Food and Chemical Toxicology 74 (2014) 35–44

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Use of urinary metabolomics to evaluate the effect of hyperuricemia on the kidney

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ARTICLE INFO

Article history:
Received 21 December 2013
Accepted 29 August 2014
Available online 6 September 2014

Keywords:
Urinary metabolomics
Hyperuricemia
Kidney injury
IL-6/STAT3 signaling

ABSTRACT

Clinical studies show that hyperuricemia is a risk factor in the progression and development of cardiovascular and metabolic disease. Elevated serum levels of uric acid induce renal injury via an inflammation response, but the detailed mechanism is still under study. To better understand the effect of hyperuricemia on the kidney, we used gas chromatography–mass spectrometry-based metabolomics to investigate the role of uric acid in the mouse kidney. Partial least-squares discriminant analysis revealed significant differences between control and hyperuricemia groups in urine metabolic profiles. We identified 33 metabolites from 76 highly reproducible peaks and found abnormal uric acid levels related to comprehensive kidney injury, including excretive function and energy metabolism. Additionally, inflammation induced by the interleukin 6/signal transducer and activator of transcription 3 signaling pathway participated in hyperuricemia-induced kidney injury. This study helps understand the relationship between hyperuricemia and kidney injury. Metabolomics may be a useful strategy for early diagnosis of kidney damage.
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1. Introduction

Uric acid is the end metabolite of purine; it exists in an ionized form (urate) under physiological pH in humans. Unlike other mammals, humans have higher serum uric acid levels because of lack of uricase. Uricase is an oxidation enzyme that can further catalyze uric acid into a more soluble end product, allantoin (Johnson et al., 2003). Uric acid is primarily excreted in urine, but only 8% to 10% of the filtered urate is excreted because of efficient reabsorption in the proximal tubule (Shekarriz and Stoller, 2002). Uric acid has a dual role in the physiological condition: as an antioxidant or a pro-oxidant (Sautin and Johnson, 2008). As a powerful antioxidant, it scavenges free radicals in hydrophilic biological fluids (primarily in plasma) (Maples and Mason, 1988; Vásquez-Vivar et al., 1996). However, it can induce an oxidative reaction within cells and may be a mediator in the pathogenesis of some diseases (Santos et al., 1999). Recently, uric acid has been proposed to be involved in human systemic inflammation (Lyngdoh et al., 2011; Ruggiero et al., 2006) and to induce endothelial dysfunction by inhibiting nitric oxide production (Khosla et al., 2005).

An abnormally high level of blood uric acid is called hyperuricemia, which can be classified as primary (due to purine metabolism) or secondary (due to another disease). The condition leads to deposition of sodium urate crystals in tissues and causes serious pathological changes in the joints and kidney. It may damage the kidney by forming renal stones, blocking tubules, and contributing to chronic interstitial nephritis. Hyperuricemia may induce three types of kidney diseases: acute uric acid nephropathy, chronic uric acid nephropathy, and uric acid nephrolithiasis (Bellomo et al., 2010). Although the role of uric acid and its association with the pathogenesis and progression of chronic kidney disease remains controversial, numerous epidemiological studies have shown that a long period of asymptomatic hyperuricemia is an independent risk factor of arterial hypertension, cardiovascular events and mortality (Kang and Chen, 2011). Acute uric acid nephropathy usually occurs with increased purine metabolism because of rapid cell turnover or cell lysis such as with leukemia and lymphoma, chemotherapy, rhabdomyolysis, or missing metabolic enzymes (Idle and Gonzalez, 2007). The result is urate crystal deposition, tubular obstruction, and acute renal failure. Treatment of hyperuricemia-induced nephritis and renal failure by dialysis and transplantation has become an important medical burden in modern society.

Metabolomics is a highly attractive systemic approach used in many fields such as botanical science, disease diagnosis, and toxicology to understand changes in metabolites and the metabolic state

Abbreviations: BUN, blood urea nitrogen; GC/MS, gas chromatography/mass spectrometry; IL-6/STAT3, interleukin-6/signal transducer and activator of transcription 3; PCA, principal component analysis; PLS-DA, partial least-squares discriminant; PO, potassium oxonate.

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http://dx.doi.org/10.1016/j.fct.2014.08.017
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of biological systems (Fiehn et al., 2000; Woodcock, 2007). Metabolomics is a cost-effective and high-throughput powerful tool for drug discovery, disease diagnosis and personalized medicine (Hagberg, 1998). NMR-based metabolomic analysis offers an efficient method to aid human disease diagnosis in cancer (Burtscher and Holtås, 2001; Smith et al., 2009), diabetes (Dunckley et al., 2005; Mäkinen et al., 2008), and neurological diseases (Ibrahim and Gold, 2005; Tung et al., 2010). Gas chromatography–mass spectrometry (GC-MS)-based techniques are robust metabolomic tools for analysis of biofluid and tissue extracts. This technology provides qualitative and quantitative information on holistic changes in metabolites in response to organ-specific biochemical and histological conditions.

Traditionally, diagnostic and treatment decisions in kidney disease have been based on kidney histology, biochemical marker analysis, and clinical manifestations. Recently, systems biology has been found valuable for studying the origin of kidney disease, predicting disease progression, and recognizing early biomarkers (He et al., 2012). Therefore, to obtain more information on the effect of hyperuricemia on the kidney, we analyzed global changes in the urine metabolome and evaluated the pathophysiological outcome in the mouse kidney.

2. Materials and methods

2.1. Reagents and antibodies

Potassium oxonate (PO), 4,6-diamidino-2-phenylindole (DAPI), and all chemicals and solvents for gas chromatography (GC) were of reagent grade and purchased from Sigma Chemical (St. Louis, MO). Primary antibodies against STAT3 and p-STAT3 (Cell Signaling Technology) and actin (Chemicon) were used.

2.2. Animals

Male ICR mice (body weight about 30 g; 6 weeks old) were purchased from BioLASCO (Taiwan). Mice were given a standard laboratory diet and distilled water *ad libitum* and kept on a 12-h light/dark cycle at 22 ± 2 °C. All animal experiments followed the guidelines for animal care and use by the Institutional Animal Care and Utilization Committee of National Taiwan Sport University (IACUC-10004). A hyperuricemic mouse model was induced by intraperitoneal injection of potassium oxonate (PO) as described (Laemmli, 1970). Control mice were treated with phosphate buffered saline (PBS). Serum and urine samples were collected 3 h after injection with 50, 100 and 200 mg/kg PO.

2.3. Uric acid, creatinine, and blood urea nitrogen (BUN) determination

Blood samples were collected by submandibular venipuncture of mice at 3 h after PO administration, then centrifuged at 1400 × g at 4 °C for 15 min and serum supernatant was collected. The serum levels of uric acid, blood urea nitrogen (BUN), and creatinine were determined by use of an auto-analyzer (Hitachi 7060, Hitachi, Japan).

2.4. Urine protein analysis

Protein levels were examined in urine of mice by SDS-PAGE followed by Coomassie brilliant blue staining as described (Huang et al., 2010).

2.5. Histology and immunohistochemistry

Mice were killed and kidneys were removed 3 h after PO administration, fixed in 10% buffered formalin, then embedded in paraffin. Paraffin-embedded samples were sectioned (4 μm) and underwent hematoxylin and eosin (H&E) staining.

Immunohistochemical studies with paraffin-embedded kidney sections were as we previously described (Tung et al., 2009). Sections were heat immobilized and deparaffinized by use of xylene and rehydrated in a graded series of ethanol with a final wash in distilled water. Antigen retrieval involved Target Retrieval Solution (DakoCytomation) in a Decloaking Chamber (Biocare Medical). Sections were stained with p-STAT3 antibody and visualized with goat anti-rabbit Cy3-labeled secondary antibody (Jackson ImmunoResearch). Sections were counterstained with DAPI (1 μg/mL) and visualized under a fluorescent microscope at 400× magnification. Positive p-STAT3 cells were quantified by use of AxioVision software (Carl Zeiss MicroImaging).

2.6. Western blot analysis

Total protein from kidney tissues (0.1 g) was homogenized by use of the Bullet Blender Tissue Homogenizer (Next Advance, Averill Park, NY) for 2 min, extracted by adding 0.4 mL lysis buffer and centrifuged at 15,000 × g for 30 min at 4 °C. Protein determination and western blot analysis were as previously described (Chuang et al., 2012).

2.7. Measurement of serum IL-6 content

Serum was collected at 3 h after PO administration. Murine IL-6 content was measured by use of a conventional ELISA kit (R&D Systems, Minneapolis, MN).

2.8. Urine preparation for metabolomics study

A total of 10 mice were randomly divided into two groups ($n = 5$): control (PBS only) and PO treatment (100 mg/kg, *i.p.*, dissolved in PBS). Urine samples were collected 3 h after injection with PO from mice in metabolism cages at ambient temperature, then centrifuged at 4000 × g at 4 °C for 10 min to remove residue, then 200 μL urine was incubated with 200 U urease at 37 °C for 15 min to decompose and remove excess urea. An amount of 200 μL methanol and 20 μL ribitol stock solution (0.2 mg/mL) as an internal standard were added. The solution was vigorously extracted for 10 min and centrifuged at 14,000 × g at 4 °C for 10 min. The dried sample was further derivatized by use of N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane and heated at 70 °C for 60 min to form trimethylsilyl derivatives. The derivatization procedure was described previously (Ragan, 1989).

2.9. Urine metabolome analysis by GC-MS

All urine samples were analyzed in a random order. GC-MS analysis involved use of ThermoFinniganTrace GC 2000 installed with a Polaris Q mass detector and Xcalibur software system at the Division of Research and Analysis, Taiwan Food and Drug Administration, Taiwan. An amount of 1 μL derivatized sample was injected into a DB-5-fused silica capillary column that was 30 m × 0.25 mm (i.d.) × 0.25 μm (film thickness) chemically bonded with a 5% diphenyl–95% dimethylpolysiloxane cross-linked stationary phase (J&W Scientific). The injector and ion source temperatures were 230 and 200 °C, respectively. The oven temperature program was initiated at 80 °C for 5 min, then increased at 5 °C/min to 300 °C and held for 1 min. Helium was used as the carrier gas at 1 mL/min. The mass spectrometer operated in electron impact (EI) mode (70 eV). Acquisition of total ion currents (TICs) involved the full scan mode from 50 to 650 *m/z* with scan time 0.58 s. All GC-MS-detected peaks were identified by comparing both the MS spectra and the retention index with those available in libraries (NIST and Wiley) and commercially available reference compounds.

2.10. Multivariate statistical analysis

The acquired GC-MS TIC chromatography data underwent multivariate statistical analysis and pattern recognition with SIMCA-P12+ software (Umetrics, Sweden). The relative intensity of each peak was normalized in terms of peak area to that of ribitol on the same chromatograph and expressed as 100 times the ratio. The data matrix was arranged with the samples as observations and peaks as the response variables. Principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) were used for data analysis. Seven cross-validation groups were used to determine the number of components. Three parameters, *R2X*, *R2Y*, and *Q2Y*, were used for evaluation of the models to indicate goodness of fit and predictive ability. *R2X* explains the cumulative variation in the GC-MS response variables, and *R2Y* is the current latent variables of the sums of squares of all Xs and Ys. *Q2Y* reflects the cumulative cross-validated percentage of the total variation that can be predicted by the current latent variables. The predictive component receives a *Q2Y* value that describes its statistical significance for separating groups. Q2 > 0.5 is regarded as good and Q2 > 0.9 as excellent (Eriksson et al., 2006). High coefficient values of *R2Y* and *Q2Y* represent good discrimination.

2.11. Statistical analysis

All data are expressed as mean ± SEM and were analyzed by one-way ANOVA with SAS 9.0 (SAS Inst., Cary, NC). A Cochran–Armitage test was used for dose–effect trend analysis. $P < 0.05$ was considered statistically significant. Comparisons of metabolites and relative content in mice treated with vehicle and potassium oxonate were conducted by nonparametric Mann–Whitney U test using GraphPad Prism (GraphPad software, version 5). $P < 0.05$ was considered statistically significant.

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