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Original Contribution

A fluorescent dual labeling technique for the quantitative measurement of reduced and oxidized protein thiols in tissue samples

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ABSTRACT

Oxidative stress can result in the reversible oxidation of protein thiols. Because the activity of numerous proteins is sensitive to thiol oxidation, this has the potential to affect many cellular functions. We describe a highly sensitive, quantitative labeling technique that measures global and specific protein thiol oxidative state in skeletal muscle tissue. The technique involves labeling the reduced and oxidized protein thiols with different fluorescent dyes. The resulting sample is assayed using a 96-well plate fluorimeter, or individual protein bands are separated using SDS–PAGE. We show that artifactual oxidation during sample preparation and analysis has the potential to confound results, and techniques to prevent this are described. We tested the technique by analyzing the muscles of mdx and c57 mice and found that the muscles of mdx mice were significantly ($p<0.05$) more oxidized ($13.1 \pm 1.5\%$ oxidized thiols) than those of c57 mice ($8.9 \pm 0.7\%$ oxidized thiols). This technique provides an effective means to measure the extent to which oxidative stress affects the oxidation of protein thiols in biological tissues.

Reactive oxygen species (ROS), such as hydrogen peroxide and hydroxyl radicals, and reactive nitrogen species (RNS), such as peroxynitrite, have a diverse range of actions in cells [1]. For instance, these reactive species can disrupt cellular function by irreversibly damaging proteins. ROS and RNS can also affect protein function by oxidizing critical cysteine residues. Although oxidation has the potential to be irreversible (to sulfonic acid), it is the biologically reversible oxidations that are of interest. Biologically relevant oxidations include the formation of sulfenic acid and nitrosylation, as well as the formation of disulfide bonds with adjacent thiols on the same protein, or with glutathione (glutathionylation), or with the thiols of other proteins [2,3]. These are physiologically reversible processes, because the oxidized form can be converted back to the reduced thiol form through the action of enzymes such as glutaredoxin [1,4].

The activities of numerous proteins, including ion transport proteins, receptors, signal transduction kinases, phosphatases, transcription factors, and contractile proteins, have been demonstrated to be sensitive to thiol oxidation [5–7]. Consequently, protein thiol oxidation has the potential to influence many cellular functions and has been associated with many diseases including schizophrenia [8], Alzheimer disease [9], Parkinson disease [10], and diabetes mellitus [11]. Protein thiol oxidation has also been implicated as a causative factor in exercise-induced muscular fatigue [7] and aging [12]. As a result, there is considerable interest in analytical techniques that can detect changes in the thiol oxidation state of proteins in skeletal muscle.

Several factors limit the reliable and quantitative measurement of the protein thiol oxidation state in tissue. For example, analytical methods such as the Ellman assay [13] and numerous other thiol-reactive reagents [1] can measure the quantity of reduced thiols in a protein sample, with changes in oxidative state estimated from changes in reduced protein thiol content. However, proteins in tissue exist in a predominantly reduced state [14], so subtle changes in the oxidation state of protein thiols are difficult to detect against a substantial background signal. Sample preparation can also limit accuracy as tissue proteins can be difficult to solubilize [15–19]. Furthermore, protein solubilization procedures may cause artifactual

Abbreviations: ANOVA, analysis of variance; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; FLm, FL-*N*-(2-aminoethyl)maleimide; NEM, *N*-ethylmaleimide; ROS, reactive oxygen species; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TCEP, tris(2-carboxyethyl) phosphine; TRm, Texas red maleimide.

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oxidation of protein thiols because of their highly reactive nature [20–22].

Here we describe a fluorescent dual labeling technique for the direct quantitative measurement of both the reduced and the oxidized protein thiols in skeletal muscle. A number of strategies are described to improve protein solubilization and prevent artifactual oxidation during extraction and analysis. The technique utilizes a 96-well plate assay to determine the global protein thiol oxidation state of a complex protein sample. Furthermore, it can be extended, using techniques such as SDS–PAGE, to measure the thiol oxidation state of specific protein bands within a tissue sample.

Materials and methods

Animals

Male dystrophic (mdx) mice and nondystrophic control (c57) mice were purchased from the Animal Resources Centre, Western Australia. Mice were housed in the preclinical animal facility in accordance with the guidelines of the University of Western Australia and the National Health and Medical Research Council of Australia animal ethics. For control tests, muscle samples were taken from the hind limbs of male c57 mice. The oxidation state may differ between control experiments because of the biological variation between individual samples.

Materials

Ethanol (99.5%) and acetone (99.5%) were purchased from Redox Chemicals. Double-deionized water was used throughout. Protein molecular weight standards were purchased from Bio-Rad (Australia). Unless otherwise stated, all chemicals and reagents were obtained from Sigma–Aldrich (Castle Hill, Australia).

Dual labeling assay for the measurement of the thiol oxidation/reduction state of proteins

Sample preparation

Weighed muscle samples were crushed with a pestle and mortar submerged in liquid nitrogen. Ice-cold 20% TCA/acetone (20% w/v) was added, yielding a wet weight muscle concentration of 20 mg/ml. The sample was homogenized (Ultra-Turrax T25; Rose Scientific) on maximum for 15 s to produce an even suspension and then incubated for at least 1 h at −20 °C. To remove TCA, a 50-μl aliquot was taken and 1.5 ml of acetone (precooled to −20 °C) was added. The sample was vortexed and then centrifuged for 5 min at 8000 *g* at 4 °C. The supernatant was removed, leaving the protein pellet undisturbed. This washing procedure was repeated twice more, first with 1.5 ml of acetone and then with 1.5 ml of ethanol.

Labeling of reduced thiols

After TCA removal, the resulting protein pellet was solubilized in 50 μl of SDS buffer (0.5% SDS, 0.5 M Tris, pH 7.0) containing 540 μM BODIPY FL-*N*-(2-aminoethyl)maleimide (FLm; Invitrogen). The protein pellet was solubilized by sonication (on ice) until the pellet was completely dispersed. Care was taken during sonication to avoid frothing. Samples were then centrifuged at 8000 *g* for 5 min (4 °C) and the supernatant was retained. Then, another 50 μl of SDS buffer containing 540 μM FLm was added to the pellet, sonication and centrifugation were repeated as before, and the entire pellet was dispersed into a clear fluorescent solution. The supernatant was removed, and the two supernatants were combined. The FLm labeling reaction was continued for 30 min at room temperature in the dark, as the fluorescent tags are light sensitive.

To remove excess FLm, 1.5 ml of ethanol precooled to −20 °C was added to the FLm-labeled protein extract, briefly vortexed, and incubated at −20 °C for at least 1 h to precipitate proteins. Samples were then centrifuged for 5 min (8000 *g*, 4 °C), and the supernatant was removed, leaving the resulting protein pellet undisturbed. The ethanol rinse was repeated, and the protein pellet was resuspended in 100 μl of SDS buffer and vortexed until completely dissolved.

Samples were standardized according to their FLm (i.e., reduced protein thiol) concentration. With this approach, samples could be read at similar points along the fluorescent standard curves, thereby improving precision. Samples were assayed for FLm by aliquotting 10 μl into a 1.5-ml centrifuge tube and diluting with 310 μl of 0.1 M NaOH. An aliquot (100 μl in triplicate) was read against an FLm standard curve using a fluorescent plate reader (Fluostar Optima; BMG Labtech, Germany), with the excitation and emission wavelengths set at 485 and 520 nm, respectively. All samples were diluted to the same FLm concentration (between 50 and 100 μM) using SDS buffer.

A 50-μl FLm-labeled aliquot was reduced with the addition of 4 μl of SDS buffer containing 50 mM tris(2-carboxyethyl)phosphine (TCEP; pH 7.0). The sample was vortexed and then incubated for 60 min at room temperature in the dark. After reduction, Texas red maleimide (TRm; Invitrogen) labeling was performed by first diluting the TCEP concentration of the sample with the addition of 50 μl of SDS buffer. Then, 5 μl of 5 mM TRm was added, the sample was vortexed briefly and then incubated for 60 min at room temperature in the dark. Excess TRm was removed with the addition of 400 μl of ethanol and the samples were incubated for at least 60 min at −20 °C to precipitate proteins. The samples were then centrifuged for 5 min (8000 *g* at 4 °C) and the supernatant (and excess dye) was discarded. The sample was resuspended in 100 μl of SDS buffer and the ethanol rinsing procedure was performed twice more. After the third rinse, the remaining protein pellet was resuspended in 100 μl of SDS buffer before being assayed for FLm and TRm.

A standard curve for FLm and TRm was constructed by adding 16 μl of 1.5 mM dye to 384 μl of ovalbumin solution (20 mg/ml in SDS buffer) and then diluting with SDS buffer. The assay was performed by diluting 10 μl of sample or standard with 310 μl of 0.1 M NaOH, and 100 μl was assayed. Fluorescence was measured using a fluorescence plate reader (Fluostar Optima; FLm, ex 485 nm, em 520 nm; TRm, ex 595 nm, em 610 nm). The concentrations of FLm and TRm in the samples were calculated from a second-order polynomial standard curve. The percentage of oxidized protein thiols was calculated from the equation TRm concentration/(TRm concentration + FLm concentration) × 100.

Protein assay

The final protein concentration of the sample was determined using a modified Bio-Rad DC protein assay. The working reagent A′ and the sample were diluted twofold with double-deionized water. The assay was performed by pipetting 20 μl of sample, 70 μl of reagent A′, and 170 μl of reagent B into a clear, flat-bottom 96-well plate. Absorbance was measured at 750 nm and the protein concentration of samples was calculated using linear regression.

Ellman's assay

Ellman's assay was performed using a modified Ellman's method [13]. Crushed muscle samples were subjected to 20% TCA/acetone treatment and solubilization in SDS buffer as described previously, with the exception that no FLm was added. Samples were first diluted 1/3 with double-deionized water before being measured for reduced protein thiols and protein concentration. For the Ellman assay, 100 μl (in triplicate) was aliquotted into a 96-well plate and 100 μl of Ellman's reagent (DTNB) was added before being incubated at room temperature for 15 min and measured for absorbance at 412 nm. A molar extinction coefficient of 14,150 M^{-1} cm^{-1} was used [23–25]. A