Molecular Genetics and Metabolism xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

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

Proteomic analysis of muccopolysaccharidosis I mouse brain with two-dimensional polyacrylamide gel electrophoresis

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ARTICLE INFO

Article history:
Received 27 August 2016
Received in revised form 8 October 2016
Accepted 8 October 2016
Available online xxxx

Keywords:
Mucopolysaccharidosis
Proteomics
2D-PAGE

ABSTRACT

Mucopolysaccharidosis type I (MPS I) is due to deficiency of α-L-iduronidase (IDUA) and subsequent storage of undegraded glycosaminoglycans (GAG). The severe form of the disease, known as Hurler syndrome, is characterized by mental retardation and neurodegeneration of unknown etiology. To identify potential biomarkers and unveil the neuropathology mechanism of MPS I disease, two-dimensional polyacrylamide gel electrophoresis (PAGE) and nanoliquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) were applied to compare proteome profiling of brains from MPS I and control mice (5-month old). A total of 2055 spots were compared, and 25 spots (corresponding to 50 different proteins) with a fold change ≥3.5 and a p value <0.05 between MPS I and control mice were further analyzed by nanoLC-MS/MS. These altered proteins could be divided into three major groups based on Gene Ontology (GO) terms: proteins involved in metabolism, neurotransmission and cytoskeleton. Cytoskeletal proteins including ACTA1, ACTN4, TUBB4B and DNM1 were significantly downregulated. STXBP1, a regulator of synaptic vesicle fusion and docking was also downregulated, indicating impaired synaptic transmission. Additionally, proteins regulating Ca^{2+} and H^{+} homeostasis including ATP6V1B2 and RYR3 were downregulated, which may be related to disrupted autophagic and endocytotic pathways. Notably, there is no altered expression in proteins associated with cell death, ubiquitin or inflammation. These results for the first time highlight the important role of alterations in metabolism pathways, intracellular ionic homeostasis and the cytoskeleton in the neuropathology of MPS I disease. The proteins identified in this study would provide potential biomarkers for diagnostic and therapeutic studies of MPS I.

1. Introduction

Mucopolysaccharidosis type I (MPS I) is an autosomal recessive disease that results from deficiency of α-L-iduronidase (IDUA, E.C.3.2.1.76), which degrades the glycosaminoglycans (GAG) heparan sulfate and dermatan sulfate. The widespread accumulation of GAG leads to progressive cellular damage and organ dysfunction, with the central nervous system (CNS) being one of the primary sites of pathology. The CNS pathology in MPS I patients is manifested by learning delays, dementia, hydrocephalus and mental retardation. The etiology of neurological dysfunction in MPS I is unclear. It has been reported that neurons and glial cells accumulate GAG [1] and gangliosides [2]. Activation of glial cells [3] and alterations in oxidative status [4] in the cortex and cerebellum has been reported. It has been previously shown that MPS I mice had difficulty to habituate in the repeated open field test [5] and impaired long-term memory for aversive training [6]. Interestingly, although one study [7] reported abnormal performance of MPS I mice in Morris water maze tests, another study found inconclusive results [8]. In more recent studies, MPS I mice showed impaired learning behaviors in water T maze test [9] and spatial memory skills in the Barnes maze test [10]. These findings describe abnormal cognitive and neuropathology in MPS I, but the mechanisms are likely to be complex and remain to be elucidated.

Development of proteomics technology includes two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), which has provided a powerful tool to study the complicated biological processes in cells and rapidly profiling the global protein expression alterations. 2D-PAGE is powerful in identifying proteins and protein isoforms that may be neglected by other methods such as in-solution digestion and nanoLC-MS/MS or 1D-PAGE and nanoLC-MS/MS. As a mass spectrometric screening method, 2D-PAGE offers many advantages: high throughput, broad dynamic range, good reproducibility and adequate sensitivity. Although combing 2D-PAGE with mass spectrometry is a common proteomic approach for high throughput screening of putative biomarkers in several disorders [11,12], it has not been used in lysosomal diseases.

In this study, a comparative analysis of the proteome of MPS I and wildtype mouse brains using two dimensional gel electrophoresis

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http://dx.doi.org/10.1016/j.ymgme.2016.10.001
1096-7192/

(2D-GE) and nanoLC-ESI-MS/MS were performed. We identified 50 proteins that were differentially expressed in brains of MPS I versus wildtype mice. Bioinformatics analyses using Database for Annotation, Visualization, and Integrated Discovery (DAVID) [13], Protein ANalysis THrough Evolutionary Relationships (PANTHER) [14] and Search Tool for the Retrieval of Interacting genes (STRING) [15] databases allowed for functional classification of the detected proteins and highlighted MPS I-relevant biological pathways. This approach of screening identified potential biomarkers of MPS I that may reveal specific protein signatures indicating MPS I risk. These might also prove useful to assess prognosis, and provide outcome measures for assessing response to therapies.

2. Materials and methods

2.1. Animals and sample collection

MPS I knockout mice (*idua*-/-), a kind gift from Dr. Elizabeth Neufeld, UCLA, has been generated by insertion of neomycin resistance gene into exon 6 of the 14-exon IDUA gene on the C57BL/6 background. MPS I mice (*idua*-/-) and wildtype were genotyped by PCR. All mouse care and handling procedures were in compliance with the rules of the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota. The whole mouse brains were collected from one MPS I and one wildtype mouse (5-month old) for further analysis.

2.2. 2D-PAGE

The samples were homogenized on ice using glass/glass dounces each with 1 mL osmotic lysis buffer (containing 10X nuclease stock, phosphatase inhibitor stock and protease inhibitor stock) and 1 mL SDS boiling buffer without reducing agents. The samples were treated with 3 μL Omnicleave and heated in a boiling water bath for 5 min before the protein concentrations were determined using the BCA assay (Pierce Chemical Co., Rockford, IL, USA) [16]. The samples were diluted to 3.33 and 0.67 mg/mL in 1:1 diluted SDS boiling buffer: Urea sample buffer with reducing agents before loading. 2D-PAGE was performed with the carrier ampholine method of isoelectric focusing as previously reported [17]. Isoelectric focusing was conducted in a glass tube of inner diameter 3.3 mm using 2% pH 3–10 Isodalt Servalytes (Serva, Heidelberg, Germany) for 20,000 volt-hrs. Then, 100 ng of an IEF internal standard, tropomyosin, was added to the sample. This protein migrates as a doublet with lower polypeptide spot of MW 33,000 and pI 5.2. The enclosed tube gel pH gradient plot for this set of Servalytes was determined with a surface pH electrode. After equilibration for 10 min in Buffer 'O' (50 mM dithiothreitol, 10% glycerol, 2.3% SDS and 0.0625 M Tris, pH 6.8), each tube gel was sealed to the top of a stacking gel that overlaid a 10% acrylamide slab gel (1.00 mm thick). SDS slab gel electrophoresis was conducted for approximately 5 h at 25 mA/gel. The following proteins (Sigma Chemical Co., St. Louis, MO, USA and EMD Millipore, Billerica, MA, USA) were used as molecular weight standards: lysozyme (14,000), carbonic anhydrase (29,000), actin (43,000), catalase (60,000), phosphorylase A (94,000) and myosin (220,000). These standards appear along the basic edge of the silver-stained 10% acrylamide slab gel. The silver-stained gels were dried between sheets of cellophane with the acid edge to the left [18]. The gels were dried between sheets of cellophane paper with the acid edge to the left.

2.3. Computerized comparisons

Duplicate gels were obtained from each sample to reduce sources of variability and to detect differences with real statistical significance. The gels were scanned with a laser densitometer (Model PDSI; Molecular Dynamics Inc., Sunnyvale, CA, USA). The scanner was checked for linearity prior to scanning with a calibrated Neutral Density Filter Set (Melles Griot, Irvine, CA, USA). The images were analyzed using Progenesis Same Spots software (version 4.5, 2011; Nonlinear Dynamics, Durham, NC, USA) and Progenesis PG240 software (version 2006; Nonlinear Dynamics). The general method of computerized analysis for these pairs included image warping followed by spot finding, background subtraction (average on boundary), matching, and quantification in conjunction with detailed manual checking. A p value (Student's *t*-test, $n = 2$ gels/sample) is calculated to help assess whether corresponding spots are different. As background is a factor, spot differences are checked by eye. Spot % is equal to spot integrated density above background (volume) expressed as a percentage of total density above background of all spots measured. Difference is defined as fold change of spot percentages. For instance, if corresponding protein spots from different samples (e.g. MPS I versus wildtype) have the same spot %, the difference field will show 1.0; if the spot % from MPS I is twice as large as wildtype, the difference field will display 2.0 indicating two fold up-regulation. If the spot % from MPS I has a value half as large, the difference field will display – 2.0 indicating a twofold downregulation.

2.4. Spot picking and in-gel digestion

Protein spots of interest were selected based on a fold increase or decrease of ≥3.5 and p-value <0.05. Picked spots were excised and subjected to in-gel tryptic digestion and peptide extraction for protein identification by nanoLC-MS/MS analysis, as described before [19]. Briefly, spots were washed in HPLC grade water, 50 mM ammonium bicarbonate (ABC), 50% acetonitrile (ACN)/50% ABC for each 15 min under moderate shaking at room temperature. Next, the gel pieces were dehydrated with 100% ACN and dried under speed vac. Reduction and alkylation were carried out with 10 mM dithiothreitol (DTT) in 25 mM ABC for 30 min at 60 °C and with 100 mM iodoacetamide in 25 mM ABC for 45 min in the dark respectively. Gel pieces were then dehydrated again, dried and rehydrated in 20 μL of a trypsin solution (10 ng/μL) overnight at 37 °C. After incubation, peptide extraction was carried out with 5% formic acid (FA)/50 mM ABC/50% ACN and with 5% FA/100% ACN (20 min each). Extracted peptides were dried and submitted to a zip tip step for purification (EMD Millipore).

2.5. LC-MS/MS

The extracted peptides mixture was analyzed by reversed phase liquid chromatography (LC) and MS (LC-MS/MS) using a NanoAcuity UPLC (Micromass/Waters, Milford, MA) coupled to a Q-TOF Ultima API MS (Micromass/Waters, Milford, MA), according to published procedures [20]. Briefly, the peptides were loaded onto a 100 μm × 10 mm NanoAquity BEH130 C18 1.7 μm UPLC column (Waters, Milford, MA) and eluted over a 150-minute gradient of 2–80% organic solvent (ACN containing 0.1% FA) at a flow rate of 400 nL/min. The aqueous solvent was 0.1% FA in HPLC water. The column was coupled to a Picotip Emitter Silicatip nano-electrospray needle (New Objective, Woburn, MA). MS data acquisition involved survey MS scans and automatic data dependent analysis (DDA) of the top three ions with the highest intensity ions with the charge of 2+, 3+ or 4+ ions. The MS/MS was triggered when the MS signal intensity exceeded 10 counts/s. In survey MS scans, the three most intense peaks were selected for collision-induced dissociation (CID) and fragmented until the total MS/MS ion counts reached 10,000 or for up to 6 s each. Calibration was performed for both precursor and product ions using 1 pmol GluFib (Glu1-Fibrinopeptide B) standard peptide with the sequence EGVNDNEEGFFSAR and the monoisotopic doubly-charged peak with m/z of 785.84.

2.6. Data processing and protein identification

The raw data were processed using ProteinLynx Global Server (PLGS, version 2.4) software as previously described [21]. The following parameters were used: background subtraction of polynomial order 5 adaptive with a threshold of 30%, two smoothings with a window of

Please cite this article as: L. Ou, et al., Proteomic analysis of muccopolysaccharidosis I mouse brain with two-dimensional polyacrylamide gel electrophoresis, Mol. Genet. Metab. (2016), http://dx.doi.org/10.1016/j.ymgme.2016.10.001