Dual Labeling Biotin Switch Assay to Reduce Bias Derived From Different Cysteine Subpopulations
A Method to Maximize S-Nitrosoylation Detection


Rationale: S-nitrosoylation (SNO), an oxidative post-translational modification of cysteine residues, responds to changes in the cardiac redox-environment. Classic biotin-switch assay and its derivatives are the most common methods used for detecting SNO. In this approach, the labile SNO group is selectively replaced with a single stable tag. To date, a variety of thiol-reactive tags have been introduced. However, these methods have not produced a consistent data set, which suggests an incomplete capture by a single tag and potentially the presence of different cysteine subpopulations.

Objective: To investigate potential labeling bias in the existing methods with a single tag to detect SNO, explore if there are distinct cysteine subpopulations, and then, develop a strategy to maximize the coverage of SNO proteome.

Methods and Results: We obtained SNO-modified cysteine data sets for wild-type and S-nitrosoglutathione reductase knockout mouse hearts (S-nitrosoglutathione reductase is a negative regulator of S-nitrosoglutathione production) and nitric oxide–induced human embryonic kidney cell using 2 labeling reagents: the cysteine-reactive pyridylidithiol and iodoacetyl based tandem mass tags. Comparison revealed that <30% of the SNO-modified residues were detected by both tags, whereas the remaining SNO sites were only labeled by 1 reagent. Characterization of the 2 distinct subpopulations of SNO residues indicated that pyridylidithiol reagent preferentially labels cysteine residues that are more basic and hydrophobic. On the basis of this observation, we proposed a parallel dual-labeling strategy followed by an optimized proteomics workflow. This enabled the profiling of 493 SNO sites in S-nitrosoglutathione reductase knockout hearts.

Conclusions: Using a protocol comprising 2 tags for dual-labeling maximizes overall detection of SNO by reducing the previously unrecognized labeling bias derived from different cysteine subpopulations. (Circ Res. 2015;117:846-857. DOI: 10.1161/CIRCRESAHA.115.307336)

Key Words: nitric oxide ■ oxidation-reduction ■ proteomics ■ S-nitrosothiols

Nitric oxide is well known to play important roles in the regulation of redox signaling through cGMP-dependent pathway. In the past decade, however, it also has been determined to signal through an oxidative post-translational modification of cysteine thiols, S-nitrosylation (SNO, also known as S-nitrosation). SNO has been proposed to trigger molecular redox-switches, which respond to changes in the redox-environment by regulating protein function. This modification has been implicated in a broad range of physiology and pathophysiology in the cardiovascular systems.

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This labile cysteine modification is most commonly detected using variations of the biotin-switch assay, originally described in 2001. In this approach, unmodified cysteine residues are blocked with a thiol-reactive agent, then SNO-modified

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residues are selectively reduced with ascorbate and labeled with biotin-HPDP, allowing the formerly SNO-modified proteins or peptides to be enriched using affinity chromatography. Since then, various labeling reagents\textsuperscript{10–19} have been introduced into the biotin-switch technique with the underlying assumption that labeling of free cysteine residues after the reduction of SNO groups is complete. However, we have observed that there is little overlap in the SNO sites obtained from different proteomic switch experiments,\textsuperscript{20} although at the time, these differences were not recognized, indicating that there could be differences in the chemistry between the various labeling reagents and the individual SNO-modifiable cysteine residues.

To investigate this potential labeling bias, we compared SNO-modified cysteine data sets equivalently handled but detected using 2 recently developed isobaric reagents that share same chemical structure except their cysteine-reactive group; cysteine-reactive tandem mass tag (cysTMT)\textsuperscript{18,21,22} and iodoacetyl tandem mass tag (iodoTMT)\textsuperscript{19,23} (Figure 1). Comparison of these reagents in both cultured cells and cardiac tissues revealed a systemic labeling bias in biotin-switch-style assays. Since then, various labeling reagents\textsuperscript{10–19} have been introduced into the biotin-switch technique with the underlying assumption that labeling of free cysteine residues after the reduction of SNO groups is complete. However, we have observed that there is little overlap in the SNO sites obtained from different proteomic switch experiments,\textsuperscript{20} although at the time, these differences were not recognized, indicating that there could be differences in the chemistry between the various labeling reagents and the individual SNO-modifiable cysteine residues.

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**Methods**

An extended Methods section is available in the Online Data Supplement.

**Cell Lysates and Heart Homogenates Preparation**

Human embryonic kidney (HEK) 293 cells were lysed in lysis buffer (PBS pH 7.4, 1 mmol/L EDTA, and 0.1 mmol/L n-octoicpronine). For parallel labeling, 400 μg of HEK cell lysate per condition in PEN buffer was treated with 0.1 mmol/L GSNO or 3 different control treatments (untreated vehicle, 0.1 mmol/L reduced glutathione, and 0.1 mmol/L oxidized glutathione for negative controls and untreated vehicle as a positive control) for 20 minutes at 37°C (n=4, from separately cultured plates). GSNO was removed using a Zeba desalt spin column (Thermo) equilibrated with PEN pH 7.4 according to the manufacturer’s protocol. The solution of remaining free thiols diluted to 0.5 g/L was blocked with 20 mmol/L of N-ethylmaleimide (NEM) in the presence of 2.5% (wt/vol) SDS and incubated for 20 minutes at 50°C. Excess NEM was removed by the PEN pH 8.0 equilibrated Zeba desalt spin column. As a positive control, an additional untreated sample (400 μg) was processed but not blocked with NEM. GSNO-treated and control samples were split for labeling with either cys- or iodoTMT\textsuperscript{6} (200 μg/each) and half of them (each n=4) were labeled with each sixplex (126–131) of cys- or iodoTMT\textsuperscript{6}, individually (n=4 for cysTMT and n=4 for iodoTMT). For cysTMT\textsuperscript{6} labeling, samples were diluted to 0.41 g/L in PEN pH 7.4, 1 mmol/L sodium ascorbate, 1 mmol/L CuSO\textsubscript{4}, and 0.3 mmol/L cysTMT\textsuperscript{6} (cysTMT\textsuperscript{6} in the case of the positive control) and incubated for 2 h at 37°C. For iodoTMT\textsuperscript{6} labeling, samples were diluted to 0.41 g/L in PEN pH 8.0, 5 mmol/L sodium ascorbate, and 0.3 mmol/L iodoTMT\textsuperscript{6} with equivalent incubation. Excess label was removed by acetone precipitation, and the resultant pellets were carefully washed with an additional volume of acetone. Pellets were resuspended to 1 g/L with PEN and 0.5% of SDS.

Sequential labeling was performed in the iodoTMT\textsuperscript{6}-labeling condition. After acetone precipitation, pellets were resuspended with cysTMT\textsuperscript{6}-labeling buffer as described and incubated for 2 hours for the second labeling. For simultaneous labeling, samples were incubated with equal concentration of cysTMT\textsuperscript{6} and iodoTMT\textsuperscript{6}, 5 mmol/L sodium ascorbate, and trace of metals as described in Online Figure VIII. TMT switch assay with individual recombiant proteins or heart homogenates (450 μg/each) was performed in the same manner as parallel labeling.

TMT switch assay in identical labeling conditions on the 2 labels was performed following the methods for iodoTMT\textsuperscript{6} as described above (PEN pH 8.0, 5 mmol/L sodium ascorbate, and 0.3 mmol/L cys- or iodoTMT\textsuperscript{6}), with recombiant protein, HEK cell lysates, and heart homogenates. In these sets of assays, nonreduced condition (no-sodium ascorbate) was used as a negative control. All steps were protected from light.

**SNO Peptides Enrichment for Mass Spectrometry Analysis**

Elutions from parallel labeling experiments were performed in 2 ways: unlabelled peptides were removed by washing with TBS, and TMT-labeled peptides were eluted with 500 mmol/L triethylammonium bicarbonate pH 8.5 buffer containing 10 mmol/L N,N-diisopropylethylamine (n=2 in separate 6plexes for both labels). Or elution was performed as described previously with 50% (v/v) acetoniitrile/ 0.4% (v/v) trifluoroacetic acid\textsuperscript{18} (n=2 in separate 6plexes for both labels). Both elution methods were applied alternatively in different replicates to increase TMT peptide identification.

**Data Analysis**

Raw mass spectrometry (MS) data were searched using OMSSA (version 2.1.9)\textsuperscript{30} against the human or mouse Uniprot database. The MS spectra were also searched using the X!Tandem algorithm (version TPF v4.4, rev 1).\textsuperscript{26} SNO sites were searched based on FASTA sequence in Uniprot using customized code. Quantitative values for the TMT reporter ions were collected with the Libra module of the transproteome pipeline using a custom condition file (Online Figure IX). The fold increase in TMT intensity of GSNO over maximum background signal was indicative of the detected extent of SNO modification and used as intensity index. For experiments performed with nonreduced control (no ascorbate), a site of SNO modification was determined if the TMT intensity in the ascorbate treated sample was greater than the no-ascorbate sample.

The MS data have been deposited to the ProteomeXchange (http://www.proteomexchange.org) via the PRIDE partner repository\textsuperscript{27} (PXD000741).

**Functional Properties Analysis**

Gene ontology–enrichment analysis, molecular function, biological process, and cellular compartment were executed using cytoscape\textsuperscript{30} with the Bingo plug-in.\textsuperscript{29} The statistical test used was the hypergeometric test with a Benjamini and Hochberg false discovery rate correction with a selected significance level of 0.05. The results were compared between groups, and unique ontology categories between...
the groups were analyzed. Spindle graphs of the enriched gene ontologies were created using a custom script and cytoscape.28

Statistics
Difference of isoelectric point was analyzed using a t test using GraphPad Prism 5. Data in bar graphs represent mean±SEM. P values <0.05 were considered significant.

Results
Detection and Site Mapping of SNO Modifications Using CysTMT or IodoTMT
In our initial investigations, we found that although the newly developed iodoTMT was effective at labeling cysteine residues, it could not be simply substituted into the previously reported cysTMT switch protocol18 for SNO modifications (Figure 1A; Online Figure IA). The cysTMT multiplex reagent shares a similar chemical structure that releases the same reporter ions (Figure 1B and 1C), and both reagents are enriched by the TMT affinity chromatography. However, cysTMT has a pyridyldithiol-reactive group that forms a reversible disulfide bond with an available cysteine, whereas iodoTMT uses an iodoacetyl group to irreversibly alkylate cysteines. IodoTMT was recently developed to circumvent the reversible cysTMT modification, which would allow iodoTMT-labeled samples to undergo routine reduction and alkylation before MS analysis.19 To achieve effective incorporation of iodoTMT into the assay, various labeling conditions were evaluated including potential radical initiators or propagators (Online Figure I). Independently optimized protocols (Online Table I) were used to detect SNO modifications using the iodoTMT and cysTMT reagents. TMT switch assays were performed on HEK 293 cell lysates treated with 5 different conditions; GSNO to induce SNO, 3 negative control conditions (untreated, reduced glutathione, and

Figure 1. Comparison of the cysteine-reactive tandem mass tag (cysTMT)– and iodoacetyl tandem mass tag (iodoTMT)–labeling reagents. A, Reaction scheme for the TMT switch assay. *Reactions performed in situ. B and C, Chemical structure of cysTMT (B) and iodoTMT (C). D, Western blot analysis indicating cysTMT and iodoTMT labeling. Both TMTs label S-nitrosylation (SNO) proteins under nitric oxide–inducing conditions. Reduced glutathione (GSH) and S-nitrosoglutathione reductase (GSSG) were used as negative controls. E and F, MS2 spectra (inset) and reporter ions clusters for the same peptide (PC[TMT]SEETPAISPSK, dUTPase nuclear isoform) identified by cysTMT (E) and iodoTMT (F). SNO modification is determined when the S-nitrosoglutathione (GSNO)–treated reporter ion intensity (129 Da) is at least 2-fold greater than greatest control condition: untreated (126 Da), GSH (127 Da), or GSSG (128 Da).
oxidized glutathione), and unblocked positive control (labeling of all available cysteine residues). Samples were divided and labeled with cysTMT6 as previously established or iodoTMT6 followed by the optimized protocol (see Methods section of this article), in a multiplex format. Then they were subsequently examined by Western blot analysis (Figure 1D) or digested, desalted, and analyzed by MS (Figure 1E and 1F). Analysis of the complete spectral data set resulted in the identification of 1008 unique SNO-modified residues on 773 proteins (Online Tables II and III). The iodoTMT6 reagent identified 648 SNO-modified cysteine residues (corresponding to 495 proteins), whereas cysTMT6 identified 731 SNO-modified sites in 557 proteins in the same biological samples, handled equivalently (Figure 2A).

Approximately one third of the SNO-modified residues (371 sites) were detected by both of the reagents. A total of 277 SNO-modified cysteine residues were identified exclusively with iodoTMT6 and this included 200 proteins whose SNO sites were only detected using iodoTMT6 (Figure 2A). When the samples were labeled with either cysTMT6 or iodoTMT6 under an identical reducing condition (Methods in the Online Data Supplement), less than one third of the total SNO sites were identified by both of the reagents (Online Table IV), whereas >60% of the sites were commonly detected between replicates of the same label (Online Figure II).

**Labeling Bias in Classic Biotin-Switch-Style Assay Reveals 2 Subpopulations of cysteine Residues**

We have shown that cysTMT6 and iodoTMT6 target different cysteine residues in the same experiment, suggesting the possibility for distinct subclasses of cysteine residues. Our data set contained numerous examples, highlighting the specificity of the labeling bias including Cys8 of dynein light chain Tctex-type 3, which was labeled preferentially by cysTMT6 (Figure 2C). Other sites, such as Cys49 of pyruvate kinase PKM, were preferentially labeled by iodoTMT6, whereas Cys357 of T-complex protein 1α reacted equivalently to both reagents (Figure 2B and 2C).

This labeling bias was validated by a TMT switch assay with recombinant proteins. For example, SNO cysteine residues on human actin was only labeled with iodoTMT, whereas SNO-modified bovine actin was preferentially detected by cysTMT (Figure 2D; Online Figure III).

**Validation of SNO-Labeling Bias in Mouse Hearts**

Next, we applied the dual-reagent protocol and optimized proteomics workflow for MS (discussed in a later section) to identify SNO modifications in mouse hearts. Left ventricular tissue from intact mouse hearts was homogenized, labeled by either iodo- or cysTMT6 and analyzed through the optimized pipeline as described later. As observed in HEK cell analysis, of the 315 total SNO sites identified, 98 sites were detected with both reagents, whereas 113 sites and 104 sites were...
Figure 3. Sequential cysteine-reactive tandem mass tag (cysTMT) and iodoacetyl tandem mass tag (iodoTMT) labeling for the TMT switch assay in human embryonic kidney cells. A, Scheme depicting sequential labeling methods. B, Mass spectrometry analysis of the sequential labeling found 14% of the S-nitrosylation (SNO) sites were detected by the second label, cysTMT, indicating incomplete labeling by iodoTMT and suggesting distinct subclasses of cysteine residues. NEM indicates N-ethylmaleimide.

Table. S-Nitrosylation Sites From Hearts

<table>
<thead>
<tr>
<th>Protein Group Description</th>
<th>Site</th>
<th>Labeled by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aconitate hydratase, mitochondrial</td>
<td>592</td>
<td>...</td>
</tr>
<tr>
<td>Actin, alpha cardiac muscle 1</td>
<td>219</td>
<td>Cys</td>
</tr>
<tr>
<td>Actin, alpha cardiac muscle 1</td>
<td>259</td>
<td>Cys</td>
</tr>
<tr>
<td>Acyl-coenzyme A thioesterase 2, mitochondrial</td>
<td>55</td>
<td>...</td>
</tr>
<tr>
<td>ADP/ATP translocase 1</td>
<td>257</td>
<td>Cys</td>
</tr>
<tr>
<td>Aspartate aminotransferase, cytoplasmic</td>
<td>160</td>
<td>...</td>
</tr>
<tr>
<td>ATP synthase subunit d, mitochondrial</td>
<td>101</td>
<td>Cys</td>
</tr>
<tr>
<td>Creatine kinase S-type, mitochondrial</td>
<td>180</td>
<td>Cys</td>
</tr>
<tr>
<td>Cytochrome b-c1 complex subunit 2, mitochondrial</td>
<td>192</td>
<td>Cys</td>
</tr>
<tr>
<td>Dihydrolipoyl dehydrogenase, mitochondrial</td>
<td>484</td>
<td>...</td>
</tr>
<tr>
<td>Elongation factor 2</td>
<td>693</td>
<td>Cys</td>
</tr>
<tr>
<td>Heat shock cognate 71 kDa protein</td>
<td>17</td>
<td>...</td>
</tr>
<tr>
<td>Isoform M1 of pyruvate kinase PKM</td>
<td>474</td>
<td>...</td>
</tr>
<tr>
<td>Malate dehydrogenase, mitochondrial</td>
<td>89</td>
<td>Cys</td>
</tr>
<tr>
<td>Myosin-6</td>
<td>1750</td>
<td>Cys</td>
</tr>
<tr>
<td>Protein kinase C and casein kinase II substrate protein 3</td>
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<td>Cys</td>
</tr>
<tr>
<td>Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial</td>
<td>536</td>
<td>Cys</td>
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<tr>
<td>Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial</td>
<td>245</td>
<td>Cys</td>
</tr>
<tr>
<td>Troponin I, cardiac muscle</td>
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<td>...</td>
</tr>
<tr>
<td>Ubiquitin-conjugating enzyme E2 D3</td>
<td>85</td>
<td>Cys</td>
</tr>
</tbody>
</table>

Selected examples of endogenous S-nitrosylation sites, which were detected from cardiac tissues of wild-type mice.

identified only with cys or iodoTMT respectively (Online Table VI). We also performed the switch assay under identical reducing conditions with 4- to 5-month-old wild-type mouse hearts (Online Table VII). More than 70% of TMT-labeled sites were identified with only 1 tag. Additional 44 sites not identified with cysTMT were determined with iodoTMT, confirming the labeling bias. SNO of muscle proteins such as cardiac troponin I (cTnI) (Cys81), α-actinin-2 (Cys781), and isoform 3 of LIM domain-binding protein 3 (Cys511/Cys514) were exclusively identified via iodoTMT labeling.

Using our 2-label strategy, we were able to confirm SNO modifications previously identified in untreated cardiac tissue and identify several novel modified sites (selected sites are listed in the Table). For example, cardiac isoform sarcoplasmic/endoplasmic reticulum Ca2+ ATPase (SERCA2A), a key protein for Ca2+ handling, was identified to be S-nitrosylated in this study. It has been reported that the SNO increases the activity of SERCA2A and thus enhances Ca2+ uptake into sarcoplasmic reticulum and decreases cytosolic Ca2+ during ischemia/reperfusion, suggesting SNO-SERCA2A as a potential mediator for cardioprotection. SERCA2A had several SNO-modified cysteine residues previously reported such as Cys364 or Cys471 and also new sites Cys344 and Cys349 were identified in this study, which were exclusively labeled with either of TMT reagents. The other examples, the sites of the muscle proteins described above, Cys781 of α-actinin-2 and Cys511/Cys514 of isoform 3 of LIM domain-binding protein 3, were newly identified to be S-nitrosylated. Without the use of both tags, the detection of SNO modification would be incomplete. On the basis of these data, it is...
clear that the addition of an alternate cysteine-reactive tag is required to reduce the labeling bias and thus increase SNO proteome coverage.

Proteomic Profiling of SNO in GSNOR Knockout Mouse

GSNOR is a negative regulator of GSNO in the cell and is thought to govern the SNO level. It has also been identified as a key regulator of cardiac function. Therefore, the GSNOR knockout mouse model was chosen to perform a high-throughput quantitative analysis of SNO modification in mouse hearts and also to highlight the labeling bias across different pools of modified cysteines. By applying the dual-reagent protocol, SNO sites in GSNOR knockout hearts were profiled for the first time; a total of 493 SNO sites were identified with either or both of the reagents (Online Table VIII) and a set of the sites partially overlapped with the sites of wild-type (Figure 4A). Of these, SNO sites of widely studied cardiac proteins in the GSNOR knockout were determined.

cTnI is the inhibitory subunit of the troponin complex, which regulates muscle contraction, and is extensively regulated by phosphorylation. cTnI exists as a complex with troponin T and troponin C (cTnC), which is the Ca\(^{2+}\)-binding subunit. The binding of Ca\(^{2+}\) to regulatory site of TnC induces a conformational change, which blocks the action of cTnI and generates muscle contraction. Although the functional roles of SNO in those proteins are not well characterized, we site-mapped Cys81 and Cys98 of cTnI and Cys35 of cTnC as S-nitrosylated in GSNOR knockout mice. In addition, 8 SNO sites of SERCA2A and 1 site of ryanodine receptor 2, one of the Ca\(^{2+}\) handling proteins, were found to be nitrosylated in this model. The SNO proteomes in WT and the GSNOR knockout were further compared based on gene ontology enrichment (Online Table XV).

Interestingly, the magnitudes of SNO-shift in GSNOR knockout hearts varied across sites because some were hypernitrosylated, whereas others were hyponitrosylated when compared with wild-type. As an example, Cys694 of ceruloplasmin displayed an SNO increase, whereas Cys376 of tubulin \(\alpha\)-4A and Cys349 of cardiac isoform SERCA2A were less nitrosylated in GSNOR knockout (Figure 4B). A total of 345 sites were hypernitrosylated, and 365 sites were less nitrosylated in the GSNOR-deficient model (Figure 4C). These 2 hyper-SNO and hypo-SNO groups were further analyzed based on gene ontology enrichment. Proteins with hyponitrosylated sites in GSNOR knockout were clustered to unique categories, including proteins that have channel or transporter activity or proteins located in extracellular region (Figure 4D; Online Figure IV).
The labeling bias in GSNOR knockout hearts was observed to be dominant with cysTMT-labeling over iodoTMT. Presumably, the biased preference to 1 tag occurs by specific cellular chemical environments, which may be different across various biological samples.

**Chemical Properties of the 2 Subpopulations**
To further investigate the potential subclasses of SNO sites detected by the different reagents, we analyzed the physicochemical properties of the cys- and iodoTMT-specific residues in the HEK and mouse hearts data sets. We hypothesized that a cysteine’s reactivity to a particular TMT labeling reagent is dictated by its surrounding chemical environment. Analogous to the selectivity of SNO formation and the chemical properties of neighboring amino acids may affect the likelihood that each cysteine residue will react preferentially with either reagent, whereas the protein’s structure determines accessibility. Although these properties were not enough to represent all elements for the environment of a certain reactive cysteine residue, they provide insight into the physicochemical effect.

All amino acid residues flanking each side of the cysteine residues that were specifically reactive to either cys- or iodoTMT from mouse hearts and HEK cell were compared. It revealed that cysTMT-specific sites from hearts had a higher proportion of high theoretical isoelectric point overall than the iodoTMT sites (Figure 5A; Online Table IX). The overall distributions for the isoelectric point were slightly different between the 2 reagents from HEK cell (Online Figure V; Online Table IX). The top 15 reactive (or intense) cysteine residues unique to each reagent were compared; the cysTMT-specific sites were found to have a higher proportion of neighboring positively charged residues (arginine/lysine) than the iodoTMT sites (Figure 5B; Online Figure VB; Online Table IX).

Next, more global characteristics were investigated for the proteins containing the most reactive (or intense) cysteine that were exclusively labeled by one or the other TMT reagent (Figure 5C and 5D; Online Table X). Analysis of aliphatic index revealed that proteins uniquely targeted by cysTMT had a higher number of aliphatic amino acid residues (alanine, valine, isoleucine, and leucine) than those by iodoTMT (Figure 5C). Analysis of molecular weight revealed that iodoTMT favored proteins with lower molecular weight than cysTMT group (Figure 5D). The analysis with data sets from samples labeled under identical conditions with control groups represented corresponding trends (Online Figure VI). This suggests a synergic effect between basic sequences and a more local/global hydrophobic structure may produce conditions more favorable to cysTMT than to iodoTMT. The iodoTMT reagent may be more accessible and reactive with cysteine residues located within smaller proteins, local regions with lower pKa, or more global polar character of the intact protein, in both HEK cells and mouse hearts.

**Figure 5. Physiochemical properties analysis of 2 subpopulations.** A, The theoretical isoelectric points (pl) of 113 exclusively cysteine-reactive tandem mass tag (cysTMT)-labeled residues and 104 iodoacetyl tandem mass tag (iodoTMT)-specific residues from mouse hearts were analyzed and illustrated using a bar graph and a color scale from low (red) to high (green) pl (left, cysTMT; right, iodoTMT). Higher proportion of low pl was observed in iodoTMT-specific group (n=3 per group). **P<0.01. B, The frequency of amino acids surrounding the cysteine residues of 2 groups was analyzed (left, cysTMT; right, iodoTMT). Valine/leucine and positively charged lysine/arginine were more frequently observed in cysTMT-specific group. Reactive cysteine is highlighted in green at position 11. C, Aliphatic index for proteins (whose cysteine residues were reactive; Online Table X) were computed based on the amino acid sequence and compared between protein groups, which were detected with only 1 TMT reagent. First color scale represents aliphatic index of proteins from human embryonic kidney cell (left, cysTMT; right, iodoTMT), and second color scale displays it from mouse hearts. Overall, cysTMT group represented more distribution of higher aliphatic characters. D, Molecular weight of the same proteins for (C) was analyzed and represented in the same way. IodoTMT group had higher proportion of low molecular weight proteins than cysTMT group.
cardiac tissue. Although a specific mechanism could not be determined, our findings demonstrate that the reagents can influence selectivity for labeling-specific classes of cysteine residues, presenting at least 2 classes of SNO-modifiable cysteine residues.

**Further Optimization of Experimental Strategies to Maximize SNO Detection**

To expand the SNO proteome coverage and ensure maximal capture of both subclasses of modified sites, different experimental configurations using both cysteine reactive tandem mass tag (cysTMT) and iodoacetyl tandem mass tag (iodoTMT) were evaluated: sequential, simultaneous, and parallel. Sequential labeling starting with either cys- or iodoTMT followed by the other second reagent was determined not to be efficient (Figure 3B; Online Figure VII; Online Table V). There was reduced TMT intensity for the second reagent along with an increased nonspecific labeling when compared with the single TMT-labeling experiments (Online Figure VII). This loss of reactivity is likely because of oxidation that accumulates during the additional labeling step. For simultaneous labeling experiments, the reaction conditions needed for efficient iodoTMT labeling was not compatible with those required for optimal cysTMT labeling (Figure 6A; Online Figure VIII). When adapting the cysTMT-labeling conditions to be compatible with iodoTMT, the simultaneous labeling was found to be less intense than under the optimized conditions for each label, because of poor iodoTMT labeling in both iodoTMT-compatible/incompatible buffers (right). Summary of a mass spectrometry analysis from a simultaneous labeling experiment demonstrating poor iodoTMT labeling efficiency. The majority of the detected TMT peptides were labeled with cysTMT. NEM indicates N-ethylmaleimide.

![Diagram of simultaneous labeling protocol](image)

**Figure 6. Simultaneous cysteine-reactive tandem mass tag (cysTMT) or iodoacetyl tandem mass tag (iodoTMT) labeling for the TMT switch assay in human embryonic kidney cells.** A, Scheme of simultaneous labeling protocol. B, The efficiency of iodoTMT labeling was assessed for simultaneous labeling. Nonreduced samples are shown in the left and dithiothreitol (DTT)-treated samples are shown in the right. DTT treatment after a coreagent labeling protocol would remove cysTMT (disulfide linked) but not iodoTMT (irreversibly alkylated), revealing the extent to iodoTMT labeling. IodoTMT was found to be less efficient in simultaneous labeling (indicated as combo [2×]) when compared with the single labeling. Total labeled S-nitrosylation (SNO) in simultaneous labeling approach was shown to increase than either cys- or iodoTMT single labeling when the concentration of TMT was double (left) but the efficiency of individual labels decreased than under the optimized conditions for each label, because of poor iodoTMT labeling in both iodoTMT-compatible/incompatible buffers (right). C, Summary of a mass spectrometry analysis from a simultaneous labeling experiment demonstrating poor iodoTMT labeling efficiency. The majority of the detected TMT peptides were labeled with cysTMT. NEM indicates N-ethylmaleimide.

for assignment of the MS spectra (Online Table XII) and the application of new enrichment protocol using a TMT analogue for elution (Online Table XIII). With MS-based analysis, a total of 1008 SNO-modified residues were identified, through OMSSA search (Figure 2A). A total of 731 SNO sites in 557 proteins and 648 sites corresponding to 495 proteins were identified with cys- and iodoTMT labeling, respectively (371 sites were detected by both of the reagents). When X!Tandem search engine was applied to the same mass spectra, 315 sites from 271 proteins were found to be SNO with cysTMT labeling and 338 SNO sites corresponding to 274 proteins were identified with iodoTMT labeling (159 sites detected by both TMTs). Overall, OMSSA identified higher number of SNO sites than X!Tandem and had >80% agreement with the X!Tandem data set (Online Table XII).

Unlike biotin-based labeling, whose labels are removed by reducing agent at enrichment step before MS analysis,9,14 the elution protocol of TMT preserves the tag during MS analysis. Previously, TMT-tagged peptides were eluted from immune-affinity chromatography by a low pH buffer to denature the interaction between the resin and the TMT peptides.18 In a new elution protocol, we introduced a small TMT analogue, which interacts with affinity resin to compete the bound TMT peptides. As shown in Online Table XIII, the number of SNO sites when using the TMT analogue was increased ≈38% over the low pH elution. We applied the new elution protocol in addition to the previous low pH elution method in this study to maximize coverage.

Further investigations indicated the majority of the SNO-modified proteins with a single SNO site and the subcellular locations and molecular functions of them, whose distributions are similar to those of other studies with a single tag (Figure 7; Online Table XIV).19,20 On the basis of this, our increased number of total SNO proteins was not derived from the specific detection of a certain functional class of proteins (eg, localized exclusively), and our method increased the depth of proteome coverage.
Significant roles of SNO have been proposed in cardiac physiology and pathophysiology. The modification is thought to affect protein function directly by increasing or inhibiting the activity or shielding the thiol groups on critical cysteine residues from further oxidation during severe oxidative stress conditions such as myocardial ischemia and reperfusion. It is expected that there are other SNO proteins for cardioprotection not identified yet, especially if they are low abundant. In addition, disruption of SNO in myocytes has been recognized as a critical effect in failing hearts. For example, Cys294 of ATP synthase was found less S-nitrosylated in dyssynchronous heart failure hearts than in cardiac resynchronization therapy hearts. Another example, hyponitrosylation of ryanodine receptor 2 because of imbalance redox, was observed in heart failure and suggested to contribute to diastolic Ca\(^2\) leak.

However, molecular mechanisms and potential therapeutic strategies by SNO in numerous cardiovascular diseases remain incompletely understood.

In the aid of better selection among the many existing SNO-labeling reagents such as biotin-HPDP, ICAT\(^{10,17}\), SNOSID\(^{11}\), SNOCAP\(^{13}\), SNO-RAC\(^{14}\), d\(_7\)-NEM\(^{16}\), cysTMT\(^{18}\) and iodoTMT\(^{19}\), sets of proteins from 2 different approaches have been compared.\(^{47}\) We have analyzed the data sets studied with 3 prevalent tags and different experimental protocols, but found little overlaps between them.\(^{20}\) Thus, in this study, we used same samples with controlled setting where the 2 labeling regents were identical except for their thiol-reactive groups as thioether or alkyl iodide, which are also common in most other labeling tags. In addition, we analyzed data sets at an amino acid site level for a more accurate comparison. It has been a fundamental assumption of previous studies that these tags and all reactions proceeded to completion, and thus all ascorbate-exposed cysteine were labeled. However, the result of our study demonstrated unsaturation of labeling by an individual label, which has underestimated the number of SNO-modified site. In light of this work, it is important to find a method that allows capture of more sites. Our bioinformatics approach to extract the position of each site allowed site-identification for the corresponding peptides. This has allowed removing peptides’ redundancy resulted from miscleavage of peptides, producing a more accurate comparison between the pools of cys- and iodoTMT-modified sites. More importantly, this enabled site-level quantitation, which provided better accuracy over the traditional peptide-level quantitation. Finally, this site-identification has made it possible to extract flanking amino acid sequences of cysteine, which was used for the analysis of consensus sequence and chemical properties.

This study discovered that there are at least 2 unique populations of SNO-modifiable cysteine residues with HEK and cardiac muscles that have distinct reactivity to 2 chemically classified tags. On the basis of this, it is clear that the use of a single tag strategy to detect SNO modifications will introduce a labeling bias. This could affect the interpretation of data and reduce the probability of identification of key modification of proteins because the reactivity of specific set of cysteine residues in a certain environment currently cannot be predicted. To date, all studies on SNO proteomics in the cardiovascular field used only 1 tag, which has led to a presumption that there is only a single SNO-reactive population. Thus, using both reagents will be required to ensure the detection and quantification of 2 subpopulations of an SNO-modifiable cysteine with different chemical characteristics. On the basis of this observation, our optimized parallel workflow is a generalizable method and can be applied to other proteomic investigations using a thiol-labeling switch strategy.
Using the dual-labeling strategy, we performed a proteomic profiling of SNO in GSNOR knockout mouse hearts. Previously, most studies have targeted specific SNO modifications. For example, SNO of β- Arrestin 2–promoted protein–protein interactions with clathrin in GSNOR knockout mouse spleen. However, few studies have investigated a global change of the entire SNO proteome in a GSNOR-deficient model. On the basis of function of GSNOR, levels of SNO in the knockout models were expected to increase overall, but it was not clear whether this would be true across all modified cysteines. Analysis of the ≈500 SNO-modified cysteine residues revealed that although the majority of sites had increased levels of modifications, more than one third were reduced. This implies that SNO regulation is a complex process, and other factors are involved in determining this redox-modification status. Our bioinformatics analysis with the hypo- and hyper-SNO sites suggests that protein functions or subcellular locations may be one of these factors. A set of proteins that contain hypo-SNO sites was uniquely clustered as transporter proteins by gene ontology–enrichment analysis, and this set of proteins was mainly located in membrane or mitochondrion and has metal or nucleotide-binding regions.

In the study, we determined that iodoTMT did not label SNO in the previously reported cysTMT switch protocol. To achieve effective incorporation of iodoTMT into this assay, various labeling conditions were tested and, although we were unable to determine the exact factors that inhibited labeling of iodoTMT compared with cysTMT, our studies successfully optimized conditions for effective iodoTMT labeling. One critical factor was the use of copper during labeling, which has been reported to accelerate ascorbate-dependent reduction of SNO without effecting specificity. However, for iodo-based TMT switch protocols with nitric oxide–inducing conditions, copper over a certain range inhibited iodoTMT labeling, while enhancing cysTMT labeling (Online Figure I). The alkyl halide moiety in iodoTMT has been indicated to be labile during radical-generating conditions, and labeling was recovered in the presence of 1 mmol/L copper by the addition of glycercol, a known radical scavenger in this study (Online Figure I). There are several candidates for a radical initiator or propagator in the TMT switch protocol. Some metals have been indicated as a source of radicals similar to those generated in the Fenton-like reaction, and it was reported hydroxyl radicals generated by Cu²⁺/H₂O₂ were stimulated specifically in HEPES buffer. This could explain the different labeling efficiency observed when labeling was performed in PBS or HEPES buffer. The TMT labeling step is coupled with the reduction of the SNO group by ascorbate, which has been shown to generate hydroxyl radical in copper-contaminated water, suggesting the formation of free radicals that could inhibit labeling.

In conclusion, the parallel combination of both the cysTMT and iodoTMT increased the coverage of SNO modifications identified; however, comparison of the individual reagent data sets indicated a clear bias in cysteine labeling between the 2 reagents. Each of the TMT reagents has thiol-reactive groups commonly used in the biotin-switch-style assays but have never been rigorously compared. The pyridylidithiol and iodoacetyl reactive groups in the different TMT reagents were found to label distinct but partially overlapping subclasses of cysteine residues. Our findings demonstrate an important but overlooked aspect of labeling in the classic switch assay, which if accounted for, can expand the accessible cysteine proteome.

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Disclosures

None.

References


What Is Known?

• S-nitrosylation (SNO) is a post-translational modification that serves as a key regulator of protein activity in cellular redox status and has many roles in cardiovascular disease, yet the identification and quantification of SNO-modified residues remain limited.
• The biotin-switch technique, which first reduces SNO with ascorbate and then chemically labels newly exposed cysteine residues with a stable tag, has been the most general strategy in this field.

What New Information Does This Article Contribute?

• When using 2 chemically different mass spectrometry–based tags to label the ascorbate-exposed cysteine residues, there are subpopulations of SNO-modified amino acid residues that are preferentially detected by one or the other of the tags, indicating that there has been an unrecognized labeling bias in the common biotin-switch assays.
• An optimized proteomic mass spectrometry–based workflow using 2 (chemically different) tags ensures the detection and quantification of the 2 subpopulations of SNO-modifiable cysteine with different chemical characteristics on the technical side.
• This labeling strategy enables a profiling of nearly 500 SNO sites in cardiac tissue of S-nitrosoglutathione reductase knockout mouse, which is an interesting model for studying redox regulation and reveals that SNO was reduced on one third of the sites, while being increased in the majority.

Investigating site-specific SNO quantification is important because SNO is a protein post-translational modification, which regulates protein activity and responds to cellular redox status. Therefore, the technique to detect SNO has been a long interest in cardiovascular research, and classic biotin-switch style technique has been a widely used method for this. This biotin-switch style techniques shared by a variety of labeling strategies assume that the labeling tag captures all available SNO. However, we show that this is not the case and the labeling bias, which has been unrecognized, exists in the commonly used biotin-switch style assays, allowing to detect only subsets of the SNO-sites. By more accurate amino acid site-level comparison between SNO sites detected by 2 chemically different tags, we provide the evidence that there are different subpopulations of SNO-modifiable cysteine residues, which can only (or preferentially) be detected by 1 labeling reagent. Collectively, we optimize a proteomic workflow using chemically different tags to maximize coverage of the SNO proteome of complex samples such as mouse hearts. We describe an important method that may have a large impact on research practice of all future SNO-based studies, the interpretation of existing SNO data, and our understanding of redox regulation in cardiovascular studies in an unbiased manner.

Novelty and Significance
Dual Labeling Biotin Switch Assay to Reduce Bias Derived From Different Cysteine Subpopulations: A Method to Maximize S-Nitrosylation Detection

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SUPPLEMENTAL MATERIAL

Dual labeling biotin switch assay to reduce bias derived from different cysteine subpopulations: A method to maximize S-nitrosylation detection

Heaseung Sophia Chung¹,³, Christopher I. Murray¹,⁸, Vidya Venkatraman²,³,⁶, Erin L. Crowgey⁶, Peter P. Rainer²,⁹, Robert N. Cole¹,⁴, Ryan D. Bomgarden⁵, John C. Rogers⁵, Wayne Balkan⁷, Joshua M. Hare⁷, David A. Kass²,³, Jennifer E. Van Eyk¹,²,³,⁶

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Detailed Methods

Reagents. IodoTMT®, cysTMT®, cysTMT0 (a non-multiplex version), antibody to TMT, anti-TMT affinity resin and Zeba desalt spin column were from Thermo Fisher Scientific. All other reagents and chemicals including N-ethylmaleimide (NEM), reduced glutathione (GSH), oxidized glutathione (GSSG), S-nitrosoglutathione (GSNO) and triethylammonium bicarbonate buffer (TEAB) pH 8.5 were obtained from Sigma-Aldrich and solution of GSNO was prepared fresh before each experiment. SOD1 recombinant protein was purchased from ProSpec and bovine actin and human beta-actin were from Cytoskeleton.

Cell culture. Human embryonic kidney (HEK) 293 cells were grown in Dulbecco’s modification of Eagle’s medium with 4.5 g/L glucose, L-glutamine, & sodium pyruvate (Corning Cellgro) at 37°C in a humidified atmosphere containing 5% CO₂. Cells were lysed in PBS pH 7.4, 0.02 mmol/L neocuproine, 0.2 mmol/L EDTA, 150 mmol/L NaCl and 1x protease inhibitor (Roche) containing 1.0% (v/v) triton X-100 using a probe sonicator and centrifuged for 10 min at 2000 X g. The protein concentration of the resulting supernatant was determined by BCA assay kit (Pierce).

Mice. S-nitrosoglutathione reductase (GSNOR) knock-out mice were generated as previously described. Wild-type C57BL/6J mice were purchased from the Jackson Laboratories. 4 to 5-month-old GSNOR knock-out and C57BL/6J mice (male and female) were used in the study performed under same reducing conditions (PEN pH 8.0, 5 mmol/L sodium ascorbate and 0.3 mmol/L cys- or iodoTMT6) for two labels. For the study in optimized conditions for parallel labeling (1 mmol/L sodium ascorbate, 1 mmol/L CuSO4 for cysTMT label, 5 mmol/L sodium ascorbate for iodoTMT), 8-month-old male C57BL/6 were used.

Whole-heart Homogenates Preparation. Animal studies were performed in accordance with National Institutes of Health Guidelines for the Care and Use of Animals and approved by the Institutional Animal Care and Use Committee at Johns Hopkins University. In the dark, left ventricles of C57BL/6 and GSNOR knock-out mouse were excised, immediately washed and homogenized in the homogenization buffer (on ice) containing (in mmol/L) sucrose (300), HEPES-NaOH 8.0 (250), EDTA (1), Neocuproine (0.1), N-ethylmaleimide (NEM) (20) and EDTA-free protease inhibitor tablet 1X (Roche) and centrifuged for 2 min at 1,500 g. All procedures were performed in the dark. Protein concentration was determined using the BCA assay.

Western Blot Analysis. General western blotting procedures were followed as described in a previous study for all TMT labeled samples. Exceptionally, half of each sample for one experiment of simultaneous labeling (Online Figure VIIC) was reduced by DTT prior to gel loading. Separated by SDS-PAGE and then transferred iodoTMT or cysTMT labeled proteins to nitrocellulose membranes were blocked with 5% (w/v) nonfat milk powder in Tris-buffered saline completed with 0.1% (v/v) Tween 20. They were detected using the primary antibody to TMT at room temperature for 1h followed by alkaline phosphatase conjugated secondary antibody to mouse (Jackson Immunoresearch Inc.) or anti-mouse horseradish peroxidase-conjugated secondary antibody (Jackson Immunoresearch Inc.) at room temperature for 30 min. Blots were washed and developed using immun-star substrate (Bio-Rad) or ECL substrate (GE Healthcare).

Cys and IodoTMT® Switch Assay in identical conditions with ‘no ascorbate’ control group. HEK cell lysates (or recombinant proteins) were diluted to 0.8 g/l in PEN buffer (PBS pH
7.4, 1 mmol/L EDTA and 0.1 mmol/L neocuproine). For parallel labeling, 520 µg of HEK cell lysate per condition in PEN buffer was treated with 0.1 mmol/L S-nitrosoglutathione (GSNO) or vehicle as a control for 20 min at 37 °C (n=4, from separately cultured plates). GSNO was removed using a Zeba desalt spin column (Thermo) equilibrated with PEN pH 7.4 according to the manufacturer’s protocol. The solution of remaining free thiols diluted to 0.5 g/l was blocked with 20 mmol/L of NEM in the presence of 2.5% (w/v) SDS and incubated for 20 min at 50°C. Excess NEM was removed by the PEN pH 8.0 equilibrated Zeba desalt spin column. GSNO-treated and control samples were split and half of them (260 µg/each) were diluted to 0.41 g/l in PEN pH 8.0, 5 mmol/L sodium ascorbate and 0.3 mmol/L cysTMT6 for 2 h at 37 °C. The other half were diluted with equivalent incubation without sodium ascorbate. TMT switch assay with iodoTMT6 was performed in the same manner as cysTMT6 labeling.

For TMT switch assay with heart homogenates, non-reduced condition (no-sodium ascorbate) was used as a negative control (450 µg/each).

All steps were protected from light.

**SNO-peptides Preparation for Mass Spectrometry (MS) Analysis.** 200 µg of each iodoTMT6 (or cysTMT6) sample were combined, desalted and digested with trypsin (Promega, 1: 40 of trypsin: protein)². A positive control (all available cysteine residues) sample was processed separately. The digested peptides were incubated with 600 µl of the anti-TMT antibody-affinity resin at room temperature for 2 h. Elutions were performed in two ways: Unlabeled peptides were removed by washing with 5 x 5 ml of TBS and TMT labeled peptides were eluted with 3 x 0.6 ml of 500 mmol/L TEAB pH 8.5 buffer containing 10 mmol/L N,N-diisopropylethylamine. Or elution was performed as described previously with 50% (v/v) acetonitrile /0.4% (v/v) trifluoroacetic acid². Both elution methods were applied alternatively in different replicates to increase TMT peptide identification. Samples were then desalted using solid phase extraction with Oasis HLB (Waters).

**Peptide Identification by Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS).** LC/MS/MS analysis was performed using an LTQ Orbitrap Velos MS (Thermo Fisher Scientific) interfaced with a nanoAcquity UPLC system (Waters). Peptides from the parallel labeling were fractionated by reverse-phase HPLC on a 75 µm x 15 cm PicoFrit column with a 15 µm emitter (PF3360-75-15-N-5, New Objective) packed in-house with Magic C18AQ (5 µm, 120Å, Michrom) using 0–60% acetonitrile/0.1% formic acid gradient over 90 min at 300 nl/min⁻¹. Eluting peptides were sprayed directly into an LTQ Orbitrap Velos at 2.0 kV. Isolation width was set to 1.3 Da and normalized collision energy was set to 38. Precursors were acquired at 30,000 resolution and 15,000 and 7,500 for the fragment ions. High energy collision dissociation was used exclusively with a lock mass of the polysiloxane at 371.101230 for MS1 and MS2.

For peptides from sequential and simultaneous labeling and TMT- labeled samples described in in this Online supplemental, LC/MS/MS analysis was performed using an Easy-nLC 1000 (Thermo Scientific, mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1 % formic acid in acetonitrile) connected to an Orbitrap Elite mass spectrometer (Thermo Scientific) equipped with a nanoelectrospray ion source. Peptides were loaded onto a Dionex Acclaim® PepMap100 trap column (Thermo, 75 µm x 2 cm, C18 3 µm 100Å) and fractionized on a Dionex Acclaim® PepMap RSLC analytical column (Thermo, 50 µm x 15 cm, C18 2 µm 100Å) at a flow rate of 300 nLmin⁻¹ using a linear gradient of 2–15% B for 70 minutes, 15–25% for 15 min, 25–35% B for 5 minutes, 35–98% B for 2 minutes then holding at 98% for 8 minutes. The nano-source capillary temperature was set to 275 °C and the spray voltage was set to 2.0 kV. MS1 scans were acquired at a resolution of 60,000 FWHM (380–2000 m/z) with
an AGC target of $1 \times 10^6$ ions over a maximum of 250 ms. MS2 spectra were acquired for the top 10 ions from each MS1 scan in the Orbitrap at a resolution 30,000 FWHM with a target setting of $3 \times 10^4$ ions, accumulation time of 200 ms, and an isolation width of 1.2 Da. The normalized collision energy was 33% for 0.1 ms activation time and one microscan was acquired for each spectra. Monoisotopic precursor selection was enabled and only MS1 signals exceeding 500 counts triggered the MS2 scans, with +1 and unassigned charge states not being selected for MS2 analysis. Dynamic exclusion was enabled with a repeat count of 1, repeat duration of 30 seconds and exclusion duration of 80 seconds.

**Data Analysis.** Raw MS data was converted to mzXML format and searched using OMSSA (version 2.1.9)\(^3\) against the concatenated target/decoy human or mouse Uniprot database. Search parameters included full digestion with trypsin with up to 2 missed cleavages, a parent mass error of 10 ppm and a fragment mass error of 0.03 Da with the variable modifications as NEM, cysTMT\(^6\), cysTMT\(^0\) or iodoTMT\(^6\) for (C) and oxidation (M). For reduced and alkylated samples, IAA (C) was additionally set as variable modification. Software msConvert (version 2.1.2132)\(^4\) from ProteoWizard was used for peaklist generation. Search results were processed using the trans-proteome pipeline (TPP, version v4.4, rev 1)\(^5\) implementing the PeptideProphet\(^6\), iProphet\(^7\) and ProteinProphet\(^8\) algorithms. Peptide and protein identifications were accepted if the peptide confidence was greater than 95.0% as specified by the PeptideProphet\(^6\) algorithm and a protein group probability greater than 95.0% as assigned by the ProteinProphet\(^8\) algorithm. False discovery rates (FDR) were calculated by the ProteinProphet\(^8\). FDRs were less than 1.0 % using a minimum probability of 0.95 for peptide and protein group as a threshold. After search, TMT-modified peptides were filtered and considered for the SNO-modification. The MS spectra was also searched using the X!Tandem algorithm (version TPP v4.4, rev 1)\(^9\) with the k-score plug-in. X!Tandem\(^9\) only accepts one variable modification to a particular amino acid. As a result we chose to set TMT as a fixed modification to utilize in order to compare the OMSSA\(^3\) search results. For sequential and simultaneous labeling, updated versions of msConvert (version 3.0.3858)\(^4\) and TPP (version v4.6, rev 1)\(^5\) were used. All peptide IDs from independent replicates of HEK cells were combined and analyzed. Values from the independent replicates and peptides for an individual cysteine residue were averaged and peptide probability, mass difference (Da), charge and observed peptide mass (Da) were assigned from those of the best identified peptide. A specific site of SNO-modification was accepted only if the value of GSNO-condition was at least two fold (one fold, for sequential and simultaneous labeling) greater than the largest control condition (untreated, GSH, GSSG). For experiments performed without ascorbate (described in Online supplemental), a site of SNO-modification was determined if the TMT intensity in the ascorbate treated sample was greater than controls (HEK cells: no ascorbate/untreated, no ascorbate/GSNO-treated and ascorbate/untreated; Tissue samples: no ascorbate condition). When the fold change was calculated, the values of GSNO-condition (reduced with ascorbate) were > 10-fold greater than the largest control condition on average and in some sites it was >6000-fold greater. However, when background signals were observed in a very few sites, we speculate that this is due to 1) default background noise in MS spectra 2) incomplete distribution of isobaric tag and/or 3) the higher sensitivity of MS over western blot analysis. Quantitative values for the TMT reporter ions were collected with the Libra module of the TPP using a custom condition file. Subcellular locations and functional analysis of proteins were performed based on Gene Ontology

**Physiochemical Properties Analysis.** 21 amino acid sequences flanking the N- and C-terminus of the SNO-modified cysteine residues were obtained based on Uniprot FASTA sequences. pI prediction for the flanking residues in cys- or iodoTMT specific labeled groups
was performed with ExPASy server\textsuperscript{10} (http://web.expasy.org/compute_pi/) and the distribution of their pI was represented as color scale from low (red) to high (green) pI and grouped bar graph (Figure 5A) The top 15 of flanking residues whose SNO-cysteine residues were extremely reactive (or had the highest intensity) to either of TMT reagents was selected and the frequencies of the amino acids of 15 flanking residues in each group were computed with WebLogo\textsuperscript{11} (http://weblogo.berkeley.edu/logo.cgi). For global proteins' properties analysis, top 20 proteins whose SNO-cysteines were most reactive (or had the highest intensity) and whose all cysteine residues were exclusively labeled with either of TMT were selected. Aliphatic index and molecular weights were computed with ProtParam tool\textsuperscript{10} (http://web.expasy.org/protparam/) and represented as grouped color scale (Figure 5C and 5D).
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**Online Table I. Experimental conditions modified during optimization of IodoTMT labeling.** Although IodoTMT₆ has similar mass and structure to cystTMT₆, it could not be simply substituted into the previous TMT-switch protocol. In this study, we establish an optimized protocol for IodoTMT₆ specifically for the switch assay. The multi-step nature of the TMT-switch assay required optimizing several factors to achieve peak efficiency: including concentration of metal, maximum concentration of protein and labeling buffer.
Online Figure I. Troubleshooting for iodoTMT labeling. TMT-switch assay was performed with HEK cell lysates. A, TMT-switch assay using cys- and iodoTMT in conditions previously described for cysTMT. Using these conditions, SNO-modified proteins were not labeled with iodoTMT. B, Comparison of cys- and iodoTMT in the TMT-switch assay using optimized conditions (PBS at pH 8.0). The addition of 1mM of copper increased labeling for cysTMT but inhibited iodoTMT labeling. C, TMT-switch assay in the presence of the metal chelator, neocuproine (0.1 and 1 mM) demonstrating the quantitative effect of metal ions on cys- and iodoTMT labeling. D, Decreased iodoTMT labeling by additional 1mM copper was recovered by the addition of 20 mM glycerol, a radical scavenger. E, Various conditions to optimize iodoTMT-switch assay were tested: optimal concentration of iodoTMT, ascorbate and SDS, addition of copper, protein: buffer used for blocking and/or labeling and effect of acetone precipitation.
Online Figure II. Comparison of SNO-sites. A, More than 60% of the sites were commonly detected between two replicates of the same cysTMT. B, Less than 30% of the total SNO-sites from HEK cells were identified by both of the reagents. SNO-sites were labeled with either cysTMT or iodoTMT, under identical conditions.
Online Figure III. Western blot analysis of TMT-labeling on recombinant human and bovine actin after cys or iodoTMT-switch assay. A, Western blot analysis of different TMT labeling on recombinant bovine actin after cys- or iodoTMT switch assay using the optimized protocol (1mM copper, 1mM ascorbate for cysTMT labeling and 5mM copper for iodoTMT labeling). B, TMT-switch assay with individual recombinant human actin was performed in the same manner for cys- and iodoTMT labeling (right, 5mM ascorbate for both TMT labeling). SNO-modified actin was detected preferentially by iodoTMT.
Online Figure IV. Cellular compartment analysis. Gene Ontology-based analysis of SNO-proteome of the two subpopulations in GSNOR knock-out heart: hyper-nitrosylated group (red) and hypo-nitrosylated group (green). Proteins that were clustered to unique categories.
Online Figure V. Physiochemical properties analysis of two subpopulations. SNO sites from cys- and iodoTMT-switch using the optimized conditions were analyzed. **A**, The theoretical pIs of 360 exclusively cysTMT\(^6\)-labeled residues and 277 iodoTMT\(^6\)-specific residues from HEK cells were analyzed and illustrated using a color scale from low (red) to high (green) pI (left, cysTMT\(^6\); right, iodoTMT\(^6\)). **B**, The frequency of amino acids surrounding cysteine residues of two groups were analyzed (top, cysTMT\(^6\); bottom, iodoTMT\(^6\)). The pool of fifteen SNO residues from mouse hearts and fifteen SNO residues from HEK cell proteome were used for the analysis. Positively charged lysine/arginine were more frequently observed in cysTMT\(^6\) specific group. Reactive cysteine is highlighted in green at position 11.
Online Figure VI. Physiochemical properties analysis of two subpopulations. The pool of datasets of SNO-modified sites labeled under optimized conditions and identical conditions were analyzed. A, The theoretical pIs of all cysTMT<sup>6</sup>-labeled residues and iodoTMT<sup>6</sup>-specific residues from HEK cell (left) and WT mouse hearts were illustrated using a color scale from low (red) to high (green) pI (left, cysTMT<sup>6</sup>; right, iodoTMT<sup>6</sup>). Higher proportion of high pI was observed in iodoTMT<sup>6</sup> specific group. B, Aliphatic index for proteins (whose cysteine residues were reactive) were computed based on the amino acid sequence and compared between protein groups which were detected with only one TMT reagent. The color scale represents aliphatic index of proteins from HEK cell (left, cysTMT<sup>6</sup>; right, iodoTMT<sup>6</sup>). C, Molecular weight of the same proteins for (B) was analyzed and represented in the same way. IodoTMT<sup>6</sup> group had higher proportion of low molecular weight proteins compared to cysTMT<sup>6</sup> group. D, The frequency of amino acids surrounding the cysteine residues of two groups was analyzed (top, cysTMT<sup>6</sup>; bottom, iodoTMT<sup>6</sup>). Reactive cysteine is highlighted in green at position 11.
Online Figure VII. Analysis of sequential labeling in the TMT-switch assay. A, Comparison of TMT-SNO labeling using the different labeling protocols as calculated by the fold difference of SNO reporter ion intensity over the background reporter ion intensity. In sequential labeling protocols, the average of SNO intensity detected by iodoTMT labeling was only slightly reduced compared to labeling experiments done in parallel while cysTMT SNO intensity was significantly reduced in the sequential protocol compared to the parallel experiments. B, Western blot (WB)-analysis of TMT-switch assay indicating non-specific labeling was increased with the sequential labeling protocol.
Online Figure VIII. Analysis of simultaneous cys- and iodoTMT labeling in the TMT-switch assay. A, Simultaneous labeling was evaluated in various conditions including the addition of metals to augment ascorbate reduction\textsuperscript{12} best optimized for cysTMT. These conditions were not compatible; labeling intensity was reduced compared to the sum of the optimized single cys- and iodoTMT labeling conditions. Additionally, non-specific labeling increased in simultaneous labeling. ‘Combo’ experiments were performed using 0.5x the standard concentration for each reagent, while ‘combo (2X)’ experiments used concentrations for each reagent that matched those of a single label experiment. Both cys- and iodoTMT were immunobotted with the anti-TMT antibody. Metal concentrations were 1 mmol/L CuSO\textsubscript{4}, 0.1 \( \mu \)mol/L FeSO\textsubscript{4} or combination of 10 \( \mu \)mol/L CuSO\textsubscript{4} and 0.1 \( \mu \)mol/L FeSO\textsubscript{4}. B, Summary of WB analysis for various labeling conditions anti-TMT signal from WB-analysis was indicated as +; ‘combo’ denotes simultaneous labeling by cys- and iodoTMT. Effective labeling was determined by comparison of the untreated control signal with the NO-induced.
When the isobaric tags are released from the manufacturer, each isobaric tag is not 100% pure. Based on the information provided by the manufacturer, correction factors were included in the libra condition file when searching for TMT reporter ion intensities (A: cysTMT labeled samples, B: iodoTMT labeled samples).

Online Figure IX. Libra condition file. When the isobaric tags are released from the manufacturer, each isobaric tag is not 100% pure. Based on the information provided by the manufacturer, correction factors were included in the libra condition file when searching for TMT reporter ion intensities (A: cysTMT labeled samples, B: iodoTMT labeled samples).
References


