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Journal of Nutritional Biochemistry 31 (2016) 38–44

Journal of Nutritional Biochemistry

Metabolic disturbances in plasma as biomarkers for Huntington's disease☆

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Received 17 June 2015; received in revised form 3 December 2015; accepted 8 December 2015

Abstract

Huntington's disease (HD), caused by expanded CAG repeats encoding a polyglutamine tract in the huntingtin protein, presents with a predominant degeneration of neurons in the striatum and cortex. Although a few studies have identified substantial metabolite alterations in plasma, the picture of plasma metabolomics of HD has not been clearly depicted yet. Using a global metabolomics screening for plasma from 15 HD patients and 17 controls, HD patient group was separated from the control group by a panel of metabolites belonging to carnitine, amino acid and phosphatidylcholine species. The quantification of 184 related metabolites (including carnitine, amino acid and phosphatidylcholine species) in 29 HD patients, 9 presymptomatic HD carriers and 44 controls further showed one up-regulated (glycine) and 9 down-regulated metabolites (taurine, serotonin, valine, isoleucine, phosphatidylcholine acyl-alkyl C36:0 and C34:0 and lysophosphatidylcholine acyl C20:3). To understand the biosynthetic alterations of phosphatidylcholine in HD, we examined the expression levels and activities of a panel of key enzymes responsible for phosphatidylcholine metabolism. The results showed down-regulation of *PCYT1A* and increased activity of phospholipase A_2 in HD leukocytes. These metabolic profiles strongly indicate that disturbed metabolism is involved in pathogenesis of HD and provide clue for the development of novel treatment strategies for HD.
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Keywords: Huntington's disease; Biomarkers; Metabolomics; Amino acid; Phosphatidylcholine; Phospholipase A_2; *PCYT1A*

1. Introduction

Huntington's disease (HD) is an autosomal dominant, progressive neurodegenerative disorder, characterized by an array of different psychiatric manifestations, cognitive decline and choreiform movements [1]. The causative gene mutation for HD is an unstable CAG trinucleotide repeat sequence encoding a polyglutamine (polyQ) tract in the huntingtin (HTT) protein [1], resulting in neuronal dysfunction and death predominantly in the striatum and cortex [2]. The polyQ expansion can cause a conformational change in the mutant protein leading to intranuclear and intracytoplasmic aggregates, which may subsequently lead to impaired proteasome activity [3], transcriptional dysregulation [4], oxidative stress [5], mitochondrial and metabolic dysfunction [5] and abnormal protein–protein interaction [6]. These abnormalities may disturb cellular metabolism and subsequently produce remarkable molecular fingerprints in various tissues [7–9]. Given that HTT is expressed ubiquitously [10], these unique molecular profiles may be detected not only in nervous but also in peripheral tissues. Searching for biomarkers in peripheral tissue, especially from blood, could be more accessible as indicators for the disease status monitoring.

Metabolomics is the quantitative measurement of a large number of low-molecular-weight molecules within a particular sample type and the organization of the data into formats for data mining and informatics [11]. A metabolic biomarker that predicts disease, measures progression or monitors therapy potentially could be a single molecule, as well as a pattern of several molecules. Recently, metabolic profiling has proved to be useful by several groups to study a number of diseases. Different analytical platforms have been applied to study potential metabolic biomarkers associated with neurodegenerative diseases including motor neuron disease [12], Parkinson's disease [13] and Alzheimer's disease (AD, reviewed by Ibanez *et al.*) [14]. The metabolic characterization of brain tissues, serum and urine of the R6/2 HD mice also highlights its usefulness to investigate disease pathogenesis and identify potential biomarkers [15]. The metabolomics analysis in the serum was able to distinguish the rat HD mice from the wild type [16]. Underwood *et al.* have found a number of HD-associated metabolic signatures in mouse and human, with changes in various molecules of fatty acid breakdown (glycerol and malonate) and in aliphatic amino acids [9]. However, a few variables, such as ethnic, dietary supplementation, smoking, tea and coffee consumption and medication, have shown to affect metabolite profiles, raising the need to reexamine the metabolic profiles of HD patients under more stringent environmental control. Herein, we analyzed the metabolic

☆ All authors are employers of Chang Gung Memorial Hospital and Chang Gung University and report no financial disclosures.

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http://dx.doi.org/10.1016/j.jnutbio.2015.12.001
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profiles in the plasma of HD patients under a homogeneous dietary condition and excluding unnecessary medication, using a combination of liquid chromatography time-of-flight mass spectrometer (LC-TOFMS) analysis and the AbsoluteIDQ p180 Kit. We found that HD patients displayed a significantly distinctive metabolic pattern composed of the changes in various markers of amino acids and phosphatidylcholines. To further delineate the metabolic details of correlated alterations in phosphatidylcholines, we examined the expression levels and activities of a panel of enzymes responsible for the metabolism of phosphatidylcholines. Our findings revealed several new biomarker candidates and potential therapeutic targets for HD.

2. Materials and methods

2.1. Ethics statement and study populations

This study was performed under a protocol approved by the institutional review boards of Chang Gung Memorial Hospital and all examinations were performed after obtaining written informed consents. The diagnosis of HD patients and presymptomatic HD (preHD) carriers was established by a neurological examination and genetic assessment showing expanded CAG repeats in the exon 1 region of the *HTT* [1]. Unified Huntington's Disease Rating Scale (UHDRS) was recorded for each patient [17]. The scale ranges (normal to most severe) of UHDRS include total motor score (0 to 124), independence score (100 to 10) and total functional capacity (13 to 0). HD and preHD carriers were recruited for hospitalization in Chang Gung Memorial Hospital under a standard protein diet composed of 15% of energy from protein, 30% from fat and 55% from carbohydrates with 33.3 kcal/kg per day for 5 days, which was matched to the dietary characteristic of adult Taiwanese [18]. Patients and controls were asked to discontinue the intake of nutritional supplements (including creatine and Q10), smoking, coffee and alcohols for at least 1 month before being enrolled in this study. Patients and controls with liver or renal dysfunctions, cardiac and pulmonary disease, infection, autoimmune diseases, malignancy or pregnancy were excluded. Blood samples for metabolite analysis were collected from HD patients and controls who were asked to be on fasting overnight for 12 h.

HD group displayed similar demographic and laboratory backgrounds (Table 1), including gender distribution, body mass index, blood levels of triglyceride, cholesterol, pre-prandial glucose and creatinine when compared to the controls. The age of preHD carriers (31.50±8.83 years) is significantly younger than controls (44.67±13.00 years) and HD patients (44.97±11.26 years), whereas the age of HD patients is not different from that of the controls in the quantification analysis.

2.2. Untargeted analysis of plasma global metabolites by LC-TOFMS

Plasma of 15 patients with HD and 17 healthy controls were obtained after 5 days of standard diet on the day 6 before breakfast (Table 1). To 50-μl plasma, 200 μl acetonitrile was added. The mixture was vortexed for 30 s, sonicated for 15 min and centrifuged at 12,000 rpm for 30 min. The supernatant was collected into a separate microcentrifuge tube. The pellets were reextracted once with 50% methanol. The aqueous methanolic supernatant and acetonitrile supernatant were pooled and dried in a nitrogen evaporator. Residues were suspended in 100 μl of 95:5 water/acetonitrile for LC-TOFMS analysis.

Liquid chromatographic separation was achieved on a 100 mm×2.1 mm Acquity 1.7-μm C8 column (Waters Corp., Milford, MA) using an ACQUITY TM ultra performance liquid chromatography (UPLC) system (Waters, USA). The column was maintained at 45°C and at a flow rate of 0.5 ml/min. Samples were eluted from LC column with a linear gradient: 0–1.25 min, 1–50% B; 1.25–2.5 min, 50–99% B; 2.5–5 min, 99% B; 5.1–7 min, 1% B for reequilibration. Solvent A was water and solvent B was acetonitrile; both solvents containing 0.1% formic acid. The eluent was introduced by positive electrospray ionization into the mass spectrometer (Waters QTOF Synapt). The desolvation gas was set to 700 L/h at 300°C, the cone gas was set at 25 L/h and the source temperature was set at 80°C. The capillary voltage and cone voltage were set to 3000 and 35 V, respectively. To maintain mass accuracy, sulfadimethoxine ($[M+H]^+=$ 311.0814) was used as the lock mass at a concentration of 60 ng/ml and a flow rate of 6 ml/min. For mass spectrometry (MS) scanning, data were acquired in centroid mode from 50 to 990 m/z and acquisition rate was set at 0.1 s. The mass chromatographic data were analyzed by Marker Lynx software (Waters) to generate a multivariate data matrix (retention time, m/z, ion intensities) for analysis by SIMCA-P software (version 13.0; Umetrics AB, Umea, Sweden). For identification of untargeted metabolites, MS/MS spectra were collected per 0.3 s, and the collision energy was ramped from 6 to 35 V.

2.3. Determining concentrations of targeted plasma metabolites

A total 82 plasma samples were analyzed with the AbsoluteIDQ p180 Kit (BIOCRATES Life Sciences AG, Austria) using the protocol in the AbsoluteIDQ user manual. The kit was used to quantify 184 metabolites within 5 compound classes. These metabolites include glycerophospholipids and sphingolipids (76 phosphatidylcholines, 14 lysophosphatidylcholines and 15 sphingomyelins), 19 biogenic amines, 40 acyl carnitines, 19 amino acids and hexose. All reagents used in this analysis were of LC-MS grade. This method involves derivatization and extraction of analyses from plasma, along with selective mass spectrometric detection and quantification *via* multiple reactions monitoring. Isotope-labeled internal standards are integrated into the kit plate filter to facilitate metabolite quantification. All reagents used in this analysis were of LC-MS grade. A quality control (QC) procedure was performed with QC samples provided in the kit. Different levels of QC samples (known as low, medium and high levels) were included in each sample plate and were analyzed along with test samples in the same plate. The values of coefficient of variation (standard deviation/mean×100%) of QC samples from four individual plates were analyzed and are shown in Supplementary Table 1. Each plasma sample of 10 μl aliquot was mixed with isotope-labeled internal standards and loaded onto the provided 96-well plate and then dried under nitrogen. Subsequently, 20 μl of 5% phenylisothiocyanate was added for amino acids and biogenic amines derivatization and was dried under nitrogen after a 20-min incubation. Extraction of the metabolites was then achieved using methanol containing 5 mM ammonium acetate. The extracts were analyzed using a Xevo TQ (Waters Corp., Manchester, UK) mass spectrometer equipped with a UPLC system. For the amino acids and biogenic amines, an LC-MS/MS method in positive mode was applied as described in the kit user manual. For flow injection analysis consisting of two 20-μl injections, we applied one for the positive-ion detection mode and one for the negative-ion detection mode. The MetIDQ software was used to control the entire assay workflow, from sample registration to automated calculation of metabolite concentrations.

Table 1
Demographic characteristics and blood biochemical parameters of the controls and the patients with HD

	Global screening		Quantification using the AbsoluteIDQ p180 Kit		
	Controls (n=17)	HD (n=15)	Controls (n=44)	PreHD (n=9)	HD (n=29)
Age (years)	43.88±13.06	42.20±11.53	44.67±13.00	31.50±8.83 *	44.97±11.26
Male (%)	11 (64.71)	9 (60.00)	21 (57.45)	3 (37.50)	13 (44.83)
Body mass index	23.36±2.82	22.00±2.75	23.86±2.97	21.45±2.55	22.31±2.31
Triglyceride (mg/dl)	94.73±24.04	72.87±25.72	117.72±43.40		87.38±49.77
Cholesterol (mg/dl)	189.83±25.14	184.53±18.95	201.80±31.19		194.55±24.59
Preprandial glucose (mg/dl)	95.87±12.51	90.13±6.11	102.67±9.39		94.68±10.67
Creatinine	0.92±0.16	0.87±0.16	0.95±0.21		0.91±0.19
HD duration (years)		4.6±3.03			3.90±2.78
Expanded CAG repeat number		46.67±6.52		41.63±2.39	44.86±5.30
Drugs (%)					
Dopamine antagonist	0 (0)	8 (53.33)	0 (0)	0 (0)	18 (62.07)
SSRI	0 (0)	5 (33.33)	0 (0)	0 (0)	7 (24.14)
Amantadine	0 (0)	5 (33.33)	0 (0)	0 (0)	8 (27.59)
UHDRS					
Motor score		26.13±17.31		0	25.24±18.72
Independence scale		84.00±18.18		100	83.79±21.08
Functional capacity		9.6±3.42		13	9.86±3.75

PreHD, HD carriers; SSRI, selective serotonin reuptake inhibitors. Scale ranges (normal to most severe) include motor score (0 to 124), independence scale (100 to 10) and functional capacity (13 to 0).
* Statistically significant in comparison with HD patients and controls. $P<.05$.

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