Review

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Review

Quantitative Proteomic Approaches to Study Redox Post-Translational Modifications in Skeletal Muscle

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Abstract

Reactive oxygen species are essential signalling molecules that regulate numerous aspects of skeletal muscle physiology. These effects are mediated through redox post-translational modifications on protein cysteine thiols, which influence the structure and function of redox-sensitive proteins. Mass spectrometry–based redox proteomic approaches have greatly advanced our ability to detect and characterise cysteine redox modifications, revealing a broad network of redox-sensitive proteins and pathways in skeletal muscle. Recent methodological developments enable quantification of the stoichiometry of reversible oxidative modifications at specific cysteine residues, providing critical insight into the extent and functional relevance of site-specific redox regulation. Redox proteomic approaches are being employed to improve our understanding of the specific redox protein modifications underlying physiological and pathophysiological processes in skeletal muscle. This review summarises current proteomic strategies for quantifying redox post-translational modifications and their application to study redox signalling in skeletal muscle. Emerging experimental approaches that offer the potential to study the specific roles of site-specific redox modifications in muscle physiology are also discussed. Collectively, these technologies present exciting opportunities to define the mechanistic roles of individual cysteine residues in muscle biology and help uncover new therapeutic avenues for conditions characterised by impaired redox homeostasis.

Keywords: reactive oxygen species; skeletal muscle; redox proteomics

1. Introduction

Reactive oxygen species (ROS) and related reactive nitrogen and sulphur species (RNS/RSS) play a central role in skeletal muscle health and disease [1,2]. Under conditions of oxidative distress, excessive ROS formation can cause irreversible damage to proteins, lipids and DNA, altering the structure and function of biomolecules [3–6]. As a result, oxidative distress contributes to the development of skeletal muscle dysfunction and has been implicated in the pathogenesis of several skeletal muscle-related diseases [7–9]. Beyond causing oxidative damage, ROS, specifically hydrogen peroxide (H2O2), play an important role in regulating skeletal muscle physiology through reversible redox post-translational modifications (PTMs) [10]. These modifications alter the redox state of thiol groups on protein cysteine residues, providing a mechanism for cells to rapidly respond to changes in the cellular redox state. Redox PTMs influence diverse aspects of protein function, including

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catalytic or ligand binding activities, protein–protein interactions, and protein stability, while also regulating the formation of other PTMs associated with physiological signalling [11].

Many metabolic and myofibrillar proteins in skeletal muscle contain redox-sensitive cysteine residues [12,13], implicating reversible redox PTMs in the regulation of muscle metabolism and force production. Changes in the cellular redox state also enable skeletal muscle to adapt to diverse stimuli, including contractile activity, the prevailing loading conditions, substrate availability and environmental factors (e.g., hypoxia, heat and cold stress). Muscle contraction results in transient increases in the cytosolic H2O2 content of muscle fibres, which, in part, mediate many of the beneficial adaptive effects of exercise through the activation of redox-sensitive signalling pathways [14,15]. Conversely, dysregulated skeletal muscle ROS production and redox signalling underpin some of the pathological changes associated with muscle-related diseases including sarcopenia [8], muscular dystrophies [16], and type 2 diabetes [7].

Despite the importance of redox regulation in skeletal muscle physiology and pathophysiology, our understanding of the specific redox PTMs that explain the molecular basis for these processes is limited, in part because methods for studying redox PTMs have typically not provided absolute stoichiometric quantitation and/or cover a small proportion of the redox proteome. Furthermore, experimental approaches to investigate causal links between redox PTMs and biological outcomes lack precision and can disrupt non-redox cysteine roles. However, in recent years, advances in redox proteomic approaches have enabled the quantification of reversible oxidative modifications on specific protein cysteine residues in a range of tissues, including skeletal muscle, with increasing coverage of the cysteine proteome. In this review, we discuss proteomic strategies to quantify redox PTMs and their application to study redox signalling in skeletal muscle. We also highlight emerging methodologies that selectively induce or block cysteine redox modifications and offer the potential to study the role of site-specific modifications in muscle physiology.

2. Regulation of the Skeletal Muscle Redox State

Skeletal muscle ROS levels are governed by the balance between ROS production and antioxidant scavenging systems (Figure 1). A variety of ROS have been detected in skeletal muscle with superoxide being the primary species. Superoxide is formed by the one-electron reduction of molecular oxygen and is produced by several organelles, including mitochondria, the endoplasmic reticulum and peroxisomes, as well as by enzymes such as NADPH oxidases (NOX) and phospholipases. Superoxide is rapidly converted to H2O2, either spontaneously or via the catalytic action of compartmentalised superoxide dismutase (SOD) isoforms. H2O2 displays the greatest stability compared with other physiologically relevant ROS and can traverse across membranes by either passive diffusion or facilitated transport via channels, such as aquaporins [17]. H2O2 exhibits low overall reactivity but high selectivity for the thiol group of cysteine residues, providing the basis for a highly selective chemical messenger. H2O2 is converted to water, or water and oxygen, by compartment-specific peroxidases including catalase (CAT), peroxiredoxins (PRDX), and glutathione peroxidases (GPX).

To date, research has focussed on understanding the role of H2O2 derived from mitochondria and NOX enzymes in skeletal muscle redox signalling. The mitochondrial electron transport chain (ETC) is a major source of superoxide [18]. Consequently, mitochondria are an important cellular redox signalling node [19]. Mitochondrial sites that leak electrons to oxygen to form superoxide include sites at complexes I, II and III (CI, CII, and CIII), four dehydrogenases in the matrix compartment, and others in the intermembrane space, including glycerol-3-phosphate and dihydroorotate dehydrogenases [20]. In resting skeletal muscle, the concentration of ADP in cells is low relative to ATP, which supresses mitochondrial oxidative phosphorylation and promotes a highly reduced redox environment (i.e., high NADH/NAD+). Surplus electron flow into the ubiquinone (Q) complex in the ETC reduces the Q pool, which, in combination with a high proton motive force, promotes electron leak from the ETC and the generation of superoxide. During exercise, the ADP:ATP ratio in the contracting muscles increases, reducing pressure at electron entry points of

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the ETC and attenuating superoxide production. While evidence from both animal and human studies suggests that muscle contraction impacts the redox state of mitochondrial proteins [21,22], current experimental evidence indicates mitochondria are not the main source of increased ROS during muscle contraction [23]. However, redox signalling by mitochondrial ROS has been implicated in a wide range of biologically important areas [19] and perturbations in mitochondrial redox homeostasis have been linked to muscle atrophy [24] and insulin resistance [25].

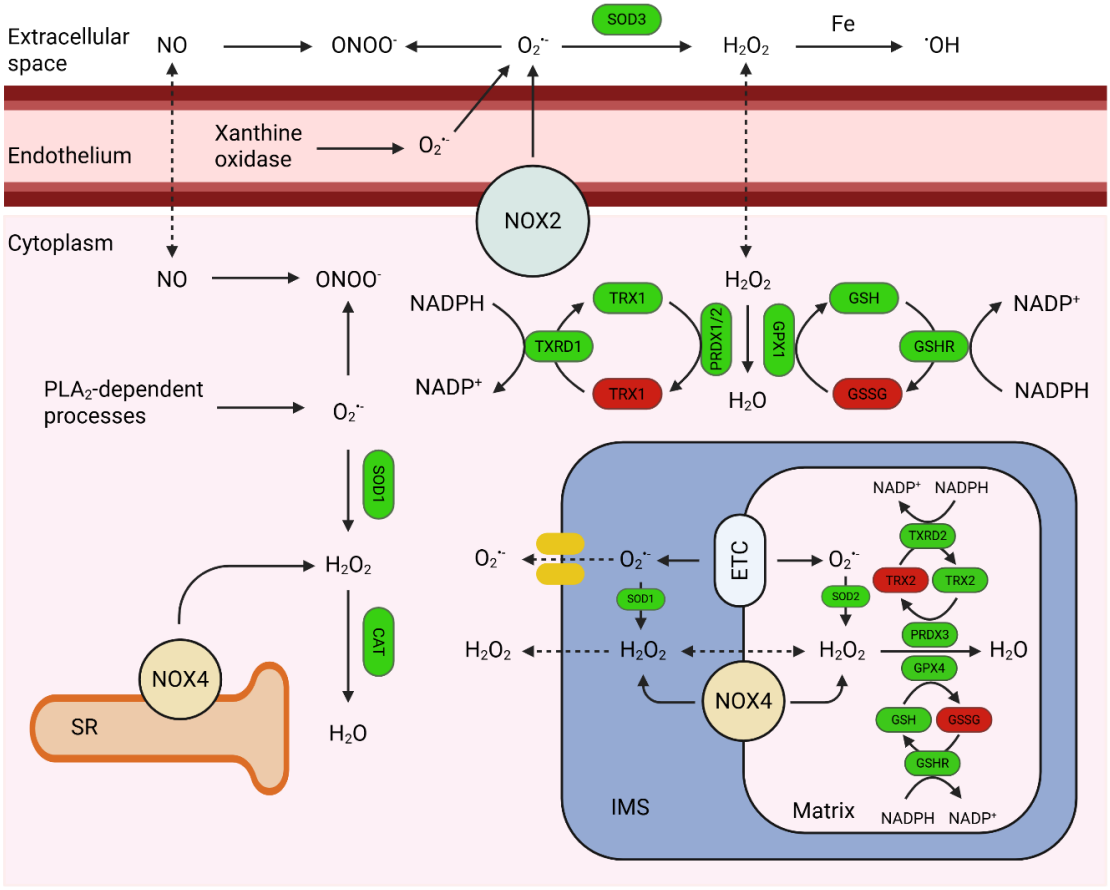

Figure 1. Overview of primary sources of ROS in skeletal muscle and the endogenous antioxidants that facilitate their removal. NO, Nitric oxide; $ONOO^-$, Peroxynitrite; $O_2^{\bullet-}$, Superoxide; H_2O_2, Hydrogen peroxide; •OH, Hydroxyl radical; Fe, Iron; PLA_2 – Phospholipase A_2; NOX, NADPH oxidase; ETC, Electron transport chain; IMS, Intermembrane space; SOD, Superoxide dismutase; CAT, Catalase; TRX, Thioredoxin; TXRD, Thioredoxin reductase; PRDX; Peroxiredoxin; GSH, Reduced glutathione; GSSG, Oxidised glutathione; GSHR, Glutathione reductase; GPX, Glutathione peroxidase; SR, Sarcoplasmic reticulum. Figure created with BioRender.

NOX2 and 4 are the main NOX isoforms expressed in skeletal muscle. The fully assembled and functionally active NOX2 enzyme is localised to the sarcolemma and T-tubules and is responsible for the transfer of electrons from NADPH to oxygen to generate superoxide [26]. NOX2 complex activation is stimulated by electrically induced muscle contractions [27] and exercise [28] in mice. Furthermore, exercise-induced increases in cytosolic H2O2 are lower in mice lacking NOX2 regulatory subunits [23], indicating that NOX2 contributes to exercise-induced increases in H2O2 in skeletal muscle. H2O2 derived from NOX2 plays a key role in exercise-induced redox signalling and modulates skeletal muscle glucose uptake during acute exercise [23] and mitochondrial adaptations to exercise training [29]. Paradoxically, ROS production by NOX2 has also been linked to muscle

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atrophy [30] and skeletal muscle insulin resistance [31], underscoring the divergent effects of ROS generated from the same source under distinct physiological and pathophysiological conditions.

The H2O2 producing NOX4 colocalises with the ryanodine receptor (RyR) in the sarcoplasmic reticulum and is present in mitochondria in isolated mouse single muscle fibers [27]. While NOX4 is constitutively active, its activity is modulated by p22phox and Polymerase δ-Interacting Protein 2 (POLDIP2). H2O2 produced by NOX4 during exercise regulates adaptive responses that promote muscle function, maintain redox balance, and prevent the development of insulin resistance [32], highlighting a role for NOX4 in exercise-induced redox signalling. Furthermore, global and endothelial specific deletion of NOX4 attenuates acute exercise-induced muscle glucose and fatty acid oxidation [33], suggesting H2O2 produced by NOX4 in non-muscle tissues during exercise may be involved in inter-tissue crosstalk.

3. Redox Signalling Mechanisms

H2O2 mediates its biological effects through the reversible oxidation of thiol groups on cysteines in target proteins. Cysteine is the least abundant amino acid in proteins, yet a high proportion are evolutionarily conserved, attributed to their important role in redox signalling [34]. Cysteine residues have several functional roles. Cysteines within a protein tend to perform structural roles, forming intramolecular disulfides as determinants of protein folding or coordinating prosthetic groups, such as iron-sulphur clusters. Exposed cysteines that are present on the protein surface are more likely to be redox-active as they are accessible to external signals or can engage in the formation of mixed disulfides with binding partners. Redox-sensitive cysteine thiols can undergo a wide range of reversible and irreversible redox modifications, including sulfenylation, disulfide formation, nitrosylation, and glutathionylation (Figure 2). Sulfenylated thiols can undergo further oxidation to form stable sulfinic or sulfonic acids, which can be used as markers of oxidative damage.

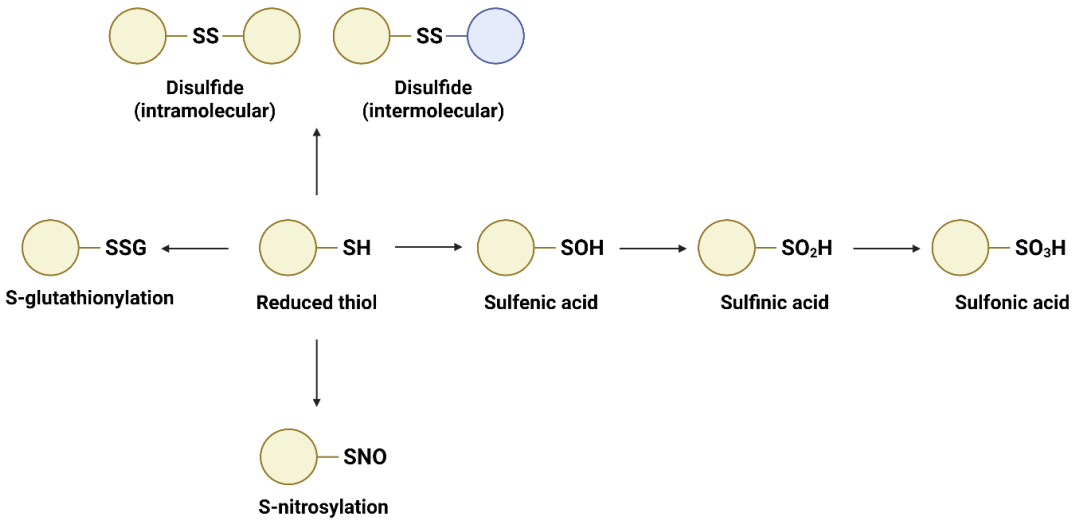

Figure 2. Oxidative modifications of protein cysteine thiols. Reduced thiols can be subjected to multiple types of oxidative modifications. Disulfides can be formed intramolecularly within the same protein or intermolecularly through exchange with a sulfur group on another protein or molecule. Sulfinylation and sulfonylation occur as subsequent, irreversible oxidative modifications of cysteine thiols that are sulfenylated. Apart from sulfinylation and sulfonylation, all modifications illustrated here are considered reversible. Figure created with BioRender.

Protein cysteine redox modifications modulate enzyme activities and protein functions across diverse cellular processes, including cell signalling, metabolism, and gene expression [35]. However, the mechanisms through which redox signalling achieves its specificity and reactivity are unclear. The reactivity of redox regulated proteins to H2O2 is generally considered to be significantly lower

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than that of H2O2-scavenging peroxidases. Thus, several scenarios could explain the potential mechanism by which H2O2 oxidises low-reactivity target proteins. Transient inactivation of PRDXs by reversible hyperoxidation or phosphorylation may result in increases in intracellular H2O2 that allow direct oxidation of protein thiols with low H2O2 reactivity (known as the "floodgate" theory). The other potential mechanism whereby H2O2 may oxidise low-reactivity target proteins is known as a "redox relay", which involves PRDXs transferring oxidising equivalents to less reactive thiol proteins in redox signalling pathways. Redox relays have been identified with the transcription factors apoptosis signal-regulating kinase 1 (ASK1) and signal transducer and activator of transcription 3 (STAT3) with PRDX1 [36] and PRDX2 [37], respectively. The five 2-cysteine PRDXs also exhibit distinct substrate specificity for redox relay reactions [38]. Therefore, the spatial and temporal restriction of H2O2 in subcellular domains and the presence of specific PRDXs that mediate target-specific oxidation may be important factors regulating the specificity of redox signalling.

4. Quantitative Proteomic Approaches for Measuring Reversible Redox PTMs: Application to Skeletal Muscle Physiology

While redox regulation plays a fundamental role in skeletal muscle physiology and pathophysiology, the underlying protein modifications remain poorly defined. Efforts to characterise the skeletal muscle redox proteome have been constrained because redox proteomics methods have typically not provided absolute stoichiometric quantitation of cysteine oxidation, preventing determination of the percentage occupancy of oxidation at specific cysteine sites. PTM site stoichiometry, also referred to as PTM site occupancy or fractional PTM occupancy, indicates the fraction of a protein that is modified with a given PTM at a specific site [39] (Figure 3). Quantitative analysis of PTMs offers deeper insight into signalling pathways by providing precise measurements that distinguish changes between steady state and a perturbation. Because many redox-sensitive cysteine residues function as allosteric or structural switches [34], small shifts in oxidation stoichiometry can have large effects on muscle function. Therefore, accurately quantifying the extent of reversible cysteine oxidation is essential to interpret how redox signals are translated into physiological responses and to identify key functional sites through which ROS and related species exert their physiological roles. Current stoichiometric approaches generally provide limited coverage of the cysteine proteome, identifying only a fraction of cysteine-containing peptides or proteins, which limits identification of key functional cysteine redox switches. Furthermore, few redox proteomic approaches allow for the determination of specific reversible redox modifications, such as S-glutathionylation and S-nitrosylation. These technical challenges have limited our ability to quantitatively interrogate how protein redox modifications regulate muscle physiology. However, advances in MS-based redox proteomics have made it possible to quantify redox PTMs on protein cysteine thiols with increasing proteome coverage.

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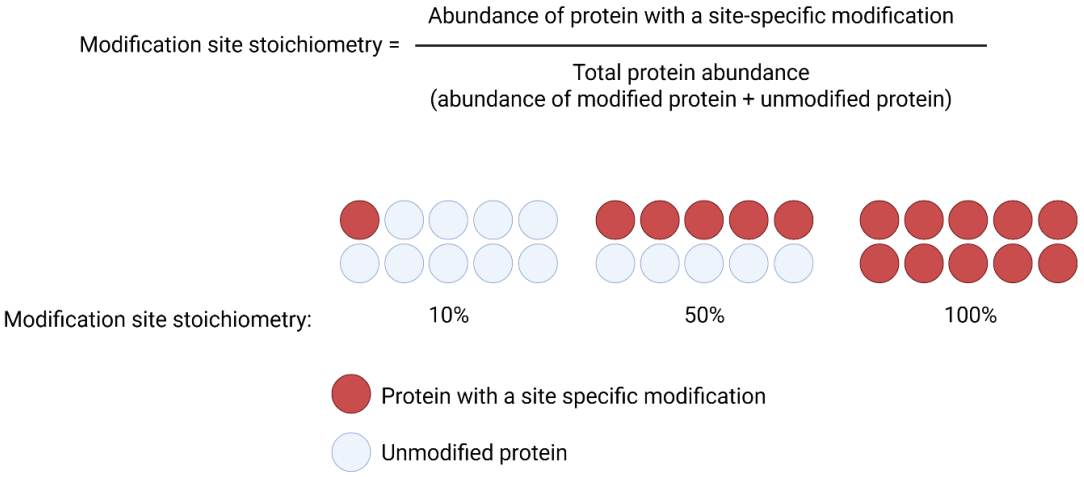

Figure 3. Protein modification site stoichiometry. The concept of PTM site stoichiometry is illustrated using three examples with different PTM site stoichiometry. Unmodified and PTM-containing proteins are marked as indicated. PTM, post-translational modification. Figure created with BioRender.

There are several approaches to make proteomics quantitative, including label-free approaches and isotopic and isobaric labelling. Label-free proteomics measures protein abundance directly from the MS signal intensity or spectral counts across separate liquid chromatography (LC)-MS runs. Quantitative proteomic analyses are most commonly performed using data-dependent acquisition (DDA), in which precursor ions are selected for fragmentation based on intensity and identified using spectral libraries. However, data-independent acquisition (DIA) has become increasingly important due to its improved reproducibility and depth, particularly in enriched samples such as those generated by redox proteomic workflows [40]. In isotopic labelling, stable isotopes are incorporated into proteins or peptides, creating a defined mass shift that allows relative quantification based on signal intensity. Isobaric labelling methods use chemical tags, such as tandem mass tags (TMT) [41] or isobaric tags for relative and absolute quantitation (iTRAQ) [42], which release distinct reporter ions upon fragmentation, enabling multiplexed quantification of protein abundances in several samples in a single run, increasing throughput and reducing LC-MS/MS run variability. To date, most studies that have used isobaric labelling to quantify cysteine oxidation in skeletal muscle have employed TMT labelling. In this approach, each peptide sample is chemically labelled with TMT consisting of three parts: a reporter group that differs in isotopic composition, a balance group that equalizes the total mass across tags, and a reactive group that covalently binds to peptide N-termini or lysine residues. After labelling, the samples are combined and analysed by LC–MS/MS. Because the labelled peptides are isobaric, they appear as a single precursor ion, ensuring identical chromatographic and ionization behaviour. During fragmentation, each tag releases its unique reporter ion, and the relative intensities of these reporter ions reflect the abundance of the peptide in each original sample. This approach provides precise, high-throughput quantification across multiple biological conditions.

Differential Cysteine Labelling

Differential labelling techniques involve first blocking free cysteine thiols with an alkylating reagent followed by reduction of reversibly oxidised cysteines and a second alkylation step using a chemically distinct or isotopically labelled reagent. This two-step labelling process allows reduced and oxidised cysteines to be distinguished by MS based on their differential mass tags, enabling site-specific quantification of redox-sensitive cysteine residues (Figure 4). Isotope-coded affinity tag (ICAT) reagent is a chemical probe that labels reduced thiols due to its iodoacetamide moiety, which rapidly and irreversibly reacts with free thiols [43]. Once free thiols are labelled with the light variant

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of the ICAT reagent, reversibly oxidised thiols in the same sample are reduced and labelled with the heavy ICAT reagent. Following digestion and purification of the labelled peptides by their biotin tags, the enriched sample is run on a tandem MS/MS, where the reduced and oxidised peptides can be resolved based on their mass shift of 9 Da, which is visible on the spectra. Finally, the stoichiometry of oxidised versus reduced proteins is determined by the ratio of heavy versus light peptide signal intensities.

Another differential labelling approach involves alkylating free thiols using N-Ethylmaleimide (d0-NEM) before reducing reversibly oxidised cysteines and labelling the newly formed free thiols with heavy NEM (d5-NEM) [12]. Relative quantification of cysteine containing peptides identified as reduced (d0-NEM labelled) and reversibly oxidised (d5-NEM labelled) is then performed using the intensity of their precursor ions. Differential cysteine labelling has been combined with label-free proteomics to quantify age-related changes in protein abundance and the reversible oxidation state of redox-sensitive cysteines in mouse skeletal muscle [12,44]. Many mitochondrial and myofibrillar proteins were among the proteins with redox sensitive thiols detected in these studies. While there was greater oxidation of some cysteines in aged muscle, others shifted toward to a more reduced state, highlighting that ageing is associated with remodelling of the redox proteome rather than a global increase in oxidation. Notably, changes in the redox proteome appear to be well preserved across gastrocnemius, quadriceps, and soleus muscles in rodents with 23 cysteine residues showing altered redox states in all three despite their different fibre type compositions and susceptibility to ageing. The total number of proteins containing redox-sensitive cysteines decreased in aged muscle, suggesting a loss of redox signalling flexibility. For example, in the gastrocnemius, proteins that were identified as containing redox-sensitive cysteine residues in muscle from adult mice only included proteins involved in glucose metabolism, such as phosphofructokinase (PFK), glucose 6-phosphate isomerase (GPI), glycogen phosphorylase (GP), phosphoglycerate mutase 1 (PGAM1), and phosphoglucomutase 2 (PGM2) [12]. Cysteine residues regulate the activity of many of these enzymes and the loss of redox sensitivity at these sites in aged muscle may reflect reduced flexibility in metabolic redox regulation with age. This loss of flexibility in redox regulation is mirrored by a loss of skeletal muscle metabolic flexibility with ageing in which the capacity to switch from carbohydrate- and fat-based fuels in response to the prevailing hormonal milieu is diminished [45].

Differential alkylation using NEM has also been employed to investigate the effects of ageing and exercise on the human skeletal muscle redox proteome [22]. Compared with mice, humans demonstrate fewer age-related shifts in the redox state of individual cysteines. All significant differences observed at rest showed a decrease in relative cysteine oxidation in aged muscle (i.e., cysteines were in a more reduced state). Ageing was associated with decreased oxidation of seven cysteines from five different proteins, including glycogen phosphorylase (GP), phosphoglycerate mutase 2 (PGAM2), PRDX6, ubiquitin-conjugating enzyme E2 N (UBE2N), and Rab GDP dissociation inhibitor (Rab GDI). The shift in specific cysteine residues toward a more reduced state appears counterintuitive as previous studies have reported increased irreversible protein oxidation in aged muscle [3]. However, such an observation is consistent with studies in mouse muscle showing age-related shifts to a more reduced state in cytosolic proteins, such as PRDX2 [21]. The mechanisms underpinning this greater reduction of cysteine residues in aged muscle are unclear but may involve adaptive upregulation of cytoprotective antioxidant proteins in response to elevated mitochondrial oxidative stress with age [46].

While there appear to be few differences in protein cysteine oxidation between young and old individuals at rest, there is a greater disparity in the response to an acute bout of high-intensity interval exercise. In muscle from both young and older individuals, four cysteines from three mitochondrial proteins (dihydrolipoamide dehydrogenase (DLD), cytochrome b-c1 complex subunit 1 (UQCRH), and PRDX3) exhibited increased oxidation in response to exercise, while one cysteine showed a reduction. A further 23 cysteines from 13 proteins exhibited exercise-induced oxidation in muscle from older individuals only, including proteins involved in glycolysis (aldolase A (ALDOA), beta-enolase (ENO3), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and triosephosphate

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isomerase (TPI1)), ROS detoxification (CAT, PRDX6, and SOD1), and creatine kinase activity (creatine kinase M-type (KCRM) and S-type (KCRS)). Nine of these proteins are cytosolic, while the rest are mitochondrial or located within the peroxisome, indicating exercise results in changes in the redox state of proteins localised to several subcellular compartments in aged muscle. Taken together, these findings suggest that redox homeostasis is largely preserved during exercise in young muscle, whereas there appears to be a disruption of the redox proteome during exercise in aged muscle.

Differential cysteine labelling using NEM has provided insight into age- and exercise-related changes in protein cysteine oxidation in skeletal muscle. However, compared to techniques that purify/enrich modified cysteine containing peptides, this technique has a relatively poor sensitivity for the detection of low-abundance proteins as labelled peptides compete with extremely abundant sarcomeric proteins, reducing the dynamic range. Indeed, this approach only identified 75 redox cysteine peptides on 45 proteins in adult mouse quadriceps [44] and in the human muscle biopsy study 154 redox cysteine peptides in 57 proteins were reported [22]. Increasing the sensitivity of detection by sample fractionation or data independent approaches would increase the accuracy of both detection and quantification of redox specific changes. Differential cysteine labelling using NEM is not suitable for multiplexing, which limits analysis to only two samples at a time, decreasing throughput and potentially increasing LC-MS/MS run variability. Furthermore, precursor-level quantification (MS1) used in the NEM differential alkylation workflow has lower precision than TMT/MS2-based quantification, particularly for detecting small redox shifts. These differential alkylation approaches do not allow for determination of specific reversible redox modifications, limiting the capacity to elucidate the specific protein modifications underlying muscle physiology.

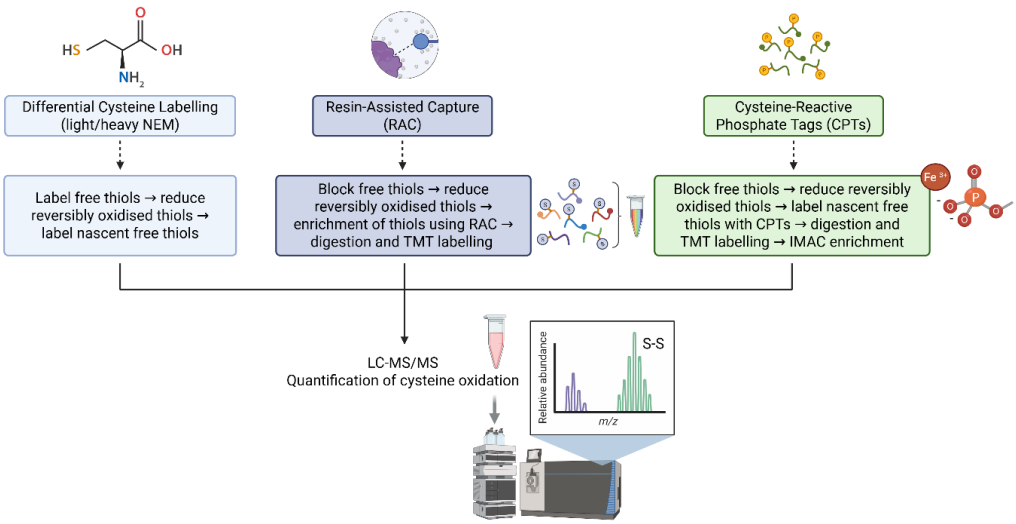

Figure 4. Quantitative redox proteomic workflows to study protein cysteine oxidation in skeletal muscle. The differential cysteine labelling using NEM approach involves labelling free thiols using N-Ethylmaleimide (d0-NEM) before reversibly oxidised cysteines are reduced and the newly formed free thiols are labelled with heavy NEM (d5-NEM). Relative quantification of cysteine containing peptides identified as reduced (d0-NEM labelled) and reversibly oxidised (d5-NEM labelled) is then performed using the intensity of their precursor ions. In the cysteine-reactive phosphate tag (CPT) and resin assisted capture (RAC) methods, total thiols (i.e., both oxidised and reduced protein thiols) are measured in a separate sample by omitting the initial step to block free thiols for stoichiometric measurements. Measurements are based on reporter ion intensities. In the RAC workflow, free thiols are alkylated, and then oxidised thiols are totally or selectively reduced (if probing for a specific modification). RAC of nascent free thiols using a thiopropyl sepharose resin is followed by on-resin digestion and labelling with tandem mass tags (TMT). Finally, peptides are eluted off the resin prior to analysis by mass spectrometry (MS). The oxidation occupancy is determined by taking the ratio of reporter ion intensities in the oxidised and total thiol channels to evaluate the modification stoichiometry. In the CPT workflow, free thiols are alkylated and oxidised thiols are totally or selectively reduced. Endogenous phosphorylation is removed to

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avoid the recovery of phosphorylated peptides during immobilised metal affinity chromatography (IMAC). Nascent free thiols are labelled with CPTs before the proteins are digested and the peptides are labelled with TMT. The CPTs allow the peptides to be enriched by IMAC and are eluted off the column prior to analysis by MS. The oxidation occupancy is determined by taking the ratio of reporter ion intensities in the oxidised and total thiol channels to evaluate the modification stoichiometry. Figure created with BioRender.

Resin-Assisted Capture

As discussed, maximising cysteine proteome coverage is a major technical challenge in the field of redox proteomics. Resin-assisted capture (RAC) enables the enrichment of oxidatively modified proteins, increasing the coverage of downstream proteomic analysis [47]. In this approach, protein free thiols are initially blocked with NEM before reversibly oxidised thiols are reduced and proteins with nascent free thiols are enriched using a thiol-affinity resin (Figure 4). RAC has the versatility to profile specific modifications such as glutathionylation or nitrosylation by using specific reagents to selectively reduce them [13]. The captured proteins are digested on-resin with trypsin, and the peptides are labelled on-resin with TMT. Direct capture of thiol-containing proteins reduces sample complexity and non-specific background, improving digestion efficiency and TMT incorporation onto the remaining cysteine containing peptides. When RAC is combined with TMT labelling (RAC-TMT), changes in the redox state of oxidatively modified proteins can be evaluated quantitatively.

The RAC-TMT workflow has been employed to investigate the effect of contractile activity on protein S-glutathionylation in mouse muscle [5]. Over 2,200 sites of S-glutathionylation modifications were detected, of which 1290 were significantly increased after contractions. Regulatory cysteines on metabolic proteins were S-glutathionylated, including mitochondrial ETC complexes I, II, and V (ATP synthase), GAPDH, malate dehydrogenase 1 (MDH1), and aconitase 2 (ACO2). S-glutathionylation of these proteins is known to regulate their activity. For example, contraction resulted in S-glutathionylation of Cys150 in the key glycolytic enzyme GAPDH, which has been shown to inactivate the enzyme and alter its tertiary structure [37]. Furthermore, ATP synthase, which is responsible for utilising the proton-motive force for ATP production, was S-glutathionylated at Cys244 and Cys294 of the alpha subunit following contractions. S-glutathionylation of Cys294, although initially inhibitory, provides protection from the irreversible modification of sulfenic to sulfinic to sulfonic acid formation [38]. Muscle contractions also resulted in S-glutathionylation of excitation–contraction coupling proteins RyR1 and sarco/endoplasmic reticulum calcium ATPase 1 (SERCA1), and myofibrillar proteins titin (TTN) and troponin I2 (TNNI2). Oxidative modification of excitation–contraction coupling and myofibrillar proteins can have an immediate effect on contraction and may be responsible for some of the changes in contraction kinetics observed during exercise-induced fatigue. For example, S-glutathionylation of SERCA1 at Cys674 can disrupt calcium reuptake into the sarcoplasmic reticulum and impact muscle relaxation [48]. The largest fold changes in the S-glutathionylated proteome after contractions occurred on signalling proteins such as 14-3-3 protein gamma (YWHAG) and mitogen-activated protein kinase 4 (MAPK4), as well as proteins like SERCA and NADH:ubiquinone oxidoreductase subunit V2 of mitochondrial complex I (NDUFV2). Taken together, these findings highlight the important role of redox regulation in controlling muscle physiology, metabolism, and the adaptive response to exercise.

Using the RAC-TMT approach, Campbell and colleagues demonstrated that ageing is also associated with increased S-glutathionylation of protein cysteine thiols in mouse skeletal muscle [49]. Among the biological processes affected, the TCA cycle showed the highest proportion of proteins with significantly altered S-glutathionylation, followed by pathways involved in sarcomeric structure and assembly. Proteins mediating interactions between the intracellular and extracellular environments were especially vulnerable to age-related shifts in redox status. Specifically, proteins associated with focal adhesion, the structural link between the plasma membrane and the extracellular matrix, as well as components of the myelin sheath in motor neurons, were highly modified in aged muscle. Focal adhesion molecules play a role in linking the sarcolemma to the extracellular matrix, which is critical for formation and stabilization of the neuromuscular junction

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[50]. This is of particular interest given the growing recognition of the importance of loss of motor units and disruption of the neuromuscular junction in sarcopenia [51]. Mitochondrial proteins were also highly sensitive to age-related increases in S-glutathionylation with almost one-third of all cysteine sites displaying significant alterations in S-glutathionylation located in or associated with the mitochondria. Notably, treatment of aged mice with the mitochondria-targeted compound SS-31 (Elamipretide) restored redox homeostasis in skeletal muscle and the profile of S-glutathionylation on skeletal muscle proteins in aged mice treated with SS-31 closely resembled that of young mice. Reversal of these modifications by SS-31 treatment strongly supports an important role for mitochondrial redox regulation in age-related protein modifications.

To enhance coverage of the redox proteome, the RAC-TMT workflow has been modified to incorporate a microscale fractionation scheme to further reduce sample complexity and improve the detection of redox-sensitive peptides [52]. This workflow has been applied to investigate changes in protein cysteine oxidation in a mouse model of muscular dystrophy [52]. In-depth coverage of the redox proteome was achieved with >18,000 Cys sites from 5,608 proteins in muscle being quantified. Notably, this level of coverage was enabled by the incorporation of offline fractionation strategies, which are increasingly essential for achieving deep and reproducible coverage in redox proteomics and may also be required to maximise site depth in cysteine-reactive phosphate tag–based workflows. Compared to the control group, *mdx* mice exhibited a marked increase in cysteine oxidation, where a ~2% shift in the median oxidation occupancy was observed. Marked changes in cysteine oxidation were seen in proteins associated with coagulation in *mdx* mice. Specifically, cysteines on proteins involved in the formation of blood clots, including fibrinogen beta/gamma chain (FGB/FGG), von Willebrand factor (VWF), plasminogen (PLG), prothrombin (F2), and thrombomodulin (THBD), displayed greater oxidation in *mdx* mice. Abnormalities or disorders in blood coagulation are side effects of Duchenne muscular dystrophy (DMD) [53]. While the underlying molecular mechanisms linking DMD and blood coagulation abnormalities are unclear, these findings suggest that oxidative stress may be involved. Increased cysteine oxidation was also observed in proteins involved in phagosome degradation by lysosomes, which may contribute to autophagic dysfunction in *mdx* mice. Autophagy is vital for homeostasis of muscle tissue and skeletal muscle may succumb to degeneration when autophagy is impaired.

The RAC-TMT workflow has been employed to investigate associations between skeletal muscle protein cysteine oxidation and physical performance in older individuals. Day and colleagues [43] quantified the oxidation of 15,049 cysteine sites, covering 4,354 proteins in skeletal muscle. Higher levels of cysteine oxidation on muscle contractile proteins were generally negatively associated with measures of muscle function, physical performance and fitness. For example, higher oxidation of cysteine sites in nebulin was associated with lower peak oxygen uptake (VO_2peak). Nebulin regulates the length of the sarcomeric thin filament by acting as a molecular ruler to establish a minimal length for filamentous actin [54]. Thus, oxidation of cysteines on nebulin may disrupt these essential interactions and dysregulate the organisation of actomyosin filaments with downstream implications for muscle contraction. Greater oxidation of cysteine sites in myomesin-1 (MYOM1), myomesin-2 (MYOM2), and nebulin was associated with slower walking speed and higher oxidation of cysteine sites in myomesin-2 (MYOM2) and alpha-actinin-2 (ACTN2) was associated with lower leg power and strength. Of note, oxidation of alpha-actinin-3 (ACTN3) was positively associated with peak isotonic leg power. ACTN proteins bind and crosslink overlapping F-actin filaments at the Z-line in the sarcomere [55], making them important proteins for muscle integrity and quality. Therefore, oxidation in the different functional domains of ACTN3 may regulate its function in generating maximum contractile power. Despite this observation, oxidation of cysteines on muscle proteins was generally associated with impaired muscle function and physical performance in older individuals. These findings provide a potential mechanism underlying the links between oxidative stress and declines in muscle function and mobility during ageing.

Cysteine-Reactive Phosphate Tags

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While redox proteomics typically identifies up to several thousand cysteine sites, phosphoproteomic approaches achieve greater coverage, highlighting significant differences in the capacity of these approaches to recover proteins of interest. To improve the enrichment of cysteine-containing proteins from tissue extracts, cysteine-reactive reagents have been chemically modified to incorporate tags that facilitate their selective capture (Figure 4). Notably, the cysteine-reactive compound iodoacetamide has been modified to carry a phosphate group, allowing labelled peptides to be purified using immobilised metal affinity chromatography (IMAC) – a technique commonly used for phosphopeptide enrichment. IMAC-based enrichment enhances coverage because the phosphate group on the modified cysteine provides a strong, specific affinity handle for metal ions such as Fe^{3+} or Ti^{4+}, enabling efficient and selective pull-down of labelled peptides. The use of cysteine-reactive phosphate tags (CPTs) improves recovery of low-abundance cysteine-containing peptides, thereby increasing coverage of the redox proteome. Furthermore, while this technique has only been used to detect total oxidised cysteines, it could also be used to enrich thiols that have been selectively reduced to probe for a specific redox modification.

Using CPTs combined with TMT multiplexing, Xiao and colleagues measured protein cysteine oxidation across 10 mouse tissues to investigate age-associated redox changes [43]. They quantified the oxidation of ~34,000 unique cysteine sites on ~9,400 proteins across all tissues, including ~4,500 cysteine sites in skeletal muscle. Similar to previous studies employing differential cysteine labelling techniques[12,44], their data revealed that redox-regulated cysteines involved in tissue-specific regulation were lost with age. Moreover, aged tissues displayed distinct, tissue-specific remodelling of the redox signalling landscape. In skeletal muscle, ageing was associated with greater oxidation of cysteines on protein drivers of muscular dystrophies, including Cys774 on collagen alpha-3(VI) chain (COL6A3), Cys630 and Cys1981 on dysferlin (DYSF), Cys321 on myotilin (MYOT), and several sites in TTN – Cys5368, Cys6920, Cys7512, Cys13231, Cys21834, Cys20367, and Cys21689. These results provide a molecular basis for the proposed links between redox dysregulation and specific functional age-related declines in skeletal muscle.

CPTs have also been employed to investigate the role of redox signalling in insulin-stimulated glucose uptake in skeletal muscle [56]. Despite inconsistent evidence, there is a longstanding belief that insulin signalling increases H_2O_2 production, leading to reversible oxidation of cysteine thiols and stimulation of intracellular phospho-signalling downstream of the insulin receptor. However, the results of a recent study that utilised advanced redox tools to study the effects insulin stimulation on H_2O_2 levels and protein cysteine oxidation in mouse skeletal muscle challenged this notion. Using genetically encoded H_2O_2 sensors and non-reducing immunoblotting, Henriquez-Olguin and colleagues observed no increase in skeletal muscle subcellular H_2O_2 levels following insulin stimulation [56]. Furthermore, stoichiometric cysteine proteome analyses using CPTs combined with TMT multiplexing revealed a selective pro-reductive shift in cysteine modifications affecting insulin transduction related proteins. Specifically, Cys179 on glycogen synthase kinase-3 beta (GSK3B), a classical insulin-inhibited kinase, was selectively reduced by insulin, in addition to other cysteines on known insulin-action–related proteins such as Cys416 on Ras and Rab interactor 2 (RIN2) and Cys450 on supervillin (SVIL). The selective reduction of these proteins correlated with the glucose-lowering effect of insulin. Together, these findings suggest that an insulin-stimulated pro-reductive shift modulates specific aspects of insulin signal transduction.

Challenges with Redox Proteomic Approaches

One of the major difficulties in the application of redox proteomic techniques is the prevention of artificial oxidation during sample collection, storage, and processing. To prevent oxidation of proteins during sample processing, it is essential to block native free thiols with an alkylating agent such as NEM. Following the selective reduction of reversibly oxidised thiols, it is still possible that the nascent free thiols can be re-oxidised during subsequent sample processing steps to form inter- or intra-molecular disulfides. This is an ever-present issue for the methods described above as they all employ selective reduction of oxidised thiols. In the case of RAC, a small concentration of DTT is

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retained in the buffer during the capture step to prevent such issues and to ensure high recovery of formerly oxidised peptides.

Another limitation of redox proteomic approaches is their relative inaccessibility to most researchers. Microplate assays that can measure protein thiol redox state have been developed and present an alternative method to study redox signalling in skeletal muscle. The Antibody-Linked Oxi-State Assay (ALISA) is an ELISA-based method designed to measure target-specific protein thiol redox state [57]. In this approach, reduced thiols are alkylated to preserve the native redox status. Reversibly oxidised thiols can then be selectively reduced and labelled with fluorescent maleimide (F-MAL). Samples are then incubated with a capture antibody, and the capture antibody-target complex is labelled with an amine-reactive fluorescent *N*-hydroxysuccinimide ester (F–NHS). The target is then selectively eluted and target specific F-MAL and F-NHS fluorescence is measured. Target specific protein thiol redox state can then be ratiometrically calculated as F-MAL/F-NHS. Alternatively, the RedoxiFluor assay uses two thiol-reactive fluorescent reporters to measure target-specific protein thiol redox state in percentages and moles [58], providing enhanced interpretation compared to fold changes provided by the ALISA. Reduced thiols are initially labelled with a fluorescent maleimide (F-MAL1) reporter and reversibly oxidised thiols are then reduced and labelled with a spectrally distinct fluorescent maleimide reporter (F-MAL2). A capture antibody functionalised solid support is used to bind the target protein thiol from a biological sample. The thiol redox state encoded F-MAL reporters enable quantification of target-specific protein thiol redox state in percentages while a biotin-conjugated detector antibody and recombinant protein standard curve enable calculation of target-specific protein thiol redox state in percentages and moles. RedoxiFluor can also be used in array mode to quantify the redox state of several proteins in percentages in a single assay. To date, these methods have been used to detect changes in target-specific protein thiol oxidation in response to fertilisation in *X. laevis* eggs [57] and lipopolysaccharide treatment in human monocytes [58]. Future studies applying these methods to study redox signalling in skeletal muscle will be of interest.

5. Approaches to Study the Role of Redox PTMs

Beyond identifying redox modifications, causal validation of cysteine redox modifications is critical to understand their role in biology. Redox-dead transgenic mouse models in which critical cysteine residues are replaced by amino acids, usually serine or alanine so they can no longer undergo reversible oxidation, provide an approach to study the biological role of protein cysteine oxidation in vivo. For example, loss of PKARIα oxidation in redox-dead Cys17Ser PKARIα knock-in mice impairs blood vessel re-growth after hind-limb ischemia and reduces tumour growth and vascularization compared with wild-type mice [59], highlighting a role for PKARIα oxidation in ROS-mediated angiogenesis.

While the generation of redox-dead transgenic mice provides a mechanism to study the biological roles of protein cysteine oxidation, a limitation of this approach is that cysteine substitution also disrupts non-redox cysteine roles (i.e., structural disulfide or metal binding). Emerging chemical biology strategies that selectively induce or block specific cysteine redox modifications offer the potential to study the role of site-specific modifications in cellular processes without perturbing other non-redox processes [60]. Bioorthogonal cleavage chemistry, combined with genetic code expansion, has emerged as a transformative tool for precise protein manipulation in living systems [61]. This approach enables incorporation of unnatural amino acids into proteins at defined sites and controlled activation of proteins by releasing functional groups on demand [62] and could be leveraged to achieve site-specific incorporation of sulfenic acid (SOH) modifications within a protein of interest, presenting a gain-of-function model to study the role of protein cysteine modification. By replacing a target cysteine residue with chemically "caged" unnatural amino acids, the redox-sensitive SOH modification could be masked during protein synthesis and later activated via bioorthogonal cleavage chemistry under controlled conditions [60]. Alternatively, the development of redox-targeted covalent inhibitors (TCIs) provides a means to selectively capture and block SOH-modified

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proteins, thereby preventing downstream redox-dependent signalling steps [60]. However, existing tools are primarily designed for broad profiling of SOH across the proteome, rather than selective targeting. Together, these strategies may facilitate causal validation of SOH-mediated redox signalling, providing mechanistic insight into the roles of cysteine oxidation in redox regulation in biological processes.

6. Conclusion and Future Directions

Redox proteomic approaches have enabled detailed characterisation of protein cysteine oxidation patterns in skeletal muscle, providing valuable insight into the molecular mechanisms underlying muscle function, adaptation, and pathology. Future studies that combine redox proteomics with techniques for site-specific manipulation of cysteine residues will allow for more direct investigation of how individual redox modifications influence protein function. By linking changes in redox state with functional outcomes, these approaches will help to delineate the precise role of cysteine oxidation in muscle physiology and may reveal key pathways altered in pathological conditions, opening new avenues for targeted therapeutic intervention. Integrating redox proteomics with other omics technologies, including phosphoproteomics and transcriptomics, will further enhance our understanding of skeletal muscle redox regulatory networks. Mapping the links between cysteine oxidation and other PTMs will enable the identification of broader regulatory networks that coordinate muscle function in both health and disease. Given the central role of redox signalling in regulating muscle function, targeting cysteine oxidation represents a promising therapeutic strategy for diseases linked to impaired redox regulation (e.g., muscular dystrophy). Future research should explore redox-based interventions, including antioxidants or compounds that selectively modulate cysteine oxidation, to prevent or treat muscle-related diseases. The development of small molecules that target redox-sensitive cysteine residues or restore redox homeostasis could provide novel therapeutic options. Finally, because the functional consequences of cysteine oxidation are often difficult to predict due to the complexity of redox modifications and their effects on protein conformation, advanced computational approaches, such as molecular dynamics simulations and structural bioinformatics, offer powerful tools to model these changes. Given that contemporary redox proteomic approaches provide large amounts of data, these approaches will be important to indicate functional effects for further study. Leveraging these technologies will allow researchers to predict how specific cysteine oxidation events influence the structural and functional dynamics of key skeletal muscle proteins, providing mechanistic insight into redox regulation in muscle physiology.

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