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.


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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 calorie 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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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% NaNO3. 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 with 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 acetonitril (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.

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

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