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A fluorometric method to quantify protein glutathionylation using glutathione derivatization with 2,3-naphthalenedicarboxaldehyde

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ABSTRACT

This study reports the development of a new assay for the rapid determination of protein glutathionylation in tissues and cell lines using commercially available reagents and standard instrumentation. In this method cells are homogenized in the presence of *N*-ethylmaleimide to eliminate free thiols and the proteins are precipitated with acetone. Subsequently, the disulfide-bound glutathione is eluted from the protein by the addition of tris(2-carboxyethyl)phosphine and reacted with 2,3-napthalenedicarboxaldehyde to generate a highly fluorescent product. Lymphoblastoid cell lines were found to have glutathionylation levels in the range of 0.3–3 nmol/mg protein, which were significantly elevated after treatment of the cells with *S*-nitrosoglutathione. Mouse tissues including liver, kidney, lung, heart, brain, spleen, and testes were found to have glutathionylation levels between 1 and 2.5 nmol/mg protein and the levels tended to increase after treatment of mice with doxorubicin. In contrast, mouse skeletal muscle glutathionylation was significantly higher (4.2 ± 0.33 nmol/mg, $p < 0.001$) than in other tissues in untreated mice and decreased to 1.9 ± 0.15 nmol/mg after doxorubicin treatment. This new method allows rapid measurement of cellular glutathionylation in a high-throughput 96-well plate format.

Glutathione (GSH) is the major antioxidant in most cells and can be reversibly oxidized to a disulfide (GSSG) as the result of oxidative stress. Glutathionylation is defined as the specific posttranslational addition of GSH to protein cysteine residues to form a mixed disulfide, Pr–SSG [1]. Protein glutathionylation is increased during cellular oxidative and nitrosative stress and since it is largely reversible, glutathionylation clearly serves to protect essential cysteine thiols from irreversible oxidation to sulfonic (RSO_3H) acids and helps maintain redox homeostasis [2,3]. Many proteins contain cysteine residues that could be subjected to glutathionylation, and a rapidly increasing number of proteins have been shown to be subject to this posttranslational modification [3,4]. Although the mechanism for the glutathionylation of proteins is not clear, there is good evidence that glutaredoxins, thioredoxins, and sulfiredoxins can catalyze the deglutathionylation of proteins [2,3,5]. Glutathionylation can significantly alter protein function and this has been compared with phosphorylation as a molecular switch or rheostat [2].

In recent years there has been increasing interest in the role of glutathionylation in a wide range of cellular processes including energy metabolism, signaling and apoptosis, cell cycle regulation, ion channel modulation, calcium homeostasis, regulation of cytoskeletal structures, protein folding, and gene regulation (reviewed in [1,3,6]). As a result of its impact on cellular processes, aberrant glutathionylation has been implicated in the pathology of a number of clinically significant diseases, including Alzheimer disease, type 2 diabetes, cystic fibrosis, cataracts, and cancer [6].

Because of the increasing recognition of the biological and clinical importance of glutathionylation, a range of techniques has been developed to identify and locate glutathionylated proteins and to determine the level of glutathionylation [7–17]. Antibodies that recognize glutathione specifically bound to protein have been used to identify glutathionylated proteins after Western blotting of cell extracts and in tissue sections [9,15,16]. In other studies, recombinant biotinylated glutathione transferase has been used as an affinity probe to specifically detect glutathiolated proteins and, in some proteomic studies, radiolabeled glutathione has been used to detect glutathionylation [12]. The quantification of glutathionylation in tissues has also been attempted using a variety of techniques. The same antibodies used to detect glutathionylated proteins on Western blots have also been used in an enzyme-linked immunosorbent assay format to measure the level of glutathionylation [13]. Other methods have eluted the disulfide-bound glutathione for subsequent measurement [17,18]. These approaches require specificity either in the elution of GSH from the protein or in the detection of the eluted GSH. The specific elution of GSH from glutathionylated protein has been achieved enzymatically by the use of recombinant glutaredoxin [17], but some methods have used nonenzymatic reduction of the Pr–SSG

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disulfide with general reducing agents such as dithiothreitol or sodium borohydride [18]. Detection of eluted GSH has been achieved previously with glutathione reductase/5,5′-dithiobis(2-nitrobenzoic acid)-based recycling assays; however, these assays can be affected by the presence of other reducing agents. The detection and quantification of eluted GSH have also been achieved by fractionation of derivatized samples by HPLC, mass spectroscopy, or electrophoresis [17]. These fractionation-based approaches can be very sensitive but limit the number of samples that can be processed. Recently a new quantitative technique was described that used recombinant glutaredoxin-3 to reduce protein–glutathione Pr–SSGs and capillary gel electrophoresis to fractionate and measure the eluted GSH [17]. While the method is sensitive and provides a valuable advance in accurate quantification, it relies on the availability of recombinant glutaredoxin-3, which is limited and requires a relatively slow capillary electrophoresis fractionation step.

Previous studies have shown that under alkaline conditions GSH and its precursor γ-glutamylcysteine react specifically with 2,3-naphthalenedicarboxaldehyde (NDA) to yield a highly fluorescent product in the presence of other thiols such as cysteine or reducing agents such as dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP) [19]. The specificity of NDA has allowed the development of sensitive assays for glutathione, glutamate–cysteine ligase, and γ-glutamylcyclotransferase [19–21]. To evaluate the dynamic posttranslational glutathionylation of proteins in cell lines and in animals in response to oxidative stress, we have now exploited the reaction of GSH with NDA [19] to develop a new, specific, and quantitative assay for the determination of total protein glutathionylation in cell lysates or tissue homogenates. The assay can be undertaken in a 96-well plate format using commercially available reagents and a standard fluorescence plate reader. In this assay, GSH is eluted from glutathionylated proteins with TCEP and quantified directly by measuring its fluorescence after reaction with NDA.

Materials and methods

Chemicals

Bovine serum albumin (BSA), GSH, NDA, ethylenediaminetetraacetate (EDTA), sulfosalicylic acid, *N*-ethylmaleimide (NEM), Tris, sodium hydroxide, TCEP, acetone, Triton X-100, dimethyl sulfoxide, hydrogen peroxide (H_2O_2), and doxorubicin were purchased from Sigma Aldrich (USA). *S*-nitrosoglutathione (GSNO) was prepared by a previously described procedure [22,23].

Preparation of purified glutathionylated BSA (BSA–SSG)

Glutathionylated BSA was prepared by treating the protein with 10 mM reduced glutathione and 200 mM hydrogen peroxide for 30 min at 37 °C. The glutathionylated protein was recovered by precipitation with ice-cold acetone for 2 h at −20 °C and was resuspended in 20 mM Tris, pH 8.0 (details described below). While this procedure provided highly glutathionylated BSA, the extent of glutathionylation varied between batches and the precise cysteine residues that were glutathionylated were not known. Consequently the BSA–SSG cannot be used as a quantitative standard and was used only to optimize reagent concentrations and to demonstrate the reproducibility and linearity of the method.

Cell culture

Epstein–Barr virus-transformed lymphoblastoid cell lines (LCLs) generated from anonymous Red Cross Blood Bank donors were a gift from Dr. Juleen Cavanaugh and were cultured in RPMI 1640, 15% fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 0.01 mg/ml streptomycin, and 10% CO_2 at 37 °C. LCLs (1×10^6) were seeded overnight in complete medium and then treated with 1 mM GSNO (freshly prepared) for 60 min and lysed in nonreducing lysis buffer.

Animals

Balb/c male mice (ages 8–10 weeks) were maintained at the ANU Biosciences Facility under controlled animal room conditions. The animals were fed regular mouse chow and water ad libitum. All animal procedures including drug administration were approved by the Australian National University Animal Ethics Committee.

Drug administration

Doxorubicin was diluted in sterile saline and 20 mg/kg was administered intraperitoneally. Control animals were injected with an equal volume of saline. Animals were regularly monitored and sacrificed at 24 h. Organs including heart, liver, lungs, brain, kidneys, testes, and spleen were harvested and immediately washed in sterile saline and homogenized in a nonreducing buffer. No significant weight loss was observed in the treated mice during this experiment.

Sample preparation

The steps involved in this assay are shown schematically in Fig. 1. In this assay determining protein glutathionylation, free thiols (predominantly GSH) are blocked by the inclusion of 10 mM NEM in the tissue-homogenizing buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 1% NP-40, 2 mM EDTA, 10 mM NEM, 2 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) protease inhibitor), 2 mM serine, and 20 mM borate. Typically 100 mg of tissue was homogenized in 500 μl of homogenizing buffer for 10–20 s with an Ultra Turrux homogenizer. Tissue homogenates were centrifuged at 20,000*g* for 20 min and the protein concentration of the supernatant was determined by the Lowry procedure [24]. For a typical assay, a sample of tissue extract containing 100 μg of protein was precipitated with twice the volume of ice-cold acetone at −20 °C for 60 min to remove the alkylated cellular free thiols (GSH) and excess NEM. After centrifugation at 20,000*g* for 15 min, the supernatant was discarded and the precipitated proteins were washed again in ice-cold acetone and allowed to air dry. The pellet was suspended for 5–10 min at room temperature in 40 μl of 0.5 M Tris–HCl, pH 8.0, containing 0.1% Triton X-100 with gentle pipetting.

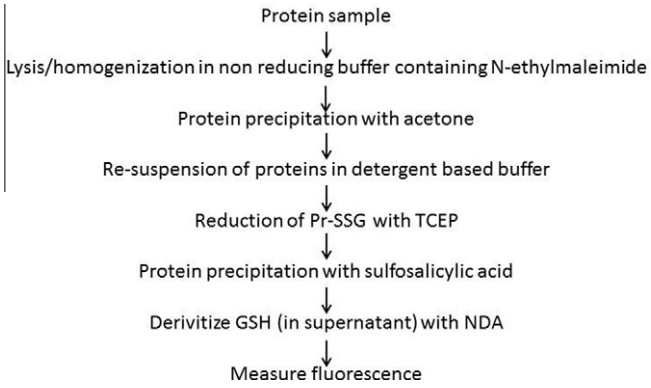

Fig.1. A scheme showing the individual steps undertaken in this glutathionylation assay.