

Review

Fiber type diversity in skeletal muscle explored by mass spectrometry-based single fiber proteomics

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Summary. Mammalian skeletal muscles are composed of a variety of muscle fibers with specialized functional properties. Slow fibers are suited for long lasting and low intensity contractile activity, while various subtypes of fast fibers are optimized to produce high force and power even with a significant fatigue. The functional specialization of muscle fibers is based on selective gene expression regulation, which provides each fiber with a specific protein complement.

The recent refinement of small-scale sample preparation, combined with the development of mass spectrometers characterized by high sensitivity, sequencing speed and mass accuracy, has allowed the characterization of the proteome of single muscle fibers with an unprecedented resolution. In the last few years, the first studies on the global proteomics of individual fibers of different types have been published. In this short review we discuss the methodological advancements which have opened the way to single fiber proteomics and the discovery power of this approach. We provide examples of how specific features of single fibers can be overlooked when whole muscle or multi-fiber samples are analyzed and can only be detected when a single fiber proteome is analyzed. Thus, novel subtype-specific metabolic features, most prominently mitochondrial specialization of fiber types, have been revealed by single fiber proteomics. In the same way,

specific adaptive responses of single fibers to aging or loss of neural input have been detected when single fibers were individually analyzed. We conclude that the fiber type-resolved proteomes represent a powerful tool which can be applied to a variety of physiological and pathological conditions.

Key words: Skeletal muscle, Muscle fiber types, Single muscle fiber proteomics, Mass spectrometry

Introduction

A distinctive feature of skeletal muscle is the diversity and specialization of fiber types, which allow muscles to fulfill a variety of functional tasks by selective recruitment of the most suitable fibers: fatigue resistant slow fibers for long lasting activity, powerful and fatiguable fast fibers for short bursts of high intensity work. Biochemical studies of selected muscles with relatively homogeneous fiber type composition, which are found in some species, have initially provided some basic information about the molecular features of muscle type-specificity. For example, the seminal studies by Bárány (1967) based on a comparative study of fast and slow muscles from different species, revealed for the first time the relationship between maximum myosin ATPase activity and the speed of contraction (see also Moss and Solaro, 2019). However, the mixed fiber type composition of most skeletal muscles, particularly in two species, mouse and human, most relevant for biomedical research, complicates the interpretation of biochemical

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studies at the whole tissue level. Data derived from whole muscle studies reflect in fact both the proportion of the different fiber types and the value of the parameters analyzed in each fiber type. In addition, whole muscle analyses also include non-muscle cells, which are a minor component in terms of global protein composition but a major component in terms of proteins present in the nucleus, as myonuclei represent less than 50% of total nuclei present in muscle tissue (Schmalbruch and Hellhammer, 1977).

To address this issue and identify the proteins expressed in specific fiber types, two approaches were subsequently used. The first approach is based on enzyme histochemistry and immunohistochemistry analyses of muscle sections. The second approach involves the dissection of single fibers from skeletal muscles and the analysis of the protein composition by SDS-PAGE and Western blotting. A crucial step in the study of muscle fiber types was the demonstration that skeletal muscles contain different myosin heavy chains (MYH) isoforms, which can be identified by specific antibodies and separated by appropriate SDS-PAGE protocols. MYHs thus became a standard marker to identify fiber types in muscle sections and in isolated fibers. These studies led to the current view that, based on MYH composition, mouse muscles contain four major fiber types, slow type 1 and fast 2A, 2X and 2B, while human muscles contain three major fiber types, slow type 1 and fast 2A and 2X (Schiaffino and Reggiani, 2011). Additional fiber types, defined by unique MYH isoforms, are confined to specialized muscles, such as cranial muscles: for example, slow-tonic fibers, which respond to stimulation with a contracture rather than a twitch, are present in extraocular muscles but not in most trunk and limb muscles (Rossi et al., 2010).

Two-dimensional gel electrophoresis combined with mass spectrometry (MS) allowed to identify a significant number of proteins, up to a few hundred, in skeletal muscle samples (see Murphy et al., 2016; Capitano et al., 2017). However, many muscle proteins, e.g. MYHs, cannot be analyzed because they do not enter the gel in the first dimension (isoelectric focusing, IF). In addition, two-dimensional gel electrophoresis, which requires about 100 μg of protein, cannot be applied to the study of single muscle fibers, which contain around 1 μg of protein. This limitation also applies to other approaches involving the isolation of specific muscle fiber structures, such as contractile proteins or mitochondria, and the analysis of these proteins by MS. Sarcomeric protein-enriched extracts of skeletal muscles, derived from 5 mg of muscle tissue, have been analyzed by top-down targeted proteomics to determine alternative splicing and post-translational modifications of contractile proteins (Jin et al., 2019). Furthermore, gel-based techniques are laborious and require long hands-on time per sample, thus preventing multiplexing of the procedure to high sample numbers.

The proteomic analysis of isolated single muscle

fibers was made possible by recent advances in proteomics techniques, in particular the down scaling of the sample size through the development of shotgun MS approaches (see below), which allow the identification of thousands of proteins in a single muscle fiber. In this short review we discuss the methodological basis of this approach and the main results obtained in studies on mouse and human skeletal muscles.

Methodological aspects

A major challenge encountered in trying to apply modern proteomics techniques to single muscle fibers is the very low amount of proteins contained in a single muscle fiber or, in the case of human muscles, in a segment of fiber as can be dissected from a biopsy sample. The estimated amount of protein in a single muscle fiber isolated from typical mouse muscles, such as the slow soleus or the fast extensor digitorum longus (EDL) is about 0.5 μg , assuming a cross sectional area of 1000 μm^2 , a length of 5 mm and a cylindrical shape. As to human samples, due to thicker cross-sectional area, similar calculations lead to 0.5 μg per mm length and thus approximately 1.5 μg with an average length of 3 mm in a biopsy taken with a Bergstrom needle. These minute sample amounts are not suitable for IF + SDS-PAGE-based approaches. The problem has been addressed by optimizing an in-solution digestion workflow, whereby all preparation steps, from sample lysis to trypsin digestion, are carried out in one buffer and confined to a single vessel. The latter is subsequently also used for peptide purification, thus minimizing sample loss (Kulak et al., 2014). In our studies we applied this approach to single fibers, which were manually isolated and immediately snap-frozen (Murgia et al., 2015, 2017). Other groups have used collagenase digestion for muscle fiber isolation (Lang et al., 2018).

A simplified workflow of the single-fiber proteomics used in our studies is presented in Fig. 1. An indication of the relative concentration of the various proteins in the muscle fibers can be derived from the corresponding IBAQ (Intensity-Based Absolute Quantification) values, an output of the MaxQuant analysis software (Cox and Mann, 2008; Tyanova et al., 2016). IBAQ values are the MS signal intensities divided by the number of theoretical peptides, thus are roughly proportional to the molar quantities of the proteins. In our studies IBAQ values of individual proteins were normalized to the IBAQ value of α -skeletal actin to account for the variable cross-sectional area and length of the fibers analyzed.

A further methodological challenge in single-fiber proteomics is the wide dynamic range of protein expression in muscle fibers, which spans several orders of magnitude, from the highly expressed contractile proteins, such as myosin and actin, to low abundance proteins, such as transcription factors. In our MS-based shotgun proteomics workflow, we measure the

Single muscle fiber proteomics

abundance of individual peptides after elution in a nanoflow liquid chromatography gradient and electrospray ionization into the mass spectrometer. The same peptides are not always picked for sequencing by the mass spectrometer, making detection partially stochastic. Because of the dramatic difference in the expression level of sarcomeric proteins compared to the rest of the muscle proteome, peptides from a few highly abundant proteins are more often selected for sequencing, thus interfering with the identification of less abundant proteins. As a consequence, the overall number of identifications and the coverage of comparably under-represented proteins (such as transcription factors) are reduced. Unlike muscle, other tissues and cell lines have a large fraction of proteins of similar abundance range and, therefore, the quantification of a large fraction of their proteome is technically easier.

A way to address this issue and identify low abundance proteins is to combine the MS analysis of single fibers with that of a “library”, consisting of a deep proteome of whole muscle homogenates, extensively fractionated to reduce proteome complexity. Such a library contains sequence information for peptides that are detected but not sequenced in the single muscle

fibers. Using the ‘match between runs’ feature of the MaxQuant analysis software it is possible to transfer peptide identifications from the deep muscle proteome, where a given peptide is much more likely to have been fragmented and sequenced, to a single fiber, where often only the intact peptide has been measured (Deshmukh et al., 2015). As a result, the number of quantified proteins in muscle fibers increases, allowing the analysis of a larger fraction of the single muscle fiber proteome (Murgia et al., 2015). The number of peptides which are identified using ‘match between runs’ with the help of a “library” is about double those identified without matching.

An additional issue to be considered is that some muscle protein families, such as MYHs, consist of isoforms with extremely similar amino acid sequences. Therefore, the number of peptides which can be used to distinguish the isoforms is only a fraction of the total peptide number measured for these proteins. As a consequence, the precise quantification of each isoform can be challenging using the algorithms used for the analysis of MS data in the standard mode, which attributes common peptides to the “protein group” with the largest number of total peptides identified. To avoid the risk of incorrect attribution of MYH peptides to the

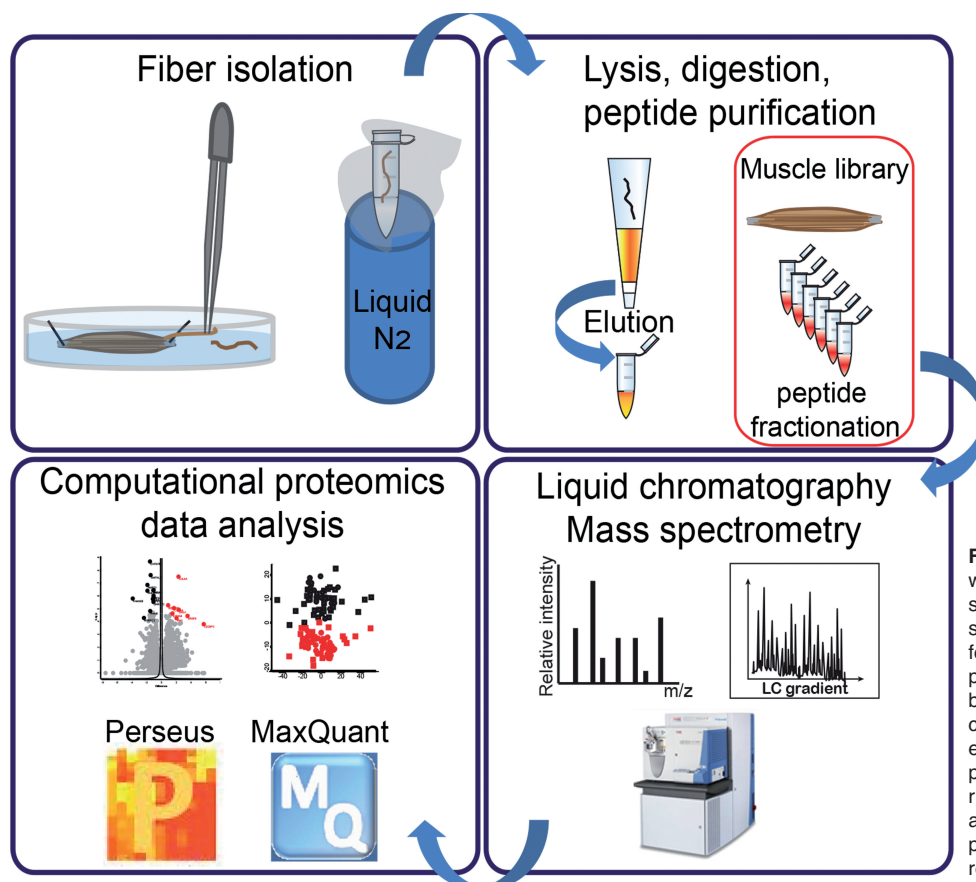


Fig. 1. Graphical illustration of the workflow for mass spectrometry-based single muscle fiber proteomics. Manual single fiber isolation and snap-freezing is followed by a single buffer/single vessel procedure for protein digestion, followed by peptide purification. Liquid chromatography connected to nano-electrospray precedes MS analysis of peptides. The corresponding spectra in raw files are analyzed using the freely available MaxQuant computational proteomics software platform and the related Perseus framework.

different isoforms, only unique peptides, i.e. peptides which are present only in a given isoform, must be used for quantification of MYHs.

The correct identification and quantification of the MYH isoforms has provided specific markers to define the protein profile of the different fiber types. In agreement with previous immuno-histochemical and biochemical (SDS-PAGE) studies, proteomic analyses also show that a significant number of fibers have a hybrid MYH composition. However, we found that both human and murine muscles contain a high percentage of fibers with a largely predominant isoform (more than 80% of the total) and thus can be safely attributed to a given type. One can thus compare the fiber type profile

of relatively pure muscle fiber types. In the following sections of this review we highlight the power of single-fiber proteomics to reveal novel aspects of mouse and human muscle biology.

Fiber-type-specific differences in mitochondrial proteins

The discovery power of single-fiber proteomics is clearly illustrated by the study of mitochondrial proteins. A large number of mitochondrial proteins was detected in the single-fiber proteome datasets of the four fiber types present in mouse skeletal muscles (Murgia et al., 2015). The comparison of these datasets shows that most

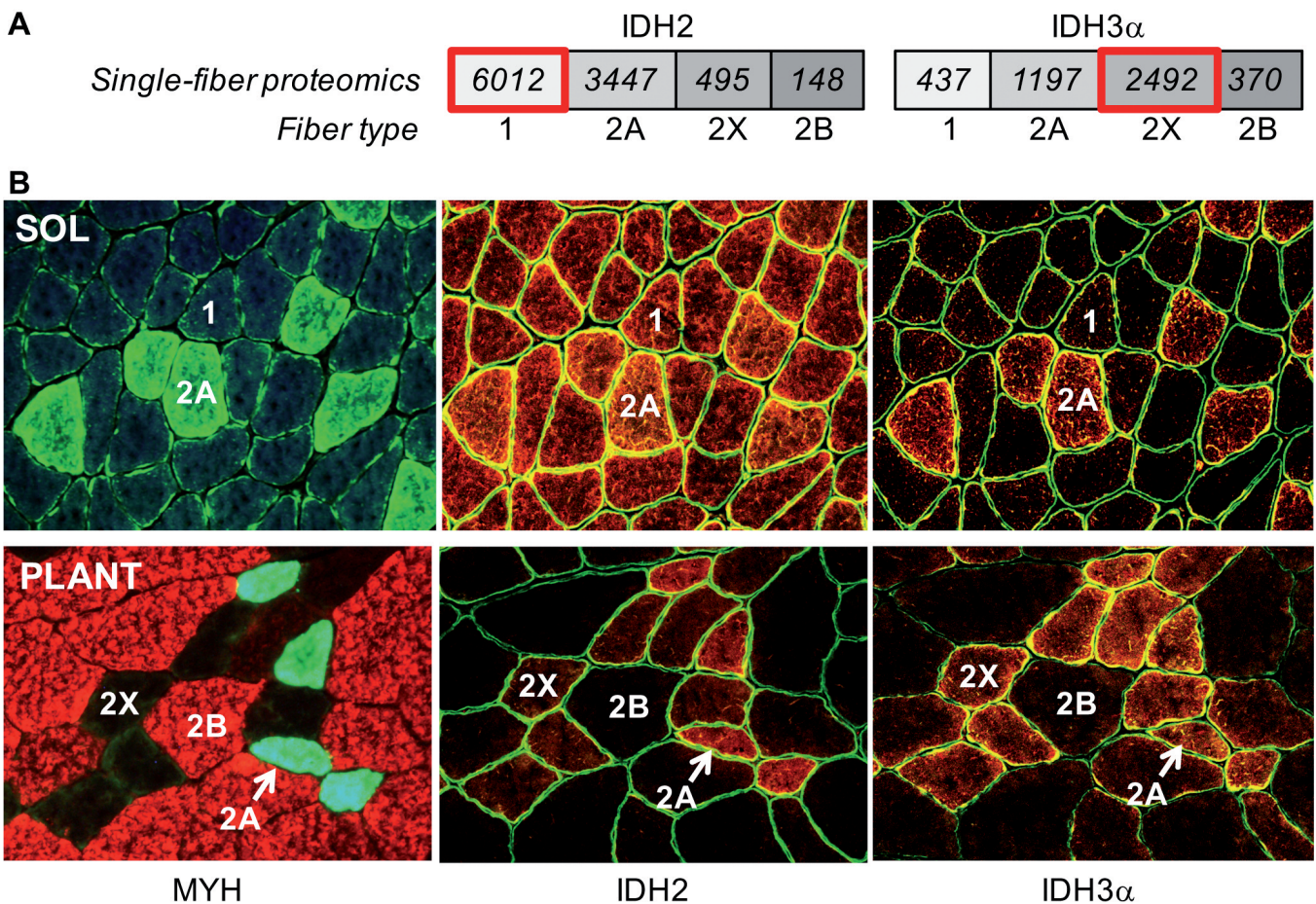


Fig. 2. Relative abundance of isocitrate dehydrogenase 2 (IDH2) and IDH3 α proteins in the four fiber types present in mouse skeletal muscle, as determined by single-fiber proteomics and immunohistochemistry. **A.** Single-fiber proteomics shows that IDH2 has highest levels in type 1 and very low levels in type 2X fibers; in contrast, IDH3 α has highest levels in type 2X and very low levels in type 1 fibers. Type 2A fibers show relatively high levels of both IDH2 and IDH3 α , while 2B fibers have the lowest levels of both isoforms due to their poor mitochondrial content. **B.** Fiber-type-specific distribution of IDH2 and IDH3 α in the slow soleus (SOL) and fast plantaris (PLANT) mouse skeletal muscles, as determined by immunohistochemistry with specific antibodies. Left panels: Transverse sections were stained with monoclonal antibodies specific for type 1, 2A and 2B myosin heavy chains (MYH) to identify the different fiber types; type 2X fibers are unstained. Middle and right panels: Serial sections were stained for IDH2 or IDH3 α and co-stained for dystrophin (green) to highlight the plasma membrane. IDH2 is more abundant in type 1 and 2A fibers, less abundant in 2X and almost undetectable in 2B fibers. In contrast, IDH3 α is expressed at much higher levels in 2X and 2A fibers compared to type 1 and 2B fibers. This pattern of expression closely corresponds to the MS values derived from single-fiber proteomics shown in A. Adapted from Schiaffino et al., 2015.

mitochondrial proteins are more abundant in type 2A and 2X fibers compared to type 1 fibers, while, not surprisingly, the glycolytic 2B fibers showed the lowest values. For example, the relative expression levels of 43 proteins from complex I, III, IV, and V (ATPase), which were represented in all our selected fibers, showed the following coefficients of OXPHOS quantity: 1 (type 2A), 0.91 (type 2X), 0.63 (type 1), and 0.21 (type 2B) (Schiaffino et al., 2015). A similar distribution of relative values was seen for the proteins of the TCA cycle, with a tendency for type 2X fibers to show the highest values. However, isocitrate dehydrogenase (IDH) showed a unique pattern of fiber type distribution, different from that of other components of the TCA cycle.

IDH is an enzyme that catalyzes the oxidative decarboxylation of isocitrate, producing α -ketoglutarate and CO_2 in a two-step process, which involves oxidation of isocitrate to oxalosuccinate, followed by the decarboxylation, forming α -ketoglutarate. In mammals, IDH exists in two isoforms: IDH3 is a tetramer composed by two α subunits, one β subunit and one γ subunit and catalyzes the oxidative decarboxylation of isocitrate to α -ketoglutarate coupled with the reduction of NAD^+ to NADH, whereas IDH2 is a homodimer and catalyzes the same reaction using NADP^+ as a cofactor instead of NAD^+ . Single muscle fiber proteomics has shown that the distribution of IDH2 and IDH3 varies significantly according to fiber type in murine skeletal muscles (Fig. 2A). In particular, IDH2 levels do not correlate with OXPHOS levels, as they are highest in type 1 fibers, slightly lower in 2A and much lower in 2X and 2B fibers. In contrast, IDH3 α , as well as IDH3 γ , is abundant in 2X and 2A fibers but shows very low levels in type 1 fibers. Similar findings were recently reported by comparing slow and fast 2A fibers in mouse soleus (Lang et al., 2018).

The differential distribution of IDH isoforms was confirmed by immunohistochemistry (Fig. 2B). The antibodies to IDH2 give a strong signal in type 1 and 2A fibers, weaker in 2X and weakest in 2B fibers, whereas antibodies to IDH3 α stain strongly 2X and 2A and very weakly type 1 fibers. This differential distribution likely has an important functional counterpart, as IDH2 supports the reduction of NADP^+ to NADPH and thus provides a reduced cofactor for glutathione sulphide reductase and thioredoxin reductase, namely the enzymes essential for controlling the buffering of superoxide via glutathione peroxidase, peroxiredoxin and superoxide dismutase. The abundance of IDH2 in slow fibers would thus improve their ability to control redox state during continuous mitochondrial ATP generation (Fig. 3). This interpretation was confirmed by the finding that nicotinamide nucleotide transhydrogenase (NNT), the enzyme that couples the hydride transfer between reduced NADH and NADP^+ to proton translocation across the inner mitochondrial membrane, is also 10 times more abundant in type 1 fibers compared to 2X fibers, in spite of the greater abundance of OXPHOS and most other mitochondrial proteins in 2X fibers (Schiaffino et al., 2015). IDH2 and NNT are the major mitochondrial enzymes involved in NADPH generation. Their abundance in the slow type 1 fibers thus supports the idea that the continuous activity of the slow fibers causes a greater ROS production which is counterbalanced by a greater abundance of NADPH generating enzymes.

Fiber-type-specific differences in the adaptive response of skeletal muscles

The diversity among single muscle fibers is not restricted to their specialization in ATP regeneration or

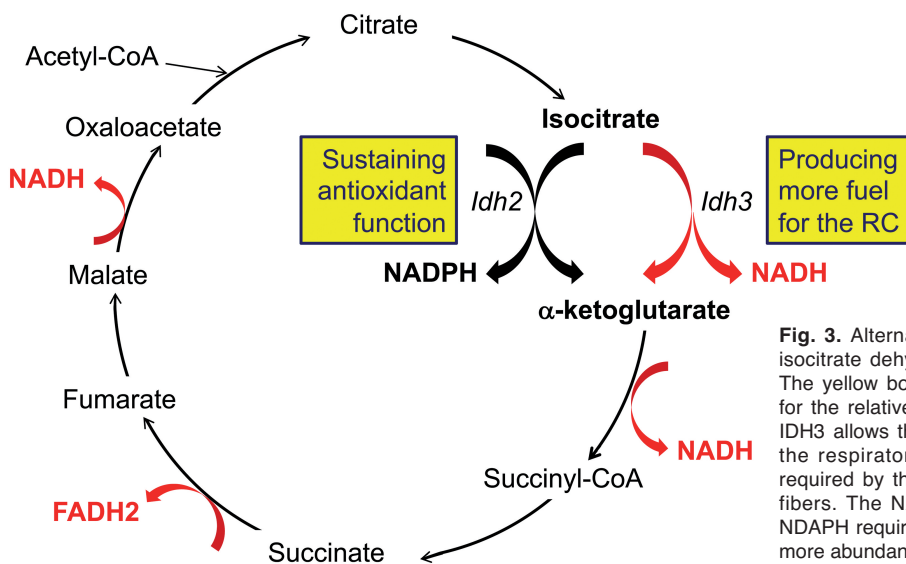


Fig. 3. Alternative pathways of the TCA cycle at the level of isocitrate dehydrogenase revealed by single-fiber proteomics. The yellow boxes suggest a possible interpretation to account for the relative role of the two pathways. The NAD^+ -dependent IDH3 allows the generation of more NADH, thus more fuel for the respiratory chain (RC) and increased ATP production, required by the more expensive myosin motor of the type 2X fibers. The NADP^+ -dependent IDH2 allows the generation of NADPH required by the antioxidant mitochondrial system that is more abundant in the type 1 fibers.

to different contractile performance. The diversity involves also regulatory and adaptive processes. Thus, the single fiber analysis can reveal adaptive changes that might remain undetected when muscle samples with heterogeneous composition in muscle fibers are analysed.

A striking example comes from the study of fiber type specific adaptations during aging of human muscles (Murgia et al., 2017). Single fiber proteomics confirmed the expected age-related decline in mitochondrial enzymes for both slow and fast 2A fibers, but showed unexpected variations in the complement of enzymes of the glycolytic pathway and of glycogen synthesis and degradation. Enzymes of glycolysis and glycogen metabolism were found upregulated in slow muscle fibers of elderly subjects (average 70 years-old) compared to young subjects (average 24 years-old), as shown in Fig. 4. One could hypothesize that the increase in glycolytic enzymes is a compensation for the loss of mitochondrial function. Alternatively, it is possible that the main effect of this metabolic shift is the control of muscle fiber trophism. In this view, glycolytic intermediates could be diverted to precursors of nucleotides, amino acids, and fatty acids to sustain muscle protein synthesis in aged slow fibers. This could underlie the observed striking difference in muscle fiber trophism during aging. Indeed, many previous studies show that the fast fibers of elderly subjects are distinctly atrophic, whereas slow fibers characteristically maintain their size during aging. (e.g. Lexell and Taylor 1991; Callahan et al 2014). Interestingly, the above mentioned isoforms of IDH show a different behaviour with aging, characterized by a decrease in IDH2 in slow fibers and stable values if not increased in fast 2A fibers.

Other interesting examples of how single fiber proteomics can reveal changes in opposite directions of different fiber types coexisting in the same muscle are given in the study of murine soleus muscle denervation

(Lang et al., 2018). Single fiber quantitative analysis revealed opposing regulation of SERCA2 (coded by ATP2A2) in slow and in fast 2A fibers. Removal of neuronal innervation tends to convert slow fibers into faster fibers and fast fibers into slower fibers. In the murine soleus muscle, the approximate composition recognizes a 50%-50% proportion of slow and fast (mainly 2A) fibers. Thus, the impact of denervation on a very abundant protein as the sarcoplasmic/endoplasmic reticulum calcium ATPases might remain undetected when whole muscles are analyzed, and only the single fiber quantitative analysis can reveal opposing regulation of SERCA2 in slow and in fast 2A fibers (Lang et al., 2018).

Conclusions and perspectives

The distinctive feature of single muscle fiber proteomics is that, in contrast to the traditional immunohistochemical and electrophoretic methods focused on specific protein components, this technique provides a global and unbiased portrait of the whole myofiber protein profile. For the first time it is thus possible to compare, within the same fiber, proteins associated with different cell structures, from the plasma membrane to mitochondria, sarcoplasmic reticulum, myofibrils and nucleus, as well as the relative distribution of enzymes involved in various metabolic pathways. Other omic approaches, such as transcriptomics, have also been applied to single muscle fibers (see Chemello et al., 2011, 2019), however changes in gene expression do not always reflect changes in protein abundance (see Andersen and Schiaffino, 1997, 1999), thus only protein levels can be used to draw meaningful physiological correlations (see also Schiaffino et al., 2019).

Single muscle fiber proteomics can be a discovery tool to define the function of known muscle proteins, as

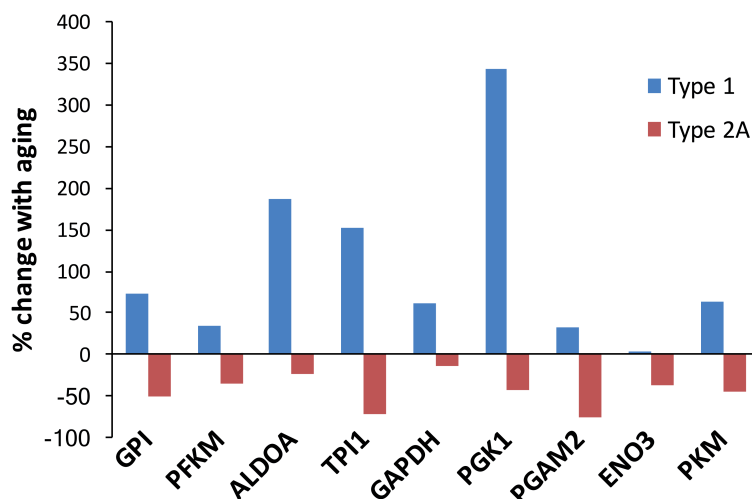


Fig. 4. Age-dependent changes in expression of glycolytic enzymes in type 1 and 2A human muscle fibers. Single fibers, isolated from vastus lateralis muscle biopsies of younger (age 22–27) and older (age 65–75) individuals, were processed for single fiber proteomics. The percentage fold change (older/younger ratio) is shown for each protein in the two fiber types. Glycolytic enzymes are indicated by the corresponding gene names. Data from Murgia et al. (2017).

illustrated by the fiber-type-specific variations in the IDH isoform profile referred to above, and to identify new fiber-type-specific proteins. Indeed, dozens of proteins, previously not considered in skeletal muscle studies, appear to be selectively expressed in one or another fiber type (see Table S2 in Murgia et al., 2015, and Table S6 in Murgia et al., 2017). Proteomic data thus open a new window to explore muscle fiber diversity, by generating hypotheses and suggesting experiments to validate these hypotheses. For example, knockdown of IDH2, especially when combined with the lack of NNT that occurs spontaneously in certain mouse strains (see Schiaffino et al., 2015), would be expected to cause increased ROS production in slow type 1 but not in fast type 2 fibers in response to electrical stimulation or forced exercise (see Schiaffino et al., 2019).

A limitation of single muscle fiber proteomics based on shotgun MS is that it is unable to resolve all the protein variants derived by alternative splicing. Top-down MS-based proteomics, in which intact proteins are analyzed rather than peptides, allows to better distinguish protein isoforms derived from homologous genes and proteins derived by alternative splicing of the same gene. However, absolute quantification of selected proteins can only be obtained by targeted proteomics, adding precise amounts of specific proteins labeled by stable isotopes to the sample to be examined. Stable isotope incorporation introduces a small mass difference to identical peptides so that they can be distinguished by MS. Different methods for absolute quantification have emerged over the last years including Protein Epitope Signature Tags (PrESTs) (Zeiler et al., 2012) and absolute quantification (AQUA) (see Lindermann et al., 2017). They all rely on either spiking in heavy labeled peptides or heavy labeled full length proteins.

Different proteomic approaches can be also be used to analyze the post-translational modifications that proteins undergo in muscle fibers, as illustrated in a recent phosphoproteomics study on the effect of exercise in human skeletal muscle (Hoffman et al., 2015). Recent methodological developments have shown that sample preparation for phosphoproteomics can be considerably scaled down and multiplexed, making it amenable to the analysis of biological samples of small size (Humphrey et al., 2018). However, a phosphoproteomics study of single muscle fibers is not yet possible with available techniques, due to the tiny amounts of proteins present in single muscle fibers. In conclusion, a likely scenario of the future development of muscle fiber proteomics is that shotgun MS will continue to provide a global picture of the myofiber protein profile and will be complemented by top-down MS and targeted proteomics approaches to define protein variants generated by alternative splicing and post-translational modifications.

A final point that must be kept in mind is that available databases required for MS searches do not include the large and continuously increasing number of previously undetected micropeptides, expressed from long non coding RNAs (lncRNAs) and circular RNAs

(circRNAs), many of which have been recently discovered in skeletal and cardiac muscle (Makarewich and Olson, 2017; van Heesch et al., 2019). The discovery of micropeptides further increases the complexity of the “proteoforms”, i.e. the molecular forms of expressed proteins, that are present in any tissue, including skeletal muscle fibers (see Aebersold et al., 2018).

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