Biomedical Data Mining

Extended Abstract

Dynamic changes in the skeletal muscle proteome during denervation-induced atrophy

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Abstract

Loss of neuronal stimulation enhances protein breakdown and diminishes protein union, causing fast bulk misfortune. To explain the pathophysiological variations that happen in decaying muscles, we utilized stable isotope marking and mass spectrometry to precisely measure protein articulation changes during denervation-instigated decay after sciatic nerve area in skeletal muscle. Moreover, mice were taken care of a SILAC diet containing 13C6 lysine for four, seven, or eleven days to compute relative degrees of protein combination in denervated and control muscles. Ubiquitin remainder peptides (K-ε-GG) were profiled by immunoaffinity advancement to recognize likely substrates of the ubiquitin proteasomal pathway. Other than a protein articulation profiling we utilized a heartbeat SILAC naming way to deal with recognize differential Lys6 joining rates among control and denervated muscle. Enhancement of diglycine remainders distinguished 2100 endogenous ubiquitination locales and uncovered a metabolic and myofibrillar protein diglycine signature, including myosin substantial chains (MyHC), myomesins and titin, during denervation.

Near examination of these proteomic datasets with known atrogenes utilizing an arbitrary timberland approach recognized 92 proteins subject to atrogene-like guideline that have not recently been straightforwardly connected with denervation-prompted decay. Correlation of protein amalgamation and proteomic information showed upregulation of explicit proteins in light of denervation is basically accomplished by protein adjustment. This investigation gives the principal incorporated examination of protein articulation, amalgamation and ubiquitin marks during solid decay in a living creature.

Keywords: Muscle atrophy, Denervation, Pulsed SILAC, Ubiquitination, Random forest

INTRODUCTION

Numerous experiments have demonstrated that the metabolic and contractile properties of skeletal muscles can change rapidly in response to environmental influences (Cohen et al., 2015; Flück and Hoppeler, 2003). For example, physical exercise can induce muscle growth and activates signalling pathways that modulate mitochondrial activity, calcium homeostasis and muscle contractility (Matsakas and Patel, 2009). Conversely, ageing-associated diseases, such as cancer, type 2 diabetes mellitus and neurodegeneration, can lead to marked loss of muscle mass.

Skeletal muscle function is closely associated with motorneuron innervation. Reduced muscle recruitment, such as occurs during extended bed rest or spinal cord injury, results in a severe loss of muscle mass, which is also termed muscle atrophy (Gutmann, 1962; Jackman and Kandarian, 2004). Protein synthesis decreases, and processes that regulate protein degradation are enhanced during muscle wasting. Depletion of proteins is coordinated by both the ubiquitin-proteasome system (UPS) and autophagy-related processes (Lecker et al., 1999). Additionally, the members of the calcium-dependent nonlysosomal protease family (calpains) facilitate rapid degradation of myofibrillar proteins (Huang and Forsberg, 1998), and cellular organelles, such as mitochondria, are degraded via mitophagy, a specific form of autophagy (O'Leary et al., 2012; Vainshtein et al., 2015).

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Unilateral sciatic nerve section is a well-established animal model of reduced neuronal stimulation (Goldspink, 1976; Gutmann, 1962; Medina et al., 1991). Previous studies demonstrated that denervation-induced atrophy elevates the cytoplasmic calcium concentration, activates the UPS and leads to remodelling of myosin heavy chain (MyHC) composition within individual muscle fibres (Ciciliot et al., 2013; Mitch and Goldberg, 1996; Zeman et al., 1986).

Analysis of the ubiquitin conjugation cascade in atrophying muscles demonstrated enhanced expression of several E2 conjugation enzymes and E3 ubiquitin ligases, each of which targets specific protein substrates for destruction via the proteasome. For example, the E3 ubiquitin ligases MURF1, atrogin-1 (MAFbx) and TRIM32 proteins are the key E3 ubiquitin ligases that mediate protein degradation during muscular atrophy (Bodine et al., 2001; Cohen et al., 2012; Gomes et al., 2001). Notably, MURF1 and TRIM32 are responsible for degradation of myofibrillar proteins, whereas atrogin-1 leads to degradation of MYOD, myogenin and the eukaryotic initiation factor 3 (eIF3).

Systematic microarray analysis of different muscle-wasting conditions, including starvation, diabetes, uraemia and denervation, has identified a specific set of genes called atrogenes, which are either up- or downregulated during muscle atrophy. So far, ~120 atrogenes have been found, but it seems likely that this list is not complete (Lecker et al., 2004; Raffaello et al., 2006; Sacheck et al., 2007). Although mRNA expression studies have provided detailed insight into gene activity during atrophy, there have been few unbiased and systematic investigations of the changes in protein expression, protein synthesis and post-translational modifications during neuronal denervation of muscle tissue (Argadine et al., 2009; Goldspink, 1976; Ryder et al., 2015).

Recently, mass spectrometric (MS)-based proteomics has become an indispensable tool to measure proteins and their post-translational modifications. As a result of the development of powerful, high-resolution MS instruments, it is now possible to quantify the expression of vast numbers of proteins in complex biological samples (Cox and Mann, 2011; Mann et al., 2013). More importantly, the combination of MS-based proteomics with several enrichment strategies for specific modifications has enabled quantitative analysis of thousands of reversible protein modifications, including phosphorylation, acetylation and ubiquitination (Olsen et al., 2010). Public databases contain data on >500,000 post-translational modifications, demonstrating that individual proteins are highly engineered via diverse sets of modifications (Olsen and Mann, 2013).

The development of metabolic labelling approaches has facilitated accurate protein quantification and has been used to analyse protein turnover in both cell culture models and living animals (Schwanhäusser et al., 2009). The stable isotope labelling of amino acids in cell culture (SILAC) approach is based on metabolic incorporation of stable amino acids, for instance lysine and arginine, into the proteome of cultured cells or living animals (Krüger et al., 2008; Ong et al., 2002). To enable relative quantification of proteins between two different conditions, completely SILAC-labelled samples (condition one) can be combined with a nonlabelled cell population (condition two) and then subjected to combined MS analysis. The intensity of the nonlabelled (light) and labelled (heavy) peptide peaks can be used to assess the relative abundance of individual proteins of interest in each condition.

In pulsed SILAC experiments, labelled amino acids are added to the cell culture media for a short period of time. Newly synthesized proteins incorporate the labelled amino acids, and the incorporation rate can be used to compare relative protein turnover between two different conditions. SILAC labelling of living animals can be achieved by administration of specific diets containing a SILAC amino acid, such as 13C6-lysine, named Lys6. Uptake of the labelled food leads to incorporation of the labelled amino acid into the proteome, which enables protein synthesis to be monitored over time in living animals. For instance, a pulsed SILAC approach demonstrated that several lysosomal degradation substrates had reduced Lys6 incorporation and increased protein levels in a heart-specific atrogin-1-deficient mouse model (Zaglia et al., 2014). Direct incorporation of labelled amino acids can be used to compare the relative rates of protein synthesis in living animals (Nolte et al., 2015). However, it should be noted that relative isotope abundance, which reflects the ratio of natural and labelled lysine isotopes in the body (Claydon et al., 2012), must be calculated to estimate absolute protein turnover rates (t½).

MATERIALS AND METHODS

Generation of SILAC mice

C57BL/6 mice (male, 9 weeks of age) were fed a 13C6-lysine (Lys6)-containing mouse diet (Silantes GmbH, München, Germany) to generate the labelled SILAC mouse colony, as previously described (Krüger et al., 2008). Administration of a diet containing Lys6 leads to a complete exchange of naturally occurring 12C6-lysine (Lys0) over one generation (Hölper et al., 2014). Isolated tissues and extracted proteins from SILAC mice were used as a heavy 'spike-in' standard for accurate quantification of the experimental conditions.

Muscle denervation and tissue lysis

Section of the left sciatic nerve was used to induce muscular atrophy (Lecker et al., 2004). After isolation of the control and denervated gastrocnemius (GAST) muscles, samples were snap frozen in liquid nitrogen. Frozen muscle tissue was ground to a fine powder using a mortar and pestle. Tissue powder from all experimental conditions was extracted with SDS lysis buffer (4% SDS in 100 mM Tris/HCl, pH 7.6). Lysates were homogenized, heated at 70°C for 10 min and clarified by centrifugation. Protein concentrations were determined using the Bio-Rad DC assay.

LysC protein digestion

For analysis of the proteome, 20 µg of nonlabelled and labelled protein extracts were mixed and loaded onto a 4-12% Bis-Tris gel (Invitrogen, Carlsbad, CA, USA), separated by SDS-PAGE and stained using the Colloidal Blue staining kit (Invitrogen). Each lane of the gel was cut into ten slices, and proteins were digested in-gel with LysC (Wako Pure Chemical Industries, Richmond, VA, USA) overnight at 37°C, as previously described (Shevchenko et al., 2006). Briefly, the gel pieces were destained in 50 mM ammonium bicarbonate and ethanol, reduced with 10 mM dithiothreitol (DTT) at 56°C for 45 min, carbamidomethylated with 55 mM iodoacetamide in the dark for 30 min at room temperature, and digested with 12 ng/µl LysC at 37°C overnight. The next day, digestion was stopped by acidification with trifluoracetic acid (TFA), and peptides were extracted from the gel pieces with increasing concentrations of acetonitrile (ACN). The organic solvent was vacuum evaporated using a SpeedVac concentrator plus (Eppendorf, Hamburg, Germany), and peptides were desalted using C18-based Stop and Go Extraction Tips (Rappsilber et al., 2007).

Trypsin digestion and peptide purification

For analysis of diglycine-containing peptides, 7.5 mg of the SILAC spike-in was mixed with equal protein amounts of nonlabelled control and denervated samples. After precipitation with acetone for 2 h at −20°C, samples were resuspended in urea buffer (6 M urea, 2 M thiourea in 10 mM HEPES, pH 7.6). Proteins were reduced with 5 mM DTT for 30 min at room temperature, carbamidomethylated with 10 mM iodoacetamide for 30 min in the dark at room temperature and subsequently digested with LysC at an enzyme:substrate ratio of 1:100 for 3 h at room temperature. Protein samples were diluted to 2 M urea using 50 mM ammonium bicarbonate and digested overnight at room temperature using sequencing-grade trypsin (Promega, Fitchburg, WI, USA) at an enzyme:substrate ratio of 1:100. After digestion, peptides were acidified with formic acid (FA) and subsequently desalted using a 500 mg C18 Sep-Pak SPE cartridge (Waters, Milford, MA, USA). C18 cartridges were preconditioned with 5 ml of ACN, followed by 5 ml of 50% ACN, 0.1% FA and 15 ml of 0.1% TFA. After acidification and clarification by centrifugation, samples were loaded onto the conditioned C18 cartridges and washed with 20 ml of 0.1% TFA. Bound peptides were eluted thrice with 2 ml of 50% ACN/0.1% FA and concentrated on a SpeedVac concentrator plus (Eppendorf) to ~100 µl.

Peptide fractionation by high pH RPLC

Off-line high pH RPLC was performed using an XBridge BEH300 C18 3.5 µm column on an ÄKTA Purifier (GE Healthcare Life Sciences, Little Chalfont, UK). Fractionation was performed as described previously (Udeshi et al., 2012). Specifically, concentrated samples were resuspended in RPLC buffer A (5 mM ammonium formate in 2% ACN, pH 10) and injected at a flow rate of 0.25 ml/min. Peptides were fractionated using a 64 min gradient that started by increasing the concentration of RPLC buffer B (5 mM ammonium formate in 90%

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ACN, pH 10) to 8% at a rate of 1.1% B/min, followed by a 38 min linear gradient from 8 to 27% B. The gradient was then ramped successively to 31% B at 1% B/min, 39% B at 0.5% B/min and 60% B at 3% B/min. During the entire fractionation, the flow rate was 0.25 ml/min. In all, 75-80 fractions were collected, and pooled in a non-contiguous manner to obtain ten fractions that were concentrated to ~100 µl on a SpeedVac concentrator.

Enrichment of ubiquitinated (K-ε-GG) peptides

The anti-K-ε-GG antibody was obtained from the PTMScan® ubiquitin remnant motif kit (Cell Signaling Technology, kit #5562). The antibody was cross-linked before use, as described previously (Udeshi et al., 2012). Antibody beads were washed twice with 1 ml of 100 mM sodium borate (pH 9.0) at 4°C and then cross-linked using 1 ml of 20 mM dimethyl pimelimidate (DMP) on a rotating wheel for 30 min at room temperature. Cross-linked beads were washed twice with 1 ml of 200 mM ethanolamine (pH 8.0) at 4°C, then cross-linking was blocked with 1 ml of 200 mM ethanolamine on a rotating wheel for 2 h at 4°C. Subsequently, the antibody beads were washed twice with 1 ml IAP Buffer (50 mM MOPS, pH 7.2, 10 mM sodium phosphate, 50 mM NaCl) at 4°C and resuspended in IAP buffer. Concentrated peptide fractions obtained from RPLC were resuspended in 1 ml IAP buffer and incubated with cross-linked antibody beads (divided equally for ten fractions) for 2 h on a rotating wheel at 4°C. After immunoprecipitation, beads were washed twice with 1 ml ice-cold PBS and 1 ml ice-cold water. Bound K-ε-GG peptides were eluted twice with 100 µl of 0.15% TFA and purified using C18-based Stop and Go Extraction Tips.

Lys6 incorporation

Mice were denervated as described above and immediately switched to a mouse diet containing 13C6-lysine (3 g/day) until they were sacrificed at specific time points for extraction of the GAST muscles from both control and denervated legs. Tissue lysis and in-gel protein digestion with LysC were performed as described above.

LC-MS/MS analysis

RPLC of peptides and MS/MS analysis were performed using an Easy nLC 1000 UHPLC coupled to a QExactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Peptides were resuspended in Solvent A (0.1% FA), picked up with an autosampler and loaded onto in-house made 50 cm fused silica emitters (75 µm diameter) packed with 1.9 µm C18-AQ Reprosil Pur beads (Dr Maisch GmbH, Ammerbuch, Germany). Samples were loaded at a flow rate of 750 nl/min. A 150 min segmented gradient of 10-38% Solvent B (80% ACN in 0.1% FA) over 120 min and 38-60% Solvent B over 7 min at a flow rate of 250 nl/min was used to elute peptides. Eluted peptides were sprayed into the heated transfer capillary of the mass spectrometer using a nano-electrospray ion source (Thermo Fisher Scientific). The mass spectrometer was operated in a data-dependent mode, where the Orbitrap acquired full MS scans (300-1750 m/z) at a resolution (R) of 70,000 with an automated gain control (AGC) target of 3×106 ions collected within 20 ms. The dynamic exclusion time was set to 20 s. From the full MS scan, the ten most intense peaks ($z \geq 2$) were fragmented in the high-energy collision-induced dissociation (HCD) cell. The HCD normalized collision energy was set to 25%. MS/MS scans with an ion target of 5×105 ions were acquired with a resolution R=35,000, with a maximal fill time of 120 ms, isolation width of 1.8 m/z, capillary temperature of 280°C, and spray voltage of 1.8 kV.

MS data processing

MS raw data were analysed using MaxQuant software version 1.4.1.2 (Cox and Mann, 2008). Peptides were searched using the Andromeda search engine (Cox et al., 2011) using the mouse UniProt database containing 73,921 entries. Multiplicity was set to 2, and Lys6 was set as the labelled amino acid to quantify SILAC peptide pairs. LysC was chosen as the digestion enzyme for protein identification and trypsin for ubiquitinated peptide identification, both with allowance of cleavage N-terminal to proline. The maximal number of missed cleavages allowed was two. Cysteine carbamidomethylation was set as a fixed modification; and methionine oxidation, N-terminal protein acetylation and Gly-Gly addition to lysines as variable modifications. The maximal mass tolerance was 20 ppm, initial precursor ion mass deviation, 7 ppm and MS/MS tolerance, 0.5 Da. An FDR of 1% and minimal peptide length of seven amino acids were applied for peptide identifications. For quantification of SILAC peptides, a minimal ratio count of two was applied. Ubiquitinated

site identification and quantitative information were obtained from the MaxQuant GlyGly(K) sites table. Statistical data analysis and t-tests were performed using Perseus (version 1.3.8.3).

Data analysis

Soft clustering of z-score normalized log2 fold-change of proteomic data was performed using the R-package Mfuzz (Kumar and Futschik, 2007). The parameter c (number of clusters) was set to six, and the parameter m (fuzzifier) was estimated (based on the data) to be 2.54. The AUC of the incorporation-time profile was calculated using the R-package MESS. To analyse proteome and ubiquitination data, a two-sided t-test was performed using a permutation-based FDR of 0.05 (fudge factor S0=0.1) in Perseus. Statistical analysis of incorporation data was performed using one-sample tests in Perseus. Multiple testing correction was performed using the R-package qvalue, calculating q-values at an FDR <0.05.

Random forest analysis

A random forest approach using the comprehensive caret (classification and regression training) R package (Kuhn et al., 2008) was used to identify potential atrogenes in the proteomics data set. First, we performed a literature search and screened transcriptomics data for known atrogenes, and classified the corresponding proteins into the following two classes: Class 1, upregulated upon denervation; and Class 2, downregulated following denervation. Then, we generated a negative data set (Class 3) by shuffling the complete proteomics data set (time course experiment after denervation). We tuned the trained random forest by maximizing Cohen's kappa (statistic) separately for Class 1 and Class 2 against Class 3. Tuning of the random forest was performed on the parameter mtry (e.g. randomly selected features at each split). Given that the negative data set could introduce bias by chance, we trained a total of 50 random forest for each class, and the probabilities for Class 1 and 2 were averaged, respectively. For visualization, we plotted the probability for Class 1 versus the Delta Score (Class 1−Class 2).

Western blotting and antibodies

Equal amounts of protein from each muscle lysate (~60 µg) were separated using the TGX stain-free sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) system from Bio-Rad (Hercules, CA, USA). Proteins were transferred onto a polyvinylidene difluoride membrane using the Bio-Rad TransBlot Turbo system and detected with the following commercial antibodies: rabbit polyclonal anti-Akt (Cell Signaling, #9272, 1:1000), rabbit monoclonal USP14 (Cell Signaling, #11931, 1:1000), mouse monoclonal anti-α-tubulin (Sigma Aldrich, T9026, 1:500) and rabbit monoclonal anti-TRIM25 (Abcam, ab167154, 1:000). An anti-dystrophin antibody (Dys1, Novocastra, NCL-DYS1) was used as a loading control. Proteins were visualized using a chemiluminescence system (Amersham ECL Prime) from GE Healthcare.

RESULTS

Unilateral section of the sciatic nerve was performed in wild-type C56BL/6J mice to induce muscle atrophy. The GAST was analysed at several time points over the first 14 days after denervation. The workflow of the MS analysis is indicated. At 7 days after sciatic nerve section, a significant loss (~20% reduction; P=0.02) in muscle weight was observed compared with control muscles, similar to earlier reports (Sacheck et al., 2007). To investigate changes in protein expression during atrophy, we isolated the GAST after 1, 4, 7 and 14 days and performed relative protein quantification between control and denervated muscles.

To enable accurate protein quantification, GAST muscles were isolated from Lys6-labelled SILAC mice. These animals were completely labelled with Lys6 over at least two generations and functioned as a spike-in standard to quantify nonlabelled peptides from control and denervated GAST muscles (Hölper et al., 2014). Each protein sample was mixed in a 1:1 ratio (based on total protein concentrations) with the SILAC GAST and subjected to in-gel digestion and LC-MS (liquid chromatography-mass spectrometry) analysis. Here, we identified 45,644 unique peptides representing 4279 identified protein groups at a false-discovery rate (FDR) <1% at the peptide and protein level. Clustering analysis using the Pearson coefficient (r) and determining the

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Euclidean distances for different time points confirmed high reproducibility of our protein quantification between biological replicates (n=3. Likewise, principal component analysis (PCA) clearly confirmed the separation of protein intensities for control and denervated samples at different time points.

To identify whether groups of proteins that participate in the same pathways are regulated in a similar manner during atrophy, we performed one-dimensional enrichment of the log2 ratios of control and denervated muscles on day 4 after section. Boxplots of enriched gene ontology (GO) terms revealed that groups associated with 'fatty acid catabolic process' (GO: 0006631) and 'myosin complex' (GO: 0016459) were significantly downregulated at this early time point after denervation. Conversely, we observed that 32 proteins associated with the GO term 'spliceosomal complex' (GO: 0005681) and 14 proteins related to the 'proteasome accessory complex' (GO: 0022624) were upregulated at 4 days after denervation. Next, volcano plots were generated using two-sided t-tests to follow time-dependent protein changes in response to denervation. Although no significant changes in protein expression were observed on day 1 after denervation, 174 and 583 proteins were significantly regulated in a different manner at 4 and 7 days after denervation, respectively, at a permutation-based FDR estimate of 0.05 (Fig. 1F,G). Over the longer term (14 days), denervation resulted in significantly differential expression of 863 proteins (FDR<0.05), indicating marked remodelling of the denervated GAST.

The GAST contains a mixture of type I and II fibres that express distinct sets of 'slow' and 'fast' myosin heavy chain (MyHC) proteins. A label-free protein quantification method based on unique MyHC peptides reflected the MyHC distributions reported in previous studies (Mänttäri and Järvilehto, 2005; Schiaffino and Reggiani, 2011). Next, we used a published list of proteins expressed specifically in the soleus and extensor digitorum longus (EDL) muscles to identify slow and fast fibre-specific proteins (Drexler et al., 2012), as shown. Comparison of this catalogue with our protein expression data set revealed that 27% of fast fibre proteins were significantly downregulated after denervation of the GAST, whereas most proteins expressed in the slow soleus muscle were significantly upregulated on day 7. This indicates that the slow type I fibres within the GAST are more resistant to denervation than type IIb fibres.

Sciatic nerve section is known to activate proteolytic pathways. Accordingly, we observed that 367 of the 2233 proteins quantified were significantly downregulated at 7 days after denervation. For example, the protein levels of neurofilament chains (NEFM, NEFL) and myelin proteins, such as myelin protein zero (MPZ) and the myelin basic protein (MBP), were significantly downregulated, indicating loss of neuronal innervation. By contrast, neuronal cellular adhesion molecule (NCAM) was ~5-fold upregulated (P<10−4) at 7 days after denervation. Enhanced NCAM expression is associated with neuromuscular diseases and might be required to recruit axons to neuromuscular junctions (Cashman et al., 1987).

The histone deacetylase HDAC4 positively regulates genes associated with synaptogenesis and suppresses glycolytic enzymes after denervation (Tang et al., 2009). Here, we detected increased HDAC4 protein expression in denervated muscles, with no corresponding peptides detected in control muscles.

DISCUSSIONS

The trophic effects of neuronal innervation have been studied using a plethora of morphological, physiological and biochemical approaches (Gutmann, 1962). To extend our knowledge of alterations at the protein level, we determined the quantitative and temporal changes in protein expression, Lys6 incorporation and diglycine signatures during denervation-induced muscle atrophy in the mouse GAST. This analysis reveals the activation of several pathways and extends the catalogue of proteins that might contribute to the remodelling of the skeletal muscle proteome during denervation-induced muscle atrophy.

Previous transcriptional profiling studies revealed that a specific programme of genes is activated during muscular atrophy, with a set of ~50 atrogenes commonly associated with catabolic states, including muscle denervation (Sacheck et al., 2007). Comparison of these genes with our data sets enabled us to generate a training set of 27 candidates by selecting known atrogenes that were congruently up- or downregulated in our data sets. Systematic random forest analysis revealed similar

'atrogene-like' protein expression profiles for 143 upregulated proteins after denervation. Furthermore, upregulation of several proteins that participate in the UPS or autophagy, or function as cathepsin proteases after denervation was confirmed. In addition, we identified that a broad range of proteins, which have not previously been described to be involved in muscular atrophy, were differentially regulated after denervation. Hence, our protein expression data set provided a starting point from which we aimed to identify the individual proteins and networks that are potentially involved in early remodelling processes after denervation. However, it is necessary to investigate whether the known atrogenes and candidate proteins identified in this study are also differentially regulated in other muscle-wasting conditions.

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