Proteomic Differential Display Analysis Reveals Decreased Expression of PEA-15 and Vimentin in FABP7-Deficient Astrocytes

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Abstract

Fatty acid binding proteins (FABPs) are intracellular lipid chaperones which mediate the uptake, transportation and signaling roles of fatty acids. Former studies suggest that FABP7 regulates proliferation and differentiation of radial glia and astrocytes and is associated with various central nervous system diseases including glioma and neuropsychiatric diseases. However, the underlying mechanism is poorly understood. To further explore the mechanistic roles of FABP7 on astrocyte function, the proteome of astrocytes cultured from Fabp7 KO mice was compared with wild-type counterparts by two-dimensional gel electrophoresis (2-DE). We found that 16 protein spots showed differential expression intensity on 2-DE gels. Among them, seven spots appeared elevated, and nine spots appeared decreased in Fabp7 KO astrocytes. Selected spots were analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS) and their protein identity was subsequently revealed using protein databases. Four spots including phosphoprotein enriched in astrocytes 15 (PEA-15), GFAP vimentin and FABP7 were selected for further confirmation by Western blotting. Consistently, there was a significant decrease in expression of PEA-15 and vimentin, both of which play significant roles in proliferation, differentiation, maturation, and survival of astrocytes. These results suggest the molecular mechanism how FABP7 controls astrocyte function and provide new insight to explain the role of FABP7 in glioma and reactive gliosis.

Keywords: 2-DE; Astrocyte; FABP7; Glioma; LC-MS/MS; PEA-15; Vimentin

Abbreviations: 2-DE: Two-dimensional Gel Electrophoresis; ACN: Acetonitrile; CNS: Central Nervous System; D-PBS: Dulbecco’s Phosphate Buffered Saline; DTT: Dithiothreitol; EMT: Epithelial-Mesenchymal Transition; FA: Fatty Acid; FABP: Fatty Acid Binding Protein; FDR: False Discovery Rate; GFAP: Glial Filibrillary Acidic Protein; IEF: Isoelectric Focusing; IPG: Immobilized pH Gradient; KO: Knockout; LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry; MS: Mass Spectrometry; OPCs: Oligodendrocyte Progenitor Cells; PEA-15: Phosphoprotein Enriched in Astrocytes 15; SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; WT: Wild-type

Introduction

Fatty acid binding proteins (FABPs) are intracellular lipid chaperones with relatively high tissue/cell type expression patterns [1]. FABPs control intracellular fatty acids (FAs) trafficking and thereby mediate various aspects of fatty acid homeostasis such as uptake, storage, oxidation, and enzymatic and transcriptional regulations. The growing evidence regarding the association of FABPs with various diseases have turned these molecules to attractive targets for basic functional studies by using several lines of FABP knockout mice [2].

FABP7 also called as brain-type FABP (B-FABP) is abundantly expressed in radial glia and neural stem/progenitor cells (NSPCs) in the developing brains and is involved in embryonic and postnatal neurogenesis [3-7]. In the adult brain, FABP7 is localized in astrocytes and oligodendrocyte progenitor cells (OPCs) [8]. We have previously shown that FABP7 regulates the reactivity, proliferation and FA uptake in astrocytes [8] and controls proliferation and differentiation of OPCs [9].

Several lines of evidence have shown the association of FABP7 in major central nervous system (CNS) diseases such as neuropsychiatric disorders [6], neurodegenerative disorders [10,11] and glioma [12,13] which suggest the regulatory roles of FABP7 in astrocytes. However, the molecular mechanism by which FABP7 is associated with the function of astrocytes is still poorly understood.

Along with our studies to explore the regulatory roles of FABP7 in astrocytes, we needed to test the possible impacts of FABP7 deficiency on astrocyte proteome. Looking for a proof about the hypothetical influence of FABP7 on astrocyte proteomic signature, in this study we compared the proteome of cultured astrocytes from wild-type (WT) and FABP7-deficient mice using two-dimensional gel electrophoresis (2-DE) followed by liquid chromatography mass spectrometry (LC-MS) and finally confirmed the results by Western blotting.

Materials and Methods

Animals

Postnatal day 0-1 mice from WT (C57BL/6) and Fabp7 KO [14] mice were used for primary astrocyte cultures. Our experimental protocols were reviewed by the Ethics Committee for Animal Experimentation of Yamaguchi University School of Medicine. All experiments were performed in accordance with the Guidelines for

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Animal Experimentation of Yamaguchi University School of Medicine and under the Law and notification requirements of the Japanese Government.

**Purified astrocyte culture**

Primary astrocyte culture was performed as previously described [15], with slight modifications. The cerebral cortices of postnatal day 0-1 mice (Fabp7 KO and WT mice) were dissected in Hank’s balanced salt solution (Sigma-Aldrich, St. Louis, MO, USA) containing 20 mM D-glucose (Wako Pure Chemical Industries, Osaka, Japan), and 1% penicillin-streptomycin (Sigma-Aldrich). Following complete removal of the meninges, tissues were dissected into smaller pieces and then treated by trypsin (0.25%; Invitrogen, Carlsbad, CA, USA) at 37°C for 20 min. Homogenized cell suspension was produced by trituration with a plastic serological pipette (Greiner Bio-One, Frickenhausen, Germany) and filtration through a 100 µm mesh (Becton Dickinson Labware, Franklin Lakes, NJ, USA). The cell suspension was centrifuged and pellet was resuspended in Dulbecco’s modified Eagle’s medium (Invitrogen) enriched with 10% heat inactivated fetal bovine serum (Invitrogen), 1% penicillin-streptomycin (Invitrogen) and amphotericin B (Sigma-Aldrich). Then, cells were seeded at a density of 2 x 10^5 cells per T75 flask (Becton Dickinson Labware) and were incubated till confluency (approximately 2 weeks). To eliminate contaminating microglia and OPCs in culture, the culture flasks were shaken on an orbital shaker (BR-40LF; TAIYEC, Koshigaya, Japan) at 200 rpm for 24 h at 37°C. Subsequently, the cells were washed three times with D-PBS (Nissui Pharmaceutical, Tokyo, Japan) and incubated in refreshed culture medium for more 2 days.

To perform passage, the cells were detached by treatment with trypsin (0.05%; Invitrogen). Harvested cells were seeded at a density of 1.5 x 10^5 cells/ml in 10 cm culture dishes (Becton Dickinson Labware), and incubated till the cells became confluent (approximately 10 days). The purity of astrocyte culture was confirmed to be > 95% by immunostaining with specific astrocyte markers (GFAP, S100β; data not shown). Following trypsinization (0.05%; Invitrogen), washing with D-PBS (Nissui Pharmaceutical), and centrifugation, the cells from three 10 cm dishes were collected in a microtube and stored in -80°C until use. Totally five independent cultures were performed from each genotype.

**Preparation of protein samples**

The harvested pellets from Fabp7 KO and WT astrocyte were homogenized in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 165 mM sodium chloride, 10 mM sodium fluoride, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM EDTA, 10 mg/ml aprotinin, 10 mg/ml leupeptin (all from Sigma-Aldrich), and 1% NP-40 (Nacalai Tesque, Kyoto, Japan) on ice. Cell suspensions were incubated for 1 h at 4°C, centrifuged at 21,500 x g for 30 min at 4°C, and the supernatant was stored at -80°C until use. Totally five independent cultures were performed from each genotype.

**2-DE analysis**

To perform each 2-DE, 80 micrograms of isolated protein was used. For the first dimension, isoelectric focusing (IEF) was accomplished in an IPI Ghor 3 IEF unit (GE Healthcare, Buckinghamshire, UK) on 11 cm, immobilized, linear gradient strips (pH 3-10; Bio-Rad, Hercules, CA, USA) at 50 µA/strip. At first protein samples were dissolved in 200 µl of rehydration buffer [consist of 8M urea (Sigma-Aldrich), 2% CHAPS (Sigma-Aldrich), 0.01% bromophenol blue (Wako Pure Chemical Industries), and 1.2% Destrack reagent (GE Healthcare)] and 0.5% IPG (immobilized pH gradient) buffer (GE Healthcare), and then loaded into the IPGghor strip holder (GE Healthcare). IEF was done according to the following voltage program: rehydration for 10 h (no voltage), 0 to 500 V for 4 h, 500 to 1000 V for 1 h, 1000 to 8000 V for 4 h, 8000 V for 20 min, and a final phase of 500 V from 20,000 to 30,000 Vh. For the second dimension the IPG strips were then transferred onto precast polyacrylamide gels with a linear concentration gradient of 5-20% (Bio-Rad) and run for 1 h at 200 V [17].

Following the 2-DE, the SDS–PAGE gels were fixed using a fixing solution consists of 40% ethanol (Wako Pure Chemical Industries) and 10% acetic acid (Wako Pure Chemical Industries) for more than 2 h. To label proteins with fluorescent dye, gels were stained with Flamingo™ Fluorescent Gel Stain (Bio-Rad) overnight [17].

**Image analysis and spot picking**

To evaluate the protein spots, stained gels were washed with Milli-Q water three times each for 5 min and then were observed using the ProEXPRESS 2D Proteomic Imaging System (PerkinElmer, Waltham, MA, USA). The intensity of each spot was quantified with Progenesis Samespots software (Nonlinear, Newcastle, upon Tyne, UK) [18]. The process of image analysis consisted of steps of spots matching and statistics. For each spot, its intensity was normalized by the total valid spot volume. Using five biological replicates and two technical replicates (n = 10 gels/genotype), only spots showing statistically significant intensities (>1 fold-change; P < 0.05, one way ANOVA analysis) between WT and Fabp7 KO astrocytes (between groups) were selected for further MS analysis. The gels were re-stained with See Pico™ (Benebios, Seoul, Korea), and the selected spots with differential intensity between the two genotypes were cut and removed for the MS analysis.

**In gel digestion**

To remove See Pico™ dye (Benebiosi), gel pieces were washed three times in 60% methanol (Sigma-Aldrich), 50 mM ammonium bicarbonate, and 5 mM DTT (both from Wako Pure Chemical Industries) for 15 min. The sample in the gel pieces was reduced twice in 50% ACN (Sigma-Aldrich), 50 mM ammonium bicarbonate, and 5 mM DTT for 10 min. The gel pieces were dehydrated in 100% ACN twice for 30 min, and then in-gel digestion was done with a reagent containing 10 µg/ml sequencing grade-modified trypsin (Promega, Madison, WI) in 30% ACN, 50 mM ammonium bicarbonate, and 5 mM DTT. The in-gel digestion procedure was completed at 30°C overnight. Then, digested samples were lyophilized overnight using a lyophilizer (Lyphe-lock 11. Model 77400, Labconco, Kansas, MO, USA).

**LC-MS/MS analysis**

The lyophilized samples were dissolved in 0.1% formic acid (Sigma-Aldrich), centrifuged at 21,500 x g for 5 min, and the supernatant was used for MS analysis. Twenty-five microliters of each sample was analyzed using a LC-MS/MS system (Agilent 1100 LC-MSD Trap XCT, Agilent Technologies, Palo Alto, CA, USA). Protein identification procedure was performed using Agilent Spectrum MILL MS proteomics workbench against the Swiss-Prot protein database search engine (http://kr.expasy.org/sprot/) and MASCOT MS/MS Ions Search engine (http://www.matrixscience.com/ search_form_select. html). The criteria for positive identification of proteins were set as follows: filter by protein score > 10, filter peptide by score > 8, and percentage scored peak intensity (%SPI) > 70 [18]. In addition by searching against a randomized decoy database, the false discovery rate (FDR) was estimated in MASCOT and FDR < 1% was considered as acceptable inclusion criteria for protein identifications.
Western blotting

The same sets of protein samples with those for 2-DE analysis were used for western blotting. Ten micrograms of protein from WT and Fabp7 KO astrocyte lysate were separated on a SDS-PAGE gel (15%), transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA), and blocked with tris-buffered saline (TBS) containing 0.1% Tween 20 (Sigma-Aldrich) and 5% skimmed milk for 1 h at room temperature. Membranes were incubated with specific primary antibodies, including rabbit anti-FABP7 (0.125 µg/ml) [14], rabbit anti-PEA-15 (1:500; Santa Cruz Biotech, Santa Cruz, CA, USA), mouse anti-vimentin (1:500; Abcam, Cambridge, UK), rat anti-GFAP (1:2500; Invitrogen), and mouse anti-β-actin (1:5000; Santa Cruz Biotech) overnight at 4°C. Subsequently, membranes were incubated with HRP-conjugated secondary antibodies, including goat anti-rabbit (1:2000; Chemicon, Temecula, CA), goat anti-rat (1:1000; Chemicon), and goat anti-mouse (1:5000; Chemicon) for 1 h at room temperature, and developed with an ECL Western blot detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The images of blots were captured using Image Reader LAS-1000 (Fujifilm, Tokyo, Japan) and the intensity of protein bands were analyzed using Multi Gauge software (Fujifilm).

Totally two sets of western blot (at least three biological replicates per each experiment) were performed and expression levels of proteins of interest were normalized to the expression of β-actin, as a control for loading.

Statistical analysis

The western blotting results are presented as mean ± SEM of two independent experiments with at least three biological replicates per each experiment. Statistical analyses were conducted with SPSS software (version 13.0; SPSS Inc, Chicago, IL). The nonparametric Mann-Whitney U test was applied to compare protein expression levels between Fabp7 KO and WT astrocyte samples. P < 0.05 was considered as statistically significant.

Results

Detection of spots with differential expression between WT and Fabp7 KO astrocytes on 2-DE gels

Protein expressions by primary cultured astrocytes from WT and Fabp7 KO mice were assessed by 2-DE (five biological and two technical replicates for each genotype). Protein spots were visualized with a fluorescent gel staining and differences in the spot intensities between the two genotypes were analyzed and quantified by Progenesis Samespots software. At least 16 protein spots showed altered expression in Fabp7 KO astrocytes (Figure 1). By visual assessment one spot was almost absent in Fabp7 KO gels (spot number 16) which was expected to be FABP7 (Figure 1A). Seven spots showed increased intensities and nine spots showed decreased intensities in Fabp7 KO astrocytes (Figure 1A and 1B).

Identification of proteins

The spots with differential expressions between the two genotypes were digested and forwarded for identification by LC-MS/MS. The result of protein identification is summarized in Table 1. Among 16 differentially expressed spots, four spots with more than two distinct peptides (≥ 2) or MS/MS search scores over 30 were selected for further confirmation by Western blotting. These proteins included FABP7, GFAP, PEA-15, and vimentin.

Decreased expression of vimentin and PEA-15 in Fabp7 KO astrocytes

Western blotting analysis was performed and confirmed the significant decrease in the expression of vimentin (1.57 ± 0.07 in WT astrocytes vs. 0.52 ± 0.19 in Fabp7 KO astrocytes, P = 0.03; Figure 2A and 2B) and PEA-15 (1.51 ± 0.18 in WT astrocytes vs. 0.8 ± 0.09 in Fabp7 KO astrocytes, P = 0.03; Figure 2A,B) proteins in Fabp7 KO astrocytes compared to WT astrocytes. Moreover, expression of FABP7 was not discerned in Fabp7 KO astrocytes which further confirmed the authenticity of our experiments (Figure 2A). However, Western blotting results did not confirm GFAP upregulation in Fabp7 KO astrocytes and no change was detected in the expression of GFAP between Fabp7 KO and WT astrocytes (2.03 ± 0.52 in WT astrocytes vs. 2.11 ± 0.39 in Fabp7 KO astrocytes, P = 0.89; Figure 2A and 2B).

Discussion

In this study we showed proteomic differential display analysis of Fabp7 KO astrocytes compared with WT astrocytes. Except for FABP7, none of the proteins were found to be absent in Fabp7 KO astrocytes. However, we found that at least two proteins, vimentin and PEA-15 are significantly decreased in FABP7-deficient astrocytes. Our findings provide a proof of the principle that FABP7 deficiency can alter the astrocyte proteome. As the first report of proteomic screening on isolated cells from Fabp7 KO mice, our study suggests the application
of such strategies for clarifying the regulatory roles of FABP7 in the brain development and the various brain diseases.

We showed the decreased expression of PEA-15 in FABP7-deficient astrocytes. PEA-15 is a ubiquitously expressed protein with high levels of expression in astrocytes [19]. This protein of low molecular weight (15 kDa) is considered a multi-protein binding molecule serving as an endogenous substrate and molecular adaptor which interacts with various key cellular effectors including protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II (CAM kinase II), Akt, and ERK and thereby modulates major cell functions such as proliferation, apoptosis, and glucose metabolism [19].

PEA-15 is involved in epithelial-mesenchymal transition (EMT) [20] and high levels of PEA-15 expression is linked with development of malignancy [19]. PEA 15 is reported to be overexpressed in gliomas and mammary carcinomas and may regulate chemoresistance [19]. Interestingly, FABP7 is also upregulated in a variety of malignancies, including malignant glioma and mammary carcinoma [12,21]. Furthermore, increased expression of PEA-15 has been reported in reactive astrocytes in postmortem human Alzheimer’s disease brain, as well as in the brains of a mouse model of Alzheimer’s disease [22] and as amyotrophic lateral sclerosis [23]. Consistently, FABP7 is upregulated in reactive astrocytes [8] and is increased in neurodegenerative disorders such as Alzheimer’s disease [10,11].

Although the mechanism how FABP7 regulates PEA-15 expression remains to be further studied, it is of note that transcriptional regulation of PEA-15 by peroxisome proliferator-activated receptor gamma (PPARγ), a transcription factor activated by FA–FABP complex, has been reported [24,25]. Together with the known evidence from the literature, our finding suggest that the known associations of FABP7 with glioma, reactive astrogliosis and neurodegenerative disorders such as Alzheimer’s disease [10,11].
FABP7-deficient astrocytes. Vimentin is an intermediate filament abundantly expressed in radial glia and immature astrocytes during early development, and it is downregulated towards maturation [26,27] and is upregulated in reactive astrocytes [28] and glioma [29]. This expression pattern highly mimics that of FABP7, suggesting involvement of both molecules in common regulatory networks and supports the FABP7-mediated regulation of vimentin expression. In contrast to vimentin, expression of GFAP did not markedly change in Fabp7 KO astrocytes as confirmed by Western blotting. Regarding this issue, it has been shown that elevated expression of GFAP and decreased vimentin expression represents astrocytes differentiation [30]; vimentin-deficient cells exhibited a slower rate of cell proliferation and DNA synthesis compared with WT cells [31]; vimentin-deficient astrocytes were predominantly found to be in G0/G1 stage of cell cycle [32]. These data indicate that vimentin downregulation in Fabp7 KO astrocytes is partly associated with their lower proliferation capacity, and it is interesting to note that Fabp7 KO astrocytes show the decreased proliferation compared with WT astrocytes in our previous study [8]. Therefore, it is likely that FABP7 is involved in the control of differentiation status of astrocytes possibly through regulation of vimentin expression.

Vimentin is also known as a marker for EMT. Overexpression of vimentin is linked with poor prognosis, invasion and tumor growth [33]. Known association of vimentin in tumorgenesis in several malignancies including CNS cancers has turned vimentin to an attractive diagnostic and therapeutic target in cancer [33]. Thus, FABP7-mediated regulation of vimentin can highlight the diagnostic and therapeutic potentials of FABP7 in malignancies such as glioblastoma.

Taken together, as the first proteomic screening on isolated astrocytes from Fabp7 KO mice, our study suggests the regulatory roles of FABP7 on the astrocytic expression of PEA-15 and vimentin, both of which are known to be associated with EMT, tumorgenesis of glioma, and reactive astrogliosis. Our findings provide novel insights and hypotheses regarding to the association of FABP7 with CNS diseases.

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