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ORIGINAL

Metabonomic Analysis of Urine from Chronic Unpredictable Mild Stress Rats Using Gas Chromatography–Mass Spectrometry

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Abstract Depression is a prevalent complex psychiatric disorder and its pathophysiological mechanism is not yet well understood. In this study, we investigated the metabolic profiling of urine samples from chronic unpredictable mild stress (CUMS) rats to find potential disease biomarkers and research pathology of depression. Metabolome in urine was analyzed using gas chromatography/mass spectrometry (GC/MS) in conjunction with multivariate statistical techniques. The urine samples of male Sprague–Dawley rats were collected at different time points and then were derivatized by methoximation/silylation. Clear separation between the model and control group was achieved, and 15 metabolites were identified, which suggested that the depressed state may be related to neurotransmitter, energy metabolism and immunity. The time-dependent trajectory of metabolites pattern revealed that the maximum biochemical change happened on the 21st day, which was consistent with the results of behavior tests. The study suggested that the metabonomic approach could be used as a potentially powerful tool to investigate the biochemical change in certain physiopathological conditions, such as depression, as an early diagnostic means.

Keywords Gas chromatography/mass spectrometry · Depression · Metabonomic · Urine · Time-dependent trajectory

Introduction

Depression is one of the most common psychiatric disorders in the world. It is a major cause of disability, suicide and physical disorders [1]. It amounts to 12.3% of the global burden of disease and has been predicted to rise up to 15% by 2020 [2]. Chronic unpredictable mild stress (CUMS), a well-validated animal model, has been used widely for studying clinical depression as well as evaluating antidepressant effects of diverse drugs [3, 4]. Much of the work has been done successfully in individual gene expression, protein structure and function, as well as biochemical studies on sympathetic nervous system such as hypothalamic–pituitary–adrenocortical axis, and noradrenergic and immunological systems [5–8]. However, little is known about the change of the whole metabolome in organisms during the pathological procedure.

Metabonomic approach is a high-throughput one. Recently, it has been successfully applied to analyze various biological samples. Combined with multivariate statistics, it can extract meaningful biological information from the resultant complex and huge data sets [9, 10]. It can provide much valuable information on stimuli-induced biochemical perturbations, with metabolic profiles carrying mechanism-

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related information. The approach can be regarded as complementary to genomics and proteomics approaches [11, 12]. It has been increasingly used as a versatile tool for the discovery of molecular biomarkers in many areas such as in the diagnosing or prognosing clinical diseases, exploring the potential mechanism of diverse diseases and assessing therapeutic effects of drugs [13–15]. GC–MS has long been used for metabolic profiling study due to its high sensitivity, reliability and the ease of metabolome identification [16]. Combined with the easily accessible database of NIST (http://www.nist.gov), GC–MS has gained more application in different fields [17]. Metabonomic analysis generates large and complex data sets. Therefore, chemometric analysis has become an integral part of metabolic profiling techniques due to its ability to provide interpretable models for complex intercorrelated data [4]. Multivariate projection methods allow the identification of groups of variables that are interrelated via phenomena that cannot be directly observed. Partial least squares discriminant analysis (PLS-DA) or (orthogonal partial least squares linear discrimination analysis) OPLS-DA can obtain a list of potential biomarkers, which are statistically significant and which separate one class from another [4]. In this study, PLS-DA and OPLS-DA were utilized.

Although urine sample has its disadvantages, such as high salt concentration, it has been heavily used in metabonomic studies because of being minimally invasive to animals or human and primarily revealing the overall metabolic state of the given organism. In addition, the analysis of urine samples in different time points can get the metabolic change of time-related trajectory because of the easily dynamic samples' collection at different time points [18]. The primary goal of this work is to characterize metabolic abnormalities in urine and the changes in its time-related trajectory. GC/MS was applied to reveal the metabolic profiling of the urine samples from both the control group and the CUMS group. The time-related trajectory of metabolites' pattern during the model building period is illustrated to dynamically monitor the response of CUMS, and to find the metabolic variations with time. Endogenous metabolome for discrimination between the CUMS and control groups have been found to be potential urine biomarkers for depression. This work, which will expand our understanding of molecular mechanism for depression, is a descriptive study on metabolome detected in the urine of a rodent model, which may reveal valuable information for the early diagnosis of depression.

Materials and Methods

Reagents and Animals

Pyridine, acetonitrile, *N*-heptane and methoxylamine hydrochloride (*O*-methyl hydroxylamine) were of analytical grade and obtained from China National Pharmaceutical Group Corporation (Shanghai, China). *N*-Methyl-*N*- (trimethylsilyl) trifluoracetamide (MSTFA) containing 1% trimethylchlorosilane (TMCS) was purchased from Pierce Chemical Company (Rockford, USA). *N*-Tetracosane, purchased from Johnson Matthey Company (Shanghai, China), was used as an internal quality standard. Alanine, valine, isoleucine, proline, glycine, serine, threonine, glutamic acid, phenylalanine, fructose, galactose, glucose, tyrosine and tryptophan were purchased from Solarbio (Shanghai, China) and used as standard substances.

The animal experiments were approved by national legislations of China and local guidelines. A total of 16 male Sprague–Dawley (SD) rats (200 ± 20 g) from the experimental animal Center of The National Institute for the Control of Pharmaceutical and Biological Products were employed in this study (No. SCXK2005-0004).

Chronic Unpredictable Mild Stress Procedure

After 2 weeks of habituation, all the rats were divided into the following two groups, CUMS and control groups ($n = 8$), according to the body weights and behavior scores in the open-field experiment. The CUMS procedures include nine different kinds of stressors, which are provided in the Supplementary materials.

Behavior Test

Open-field test and sucrose preference test were conducted as previously described [19]. The experimental details and statistical analysis are provided in the Supplementary materials.

Sample Collection and Preparation

Urine samples were collected overnight (12 h) in metabolism cages from all the rats on the 0, 7th, 14th and 21st day. Sodiumazide was added to the collection vessels as an antibacterial agent. After centrifugation at 5,000*g* for 10 min to remove residues, urine samples were immediately stored in aliquots at −80 °C until GC/MS analysis.

Sample preparation for GC/MS analysis was based on methods developed for targeted GC/MS analysis of human urinary metabolome [20, 21]. In brief, 150 μL of urine was incubated with 30 units of urease (Type C-3, Sigma) at 37 °C for 15 min. Then, 600 μL of methanol was added, mixed and centrifuged to precipitate protein; 300 μL of the supernatant was vacuum dried. The residue was chemically derivatized to increase the volatility and thermal stability, using 30 μL *O*-methylhydroxylamine (15 mg/mL in pyridine) at 70 °C for 1 h to enhance oxime formation, then 50 μL MSTFA + 1% TMCS (Sigma) at 40 °C for 90 min

to enhance trimethylsilylation. The final solution was vortex mixed with 1,200 μL heptane (0.1 mg/mL tetracosane dissolved in heptane).

GC/MS Method

GC/MS analysis was performed using a Polaris Q ion trap mass spectrometer (Thermo Fisher Scientific Inc., USA). Chromatography was performed on a DB-5MS capillary column (30 m × 250 μm i.d., 0.25 μm film thickness; 5% diphenyl cross-linked 95% dimethylpolysiloxane; Agilent J&W Scientific, Folsom, CA, USA). Helium carrier gas was used at a constant flow rate of 1 mL/min and 1.0 μL derivatized samples was injected into the GC/MS instrument. To acquire a well separation, the column temperature was initially maintained at 60 °C for 3 min, and then increased from 60 to 140 °C at a rate of 7 °C/min for 4 min. Then, the column temperature was increased to180 °C at 5 °C/min for another 6 min. After that, the temperature was increased to 280 °C at 5 °C/min, and held for 2 min. The injection, interface and source temperatures were set at 260, 280 and 200 °C, respectively. After a solvent delay of 9 min, MS detection was implemented with electron ionization mode (electron energy of 70 eV) and full scan mode (*m/z* 50-650).

Identification of the Endogenous Metabolome

All collected urine samples were analyzed, and low molecular weight metabolomes were represented as the chromatographic peaks in the GC total ion current (TIC) chromatograms. Peaks with intensity higher than tenfold of the signal-to-noise (S/N) ratio were recorded and integrated. EI-MS spectra of these peaks were interpreted using AMDIS (version 2.1, DTRA/NIST, USA) software, and identification of metabolites was based on the NIST library 2005, some of which were further confirmed using the commercially available standards by comparing their MS spectra and retention times.

Data Analysis

All the GC–MS raw files were converted to NetCDF format via Xcalibur (Thermo Fisher Scientific Inc., USA) and subsequently processed by the XCMS toolbox (http://metlin.scripps.edu/download/) using XCMS's default settings with the following exceptions: xcmsset (full width at half-maximum: fwhm = 4; S/N cutoff value: snthresh = 10, max = 20), group (bw = 10). The resulting table was exported into Matlab software 7.0 (The MathWorks, Inc.), where normalization was performed prior to multivariate analyses. The resulting two-dimensional matrix involving peak index (RT-m/z pair), sample names (observations) and normalized peak area percent was introduced into the SIMCA-P 11.0 software package (Umetrics AB, Sweden), where PLS-DA, OPLS-DA and VIP statistics were performed to show the possible presence of cluster and to extract novel potential biomarkers.

Results and Discussion

Effect on Open-Field Activity Scores, Body Weight and Sucrose Preference Test

Open-field test, body weight and sucrose preference test were measured during the experimental period. In these tests, there were significant differences between the rats in the CUMS and control groups (Table 1). After 3 weeks of experiment, rats in the CUMS group showed a significant decrease in the number of rearing and crossing ($P < 0.01$), in the sucrose preference test ($P < 0.01$) and also in the body weight ($P < 0.01$) compared with the control group, indicating the stress-related effects on the rats.

In summary, the marked decrease in the body weight gain, sucrose preference percentage, crossing and rearing numbers, as well as the significantly increased immobility time [22, 23], all similar to the clinical symptoms of depression in humans, suggested that rat depression models were achieved after 3 weeks of CUMS treatment.

GC/MS Spectra of the Two Groups and the Metabolic Profiling of Urine

Urine metabolic profiles of six CUMS rats and six control rats were obtained by GC/MS with the method as described above. Typical GC/MS total ion current (TIC) chromatograms of urine sample of the control group and the model group are illustrated in Fig. 1. Visual inspection of these spectra revealed significant differences in the TIC profile between control and model groups, indicating that the endogenous metabolite levels were perturbed by CUMS. Many peaks representing components with different concentration levels were present in the GC/MS spectra.

The identification of endogenous metabolome was based on comparing with the corresponding standards according to their retention times and mass spectra characteristics or searching the mass spectral database library NIST 2005. In all, 43 metabolites were identified in the urine profiling in this study (shown in Fig. 2), including amino acids, fatty acids, sugars and organic acids. The peaks in TICs of the urine samples represented the endogenous metabolome in urine. Therefore, each TIC could be considered as a fingerprint of endogenous metabolome in urine.

Table 1 The dynamical changes of behavior scores of healthy control and model group

Test items	0 days	7 days	14 days	21 days
Body weight (g)				
NS	239.81 ± 9.92	284.56 ± 13.50	294.81 ± 32.80	314.12 ± 28.28**
MS	235.56 ± 18.16	264.75 ± 25.76	268.18 ± 26.75	262.13 ± 24.69
Sucrose preference (%)				
NS	79.69 ± 32.21	76.84 ± 2.83	76.71 ± 10.42	79.00 ± 7.07**
MS	73.73 ± 11.83	78.29 ± 5.35	66.50 ± 7.07	54.29 ± 4.89
Open-field test				
Crossing numbers				
NS	71.50 ± 37.96	63.00 ± 40.41*	55.00 ± 28.00*	47.00 ± 12.06**
MS	90.13 ± 17.43	20.1 ± 14.43	19.37 ± 11.41	13.87 ± 9.53
Rearing numbers				
NS	11.66 ± 6.83	10.00 ± 7.48*	9.00 ± 5.04*	5.66 ± 2.42**
MS	13.30 ± 4.52	2.20 ± 2.74	2.87 ± 2.41	0.67 ± 0.81
Immobility time (s)				
NS	18.60 ± 44.42	49.32 ± 10.23	18.88 ± 23.59**	10.52 ± 19.12**
MS	0.14 ± 0.39	70.23 ± 54.59	129.25 ± 59.95	159.11 ± 84.8

The behavior scores in the control group (NS) and CUMS model group (MS) were expressed as mean ± SD ($n = 8$). Compared with the CUMS group: * $p < 0.05$, ** $p < 0.01$

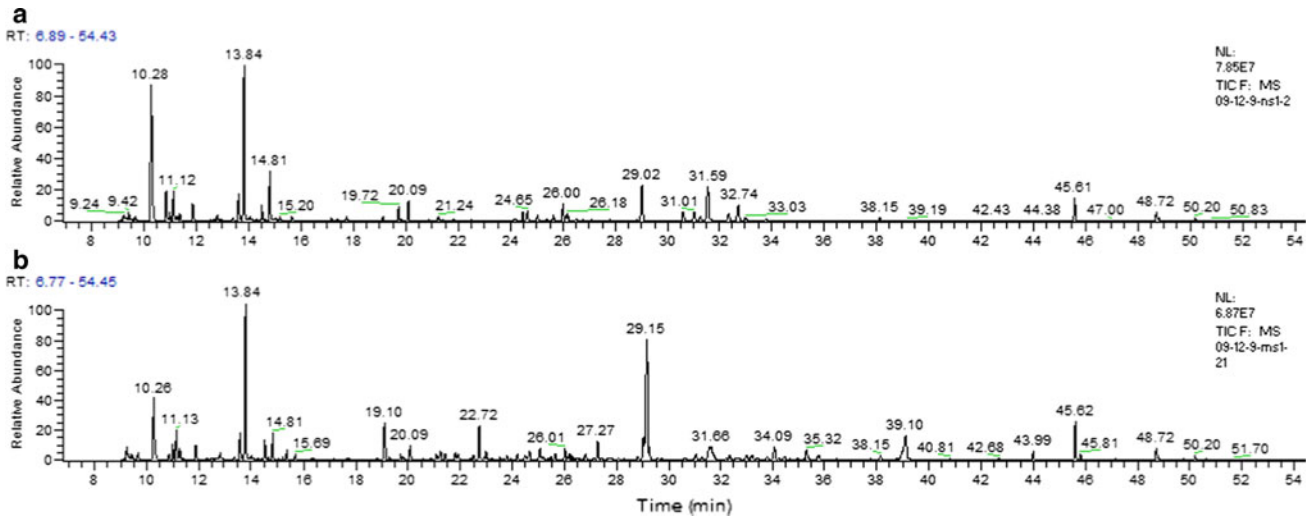

Fig. 1 Typical GC/MS total ion current chromatograms (TIC) of the urine from rats in **a** the control group and **b** the chronic unpredictable mild stress (CUMS)-treated model group on the 21st day

Multivariate Statistical Analysis and Potential Biomarkers

PLS-DA was applied to maximize the metabolites' difference between the model group and control group on the basis of the GC/MS spectra. The scores plot reveals any inherent clustering of groups of data, based purely on the closeness or similarity of their input coordinates. Thus, the analysis provides a convenient and objective means of visualizing groups and classifying them. The PLS-DA score plot showed a better discrimination with Q^2 value of 0.989 ($R^2X = 0.826$, $R^2Y = 1$), suggesting that the model was reliable and good for prediction. The score plot clearly distinguishes the model group from the control group (Fig. 3d).

These findings suggested that urine metabolic pattern was significantly changed under CUMS treatment. After 21 days' stimuli, rats in the model group could be distinguished from those in the control group based on urine samples. The difference between the model and control groups was more remarkable than the intra-group difference. It showed that the urinary metabolic pattern was significantly changed in the model group and the CUMS was built successfully, which was consistent with the result of behavior tests.

The important variables accountable for such significant separation could be extracted from loadings plot or

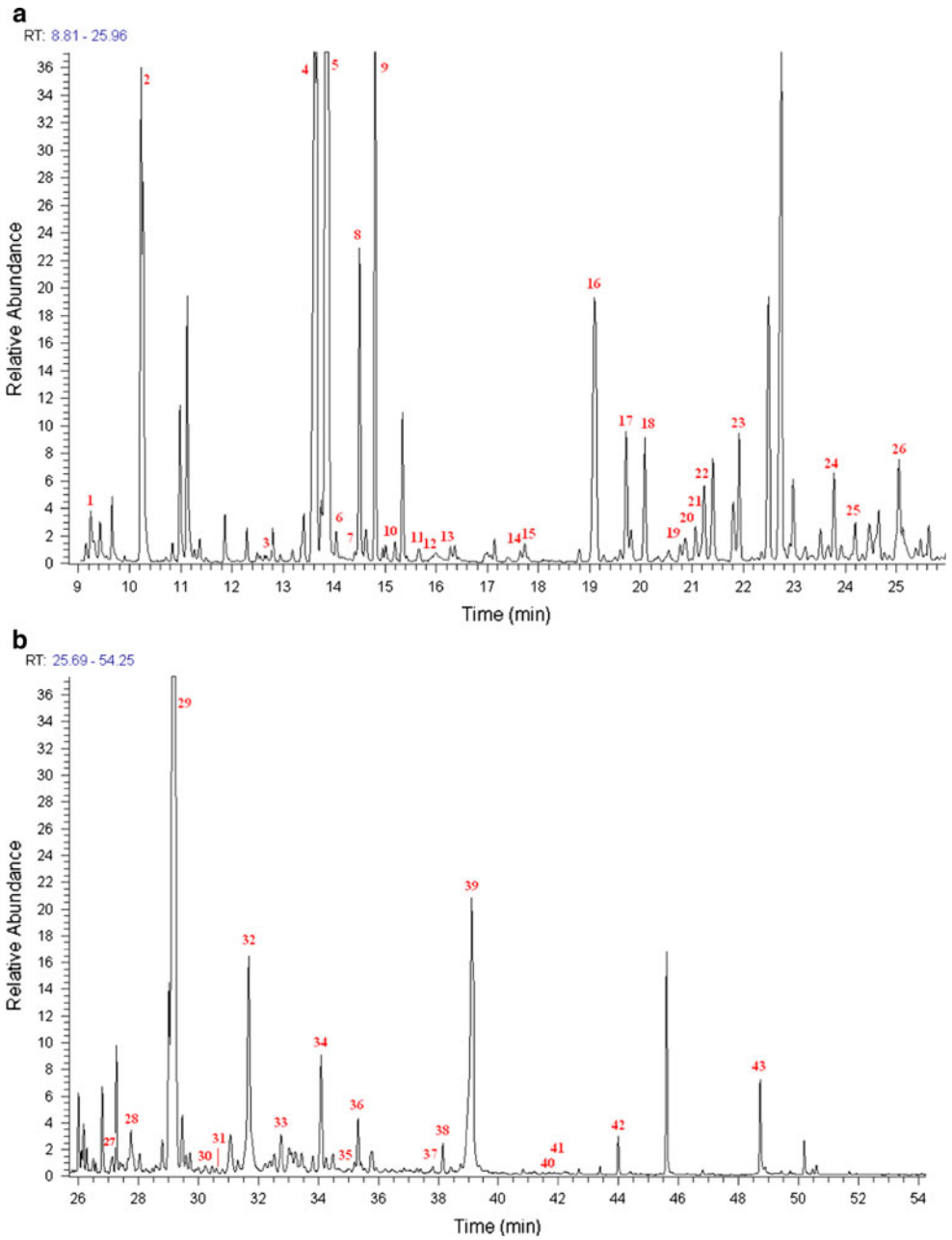

Fig. 2 Chromatographic profiles of the derivatized urine. *Peaks 1* lactic acid, *2* alanine, *3* valine, *4* benzoic acid, *5* urea, *6* phosphoric acid, *7* isoleucine, *8* glycine, *9* butanedioic acid, *10* 2,3-dihydroxypropanoic acid, *11* fumaric acid, *12* serine, *13* threonine, *14* β-alanine, *15* 3,4-dihydroxypropanoic acid, *16* 2-aminopropanoic acid, *17* malic acid, *18* threitol, *19* aspartic acid, *20* pyroglutamic acid, *21* glutamine, *22* 2,3,4-trihydroxybutyric acid, *23* ketoglutaric acid, *24* glutamic acid, *25* phenylalanine, *26* ribose, *27* aconitic acid, *28* hydroxyphenylpropionic acid, *29* isocitric acid, *30* deoxyglucose, *31* fructose, *32* glucose, *33* Sorbitol, *34* gluconic acid, *35* indoleacetic acid, *36* glalactonic acid, *37* hexadecanoic acid, *38* myo-inositol, *39* linoleate, *40* oleate, *41* uric acid, *42* uridine, *43* glucopyranoside

VIP statistics of PLS-DA, respectively. According to the criterion for VIP statistics, variables with VIP value >1.0 are considered as candidate biomarkers. But in the VIP list, some metabolites showed great confidence intervals, suggesting that their contribution to the PLS-DA model might be caused by analytical variation. Such metabolites were excluded from the list. Finally, 15 metabolites were generated as biomarker candidates: hexadecanoate, aconitate, succinate, isocitrate, glycine, glutamic acid, glucose, ribose, valine, aspartic acid, serine, phenylalanine, oxoglutaric acid, indoleacetic acid (IAA) and β-alanine. These metabolites are given in Table 2 together with the PLS-DA correlation coefficients indicating the relative contributions of potential markers to the stimuli-perturbed profiles. The table shows that the levels of glycine, ribose and indoleacetic acid increase (IAA) and the levels of serine, β-alanine, aspartic acid, oxoglutaric acid, glutamic acid, phenylalanine, alanine, valine, succinate, aconitate, isocitrate and hexadecanoic acid decrease in the model group compared with those in the control group. These metabolites are endogenous metabolomes such as amino acids and organic acids, which are involved in multiple biochemical processes. The changes in these endogenous substances suggested that biochemical perturbation was induced by CUMS, which was detected.

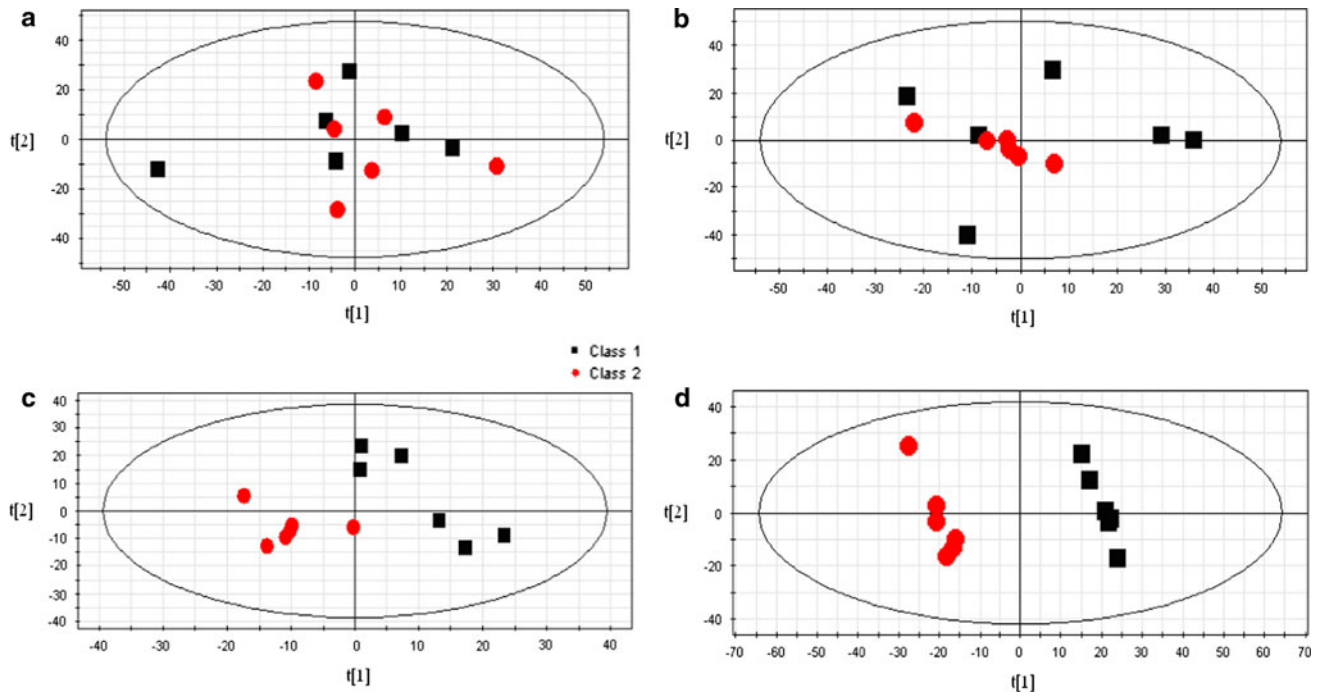

Fig. 3 PLS-DA scores plot comparing the control group (*black squares*) and chronic unpredictable mild stress group (*black circles*) on day 0 (**a**), 7 (**b**), 14 (**c**) and 21 (**d**)

Table 2 List of potential biomarker metabolites relevant to CUMS rats

	Retention time (min)	Mass (m/z)	Metabolites	CUMS versus control (correlation coefficient)
1	10.26	116.16	Alanine	−0.0034
2	12.64	144.21	Valine	−0.0014
3	14.50	277.16	Glycine	0.0049
4	14.81	147.19	Succinate	−0.0031
5	15.68	188.19	Serine	−0.0024
6	17.64	174.18	*β*-alanine	−0.0030
7	20.77	232.22	Aspartic acid	−0.0030
8	21.94	254.22	Oxoglutaric acid	−0.0021
9	23.79	246.20	Glutamic acid	−0.0019
10	24.01	218.17	Phenylalanine	−0.0035
11	25.07	258.21	Ribose	0.0044
12	27.28	229.17	Aconitate	−0.0027
13	29.24	347.14	Isocitrate	−0.00132
14	35.20	202.22	Indoleacetic acid (IAA)	0.00033
15	37.75	313.28	Hexadecanoic acid	−0.00045

+ relatively higher in the model group, − relatively lower in the model group

Time-Dependent Metabolic Trajectory

After GC/MS determination and data analysis, a two-dimensional PLS-DA scores plot was used to depict the general variation of the metabolic pattern between the chronic stress and control groups. No separation between the two groups was observed in the PLS-DA scores plot on day 0. The difference increased over time. Following 21 days of exposure to CUMS, a clear separation between the chronic stress and control groups was observed in the PLS-DA scores plot (Fig. 3), suggesting that exposure to chronic unpredictable mild stress may lead to systemic metabolic variation. Also, OPLS-DA was applied to show the time-related trajectory of metabolome patterns from day 0 to day 21 in the model and control groups, respectively. Clear separation of metabolic states after 0, 7, 14 and 21 days of CUMS treatment was observed in the model group (Fig. 4a), suggesting that exposure to unpredictable

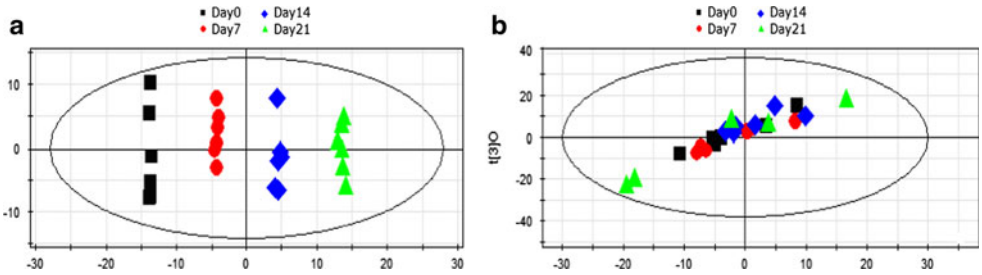

Fig. 4 OPLS-DA scores plot of variation in urinary metabolites of rats during 21 days of chronic stress. **a** CUMS group, **b** control group

chronic stress might lead to a gradual metabolic variation. However, metabolic variations with time in the control rats could not be distinguished clearly (Fig. 4b).

Metabolic Pathway of Biomarker

After exposure to CUMS, there was alteration in 15 important metabolites contributing to a significantly different metabolic profile of the model group compared to the control group. The mechanism of depression was related to the metabolic pathway of these biomarkers, seven of them were amino acids, four were organic acids, three metabolic products of these were amino acids or organic acids, and the last one was ribose. All the analyses showed that depression was related to neurotransmitters, energy metabolism and glycometabolism (Fig. 5).

Neurotransmitters

Glutamic acid and aspartic acid are the excitatory neurotransmitters in the mammalian nervous system [24], which significantly decreased at day 21 in the model group. Glycine is the inhibitory neurotransmitter [17] that significantly increased at day 21 in the model group. The increased urinary level of glycine in CUMS rats may suggest the injury of hepatic mitochondria, which causes suppressed dynamic glycine cleavage system [25]. The above results indicated that the function of the nervous system of the model group rat might be lower under prolonged stress condition.

Energy Metabolism

Glutamic acid is converted to oxoglutaric acid by glutamate dehydrogenase, aspartic acid is directly converted to oxaloacetic acid by aminotransferase, and alanine and glycine are converted to pyruvic acid by transamination and the glycine cleavage system, respectively. Oxaloacetic acid and oxoglutaric acid are the important intermediates in tricarboxylic acid cycle (TCA); therefore, the decreased level of glutamic acid, aspartic acid and alanine indicates the dysfunction of the energy metabolism. In addition, aconitate, succinate and isocitrate are the important

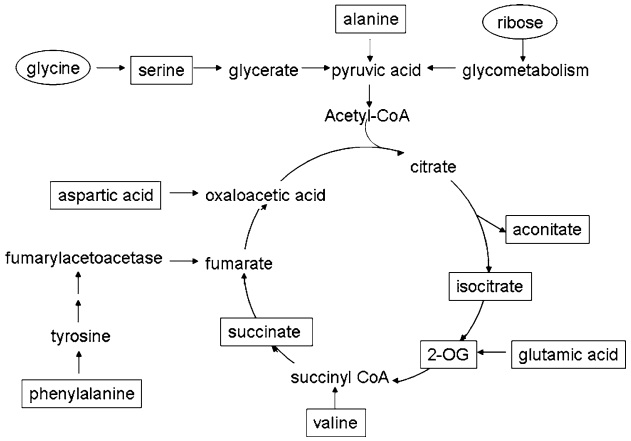

Fig. 5 The interrelation between the biomarkers and the relevant pathways leads to depression. Significantly increased metabolites detected in this study are shown in the *ellipse*. Significantly decreased metabolites detected in this study are shown in the *square*

intermediates in TCA. Their decreased level also indicates the dysfunction of the energy metabolism [15, 22]. The reduced activity of the TCA leads to reduced ATP generation in the mitochondria, potentially leading to fatigue which is a frequent symptom of depression [26], which was also found in the animal model used in our study.

Glycometabolism

The concentration of ribose and glucose in the CUMS rats were increased significantly. It was reported that depression was associated with glucose metabolism in the biological mechanisms, and glucose metabolism may be affected by the abnormal secretion of depression-related hormone [27].

Others

In this study, the decrease in the valine levels may cause disorder in the function of the central nervous system; it may be caused by stressors of CUMS related to depression [28, 29].

In conclusion, we applied GC/MS to the metabonomic analysis of urine obtained from CUMS rats, aiming to observe the physiological changes with the change of time

and investigate the biomarker of depression. Forty-three metabolites were identified among the detected compounds from TIC chromatograms using the authentic standards and the NIST 2005 mass spectral database. The concentrations of 15 metabolites were observed to be significantly changed in the CUMS group when compared with the controls. The elevated or decreased endogenous metabolites in the urine of CUMS rats suggested a different metabolic pattern between the CUMS and control groups. Using the KEGG pathway database [30], it was found that neurotransmitters, energy metabolism and glycometabolism were affected after the CUMS treatment. Time-dependent metabolic trajectory and behavior tests reached the same conclusion, suggesting that exposure to chronic unpredictable mild stress may lead to systemic metabolic variation. The determination of potential biomarkers of depression and the time-dependent metabolic trajectory may be useful for the early clinical diagnosis of depression, and for evaluating the treatment strategy and measuring the outcomes.

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