resistant protein complexes are known, which are dissociated only at high temperatures. Again, current methods to detect and identify SNO-proteins are based on the chemistry of a simple SNO and are aimed at differentiating between SNOs and other cysteine redox modifications, in particular sulfenic and disulfides. In light of these new studies,13,20 SNO-detection protocols should be modified to optimize identification of multiple SNO-populations that exist in biological samples.

Cellular S-nitrosylation levels, and hence the SNO-proteome, are regulated in substantial part by denitrosylases. GSNO shifts the equilibrium toward protein denitrosylation by reducing GSNO and thereby regulates cardiovascular function and cardioprotection.24,25 A GSNOR−/− mouse has inherently higher levels of endogenous protein S-nitrosylation and consequently can be used in proteomic studies to facilitate the identification of SNO-sites. Chung et al17 identified 493 SNO-sites in GSNOR−/− hearts, and many of these were hypernitrosylated compared with wild-type hearts. Irie et al20 established the specificity of a subset of SNO-proteins identified in their study by comparing SNO-levels in wild-type with SNO-levels in transgenic mice, in which GSNOR was overexpressed. As expected, S-nitrosylation of target proteins was diminished in the transgenic mouse samples. That levels of SNO proteins are increased on knockout of GSNOR and decreased by over-expression of GSNOR as assessed by both biotin-switch and
phenylmercury methods represents a powerful genetic demonstration of the specificity of the current methodologies toward SNOs.

S-nitrosylation in the cardiac system is precisely regulated, but the factors influencing the cardiac SNO-proteome are not fully understood. Chung et al\textsuperscript{13} found not only hyper-nitrosylated SNO-sites in GSNOR-knockout mouse hearts but also a significant number of hyponitrosylated SNO-sites. S-nitrosylation is known to regulate transcription and to modulate stability of proteins. There are numerous examples of S-nitrosylation facilitating degradation of proteins, including the phosphatase and tensin homolog,\textsuperscript{26} iron-regulatory protein-2,\textsuperscript{27} and Pias3.\textsuperscript{28} However, S-nitrosylation also increases the stability of many proteins, including membrane repair protein TRIM72,\textsuperscript{4} HIF-1α,\textsuperscript{29} and Bcl2.\textsuperscript{30} Because S-nitrosylation can alter protein production and stability and thereby protein abundance, it may be informative, although often overlooked, to evaluate SNO-protein levels relative to protein abundance under conditions of altered S-nitrosylation.

The issues faced in the identification of the SNO-proteome are largely reminiscent of the challenges faced in identification of the phosphoproteome. Different phosphosite sub-populations of SNO-proteins

![Figure. Hypothetical situations leading to differential identification of subpopulations of S-nitrosothiol by different methods.](http://circres.ahajournals.org/)

because of multiple factors (e.g., differential enrichment of diverse subsets of phosphoproteins and interference with proteolytic digestion by phosphorylation itself). The use of multiple proteases and enrichment methods has been proposed to facilitate identification of the phosphoproteome.

The methodologies used to identify the SNO-proteome are rapidly evolving and have led to the identification of thousands of SNO-proteins in various tissues and conditions. The results of Chung et al\textsuperscript{13} emphasize the wisdom of using >1 strategy to assure adequate coverage of the SNO-proteome. More generally, however, inasmuch as SNOs differing with respect to structure and source of NO group can be predicted to have different effects on protein function,\textsuperscript{32,33} more specific methods that can distinguish between different SNO forms will be needed for understanding the broader repertoire of NO-based protein modification and the molecular code through which alternative redox-based modifications elicit different effects. Careful validation of current methods with respect to different SNO subtypes and new approaches to detect specific SNOs will potentially reveal the full extent of regulation of cell signaling by S-nitrosylation and thus elucidate its essential role in normal physiology and disease causation.
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References


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