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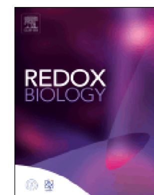

Research Paper

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

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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 muscleswere 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 fatiguing contractions. 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), thiyl radicals (-S.), or S-nitroso

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(-SNO), thereby converting these oxidized forms to a more stable modification [6–8] and preventing further thiol oxidation to sulfinic and sulfonic acid. This is significant because sulfinylation and sulfonylation are largely irreversible modifications with the exception of reduction of 2-Cys PRX sulfinylation by sulfiredoxin [9,10]. In addition to this protective effect, P-SSG is considered an important type of oxidative modification that regulates transcription, mitochondrial metabolism, 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, NFkB, AMPK, and NRF2, to promote exercise recovery, biogenesis, protein turnover, and cyto-protective 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, mitochondrially-targeted antioxidants, such as elamipretide (SS-31) and AAV-mCAT, improve mitochondrial deficits, fatigue resistance, 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 contraction 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-mitochondrial sources [18], we hypothesized that mitochondrial proteins would undergo relatively less redox modification than cytoplasmic and sarcolemmal proteins following an acute bout of contraction. In this study, in vivo electrical stimulation of skeletal muscle was used to induce fatiguing contractions in adult CB6F1 mice. Fatigue increased the P-SSG levels of many new and previously reported Cys sites on mitochondrial, 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 isofluorane 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 stimulation 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 stimulator (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 allowed 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 homogenization 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 reagents 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 S-glutathionylated thiol residues using GRX1 enzyme cocktail containing 2.5 μg/mL GRX1M (C14S mutant from E. coli), 0.25 mM GSSG, 1 mM NADPH, and 4 U/mL glutathione reductase [33] 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 enriched 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 performed (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% $NH_2OH{\cdot}HCl$ in 200 mM TEAB buffer for 15 min. The excess TMT reagents 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 cm × 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 2×10^5. MS/MS was performed in the data-dependent mode with orbitrap resolution of 30 K, an AGC target value of 1×10^5, 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]

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