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REVIEW ARTICLE

Development and application of site-specific proteomic approach for study protein S-nitrosylation

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Abstract Protein S-nitrosylation is the covalent redox-related modification of cysteine sulfhydryl groups with nitric oxide, creating a regulatory impact similar to phosphorylation. Recent studies have reported a growing number of proteins to be S-nitrosylated in vivo resulting in altered functions. These studies support S-nitrosylation as a critical regulatory mechanism, fine-tuning protein activities within diverse cellular processes and biochemical pathways. In addition, S-nitrosylation appears to have key roles in the etiology of a broad range of human diseases. In this review, we discuss recent advances in proteomic approaches for the enrichment, identification, and quantitation of cysteine S-nitrosylated proteins and peptides. These advances have provided analytical tools with the power to interpret the impact of S-nitrosylation at the system level, providing a new platform for drug discovery and the identification of diagnostic markers for human diseases.

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Introduction

The discovery of nitric oxide (NO) as a regulator of cellular redox has emerged as a key cellular mechanism for scientists to study, particularly its impact on signal transduction and cellular function (Tuteja et al. 2004). In mammalian cells, NO is mainly produced by three types of NO synthases (NOSs): neuronal NOS (nNOS), endothelial (eNOS) and inducible NOS (iNOS), through the conversion of L-arginine to NO and L-citrulline (Huerta et al. 2008). NO was involved in the physiological regulation of most major mammalian systems, including cardiovascular, respiratory, gastrointestinal, and reproductive as well as host defense (Derakhshan et al. 2007). Initially, NO was thought to interact with transition metal ions such as the heme-ion in soluble guanylate cyclase. In recent years, protein S-nitrosylation, the covalent modification of NO on cysteine thiol, has been recognized as a main mechanism by which NO modulates protein activity and transduces NO bioactivity (Hess et al. 2005). Protein S-nitrosylation has been reported to regulate diverse pathways such as G protein-coupled receptor signaling, death receptor-mediated apoptosis, and glutamate-dependent neurotransmission (Forrester et al. 2009a). It is now understood that aberrant S-nitrosylation of specific protein targets contributes to disease pathogenesis (Foster et al. 2003, 2009b). For example, aberrant S-nitrosylation is implicated in cardiopulmonary, inflammatory, skeletomuscular and neurodegenerative diseases (Foster et al. 2003, 2009b). In addition, deregulation of S-nitrosylation is implicated in tumor initiation and progression (Lim et al. 2008; Forrester et al. 2009a). The increasing prominence of the role of

S-nitrosylation in disease supports a need for improved analytical methods to identify and quantify S-nitrosylated proteins under various physiological and pathophysiological conditions for investigative studies and clinical diagnosis.

Detection and identification of S-nitrosylated proteins and peptides

Mass spectrometry (MS)-based proteomics holds tremendous potential for detecting and mapping covalent protein post-translational modifications (PTMs) and quantifying their changes (Witze et al. 2007). However, direct analysis of protein S-nitrosylation by MS has been challenging, primarily owing to the inherent chemical instability of the S–NO bond. S-nitrosylated proteins are sensitive to UV light, reducing conditions especially in the presence of heavy metals, and are difficult to observe by MS analysis (Forrester et al. 2009a). The S–NO bond can be lost or gained artificially during sample preparation (Mannick and Schonhoff 2008).

Direct identification of protein S-nitrosylation by MS

Direct identification of the S-nitrosylation site by MS is always preferred as the identification results would authenticate the occurrence of S-nitrosylation, minimize the chance of inducing false-positives during sample preparation and better reflect the in vivo situation. Pioneering work in this field has shown that the S–NO bond is UV sensitive, preventing the analysis of S-nitrosylated peptides by matrix-assisted laser desorption ionization (MALDI) (Kaneko and Wada 2003). Published reports have suggested that fine-tuning of MS instrument parameters could enable the direct determination of peptide S-nitrosylation sites under soft electrospray ionization (ESI) conditions. S-nitrosylated peptides have been observed as a +29 Da ion (M-H + 30) over the unmodified peptide ions for each S-nitrosylated cysteine (Kaneko and Wada 2003). For direct analysis of S-nitrosylated peptides, several reports have been published using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) on a variety of MS instruments (Mirza et al. 1995; Taldone et al. 2005; Chen et al. 2007; Hao and Gross 2006; Hao et al. 2004; Wang et al. 2008; Liu et al. 2010). The drawback of these approaches is that all reported examples focused on a few synthetic S-nitrosylated peptides or on a single and well-characterized S-nitrosylated protein digests. They have not been shown to be suitable for high-throughput analysis of unknown S-nitrosylation sites in complex biological samples.

Indirect identification of protein S-nitrosylation by MS

The majority of other approaches for detecting protein S-nitrosylation by MS involve indirect methods, such as measuring free NO levels after cleavage of the S–NO bond or converting the S–NO bond into an S-detectable tag (Torta et al. 2008). In 2001, a strategy called biotin switch technique (BST) was designed to purify and detect S-nitrosylated proteins (Jaffrey and Snyder 2001), which lead to subsequent studies probing S-nitrosylation using standard molecular methods such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), immunodetection, and MS (Jaffrey and Snyder 2001; Forrester et al. 2009a). BST consists of three principal steps: (1) blocking of free cysteine thiols by S-methylthiolation with methyl methane thiosulfate (MMTS), a reactive thiosulfonate; (2) conversion of SNOs to thiols via transnitrosation with ascorbate; and (3) S-biotinylation of the nascent thiols with *N*-[6-(biotinamido)hexyl]-3′-(2′-pyridyldithio) propionamide (biotin-HPDP) (Jaffrey and Snyder 2001). The key to this methodology is the substitution of the NO group with a molecule bound to a biotin tag that enables one to select and enrich the modified proteins by affinity chromatography on streptavidin column. Although the identification of protein S-nitrosylation is performed indirectly, one major advantage of BST is that it enables the enrichment of low abundant post-translationally modified proteins, increasing the likelihood of their detection. Recently, some variations of this method have been developed that go deeper in the characterization of S-nitrosylated proteins with the aim to improve the detection sensitivity, to unambiguously recognize the modified cysteine sites and to design a proper control to minimize the false-positive identifications. These steps in BST are illustrated in Fig. 1 and are discussed below.

Improving the blocking efficiency of free thiols

Effective blocking of free thiols is an essential step for BST and other similar approaches. In the very first step of BST, combination of heat and SDS functions to denature proteins, thus granting MMTS optimal access to natively buried protein thiols (Forrester et al. 2009a). However, detergent such as SDS is notoriously detrimental in ESI–LC–MS/MS analysis and is difficult to fully remove from a protein/peptide preparation. As an alternative, urea has been used to denature the protein (Han and Chen 2008). By applying the detergent-free BST combined with LC–MS/MS analysis, Han and Chen (2008) were able to identify 44 S-nitrosylation sites on 35 distinct proteins with good reproducibility in S-nitrosoglutathione (GSNO)-treated HeLa cell extracts. Regardless of the use of the detergent or urea, most of the blocking strategies are still performed on

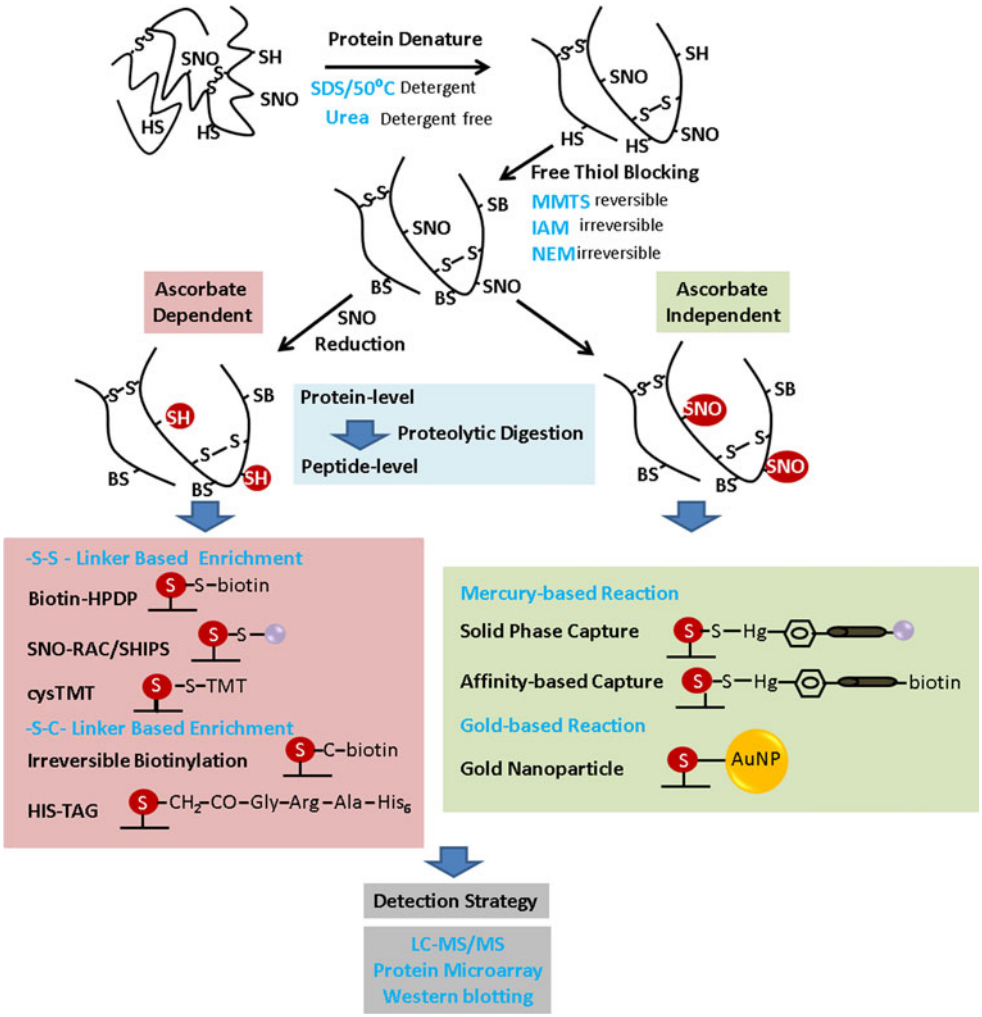

Fig. 1 Site-specific proteomic strategies for protein S-nitrosylation analysis

a partially unfolded protein structure as the reduction of intermolecular disulfide bonds is generally avoided. It is believed that the reduction of disulfide bond may also cause dissociation of NO from S-nitrosylated cysteine, thus compromising the sensitivity of the assay (Retamal et al. 2006). Moreover, as an alternative to MMTS, it was suggested to use the S-alkylating agent such as the *N*-ethylmaleimide (NEM) (Murray et al. 2011b) or iodoacetamide (IAM) (Chen et al. 2010) to block the free thiols. Thus, an irretrievable bond could be formed on free cysteine thiol instead of a disulfide bond linker. Lower reaction temperature thus could also be applied to avoid thermolysis of SNO (Chen et al. 2010). In spite of these adjustments, some protein thiols could be still resistant to complete blocking, which will lead to "false-positive" identifications. Regarding this issue, several controls have been designed for exclusion of the false-positive signals that may result from incomplete blocking, which will be discussed later.

Ascorbate dependent enrichment of S-nitrosylated proteins/peptides

The second step of BST is the conversion of protein-SNO to a free thiol (Forrester et al. 2009a). This is achieved by treating with ascorbate to promote a transnitrosation reaction with the protein-SNO. The specificity of the reaction is supported by thermodynamic measurements and by several experimental validations (Forrester et al. 2009a). Based on the ascorbate-reduced free thiol reaction, several strategies have been designed for the enrichment of S-nitrosylated proteins/peptides.

An "–S–S–" linker-based enrichment

In the original design of BST, a disulfide bond linkage was formed between the new formed thiol and biotin-HPDP (Jaffrey and Snyder 2001). The S-biotinylated proteins could then either be detected by western blotting or be

purified and enriched for subsequent analysis (Jaffrey and Snyder 2001). Recently, a modified BST assay using an anti-biotin antibody and a fluorescent-labeled secondary antibody has been reported for detecting SNO-proteins through microarray-based analysis (Foster et al. 2009a). The protein microarray study provides several advantages for studying protein S-nitrosylation, including the independence of MS for high-throughput analysis and the ability to perform relative quantification (Foster et al. 2009a). To extend the capability of the BST approach for site-specific identification, Hao et al. (2006) designed the SNOSID approach by simply introducing a proteolytic digestion step before avidin capture. This added step provides the selective isolation of peptides that previously contained SNO-Cys residues, rather than intact S-nitrosylated proteins (Hao et al. 2006). A total of 68 S-nitrosylated peptides from 56 proteins were identified in rat cerebellar after the treatment with GSNO (Hao et al. 2006).

As an alternative to the biotin-HPDP based affinity purification strategy, a thiol-reactive resin was utilized to capture and enrich the S-nitrosylated proteins/peptides generated by ascorbate treatment. The method was termed SNO-RAC (Forrester et al. 2009b). It was suggested that by combining the obligatory 'labeling' and 'pulldown' steps, SNO-RAC required fewer steps and could detect high-mass S-nitrosylated proteins more efficiently (Forrester et al. 2009b). An additional advantage of SNO-RAC is that a covalent disulfide linkage is directly formed between the reduced SNO site on the protein and the resin making the preparation amenable to 'on-resin' trypsinization and peptide labeling, which subserve mass spectrometric methodologies (Forrester et al. 2009b).

Reductant-dependent elution of S-nitrosylated protiens/peptides

Despite the improvements in method design for S-nitrosylation analysis, the ambiguity in site identification remains as an issue. One concern is that some protein disulfide bonds may be falsely identified as S-nitrosylation sites, because the BST, SNOSID and SNO-RAC all rely on the formation of disulfide bond to enrich the S-nitrosylated proteins/peptides. When MMTS was used as the blocking agent and the reductant such as dithiothreitol (DTT) or β-mercaptoethanol (ME) was used to elute the purified S-nitrosylated proteins/peptides, it was impossible to distinguish the S-nitrosylated cysteine from the cysteine originating from the intermolecular disulfide bond. The unambiguous identification of the modified cysteine is crucial for understanding how S-nitrosylation can modulate protein activity. To address this issue, we have designed a novel proteomic approach for site-specific high-throughput identification of protein S-nitrosylation (SHIPS) (Liu et al. 2010). In the first step of the SHIPS, DTT is added to the protein sample to reduce the intermolecular disulfide bonds. Free thiols and thiols originated from the intermolecular disulfide bonds are then alkylated with acrylamide. Followed by proteolytic digestion with trypsin, S-nitrosylated peptides are then selectively reduced with ascorbate and get enriched using a thiol affinity resin. The peptides are eluted by incubating with DTT and then alkylating a second time with IAM. These two commonly used alkylation agents yield differently added mass thus free and disulfide-bonded cysteines could be differentiated from the S-nitrosylated ones. We found that about 17 % of the identified peptides contain more than two cysteines. Application of two different alkylation agents offers a simple and effective way to accurately localize the protein S-nitrosylation sites and it reduces the possibility of false-positive identification from the cysteine-containing peptides which non-specifically bind to the cysteine affinity resin. Though DTT is accepted as a quantitative dithiol reducing agent (Cleland 1964), it is conceivable that a small fraction of the disulfides in a complex proteome would not be reduced. If those dithiols were possibly reduced by ascorbate, then we would see those peptides as false-positives in our method as well. In addition, it has been reported that DTT is capable of reducing S-nitrosylated thiols (Derakhshan et al. 2007). Though this is of concern for our method, we have identified most of the currently accepted S-nitrosylated peptides using our method and we have found substantially more S-nitrosylated peptides than other methods currently in use. The relatively high number of S-nitrosylation sites reported in our study is largely attributed to the superior performance of our in-house developed nanoscale LC system, which was originally described for use in highly sensitive phosphoproteomic analysis (Zhao et al. 2009).

Reductant-independent elution of S-nitrosylated proteins/peptides

In order to accurately localize the S-nitrosylation site, the interference of intermolecular disulfide bonds in the original design of BST can be readily ruled out when the biotin label is not removed during analysis. For instance, acid can be used as the elution buffer instead of reductant. This will keep the mixed disulfide bond untouched. The S-nitrosylation site thus could be recognized with a mass shift of 428 Da provided by the biotin-HPDP tag. This strategy has been used to identify 20 S-nitrosylated peptides belonging to 18 proteins in human aortic smooth muscle cells upon exposure to Cys-NO and NONOate, two commonly used NO donor (Greco et al. 2006). Recently, a new isotope-coded cysteine thiol-reactive multiplex reagent, called the cysteine reactive tandem mass tag

(cysTMT), was used in place of biotin (Murray et al. 2011b). The reaction is still based on ascorbate-reduced free thiol and the formation of disulfide bonds. TMT-labeled peptides could be captured with a TMT affinity resin and eluted with a distinguishingly added mass of 304 Da without breaking the disulfide bond. There are up to 6 isotopically balanced reporter ions between 126 and 131 Da thus providing a lower added mass for S-nitrosylation analysis (Murray et al. 2011b). For protein-level identification, the cysTMT labeled proteins could be detected by incubating with anti-TMT primary antibody followed by an anti-mouse alkaline phosphatase conjugated secondary antibody (Murray et al. 2011b).

An "–S–C–" linker-based enrichment

Besides the above-mentioned disulfide bond formation (–S–S-linker)-based enrichment strategies, other enrichment strategies for S-nitrosylation have been developed, providing an distinguished added mass for accurate identification of the S-nitrosylation site. For example, Huang et al. described an irreversible biotinylation procedure (IBP) for S-nitrosylation study (Huang and Chen 2010). In their study, biotin-maleimide was used instead of biotin-HPDP (Huang and Chen 2010). Thus, an –S–C-linker–biotin complex was formed instead of an –S–S-linker–biotin complex. Then in the next step, biotinylated samples could be reduced with DTT to break any intermolecular disulfide bonds before the biotinylated proteins are purified, thus removing the possibility of interference from intermolecular disulfide bonds (Huang and Chen 2010). Similarly, in the study of Chen et al., they also describe a PEO-iodoacetyl-biotin that could react with thiols to form an –S–C–biotin (Chen et al. 2010). Although these methods are capable of differentiating the disulfide-bonded cysteines from the S-nitrosylated ones, the added mass of these biotin tags tends to be very large and they sometimes undergo a neutral loss during the fragmentation process, which complicates the downstream MS data analysis. In the study of Camerini et al., an alkylating molecule (I-CH2-CO-Gly-Arg-Ala-His6) containing a His-tag was utilized to specifically bind and enrich the S-nitrosylated cysteines. This method was termed the His-tag Switch (Camerini et al. 2007). This alkylating reagent also binds irreversibly to the ascorbate-reduced cysteine residues to form an -S-CH2-CO-Gly-Arg-Ala-His6 structure (Camerini et al. 2007). The tagged proteins/peptides thus could be purified by standard affinity chromatography. Moreover, this molecule was designed to be sensitive to trypsin digestion, so that the alkylating label on S-nitrosylated cysteines can be detected as a mass shift of only 271.12 Da (-CH_2-CO-Gly-Arg), which simplifies the mass spectrometric analysis (Camerini et al. 2007).

Ascorbate independent enrichment of S-nitrosylated proteins/peptides

In spite of the wide application of the BST and the methods development based on it, there are still some concerns about the specificity of the ascorbate. It was suggested that ascorbate may reduce disulfide bonds or other oxidative modifications (Giustarini et al. 2008; Murray et al. 2011b). As an alternative strategy, Doulias et al. (2010) directly reacted phenylmercury compounds with S-nitrosocysteine forming a relatively stable thiol–mercury bond, thereby not requiring the SNO reduction step for analysis. In this study, ρ-amino-phenylmercuric acetate was conjugated to *N*-hydroxysuccinimide-activated Affi-Gel 10 agarose beads to synthesize an organomercury resin (MRC) which was designed for solid phase-based capture of S-nitrosylated proteins/peptides. Alternatively, a phenylmercury-polyethyleneglycol-biotin compound was designed to capture S-nitrosylated proteins/peptides through biotin-avidin affinity purification (Doulias et al. 2010). In addition to the mercury-based SNO reaction, Faccenda et al. (2010) described a gold nanoparticle (AuNP) that could be used to directly enrich the protein S-nitrosylated protein. It was demonstrated that thiols and thioethers have a higher affinity for gold than other functional groups in proteins (Faccenda et al. 2010). Thus, AuNP may have the potential to discriminate the disulfide bond and other cysteine modifications from the S-nitrosylated one.

Proper control design for S-nitrosylation identification

Despite the promising results, the specificity of both the organomercury compounds and AuNP requires further investigation. A properly designed control in addition to the replacement of ascorbate could help the concern of specificity and exclude the false-positives arising from incomplete blocking of free cysteines. Two types of controls (negative and positive) can be used to confirm the origin of the S-nitrosylation site. A negative control can be established by selectively decomposing the S–NO bonds in the sample, suppressing or abolishing detection of the S-nitrosylated peptide ion by MS (Derakhshan et al. 2007). There are a number of different ways to achieve this goal. For example, incubation with an excess quantity of a low molecular weight thiol (e.g., 5 mM DTT) was used to selectively denitrosylate proteins (Derakhshan et al. 2007). The rationale is that the low molecular weight thiol can serve in transnitrosation reactions as an acceptor of NO from proteins (Derakhshan et al. 2007). However, when the samples are very complex, the endogenous protein disulfides or adventitious oxidation might influence the ability of DTT to alter the pattern of S-nitrosylation (Foster et al. 2009a). Alternatively, decomposition of S–NO bonds could

be achieved by the UV photolysis (Derakhshan et al. 2007). When an aliquot of each sample was pre-exposed to UV light, the signals that originate from authentic S-nitrosylated proteins will predictably be suppressed by UV illumination, whereas false-positive signals that do not originate from S-nitrosylated proteins would predictably be unaffected. The inhibition of NOS may also serve for the same purpose (Ravi et al. 2004). A positive control that increases the abundance of the SNO contents can also be designed to control the false-positive signals (Derakhshan et al. 2007). A positive control may be created by treatment of samples with NO donor compound or through the overexpression of the NOS (Derakhshan et al. 2007). In this situation, MS ion signals that originate from authentic S-nitrosylated proteins will predictably be increased, whereas false-positive signals that do not originate from S-nitrosylated proteins would predictably be unaffected.

Protein-level versus peptide-level enrichment strategy for S-nitrosylation analysis

To date, most proteomic studies of S-nitrosylation have employed enrichment strategies at the protein level. One of the advantages of SNO enrichment at the protein level is that both non-S-nitrosylated peptides and S-nitrosylated peptides derived from targeted proteins can be identified by MS analysis, which improves the sequence coverage of the identified SNO-modified protein. In addition, SNO-protein binding partners can potentially be identified by the protein enrichment approach. Protein–protein interactions are an important feature of cell physiology and identification of proteins that associate with SNO-proteins could reveal SNO-dependent protein–protein interactions. Such interactions may be as important as cysteine nitrosylation itself. However, the potential for detecting non-nitrosylated proteins introduces a complication into the analysis of S-nitrosylated proteins (Liu et al. 2010). The only way to distinguish between S-nitrosylated proteins and their binding partners is to identify the S-nitrosylation sites. However, using peptide-level enrichment strategy, protein identification will be compromised because many of the proteins are identified by only one peptide (one-hit wonders). Combining the identification result of both the protein and site identifications will be optimal (Huang and Chen 2010).

Quantitative approaches for protein S-nitrosylation analysis

Elucidation of the role of NO under various physiological and pathological conditions requires quantitation of the dynamic changes of protein S-nitrosylation. Quantitative analysis can also yield information about the reaction profile of different cysteine residues that undergo redox-based S-nitrosylation. For gel or array-based S-nitrosylation profiling, quantitation of S-nitrosylation state can be carried out using western blotting or fluorescence-based signals (Liu et al. 2010; Foster et al. 2009a). A rough measurement of cysteine S-nitrosylated protein abundance change can be achieved by comparison of the protein band/spot intensities among different sample gels. In-gel digestion and MS analysis can be used to identify proteins of interest followed by excision of the protein bands/spots. The advantage of these approaches for quantitative S-nitrosylation analysis includes simplicity, reliability, high information content and ready accessibility to researchers. In addition, these approaches are well suited to differentiate the cellular response of changing protein expression from changing S-nitrosylation. For example, Wiktorowicz et al. (2011) reported a novel strategy for specifically labeling, detecting, and quantifying protein S-nitrosylation by fluorescence saturation (SNOFlo). In this approach, the total cysteine content of the cell extracts is determined by amino acid analysis. This is followed by denaturation and division of the extract into two equal fractions. One fraction is labeled with BODIPY® FL maleimide (BD), an uncharged cysteine-specific fluorescent dye. The second fraction is treated with ascorbate to reduce the S-nitrosylation and is likewise labeled with BD. Subsequent two dimensional gel electrophoresis with fluorescence quantification permits the ratiometric determination of the ratio of free cysteines to S-nitrosylated cysteines between the control and experimental samples that were treated with ascorbate. It is also possible to determine the change in the ratio of S-nitrosylation in the fractions not treated with ascorbate. This "ratio of ratios" yields the change in S-nitrosylation normalized to the change, if any, in protein abundance (Wiktorowicz et al. 2011).

However, few S-nitrosylation sites on proteins have been identified by this approach presumably due to their low abundance and to the low recovery of S-nitrosylated peptides from in-gel digestion (Torta et al. 2008). Therefore, gel-free proteomic strategies for quantitative S-nitrosylation analysis have been developed and summarized in Fig. 2. Overall, quantitative proteomic analysis of S-nitrosylation can be realized by adopting existing general stable isotope labeling (SIL) methods or by developing new S-nitrosylation specific SIL methods.

Adopting existing SIL methods for quantitative S-nitrosylation analysis

Forrester et al. (2009b), combined isobaric tags for relative and absolute quantitation (iTRAQ) techniques with the described SNO-RAC strategy to analyze proteome-wide

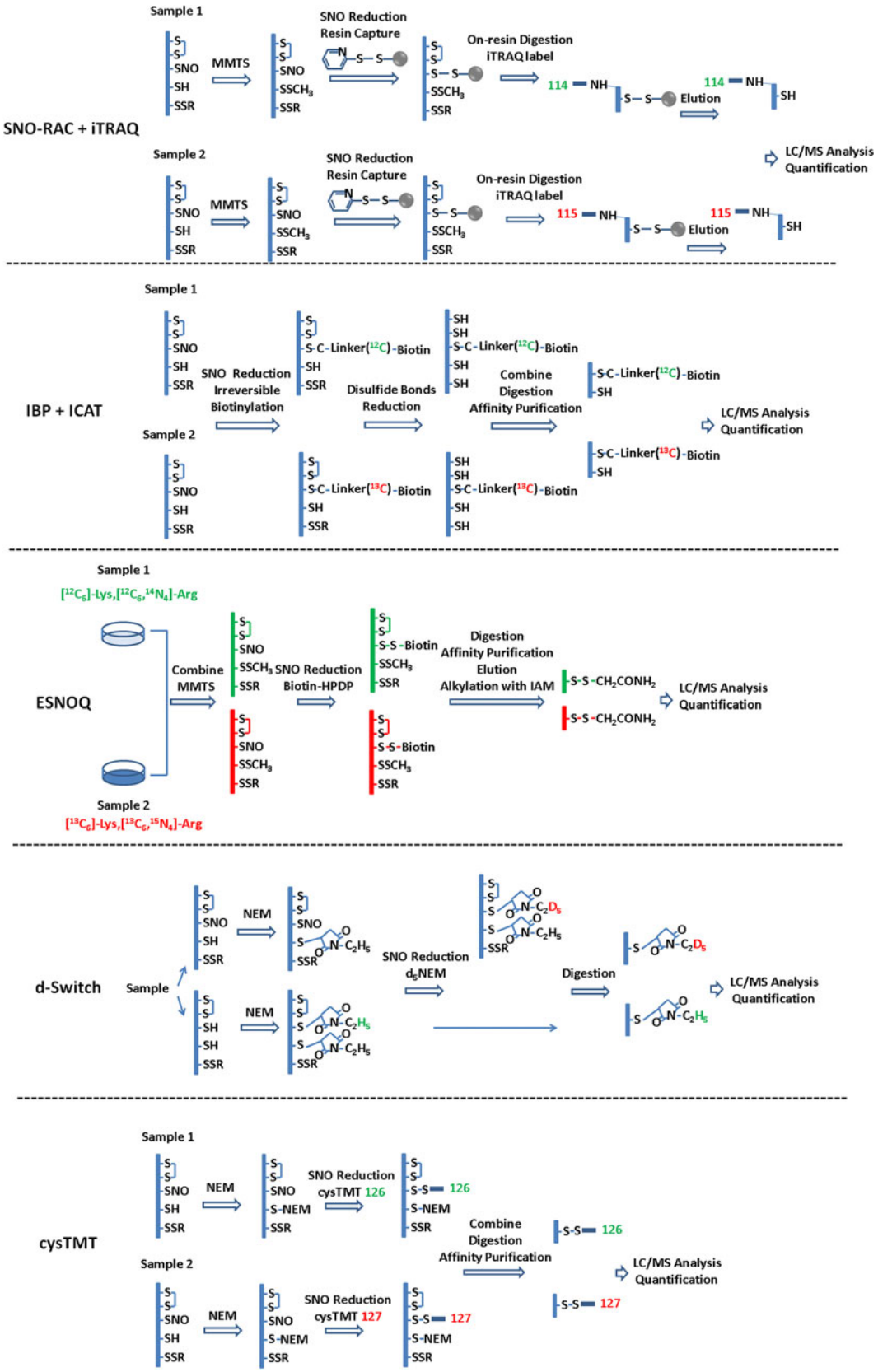

Fig. 2 Mass spectrometry-based quantification strategies for protein S-nitrosylation analysis

quantitative S-nitrosylation. The iTRAQ can be broadly applicable for any digestible proteome samples and offer the choices of 4-plex and 8-plex labeling kits. This strategy is desirable for quantitative comparison of multiplex samples. Applying this strategy, the author confirmed the global occurrence of protein denitrosylation (Forrester et al. 2009b). Huang et al. (2010), described a quantitative proteomic approach for S-nitrosylation analysis, which combines IBP with the isotope-coded affinity tag (ICAT). A light ICAT reagent (with nine 12C atoms) and a heavy ICAT reagent (with nine 13C atoms) were employed to quantify the S-nitrosylation ratio of two samples (Huang and Chen 2010). To minimize the systematic error that may be introduced during sample preparation, the ICAT-labeled proteins were combined before the enzymatic digestion and purification (Huang and Chen 2010). Zhou et al. (2010) described the endogenous SNO quantification method (ESNOQ) that combines the stable isotope labeling by amino acids in cell culture (SILAC) with the detergent-free biotin switch assay and LC–MS/MS. Light isotopes, [12C6]-Lys, [12C6, 14N4]-Arg, and heavy isotopes, [13C6]-Lys (+6 Da), [13C6, 15N4]-Arg (+10 Da), were incorporated separately into RAW264.7 cells, and then cells labeled with light isotopes were treated with LPS/IFN-c to induce iNOS expression. To avoid excess utilization of Arg by iNOS during nitric oxide synthesis, the light isotope-labeled cells were used as the treatment group, while cells labeled with heavy isotopes were used as controls (Zhou et al. 2010). The major advantage with SILAC labeling is that samples can be mixed before the blocking and purification steps thus reducing the errors arising from sample preparation steps and providing a more accurate quantification ratio. Using this method, the authors were able to confirm a total of 27 S-nitrosylated protein targets, and quantitative information was obtained on the S-nitrosylation site (Zhou et al. 2010).

Developing new SIL methods for quantitative S-nitrosylation analysis

Operating under the conception that the degree of protein S-nitosylation on a given protein varied under different conditions, Sinha et al. (2010), described the d-Switch approach to quantify the absolute ratio of S-nitrosylation to non-nitrosylation on individual proteins. *N*-ethylmaleimide (NEM) was used in place of the MMTS for free thiol blocking, and d5-NEM was used to label the ascorbate-reduced SNO site (Sinha et al. 2010). Because both the NEM and the d5-NEM labeled peptides have almost identical retention time and identical ionization efficiency, the quantification of S-nitrosylation changes could be easily performed based on the peak area ratios of the two differentially labeled peptides (Sinha et al. 2010). Coupled with MS analysis, Sinha et al. (2010) quantified the percentage of S-nitrosylation in purified proteins under five different NO donors treatment, as well as for cultured cells under different concentration of a NO donor treatment. However, no enrichment strategy was used in this study, limiting the application of d-Switch approach for identifying and quantifying the low abundant S-nitrosylated proteins from complex biological samples. Recently, a thiol-reactive version of tandem mass tag, cycTMT, was utilized to enrich and quantify the S-nitrosylation sites (Murray et al. 2011b). This new reagent not only fulfilled the need for enrichment of low abundant S-nitrosylated proteins/peptides but also for the use of up to six isotopically balanced reporter ions permitting multiplex quantification. Using this technique, the authors demonstrated specific detection of S-nitrosylation sites and quantified the response of individual cysteine residues to GSNO treatment by mapping the continuum of protein thiol-reactivity to SNO-modification (Murray et al. 2011b). By quantifying the change of S-nitrosylation after different NO donor treatments, this approach made it possible to discriminate potential false positive results including non-SNO-modifications and misassigned ones.

Biological applications of S-nitrosylation analysis

The emerging activity profile reveals that hypo- or hyper-S-nitrosylation can alter protein functions directly implicated in the etiology and symptomology of numerous human diseases in clinical conditions (Foster et al. 2003, 2009b). In these cases, specific cysteine residues have been identified as the loci of (patho)physiological regulation by S-nitrosylation (Foster et al. 2009b). Increasing attention have been given to studies aiming to decipher the molecular mechanisms underlying the (dys)regulation of S-nitrosylation and possible approaches to therapeutically altering protein S-nitrosylation levels (Foster et al. 2009b).

Neurodegenerative diseases

NO is the first gaseous molecule that is considered to be neurotransmitter in the nervous system (Snyder 1992). Studies have implicated NO as a key mediator of neurodegeneration in numerous diseases of the nervous system, including Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, Huntington's disease, and ischemic brain injury (stroke) (Knott and Bossy-Wetzel 2009). Increasing evidence suggested that S-nitrosylation is involved in the pathogenic process of various neurodegenerative disorders (Chung 2006). Therefore, a thorough understanding of how protein S-nitrosylation modulates the function of important proteins that are involved in different

physiological and cellular pathways may identify potential new pharmacological targets for neurodegenerative diseases in the future.

Cardiovascular diseases

NO also plays an important role in the regulation of cardiac function (Sun et al. 2006; Sun and Murphy 2010). It has been suggested that under physiological oxidative stress, NO might provide protection to cells by S-nitrosylation of some critical protein thiols, thereby protecting them from further oxidative modification by reactive oxygen species (Sun and Murphy 2010). To identify the NO susceptible pathways and novel mechanisms of oxidative cardioprotection, Murray et al. (2011a), assessed mitochondrial S-nitrosylation-modifiable cysteines in rat cardiac mitochondrial lysates after GSNO treatment. The authors identified a total of 83 S-nitrosylation sites on 60 proteins with cardioprotective potential and involved in electron transport chain, tricarboxylic acid cycle, oxidative stress defense, fatty acid and amino acid metabolism (Murray et al. 2011a). Kohr et al. (2011), identified a total of 116 potentially S-nitrosylated proteins in mouse myocardium under basal conditions and 951 potentially S-nitrosylated proteins after treatment with GSNO, using a modified SNO-RAC approach. This study provided useful information regarding the constitutively S-nitrosylated proteins in the myocardium, as well as the potential myocardial S-nitrosylation sites on key proteins involved in myocardial contraction, metabolism, and cellular signaling (Kohr et al. 2011).

Inflammation, immunity and cancer

NO has been show to play an important if not complex role in tumor biology, host immune response and inflammation-related diseases (Fukumura et al. 2006; Tripathi et al. 2007; Bogdan 2001). NO can either positively or negatively regulate cellular processes depending on conditions such as the genetic make-up of the cells, the local concentration of NO, and the presence of other regulators such as NO scavengers (Shi et al. 2000). NO also plays a functional role in tumor progression, affecting tumor cell proliferation, survival, migration, and invasiveness, as well as the ability of immune cells to infiltrate tumors and endothelial-cell progenitors to induce angiogenesis. The proteomic strategies are well suited to dissect the complex roles of tumor-derived NO in tumor progression and the results will help explain much of the conflict in published findings of a positive as well as a negative association of NO with tumor progression and metastasis, which has been documented in both human and experimental models (Xie and Fidler 1998). Integration of MS with improved strategies for specific characterization of proteins and their S-nitrosylation sites will significantly improve current limited knowledge of the essential role of NO in tumor progression and metastasis as well as in immunosuppression.

Conclusions and future directions

Rapid progress has been made in development and application of proteomic approaches for S-nitrosylation analysis, resulting in the exponential growth of S-nitrosylated proteins reported in the literature. Furthermore, evidence increasingly shows the important role of dysregulated S-nitrosylation in a wide spectrum of human diseases by which proteomic analysis has the untapped potential to yield therapeutic targets or diagnostic biomarkers, especially utilizing advanced quantitative proteomic approaches. Finally, we need to direct future research toward the identification of the biological roles of S-nitrosylation sites and the impact of S-nitrosylation on protein activities, subcellular localization, and the dynamics of signaling and protein-interaction networks. In addition to traditional biochemical approaches such as site-specific mutations, novel analytical approaches are required to examine the specific roles of S-nitrosylation on protein activities and protein–protein/DNA interactions, preferably in a high-throughput fashion. Determination of other combinatory PTMs that occur on the S-nitrosylated protein targets such as phosphorylation by MS may shed important light into the change of protein activity status after S-nitrosylation. In summary, proteomic study of S-nitrosylation can significantly advance our knowledge about the role of NO in complex biological systems, which in turn can be used to generate testable hypotheses and ultimately provide improved and more targeted diagnoses and therapies.

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