ESNOQ, Proteomic Quantification of Endogenous S-Nitrosation

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Abstract

S-nitrosation is a post-translational protein modification and is one of the most important mechanisms of NO signaling. Endogenous S-nitrosothiol (SNO) quantification is a challenge for detailed functional studies. Here we developed an ESNOQ (Endogenous SNO Quantification) method which combines the stable isotope labeling by amino acids in cell culture (SILAC) technique with the detergent-free biotin-switch assay and LC-MS/MS. After confirming the accuracy of quantification in this method, we obtained an endogenous S-nitrosation proteome for LPS/IFN-γ induced RAW264.7 cells. 27 S-nitrosated protein targets were confirmed and using our method we were able to obtain quantitative information on the level of S-nitrosation on each modified Cys. With this quantitative information, over 15 more S-nitrosated targets were identified than in previous studies. Based on the quantification results, we found that the S-nitrosation levels of different cysteines varied within one protein, providing direct evidence for differences in the sensitivity of cysteine residues to reactive nitrosative stress and that S-nitrosation is a site-specific modification. Gene ontology clustering shows that S-nitrosation targets in the LPS/IFN-γ induced RAW264.7 cell model were functionally enriched in protein translation and glycolysis, suggesting that S-nitrosation may function by regulating multiple pathways. The ESNOQ method described here thus provides a solution for quantification of multiple endogenous S-nitrosation events, and makes it possible to elucidate the network of relationships between endogenous S-nitrosation targets involved in different cellular processes.

Introduction

S-nitrosation (commonly referred to as S-nitrosylation) has emerged as an important post-translational modification regulating protein functions, and is believed to be ubiquitously involved in cell signaling [1]. Although there are some methods, such as biotin-switch and SNOSID, for studying S-nitrosation [2,3], a high-throughput method for quantifying the level of S-nitrosation has not yet been developed. At the present time, there are several limitations associated with current methods for S-nitrosation, including: (I) Elimination of false negatives during identification of S-nitrosation targets. The criterion for identifying positive S-nitrosation targets in the biotin-switch assay is a significant increase in signal relative to the negative control. However, if an S-nitrosated target protein is identified in both the sample and the negative control, it must be eliminated from the candidate list without obtaining further information on relative quantification as it is technically difficult to confirm whether there is a significant increase in sample signal relative to the negative control. (II) In endogenous studies the above problem is even more serious because of the low abundance of endogenous S-nitrosated proteins. It is very hard to determine S-nitrosation of sample proteins because of the background present in the control. (III) In studies of the dynamics of S-nitrosation it is not sufficient just to know which protein is S-nitrosated; rather it is necessary to determine quantitative changes in S-nitrosation during biological processes. Quantitative information, which reflects the degree of change in S-nitrosated proteins over time, is essential for further study on the functions of S-nitrosated proteins. (IV) In addition, S-nitrosation usually occurs on multiple targets in vivo. Therefore, to study the role of S-nitrosation in a cellular process, multiple targets should be considered simultaneously as an integrated network. Taking these factors into account it is clear that there is an urgent need for a quantitative high-throughput method for studying S-nitrosation.

Biotin-switch assay is a method for indirect S-nitrosation detection. Free thiols were blocked by methylmethanethiosulfonate (MMTS). Then S-nitrosothiols were reduced by ascorbate and labeled by biotin-HPDP. S-nitrosated cysteines are converted to biotinylated cysteines accordingly. Stable isotope labeling by amino acids in cell culture (SILAC) [4] is a metabolic labeling strategy that relies on the incorporation of amino acids containing substituted stable isotopes into proteins in living cells for relative quantification by mass spectrometry. SILAC has been widely used for studying dynamics of protein abundance [5] and post-translational modifications [6], such as methylation [7,8] and phosphorylation [9,10]. In this work we developed a method of protein S-nitrosation quantification termed ESNOQ (Endogenous S-nitrosothiol [SNO] Quantification) based on combining the detergent-free biotin-switch method [11], SILAC and LC-MS/
MS. Using this method, we have measured the endogenous $S$-nitrosation proteome in LPS/IFN-$\gamma$ induced RAW264.7 cells [12,13].

**Results**

**Development of the ESNOQ method**

The ESNOQ strategy is shown schematically in Fig. 1. In order to quantify endogenous $S$-nitrosated cysteines via a mass spectrometry approach, we introduced a mass difference between sample proteins and the control. First, light isotopes, $[^{12}\text{C}_6]$-Lys, $[^{12}\text{C}_6,^{14}\text{N}_4]$-Arg, and heavy isotopes, $[^{13}\text{C}_6]$-Lys (+6 Da), $[^{13}\text{C}_6,\text{N}_4]$-Arg (+10 Da), were incorporated separately into RAW264.7 cells. This was termed “metabolic labeling”. Cells labeled with light isotopes were then treated with LPS/IFN-$\gamma$ for 18 h, while cells labeled with heavy isotopes were used as controls. The reason why light isotope-labeled cells were used as the treatment group is to avoid excess utilization of Arg by iNOS during nitric oxide synthesis. Treated cells were then mixed with the control group at a ratio of 1:2 on the basis of cell number. We used the ratio 1:2 instead of 1:1 to increase the level of the signal in the control group in order to increase the likelihood of detecting $S$-nitrosation events in the control and thus obtain quantifiable results. The mixed cells were then lysed and proteins were extracted in detergent-free conditions in order to optimize LC-MS/MS analysis [11]. After biotin-switch, tryptic digestion and purification, the peptides which now contain both quantitative and site-specific information were then identified by mass spectrometry.

Before the ESNOQ assay, we confirmed that nitric oxide synthase was significantly induced in RAW264.7 cells by LPS/IFN-$\gamma$ [14] using Western-blotting and proved that isotope labeling had no influence on cell growth and properties, in agreement with previous reports [15]. The incorporation ratio of heavy isotopes in every cell generation was also monitored by LC-MS/MS (see Fig. S1 in Supporting Information). In the 6th generation, the signal of light isotope-labeled peptides was below the level of detection for MS, indicating the full incorporation of heavy isotope labeled amino acids.

**Validation of the ESNOQ method**

In the ESNOQ assay it is important to preserve accurate $S$-nitrosation quantitative information after the biotin-switch and purification steps. To test whether our method does preserve accuracy, the same concentration of proteins extracted from RAW264.7 cells labeled with light and heavy isotopes were both treated with 1 mM S-nitrosoglutathione (GSNO) in the dark for 1 h. Then the light and heavy-isotope labeled samples were mixed at ratios of 3:1, 1:1 and 1:3. After ESNOQ analysis and database searching, data were validated by Peptideprophet [16]: peptides were accepted when they satisfied all of the following conditions: Peptideprophet $p > 0.8$; peptides in both the heavy and light forms had the same elution time; more than four continuous $b$ ions or $y$ ions were matched. All data were manually checked. Fig. 2 shows the mean and standard deviation (SD) of each sample. For example, in the L:H 1:1 group, the solid circle shows the mean of the actual MS quantification results of light/heavy of all peptides in this group, which is expected to be equaled to 1. The standard deviation of this sample is about 0.5. The standard deviations

![Figure 1. Schematic overview of the ESNOQ strategy.](image)

![Figure 2. Accuracy of the ESNOQ method.](image)
of the L:H 3:1 group and the L:H 1:3 group are about 1 and 0.1, respectively.

Quantification of Endogenous S-nitrosation in RAW264.7 Cells induced by LPS/IFN-γ with the ESNQQ Method

Samples were prepared as shown in Fig. 1 and LC-MS/MS detection was performed followed by database searching, data validation and quantification. SEQUEST™ was used for data searching with the IPI mouse 3.29 database. Peptides were then validated using PeptideProphet: peptides containing cysteine and having a PeptideProphet probability higher than 0.80 were accepted (Due to the limitation of purification efficiency, there were about 20% non-cysteine containing peptides in this study). With this restriction, the sensitivity of peptide identification was above 50% (i.e. more than 50% “true” peptides were identified) and the error rate was 4% (i.e. 4% of the peptides in the results could be errors). ASAPRatio [17] was used to integrate the peak of the peptide in the extracted ion current (XIC) for quantifying the signals. Taking the peptide TFCQLILDPIFK from Elongation factor 2 (Swiss-prot, P58252) as an example, the three left peaks in red in Fig. 3A are the MS1 signals of light isotope-labeled peptides. The heavy isotope-labeled peptides in green have a +3 m/z shift because the [15C0]-Lys is +6 Da heavier in mass compared to [13C0]-Lys. The ratio calculated by ASAPRatio was 1.0. Thus the quantity of S-nitrosation of the Cys in TFCQLILDPIFK from Elongation Factor 2 was 2.0 since the treated cells were mixed with control group cells in a ratio of 1:2 based on cell number, indicating that the S-nitrosation of this cysteine increased two-fold after LPS/IFN-γ treatment. This peptide sequence was identified by database searching using results from MS5 as shown in Fig. 3B. The precursor ion was the heavy isotope-labeled peptide, all single charged y ions had a +6 m/z shift, and the cysteine also matched with a carboxyamidomethylation with a +57 m/z shift due to alkylation with iodoacetamide. The peptide sequence and the isotope labeling status (light or heavy) were obtained by database search. Then the m/z of its partner peptide was calculated automatically by ASAPRatio according to the peak areas of the extracted ion current of the peptide pairs. After manually checking each set of data, all together 27 protein targets, including 31 different S-nitrosation sites, were identified as shown in Table 1. Over 15 S-nitrosated proteins were found here for the first time and six ribosomal proteins were newly identified in LPS/IFN-γ induced RAW264.7 cells. Endogenous S-nitrosation increased up to 10-fold in this model. MS and MS² information of all peptides enlisted in Table 1 can be found in Supplemental Information (Fig. S2). We have also blasted all the peptides listed in Table 1 and found no peptides were shared by different proteins. We would like to point out that there are two cysteine residues in the peptide IVSNASCTTINCLAPLAK of GAPDH, which the S-nitrosation of Cys increased 6.3 times. We are still not able to conclude that which Cys on this peptide was the S-nitrosation site or both are. One way to resolve this issue is to use another digestion method that can cut the peptide between these two Cys residues, and combine with a relevant isotope-labeling strategy.

In addition, we also quantified S-nitrosation of 10 μM GSNO treated RAW264.7 cells with the ESNQQ method. The results can be found in Supplemental Information (Table S1). About 90 S-nitrosation proteins were quantified and the S-nitrosation level increased with the ratios from 1.5 to about 8.0. We found that some S-nitrosated proteins such as pyruvate kinase, elongation factor and ribosomal proteins were also S-nitrosated targets in the LPS/IFN treated RAW264.7 cells, and the S-nitrosation sites are the same. These results proved the reliability of the ESNQQ method.

Bioinformatics analysis of the S-nitrosated proteins

The S-nitrosated proteins were queried in the Gene Ontology Database (http://www.ebi.ac.uk/ego/) and clustered according to biological processes. As shown in Fig. 4A, the S-nitrosated targets glycolaldehyde 3-phosphate dehydrogenase (GAPDH) and pyruvate kinase (PK) are involved in glycolysis. Four proteins, for example aspartate aminotransferase, were involved in biosynthetic processes. Six ribosomal proteins and two elongation factors were involved in translation. Consistent with the above results, KEGG pathway analysis [18] also revealed two notable S-nitrosation-rich pathways: glycolysis and protein translation. The glycolysis pathway, from glyceraldehyde-3P to pyruvate, involves five enzymes, two of which are S-nitrosated (in red) (Fig. 4B). Of the

Figure 3. Quantification and identification of S-nitrosation targets in RAW264.7 cells induced by LPS/IFN-γ. Peptide TFCQLILDPIFK from Elongation Factor 2 (Swiss-prot: P58252) is shown as an example. (A) Peaks of light and heavy precursor ions in the same scan. The light and heavy peaks, and even individual isotope peaks, were clearly separated. The relative abundance of the peaks corresponds to the amount of S-nitrosation. (B) Peptide sequence identification by MS5. The precursor ion was the heavy isotope labeled peptide, all single charged y ions had a +6 m/z shift, and the cysteine with a +57 m/z shift was matched with carboxyamidomethylation due to alkylation with iodoacetamide.

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27 \textit{S}-nitrosated proteins detected in this study, GAPDH and PK had high levels of \textit{S}-nitrosation variation. 8 proteins were enriched in the protein translation process.

**Discussion**

In this work, we have developed the ESNOQ method and applied it to obtain an endogenous \textit{S}-nitrosated proteome in the LPS/IFN-\textgamma-induced RAW264.7 cell model. Our results have shown that this method is a powerful tool for effectively and accurately quantifying endogenous \textit{S}-nitrosation with specific modified site information. Using ESNOQ, many of the limitations frequently encountered in studies of \textit{S}-nitrosation can be solved: 1) The quantitative information obtained allows identification of \textit{S}-
nitrosation protein targets that would otherwise be excluded as false negatives. 2) In endogenous studies, the low abundance of endogenous \textit{S}-nitrosated proteins can be distinguished from the control. 3) More \textit{S}-nitrosated protein targets can be identified with ESNOQ: here we identified 41 different endogenous \textit{S}-nitrosation targets. By way of comparison, only four endogenous \textit{S}-nitrosated protein targets were identified in a similar study using SNOSID [2]. 4) Multiple \textit{S}-nitrosated targets in a given cellular process can be considered simultaneously with this high-throughput method.

In brief, \textit{S}-nitrosation signaling mechanisms and pathways can be studied systematically, quantitatively, and site-specifically with this method.

Results from this study are consistent with previous reports; glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and...
that protein, providing direct evidence for different sensitivity of
that the LPS/IFN-γ-nitrosation levels of different cysteines varied within one protein. This indicates that the ESNOQ method is reliable. Over 15 of the new S-nitrosation targets detected using our method in LPS/IFN-γ treated RAW264.7 cells have not previously been reported [2]. The quantification results showed that the level of S-nitrosation of newly identified protein targets increased 1.5 to 2.5 times, while it was previously almost impossible to distinguish these endogenous S-nitrosation targets without accurate quantification information.

Quantitative information on site-specific S-nitrosation is necessary for in depth functional studies. Using the ESNOQ method, 45 different S-nitrosation sites on 41 protein targets were identified in LPS/IFN-γ treated RAW264.7 cells. More interesting, we found that the S-nitrosation levels of different cysteine residues varied within one protein, providing direct evidence for different sensitivity of cysteine residues to reactive nitrosative stress (RNS) and suggesting that S-nitrosation is a site-specific modification. Taking GAPDH as an example, the S-nitrosation ratio of Cys in the peptide IVSNASCTTNCLAPLAK increased 6.3 times, while S-nitrosation increased only 3.1 times at another Cys site (VPTFNVSVVDLTCR). It has been reported that a Cys residue is more easily S-nitrosated when it has a lower pKa [1]. Here we calculated the pKa’s of cysteines within one protein using PROPKA [19] and found that up-regulation of S-nitrosation is tightly correlated with cysteine pKa in the same protein target. In pyruvate kinase, the calculated pKa value of C48 is 11.90, and its S-nitrosation ratio is 2.7, while the pKa of C422 is 8.62, significantly lower than that of C48, and its S-nitrosation ratio is 6.9, much higher than that of C48. It is the same in GAPDH: C246 has a pKa of 9.24 and an S-nitrosation ratio of 3.1, while C151 has an exceptionally low pKa of 5.27 and a high S-nitrosation ratio of 6.3. That means, if one protein is known to be an S-nitrosation target, the cysteine with the lowest pKa may be the S-nitrosation site.

With the benefits of high-throughput quantification, the landscape of endogenous S-nitrosation has been revealed, which is very important for research on signal transduction mechanisms. Gene ontology clustering of biological processes showed that S-nitrosation targets were mainly related to translation and cell metabolism, including biosynthetic processes (e.g. Asparatate aminotransferase), glycolysis (e.g. GAPDH) and proteolysis (e.g. Cathepsin B). In the glycolytic process, 2 enzymes in the same pathway have been identified as S-nitrosation targets, and their S-nitrosation levels were relatively higher than the other targets. In the translation process, 8 proteins, including 6 ribosomal proteins and 2 elongation factors, were identified as S-nitrosation targets. These results indicate that S-nitrosation may function by regulating multiple pathways.

Recently an iTRAQ-based quantitative method for S-nitrosation detection has been reported [20], however, it has not yet been applied to endogenous analysis. The advantage of iTRAQ approach is that it can be widely used for analysis of cell, tissue and animal samples. However, since the labeling strategy on peptide was carried out after multi-steps of sample preparation, which may introduce significant quantification error, the parallel and accuracy of quantification were compromised. Being different from it, our SILAC-based ESNOQ method shows significant advantages in the parallel and accuracy of quantification because treatment and control group cells can be mixed as intact cells and processed together throughout the experimental procedure. Therefore, sample losses at a particular step do not affect the quantitative accuracy. The follow-up steps including blocking, reducing, labeling and LC-MS analysis are all performed on the same sample. Therefore, ESNOQ has high accuracy for quantification of endogenous SNOs. The disadvantage of our method is that it can not be easily used for animal and tissue samples.

The ESNOQ method described here may be used for analyzing S-nitrosation profiles in cellular processes such as apoptosis or differentiation. It could also be used for dynamic studies by labeling with a range of different isotopes. Moreover, the ESNOQ method lends itself to the study of S-nitrosated modification networks since multiple SNO targets can now be evaluated using the quantitative information obtained. Thus, the ESNOQ method takes us one step closer to revealing the dynamic endogenous roles of S-nitrosation.

Materials and Methods

Materials

SILAC™ protein identification and quantitation kits were purchased from Invitrogen (Cat. No. MS10030, USA). S-nitro-
soglutathione (GSNO) was synthesized as described [21]. Methyl methanethiosulfonate (MMTS), biotin-HPDP (HPDP: N-[6-(biotinamido)hexyl]-3-(2-pyridyldithio)propionamido), the BCA™ protein assay kit, and the Slide-A-Lyzer dialysis cassette (0.5 ml to 3 ml, 7 kDa molecular-weight cutoff) were from Pierce (Rockford, IL, USA). PlusOne™ urea was from GE Healthcare (Piscataway, NJ, USA). Protease inhibitor cocktail tablets (Complete-Mini, EDTA-free) were from Roche Applied Sciences (Indianapolis, IN, USA). Sequencing-grade modified trypsin (Complete-Mini, EDTA-free) were from Roche Applied Sciences (Indianapolis, IN, USA). Protease inhibitor cocktail tablets (Comple-
Endogenous SNO Quantification

Figure S2  ASAPRatio quantification and MS/MS analysis of all peptides listed in Table 1. Found at: doi:10.1371/journal.pone.0010015.s002 (3.17 MB DOC)

Table S1  S-nitrosation quantification with the ESN0Q approach of GSNO treated RAW264.7 cells. RAW264.7 cells labeled with heavy amino acids were treated with 10 μM GSNO for 1 h. Cells labeled with light amino acids were used as control group with the solvent as control treatment. First, cell lysis was blocked with MMTS to avoid the trans-nitrosation, and then the two groups were mixed into one sample. Purification and MS/MS analysis were the same as described in the manuscript. The ratios in this table represent the quantification results of Heavy/Light, i.e. the increasing of S-nitrosation after GSNO treatment. Found at: doi:10.1371/journal.pone.0010015.s003 (0.20 MB DOC)

Author Contributions
Conceived and designed the experiments: XZ PH CC. Performed the experiments: XZ PH JL HQR. Analyzed the data: XZ XZ BH CC. Wrote the paper: XZ CC.

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