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Nitrosative Stress-Induced S-Glutathionylation of Protein Disulfide Isomerase

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Abstract

Oxidative and nitrosative stress result in the accumulation of reactive oxygen and nitrogen species (ROS/RNS) which trigger redox-mediated signaling cascades through posttranslational modifications on cysteine residues, including S-nitrosylation (P-SNO) and S-glutathionylation (P-SSG). Protein disulfide isomerase (PDI) is the most abundant chaperone in the endoplasmic reticulum and facilitates protein folding via oxidoreductase activity. Prolonged or acute nitrosative stress blunts the activity of PDI through the formation of PDI-SNO and PDI-SSG. The functional implication is that reduced activity for the period of time leads to an accumulation of misfolded or unfolded proteins and activation of the unfolded protein response. Redox regulation of PDI and downstream signaling events provides an integration point for the functional determination of cell survival pathways. Herein, we describe the methodologies to globally identify S-glutathionylated targets of ROS/RNS; validate and identify the specific cysteine targets and characterize the structural and functional consequences.

1. Introduction

Oxidation and reduction (redox) reactions play an essential role in numerous cell-signaling cascades including those associated with proliferation, apoptosis, and inflammatory responses. Oxidative and nitrosative stress results from the imbalance between the production of oxidants and their removal by antioxidants, leading to the accumulation of reactive oxygen and nitrogen species (ROS/RNS). Elevated levels of nitric oxide (NO) provide the primary source of RNS. NO is an endogenous, diffusible, transcellular messenger shown to participate in survival and death pathways (Moncada, 1997) and can alter protein function directly through posttranslational modifications (nitration or S-nitrosylation) or indirectly through interactions with oxygen, superoxide, thiols, and heavy metals, the products of which cause protein S-glutathionylation. Altered NO homeostasis can lead to the release of RNS and ROS, each of which have been implicated in a number of human pathologies, including neurodegenerative disorders, cystic fibrosis, cancer, and aging (Ilic *et al.*, 1999; Tieu *et al.*, 2003; Townsend and Tew, 2003).

Glutathione (GSH), a tripeptide of cysteine, glutamic acid, and glycine, represents one of the most prevalent and important antioxidant buffers in the cell. The ratio of GSH (reduced) and its disulfide, GSSG (oxidized), contributes to the redox potential of the cell and thereby, is key in defining redox homeostasis. Oxidative or nitrosative stress induced by physiological or pathological conditions leads to a decreased ratio of GSH/GSSG. As a consequence,

reduced cysteine residues (-SH) that have a low pK_a are reactive and can become oxidized into protein sulfenic acids (P-SOH), nitrosylated (P-SNO), or S-glutathionylated (P-SSG). These proteins are referred to as redox sensors and the corresponding posttranslational modifications lead to structural and functional changes that govern signal transduction pathways.

Unlike the cytosol where GSH/GSSG ~100:1, the endoplasmic reticulum (ER) favors an oxidizing environment (~3:1) which facilitates protein folding. This unique ER environment also provides a platform to sense oxidative and nitrosative stress. Stress upon the ER results in the accumulation of misfolded proteins, leading to activation of the unfolded protein response (UPR). The UPR has three primary functions: (1) restore normal function of the cell by halting protein translation, (2) activate the signaling pathways that lead to increased production of molecular chaperones involved in protein folding, and (3) trigger the degradation of terminally misfolded proteins (Townsend, 2007).

The ER contains several key chaperones that catalytically mediate protein folding and prevent aggregation of proteins as they undergo maturation. The most abundant chaperone in the lumen of the ER is protein disulfide isomerase (PDI). It is not surprising that PDI acts as a redox sensor and can be S-nitrosylated (Uehara *et al.*, 2006) and/or S-glutathionylated (Townsend *et al.*, 2006, 2009b) following nitrosative stress (Fig. 18.1). PDI is organized into five domains (a, b, b', a', and c) and the C-terminal KDEL sequence retains it to the ER. The crystal structure of yeast PDI suggests that the four thioredoxin domains (a, b, b', a') form a twisted U shape with the catalytic domains (a and a') facing each other and an internal hydrophobic surface that interacts with misfolded proteins (Tian *et al.*, 2006). The cysteine residues within the a and a' domains (catalytic) are targets for redox regulation (Fig. 18.1). The functional implication is that PDI has a reduced activity for the period of time that the cysteine residue is S-glutathionylated, resulting in accumulated unfolded/misfolded proteins hence triggering activation of the UPR. The coordination of PDI and downstream signaling events provides an integration point for the functional determination of cell survival pathways. Here, we describe the methodology for the detection, confirmation, and validation of the functional consequences of S-glutathionylation of PDI.

2. Identification and Confirmation of S-Glutathionylated Proteins in Cells

S-Glutathionylation is an important posttranslational modification that governs redox-mediated signaling events. Identification and validation of target cysteine residues have been limited by detection methods that are not compatible with reducing agents. A two-pronged approach was utilized to identify and confirm that PDI is S-glutathionylated (Townsend *et al.*, 2006, 2009b). First, using two-dimension isoelectric focusing under nonreducing conditions PDI was isolated and identified by mass spectrometry as a target for S-glutathionylation (Townsend *et al.*, 2006). Second, S-glutathionylation of PDI was confirmed via immunoprecipitation of PDI and Western blot analysis for P-SSG. This general approach is outlined in Fig. 18.2 and has been used *in vitro* and *in vivo* to identify S-glutathionylated proteins following both oxidative and nitrosative stress (Findlay *et al.*, 2006; Townsend *et al.*, 2006, 2008, 2009a,b). *In situ* detection of S-glutathionylated proteins

has been recently reported (Aesif *et al.*) and collectively these tools can define the role of PDI in multiple pathologies associated with misfolded proteins.

2.1. Materials

PABA/NO; GSH; 20 mM PBS; protein A/G Plus agarose; IgG; anti-PDI (Affinity BioReagents, Golden, CO); anti-SSG (Virogen, Watertown, MA); lysis buffer (20 mM Tris-HCl, pH 7.5, 15 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, and 1 mM β -glycerophosphate with freshly added protease and phosphatase inhibitors, 5 mM NaF and 1 mM Na_3VO_4); nonreducing loading sample buffer; 10% SDS-PAGE gel 100 μg whole cell protein; 2-D lysis buffer (8M urea, 2 M thiourea, 4% CHAPS, 20 mM Spermine, 1 mM PMSF); rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer); equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl, pH 8.8).

2.2. Methods

2.2.1. Induction of S-glutathionylation in cells—Prior to identifying specific protein targets of S-glutathionylation, a dose- and time-curve is performed using the anti-glutathionylation (anti-SSG) antibody as the final readout. For the time course, $\sim 5 \times 10^5$ HL60 cells per treatment group are seeded and treated with 25 μM PABA/NO (IC_{50}) for 0–8 h with 30-min to 1-h increments. Wash cells prior to harvest with PBS and resuspend the pellets in lysis buffer and incubate on ice for 30 min. Sonicate lysates for 10 s and centrifuge for 30 min at $10,000\times g$ at 4 °C. Determine the protein concentration with the Bradford reagent using IgG as a standard. Do not freeze cell lysate. Add 50 μg cell lysate in nonreducing sample loading buffer and heat to ~ 95 °C for 10 min. Separate the proteins under nonreducing conditions on 10% SDS-PAGE gels. Transfer proteins onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). Block nonspecific binding with blocking buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 1 μM protease inhibitors, 5 mM NaF, and 1 mM Na_3VO_4) containing 10% nonfat dried milk for 1 h. Incubate the membrane with anti-SSG (1:100) overnight at 4 °C. Wash the membrane 3 $\times$ with PBS for 15 min and incubate with secondary antibody conjugated to horseradish peroxidase for 1 h. Remove the secondary antibody with 3 $\times$ washes using PBS. Develop the blots with enhanced chemiluminescence detection reagents and scan using a transilluminator. Strip the membrane and reprobe with anti-actin. Calculate the relative abundance of P-SSG to actin for each treatment group.

For the dose-curve, treat $\sim 5 \times 10^5$ cells with 0–50 μM PABA/NO for the time at which peak P-SSG levels were observed and perform immunoblot analysis as described above. To identify specific proteins, it is necessary to run two-dimensional gels and excise bands using the optimal dose/time regime determined in this series of experiments.

2.2.2. Identification of S-glutathionylated proteins via 2-D SDS-PAGE and MALDI-TOF mass spectrometry—From the dose- and time-curve, treat the cells with an IC_{50} dose for the time to reach maximal global S-glutathionylation. For SKOV3 or HL60 cells, treat cells with diluent or 25 μM PABA/NO for 1 h. Wash cells prior to harvest with PBS and resuspend the pellets in lysis buffer and incubate on ice for 30 min. Sonicate lysates for 10 s and centrifuge for 30 min at $10,000\times g$ at 4 °C. Determine the protein

concentration with the Bradford reagent using IgG as a standard. (Two gels per treatment group are needed for Coomassie staining and Western blot.)

For the first dimension, 200 µg of whole cell protein will be resuspended in 2-D-lysis buffer. Prepare the pH gradient strips according to the manufacturers' suggestion in rehydration buffer without DTT. The suspension will be divided equally and run on immobilized pH gradients covering exponentially the pH range from 3 to 10 as follows: rehydration for 12 h; 50 µA/strip at 500 V for 1 h, 1000 V for 1 h, and then 8000 V for ~3 h. Equilibrate according to manufacturers' directions but do not include DTT in EQ Buffer 1 or P-SSG modifications will be lost.

For the second dimension, run the immobilized pH gradient strip on a 10% polyacrylamide gel for ~15 min at 10 mA/gel then increase to ~25 mA/gel for 4 h or until the band reaches the bottom of the gel. One gel will be stained using Coomassie brilliant blue R250 or G250 which is mass spectrometry compatible. The other gel will be transferred and blotted for P-SSG. Place the Coomassie-stained gel in HPLC grade water until the Western blot for P-SSG is complete. Place the developed film for P-SSG onto a light box and align the gel. Excise the band(s) of interest with a clean scalpel and transfer to a microcentrifuge tube. Following trypsin digestion, peptide mass analysis can be performed by MALD-TOF mass spectrometry and the protein identification can be assessed using software from the National Center for Biotechnology Information protein database.

2.2.3. Confirmation of S-glutathionylated PDI—Pre-clear 800 µg cell lysate from control and treated cells with protein A/G plus agarose. Precipitate PDI with the anti-PDI antibody (1:100) overnight at 4 °C. Spin and wash the precipitate three times with lysis buffer. Resuspend the precipitated PDI in nonreducing sample loading buffer and heat to ~95 °C for 10 min. Separate the proteins under nonreducing conditions on 10% SDS-PAGE gels. Transfer proteins onto a nitrocellulose membrane (Bio-Rad). Block nonspecific binding with blocking buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 1 µM protease inhibitors, 5 mM NaF, and 1 mM Na₃VO₄) containing 10% nonfat dried milk for 1 h. Incubate the membrane with anti-SSG (1:100) overnight at 4 °C. Wash the membrane 3× with PBS for 15 min and incubate with secondary antibody conjugated to horseradish peroxidase for 1 h. Remove the secondary antibody with 3× washes using PBS. Develop the blots with enhanced chemiluminescence detection reagents and scan using a transilluminator. Strip the membrane and reprobe with anti-PDI.

3. Identification of Target Cysteine Residues

Specificity is critical in ascribing any posttranslational modification. Unlike phosphorylation, there is no sequence signature that is a hallmark for S-glutathionylation. However, reduced cysteine residues (-SH) that have a low pK_a (vicinal or close to basic amino acid residues) are reactive and can become oxidized into protein sulfenic acids (P-SOH), nitrosylated (P-SNO), or S-glutathionylated (P-SSG). As such, it is important to search the database for each of these modifications concurrently. Using the biotin switch assay, Uehara *et al.* (2006) reported that PDI is S-nitrosylated in neuronal cells and tissue from PD and AD patients. Oxidation or S-glutathionylation was not investigated in these

samples. Both P-SNO and P-SSG of PDI lead to diminished isomerase activity and activation of the UPR. Mass spectrometry analysis of PDI following nitrosative stress showed that both active site cysteines are *S*-glutathionylated; however, P-SNO was not detected, suggesting that P-SNO residues are subject to rapid conversion to a P-SSG product (Townsend *et al.*, 2009b). The following technique utilizes recombinant PDI-SSG as a positive control in the mass spectrometry analysis of PDI in cells following nitrosative stress.

3.1. Materials

Recombinant PDI; PABA/NO; GSH; 20 mM PBS; control and PABA/NO-treated cell pellets; protein A/G Plus agarose; IgG; anti-PDI (Affinity BioReagents); anti-SSG (Virogen); lysis buffer (20 mM Tris-HCl, pH 7.5, 15 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, and 1 mM β -glycerophosphate with freshly added protease and phosphatase inhibitors, 5 mM NaF and 1 mM Na_3VO_4); nonreducing loading sample buffer; 10% SDS-PAGE gel; LysC. Biospin size exclusion micro-spin columns (Bio-Rad).

3.2. Methods

3.2.1. *In vitro* S-glutathionylation of PDI—Incubate 2 mg/mL PDI (>95% homogeneous) in 20 mM phosphate buffer (pH 7.4) for 1 h at room temperature as follows: (a) control; (b) 25 μM PABA/NO and 1 mM GSH; (c) 25 μM PABA/NO; and (d) 1 mM GSH. Excess PABA/NO and GSH will be eliminated through Biospin-6 Size exclusion micro-spin columns (Bio-Rad) with 20 mM PBS. Five microliters of native and PDI-SSG will be used for immunoblot analysis and the remaining will be digested with LysC and evaluated by mass spectrometry.

3.2.2. S-glutathionylation of PDI in cells—Treat cells with 25 μM PABA/NO for 4 h. Wash cells prior to harvest with PBS and resuspend the pellets in lysis buffer and incubate on ice for 30 min. Sonicate lysates for 10 s and centrifuge for 30 min at $10,000\times g$ at 4 °C. Determine the protein concentration with the Bradford reagent using IgG as a standard. Immunoprecipitate PDI as described in Section 2.2. Divide the sample equally and load on two SDS-PAGE gels; one for transfer and immunoblot confirmation as described in Section 2.2 and the other for staining and mass spectral analysis.

3.2.3. Mass spectrometry identification of target cysteine residues—Stain the proteins using Coomassie brilliant blue R250 or G250 which is mass spectrometry compatible. Place the gel in HPLC grade water for a few hours. Place the gel onto a light box to excise the band of interest with a clean scalpel, transfer to a microcentrifuge tube, and spin down. The gel will be destained by adding 100 μl of 100 mM ammonium bicarbonate/acetonitrile (1:1, v/v) and vortexing. Depending on the stain intensity, this will require occasional vortexing for ~30 min. Remove and add 500 μl acetonitrile and incubate at room temperature until the gel pieces are clear and shrunk. Remove acetonitrile prior to digesting the protein. To digest, cover the gel piece with LysC buffer at 4 °C for 30 min. It is important to keep the gel plugs wet during the enzymatic cleavage. Incubate the tubes at 37 °C overnight in an air circulation thermostat. To extract the peptide digestion products, add

100 μ l of extraction buffer (1:2, 5% formic acid/acetonitrile) to each tube and shake for 15 min at 37 °C. Separate the digested fragments (20 μ l) by HPLC using a C18 RP column. Elute the peptides during a 30-min gradient from 2% to 70% with 0.2% formic acid using a 180 nL/min flow rate. The eluted peptides can be detected on an ion trap mass spectrometer operated in data acquisition mode with dynamic exclusion enable. Dynamic exclusion will prevent duplication of MS/MS experiments of the same precursor ions over an elution window of 3 min and thus allow lower abundance peptides to be sequenced and more complete sequence coverage. Analysis of the LysC digested protein with GPS Explorer software using Mascot showed that two fragments were detected with a molecular mass [+305.6] compatible with a single S-glutathionylation in the PABA/NO-treated samples that was not present in untreated. The S-glutathionylated fragments (43–57 and 387–401) correspond to the active sites in the α and α' domain, and each contain a CXXC motif. Both active sites of PDI are altered and thereby inhibit protein function.

4. Characterization of Structural and Functional Consequences of S-Glutathionylated PDI

Activation of the UPR is triggered by the accumulation of misfolded or unfolded proteins. PDI is a key player in protein folding and as such, its dysregulation can lead to activation of the UPR. The structural and functional consequences of S-glutathionylation of PDI can be assessed directly using recombinant protein. The functional consequences can be assessed indirectly in cells and tissues by detection of activation of the UPR concurrent with S-glutathionylation. Methods to detect activation of the UPR are reviewed in this issue. Here, we describe *in vitro* assays for assessing structural (circular dichroism (CD) and intrinsic fluorescence) and functional aspects (insulin turbidity assay) of native and modified PDI (Holmgren, 1979; Lundstrom and Holmgren, 1990).

4.1. Materials

Native or S-glutathionylated PDI (1 mg/ml in sodium phosphate buffer); 10 mg/ml insulin in 50 mM Tris–HCl, pH 7.5; 100 mM DTT; 100 mM sodium EDTA, pH 7.0.

4.2. Methods

4.2.1. Determination of the structural consequences of P-SSG—The effect of PDI-SSG on secondary structure can be examined by spectroscopic analysis (Townsend *et al.*, 2009b). *In vitro* S-glutathionylation of PDI is carried out as described in Section 3.2 and the excess GSH and PABA/NO removed by running the samples through Biospin columns. Add 1 ml PDI, PDI-SSG or diluent to a 10 $\times$ 10 $\times$ 40 mm quartz cuvette. Record protein tryptophan fluorescence on an F 2500 spectrofluorometer (Hitachi) where excitation and emission slits are 2.5 and 5.0 nm, respectively. The excitation wavelength at 295 nm will minimize the effect of protein tyrosine and phenylalanine residues. Subtract background (diluent) spectra from the final emission of the protein ($N = 3$). Remove the sample and use for CD.

CD measurements can be carried out on a 202 AVIV Associates (Lakewood, NJ) using a semi-micro quartz rectangular 1 $\times$ 10 $\times$ 40 mm cuvette. Maintain samples at 22 °C using a

Pelletier element. Record spectra while scanning in the far-ultraviolet region (190–260 nm), with bandwidth of 1.0 nm, step size of 1.0 nm, integration time of 30 s three times for buffer control, PDI, and PDI-SSG. The output of the CD spectrometer ($N = 3$) will be reported according to the protein concentration, amino acid content, and cuvette thickness into molecular ellipticity units (degrees/cm²/dmol).

4.2.2. Determination of isomerase activity—The insulin turbidity assay is based on the ability of PDI to reduce the disulfide bonds within insulin, resulting in the precipitation of an insoluble β -chain which can be followed at 650 nm (Holmgren, 1979; Lundstrom and Holmgren, 1990) (Fig. 18.3). DTT has been shown to reduce the disulfide bonds completely and is used as a positive control. Prepare a 5 ml reaction mix (4.2 ml sodium phosphate buffer, 130 μ l EDTA; 670 μ l insulin). Prepare native and *S*-glutathionylated PDI to a final dilution of 3.6 μ g PDI. Positive control (100 mM DTT); negative control (no PDI or DTT). In a 96-well plate, add the following per well (in triplicate): 75 μ l reaction mix; 23.5 μ l sodium phosphate buffer; and 1 μ l DTT. Equilibrate the plate to 25 °C and read at A_{650} . Add 1.5 μ L PDI (native or test) or 1.5 μ L DTT (positive control) or diluent (negative control) and read every 5 min for 1 h. Plot the absorbance/minute after subtracting from the negative control.

5. Summary

Redox regulation of proteins is emerging as an important aspect to signaling cascades. Identification and characterization of *S*-glutathionylated moieties will be critical to ascribing structural and functional consequences. The methodologies described herein can be used to identify and validate *S*-glutathionylated proteins in different cells and tissues following oxidative or nitrosative stress.

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Figure 18.1.

Nitrosative stress induced S-glutathionylation. Nitrosative stress leads to an alteration in the ratio of GSH/GSSG and formation of reactive nitrogen species. PDI can be posttranslationally modified to form S-nitrosylated or S-glutathionylated proteins. The cellular consequence leads to an accumulation of unfolded proteins and activation of the UPR.

Figure 18.2.

Identification, validation, and characterization of PDI-SSG and its functional consequences. A general scheme for identifying *S*-glutathionylated proteins by MALDI-TOF showed that PDI is a target for redox regulation. Subsequent confirmation in cells via immunoprecipitation validated the modification. The cysteine targets were mapped using LC-MS-ESI from recombinant protein and in cells. The structural and functional consequences were evaluated both *in vitro* and in cells.

Figure 18.3.

PDI-SSG activity is blunted as measured by the insulin turbidity assay. PDI breaks the two disulfide bonds between two insulin chains (A and B) that results in precipitation of the B chain. The turbidity is measured as an increase in the absorbance at 650 nm. RNS/ROS induces S-glutathionylation of PDI in the a and a' domains, changes the protein conformation, and abolishes its enzyme activity.