



Proteomic analysis of muscle affected by motor neuron degeneration: The wobbler mouse model of amyotrophic lateral sclerosis

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ABSTRACT

Amyotrophic lateral sclerosis is the most common form of motor neuron disease in adult patients and characterized by progressive paralysis. The wobbler mouse (phenotype WR, genotype *wr/wr*) is an established animal model of human motor neuron disease and is characterized by a large variety of cellular abnormalities including muscular atrophy. In analogy to recent proteomic studies of cerebrospinal fluid and spinal cord, we have used here fluorescence difference in-gel electrophoresis to analyze global changes in the skeletal muscle proteome from WR versus normal mice. Relative concentrations of 21 proteins were found to be increased and 3 proteins were decreased. Mass spectrometric analysis identified these proteins to be associated with key metabolic pathways, the contractile apparatus, intermediate filaments and the cellular stress response. Drastically increased levels of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase were confirmed by immunoblotting and this finding agrees with the idea of an oxidative-to-glycolytic shift in disease-related muscular atrophy. The establishment of novel disease-specific biomarkers of motor neuron disease might be helpful in the design of improved diagnostic tools and the identification of novel therapeutic targets.

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1. Introduction

Motor neuron diseases are a heterogeneous group of fatal neurological diseases [1]. Disorders with lower motor neuron degeneration are spinal muscular atrophy, an autosomal recessive disorder that is caused by mutations in the survival motor neuron SMN1 gene, spinal bulbar muscular atrophy, a slowly progressive adult-onset disorder triggered by the expansion of a polyglutamine tract in the androgen receptor gene and Charcot-Marie-Tooth disease, a diverse group of inherited neuropathies with mutations that cause defects in various neuronal proteins [2]. Amyotrophic lateral sclerosis (ALS), which can be sporadic or be of genetic origin, is the most progressive and most frequent form of adult-onset motor neuron disease [3]. Genetic abnormalities in ALS have been shown to occur in the CuZn-superoxide dismutase encoding *SOD1* gene and the TDP-43 encoding *TARDBP* gene [3]. The degeneration of motor neurons leads to contractile weakness of the limb, bulbar and respiratory muscle systems. Paralysis is highly progressive and is usually fatal due to respiratory failure within 2–3 years for bulbar-onset pathologies and within 3–5 years for limb-onset cases [4]. Currently only one drug, the glutamate antagonist riluzole, has regula-

tory approval for the treatment of ALS, but prolongs the survival time in the treatment of adult-onset motor neuron degeneration by only 3–4 months [5]. Since patients afflicted with ALS have a very poor prognosis, detailed biomedical investigations into the molecular pathogenesis of this fatal neurodegenerative disease and an intensive search for novel therapeutic targets to treat aggressive disorders of the neuromuscular system are warranted.

In this respect, animal models that mimic key symptoms of neurodegeneration and the progressive nature of ALS may be helpful for studying the underlying mechanisms of muscle paralysis [6]. The wobbler mouse (genotype *wr/wr*, phenotype WR) [7] has been used during the last decades as an animal model of motor neuron disease. The recessive mutation *wr* has been mapped to mouse chromosome 11 [8] and was identified as a missense mutation in the ubiquitously expressed gene *Vps54* [9]. The corresponding protein, vesicular protein sorting factor VPS54, is a component of the hetero-trimeric GARP complex involved in retrograde vesicle transport to the Golgi apparatus, and thus plays a crucial part in intracellular transport. The *wr* allele causes a defect in spermiogenesis [10] in addition to motoneuron degeneration. Knock out of the *Vps54* gene leads to embryonic lethality [9]. Recently, it was found that the *wr* missense mutation destabilizes the VPS54 protein and thereby leads to a reduction of its concentration [11]. Although there is no human neurodegenerative disease known in which the gene for VPS54 (chromosome 2p14-15) is affected [12], the WR mouse with its rapid disease progression nevertheless represents a highly

Abbreviations: 2D-GE, two-dimensional gel electrophoresis; ALS, amyotrophic lateral sclerosis; DIGE, difference in-gel electrophoresis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MS, mass spectrometry; MyHC, myosin heavy chain.

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valuable model for many aspects of neurodegeneration, including neuroinflammatory processes [13]. The WR mouse is also widely employed for evaluating novel treatment options to counteract symptoms of ALS [14].

Over the last few years, mass spectrometry-based proteomics has been instrumental to perform global surveys of changed protein expression patterns in normal and pathological skeletal muscle tissues [15]. The identification of novel disease-specific biomarker signatures of neuromuscular diseases might be helpful in the design of improved diagnostic tools and the establishment of new therapeutic targets, as well as the superior monitoring of disease progression and determination of adverse effects of experimental drug treatments. The current international effort to discover reliable biomarkers of ALS [16] includes the verification of both suitable metabolites [17] and protein factors [18]. Recent proteomic profiling studies of ALS patient samples and the WR animal model have focused on the analysis of cerebrospinal fluid [19–21] and cervical and lumbar spinal cord [22,23], respectively. The studies on the WR mouse showed that the majority of differentially expressed proteins were related to the glutamate–glutamine cycle, energy transduction, redox functions and astrogliosis [22], thus confirming previous studies [24]. Here, we have applied comparative proteomics to the analysis of skeletal muscle from the WR mouse and established changes in proteins involved in metabolic pathways, the contractile apparatus, intermediate filaments and the cellular stress response.

2. Materials and methods

2.1. Chemicals and materials

CyDye DIGE Fluor minimal dyes Cy3 and Cy5, Coomassie Blue, ampholytes, cover fluid, acetonitrile and immobilised pH gradient (IPG) drystrips pH 3–10 were obtained from Amersham Biosciences/GE Healthcare (Little Chalfont, Buckinghamshire, UK). Sequencing grade-modified trypsin was obtained from Promega (Madison, WI). LC–MS Chromasolv water and formic acid were from Fluka (Milwaukee, WI). Spin filters were obtained from Fisher Scientific (U.K). Protein molecular weight ladders, Laemmli-type buffer and Bradford reagent for protein quantification were purchased from Biorad Laboratories (Hemel-Hempstead, Hertfordshire, UK). Protease inhibitors were from Roche Diagnostics (Mannheim, Germany). Acrylamide stock solutions were obtained as ultrapure Protogel from National Diagnostics (Atlanta, GA). Ultrapure lysine for quenching the DIGE labeling reaction and all general reagents were obtained from Sigma Chemical Company (Dorset, UK).

2.2. Preparation of muscle extracts from WR and control mice

The WR mouse represents an established animal model of motor neuron disease [7]. The origin of the WR stock and breeding of mice at the University of Bielefeld has been previously described [8,9]. Animals were sacrificed by cervical dislocation and biochemical analyses were performed with post-mortem tissue samples. WR mutants were bred in accordance with German law on the protection of laboratory animals and approved by the local authorities. *Tibialis anterior*, *gastrocnemius*, *soleus* and *vastus lateralis* muscles from 9-week old WR mice and their counter-parts from age-matched normal C57 mice were freshly dissected and then quick-frozen in liquid nitrogen. WR and control mice had an average body weight of 9.6 ± 1.6 g ($n = 9$) and 23.2 ± 3.0 g ($n = 5$), respectively. The combined wet weights of the analyzed muscles from both legs of the WR and normal mice were 124 ± 44 mg ($n = 9$) and 314 ± 71 mg ($n = 5$), respectively. Samples were transported on dry ice and stored at -80 °C prior to usage. For the prep-

aration of muscle extracts, tissue specimens were ground, in the presence of liquid nitrogen, into a fine powder using pestle and mortar. For comparative proteomic studies, equal amounts of WR and normal muscle tissue (100 mg wet weight) were used. Muscle powder was suspended in 1 ml of DIGE lysis buffer and urea-soluble proteins extracted as previously described in detail [25].

2.3. Fluorescence DIGE analysis of muscle extracts

A 50 mg protein sample was fluorescently labeled for each of the protein samples being studied. Normal muscle extracts and WR muscle extracts were each labeled with Cy3 DIGE Fluor dye and the pooled internal standard with Cy5 DIGE Fluor dye, as previously described in detail [25]. The 2D-GE separation of fluorescently labeled proteins was carried out by using a total of 100 mg protein per DIGE gel. Isoelectric focusing was performed using 24 cm strips in a IPGphor system from Amersham Biosciences/GE Healthcare (Little Chalfont, Buckinghamshire, UK) using running conditions as previously described [26]. Following first-dimension separation, gel strips were equilibrated for 20 min in equilibration buffer containing 100 mM dithiothreitol, followed by 10 min of equilibration in buffer containing 250 mM imidazole. Using the Amersham EttanDalt–twelve system, the gel electrophoretic separation of muscle-associated proteins in the second dimension was performed with standard 12.5% (w/v) slab gels [25]. Twelve slab gels were run in parallel at 0.5 W/gel for 60 min and then 15 W/gel until the blue dye front had disappeared from the bottom of the gel. Fluorescently labeled proteins were visualized using a Typhoon Trio variable mode imager. Protein expression changes between WR and control samples were analyzed using Progenesis SameSpots analysis software from Non Linear Dynamics (Newcastle upon Tyne, UK). Prior to analysis, individual gels were warped to a single master gel. SameSpots analysis was used to identify proteins of interest using the following parameters: $n = 4$; t -test $p < 0.05$; and a power value of >0.8 . Proteins with a significantly changed abundance were picked for tryptic digestion from Coomassie Blue-stained preparative gels [25].

2.4. Mass spectrometric identification of muscle proteins and data verification

The mass spectrometric identification of proteins of interest was performed with 2-D protein spots from Coomassie-stained pick gels, matched to a DIGE-labeled master gel. Standardized in-gel tryptic digestion was used for the generation of peptides and mass spectrometric analysis was carried out on a Model 6430 Ion Trap LC/MS apparatus from Agilent Technologies (Santa Clara, CA). Excision, washing, destaining and treatment with trypsin were performed by previously optimized methods [25]. Peptide samples were dried through vacuum centrifugation and then resuspended in mass spectrometry-grade distilled water and 0.1% (v/v) formic acid, spun down through spin filter and added to LC–MS vials for identification by ion trap LC–MS analysis [25]. Separation was performed with a nanoflow Agilent 1200 series system. Samples were loaded into the enrichment at a capillary flow rate set to 2 μ l/min with a mix of 0.1% (v/v) formic acid and 50% (v/v) acetonitrile and formic acid at a ratio of 19:1. The voltage was set to 1700 V. Database searches were carried out with Mascot MS/MS Ion search (Matrix Science, London, UK). For data verification, immunoblotting was carried out as previously described in detail [26].

3. Results and discussion

The underlying objective of our proteomic profiling study of the WR mouse was the establishment of global effects of motor neuron

disease on the protein complement from skeletal muscle tissue. Since conventional protein staining procedures have a limited dynamic range, we employed fluorescence DIGE analysis for the detailed characterization of global changes in the urea-soluble WR muscle proteome. The most prominent changes in gene expression in muscles affected by motor neuron degeneration are expected to reflect physiological denervation. In fact, an increase of the mRNA for the α -subunit of the nicotinic acetylcholine receptor, a typical sign of denervation, has been found in WR muscle [27]. In contrast to myotonic mouse muscle, in which there is a shift from the dominant myosin heavy chain (MyHC) isoform IIB (fast-glycolytic) to IIX and IIA (fast-oxidative), in WR muscle there is an opposite shift to an even higher proportion of MyHC IIB and a glycolytic phenotype [28]. In addition, effects related to muscular atrophy are expected. Importantly, since the WR pathology is a long-term and continuous process, as opposed to surgical denervation, intrinsic effects of the partial deficiency in VPS54 may cause proteome-wide alterations in skeletal muscle.

Following differential fluorescent tagging, gel electrophoretic separation, densitometric scanning and image analysis, 24 protein spots were identified in WR muscle tissue that exhibit a significant change in concentration levels. An overview of our DIGE analysis is given in Fig. 1, which illustrates the Cy3 (WR or wild type sample) and Cy5 (pooled standard) fluor labeling approach. A representative master gel is shown for the pH 3–10 range in Fig. 2. WR-associated changes in the concentration of proteins included two-dimensional spots that covered a *pI*-range from approximately 4.1 (Troponin C) to 8.6 (GAPDH) and ranged in molecular mass from apparent 16 kDa (Cu/Zn superoxide dismutase) to 128 kDa

(Myosin binding protein C). The findings from the mass spectrometric identification of the 24 protein spots with a changed abundance is presented in Table 1, listing protein name, accession number, *pI*-value, molecular mass, number of matched peptide sequences, Mascot score, percentage sequence coverage, Anova score and relative concentrations of individual proteins affected in the WR mouse.

Identified proteins are associated with key metabolic pathways, the contractile apparatus, intermediate filaments and the cellular stress response. Expression levels were reduced in the case of 3 protein species and increased for 21 proteins. The mass spectrometric hit with the highest increase of 6.5 relative concentration is not listed in Table 1, since this protein was only recognized by 1 peptide (MPSSMGGGGGGSPVELR) as histone deacetylase 5 (gi|220941642], *pI* 9.07, MW 20,096, Mascot score 28, 10% coverage, 0.001 Anova). The second highest relative concentration was found for the glycolytic enzyme aldolase (spot 1, Table 1), followed by GAPDH (spot 2). These two enzymes with increased concentrations, in accordance with the previously reported increase in the MyHC isoform IIB [28] reflect a shift towards an extreme glycolytic muscle fiber type. Other increased proteins are represented by actin (spots 4, 9, 16, 19), desmin (spots 5, 7, 11), myoglobin (spots 6, 12), myozenin (spot 8), troponin C (spot 14), actinin (spot 15), Cu/Zn superoxide dismutase (spot 20) and fast myosin light chain 1f (spot 21). Subtypes of creatine kinase M-type, possibly representing differently phosphorylated isoforms, showed differential changes in a variety of protein spots. Five spots exhibited an increased abundance (spots 3, 10, 13, 17, 18) and one spot a decreased concentration (spot 22). A decrease was shown for 2

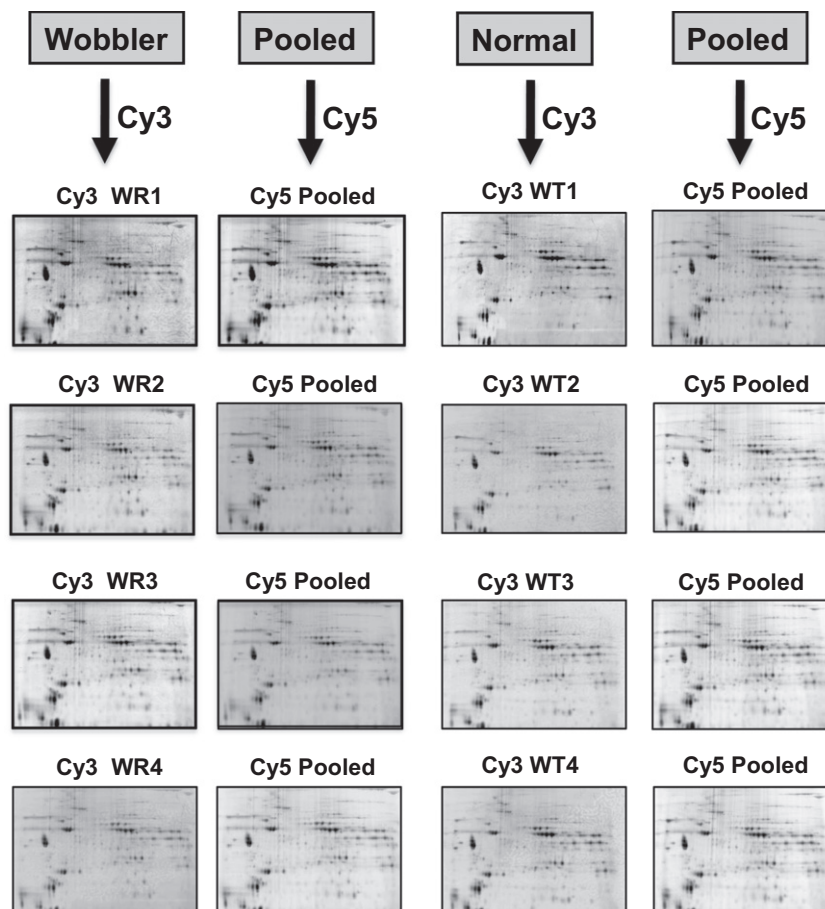


Fig. 1. Two-dimensional DIGE analysis of wobbler versus normal skeletal muscle. Shown are Cy3-labeled gels of total muscle extracts from wobbler (WR1 to WR4) versus normal wild type (WT1 to WT4) mice, as well as Cy5-labeled gels containing pooled standards. DIGE images are shown for the pH 3–10 range.

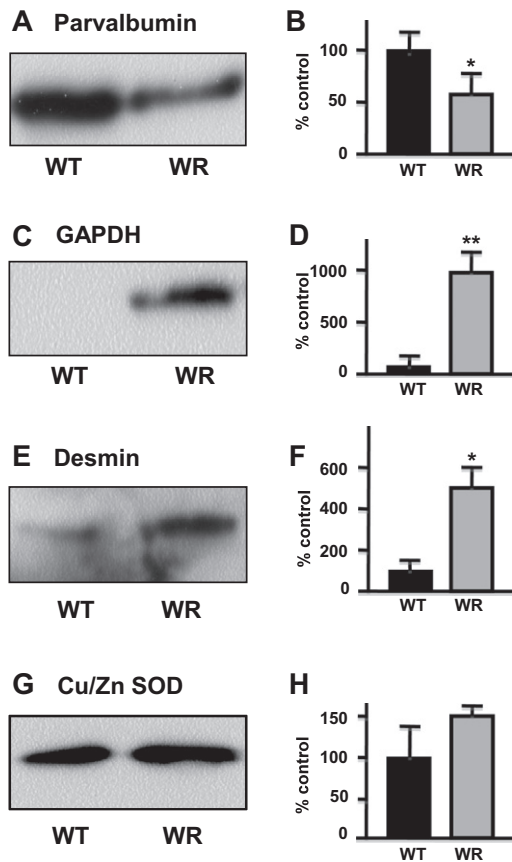


Fig. 3. Immunoblot analysis of wobbler mouse muscle. Shown are representative immunoblots with expanded views of immuno-decorated bands labeled with antibodies to parvalbumin (A and B), GAPDH (C and D), desmin (E and F) and Cu/Zn superoxide dismutase (SOD) (G and H). Lanes 1 and 2 represent normal wild type (WT) versus WR preparations, respectively. The comparative immunoblot analysis was statistically evaluated using an unpaired Student's *t*-test ($n = 5$; * $p < 0.05$; ** $p < 0.01$).

myozenin, myosin light chain and myosin binding protein suggest atrophy-related modulations of the contractile apparatus and the increase in desmin possibly a compensatory stabilization of the cytoskeletal network. The drastic increase in almost all subtypes of creatine kinase imply crucial alterations in creatine phosphate metabolism that might be related to metabolic shifts in WR muscle tissue. Disease-induced atrophy appears to trigger similar slow-to-fast transition processes as previously described for disuse atrophy [15]. Diminished neuromuscular activity or the lack of proper loading has distinct effects on the integrity of the skeletal musculature. Immobilization, disuse or denervation triggers muscular atrophy, which clearly shows that neuromuscular innervation patterns are directly linked to gene expression, protein abundance and isoform patterns in skeletal muscles [29].

In analogy to the recent proteomic profiling of cerebrospinal fluids from patients suffering from ALS and the spinal cord from the wobbler mouse [19–23], this study describes the findings of the MS-based proteomic survey of skeletal muscle from the WR mouse. From the detailed analysis of the isoform patterns of the sarcomeric MyHCs it became clear that the response of gene expression in skeletal muscle to denervation is characteristically different from that to hyper-excitability and muscular dystrophy [28]. Thus, studying changes in the WR muscle proteome has the potential to contribute crucial information on disease process during denervation-induced muscular atrophy. Muscular atrophy is associated with a decreased fiber size, a drastic loss in muscle tis-

sue mass and a decrease in isometric contractile force. In general, muscular atrophy is observed in skeletal muscles under conditions of microgravity and following extended periods of bed rest, traumatic injury to motor units or pathological damage to motor neurons [29]. Insufficient loading, denervation or disuse is usually associated with slow-to-fast muscle transitions, which has been confirmed by recent MS-based proteomic studies [30–35]. The proteomic profiling of muscular atrophy has revealed an increase in key enzymes of the glycolytic pathway and a concomitant decrease in slow contractile proteins, such as the cardiac isoform of myosin heavy chain, slow troponin T and slow myosin light chain [30–35]. In analogy, the proteomic analysis of the WR mouse, which represents an animal model of disease-induced muscular atrophy, showed an increase in the abundance of key glycolytic enzymes. Thus, the proteomic analysis presented here supports the general idea of disuse- and atrophy-associated oxidative-to-glycolytic transformation processes. In the long-term, the newly identified global changes in the soluble skeletal muscle proteome may be useful candidates for the establishment of a biomarker signature of motor neuron disease [18].

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