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Comparative Proteomics for Studying Muscular Dystrophy: Intrinsic Biological and Analytical Issues Associated with the Systematic Utilization of Tissue Specimens

Ashling Holland and Kay Ohlendieck*

Muscle Biology Laboratory, Department of Biology, Maynooth University, National University of Ireland, Maynooth, Co. Kildare, Ireland

Abstract

Over the past decade, mass spectrometry-based proteomics has been instrumental for the detailed elucidation of pathobiochemical mechanisms involved in major neuromuscular diseases. Although the identification of muscle-derived proteins in biofluids is the main focus of diagnostic biomarker research, the large-scale proteomic analysis of pathological muscle tissue is of central importance for furthering our general understanding of the dysregulation that underlies complex muscle diseases. Here, we discuss intrinsic biological issues and bioanalytical difficulties that are generally associated with comparative muscle tissue proteomics. The systematic utilization of cellular mixtures or whole tissue specimens as starting material for studying neuromuscular pathology is seriously complicated by the cellular heterogeneity and physiological plasticity of contractile tissues. The comprehensive biochemical analysis of the skeletal muscle proteome is often hampered by the wide dynamic expression range of proteins, the greatly differing physicochemical properties of dissimilar muscle protein species and the potential cross-contamination of samples by highly abundant proteins. Thus, neither gel electrophoretic methodology nor liquid chromatography, is capable of appropriately separating all constituents of the skeletal muscle proteome. However, the application of advanced extraction strategies, the usage of subcellular fractionation protocols to reduce sample complexity and the affinity purification of distinct protein fractions prior to mass spectrometric analysis promises to overcome some of the inherent problems associated with muscle tissue proteomics.

Keywords: Gel electrophoresis; Mass spectrometry; Muscle pathology; Muscular dystrophy

Introduction

Proteomics has undisputedly revolutionized the global characterization of time-dependent and tissue-related gene expression patterns in health and disease [1]. Proteomic profiling has been instrumental in determining the cellular dynamics in protein concentration changes and post-translational modifications [2], making it a fundamental analytical part of the modern biosciences [3]. The recent establishment of a mass spectrometry-based draft map of the human proteome [4,5], together with antibody-based affinity proteomics initiatives [6] and the genome-wide characterization of the human proteome by chromosome-centric projects [7-9], have produced a large catalogue of over 18,000 proteins. This information has given unprecedented insight into the complexity of protein expression levels in different cell types, tissues and organs. However, the approximately 20,700 protein-coding genes [10] have been estimated to translate into more than 100,000 different protein isoforms during the formation of trillions of cells in the adult human body. The various proteins belonging to the dystrophin-glycoprotein complex [11], which are intrinsically involved in several neuromuscular diseases [12], are an excellent example of this diversity at the proteome level. The core anchoring protein dystrophin of this sarcolemmal complex exists in an extraordinary variety of isoforms and its associated dystroglycans derive as cleavage products from a single peptide chain [11]. This is due to the fact that multiple dystrophin gene promoters express three full-length transcripts and four shorter isoforms in various cellular locations [13]. The dystroglycan proteins α-dystroglycan and β-dystroglycan are translated from a single mRNA molecule as a 97 kDa protein that is subsequently cleaved into 2 proteolytic protein products [14].

Another illustration of how differential splicing results in a single gene coding for more than one muscle protein product, in conjunction with an added layer of complexity due to different types of post-translational modifications, is the abundant class of Ca^{2+}-pumping ATPases from the sarcoplasmic reticulum [15]. Of the 3 genes that encode the protein family of sarco(endo)plasmic reticulum Ca^{2+}-ATPases (SERCA), skeletal muscle fibers express the *SERCA1* and *SERCA2* genes [16]. These 2 *SERCA* genes produce more than 10 distinguishable SERCA protein isoforms [17] via alternative splicing of the transcripts and various post-translational modifications. This exemplifies the point that distinct genes may produce several fold more muscle protein isoforms and thereby complicate the relationship between genome biology and skeletal muscle proteomics. Since primary or secondary changes in dystrophin and its associated glycoproteins are linked to the most frequent forms of inherited muscular dystrophies, tissue proteomics suggests itself as a highly suitable bioanalytical tool to study dystrophinopathies [11]. Here, we outline biological issues and common bioanalytical problems that are frequently associated with the systematic utilization of tissue specimens for studying muscular dystrophy and related neuromuscular disorders by comparative proteomics.

***Corresponding author:** Kay Ohlendieck, Professor & Chair, Department of Biology, Maynooth University, National University of Ireland, Maynooth, Co. Kildare, Ireland, Tel: (353) (1) 708-3842; Fax (353) (1) 708-3845; E-mail: kay.ohlendieck@nuim.ie

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Diagnostic and investigative value of skeletal muscle biopsies

Although magnetic resonance imaging and spectroscopy is increasingly used to substantiate the diagnosis of certain neuromuscular pathologies by non-invasive means [18], the requirement for specialized facilities and relatively high costs make this advanced approach not yet suitable for routine diagnostic or prognostic screening in muscle pathology. The histological, histochemical and immunohistochemical assessment of muscle biopsy specimens is still of central importance for the conventional diagnosis of muscular abnormalities [19]. Ideally the portion of a skeletal muscle selected for a biopsy procedure should be free of unrelated pathologies, not be affected by previous trauma and show moderate weakness for accurate testing. In conjunction with physical examinations, genetic testing, the evaluation of clinical history and routine tests of motor assessment, studying histological changes in patient muscle samples is crucial for the overall determination of the pathological status of the neuromuscular system. In early-onset muscular dystrophies, histological hallmarks include considerable variations in fiber diameter, extensive connective tissue proliferation in the perimysium and endomysium, increased numbers of central nuclei and other cytoarchitectural alterations, fiber splitting and degeneration-regeneration cycles in affected myofibers [12].

Biochemical tests are regularly employed in the form of enzyme assays for the detection of tissue damage markers, which usually includes muscle enzyme-related serum activity [20,21]. However, these muscle-derived biomarkers are relatively non-specific [22] and there is a major research drive to identify novel disease-specific protein indicators that can be employed for the improved diagnosis, prognosis and/or therapy monitoring of common muscle diseases [23]. The recent development of high-throughput biochemical screening methods that combine large-scale separation techniques, such as gel electrophoresis and/or liquid chromatography, with highly sensitive mass spectrometry have given skeletal muscle biopsies a greater investigative value [24]. Routine proteomic screening can now be carried out with relatively small amounts of contractile tissue to generate meaningful data on global changes in the entire protein constellation of pathological muscle fibers [25,26]. As outlined in the flowchart of Figure 1, the systematic screening of the assessable muscle proteome can be used both for the swift identification of a new biomarker signature and

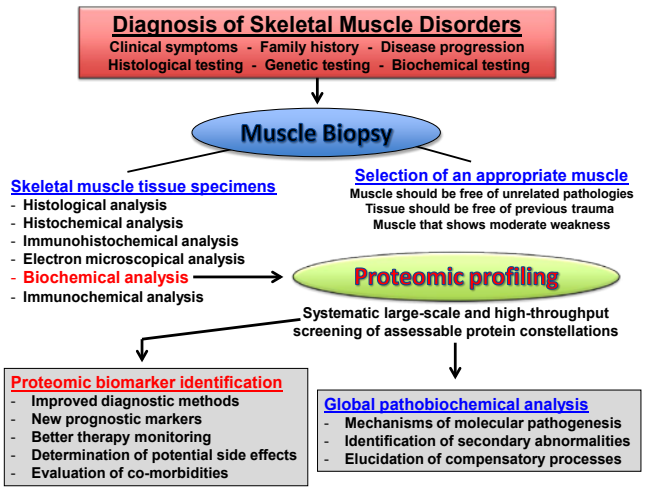

Figure 1: Summary of routine analyses performed with muscle biopsy specimens and the new bioanalytical value of skeletal muscle samples for the proteomic profiling of neuromuscular diseases.

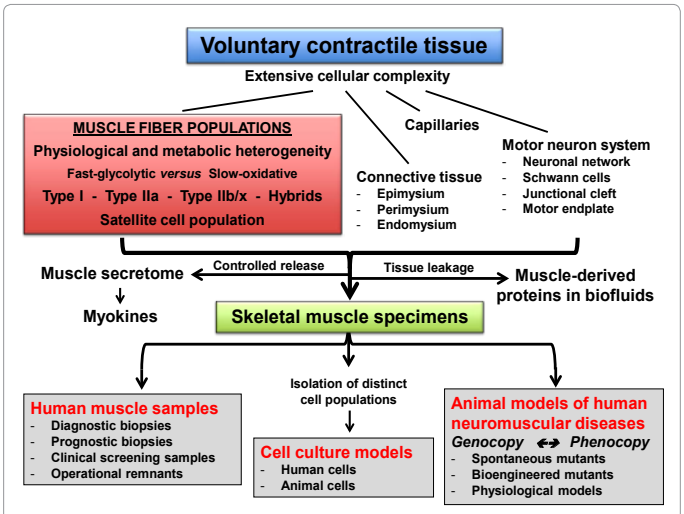

Figure 2: Diagram outlining the cellular complexity of skeletal muscle tissue and the physiological heterogeneity of muscle fibre populations used in the analysis of human biopsy material or biochemical studies with animal models of neuromuscular disorders.

the pathobiochemical analysis of cellular dysregulation and potential compensatory processes. Due to its unparalleled capacity to identify changes in distinct protein isoforms, mass spectrometry-based proteomics is especially suitable for the establishment of potential differences between moderately versus severely affected muscle tissues.

Tissue complexity and heterogeneity in skeletal muscles

Voluntary contractile tissues, representing one of the most abundant cellular entities in the human body, maintain the excitation-contraction-relaxation cycle via the biochemical conversion of potential chemical energy into mechanical filament sliding [27]. Other major biochemical tasks include the regulation of heat homeostasis and metabolic integration. The fact that skeletal muscles are an extremely tough type of tissue that consists of overlapping filamentous structures surrounded by several layers of connective tissue, makes it difficult to study certain pathological changes in biopsy samples by routine homogenization approaches, extraction methods and biochemical separation techniques. Muscle fiber populations are characterized by their physiological and metabolic heterogeneity spanning from fast-glycolytic to slow-oxidative cell types [28]. Besides the main type I, type IIa and type IIb/x fibers [29], most muscles also contain a variety of hybrid fibers and a myo-specific stem cell pool consisting of satellite cells [30]. Thus, the cellular complexity of crude muscle samples has to be taken into consideration when the results from biochemical analyses of human biopsy, autopsy material or operational remnants are interpreted. Besides the highly complex combination of muscle fiber types within an individual skeletal muscle, other tissue classes are represented by capillaries, the epimysium, the perimysium, the endomysium and the abundant motor neuron system (Figure 2).

For example, extensive fibrosis or infiltration by fatty cells in diseased muscle tissue might make the direct biochemical comparison of normal versus affected specimens difficult due to the introduction of potential artifacts during the homogenization procedure or subcellular fractionation steps. The appearance of extensive amounts of collagen fibrils or fatty deposits in tissue homogenates may cause differential protein adsorption or entrapment processes in control versus pathological samples. It is therefore crucial to verify biochemical and

proteomic findings from routine screening studies by independent bioanalytical methods, such as immunofluorescence microscopy, immunoblotting surveys or functional assays [24]. In the case of studies with genetic animal models of human muscle diseases the differing fine structure of the neuromuscular system, but also dissimilarities in the immune system, physiological adaptations and metabolic regulation, have to be taken into account when attempting to properly extrapolate findings from animal testing to human pathology [31].

Complexity of the skeletal muscle proteome

In biological terms, it is difficult to precisely delineate the exact protein constellation that defines the skeletal muscle proteome, since muscle tissue is exceedingly heterogeneous in its composition and highly plastic with respect to quickly adapting to changed functional and metabolic demands. Hence, in contrast to the comparatively stable genome, the muscle tissue proteome is constantly fluctuating to adjust its protein repertoire to the physiological challenges of the body. Numerous attempts have been made to separate the assessable protein constellation from total muscle extracts in order to establish the near-to-complete proteome of distinct skeletal muscles [32-35]. Both two-dimensional gel electrophoresis and liquid chromatography were used for the large-scale protein separation from human muscle specimens and various animal muscle preparations of biomedical or agricultural importance. The systematic application of tissue proteomics has so far resulted in the identification of more than 2,000 muscle-associated proteins [34]. Interestingly, mitochondrial proteins accounted for 22% of the accessible skeletal muscle proteome, including 55 subunits of the respiratory complexes I to V [32], clearly confirming the crucial importance of oxidative metabolism in muscle tissue [36]. The comparative analysis of predominantly fast-twitching versus slow-twitching muscle has shown that several hundred proteins are differentially expressed in contractile tissue with differing fiber specification [37-40]. Fiber type-specific expression patterns were shown to encompass especially protein families involved in the contraction-relaxation cycle, ion handling, glycolysis, oxidative phosphorylation and cellular signaling [40]. Numerous studies have catalogued a large cohort of organelle-specific muscle proteins [41], including protein isoforms expressed predominantly in mitochondria [42,43], the sarcoplasmic reticulum [44,45] or the sarcolemma [46]. The most abundant proteins of the diffusible fraction of the skeletal muscle proteome were shown to be the enzymes that mediate the glycolytic pathway [47,48] and recent studies have revealed that the skeletal muscle secretome contains a highly complex mixture of fiber-derived proteins and a novel class of signaling proteins named myokines [49-51].

Bioanalytical challenges and opportunities in comparative tissue proteomics

Since no single set of protein biochemical methods is currently capable of separating and analyzing the entire spectrum of proteins contained in a complex tissue proteome, this technical obstacle is the most significant limiting factor in most comparative proteomic studies. The wide range of concentration levels of individual protein species and the greatly differing physicochemical properties of proteins with respect to hydrophobicity make global biochemical analyses technically challenging. The large-scale protein separation by gel electrophoresis or liquid chromatography is inevitably biased towards certain subtypes of proteins based mostly on their molecular size and overall electric charge. Although two-dimensional gel electrophoresis can readily separate a large portion of the skeletal muscle proteome, including contractile proteins, metabolic enzymes and molecular chaperones [52-54], this standard technique of proteomics underrepresents low copy number proteins, integral membrane proteins and high-molecular-mass proteins [55-57]. An additional problem encountered with routine gel electrophoresis is the fact that proteins with extensive post-translational modifications are often presented by less defined spots or bands, and that proteins exhibiting extreme p*I*-values do not properly resolve at the edge of analytical gels with a wide pH-range [58].

Cross-contamination of separated low copy number proteins by highly abundant proteins is also a potential problem in muscle proteomics. This includes the presence of myosin heavy and light chains, actins, tropomyosins and troponins in crude tissue extracts or highly abundant organelle-specific proteins such as the sarcoplasmic reticulum Ca^{2+}-ATPases in subcellular membrane fractions. To overcome some of these technical issues, the comprehensive proteomic screening of muscle specimens should ideally be carried out with both crude tissue extracts and isolated organelles as starting material. Figure 3 outlines the most commonly employed proteomic workflows and analytical techniques for comparative muscle tissue proteomics. For the analysis of the near-to-complete proteome, the urea- or detergent-based extraction of total tissue specimens is usually employed and can be combined with a variety of label-free or label-based strategies for protein identification. In order to reduce sample complexity, subcellular fractionation protocols are commonly used in organelle proteomics [41,59-61] and affinity purification methods are highly suitable to enrich supramolecular protein complexes prior to their mass spectrometric analysis [44,46,62-65]. The protein composition of crude extracts can be determined by label-free mass spectrometry or electrophoretic labeling approaches combined with mass spectrometry.

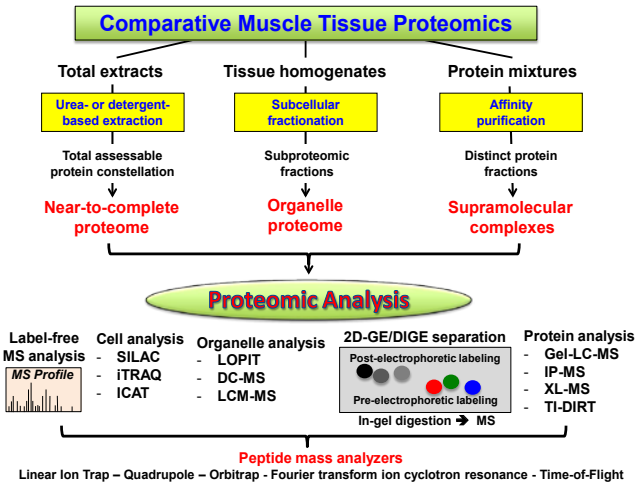

Figure 3: Flow chart outlining the various bioanalytical strategies used in comparative muscle tissue proteomics. Cellular analyses are often carried out by relative quantitation methods such as SILAC (Stable Isotope Labeling with Amino acids in Cell culture), iTRAQ (isobaric Tags for Relative and Absolute Quantitation) or ICAT (Isotope-Coded Affinity Tag). Organelle proteomics encompasses LOPIT (Localization of Organelle Proteins by Isotope Tagging), laser capture microscopy (LCM) and differential centrifugation (DC) approaches. The most powerful two-dimensional gel electrophoresis (2D-GE) technique used in comparative muscle proteomics is represented by fluorescence difference in-gel electrophoresis (DIGE). Protein affinity purification methods include immuno precipitation (IP), chemical crosslinking (XL) and the TI-DIRT method (Transient Isotopic Differentiation of Interactions as Random or Targeted). Distinct protein populations can also be conveniently analyzed by a combination of one-dimensional gel electrophoresis and liquid chromatography (LC), followed by mass spectrometry (MS).

The fluorescence difference in-gel electrophoresis method [66] has been widely applied to study neuromuscular disorders [52-54,67].

Quantitative proteomic analyses can be performed with metabolic or chemical labeling and often involve the isotopic tagging of peptides or proteins prior to differential analysis by mass spectrometry [68]. Cellular analyses are frequently carried out by relative quantitation methods such as SILAC (Stable Isotope Labeling with Amino acids in Cell culture), iTRAQ (isobaric Tags for Relative and Absolute Quantitation) or ICAT (Isotope-Coded Affinity Tag) [69]. Organelle proteomics employing isotope tagging is known as LOPIT (localization of organelle proteins by isotope tagging) [61,70]. Interestingly, laser capture microscopy has been successfully used for pre-fractionation steps in subproteomic studies [71], positioning this technique as a suitable alternative to differential centrifugation in the field of subproteomics. Technical details on the application of specific peptide mass analyzers using linear ion traps, quadrupole, orbitrap, fourier transform ion cyclotron resonance or time-of-flight methodology have been described in several extensive reviews [72-74]. The performance comparison of individual classes of mass spectrometers with respect to resolving power, mass accuracy, sensitivity, dynamic range, throughput capacity and available fragmentation modes has been recently discussed by Zhang et al. [69]. For the proteomic analysis of protein complexes involved in muscular dystrophy, both affinity purification and co-immuno precipitation techniques have been employed [46,75,76]. A new approach for the identification of transiently interacting proteins is represented by the TI-DIRT method (transient isotopic differentiation of interactions as random or targeted) [64], which future application in muscle proteomics might identify novel transient binding partners of the membrane cytoskeletal protein dystrophin [11]. A widely used protein biochemical method, chemical crosslinking [62], has also been applied to the mass spectrometric analysis of distinct coupling sites within the structure of proteins and protein complexes [63,65]. In the field of muscle biology, the usuage of a trifunctional crosslinker agent has helped in the elucidation of the interactions between the Ca^{2+}-dependent complex between calmodulin and the C-terminal sequence of the skeletal muscle myosin light chain kinase [77]. Combining chemical crosslinking analysis and advanced mass spectrometry will hopefully also be useful for the detailed analysis of changes in the dystrophin-associated glycoprotein complex in X-linked muscular dystrophy.

Successful applications of tissue proteomics for studying muscular dystrophy

Despite the above outlined technical problems and biological issues that may complicate comprehensive tissue profiling by comparative proteomics, large-scale protein separation in combination with mass spectrometry was successfully applied for studying many crucial aspects of muscular dystrophy [23]. Over the last few years, the systematic usage of gel electrophoresis, liquid chromatography and various mass spectrometric techniques has decisively advanced the field of muscular dystrophy research [11]. The identification of novel proteome-wide changes in dystrophic tissues has drastically increased our general understanding of the molecular pathogenesis of dystrophinopathies. The proteomic characterization of isolated sarcolemma vesicles and the mass spectrometric analysis of the purified dystrophin-glycoprotein complex from skeletal muscle has confirmed the close interactions between dystrophin and dystroglycans, sarcoglycans, dystrobrevins, syntrophins and sarcospan [46,76]. Interestingly, the proteomic analysis of the immunoprecipitated cardiac dystrophin complex revealed no interactions with the signaling enzyme nNOS, differing compositions of dystrobrevins and syntrophins and additional binding partners, such as Cypher, Cryab, Cavin-1 and Ahnak-1 [75]. Thus, proteomics was extremely useful in determining differences between the compositions of dystrophin-associated complexes from skeletal muscles versus the heart.

The proteomic survey of total tissue extracts from dystrophic *mdx* hindlimb muscle showed elevated levels of the AK1 isoform of the enzyme adenylate kinase [78,79], which had not previously been shown by the conventional biochemical analysis of dystrophin-deficient muscles. This finding suggests abnormal nucleotide ratios and an impaired regulation of bioenergetic processes in muscular dystrophy. Biochemical surveys focusing on ion-regulatory proteins have shown drastic reductions in the Ca^{2+}-binding proteins calsequestrin of the sarcoplasmic reticulum [54,80] and regucalcin [81] and parvalbumin of the cytosol [67]. These altered expression levels of important Ca^{2+}-handling proteins agree with pathophysiological impairments of cytosolic ion cycling and a considerably lower capacity for luminal Ca^{2+}-buffering [82]. The diminished ability to properly sequester excess Ca^{2+}-ions in dystrophic fibers, in conjunction with chronic Ca^{2+}-leakage through the ruptured and inadequately repaired plasma membrane [83] and an enhanced proteolytic degradation of sensitive muscle proteins [84], probably exacerbates the dystrophic phenotype [85]. The proteomic analysis of total tissue extracts from young *mdx* hindlimb muscle revealed changes in vimentin, desmin, tubulin, annexin, glycolytic enzymes and mitochondrial proteins involved in oxidative phosphorylation [86,87]. These findings suggest alterations in energy metabolism and compensatory remodeling of the cytoskeletal network in dystrophin-deficient muscle cells.

In stark contrast to mildly affected extraocular and *interosseus mdx* muscles [88,89], the severely dystrophic *mdx* diaphragm exhibited considerable changes in many proteins involved in energy metabolism, the contractile apparatus and the cellular stress response [52,54,67]. The up-regulation of molecular chaperones was shown to be a general hallmark of muscular dystrophy [52,90] and suggests high levels of cellular stress in dystrophinopathy with the constant need for refolding or efficient removal of misfolded proteins [91]. The proteomic analysis of experimental skipping of exon 23 in the *mdx* mouse model of Duchenne muscular dystrophy demonstrated that this novel therapeutic approach reversed the high concentration levels of muscle-specific heat shock proteins [54]. Although this is an encouraging finding with respect to developing new treatment strategies, one of the major obstacles for successful delivery of pharmacological or gene therapeutic agents is muscular dystrophy-associated fibrosis [92-94]. Exon skipping therapy might be seriously complicated by the presence of muscle tissue scarring. Proteomics has clearly confirmed the extensive accumulation of collagen in the extracellular matrix region of dystrophin-deficient muscle [67], whereby aging exacerbates the extent of fibrotic changes [95,96]. An extensive deposition of extracellular matrix proteins was also shown to occur in laminin α2 chain-deficient muscle by quantitative proteomics [97], confirming that congenital muscular dystrophy is associated with widespread fibrosis [98]. Besides extensively studying the established *mdx* mouse model by proteomics, the *vastus lateralis* muscle from the dystrophic *grmd* golden retriever was also evaluated by mass spectrometric surveys. Interestingly, several metabolic proteins that are regulated by PGC1-α, were shown to be altered in *grmd* muscle fibers, suggesting considerable bioenergetics disturbances in the absence of dystrophin [99].

Since changes in post-translational modifications play an important role during muscle development, fiber maturation, physiological

adaptations, neuromuscular pathology and the natural aging process, the global evaluation of changes in glycosylation [100], phosphorylation [101], carbonylation [102], nitrosylation [103] and acetylation [104] is becoming increasingly important in the areas of muscle biochemistry and proteomic biomarker discovery research [21]. With the exception of a recent mass spectrometric analysis of carbonylation of the molecular chaperone Hsp70 in Duchenne muscle samples [105], post-translational modifications have not been intensively studied in dystrophic muscle tissue samples from X-linked muscular dystrophy. However, changes in phosphorylation, glycosylation, carbonylation and nitrosylation have been documented in a variety of neuromuscular pathologies. This included alterations in the phosphorylation patterns of the mitochondrial ATP synthase in insulin-resistant muscle [106] and a changed concentration of the phosphorylated form of myosin light chain MLC2f in hyperexcitability-related myotonic muscle [107]. Drastic alterations in post-translational modifications were shown to be involved in age-related muscle degeneration, affecting especially metabolic enzymes [108,109]. For example, the rate-limiting glycolytic enzyme pyruvate kinase was shown to exhibit a drastically reduced activity in aged muscle tissues and this functional impairment appears to be directly related to enhanced levels of N-glycosylation [110] and tyrosine nitration [111,112]. Changes in post-translational modifications probably influence overall protein stability, subcellular protein targeting, intra- and intermolecular interactions, and the biochemical coupling efficiency between substrate and active site in metabolic enzymes. In the future, the detailed bioanalytical assessment of changes in critical post-translational modifications will also be applied to the study of dystrophic fibers and hopefully enhance our understanding of the role of phosphorylation, glycosylation, carbonylation, nitrosylation, acetylation and ubiquitination in dystrophinopathy.

Conclusions

Muscle tissue proteomics is concerned with the global biochemical analysis of the protein constellation of contractile fibers and their associated cellular structures. In comparative pathoproteomic studies, the systematic extraction of the assessable protein repertoire from crude tissue extracts has the clear advantage of using a defined starting material that represents the near-to-complete muscle proteome. This approach, although it may underrepresent the presence of certain types of protein, avoids the introduction of artifacts due to extensive subcellular fractionation steps. Proteome-wide studies should ideally be carried out in conjunction with organelle and membrane proteomics that uses distinct fractions with reduced sample complexity. Importantly, since pathoproteomics measures concentration levels of proteins and identifies distinct protein isoforms, this information usually focuses on the status of distinct subspecies of proteins at a given point of time during a disease process. It is therefore not readily possible to deduce from proteomic data sets any indications on the actual mechanisms that change regulatory processes. Proteomic findings should therefore not be over-interpreted. For example, the reduction of a specific protein as determined by comparative proteomics might be due to down-regulation, controlled loss via secretion, tissue damage-related leakage or non-specific degradation. Thus, to make more broadly applicable conclusions based on proteomic data, additional cell biological and physiological characterization should be performed. Overall, tissue proteomics has clearly progressed the field of basic and applied myology and the recent development of more advanced peptide mass analyzers will certainly improve the sensitivity to detect even smaller amounts of distinct protein species in muscle biopsy samples. In the future the combination of crude tissue proteomics, organelle proteomics and affinity proteomics of isolated protein complexes promises to be the most suitable bioanalytical strategy for the comprehensive screening of pathological muscle specimens. This should result in the identification of novel biomarkers of muscle diseases and a vastly improved knowledge of the molecular pathogenesis of common disorders of the neuromuscular system.

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