Research Paper

Fatiguing contractions increase protein S-glutathionylation occupancy in mouse skeletal muscle

Philip A. Kramer, Jicheng Duan, Matthew J. Gaffrey, Anil K. Shukla, Lu Wang, Theo K. Bammler, Wei-Jun Qian, David J. Marcinek

Abstract

Protein S-glutathionylation is an important reversible post-translational modification implicated in redox signaling. Oxidative modifications to protein thiols can alter the activity of metabolic enzymes, transcription factors, kinases, phosphatases, and the function of contractile proteins. However, the extent to which muscle contraction induces oxidative modifications in redox sensitive thiols is not known. The purpose of this study was to determine the targets of S-glutathionylation redox signaling following fatiguing contractions. Anesthetized adult male CB6F1 (BALB/cBy × C57BL/6) mice were subjected to acute fatiguing contractions for 15 min using in vivo stimulations. The right (stimulated) and left (unstimulated) gastrocnemius muscles were collected 60 min after the last stimulation and processed for redox proteomics assay of S-glutathionylation. Using selective reduction with a glutaredoxin enzyme cocktail and resin-assisted enrichment technique, we quantified the levels of site-specific protein S-glutathionylation at rest and following fatigue. Redox proteomics revealed over 2200 sites of S-glutathionylation modifications, of which 1290 were significantly increased after fatiguing contractions. Muscle contraction leads to the greatest increase in S-glutathionylation in the mitochondria (1.03%) and the smallest increase in the nucleus (0.47%). Regulatory cysteines were significantly S-glutathionylated on mitochondrial complex I and II, GAPDH, MDH1, ACO2, and mitochondrial complex V among others. Similarly, S-glutathionylation of RYR1, SERCA1, titin, and troponin I2 are known to regulate muscle contractility and were significantly S-glutathionylated after just 15 min of fatiguing contractions. The largest fold changes (> 1.6) in the S-glutathionylated proteome after fatigue occurred on signaling proteins such as 14-3-3 protein gamma and MAP2K4, as well as proteins like SERCA1, and NDUV2 of mitochondrial complex I, at previously unknown glutathionylation sites. These findings highlight the important role of redox control over muscle physiology, metabolism, and the exercise adaptive response. This study lays the groundwork for future investigation into the altered exercise adaptation associated with chronic conditions, such as sarcopenia.

1. Introduction

Oxidative modifications are implicated in both the pathological damage of oxidative stress and the physiological and adaptive responses to redox signaling. The thiol group on the amino acid cysteine is a major target for oxidative modification of proteins [1]. The reactive nature of the thiol group means that they also play an important regulatory role in protein structure through disulfide bond formation, cofactor binding, and in catalytic activity [2]. Glutathione is the most abundant antioxidant molecule within cells, especially the mitochondria [3,4]. It is an essential cofactor of glutathione peroxidase, glutathione S-transferase, and glutaredoxin for the scavenging of hydrogen peroxide and the reversal of oxidative modifications to proteins [3]. Reduced or oxidized glutathione can react with oxidized protein thiols or thiolate anions to form protein S-glutathionylation (P-SSG) modifications through several enzymatic and non-enzymatic mechanisms [5]. Glutathione can also react with oxidized derivatives of protein cysteines, such as sulfenic acid (SOH), thiol radicals (S·), or S-nitroso...
(-SNO), thereby converting these oxidized forms to a more stable modification [6–8] and preventing further thiol oxidation to sulfenic and sulfonic acid. This is significant because sulfenylation and sulfo-
nylation are largely irreversible modifications with the exception of reduction of 2-Cys PRX sulfenylation by sulfiredoxin [9,10]. In addition to this protective effect, P-SSG is considered an important type of oxi-
dation modification that regulates transcription, mitochondrial meta-
bolism, apoptosis, and other critical processes [6,11]. Glutaredoxin is 
the primary enzyme responsible for the reversal of this modification and returning oxidized cysteines to their original thiol (SH) redox status [12].

Contraction of skeletal muscle results in an immediate and transient 
increase in oxidants produced by NADPH oxidase and xanthine oxidase 
[13–18]. The oxidants produced during exercise interact with redox-
sensitive signaling pathways such as P-38/MAPK, NFκB, AMPK, and 
NRF2, to promote exercise recovery, biogenesis, protein turnover, and 
cytotoxic and antioxidant responses [13,19–21]. In muscle, these 
oxidants are implicated in the regulation of metabolism, calcium 
homeostasis and sensitivity of contracting fibers through proteins like 
SERCA, troponin I, ATP synthase, and mitochondrial complexes 
[17,19,22,23]. Efforts to reduce the amount of oxidants generated with 
exercise have occasionally resulted in decreased protection against 
subsequent oxidative events (ischemia), injury, and adaptive signaling, 
emphasizing the importance of redox signaling in exercise [13,24,25]. 
In contrast, mitochondria-targeted antioxidants, such as elamipretide 
(SS-31) and AAV-mCAT, improve mitochondrial deficits, fatigue re-
sistance, and reduce oxidative stress in mice [26–28]. In addition, aged 
mice, where increased mitochondrial oxidant production leads to 
chronic oxidative stress, have an impaired adaptive response to con-
traction compared to younger mice [29,30].

The goal of this study was to determine the effect of fatiguing 
contractions on the redox sensitive S-glutathionylated proteome in 
order to provide new insights into fatigue-associated redox signaling 
involved in the muscle response to exercise. Given the evidence that the 
contraction-induced oxidant production is primarily from non-mi-
 tochondrial sources [18], we hypothesized that mitochondrial proteins 
would undergo relatively less redox modification than cytoplasmic and 
sarcosommal proteins following an acute bout of contraction. In this 
study, in vivo electrical stimulation of skeletal muscle was used to in-
duce fatiguing contractions in adult CB6F1 mice. Fatigue increased the 
P-SSG levels of many new and previously reported Cys sites on mi-
 tochondrial, sarcomeric, and calcium homeostasis proteins.

2. Materials and methods

2.1. All animal procedures described in this study were approved by the University of Washington IACUC

2.1.1. In-vivo stimulation

Five littermate male CB6F1 (BALB/cBy x C57BL/6) mice at eleven 
months of age were obtained from National Institute on Aging (NIA) 
and placed under isoflurane anesthesia just prior to and throughout 
the duration of the in vivo stimulation and recovery. The right leg of 
the mouse was secured at the knee and the foot taped to a footplate and 
force transducer (Aurora Scientific Inc. Ontario). Subdermal stimula-
tion electrodes were placed proximal and distal to the gastrocnemius. 
Stimulation voltage was optimized to produce maximum twitch force 
(20–22 V). Muscle was stimulated at 100 Hz for a 300 ms duration every 
fourth second for 15 min using a Grass stimulus (S88x, Astro-Med, 
Inc.), and torque force obtained by Aurora DMC software (V5.5), and 
analyzed by Aurora DMA software (V5.321). The muscle was then al-
lowed to rest for 1 h before the mouse was sacrificed and both the 
stimulated and unstimulated muscles were frozen in liquid nitrogen and 
stored at −80 °C (Fig. 1A). Briefly, maximal force production and the 
force-time integral for each tetanic stimulation was assessed throughout 
the 15 min of fatigue (Fig. 1B and C). The maximal rate of contraction 
and relaxation of each stimulation was measured over the course of 
fatigue (Fig. 1D, E).

2.1.2. Redox proteomics analyses of P-SSG

The redox proteomics experiments were performed as previously 
described for P-SSG [31–33]. Briefly, frozen mouse gastrocnemius 
muscles from stimulated and unstimulated legs from four mice were 
minced while frozen and incubated for 30 min on ice in the dark in the 
homogenizer buffer comprising 250 mM HEPES buffer pH 6.0, 1% v/
v SDs, 1% v/v Triton X-100 and 100 mM n-ethyl-maleimide (NEM) to 
block all free thiols. A small portion of tissues were pooled from the 
four unstimulated (C) and four stimulated (S) samples, respectively, for 
total thiol profiling (Fig. 2), in which tissue was incubated in the same 
homogenization buffer without NEM for thiol blocking. All tissue 
samples were then homogenized using a hand held homogenizer until 
completely homogenized. The resulting homogenate was pre-cleared by 
centrifugation at 14,000 rpm for 10 min at 4 °C. The 8 samples for P-
SSG profiling were further incubated with 2 mM sodium ascorbate, 
2 μM CuCl, and 1 mM SDS in the dark at 55 °C for selective reduction of 
nitrosylated cysteine residues and complete alkylation of free thiols. 
The NEM blocking and ascorbate reduction step was omitted for the 
two total thiol profiling samples. All 10 samples were then subjected to 
acetone precipitation overnight for complete removal of all excessive 
reactants such as detergents or NEM. Precipitated proteins were re-
suspended/solubilized in 250 mM HEPES buffer pH 7.0 containing 8 M 
urea and 0.1% SDS. Buffer exchange was performed twice using 
250 mM HEPES buffer pH 7.0 containing 8 M urea, resulting in a final 
volume of 30–40 μL. Protein concentrations were measured by the BCA 
assay. Approximately 480 μg of the protein solution was diluted to ~ 
500 μL by 1 M urea in 25 mM HEPES buffer, pH 7.6 and then subjected 
to selective reduction of 5-glutathionylated thiol residues using GRX1 
enzyme cocktail containing 2.5 μg/mL GRX1M (C145 mutant from E. 
coli), 0.25 mM GSSG, 1 mM NADPH, and 4 U/mL glutathione re-
ductase [32] for P-SSG or reduced with 20 mM DTT in 25 mM HEPES 
buffer pH 7.7 containing 2% v/v SDS for 30 min at 37 °C (Fig. 2A).

Following the reduction step, free thiol-containing proteins were 
practiced using Thiopropyl Sepharose 6B resin with 400 μg protein per 
sample for SSG-channels and 100 μg protein per sample for total thiol 
[32,34]. Following on-resin digestion, isobaric labeling with 10-plex 
tandem mass tag (TMT) reagents (Thermo Fisher Scientific) was per-
fomed (Fig. 2B). Briefly, 70 μL of anhydrous acetonitrile was added to 
the manufacturer-provided TMT reagent vials. Forty microliters of 
100 mM triethylammonium bicarbonate (TEAB) buffer pH 8.5 and the 
70 μL of the TMT reagent solutions were added to the resin containing 
peptides and the labeling reaction was carried out at room temperature 
for 1 h. The reaction was stopped by the addition of 8 μL of 5% 
NH₄OH/HCl in 200 mM TEAB buffer for 15 min. The excess TMT re-
agents were removed by washing five times each with 80% ACN with 
0.1% TFA and 25 mM ammonium bicarbonate. The captured, labeled 
peptides were eluted by DTT as previously described [20].

LC-MS/MS analysis of TMT-labeled cysteine-containing peptides 
was performed on an orbitrap fusion Lumos Mass spectrometer 
(Thermo Fisher Scientific). A Waters nanoACQUITY UPLC system with 
a custom packed C18 column (50 µm x 75 µm i.d., Phenomenex 
Jupiter, 3 µm particle size) and a 3h LC gradient was applied for peptide 
separation. Full MS spectra were recorded at resolution of 30 K over the 
range of m/z 400–2000 with an automated gain control (AGC) value of 
2 × 10⁵. MS/MS was performed in the data-dependent mode with or-
bitrap resolution of 30 K, an AGC target value of 1 × 10⁵, a normalized 
collision energy setting of 30 for high-energy collision dissociation 
(HCD), a dynamic cycle time of 3 s and a dynamic exclusion time of 60 s 
were used.

2.1.3. Data Analysis and statistics

LC-MS/MS raw data were converted into dta files using Bioworks 
Cluster 3.2 (Thermo Fisher Scientific), and MSGF plus algorithm [35]
Ingenuity Pathway Analysis software (IPA, http://www.ingenuity.com/) allowed for the identification of biologically relevant canonical pathways affected by stimulation. IPA core analysis was performed using a FDR cutoff of 0.05 and User Data Set was used as the Reference. Cysteine residues that corresponded to the same protein were consolidated using the maximum of absolute values of log Fold-Change. The significance values for the canonical pathways were calculated by right-tailed Fisher’s exact test (p < 0.05). The Cellular Compartment and Biological Process of the top 1290 P-SSG modifications (FDR ≤ 0.05) were identified using DAVID Bioinformatics Resources (https://david.ncifcrf.gov/).

Protein crystal structures were attained from Protein Data Bank (www.rcsb.org) using the accession number 2b05 for 14-3-3 gamma [36], 3ALO for MAP2K4 gamma [37], 5txb for NDUV2 and NDUV1 [38], and 3tlm for SERCA1 [39]. Figures were constructed using Molsoft software 3.8–6a (www.molsoft.com).

3. Results

Adult mice averaged a submaximal (100 Hz) tetanic force of 16.5 ± 1.3 millinewton-meters (mN-m) (mean ± SD) using the described in vivo stimulation protocol. Isometric fatiguing stimulations reduced the maximal torque force to 31% of initial force over the course of fatigue. Five adult (11 month) male CB6F1 mice were anesthetized and subject to a 15 min fatiguing protocol before a 1 h recovery and collection of the left and right gastrocnemius (A). 100 Hz submaximal stimulations for 300 ms every fourth second over fifteen minutes induced fatigue to approximately 30% initial force (B). The prolonged force output of type 2 fibers is observed for up to 2 min with force-time integration analysis (C). The maximum rate of contraction (D) and relaxation (E) during the 15 min of fatigue suggest a shift from fast-twitch to slow-twitch fibers.
Thiol-containing peptides were eluted from all 10 labeled samples and combined into one final sample for LC-MS/MS analysis such that each thiol-containing peptide was identified with 10 reporter ion intensities reflecting the abundance levels of P-SSG and total thiols for each condition. In total, over 2200 Cys sites of P-SSG modifications were identified and quantified across all 10 channels. The levels of P-SSG were significantly increased after fatigue for over half of the sites as illustrated in the volcano plot shown in Fig. 3A. P-SSG occupancy for each Cys site was estimated as the ratio of the average level of P-SSG (n = 4) over total thiol (n = 1 for the pooled sample) in percentage for each condition. As shown, the left unstimulated gastrocnemius had an average P-SSG occupancy of 4.5%, while the right stimulated gastrocnemius showed a 5.22% average occupancy (Fig. 3B). Despite the moderate increase, significant differences in terms of average occupancy were observed in different subcellular compartments. Of the protein Cys sites detected, with some multi-compartmental overlap, 588 sites were annotated as nuclear proteins, 1154 as cytosolic proteins, 333 as secreted proteins, 141 as endoplasmic reticulum proteins, 229 as cell membrane proteins, and 390 as mitochondrial proteins. The average change in P-SSG occupancy was 0.72%; however, mitochondria showed the most significant occupancy shift at an average of 1.03%, and nuclear proteins the lowest occupancy shift at an average of 0.47% (Fig. 3C). Additionally, secreted proteins had the largest baseline occupancy at 5.98%, while mitochondria had the smallest at 3.99%.

Ingenuity Pathway Analysis (IPA) revealed that the top significantly altered protein targets (p ≤ 0.05) were involved in sirtuin, cell junction, and actin cytoskeleton signaling, as well as mitochondrial dysfunction and oxidative phosphorylation (Fig. 4A). Sirtuin signaling encompassed many proteins associated with mitochondrial complex I and lactate dehydrogenase, among others associated with the regulation of NAD (Supplementary Table 1). S-glutathionylated proteins involved in actin cytoskeleton, cell junction, FAK, and ILK signaling are predominately cytoskeletal and sarcromeric proteins as well as several proteins associated with the MAPK pathway. Although IPA analysis indicates P-SSG of proteins in these pathways was altered by contraction, this analysis does not indicate if the P-SSG modification alters protein activity. The role of these modifications in protein function and redox signaling will require further study.

The significantly changed 1290 P-SSG sites (FDR ≤ 0.05) were assessed with DAVID Bioinformatics Resources for Biological Process and Cellular Compartment [41,42]. The Biological Processes of most affected proteins are associated with oxidative and glycolytic metabolism as well as muscle contraction and oxidative stress responses (Fig. 4B, Supplementary Table 2). The compartments to which the most affected proteins were localized include proteins extracellular to the muscle fibers, including exosomes, myelin sheath, blood micro-particle, and extracellular matrix (Fig. 4C, Supplementary Table 3). This is consistent with the observation that NADPH oxidase, a membrane protein, produces oxidants during exercise [18,24]. Mitochondria, however, was the second most oxidized compartment following fatigue.

Many studies have identified P-SSG sites as modulators of protein activity, several of which are discussed below and outlined in Table 1. However, new analytical approaches, like the ones described here, have allowed for the detection of proteins with novel P-SSG modifications. Some of these proteins were among the most affected by fatigue. We have outlined a selected list of these novel P-SSG sites in Table 2 and Fig. 5.

4. Discussion

Physical activity is one of the most effective interventions for the
prevention and treatment of muscle atrophy and promotes beneficial health outcomes in heart disease, diabetes, and many other pathologies [43,44]. Reactive oxygen/nitrogen species (ROS/RNS) generated during exercise modulate contractile function and participate in the exercise adaptive response through oxidative post-translational modifications of regulatory cysteines in skeletal muscle [9,14,19]. Protein S-glutathionylation in particular is a transient and reversible modification that modulates the function and structure of proteins and also prevents the subsequent irreversible oxidation of cysteines [20]. Many of the P-SSG sites identified in muscle are known to alter actin-myosin bridge formation, calcium homeostasis, and metabolism, and contribute to fatigue [40]. The goal of this study was to assess the S-glutathionylated proteome following fatiguing contractions. In addition to the detection of P-SSG sites known to result in physiological effects, proteomic analysis has revealed many novel sites susceptible to S-glutathionylation that may contribute to contraction induced redox signaling.

Compared to other post-translational modifications, such as phosphorylation, the number of known S-glutathionylation sites are small and their roles in regulating adaptive responses in skeletal muscle are less well-characterized. Despite the increasing effort to understand redox dependent signaling in skeletal muscle, the quantitative effect of muscle contraction on the S-glutathionylated proteome has yet to be assessed. Using a thiol-based redox proteomics technique, we detected a significant shift in P-SSG level in mouse gastrocnemius muscle 60 min after 15 min fatiguing contractions (Fig. 3). All detected P-SSG sites were present in both the unstimulated stimulated muscles with an average occupancy of 4.5% for the whole P-SSG proteome in the unstimulated leg that increased to an average occupancy of 5.22% in the stimulated gastrocnemius. This suggests that S-glutathionylation of proteins is a common post-translational modification under basal redox homeostasis; however, a significant shift of homeostasis towards oxidation would lead to higher occupancy of P-SSG. Interestingly, the proteins annotated as secretory proteins or present in the extracellular space have a higher average basal P-SSG occupancy (5.98%), while those in subcellular organelles, like the mitochondria (3.99%), have a lower basal occupancy. Proteins most sensitive to changes in P-SSG after fatiguing contractions (change > 1.4 FC and p ≤ 0.05) began with 1.67% average P-SSG occupancy and increased to 2.52% after fatigue. As previously mentioned, mitochondrial proteins had the most significant shift in occupancy, suggesting they may contribute to ROS production during fatigue or are more susceptible to oxidation (Fig. 3C), despite the evidence indicating that the contraction-induced oxidant production is primarily from non-mitochondrial sources. Indeed, the alkalinity of the mitochondrial matrix is known to promote the ionized thiolate form of cysteine [45].

Discussed below are a few of those P-SSG sites known to result in physiological effects (Table 1). More studies are required to explore the other physiological consequences of the P-SSG sites reported in this study, as well as those sites not previously described (Table 2).

5. Muscle contraction

5.1. Myofibril

Oxidative modification of contractile proteins can have an immediate effect on contraction and may be responsible for some of the
Fig. 4. Canonical Pathways, Biological Process, and Cellular Compartment analysis of proteins with significant changes of P-SSG by fatiguing contractions.

The top ten Canonical Ingenuity Pathways (IPA) involving the 1290 P-SSG sites with significant changes after fatigue (A). The significance of each pathway was determined by right-tailed Fisher's exact test (p < 0.05). DAVID Bioinformatics Resources identified the Biological Processes (B) and Cellular Compartments (C) associated with the oxidized proteins, ranked by significance.

### Table 1

Known glutathionylation sites associated with altered protein function. Table of P-SSG modifications significantly affected by fatiguing contractions sorted by Excitation/contraction Coupling, Metabolism, Redox Regulation, and Other. Significance determined by p-value (≤ 0.05). Note: Some P-SSG sites were no longer significant with FDR correction.

| Function                  | Protein                                      | Cysteine Site(s) | Log2 FC    | P value     | FDR       |
|---------------------------|----------------------------------------------|------------------|------------|-------------|-----------|
| EC Coupling               | Troponin I, fast skeletal muscle             | 134              | 0.5239111  | 0.000948    | 0.009052  |
|                           | Ryanodine receptor 1                         | 3636             | 0.309799   | 0.003931    | 0.016153  |
|                           | Sarcomplasmic/endoplasmic reticulum calcium ATPase 1 | 349            | 0.2534598  | 0.000531    | 0.007553  |
|                           | Metabolism                                  | 674              | 0.1602496  | 0.031643    | 0.054613  |
|                           | ATP-sensitive inward rectifier potassium channel 11 | 42              | 0.2226592  | 0.009947    | 0.065353  |
|                           | Glyceraldehyde-3-phosphate dehydrogenase     | 150              | 0.2984179  | 0.006478    | 0.020467  |
|                           | Creatine kinase M-type                      | 283              | 0.4612769  | 0.002407    | 0.012625  |
|                           | ATP synthase subunit alpha, mitochondrial    | 244              | 0.450853   | 0.009933    | 0.025096  |
|                           | Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial | 89             | 0.3130165  | 0.00116    | 0.009595  |
|                           | Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial | 251            | 0.334329   | 0.002124    | 0.039935  |
|                           | NADH dehydrogenase [ubiquinone] flavoprotein 1, mitochondrial | 187            | 0.394997   | 0.001392    | 0.031497  |
|                           | Aconitate hydratase, mitochondrial           | 286              | 0.285537   | 0.001949    | 0.02892   |
|                           | Electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial | 419             | 0.2692044  | 0.008289    | 0.023541  |
|                           | ADP/ATP translocase 1                       | 489              | 0.2875243  | 0.003558    | 0.015214  |
|                           | Malate dehydrogenase, cytoplasmic           | 451              | 0.2168614  | 0.00297    | 0.030181  |
|                           | Peptidyl-prolyl cis-trans isomerase F, mitochondrial (Cyclophilin D) | 52              | 0.6198489  | 0.005623    | 0.018121  |
|                           | Peroxiredoxin-1                            | 73               | 0.3667812  | 0.003675    | 0.031156  |
|                           | Thioredoxin                                 | 90               | 0.3926398  | 0.004439    | 0.016882  |
|                           | Thioredoxin, mitochondrial                  | 93               | 0.3926398  | 0.004439    | 0.016882  |
|                           | Hemoglobin subunit beta-1                   | 94               | 0.3880275  | 0.005266    | 0.018398  |
Table 2

| Protein                                           | Cysteine Site(s) | Log2 FC | P value   | FDR   |
|---------------------------------------------------|------------------|---------|-----------|-------|
| 14-3-3 protein gamma                              | 112              | 1.023413| 0.012581  | 0.029635 |
| ARL14 effector protein-like                       | 100              | 1.386748| 0.015173  | 0.032951 |
| Activating signal co-activator 1 complex subunit 3| 2004             | 0.824314| 0.032172  | 0.055351 |
| Dual specificity mitogen-activated protein kinase  | 264              | 0.854978| 0.040535  | 0.066078 |
| NADH dehydrogenase [ubiquinone] flavoprotein 2,  | 223              | 1.24754 | 0.009351  | 0.025096 |
| mitochondrial                                       |                  |         |           |       |
| Phosphomannomutase 2                               | 224              | 1.24754 | 0.009351  | 0.025096 |
| Ribosome-binding protein 1                         | 1415             | 0.786351| 0.03373   | 0.057513 |
| Sarbcalumenin                                      | 335              | 0.775664| 0.012484  | 0.029529 |
| Synaptophysin-like protein 2                       | 155              | 0.775554| 0.048596  | 0.075513 |

This modification was increased 1.44 fold (p = 0.0095) in the fatigued muscle compared to its unstimulated control. Post-translational modifications of titin, the largest known mammalian protein, has an increasingly appreciated role in the regulation of myofibril stiffness [49]. While disulfide formation of cryptic cysteines in titin’s I-band Ig domains increases titin stiffness, glutathionylation of cysteines exposed during stretch decrease protein folding, thus enhancing elasticity [50,51]. In this study, over 200 P-SSG modifications were observed on titin in the mouse gastrocnemius, and 50 of them had a significant increase in glutathionylation after fatiguing contractions. Many of these P-SSG sites were in titin’s I-band Ig domains.

5.2. E-C coupling

Calcium release and reuptake can also be altered by P-SSG of E-C coupling proteins in response to muscle contraction. P-SSG and S-nitrosylation of ryanodine receptor 1 (RYR) at Cys3636 alter the sensitivity of the receptor to voltage and calcium/calmodulin binding [46,52–54]. In this study, P-SSG occupancy of Cys 3636 of RYR-1 was significantly altered after stimulation, but only by 1.23 fold. Sarco-plasmic/endo-plasmic reticulum calcium ATPase 1 (SERCA1) P-SSG at Cys674 can disrupt calcium reuptake into the SR, affecting relaxation time [46,52,55]. This cysteine is also susceptible to irreversible oxidation, which would permanently impair calcium reuptake, suggesting that P-SSG may serve to protect against oxidative damage from increased oxidant production during muscle contraction [56]. Similarly affecting calcium homeostasis, the activity of ATP-sensitive inward rectifier potassium channel 11 (KCNJ11) is reportedly regulated by Cys42 oxidation and is restored by DTT [52,57]. This cysteine was moderately S-glutathionylated 60 min after fatigue in this study (FDR = 0.065, Log2FC = 0.22).

6. Metabolism

In contrast to our hypothesis, mitochondrial proteins underwent one of the greatest increases in P-SSG following an acute bout of...
contractions. Mitochondrial and glycolytic metabolism are essential for contraction, energy balance, and regulation of active electrolyte transport in muscle. Mitochondria are a regular source of oxidants in cells [58,59], though not considered to be a major source during exercise [13,17,24]. They contain a robust antioxidant defense with 10–15 mM glutathione and mitochondrial-specific peroxiredoxin (PRX3), superoxide dismutase (MnSOD), thioredoxin-2 (TRX2), GSH peroxidase (GPX4), and glutaredoxin (GRX2), indicating that the redox state of mitochondrial proteins are highly regulated [60]. P-SSG in mitochondria can temporarily regulate the activity of key metabolic enzymes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key glycolytic enzyme, is inhibited by S-glutathionylation of Cys150, a modification also increased with fatigue in this study [61–63]. Creatine kinase (M-type) is essential for the production of phosphocreatine and ADP and the reverse reaction, supplying muscle with ATP during contraction. S-glutathionylation at creatine kinase Cys283 was associated with a loss of enzyme activity during ischemia and was identified as redox sensitive through Isotope-Coded Affinity Tag (ICAT) labeling and proteomics [64,65]. This modification, as well as Cys317 of creatine kinase (mitochondrial, S-Type) was also observed to be a redox sensitive site in this study and others [66].

6.1. TCA cycle

Within the TCA cycle, malate dehydrogenase (MDH1) Cys137 as well as aconitase (ACO2) at Cys385 were significantly S-glutathionylated with fatigue [66–69]. When modified, the cysteine on ACO2 is reported to reduce enzyme activity. Like Cys385, Cys448 and Cys451 are also significantly modified with fatigue, and are associated with the 4Fe-4S cluster.

6.2. Electron transport system

Oxidative modifications of protein complexes in the electron transport chain can regulate superoxide and ATP production, and are implicated in the detrimental effects of oxidative stress on bioenergetics [67,70,71]. Cys125 and Cys142 of NADH dehydrogenase flavoprotein 1 (NDUFV1) are reported to be S-glutathionylated and possibly form disulfide linkages, but their S-glutathionylation occupancy was not significantly increased under fatiguing conditions [69,72]. However, Cys187 and 206 were also reported as possible disulfide linkages by Zhang et al., and these were significantly S-glutathionylated with fatigue [72,73]. Additionally, Cys59 of NDUFB7, Cys463, Cys64, and Cys92 of NDUFV1, and Cys347 of NDUFV2 were among the complex I subunit cysteines to significantly change in P-SSG and were previously reported to be redox sensitive by Danielson et al. [74]. Succinate dehydrogenase (SDHA) S-glutathionylation on Cys89 increases enzyme activity and reduces superoxide production in purified protein and in bovine and ischemic rat hearts [46,69,75,76]. ATP synthase, responsible for utilizing the proton-motive force for ATP production, was S-glutathionylated at Cys244 and 294 of the alpha subunit with fatigue. S-glutathionylation of Cys294, although transiently inhibitory, is shown to protect the enzyme from irreversible oxidation, while S-sulfhydration is reported to improve activity [67,69,77,78].

7. Novel P-SSG sites

Advances in proteomics and enrichment techniques have increased the detection of novel S-glutathionylation sites. We report several new sites of P-SSG on proteins involved in common signaling pathways, metabolic pathways, and proteins associated with excitation/contraction coupling. The sites listed in Table 2 had a minimum fold change of 1.68 and were significantly affected by fatigue (p ≤ 0.05). Briefly, we will describe a few of the most interesting novel P-SSG modifications and speculate on possible downstream effects.

Protein 14-3-3 gamma has been shown to associate with and regulate the activity, localization, and interactions of over a hundred proteins, including phosphatases and kinases like PKA and PKC [79–81]. These functions are mediated through its specific phosphoserine/phosphothreonine binding activity on target proteins. Two of the most significantly affected S-glutathionylation sites associated with fatigue (C112, C97) reside in or near this phospho-peptide binding domain (Fig. 5A). As these S-glutathionylation sites are previously unreported, it is unknown whether these oxidative modifications affect its function.

Similarly S-glutathionylated after fatiguing contractions, Cys264 is adjacent to the activating phosphosites of S255 and T259 of MAP2K4, a member of the mitogen-activated protein kinase family, and may be a mechanism by which ROS regulates the MAPK pathway (Fig. 5B) [82]. Two cysteines, 223 and 224, on the N1A iron-sulfur cluster of complex I flavoprotein 2 subunit (NDUV2) were among the most sensitive site of glutathionylation following contraction. This protein is believed to act as an antioxidant by capturing one of the two electrons donated to the complex by NADH [83]. Hinchliffe et al. proposes that, in doing so, it prevents the excess reduction of the FMN site in the NDUV1 subunit and the formation of reactive oxygen species. It is possible that the oxidation of the conserved cysteine, Cys224, can form a previously unknown disulfide bond with a similarly oxidized cysteine on the adjacent complex (Cys125 of NDUV1) approximately 5 angstroms away (Fig. 5C), a cysteine already known to participate in disulfide bonds [72]. Further study is required to determine the role of this modification in complex I function with fatigue. Not listed on Table 2, but also among the most significantly S-glutathionylated cysteines (1.64 fold change), was Cys910 of SERCA, the calcium transporter that functions to reuptake calcium into the sarcoplasmic reticulum. Cysteine 910, another previously unreported glutathionylation site, lies in the transmembrane domain next to the glutamic acid residue (Glu908) that assists calcium into the SR (Fig. 5D). Further study is required to determine if this modification might alter calcium uptake and delay muscle relaxation.

There are several potential limitations regarding the current redox proteomics approach. First, there is a possibility of thiol oxidation occurring in the minutes before freezing, during storage, or immediately following the thaw, despite the use of a high concentration of NEM (100 mM) in the first stage of sample processing. Moreover, the indirect nature of P-SSG measurement is considered a surrogate of thiol oxidation since the glutaredox reaction does not offer perfect specificity for P-SSG. For example, some protein disulfide bonds or SOH may also be reduced during glutaredoxin treatment and therefore contribute to the P-SSG signal in our analyses [32,84]. Indeed, we have previously performed several negative controls to evaluate the specificity of reduction where nearly zero background signals were observed when no reducing agents were applied and a minimal signal (estimated to be < 10%) of non-specific reduction was observed for the most proteins when GRX was omitted in the reduction cocktail still containing GSH, NADPH, and glutathione reductase [32]. These data suggest a relative good specificity for the GRX reduction. Finally, the time course of changes in the thiol redox proteome following exercise is not known, and further optimization of post-fatigue analysis would be required to capture the dynamic P-SSG levels.

In conclusion, mapping the S-glutathionylation proteome in muscle has revealed many unreported glutathionylation sites, which are highly susceptible to modification with fatigue. Further study will be required to test the physiological relevance of the novel P-SSG sites through computational means or site-directed mutagenesis [85]. Pathway and cellular compartment analyses implicate P-SSG regulatory control over metabolism, contraction kinetics, and many signal transduction pathways. Nearly all modifications had a relatively small change in glutathionylation (< 1.6 fold), which suggests that exercise-induced adaptive responses are regulated by relatively subtle changes of in situ P-SSG modifications, that 60 min delay between contraction and biopsy misses the window of peak redox signaling, or downstream adaptive responses.
require repeated bouts of contraction. On the other hand, some of the P-SSG sites may not play a substantial role in exercise adaptation signaling. However, their identification as reoxidon sensitive may implicate a functional role under more oxidative conditions such as protection from further irreversible modifications. The in vivo stimulation method described in this study provides an effective means of reliably reproducing fatigue and studying redox signaling in stimulated and unstimulated muscle through global quantitative redox proteomics methods for advancing our understanding of redox signaling in health and disease [14,22,23]. Thus, the data reported here provide insights into the potential effects on muscle function and adaptive signaling resulting from oxidants produced during exercise.

Funding and conflicts

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2018.05.011.

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P.A. Kramer et al.

Redox Biology 17 (2018) 367–376

376