

Research on plasma metabolomics of hypertensive rats with liver–fire hyperactivity syndrome

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This paper reports a study of plasma metabolites in hypertensive rats with syndrome of liver-fire hyperactivity in order to elucidate the biological basis of hypertension with syndrome of liver-fire hyperactivity. ¹H-NMR method was used to identify and compare the small-molecule endogenous metabolites in the plasma of spontaneous hypertensive rats with liver-fire hyperactivity syndrome and normal rats. The results of ¹H-NMR pattern recognition showed that hypertensive rats with liver-fire hyperactivity were significantly different from those of the control group. The results of ¹H-NMR analysis indicated that there are close relationships between plasma metabolites and TCM syndromes, which is expected to be an objective diagnostic indicator in the further studies.

Keywords: Plasma metabolomics, Hypertension, Liver–fire hyperactivity syndrome

INTRODUCTION

The high incidence, high mortality and high morbidity of hypertension have great influence on human health [1-3]. Hypertension is the leading cause of cardiovascular disease and premature death worldwide [4]. More than 60% of the risk factors for hypertension are associated with metabolic disturbances. Metabolic abnormalities increase the risk for hypertension and cause high blood pressure. Improving metabolic disturbances is beneficial for hypertension treatment [5]. Therefore research on relationship of clinical classification of hypertension and the metabolic abnormalities become very important since traditional Chinese medicine lies primarily in "treatment based on syndromes differentiation of the patients" [6]. Although recent research indicated that the pathogenesis of hypertension is closely related to biochemical and metabolic disorders, the pathogenesis and mechanism of hypertension are still unclear [7]. Liver-fire hyperactivity is one of the most common syndromes of hypertension. On the basis of our previous study we found that spontaneous hypertensive 14-18 weeks old rats mainly manifested the liver-fire hyperactivity syndrome [8, 9]. Therefore, in this study ¹H nuclear magnetic resonance (¹H-NMR) technology [10-11] was used

to comprehensively identify and analyze the differences of small endogenous molecule metabolites in plasma of hypertensive rats with liver-fire hyperactivity syndrome, to find the metabolic markers and provide a basis for its objective diagnosis and treatment.

EXPERIMENTAL

Instruments and reagents

Japan's Softron BP-98A rat noninvasive tail vein arterial pressure meter; open field reaction box; animal behavior record analysis system (EthoVision 3.1, Noldus Netherlands); colorimetric card: Casmtch Color Card (BEAR Medic Japan). CNU-VNMRS 600MHz superconducting Fourier transform nuclear magnetic resonance spectrometer (Varian, USA), superconducting magnet with a field strength of 14.1T (600MHz), equipped with a ¹H/¹³C/³¹P/¹⁹F quad core probe, gradient inversion probe (109Ag-³¹P) (Thermo Revo Value Plus ULT-2586-4-V, Thermo Corporation, USA), and a small high-speed refrigerated centrifuge (Sigma-15PK, SIGMA, Germany). Reagent: 98% heavy water solution (D₂O) purchased from Beijing Jingju Chemical Industry & Trade Co., Ltd.

General experimental procedure

A total of 20 rats, 10 spontaneously hypertensive rats (SHR), 13 weeks old, male; and 10 Kyoto Wistar rats, 13 weeks old, male, clean grade (all purchased from Beijing Weitong Lihua Experimental Animal Technology Co., Ltd.) were used. The rats were kept

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in the animal room of the Research Center of Beijing University of Traditional Chinese Medicine at ambient temperature (20-25 °C). Light rhythm was 12L: 12D (6:00-18:00) with conventional food feeding. Preliminary experiments were performed in our group [8,9], through comprehensive and dynamic collection of spontaneous hypertensive rats macroscopic characterization and behavioral tests (open-field test, irritability score, rotation tolerance time determination). About 2 ml blood samples were *in vitro* collected, centrifuged at 4 °C, and stored at -80 °C.

1H-NMR data acquisition and processing

The supernatant was centrifuged at 4 °C for 10 min and 200 µl of the supernatant was added to a 1.5-ml EP tube. To the EP tube, 400 µl of a heavy water solution was added and the mixture was centrifuged at 4 °C, 14000 r/min speed for 10 min; 550 µl of the mixture were transferred to the nuclear magnetic tube. Plasma samples were analyzed using a Varian VNMRs 600M superconducting Fourier transform nuclear magnetic resonance spectrometer to observe the relaxation of small molecule signal (CPMG) pulse sequences. The saturation time was 2 s, the mixing time was 0.15 s, the spectral width was 8012.8 Hz, the number of sampling points was 32 k, the number of accumulation was 64, and the pre-saturation frequency and center frequency were all in the water peak position. The free induction decay (FID) signal was converted to a one-dimensional 1H-NMR spectrum by 32-k Fourier transform. The 1H-NMR spectra were processed using MestReNova software. (4.7 to 5.2 ppm); automatic adjustment of the phase and baseline; lactic acid as the chemical shift reference peak, set to 1.33 ppm; 0.5-5.5 ppm (CPMG experiment) range of sub-integral, each 0.001 ppm. The integral was normalized by the total integral intensity of each spectrum and scaled by the median method, the resulting data output was saved in Excel format.

1H-NMR data analysis

The data were processed in SIMCA-P 12.0 software (Umetrics, Umea, Sweden). PCA was used to calculate the principal components (PC), and the plasma metabolic components were analyzed by orthogonal partial least-squares discriminant analysis (PC-PCA) using PC. The data were analyzed by Pareto scale (PCA) OPLS-DA to obtain the OPLS-DA scatter plot and loading plot of the corresponding plasma samples. The data of the metabolites of each group of blood samples were visually displayed and the characteristic difference metabolites were identified. OPLS-DA is a newly developed data analysis method. Orthogonal signal correction (OSC) and partial least squares (PLS) can be combined to modify PLS. In the metabolomics

study. The color linear load map was drawn by MATLAB software, and the different metabolites of each group were visually expressed. The difference was statistically significant ($P < 0.05$).

RESULTS AND DISCUSSION

Spontaneously hypertensive 14-18 weeks old rats manifesting liver-fire hyperactivity syndrome

Spontaneously hypertensive 14-18 weeks old rats have the following characteristics: significantly increased irritability score, shortened rotation tolerance, increased number of small vessels in ear and claw, increased image R-value of tongue and claw, dry tongue, dry and hard stool, difficult and prolonged defecation, significantly increased total activity distance and increased grid crossing times in open field test. SHR rats at the age of 14-18 weeks manifested liver-fire hyperactivity according to the diagnostic criteria of hypertension with liver-fire hyperactivity syndrome [9].

Plasma 1H-NMR spectrum metabolite analysis of hypertensive Wistar rats and normal rats with syndrome of liver-fire hyperactivity

The endogenous differences in the 1H-NMR spectra of hypertensive rats with liver-fire hyperactivity were compared with those of the normal control group. The t-test/non-parametric test was used for statistical analysis after normalized treatment of the integral values of the small molecule substances. There are 21 substances, including tryptophan, sarcosine, glycerol, choline, urea, formic acid, isoleucine, leucine, methionine, β -hydroxybutyric acid, β -glucose, α -glucose, β -hydroxyisobutyric acid, glycoprotein, glutamine and carnitine (see Table 1).

Results of PLS-DA analysis of spontaneously hypertensive rats with liver-fire hyperactivity

The OPLS / O2PLS-DA integral matrix and the PLS-DA integral matrix of the plasma CPMG metabolites in spontaneously hypertensive 18 weeks old rats with liver-fire hyperactivity syndrome and in the normal group were measured. The results are shown in Figures 1 and 2. Spontaneously hypertensive rats with liver-fire hyperactivity and normal rats were completely separated along the t (1) axis of the first principal component, with no obvious cross or overlap. Corresponding load matrix diagram (Figure 3) and colored linear load map (Figure 4) intuitively expressed the different metabolic substances in the plasma of spontaneously hypertensive rats with liver-fire hyperactivity and normal rats, such as tryptophan, thiazine, leucine, histidine, proline, α -glucose, β -glucose, β -hydroxyisobutyrate, glutamate.

Table 1. Comparison of plasma metabolites of spontaneously hypertensive rats with liver-fire hyperactivity syndrome

Metabolites	Ascription	δ (1H)	Multiplicity	p
Tyrosine	CH2	3.20	ABX	0.003
Trimethylamine-N-oxide	N(CH3)3	3.27	S	0.012
Myo-Inositol	H5	3.28	T	0.009
Tryptophan	CH2	3.49	ABX	0.034
Sarcosine	CH2	3.61	S	0.012
Glycerol	CH2	3.65	ABX	0.038
Choline	OCH2	4.07	M	0.011
Urea	NH2	5.78	S	0.019
Formate	CH	8.46	S	0.000
Isoleucine	δ -CH3 , γ -CH2 , α -CH	0.94,1.26,3.68	t , m , d	0.009
Leucine	δ -CH3 , α -CH	0.96,0.97,3.73	T	0.012
Methionine	S- CH3 , α -CH	2.14 , 3.86	s	0.006
Acetoacetate	CH3 , CH2	2.29 , 3.45	s	0.003
β -Hydroxybutyrate	CH	2.31 , 4.16	ABX	0.000
Phenylalanine	b-CH2	3.13 , 3.28	m	0.013
β -Glucose	C-H4 , C-H3 ,	3.4 ,3.47 ,3.49	t , ddd	0.030
α -Glucose	C-H2 ,C-H6 ,C-H5 ,CH1	3.53,3.84,5.23	ddm , ddd , d	0.011
β -Hydroxyisobutyrate	CH3	1.20	d	0.000
Glycoproteins	CH3	2.05	s	0.006
Glutamine	b-CH2	2.142	m	0.004
Carnitine	CH2(COO)	2.44	dd	0.045

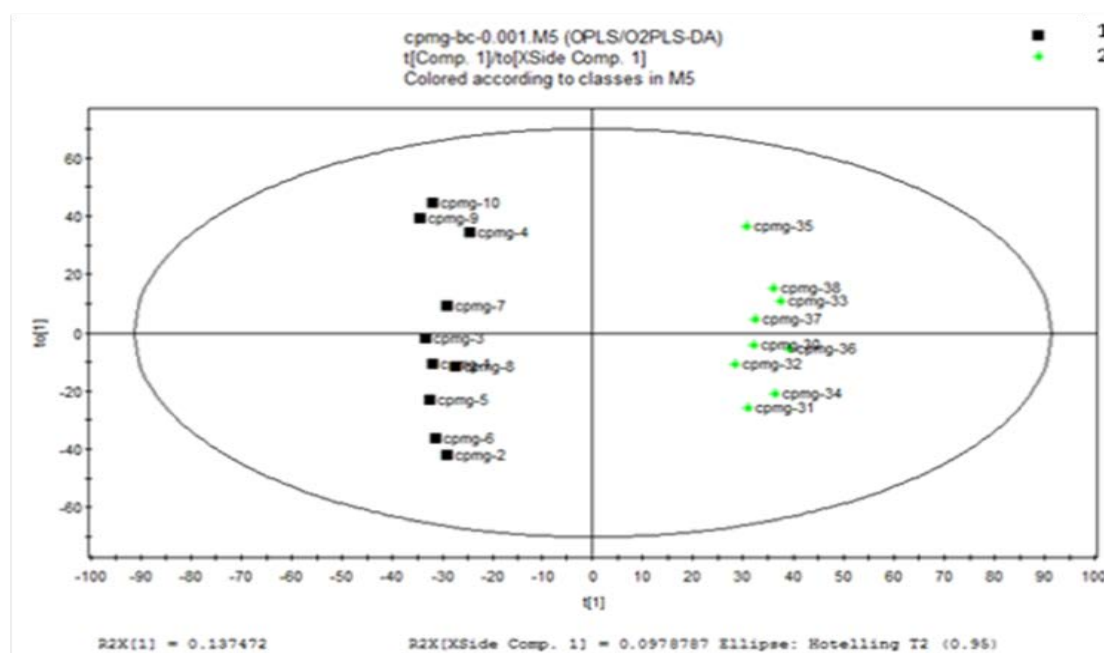


Fig. 1. OPLS/O2PLS-DA score graph of plasma samples of spontaneously hypertensive rats with liver-fire hyperactivity and normal rats. Note: 1= group of spontaneously hypertensive rats with liver-fire hyperactivity , 2= normal group, Wistar rats

At present, metabolomics-based technique has been demonstrated in the process of TCM syndrome research [12], which showed significant advantages compared to other techniques in the past. Although there are few works applying metabolic methods in the study of hypertension, with the development of metabolomics, the combination of metabolomics and TCM as a whole system thinking will provide

more efficient techniques and methods for standardization of TCM syndrome differentiation, diagnosis and basic research of syndrome biology. In this study, metabolomics techniques were used to explore the differential metabolites of plasma in spontaneously hypertensive rats with liver-fire hyperactivity and normal rats, and to further explore the biological basis of liver-fire hyperactivity.

The results of this study suggested that the different metabolites in the plasma of spontaneously hypertensive rats with liver-fire hyperactivity are tryptophan, tyrosine, leucine, histidine, proline, α -glucose, β -glucose, glutamic acid. Peripheral tryptophan exists in two forms in circulating blood, one is bound, TRP relaxation and albumin in the blood binding, fatty acid, FA can be competitive with albumin; the second is free type that is not bound to albumin, which can be transported through the blood-brain barrier. To penetrate the blood-brain barrier f-TRP must rely on a special carrier, which in turn can be combined with other neutral amino acids, especially branched-chain amino acids (BCAA), including leucine, isoleucine and valine. Therefore, the concentration of f-TRP in the brain depends on the concentrations of f-TRP and BCAA in the plasma and their ratio, that is, the increase of 5-HT synthesis in the brain is closely related to the increase in f-TRP in the blood [13]. Central 5-HT is the most potent vasoconstrictor in the brain circulation. It is formed by the hydroxylation of L-tryptophan through mitochondrial tryptophan hydroxylase (TPH) to form 5-hydroxytryptophan (5-HTP) [14],

5-hydroxytryptophan is transported through the cytoplasm of L-aromatic amino decarboxylase (ADD) decarboxylation to produce 5-HT. In addition, being recovered in the synaptic cleft, 5-HT is also oxidized by monoamine oxidase to dehydroxylate and form 5-oxindole acetaldehyde, which is then inactivated by aldehyde dehydrogenase oxidation to 5-hydroxyindole acetic acid (5-HIAA) [15]. The concentration of 5-HT in the brain affects the activity of tryptophan hydroxylase, and thus plays a feedback self-regulatory role in 5-HT. In this study, the results of 1H-NMR analysis showed that the content of tryptophan increased, and the increase in plasma tyrosine level in spontaneously hypertensive rats with liver-fire hyperactivity may be a substitute for their irritability indicators of increased irritability score. Open-field activity increased, because tyrosine is one of the precursors of neurotransmitters, which can increase the body fluid neurotransmitters, especially dopamine and norepinephrine content. Emotional impact is mainly reflected in the high stress of the crowd [16], while the literature reported dopaminergic hyperactivity, the animal will quickly explore the activities of a substantial increase in exercise.

Due to the limitation of current metabolomics techniques, still many metabolites cannot be identified, although some have significantly different content of compounds. However, it is certain that there must be some relationship between these specific different metabolic groups and syndromes, which can be expected to become

indicators of objective diagnosis of syndromes, some of which may be eventually determined as candidate biomarkers.

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