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ORIGINAL ARTICLE

SNO spectral counting (SNOSC), a label-free proteomic method for quantification of changes in levels of protein S-nitrosation

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Abstract

S-Nitrosation plays an important role in regulation of protein function and signal transduction. Discovering S-nitrosated targets is a prerequisite for further functional study. However, current proteomic methods used to quantify S-nitrosation are limited in their applicability to certain types of samples, or by the need for special reagents and complex procedures to obtain the results. Here we devised a label-free proteomic method for quantification of changes in the level of protein S-nitrosation on the basis of a spectral counting strategy, called S-nitrosothiol (SNO) spectral counting (SNOSC). With this method, samples can be from any source (cells, tissues); there is no need for labelling reagents or procedures, and the results yield quantitative information. Moreover, as it is based on the irreversible biotinylation procedure (IBP) for S-nitrosation protein enrichment, false positive targets caused by the interference of intermolecular disulphide bonds are ruled out. Using SNOSC we studied S-nitrosation in the cell line RAW264.7 induced exogenously with S-nitrosoglutathione (GSNO), or induced endogenously by lipopolysaccharides/interferon-gamma (LPS/IFN-γ). We detected a significant increase in S-nitrosation of 50 proteins after exogenous induction and 17 proteins after endogenous induction. We thus demonstrate that SNOSC is a widely applicable proteomic method for fast screening of SNO proteins.

Keywords: S-nitrosation, S-nitrosothiol (SNO), spectral counting, irreversible biotinylation procedures (IBP), quantification

Introduction

S-Nitrosation is a ubiquitous redox-based post-translational modification of protein cysteine thiols by nitric oxide or its derivatives, which transduces the bioactivity of nitric oxide by regulation of protein properties such as activity, conformation, stability, location or protein–protein interactions. S-Nitrosation plays an important role in global regulation of protein function [1]. In recent years, increasing numbers of proteins have been identified as endogenous S-nitrosation targets in physiological and pathological processes. Therefore, systematic research using '-omic' methods is critical for understanding the role of S-nitrosation in biological processes [2]. Several quantitative S-nitrosoproteomic methods have recently been developed, including endogenous S-nitrosothiol (SNO) quantification (ESNOQ) [3], isotope coded affinity tag switch (ICAT switch) [4], isobaric tags for relative and absolute quantitation-SNO resin-assisted capture (iTRAQ-SNO RAC) [5] and some two-dimensional gel electrophoresis (2DE)-based strategies [6,7]. All of these methods can quantify changes in S-nitrosation levels successfully, but unfortunately, all of them have some limitations. ESNOQ is based on stable isotope labelling by amino acids in cell culture (SILAC) and is useful for quantifying endogenous SNO levels in parallel samples, but it can only be used in cell models, and requires isotope incorporation in cell culture. ICAT switch and iTRAQ-SNO RAC can be used for any type of sample but they both require additional steps, which makes the experimental procedure more complex. 2DE-based strategies have an advantage in terms of reliability of the data, but sample preparation can be difficult, especially for hydrophobic proteins or proteins that are extremely acidic or basic. Therefore, a simple, specific and quantitative proteomic screening method is very much needed.

Spectral counting, a label-free quantitative proteomic method, compares the number of spectra of tandem mass spectrometry (MS/MS) assigned to each protein [8]. Sample preparation for this strategy is straightforward, and there is no limitation in terms of sample type. In addition, it has been successfully applied in proteomic quantification of protein palmitoylation [9]. We recently reported that intermolecular disulphide bonds have been found to interfere with the accuracy of the identification of S-nitrosated proteins: proteins which are linked to S-nitrosated proteins by intermolecular disulphide bonds can be falsely detected as S-nitrosated targets. The irreversible biotinylation procedure (IBP) can prevent this interference [10]. In this study, we used the spectral counting strategy after two-dimensional liquid chromatography–tandem mass spectrometry (2D-LC-MS/MS) to compare the abundance of

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S-nitrosated proteins purified using IBP. We term the new method described here SNO spectral counting (SNOSC).

Using SNOSC, we studied S-nitrosation in the cell line RAW264.7 induced exogenously with S-nitrosoglutathione (GSNO) or induced endogenously by LPS/IFN-γ. We demonstrate that SNOSC is a widely applicable proteomic method for fast screening of SNO proteins.

Methods

Materials

GSNO was synthesized as described [11]. The concentration was determined by its optical density (O.D.) value at 334 nm (with extinction coefficient 800 M^{-1} cm^{-1}). Neocuproine, methylmethanethiosulfonate (MMTS), biotin-maleimide (biotin-M) and streptavidin-agarose were purchased from Sigma (St. Louis, MO). Protease inhibitor cocktail was from Roche. Urea was purchased from Amresco (Solon, OH). Solvents used in LC/MS analysis, including formic acid (FA), acetonitrile (ACN) and methanol, were from J.T. Baker Chemicals (Philipsburg, NJ, USA). Sequencing-grade modified trypsin (V5111) was from Promega (Madison, WI, USA). Rabbit β-actin, elongation factor 2, annexin2 and glyceraldehyde-3-phosphate dehydrogenase antibodies were from Santa Cruz Biotech, as were mouse and rabbit secondary antibodies tagged with horseradish peroxidase (HRP). Other chemicals were from Sigma–Aldrich (St. Louis, MO, USA).

Cell culture and extract preparation

The RAW264.7 cell line was purchased from Peking Union Medical College (Beijing, China) and cultured in Dulbecco's modified Eagle's medium supplemented with 10 mM HEPES, 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin, maintained in a 5% CO_2 incubator at 37°C in dark. After treatment with 100 ng/mL LPS and 1 ng/mL IFN-γ for 18 hour, cells were lysed in lysis buffer (50 mM HEPES, pH 7.7, 2.5 mM EDTA, 0.1 mM neocuproine, 8 M urea and protease inhibitor cocktail).

Enrichment of S-nitrosated proteins using IBP

Lysate of RAW264.7 cells was incubated with or without 1 mM GSNO for 30 minutes in dark at room temperature. This sample and the RAW264.7 cell sample treated with LPS/IFN-γ were analysed using IBP [10]. Briefly, free cysteines in samples were blocked in blocking buffer (250 mM HEPES pH 7.7, 10 mM EDTA, 0.1 mM neocuproine (HEN buffer) with 8 M urea and 20 mM MMTS). After TCA precipitation and twice ice-cold acetone precipitation, samples were recovered in HENS (HEN buffer with 1% SDS (w/v)) buffer containing 8 M urea with 0.2 mM biotin-maleimide (biotin-M) and 10 mM ascorbate and incubated for 2 hour at room temperature. In this step, S-nitroso-cysteines were reduced by ascorbate and labelled irreversibly with biotin-maleimide. All of the procedures above were performed in dark. After another TCA/acetone precipitation to remove excess biotin-M, the protein pellet was resuspended in HEN buffer containing 6 M urea and 200 mM DTT, and incubated for 15 minutes at 100°C to reduce any intermolecular disulphide bonds. Two volumes of neutralisation buffer (HEN buffer containing NaCl) were added and streptavidin-agarose was used to purify the biotinylated proteins (4°C, overnight). After washing five times with 20 mM HEPES (pH 7.7) containing 1 mM EDTA and 1 M NaCl and then washing with 5 mM NH_4HCO_3 containing 20% ACN, biotinylated proteins were eluted by incubating the agarose with elution buffer (30% ACN containing 0.4% TFA) at 95°C for 15 minutes.

In-solution digestion

The enriched proteins were lyophilised to near dryness in a SpeedVac (ThermoFisher) to reach a volume of approximate 100 μL. Nine volumes of digestion buffer (2 M urea, 20 mM NH_4HCO_3, pH 8.3) were added. Purified proteins were then trypsinised in solution for 12–16 hour using a 1:50 ratio of protein:trypsin.

Two-dimensional liquid chromatography–tandem mass spectrometry (2D-LC-MS/MS) analysis

The digested peptide mixtures were lyophilised by SpeedVac and dissolved in 0.1% FA. After centrifugation at 13,000*g* for 10 minutes, the supernatant was analysed using a high-throughput tandem mass spectrometer LTQ ion trap (Thermo Fisher Scientific, Waltham, MA) equipped with a nanoelectrospray device that was built in-house. The HPLC system used was equipped with a Surveyor pump (Thermo Fisher Scientific, Waltham, MA). Peptide mixtures were pressure-loaded onto a two-dimensional capillary column (200 μm i.d.) packed with 3 cm of a C18 resin (Sunchrom 5 μm, Germany) and 3 cm of a strong cation exchange resin (Luna 5 μm strong cation exchange (SCX) 100A, Phenomenex). The buffer solutions used were 5% ACN/0.1% FA (buffer A), 80% ACN/0.1% FA (buffer B) and 500 mM ammonium acetate/5% ACN/0.1% FA (buffer C). The two-dimensional column was first desalted with buffer A and then eluted using a four-step (0% buffer C, 30% buffer C, 60% buffer C and 100% buffer C) salt gradient ranging from 0 to 500 mM ammonium acetate. The eluted peptides from the two-phase column from each step were directed onto a 10-cm C18 analytical column (100 μm i.d.) using a 3–5-μm spray tip. Step 1 consisted of a 80-minute gradient from 0% to 100% buffer B. Steps 2–4 had the following profile: 3 minutes of 100% buffer A, 5 minutes of x% buffer C (x representing the percentage of buffer C referred to above), a 5-minute gradient from 0% to 10% buffer B, a 77-minute gradient from 10% to 45% buffer B, a 10-minute gradient from 45% to 100% buffer B and a final 10-minutes of 100% buffer B. Nanoelectrospray ionisation was accomplished with a spray voltage of 2.2 kV. A cycle

of one full-scan mass spectrum (400–2000 m/z) followed by five data-dependent tandem mass spectra was repeated for each step of the multidimensional separation.

Analysis of MS/MS data

MS/MS spectra were searched by SEQUEST (licensed to Thermo Finnigan) [12] against the ipi mouse database v3.70 with the following parameters: the tolerance of the precursor ion and fragment ion were 2 and 1 amu, respectively. Peptides were permitted to have up to two missed cleavages. A variable modification of 15.99 was set on methionines. DTASelect [13] (version 2.0.39) was used to filter the SEQUEST results according to the following parameters: minimum cross correlation coefficients of 1.8, 2.5 and 3.5 for singly, doubly and triply charged precursor ions, respectively, and minimum ΔCn of 0.08. Spectral counting was carried out using the software Census 1.57 [14] by inputting the files produced by DTASelect. In order to reduce the influence of random identification, proteins whose sum spectral counts in the two groups were below 10 were removed. Spectral counts for every protein in two comparable groups were plotted in a scatter diagram using statistics software R 2.10.

Statistical analysis of S-nitrosated proteins

A RAW264.7 cell sample (GSNO treated) after IBP was divided into two equal samples. The two samples, which contained equivalent numbers of S-nitrosated proteins were analysed by 2D-nanoLC-MS/MS and subsequent data processing steps. In theory, spectral counting for every protein in these two samples should be nearly equal. A linear regression analysis was conducted for the counts of proteins in the two samples. The fitted regression line and prediction bands (the area between the upper and lower bands) for linear regression were indicated in the scatter diagram. Spots representing proteins without a significant change in S-nitrosation level would fall in the area between the upper and lower bands according to a given confidence degree. Spots outside of the prediction bands represent the proteins, which show a significant change in S-nitrosation level between the two groups. The statistical analysis and graphical presentation were conducted using the software R.

Western blot

The gels were Western blotted to nitrocellulose (NC) membranes and blocked with 5% non-fat milk with 0.05% Tween-20 in Tris buffered saline (TTBS) for 1 hour. Then the membranes were incubated with different primary antibodies at a 1:2000 dilution (0.5% non-fat milk in TTBS) for 1 hour. After washing the primary antibodies with TTBS, the membranes were incubated with the corresponding secondary antibodies tagged with HRP (1:10,000 dilution, 0.5% non-fat milk in TTBS). After washing, the signals were detected by enhanced chemiluminescence detection (Pierce).

Results

Design of the SNOSC method

The SNOSC strategy is shown schematically in Figure 1. S-Nitrosated proteins in the samples for each group were enriched using the IBP method. Spectral counts of every protein in the two groups were plotted in a scatter diagram. The spots representing proteins whose S-nitrosation level did not change are distributed near the diagonal line (red spots in the diagram). The spots whose S-nitrosation level changed markedly are distributed near the coordinate axes (blue spots in the diagram). By the difference in distribution, we could detect proteins whose level of S-nitrosation changed significantly. To test this, we analysed S-nitrosated proteins in two identical samples, which were produced by dividing a single sample into equal parts. The spectral counts of proteins enriched with IBP from the two samples are shown in Figure 2.

When using the spectral counts of proteins in sample 1 as the x-axis and counts of proteins in sample 2 as the y-axis, most (235/245) of the proteins (represented in blue) were near the diagonal (red) line. A linear regression analysis was conducted shown as the blue solid line, with a slope 1.06 (K1). A similar linear regression was conducted after we exchanged the x- and y-axes (represented using green colour), and the slope K2 was 0.825. The result is consistent with our expectation (i.e. the slopes K1 and K2 were approximately 1). Then based on the two regression lines, we can get two prediction bands with confidence level 0.95, shown as the areas between the two dashed blue lines and the two dashed green lines, respectively. The area between the upper dashed blue line and the lower dashed green line can be used as the 'prediction area', with which we could make the following inference: if a spot whose S-nitrosation level did not change between samples, it would lie inside the 'prediction area' with a confidence level of 0.95. In other words, a spot whose S-nitrosation level changed between samples should fall outside the 'prediction area' with a confidence level of 0.95. Finally, we can consider (with a confidence level of 0.95) that spots outside of the 'prediction area' are proteins, which show a change in level of S-nitrosation between the two groups.

Detecting proteins with a change in S-nitrosation level after exogenous treatment with GSNO

We used the SNOSC method to analyse proteins whose S-nitrosation level changed after treatment with exogenous GSNO. In the experimental group, the lysate of RAW264.7 cells was treated with 1 mM GSNO for 30 minutes. An equivalent quantity of RAW264.7 cell lysate was used as the control group. For each protein identified, spectral counts from the GSNO treated sample (x-axis) were plotted against the control sample spectral counts (y-axis), as shown in Figure 3A. The expanded views of the indicated regions are shown to the right of each panel. Using the 'prediction area' (confidence level 0.95),

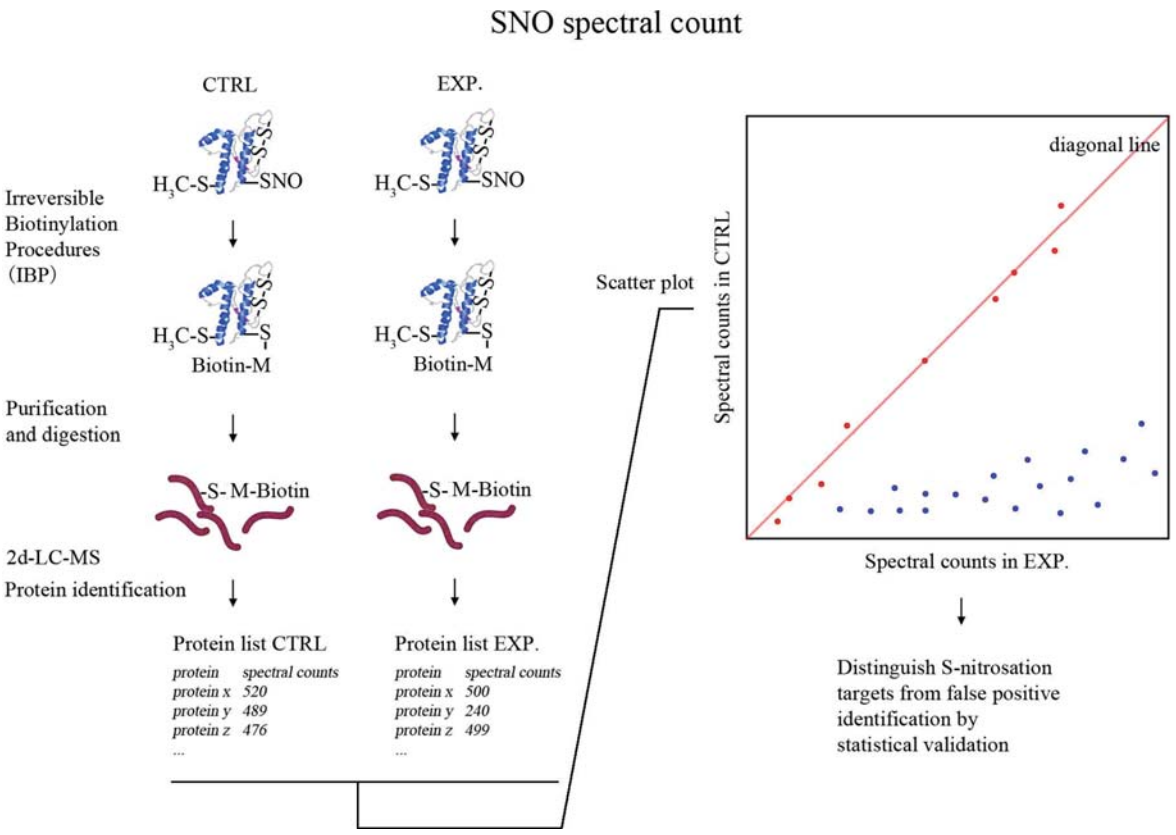

Figure 1. Schematic diagram of SNO spectral counting. Proteins were extracted from control and experimental groups. Free cysteines were blocked by being methylthiolated with MMTS. S-Nitroso-cysteines were reduced by ascorbate and labelled with the irreversible labelling reagent biotin-maleimide. After reducing potential intermolecular disulphide bonds using DTT, the labelled proteins were enriched using streptavidin-agarose and digested with trypsin. Peptide mixtures were analysed by 2D-LC-MS/MS. Lists containing spectral counts for both groups were generated and spectral counts for every protein in the two groups were plotted in a scatter diagram. The spots whose S-nitrosation level changed markedly lie near the coordinate axes.

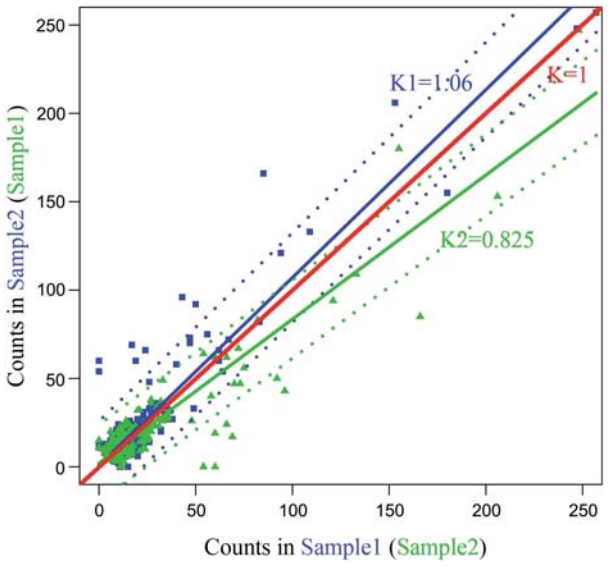

Figure 2. Statistical model used for detecting proteins with a change in level of S-nitrosation. The spectral counts of proteins enriched from the two groups (which were derived from a single sample divided equally) are depicted as blue spots (sample 2 vs. sample 1) and green spots (sample 1 vs. sample 2). Linear regression analysis for both sets of spots was conducted separately. The upper dashed blue line and the lower dashed green line can be used as the 'prediction area' (confidence level = 0.95), which can be used to detect proteins with a change in level of S-nitrosation.

several proteins (red spots) were detected as proteins whose S-nitrosation level changed significantly. When we lowered the confidence level to 0.8, more S-nitrosated proteins were detected (yellow spots). The purple line is the regression line of the spots.

To test the sensitivity of SNOSC, we use this method to analyse a sample, which contained lower amounts of exogenously S-nitrosated proteins. The GSNO treated RAW264.7 cell lysate mentioned above was diluted 10-fold with normal RAW264.7 cell lysate. We analysed the diluted sample against the normal RAW264.7 cell sample. The results of this experiment are shown in Figure 3B (a replicate experiment was carried out and the results are shown in Supplementary Figure 1 to be found online at http://www.informahealthcare.com/doi/10.3109/10715762.2012.684244). Proteins identified as showing a change in S-nitrosation level in the above four experiments are combined and listed in Supplementary Table I to be found online at http://www.informahealthcare.com/doi/10.3109/10715762.2012.684244.

It is better to repeat the experiments more times to repeat the results and be more confident with the identified S-nitrosated proteins. If one protein target was identified in more than one independent experiment, the confidence of this protein will be higher, which is a big advantage for repeat experiments compared with only one experiment.

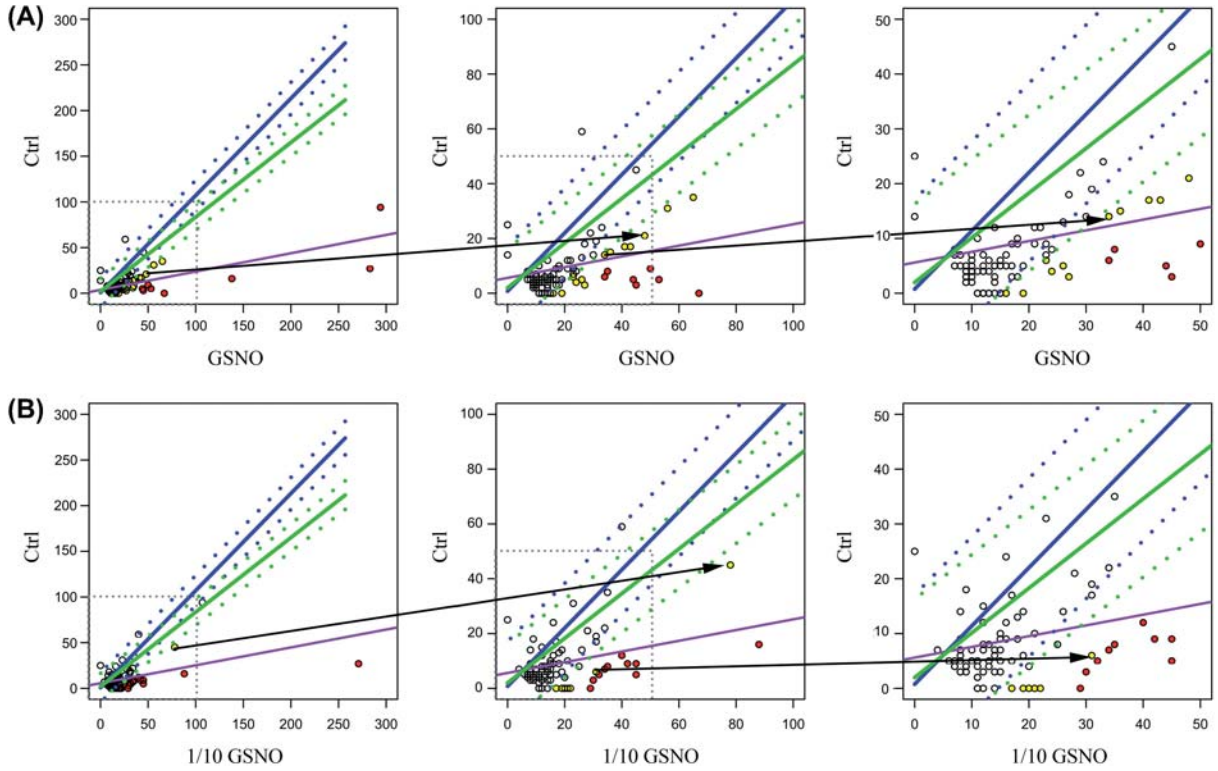

Figure 3. Proteins with a change in S-nitrosation level after GSNO treatment. (A) Proteins with a change in level of S-nitrosation in the GSNO treatment group (x-axis) versus the control group (y-axis). Red and yellow spots represent proteins detected as having a change in level of S-nitrosation, with confidence levels of 0.95 and 0.8, respectively. The purple line is the regression line of the spots with changed S-nitrosation level. (B) Proteins with a change in level of S-nitrosation after 1/10 dilution of the GSNO treatment group (x-axis) versus the control group (y-axis). The colours used for spots and lines were the same as in (A).

However, since we are not meant to cover all of the candidate proteins in the present work, but to build a method with the new strategy with a statistic confidence (0.8 or 0.95), thus we did four times replications.

Detecting proteins with changes in level of S-nitrosation caused by endogenous induction of S-nitrosation

When RAW264.7 cells were treated with LPS/IFN-γ for 18 hour, protein S-nitrosation in the cell is enhanced due to the induction of iNOS. This is a typical model for studying endogenous S-nitrosation. We tested the SNOSC method in this model. The sample of RAW264.7 cells treated with LPS/IFN-γ was used as the experimental group, and the spectral counts of the proteins identified in this group were depicted on the x-axis. The sample of normal RAW264.7 cells was used as the control group, and the spectral counts of the proteins identified in this group were depicted on the y-axis. The expanded views of the indicated regions are shown to the right of each panel. The results of this experiment are shown in Figure 4. Using the 'prediction area', we detected some proteins whose S-nitrosation level was changed by endogenous NO according to the corresponding confidence level. The purple line is the regression line of the spots. A replicate experiment was carried out and the results are shown in Supplementary Figure 2 to be found online at http://www.informahealthcare.com/doi/10.3109/10715762.2012.684244. Proteins identified as showing a change in S-nitrosation level in the above two experiments are combined and listed in Supplementary Table II to be found online at http://www.informahealthcare.com/doi/10.3109/10715762.2012.684244.

Validation of the model and the results

The samples after the IBP step were detected by Western blot using a biotin antibody (Supplementary Figure 3A to be found online at http://www.informahealthcare.com/doi/10.3109/10715762.2012.684244). The sample treated exogenously with GSNO had the strongest enhancement in S-nitrosation. The S-nitrosation enhancement caused by endogenous NO was a little weaker than the diluted GSNO treated sample. We also detected the effect of LPS/IFN-γ on the iNOS expression level (see Supplementary Figure 3B to be found online at http://www.informahealthcare.com/doi/10.3109/10715762.2012.684244) and found that iNOS was induced significantly by the stimulation, indicating the endogenous S-nitrosation model is reliable. To test the reliability of SNOSC in detecting proteins with a change S-nitrosation level, four proteins (actin, elongation factor 2, annexin 2 and glyceraldehyde-3-phosphate dehydrogenase) detected in the endogenous S-nitrosation group were confirmed using Western blot. The S-nitrosated proteins purified by IBP were detected using corresponding antibodies. The S-nitrosation level of all proteins was increased when RAW264.7 cells were treated with

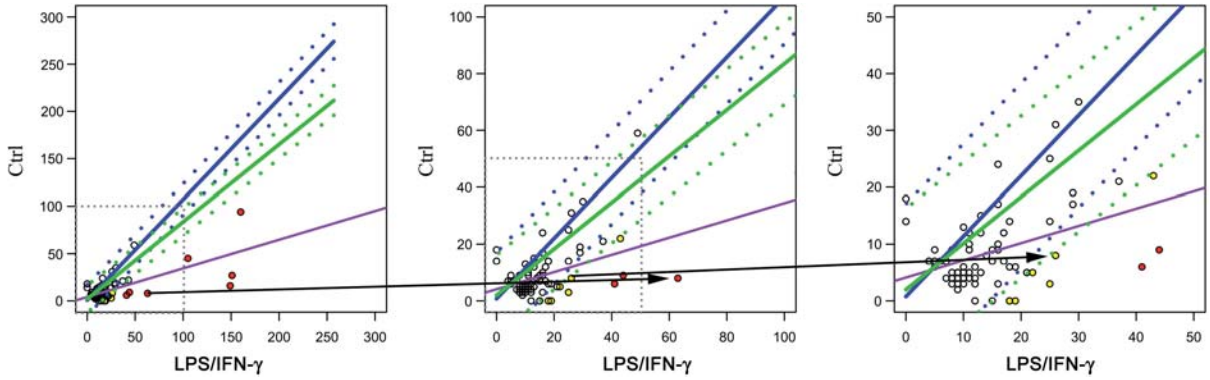

Figure 4. Proteins with a change in level of S-nitrosation caused by endogenous NO stimulation. Proteins with a change in level of S-nitrosation in RAW264.7 cells treated with LPS/IFN-γ (x-axis) versus the control group (y-axis). Red and yellow spots represent proteins detected as having a change in level of S-nitrosation with a confidence level of 0.95 and 0.8, respectively. The purple line is the regression line of the spots with changed S-nitrosation level.

LPS/IFN-γ (Figure 5A). This result indicates that the SNO spectral counting method is reliable. Nearly all the proteins (16/17 proteins), which showed a change in S-nitrosation level after endogenous induction with LPS/IFN-γ were also detected as S-nitrosated targets in the sample treated exogenously with GSNO (Figure 5B).

Discussion

In this study, we developed a method, which we term SNO spectral counting (SNOSC) for detection of proteins, which show a change in level of S-nitrosation between two groups. Using SNOSC, we studied S-nitrosation in the cell line RAW264.7 treated exogenously with GSNO or treated endogenously with LPS/IFN-γ, leading to detection of 50 and 17 proteins with a significant increase in level of S-nitrosation, respectively, in the two induction systems. Four endogenous targets were chosen to be further confirmed with IBP combined with Western blot and consistent results were obtained. Many other endogenous targets had been identified as S-nitrosation targets in other single-target works (e.g. Cltc [15], Plec1 [16]) and SNO proteomic works (e.g. Pkm2 [6], Eno1 [6, 17, 18], Eef1a1 [6]) as well.

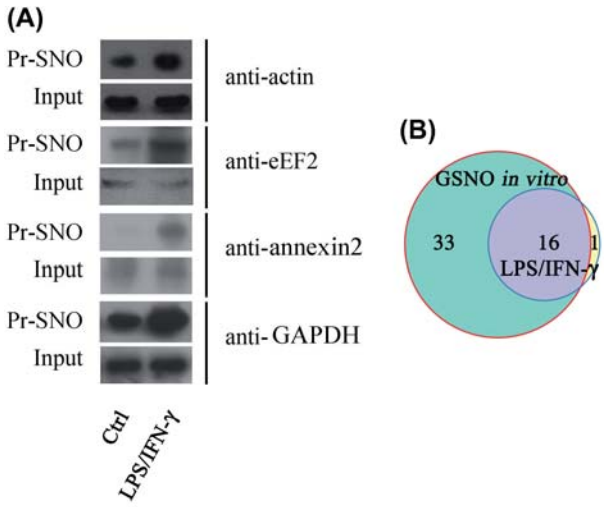

Figure 5. Validation of proteins with a change in level of S-nitrosation and comparison of effects of exogenous and endogenous NO treatment. (A) Four proteins detected by SNOSC as showing a change in level of S-nitrosation in the endogenous NO treatment group were confirmed by Western blot. All four proteins showed an obvious change in level of S-nitrosation after stimulation with LPS/IFN-γ. (B) Venn diagrams showing the number of proteins with a change in level of S-nitrosation detected by SNOSC in the exogenous and endogenous treatment groups. Most of the proteins detected in the endogenous treatment group were also detected in the exogenous group.

The advantages of SNOSC are summarised as follows: (1) there is no limitation in sample choice: it can be applied to any type of samples such as extracted proteins, cell cultures or tissues. (2) There is no need to purchase special labelling reagents and there is no problem of isotope labelling efficiency. (3) There are no additional procedures that must be introduced into the work flow, so the loss of S-nitrosated proteins due to complicated procedures and multiple steps can be reduced. At the same time, the high labelling efficiency of biotin-M was retained during enrichment of S-nitrosated proteins. (4) Since IBP was used to purify the S-nitrosated proteins, false positive targets caused by the interference of intermolecular disulphide bonds are ruled out.

Besides the advantages of SNOSC, there are also major disadvantages of SNOSC method inherited from the strategy of label-free based proteomic approaches. First, a label-free based proteomic method relays on a stable mass spectrometry environment to support the comparison of spectra counts between different samples analysed by LC-ESI-MS/MS. Second, SNOSC is indeed a semi-quantitative proteomic method with higher variant of quantity during independent experiments, not like a label-based quantitative method such as SILAC or iTRAQ. SNOSC is mainly used for screening. Third, since we delete all the proteins with spectra counts less than 10 in either sample compared, the protein targets screened out were prone to be abundant proteins with higher spectra counts.

In cases that the researchers want to know whether there is a change in level of S-nitrosation between samples

and to identify what the targets are, SNOSC is a good choice. It should be noted that when using SNOSC, the results are always related to the confidence level, and an appropriate value should be chosen. Researchers need to set their own appropriate confidence level in a specific set of experiments. A higher 'confidence level' means a less chance to be a false positive identification but will also so lead to more false negative identification. Due to the statistic approach that we used in this paper, the confidence of the identified protein targets was 0.8 or 0.95 in an individual experiment, that is, 20% or 5% possibility to be a false positive identification. Replicate analyses are required for higher confidence of correct identification of nitrosation. In our study, a confidence level of 0.8 was appropriate. The targets were confirmed by Western blot in Figure 5 and the amount of S-nitrosated protein detected is similar to other quantitative S-nitrosation proteomic methods.

In conclusion, the present work introduced a new strategy different from label-based proteomic methods for S-nitrosation detection, which is an alternative way for proteomic study of S-nitrosation. SNOSC can be a widely applicable proteomic method for fast screening of SNO proteins in different physiological and pathological processes.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. This work was supported by the '973 Program' (2011CB910900, 2011CB503900, 2012CB911000) and the National Natural Sciences Foundation of China (31030023).

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Supplementary data is available online

Supplementary Figures 1 to 3.
Supplementary Tables I and II.