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Original Contribution

Proteomic identification and quantification of S-glutathionylation in mouse macrophages using resin-assisted enrichment and isobaric labeling

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ABSTRACT

S-Glutathionylation (SSG) is an important regulatory posttranslational modification on protein cysteine (Cys) thiols, yet the role of specific cysteine residues as targets of modification is poorly understood. We report a novel quantitative mass spectrometry (MS)-based proteomic method for site-specific identification and quantification of S-glutathionylation across different conditions. Briefly, this approach consists of initial blocking of free thiols by alkylation, selective reduction of glutathionylated thiols, and covalent capture of reduced thiols using thiol affinity resins, followed by on-resin tryptic digestion and isobaric labeling with iTRAQ (isobaric tags for relative and absolute quantitation) for MS-based identification and quantification. The overall approach was initially validated by application to RAW 264.7 mouse macrophages treated with different doses of diamide to induce glutathionylation. A total of 1071 Cys sites from 690 proteins were identified in response to diamide treatment, with ~90% of the sites displaying >2-fold increases in SSG modification compared to controls. This approach was extended to identify potential SSG-modified Cys sites in response to H_2O_2, an endogenous oxidant produced by activated macrophages and many pathophysiological stimuli. The results revealed 364 Cys sites from 265 proteins that were sensitive to S-glutathionylation in response to H_2O_2 treatment, thus providing a database of proteins and Cys sites susceptible to this modification under oxidative stress. Functional analysis revealed that the most significantly enriched molecular function categories for proteins sensitive to SSG modifications were free radical scavenging and cell death/survival. Overall the results demonstrate that our approach is effective for site-specific identification and quantification of SSG-modified proteins. The analytical strategy also provides a unique approach to determining the major pathways and cellular processes most susceptible to S-glutathionylation under stress conditions.

Introduction

The importance of reactive oxygen species (ROS) and reactive nitrogen species (RNS) as second messengers in signal transduction has recently gained recognition [1,2]. Reversible posttranslational modifications of protein cysteine thiols represent a major form of cellular regulation mediated by ROS and RNS in redox signaling [1,3–5]. The formation of mixed disulfides between protein cysteine thiols and cellular glutathione (GSH), known as protein S-glutathionylation (SSG), is one of the most prevalent forms of reversible posttranslational modifications of protein thiols. Emerging evidence supports the significance of protein-SSG in regulating a variety of cellular processes from bacteria to mammals, including human pathologies under oxidative and nitrosative stress [6–9].

Protein-SSG can be induced by ROS or RNS under physiological or pathological conditions. Although not exactly resolved, a number of potential mechanisms that either occur spontaneously or are catalyzed by enzymes such as glutaredoxins (Grx) have been recognized for the formation of protein-SSG [1,8,10], including: (1) protein thiols react with glutathione disulfide (GSSG) via thiol-disulfide exchange

Abbreviations: SSG, S-glutathionylation or S-glutathionylated; ROS, reactive oxygen species; RNS, reactive nitrogen species; GSH, glutathione; GSSG, glutathione disulfide; Grx, glutaredoxin; GR, glutathione reductase; iTRAQ, isobaric tags for relative and absolute quantitation; NEM, *N*-ethylmaleimide; BST, biotin switch technique

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reaction; (2) protein thiol or GSH reacts with the corresponding oxidized thiol derivatives (e.g., *S*-nitroso, sulfenic acid, and thiyl radical). Conversely, the protein SSG modification can be reversed by means of reactions catalyzed by the thiol-disulfide oxidoreductases glutaredoxins (Grx), and potentially other enzymes [10].

A growing number of proteins have been identified as regulated by SSG covering a wide spectrum of cellular signaling pathways [6,11]. Examples of reported SSG-modified proteins include enzymes with active-site thiols such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [12] and caspase-3 [13], signaling proteins such as protein kinase A [14] and protein kinase C [15], transcription factors c-Jun and NF-κB [16–18], ion channels and calcium-dependent proteins such as sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) [19,20], and apoptotic death receptor protein Fas (CD95) [21]. Nevertheless, our knowledge of the relevance of S-glutathionylation in physiological and pathological processes is still limited due to the lack of effective approaches for the identification and quantification of protein-SSGs and their specific modification sites. One early established method utilizes *in vivo* metabolic labeling of GSH with [35S] cysteine coupled with SDS-PAGE separation and autoradiography for the detection of modified thiols [22–24]. Another conventional method is based on Western blot coupled with anti-GSH antibodies [25] or biotinylated glutathione *S*-transferase and anti-biotin antibodies [26]. However, these methods have limited specificity and sensitivity and are unable to distinguish individual S-glutathionylated (SSG) sites within a target protein which may have different functional consequences.

Mass spectrometry (MS)-based proteomics coupled with affinity or chemical enrichment strategies can overcome these limitations and enable large-scale identification of specific sites subject to modifications. Recent approaches for identification of glutathionylated proteins have been reported which incorporate a biotin tag via an exogenous glutathionylation reagent [27–29] or via a modified biotin-switch technique involving selective reduction and immediate alkylation of protein-SSG sites [30] followed by avidin-biotin-based enrichment. These former methods involve the reaction of cysteine thiols with biotinylated GSSG or similar reagents to form protein-SSGs, which might not reflect the true endogenous level of SSG modifications. The effectiveness of the modified biotin switch technique was also not demonstrated for identification of specific sites of modification. Moreover, there are currently no effective approaches for quantitative measurement of the dynamic changes of S-glutathionylation at a broad proteome scale. It will contribute greatly to an increased understanding of the biological role of S-glutathionylation if a more sensitive detection method for site-specific identification and quantification of SSG modified proteins is available.

Herein we report a quantitative MS-based proteomic method for profiling protein-SSGs and their specific modification sites by adapting a recently developed resin-assisted enrichment method used for S-nitrosylation [31,32] with on-resin isobaric labeling with iTRAQ (isobaric tags for relative and absolute quantitation) reagents. A number of previous studies have reported that the resin-assisted covalent enrichment offers a simpler, more efficient means of capturing cysteine-containing peptides [33] and other PTMs such as S-nitrosylation [32,34]. The resin-assisted enrichment minimizes the degree of nonspecific binding that is often encountered with noncovalent avidin-biotin enrichment, thus providing an overall better specificity and sensitivity [31–33]. This approach was initially validated and applied to RAW 264.7 macrophage cells treated with diamide and H_2O_2 to identify potential cysteine redox switches that are sensitive to S-glutathionylation. Macrophage cells are selected as a model due to the potential significance of redox regulation in oxidative stress response and inflammation [16]. The capacity of macrophages to generate substantial amounts of ROS is an important property of their activation by foreign particulates and pathogens. Although macrophages must deal with high oxidative stress levels, surprisingly little is known about the specific macrophage proteins susceptible to SSG modification and the potential signaling pathways impacted. We identified 364 SSG-modified Cys sites from 265 proteins in macrophages that were sensitive to S-glutathionylation in response to H_2O_2 treatment. These SSG-modified proteins cover a range of enzymes involved in ROS metabolism, stress response signaling, and apoptosis pathways.

Materials and methods

Materials

E. coli glutaredoxin (Grx 3) [C14S/C65Y] was from IMCO Corporation Ltd AB (Stockholm, Sweden). Glutathione reductase (GR) was from Roche Diagnostics Corporation (Indianapolis, IN). NADPH tetrasodium salt (β-NADPH · 4Na, β-nicotinamide adenine dinucleotide phosphate (reduced form) · 4Na), BCA protein assay reagents, silver stain kit, spin columns, cell culture RPMI-1640 media and reagents (penicillin, streptomycin, L-glutamine), and hydrogen peroxide were purchased from Thermo Fisher Scientific (Rockford, IL). Sequencing grade modified porcine trypsin was from Promega (Madison, WI). iTRAQ (Isobaric Tags for Relative and Absolute Quantitation) reagents were from AB SCIEX (Redwood City, CA). The SeeBlue Plus2 protein standard was from Invitrogen (Carlsbad, CA). Thiol-affinity resin (thiopropyl Sepharose 6B) was from GE Healthcare (Uppsala, Sweden). Tris/glycine/SDS (TGS) buffer, Laemmli sample loading buffer, and precast Tris-HCl 4–20% gradient gels were all from Bio-Rad Laboratories (Hercules, CA). Unless otherwise noted, all other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture, diamide and hydrogen peroxide treatments, and protein extraction

Murine RAW 264.7 macrophages (TIB-71) from American Type Culture Collection (ATCC) (Manassas, VA, USA) were cultured and maintained in 100 mm culture plates with RPMI-1640 media containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. Cells were cultured at 37 °C with 5% CO_2. Prior to treatment, cells were seeded into 100 mm culture plates and grown until 80% confluent. Original growth media were removed and replaced with media containing either hydrogen peroxide or diamide at the desired concentration for 30 min. After treatment, cells were rinsed twice with cold RPMI-1640 media containing no supplements and harvested in lysis buffer (50 mM Hepes, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, pH 7.7) containing freshly prepared 50 mM *N*-ethylmaleimide (NEM). Cell lysates were centrifuged at 14,000 rpm at 4 °C, for 10 min and soluble protein fraction was retained. Protein concentration was determined using the BCA assay.

Denitrosylation and alkylation of free thiols

To block free protein thiols, ~1 mg of the above lysates was resuspended to a final protein concentration of 0.5 μg/μL in alkylation buffer consisting of 250 mM Hepes (pH 7.7), 20 mM NEM, and 2% SDS. 1 mM sodium ascorbate (NaASC) and 1 μM CuCl were added to the alkylation buffer to reduce S-nitrosylated cysteines back to free cysteines. Both free cysteines and S-nitrosylated cysteines (reduced to free cysteines) were alkylated. The alkylation reaction was carried out in 4 mL Amicon Ultra 30 K molecular weight cutoff (MWCO) filter units (EMD Millipore,