

Screening for increased protein thiol oxidation in oxidatively stressed muscle tissue

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Abstract

Elevated oxidative stress can alter the function of proteins through the reversible oxidation of the thiol groups of key cysteine residues. This study evaluated a method to scan for reversible protein thiol oxidation in tissue by measuring reduced and oxidized protein thiols. It assessed the responsiveness of protein thiols to oxidative stress *in vivo* using a dystrophic (mdx) mouse model and compared the changes to commonly used oxidative biomarkers. In mdx mice, protein thiol oxidation was significantly elevated in the diaphragm, gastrocnemius and quadriceps muscles. Neither malondialdehyde nor degree of glutathione oxidation was elevated in mdx muscles. Protein carbonyl content was elevated, but changes in protein carbonyl did not reflect changes in protein thiol oxidation. Collectively, these data indicate that where there is an interest in protein thiol oxidation as a mechanism to cause or exacerbate pathology, the direct measurement of protein thiols in tissue would be the most appropriate screening tool.

Keywords: Protein thiol, muscle, mdx, glutathione, carbonyl

Abbreviations: DMD, Duchenne's muscular dystrophy; DNPH, 2,4-dinitrophenylhydrazine; E, Reduction potential of GSSG/GSH; ex, Excitation; em, Emission; GSH, Reduced glutathione; GSSG, Oxidized glutathione; HCl, Hydrochloric acid; HClO₄, Perchloric acid; HPLC, High performance liquid chromatography; MDA, Malondialdehyde; mdx, Mutant dystrophin deficient x-linked; MEKK1, MAPK/ERK kinase kinase 1; PSH, Reduced protein thiol; PSox, Oxidized protein thiol; PSox%, Percentage protein thiol oxidation; PStot, Total protein thiol; ROS, Reactive oxygen species; SDS, Sodium dodecyl sulphate; SE, Standard error of the mean; TBA, Thiobarbituric acid; TCA, Trichloroacetic acid; TCEP, Tris(2-carboxy-ethyl)phosphine hydrochloride; Tris, Tris[hydroxymethyl]aminomethane.

Introduction

During physiological homeostasis, an overall oxidative balance is maintained in tissues by utilizing a variety of antioxidant systems to remove reactive oxygen species (ROS) generated from a variety of sources. Disruption of this oxidative balance, often referred to as oxidative stress, is evident in many chronic disease states including muscle wasting conditions and may contribute to the pathology [1]. However, oxidative stress is a broad descriptor and so the biological response will vary depending on the specific ROS involved.

Two broad modes of action can be identified for ROS. One involves irreversible damage to macromolecules such as DNA, membrane lipids and proteins. A number of analytical techniques have been developed to measure the oxidation products directly (e.g. carbonyl assay for oxidized proteins) or the resultant degradation products (e.g. malondialdehyde for lipid peroxidation) [2]. These oxidation products can be used as biomarkers in tissue or plasma to monitor the irreversible consequences of oxidative stress in animal models [3,4] and humans [5,6].

An alternate mode of action of ROS involves altering protein function through reversible thiol

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modifications of protein cysteine residues. Reversible protein thiol oxidation has been demonstrated in proteins with a range of functions including signal transduction, ion transport, contractility, metabolism, protein synthesis and protein catabolism [1,7]. Alterations in protein function caused by protein thiol oxidation can profoundly affect cell function. For example, changes in the thiol oxidation state of the signal transduction protein MEKK1 has been linked to increased apoptosis [8].

Several proteomic methods have been developed to identify specific proteins modified by oxidative stress [7,9,10]. These methods are generally time consuming, require specialized equipment and involve particular technical skills. Consequently, a biomarker of protein thiol oxidation in tissue would be a useful screening tool in many biomedical experimental and clinical situations. We have developed a sensitive technique to measure the status of reversible global protein thiol oxidation in tissue by labelling both reduced and oxidized (following reduction with a disulphide reducing agent) protein thiols with two different fluorescent tags in the same tissue sample [11]. The result is a highly sensitive ratiometric measure that is capable of detecting small changes in the oxidative state of protein thiols. This simple technique is suitable for analysing large numbers of biological samples to assess conditions or pathological states in which protein thiol oxidation state may change.

In this study, our objective was to establish whether measuring global protein thiol oxidation would be a useful biomarker of protein thiol oxidation *in vivo*. Our first aim was to measure the extent to which protein thiols were oxidized *in vivo*. We utilized muscle tissue from mice because we have expertise in working with *in vivo* mouse models of muscle disorders [12]. Our second aim was to establish whether a global assay for protein thiols would be sufficiently responsive to oxidative stress *in vivo*. We used muscle from mdx mice since oxidative stress has been reported in dystrophic muscle [3,13] and mdx mice are a model of the human X-linked Duchenne muscular dystrophy (DMD) caused by mutations in the dystrophin gene [12]. Our third aim was to assess whether other commonly used biomarkers of oxidative stress could be used as alternatives to the direct measurement of protein thiol oxidation. In particular, we examined whether changes in the degree of glutathione oxidation, a commonly used measure of thiol redox status, reflected changes in the level of protein thiol oxidation.

Methods

Animals

Dystrophic male mdx (C57Bl/10ScSn^{mdx/mdx}) mice and non-dystrophic control C57 (C57Bl/10ScSn) mice (the parental strain for mdx) were purchased

from the Animal Resources Centre, Western Australia. Mice were housed in the pre-clinical animal facility at the University of Western Australia (UWA) and all procedures were conducted in accordance with the Animal Ethics guidelines and approvals of the National Health and Medical Research Council of Australia and UWA. Mdx and C57 mice were sacrificed at 24 days and 12 weeks of age. Mice were anaesthetized (2% v/v Attane isoflurane, Bomac, Australia) and were maintained under general anaesthesia as the blood and muscles were collected. Blood was centrifuged at 8000 g for 10–15 min to obtain plasma. The muscles were placed into polyethylene tubes and immediately quenched in liquid nitrogen. All samples were stored at -80°C until analysis. The muscles used in this study were quadriceps, gastrocnemius, triceps and diaphragm.

Materials

2,4-dinitrophenyl hydrazine, 1,1,3,3-tetraethoxypropane, guanidine hydrochloride, dansyl chloride, thiobarbituric acid, trichloroacetic acid, butylated hydroxytoluene, tris(2-carboxy-ethyl)phosphine hydrochloride, iodoacetic acid, γ -glutamylglutamate (γ -Glu-Glu), GSH and GSSG were obtained from Sigma-Aldrich Chemical (St Louis, MO). Potassium hydroxide, sodium acetate trihydrate, potassium dihydrogen phosphate, perchloric acid and hydrochloric acid were purchased from Analar (Victoria, Australia). Tris(hydroxymethyl)aminomethane was from Chem-Supply (Gillman, Australia). HPLC grade methanol, butanol and chloroform were from LAB-SCAN (Bangkok, Thailand). BODIPY FL-N-(2-aminoethyl) maleimide and TEXAS RED-C2-maleimide were from Invitrogen (Victoria, Australia). Bovine serum albumin was from Roche Diagnostics (Indiana, USA).

Protein carbonylation. Protein carbonyl content was determined using 2,4-dinitrophenylhydrazine (DNPH) as described by Hawkins et al. [14] and Levine et al. [15]. In brief, muscles were homogenized on ice in 20% TCA in acetone, the protein pellets were precipitated by centrifugation at 8000 g for 10 min at 4°C , pellets were dried and resuspended in 10 mM DNPH in 2 M HCl and incubated for 30 min at room temperature in the dark. Proteins were washed with ethyl acetate/ethanol (1:1) and dissolved in 6 M guanidine hydrochloride and absorbance was measured at 370 nm. Protein concentration (mg/ml) was determined using the Bio-Rad protein assay. Carbonyl concentrations are expressed as nmol of carbonyl per mg soluble protein.

Lipid peroxidation measured by malondialdehyde (MDA). Muscles were homogenized in 0.1 M HClO_4 as an

aqueous acid extraction medium [16]. Sample supernatant (150 μ l) or MDA standard was derivatized by thiobarbituric acid (TBA) at 50°C for 1 h as described by Agarwal and Chase [17]. After cooling, 200 μ l of butanol was added and mixed vigorously. The butanol layer was separated by centrifugation at 8000 g for 5 min, transferred to autosampler vials and analysed by HPLC (UltiMate 3000 LC system, Dionex). HPLC was used to detect the (TBA)₂-MDA adduct because of its high analytical sensitivity and specificity [18]. HPLC was performed on a reverse phase column (Acclaim 120; C18 column; 5 μ m; 4.6 \times 150 mm; Dionex) as described by Agarwal and Chase [17]. The MDA stock standard solution was prepared following the procedure of Wang et al. [19]. The limit of detection was 0.02 μ M (based on three standard deviations of the blank measurements), with MDA concentrations in tissue samples ranging between 0.3–0.9 μ M. All samples were run in duplicate and the level of MDA was expressed as nmol/mg soluble protein.

Glutathione oxidation. Tissue was homogenized in liquid nitrogen in the presence of 5% HClO₄ and the homogenates centrifuged at 8000 g for 10 min at 4°C. An aliquot (300 μ l) of the resulting supernatant was derivatized by dansyl chloride solution as described by Jones et al. [20]. Chloroform (300 μ l) was added and mixed vigorously. The aqueous layer was separated by centrifugation at 8000 g for 5 min and transferred to autosampler vials and analysed by HPLC, as described by Jones et al. [20]. HPLC was performed on a Luna 3 μ m NH₂ 100A column (100 \times 20 mm; Phenomenex). All samples were run in duplicate and the amounts of GSH and GSSG were obtained by integration relative to the internal standard (γ -Glu-Glu). Glutathione content was measured in the quadriceps, triceps and gastrocnemius muscles, but not diaphragm as there was insufficient muscle following assays for protein thiols and protein carbonyl.

Two tag fluorescence labelling of thiol proteins. The fluorescent two-tag labelling technique involved the sequential labelling of reduced and oxidized protein thiol groups using two separate fluorescent tags on the same protein sample [11]. The protein pellets were extracted with 20% TCA in acetone as previously described, then re-suspended in 50 μ l of SDS buffer (0.5% SDS and 0.5 M Tris pH 7.0), in the presence of 0.5 mM BODIPY FL-N-(2-aminoethyl) maleimide (tag1) in the dark. All samples were sonicated and incubated for 30 min at room temperature in the dark with continuous vortexing. After centrifuging, the labelled protein solution was precipitated with cold ethanol to remove excess dye (tag 1), then re-dissolved in 50 μ l of SDS buffer (pH 7.0). Samples were reduced with the addition of 4 mM tris (2-carboxy-ethyl) phosphine hydrochloride (TCEP)

and incubated for 1 h at room temperature in the dark. After reduction, samples were labelled with 0.25 mM Texas red - C2-maleimide (tag 2) and incubated for 30 min at room temperature in the dark. Ethanol precipitation and re-solubilisation in SDS buffer was repeated four times to remove excess un-reacted dye (tag 2). Samples were re-solubilized in 100 μ L of SDS Buffer and the intensity of the fluorescence was measured for both tag 1 and tag 2 (tag 1: λ_{ex} 485 nm, λ_{em} 520 nm, and tag 2: λ_{ex} 595 nm, λ_{em} 610 nm). Ovalbumin pre-labelled with a known quantity of tag 1 and 2 was used as a standard to quantify labelled protein thiols. Protein concentration (mg/ml) was determined using the Bio-Rad DC Protein Assay. The percentage of protein thiol oxidation was expressed as the concentration of oxidized thiols (tag 2) to total protein thiols (tag 1 and tag 2). The limit of detection was 0.05 μ M, based on three standard deviations of the blank measurements [21], with PSox concentrations in tissue samples ranging between 1.3–4.7 μ M. The inter-assay coefficient of variation was 8% with an intra-assay coefficient of variation of 6%.

Statistical methods

All data are expressed as mean \pm SE ($n=8$ unless otherwise stated). Statistical analyses used GraphPad InStat software (version 3.06). Means were compared using *t*-tests or one-way ANOVA with Tukey post-hoc testing where appropriate and differences were considered to be statistically significant at *p* less than 0.05.

Results

Protein thiol content in muscles of adult (12 week) mice

To generate baseline data, reduced (PSH) and oxidized (PSox) protein thiol content was measured in the quadriceps, gastrocnemius, triceps and diaphragm muscles of male C57 mice (Figure 1). There were significant differences in reduced protein thiol content between muscle types, ranging from 23 nmol/mg in triceps to 39 nmol/mg in gastrocnemius muscles (Figure 1A). Oxidized protein thiol content was ~10-times lower than reduced protein thiol content (Figure 1B). Oxidized protein thiol content was comparable in quadriceps, gastrocnemius and diaphragm muscles, but 40% lower ($p < 0.05$) in triceps muscles (Figure 1B).

Two calculated parameters were also examined: total protein thiol content (PStot = PSH + PSox) and percentage protein thiol oxidation (PSox% = {PSox/[PSH + PSox]} \times 100). Total protein thiol content (reduced plus oxidized) ranged from 25–45 nmol protein thiol/mg protein (Figure 1C). Total protein thiol content was comparable for quadriceps and

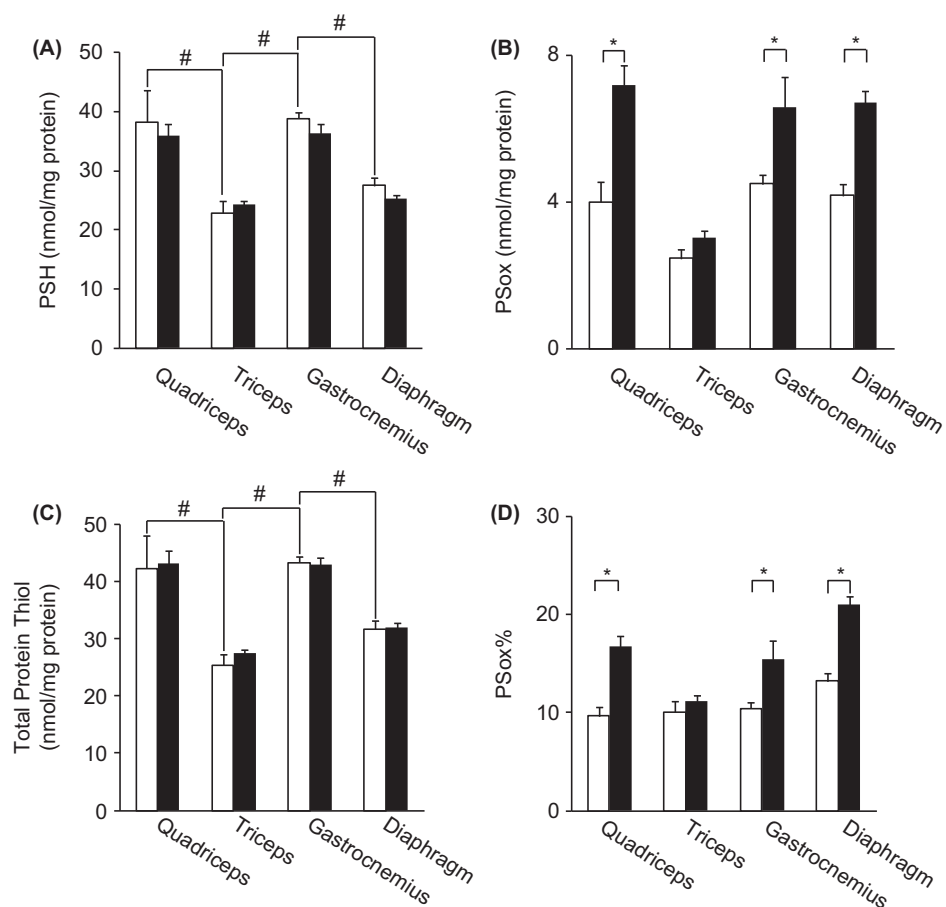


Figure 1. Protein thiol content in C57 and mdx muscles. (A) Reduced protein thiol content (PSH), (B) oxidized protein thiol content (PSox), (C) Total protein thiol content and (D) percentage protein thiol oxidation (PSox%). Bars are means \pm SE of eight C57 male mice (blank bars) and eight mdx male mice (black bars), at 12 weeks of age. #Significant differences ($p < 0.05$) between various C57 muscles (A and C). *Significant differences ($p < 0.05$) between C57 and mdx mice (B and D).

gastrocnemius muscles, but was significantly lower for triceps and diaphragm muscles (Figure 1C). The percentage of protein thiol oxidation ranged from ~10–13% for the different muscles from male C57 mice (Figure 1D). Overall, these data indicated that total protein thiol content could vary between muscle types and that protein thiols were primarily in the reduced form in resting normal mouse skeletal muscles.

There is evidence that oxidative stress occurs in the muscles of mdx mice, but the extent to which this affects protein thiol oxidation in mdx mice has not been assessed [3,13,22]. We therefore examined four different muscles (quadriceps, gastrocnemius, triceps and diaphragm) from mdx mice for evidence of protein thiol oxidation. When reduced protein thiol content was compared, there were no significant differences between muscles of C57 and mdx mice (Figure 1A). However, oxidized protein thiol content was significantly higher for diaphragm, gastrocnemius and quadriceps muscles of mdx mice (Figure 1B). These differences were reflected in the calculated parameter of percentage protein thiol oxidation which ranged from ~15–21% in the diaphragm, gastrocnemius and quadriceps muscles of mdx mice (Figure 1D).

Elevated protein thiol oxidation was not evident in all muscle types, as the percentage protein thiol oxidation in triceps muscles from mdx and C57 mice were comparable (Figure 1D). The increase in percentage protein thiol oxidation for muscles from mdx mice reflected an increase in oxidized protein thiol content (Figure 1B). As total protein thiols did not significantly increase (Figure 1C), it is possible that the increase in oxidized protein thiol content reflected the conversion of reduced protein thiols to oxidized protein thiols.

Glutathione oxidation in muscles of adult (12 week) mice

The ratio of oxidized glutathione (GSSG) to reduced glutathione (GSH) has been used to indicate the non-protein thiol redox state of tissue [23]. To assess whether the non-protein thiol redox state of tissue could be used to report changes in protein thiol redox state we measured glutathione in gastrocnemius, quadriceps muscle and triceps muscles of C57 mice and mdx mice. Reduced glutathione (GSH) content between muscle types ranged from ~4.4–6.3 nmol/mg

protein (Figure 2A). Oxidized glutathione (GSSG) and percentage of oxidized glutathione (GSSG%) were comparable in quadriceps and triceps muscles, but significantly higher ($p < 0.05$) in gastrocnemius muscles (Figures 2B and D).

GSH, GSSG and total glutathione in gastrocnemius, quadriceps and triceps muscles were comparable between mdx and C57 mice (Figures 2A–C). To establish whether there were any relationships between the degree of glutathione oxidation and

protein thiol oxidation, we used regression analysis of all muscle types for the following associations: percentage protein thiol oxidized to percentage oxidized glutathione, $r^2 = 0.004$, $n = 48$ (Figure 2E); GSH (nmol/mg protein) to protein thiol (nmol/mg protein), $r^2 = 0.013$, $n = 48$; GSSG (nmol/mg protein) to oxidized protein thiol (nmol/mg protein), $r^2 = 0.013$, $n = 48$; and GSSG/GSH ratio to PSox/PSH ratio, $r^2 = 0.005$, $n = 48$. For all relationships tested, there were no significant correlations, indicating

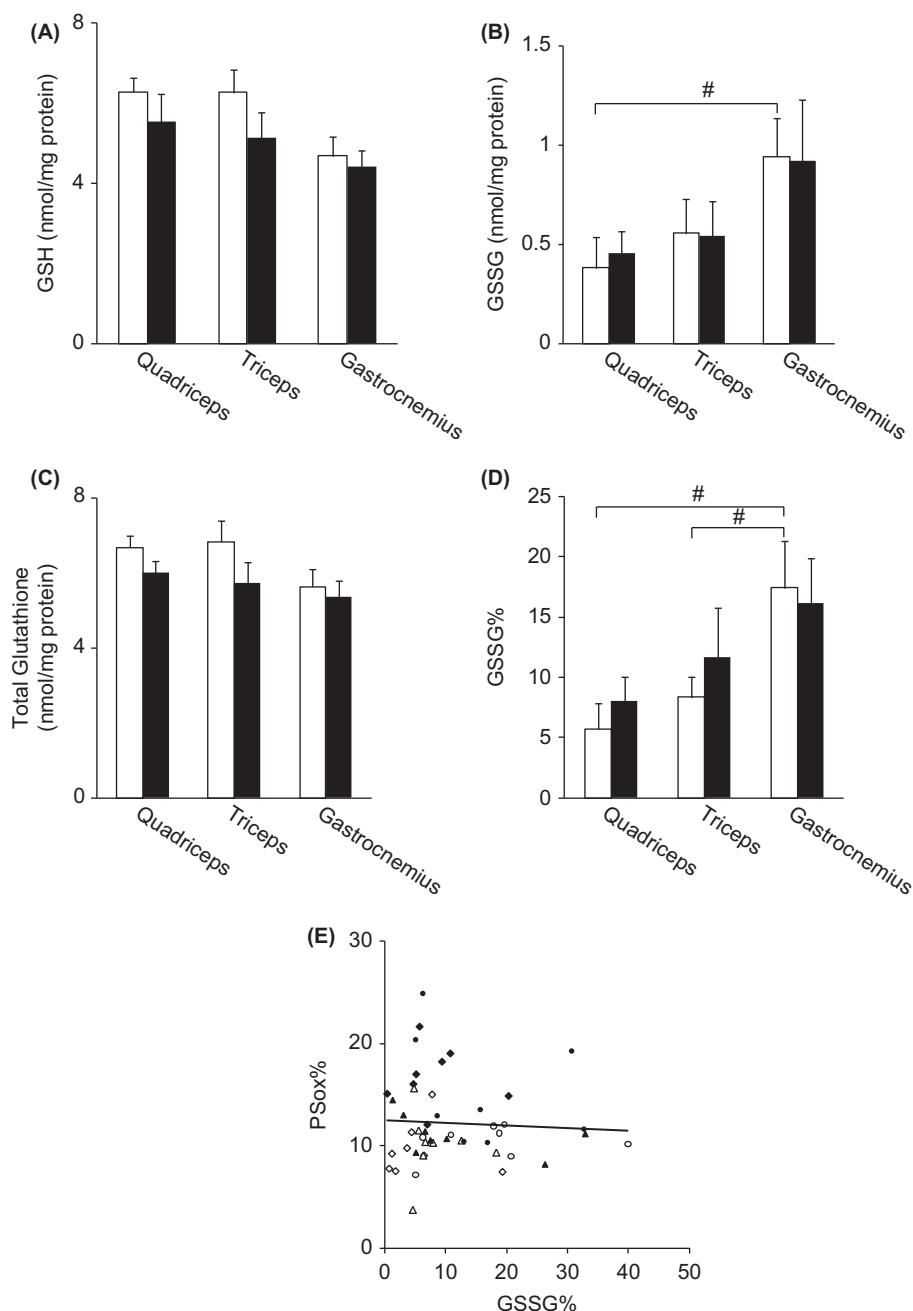


Figure 2. Glutathione content in C57 and mdx muscle tissues. (A) Reduced glutathione content (GSH), (B) oxidized glutathione content (GSSG), (C) Total glutathione (GSSG + GSH) and (D) percentage glutathione oxidation (GSSG%). Bars are means \pm SE of eight C57 (blank bars) and eight mdx (black bars) male mice at 12 weeks of age. #Significant differences ($p < 0.05$) between various C57 muscles (B and D). There were no significant differences between C57 and mdx mice. (E) Relationship (regression line shown, $r^2 = 0.004$, $p = 0.68$) between percentage of protein thiols oxidized (PSox%) and percentage of glutathione oxidized (GSSG%). Data are shown for gastrocnemius (O, ●), triceps (Δ , \blacktriangle) and quadriceps (\diamond , \blacklozenge) muscles from C57 (O, Δ , \diamond) and mdx mice (●, \blacktriangle , \blacklozenge) at 12 weeks of age.

that changes in protein thiol redox were independent of changes in glutathione oxidation.

Protein carbonyls in muscles of adult (12 week) mice

As oxidative stress can result in irreversible damage to proteins, we examined whether there was any relationship between oxidative damage of proteins and the degree of reversible protein thiol oxidation. The protein carbonyl assay [15,23] is commonly used as an indicator of oxidative damage to proteins. For adult C57 mice, protein carbonyl content in muscles ranged from ~1.8–2.5 nmol/mg protein, which is in agreement with previously published data [4,24] (Figure 3A).

In mdx mice, protein carbonyls were significantly elevated in the diaphragm, gastrocnemius, quadriceps, but not triceps muscles (Figure 3A). The extent to which protein damage occurred in the various muscles from mdx mice did not consistently mirror the extent to which protein thiol oxidation was elevated in muscles of mdx mice. Maximum protein thiol oxidation occurred in the diaphragm, whereas protein carbonylation was maximal in the gastrocnemius and quadriceps muscles. Furthermore, there was no apparent relationship between percentage protein thiol oxidation and carbonyl content ($r^2 = 0.008$, $n = 78$; Figure 3B). These data indicate there was no direct relationship between the degree of protein oxidative damage and the level of protein thiol oxidation.

Malondialdehyde in muscles and plasma of adult (12 week) mice

Malondialdehyde (MDA) is generated as a consequence of peroxidation damage to membrane lipids and is a commonly used marker of oxidative stress. We

examined whether changes in MDA content were associated with changes in protein thiol oxidation. MDA content was comparable between C57 and mdx mice for gastrocnemius, quadriceps and triceps muscles (Figure 4A). These data imply that lipid peroxidation was not elevated in mdx muscles in contrast with the elevated protein thiol oxidation and elevated protein carbonyl content in muscles of mdx mice. MDA measured in plasma is also used as a guide to oxidative stress [25]. We measured MDA in plasma and found that the plasma MDA content was comparable between C57 and mdx mice, a result consistent with the muscle measurements (Figure 4B). We conclude that there is not necessarily an association between protein thiol oxidation and malondialdehyde content.

Oxidative stress in young (24 day old) mice

Mdx mice are characterized by an acute period of high myofibre necrosis and subsequent regeneration beginning at ~21 days after birth and decreasing by 6 weeks to a low level in adult mice [12,26]. Oxidative stress has been hypothesized to be a primary mechanism linking the underlying genetic defect with myofibre necrosis [13]. Consistent with this hypothesis, protein carbonyl content was 3-fold higher in quadriceps muscle from young (24 days) mdx mice relative to age-matched C57 control mice (Figure 5A). In contrast, MDA content was comparable between young C57 and mdx mice, as was seen for adult mice (Figure 5B).

We examined whether protein thiol oxidation was elevated at 24 days. Total protein thiol content was comparable between mdx and C57 mice at 24 days (Figure 5C), as for adult mice (Figure 1C). The percentage oxidized protein thiol content was 2-fold

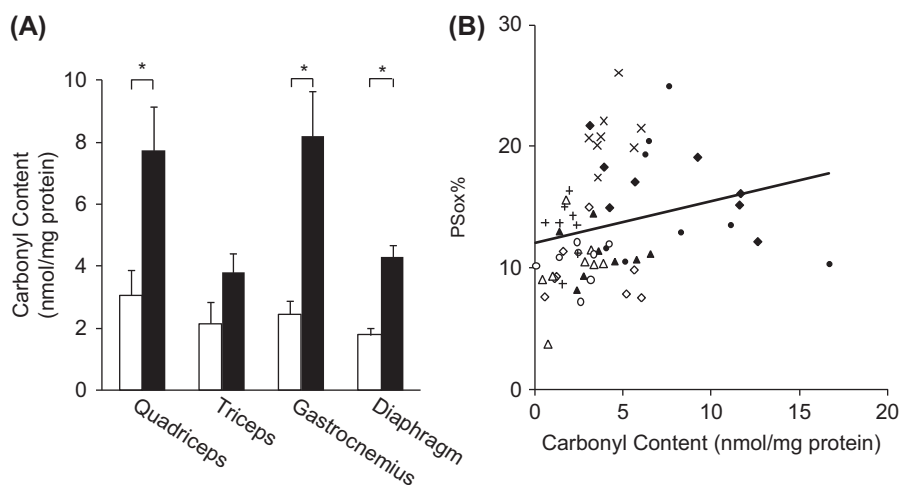


Figure 3. Protein carbonyl content in C57 and mdx muscles. (A) Bars are means \pm SE of eight C57 (blank bars) and eight mdx (black bars) male mice at 12 weeks of age. *Significant differences between C57 and mdx mice ($p < 0.05$). (B) Relationship (regression line shown, $r^2 = 0.05$, $p = 0.063$) between percentage of protein thiols oxidized (PSox%) and protein carbonyl content (nmol/mg protein). Data are shown for gastrocnemius (○, ●), triceps (△, ▲), quadriceps (◇, ◆) and diaphragm (+, ×) muscles from C57 (○, △, ◇, +) and mdx mice (●, ▲, ◆, ×) at 12 weeks of age.

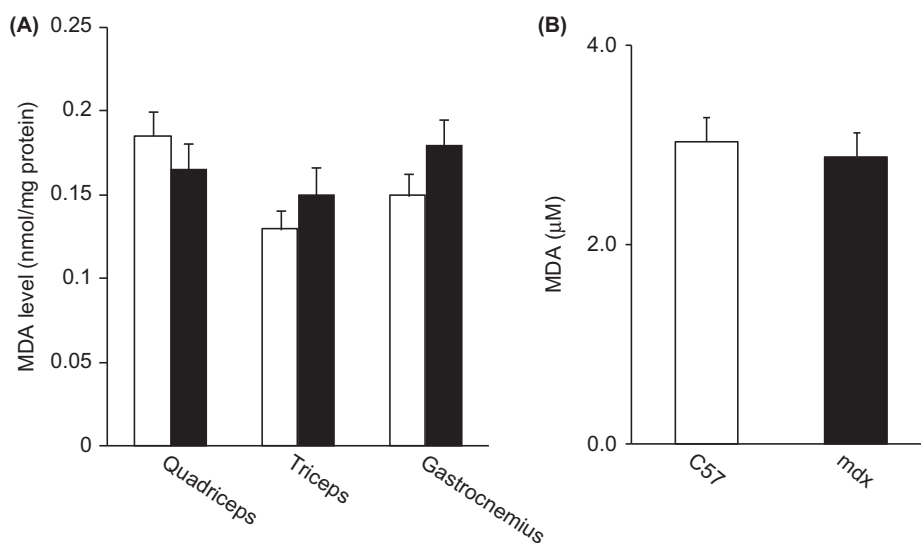


Figure 4. Malondialdehyde (MDA) content in muscle (A) and plasma (B) of 12 week old C57 and mdx male mice. Bars are means \pm SE of eight C57 (blank bars) and eight mdx (black bars) mice. There were no significant differences between C57 and mdx mice.

higher in young (24 days) mdx mice relative to age-matched C57 control mice (Figure 5D). This difference between strains was comparable to that seen at 12 weeks of age (Figure 1D). Together, these observations indicate that reversible protein thiol oxidation is a possible pathway by which oxidative stress could be contributing to muscle necrosis.

Discussion

Reduced protein thiols have previously been measured in skeletal muscle tissue [27–29] but, to our

knowledge, the magnitude of reversible protein thiol oxidation has not been previously quantified or compared with other measures of oxidative stress. This study established in control C57 mice that the level of reversible protein thiol oxidation was relatively consistent at 10–13% of total protein thiols for different muscle groups, despite variation in total protein thiols between different muscles. The higher level of protein thiol oxidation in mdx muscles, ~21%, indicates that such pathological conditions can affect protein thiol oxidation and it can be detected using a global measure.

Measuring both reduced and oxidized protein thiols will be useful biomarkers of the potential of oxidative stress to affect protein function through reversible thiol modifications of protein cysteine residues. Consequently, it would be informative to compare levels of protein oxidation measured by different laboratories for a range of diverse conditions and diseases. One approach would be to only report reduced protein thiol content and oxidized protein thiol content. However, data between different laboratories may not be directly comparable if, as commonly practiced, thiols are reported relative to protein content, wet tissue weight or dry tissue weight [30,31]. For protein thiols reported relative to protein content, the measured protein content may vary depending on the efficacy of protein extraction, the type of protein assay used and the particular protein used to generate a standard curve [32]. This may explain why our measured protein thiol content (39 nmol/mg protein, 12 weeks of age) is lower than a previous estimate [27] (~55 nmol/mg protein, 5–11 months of age) in gastrocnemius of quiescent mice.

An alternate approach to reporting oxidized protein thiol content is to express oxidized proteins thiols relative to total protein thiols. This has the advantage of eliminating variability introduced by a loss of

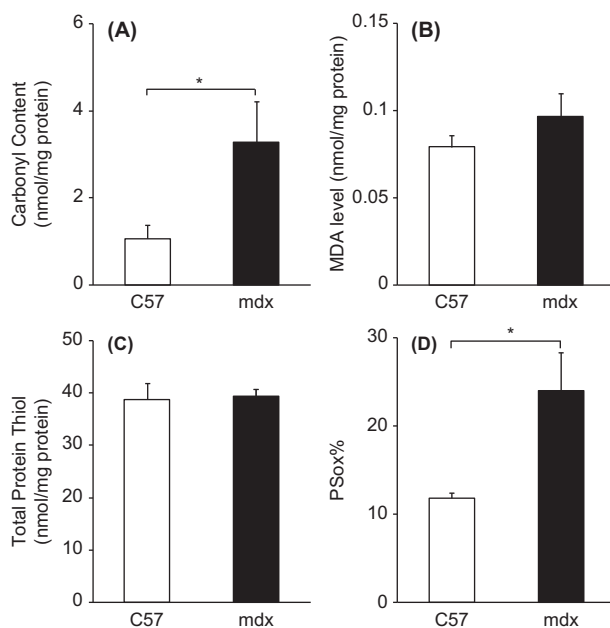


Figure 5. Oxidative stress in 24 day old C57 and mdx quadriceps muscle. (A) Protein carbonyl content, (B) MDA content, (C) total protein thiol content and (D) percentage protein thiol oxidation (PSox%). Bars are means \pm SE of eight C57 and eight mdx mice. *Significant differences between C57 and mdx mice ($p < 0.05$).

precision in measuring protein content or tissue weight and has the advantage that it is an easy concept to grasp. Reporting the percentage protein oxidized is analogous to reporting glutathione ratios (e.g. GSH/GSSG) [33,34]. For protein thiol oxidation the situation is much more complex because the susceptibility of protein thiols to oxidation depends, in part, on the micro-environment [35]. This interpretative approach is also reliant on total protein thiols not being affected by the pathological state. At least for the muscles of mdx mice, total protein thiols were comparable to total protein thiols in the muscles of normal mice of the same parental strain. Consequently, reporting percentage protein thiol oxidized appears to be a useful practical concept.

A widely used method of assessing the sensitivity of thiols to oxidative stress is to measure the relative oxidation of glutathione. Glutathione is a major thiol containing peptide in muscle tissue and serves multiple functions in protecting tissues from ROS and contributes to maintaining the reduced state of the intra-cellular environment [36]. The extent to which oxidative stress affects thiol oxidation in tissue has been assessed by measuring reduced and oxidized glutathione. Data are reported as the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) or a redox potential can be calculated from the Nernst equation [$E = -240 - (59.1/2) \log [(GSH)^2 / (GSSG)]$ at 25°C, pH 7.0] [33,34]. Based on thermodynamic considerations, it has been proposed that the degree of glutathione oxidation is a good indicator for thiol oxidation, although this concept has been challenged on theoretical grounds [37]. Our data show that protein thiol oxidation is more sensitive to oxidative stress than is glutathione oxidation, which means that the level of glutathione oxidation is not necessarily a useful guide to the extent of protein thiol oxidation in tissue.

Assays such as those for protein carbonyls or MDA have been used as generalized biomarkers of oxidative stress [6,23,38]. The differential response of protein carbonyls (which changed) and MDA (which did not) has also been observed previously, where protein carbonyls did not change but MDA increased following exercise [39]. These differential responses presumably reflect differences in the ROS and their targets [2]. However, neither measure was a useful biomarker for the extent of protein thiol oxidation in dystrophic tissues. Plasma MDA has also been used as a systemic marker of oxidative stress [6,25]. Our data indicate that plasma MDA did not reflect and therefore is not a suitable biomarker for protein thiol oxidation in muscle tissue. In a broader context, the lack of consistency between various biomarkers of oxidative stress has been noted in previous reports examining the use of biomarkers in plasma [6].

Overall, our data show that oxidative stress resulting from disease pathology (in this case, dystrophy) does

cause detectable changes in the level of protein thiol oxidation in muscle tissue. Our data also show that at least some of the generalized biomarkers of oxidative stress do not necessarily reflect the extent of protein thiol oxidation in tissue. Instead, where there is an interest in protein thiol oxidation, the direct measurement of protein thiols would be the most appropriate screening tool. Where changes in the level of global protein thiol oxidation are detected, further work using more specific proteomic techniques can be used to identify the particular proteins that have been modified by such oxidative stress [7,9,40,41].

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Declaration of interest

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