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Review Article

Proteomic strategies to unravel age-related redox signalling defects in skeletal muscle

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ABSTRACT

Increased oxidative damage and disrupted redox signalling are consistently associated with age-related loss of skeletal muscle mass and function. Redox signalling can directly regulate biogenesis and degradation pathways and indirectly via activation of key transcription factors. Contracting skeletal muscle fibres endogenously generate free radicals (e.g. superoxide) and non-radical derivatives (e.g. hydrogen peroxide). Exercise induced redox signalling can promote beneficial adaptive responses that are disrupted by age-related redox changes. Identifying and quantifying the redox signalling pathways responsible for successful adaptation to exercise makes skeletal muscle an attractive physiological model for redox proteomic approaches. Site specific identification of the redox modification and quantification of site occupancy in the context of protein abundance remains a crucial concept for redox proteomics approaches. Notwithstanding, the technical limitations associated with skeletal muscle for proteomic analysis, we discuss current approaches for the identification and quantification of transient and stable redox modifications that have been employed to date in ageing research. We also discuss recent developments in proteomic approaches in skeletal muscle and potential implications and opportunities for investigating disrupted redox signalling in skeletal muscle ageing.

1. Age-related loss of skeletal muscle mass and function

The age-related loss of skeletal muscle mass and function is a major contributor to morbidity and has a profound effect on the quality of life of older people. The reduction in muscle strength with age leads to instability, increased risk of falls and consequently an increased demand for medical and social care [1]. Decline in skeletal muscle strength becomes apparent from the age of 40 and continues until the end of life [2]. Human studies have reported that by the age of 70 years, there is a 25–30% reduction in the cross sectional area of skeletal muscle and a decline in muscle strength by 30–40% [3].

Age-related skeletal muscle atrophy is associated with a number of neurological abnormalities including motor unit loss [4,5], degeneration and structural alterations of neuromuscular junctions (NMJ) [6–8], a decline in motor nerve function (partial denervation) [7,9–11], impaired nerve redox signalling [12] and increased fibre type shifting due to denervation/reinnervation cycling [13]. While physical activity can undoubtedly modify the rate of skeletal muscle functional deficits [14], even physically active older adults exhibit an age-related decline in skeletal muscle mass and function [15,16]. Age-related skeletal muscle atrophy and weakness is a life-long process with a multifactorial and complex etiology that involves both extrinsic and intrinsic factors [13]. An altered redox environment within skeletal muscle fibres is associated with the primary molecular and biochemical mechanisms underlying age-related deficits in neuromuscular integrity and function [17,18].

1.1. Redox homeostasis and neuromuscular ageing

Skeletal muscle generates reactive oxygen species (ROS), in particular superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), from a variety of intracellular sources [19]. Experimental evidence in humans [20–22] and rodents [23,24] demonstrates that skeletal muscle exhibits age-dependent increases in the products of oxidative damage to biomolecules including proteins, lipids and nucleic acids. Recent reports have provided evidence that increased oxidative damage inherent with ageing is associated with age-related changes in ROS, with skeletal muscle fibres from old rodents exhibiting elevated intracellular ROS

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levels compared to young/adult rodents [24].

Identifying the mechanisms underlying the structural and functional changes that occur in skeletal muscle during neuromuscular ageing has stimulated the interest of many laboratories to investigate the protein content or skeletal muscle proteome and in particular reversible and irreversible ROS induced oxidative modifications. Redox modifications of key metabolic proteins can alter a wide variety of metabolic pathways influencing related post-translational modifications (PTMs) such as phosphorylation, ubiquitination and acetylation. Redox signalling can modify the activity of key metabolic proteins such as AMP-activated protein kinase (AMPK), protein kinase C (PKC), sirtuin 1 and mammalian target of rapamycin (mTOR) [25]. Redox signals generated during lengthening contractions *ex vivo* are essential for the adaptive response to contractile activity [26] and skeletal muscle has repeatedly been shown to develop a disrupted redox response defined by defective signalling with ageing [25,27]. Evidence suggests ROS can alter myofilament structure and function [28]. Several myofilament proteins including Troponin C [29], Actin [30], α-Actinin [27,28], and Myosin heavy chains [30–32] are susceptible to oxidative modifications, affecting Ca^{2+} dynamics and sensitivity [33], and inevitably cross-bridge kinetics [29], which may result in contractile dysfunction. In addition, irreversible oxidative modifications can lead to formation of insoluble protein aggregates and impaired protein degradation, which have been reported to increase in neurodegenerative diseases and ageing [34].

2. Exercise attenuates age related atrophy

Exercise training counteracts age-related declines in aerobic capacity, skeletal muscle mass and strength. The adaptive responses to exercise reflects the type of training undertaken. Typically, endurance type exercise training improves cardiovascular adaptations whereas resistance type exercise training improves skeletal muscle mass and strength [35]. Endurance exercise training improves maximal oxygen uptake (VO_{2max}), a marker of aerobic capacity, in the elderly. Cross-sectional studies demonstrate that lifelong endurance training is associated with preserved VO_{2max} [36], corresponding to a difference of ~25–30 ml kg^{-1} min^{-1} in VO_{2max} between trained and untrained individuals between 25 and 65 years [37]. Longitudinal studies reveal that endurance training improves VO_{2max} in the elderly [38]. Improved VO_{2max} is significant because aerobic capacity is a predictor of all-cause mortality. Intriguingly, endurance type exercise also improves muscle mass and strength in the elderly. Harber et al. [39] found that 12 weeks of cycling training improved aerobic capacity by 30% and quadriceps size by 12% in older women and their finding have been recapitulated (reviewed in [40]). Resistance type exercise potently enhances skeletal muscle mass in the elderly to attenuate age-related skeletal muscle atrophy [41]. Leenders et al. [42] report that 6 months of resistance exercise improves quadriceps cross sectional area by 9% in the elderly. As Witard et al. [40] remark nutritional strategies (e.g. protein ingestion) can potentiate the adaptive responses to resistance exercise in the elderly. Analogous to endurance type exercise, Melov et al. [43] report that resistance type exercise reverses mitochondrial dysfunction at the transcriptional level in the elderly. In summary, exercise training is an effective strategy to counteract age-related declines in skeletal muscle mass.

2.1. Endogenous ROS generation in quiescent and exercised skeletal muscle fibres

Skeletal muscle fibres produce ROS, notably $O_2^{\cdot-}$ and H_2O_2 [18], their biochemistry is comprehensively reviewed elsewhere [44]. Key $O_2^{\cdot-}/H_2O_2$ producers in skeletal muscle include mitochondria and NADPH oxidase (NOX) isoforms (Fig. 1), as well as, cytochrome P450 enzymes, cyclooxygenases and lipoxygenases (see [19] for a comprehensive review). Site specific increases in endogenously generated ROS can activate/inactivate a number of key cellular pathways including

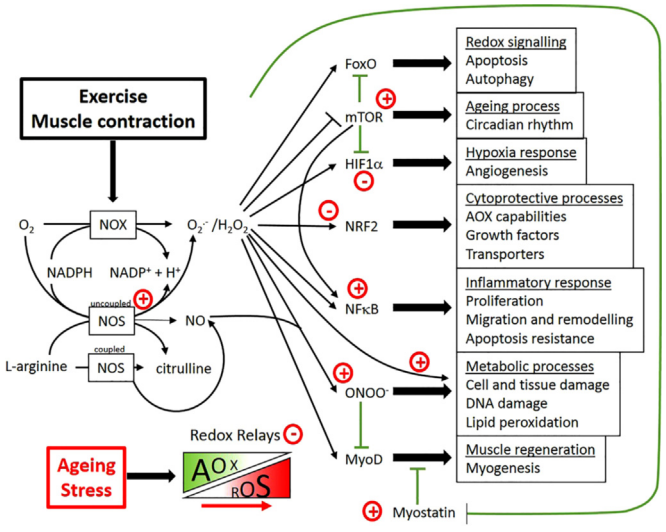

Fig. 1. Major cellular events associated with redox signalling under exercise and stress conditions. Normal homeostatic redox coupling ($O_2^{\cdot-}/H_2O_2$ and NO via NOX and NOS) leads to activation of numerous gene activation pathways such as muscle regeneration (MyoD), detoxification (FoxO), hypoxic responses (HIF1a), cytoprotective processes (NRF-2), activation of genes involved in inflammation and proliferation (NF-kB), and inhibition (green lines) of genes associated with ageing (mTOR). Stressors such as the ageing process (red) imbalances this system whereby ROS and RNS levels are chronically activated e.g. uncoupled NOS activity and mitochondrial H_2O_2 release, blunting redox signalling and resulting in an imbalance of mTOR/AMPK pathway, which in turn inhibits events under control of FoxO and HIF1α, and elevates the response by NF-kB. Simultaneously, ageing-associated myostatin activation inhibits the MyoD effects, and the quantitative rise of reactive molecule species leads ultimately to damaging metabolic events.

cytoprotective (Nrf2), inflammation (NFκB) and muscle regeneration (MyoD) (Fig. 1). It is widely held that mitochondria produce the bulk of $O_2^{\cdot-}/H_2O_2$ in quiescent skeletal muscle fibres [19]. Such a view is coherent with a body of work in isolated mitochondria [45] suggesting low ATP demand coupled with increased [NADH] increases mitochondrial $O_2^{\cdot-}$ production at discrete sites (primarily in complex I and III [46]), one would expect such conditions in inactive skeletal muscle fibres. Consistent with this, Brand's group [47] demonstrate that mitochondrial H_2O_2 release is 340 pmol min^{-1} mg^{-1} protein when rat skeletal muscle mitochondria are incubated with a native substrate mix designed to mimic the resting situation compared with 50 pmol min^{-1} mg^{-1} protein during conditions designed to mimic intense exercise. The absolute contribution of different intracellular sources to $O_2^{\cdot-}/H_2O_2$ production in quiescent skeletal muscle fibres is unclear because of the technical difficulties associated with measuring and quantifying $O_2^{\cdot-}/H_2O_2$ [48].

Evidence consistently suggests that exercise increases biochemical surrogates of $[O_2^{\cdot-}/H_2O_2]$ in skeletal muscle that can potentially activate or inhibit a number of key signalling pathways within skeletal muscle (Fig. 1) [18]. It was traditionally considered that the increase in skeletal muscle O_2 uptake concomitantly increased $O_2^{\cdot-}/H_2O_2$ production during exercise [18,49]. However, exercise decreases mitochondrial $O_2^{\cdot-}/H_2O_2$ production [47], a probable corollary of increased ATP demand, respiration and decreased [NADH] [45]. Nevertheless, mitochondrial dysfunction (e.g. mito-nuclear DNA mismatch or mutations [50]) may raise mitochondrial $O_2^{\cdot-}/H_2O_2$ production in the exercised state, which may be important in an ageing context given mitochondrial heteroplasmy [17]. The principal contributor to exercise induced $O_2^{\cdot-}$ production appears to be NOX isoforms [19,51]. Evidence has shown [24] that NOX isoforms, particularly NOX2 in the sarcolemma and transverse t-tubules [52], drives exercise induced increase in cytosolic $O_2^{\cdot-}$ in isolated skeletal muscle fibres. The interesting question of

how contracting skeletal muscle fibres sustain NOX isoform mediated $O_2^{\cdot-}$ production, which requires NADPH, Rac1 and a multi-protein membrane complex [53] during exercise is unclear. In addition, the role of other non-mitochondrial sources of $O_2^{\cdot-}$—for example molecules interacting with CasL [54]—to the exercise induced increase in $O_2^{\cdot-}/H_2O_2$ is relatively unexplored. Further research is required to gain a better understanding of the quiescent and exercise induced $O_2^{\cdot-}/H_2O_2$ production in skeletal muscle.

2.2. Exercise induced redox signalling inducts beneficial adaptive responses

Given the resources needed to deliberately sustain exercise induced NOX activity (e.g. NADPH), it is likely that the $O_2^{\cdot-}/H_2O_2$ generated play meaningful biological roles [55]. Accordingly, $O_2^{\cdot-}/H_2O_2$ are permissive for optimal muscle force generation [56]. However, beyond a certain point, $O_2^{\cdot-}/H_2O_2$ induce skeletal muscle fatigue [57] which has been suggested to provide a protective brake preventing excessive damage [58]. Exercise induced $O_2^{\cdot-}/H_2O_2$ herald a number of beneficial adaptive responses (Fig. 1) [59–62]. Evidence for an adaptive role for $O_2^{\cdot-}/H_2O_2$ is in part derived from studies showing that alpha tocopherol and ascorbate supplementation blunts exercise adaptations [63–65]. For example, alpha tocopherol and ascorbate supplementation abrogates cytochrome c oxidase gene expression following endurance training [65]. Kinetic constraints limit the direct reaction of both alpha tocopherol and ascorbate with $O_2^{\cdot-}/H_2O_2$ [66], how they interfere with redox signalling is unclear. Notwithstanding, pharmacologically inhibiting NOX2 suppresses early transcriptional responses to exercise in mice [67]. Moreover, individuals with the lowest change in oxidative stress biomarkers post exercise experienced the lowest response to exercise training whereas those with the highest response improved to the greatest extent [68].

How $O_2^{\cdot-}/H_2O_2$ stimulate exercise adaptations referred to in Fig. 1 are unresolved. The tacit view is that $O_2^{\cdot-}/H_2O_2$ signal beneficial adaptive responses by post translationally modifying signalling proteins [69], typically at critical cysteine and methionine residues (discussed below). Because H_2O_2 reacts slowly (k ~1–10 M^{-1} s^{-1}) with most signalling proteins [70,71], redox signalling likely involves more kinetically favourable redox relays [72,73]. Redox relays define situations wherein H_2O_2 reacts with a rapid (e.g. peroxiredoxin isoforms) reactant, before the rapid reactant transfers H_2O_2 derived oxidising equivalents to a signalling protein [73], which may afford specificity in redox signalling via choreographed protein-protein interaction. Such a mechanism has been demonstrated for STAT3 [73] and is now thought to be pervasive [71]. Direct evidence for redox relays in an exercise context is still lacking [66]. Regardless of the mechanism, exercise associated redox signalling seems to converge on the transcription factors Nrf-2, AP-1 and NF-kB [74]. Redox sensitive transcription factors upregulate a number of antioxidant enzymes (e.g. peroxiredoxins). Nrf-2 provides a paradigmatic example. Under quiescent conditions, KEAP1 sequesters Nrf-2 in the cytoplasm, which enables the E3 ubiquitin ligase cullin 3 to tag Nrf-2 for degradation by the 20 S proteasome. Disrupted redox homeostasis, can activate Nrf-2 signalling via inhibiting KEAP1 secondary to oxidative modification (typically alkylation) of conserved cysteine residues (whether such modification is direct or mediated through relays is unclear in an exercise context). Nrf-2 can then enter the nucleus to transcribe cyto-protective genes.

2.3. The influence of ageing on exercise induced redox signalling: evidence for failed adaptive responses

Several studies suggest ageing attenuates redox regulated adaptive responses to acute exercise in sedentary mice (reviewed in [16]). Attenuated responses include an inability to up-regulate antioxidant enzymes and heat shock proteins owing to blunted NF-kB and AP-1 activity, which may be related to insufficient exercise induced $O_2^{\cdot-}/H_2O_2$ production [75]. In addition, nuclear translocation of Nrf-2 is impaired in older adults following acute exercise [76], consistent with age-related decrements in Nrf-2 activity [77,78]. The nature of the defect is unclear—it could relate to an inability to use NOX isoforms to generate $O_2^{\cdot-}/H_2O_2$ or execute redox relays (Fig. 1). A further possibility is that adaptive responses to acute exercise are blunted because the elderly already have the adaptation at baseline [16]. That is, stress (e.g. DNA damage [20]) in quiescent skeletal muscle necessitates adaptive responses without exercise, such that little scope for exercise to have further effect exists (Fig. 1). A similar situation seems to characterise ageing (reviewed in [79]). Perhaps, the inability to mount redox responses to acute exercise may reflect a lifetime of sedentary living [80]. However, lifelong endurance type exercise is unable to preserve *some* acute translational responses to acute exercise in human skeletal muscle [21], suggesting age *per se,* as opposed to inactivity, abrogates exercise induced redox signalling [16]. In an exercise context, defects seem specific to redox responses because older individuals retain their ability to mount appropriate mitochondrial remodelling associated transcriptional and translational responses to acute exercise [81,82]. Further research is required to understand how exercise induced redox signalling fails, proteomic approaches are ideally suited to this end.

3. Limitations in proteomic analysis of skeletal muscle

Proteomics is a powerful analytical technique for the analysis of the skeletal muscle proteome in response to exercise, ageing and pharmacological interventions [83]. Fig. 2 shows an overview of the proteomic strategies used to separate, acquire data and identification of proteins from the skeletal muscle proteome. In most examples proteomics is used as a hypothesis generating or discovery approach that complements conventional physiological and histological measurements to gain molecular insights. Due to the composition of the sarcomere with an abundance of contractile proteins (alpha actin and myosin), the quantification and analysis of the skeletal muscle proteome has proved to be challenging when applying conventional approaches. It has been estimated that sarcomeric and cytoskeletal proteins account for more than 50% of the skeletal muscle proteome [84]. Proteomic analysis is further complicated by large numbers of membrane associated proteins, major protein complexes and large proteins, notably nebulin (~600–900 kDa) and titin (~3.5 MDA) [85]. Many of the regulatory proteins involved in cellular transcription and signalling (e.g. peroxisome proliferator activated receptor co-activator one alpha) are expressed at low concentrations meaning that comparison of low to high abundant proteins can span up to seven orders of magnitude [86]. Protein synthesis, degradation and cross-talk between PTMs, effectively places the proteome in a constant state of flux. In relation to redox proteomics, the large number of both transient and stable protein PTMs and their role in cellular signalling further complicates the analysis. Many known cysteine PTMs lack functional annotation—making it difficult to discern if the modification identified is biologically meaningful [87]. Individual PTMs of catalytic sites or regulating co-factor binding can potentially directly regulate protein activity, signalling cascades or selection of proteins for degradation/secretion [88].

3.1. Data dependent and independent acquisition

A number of approaches using 2D electrophoresis and gel free mass spectrometry have been used to analyse the skeletal muscle proteome under a variety of conditions with highly abundant proteins being regularly identified and quantified [89]. Gel free mass spectrometry approaches have generally been performed using an unbiased shotgun or bottom up proteomic approach, whereby the proteome is digested using a common protease (usually the serine protease trypsin) and peptide ions are separated and fragmented using MS/MS. Typically, the fragmentation data generated is searched against the relevant protein database to get the most probable sequence of the parent ion of the peptide and subsequent identification of the proteins originally present

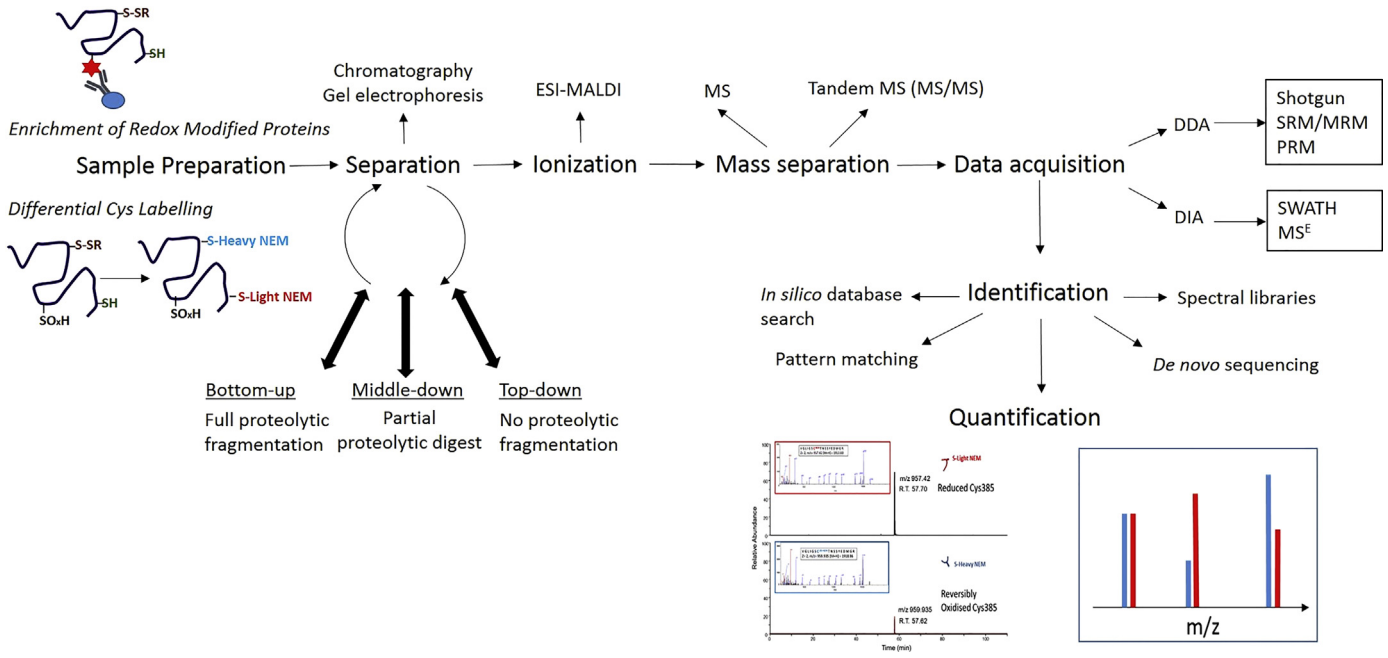

Fig. 2. Schematic overview of mass spectrometry strategies for analysis of the skeletal muscle proteome. Identification of redox modified proteins can require specific enrichment and/or labelling strategies during sample preparation.

in the sample. Label free relative quantification of protein abundance between conditions can be estimated using the relative intensity of unique peptides of a particular protein, or peptide labelling with isobaric tags to quantify multiple samples in a single analysis. These approaches up until recently have generally been performed using data dependent acquisition (DDA), also referred to as information dependent acquisition (IDA), which essentially means that during the cycle times of the instrument a number of peptide precursor ions are selected for fragmentation in the order of decreasing relative intensity. In skeletal muscle, highly abundant proteins have peptide ions that are identified multiple times while low abundant proteins might remain unidentified. That is, DDA preferentially selects highly abundant proteins for analysis and hence identification and quantification. To overcome the large dynamic range within skeletal muscle approaches such as subcellular fractionation or organelle preparations can increase sensitivity [90]. Large scale fractionation of the proteome can also increase proteome coverage [86].

Approaches to overcome the constraints of DDA include using targeted analysis such as selected reaction monitoring (SRM) or parallel reaction monitoring (PRM) [91,92]. Both approaches are hypothesis driven and information on the peptide ions from the target proteins are required before analysis. One does, therefore, sacrifice hypothesis free analysis. As the resolution and cycling times of MS equipment have improved, there has been an increased focus on employing SWATH (sequential window acquisition of all theoretical fragment ion spectra) approaches where all peptide precursors detected are fragmented regardless of their relative intensity, improving the dynamic range. The data obtained is subsequently mined for the protein of interest using peptide libraries of the protein of interest, potentially this technique has numerous possibilities for the analysis of the proteome and particularly the redox proteome. The data generated from the analysis of large numbers of samples by dedicated laboratories using standardised protocols are now routinely deposited in open access repositories allowing secondary data use by researchers who lack access or resources. If detailed sample preparation protocols are included that are amenable for the detection of particular PTMs it would allow post analysis of a large number of high quality data files from independent laboratories.

3.2. Protein turnover in skeletal muscle

Another consideration for the proteomic analysis of skeletal muscle in ageing is protein turnover. The dynamic flow of molecules from the protein to the organelle level is regulated by the integration of functionally important pathways, underlying the true biological phenotype. The intracellular milieu is in constant flux responding to cellular signalling through the spatial, temporal and chemical environment [93–95]. Ageing is a complex multifactorial process involving biochemical and morphological changes at the cellular level that are ultimately reflected in an altered phenotype. As a result, skeletal muscle inherently has a number of limitations for a steady state or snapshot approach as fibres consist of a variety of long lived structural proteins but also relatively short lived glycolytic and cytoplasmic enzymes. The flux of individual proteins is dependent on rates of biosynthesis and degradation, such that, changes in turnover can occur in the absence of changes in protein abundance. The constant flux and turnover that is regulated by the synthesis and degradation of proteins means the proteasome and autophagy are essential for the response to stimuli. Impaired autophagy contributes to skeletal muscle ageing [96,97]. Biosynthesis and degradation of proteins is regulated by a wide range of proteins such as kinases, (de)acetylases, proteases, ubiquitin ligases, molecular chaperones and hydrolases. Turnover rates of individual proteins are responsible for metabolic shifts within a cell and critical for tissue remodelling.

Acute exercise has been shown to induce autophagy in skeletal and cardiac muscle and the adaptive response to exercise requires the autophagic degradation of cellular components, allowing the skeletal muscle fibre to rebuild and respond to repetitive bouts of exercise [96]. Autophagy is, therefore, a key house keeping mechanism that regulates cell protein homeostasis, preventing accumulation of damaged proteins or cellular organelles [98]. However, excessive autophagy can be detrimental because it can cause atrophy, which has been reported to occur in a number of cancers and muscular dystrophies [98]. Impaired autophagy and proteasome degradation with age has led to a paradigm shift over the past number of years, intracellular structural components are no longer considered unchanging and replaced only when damaged but are considered to be in dynamic equilibrium. As a result, quantitative analysis of protein expression in skeletal muscle under different

conditions requires high level of resolution for the analysis of changes in dynamic range coupled with sophisticated bioinformatic tools to quantify turnover of individual proteins [93,95,99].

3.3. Future directions of skeletal muscle proteomics

Aside from data independent approaches to investigate the skeletal muscle proteome, the proteomic analysis of skeletal muscle components will also be enhanced by complementary techniques such as imaging mass spectrometry coupled with laser capture microscopy, permitting the analysis of the protein composition of specific muscle components (e.g. mitochondria). A combination of such techniques would allow the identification and relative quantification of the protein composition of particular regions of interest. It is also increasingly recognised that skeletal muscle is a signalling organ that secretes a range of cytokines, peptides and proteins that can have effects on a host of different systems within the body. Proteomic analysis of the secretome of skeletal muscle has identified a range of muscle specific myokines. Furthermore, extracellular vesicles (EVs) released from skeletal muscle during exercise, development and regeneration offer a means of integrating the cellular response between tissues [100]. EVs are increasingly recognised as key signalling communication tools delivering packaged proteins, lipids, mRNA and microRNAs to neighbouring cells. Recent studies have examined the release of EVs from muscle during exercise that provides a means of crosstalk between tissues [100,101]. Purification of EVs and subsequent proteomic analysis has revealed that EVs are typically enriched for structural and biogenesis proteins, protein involved in localisation, transport, vesicle mediated transport and protein targeting [100]. Identification of the cargo contained within those vesicles has identified a number of key proteins required for delivery and inter tissue communication. Further research is required to discover the effects of age on EV communication in skeletal muscle.

4. Redox proteomics in skeletal muscle ageing

Although there are a number of limitations for the proteomic analysis of skeletal muscle (discussed above), the endogenous generation of ROS in skeletal muscle during exercise makes it an ideal model for studying redox signalling using proteomic approaches [102]. It is over 30 years since it was demonstrated using electron paramagnetic resonance spectrometry that contracting skeletal muscle fibres generate ROS [103], which potentially play an important signalling role [66]. Since these landmark studies a number of studies have proposed the mitochondrial and non-mitochondrial sites and enzymes responsible for the contraction induced increase in endogenous ROS generation (see above [19]). Skeletal muscle from older individuals have consistently been reported to have chronically higher levels of intracellular ROS and overall a higher oxidative status potentially interfering with the skeletal muscle redox signalling responses (reviewed in [18]) but also directly affecting the function of proteins involved in contractile machinery [104]. Skeletal muscle function can be directly affected by reversible and irreversible ROS induced protein modifications potentially affecting proteins regulating Ca^{2+} release from the sarcoplasmic reticulum, Ca^{2+} binding on Troponin or the direct oxidation of contractile proteins (e.g. Myosin) [105,106]. The relevant advantages and disadvantages of techniques employed to study the redox proteome are included in Table 1. Site specific identification and relative quantitation of ROS induced modifications by redox proteomics would complement physiological measurements of skeletal muscle function (e.g. specific force) [55,107,108].

4.1. Stable redox modifications in skeletal muscle

Considerable interest in the relationship between the skeletal muscle redox proteome and ageing exists (reviewed in [25]). Studies on the effects of ageing on skeletal muscle from both humans and rodents have consistently identified an increase in irreversible redox modifications of proteins such as carbonylation and 3-nitrotyrosine (nitration of tryptophan and phenylanine is also possible [109]) [110]. Initially changes in carbonylation levels relied on global oxidation levels revealed by western blotting but have been refined to identify modified proteins utilising mass spectrometry [110]. In skeletal muscle, highly abundant metabolic, contractile and mitochondrial proteins have been identified as carbonylated during ageing; including: components of the electron transport chain, Troponin, Creatine kinase M-type, Myosin binding protein C and Myosin [31,111,112]. Although not as widespread as carbonylation, 3-nitrotyrosine formation on proteins is another indicator of increased oxidative status and has been identified to increase in ageing skeletal muscle, modified proteins include: SERCA ATPase, carbonic anhydrase, aconitase and enolase [113,114]. The identification and label free quantification of a protein by mass spectrometry using data from peptide fragmentation is dependent on the identification and fragmentation of a number of unique peptides. However, the site specific identification of oxidative-PTMs such as carbonylation and tyrosine nitration by directly searching against a protein database is challenging. Site occupancy may be relatively low in comparison with total protein abundance and would require high resolution MS instrumentation. Further, the redox modification itself can result in an altered fragmentation profile with the result that no identification may be obtained or false positive identification. For in depth reviews of mass spectrometry approaches for the identification of carbonylation and tyrosine nitrated proteins see [110,115,116].

4.2. Reversible redox modifications in skeletal muscle

To understand the effects of ageing on redox signalling in skeletal muscle many redox proteomic approaches have focused on the reversible and transient oxidation of Cys residues, notable PTMs include disulfide bond formation, S-glutathionylation and S-nitrosylation [117]. This can be particularly challenging due to the labile and transient nature of redox PTMs [88], especially in skeletal muscle tissue that requires extensive tissue homogenisation. Approaches to identify proteins involved in disulfides have typically employed an alkylating reagent (e.g. iodoacetamide or N-ethylmaleimide) in the lysis buffer to block free thiol groups and prevent *ex vivo* oxidation [118,119]. Samples can be desalted to remove the excess alkylating reagent and a reducing agent added (e.g. DTT) to break disulfide bonds regenerating free thiols. Proteins containing newly reduced free thiols can be tagged using biotin [120] or purified using an activated thiol sepharose column [121] with subsequent identification by mass spectrometry. Although this approach will reduce all Cys residues involved in disulfide bonds including S-glutathionylated residues to preclude identifying PTM type, it will identify residues involved in disulfide bond formation. Combining a differential Cys labelling step, employing a light and heavy alkylating reagent allows labelling of Cys residues that were originally in a reduced state and following reduction those involved in disulfide bonds [27]. A targeted analysis of identical peptides labelled with the light and heavy reagents can be performed for the relative quantification of the oxidation state of individual Cys residues [27]. The advantage of this approach is that not only does it allow relative quantification of individual Cys residues but also the relative quantification of protein abundance [27]. This is particularly important when investigating changes that occur between samples with substantial intracellular changes such as skeletal muscle ageing, where changes in the redox state may also be accompanied by significant changes in protein abundance. A recent review outlines considerations to be taken into account for the analysis of disulfide bonds including sample preparation, fragmentation techniques and approaches for identification of disulfide bonds [122].

Recently, a redox proteomic approach analysed the effects of age on two metabolically distinct skeletal muscles that have different susceptibility to age-related atrophy, the oxidative soleus and more

Table 1
Key advantages and disadvantages of proteomic approaches in skeletal muscle, with particular reference to the human skeletal muscle biopsy setting.

Approach	Advantage	Disadvantage
Global shot-gun proteomics	• Hypothesis free (unbiased) • Compatible with multiple sample work up techniques • Multiplex global analysis • Availability of experimental workflows • Sensitive • Amenable to quantitative analysis	• DDA • Often requires complementary techniques to confirm (e.g. western blot or protein activity assay) • Sample work up requires time • Often misses membrane and hydrophobic proteins • Proteome coverage can be limited
MRM/SRM	• Overcomes DDA constraints on dynamic range • Reduces analysis time • Able to analyse low abundant signalling proteins	• Hypothesis driven • Requires knowledge of protein of interest fragmentation behaviour • Loss of global coverage
Redox proteomics in general	• Insight into potential signalling events, particularly with regard to potential scale • Global and multiplex analysis • Availability of workflows • Known annotation of some modified proteins • Methods exist to avoid *ex vivo* oxidation	• Requires additional sample work-up steps that differ according to the modification of interest. • Some modifications are labile. • Specific modifications may serve no signalling role-parallel mechanistic studies required to resolve • Usually % modified site is low and may be occupied by other modifications.
Top-down redox proteomics	• Mass change corresponding to specific redox modification.	• No information on redox modified amino acid.

glycolytic quadriceps [123]. Global proteomic analysis revealed a significant up regulation of contractile proteins in the quadriceps and loss of mitochondrial proteins in soleus, however, the redox sensitive proteins appear to be preserved between the metabolically distinct tissues and were involved in metabolism and contractile machinery [123]. Consistent across a number of different skeletal muscles including the gastrocnemius [27], soleus [123], diaphragm [108], a reduction in the number of redox sensitive proteins detected occurs with age, which could affect redox signalling within skeletal muscle fibres. It would be expected that if there is a decrease in reversible modified Cys residues a corresponding increase in irreversibly oxidised Cys residues would occur, however, this was not always the case. As these studies were performed using DDA, potentially the overall changes in the proteome with an increase in contractile proteins may mask the detection of lower and more subtle changes in the redox proteome.

4.3. Top down redox proteomics

Many of the approaches discussed employed a bottom up or shotgun methods, whereby the protein is digested into peptides, which are then used to identify the respective proteins by mass spectrometry. Recently, a top down proteomic approach investigated age related redox specific changes in a number of contractile proteins from representative fast and slow skeletal muscles from rat, identifying Troponin I as S-glutathionylated by the increased molecular weight due to glutathione moiety (+305.3). Although this approach does not identify the residue modified it is specific for the redox PTM. Cys^{134} of Troponin I has previously been identified as S-glutathionylated and S-nitrosylated with contrasting effects on Ca^{2+} sensitivity, S-glutathionylation increasing and S-nitrosylation decreasing Ca^{2+} sensitivity of the protein [124]. The alternative effects on the functionality of the protein from different redox modifications on the same residue highlights the complexity of redox responses, whereby redox modifications on the same residue can result in distinct effects on enzymatic activity —and the flexibility redox signalling offers [87].

5. Conclusions and perspectives

Redox proteomic approaches that allow site specific identification and quantification of ROS induced proteomic modifications will allow elucidation of the effects of ageing on redox signalling processes in skeletal muscle. It is unlikely that a particular protein is exclusively modified by a single redox modification but exists in a balance of different oxidation states dependent on the overall intracellular redox environment. Therefore, the quantification of the proportion of residues modified in conjunction with overall protein abundance will allow greater insight into crosstalk of PTMs and signalling mechanisms. Integration of approaches that account for the dynamic flux of the proteome through altered turnover and biogenesis will allow for a more in depth understanding of the skeletal muscle proteome and the signalling pathways most susceptible to altered redox states in response to age-related skeletal muscle atrophy or exercise.

Skeletal muscle is an endocrine organ that can secrete myokines and EVs which would provide an attractive conduit for the integration of inter and intra cellular redox signalling. Redox secreted factors form an integral role in regulating intra tissue signalling. Regulation of circadian rhythms in different tissues has been proposed to be regulated by the redox state of circulating peroxiredoxins [125], in a mechanism that couples circadian rhythms with metabolism by modulating mitochondrial H_2O_2 generation [126,127]. However, there is a disruption of the circadian clock during ageing and loss of the core clock gene *Bmal1* leads to an accelerated sarcopenic phenotype in rodents [128]. Nagy and Reddy contend that the increasing importance of circadian oscillations mean it is time to re-think redox interventions [129]. The circadian clock plays an important role in skeletal muscle performance, it has been demonstrated that peak muscle performance assessed by strength and power is between 16.00 and 18.00 [130]. Skeletal muscle receives cues from the central clock located in the suprachiasmatic nucleus (SCN) but also maintains a peripheral clock that receives external cues or “Zeitgebers” such as from exercise and feeding [131,132], that are also involved in localised ROS generation. Integration of intra tissue crosstalk by skeletal muscle via redox secreted factors contained within EVs would be an inviting mechanism for regulating intra tissue signalling during exercise and analysis by redox proteomic approaches. Last, it will be of interest to combine proteomic approaches in older skeletal muscle with analysis of motor neurons given their susceptibility to oxidative stress [133,134], with the role of mitochondria in denervation representing an attractive starting point [13,135]. Overall, continuing improvements in the resolution of instrumentation in proteomics and development of techniques such as SWATH, offer exciting research possibilities for the site specific identification and quantification of redox modifications that play significant roles in altered redox signalling pathways in skeletal muscle ageing.

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