A proteomic approach for the analysis of S-nitrosylated proteins using a fluorescence labeling technique

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SUMMARY

S-nitrosylation, a post-translational modification of the thiol group of cysteine residues by nitric oxide (NO), has emerged as a new mode of signal transduction and regulation of protein function. It has recently been shown that S-nitrosylation may result in various protein dysfunctions. However, an improved S-nitrosylation analysis method is needed to achieve high sensitivity and quantitative accuracy. We hypothesized that an analysis method using fluorescence dye could detect S-nitrosylated proteins at a higher sensitivity than that of the conventional method. In this study, we developed a procedure for analyzing S-nitrosylated proteins using CyDye (the CyDye switch method). This CyDye switch method for detecting S-nitrosylated proteins was developed based on the biotin-switch method for analyzing S-nitrosylated proteins. We analyzed NO donor-induced S-nitrosylated proteins in a model protein and at the cellular level. We demonstrated that this CyDye switch method could detect specific S-nitrosylated proteins using SDS-PAGE and mass spectrometry. Our results indicate that the optimized CyDye switch method is suitable for the detection of the post-translational S-nitrosylation of proteins.

Key words: S-nitrosylation, CyDye switch method

INTRODUCTION

Nitric oxide (NO), a well-known free radical, is produced under the action of nitric oxide synthase (NOS). NO has been shown to exert a variety of physiological/biochemical effects, including blood pressure control, activation of guanylyl cyclase, synaptic signal transmission, and inflammatory response1-4. S-nitrosylation, a post-translational modification of the thiol group of cysteine residues by NO, has recently been clarified as an important post-translational modification that may regulate signal transduction and proteins function by causing protein misfolding and/or degradation, mitochondrial fragmentation, and apoptosis5-8. Studies have demonstrated that S-nitrosylation may inhibit the enzymatic activity of protein disulfide isomerase (PDI), leading to the accumulation of denatured proteins9,10.

A detection method, named the biotin-switch method (BSM), was developed in 2001 for the identification of S-nitrosylated proteins11. This method involves three steps: i) blocking free thiol groups with sulphydryl-reactive reagents such as methyl methanethiosulfonate (MMTS), ii) converting S-nitrosothiols to free thiols using ascorbic acid, and iii) labeling converted free thiols with N-[6-(biotinamido)hexyl]-3'-(2'-pyridyl)dithio) propionamide (Biotin-HPDP), followed by detection via western blotting or concentration using agarose beads. This method, however, has some drawbacks: it is cumbersome and error-prone, does not easily yield quantitative data, and is low-throughput.

In this study, we employed a fluorescence labeling technique to develop a method for the detection of S-nitrosylated proteins. This fluorescence labeling method has several advantages over the BSM, including high sensitivity, quantitative accuracy, and simplicity. Moreover, multiple fluorescence dyes may be employed in the fluorescence labeling to allow for differential analysis of proteins expression by two-dimensional electrophoresis (2-DE). Although the CyDye switch method has several advantages, it has not been established as a detection method for S-nitrosylated proteins.
Some researchers have attempted to detect S-nitrosylated proteins using fluorescence-labeling techniques, but these different methods had variable sensitivities\(^{12-14}\). Thus, in this study, we attempted to optimize the CyDye switch method.

**MATERIALS AND METHODS**

**Materials**

Dulbecco’s modified Eagle’s medium (DMEM), \((+)-\)ascorbic acid sodium salt, and penicillin-streptomycin solution (x100) were purchased from Wako (Osaka, Japan). Fetal bovine serum (FBS) was from Thermo Scientific (Waltham, MA, USA). Cy5-maleimide was purchased from Dojindo (Kumamoto, Japan). N-ethylmaleimide (NEM) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Trypsin gold was from Promega (Fitchburg, WI, USA). Cyclooxygenase-2 (COX-2) antibody and anti-mouse IgG HRP-linked antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Copper (II) sulfate pentahydrate was from Nacalai Tesque (Kyoto, Japan). TRIzol® Reagent was purchased from Life Technologies (Pitam Pura, Delhi, India).

**Cell culture**

A549 human lung carcinoma cells and RAW264.7 mouse leukemic monocyte-macrophage cells were routinely cultured in DMEM containing 10% heat-inactivated FBS, 100 µg/mL penicillin, and 100 µg/mL streptomycin. The cells were maintained in a CO\(_2\) incubator at 37°C under a humid atmosphere containing 95% air and 5% CO\(_2\).

**Protein S-nitrosylation**

S-nitrosocysteine (CysNO), an NO donor, was synthesized by mixing 0.2 M NaNO\(_2\) with 0.2 M cysteine in 0.2 N HCl, followed by neutralization with NaOH. The concentration of the resulting CysNO was calculated using an extinction coefficient \(\varepsilon_{338}=900 \text{ M}^{-1} \text{ cm}^{-1}\)\(^{13}\). Another NO donor, GSNO, obtained commercially was prepared in a 20 mM solution (x100) were purchased from Wako (Osaka, Japan), and the luminescence signal was detected using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL, USA).

**Griess assay for NO measurement**

NO production was measured by detecting its nitrite by photometrically at 540 nm using a UV spectrophotometer Model UV-1800 (SHIMADZU, Kyoto, Japan).

**Detection of iNOS mRNA expression**

Total RNA was isolated using the TRizol® Reagent according to the manufacturer’s instructions. First-strand cDNA was synthesized using the First-Strand cDNA Synthesis Kit (TOYOBO, Osaka, Japan) with the isolated total RNA as the template. Polymerase chain reaction (PCR) was performed in a 20 µl reaction mixture using mouse-iNOS sense (5’-AGTCCGAAGCAACATCAC-3’) and mouse-iNOS antisense (5’-TAATGTCCAGGAATAGGTG-3’) primers under the action of Taq polymerase. The PCR conditions were 95°C for 5 min, followed by 30 cycles of 15 sec at 95°C, 30 sec at 50°C, 20 sec at 68°C and a final incubation at 68°C for 5 min. The amplified PCR product was separated by electrophoresis using a 2.0% agarose gel. The gel image was detected by UV (302/312-nm) using a Molecular Imager ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA).

**Detection of COX-2 protein expression using western blotting**

Inflammatory marker, COX-2, was detected by western blotting. Cell lysates prepared from A549 and RAW264.7 cells were separated by 10% SDS-PAGE, and electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking the transferred membrane with 3% skim milk in PBS containing 0.1% (v/v) Tween 20 (TPBS), the membrane was probed with the primary antibody. Following washing and blocking the probed membrane with TPBS and 3% skim milk in TPBS, the membrane was treated with HRP-conjugated secondary antibody. The antibody-labeled proteins were visualized by enhanced Immunostar LD (Wako, Osaka, Japan), and the luminescence signal was detected using a Molecular Imager ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA).

**Optimized CyDye switch method and image analysis**

Protein S-nitrosylation was detected using the optimized CyDye switch method. Briefly, proteins and cells were solubilized in a TUCD buffer composed of 8 M Urea, 30 mM Tris-HCl, pH 7.3, 2% CHAPS, 2% n-dodecyl-β-D-maltoside, and 1 M DTPA. Free thiols were blocked with 100 mM N-ethylmaleimide (NEM) in TUCD buffer at 50°C for 60 min. Excess NEM was removed by precipitation with 15% TCA for 20 min, and precipitates were collected by centrifugation at 20,000 \(\times\) g for 10 min. The pellets were washed three times with 15% TCA. Afterwards, the pellets were lysed in TUC buffer composed of 8 M Urea, 30 mM Tris-HCl, pH 7.3, 4% w/v CHAPS. The lysates were reduced by 100 µM HCl, followed by neutralization with NaOH. The concentration of the resulting CysNO was calculated using an extinction coefficient \(\varepsilon_{338}=900 \text{ M}^{-1} \text{ cm}^{-1}\)\(^{13}\). Another NO donor, GSNO, obtained commercially was prepared in a 20 mM solution (x100) were purchased from Wako (Osaka, Japan), and the luminescence signal was detected using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL, USA).

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Two-dimensional electrophoresis

The cell lysate (100 µg) were separated by isoelectric focusing (IEF) using a 12 cm IPG strip gel (pH 3–10). After IEF, the IPG strip gel was incubated in an equilibration buffer composed of 6 M Urea, 377 mM Tris-HCl, pH 8.8, 2% SDS, 20% glycerol at room temperature for 60 min. Proteins distributed on the IPG strip gel were further separated by 10% SDS-PAGE. Afterwards, fluorescence images were obtained using a Typhoon FLA 9500 imager (GE Healthcare Japan, Tokyo, Japan). Fluorescence band detected following SDS-PAGE was quantified using the Image Quant TL software (GE Healthcare Japan, Tokyo, Japan).

Proteins digestion, determination of the S-nitrosylation site, and identification of S-nitrosylated proteins by mass spectrometry

A model protein; S-nitrosylated BSA, was digested in a trypsin digestion solution (10 ng/mL trypsin (Trypsin Gold, Mass Spec Grade; Promega KK, Fitchburg, WI, USA), 0.1% n-Octyl-β-D-glucoside 50 mM ammonium bicarbonate) using at 37°C for 12 h. Peptides generated following trypsin digestion were separated using a reverse phase C18 LC column (Thermo, Waltham, MA, USA) and analyzed using MS using Q Exactive hybrid quadrupole-orbitrap mass spectrometer (Thermo, Waltham, MA, USA). MS spectrum obtained was searched against the SwissProt database in Proteome Discoverer software using the SEQUEST search engine (Thermo, Waltham, MA, USA). Cy5-maldeimide (605.349) and NEM (125.048) were selected for analysis of modification sites as dynamic modification. Fig. 1C shows the Cy5-maleimide at room temperature for 60 min. Excess fluorescence dye and salt were removed by TCA-DOC-acetone precipitation. Precipitated proteins pellets were solubilized in a lysis buffer composed of 7 M Urea, 2 M Thiourea, 2% w/v CHAPS, 2% w/v n-dodecyl-β-D-maltoside and separated on SDS-PAGE. Fluorescence images were obtained using a Typhoon FLA 9500 imager (GE Healthcare Japan, Tokyo, Japan). Fluorescence band detected following SDS-PAGE was quantified using the Image Quant TL software (GE Healthcare Japan, Tokyo, Japan).

RESULTS

Detection of S-nitrosylated model proteins using optimized CyDye switch method and determination of S-nitrosylation site

In this study, we first attempted to optimize the CyDye switch method for the detection of S-nitrosylated proteins. It has been suggested that this is a high performance method, in comparison with the biotin switch method (BSM), since the fluorescence reagent, CyDye-maleimide, is stable even under reducing conditions. The CyDye switch method thus has a high sensitivity and has quantitative accuracy for directly labeling S-nitrosylated proteins. Moreover, differential analysis can be performed using multiple fluorescence dyes at reduced speed and costs. In the CyDye switch method, the fluorescence dyes labels the reduced thiol group of S-nitrosocysteine in the presence of sodium l-ascorbate. Since MMTS, which is utilized in BSM to block free thiol groups, is unstable under reducing conditions, N-ethylmaleimide (NEM) was employed to block free thiol groups in our study. Moreover, while acetone precipitation has been utilized to remove excessive un-reactive blocking reagent, we employed TCA precipitation to ensure a low background. Furthermore, although sodium l-ascorbate alone has been utilized to reduce nitrosothiols on S-nitrosylated proteins, we employed a combination of sodium l-ascorbate and copper (II) sulfate pentahydrate in order to increase detection sensitivity. Therefore, S-nitrosylated proteins were selectively reduced under aforementioned conditions and subsequently labeled using CyDye-maleimide, forming stable CyDye labeling complexes.

To assess the detection of S-nitrosylated proteins using the modified CyDye switch method, 100 µg bovine serum albumin (BSA) was exposed to 200 µM CysNO at room temperature for 30 min, and the resulting CyDye labeled S-nitrosylated proteins were separated by SDS-PAGE. As shown in Fig. 1A and B, the modified CyDye switch method was able to detect fluorescent image only for BSA treated with CysNO, but not untreated BSA. Additionally, the use of sodium l-ascorbate alone or copper (II) sulfate pentahydrate alone as reduction agent failed to show any fluorescence images. We further attempted to determine the S-nitrosylation site in nitrosylated BSA. S-nitrosylated BSA was subjected to trypsin digestion and the trypsin digested S-nitrosylated BSA peptides were separated and detected by LC/MS/MS. Separated peptides were analyzed using the SEQUEST algorithm. Cy5-maldeimide (605.349) and NEM (125.048) were selected for analysis of modification sites as dynamic modification. Fig. 1C shows the Cy5-maleimide...
blocking reagent, acetone precipitation for removing excess reagents, and 1 mM ascorbate and copper (II) sulfate pentahydrate for reducing $S$-nitrosothiols. In method 3 (our optimized CyDye switch method), we employed a different reaction buffer called the TUCD buffer, which is composed of 8 M urea, 30 mM Tris-HCl, pH 7.3, 2% CHAPS, 2% n-dodecyl-$\beta$-D-maltoside, and 1 mM DTPA. This method utilizes 100 mM NEM as the blocking reagent and 10% TCA precipitation for removing excess reagents. These three methods were compared using A549 cell lysate exposed to GSNO at room temperature for 30 min. Results shown in Fig. 2 indicated that our optimized CyDye switch method was able to detect $S$-nitrosylated proteins with higher sensitivity and lower background than the other two methods. The results of the quantitative analysis using Image Quant TL software indicated that the fluorescent signal detected binding sites detected in $S$-nitrosylated BSA fragments. Collectively, the above-mentioned results demonstrated that the modified CyDye switch method was able to label and detect $S$-nitrosylated proteins with improved specificity and sensitivity.

We next tried to compare our method with two previous reported methods that also utilize fluorescence dye for the detection of $S$-nitrosylated proteins. Method 1, reported by Huang et al. in 2009, employs the HEN buffer, composed of 250 mM HEPES-NaOH pH 7.7, 1 mM EDTA, 0.1 mM Neocuproine containing 1% Triton X-100, 0.1% SDS, and 1% of a protease inhibitor cocktail, as the reaction buffer. This method utilized 20 mM MMTS as the blocking reagent, acetone precipitation for removing excess reagents, and 1 mM ascorbate for reducing $S$-nitrosothiols. Method 2, reported by Kettenhofen et al. in 2008, employs 50 mM NEM as the blocking reagent, acetone precipitation for removing excess reagents, and 1 mM ascorbate and copper (II) sulfate pentahydrate for reducing $S$-nitrosothiols. In method 3 (our optimized CyDye switch method), we employed a different reaction buffer called the TUCD buffer, which is composed of 8 M urea, 30 mM Tris-HCl, pH 7.3, 2% CHAPS, 2% n-dodecyl-$\beta$-D-maltoside, and 1 mM DTPA. This method utilizes 100 mM NEM as the blocking reagent and 10% TCA precipitation for removing excess reagents. These three methods were compared using A549 cell lysate exposed to GSNO at room temperature for 30 min. Results shown in Fig. 2 indicated that our optimized CyDye switch method was able to detect $S$-nitrosylated proteins with higher sensitivity and lower background than the other two methods. The results of the quantitative analysis using Image Quant TL software indicated that the fluorescent signal detected

Fig. 1. Detection of model protein $S$-nitrosylated bovine serum albumin using CyDye switch method, and determination of CyDye labeling site.

A: The BSA (100 $\mu$g) was induced by $S$-nitrosylation using 100 $\mu$M CysNO, and SNO-BSA was detected by CyDye switch method. Fluorescence labeling BSA sample was separated by SDS-PAGE and Cy5 fluorescent signals were detected in photomultiplier tube (PMT) voltage of 500 using Typhoon FLA 9500 (GE healthcare japan). Control was not treated CysNO, and was performed CyDye switch method.

B: SNO-BSA was digested using trypsin gold, mass spec grade in solution. The trypsinized peptides were analyzed with the SwissProt database using the SEQUET algorithm by setting a peptide tolerance of ppm. CyDye labeling peptides were determined with a mass shift of 684. Da (Cy5-Malimide).

C: The $S$-nitrosylated cysteine was indicated under line in the identified peptide. The MS/MS analysis of CyDye labeling peptide is shown as a peak list.
teration of S-nitrosylated proteins in A549 cells. A549 cells, grown in different wells of a 24-well plate, were exposed to 200 µM CysNO at 37°C for 60 min, and thereafter incubated in fresh FBS-free DMEM. Afterwards, A549 cells were collected and lysed at 0, 1, 12 and 24 h, and analyzed using the optimized CyDye switch method. As shown in Fig. 3E and F, the fluorescent signals were strongest for cell lysate prepared at 0 h following incubation in FBS-free DMEM. Thereafter, the fluorescent signals were attenuated in a time-dependent manner. Fluorescent signals of 24 h after switching to exchange medium were reduced to almost the same fluorescent level as intact A549 cells. Collectively, these results indicated that the detection of S-nitrosylated proteins generated in CysNO-treated A549 cells in both time- and dose-dependent manners, with high sensitivity and specificity.

We next performed a comprehensive analysis of S-nitrosylated proteins generated in CysNO (200 µM)-treated A549 cells. Cell lysate prepared was separated by two-dimensional electrophoresis, followed by detection using the CyDye switch method (Fig. 4). Detected fluorescent spots were trypsinized, and trypsin-digested peptides were analyzed by peptide mass fingerprinting (PMF) using MALDI-TOF/MS autoflex. The mass spectra obtained were processed without smoothing using the Flexanalysis software. The mass data were used to search the SwissProt database using the Mascot search. As shown in Table 1, most of the identified proteins were the ones that had previously been reported as targets for S-nitrosylation. Prominently among these pro-

![Image](image-url)

**Fig. 2.** Comparison of our optimized method with another method using fluorescence dye

Solubilized A549 cell by each buffer were treated 200 µM CysNO at room temperature for 30 min. HEN buffer was utilized as lysis buffer in the method 1. 20 mM MMTS and acetone were used as blocking reagent and removing excess blocking reagents, and 1 mM ascorbate was used as reducing S-nitrosothiols in method 1. 50 mM NEM and acetone were used as blocking reagent and removing excess reagent, and 1 mM sodium l-ascorbate and 10 µM copper (II) sulfate pentahydrate were used as reducing S-nitrosothiols in method 2. TUCD buffer was utilized as lysis buffer in the method 3. 100 mM NEM and 10% TCA were used as blocking reagent and washing excess blocking reagent in the method 3. 100 µM sodium l-ascorbate and 10 µM copper (II) sulfate pentahydrate were used as reducing S-nitrosothiols in method 3. (a) Cy5 fluorescent signals were detected in photomultiplier tube (PMT) voltage of 500 using Typhoon FLA 9500 (GE healthcare japan) (b) Cy5 fluorescent volumes were quantified using Image Quant TL software (GE healthcare japan). Fluorescent volumes were calculated relative value as a % of GSH treatment in method 1. Data were presented as the mean±S.D. derived from three determinations.

with our optimized method was more than one-and-a-half times higher than with the other methods.

**Detection of S-nitrosylated proteins generated in A549 cells following exposure to CysNO**

While the above-mentioned optimized CyDye switch method was able to detect S-nitrosylated proteins with high sensitivity and specificity, it is important to demonstrate that this method can be applied to detect S-nitrosylated proteins generated in the cells. Thus, it is necessary for analysis of S-nitrosylated proteins in cellular level. We tried to detect S-nitrosylated proteins in A549 cellular level exposed to CysNO and performed monitoring of time-dependent and dose-dependent S-nitrosylation exposed to NO donor. Cultured A549 cells were treated with 200 µM CysNO at 37°C for 60 min in a CO₂ incubator. Treated A549 cells were fixed using 10% TCA and lysed in the TUCD buffer. S-nitrosylated proteins present in the cell lysate were labeled using the optimized CyDye switch method. As shown in Fig. 3A and B, fluorescent signals, quantitatively analyzed using the Image Quant TL software, were detected only in A549 cells treated with CysNO. These results indicated that S-nitrosylated proteins could be detected in A549 cells exposed to CysNO. To assess the sensitivity of detection for the optimized CyDye switch method, a CysNO dose-dependent experiment was performed. A549 cells, grown in different wells of a 24-well plate, were exposed to different concentrations (0, 10, 20, 50, 100 and 200 µM) of CysNO and incubated at 37°C for 60 min. As shown in Fig. 3C and D, S-nitrosylated proteins were detected in a CysNO dose-dependent manner. We next examined the time course alteration of S-nitrosylated proteins in A549 cells. A549 cells, grown in different wells of a 24-well plate, were exposed to 200 µM CysNO at 37°C for 60 min, and thereafter incubated in fresh FBS-free DMEM. Afterwards, A549 cells were collected and lysed at 0, 1, 12 and 24 h, and analyzed using the optimized CyDye switch method. As shown in Fig. 3E and F, the fluorescent signals were strongest for cell lysate prepared at 0 h following incubation in FBS-free DMEM. Thereafter, the fluorescent signals were attenuated in a time-dependent manner. Fluorescent signals of 24 h after switching to exchange medium were reduced to almost the same fluorescent level as intact A549 cells. Collectively, these results indicated that the detection of S-nitrosylated proteins the optimized CyDye switch method was able to detected S-nitrosylated proteins generated in CysNO-treated A549 cells in both time- and dose-dependent manners, with high sensitivity and specificity.

We next performed a comprehensive analysis of S-nitrosylated proteins generated in CysNO (200 µM)-treated A549 cells. Cell lysate prepared was separated by two-dimensional electrophoresis, followed by detection using the CyDye switch method (Fig. 4). Detected fluorescent spots were trypsinized, and trypsin-digested peptides were analyzed by peptide mass fingerprinting (PMF) using MALDI-TOF/MS autoflex. The mass spectra obtained were processed without smoothing using the Flexanalysis software. Mass lists were obtained using the Biotools software, and the mass data were used to search the SwissProt database using the Mascot search. As shown in Table 1, most of the identified proteins were the ones that had previously been reported as targets for S-nitrosylation. Prominently among these pro-
indicated clearly that the optimized CyDye switch method could be used for the comprehensive analysis of S-nitrosylated proteins.

Detection of S-nitrosylated proteins generated in RAW264.7 cells exposed to LPS

We next attempted to detect S-nitrosylated proteins generated in RAW264.7 cells exposed to LPS.

Fig. 3. Detection and quantification of A549 cells S-nitrosylated proteins in cellular level by CyDye switch method.

A549 cells were treated CysNO in FBS-free DMEM containing 100 U/mL penicillin, and 100 µg/mL streptomycin and incubated in CO₂ incubator at 37°C for 30 min, and were induced S-nitrosylation. A: 200 µM CysNO induced S-nitrosylation A549 cells were lysed TUCD buffer, and lysed proteins were separated by SDS-PAGE. B: Dose dependent effect of CysNO on A549 cells S-nitrosylation levels. A549 cells were treated or untreated with 10, 20, 50, 100 and 200 µM CysNO. C: Time dependent effect in A549 cells S-nitrosylation levels. A549 cells were treated or untreated with 200 µM CysNO for 1 h, followed exchange FBS-free DMEM. After exchange medium 0, 1, 12 and 24 h, A549 cells S-nitrosylated proteins were collected, and were detected by CyDye switch method.

(a) Cy5 fluorescent signals were detected in photomultiplier tube (PMT) voltage of 500 using Typhoon FLA 9500 (GE healthcare japan) (b) Cy5 fluorescent volumes were quantified using Image Quant TL software (GE healthcare japan). Fluorescent volumes were calculated relative value as a % of control. Data were presented as the mean±S.D. derived from three determinations.

Proteins are the heat shock protein, protein disulfide isomerase, and actin that had previously been reported as targets for S-nitrosylation, and have been extensively studied in regard to the regulation of their protein function by S-nitrosylation. In addition, several previously unidentified S-nitrosylated proteins such as ATP-citrate synthase and pyruvate kinase PKM were also detected. These results indicated clearly that the optimized CyDye switch method could be used for the comprehensive analysis of S-nitrosylated proteins.
was able to detect S-nitrosylated proteins generated in LPS treated RAW264.7 cells. A time-course experiment was then conducted, and after 0, 1, 12 and 24 h of exposure to LPS, S-nitrosylated proteins present in RAW264.7 cell lysates were labeled with Cy5-maleimide using the CyDye switch method. For peptide mass fingerprinting (PMF) procedure, each mass spectrum was obtained from signals generated from at least 4000 laser shots. The obtained mass spectra were processed without smoothing using Flexanalysis software (Bruker Daltonics) and mass lists were obtained by Biotools software; the mass data were used to search the SwissProt database using the Mascot search.

Table 1. Identified S-nitrosylated proteins

| Spot Number | Accession Number | Short Name       | Protein Name                | Mass    | Mascot Score | Sequence Coverage (%) |
|-------------|------------------|------------------|----------------------------|---------|--------------|----------------------|
| 1           | Q14764           | MVP_HUMAN        | Major vault protein         | 92666   | 80           | 16                   |
| 2           | P55072           | TERA_HUMAN       | Transitional endoplasmic reticulum ATPase | 82666   | 70           | 16                   |
| 3           | P08238           | HSP90B_HUMAN     | Heat shock protein HSP 90-beta | 8312    | 74           | 12                   |
| 4           | P11021           | GRP78_HUMAN      | Heat shock cognate 71 kDa protein | 72288   | 104          | 14                   |
| 5           | P11142           | HSP70_HUMAN      | Heat shock cognate 70 kDa protein | 70854   | 113          | 27                   |
| 6           | P08107           | PDI1_HUMAN       | Protein disulfide-isomerase A1 | 70009   | 126          | 28                   |
| 7           | P07327           | Vimentin         |                                  | 57081   | 105          | 19                   |
| 8           | P14618           | KYM_HUMAN        |                                  | 57900   | 106          | 21                   |
| 9           | P06709           | ACTB_HUMAN       | Actin, cytoplasmic 1          | 41710   | 63           | 10                   |
| 10          | P53396           | ACLY_HUMAN       | ATP-citrate synthase          | 120762  | 69           | 8                    |
| 11          | P13639           | EF2_HUMAN        | Elongation factor 2           | 95277   | 64           | 12                   |
| 12          | P13639           | EF2_HUMAN        | Elongation factor 2           | 95277   | 59           | 14                   |
| 13          | P00352           | ALA1_HUMAN       | Retinal dehydrogenase 1       | 54827   | 62           | 18                   |
| 14          | P00352           | ALA1_HUMAN       | Retinal dehydrogenase 1       | 54827   | 58           | 21                   |
| 15          | P00352           | ALA1_HUMAN       | Retinal dehydrogenase 1       | 54827   | 73           | 21                   |
| 16          | P06733           | ENOA_HUMAN       | Alpha-enolase                 | 47139   | 69           | 20                   |
| 17          | P14618           | KYM_HUMAN        | Pyruvate kinase PKM           | 57900   | 82           | 21                   |
| 18          | Q6GPBH4          | XAF1_HUMAN       | XIAP-associated factor 1      | 34603   | 40           | 7                    |
| 19          | O60218           | AK1_B_HUMAN      | Aldo-keto reductase family 1 member B10 | 35997   | 62           | 30                   |

was able to detect S-nitrosylated proteins generated in LPS treated RAW264.7 cells.

A time-course experiment was then conducted, and after 0, 1, 12 and 24 h of exposure to LPS, S-nitrosylated proteins present in RAW264.7 cell lysates were labeled with Cys-maleimide using the CyDye switch method. As shown in Fig. 5D and E, the strongest fluorescent signals were detected following 12 h of treatment with LPS. After 24 h of treatment with LPS, the fluorescent signal decreased to almost the same level as in intact cells. The expression of reductase may have been involved in the decrease in S-nitrosylated proteins after 24 h of treatment with LPS. Therefore, we detected S-nitrosylated proteins using the CyDye switch method under physiological conditions. The results indicated that the CyDye switch method was a suitable method for the analysis method of S-nitrosylated proteins.
S-nitrosylation and various diseases\textsuperscript{19}). A sensitive and specific method for the detection of S-nitrosylated proteins is therefore of critical importance. In the present study, we developed an optimized CyDye switch method for detecting S-nitrosylated proteins with high sensitivity and specificity.

**DISCUSSION**

Many recent studies have indicated that protein S-nitrosylation, a post-translational cysteine modification caused by NO, plays an important role in the regulation of protein functions, suggesting a link between protein S-nitrosylation and various diseases\textsuperscript{19}). A sensitive and specific method for the detection of S-nitrosylated proteins is therefore of critical importance. In the present study, we developed an optimized CyDye switch method for detecting S-nitrosylated proteins with high sensitivity and specificity. The developed method was shown to be capable of detecting...
SNO-BSA and determining the S-nitrosylation site in SNO-BSA by LC-MS/MS analysis. In addition, it was demonstrated that the optimized CyDye switch method was able to detect S-nitrosylated proteins in cultured cells either treated with CysNO or exposed to LPS stimulation.

The fluorescent labeling by CyDye-maleimide was superior to that of Biotin-HPDP as used in the BSM. These fluorescent dyes specifically label thiol groups of free cysteine and form stable bonds in the presence of reduction reagents such as DTT and 2-mercaptoethanol. Therefore, CyDye-labeling is not lost in samples following reduction during their preparation and analysis of sample, such as protein precipitation and electrophoresis. The CyDye switch method allows the detection of labeled proteins easily and rapidly using a fluorescent imager, and there is no need to perform the immunoblotting and concentration using affinity beads such as in the BSM. In addition, fluorescent labeling allows differential quantitative analysis within one gel using analysis software. This method may be a useful tool for analyzing the alteration of S-nitrosylated proteins under various conditions.

Our data indicated that fluorescent signals were detected only following treatment with sodium l-ascorbate and copper (II) sulfate pentahydrate (Fig. 1A). However, originally, the BSM was reported specifically detect S-nitrosylated proteins using only ascorbic acid in the reduction step. Interestingly, previous studies have reported that S-nitrosylation is subjected to the reduction by ascorbic acid and copper ions. Ascorbic acid reduces a copper ion, Cu\(^{2+}\), to a catalytically active redox form, Cu\(^{+}\), which releases the NO radical from S-nitrosothiols. These results suggest that the sensitivity of detecting S-nitrosylated proteins may vary in the reduction step. We attempted to detect S-nitrosylated proteins using ascorbic acid alone in the reduction step, but no fluorescent signals were detected. The BSM method using ascorbic acid as a sole reducing reagent can likely to detect S-nitrosylated proteins following concentration by affinity beads. We hypothesized that this method was possible to detect by low-reduction capacity such as ascorbic acid for detecting concentrated S-nitrosylated proteins. Thus, our results suggested that the CyDye switch method may have a higher sensitivity for detecting S-nitrosylated proteins.

In a comprehensive analysis using two-dimensional electrophoresis, the CyDye switch method detected not only previously reported S-nitrosylated proteins, but also new targets for S-nitrosylation, including ATP-citrate synthase and Pyruvate kinase PKM. These two proteins are involved in citric acid cycle and glycolytic pathway, respectively. Further studies are warranted in order to reveal the mechanism of S-nitrosylation of these newly identified proteins and relevant pathophysiological implications.

At the cellular level and under physiological conditions, the CyDye switch method successfully detected S-nitrosylated proteins. In LPS-stimulated RAW264.7 cells, the detected fluorescent signals were highest after 12 h of exposure to LPS. This result suggested that protein S-nitrosylation was detected to produce NO by iNOS protein expression after 12 h of treatment with LPS. A previous study has shown that NO production and inflammation caused by LPS induced the expression of redox proteins such as the peroxiredoxin family. Fluorescent signals may be therefore decreased after 24 h of treatment. The concentration of NO was detected about 25 \(\mu\)M after 12 h of exposure to LPS (Fig. 5B). The relative intensity of the fluorescent signals was detected 150% of the control value following the treatment with the NO donor at 20 \(\mu\)M (Fig. 3B). Thus, under physiological conditions, the results indicated that the CyDye-switch method was able to detected S-nitrosylated proteins with high sensitivity and quantitative accuracy.

Recently, Majmudar et al. reported a new method for the detection of S-nitrosylated proteins and S-sulfonated proteins using fluorescent dye. This method involves direct and selective labeling of S-nitrosylated proteins using biotin-SO\(_2\)H, which reacts with S-nitrosothiols. While this newly reported method is innovative in terms of directly labeling S-nitrosothiols, it employs nonreducing SDS-PAGE and streptavidin-Cy5 blot in order to detect fluorescent signals. These method, however, does not easily yield quantitative data and is low-throughput for employs blot procedure. Thus, this method may require the complicated procedure and limit the further application. In contrast, our optimized method takes advantages for immediately detection after labeling fluorescent dye. It has advantages of i) rapid and direct detection of the reduced S-nitrosothiols using fluorescent dye. ii) flexibility in the differential analysis of proteins expression using multiple fluorescence dyes.

In summary, our optimized CyDye switch method is a powerful procedure to profile S-nitrosylated proteins under the various conditions such as in diseases and investigate the screening of potential drug/food components to attenuate S-nitrosylated proteins.

**ABBREVIATIONS**

BSM, Biotin switch method; COX-2, Cyclooxygenase-2; Cy5-maleimide, Cyanine5-maleimide; CysNO, S-nitrosocysteine; DDM, n-dodecyl-β-D-maltoside; DMEM, Dulbecco’s modified eagle medium; DMSO, Dimethyl sulfoxide; DOC, Sodium deoxycholate; DTPA, Diethylene triamine pentaacetic acid; FBS, Fetal bovine serum; GSH, Glutathione; GSNO, S-nitrosoglutathion; LPS, Lipopolysaccharide; MMTS, Methyl methanethiosulfonate; NEM, N-ethylmaleimide; NO, Nitric oxide; NOS, Nitric oxide synthase; ROS, Reactive oxygen species; PCR, Polymerase chain reaction; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, Trichloroacetic acid
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