

Nitrosative Stress in the Nervous System: Guidelines for Designing Experimental Strategies to Study Protein S-Nitrosylation

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Received: 26 March 2015 / Revised: 11 June 2015 / Accepted: 12 June 2015 / Published online: 29 June 2015
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Abstract Reactive nitrogen species, such as nitric oxide (NO), exert their biological activity in large part through post-translational modification of cysteine residues, forming S-nitrosothiols. This chemical reaction proceeds via a process that we and our colleagues have termed protein S-nitrosylation. Under conditions of normal NO production, S-nitrosylation regulates the activity of many normal proteins. However, in degenerative conditions characterized by nitrosative stress, increased levels of NO lead to aberrant S-nitrosylation that contributes to the pathology of the disease. Thus, S-nitrosylation has been implicated in a wide range of cellular mechanisms, including mitochondrial function, proteostasis, transcriptional regulation, synaptic activity, and cell survival. In recent years, the research area of protein S-nitrosylation has become prominent due to improvements in the detection systems as well as the demonstration that protein S-nitrosylation plays a critical role in the pathogenesis of neurodegenerative and other neurological disorders. To further promote our understanding of how protein S-nitrosylation affects cellular systems, guidelines for the design and conduct of research on S-nitrosylated (or SNO-)proteins would be

highly desirable, especially for those newly entering the field. In this review article, we provide a strategic overview of designing experimental approaches to study protein S-nitrosylation. We specifically focus on methods that can provide critical data to demonstrate that an S-nitrosylated protein plays a (patho-)physiologically-relevant role in a biological process. Hence, the implementation of the approaches described herein will contribute to further advancement of the study of S-nitrosylated proteins, not only in neuroscience but also in other research fields.

Keywords S-Nitrosylation · Reactive nitrogen species · Reactive oxygen species · Nitric oxide · Neurodegeneration · Biotin-switch assay

Abbreviations

GSNO	S-Nitrosoglutathione
MS	Mass spectrometry
NO	Nitric oxide
NOS	NO synthase
SNO	S-Nitrosylation or S-nitrosothiol
SNOC	S-Nitrosocysteine

Special Issue: In honor of Dr. Philip Beart.

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Background on Protein S-Nitrosylation

In mammalian cells, three nitric oxide synthases (NOSs), namely NOS1 (or neuronal NOS), NOS2 (or inducible NOS) and NOS3 (endothelial NOS), are responsible for NO production from arginine [1]. In large part, the biological effects of NO are mediated through a redox reaction, S-nitrosation, leading to the post-translational modification of S-nitrosylation, which affects protein function in many ways analogous to phosphorylation. In this reaction, an NO moiety interacts with the thiol (–SH) group, or perhaps

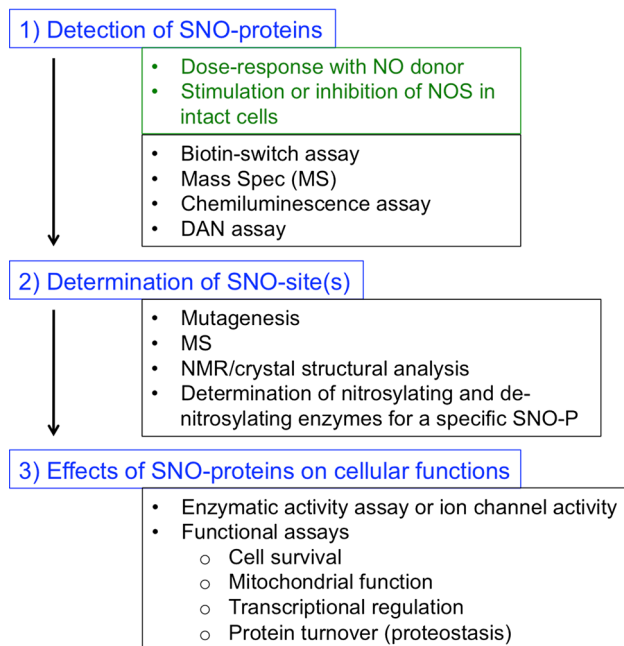


Fig. 2 Proposed experimental scheme to study protein S-nitrosylation. Initially, presence of a S-nitrosylated protein (SNO-protein) of interest can be detected by the biotin-switch assay (best if performed in human tissue for relevance), mass spectrometry (MS), or other methods. In order to observe SNO-protein formation under physiological conditions, a dose-response with NO donors (e.g., SNOC or GSNO) or, even better, endogenous NO should be employed. For the mapping of S-nitrosylation site(s), site-directed mutagenesis is necessary of the putative critical cysteine residue, followed by MS, NMR, and crystal structure analysis. Lastly, in order to demonstrate that a specific SNO-protein significantly contributes to a biological process, functional assays should be explored preferably with a non-nitrosylatable cysteine mutant. Determining the endogenous enzymatic system (S-nitrosylase and denitrosylase) for a given SNO-protein is also a critical experimental avenue

study. Physiological concentrations of NO are believed to be less than 1 μM [22, 23]. In order to avoid non-physiological, artifactual generation of SNO-proteins, cells should be exposed to <200 μM exogenous SNOC or GSNO and a careful dose-response curve for producing S-nitrosylated protein should be performed. Importantly, the biotin-switch assay should be employed to verify that ‘endogenously produced NO’ can give rise to the formation of SNO-proteins in cellular or animal models of the disease of interest (if possible, in patient-derived tissue samples), strengthening the notion that the SNO-proteins play a patho-physiological role. When detecting endogenous SNO-proteins, proper controls should be included in the biotin-switch assay to validate the quality of the result, as this assay can generate a SNO-independent biotinylation (i.e., false positive) result [24]. For example, omission of ascorbate or ‘pre-photolysis of the S–NO bond’ should attenuate the biotinylation of S-nitrosylated cysteine residues, validating that the target protein was indeed

S-nitrosylated. For troubleshooting the biotin-switch assay, readers are referred to the excellent articles by Stamler and colleagues [24, 25]. Other important assays for SNO-proteins include, for example, the 2,3-diaminonaphthalene (DAN) assay on purified or recombinant protein, and chemiluminescent assays [2, 12, 19].

Note that commercially available antibodies against S-nitrosylated cysteine often suffer from non-specific binding. Thus, if one decides to use SNO-antibodies, experiments should include carefully-designed negative controls to verify that the candidate protein is really S-nitrosylated. Moreover, the recruitment of a confirmatory experiment with another assay, such as the biotin-switch method, is strongly encouraged. In our hands and those of many of our colleagues, commercially available SNO-antibodies are in general not reliable and may yield artifactual results.

Mapping SNO Site(s) on Proteins

As alluded to earlier, endogenously produced NO can modify specific cysteine residues. In order to determine the target cysteine residue(s), researchers often adopt a site-directed mutagenesis technique, specifically substituting cysteine residues with another conservatively-changed amino acid, such as alanine or serine. When a critical cysteine residue is mutated, this should significantly diminish the SNO signal on the biotin-switch assay, thus indicating the SNO site. However, some caveats are known to be associated with this approach. Mutation of a cysteine residue may perturb the overall structure of the protein, potentially altering the sensitivity of other cysteine residues to S-nitrosylation. In addition, because the biotin-switch assay is not a quantitative method, if a protein contains two or more S-nitrosylation sites, the removal of a single SNO site may not significantly decrease the SNO signal. To overcome these potential problems, mass spectrometry (MS), NMR structure, and crystal structure analysis of wild-type SNO-protein (non-mutated with non-nitrosylatable mutant as a control) may be alternative approaches, albeit these assays often require the presence of special instruments and core facilities. Typically, purified proteins are used for these alternative analyses, as it is extremely challenging to observe endogenous S-nitrosylation of a protein in a ‘top-down’ fashion. Additionally, concerning MS analysis, a more mild, electron transfer dissociation technique (ETD), in lieu of the more harsh, conventional collision-induced dissociation technique (CID), should be explored because of the lability of SNO modifications [14]. NO is often a good ‘leaving group’ and thus it may also be replaced by reactive oxygen species (ROS), leading to sulfenated (RSOH), sulfinated (ROS₂H), or sulfonated

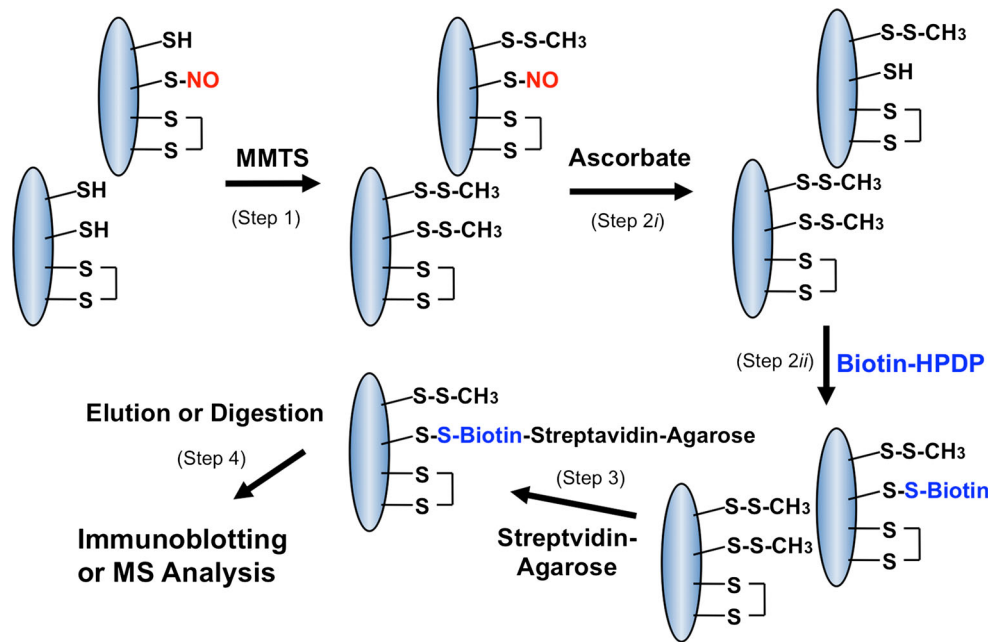


Fig. 3 Steps in the biotin-switch assay for the detection of S-nitrosylated proteins. *Step 1:* After preparation of cell or tissue lysates, free thiols are blocked by incubation with methemethanesulfonate (MMTS) under denaturing conditions. The unreacted MMTS is then removed by acetone precipitation. *Step 2:* S-nitrosothiols are selectively reduced by ascorbate, and then the reformed free thiols are

labeled with *N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide (biotin-HPDP). *Step 3:* The biotinylated proteins are collected by a biotin-avidin affinity purification procedure, e.g., on beads. *Step 4:* Proteins eluted from the beads are separated by SDS-PAGE and subjected to immunoblot analysis. Alternatively, 'proteins on beads' or 'proteins eluted from beads' can be digested by trypsin for MS analysis

(RSO₃H) adducts; these may be physiologically or pathologically relevant substitutions depending on the conditions under which they were observed [16, 26].

Effects of S-Nitrosylation on Protein Function

After determining the SNO-site, the next step is to study the effect of S-nitrosylation on protein activity. If the target protein possesses known catalytic properties, these experiments might entail assays of enzymatic activity *in vitro* and *in situ*. Alternatively, proteins functioning as ion channels or carriers, transcription factors, chaperones, and binding partners can all be affected by S-nitrosylation. S-Nitrosylation can inhibit or increase protein activity, depending on the target. For example, NO may modify a catalytically-active cysteine to form an S-nitrosothiol, abrogating its enzymatic activity [9, 11, 16, 26]. Alternatively, S-nitrosylation can positively or negatively regulate protein activity in an allosteric manner [12, 15]. In addition, one should evaluate if the SNO-protein influences downstream biological signaling. One approach involves expressing a non-nitrosylatable cysteine mutant in specific cells *in vitro* or in the whole animal. In particular, in cases of allosteric regulation of protein function by S-nitrosylation, many studies have

shown that mutation of the target cysteine residue typically spares basal activity. The prediction would be that cells expressing non-nitrosylatable mutant would remain unresponsive to NO compared to wild-type transfected cells, strongly suggesting a causal link between SNO-protein formation and the biological processes in question (see References [12, 15] as examples). Recent advancements in gene editing technologies, including ZFNs (zinc-finger nucleases), TALENs, and CRISPR/Cas9 [27], provide tools to introduce site-specific genome modifications with high precision. Future studies should investigate the role of endogenous nitrosylation using these techniques to introduce the non-nitrosylatable mutant.

In contrast to allosteric modulation, when analyzing a SNO-protein whose S-nitrosylation occurs on an active-site cysteine, mutation may not provide a mechanistic link between SNO-protein formation and downstream effect because mutation of this cysteine itself leads to the generation of an activity-dead enzyme in the absence of NO. As an alternative, researchers are encouraged to employ additional approaches. For instance, RNAi-mediated knockdown of the target protein may mimic the effects of S-nitrosylation. Reduced expression of target protein by genetic alteration may render cells or animals insensitive to a particular effect of NO due to lack of the target cysteine.

Conclusions

The goal of this ‘COMMENTS’ article is to provide an overview of experimental procedures to investigate protein S-nitrosylation. Strategic considerations for experimental design are presented in an attempt to standardize the procedures for investigating SNO-proteins and avoiding artifacts. In the coming years, a number of improved or novel methods are expected to emerge, allowing further refinement of the approach to redox-mediated chemical biology in general and S-nitrosylation in particular.

Acknowledgments This work was supported in part by NIH Grants P30 NS076411, R01 NS086890, R01 ES017462, P01 HD029587, and R21 NS080799 (SAL), the Brain and Behavior Research Foundation (SAL), and the Michael J. Fox Foundation (SAL and TN).

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