

Site-specific S-nitrosylation in Mouse Muscle Profiled by Cys-Enrichment Coupled with LC-MS/MS

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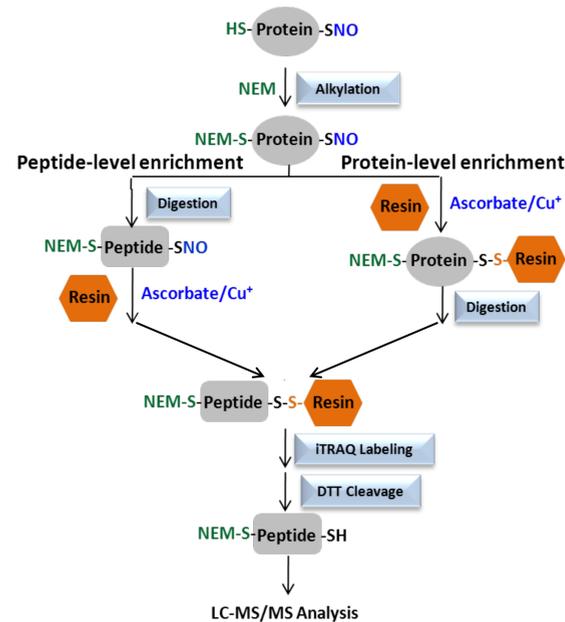
Overview

- A mass spectrometry (MS)-based proteomics approach coupled with resin-assisted cysteine peptide enrichment for site-specific identification and quantification of S-nitrosylation (SNO) was improved and optimized.
- Protein- and peptide-level enrichment protocols provided comparable specificity and coverage of S-nitrosylated peptide identifications.
- 275 SNO-sensitive sites from 142 proteins were identified from mouse muscle by comparing the SNO response to the two different doses of nitric oxide (NO) donor.
- The results reveal that subcellular components (i.e., mitochondria, contractile fiber, and actin cytoskeleton) and pathways (i.e., metabolic pathways) in mouse muscle are particularly susceptible to S-nitrosylation.

Introduction

- S-nitrosylation (SNO) along with S-glutathionylation (SSG), and S-sulfenic acid (SOH) have been increasingly emphasized for their roles in mediating cellular signaling pathways.
- Site-specific SNO detection is challenging mainly due to the labile nature and the low-abundance of S-nitrosylation.¹
- Many existing site-specific SNO detection methods adopted the biotin-switch technique (BST) introduced by Jaffrey *et al.*²; however, a number of issues related to detection specificity and sensitivity remain unresolved.
- A resin-assisted capture strategy for site-specific SNO identifications was recently reported.³ In this work, we adapted this approach with further improvement and optimization to assess protein-level vs. peptide-level enrichment.
- The optimized approach was applied for quantitative site reactivity profiling for S-nitrosylation in mouse muscle, an important tissue that has significant roles in many diseases.

Methods



Sample preparation. Homogenous mouse muscle samples were treated with GSNO, alkylated with N-ethylmaleimide (NEM), reduced with ascorbate coupled with Cu⁺, captured with thiol-activated resin.⁴

Quantitative profiling. Enriched Cys-peptides were labeled with 4-plex iTRAQ mass tags on-bead.

Relative reactivity profiling. The relative reactivity of specific thiol-sites was evaluated based on the relative SNO levels by treating samples with two different doses (10 μM and 100 μM) of NO donor S-nitrosoglutathione (GSNO).

LC-MS(/MS) analysis. Analyses were performed using a 75-μm i.d. reversed phase capillary column and 100-min separation, and an LTQ-Orbitrap Velos. The six most abundant parent ions were selected for MS/MS using high-energy collisional dissociation (HCD). The extent of SNO modification was quantified based on iTRAQ reporter ion intensities from MS/MS spectra.

Data analysis. MS/MS data were analyzed by SEQUEST algorithm was used to search all MS/MS spectra against the Uniprot mouse genome sequence database. The decoy-database searching methodology was used to control the FDR at the unique peptide level to <1% with MS Generating-Function (MSGF) score as the main criteria.

Results

Protein- vs. peptide-level enrichments

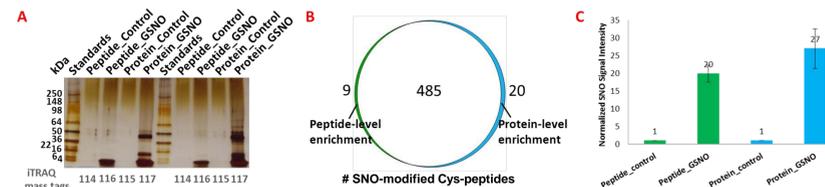


Figure 1. Comparison of peptide- and protein-level enrichment results. A) Silver staining image of SDS-PAGE of enriched Cys-peptides. B) Venn diagrams of SNO-modified Cys-peptides identified from the peptide- and protein-level enrichment protocols within the same multiplexed iTRAQ experiment. C) SNO signal fold-increases from the peptide- and protein-level enriched samples.

Relative reactivity

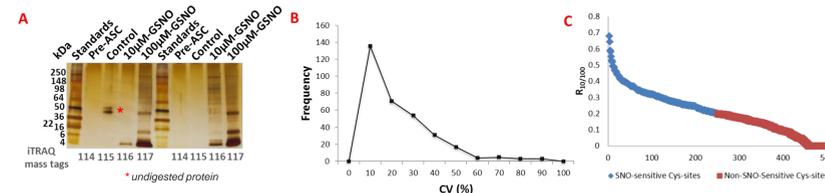


Figure 2. Assessment of the relative reactivity of SNO-modified Cys-peptides. A) Silver staining image of SDS-PAGE assay of enriched Cys-peptides from two independent experiments. B) CV distribution of measured ratios of R_{10/100} values between two independent experiments for commonly identified SNO-modified Cys-peptides. C) Relative reactivity of SNO-modified Cys-peptides. The y-axis values are the average of R_{10/100} values of the two independent experiments. SNO-sensitive Cys-peptides are depicted in blue with average R_{10/100} values >0.2.

Table 1. SNO-sensitive peptides from selected proteins.

Peptide	Reference	C-site	Avg R _{10/100}	R _{10/100} a	R _{10/100} b	Stdev R _{10/100}
K.GLVLIAFSQYIQKCSYDEHAK.L	ALBU_MOUSE	58	---	---	0.68	---
R.YNDLGEQHFHGLVLIQVYQKSYDEHAK.L	ALBU_MOUSE	58	---	---	0.64	---
R.SLPSVETLGTGTSVICSDK.T	AT2A1_MOUSE	349	0.45	0.36	0.53	0.12
R.SLPSVETLGTGTSVICSDK.T	AT2A1_MOUSE	344	0.43	0.34	0.53	0.13
R.ACCFCAR.V	AT2A1_MOUSE	675	0.42	0.39	0.44	0.03
R.ACCFCAR.V	AT2A1_MOUSE	674	0.40	0.36	0.44	0.06
K.EVTGSIQLCR.D	AT2A1_MOUSE	614	0.40	0.29	0.51	0.16
R.ANACNSVIR.Q	AT2A1_MOUSE	471	0.39	0.31	0.47	0.11
K.GAPFEGVIDRCNVR.V	AT2A1_MOUSE	525	0.37	0.37	0.36	0.01
R.KSM5VYCSPAK.S	AT2A1_MOUSE	498	0.34	0.30	0.37	0.05
R.ACCFCAR.V	AT2A1_MOUSE	674,675	0.33	0.27	0.38	0.08
R.ACCFCAR.V	AT2A1_MOUSE	674,675	0.33	0.26	0.39	0.09
R.SLPSVETLGTGTSVICSDK.T	AT2A1_MOUSE	344,349	0.32	0.25	0.39	0.10
R.SLPSVETLGTGTSVICSDKTGLTTLNQMSVCK.M	AT2A1_MOUSE	344,349	0.32	0.32	0.32	0.00
K.SMSVYCSPAK.S	AT2A1_MOUSE	498	0.29	0.23	0.36	0.09
R.EFDLPLAEGREAR.R	AT2A1_MOUSE	670	0.29	0.36	0.22	0.09
K.STEELSYFVGSSETGTLTPDQVKR.R	AT2A1_MOUSE	12	0.28	0.20	0.26	0.12
R.AGGYDGLVELATICALNDSSLDLDFNETK.G	AT2A1_MOUSE	420	0.27	0.33	0.21	0.09
R.AGGYDGLVELATICALNDSSLDLDFNETK.G	AT2A1_MOUSE	417	0.24	0.11	0.38	0.20
K.STEELSYFVGSSETGTLTPDQVKR.H	AT2A1_MOUSE	12	0.23	0.14	0.31	0.12
K.MFIIDKVDGDCSLNFEISGTSTYAPEGEVLK.N	AT2A1_MOUSE	377	0.22	0.16	0.28	0.09
K.TGTLTTLNQMSVCK.M	AT2A1_MOUSE	364	0.20	0.15	0.25	0.08
R.AAICSGK.V	G3P_MOUSE	22	0.59	0.49	0.69	0.14
R.VPTPNVSDVLTCLRLEPAK.Y	G3P_MOUSE	245	0.46	0.50	0.42	0.06
K.IVSNASCHFTTNCLAPLAK.V	G3P_MOUSE	154	0.44	0.46	0.42	0.03
K.IVSNASCTTNCLAPLAK.V	G3P_MOUSE	150,154	0.29	0.31	0.28	0.02

ALBU (Serum albumin), AT2A1 (Sarcoplasmic/endoplasmic reticulum calcium ATPase 1), and G3P (GAPDH) proteins. Average R_{10/100} and STDEV values were calculated from two independent experiments. # designates NEM modification indicating not an SNO-modified Cys-site.

Biological functions and pathways

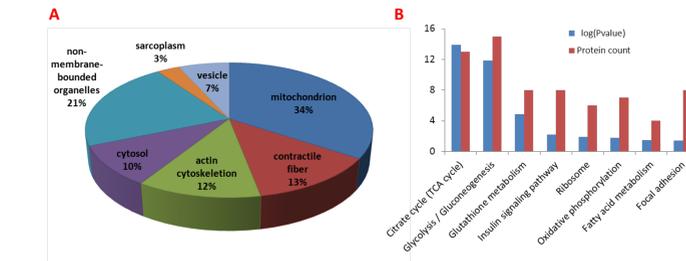


Figure 3. Enriched subcellular components and biological pathways of identified SNO-sensitive proteins.

Table 2. Selected SNO-sensitive proteins in different functional categories.

Reference	Description	Cys-site
Metabolic enzyme		
DLDH_MOUSE	O08749 Dihydropyridyl dehydrogenase, mitochondrial	69
IDH3A_MOUSE	Q9D6R2 Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	331
ACSL1_MOUSE	P41216 Long-chain-fatty-acid-CoA ligase 1	109
ACADS_MOUSE	Q07417 Short-chain specific acyl-CoA dehydrogenase, mitochondrial	246
ACADL_MOUSE	P51174 Long-chain specific acyl-CoA dehydrogenase, mitochondrial	351
Oxidative phosphorylation		
NDU51_MOUSE	Q91VD9 NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	75
AT12A_MOUSE	Q9Z1W8 Potassium-transporting ATPase alpha chain 2	695
ATP6_MOUSE	Q91VR2 ATP synthase subunit gamma, mitochondrial	103
NDUV1_MOUSE	Q91Y10 NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial	206
ACPM_MOUSE	Q9CR21 Acyl carrier protein, mitochondrial	140
Kinase and phosphatase		
SPEG_MOUSE	Q62407 Striated muscle-specific serine/threonine-protein kinase	1697
ADK_MOUSE	P55264 Adenosine kinase	352
KPBB_MOUSE	Q775H2 Phosphorylase b kinase regulatory subunit beta	1058
PHKG1_MOUSE	P07934 Phosphorylase b kinase gamma catalytic chain, skeletal muscle isoform	277
2AAA_MOUSE	Q76M23 Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	294,390
Stress signaling protein		
HSP74_MOUSE	Q61316 Heat shock 70 kDa protein 4	417
HSP90_MOUSE	P11499 Heat shock protein HSP 90-beta	521
HSP71_MOUSE	Q61696 Heat shock 70 kDa protein 1A	306
HSP7C_MOUSE	Q3U9G0 Heat shock cognate 71 kDa protein	17,603

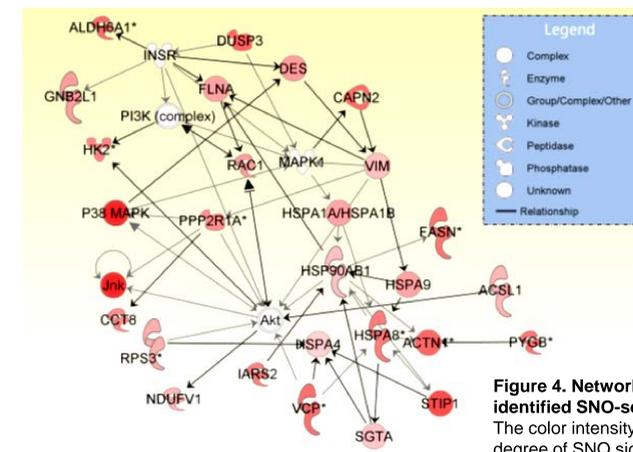


Figure 4. Network depiction of identified SNO-sensitive proteins. The color intensity indicates the degree of SNO signal increase.

Conclusions

- The optimized resin-assisted cysteine-peptide enrichment approach for site-specific S-nitrosylation profiling provides comparable sensitivity and specificity for both peptide- and protein-level enrichment.
- Quantitative reactivity profiling of S-nitrosylation in mouse muscle samples led to identification of a total 467 SNO sites from 197 proteins, and among them 275 Cys-sites from 142 proteins were revealed to be more sensitive to SNO modifications.
- SNO-sensitive proteins appeared preferentially localized in mitochondria, contractile fiber, and actin cytoskeleton, which suggested the susceptibility of these subcellular compartments to redox regulation.
- Many of the SNO-sensitive proteins are metabolic enzymes associated with TCA cycle, glycolysis/gluconeogenesis, glutathione metabolism, and fatty acid metabolism, highlighting the importance of redox regulation in metabolism.
- A number of signaling proteins were also identified as SNO-sensitive proteins, suggesting the role of S-nitrosylation in signal transduction.
- Discovery of the SNO-sensitive sites in mouse muscle would provide valuable information for understanding the nature of S-nitrosylation in cellular signaling pathways and in pathophysiology, such as insulin resistance.

Acknowledgements

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