Journal of Neuroimmunology 261 (2013) 141–145

Contents lists available at SciVerse ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Short communication

Proteomic analysis of rat tibialis anterior muscles at different stages of experimental autoimmune myasthenia gravis

Alejandro M. Gomez [a,1], Annelies Vanheel [b,1], Mario Losen [a], Peter C. Molenaar [a], Marc H. De Baets [a,b], Jean-Paul Noben [b], Niels Hellings [b], Pilar Martinez-Martinez [a,*]

[a] Division Neuroscience, School for Mental Health and Neuroscience, Maastricht University, 6200 MD Maastricht, the Netherlands
[b] Biomedical Research Institute, Hasselt University and Transnationale Universiteit Limburg, School of Life Sciences, 3500 Hasselt, Belgium

ARTICLE INFO

Article history:
Received 18 April 2013
Accepted 10 May 2013

Keywords:
Myasthenia gravis
Muscle proteomics
2D-DIGE
Autoimmunity

ABSTRACT

Myasthenia gravis (MG) is an autoimmune disease in which autoantibodies, most commonly directed against the acetylcholine receptor (AChR), impair neuromuscular transmission and cause muscle weakness. In this study, we utilized two-dimensional difference in-gel electrophoresis (2D-DIGE) to analyze the muscle's proteomic profile at different stages of experimental autoimmune myasthenia gravis (EAMG). We identified twenty-two differentially expressed proteins, mainly related to metabolic and stress-response pathways. Interestingly, these identified proteins have also been associated with other contraction-impairing muscle pathologies (e.g. inclusion body myositis), suggesting a similar response of the muscle to such conditions.

1. Introduction

Myasthenia gravis (MG) is one of the best characterized antibody-mediated autoimmune diseases, and its symptoms include muscle weakness and fatigability. The autoantibodies in MG are primarily directed to proteins of the neuromuscular junction (NMJ), such as the acetylcholine receptor (AChR-MG, 85% cases), muscle-specific kinase (MuSK-MG, 10% cases) and low-density lipoprotein receptor-related protein 4 (Lrp4-MG, ~2% cases) (Richman, 2012; Silvestri and Wolfe, 2012). In AChR-MG, autoantibodies cause extensive damage at the NMJ, mainly by activation of the complement system and by antigenic modulation of the AChR (Gomez et al., 2010). This autoimmune attack leads to a reduction of AChRs, and other AChR-related proteins (Conti-Fine et al., 2006; Martinez-Martinez et al., 2007), which further contributes to the severity of symptoms. Consequently, such AChR-related proteins could represent novel targets for symptomatic treatments. However, the precise number and identity of muscle proteins affected by the autoimmune attack in MG remains largely unknown.

In recent years, the advent of the two-dimensional difference in-gel electrophoresis (2D-DIGE) technology has allowed a more sensitive and accurate quantification of differential protein expression and/or protein modifications affecting size and charge in biological samples (Lilley and Friedman, 2004). In this study, we used the experimental autoimmune myasthenia gravis (EAMG) rat model and 2D-DIGE to evaluate the muscle's proteomic profile at different disease stages of EAMG.

2. Material and methods

2.1. Animals, induction of EAMG and tissue preparation

Seven-week-old female Lewis rats were obtained from Charles River laboratories (Cologne, Germany). Chronic EAMG was induced by immunization with AChRs purified from *T. californica,* the severity of EAMG symptoms was assessed three times a week with the paw-grip test for muscle weakness as described (Gomez et al., 2011). Animals were sacrificed between 5 and 8 weeks after immunization according to their disease score, to have a representative number of muscles from each score (0, no weakness; 0/1, mild weakness after testing; 1, weakness after testing; 2, clinical signs of EAMG present before testing; 3, severe clinical signs of EAMG, moribund). Animals were perfused to obtain blood-free tibialis anterior muscle [5 mM EDTA in PBS pH 7.2, containing Complete Protease Inhibitor Cocktail (Roche, Almere, the Netherlands)]. Muscles were processed to remove their connective membranes and tendons, frozen in liquid nitrogen and subsequently stored at −80 °C. All experiments were done with permission from the Committee on Animal Welfare of Maastricht University, according to Dutch governmental rules.

* Corresponding author at: Division Neuroscience, School for Mental Health and Neuroscience, Maastricht University, Universiteitssingel 40, 6229 ER, Maastricht, the Netherlands. Tel.: +31 43 3881042; fax: +31 43 3671096.
E-mail address: p.martinez@maastrichtuniversity.nl (P. Martinez-Martinez).
[1] These authors contributed equally to this work.

0165-5728/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jneuroim.2013.05.008

2.2. Protein extraction

Proteins were extracted as described (Sizova et al., 2007). Briefly, muscle samples were lyophilized, crushed (GentleMACS, Miltenyi Biotec, Leiden, the Netherlands) and proteins solubilized before ultracentrifugation. Samples were then desalted and the buffer was exchanged to labeling buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS in 30 mM Tris HCl pH 8.5) using Amicon Ultra filters (Millipore, 3 kDa cut-off). Protein concentration was determined using the 2D Quant kit (GE Healthcare, Diegem, Belgium) and aliquots were stored at −80 °C. For Western blotting experiments, additional muscles from animals described in Gomez et al. (2011) were homogenized with a Mini BeadBeater (Biospec Products, Bartlesville, OK) in lysis buffer (30 mM triethanolamine, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 1 mM Na-orthovanadate, 1 mM benzamidine, 1 mM Na-tetrathionate, 1 mM PMSF, pH 9.5) containing HALT Protease Inhibitor Cocktail (Thermo Scientific, Rockford, IL) and Triton X-100 (1%).

2.3. 2D-DIGE

Minimal labeling with N-hydroxysuccinimidyl-ester dyes Cy2, Cy3 and Cy5 (GE Healthcare, Diegem, Belgium) and 2D-GE were performed as described in Vanheel et al. (2012). CyDye-labeled 2D-DIGE gels were scanned on the Ettan DIGE imager (GE Healthcare). Gel images from all three CyDyes were loaded into DeCyder 7.0 software (GE Healthcare) and analyzed. Statistical significance was calculated using analysis of variance (ANOVA) and multiple comparison test. Spots present in 85% of the gel images, and with $p \leq 0.05$, were considered for further analysis.

2.4. Spotpicking, protein digestion and identification

For spot picking (ProPicII, Isogen Life Science, PW De Meern, the Netherlands) a 12.5% acrylamide gel was loaded with 200 μg of an unlabeled internal standard and 50 μg of the Cy2 labeled internal standard. In-gel digestion using trypsin (Promega, Leiden, the Netherlands; Shevchenko et al., 1996) was performed followed by protein identification by mass spectrometry (Vanheel et al., 2012).

2.5. Western blotting

To validate the results obtained by 2D-DIGE, we performed Western blotting both with muscle extracts used for the 2D-DIGE analysis and muscle extracts from control and EAMG animals characterized in a previous study (Gomez et al., 2011). Protein extracts were separated by SDS polyacrilamide gel electrophoresis (SDS-PAGE) and membranes were incubated with either rabbit anti-β-enolase (W-25, Santa Cruz Biotechnology, CA; 1:800) or goat anti-carbonic anhydrase III (CAIII) (E-19, Santa Cruz Biotechnology; 1:800), and mouse anti-GAPDH (10R-G109a, Fitzgerald, MA; 1:5000000). Secondary antibodies were donkey anti-mouse IgG IRDye 680, goat anti-rabbit IgG IRDye 800 and donkey anti-goat IgG IRDye 800 (926–32222, 926–32211 and 926–32214 respectively; LI-COR Biosciences, Lincoln, NE), diluted 1:10000. Membranes were imaged with Odyssey Infrared Imaging System (LI-COR Biosciences) and bands were quantified with ImageJ software (mean intensity), normalizing for GAPDH mean intensity. Protein levels were compared between groups with an unpaired *t*-test using GraphPad Prism 4 software.

3. Results

A 2D-DIGE proteomics experiment was performed to identify proteins that are affected at various disease stages of EAMG. Proteins were extracted from blood-free tibialis anterior muscles from each of the experimental groups (control, clinical scores 0, 0–1, 1, 2, 3; $n = 4$). A 2D spotmap with an average of 3342 protein spots per sample was obtained and analyzed in the DeCyder 7.0 software.

Twenty-six protein spots with significantly different fluorescence intensities, at least between two of the experimental groups, were identified (1w-ANOVA, $p \leq 0.05$) (Fig. 1S). Unequivocal protein identification by mass spectrometry was achieved for seventeen of the selected spots. There were spots containing more than one protein due to co-migration, so a total of twenty-seven proteins were identified. In this study, a maximum of three proteins per spot were found. Two proteins, serum albumin and pyruvate kinase were present in three spots, leading to the identification of twenty two unique proteins in EAMG (Table 1).

As expected, most identified proteins detected by 2D-DIGE were cytoplasmic, probably due to the bias of detecting soluble proteins using this technique. We observed changes by 2D-DIGE in β-enolase and carbonic anhydrase III (CAIII) (Fig. 1 A, B), and quantified their protein levels in control and EAMG animals by Western blotting. Protein levels of β-enolase were significantly reduced in EAMG muscles compared with controls ($n = 6$ for both groups, $p < 0.05$), with an average reduction of approximately 25% (Fig. 1C). CAIII protein levels were increased in all EAMG stages when quantified by immunoblotting (data not shown). Interestingly, only severely affected animals (score 3) had significantly higher levels of CAIII protein compared with controls. In such muscles, average levels of CAIII were increased by approximately 35% compared with control muscles ($p < 0.01$; $n = 5$ for score 3 EAMG and $n = 10$ for control) (Fig. 1D).

4. Discussion

We analyzed the muscle proteome at different EAMG disease stages by 2D-DIGE and identified twenty-two differentially expressed proteins. The majority of these identified proteins are involved in metabolic pathways (glycolysis and the citric acid cycle), while others are related to cellular-stress responses (e.g. glutathione S-transferase Yb3, 60 KDa heat shock protein), or are contractile proteins (myosin-4 and myozenin-1). Overall, we observed a reduction of the glycolytic capacity and of fast-twitch myosin isoforms in EAMG tibialis anterior muscles, which suggests a switch from fast- to slow-twitch fibers. Similar proteomic profiles were previously described in conditions that impair muscle contraction both in animals and human disease, such as denervation (Isfort et al., 2000; Li et al., 2005), inclusion body myositis (Parker et al., 2009), immobilization (Isfort et al., 2002) and aging (Ohlendieck, 2011). In such studies, relatively few differentially expressed proteins were identified (between 17 and 73), most of them also related to cellular-stress responses and to changes in the type of muscle fibers. Therefore, it appears that the most evident protein alterations in EAMG are a consequence of the impaired muscle-nerve signaling and, possibly, of the atrophy (or loss) of fast-twitch fibers; as it was previously demonstrated in muscle biopsies from MG patients (Coers and Telerman-Toppet, 1976; Martignago et al., 2009; Zamecnik et al., 2009). In this connection it might be relevant that enhancing the response of fast-twitch muscle cells with a selective troponin activator improves muscle strength in EAMG (Russell et al., 2012).

Most of the proteins found in our study are not specific for MG, since they are also affected in other pathological (and physiological) conditions of the muscle. Nonetheless, β-enolase and CAIII have been associated with muscle regeneration and autoimmunity respectively. β-enolase is a muscle-specific metabolic enzyme very sensible to physiological stimuli (Keller et al., 1995), with an important role in developing and regenerating muscles (Merkulova et al., 2000). Moreover, its deficiency leads to severe myalgias, muscle weakness and fatigability in affected individuals (Comi et al., 2001). CAIII is a muscle-specific enzyme that catalyzes the hydration of carbon dioxide and can protect the cell from oxidative damage (Raisanen et al., 1999). It has been described as an auto-antigen in several autoimmune