Analysis of S-glutathionylated proteins during adipocyte differentiation using eosin-glutathione and glutaredoxin 1

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**INTRODUCTION**

Protein S-glutathionylation is a reversible post-translational modification on cysteine residues forming a mixed disulfide with glutathione. S-glutathionylation, not only protects proteins from oxidation but also regulates the functions of proteins involved in various cellular signaling pathways. In this study, we developed a method for the detection of S-glutathionylated proteins (ProSSG) using eosin-glutathione (E-GSH) and mouse glutaredoxin 1 (mGrx1). ProSSG was efficiently and specifically labeled with E-GSH to form ProSSG-E via thiol-disulfide exchange. ProSSG-E was readily luminescent allowing the detection of ProSSG with semi-quantitative determination. In addition, a glutathionylation enzyme mGrx1 specifically released E-GSH from ProSSG-E, which increased fluorescence allowing a sensitive determination of ProSSG levels. Application of the method to the adipocyte differentiation of 3T3-L1 cells showed specific detection of ProSSG and its increase upon differentiation induction, which was consistent with the result obtained by conventional immunoblot analysis, but with greater specificity and sensitivity. [BMB Reports 2022; 55(3): 154-159]

Protein S-glutathionylation has been extensively studied in human diseases including cancer, obesity, and diabetes, with the aim of targeting glutathionylated proteins (ProSSG) for disease treatment (2, 3).

However, studies on S-glutathionylation in cells are hampered by difficulties associated with the detection of ProSSG, which is generally present at low levels. Immunoblot analysis using anti-GSH antibody is most commonly used to detect ProSSG, but its specificity and sensitivity have been questioned (4, 5). Other methods are indirect detection with complicated and time-consuming processes. Such methods involve the reduction of ProSSG to free thiols and subsequent labeling with thiol-reactive probes or affinity tags and further enrichment (6, 7). Although various methods for the analysis of ProSSG have been developed, most current methods have technical issues of complexity, detection specificity, sensitivity and physiological relevance.

Previously, fluorescent eosin-GSH (E-GSH) was used in de-glutathionylation enzyme glutaredoxin1 (Grx1) and protein disulfide reductase thioredoxin (Trx) activity assays (8, 9). E-GSH was strongly fluorescent, but its oxidized disulfide form diE-GSSG was insignificantly fluorescent. Grx1 and Trx reduced diE-GSSG to E-GSH, which increased fluorescence and allowed to measure enzyme activities. Moreover, the fluorescence of E-GSH was significantly quenched by forming BSASSG-E, a protein mixed disulfide with bovine serum albumin (BSA) on cysteine residues (9). Grx1 released E-GSH from BSASSG-E and increased fluorescence, which allowed the determination of deglutathionylation enzyme. However, this Grx1-catalyzed deglutathionylation was performed in an assay system containing the enzyme cofactor GSH, which could cause spontaneous deglutathionylation via thiol-disulfide exchange.

In the present study, we developed a method for the detection of ProSSG using E-GSH and mouse glutaredoxin 1 (mGrx1). A standard glutathionylated protein BSASSG-E was efficiently and specifically labeled with E-GSH via thiol-exchange forming BSASSG-E, and the labeled protein was readily luminescent under UV-light. Moreover, in the absence of the enzyme cofactor GSH, mGrx1 released E-GSH from BSASSG-E, which increased fluorescence and allowed sensitive quantitation. Under optimized conditions, ProSSG in 3T3-L1 cells was specifically labeled with E-GSH. The labeled ProSSG-E was determined by measuring luminescence and fluorescence by mGrx1-mediated...
RESULTS

Eosin-glutathione fluorescence and luminescence and thiol-disulfide exchange

E-GSH exhibited strong fluorescence upon excitation at 490 nm with maximum emission at 540 nm (Fig. 1A lower). The fluorescence of E-GSH was significantly quenched in the disulfide form diE-GSSG and this was recovered by reduction with DTT (Fig. 1A upper). E-GSH was also luminescent under UV-light (Fig. 1B inset). On the other hand, DiE-GSSG was insignificantly luminescent, but reduction with DTT induced strong luminescence (Fig. 1C inset). Incubation with excess thiols, cysteine (CysSH), and reduced glutathione (GSH), did not change the fluorescence of E-GSH (Fig. 1B). Consistently, thiols did not change the luminescence of E-GSH (Fig. 1B inset). However, incubation with disulfides, cystine (Cyss) and oxidized glutathione (GSSG) reduced fluorescence and luminescence, which indicated thiol-disulfide exchange between E-GSH and disulfides and the formation of corresponding mixed disulfides (Fig. 1B). Similar thiol-disulfide exchange was observed for diE-GSSG (Fig. 1C). The incubation of diE-GSSG with thiols, CysSH and GSH, substantially increased fluorescence and luminescence, whereas incubation with disulfides (Cys and GSSG) did not induce any change.

Efficient labeling of glutathionylated BSA with E-GSH via thiol-disulfide exchange

To prepare a standard glutathionylated protein, bovine serum albumin (BSA) was incubated with GSSG or E-GSSG, as described in the supplementary method. Determination of reduced cysteine residues showed that BSA was efficiently glutathionylated by GSH and E-GSH, forming BSASSG and BSASSG-E, respectively (Supplementary Fig. 1). BSASSG-E was readily luminescent under UV-light (Fig. 2B and Supplementary Fig. 1).

Labeling of BSASSG with E-GSH was examined by incubation of the protein with increased concentrations of E-GSH. BSASSG was not luminescent, but protein luminescence was increased dose-dependently by incubation with E-GSH, indicating the formation of BSASSG-E (Fig. 2A). Whereas the incubation of BSASSG with diE-GSSG did not show significant luminescence. These results indicated that GSH in BSASSG was replaced by E-GSH to form BSASSG-E via thiol-disulfide exchange. Complete replacement of GSH by E-GSH was obtained at a 50-fold molar excess of E-GSH (E-GSH/BSASSG) (Fig. 2A, C). In another experiment, BSASSG-E was incubated with GSH. The luminescence of BSASSG-E was reduced by incubation with increasing concentrations of GSH, while GSSG did not change the luminescence of BSASSG-E (Fig. 2B). These results indicated thiol-disulfide exchange between BSASSG-E and GSH to form BSASSG. However, the complete replacement of E-GSH in BSASSG-E by GSH was achieved at a 5,000-fold molar excess of GSH (GSH/BSASSG-E) (Fig. 2B, C).

mGrx1-mediated deglutathionylation of BSASSG-E

The fluorescence of BSASSG-E was significantly increased by DTT reduction (Fig. 2D), indicating E-GSH fluorescence was quenched in BSASSG-E, as previously reported (9). Deglutathionylation of BSASSG-E was examined using mouse glutaredoxin1 (mGrx1) (preparation in the supplementary method) in the absence of enzyme cofactor GSH. This mGrx1-mediated deglutathionylation released E-GSH from BSASSG-E and rapidly increased fluorescence in a manner proportional to the amount of mGrx1 in deglutathionylation reactions (Fig. 2F). Complete deglutathionylation of BSASSG-E was reached at a >20-fold molar excess of mGrx1 (mGrx1/BSASSG-E). Deglutathionylation was confirmed by measuring decreases in BSASSG-E luminescence (Fig. 2F).

Labeling of glutathionylated proteins in 3T3-L1 cells with E-GSH

Labeling of cellular glutathionylated proteins (ProSSGs) with E-GSH was investigated using proteins extracted from 3T3-L1 cells. Incubation of cellular proteins with diE-GSSG and non-reducing SDS-PAGE showed strong luminescence indicating the formation of ProSSG-E (Fig. 3A, E). However, pre-alkylation of cellular proteins with NEM significantly inhibited ProSSG-E deglutathionylation, which allowed sensitive analysis of cellular ProSSG. Application of these methods to the adipocyte differentiation of 3T3-L1 cells showed increases in ProSSG levels upon differentiation induction, which was consistent with the result obtained by immunoblot analysis, but with greater specificity and sensitivity.
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Fig. 2. Labeling glutathionylated BSA with E-GSH via thiol-disulfide exchange. Luminescence intensities of 10 μg/10 μl (15 μM) BSASSG (A) and 10 μg/10 μl (15 μM) BSASSG-E (B) incubated with the indicated concentrations of E-GSH/GSH and diE-GSSG/GSSG. (C) Luminescence intensities of A and B. (D) Fluorescence spectra of BSASSG and BSASSG-E reduced with 1 mM DTT. (E) Fluorescence at 540 nm for deglutathionylation of 10 μg BSASSG-E by mGrx1 (filled circles, 0 μg; open circles, 1 μg; filled triangles, 10 μg; open triangles, 20 μg; filled squares, 40 μg; open squares, 1 mM DTT). (F) Fluorescence increase for the reactions shown in E. The inset shows luminescence intensities for the reactions shown in E. Asterisks indicate significant differences (P < 0.01).

Fig. 3. Labeling glutathionylated proteins in 3T3-L1 cells. Luminescence of cellular proteins pre-alkylated at the indicated NEM concentrations and incubated with diE-GSSG (A and E-GSH (E), (B and F) Luminescence intensities determined for A and E, respectively. (C) Fluorescence at 540 nm for deglutathionylation of 20 μg ProSSG-E by mGrx1 (filled circles, 0 μg; open circles, 10 μg; filled triangles, 20 μg; open triangles, 40 μg). Fluorescence (D) and luminescence (G) increases observed for the reactions shown in E. Asterisks indicate significant differences (P < 0.01).

formation showing markedly reduced luminescence. Complete inhibition was obtained at 100 mM NEM, indicating that ProSSG-E was formed via the thiol-disulfide exchange between thiols in cellular proteins and diE-GSSG (3A, B). In another experiment, incubation of cellular proteins with E-GSH also formed ProSSG-E showing luminescence, but significantly less than observed with E-GSSG (3E). Pre-alkylation of cellular proteins inhibited ProSSG-E formation in the incubation with E-GSH, however, complete inhibition was not obtained even at ≥ 100 mM NEM (Fig. 3E, F). These results indicated that ProSSG-E was formed via the thiol-disulfide exchange between E-GSH and disulfides in cell proteins.

mGrx1-mediated deglutathionylation was examined using ProSSG-E formed with E-GSH by incubating cellular proteins pre-alkylated at 100 mM NEM (Fig. 3C). mGrx1 rapidly released E-GSH from ProSSG-E showing fluorescence increase that was proportional to the amount of the enzyme in deglutathionylation reactions (Fig. 3C, D). Complete deglutathionylation was obtained with ≥ 20 μg mGrx1, which was confirmed by measuring the disappearance of ProSSG-E luminescence (Fig. 3G). These results indicated that mGrx1 accepted ProSSG-E, as a substrate, and ProSSG-E was likely produced by labeling of ProSSG with E-GSH.

Analysis of glutathionylated proteins during the adipocyte differentiation of 3T3-L1 cells
Intracellular ROS was significantly increased upon inducing the adipocyte differentiation of 3T3-L1 cells (Fig. 4A). ROS levels reached a maximum level on the day 2 post-induction and then decreased to a basal level on the day 8 differentiation (Fig. 4A, B). Immunoblot analysis using anti-GSH antibody showed increases in ProSSG levels upon differentiation induction, which decreased on the day 8 differentiation (Fig. 4C). The most significant ProSSG bands were detected at molecular masses of 60 and 40 kDa. ProSSGs in the adipocyte differentiation of 3T3-L1 cells were also analyzed by pre-alky-
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...ting cellular proteins and labeling with E-GSH. Luminescence analysis showed increases in ProSSG-E upon induction of adipocyte differentiation, consistently indicating increases in ProSSG levels (Fig. 4D). Significant ProSSG-E bands were similarly detected at ∼60 and ∼40 kDa, however, overall band patterns appeared to differ from those of ProSSG obtained by immunoblot analysis. Quantitative determination of ProSSG-E luminescence showed ProSSG levels peaked on day 1 post-induction (Fig. 4E). In addition, mGrx1-mediated deglutathionylation showed consistent increases in ProSSG-E levels with higher sensitivity (Fig. 4F).

**DISCUSSION**

S-Glutathionylation regulates the structures and functions of proteins involved in cellular signaling and redox regulation. For instance, cancer cells generally exhibit higher resistance to ROS than normal cells and use S-glutathionylation to protect many proteins from excessive ROS and to regulate protein functions (1, 3). The tumor suppressor p53 is an example protein regulated by S-glutathionylation. The level of S-glutathionylated p53 was low, but increased after treatment of cancer cells with oxidant or anticancer drugs showing ROS resistance (10). Therefore, the detection of S-glutathionylation target proteins have attracted to identify new target proteins in disease treatment.

Various methods have been developed for the detection of ProSSG to identify proteins regulated by S-glutathionylation and to investigate the regulation mechanism involved in human diseases. However, currently available methods include complicated processes and have limited specificity, sensitivity, and/or physiological relevance (4, 5). Accordingly, we developed a method for the analysis of ProSSG using E-GSH and mGrx1-mediated deglutathionylation. E-GSH efficiently labeled, not only a standard glutathionylated protein BSASSG but also glutathionylated cellular proteins ProSSG to form luminescent proteins simply detectable by non-reducing SDS-PAGE using UV-light (Fig. 2A and 3E). This protein luminescence completely disappeared after reduction with DTT, which indicated the formation of mixed disulfides between glutathionylated proteins and E-GSH via thiol-disulfide exchange (Fig. 2F and 3G).

E-GSH appeared to replace CysSH in free CysS to produce CysSS-G-E, and further to form dE-GSSG, showing decreases in fluorescence and luminescence (Fig. 1B). This observation might be an argument for the specific detection of ProSSG, as E-GSH could label protein cysteine disulfides. However, cysteine disulfides stabilizing protein structures are generally located in the hydrophobic cores of proteins, where the bulky ring group of E-GSH is unlikely accessible. Whereas reactive cysteine disulfides and the reduced cysteines of S-glutathionylation targets are exposed on protein surfaces (11, 12). In fact, BSA contains 35 cysteine residues and most of them are in disulfide forms except for one in thiol form. When BSA labeled with E-GSH in its native form showed very low efficiency of labeling (Supplementary Fig. 1) (13). More importantly, mGrx1-mediated deglutathionylation completely released E-GSH from ProSSG-E, which indicated E-GSH-labeled proteins were accepted as an mGrx1 substrate (Fig. 3G). Therefore, ProSSG-E were most likely derived from ProSSG, or at least from active cysteine disulfides of S-glutathionylation targets, by E-GSH labeling.

The fluorescence of E-GSH was strongly quenched in BSASSG-E but recovered after its release from the protein (Fig. 2D-F). This quenching effect was not specific for BSASSG-E, as ProSSG-E also showed similar fluorescence quenching, and fluorescence increase after E-GSH release (Fig. 3C, D). We speculate that the quenching effect is unlikely to be specific for a local amino acid sequence, but rather due to energy transfer from the eosin moiety to electrophilic peptide bonds (14). Grx1 is a well-studied enzyme known to catalyze deglutathionylation and GSH release from ProSSG (15). The catalytic cysteine thiol of Grx1 is oxidized during deglutathionylation and subsequently reduced by GSH. In this study, we excluded GSH in mGrx1-mediated deglutathionylation reaction, because GSH chemically released E-GSH from ProSSG-E (Fig. 2B). Therefore, deglutathionylation was specifically mediated by mGrx1 and ProSSG-E should be mGrx1 substrate derived from ProSSG by E-GSH labeling.

ROS stimulates adipocyte differentiation and is believed to accelerate adipose tissue dysfunction in metabolic disorders (16). In addition, S-glutathionylation is considered to regulate various proteins during adipocyte differentiation, although the relationship between ROS and S-glutathionylation is not clearly understood. The conventional immunoblot analysis showed increases in ProSSG levels (Fig. 4C), consistent with previous reports (17, 18). However, relative ProSSG levels were not significantly different between the days post-induction of adipocyte differentiation, likely due to limited detection sensitivity and specificity. E-GSH-labeling showed similar increases in ProSSG levels, but with higher sensitivity showing significantly high level on the day 1 (Fig. 4C, D). ProSSG levels appeared to be not proportional to intracellular ROS levels, showing the peaks on the day 1 and the day 2 post-induction, respectively. Although it is not yet completely understood, ROS and ProSSG are not essentially proportional due to complex regulatory systems in cells. We speculate that enzymes such as glutaredoxin and glutathione transferases are likely involved in the regulation of ProSSG levels in the early phase of adipocyte differentiation, as suggested in the previous reports (18-20). The luminescence of ∼40 kDa proteins peaked on the day 1 post-induction and then decreased, indicating that E-GSH-labeling has different detection specificity from immunoblot analysis. A recent study revealed that the early adipogenic transcription factor C/EBP-β (∼40 kDa) is regulated by S-glutathionylation, which is consistent with our result, although further identification and confirmation are required (18). mGrx1-mediated deglutathionylation allowed more sensitive quantification of ProSSG-E derived from ProSSG by labeling with E-GSH (Fig. 4F), and after deglutathionylation, no significant luminescent protein was detected (data not shown). These data indicate that the E-GSH-labeling method is more sensitive and spe-
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Specific to detect ProSSG than conventional the immunoblot assay.

In summary, we developed a straightforward method for the analysis of ProSSG using E-GSH and mGrx1-mediated deglutathionylation. ProSSG was efficiently and specifically labeled by E-GSH via thiol-disulfide exchange to form luminescent ProSSG-E, which enabled the semi-quantitative analysis of ProSSG. Furthermore, mGrx1-mediated glutathionylation allowed the sensitive quantitation of ProSSG based on the fluorescence increased by the release of E-GSH from ProSSG-E. The study shows E-GSH-labeling and mGrx1-mediated deglutathionylation could be applied for the analysis of ProSSG during the adipocyte differentiation of 3T3-L1 cells and that it has greater sensitivity and specificity than conventional immunoblot analysis. Although the E-GSH-labeling requires further enhancement, in particular, for the identification of ProSSG, these results suggest the method developed in this study provides an alternative means of ProSSG analysis that overcomes technical problems of currently available methods.

MATERIALS AND METHODS

Preparation of E-GSH
Di-eosin-glutathione disulfide (diE-GSSG) was prepared in the reaction of glutathione disulfide (GSSG, Sigma) and eosin-5-isothiocyanate (Sigma) (Supplementary Fig. 2) as previously described (8, 21). Eosin-glutathione (E-GSH) was prepared by reducing diE-GSSG with dithiothreitol (DTT), and excess DTT was removed by precipitation of E-GSH in ice-chilled acetonitrile (8, 21). Concentrations of diE-GSSG and E-GSH were determined using extinction coefficients ε525 nm = 112 mM⁻¹ cm⁻¹ and ε519 nm = 88 mM⁻¹ cm⁻¹, respectively (21, 22). Fluorescence spectra were obtained using an LS55 (Perkin Elmer) at an excitation wavelength of 490 nm and luminescence intensities were measured under UV-light using a GelDoc (BioRad).

Thiol-disulfide exchange between glutathionylated BSA and glutathione
Glutathionylated BSA (BSASSG or BSASSG-E) 10 µg/10 µl (15 µM) in 50 mM HEPES pH 7.4 and 150 mM NaCl containing the indicated concentrations of GSH, E-GSH, GSSG, or diE-GSSG were incubated for 1 h at room temperature. After non-reducing SDS-PAGE, BSASSG-E formation was estimated by measuring luminescence intensities under UV-light, and then gels were stained with Coomassie brilliant blue to confirm equal protein loadings. Luminescence intensities were determined using Image J software.

Cell culture and preparation of intracellular proteins
Preadipocytes 3T3-L1 were maintained in DMEM (Hyclone) supplemented with 10% calf serum (Gibco) in a humidified 5% CO₂ incubator at 37°C. Adipocyte differentiation was performed as previously described (23). The differentiation of post-confluent 3T3-L1 cells was induced by the addition of adipogenic media: DMEM containing 10% FBS (Welgene) with 0.5 mM 3-isobutyl-1-methylxanthine, 1 µM dexamethasone and 5 µg/ml insulin (MDI) for 2 days. Cells were then cultured in DMEM containing 10% FBS and 5 µg/ml insulin for 8 days (the medium was replaced every 2 days). ROS generation was estimated using 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (H2-DCFDA) (24), and adipocyte differentiation was confirmed by oil red O staining (23, 25).

Cells harvested at the indicated times were lysed in 50 mM HEPES pH 7.4 and 150 mM NaCl, 1 mM PMSF, 1% Triton X-100, 100 mM N-ethylmaleimide (NEM), and 1x protease inhibitor cocktail (Roche) by incubation for 30 min at room temperature. For efficient alkylation of proteins with NEM to block the free thiols, the cell lysis was performed at room temperature. Cell lysates were centrifuged at 13,200 rpm for 30 min at 4°C, and protein supernatants were exchanged with 50 mM HEPES pH 7.4 and 150 mM NaCl using a Zeba spin desalting column (7K MWCO, Thermo). Protein concentrations were determined using a BCA assay kit (Thermo). Cellular proteins (1.0 mg/ml) were then incubated with 0.5 mM E-GSH or 0.5 mM diE-GSSG for 1 hr at room temperature. Excess E-GSH or diE-GSSG was removed by buffer exchange using a spin column (Thermo). After non-reducing SDS PAGE, the formation of E-GSH-labeled cellular proteins was estimated by measuring luminescence intensities under a UV light, and luminescence intensities were determined using Image J software.

mGrx1-mediated deglutathionylation and determination of glutathionylated proteins
mGrx1-mediated deglutathionylation was performed using 10 µg BSASSG-E or 20 µg ProSSG-E in 50 mM HEPES pH 7.4 and 150 mM NaCl. Deglutathionylation was initiated by adding 40 µg of mGrx1 and incubating for 30 min at room temperature. The release of E-GSH was monitored by measuring fluorescence at excitation/emission wavelengths of 490/540 nm using a microplate reader Infinite M200 (Tecan). To determine the relative amounts of glutathionylated proteins, fluorescence differences were calculated by subtracting initial values from final values.

Statistical analysis
The significance of differences in ROS, luminescence and Δfluorescence compared to the day 0 control values was analyzed by an unpaired Student’s t-test. A P value < 0.05 was considered statistically significant.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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