Quantitative Proteomics Analysis of Protein S-Nitrosylation using Sixplex iodoTMT

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INTRODUCTION

S-Nitrosylation is a redox-based covalent post-translational modification of specific cysteine residues by nitric oxide (NO) and is involved in a wide-range of cellular processes. To identify S-nitrosylated proteins, Jaffrey and Synder (2001) developed a biotin switch method which exchanges S-nitrosothiols with a reversible biotinylated, thiol-reactive reagent for detection. This method was further extended for proteomic profiling of S-nitrosylated proteins using various mass spectrometry (MS) compatible techniques. Recently, a sixplex cysTMT reagent was used for multiplexed quantification of protein S-nitrosylation (Murray et al., 2011). However, due to the reversible nature of cysTMT labeling, extensive reduction/alkylation of proteins was not achievable, thus hindering the efficiency of MS detection. Here, we introduce a novel cysteine-reactive, iodoacetyl TMT (iodoTMT™) sixplex reagent, which enables irreversible labeling of S-nitrosylated proteins for enrichment and quantification.

Activation of microglia is known to respond to endotoxin lipopolysaccharides (LPS) to induce protein S-nitrosylation and associated with inflammatory responses to brain injury as a converging signal event in neurodegenerative disorders. A comprehensive investigation of proteins affected by S-nitrosylation in activated microglial cells could provide an insight into the molecular mechanisms of NO signaling in disease. Here, we investigated protein S-nitrosylation under both *in vitro* and *in vivo* conditions in the immortalized murine microglial BV-2 cells.

METHODS

- **S-nitrosylation induction**
  In vitro protein S-nitrosylation was induced by exposing microglial BV-2 cell lysates to various concentrations of the physiological NO donor, S-nitroso-cysteine (SNOC), for 30 minutes at room temperature. For *in vivo* studies, BV-2 cells were stimulated by 100 ng/ml LPS for 20 hours before lysis.
- **iodoTMT switch labeling**
  Protein samples were blocked using *S*-methylmethanethiosulfonate (MMTS) and then labeled with 1 mM iodoTMT reagents (iodoTMTzero or iodoTMTsixplex; Figure 1) in the presence of 5 mM ascorbate.
- **Digestion and enrichment of iodoTMT labeled peptides**
  iodoTMT-labeled samples were reduced with 10 mM DTT at 55˚C for 1 hour, alkylated with 25 mM iodoacetamide at 37˚C for 1 hour and digested with trypsin at 37˚C for 4 hours. For enrichment, labeled peptides (25-100 µg) were incubated with immobilized anti-TMT antibody resin (20-100 µL) overnight at 4˚C. Peptides were eluted with 50% acetonitrile, 0.4% TFA.
- **MS/MS analysis**
  Enriched peptides were analyzed using a Thermo Scientific LTQ™ Orbitrap-$_{XL}$ or Elite mass spectrometers. Data analysis was performed with Thermo Scientific Proteome Discoverer™ 1.3 software.

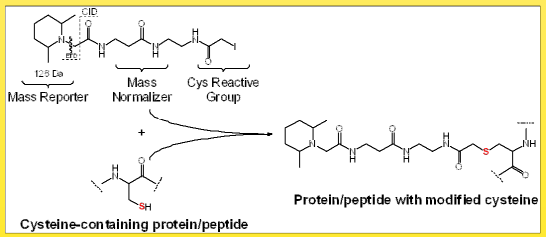

Figure 1. iodoTMT reagent and labeling reaction.

RESULTS

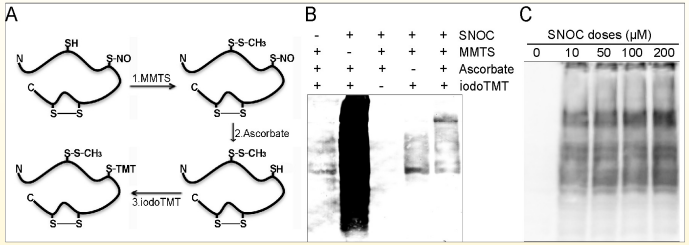

Figure 2. iodoTMT specifically labels S-nitrosylated proteins.
(**A**) Schematic of labeling *S*-nitrosylated protein using iodoTMT reagent.
(**B**) BV-2 cell lysates were exposed to 200 µM SNOC and labeled with iodoTMTzero. Western blotting (anti-TMT) with labeled protein samples showed the specificity of iodoTMT labeling.
(**C**) The sensitivity of iodoTMT labeling. A series of SNOC dosages were applied to BV-2 cell lysates, and anti-TMT western blot showed iodoTMT could detect *S*-nitrosylated proteins in response to as low as 10 µM SNOC treatment.

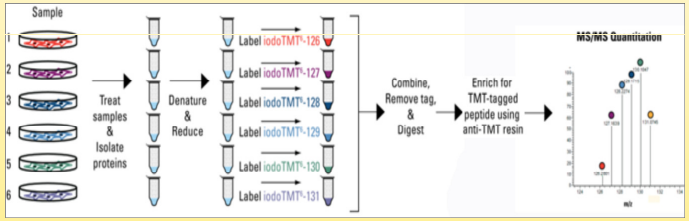

Figure 3. Workflow for quantitative proteomics analysis of protein S-nitrosylation using iodoTMT sixplex reagents.
Six different sample conditions can be prepared for iodoTMT reagent labeling. Labeled proteins are combined before iodoTMT peptide enrichment using immobilized anti-TMT antibody resin and subsequent LC-MS/MS analysis of isobaric reporter ions.

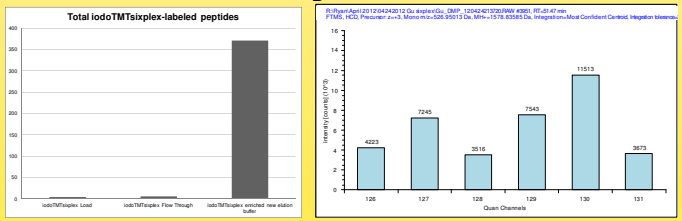

Figure 4. Analysis of protein S-nitrosylation induced by SNOC *in vitro*.
SNOC-treated and -untreated BV-2 cell lysates groups in triplicate were labeled by iodoTMTsixplex reagents: untreated-126, SNOC-treated-127, untreated-128, SNOC-treated-129, SNOC-treated-130, untreated-131.
(**A**) After trypsin digestion and enrichment, 371 iodoTMT-labeled peptides were identified by MS/MS analysis, which is 21.4% of total detected peptides.
(**B**) Totally 558 unique proteins were found, out of which 79 iodoTMT-labled proteins were able to be quantified. An example of quantified proteins was given here. Peptide sequence: YVDIAIPCNNK; Modification C8-iodoTMT6 (329.22660 Da); Identified with: SEQUEST (v1.20); Protein references: - 40S ribosomal protein SA.

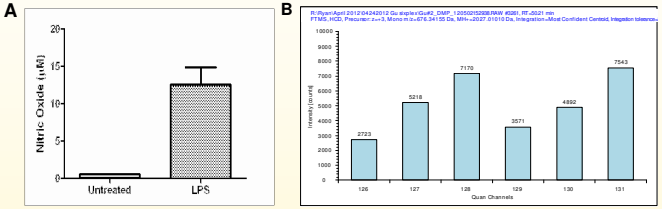

Figure 5. Analysis of protein S-nitrosylation in LPS-stimulated BV-2 cells.
(**A**) BV-2 cells were treated or untreated with LPS for 20 hours. NO production was measured by Griess assay. SNOC-treated BV-2 cell lysates sample was used as positive control. Duplicate protein sample set was labeled as following: untreated-126, LPS-treated-127, SNOC-treated-128, untreated-129, LPS-treated-130, SNOC-treated-131.
(**B**) 65 iodoTMT-labeled proteins were detected and quantified. Here shows an example of quantified proteins. Identified peptide sequence: GCITIIGGGDTATCCAK; Modification: C2-Carbamidomethyl (57.02146 Da), C14-iodoTMT6 (329.22660 Da), C15-Carbamidomethyl (57.02146 Da); Identified with: SEQUEST (v1.20); Protein references: Phosphoglycerate kinase 1.

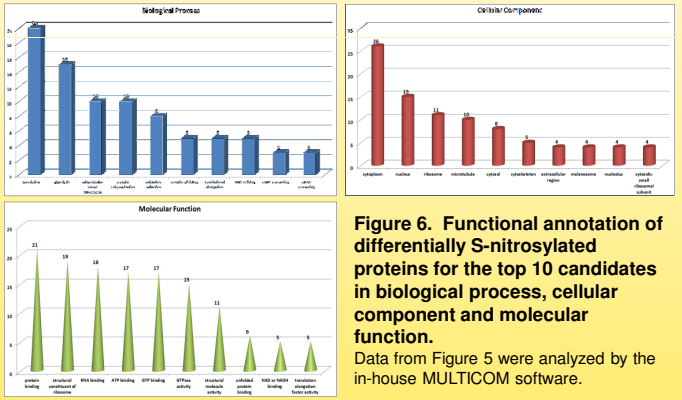

Figure 6. Functional annotation of differentially S-nitrosylated proteins for the top 10 candidates in biological process, cellular component and molecular function.
Data from Figure 5 were analyzed by the in-house MULTICOM software.

CONCLUSIONS

- **iodoTMT reagent could specifically label S-nitrosylated proteins.**
- **Anti-TMT resin successfully enriches iodoTMT-labeled peptides.**
- **Both S-nitrosylated proteins and modified cysteine sites could be indentified by iodoTMT switch labeling.**
- **iodoTMT switch assay gains high output. 79 and 65 unique S-nitrosylated proteins were detected and quantified from *in vitro* and *in vivo* studies, respectively.**
- **iodoTMT switch assay is able to profile global S-nitrosylated proteins and compare protein S-nitrosylation levels under different conditions as well. Therefore, this is an effective proteomic approach for quantitative analysis of protein S-nitrosylation.**

FUNDING

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