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

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A B S T R A C T
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.
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 (Burschert and Hölzlès, 2001; Smith et al., 2009), diabetes (Duncanley 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

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.

2.9. Urine metabolome analysis by GC-MS

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).

2.10. Multivariate statistical analysis

The acquired GC-MS TIC chromatography data underwent multivariate statistical analysis and pattern recognition with SIMCA-P+12 software (Umetrics, Sweden). The relative intensity of each peak was normalized in terms of peak area to that of ribitol on the same chromatogram 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 Q2 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.
3. Results

3.1. Potassium oxonate (PO)-induced hyperuricemia significantly increased the serum levels of uric acid, blood urea nitrogen (BUN), and creatinine

PO at 200 mg/kg significantly increased serum uric acid production in mice (Fig. 1A). BUN and serum creatinine are nitrogenous end products of protein metabolism and their levels are valuable markers of renal function. Level of BUN was higher with PO, 50, 100, and 200 mg/kg than control treatment (20.5 ± 1.1, 39.7 ± 3.5, and 63.7 ± 1.9 mg/dL, respectively, vs. 14.2 ± 1.1; Fig. 1B). Trend analysis revealed a significant dose-dependent effect on BUN level with 100 and 200 mg/kg PO (P < 0.0001). Furthermore, serum creatinine levels with 100 and 200 mg/kg PO were significantly higher, by 2.00- (P = 0.0169) and 5.67-fold (P < 0.0001), respectively, than control treatment (Fig. 1C). Therefore, hyperuricemia could cause serious renal function failure.

3.2. Hyperuricemia induced proteinuria and nephron injury

Proteinuria is a nonspecific renal function marker and can provide information regarding pathophysiological changes in affected nephrons. The molecular weight of excreted protein indicates renal injury occurs with altered the glomerular permeability and/or the tubules reabsorption (Nechemia-Arbely et al., 2008). Albumin is the main component of total urinary protein and represents more than 70% of the content. PO dose-dependently increased excretion of both high- and low-molecular-weight protein, especially albumin (68 kDa) (Fig. 2). Large proteins (>65 kDa) in urine indicate loss of function of the glomerulus to retaining protein, and tubular proteinuria indicates that low-molecular-weight proteins cannot be reabsorbed by renal tubules. Therefore, hyperuricemia significantly induced nephron injury both in the glomerulus and tubules.

3.3. GC-MS analysis of effect of hyperuricemia on urine metabolome

According to the biochemical values obtained above, we analyzed urine metabolites to further verify the effect of hyperuricemia on kidney. Typical GC-MS TIC chromatograms of urine samples obtained from control and hyperuricemia mice are shown in Fig. 3A. After alignment all of sample profiles, we detected 76 peaks that were normalized to the internal standard (ribitol). The data matrix was constructed with samples as observations and peaks as response variables for multivariate statistical analysis. Initially, principal component analysis (PCA) was used to decipher whether an unsupervised method of multivariate statistical analysis could discriminate groups. Two principal-component models could explain 76.8% (R2X) of the data. PC1 accounted for 50.9% of the variation in all PO-treated samples compared with vehicle-treated samples (Fig. 3B). PC2 accounted for the biological variability among individual samples and environmental background (25.9%). The high dispersion of PO-treated samples indicated different responses of individuals to PO.

3.4. Identification of urinary metabolites related to the effect of hyperuricemia on nephron injury

PLS-DA yields a graphical display and offers an interpretation tool that enables investigation of the key metabolites of hyperuricemia on the kidney. To further elucidate the effect of hyperuricemia on the kidney, we used PLS-DA to model and cluster the maximum covariance between groups of observations. The score plot of the two-component PLS-DA model showed that PO-treated groups could be separated from the vehicle-treated group (Fig. 4A). The distances between dots represent the similarity of the sample’s
metabolite composition. Data for PO-treated mice tended to cluster to the left part of the figure, whereas that for vehicle-treated mice clustered to the right. This model provided good group separation, with \( R^2X \) value higher than 0.76 and \( R^2Y \) value higher than 0.96, which indicated potent statistical models to predict 91.4% (Q2Y) of the data according to the cross-validation.

A PLS-DA loading plot was created to display the specific metabolites that differed in expression between the two groups of mice. The variables to the right side of Fig. 4B indicate metabolites with greater levels in vehicle- than PO-treated mice and vice versa. Variables closer to the center of the loading plot are less differentiated in level. The ratio of the level of uric acid to creatinine was >1 in PO-treated mice, which indicates signs and symptoms of gouty nephropathy (Table 1). The loading plot indicated significantly increased urinary expression of valine, glycine, threonine, glutamate, glucose, inositol, lactate, and phosphate. Glucose, amino acids, and lactate are well known to be reabsorbed through the proximal tubule. Therefore, hyperuricemia-induced dysfunction of the renal proximal tubule affected reabsorption of glucose, amino acids, and lactate, which led to glucosuria, aminoaciduria, and lactic aciduria. Furthermore, the reduced reabsorption of phosphate would cause hypophosphatemia. The variables to the right of the loading plot indicated metabolites with significantly reduced levels in PO-treated mice. The decreased expression of succinate, malate, aspartate, citrate, and

### Table 1

<table>
<thead>
<tr>
<th>Peak ID</th>
<th>Identified metabolites</th>
<th>Control</th>
<th>Hyperuricemia</th>
<th>( P ) value (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Content increased in hyperuricemia mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Lactate</td>
<td>0.99 ± 0.39</td>
<td>5.91 ± 0.86*</td>
<td>0.0008 (5.97-)*</td>
</tr>
<tr>
<td>3</td>
<td>Oxalate</td>
<td>0.17 ± 0.03</td>
<td>2.49 ± 0.73*</td>
<td>0.0329 (14.7-)*</td>
</tr>
<tr>
<td>4</td>
<td>2-Hydroxybutyric acid</td>
<td>ND</td>
<td>1.13 ± 0.20*</td>
<td>0.0049</td>
</tr>
<tr>
<td>5</td>
<td>Valine</td>
<td>ND</td>
<td>0.38 ± 0.12*</td>
<td>0.0318</td>
</tr>
<tr>
<td>6</td>
<td>Phosphate</td>
<td>1697 ± 210</td>
<td>3528 ± 406*</td>
<td>0.0040 (2.08-)*</td>
</tr>
<tr>
<td>7</td>
<td>Glycine</td>
<td>0.40 ± 0.08</td>
<td>1.98 ± 0.36*</td>
<td>0.0110 (4.95-)*</td>
</tr>
<tr>
<td>12</td>
<td>Threonine</td>
<td>0.05 ± 0.02</td>
<td>0.26 ± 0.10*</td>
<td>0.0086 (5.20-)*</td>
</tr>
<tr>
<td>15</td>
<td>Glutamate</td>
<td>0.42 ± 0.08</td>
<td>1.03 ± 0.11*</td>
<td>0.0018 (2.45-)*</td>
</tr>
<tr>
<td>33</td>
<td>Fructose</td>
<td>0.43 ± 0.09</td>
<td>1.46 ± 0.22*</td>
<td>0.0023 (1.40-)*</td>
</tr>
<tr>
<td>36</td>
<td>α-Glucose</td>
<td>4.15 ± 100</td>
<td>274 ± 63*</td>
<td>0.0126 (66.0-)*</td>
</tr>
<tr>
<td>44</td>
<td>β-Glucose</td>
<td>7.18 ± 100</td>
<td>648 ± 126*</td>
<td>0.0070 (90.3-)*</td>
</tr>
<tr>
<td>50</td>
<td>Inositol</td>
<td>5.46 ± 0.69</td>
<td>14.15 ± 1.89*</td>
<td>0.0025 (2.59-)*</td>
</tr>
<tr>
<td>65</td>
<td>Disaccharide</td>
<td>23.76 ± 1.71</td>
<td>42.26 ± 4.33*</td>
<td>0.0041 (1.78-)*</td>
</tr>
<tr>
<td>Content decreased in hyperuricemia mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Acetate</td>
<td>0.21 ± 0.05</td>
<td>ND*</td>
<td>0.0182</td>
</tr>
<tr>
<td>9</td>
<td>Succinate</td>
<td>3.91 ± 1.04</td>
<td>0.27 ± 0.11*</td>
<td>0.0240 (0.70-)*</td>
</tr>
<tr>
<td>10</td>
<td>Propanoic acid</td>
<td>0.76 ± 0.13</td>
<td>0.32 ± 0.04*</td>
<td>0.0282 (0.42-)*</td>
</tr>
<tr>
<td>11</td>
<td>2,4-Dihydroxyxypirinate</td>
<td>0.18 ± 0.03</td>
<td>ND*</td>
<td>0.0069</td>
</tr>
<tr>
<td>13</td>
<td>Malate</td>
<td>0.92 ± 0.18</td>
<td>0.29 ± 0.07*</td>
<td>0.0117 (0.32-)*</td>
</tr>
<tr>
<td>19</td>
<td>Trihydroxybutyric acid</td>
<td>2.85 ± 0.35</td>
<td>0.92 ± 0.07*</td>
<td>0.0045 (0.32-)*</td>
</tr>
<tr>
<td>30</td>
<td>Acetonic acid</td>
<td>1.42 ± 0.12</td>
<td>0.23 ± 0.06*</td>
<td>&lt;0.0001 (0.16-)*</td>
</tr>
<tr>
<td>34</td>
<td>Citric acid</td>
<td>123.5 ± 29.3</td>
<td>21.19 ± 4.44*</td>
<td>0.0243 (0.17-)*</td>
</tr>
<tr>
<td>35</td>
<td>Hippuric acid</td>
<td>5.69 ± 0.36</td>
<td>2.48 ± 0.48*</td>
<td>0.0007 (0.44-)*</td>
</tr>
<tr>
<td>No significant difference</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Creatinine</td>
<td>2.65 ± 0.39</td>
<td>2.59 ± 0.21</td>
<td>0.8872</td>
</tr>
<tr>
<td>20</td>
<td>Ketonolactate</td>
<td>3.60 ± 1.32</td>
<td>0.52 ± 0.16</td>
<td>0.0794</td>
</tr>
<tr>
<td>23</td>
<td>Hydroindole</td>
<td>1.02 ± 0.27</td>
<td>0.37 ± 0.07</td>
<td>0.0733</td>
</tr>
<tr>
<td>31</td>
<td>Glycerocephosphate</td>
<td>2.04 ± 0.41</td>
<td>3.23 ± 0.48</td>
<td>0.0989</td>
</tr>
<tr>
<td>32</td>
<td>Hydroxyxymandelic acid</td>
<td>0.32 ± 0.05</td>
<td>0.48 ± 0.11</td>
<td>0.2050</td>
</tr>
<tr>
<td>39</td>
<td>Glucitol</td>
<td>11.88 ± 2.89</td>
<td>14.11 ± 5.00</td>
<td>0.7097</td>
</tr>
<tr>
<td>47</td>
<td>Glucaric acid</td>
<td>1.58 ± 0.24</td>
<td>1.42 ± 0.58</td>
<td>0.8129</td>
</tr>
<tr>
<td>49</td>
<td>Glucuronic acid</td>
<td>2.01 ± 0.45</td>
<td>2.54 ± 0.50</td>
<td>0.4499</td>
</tr>
<tr>
<td>52</td>
<td>Uric acid</td>
<td>4.18 ± 0.89</td>
<td>12.53 ± 6.17</td>
<td>0.2490</td>
</tr>
<tr>
<td>53</td>
<td>5-Hydroxyindoleacetic acid</td>
<td>0.20 ± 0.07</td>
<td>0.28 ± 0.05</td>
<td>0.3616</td>
</tr>
<tr>
<td>64</td>
<td>Lactose</td>
<td>12.16 ± 1.27</td>
<td>11.78 ± 0.77</td>
<td>0.8045</td>
</tr>
</tbody>
</table>

The relative intensity of each metabolite was expressed as 100 times the ratio of its peak area to that of ribitol (internal standard) on the same chromatograph. ND, not detected. Data are mean ± SEM. The \( P \) value was obtained using the Mann–Whitney \( U \) test.

* \( P < 0.05 \) compared with control group.
hippurate indicate reduced efficacy of tricarboxylic acid cycle activity. This finding suggests reduced energy supply to the kidney system. The metabolites identified according to the similarity between our MS data and that in the NIST database for both groups are in Table 1.

3.5. Effect of hyperuricemia on kidney tissue

To verify the results obtained from serum and urine studies, we further examined the kidney morphologic features of mice. Hematoxylin and eosin staining of kidney sections revealed characteristic tubular atrophy and proteinaceous casts in PO-treated mice tubules (Fig. 5), but no uric-acid crystal accumulation in renal tissue. Therefore, hyperuricemia-induced acute nephron injury was not caused by the uric acid nephrolithiasis.

3.6. Interleukin 6/signal transducer and activator of transcription 3 (IL-6/STAT3) signaling was dose-dependently increased in hyperuricemia-induced renal injury

IL-6/STAT3 activation has been found responsible for toxin-induced acute kidney injury in the early phase (Gartland et al., 1990). To verify whether hyperuricemia-induced renal injury has a similar mechanism, we investigated IL-6 and STAT3 expression in renal tissue. Administration of PO to mice dose-dependently increased serum IL-6 levels by 10-, 34- and 145-fold with 50, 100 and 200 mg/kg, respectively (Fig. 6A). Together with the increase in IL-6 levels, PO-induced phosphorylation of STAT3 (p-STAT3) was significantly increased in mouse kidneys challenged with doses of PO, especially 100 and 200 mg/kg (Fig. 6B). We examined the effect on p-STAT3 protein expression in kidney by immunofluorescence study. Kidney sections were stained with fluorescence-labeled antibodies against

![Fig. 3](https://example.com/fig3.png)

(A) Representative gas chromatography–mass spectrometry total ion current (TIC) chromatographs of urine metabolites from control and hyperuricemic mice. Upper panel: urine metabolites profile of the control mice. Lower panel: urine metabolites profile of the hyperuricemia mice. Each peak indicates a metabolite, and peaks are identified by comparing their retention times and MS spectra with data from the NIST and Wiley databases. The detail informations of identified metabolites were listed in Table 1. [B] Data clustering and visualization by principal component analysis plots for control and hyperuricemia mice. The X and Y axes represent PC1 and PC2, respectively.
p-STAT3 and DAPI to distinguish nuclei. p-STAT3 expression was increased in kidney tissue of PO-treated mice and was dose-dependently increased in the nuclei of PO-treated groups (Fig. 6C). Therefore, IL-6/STAT3 signaling was increased during hyperuricemia-induced kidney injury.

4. Discussion

Hyperuricemia-induced kidney injury represents an important part of clinical practice. Measurement of traditional renal function markers, such as BUN and serum creatinine, is approximate and
limited. Therefore, the use of new technologies to evaluate kidney damage and to explore better biomarkers has rapidly grown in the past few years. Metabolomics technology is a powerful strategy to provide a global view for rapid evaluation of renal function by evaluating change in metabolites. The change in metabolites in biofluids collected from experimental samples reflects the metabolic deregangement of organs, for proper application in preclinical and clinical situations. In this study, we first analyzed the urine metabolome of PO-induced hyperuricemia in mice and then demonstrated that hyperuricemia caused nephron injury through the IL-6/STAT3 signaling pathway.

Typical GC-MS profiles and PCA score plots revealed critical differences in vehicle- and PO-treated groups (Fig. 3). Further PLS-DA analysis (Fig. 4) and statistical analysis of identified metabolites (Table 1) revealed >80-fold increase in glucose in PO-treated as compared with vehicle-treated mice. The levels of lactate and phosphate were elevated six- and twofold, respectively. Additionally, the content of amino acids (e.g., glycine, threonine, and glutamine) was also elevated two- to fivefold in PO-treated mice. The nephron, composed of a renal corpuscle and renal tubule, is the fundamental structural and functional unit of the kidney. This system helps control the quantity of water, salts, glucose, urea and other minerals via glomerular filtration and tubule reabsorption/secretion. Therefore, our findings of severe glucosuria, aminonria and aciduria with hyperuricemia indicated that injured proximal tubules led to significantly decreased reabsorption/secretion function in PO-treated group. The urine output was dose-dependently reduced (data not shown), which also reflected damaged glomerular filtration. This speculation was supported by the results of urine protein analysis showing PO-treated mice with severe mixed proteinuria (Fig. 2). Therefore, hyperuricemia could cause acute renal dysfunction. Consequently, the levels of TCA cycle-related components, including succinate, malate, and citrate, were significantly decreased in PO-treated mice (Table 1). The decrease in the TCA cycle efficiency further demonstrated destroyed kidney function. Compared to serum uric acid and creatinine levels showing no significant differences at the same dose (100 mg/kg) (Fig. 1A), urine metabolite profile analysis revealed significant changes between the vehicle- and PO-treated groups. Early work conducted at the Imperial College in London suggested that urinary metabolic profiles established by 1H-NMR can distinguish the localization of nephrotoxicity (Klein et al., 2008). By establishing a series of metabolite profiles with nephrotoxic drug administration, the relationship of injury location and nephrotoxins was verified (Lindon et al., 2006; Stavric and Nera, 1978). Compared to these previous studies, our urine metabolomics study also demonstrated that hyperuricemia and nephrotoxins have the same characteristics at nephron injury sites.

PO is a competitive uricase inhibitor of hepatic uricase for use as an effective animal model in hyperuricemia-related studies (Yamashita et al., 2006). In the clinic, PO is a pyrimidine phosphoribosyltransferase (EC 2.4.2.10) competitive inhibitor used as a modulator with a novel antineoplastic combination agent, S-1 (TS-1, Taiho Pharmaceutical Co., Tokyo) (Peters et al., 2003). Pharmacokinetic study of PO indicated high compartmentalization in the body, which suggests high tissue accumulation (Shirasaka et al., 1996). PO accumulates specifically in the gut after oral administration (van Groeningen et al., 2000; Yoshisue et al., 2000), then is transformed into cyanoacetic acid in the gastrointestinal tract by two metabolic pathways: transformation to cyanoacetic acid by cecal microflora and degradation to 5-azauracil in the stomach and then conversion to cyanoacetic acid via xanthine oxidase or aldehyde oxidase (Martinon et al., 2006). Although cyanoacetic acid can form an insoluble complex with melamine, inducing tubular obstruction and tubular epithelial cell injury, no experiences have indicated the cyanoacetic acid are formed renal crystals in previous studies. Accordingly, our results of hyperuricemia-induced nephron injury were mainly caused by hyperuricemia, not PO toxicity.

Uric acid crystal triggers inflammation, and the acute process of gout has been demonstrated via activation of NALP3 inflammasome and production of IL-1β (Martinon, 2010; Wu and Wu, 2008). Additionally, uric acid stimulates the release of several inflammatory factors, such as IL-6, tumor necrosis factor α, C-reactive protein, and monocyte chemottractant protein 1 (MCP-1) (Lyngdoh et al., 2011; Ruggiero et al., 2006). Thus, uric acid-induced inflammation which is considered a risk signal is as highly correlated with the development of many chronic diseases (Jalal et al., 2011; Meotti et al., 2011; Roncal et al., 2007). Hyperuricemia plays a significant role in renal injury; however, the detailed mechanisms remain unclear. Uric acid induces chemokines such as MCP-1 in the kidney and cyclooxygenase 2 in blood vessels (Kang et al., 2002; Zhou et al., 2012). Recently, uric acid was found to induce renal inflammation via NF-κB signaling (Suganuma et al., 2002). IL-6 is a pleiotropic cytokine regulating a variety of biological functions; types of cells that can respond include endothelial cells, hepatocytes, fibroblasts, and mesangial cells (kidney) (Bonventre and Zuck, 2004). Previous studies indicated that inflammation plays a major role in the pathophysiological features of acute kidney injury (AKI) (Friedewald and Rabb, 2004; Kieler et al., 2005), and IL-6-mediated inflammatory response is a common feature of organ failure and lung injury in mice with AKI (Klein et al., 2008). Recently, the IL-6/STAT3 pathway was demonstrated to participate in HgCl₂-induced acute renal injury (Gartland et al., 1990). We found that hyperuricemia induced IL-6 expression and downregulated p-STAT3 expression dose-dependently, which suggests that the IL-6/STAT3 signaling pathway participated in the hyperuricemia-induced development of AKI (Fig. 6).

In the past decade, the relationship between hyperuricemia and renal disease progression has been widely observed in clinical and experimental studies. Using metabolomics, we revealed that the effect of hyperuricemia on kidney function is similar to nephrotoxins. We also demonstrated that hyperuricemia induced renal inflammation via activating IL-6/STAT3 signaling. Besides examining the traditional serum markers BUN, creatinine, and uric acid, determining urine metabolite profiles and serum IL-6 level would be useful for early diagnosis of hyperuricemia-induced kidney injury. This study shed light on a new perspective of the mechanism and diagnosis methods of hyperuricemia in kidney injury and can be useful in the clinical study of hyperuricemia-related diseases.
Fig. 6. Hyperuricemia induces interleukin 6 (IL-6) expression and signal transducer and activator of transcription 3 (STAT3) phosphorylation. (A) ELISA analysis of IL-6 expression in serum samples from mice treated with PO (0, 50, 100 and 200 mg/kg). Data are mean ± SEM (n = 5). Different letters indicate significant difference at P < 0.05 (one-way ANOVA). (B) Western blot analysis of phosphorylated (p)-STAT3 and total STAT3 protein level in the kidney of PO-treated mice 3 h after challenge. (C) p-STAT3 immunostaining in mouse kidney 3 h after PO-induced injury reveals extensive STAT3 activation (red nuclear staining; arrows) in renal tubular epithelial cells. Magnification, × 200. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Additionally, inflammation through IL-6/STAT3 signaling partici-
pated in the mechanism of hyperuricemia-induced kidney injury. This report will lead to a better understanding of the rela-
tionship between hyperuricemia and kidney injury and suggests that urinary metabolomics can be useful for early diagnosis of kidney disease.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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