Proteome Analysis of the Cerebellum Tissue in Chronically Alcohol-Fed Rats

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**Received:** September 16, 2016; **Accepted:** October 13, 2016; **Published:** October 20, 2016


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**Abstract**

**Background:** Cerebellar degeneration is one of the most common effects of chronic alcohol exposure, and Purkinje cells are the main targets of alcohol-induced cerebellum neuropathology, but the underlying mechanism remains unclear.

**Methods:** Eight rats were fed for 8 weeks with a nutritionally adequate liquid diet containing either ethanol as 36% of the total caloric content or an isocaloric control diet. Rat cerebellum homogenates were subjected to agarose two-dimensional electrophoresis (2-DE), and the protein expression profiles in chronically alcohol-fed rats and the pair-fed controls were compared. The observed changes in the protein expression levels were confirmed using immunoblotting analysis.

**Results:** Three protein spots changed significantly in intensity according to 2-DE. Based on immunoblotting analysis, low expression levels of microtubule-associated protein-2 (MAP2) and the overexpression of voltage-dependent anion channel protein 1 (VDAC1) were observed in the cerebellum of alcohol-fed rats. The expression levels of both proteins did not change in other parts of the brain.

**Conclusions:** Low expression levels of MAP2 and overexpression of VDAC1 were detected using proteome analysis of the cerebellum tissue from chronically alcohol-fed rats. Changes in the expression of these proteins may be related to cerebellar degeneration following chronic alcohol consumption.

**Keywords:** Cerebellum; Chronically alcohol-fed rats; Proteome analysis

**Abbreviation:** IEF: Isoelectric Focusing; 2-DE: 2-Dimensional Electrophoresis; SDS-PAGE: Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis; IPG: Immobilized pH Gradient; CBB: COOMASSIE Brilliant Blue; CAN: Acetonitrile; MS: Mass Spectrometry; HPLC: High Performance Liquid Chromatography; TBS-T: Tris Buffer Saline-0.1% Tween 20; MAP2: Microtubule-Associated Protein 2; VDAC1: Voltage-Dependent Anion Channel Protein 1; ACON: Acetoin Hydratase; Mitochondrial; AD: Alzheimer’s Disease

**Introduction**

Alcohol abuse is a serious health problem worldwide, which has severe detrimental effects on the structure and function of many organs, including the brain [1]. Cerebellar degeneration is the most common effect of alcoholism [2,3]. The findings of recent clinical studies indicate that up to two thirds of chronic alcoholics exhibit signs of cerebellar degeneration [4]. Moreover, approximately 50% of alcoholic’s exhibit cerebellar degeneration without clinical signs, according to a large-scale postmortem study conducted in Japan [5]. The Purkinje cells are the main targets of alcohol-induced cerebellar degeneration [6,7]. Thus, the loss and/or atrophy of Purkinje cells and reductions in the dendrite volume in the molecular layer may contribute to cerebellar atrophy [5,8].

Malnutrition, mainly in the form of thiamine deficiency, is also considered as a contributing factor to ethanol-induced neuropathological changes in the cerebellum [9,10]. However, 33% of chronic alcoholics without thiamine deficiency exhibited cerebellar atrophy according to an imaging study [11], and significant decreases in the size and density of Purkinje cells were also found in chronic alcoholics with no clinical signs of Wernicke’s encephalopathy [6].

Proteomic analysis of the human brain has been employed in postmortem studies of chronic alcoholism [12,13], but the results were complicated by multifactorial effects on the human brain.

A rat model is suitable for the study of alcoholism. Alcohol exposure (dose and patterns) can be easily controlled to facilitate direct examination of the effects of ethanol on the central nervous system. In this study, we assessed changes in the protein expression profiles in the cerebellum of rats after chronic exposure to alcohol compared with controls using the Lieber–DeCarli model because it is reliable for effectively controlling the nutritional status [14]. Furthermore, this model has been used to study the brain damage caused by chronic alcohol consumption [15-17].
Materials and Methods

Animal treatment

Eight 4-week-old male Sprague-Dawley rats weighing 140-150 g were purchased from Charles River Japan (Yokohama, Japan) and housed in individual cages in the same room at the laboratory animal facility of the Chiba University Graduate School of Medicine under a 12/12 h light/dark cycle at a constant temperature of 24°C ± 1°C. A total of eight rats were pair-fed with a nutritionally adequate liquid diet containing ethanol as 36% of the total caloric content (5% ethanol dose) or an isocaloric control diet (Oriental Yeast Co., Tokyo, Japan) for 8 weeks, as described by Lieber and DeCarli [14]. The pair-feeding method used in this study was originally designed for studies of effects of chronic alcohol consumption on the liver. All of the experiments were performed in accordance with the Animal Experimentation Guidelines of Chiba University and the study protocol was approved by the Ethics Committee of Chiba University Graduate School of Medicine.

Tissue preparation

At the end of the ethanol exposure period, all of the rats were sacrificed separately using decapitation under light ether anesthesia. The brains were removed and the cerebellum, hippocampus, frontal cortex, striatum, amygdala, and hypothalamus were then dissected separately, cut into small pieces, and stored at −80°C until further processing.

Protein extraction

Frozen tissues (approximately 50 mg) were homogenized in 20 volumes of buffer [7 M urea, 2 M thiourea, 2% (w/v) 3-(3-cholamidopropyl) dimethylammoniumpropane sulfonate, 0.1 M dithiothreitol, 2.5% pharmalyte (pH 3–10), and protease inhibitors (Complete Mini EDTA-free; Roche Diagnostics Deutschland GmbH, Mannheim, Germany)]. The homogenates were centrifuged at 112,000 × g (Optima TLX Ultracentrifuge; Beckman Coulter Inc., Brea, CA, USA) at 4°C for 60 min, and the clear supernatants were transferred to new microcentrifuge tubes. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 4°C for 60 min. The peptide fragments remaining in the gel were subjected to isoelectric focusing (IEF) analysis. Agarose gels were used for the first dimension of the proteomic-grade trypsin (Roche Diagnostics Deutschland GmbH) for 45 min at 4°C. After removing the unabsorbed solution, the gel pieces were incubated in 10–30 μL of 25 mM NH4HCO3 buffer for 24 ± h. The solution containing digested protein fragments was transferred into a new tube and stored at 4°C.

2-DE gel image analysis

All of the 2-DE agarose gels were scanned using an Epson ES-10000G scanner equipped with a transparency adapter (Seiko Epson Corp., Suwa, Japan). Images were acquired and processed using Progenesis SameSpots image analysis software (version 3.3; Nonlinear Dynamics, Durham, NC, USA) to determine the integrated densities of the protein spots on the same 2-DE agarose gel. The statistical significance of the observed differences in each spot was assessed by Student’s t-test.

In-gel protein digestion

The proteins separated by 2-DE were identified by in-gel tryptic digestion, followed by mass spectrometry (MS). The protein spots were fragmented into peptides as described by Satoh et al. [20]. Briefly, each of the protein spots was individually excised from the CBB-stained gel in approximately 1 mm squares and destained in 50% (v/v) acetonitrile (ACN) containing 50 mM NH4HCO3. The gel pieces were dehydrated in 100% ACN for 15 min and then dried using a TOMY CC-105 microcentrifugal vacuum concentrator (Tomy Seiko Co. Ltd, Tokyo, Japan) for at least 60 min. The gel pieces were rehydrated and immersed in 10–30 μL of 25 mM NH4HCO3 containing 50 ng/μL of proteomic-grade trypsin (Roche Diagnostics Deutschland GmbH) for 45 min at 4°C. After removing the unabsorbed solution, the gel pieces were fragmented into peptides as described by Satoh et al. [20]. Briefly, each of the protein spots was individually excised from the CBB-stained gel in approximately 1 mm squares and destained in 50% (v/v) acetonitrile (ACN) containing 50 mM NH4HCO3. The gel pieces were dehydrated in 100% ACN for 15 min and then dried using a TOMY CC-105 microcentrifugal vacuum concentrator (Tomy Seiko Co. Ltd, Tokyo, Japan) for at least 60 min. The gel pieces were rehydrated and immersed in 10–30 μL of 25 mM NH4HCO3 containing 50 ng/μL of proteomic-grade trypsin (Roche Diagnostics Deutschland GmbH) for 45 min at 4°C. After removing the unabsorbed solution, the gel pieces were incubated in 10–30 μL of 25 mM NH4HCO3 buffer for 24 ± h. The solution containing digested protein fragments was transferred into a new tube and stored at 4°C. The peptide fragments remaining in the gel were extracted in a minimal volume of 5% (v/v) formic acid containing 50% (v/v) ACN for 20 min at room temperature.

MS analysis and database search

The digested peptides were desalted and selectively enriched with C18-StageTips [21]. Next, the enriched samples were injected into a trap column (C18, 0.3 × 5 mm; Dionex Corporation, Sunnyvale, CA, USA) and an analytical column (C18, 0.075 × 120 mm; Nikkyo Technos Co. Ltd, Tokyo, Japan), which were attached to an UltiMate 3000 high-performance liquid chromatography (HPLC) system (Dionex Corporation). The flow rate of the mobile phase was 300 nL/min. The solvent composition of the mobile phase was programmed to change over a 120 min cycle with variable mixing ratios of solvent A (2% v/v CH3CN and 0.1% v/v HCOOH) relative to solvent B (90% v/v CH3CN and 0.1% v/v HCOOH): 5–10% B for 5 min, 10–13.5% B for 35 min, 13.5–35% B for 65 min, 35–90% B for 4 min, 90% B for 0.5 min, 90–5% B for 0.5 min, and 5% B for 10 min. Purified peptides were transferred from the HPLC system to a LTQ-Orbitrap XL hybrid ion-trap Fourier transform mass spectrometer (Thermo Scientific, San Jose, CA, USA). The Mascot search engine (version 2.2.6; Matrix Science Ltd., London, UK) was used to identify proteins from the mass and tandem mass spectra of peptides. Peptide mass data were matched by searching the UniProtKB Rattus database (SwissProt 2011x, August 2011, 7645 entries), where the database search parameters were as follows: Peptide mass tolerance=2 ppm, fragment tolerance=0.6 Da, enzyme=trypsin, up to one missed cleavage was allowed, and variable modifications=methionine oxidation. The minimum criterion for protein identification was set as a false discovery rate of <1%, which was estimated by searching against a randomized decoy database created by the Mascot Perl program supplied by Matrix Science Ltd.

Western blotting

Western blotting analysis of the proteins identified by 2-DE was performed to confirm the differences in the protein expression levels in
cerebellum between the alcohol-fed and pair-fed control groups. Same amount of protein extracts (5 µg per lane) were separated by SDS-PAGE on 10–20% gradient gels or a 7.5% gel (XV PANTERA Gel; DRC, Tokyo, Japan) for 18 min at 240 V, and then transferred onto polyvinylidene fluoride membranes (EMD Millipore Corporation, Bedford, MA, USA) in a tank-transfer apparatus (Bio-Rad Laboratories, Inc.) at 10 V overnight (17 h). The membrane was blocked with 0.5% low-fat skim milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 1 h at room temperature. The membranes were incubated with the following primary antibodies (all from Abcam plc., Tokyo, Japan) in 0.5% low-fat milk in TBS-T: (a) Chicken anti-rat microtubule-associated protein 2 (MAP2) antibody (ab5392; dilution, 1:100,000), (b) Mouse anti-rat voltage-dependent anion channel 1 (VDAC1) antibody (ab14734; dilution, 1:10,000), (c) Rabbit anti-rat aconitate hydratase mitochondrial (ACON) antibody (ab129105; dilution, 1:50,000), and (d) Mouse anti-rat β-actin antibody (ab49846; dilution, 1:1,000,000). The blots were washed three times with TBS-T, each for 5 min and then incubated with the following secondary antibodies in blocking buffer for 1 h: (a) Anti-chicken antibody (dilution, 1:10,000; GenWay Biotech, Inc., San Diego, CA, USA), (b) Anti-mouse antibody (dilution, 1:1000; Dako Denmark A/S, Glostrup, Denmark), and (c) Anti-rabbit antibody (dilution, 1:5000; Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Antigens bound to the membrane were detected using ECL plus enhanced chemiluminescence detection reagents (GE Healthcare UK Ltd., Little Chalfont, Buckinghamshire, UK), and the band intensities were quantified using LPR-400EX chemiluminescence imager imaging analysis software (Taitec, Tokyo, Japan).

Statistical analysis

All of the numerical data were expressed as means ± standard deviations. The statistical significance of differences was assessed using paired t-tests. *p*<0.05 was considered statistically significant.

Results

Animal data

Before feeding for 8 weeks, the Sprague-Dawley rats weighed 140–150 g. At the end of feeding, the weights of the control and alcohol-fed rats were 357 ± 19 g and 362 ± 29 g, respectively. The total caloric intake of the control and alcohol-fed rats was 3995 ± 289 kcal and 3993 ± 291 kcal, respectively. There were no significant differences in body weight or caloric intake between the alcohol-fed groups and pair-fed controls during the period of alcohol exposure. There were no significant cerebellar symptoms, such as ataxic gate or shaking, in the alcohol-fed rats. All of the alcohol-fed rats had alcoholic hepatic steatosis (Supplementary Figure 1).

Identification of proteins with significantly changed expression profiles after alcohol exposure

A representative 2-DE pattern of the protein extract from a control rat is shown in Figure 1A. In total, 615 protein spots were detected in each of the eight gels. Protein spots with significant changes in intensity (Student’s t-test; *p*<0.05) 2.14-fold are shown in Figure 1B. MAP2, ACON, and VDAC1 were identified in spots 1, 2, and 3, respectively, by MS analysis and database search (Table 1). MAP2 is known as a major cytoskeletal protein, ACON as an enzyme that catalyzes the isomerization of citrate to isocitrate via cis-aconitate in mitochondria, and VDAC1 as a channel at the outer mitochondrial membrane.

Validation of differentially expressed proteins in cerebellar tissues and other brain region tissues in control and alcohol-fed rats

Western blotting indicated MAP2 expression was significantly lower (control: 0.3 ± 0.08; alcohol: 0.18 ± 0.03; *p*=0.0396) and VDAC1 expression was significantly higher (control: 0.67 ± 0.26; alcohol: 0.73 ± 0.26; *p*=0.0264) in the cerebellums of alcohol-fed rats compared with the pair-fed controls (Figure 2). However, there were no significant differences in the ACON expression levels between the two groups (Figure 2). Western blotting was performed for MAP2 and VDAC1 in other brain regions, including the hippocampus, frontal cortex, striatum, amygdala, and hypothalamus, for the pair-fed control and alcohol-fed groups, but there was no significant change in the protein expression levels of MAP2 and VDAC1 in the alcohol-fed and pair-fed control groups (Supplementary Figure 2).

Discussion

The cerebellum is one of the regions that are most susceptible to alcohol-induced brain damage [3], but the underlying mechanisms of cerebellar degeneration following chronic alcohol exposure remain unclear.

In this study, we compared the protein expression profiles in the cerebellar tissues of control rats and chronically alcohol-fed rats based on agarose 2-DE proteomics followed by immunoblotting analysis. We employed agarose gels in the first dimension of 2-DE proteomics. This method is useful for resolving high molecular mass proteins (>150...
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In vivo studies have reported no loss of neurons in the hippocampus of alcoholics [36]; however, it is possible that longer alcohol feeding would lead the adult, and the results in our study should be carefully considered.

In this study, we detected the overexpression of VDAC1 in the cerebellum of rats after chronic alcohol treatment. Thus, the overexpression of VDAC1 in the brain might indicate apoptotic cell death due to alcohol-induced oxidative stress.

In addition, VDAC1 overexpression has also been demonstrated in other neurodegenerative disorders by proteomic analysis, such as Alzheimer’s disease (AD) [34]. Reduced VDAC1 expression was shown to have a protective role in the brain tissue of animal models of AD [35], but further research by overexpression/inhibition techniques is needed to determine whether the inhibition of VDAC1 can prevent oxidative stress and apoptosis in the cerebellum with long-term alcohol exposure.

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cerebellar damages. The alterations in MAP2 and VDAC1 expressions found in this study could be the early changes in the alcohol-induced cerebellum degeneration.

Conclusion

In conclusion, we identified changes in the expression levels of MAP2 and VDAC1 by 2-DE in the cerebellum of rats subjected to an 8-week ethanol exposure regimen using the Lieber–DeCarli liquid diet. The low expression of MAP2 and overexpression of VDAC1 in the cerebellum of rats exposed chronically to alcohol in this study may provide insights into the mechanisms of neuronal cytoskeleton alterations and neuronal apoptotic changes in the cerebellum following chronic alcohol exposure.

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