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REVIEW

The application of proteomics in muscle exercise physiology

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ABSTRACT

Introduction: Exercise offers protection from non-communicable diseases and extends healthspan by offsetting natural physiological declines that occur in older age. Striated muscle is the largest bodily organ; it underpins the capacity for physical work, and the responses of muscle to exercise convey the health benefits of a physically active lifestyle. Proteomic surveys of muscle provide a means to study the protective effects of exercise and this review summaries some key findings from literature listed in PubMed during the last 10 years that have led to new insight in muscle exercise physiology.

Areas covered: 'Bottom-up' analyses involving liquid-chromatography tandem mass spectrometry (LC-MS/MS) of peptide digests have become the mainstay of proteomic studies and have been applied to muscle mitochondrial fractions. Enrichment techniques for post-translational modifications, including phosphorylation, acetylation and ubiquitination, have evolved and the analysis of site-specific modifications has become a major area of interest in exercise proteomics. Finally, we consider emergent techniques for dynamic analysis of muscle proteomes that offer new insight to protein turnover and the contributions of synthesis and degradation to changes in protein abundance in response to exercise training.

Expert opinion: Burgeoning methods for dynamic proteome profiling offer new opportunities to study the mechanisms of muscle adaptation.

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1. Introduction

The health benefits of regular exercise link intimately with whole-body maximum aerobic capacity (VO_2max) [1], which can be assessed during an incremental exercise test [2]. Adults experience year-on year declines in VO_2max cumulating to ~6% per decade [3,4], which contributes to a rise in disease risk and mortality during later adulthood. Exercise training is effective in improving VO_2max across the lifespan and offers a proven means to extend an individual's healthspan [5]. The American College of Sports Medicine (ACSM) recommends that adults engage in moderate-intensity cardiorespiratory exercise for 150 min per week, e.g. 30 min sessions 5 days per week. Alternatively, more vigorous-intensity exercise can be done in >20 min sessions 3–4 days per week (i.e. ≥75 min/week). Various combinations of moderate- and vigorous-intensity exercise can be prescribed based on metabolic equivalents (METs), which relate the metabolic cost of an activity to the individual's resting VO_2max. For example, a mixed programme of activities that achieves a total energy expenditure of 500–1000 METs per week conveys significant health benefits [6]. Skeletal muscle is an important component of exercise capacity, and in the context of aging the maintenance of muscle mass and function becomes a key determinant of healthspan and quality-of-life [7]. To maintain and enhance muscle mass and strength, resistance exercises at an intensity of 75–85% of one repetition maximum, completed in 1–3 sets of 8–12 repetitions per session are recommended [6]. Such recommendations for exercise and a physically active lifestyle have been incorporated in to World Health Organization guidance [8] and regular exercise is recognized as being key to the prevention of more than 40 chronic diseases [9], including type 2 diabetes [10], cardiovascular disease [11] and age-related muscle wasting [7].

While the benefits to health are well established, the mechanisms that underpin the protective effects of exercise training are yet to be fully resolved. Striated muscle, including the heart, diaphragm and skeletal muscles, is the principal tissue that underpins the capacity for physical work and athletic performance. In addition to locomotion and respiration, skeletal muscle has fundamental roles in whole-body metabolism. For example, skeletal muscle is the primary site of post-prandial uptake of glucose [12] and is a vital source of amino acids that can be used to repair other tissues [13]. Furthermore, skeletal muscle is an accessible tissue in humans via percutaneous biopsy techniques and offers a rare opportunity to study complex physiology and polygenetic responses to exercise, aging and disease directly in humans. Proteomic studies of skeletal muscle are challenging, the high abundance of a relatively small number of myofibrillar proteins and metabolic enzymes mean that the depth of proteomic analyses of muscle is less than other tissues [14]. The challenges of cataloging the muscle proteome have been reviewed recently [15], whereas this review focuses on some key recent trends in the application of proteomics in muscle exercise physiology. We previously reviewed the proteomic responses of striated

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muscle to exercise in 2011 [16] and our current review draws mostly from the body of literature produced after this period. In the last decade, interest in exercise proteomics has grown substantially and the number of articles published per year has approximately doubled since pre-2011.

Early studies in exercise proteomics focused on cardiac and skeletal muscle responses to endurance exercise in sedentary but otherwise healthy populations and conducted protein abundance profiling of muscle using 2-dimensional gel electrophoresis [16]. In more recent works subcellular fractionation has been used to focus on mitochondrial adaptations, which are a key component of the response to endurance exercise. In addition, there has been a greater use of bottom-up proteomics involving liquid-chromatography tandem mass spectrometry (LC-MS/MS) of peptide digests rather than gel-based separations of proteins. Early studies primarily investigated the health benefits of endurance exercise. This focus of interest has been maintained amongst the recent literature and an emerging number of studies have also investigated muscle proteome responses to strength training. Several proteomic studies of exercise have now been conducted against backgrounds of human disease, aging or animal models of disease. Technical developments in mass spectrometer speed, resolution and mass accuracy, alongside the maturation of LC-MS /MS profiling methods, and advancements in sample preparation techniques have greatly increased the number of proteins that can be studied in muscle. A growing number of protocols for the enrichment of well-known post-translational modifications, including phosphorylation, acetylation and ubiquitination, have also evolved in recent years and the analysis of post-translational modifications has become a major area of interest in exercise proteomics. Finally, we consider emergent techniques for dynamic analysis of muscle proteomes that offer new insight to the mechanisms underpinning changes in protein abundance in response to exercise training. In particular, we highlight the emerging contributions that have used deuterium oxide labeling in vivo and peptide mass spectrometry to investigate protein-specific synthesis rates in muscle.

Exercise spans a continuum from high-force maximal/singular contractions associated with strength training to sustained periods of lower-intensity contractions associated with endurance exercise (Figure 1). Interval training, involving high-intensity bouts of exercise interspersed by short recovery periods, also develops endurance and is associated with adaptations in aerobic metabolism that afford protection against chronic diseases and the effects of aging. Exercise prescription specifies the intensity, duration, frequency and mode of exercise. Each of these attributes influence the magnitude and nature of the adaptations that occur in the heart and skeletal muscle and, therefore, cannot be ignored when interpreting data or designing exercise interventions. There are also complex interactions between exercise and the genome that may influence either baseline (innate) capacity or an individual's responsiveness (acquired) to exercise training. As such, an individual's capacity for exercise is a product of their genetic heritage as well as their recent level of habitual activity. As yet, individual variability in human muscle response to exercise has not been investigated using proteomics. However, bi-directional artificial selection has been used to generate animal models that provide highly useful substrate for investigating innate [17] and acquired [18] differences in aerobic capacity and therefore disease risk and mortality [19].

2. Proteomic studies of mitochondrial adaptations to exercise

An increase in muscle mitochondrial content is a key outcome of endurance training and mitochondrial adaptations

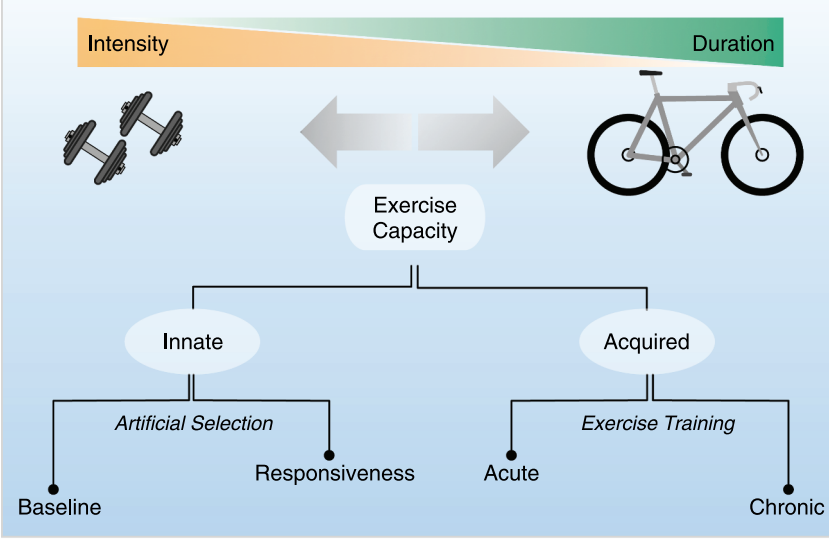

Figure 1. The exercise continuum.

Exercise stimuli span a continuum from maximal intensity contractions (e.g. associated with heavy weight training) to sustained periods of lower intensity work associated with endurance events. The intensity and duration of each exercise bout are important components that determine subsequent cellular responses and long-term adaptation to training. Indices of exercise capacity, including maximum oxygen uptake (VO_2max), muscle force etc., are the result of acquired environment factors (i.e. exercise training) and innate genetic factors that can influence either baseline levels or an individual's responsiveness to exercise training.

to exercise have important cytoprotective effects. The majority of mitochondrial proteins are synthesized in the cytosol and translocate to within mitochondria. Therefore, proteomic data on mitochondrial protein responses to exercise reported from crude homogenates of muscle can be challenging to interpret. Studies on isolated mitochondria have shed new light on changes that occur within the mitochondrial proteome of exercised muscle. Egan et al. [20] reported high-resolution 2-dimensional (2D) difference in-gel electrophoresis (DIGE) of human mitochondrial proteome responses during the first 2 weeks of an endurance training programme. After 14 consecutive days of cycling at an intensity of 80% VO_2max for 1 h per day the whole-body VO_2max in sedentary adult men increased by 18%. Timeseries analysis found the majority of proteins that changed in abundance reached an asymptote at 7 days and no further gain occurred between 7–14 d of training. There were ~1.3- to 2-fold increases in relative abundance of subunits of mitochondrial respiratory chain I and V and enzymes of the tricarboxylic acid cycle. In addition, the abundances of mitochondrial elongation factor Tu (TUFM) and Mn superoxide dismutase (SOD2) rose more steadily over the 14-d period and became statistically significant after 14 days of training. No changes in mitochondrial: nuclear DNA ratio were detected, which suggests increases in the overall mitochondrial content reported in longer duration studies [21] were yet to occur. Therefore, the proteome data reflect 'qualitative' time-dependent changes to the makeup of the mitochondrial proteome that occur prior to gains in mitochondrial abundance.

Egan et al. [20] identified gel spots that exhibited statistically significant changes in abundance but did not survey the wider mitochondrial proteome. Therefore, it is not certain whether the data represent changes in protein abundance per se or if they also include changes due to shifts in splice variation or post-translational state of mitochondrial proteins. 2D DIGE resolves proteins to proteoforms, which may reflect different combinatorial patterns of splice variants and post-translational states that may need to be considered in the interpretation of 2D gel data [22]. For example, Egan et al. [20] report an increase in protein disulfide isomerase A3 (PDIA3) in vastus lateralis of exercised humans. However, PDIA3 and many other muscle proteins were resolved as multiple proteoforms in the soleus of rats artificially selected as high-capacity runners [22]. Unsupervised analysis, using, conditional independence mapping, found networks within the data and proteoforms of PDIA3 emerged as key nodes segregating differences in aerobic capacity between high- and low-capacity groups. Three proteoforms of PDIA3 were differentially expressed between high and low-capacity groups, 2 were more abundant in the low-capacity group and 1 proteoform was more abundant in the high-capacity group. The observed shift in proteoform pattern corresponded to a shift in post-translational state of PDIA3 but it is not certain whether this also occurs during acquired gains in aerobic capacity that result from exercise training. In addition, our [22] analysis was performed on the soluble protein fraction, which includes sarcoplasmic as well as mitochondrial proteins, so further work is required to investigate whether different proteoforms of PDIA3 occupy different subcellular localizations.

In recent years, gel-based proteomic studies have largely been supplanted by LC-MS/MS analyses of muscle peptide digests. Bottom-up analyses of peptides overcomes some key challenges and technical limitations associated with handling and separating large numbers of proteins [23,24]. Almost all proteins have tryptic peptides that are unique and, therefore, can serve to indicate the relative abundance of the parent protein. LC-MS/MS profiling techniques have greatly increased the number of proteins that can be identified but this approach brings its own challenges and constraints. To date, the most comprehensive catalogs of the heart [25] and skeletal muscle [26] proteomes include 11,163 and 10,218 protein identities, respectively. However, such deep proteome coverage relies upon thorough orthogonal 2D separation and extensive MS instrument time, which may become prohibitively expensive in large multifactorial experimental designs. The complexity of tissue proteomes and the stochastic nature of common data-dependent analysis (DDA) of peptides make it challenging to resolve the same peptide in every experiment, particularly when combined with extensive pre-separation. The handling of missing data becomes a key concern in bottom-up proteomic analyses. Very large numbers of proteins can be identified but it is more challenging to rigorously match peptides that are unique to a protein in every sample analyzed. For this reason, the number of proteins quantified is always less than the total number of proteins identified. Nevertheless, when missing data are excluded, high levels of repeatability can be achieved, particularly when combined with data-independent analysis (DIA) of peptides [23]. For example, the abundance of all enzymes of the major metabolic pathways in muscle can be quantified at a coefficient of variation of <5% between technical replicates [23].

The use of subcellular fractionation to study mitochondrial responses removes the majority of highly abundant myofibrillar proteins and, therefore, facilitates deeper analysis of the mitochondrial proteome. Sollanek et al. [27] report proteomic analysis of isolated mitochondria from rat diaphragm after 2 weeks aerobic training using LC-MS/MS and label-free quantitation. Exercised rats completed a 10-day protocol that encompassed motorized treadmill running for 60 min/d at a speed that equated to ~70% VO_2max. An efficient 1D LC-MS/MS workflow identified 732 proteins in the mitochondrial fraction and subsequently 408 of these proteins were quantified after stringent filtering to exclude proteins that did not have at least 3 quantifiable unique peptides in each of the $n = 8$ control and $n = 8$ exercised biological replicates. Twenty-five mitochondrial proteins exhibited significant differences in abundance in exercised diaphragm at a statistical threshold of $P < 0.05$ and false discovery rate (FDR) of 10%. Subunits of the pyruvate dehydrogenase complex were more abundant in mitochondria from exercised diaphragm, consistent with responses of human mitochondria to aerobic exercise [20] and innate gains in aerobic capacity that result from artificial selection for high running capacity [22]. Label-free quantitation also highlighted a greater abundance of pyruvate

dehydrogenase kinase 4, which was not detected in the gel-based analyses [20,22]. Increases in the abundance of novel cytoprotective proteins were also discovered, including the antioxidants mercaptopyruvate sulfotransferase (Mpst) and alpha-keto reductase (Akr1b10) which catalyses NAPDH-dependant reduction of carbonyl-containing compounds. Relaxing of the stringent criteria to include proteins with only 1 quantifiable peptide in every sample expanded the list to include significant increases in the abundance of the monocarboxylate transporter 1 and uncoupling proteins 2 and 3 in mitochondria from the diaphragm of exercised animals.

The above findings point to an enhanced capacity for the oxidation of pyruvate in mitochondria of exercise-trained animals. Parallel analysis was also conducted on the diaphragm soluble fraction (i.e. encompassing mitochondrial and sarcoplasmic proteins) and highlighted a further 70 significant differences [27], including increases in numerous mitochondrial proteins reflecting an overall gain in mitochondrial abundance in the diaphragm of exercise-trained rats. PDIA3 was detected in the soluble protein fraction, rather than from the mitochondrial fraction but no change in PDIA3 abundance was found in exercised diaphragm. LC-MS/MS data reported in Sollanek et al. [27] did not include changes in NADH complex I of the electron transport chain as reported in Egan et al. [20], nor were there detectable changes in SOD either by proteomics or western blot analysis of diaphragm mitochondria. The exercise stimuli of Egan et al. [20] and Sollanek et al. [27] do not appear to be too dissimilar, i.e. each were ~2-week interventions of continuous aerobic exercise performed for 1 h per day but these investigations used locomotor or respiratory muscle and were conducted in different species. Nevertheless, some of the inconsistencies between the findings likely reflect regulation of the post translation state of proteins (e.g. PDIA3) as opposed to a tissue- or species-specific response to exercise.

Overmyer et al. [28] reports LC-MS/MS analysis of mitochondria from HCR/LCR extensor digitorum longus (EDL) muscle, using tandem mass tagging (TMT) labeling to investigate differences in protein abundance. The number of mitochondrial proteins quantified (428) was almost identical to Sollanek et al. [27] but overlap between the studies was low (188 proteins, equating to ~40% of the data), likely reflectinging differences in instrument configuration and sample preparation rather than overt tissue-specific differences in the mitochondrial proteome. Overmyer et al. [28] reported few differences in protein abundance between HCR and LCR mitochondria. Subunits of pyruvate dehydrogenase were detected but did not exhibit a significant difference in abundance between HCR/LCR. HCR had greater abundance of the α and β subunits of hydroxyacyl-CoA dehydrogenase trifunctional enzyme and subunits of cytochrome c oxidase/complex IV of the elcetron transport chain, and uncoupling protein 3. The majority (137 of 158) of proteins were significantly more abundant in LCR mitochondria, including mitochondrial ribosomal subunits and subunits of NADH dehydrogenase/electron transport chain complex I and the apoptosis regulator, Bcl-2-associated X protein (Bax). Therefore, artifical selection for high aerobic capacity appears to result in subtle differences in mitochondrial phenotype compared to gains in aerobic capacity that are acquired through exercise training. Mitochondria of HCR muscle may be optimized for fatty acid β-oxidation but lack gains associated with the oxidation of pyruvate that occur in response to exercise training in previously sedentary animals.

Mitochondrial samples from HCR/LCR muscle were also enriched for phosphorylated or acetylated peptides and differences in modification status were investigated by LC-MS/MS analysis of TMT-labeled samples [28]. Acetylation rather than phosphorylation emerged as the most prominent difference between HCR and LCR mitochondria. Numerous proteins were less acetylated in HCR than LCR, and the acetylation of some proteins decreased further in HCR mitochondria after exercise. Differences in the acetylation of mitochondrial enzymes were not associated with the abundance of sirtuin-3 deacetylase and may, instead, reflect differences in NAD/NADH ratio in the mitochondria of high- versus low-capacity runners. Mitochondrial malate dehydrogenase (MDHM) emerged as a key enzyme that may be regulated by acetylation. K^{335} acetylation of MDHM was significantly less in HCR than LCR mitochondria, and K^{239} acetylation of MDHM decreased significantly after 10 min aerobic exercise specifically in HCR mitochondria. Coincidently, Souza et al. [29] reports reversible oxidation of cysteine residues in HCR and LCR plantaris muscle and found significantly greater oxidation of C^{137}/C^{154} of cytoplasmic malate dehydrogenase (MDHC) in HCR. In both Overmyer et al. [28] and Souza et al. [29] the post-translation modification of malate dehydrogenase isoforms were associated with greater enzymatic activity, which is consistent with a greater capacity to exchange reducing equivalents via the malate-aspartate shuttle in HCR skeletal muscle. These findings are consistent with human muscle responses to exercise in diabetic patients, which also included gains in the abundance of enzymes of the malate-aspartate shuttle [30]. Overall, the recent proteomic analyses of mitochondria-enriched muscle fractions suggest adaptations to aerobic training are more intricate than a general upward shift in muscle mitochondrial content.

3. Global analysis of protein post-translational modifications in exercised muscle

The proteome can be regarded as an end-product of gene transcription and the balance between the rates of protein translation and degradation. However, the proteins residing within muscle are also responsible for sensing stimuli and transducing intracellular signals and so can also be regarded as being upstream of changes in gene transcription, protein synthesis and protein degradation, depending on the experimental perspective. An important new trend in exercise proteomics has been the application of global analyses of site-specific protein post-translational modifications that occur in muscle soon after an acute bout of exercise. As discussed already, Overmyer et al. [28] and Souza et al. [29] report acetylation and cysteine oxidation, respectively. In addition Kramer et al. [31] report changes in protein S-glutathionylation in mouse skeletal muscle after an acute bout of fatiguing contractions. However, the largest body of literature relates to phosphorylation, which has

well-established roles in the transduction of intracellular signals. Several kinases, including AMP-activated protein kinase (AMPK) and complex 1 of mammalian target of rapamycin (mTORC1), are firmly established regulators of key muscle responses to exercise. AMPK is responsive to cellular energy status, whereas mTORC1 is responsive to extracellular signals and cellular nutrient availability, and each of these kinases have become key focal points for research in exercise metabolism as well as chronic human diseases. Many other kinase families are also implicated in muscle responses to exercise, including Ca^{2+}-calmodulin dependent kinase (CaMK) and mitogen-activated protein kinases (MAPK), etc. but largely, each have been studied in isolation from the other in hypothesis-led studies. Physiological responses to exercise are likely to require a coordinated response amongst signaling pathways and other, as yet unidentified, entities may also be required in the muscle response to exercise training.

Hoffman et al. [32] used non-targeted 2D LC-MS/MS profiling of TiO2 enriched phosphopeptides in muscle samples collected from 4 healthy young adult men prior to and immediately after a ~ 10 min protocol of high-intensity aerobic exercise. LC-MS/MS analysis confidently identified >8500 phosphorylation sites on 4317 proteins and 1004 phosphosites were differentially regulated by exercise *in vivo*. Many (412, 41%) of the exercise responsive phosphosites were new discoveries and had not previously been annotated in the PhosphoSite database [33]. Of the 592 phosphosites that were annotated, the majority (516) had no known upstream kinase, therefore 928 of 1004 (92%) of phosphosites regulated by exercise were yet to be associated with upstream kinases. Bioinformatic analysis identified just 5 exercise responsive phosphosites that were substrates of AMPK. In total 15 kinases had 2 or more substrates that were responsive to the endurance exercise stimulus and kinases including protein kinase A (PKA) and cyclin-dependent kinase (CDK) had greater numbers of predicted substrates than AMPK. The distribution of substrates assigned to kinases is, in part, a feature of the existing databases and algorithms used for substrate predictions. Therefore, experiments employing gain-/loss- of function strategies were used to find new AMPK substrates but surprisingly did not generate large numbers of potential targets. One target, A kinase anchor protein 1 (AKAP1) S^{107} (human) was validated as a new AMPK substrate [32]. AKAP1 was localized to mitochondria and follow-up studies in rat L6 myotubes *in vitro* established that phosphorylation of AKAP1 S^{103} (rat homologue of S^{107}) is associated with a gain in mitochondrial respiration. Hoffman et al. [32] provide an exemplar in the application of mass spectrometry to discover the role of new site-specific post-translational modifications. This work also serves to illustrate the broad and largely unexplored nature of muscle exercise responses and offers wider context that questions the weight of resources that have been focused solely to AMPK in hypothesis-led studies of exercise.

Potts et al. [34] report similar 2D LC-MS/MS analysis of phosphopeptides from mouse tibialis anterior exposed to unilateral electrical stimulation encompassing 10 sets of 6 maximal contractions performed *in situ*. Tibialis anterior from the left non-stimulated and right stimulated limb was isolated 1 h after the protocol of maximal-intensity contractions to coincide with the peak activity of mTORC1 kinase. In total 5983 phosphorylation sites were identified, and 4,858 proteins were identified in at least $n = 3$ samples per group. Maximal-intensity contractions did not alter the abundance of proteins but did result in statistically significant differences in phosphorylation of 621 sites on 313 proteins. The majority of changes (531 sites) increased in phosphorylation after exercise but less than half of the exercise responsive sties had previously been annotated. Consistent with the work reported by Hoffman et al. [32], few of the exercise responsive phosphorylation sites had known upstream kinases. Where kinases could be identified, there was a high prediction rate for MAPKs (ERK1 and ERK2) and CamKIIα. However, only 12 proteins had known upstream kinases, illustrating the rich resource of new information generated by phosphoproteomic profiling of exercised muscle. The myofibrillar proteome, in particular I-band and Z-disc regions, were identified as 'hot spots' in the phosphoproteome response of muscle to maximal-intensity contractions. Novel kinases, including striated muscle-specific serine/threonine-protein kinase (SPEG) and obscurin were identified, which share similarity with titin and may represent new components of the elusive mechanosensitive mechanisms in skeletal muscle. Indeed, proteomic analysis of rats selected for either high- or low- responsiveness to endurance exercise [35] reported a greater abundance of SPEG-beta in the gastrocnemius of high-responder animals.

Samples collected soon after the completion of exercise are likely to exhibit a complex mix of events associated with restoration of homeostasis as well as responses that instigate adaptation. Guo et al. [36] report phosphopeptide analysis of rat heart after a standardized exercise test to a physiological end-point (i.e. VO_2peak). Time-series analysis was used to disambiguate stress from adaptational responses and 1169 phosphopeptides were profiled after exercise. Eighteen animals were studied across 3 groups that were killed either immediately after or 3 h after the cessation of the exercise. Phosphoproteome responses to exercise are known to differ depending on the time of day [37], therefore non-exercised control animals were killed at a time coinciding with the average between the exercised groups. One-way ANOVA discovered 141 statistically significant differences in phosphopeptides due to exercise and unsupervised hierarchical clustering highlighted 3 different temporal responses. Cluster 1 included phospholamban, p38α MAPK and alpha β-crystallin that were highly phosphorylated immediately after exercise and returned to basal levels within 3 h of recovery. Proteins in cluster 2 exhibited sustained elevations in phosphorylation after exercise and included obscurin and SPEG beta, consistent with the phosphoproteome response of skeletal muscle to resistance exercise [34]. The third cluster contained phosphopeptides whose abundance decreased immediately post exercise but returned to basal levels within 3 h after exercise cessation, including phosphorylation of myofibrillar proteins, including muscle LIM protein. The cardiac phosphoproteome

response to exercise [36] shared a high degree of similarity, but was not entirely explained by the cardiac response to beta-adrenergic receptor stimulation [38]. In addition, changes in Akt signaling associated with cardiac hypertrophy [39] were not prominent and indicate time-series analysis is required to study several subsequent bouts of exercise to build a more complete picture of muscle responses.

Currently, phosphorylation is the most well categorized post-translational modification response to exercise, however acute changes to protein post-translation state are multifaceted and crosstalk exists amongst different post-translational modifications, including phosphorylation, acetylation [40] and ubiquitination [41]. Parker et al. [41] report LC-MS/MS analysis of diGly-modified peptides, which indicate sites of ubiquitination or ubiquitin-like post-translational modifications. Healthy males performed ~10 min high-intensity aerobic exercise consistent with the earlier investigation of muscle phosphoproteome responses reported in Hoffman et al. [32]. Biopsy samples of vastus lateralis were collected prior to, immediately after and 2 h and 5 h after exercise cessation. A total of 4395 proteins and 1536 diGly-modified peptides were quantified in all samples from n = 6 participants. Protein diGly-modifications exhibited rapid changes, 391 diGly-modified peptides changed in abundance after exercise and then returned to baseline levels within 2 h of recovery. Exercise resulted in a marked depletion of diGly-modified proteins and the majority of proteins that had greater levels of diGly-modification fell in overall abundance, consistent with proteasome activation. Integration of these data with phosphopeptide data reported in Hoffman et al. [32] identified areas of potential proximal co-regulation in 43 proteins suggesting crosstalk between phosphorylation and ubiquitination in exercised muscle. 3′,5′-cyclic adenosine monophosphate (cAMP) signaling is a regulator of proteasome activation and follow-up experiments clarified a role of protein NEDDylation in the cAMP-dependent regulation of the ubiquitin proteasome system (UPS). Experiments conducted in HEK293 embryonic kidney cells *in vitro* found the inhibitor of NEDD8-activating enzyme (Pevonedistat) prevented cAMP-dependent activation of E3 ligases in response to cAMP (forskolin) stimulation whereas proteasome activation was not affected. Protein degradation via the activation of the ubiquitin proteasome system in exercised muscle helps to explain why mRNA and protein data (e.g [21,42].) do not correlate perfectly and further highlight the dynamic nature of muscle responses to exercise.

4. The effect of exercise on muscle proteome dynamics

Exercise is a potent stimulator of muscle adaptation, but the majority of literature report static data on protein abundances or post-translational states, which do not capture the dynamic processes of change. Robinson et al. [21], reports proteomic and transcriptomic analyses of human muscle responses to different training regimens and highlights that regulation of protein translation contributes to muscle adaptations to exercise. Three exercise modes were studied in young (18–30 years) and older (65–80 years) adults, including high-intensity interval training (HIIT; cycling at >90% VO_{2max} 5 times a week), resistance training (RT; 4 sets of 8–12 repetitions, 4 times a week) or combined training (CT) encompassing cycling (30 min at 70% VO_{2max}) 5 days per week and moderate resistance exercise 4 days a week [21]. HIIT training robustly increased VO_2max, insulin sensitivity, mitochondrial respiration, fat-free mass, and muscle strength regardless of age, and emerged as a more effective recommendation in older adults. Notably, there was limited overlap between transcriptome and proteome responses, particularly for mitochondrial and ribosomal proteins. The increased ribosomal proteins and other proteins significant in the translational machinery indicate an increased translational capacity in exercised muscle. Further analysis of $^{13}C_6$-phenyaline incorporation *in vivo* found similar gains in the fractional synthesis rate (FSR) of mixed mitochondrial proteins in the muscle of exercise-trained younger and older adults [21]. These findings indicate the translational response to exercise may be maintained in older adults but raise questions regarding the link between transcriptional responses and proteome outcomes. The time course of transcriptome and proteome responses to exercise differ, which may account for some of the disconnection in samples analyzed 72 h after the last bout of exercise. However, Makhnovskii et al. [42] report transcriptome responses at 1 h or 4 h after acute exercise also exhibit a poor correlation to the proteome of exercise-trained muscle. The lack of relationship between transcriptome and proteome was particularly evident amongst mitochondrial proteins and may indicate a role for post-transcriptional regulation of changes in protein abundance that warrants further investigation.

To date, few studies have focused on the dynamic period of change (Figure 2), wherein repeated bouts of exercise drive adaptation through accumulative effects of transient processes, including cell signaling, gene expression and protein turnover. Instead, the majority of literature report data on either early (0–24 h) responses to acute exercise or the changes in muscle proteome after longer periods of training. Time series studies across the course of adaptation could provide information on the temporal or sequential stages of adaptation but they cannot bring insight to the underlying mechanisms responsible for these phenomena. Proteins are dynamic entities that exist in a state of constant renewal (turnover) and the abundance of each protein results from the balance between its rate of synthesis and degradation. Meaning any changes in protein abundance (and therefore muscle function) are brought about by modulations to the relative contributions of synthesis and degradation. Biosynthetic labeling techniques, including radio or stable-isotope labeling, fluorescent labels and derivatized amino acids, have long been used to study the dynamic processes of protein turnover (reviewed in [43]) including protein turnover in muscle (e.g [44].). The turnover or fractional synthesis rate (FSR) of protein can be calculated by comparing the incorporation of an isotope label into newly made proteins (product) against the level of isotope enrichment in the precursor pool over a defined period of time. Largely, this has been achieved by administering stable isotope-labeled amino acid in vivo, followed by hydrolysis of the muscle protein

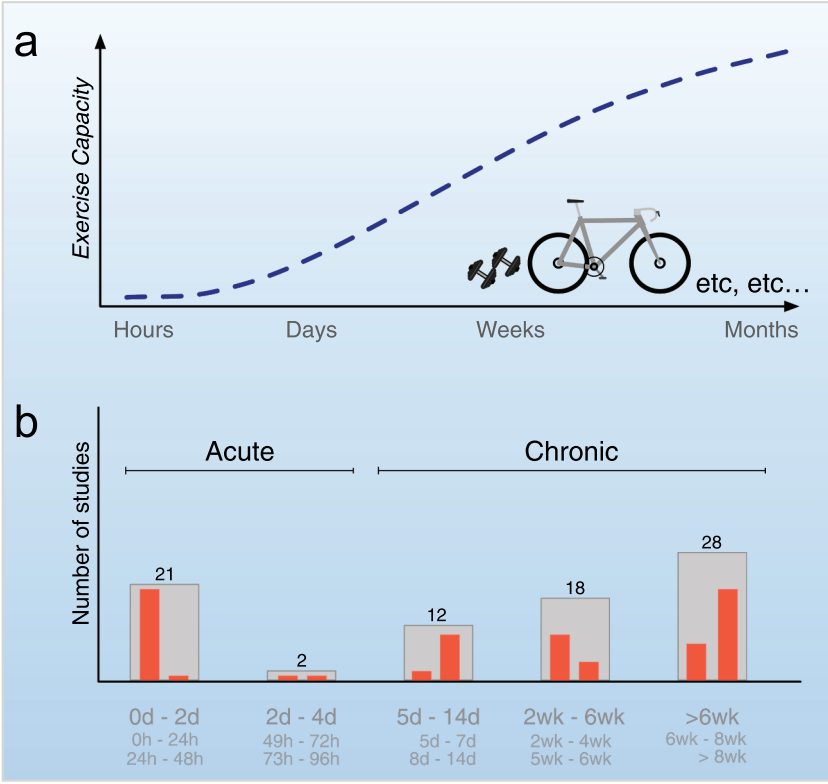

Figure 2. Distribution of proteomic literature over the time course of muscle adaptations to exercise.

Adaptations to exercise are dynamic and gains in exercise capacity develop gradually over time (A). Proteomic studies (number of publications) of muscle responses to exercise can be categorized as acute or chronic (B). Acute studies have typically investigated muscle responses within the first 24 h after a bout of exercise. These data offer insight to early adaptive responses but can also be challenging to interpret because they may contain a mix of responses that are associated with restoration of homeostasis as well as signals specific to adaptation. Studies of chronic training have typically investigated samples collected 48–96 h after the cessation of training. This approach helps focus on the outcomes of training and avoid the potentially confounding effects of the last bout of exercise.

mixture and gas chromatography-mass spectrometry (GC-MS) analysis of the amino acid of interest so that the proportion of labeled: unlabeled amino acid can be measured [45]. When combined with similar measurements of the enrichment of free amino acids in the precursor pool, the precursor: product ratio can be used to calculate the fraction of newly synthesized protein, i.e. FSR. The measurement of precursor enrichment is important, particularly when using amino acid tracers, e.g. $^{13}C_6$-phenylalanine (or in more contemporary uses, SILAC labeling of lysine or arginine) because the concentration of amino acids in blood or extracellular fluid may differ from the intracellular amino acid pool due to transporter-mediated processes and the dilution effect of amino acids released by degradation of existing intracellular proteins. Amino acid tracers have a long history in muscle metabolism research [46], but the hydrolysis of samples for GC-MS analysis severs the link between FSR data and the identity of individual proteins. Therefore, the majority of data (especially in humans) report FSR of mixtures of proteins from whole muscle or muscle subfractions and do not provide protein-specific information that can be used alongside proteomic studies of muscle adaptation.

Proteomic studies have been conducted in free-living animals by supplementing their diet with stable isotope-labeled amino acids such as deuterated valine [44] or leucine [47]. However, the palatability of synthetic diets can be low and may require lengthy periods of transition from the animals' standard chow before experimental interventions can take place. Deuterium oxide (^2H_2O; 'heavy water'), on the other hand, is a stable isotope that can be used for biosynthetic labeling experiments *in vivo* that is not associated with changes to the feeding or drinking habits of animals [48]. Deuterium oxide also obviates the requirement for intravenous infusion in humans and so lessens the burden on participants and enables studies to be conducted under free-living conditions over short (hours – days) or longer (weeks – months – years) time periods. Labeling of the precursor pool occurs intracellularly meaning experiments that use ^2H_2O are also less influenced by the metabolism or transport rates of amino acids amongst different tissues [49]. Deuterium becomes incorporated in to almost all amino acids *in vivo* and, therefore, gives a proportionally greater signal than methods that rely on labeling of a single amino acid. Metabolic labeling of proteins with deuterium results in a shift in the mass isotopomer pattern of peptides analyzed by mass spectrometry [50], which can be used to calculate the fraction of newly synthesized protein on a protein-by-protein basis. The first analyses of deuterium-labeled samples that used peptide mass spectrometry [51,52] investigated albumin synthesis in blood samples from laboratory rodents. In Hesketh et al. [53], we built from these studies and incorporated 2D gel electrophoresis

separation of muscle to investigate 8 proteins in the heart, diaphragm and fast- and slow-twitch muscles of rat using peptide mass spectrometry. The FSR of individual proteins ranked differently between different muscles [53], which warns against the extrapolation of findings across muscles that have different functions. Indeed, follow-up analysis of protein-specific FSR in fast- versus slow-twitch rat muscles [54] found the long-established paradigm that protein turnover is greater in slow- compared to fast-twitch muscle does not hold true when data are reported at the individual protein level.

An emerging body of research has used $^{2}H_2O$ labeling and peptide mass spectrometry to investigate changes to the synthesis rate of muscle proteins in response to exercise training. Shankaran et al. [55] report the FSR of 139 proteins in the muscle of at least 3 sedentary and 3 exercised participants and highlighted 20 proteins involved in glucose metabolic processes, contraction and cellular respiration had significantly greater rates of synthesis in exercised muscle. Consistent with data in rat, individual proteins in human muscle exhibited a range of different turnover rates. However, protein abundance was not measured and the general upshift in FSR in exercised muscle seems at odds with proteome studies reviewed earlier reporting some proteins increase whilst others decrease in abundance in response to training. Indeed, in rats that have free access to running wheels, the exercise stimulus impacts protein synthesis rates by decreasing as well as increasing protein-specific FSR [56]. In rat muscle, 80 of 108 proteins investigated exhibited changes in FSR ranging from −64% to +420% compared to sedentary controls [56]. Notably, exercise resulted in significantly greater synthesis rates of F1-ATP synthase and ATP synthase subunit α which are commonly reported to increase in abundance in response to endurance training. Compared to parallel data from the analysis of amino acid hydrolyzates, individual protein synthesis rates spanned a broader range than mixed protein synthesis rates [56]. For example, the synthetic rates of key mitochondrial proteins, such as cytochrome-c oxidase (2.77 ± 0.40%/d) and succinate dehydrogenase (3.30 ± 0.35%/d) were four-fold greater than key myofibrillar proteins, such as α-actin (0.77 ± 0.28%/d) and α-actinin-1 (1.55 ± 0.11%/d). These data suggest changes to muscle protein turnover occur in a highly selective manner on a protein-by-protein basis in response to endurance exercise.

Murphy et al. [57] report a similar analysis of protein FSR responses to resistance exercise in overweight males during a 2 week period of energy restriction. The FSR of 190 proteins were reported in at least n = 2 participants per group. The synthesis of mixed myofibrillar proteins was not affected by energy restriction interventions but resistance training resulted in significant gains (~26%) in myofibrillar protein synthesis integrated over the 2 week study period [57]. At the individual protein level, 175 of the 190 identified proteins exhibited a significant increase in synthesis rate in exercised muscle, including mitochondrial and sarcoplasmic proteins. Similar to Shankaran et al. [55], FSR was reported but protein abundance data were not investigated. Therefore, it is not certain whether the reported changes to protein FSR resulted in gains in protein abundance or if they represent a change to the rate of protein turnover. During the adaptation to exercise there are changes in the abundance of individual muscle proteins which must be accounted for alongside the effects of exercise on protein FSR. Traditionally, biosynthetic labeling studies have been conducted on the premise that protein abundance is stable throughout the experimental period. Under this constraint, the rate of synthesis measured by the incorporation of the label into protein is equivalent to the rate of turnover, because it was assumed synthesis and degradation were in equilibrium. The body of proteomics literature reporting exercise-induced changes in muscle protein abundance suggests the assumption of a steady-state is unlikely to hold true in the aforementioned studies [55,57].

If both protein abundance and synthesis are measured, changes in abundance that are not accounted for by changes in synthesis can be assigned to protein degradation. Likewise, changes in FSR that are not accompanied by changes in abundance can be interpreted as an effect on turnover rate, which is also an important outcome in relation to proteome health. In untrained males a schedule of 3 resistance training sessions over a 9-day period resulted in significant changes to both the abundance and synthesis rate of myofibrillar proteins and metabolic enzymes [58]. Amongst the 91 proteins identified in all samples from n = 8 trained and n = 8 control individuals, protein FSR spanned 3 orders of magnitude and ranged from 0.08%/d (pyruvate dehydrogenase E1 component subunit beta) to 23%/d (carbonic anhydrase 3). Individual proteins exhibited different patterns of response, including proteins that increased in turnover rate with no change in abundance, increases in abundance with no increase in synthesis rate or a decrease in abundance despite increases in synthesis rate. These findings generate new insight and illustrate the complexity of the initial responses of muscle to resistance training. For example, an increase in myofibrillar protein FSR is a widely acknowledged response to resistance exercise (e.g [57].). Protein-specific FSR and abundance data [58] add detail and reveal resistance exercise selectively increases the turnover of type IIa myosin heavy chain (MyHC), which aligns with the expected gain in the abundance of MyHC IIa after longer periods of resistance training.

The findings from the small number of existing studies on the dynamic response of muscle proteins to exercise warrant further investigation, and also further development of techniques for dynamic proteome profiling. Studies on the repeatability of dynamic proteome profiling [59] report the median coefficient of variation in protein FSR measurements (10%) is greater than protein abundance data (median coefficient of variation = 3.6%). However, protein FSR is more labile than protein abundance, and the effect size of exercise on muscle protein synthesis is greater than the size of effect on protein abundance. Therefore, proteome profiling investigations of abundance and synthesis responses have equal power to detect changes in response to exercise. Moreover, the repeatability of protein abundance and synthesis data in humans is robust and compares favorably against longer established label-free quantitation techniques [59]. Nevertheless, relative data such as FSR may be confounded, or challenging to

interpret if there are co-occurring changes in protein abundance, particularly in systems that exhibit overt changes in phenotype. Stansfield et al. [60] report the use of an exogenous reference protein to estimate the abundance and synthesis rate of proteins in mole rather than fractional units. During differentiation of myoblasts in to myotubes *in vitro*, the analysis of mole synthesis rates (MSR) rather than FSR gave rise to different biological interpretations. Notably, there was a weak relationship between FSR and MSR, and MSR data outperformed FSR in predicting changes in protein abundance [60].

Longer duration training studies may be more likely to be associated with co-occurring changes in muscle mass, which could yet further confound the interpretation of dynamic proteome data reported in relative protein abundances and fractional rates of protein synthesis. Hesketh et al. [61] developed an Absolute Dynamic Profiling Technique for Proteomics (Proteo-ADPT) that can be implemented to measure protein abundances within the entire muscle in laboratory animals and report absolute rates (ng/d) of synthesis, degradation and abundance on a protein-by-protein basis (Figure 3). Hesketh et al. [61] reports data from independent groups of rats (n = 3 per group) that received unilateral chronic low-frequency stimulation (CLFS; 10 Hz, 24 h/d) and $^{2}H_2O$ for 0, 10, 20, or 30 days. The extensor digitorum longus (EDL) was isolated from stimulated and contralateral non-stimulated legs. Proteomic analysis encompassed 38 myofibrillar and 46 soluble proteins and the rates of change in abundance, synthesis, and degradation were reported in absolute (ng/d) units for the entire EDL. Strikingly, changes in protein degradation contributed to the adaptation of the proteome equally as much as synthesis, including instances where a decrease in protein-specific degradation primarily accounted for the increase in the abundance of the protein. Moreover, the relative contributions of synthesis and degradation to protein-by-protein changes in abundance were different during early, mid and late periods of the experimental intervention. For example, during the early period (0 d – 10 d) of the intervention, a decrease in degradation rate accounted for 82% of the increase in the abundance of ATP synthase beta [61]. During the mid-period (10 d – 20 d) of the intervention, there was evidence of co-occurring changes in protein turnover and post-translational modifications. For instance, S^{20} phosphorylation of myosin regulatory light chain (MLRS) was associated with greater protein degradation and evidence of a diGly remnant was found on K^{137} of this phosphorylated proteoform of MLRS. These data give a glimpse of the future capabilities of proteomics in mechanistic studies of exercise-induced muscle adaptation, exploring both the 'synthetic' and 'degradative' *arms* of protein turnover and linking these data with site-specific post-translational modifications.

5. Conclusions

Over the past decade proteomic studies have added significantly to knowledge regarding exercise adaptation,

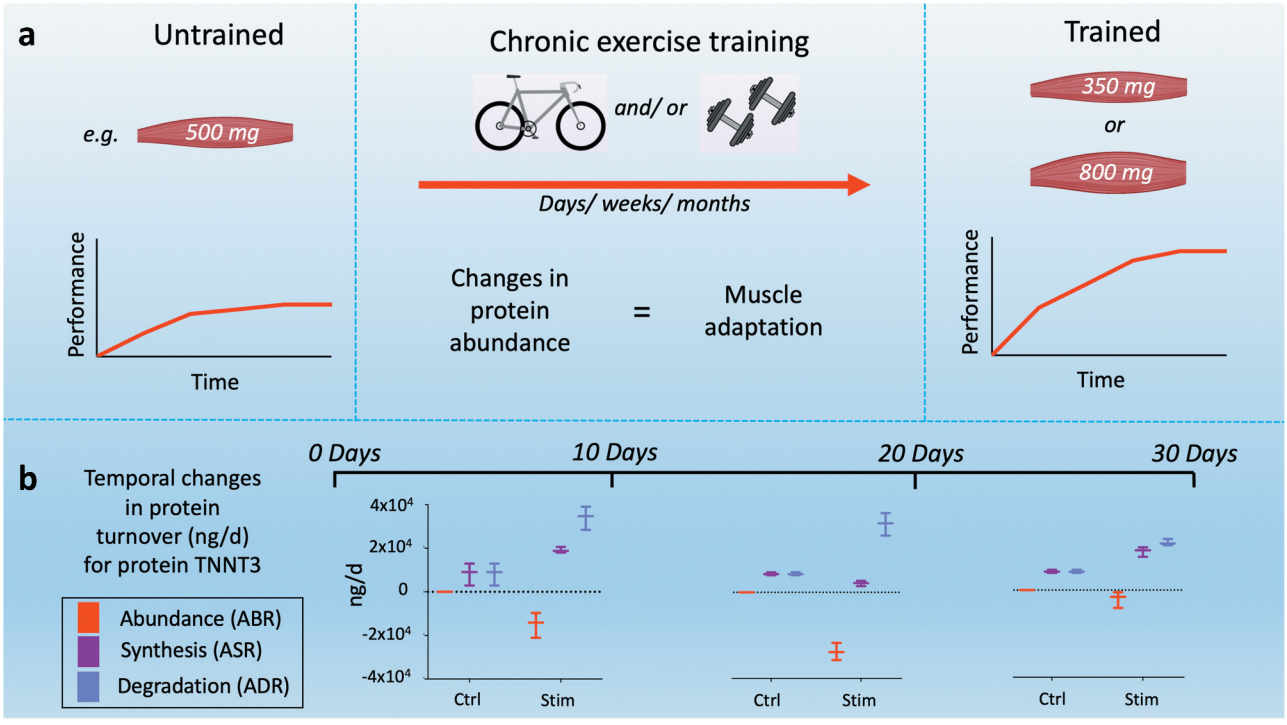

Figure 3. The 'Proteo-ADPT' method.

The Absolute Dynamic Profiling Technique for Proteomics (Proteo-ADPT) enables temporal analysis of the contributions of synthesis and degradation to changes in the abundance of individual proteins. (A) Chronic exercise training over periods of weeks or months confers overt changes in muscle mass, induced by alterations in individual protein abundances resulting in both changes in muscle phenotype and quantitative changes in muscle performance. (B) Proteo-ADPT data from [61] reporting responses of the fast isoform troponin t (TNNT3) during 30 days of unilateral chronic low-frequency stimulation. Absolute (ng/d) data are presented as mean ± SD (n = 3 per group) from stimulated (Stim) and contralateral control (Ctrl) EDL. During the period between 10 d and 20 d CLFS, the rate of change in abundance (ABR) of TNNT3 was 0 ng/d in Ctrl EDL and −27,591 ± 4000 ng/d in Stim EDL. The absolute synthesis rate (ASR) of TNNT3 was not significantly different between Ctrl (8564 ± 690 ng/d) and Stim (4174 ± 1237 ng/d). Whereas the absolute degradation rate (ADR) of TNNT3 was significantly greater in Stim (31,766 ± 5236 ng/d) than Ctrl (8564 ± 690 ng/d) and accounted for the change in TNNT3 abundance.

particularly in skeletal muscle. The datasets that have been amassed so far offer a new level of comprehension and expansive opportunities for hypothesis generation that will also serve to better inform targeted mechanistic research. Both the number and sophistication of proteomic studies has increased since conception of the field. Early literature reporting cross-sectional analyses of trained versus untrained muscle have been elaborated upon and more contemporary works offer insight to the interaction of exercise interventions with disease or aging. Consistent with the wider proteomics field, there has been a shift away from gel-based protein separation and a greater reliance upon bottom-up techniques that use LC-MS/MS analysis of peptide mixtures. The number of proteins studied has also significantly expanded, but proteome coverage is not perfectly consistent, even among studies that seemingly report similar numbers of proteins.

Proteomic studies of enriched mitochondrial fractions of skeletal muscle have been reported but studies have not yet considered differences that may exist between exercise responses of subsarcolemmal and intermyofibrillar mitochondria which have been reported in the heart [62]. Mitochondrial responses to exercise might also be expected to differ as a function of fiber type, sex, age and diet. Fiber-type specific responses to exercise have very recently been reported [63], and aging is known associated with changes to muscle fiber proteomes [64]. Proteomic studies have provided insight to the interaction between training status and aging on the skeletal muscle proteome [65] and suggest some features associated with older age may be due to lower levels of physical activity rather than aging per se. New methods for the enrichment of key post-translational modifications have been successfully exploited to discover exercise responsive sites. Phosphorylation, which is a well-established regulator of protein function and intracellular signaling, has been the focus of attention in the majority of studies. However, in mitochondria, protein acetylation emerged to be a more prominent modification that differentiates between low and high levels of aerobic capacity. Nevertheless, phosphoproteome analyses have illuminated an array of exercise responsive kinases that is broader than popular foci of interest amongst hypothesis-led studies.

Exercise is a potent stimulus for muscle adaptation but the majority of literature report static data on protein abundances or post-translational states, which do not capture the dynamic processes of change. In particular few studies have addressed the dynamic period of change that lies between the acute responses and chronic outcomes of muscle to exercise (Figure 2). Time series studies provide information on the temporal or sequential stages of adaptation but cannot bring insight to the mechanisms that are responsible for these phenomena. Proteins are dynamic entities that exist in a state of constant renewal. The abundance of each protein is controlled by the balance between its rate of synthesis and degradation. Emergent data from dynamic proteome profiling studies suggest changes to individual protein abundance (and therefore muscle function) are brought about by modulations to the relative contributions of degradation as well as synthesis. In particular, techniques such as Proteo-ADPT, are well positioned to bring significant new insight to the mechanisms of exercise-induced muscle adaptation. In the future development of the field, we expect combined analysis of proteome dynamics, and post-translational modifications (in particular ubiquitination) in muscle sub-cellular fractionations will offer important new insight to the mechanisms underlying muscle responses to exercise.

6. Expert opinion

Our review is dedicated to the application of muscle proteomics, but we readily acknowledge there is no single analytical technique or experimental design that should be regarded above all others. A full understanding of complex biological processes will require data from all levels of interrogation, including omic studies of genetics, epigenetics, transcriptomics, proteomics and metabolomics, that link molecular processes with functional outcomes at cell, tissue and whole organism levels. Advances in knowledge will come through better exploitation of the strengths of different experimental approaches, including targeted reductionist research and hypothesis-generating systems biology. The use of data harnessed from non-targeted omic datasets, represents the gold-standard for hypothesis generation and could prevent valuable resources from being allocated to research targets that may not be the most biologically pertinent.

LC-MS/MS profiling of enriched modified peptides is undoubtably a powerful discovery technique, but the functional characteristics of muscles are determined by their proteoform composition, which encompass gene isoforms, splice variants and different combinatorial patterns of post-translational states [66]. Some muscle proteins exhibit particularly complex patterns of splice variation and >60% of UniProtKB entries contain annotated evidence of post-translational modification, including covalent chemical modifications and proteolytic processing. LC-MS/MS analysis of site-specific phosphorylation or acetylation report, on average, 2–3 sites of modification per protein. Crosstalk between covalent modifications and the number of potential combinatorial of post-translational states for each protein will need to be considered to gain physiological insight from these complex molecular data [67] .

Little information currently exists regarding which changes to protein abundance are controlled by synthesis, degradation or a combination of the two. To date, the primary focus of interest has been on synthesis and/or the 'synthetic arm' including gene transcription [42] and ribosomal translation (e.g [34].). However, more recently the role of degradation in maintaining proteome health has been emphasized [41] and we found alterations to the degradation rate of proteins can be the dominant factor controlling changes in the abundance of some muscle proteins in response to an endurance exercise stimulus [61]. Synthesis and degradation are regulated independently on a protein-by-protein basis to maintain homeostasis and/or to facilitate muscle adaptation. The regulatory processes of protein degradation are convoluted, and little is known about the sequence of events linking particular stimuli to the degradation of specific individual proteins. This

contrasts sharply against our understanding of the regulators of protein synthesis, where a more tangible 'audit trail' exists between genetic regulation and the synthesis of new proteins. Protein degradation is essential to adaptation, and even during 'steady-state' conditions proteins must continuously be renewed (i.e. protein turnover) to maintain proteostasis and prevent the accumulation of damaged proteins from causing cell dysfunction.

Proteomic studies have focused on muscle adaptations to either endurance or strength training. This leads to a dichotomized perspective that does not reflect the practices of athletes, or health guidelines that emphasize the importance of both endurance and strengthening exercises in the maintenance of health [68]. Many athletic disciplines and recreational activities involve a mixture of both endurance and resistance work that are associated with different phenotypic outcomes. The comprehensive nature of proteomic analysis may offer new insight regarding optimization of such concurrent training programs, which remains an issue in exercise physiology and health-based exercise prescription.

A substantial body of proteomic data already exists and could be leveraged to make new discoveries that are beyond the scope of each individual report. For example, we have previously used systematic review methods and meta-analysis of proteomics data in human muscle and type 2 diabetes [69], which more closely match the ethos of omic/non-targeted studies. However, the assimilation of data across studies is constrained by a lack of data standardization, which needs to be addressed as the field moves forward to enable data extraction for meta-analytical techniques. Exercise or the physical capacity of participants was also not always adequately reported. The 'dose' of exercise is multifaceted construct incorporating intensity, duration, frequency but also mode of exercise, training status of the individual and responsiveness of the cohort to the training stimulus. In the future, better characterization of exercise performance using key health and performance indicators, e.g. VO_2max, will facilitate more accurate comparison of proteomic data across multiple studies.

We expect the future data available to exercise physiologists will be extensive. The plethora of health benefits conferred by acute exercise bouts and chronic exercise training have stimulated a collective attempt to bridge the gap between identifying the detailed molecular signals induced by exercise and the associated prevention of disease and health maintenance. The Molecular Transducers of Physical Activity Consortium (MoTrPAC) has been established to address this question and seeks to generate a molecular map of exercise, allowing researchers to hopefully identify the mechanisms responsible for its complex health benefits. The MoTrPAC project leverages millions of dollars in funding to investigate the mechanisms of exercise in humans and non-human animal models [70]. MoTrPAC investigates the effects of both endurance and resistance exercise across a range of ages and fitness levels by analysis of multiple tissues before and after acute and chronic exercise. From these multi-omic and bioinformatic analyses, a molecular map of exercise will be established, providing a public database that is expected to significantly advance our knowledge of the health benefits of exercise, ultimately providing further insight into how exercise effectively mitigates the many costly and devastating diseases that we currently live with.

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