2D DIGE proteomic analysis highlights delayed postnatal repression of α-fetoprotein expression in homocystinuria model mice

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1. Introduction

Elevated levels of plasma homocysteine are an independent risk factor for atherosclerotic cardiovascular diseases, stroke, peripheral arterial occlusive diseases, and venous thrombosis [1]. Hyperhomocysteinemia is caused by several genetic defects, but mainly by deficiency of cystathionine β-synthase (CBS; EC 2.1.2.1). CBS-deficient homocystinuria patients (MIM 236200) exhibit various severe clinical manifestations including thromboembolism, mental retardation, osteoporosis, and skeletal abnormalities. The molecular mechanisms by which accumulated homocysteine may promote such diseases have been the focus of numerous investigations. Endothelial dysfunction appears to play a key role in cardiovascular diseases [2], but the pathogenesis of hepatic steatosis, a sporadic feature in CBS-deficient patients [1,3], remains to be clarified. It is notable that plasma homocysteine levels are elevated in patients with non-alcoholic fatty liver disease (NAFLD) [4].

A genetic model with targeted deletion of the Cbs gene was generated in 1995 [5] and has subsequently been widely used in homocysteine-related research. Homozygous Cbs−/− mice develop fatty liver at a juvenile age (∼2 weeks old) [5,6] and display an abnormal lipoprotein profile [7], but a few escape and fortunately survive beyond this age [8]. This study examined the plasma protein profile of Cbs−/− mice using proteomic analysis with fluorescent two-dimensional difference gel electrophoresis (2D DIGE) to gain insight into the molecular background of hepatic steatosis. For comparison, we utilized mice lacking cystathionine γ-lyase (Cth, also known as CSE; EC 4.4.1.1), which also display homocysteinemia but are free of obvious abnormalities (such as fatty liver) [9]. Here, we found hyperaccumulation of α-fetoprotein (AFP) in the plasma and fatty liver of Cbs−/− mice but not of Cth−/− mice.

2. Materials and methods

2.1. Animals

Heterozygous Cbs+/− mice in a C57BL/6J background (B6.129P2-Cbstm1Unc/J) were obtained from the Jackson Laboratory.
Heterozygous Cth+/− mice were generated by our group [9] and backcrossed for 10 generations (N10) with C57BL/6Jcl [Jcl: Japan Clea, Tokyo, Japan] [10]. N10 Cbs−/− or N10 Cth+/− mice were bred to produce Cbs−/− or Cth+/− mice, and their age-matched progenies were analyzed comparatively. Mice were housed in an air-conditioned room kept on a 12-h dark/light cycle and allowed to free access to a standard dry rodent diet and water. All animal protocols were approved by the Animal Care Committee of Keio University (No. 09187-(4)).

2.2. Polycrylamide gel electrophoresis (PAGE) and western blotting

Male mice were anesthetized with diethyl ether, and blood samples were collected from beating hearts of laparotomized mice, and then EDTA plasma (or serum) was prepared. Livers were collected from beating hearts of laparotomized mice, and then EDTA-free protease inhibitor cocktail (Roche Applied Science) was added to the samples. Liver homogenates (2.5% wt/vol) were prepared using a Polytron homogenizer (Brinkmann Instruments, Westbury, NY, USA) and then EDTA plasma (or serum) was added to the samples. The samples were centrifuged at 12,000 × g for 10 min at 4°C. The supernatants were removed and used for further experiments. Each sample was snap frozen in liquid nitrogen and stored at −80°C until analysis.

2.3. Measurement of plasma albumin levels

Plasma levels of albumin were measured using a Dri-Chem 7000i biochemistry analyzer with ALB-P slides (Fujifilm, Tokyo, Japan).

2.4. 2D DIGE

Plasma (10 μL) was mixed with 90 μL lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1 mM PMSF, 1 mM Na3VO4). After adjusting the pH to 8.5 by adding 10 mM Tris–HCl (pH 8.5), 0.8 μL samples were labeled with 200 pmol of CyDye DIGE fluor, minimal labeling dye (Cy2, Cy3 or Cy5 [GE Healthcare]) at 4°C for 30 min in the dark. The reaction was stopped by adding 0.5 μL of 10 mM lysine. Labeled samples were mixed with dithiothreitol (DTT) and immobilized pH gradient (IPG) buffer (final 1% each) at 4°C for 10 min in the dark. The samples were immediately subjected to isoelectric focusing (IEF) in an Immobiline DryStrip (13 cm, pH 4–7 [GE Healthcare]) that were rehydrated for 20 h in a rehydration buffer (7 M urea, 2 M thiourea, 2% Triton X-100, 13 mM DTT, 2.5 mM acetic acid, 1% IPG buffer, and a trace amount of bromophenol blue) at 20°C. IEF was performed using a CoolPhoreStar IPG-IEF Type-px system (Anatech, Tokyo, Japan) in the following conditions: 500 V for 1.5 h, linear gradient from 500 V to 3500 V for 4.5 h, and finally 3500 V for 8 h at 20°C. Once IEF was completed, the strips were equilibrated for 30 min in reducing buffer (50 mM Tris–HCl [pH 6.8], 6 M urea, 2% SDS, 30% [v/v] glycerol, 65 mM DTT and a trace amount of bromophenol blue), followed by an alkylating buffer (reducing buffer with 4.5% iodoacetoamide instead of DTT) for an additional 15 min. The strips were sealed on the top of 10% PAGE gels using 0.5% low-melting-point agarose in a Tris–glycine electrophoresis buffer. The second dimension of protein separation was performed at a constant 200 V using an ERICA-S high-speed electrophoresis system (DRC, Tokyo, Japan) [12]. Gels were scanned using a Typhoon Trio image scanner (GE Healthcare).

2.5. Matrix-assisted laser desorption/ionization-time of flight/mass spectrometry (MALDI-TOF/MS) analysis

For silver staining, plasma (10 μL) was subjected to IEF and then SDS–PAGE without CyDye labeling. The gel was stained using a Silver Stain MS kit (Wako, Tokyo, Japan) in accordance with the manufacturer’s instructions. The gel pieces were excised, destained, washed twice with deionized water and four times with 50 mM ammonium bicarbonate: acetonitrile (1:1), and dehydrated once with acetonitrile. Then, the gel pieces were twice alternately rehydrated with 100 mM ammonium bicarbonate and dehydrated with acetonitrile, and dried by vacuum centrifugation. Protein samples were digested at 37°C for 12 h with 5 μL of 0.02 μg/μL Sequencing Grade Modified Trypsin (Promega) dissolved in 25 mM ammonium bicarbonate. Peptides were extracted from the gels in 40 μL of 1% trifluoroacetic acid/50% acetonitrile solution by sonication. Samples were spotted onto a μFocus MALDI plate (900 μm, 384 circles, Hudson Surface Technology; Old Tappan, NJ, USA) with an equal volume of matrix solution, containing 10 mM α-cyano-4-hydroxycinnamic acid in 1% trifluoroacetic acid/50% acetonitrile. Positive ion mass spectra were obtained using an AXIMA-CFR Plus (Shimadzu, Kyoto, Japan) in a reflection mode. MS spectra were acquired over a mass range of 700–4000 m/z and calibrated using peptide calibration standards (~1,000–3,200 Da, Bruker Daltonics, Yokohama, Japan) [12].

2.6. Protein database search

Proteins were identified by matching the peptide mass fingerprint with the Swiss-Prot protein database using the MASCOT Search engine (Matrix Science, http://www.matrixscience.com). Database searches were carried out using the following parameters: taxonomy, Mus musculus; enzyme, trypsin; and allowing one missed cleavage. Carbamidomethylation was selected as a fixed modification, and the oxidation of methionine was allowed as a variable. The peptide mass tolerance was set at 0.5 Da and the significance threshold was set at P < 0.05 probability based values on Mouse scores (≥55).

2.7. Quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from the liver using TRI Reagent (Molecular Research Center, Cincinnati, Ohio, USA). Total RNA (1 μg) was used to produce first-strand cDNA with a ReverTra Ace qPCR RT Kit (Toyobo, Tokyo, Japan). A total of 10 ng of cDNA from each sample was amplified via qPCR using the SYBR Green Master Mix (Toyobo), 18 primer sets (Table 1), and a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) [11]. Each mRNA level was quantified using the comparative CT method with housekeeping gene hypoxanthine guanine phosphoribosyl transferase (Hprt) levels used for normalization, and the relative expression in wild-type mice was set at 1.

2.8. Statistical analysis

Data are expressed as means ± SD of independent samples (n as indicated). Statistical analyses were performed using unpaired two-tailed Student’s t-test, where P values < 0.05 were considered significant.

3. Results

3.1. Plasma protein profiling by 2D DIGE

Cbs−/− mice suffer from hepatic dysfunction/steatosis [6,7] and start to die from 2 weeks of age, and the majority die by 4 weeks...
1-week-old mice (Fig. 1B), but were generally maintained in 1-, 2-, and 4-week-old mice [8], and thus we analyzed plasma protein profiles comparatively in wild-type and 

-8a–d), antithrombin-III (spot 9), ceruloplasmin (spots 5a–e), inter- 

| Gene   | Primer sequence | Size |
|--------|-----------------|------|
| Afp    | 5′-CGCCGACTCTGAGACGAAAG-3′ (Forward) | 218 bp |
|        | 5′-ATGAAATGGCTGGCATTCCT-3′ (Reverse) | 162 bp |
| Apoe   | 5′-TGGCTTGTTGCCATTTCTGT-3′ (Forward) | 161 bp |
|        | 5′-CTCCAGGCTTGGTAAAC-3′ (Reverse) | 120 bp |
| Aapos4 | 5′-AACTATGCGACTGGAGCT-3′ (Forward) | 127 bp |
|        | 5′-CGATGCTAGTGGTCCCGA-3′ (Reverse) | 111 bp |
| A2m    | 5′-CGAGCAAGAGCAATGACCT-3′ (Reverse) | 172 bp |
| Cps    | 5′-ACGATCCAGACATCTGGCG-3′ (Forward) | 166 bp |
|        | 5′-AGTTTGGAAGCTCAGGATG-3′ (Reverse) | 122 bp |
| Hpfs   | 5′-ATATGGCCGACACAACAA-3′ (Reverse) | 216 bp |
| Kng1   | 5′-AACCGGGCTTTCGGGAGA-3′ (Reverse) | 185 bp |
|        | 5′-TCTGAGGCAGGGGCTG-3′ (Reverse) | 125 bp |
| Serpica1c | 5′-GGATGGAGACGAGGCTT-3′ (Reverse) | 219 bp |
|        | 5′-GGATACGCGGATGATTC-3′ (Reverse) | 166 bp |
|        | 5′-CGGATGGAATGGTCCCGA-3′ (Reverse) | 172 bp |
|        | 5′-CGGTGGAGATCGTGGATGGA-3′ (Reverse) | 176 bp |
|        | 5′-CAGGAGCTGGAACAGTTTG-3′ (Reverse) | 211 bp |

MALDI-TOF/MS and Mascot search analyses identified a total of 10 genes (Table 2). Afp expression levels were similar between wild-type and 

| Gene   | Primer sequence | Size |
|--------|-----------------|------|
|        | 5′-TCCTAGGAGACGAGGCTT-3′ (Reverse) | 125 bp |
|        | 5′-GGATACGCGGATGATTC-3′ (Reverse) | 166 bp |
|        | 5′-CGGATGGAATGGTCCCGA-3′ (Reverse) | 172 bp |
|        | 5′-CGGTGGAGATCGTGGATGGA-3′ (Reverse) | 176 bp |
|        | 5′-CAGGAGCTGGAACAGTTTG-3′ (Reverse) | 211 bp |

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carcinoma (HCC), yolk sac tumors, and acute/chronic hepatitis [14,15]. The characteristic machinery of Afp repression has attracted considerable attention from researchers interested in transcriptional regulation [13,16,17]; however, despite over 50 years of research since its first discovery in liver cancer, the physiological functions of AFP still remain obscure [14,18]. The main function of AFP is considered to be the extracellular transport of small molecules including estrogens, fatty acids, and bilirubin [19,20], but AFP-deficient mice develop normally and thus AFP is dispensable for embryonic development [21]. Meanwhile AFP is required for fertility in female mice [21] (i.e. protection of the developing female brain from masculinization/defeminization by estrogens [22]) and may play important immune regulatory roles [19,20]. This is the first demonstration of AFP accumulation during juvenile development in CBS-deficient mice, a homocystinuria model that is widely utilized for homocysteine-related research [5–8,12,23]. Our results may be clinically relevant because markedly lower Cbs expression in 120 HCC specimens compared with

Fig. 1. Altered plasma protein profiles in juvenile Cbs−/− mice revealed by 2D DIGE proteomic analysis. Plasma samples from wild-type (WT) and Cbs−/− male mice (n = 3 each) at 2 (A), 1 (B), and 4 (C) weeks of age were analyzed comparatively. Representative fluorescent image in which plasma samples from WT and Cbs−/− mouse plasma was pseudocolored in green and red, respectively, are presented with approximate isoelectric points (pl) and molecular weights (kDa).
surrounding non-cancer liver cells was found to be associated with high tumor stage and serum AFP level [24], and serum AFP elevation was found in NAFLD patients [25].

Previous studies demonstrated that human AFP contains a single glycosylation site but its structure varies with developmental stage and disease state, probably by alternating glycosylation

Table 2
Differentially expressed plasma proteins between 2-week-old wild-type and Cbs–/– mice.

| Spot ID | Uniprot ID | Unigene | Protein (up or down regulated in Cbs–/–) | Mascot score | Sequence coverage (%) | Peptide matches | MWcalc | pIcalc |
|---------|------------|---------|----------------------------------------|--------------|----------------------|----------------|--------|--------|
| 1 a–d   | P02772     | Afp     | α-fetoprotein (up)                      | 218          | 64                   | 36/104         | 69,118 | 5.65   |
| 2       | P08226     | Apoe    | Apolipoprotein E (up)                   | 172          | 46                   | 20/36          | 35,901 | 5.56   |
| 3       | P06728     | Apoa4   | Apolipoprotein A-IV (up at 4 weeks)     | 145          | 61                   | 23/58          | 45,001 | 5.34   |
| 4 a–f   | Q61838     | A2m     | α-2-macroglobulin (down)                | 73           | 16                   | 14/39         | 167,116| 6.24   |
| 5 a–e   | Q61147     | Cpr     | Ceruloplasmin (down)                    | 150          | 31                   | 26/47         | 121,872| 5.53   |
| 6 a–d   | A0X035     | InhB    | Inter α-trypsin inhibitor, heavy chain 4 (down) | 100 | 30 | 22/79 | 104,765| 5.99 |
| 7 a–c   | Q01X72     | Hpx     | Hemopexin (down)                        | 115          | 42                   | 18/84         | 52,026 | 7.92   |
| 8 a–d   | O08677     | Kng1    | Kininogen-1 (down)                      | 112          | 26                   | 18/59         | 74,140 | 6.05   |
| 9       | P12261     | SerpinC | Anti-thrombin-III (down)                | 92           | 35                   | 16/49         | 52,484 | 6.10   |
| 10 a–d  | Q00896     | Serpin1c| α-1-antitrypsin 1–3 (down)              | 97           | 37                   | 11/41         | 45,966 | 5.25   |
| 11 a–c  | P29099     | Abg     | α-2-HS-glycoprotein (down)              | 72           | 13                   | 9/34          | 38,100 | 6.04   |
| 12       | P21614     | Ga      | Vitamin D-binding protein (down)        | 121          | 47                   | 16/61         | 55,162 | 5.39   |
| 13       | Q8K0E8     | Fgb     | Fibrinogen b chain (down)               | 174          | 60                   | 35/120        | 55,402 | 6.68   |
| 14 a–d  | Q8VC7M     | Fgg     | Fibrinogen γ chain (down)               | 212          | 71                   | 24/45         | 50,044 | 5.54   |
| 15 a–c  | Q80823     | Apoa1   | Apolipoprotein A-I (down)               | 195          | 50                   | 20/51         | 30,397 | 5.51   |
| 16 a–c  | P07724     | Alb     | Serum albumin (no change)               | 263          | 60                   | 31/72         | 70,700 | 5.75   |

A total 16 proteins identified from MALDI-TOF/MS analysis and Mascot searches are listed with their spot ID (in Fig. 1A), Uniprot ID, Unigene/protein names, Mascot score, sequence coverage, peptide matches, MWcalc (molecular weight calculated from identified protein sequence), and pIcalc (isoelectric point calculated from identified protein sequence).

* Protein has some variant spots and the representative data from spots with the highest Mascot score are shown.

Fig. 2. Increased α-fetoprotein (AFP) expression in 2-week-old Cbs–/– mouse plasma/serum/liver. (A) Two-dimensional PAGE/western blotting analysis of AFP variants in 2-week-old wild-type (WT) and Cbs–/– male mouse plasma (10 μL). (B) PAGE/western blotting analysis of AFP proteins in 2-week-old WT, (heterozygous) Cbs+/-, Cbs–/–, and Cth+/- mouse serum and liver (2.5 μg protein per lane). As for liver samples, GAPDH expression was examined as a loading control. Band intensities of ~79 kDa AFP proteins were densitometrically scanned and the relative values against the average AFP expression level (for serum) or the AFP/GAPDH ratio (for liver) in WT mouse samples were calculated. The representative band images are presented. Bar data show means ± SD (n = 3 each) and differences versus WT are significant at *P < 0.05 and **P < 0.01 by Student’s t-test. (C) PAGE/western blotting analysis of AFP proteins in embryonic day 15.5 (E15.5), postnatal day 0.5 (P0.5), P7.5, and P14.5 WT, Cbs–/–, and Cbs–/– mouse serum and liver (2.5 μg protein per lane). Postnatal repression of AFP expression was delayed in Cbs–/– mouse liver, and thus in Cbs–/– mouse serum.
Lens culinaris agglutinin (LCA)-reactive fraction of AFP (AFP-L3) has been considered as a more specific HCC marker [27]. In this study, we detected hyperaccumulation of all nine AFP variants in 2-week-old Cbs/C0/C0/C0 mouse plasma compared with respective wild-type samples (Fig. 2A), which was attributable to delayed repression of Afp expression in the liver (Fig. 2C). In contrast, expression of most other major plasma proteins was suppressed (Table 2 and Fig. 1A), at least partly, via transcriptional repression (Fig. 3). One plausible explanation is that increased AFP may bind and hold multiple endogenous ligands required for transcriptional activation of such liver proteins. This is because AFP belongs to a three-domain albuminoid gene family that currently consists of four members (AFP, albumin, vitamin D-binding protein, and α1-albumin) and binds steroids, fatty acids, bilirubin, retinoids, and heavy metals [19,20]. Indeed, vitamin D-binding protein, which binds/transport vitamin D and plays important roles in bone/calcium homeostasis [28], was downregulated in 2-week-old Cbs−/− mouse plasma (Table 2 and Fig. 1A). This downregulation could be related to osteoporosis and skeletal abnormalities found in Cbs−/− mice [29], although transgenic mice overexpressing human AFP were found to be generally normal [30], and hereditary persistence of AFP in two unrelated Japanese families exhibited no apparent phenotypes [31]. We reported previously about abnormal and decreased high-density lipoprotein contents in 2-week-old Cbs/C0/C0/C0 mouse serum [7], which may be associated with decreased apolipoprotein A-I levels (Table 2 and Fig. 1A).

In conclusion, we found transcriptionally regulated hyperaccumulation of AFP in fatty liver and plasma of juvenile Cbs−/− mice. Mice lacking methionine adenosyltransferase 1A also displayed...
both fatty liver and AFP accumulation [32], but our Ch^h^mice did not [9]; therefore, the methionine cycle/transsulfuration pathway may play important roles in epigenetic regulation of Afp.

Conflict of interest

The authors declare no competing financial interests.

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