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Functional Proteomics Approaches for the Identification of Transnitrosylase and Denitrosylase Targets

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Abstract

Protein S-nitrosylation is a dynamic post-translational modification (PTM) of specific cysteines within a target protein. Both proteins and small molecules are known to regulate the attachment and removal of this PTM, and proteins exhibiting such a function are transnitrosylase or denitrosylase candidates. With the advent of the biotin switch technique coupled to high-throughput proteomics workflows, the identification and quantification of large numbers of S-nitrosylated proteins and peptides is now possible. Proper analysis and interpretation of high throughput and quantitative proteomics data will help identify specific transnitrosylase and denitrosylase target peptide sequences and contribute to an understanding of the function and regulation of specific S-nitrosylation events. Here we describe the application of a quantitative proteomics approach using isotope-coded affinity tags (ICAT) in the biotin switch approach for the identification of transnitrosylation and denitrosylation targets of thioredoxin 1, an enigmatic protein with both reported transnitrosylase and denitrosylase activities.

Keywords

Biotin switch; ICAT; proteomics; S-nitrosylation; thioredoxin

1. Introduction

S-nitrosylation and denitrosylation are the covalent addition or removal of a nitric oxide (NO) to or from a cysteine thiol within proteins. Similar to phosphorylation, it has increasingly assumed importance as a ubiquitous PTM, known to regulate diverse cellular processes including signal transduction, DNA repair and neurotransmission. Protein S-nitrosylation requires either small molecule NO donors or putative protein transnitrosylases, a yet to be clearly defined class of enzymes (Table 1). This PTM is reversible. Denitrosylation is carried out by putative denitrosylases (Table 1) and small molecule NO recipients. Not all cysteines in proteins are equally accessible to transnitrosylases or denitrosylases, therefore S-nitrosylated cysteine (SNO-Cys) site modifications can display

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exquisite specificity, e.g. the ryanodine receptor [1]. Identification of SNO-Cys sites and quantification of the relative changes among S-nitrosylated peptides (SNO-peptides) is crucial to understanding the biological significance of this PTM in modulating the function of a particular protein.

An increasing awareness of the physiological and pathophysiological significance of S-nitrosylation has grown within the scientific and medical fields over the past decade. Specific protein dependent-denitrosylation studies have provided the most recent evidence of this PTM being a highly regulated, enzymatic-dependent biological process [2]. S-nitrosylation was previously described as a non-enzymatic process [3], but several lines of evidence now suggest that numerous proteins, including members of the thioredoxin (Trx) family can catalyze the specific transnitrosylation and/or denitrosylation of target proteins [4–6]. As such, the Trx system comprised of Trx1 or Trx2, cytosolic Trx reductase (TrxR), mitochondrial TrxR and NADPH, are part of a newly defined class of enzymes with denitrosylase activities (Table 1) [7]. Trx1 has also been shown to transnitrosylate select proteins under cell-free conditions in the absence of TrxR or in cells expressing *Trx1^{C32,C35S}*, a mutant with attenuated Trx1 disulfide reductase or denitrosylase functions [4]. Hence, Trx1 regulation of protein transnitrosylation is likely to be important in highly oxidizing cellular microenvironments, e.g., when Cys32 and 35 disulfide bond oxidized Trx1 (oTrx1) accumulates in cells due to either down modulation of TrxR or upregulation of thioredoxin interacting protein (Txnip), an endogenous Trx1 reductase/denitrosylase inhibitor. For example, the over expression of Txnip has been shown to elevate cellular protein nitrosylation levels [8], presumably due to Txnip's inhibition of Trx1's reductase/denitrosylase activities, which may in turn lead to an accumulation of SNO-Trx1 and downstream S-nitrosylated proteins (SNO-proteins). Indeed, oTrx1 has been observed in several normal animal tissues, including kidney and lung, and increased oTrx1 in cells is correlated with increased SNO-protein [6]. Importantly, animal models for cardiovascular disorders including hypertension and ventricular remodeling that originate from dysregulation of the Trx system could be caused, in part, by an imbalance in denitrosylation/transnitrosylation [9]. For example, transgenic mice over-expressing *Trx1^{C32,C35S}* mutant in the heart exhibit cardiac hypertrophy, which may be due to increased oxidative PTMs including disulfides and nitrosylation among specific proteins [10]. Therefore, it becomes crucial to determine whether these aberrant S-nitrosylation patterns and related pathologies are due to decreased denitrosylation or increased transnitrosylation. With this view, identification of the SNO-Cys sites within specific target SNO-peptides is crucial for understanding the biological significance of S-nitrosylation in modulating the function of signaling proteins.

Due to its labile nature this PTM has been classically difficult to study, but several indirect methods have been developed. They include the use of a SNO-Cys-specific antibody to detect *in situ* protein S-nitrosylation by immunohistochemistry [11], and the use of the biotin-switch technique (BST) coupled with immunoblotting and 2D gel electrophoresis [12–14]. However, none of these methods can readily identify specific SNO-Cys sites within proteins. To this end, BST coupled with tandem mass spectrometry (MS/MS) identification of protein SNO-Cys sites (SNOSID) method was developed [15]. In this method, SNO-Cys in peptides are converted into more stable biotinylated cysteines, which can be analyzed by LC/MS/MS methods, either with matrix-assisted laser desorption ionization (MALDI) or electrospray ionization (ESI) techniques [4, 6, 16].

Quantitative approaches have an inherent advantage over qualitative methods, such as direct MS detection [17–19] and SNOSID [15], which rely on identifying proteins with dramatically elevated S-nitrosylation. The exclusion of an SNO-peptide from downstream analysis may occur simply because it is not found solely in the biological sample; this may

miss important regulatory events whose function is manifested by gradual changes in SNO-peptide levels. Therefore, we believe that the ICAT method described below, isobaric tag for relative and absolute quantitation (iTRAQ), stable isotope labeling by amino acids in cell culture (SILAC) [20], S-nitrosothiol capture (SNO-CAP) [21], S-nitrosothiols resin-assisted capture (SNO-RAC) [22] and Tandem Mass Tags (TMT) [23] methods used by others, as well as the Multiple Reaction Monitoring (MRM) method [24], will provide crucial quantitative information essential to understanding the function of regulated S-nitrosylation in diverse biological systems. However, it is necessary to understand the advantages and disadvantages of each method when applied to a specific biological sample (Table 2). Direct MS detection enables the rapid and specific analysis of SNO-peptides or proteins without possible confounding artifacts from the indirect method involving BST [17, 19]. However, this method is not always suitable for the analysis of very complex protein mixtures derived from biological sources. Furthermore, direct MS detection often fails to localize SNO-Cys sites on SNO-peptides with multiple cysteines, due to the labile nature of the S-NO bond, which is liable to fragment more readily than the peptide backbone [25]. All other quantitative methods discussed in Table 2 involve the use of BST, which enables the localization of the exact SNO-Cys and are amenable to multidimensional LC separations for analysis of very complex protein mixtures. However, due to the relatively large size of the biotin group, MS/MS analyses of biotinylated peptides usually produces spectra that are not as easily interpretable as those of the corresponding unmodified peptides. Typically, there are numerous and dominant biotin-derived fragment ions in the MS/MS spectra, which hinder both manual and search engine-based data interpretation. By comparison, both SNO-RAC and TMT methods monitor peptides without attached biotins; these methods are likely to produce richer MS/MS spectra for peptide sequence interpretation and SNO-Cys assignments. However, for SNO-RAC, the reductants used for the elution of the SNO-peptides from capture resins may also reduce the disulfide bonds at MMTS blocking sites or other oxidatively modified cysteines, increasing false SNO-Cys identification on SNO-peptide quantification. One distinct advantage of the TMT method over most other quantitative proteomics methods for the comparison of two to three sample groups is its design to allow for up to six samples to be compared in one experiment. On the other hand, the specificity of the commercial antibody used for the enrichment of TMT-conjugated peptides is low; resulting in futile LC-limited MS/MS data acquisition time on the fragmentation of non-SNO-peptides. Compared to these methods, ICAT has its unique advantages: ICAT tags contain biotin, which can be used to directly enrich the SNO-peptides, after BST, and the biotin group can be cleaved off from the ICAT-enriched peptides before LC/MS/MS analysis, which generates much “cleaner” MS/MS spectra for the identification and quantification of SNO-peptides.

This study applies the ICAT reagents to BST and in so doing modifies an existing qualitative technology to yield quantitative information on SNO-Cys-containing peptides. Here we outline recent application of quantitative proteomics techniques in our lab to resolve the denitrosylation and transnitrosylation targets of Trx1, a 12-kDa multifunctional protein involved in cell growth, death, protein reduction, and tissue development.

2. Rationale

We previously revealed a disulfide redox status-dependent mechanism of toggling Trx1 from a transnitrosylase into a denitrosylase, and *vice versa* [4, 6]. When its catalytic Cys32 and Cys35 are both reduced into dithiols, Trx1 serves as a denitrosylase. On the other hand, when a Cys32 and Cys35 disulfide is formed, Trx1 acts as a transnitrosylase. In this report we outline the *in vitro* methods previously used to identify and quantify Trx1 transnitrosylation and subsequent Trx1 denitrosylation target peptides, a task that is rather challenging to delineate from *in vivo* systems [6]. Most steps of this method are equally

applicable to the identification of any transnitrosylase and denitrosylase target isolated from cells and tissues. We have included additional discussions on how to apply this method for more physiologically relevant systems.

3. Material and methods

3.1 Preparation of transnitrosylating S-nitrosylated Trx1 (SNO-Trx1)

Recombinant human Trx1 and chemical reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated. Acetonitrile (ACN) and HPLC-grade water were obtained from J. T. Baker (Phillipsburg, NJ, USA). Formic acid was purchased from EMD Chemicals (Merck KGaA, Darmstadt, Germany). To produce transnitrosylase-active SNO-Trx1, Cys32 and Cys35 oxidized human Trx1 (100 μ g) (Sigma, St. Louis, MO USA) was mixed with a 25-fold molar excess of S-nitrosoglutathione (GSNO) in 50 μ l of S-nitrosylation buffer (NB, 50 mM Tris, pH 7.5, 1 mM EDTA and 0.1 mM neocuproine) at 37 °C for 30 min in the dark. The resulting SNO-Trx1 was precipitated with cold acetone at -20 °C for 1 h. To separate GSNO from SNO-Trx1, the protein pellet was washed four times with cold acetone at -20 °C and dissolved in NB, and adjusted to 2 μ g/ μ l to be used as transnitrosylase. Since Trx1 is a small protein, acetone precipitation did not affect the refolding of SNO-Trx1 and its ability to transnitrosylate target proteins. Alternatively, in order to minimize the possibility of protein denaturation, SNO-Trx1 or other transnitrosylases prepared *in vitro* can be separated from excessive GSNO or other NO donors by using ultrafiltration or microdialysis.

3.2 Cell lysates preparation

A cell line or tissue with physiological relevance to the dynamic changes of SNO-proteins or their regulatory factors is preferable; here we employed the neuroblastoma cell line SH-SY5Y (ATCC; CRL-2266). SH-SY5Y cells were grown at 37 °C in DMEM/F12 media containing 10% FBS in a 5% CO₂ atmosphere, as recommended by ATCC. The cells were harvested *via* centrifugation at 500 $\times$ g for 5 min and washed with PBS. Cells were lysed in a lysis buffer (LB, 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA and 0.1 mM neocuproine) supplemented with a protease inhibitor cocktail (Sigma). After the removal of cell debris from the lysate *via* centrifugation, protein concentrations were measured using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA) and adjusted to 1 μ g/ μ l with LB.

3.3 In vitro transnitrosylation and denitrosylation of cellular proteins

Freshly made SNO-Trx1 (100 μ g) was used as a specific transnitrosylase to transnitrosylate 1 mg of proteins extracted from SH-SY5Y cells in 1 ml LB at 37 °C for 30 min in the dark. Similarly, GSNO treatment of cellular proteins can be performed as a positive control. Negative controls, such as un-nitrosylated oTrx1 or buffer alone treatment of cellular proteins can be included. Following transnitrosylation treatment, proteins were precipitated with cold acetone, washed 3 times in cold acetone and processed by BST (Section 3.4). In a separate experiment for the identification of Trx1 denitrosylase targets that were initially S-nitrosylated by SNO-Trx1, 300 μ g SNO-Trx1 treated proteins were incubated with 5 μ M Trx1, 100 nM TrxR and 200 μ M NADPH in a total volume of 0.5 ml LB at 37 °C for 30 min [20].

3.4 BST using ICAT labeling for quantitative determination of SNO-proteins

S-nitrosylation is very labile. Although direct detection by MS is possible for synthetic peptides [19], SNO-proteins are typically captured by the BST in which NO is replaced with a biotinylated thiol reaction reagent, *e.g.* N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide (biotin-HPDP) [12]. ICAT reagent is conventionally used for quantitative

proteomic research by alkylating cysteines in proteins. There is a biotin tag in the ICAT reagent for affinity capture and detection. In the following experiments, we used the ICAT reagent instead of biotin-HPDP in BST. In brief, the modified BST can be performed in the following steps:

3.4.1. Free thiol blockage—Proteins (1 mg) were denatured in 1 ml LB buffer with additional 2.5% SDS, and free thiols were alkylated using 20 mM methyl methanethiosulfonate (MMTS) (Pierce, Rockford, IL, USA) with frequent mixing on a vortexer at 50 °C for 30 min. Excess MMTS was removed by cold acetone precipitation of the proteins.

3.4.2. SNO-Cys reduction—Protein pellets were reconstituted in an HEN buffer (25 mM HEPES, pH 7.7, 1 mM EDTA and 0.1 mM neocuproine) containing 1% (w/v) SDS to a final concentration of 1 µg/µl. SNO-Cys's were reduced with 10 mM ascorbate for 1 h at RT in the dark; a condition that does not reduce disulfide bonds [Li lab, unpublished data]. The inclusion of proper controls to ensure specific detection of SNO-protein signals is essential [26]. It is recommended that a negative control reaction should be also performed; in which ascorbate is omitted and thus no SNO-Cys-derived free thiols are available for subsequent biotinylation and detection.

3.4.3. Biotinylation—The method for biotinylation using ICAT reagents was modified from Paige *et al.* 2008 [21]. Briefly, newly exposed free thiols in 600 µg of proteins reduced by ascorbate were differentially labeled with either 240 µg light (ICAT-L) or heavy (ICAT-H) ICAT reagent. Two independent ICAT-modified BST experiments were performed to detect Trx1 transnitrosylation and denitrosylation targets, respectively. First, SNO-Trx1 transnitrosylated proteins were labeled with ICAT-H, buffer treated proteins were labeled with ICAT-L. In a second experiment, SNO-Trx1 transnitrosylated proteins were first treated with Trx1/TrxR/NADPH and labeled with ICAT-L and subsequently compared with ICAT-H labeled SNO-Trx1 transnitrosylated proteins [6]. The ICAT labeling reactions were performed following ascorbate reduction of SNO-Cys's described in section 3.4.2 and incubated in the dark for 1 h at RT. Excess ICAT reagents were removed from labeled proteins by cold acetone precipitation of the proteins. Mixtures of different combinations of SNO-Trx1 transnitrosylated proteins (ICAT-H) with untreated proteins (ICAT-L) or Trx1/TrxR/NADPH denitrosylated proteins (ICAT-L) were produced to allow relative quantification of SNO-peptides changes (section 3.7).

3.5 Visualization of SNO-proteins by 1D Western blotting

To confirm proper ICAT labeling of SNO-proteins following BST (section 3.4), ICAT-labeled proteins can be visualized by Western blotting without affinity enrichment. Protein pellets (15 µg) were solubilized in a SDS-PAGE loading buffer (100 mM Tris, pH 6.8, 2% SDS, 15% glycerol, 0.01% bromophenol blue) and separated by a SDS-PAGE gel. Resolved proteins were transferred to a nitrocellulose membrane (0.45 µm; BioRad, Benicia, CA, USA), and the nonspecific antibody binding sites in the membrane were blocked with 5% milk and 0.1% Tween 20. The membrane was probed with an anti-biotin antibody (1:3,000) (Vector Laboratories, Burlingame, CA, USA) and ICAT labeled proteins were visualized with enhanced chemiluminescent substrate (PerkinElmer, Waltham, MA, USA).

3.6 Visualization of SNO-proteins by 2D Western blotting and Sypro Ruby staining

In order to improve protein resolution for global quantitative analysis, two-dimensional gel electrophoresis (2DE) can be performed. 2DE-resolved total proteins can be blotted using the anti-biotin antibody to evaluate for the changes in global SNO-protein levels. Alternatively, ICAT-labeled proteins can be enriched by an avidin column, and proteins

recovered from the affinity column can be resolved by 2DE for mass spectrometry identification of SNO-proteins. For 2D Western analysis, ICAT labeled protein pellets (100 μ g) were first dissolved in the 2DE buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT, 0.2% BioLyte, pH 3–10, and 0.01% bromophenol blue). For the isoelectric focusing (IEF) step, the protein solutions were loaded onto 11 cm IPG strips (pH 3–10 NL, BioRad, Benicia, CA, USA). IEF was performed with a Protean IEF Cell (BioRad, Benicia, CA, USA) at 20 °C, using the following settings: 12 h rehydration at 50 V, 0.5 h ramping to 250 V, 3 h ramping to 8,000 V and held at 8,000 V for 6 h. After IEF, the proteins on the IPG strips were reduced with DTT (2% w/v) for 15 min and then alkylated with iodoacetamide (2.5% w/v) in an equilibration buffer (6 M urea, 375 mM Tris-HCl, pH 8.8, 2% SDS and 20% glycerol) for 15 min. The second dimension separation was performed on 12.5% SDS-PAGE gels at 120 V. Subsequently, the proteins in each gel were either visualized by Sypro Ruby staining or transferred onto a nitrocellulose membrane (0.45 μ m; BioRad, Benicia, CA, USA); ICAT labeled SNO-proteins were detected by an anti-biotin antibody as described above. Differentially labeled proteins determined by Sypro Ruby staining or from Western blotting can be identified by tandem mass spectrometry methods described below.

3.7 ICAT labeling for quantitative determination of SNO-peptides

Although 2DE coupled with tandem MS is effective at identifying proteins whose S-nitrosylation status is altered by the activities of transnitrosylases or denitrosylases, this approach typically does not reveal the precise sites of modification. Therefore quantitative ICAT analysis by LC/MS/MS can bridge this gap. Depending on its redox state, Trx1 can act as transnitrosylase or as a denitrosylase; the latter when Trx1 is engaged with the Trx reducing system (Trx1/TrxR/NADPH)(6, 20). To delineate which proteins are targets of the two opposite functions of Trx1, SNO-proteins can be labeled with either ICAT-L or -H instead of the conventional biotin-HPDP reagent in BST.

3.7.1—For ICAT-based quantification of SNO-Trx1 transnitrosylase target peptides (section 3.3), 300 μ g of SNO-Trx1 treated proteins were processed *via* BST and labeled with 240 μ g of ICAT-H, while buffer treated control proteins were labeled with 240 μ g of ICAT-L.

3.7.2—Alternatively, for the identification of Trx1 denitrosylase targets that were initially S-nitrosylated by SNO-Trx1, 300 μ g SNO-Trx1 transnitrosylated proteins were denitrosylated by incubation with 5 μ M Trx1, 100 nM TrxR and 200 μ M NADPH (the latter are needed to maintain Trx1 denitrosylase activity) in a total volume of 0.5 ml LB [20] at 37 °C for 30 min. The resulting proteins were labeled with 240 μ g ICAT-L during BST. An equal amount of SNO-Trx1 transnitrosylated proteins were labeled with 240 μ g ICAT-H for comparison.

3.7.3—Following ICAT labeling, excess ICAT reagents were removed from each reaction mixture by ice-cold acetone precipitation and the protein pellets were dissolved in 8M urea. For each pair-wise comparison, the corresponding ICAT-H and ICAT-L labeled proteins were mixed at a 1:1 mass ratio, diluted 10-fold with 50 mM NH_4HCO_3 (pH 8.3) and subjected to trypsin digestions at a 50:1 protein/trypsin ratio at 37 °C overnight.

3.8 Affinity capture of ICAT-labeled peptides

Mixtures of ICAT-H and ICAT-L labeled peptides were enriched by the biotin affinity chromatography procedures using an avidin column provided in the cleavable ICAT kit according to the manufacturer's (AB Sciex, Foster City, CA, USA) protocol outlined below:

3.8.1—Dilute the mixture of ICAT-L and -H labeled peptides by adding 500 μ l or more of the Loading Buffer (LB, 2x PBS, pH 7.2), to adjust the pH to 7.0.

3.8.2—Slowly inject (~1 drop/second) the peptides onto the avidin cartridge and collect the flow-through. Inject 500 μ l of the LB onto the cartridge and collect the eluate.

3.8.3—Inject 1 ml of the Wash Buffer 1 (1 $\times$ PBS, pH 7.2) followed by 1 ml of the Wash Buffer 2 (50 mM NH_4HCO_3 and 20% methanol) and 1 ml of distilled water to clean the cartridge and remove nonspecifically bound peptides.

3.8.4—To elute the ICAT-labeled peptides, slowly inject (~1 drop/second) 750 μ l of the Elute Buffer (30% acetonitrile (ACN) and 0.4% trifluoroacetic acid (TFA)) onto the cartridge and collect the eluate. Evaporate the affinity-captured eluate to dryness in a speed vac.

3.8.5—Add 90 μ l of the freshly prepared Cleaving Reagent (95% TFA and 5% scavenger to minimize side reactions) to the sample tube, vortex to mix, and incubate the mixture for 2 h at 37 $^\circ\text{C}$.

3.8.6—The cleaved peptides were completely dried in a speed vac, desalted by on a C_{18} spin column (Thermo Fisher Scientific Rockford, IL, USA) and reconstituted in 5 μ l of 2% ACN and 0.1% formic acid for LC/MS/MS analysis (Section 3.9).

3.9 Identification of SNO-peptides and mapping of SNO-Cys sites

For LC/MS/MS analysis, ICAT-labeled peptides were first separated by a Dionex UltiMate® 3000 reversed phase liquid chromatography system, using a PepMap 100 C_{18} column (75 $\mu\text{m} \times 150 \text{ mm}$, 3 μM , 100 $\text{\AA}$, Dionex, Sunnyvale, CA, USA). The LC-resolved peptides were analyzed using a Waters API-US QTOF MS system with a nano-ESI source (New Objectives, Boston, USA). MS spectra (m/z 400–1,900) were acquired in the positive ion mode. Argon was used as the collision gas. The collision energy was set from 16 to 60 V, depending on the precursor ion charge state and mass to charge ratios (m/z). MS/MS spectra were acquired in the Data-Dependent Analysis (DDA) mode, in which the three most abundant precursors with two to five charges from each MS survey scan were selected for MS/MS analysis. Following data acquisition, PKL files were generated with ProteinLynx (v2.1). Peptide identification and SNO-Cys localization was accomplished by searching the PKL files against a human Swissprot protein database (containing 20,258 entries, 08/10/2010) using the Mascot search engine (v2.2); the following search parameters were used: trypsin was selected with 1 missed cleavage; mass tolerance of 200 ppm was set for precursor mass and 0.6 Da for MS/MS mass; either ICAT-C (ICAT-L) or ICAT-C:13C(9) (ICAT-H)-modified or MMTS-modified cysteines and oxidized methionines were set as variable modifications. For MS/MS identification of the peptides' S-nitrosylation site, a Mascot score threshold was set > 29 , corresponding to a confidence interval of 95% or better. The matched spectra were also manually validated for the precise location of the ICAT-modified cysteines. The peptide and protein identification false discovery rates (FDRs) were evaluated with a target-decoy database search strategy [27], with all protein and peptide FDR values from the different experiments calculated to be 0.5%. Alternatively, the LC-resolved ICAT-labeled peptides were analyzed using a LTQ-Orbitrap Velos tandem MS system with a nano-ESI source (Thermo Fisher Scientific Rockford, IL, USA). MS spectra (m/z , 400–2,000) were acquired in the positive ion mode with a resolution of 60,000 for the precursor ion scan. MS/MS spectra were acquired in the DDA mode, in which the top 10 most abundant precursor ions with at least a 300 ion count threshold from each MS survey scan were selected for MS/MS analysis. Collision-induced

dissociation (CID) was used for fragmentation with a 2 Da isolation window, at a normalized collision energy of 35%. The resulting raw spectral files were submitted to the Proteome Discoverer/Mascot (Thermo Fisher Scientific, V 1.3) for SNO-peptide identification, SNO-Cys localization and ICAT-based peptide quantification.

3.10 Quantitative data analysis

Once an ICAT-L or ICAT-H labeled peptide was identified by Mascot search, the extracted ion chromatograms for the corresponding ICAT-L and -H pairs were used to calculate the relative ratios between ICAT-H to ICAT-L labeled peptides. To estimate the analytical variation of ICAT-based peptide quantification, SH-SY5Y cell lysate was incubated with GSNO, and equal amounts of the resulting SNO-proteins were labeled using either ICAT-L or -H reagent with the modified BST protocol described above, and then mixed in a 1:1 ratio. After tryptic digestion, ICAT-labeled peptides were enriched and detected by LC/MS/MS for peptide identification (same as section 3.9) and quantification. Since ICAT-L or -H reagent labeled proteins were mixed in a 1:1 ratio, the expected ICAT (H/L) ratio was one. The experiment was repeated three times to calculate the standard deviation of the ICAT ratios to estimate analytical variation, and to establish a statistical benchmark of ICAT fold change of <0.78 for protein denitrosylation or >1.22 for transnitrosylation, both with a 95% confidence interval. Alternatively, Proteome Discoverer software can be used for automatic identification and quantification of ICAT-labeled peptides. The raw spectrum file was imputed for peptide identification using Spectrum Selector and Mascot, and Events Detector and Precursor Ions Quantifier were used for peptide quantification. In the Spectrum Selectors, the minimum and maximum precursor masses were set as 350 Da and 10,000 Da, respectively. Mascot search parameters were set up the same as described in section 3.9 except that the precursor mass tolerance was set at 10 ppm. For quantification, the mass precision was set at 2 ppm for event detection. A factory preset ICAT quantification method was used as the Precursor Ions Quantifier; the areas of the extracted ion chromatograms of ICAT-labeled peptides were used for calculating the ratios of ICAT-H/L. No normalization was applied for calculating SNO-peptide ratios. Quantitative data were expressed as mean $\pm$ standard error (SEM). Statistical analysis was performed using the two-tailed unpaired Student's t-test with Microsoft Excel. Differences were considered significant with $P < 0.05$ and ICAT ratios of >1.22 or <0.78 were considered significant for transnitrosylation or denitrosylation, respectively.

4. Results and Discussion

4.1 ICAT modified BST can effectively label SNO- proteins

In order to quantitatively detect changes in SNO-proteins from the activation of transnitrosylases and denitrosylases, we used ICAT reagents instead of biotin-HPDP during the application of the BST. The BST method involves three steps: free cysteine thiols are first alkylated with MMTS (Fig. 1A); SNO-Cys are then reduced by ascorbate without also reducing disulfide bonds or other oxidative cysteine PTMs (Fig. 1B), and lastly, the newly-exposed cysteine free thiols are alkylated by either the "light" or "heavy" version of the ICAT reagents (Fig. 1C). ICAT reagents have been proved to be good replacements for biotin-HPDP in BST [28, 29]. Labeling of SNO-peptides with the ICAT reagent provide a means to accurately quantify the extent of S-nitrosylation (Fig. 1, D to K).

To illustrate the application of ICAT-based BST for the quantification of SNO-peptide changes catalyzed by transnitrosylases, we first utilized SNO-Trx1 as a transnitrosylase to increase S-nitrosylation of proteins isolated from SH-SY5Y cells. Subsequently, we performed ICAT-based BST to label free thiols derived from the SNO-Cys sites with either light or heavy ICAT reagent; SNO-Trx1 transnitrosylated proteins were labeled with ICAT-

H and buffer-treated proteins were labeled with ICAT-L (Fig. 1C). Equal amounts of ICAT-H and ICAT-L labeled proteins were mixed together for subsequent proteomics analysis of SNO-peptide increases as the result of SNO-Trx1 transnitrosylation (Fig. 1, D–K).

In a separate experimental design, in order to demonstrate the utility of ICAT-based BST for the quantification SNO-peptide changes catalyzed by denitrosylases, an aliquot of SNO-Trx1 transnitrosylated proteins was treated with the Trx1/TrxR/NADPH denitrosylase system and the remaining SNO-proteins were labeled with ICAT-L following BST (Fig. 1C). A mixture of ICAT-H and –L labeled proteins was processed to identify and quantify the decrease of SNO-peptides following the denitrosylation reaction (Fig. 1, D–K).

4.2 Gel-based analysis of ICAT-labeled proteins revealed the presence of specific Trx1 transnitrosylation and denitrosylation targets

As each ICAT reagent has a biotin tag, the ICAT-labeled proteins can be detected by Western blotting to verify proper protein labeling during the BST procedures, using an anti-biotin antibody. Following SNO-Trx1 treatment, overall SNO-protein levels were increased over untreated protein extracts (Supplemental Fig. 1, first two lanes). Subsequent addition of TrxR/NADPH activated Trx1 demonstrated denitrosylation activity towards many SNO-Trx1 transnitrosylated proteins, reducing their S-nitrosylation levels (Supplemental Fig. 1, last two lanes). It is also apparent that not all SNO-protein signals are equally affected by the treatments with SNO-Trx1 and the Trx1 denitrosylation system, suggesting that not all proteins are transnitrosylation or denitrosylation targets of Trx1.

In order to better resolve and detect SNO-Trx1 transnitrosylation target proteins, we also analyzed the ICAT-labeled SNO-proteins by Western blotting following 2DE separations of ICAT-labeled protein. Although there are a few SNO-proteins in the untreated extracts, substantially more SNO-proteins were detected from SNO-Trx1 transnitrosylated proteins compared to buffer treatment alone (Fig. 2, A and B), with proteins in the 40 and 60 kd acidic region of 2DE being especially sensitive to SNO-Trx1 transnitrosylation.

4.3 Quantification of Trx1 transnitrosylation and denitrosylation target peptides by ICAT

The biological significance of Trx1 as a transnitrosylase or denitrosylase is its ability to modulate specific SNO-Cys modification sites within target proteins. However, the global SNO-protein quantification information obtained by 1D or 2D gel is inadequate at providing site-specific modification information. ICAT-based BST and other proteomics methods are needed to provide such essential data for downstream functional determination of the significance of dynamically regulated SNO-Cys residues in Trx1 target proteins. In the methods outlined here, we first quantified SNO-peptides increased by the incubation of neuroblastoma cell proteins with SNO-Trx1. Following the BST procedures, SNO-proteins were labeled with ICAT-H and buffer-treated proteins were labeled with ICAT-L. Both samples were mixed at a 1:1 ratio; the increase of SNO-peptides in SNO-Trx1 transnitrosylation targets was quantified by the ICAT ion signals in the LC/MS spectra, and the SNO-Cys sites were identified by MS/MS as described below. In a separate experiment setup to detect denitrosylation target SNO-peptides of Trx1, the SNO-Trx1 transnitrosylated samples were treated with the Trx1/TrxR/NADPH system, and labeled with ICAT-L in the BST. Transnitrosylated (ICAT-H labeled) and denitrosylated (ICAT-L labeled) proteins were also mixed in a 1:1 ratio. After obtaining the two mixtures of ICAT-H and -L labeled proteins, proteins were digested by trypsin and the ICAT-labeled peptides enriched by an avidin column. SNO-peptides were identified and quantified by LC/MS/MS [6]. For the quantification of ICAT labeled peptides, we used the LC extracted ion chromatogram areas of each ICAT-labeled peptide for calculating ICAT peptide abundance ratios. A significantly higher ICAT-H to ICAT-L ratio in repeat transnitrosylation experiments

suggested a SNO-peptide to be a putative SNO-Trx1 transnitrosylation target. On the other hand, for the Trx1/TrxR/NADPH denitrosylation reactions, a lower ICAT-L over ICAT-H labeled MS peak measured the degree of Trx1 denitrosylase activity towards a SNO-peptide. For example, a tryptic peptide (amino acid 17-31) derived from 60S ribosomal protein L12 (RL12_HUMAN) with a SNO-Cys¹⁷ was among the target sequences sensitive to both transnitrosylation and denitrosylation by Trx1 (Fig. 3, A–C). MS/MS analysis revealed the SNO-Cys site to be labeled with an extra mass corresponding to an ICAT-H (Fig. 3A). Following SNO-Trx1 transnitrosylation, LC/MS analysis indicated an ICAT-H/ICAT-L peptide ratio of 2.5 for RL12 peptide 17-31, suggesting that Cys¹⁷ is very sensitive to transnitrosylation by SNO-Trx1 (Fig. 3B). By comparison, an ICAT-H/ICAT-L peptide ratio of 1.6 for the same peptide following Trx1/TrxR/NADPH denitrosylation, suggests that SNO-Cys¹⁷ can be denitrosylated by Trx1 (Fig. 3C).

As expected, SNO-Trx1 was detected by ICAT analysis in both transnitrosylation and denitrosylation ICAT mixtures (Fig. 3D–F). Once SNO-Trx1 was mixed with target proteins in the transnitrosylation reaction, ICAT analysis indicated an 80% decrease of a SNO-Cys⁷³-containing Trx1 tryptic peptide (Fig. 3D–E), supporting the role of SNO-Trx1 transfer of its NO onto the recipient peptides. Furthermore, SNO-Trx1 itself is also sensitive to Trx1/TrxR/NADPH denitrosylation, as indicated by a 70% decrease of the SNO-Cys⁷³-containing Trx1 tryptic peptide (Fig. 3F). In this experiment we used relatively large amounts of SNO-Trx1 and Trx1/TrxR/NADPH in the *in vitro* transnitrosylation and denitrosylation experiments, in order to observe SNO-peptide changes for lower abundant SNO-proteins, which otherwise might not be able to compete with other proteins for the transfer of NO. However, like any proteomics experiments, the presence of higher amount of proteins could hinder the detection of lower abundant proteins. Therefore, one way to minimize the impact of interference of large amounts of transnitrosylase or denitrosylase is to use enzymes immobilized on beads that can be easily separated from the target SNO-proteins prior to tryptic digestion.

4.4 Quantitative data validation

Many of the proteins discovered from this *in vitro* study were previously reported to be either transnitrosylated or denitrosylated by Trx1 (or related oxidoreductases) *in vivo* [6, 20], supporting the effectiveness of the current quantitative proteomics approach. However, given the complexity of biological experimental systems, it is important that once an ICAT-L or ICAT-H labeled peptide is quantified, the extent of transnitrosylase or denitrosylase-mediated SNO-peptide change should be validated by repeat experiments and alternative approaches, using biotin-HPDP or other biotinylating reagents, instead of ICAT reagents. Typically, the validation is carried out by using BST processing of the SNO-proteins isolated from cells with activated transnitrosylase, denitrosylase and relevant control conditions, followed by Western Blotting of specific target proteins enriched by avidin affinity column [4, 6]. Alternatively, fluorescently-labeled and biotinylated Cy5-dyes can be used, in a method referred to as SNO-DIGE [30]. The SNO-protein changes quantified by Western blotting or similar methods should match the direction of SNO-peptide changes by ICAT quantification. As discussed previously, global protein-based quantification may not capture site-specific changes if multiple sites within a protein are differentially regulated by different transnitrosylases or denitrosylases. Site-specific nitrosylation changes can be validated by Western blotting if SNO-Cys-specific antibodies are available. On the other hand, a mass spectrometric methods called selected reaction monitoring (SRM) or the multiple-reaction monitoring (MRM) approach can be developed to quantify SNO-Cys containing peptide changes among a large number of biological samples [31].

5. Conclusions

Here we present a protocol for quantitative identification of *in vitro* Trx1 transnitrosylase and denitrosylase target peptides using a modified biotin switch method, with ICAT reagents replacing conventional biotin-HPDP. Combining this method with LC/MS/MS techniques, the SNO-Cys within target peptides can be identified and the relative changes of SNO-peptides following transnitrosylase and denitrosylase treatment can be easily determined by ICAT ratios. With this approach, we were able to identify putative Trx1-mediated transnitrosylation and denitrosylation targets in neuroblastoma cells. The results from this study have provided direct evidence that Trx1 is an enigmatic protein with both transnitrosylase and denitrosylase activities. Use of the described ICAT method coupled to BST for studying other cell and biological systems will likely provide a more comprehensive understanding of the precise role of both transnitrosylases and denitrosylases in regulating target proteins in specific physiological or pathological signal transduction pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

2DE	two-dimensional gel electrophoresis
ACN	acetonitrile
BCA	bicinchoninic acid
Biotin-HPDP	N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)-propionamide
BST	biotin-switch technique
CID	collision induced dissociation
DDA	data-dependent analysis
ESI	electrospray ionization
ENOA	human α -enolase
FDR	false discovery rate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GSNO	S-nitrosoglutathione
ICAT	isotope-coded affinity tags
ICAT-H	ICAT heavy reagent
ICAT-L	ICAT light reagent
IEF	isoelectric focusing
iTRAQ	isobaric tag for relative and absolute quantitation
LB	lysis buffer

MALDI	matrix-assisted laser desorption ionization
MMTS	methyl methanethiosulfonate
MRM	multiple-reaction monitoring
MS/MS	tandem mass spectrometry
NADPH	nicotinamide adenine dinucleotide phosphate
NB	S-nitrosylation buffer
PTM	post-translational modification
RB	resuspension buffer
SILAC	stable isotope labeling with amino acids in cell culture
SNO-Cys	S-nitrosylated cysteine
SNOSID	SNO-site identification
SNO-Trx1	S-nitrosylated thioredoxin 1
TFA	trifluoroacetic acid
TMT	Tandem Mass Tags
Trx	thioredoxin
Trx^{C32S/C35S}	Cys32 and Cys35 to Ser Trx1 mutant
TrxR	Trx reductase

References

1. Xu L, Eu JP, Meissner G, Stamler JS. Science. 1998; 279:234–237. [PubMed: 9422697]
2. Benhar M, Forrester MT, Hess DT, Stamler JS. Science. 2008; 320:1050–1054. [PubMed: 18497292]
3. Duan S, Chen C. Cell Mol Immuno. 2007; 14:353–358.
4. Wu C, Liu T, Chen W, Oka S, Fu C, Jain MR, Parrott AM, Baykal AT, Sadoshima J, Li H. Mol Cell Proteomics. 2010; 9:2262–2275. [PubMed: 20660346]
5. Wu C, Parrott AM, Fu C, Liu T, Marino SM, Gladyshev VN, Jain MR, Baykal AT, Li Q, Oka S, Sadoshima J, Beuve A, Simmons WJ, Li H. Antioxid Redox Signal. 2011; 15:2565–2604. [PubMed: 21453190]
6. Wu C, Parrott AM, Liu T, Jain MR, Yang Y, Sadoshima J, Li H. J Proteomics. 2011; 74:2498–2509. [PubMed: 21704743]
7. Benhar M, Forrester MT, Stamler JS. Nat Rev Mol Cell Biol. 2009; 10:721–732. [PubMed: 19738628]
8. Forrester MT, Seth D, Hausladen A, Eyler CE, Foster MW, Matsumoto A, Benhar M, Marshall HE, Stamler JS. J Biol Chem. 2009; 284:36160–36166. [PubMed: 19847012]
9. Ago T, Sadoshima J. J Mol Cell Cardiol. 2006; 41:41, 762–773.
10. Yamamoto M, Yang G, Hong C, Liu J, Holle E, Yu X, Wagner T, Vatner SF, Sadoshima J. J Clin Invest. 2003; 112:1395–1406. [PubMed: 14597765]
11. Gow AJ, Davis CW, Munson D, Ischiropoulos H. Methods Mol Biol. 2004; 279:167–172. [PubMed: 15199244]
12. Jaffrey SR, Snyder SH. Sci STKE. 2001; 86:pl1. [PubMed: 11752655]
13. Sun J, Morgan M, Shen RF, Steenbergen C, Murphy E. Circ Res. 2007; 101:1155–1163. [PubMed: 17916778]
14. Kuncewicz T, Sheta EA, Goldknopf IL, Kone BC. Contrib Nephrol. 2004; 141:221–230. [PubMed: 14650235]

15. Hao G, Derakhshan B, Shi L, Campagne F, Gross SS. *Proc Natl Acad Sci USA*. 2006; 103:1012–1017. [PubMed: 16418269]
16. Sayed N, Baskaran P, Ma X, van den Akker F, Beuve A. *Proc Natl Acad Sci USA*. 2007; 104:12312–12317. [PubMed: 17636120]
17. Barglow KT, Knutson CG, Wishnok JS, Tannenbaum SR, Marletta MA. *Proc Natl Acad Sci USA*. 2011; 108:E600–6. [PubMed: 21849622]
18. Chen YY, Chu HM, Pan KT, Teng CH, Wang DL, Wang AH, Khoo KH, Meng TC. *J Biol Chem*. 2008; 283:35265–35272. [PubMed: 18840608]
19. Wang Y, Liu T, Wu C, Li H. *J Am Soc Mass Spectrom*. 2008; 19:1353–1360. [PubMed: 18635375]
20. Benhar M, Thompson JW, Moseley MA, Stamler JS. *Biochemistry*. 2010; 49:6963–6969. [PubMed: 20695533]
21. Paige JS, Xu G, Stancevic B, Jaffrey SR. *Chem Biol*. 2008; 15:1307–1316. [PubMed: 19101475]
22. Forrester MT, Thompson JW, Foster MW, Nogueira L, Moseley MA, Stamler JS. *Nat Biotechnol*. 2009; 27:557–559. [PubMed: 19483679]
23. Murray CI, Uhrigshardt H, O'Meally RN, Cole RN, Van Eyk JE. *Mol Cell Proteomics*. 2012; 11:M111.013441. [PubMed: 22126794]
24. Held JM, Danielson SR, Behring JB, Atsriku C, Britton DJ, Puckett RL, Schilling B, Campisi J, Benz CC, Gibson BW. *Mol Cell Proteomics*. 2010; 9:1400–1410. [PubMed: 20233844]
25. Knipp M, Braun O, Gehrig PM, Sack R, Vasak M. *J Biol Chem*. 2003; 278:3410–3416. [PubMed: 12441345]
26. Forrester MT, Foster MW, Benhar M, Stamler JS. *Free Radic Biol Med*. 2009; 46:119–126. [PubMed: 18977293]
27. Fu C, Hu J, Liu T, Ago T, Sadoshima J, Li H. Quantitative analysis of redox-sensitive proteome with DIGE and ICAT. *J Proteome Res*. 2008; 7:3789–3802. [PubMed: 18707151]
28. Aracena-Parks P, Goonasekera SA, Gilman CP, Dirksen RT, Hidalgo C, Hamilton SL. *J Biol Chem*. 2006; 281:40354–40368. [PubMed: 17071618]
29. Zhang X, Huang B, Zhou X, Chen C. *Protein Cell*. 2010; 1:675–687. [PubMed: 21203939]
30. Chouchani ET, Hurd TR, Nadtochiy SM, Brookes PS, Fearnley IM, Lilley KS, Smith RAJ, Murphy MP. *Biochem J*. 2010; 430:49–59. [PubMed: 20533907]
31. Held JM, Gibson BW. *Mol Cell Proteomics*. 2012; (11):R111.013037. [PubMed: 22159599]
32. Nakamura T, Wang L, Wong CC, Scott FL, Eckelman BP, Han X, Tzitzilonis C, Meng F, Gu Z, Holland EA, Clemente AT, Okamoto S, Salvesen GS, Riek R, Yates JR 3rd, Lipton SA. *Mol Cell*. 2010; 39:184–195. [PubMed: 20670888]
33. Kornberg MD, Sen N, Hara MR, Juluri KR, Nguyen JV, Snowman AM, Law L, Hester LD, Snyder SH. *Nat Cell Biol*. 2010; 12:1094–1100. [PubMed: 20972425]
34. Pawloski JR, Hess DT, Stamler JS. *Nature*. 2001; 409:622–626. [PubMed: 11214321]
35. Ramachandran N, Root P, Jiang XM, Hogg PJ, Mutus B. *Proc Natl Acad Sci USA*. 2001; 98:9539–9544. [PubMed: 11493694]
36. Mitchell DA, Morton SU, Fernhoff NB, Marletta MA. *Proc Natl Acad Sci USA*. 2007; 104:11609–11614. [PubMed: 17606900]
37. Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS. *Nature*. 2001; 410:490–494. [PubMed: 11260719]
38. Bateman RL, Rau H, Tavshanjian B, Shokat KM. *J Biol Chem*. 2008; 283:35756–35762. [PubMed: 18826943]
39. Hausladen A, Gow AJ, Stamler JS. *Proc Natl Acad Sci USA*. 1998; 95:14100–14105. [PubMed: 9826660]
40. Sliskovic I, Raturi A, Mutus B. *J Biol Chem*. 2005; 280:8733–8741. [PubMed: 15611098]

Highlights

- Quantitative proteomics methods can measure changes of peptide S-nitrosylation status.
- Transnitrosylases catalyze increased S-nitrosylation of specific peptides
- Denitrosylases catalyze decreased S-nitrosylation of specific peptides
- Quantitative proteomics information can be used to characterize transnitrosylase and denitrosylase activities

Figure 1. BST/ICAT work flow for the quantification of Trx1 transnitrosylase and denitrosylase target SNO-peptides

There are nine steps involved in this procedure; **A**: Free thiols in proteins are first blocked with MMTS; **B**: Nitrosothiol reduction by ascorbate; **C**: Labeling of the newly formed free thiols with ICAT; **D**: Mixing ICAT light and heavy reagents-labeled proteins; **E**: Trypsin digestion of the protein mixtures; **F**: Enrichment of ICAT-labeled peptides using an avidin cartridge; **G**: TFA cleavage of biotins from ICAT-labeled peptides; **H**: Desalting of the cleaved peptides; **I**: Identification of SNO-Cys site and quantification of SNO-peptides by LC/MS/MS. **J**: An example MS spectrum of SNO-GAPDH [235-248] quantification by ICAT. **K**: An example MS spectrum of the identification of SNO-GAPDH [235-248] and localization of SNO-Cys²⁴⁷ by MS/MS. Modified from Wu *et al.*, 2011 (6).

Figure 2. Anti-biotin Western blotting of 2DE-resolved and ICAT-labeled proteins following SNO-Trx1 transnitrosylation

ICAT labeled protein pellets (100 μ g) were first dissolved in a 2DE buffer and loaded onto IPG strips for isoelectric focusing (IEF). After IEF, the proteins on the IPG strips were reduced with DTT and alkylated with iodoacetamide, followed by the second dimension of separation in SDS gels. Subsequently, the proteins in each gel were transferred onto a nitrocellulose membrane. ICAT labeled SNO-proteins were detected by an anti-biotin antibody. **A:** Endogenous SNO-proteins isolated from SH-SY5Y cells. **B:** Increase of select SNO-proteins following SNO-Trx1 treatment, as seen from both the number and levels of SNO-protein signals detected.

Figure 3. Identification and quantification of Trx1 target SNO-peptides

A: Example MS/MS spectrum of RL12_HUMAN peptide 17-C*TGGEVGATSALAPK-31 (M^{2+} 799.44, C*: ICAT-H-labeled cysteine). **B:** Dramatic increase of SNO-RL12 [Cys¹⁷-Lys³¹] from the MS spectrum of ICAT-L (control) and ICAT-H (SNO-Trx1 treated) ion signals indicates the degree of transnitrosylation. **C:** Dramatic decrease of SNO-RL12 [Cys¹⁷-Lys³¹] from MS spectrum of ICAT-H (control) and ICAT-L (Trx1/TrxR/NADPH treated) ion signals indicates the degree of denitrosylation. **D:** MS/MS spectrum of SNO-Trx1 peptide 73-C*MPTFQFFK-81 (M^{2+} 692.85, C*: ICAT-H-labeled cysteine). **E:** Dramatic decrease of SNO-Trx1 [Cys⁷³-Lys⁸¹] from the MS spectrum of ICAT-L (control) and ICAT-H (SNO-Trx1 treated) ion signals indicates the degree of NO transfer from SNO-Trx1 onto the target proteins. **F:** Dramatic decrease of SNO-Trx1 [Cys⁷³-Lys⁸¹] from MS spectrum of ICAT-H (control) and ICAT-L (Trx1/TrxR/NADPH treated) ion signals indicates the degree of denitrosylation of SNO-Trx1 by reacting with Trx1/TrxR/NADPH.

Table 1

Examples of proteins with transnitrosylase and denitrosylase activities.

S-transnitrosylase	Targets	Reference
Caspase 3	XIAP	[32]
GAPDH	DNA-PK, HDAC2, SIRT1	[33]
Hemoglobin	AE1	[34]
Protein disulfide isomerase	N-dansylhomocysteine	[35]
Thioredoxin 1	Procaspase 3	[36]
Denitrosylase	Targets	Reference
Alcohol dehydrogenase III	GSNO	[37]
Carbonyl reductase	GSNO	[38]
Flavohemoglobin	NO	[39]
Protein disulfide isomerase	SNO-PDI	[40]
Thioredoxin 1	SNO-Caspase 3	[2]
Thioredoxin 2	SNO-Caspase 3	[2]

Table 2

Proteomics methods for SNO-peptide and SNO-protein quantification.

Method	Starting Materials	Advantages	Disadvantages	References
Direct MS Detection	Purified peptides and proteins	Rapid and direct detection of protein S-nitrosylation without the sample loss and artifacts from complicated derivatization steps.	<ol style="list-style-type: none"> 1 Not suitable for the detection of proteins in complex mixtures; 2 Not suitable for locating SNO-Cys in peptides containing multiple cysteines. 3 Low throughput 	[17–19]
ICAT	Proteins extracted from HeLa Cells & cerebellum	<ol style="list-style-type: none"> 1 Enable the comparison of two samples in one experiment 2 Enable simultaneous SNO-Cys localization and SNO-peptide quantification 3 Amenable to multidimensional separation 4 Suitable for large scale screening 5 The cleavage step can remove biotins to produce simpler MS/MS spectra for SNO-Cys localization 	<ol style="list-style-type: none"> 1 The reagents are costly 2 The throughput is relatively low compared to other multiplex proteomics methods 	[6, 18, 21]
SILAC coupled with biotin switch	Proteins extracted from Jurkat cells	<ol style="list-style-type: none"> 1 Enable the comparison of up to three cellular extracts in one experiment 2 Enable simultaneous SNO-Cys localization and SNO-peptide quantification 3 Enable detection of <i>in vivo</i> S-nitrosylation events 4 Amenable to multidimensional separation & large scale screening 	<ol style="list-style-type: none"> 1 Not suitable for detection of protein from tissues and non-dividing primary cells 2 The cell culture process for reagent incorporation is time consuming 3 Biotinylated peptides contribute to complex MS/MS spectra, hinders automated SNO-peptide identification and SNO-Cys localization 	[20]
SNO-CAP	Proteins extracted from rat cerebellum	<ol style="list-style-type: none"> 1 Enable the comparison of two samples in one experiment 2 Enable simultaneous SNO-Cys localization and SNO-peptide quantification 3 Has similar SNO-Cys detection specificity as biotin-HPDP 4 Amenable to multidimensional separation & large scale screening 	<ol style="list-style-type: none"> 1 Reagents are not commercially available 2 Biotinylated peptides contribute to complex MS/MS spectra, hinders automated SNO-peptide identification and SNO-Cys localization 	[21]
SNO-RAC	Proteins extracted from human embryonic kidney cells	<ol style="list-style-type: none"> 1 Fewer steps than regular BST 	<ol style="list-style-type: none"> 1 Reagents are not commercially available 	[22]

Method	Starting Materials	Advantages	Disadvantages	References
		<ol style="list-style-type: none"> 2 Efficient detection of high-mass SNO-proteins 3 When coupled with isotopic or isobaric tag labeling, it allows the quantification of SNO-peptides from up to eight samples 	<ol style="list-style-type: none"> 2 MMTS, other disulfides and oxidized cysteines can also be released from the capture resins, thus could interfere with the identification and quantification of genuine SNO-peptides 	
TMT	Proteins extracted from human pulmonary arterial endothelial cells	<ol style="list-style-type: none"> 1 Enable the comparison of six samples in one experiment 2 Enable simultaneous SNO-Cys localization and SNO-peptide quantification 3 Amenable to multidimensional separation & large scale screening 	<ol style="list-style-type: none"> 1 The reagents are costly 2 The specificity of antibody enrichment of TMT tagged peptide is low. 	[23]