A Proteomic Study of S-Nitrosylation in the Rat Cardiac Proteins in Vitro

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Protein S-nitrosylation in the heart tissue has been implicated in several patho (physiological) processes. However, specific protein targets for S-nitrosylation remain largely unknown. In this study, the rat cardiac proteins were incubated in vitro with S-nitrosoglutathione (GSNO), a biologically existing nitric oxide (NO) donor and S-nitrosating agent, to induce protein S-nitrosylation, and the resulting S-nitrosylated proteins were purified by the biotin switch method, followed by two-dimensional gel electrophoresis (2-DE) separation and matrix-assisted laser desorption ionization/time of flight tandem mass spectrometry (MALDI-TOF-MS/MS) identification. Candidate Western blot analysis was also used to identify potential S-nitrosylated proteins. A total of ten proteins including triosephosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, creatine kinase, adenylate kinase 1 (AK1), enolase 1, destrin, actin, myosin, albumin and Hsp27 were unambiguously identified, among which AK1 was found as a novel target of S-nitrosylation. Further studies showed that AK1 activity in the rat heart extracts was significantly inhibited by GSNO but not oxidized glutathione (GSSG), and the inhibition was completely reversed by dithiothreitol (DTT) post-treatment, demonstrating that S-nitrosylation might serve as a new regulatory mechanism in controlling AK1 activity. This study represents an initial attempt to characterize the S-nitrosoproteome in the heart and highlights the importance of protein S-nitrosylation in cardio function regulation.

Key words S-nitrosylation; S-nitrosoglutathione; biotin switch method; S-nitrosoproteome; adenylate kinase

MATERIALS AND METHOD

Protein S-nitrosylation refers to the reversible attachment of nitric oxide (NO) moiety to specific cysteine residue(s) on selected proteins, producing labile S-nitrosothiol structure and functional alterations.1,2) S-Nitrosylation is involved in the regulation of a diverse array of protein functions and plays a dominant role in many signaling pathways. It is increasingly accepted that S-nitrosylation of proteins serves as a critical cellular regulation mechanism akin to O-phosphorylation.3) Besides the physiological importance, dysfunction in maintaining the homeostasis of S-nitrosylation has been linked to many disease states.4) Specifically in the heart, a low level of protein S-nitrosothiols was found at physiological conditions.5) During ischemic preconditioning (IPC), a significant increase of S-nitrosylation was observed.6) Of note, several heart proteins have been recently identified as targets of S-nitrosylation. For example, the L-Type Ca2+ Channel and mitochondrial complex I were demonstrated to be S-nitrosylated and this modification appears to confer protective effects against ischemia/reperfusion (IR) injury.7,8) However, the systematic investigations of the overall protein targets of S-nitrosylation in the heart, or heart S-nitrosoproteome, is still at the very beginning stage,9) and further studies are needed to validate or expand the reported results.

Recently, a novel method named the biotin switch method (BSM) has been devised to tackle the S-nitrosoproteome.10) In this three-step method, un-modified protein thiols are blocked and S-nitrosylated thiols are reduced to free thiols, and the newly generated thiols are labeled with a biotin tag, followed by avidin capture of the labeled proteins. The final products therefore represent the originally S-nitrosylated proteins, and these proteins can be identified by proteomic approaches. This method has been successfully used in various mammalian tissues, cell types, sub-cellular organelle and certain plant or bacterium.11,12) Though some authors questioned the specificity of this method,13) it appears that high specificity can be attained provided proper care is taken in handling samples.13) The BSM is in fact the only currently available methods to study protein S-nitrosylation at the proteomic level. In this study, we adopted the BSM to characterize the S-nitrosoproteome in the rat cardiac proteins in vitro. To promote S-nitrosylation, the heart proteins were incubated in vitro with S-nitrosoglutathione (GSNO), which exists biologically in vivo in NO pools and is widely used as an in vitro S-nitrosating agent.

Chemicals Streptavidin agarose, methyl methanethiosulfonate (MMTS), neocuproine, 3-([3-Cholamidopropyl]dimethylammonio)-1-propanesulfonate (CHAPS), dithiothreitol (DTT), oxidized glutathione (GSSG), rabbit anti-heart shock protein (Hsp) 27/25 antibody and mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody were obtained from Sigma. N-6-(Biotinamido)hexyl)-3’-(2’-pyridyldithio)pro-pionamide (biotin-HPDP) were purchased from Pierce. IPG-strips, pH 3—10 were from Amersham Bioscience. NADP, ADP, hexokinase and glucose-6-phosphate dehydrogenase were from Roche.

Animals Male Sprague-Dawley rats weighing 180—220g were obtained from Zhejiang Experimental Animal Center. Animal protocols were approved by The Animal Ethic Review Committees of Zhejiang University.

Synthesis of GSNO GSNO was synthesized by combining 200 mM NaNO2 and 200 mM reduced glutathione in 0.5 M HCl in the dark at room temperature for 10 min. The solution was neutralized to pH 7.2 with 1 M NaOH, and its concentration determined by absorbance at 334 nm using an extinction coefficient of 900 M−1 cm−1.14)

Preparation of Rat Heart Extracts and in Vitro S-Nitrosylation The hearts from 10 rats were homogenized in ice-cold HEN buffer (250 mM Hepes, pH 7.7, 1 mM EDTA, 0.1 mM neocuproine). After centrifuging at 2000 g for 10
min, the supernatant was recovered and protein concentration determined by the Bradford method.\(^5\) Protein concentration was adjusted to 0.8 mg/ml, and CHAPS was added to a final concentration of 0.5% to dissolve membrane constituents. Samples were stored at −80 °C before use. To induce S-nitrosylation, the cardiac proteins were incubated with freshly prepared GSNO (100 μM) in the dark for 30 min. Acetone precipitation was used to remove excess GSNO.

**Purification of the S-Nitrosylated Proteins by the BSM**

The BSM was carried out essentially as described before.\(^9\) The starting samples contained 28 mg protein. Briefly, free protein thiols were blocked with 4 volumes of HEN buffer containing 2.5% SDS and 20 mM MMTS at 50 °C for 20 min. Proteins were precipitated with cold acetone and suspended in HEN buffer containing 1% SDS. Ascorbate (2 mM) was added to specifically reduce the SNO bond to free protein thiols, which were then labeled by biotin-HPDP (4 mM) for 1 h at room temperature. Proteins were again acetone-precipitated and resuspended. Streptavidin agarose (300 μl packed volume) was added to pull-down the biotin-tagged proteins. After incubation for 1 h at room temperature, the Streptavidin agarose was washed five times with washing buffer (20 mM Hepes, pH 7.7, 600 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100). The bound proteins were recovered by incubation with 0.6 ml 100 mM DTT for 30 min. Samples were desalted by Bio-Spin columns (Bio-Rad) and concentrated using a centrifugal evaporator.

**Separation of the S-Nitrosylated Proteins by Two-Dimensional Gel Electrophoresis (2-DE)**

The enriched S-nitrosylated proteomes of 10 μg were mixed with the rehydration solution which contained 8 μl urea, 2% CHAPS, 0.5% IPG Buffer, 0.02% bromophenol blue and 50 mM DTT. Samples were applied to a 13 cm non-linear gradient IPG-strips, pH 3—10. First-dimensional electrophoresis was performed on an IPGphor isoelectronic focusing system (Amersham Bioscience). Rehydration, electrofocusing and equilibration conditions were as detailed before.\(^16\) In the second dimension, 1.0 mm thick polyacrylamide gels of 14×15 cm were used in the Hoefer™ SE 600 system. The running was started at a constant current of 10 mA/gel for 30 min, and then continued for 5 h at 20 mA/gel. Gels were visualized using a ProteoSilver™ Plus Staining Kit and the protein spots of interest were excised for matrix-assisted laser desorption ionization/time of flight tandem mass spectrometry (MALDI-TOF-MS/MS) identification.

**Protein Identification by MALDI-TOF-MS-MS and Database Searching**

In brief, silvering stained gel spots were destained, digested with trypsin of proteomics grade (Sigma) over night at 37 °C. The resulting peptides in gels were extracted twice with 50% acetonitrile and 0.1% trifluoroacetic acid. The extract solutions were pooled and dried under nitrogen gas and then mixed 1 : 1 with 20 mg/ml o-cyano-4-hydroxycinnamic acid in acetoniitrile before spotting onto a MALDI target plate. Mass spectra were collected using an ABI 4700 Proteomics Analyzer (TOF/TOF™) (Applied Biosystems, U.S.A.). To calibrate the instrument, trypsin digested peptides of horse myoglobin were used. Data were searched against the MASCOT engine (Matrix Science, London, U.K.).

**Western Blot Detection of the Candidate Proteins in the Purified S-Nitrosylated Samples**

Cardiac proteins (0.8 mg/ml; 3 ml) were treated with or without 100 μM GSNO, and the resulting S-nitrosylated proteins were purified by the BSM. Before BSM, a small portion of the starting protein samples (5 μg) were retained for Western blot. When performing BSM, some samples were not treated with ascorbate (Vc, 2 mM). Proteins were separated on 15% SDS-PAGE gels and transferred to nitrocellulose membranes, which were blocked for 1 h using 5% milk in PBS. Anti-Hsp25/27 (1 : 10000) or anti-GAPDH (1 : 5000) antibody was used to probe with the blocked membranes. After overnight incubation, the peroxidase-conjugated secondary antibody (1 : 10000) was used to visualize the immuno-reactive protein band.

**Assays of Adenylate Kinase (AK) Activity**

The ventricular tissue of rat heart was minced and homogenized at 4 °C in 100 mM Tris–HCl (pH 7.5) containing 1 mM EDTA and centrifuged at 10000 g for 10 min. The supernatant was collected and protein concentration determined. GSNO or GSSG was added to final concentrations of 100—900 μM, and the incubation was carried out at room temperature for 1 h. Samples were desalted by Bio-Spin columns to remove excessive GSNO or GSSG. To half of the samples, DTT of 1 mM was added for 15 min. AK activity (of ATP formation) was determined in a reaction mixture containing 100 mM potassium-acetate, 20 mM HEPES (pH 7.5), 20 mM glucose, 4 mM MgCl₂, 2 mM NADP, 1 mM EDTA, 2 mM ADP, 4.5 U/ml hokinoxase and 2 U/ml glucose-6-phosphate dehydrogenase. The reduction of NADP at 340 nm was recorded by a SmartSpec™ Plus spectrophotometer from Bio-Rad.

**RESULTS**

**Multiple Cardiac Proteins Were S-Nitrosylated after GSNO Treatment**

As shown in Fig. 1A, cardiac proteins not treated with GSNO presented few protein spots on 2-DE gels in the BSM assay. In theory, these very minor spots might represent the endogenously biotinylated proteins or endogenously S-nitrosylated protein, but we tend to favor the former speculation, as endogenously S-nitrosylated protein level was very low,\(^5\) possibly below the detection limit of the BSM.\(^3\) Figure 1B showed that in the GSNO treated samples, a significant amount of protein spots were observed in the BSM assay, demonstrating that a number of cardiac proteins underwent S-nitrosylation after GSNO treatment.

**Nine S-Nitrosylated Proteins Were Identified by MALDI-TOF-MS-MS**

The predominant protein spot presented in Fig. 1B were cut and subjected to MALDI-TOF-MS-MS identification. Ten protein spots indicated by the arrows were successfully identified. Note that spots 4 and 5 were found to belong to a same protein, indicating that post-translation modifications which caused a change in the PI might occur. Five of the identified proteins were metabolic enzymes, three were structural protein, and the remaining one was serum protein albumin. Though there were several other protein spots with much higher intensity than spot 6, which is of the slightest intensity we successfully identified, we can not obtain unambiguous identification for them either due to poor MS/MS signal or due to very low protein scores. Details of each identified proteins are listed in Table 1.

**Confirmations by Western Blot That GAPDH Was S-Nitrosylated after GSNO Treatment**

To confirm the iden-
Identification by MS/MS, we used Western blot to test whether one identified protein, GAPDH, is indeed presented among the purified $S$-nitrosylated proteins. As shown in Fig. 2, in the protein samples not subjected to BSM, GAPDH were readily detectable. However, after BSM, in samples not treated with GSNO, GAPDH was not detected on the Western blot, while in the GSNO treated samples, GAPDH was readily detectable, suggesting that GAPDH was indeed $S$-nitrosylated after GSNO treatment. When ascorbate (Vc) was omitted during BSM, the immuno-blot signal in GSNO-treated samples was almost completely abolished, further indicating that GAPDH underwent $S$-nitrosylation after GSNO treatment.

Identification by Western Blot That Hsp27 Was a Target for $S$-Nitrosylation

$S$-Nitrosylation of Hsp family has been recently reported, and we previously found that Hsp27 is up-regulated in rats after myocardial infarction.\textsuperscript{16} Here we tested by Western blot whether this chaperon is among the $S$-nitrosylated cardiac proteins. As shown in Fig. 2, Hsp25/27 was detectable in the protein samples before BSM. After BSM, anti-Hsp25/27 antibody gave positive signal in the GSNO treated samples but not untreated ones, and omission of Vc during BSM abolished the immuno-blot signal in GSNO treated samples, indicating that GSNO triggered $S$-nitrosylation of this chaperon in the cardiac proteins.

GSNO, But Not GSSG, Inhibited AK1 Activity in a Concentration-Dependent and DTT Reversible Manner

AK1 is a novel targets for $S$-nitrosylation we identified. Therefore we further tested whether $S$-nitrosylation could trigger functional changes in this enzyme. As shown in Fig. 3, GSNO (100—900 $\mu M$) caused a concentration-dependent inhibition on AK activity in the cardiac extracts. As the

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein identity</th>
<th>NCBI accession no.</th>
<th>Experimental (theoretical values) of pI/kD</th>
<th>Matched peptides</th>
<th>Protein score</th>
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<td>Enolase 1, alpha</td>
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<td>Creatine kinase</td>
<td>gi</td>
<td>125313</td>
<td>7.6/42 (8.8/47)</td>
<td>9</td>
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<tr>
<td>3</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>gi</td>
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<tr>
<td>4</td>
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<td>gi</td>
<td>538426</td>
<td>7.1/27 (6.5/27)</td>
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<td>7.8/19 (8.2/19)</td>
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$S$-nitrosylated proteins were separated on 2-DE gels, and the protein spots indicated by arrows in Fig. 1B were identified by MALDI-TOF-MS/MS.
GSNO we used might contain a small amount of GSSG,\(^\text{17}\) we tested whether GSSG contributes to the inhibitory effects on AK function. We found that GSSG at concentrations of 100—900 \(\mu\)M had no detectable effects on AK activity (data not shown), suggesting that the contaminating GSSG did not contribute to the inhibitory effects of GSNO. When DTT, a reagent that effectively decomposes \(\cdot\)nitrosothiol, was added to the GSNO treated samples, AK activity was completely recovered to the original level, further supporting the hypothesis that \(\cdot\)nitrosylation plays a role in controlling AK activity.

**DISCUSSION**

NO plays essential roles in regulating heart function, and disruption of NO homeostasis has been implicated in many cardiac diseases.\(^\text{18—21}\) The molecular mechanism(s) of NO under different (patho) physiological conditions, however, has not been completely understood. At the post-translational modification level, the NO related species can cause either tyrosine nitration or cysteine \(\cdot\)nitrosylation of cardiac proteins. Protein nitration in the heart has been extensively studied,\(^\text{22}\) while investigations on cardiac \(\cdot\)nitrosylation are still at the beginning stage. To reveal the biological importance of \(\cdot\)nitrosylation in the heart, the identification of the potential targets for \(\cdot\)nitrosylation is obviously of critical importance.

This study attempted to reveal the overall protein targets of \(\cdot\)nitrosylation in the heart by a proteomic approach. We successfully identified a total of ten proteins susceptible to GSNO induced \(\cdot\)nitrosylation. A majority of these proteins are metabolic enzymes involved in energy supply. As energy supply is of particular importance to the beating heart, \(\cdot\)nitrosylated of these enzymes might play critical roles in regulating cardiac functions. Among the proteins we identified, some are well established targets of \(\cdot\)nitrosylation and their functional alterations after \(\cdot\)nitrosylation are fairly clear, such as GAPDH, albumin, actin, creatine kinase (CK) and Hsp27. \(\cdot\)Nitrosylation of GAPDH, albumin, actin, creatine kinase (CK) and Hsp27. \(\cdot\)Nitrosylation of GAPDH has been extensively observed in many cell type and tissues, and \(\cdot\)nitrosylation not only alters its catalytic function,\(^\text{23}\) but also plays a central role in mediating apoptosis.\(^\text{24}\) \(\cdot\)Nitrosylation of albumin is believed to serve as a NO pool\(^\text{25}\) and is involved in vascular control.\(^\text{26}\) \(\cdot\)Nitrosylation of CK was found to inhibit the catalytic activity.\(^\text{27}\) \(\cdot\)Nitrosylation of actin decreases its capacity of polymerization and might be a means to conserve NO bioavailability.\(^\text{28}\) \(\cdot\)Nitrosylation of Hsp90 inhibits its ATPase activity and abolishes its positive control on endothelial nitric oxide synthase (eNOs) activity.\(^\text{29}\) Other proteins we identified are relatively new targets of \(\cdot\)nitrosylation, including dextrin, myosin, triosephosphate isomerase, and enolase 1, all of which are recently revealed by proteomic method in other tissues or cell types, and the functional consequences await further investigation.

The remaining protein we identified, adenylate kinase 1 (AK1), is a novel targets for \(\cdot\)nitrosylation. Adenylate kinas (AKs, EC: 2.7.4.3) are a group of ubiquitous and strongly conserved enzymes, which catalyzes the reaction of ATP+AMP\(\leftrightarrow\)2 ADP. This reaction is of critical importance in adenine nucleotide metabolism and high energy phosphoryl transfer. There are at least five isoforms of AKs in mammalian tissues, and AK1 is the major one. It harbors two cysteines and no disulfide. Modification of the cysteines by chemical reagent caused marked inhibition on AK1 catalytic activity,\(^\text{30}\) and site-directed mutagenesis study showed that mutation of the C-terminal cysteine (Cys187) caused decreased catalytic efficiency.\(^\text{31}\) It is reasonable to speculate that modification of the cysteine(s) by \(\cdot\)nitrosylation might serve as a novel mechanism in controlling AK activity.

It is also worth to note that all the proteins we identified contain cysteine(s). This is in contrast with some previous reports, in which the authors encountered non-specific signal in the BSM, \texti.e, proteins that do not contain cysteine(s) are positively identified.\(^\text{32}\) In our hand, we did not meet similar problems. As very recently pointed out by Stamler J. S. \textit{et al.}, if proper care is taken, the BSM is highly specific.\(^\text{13}\) Our results appear supportive of this notion.

Very recently, using a modified BSM, another group has also identified a number of \(\cdot\)nitrosylated heart proteins, among which several were identified in our study as well, while the others were not. On the other hand, several proteins we identified here were not reported in the previous investigations.\(^\text{9}\) These differences might reflect the different susceptibility of proteins to \(\cdot\)nitrosylation under \textit{ex vivo} conditions used in previous report\(^\text{8}\) and the \textit{in vitro} conditions used here. Also, only sleeted protein spots were subject to proteomic identification in both studies, and this could be the major reasons for the different sets of proteins identified in the two groups. It should also be pointed out that though the BSM has made proteomic characterization of \(\cdot\)nitrosylation feasible; its sensitivity is rather low. And it is likely that this method preferably detects only protein \(\cdot\)nitrosothiols that are highly sensitive to ascorbate reduction.\(^\text{12}\) A complete picture of \(\cdot\)nitrosoproteome in the heart can not be expected at this stage.

The \(\cdot\)nitrosylated proteins identified here might play important roles in cardiac diseases. It has been demonstrated that increased \(\cdot\)nitrosylation, triggered by IPC or low molecular weight \(\cdot\)nitrosothiols, exerts a critical role in cardiac protection against I/R injury.\(^\text{7,8}\) Though available data point
to the protective role of enhanced S-nitrosylation in the heart, detrimental effects of S-nitrosylation could not be dismissed. Besides I/R injury, there are many other cardio dysfunctions that are associated with nitric oxide over-production, such as cardiac damage during sepsis, after cardiac transplantation,\(^\text{13}\) or in the process of chemotherapy,\(^\text{34}\) etc. Further studies in elucidating the role of S-nitrosylation in these processes would be very insightful, and data presented here might provide important clues in choosing the candidate protein targets for future investigations.

To conclude, this study identified ten cardiac proteins susceptible to S-nitrosylation and demonstrated that S-nitrosylation is a novel mechanism for regulating AKI activity.

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**REFERENCES**


