Analysis of urinary metabolic profile in aging rats undergoing caloric restriction

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ABSTRACT. ***Background and aims:*** *While caloric restriction (CR) is associated with a prolonged lifespan in multiple species by regulating metabolism, a comprehensive profile of metabolism under CR conditions remains largely unclear. Therefore, in this study we aimed to characterize the metabolomic profiling associated with CR using a rat model.* ***Methods:*** *Rapid resolution liquid chromatography/electrospray ionization quadrupole-time of flight mass spectrometry (RRLC/ESI-Q-TOFMS) was employed to analyze metabolomic profiling of urine samples from aging rats who underwent caloric restriction (CR; n=7) or were provided a normal diet (N; n=8) for 12 weeks time. Multivariate data analysis was performed on the mass data of metabolomic profiles to uncover the differences between the CR and N groups.* ***Results:*** *CR treatment led to manifest metabolic changes in aging rats, and fifteen urinary metabolites including hypoxanthine, hippurate, dimethylglycine and creatinine were significantly different in the rat groups.* ***Conclusion:*** *Our study demonstrates the high reliability of the HPLC-based metabolomic approach towards the study of anti-aging effects induced by CR, while the urinary metabolites we identified may become potential biomarkers of aging.*

(Aging Clin Exp Res 2012; 24: 79-84)

INTRODUCTION

Calorie restriction (CR) is the only intervention that has been consistently shown to delay the rate of aging and to increase the mean and maximum lifespan in a number of species (1, 2). While accumulating evidence suggests that CR acts to reduce oxidative damage (3, 4), by promoting insulin sensitivity and optimizing glucose utilization (5-8), the exact pathways linking low energy intake to longevity remain largely unknown.

While caloric restriction (CR) is associated with a prolonged lifespan in multiple species by regulating metabolism, a comprehensive profile of metabolism under CR conditions remains largely unclear. Metabolomics has emerged as a novel non-targeted analysis of a large number of metabolites produced by the body in response to various environmental stimuli (9, 10). To the best of our knowledge, metabolomic studies have not yet been performed to assess changes in urinary metabolites following CR diets. Therefore, in this study we aimed to characterize the metabolomic profiling under CR conditions using a rat model.

METHODS

Chemicals

Reference chemicals, including dimethylglycine, hypoxanthine, hippurate and creatinine, were purchased from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile was of HPLC grade from J.T. Baker (Phillipsburg, NJ, USA). Ultrapure water was prepared from Millipore water purification system (Millipore, Miford, MA, USA). Other reagents were of analytical grade.

Animals

Twenty-four month-old specific pathogen-free male Sprague-Dawley rats were purchased from Shanghai

Key words: Aging, anti-aging, caloric restriction, metabolomics, multivariate data analysis.

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Received July 10, 2010; accepted in revised form February 3, 2011

First published ahead of print February 21, 2011 as DOI: 10.3275/7519

SLAC Laboratory Animal Co.Ltd. (Shanghai, China). They were bred and humanely cared for in the animal center of the Second Military Medical University (Shanghai, China). The animals were housed individually in polycarbonate cages with wood chip bedding, kept in an air-conditioned animal room, where the temperature and humidity were regulated at 21-22°C and 30-60%, respectively. A light cycle of 12h on/12h off was established. The rats were divided into two groups: a control group (N) and a calorie restricted one (CR). While N rats were allowed *ad libitum* access to food and tap water, CR rats were provided a vitamin and mineral fortified version of the same diet at a level of 60% of the food (by weight) consumed by the N rats during the previous week. The body weight of the rats was recorded weekly. Eight rats were included in each group. However, one rat in the CR group died during the experiment. There were 8 rats in N group and 7 rats in CR group at end of the experiment.

Blood and urine sampling collection

The rats were killed by decapitation at the end of the experiment. Blood was collected from the retro-orbital plexus with a capillary tube. Glucose (GLU), urea nitrogen (BUN) and serum creatinine (Cr) were estimated by a Biochemistry Autoanalyzer (Olympus). The day before decapitation, all the animals were kept in metabolic cages and deprived of food to eliminate contamination, while water was provided *ad libitum*. Urine sample was collected 24 h later and made up to the same urine volume by adding normal saline and 100 μL 1‰ NaN_3. Samples were then centrifuged at 13,000 rpm for 5 min and 200 μL supernatant was collected and stored at -80°C.

LC/MS Analysis

The urine samples were thawed at room temperature, mixed wth acetonitril (2:1, v/v), incubated for 10 min, and centrifuged and filtered through a syringe filter (0.2 μm). The urine samples from each animal were run for RRLC/ESI-Q-TOFMS analysis separately, using the Agilent-1200 series (Agilent, MA, USA). A C18 RP-ODS column (4.6 mm × 150 mm, 3.5 μm, Agilent, USA) and a C18 guard column (4.6 mm × 7.5 mm, 3.5 μm, Merck, Rahway, NJ, USA) were used. The mobile phases were composed of water (A) and acetonitrile (B). The gradient was as follows: 0 min, 95% A, 5% B; 3 min, 60% A, 40% B; 5 min, 45% A, 55% B; 16-18 min, 5% A, 95% B. The column compartment was kept at 25°C, and the sample injection volume was 2 μL. Elution was performed at a solvent flow rate of 0.6 mL/min, and 0.2 mL/min portion of the column effluent was delivered into the ion source of mass spectrometry. The conditions of the electrospray ionization source were as follows: drying gas N2 8 L/min, temperature 320°C, pressure of nebulizer 30 psi, capillary voltage 4000 V, and scan range 50-1000 m/z.

Multivariate Date Analysis

The LC/MS data were deconvoluted and aligned with mass and retention time tolerances using GeneSpring software (version 1.1, Agilent, USA) to generate a matrix containing information regarding mass, retention time and intensities for all the detected peaks. The peak intensities for each spectrum were then normalized to a constant integrated intensity of 1000 to partially compensate for the concentration bias of each sample, and subsequently standardized for Partial Least Square (PLS) analysis. The MATLAB 7.0 platform (The Mathworks, USA) was used to create a proprietary algorithm for PLS.

Identification of metabolites

To identify significant metabolites, we first searched the Agilent METLIN Personal Metabolite Database (Version B.01.00) by mass weight for a list of candidates and then performed tandem mass analysis to exclude those without the given mass fragment information. Finally, the structural identities of some of the candidates were confirmed by comparing the retention times and mass spectra with those of commercial standards.

RESULTS

Effect of CR Diet

As expected, there were multiple differences in body weight and biochemical parameters in the two groups (Table 1).

With regard to the weekly changes in body weight during the CR period, no significant changes in body weight were observed in the N rats. Body weight was significantly lower in the CR rats during the first two weeks of CR ($p<0.01$). There was instead a plateau in the CR rats' body weight until the end of the 12 week period during which their weight stabilized at about 430 g (Fig. 1).

In addition, the CR diet led to lower concentrations of BUN and Scr and higher serum GLU levels compared to those in normal rats. The differences in serum BUN, Scr or GLU concentrations in the two groups were not statistically significant ($p>0.01$).

Table 1 - *Changes in body weight and biochemical parameters in N and CR rats.*

Variable	n	CR
Starting weight	667.6±55.1	639.1±39.0
End weight	708.6±72.5	436.6±21.5*#
Serum GLU (mmol/L)	5.50±0.41	5.96±0.20
Serum BUN (mg/100 mL)	36.85±2.09	30.66±5.85
Serum Scr (mg/100 mL)	0.32±0.09	0.28±0.02

CR *vs* N, *$p<0.01$; starting weight *vs* end weight, #$p<0.01$.

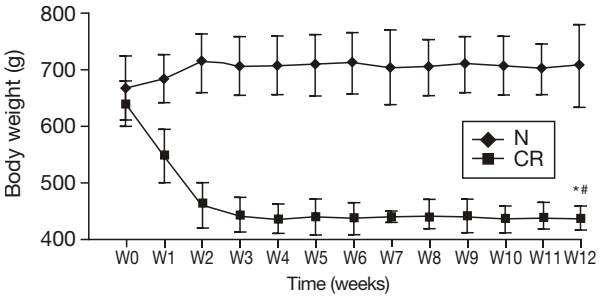

Fig. 1 - *Body changes in a control group and in a CR treated group of rats.* *p<*0.01, end weight CR* vs *N;* #p<*0.01, starting weight* vs *end weight.*

Metabolic Profiling

The operating conditions of LC/MS were optimized to analyze as many metabolites as possible in a single injection. Both positive and negative mode analyses were experimented, and the LC/MS profile in the positive mode was finally adopted because it provided many more metabolic features. The representative total ion chromatograms (TICs) of the urine samples from the N and CR rats are outlined in Figure 2. It was obvious that the TICs contained only a few overlapped peaks, and a further feature extraction procedure was thus necessary.

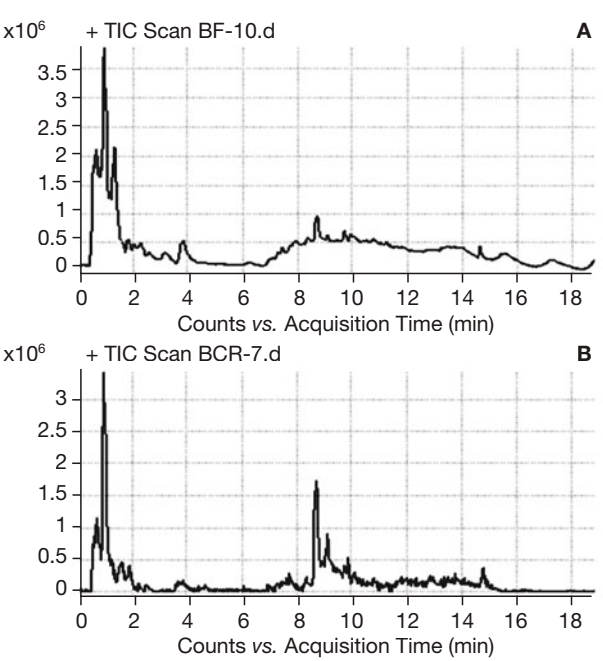

Fig. 2 - *Representative total ion chromatograms of urine samples from a control group (a) and a CR treated group (b) of rats.*

Totally 713 peaks were resolved for each sample using GeneSpring software and 233 of these were selected for further analysis based on *t*-test statistics. To summarize and visualize the differences between the control and CR treated rats, PLS was performed on the data of the metabolic profiles. The first two PLS components represented the majority of the global metabolic information (over 80% variations explained by PC1 and PC2). Figure 3A outlines the score plot, in which the profiles of N and CR rats were significantly separated from one another, especially in the direction of PC2 (39.8% variances explained). Thus we concluded that the metabolic profiles observed could reveal the physiological characteristics in the CR and normally fed rats indicating that CR markedly altered the metabolic features of aging rats.

Biomarker Identification

The PLS loading plot, often used in metabolomic studies to reveal potential biomarkers that contribute to the

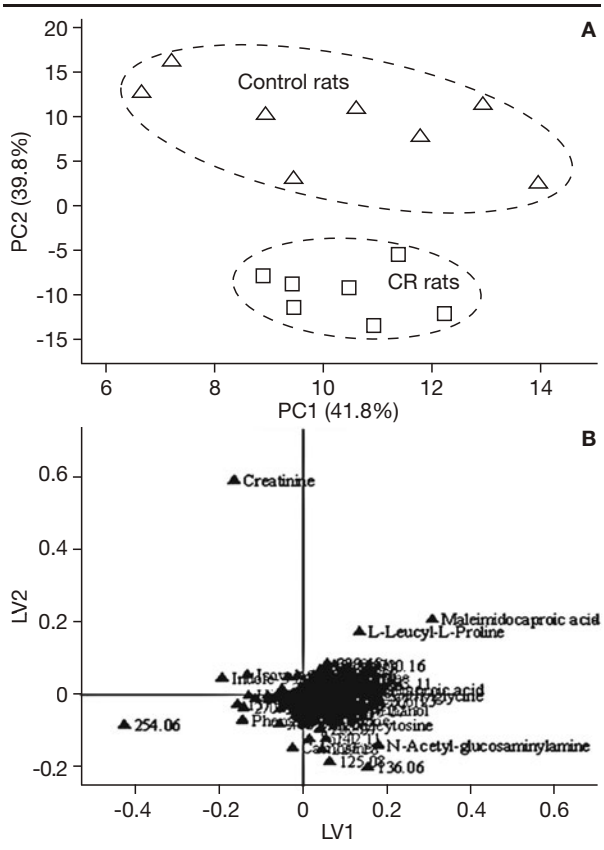

Fig. 3 - *Score plot (A) and Loading plot (B) of PLS-DA performed on the metabolomic data of samples from control and CR rats. Plot A: Scattered points represent samples from control rats (Δ) and CR rats (□); Plot B: Scattered points represent detected metabolites, partly marked with postulated chemical names or their molecular weights.*

Table 2 - *Data of MS fragments of postulated metabolites in rat urine.*

No.	RT (min)	Postulated chemicals	Elemental composition	Mass	MS fragments (m/z)		Relative Content*	
					MS1	MS2	Control	CR treated
1	0.95	Creatinine	$C_4H_7N_3O$	113.0589	114.0590	86.0710, 72.0450	147.5±15.2	221.1±21.9#
2	0.64	Betaine	$C_5H_{11}NO_2$	117.0790	118.1220	59.0820, 58.0499	2.26±0.33	4.43±1.07#
3	1.68	Isovaleryalanine	$C_8H_{15}NO_3$	173.1052	174.1202	130.0984, 87.0438, 71.0604, 60.0548	1.06±0.95	4.94±3.90#
4	1.51	L-Leucyl-L-Proline	$C_{11}H_{20}N_2O_3$	228.1474	229.1533	170.0835, 142.0840, 114.0538, 85.0632	1.31±1.45	13.15±9.48#
5	0.94	Allysine	$C_6H_{11}NO_3$	145.0739	146.1055	129.0613, 111.0468, 87.0438	3.49±0.54	4.98±2.05#
6	1.03	Indole-3-ethanol	$C_{10}H_{11}NO$	161.0841	162.1109	130.0627, 57.0670	10.05±2.47	13.76±5.24
7	0.86	6-Maleimidocaproic acid	$C_{10}H_{13}NO_4$	211.0845	212.1025	194.0410, 109.0760	30.52±9.32	41.62±9.28#
8	0.92	Unidentified	–	254.3421	255.0707	237.0788, 197.6545, 141.0667	12.1±4.54	8.01±3.91
9	7.26	Dimethylglycine	$C_4H_9NO_2$	103.0633	104.1062	61.1062, 60.0816, 59.073, 58.066	8.23±1.29	6.31±2.52#
10	1.23	Hypoxanthine	$C_5H_4N_4O$	136.0385	137.0723	119.0345, 94.0645, 110.0392, 78.0321	14.74±3.76	7.66±2.01#
11	1.18	Hippurate	$C_9H_9NO_3$	179.0582	180.0636	105.0329, 77.0385	1.23±0.84	0.75±0.54#
12	1.36	Phenylacetylglycine	$C_{10}H_{11}NO_3$	193.0739	194.0794	91.0535, 76.0397, 65.0392	25.98±10.79	15.27±13.39#
13	1.93	5-Methylcytosine	$C_5H_7N_3O$	125.0589	126.0628	109.0357, 96.0091, 83.0567	2.07±0.75	0.43±0.24#
14	9.17	1-Methylinosine	$C_{11}H_{14}N_4O_5$	282.0964	283.1222	150.0768, 73.0275	1.81±0.71	–
15	1.07	Carnosine	$C_9H_{14}N_4O_3$	226.1066	227.1247	114.0660, 44.0512	7.45±0.95	–
16	8.99	N-Acetyl-b-glucosaminylamine	$C_8H_{16}N_2O_5$	220.1059	221.1392	203.1011, 61.0111	11.30±1.89	7.61±3.85#
17	11.4	Unidentified	–	136.0652	137.0707	94.0660	13.3±5.2	4.96±3.72

–: not detected; *Relative Content was the processed peak area which was normalized and logarithm transformed, shown as mean±SD. CR *vs* N, #$p<0.01$.

separation of various groups of samples, is presented in Figure 3B. In the loading plot, tens of numerous scattered points were distributed as outliers, which are considered potential CR related biomarkers. Table 2 listed 17 metabolites including creatinine, betaine, carnosine, amino acids and some other important metabolites (two of which were unidentified by MS2 analysis). These small molecules have a mass range between 113.0590 and 282.0964 Da, and numbers 1-8 were more abundant while numbers 9-17 were less abundant in the urine of the CR rats compared to those in the controls. To validate these potential biomarkers, commercial standards of hippurate, dimethylglycine, creatinine and hypoxanthine were subjected to MS2 analysis under the same LC-MS conditions mentioned above. These significant metabolites were thus structurally confirmed by comparing the retention times and MS2 spectra. Representative MS2 spectra of creatinine in the samples and reference chemicals are outlined in Figure 4.

DISCUSSION

While multiple mechanisms including metabolism regulation hypothetically link CR with prolonged lifespan (11), no comprehensive analysis of metabolism status under CR condition has been performed. In the present study, for the first time a LC/MS based metabolomic approach was employed that identified 15 metabolites whose body weight significantly decreased in the rats during the first two weeks of CR ($p<0.01$). We found that body weight was significantly decreased in rats during the first two weeks of CR but then stabilized at a low level until the end of the 12 week period. Thus, the metabolic profiling we analyzed reflected the new metabolic state after caloric restriction rather than a transient adjustment to the new diet.

With regard to these metabolites, hypoxanthine is an intermediate product in energy metabolism. Hypoxanthine can be hydrogenised as xanthine by xanthine oxidase with the release of free radicals. It is well known that free radicals are the main source of reactive species responsible for oxidative damage, which plays a major role in aging (12-15). Therefore, hypoxanthin level may not only reflect energy consumption but can also be considered an index of oxidative damage. In this respect, the lower level of hypoxanthine in CR rat urine that we observed is consistent with decreased levels of oxidative damage and energy metabolism.

We found that the levels of many aromatic metabolites from gut microflora (16), including hippurate and dimethylglycine, were also lower in CR rats. Interestingly, hippurate is considered by some a biomarker of aging since its level is markedly increased with aging (17, 18). The concentration of dimethylglycine in the urine was also reported to show age-dependent changes, perhaps due to

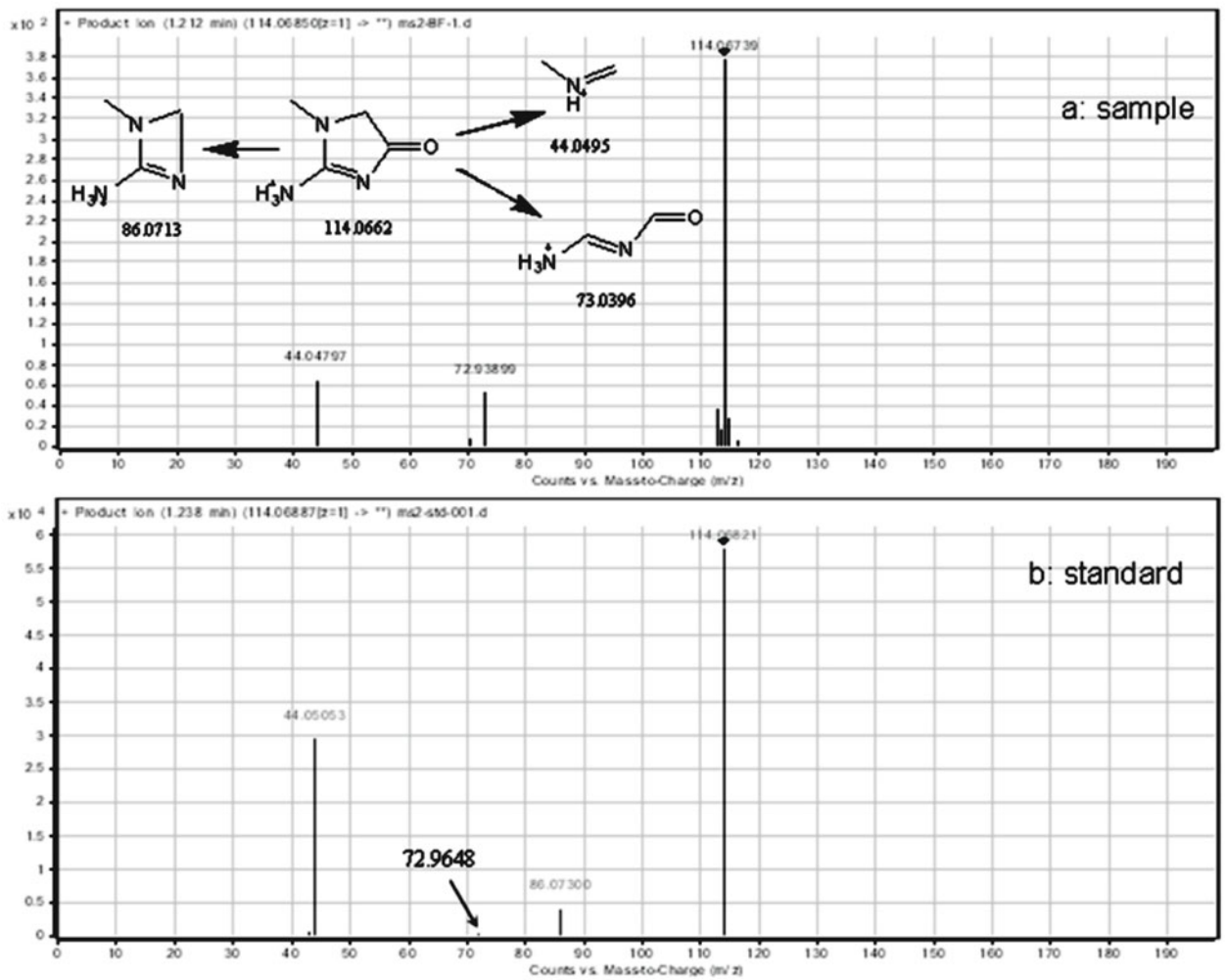

Fig. 4 - *Comparative MS2 analysis of creatinine in the urine and standard samples.*

the degradation of dietary choline by the gut microbiota (19). In CR rats, the decreased level of hippurate and dimethylglycine may be the result of modulated gut microbiota activity by CR treatment, and may reflect improved nutrient digestibility in aged mammals.

Most significantly, creatinine was the metabolite with the greatest difference in urine levels found in the two groups. As urine creatinine is directly related to muscle mass (20), the higher level of creatinine in CR rats may be indicative of decreased muscle mass in these animals. However, our findings showing that serum creatinine level is normal in CR rats excluded that possibility. Nevertheless, urinary creatinine is a marker of renal function (21, 22). While decreased urine creatinine is associated with aging in rats and dogs (18, 23), it has been suggested that renal plasma flow and glomerular filtration rate tend to decline with aging, thus contributing to lower urine creatinine levels (24, 25). The increased urine creatinine levels in the CR rats that we observed indicates that the plasma flow and glomerular filtration rate were improved, leading to higher urinary creatinine excretion. In agreement with previous studies demonstrating that CR retards kidney aging (26, 27), these data suggest that renal function in the CR rats was ameliorated. Furthermore, since creatinine clearance decreases with age without any increase in plasma creatinine (28), our data that urine creatinine levels are higher in CR rats seem to support the hypothesis that CR promotes creatinine clearance. In future experiments we will examine the creatinine clearance in CR rats to test this hypothesis.

In conclusion, to our knowledge, this is the first study

aiming to perform metabolomic characterization on the urine from aging rats. Our findings demonstrated the high reliability of the HPLC-based metabolomic approach to the study of metabolic changes induced by CR and identified several urinary metabolites including hypoxanthine, hippurate, dimethylglycine and creatinine. Further pharmacological studies to investigating the functional significance of these metabolites will hopefully classify their potential role as biomarkers of aging.

ACKNOWLEDGEMENTS

This work was supported by program for Changjiang Scholars and Innovative Research Team in University (PCSIRT), National 973 program (2007CB507400), National 863 Program (2006AA02Z338), and partly supported by Shanghai Leading Academic Discipline Project (B902).

Conflict of interest: None declared.

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