Metabolic profiling of kidney and urine in rats with lithium-induced nephrogenic diabetes insipidus by $^1$H-NMR-based metabonomics

Geum-Sook Hwang, Ji-Young Yang, Do Hyun Ryu, and Tae-Hwan Kwon

Joint Bioanalytical Research Team, Korea Basic Science Institute, Seoul; Department of Chemistry, Sungkyunkwan University, Suwon; and Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, Taegu, Korea

Submitted 10 July 2009; accepted in final form 10 November 2009

Hwang GS, Yang JY, Ryu DH, Kwon TH. Metabolic profiling of kidney and urine in rats with lithium-induced nephrogenic diabetes insipidus by $^1$H-NMR-based metabonomics. Am J Physiol Renal Physiol 298: F461–F470, 2010. First published November 18, 2009; doi:10.1152/ajprenal.00389.2009.—Lithium (Li) treatment for bipolar affective disorders is associated with a variety of renal side effects. The metabolic response of the kidney to chronic Li treatment has rarely been studied. We applied a novel method of $^1$H-nuclear magnetic resonance (NMR)-based metabonomics to integrate metabolic profiling and to identify the changes in the levels of metabolites in the kidney and urine from rats with Li-induced NDI. Metabolite profiles of urine and kidney homogenate (3 different zones, cortex, outer medulla, and inner medulla) were investigated using high-resolution NMR spectroscopy coupled with pattern recognition methods. The accurate concentrations of metabolites in kidney homogenates and urine were rapidly measured using the target-profiling procedure, and the difference in the levels of metabolites was compared using multivariate analysis, such as principal component analysis and orthogonal partial least squares–discriminant analysis. Major endogenous metabolites for kidney homogenates contained products of glycolysis (glucose, lactate) and amino acids, as well as organic osmolytes (e.g., betaine, myo-inositol, taurine, and glycerophosphocholine). Many metabolites revealed changes in their levels, including decreased levels of organic osmolytes and amino acids in the inner medulla. A number of urinary metabolites were changed in Li-induced NDI, and in particular, elevated urinary levels of acetate, lactate, allantoin, trimethylamine, and creatine could suggest Li-induced renal cell stress or injury. Taken together, metabonomics of kidney tissue and urine based on $^1$H-NMR spectroscopy could provide insight into the effects of Li-induced renal effects and cell injury.

multivariate analysis; NMR spectroscopy; urinary concentration

There are a number of metabolites in urine, plasma, cells or tissues, and altered metabolic profiling could provide important information which indicates organ dysfunction. The metabolome is defined as the collection of all exogenous and endogenous small-molecule metabolites present in a living cell or tissue (34, 45), and the recent development of new technology for the separation and identification of small molecules makes it possible to identify and quantify a number of small-molecule metabolites in complex biological samples (11, 14, 24, 31).

The kidney is an organ which metabolizes a large number of substrates (4). These substrates can be changed into metabolites by the kidney cells (e.g., tubular epithelial cells, interstitial cells, and vascular endothelial cells) which are heterogeneous in structure, function, and metabolism (10, 25). These metabolites are also secreted into tubular lumen (i.e., urine), and hence metabonomics (i.e., a metabolic profiling analysis) of both kidney tissue and urine could provide important biological information regarding the ongoing status of kidney function via quantitative determination of the intermediary metabolites.

Lithium (Li) has widely been used for the treatment of bipolar affective disorder in human patients (40). Moreover, Li treatment is recently highlighted by a study showing that Li delays disease progression in human patients with amyotrophic lateral sclerosis (16). Chronic Li treatment, however, is associated with a variety of renal side effects, including vasopressin-resistant nephrogenic diabetes insipidus (NDI) and increased urinary sodium loss (32, 40). Our previous studies demonstrated that these side effects are, to a major extent, caused by significantly downregulated aquaporin-2 (AQP2), aquaporin-3 (AQP3), and epithelial sodium channel (ENaC) protein expression in the kidney collecting duct (23, 33). Moreover, chronic Li treatment is associated with a major cellular reorganization of the collecting duct cells with a marked increase in the fraction of intercalated cells and a significant decrease in the fraction of principal cells (9).

Li-induced effects including altered tissue metabolism has rarely been studied, particularly at the biochemical level. Although there is evidence showing that Li inhibits enzymes in the inositol pathway in tissue and increases glucose transport and glycogen synthesis in insulin-sensitive cell lines and rat skeletal muscle (7, 18, 22, 26), no studies were done previously to integrate the profiling of metabolites and to identify the changes in the levels of metabolites in the kidney and urine between control rats and rats with Li-induced NDI (Li-NDI). The successful application of $^1$H-nuclear magnetic resonance (NMR) spectroscopy of tissues and biofluids for studying metabolic diseases and toxic processes has now been established, and several novel biomarkers have been discovered for organ-specific toxicity (11, 24). Thus, using a novel method of $^1$H-NMR-based metabonomics, the present study aimed 1) to integrate the metabolic profiling data in the kidney [i.e., either in the three different zones of the kidney cortex [(COR), outer medulla (OM), and inner medulla (IM)], or in the whole kidney and urine from control rats and rats with Li-NDI; and 2) to identify the changes in the levels of the metabolites to understand the changes of metabolic response of the kidney to chronic Li treatment at the biochemical level.

MATERIALS AND METHODS

Animal Protocols of Li-NDI Rats with a Fixed Amount of Food Intake

Pathogen-free male Sprague-Dawley rats (180–200 g) were obtained from Charles River (Orient Bio, Seongnam, Korea). The animal protocols were approved by the Animal Care and Use Committee of the Kyung-
pook National University, and all animal experiments were conducted according to the guidelines of Kyungpook National University.

Protocol 1. Vehicle-treated male Sprague-Dawley rats (control rats, n = 4) and Li-treated male Sprague-Dawley rats (Li-treated rats, n = 4) received daily food rations of a food mixture consisting of 15 g grinded rat chow (2018S, Harlan Teklad, Madison, WI) and 20 ml tap water, as previously described (23, 33). This was for avoiding the potential effects of different amounts of food intake per se on the metabolic profiling in kidney tissues and urine. The Li-treated rats received 0.6 mmol LiCl (L4408 Sigma) added to the food mixture per day for 7 days, and thereafter rats received 0.9 mmol LiCl (L4408 Sigma) added to the food mixture per day for the following 16 days. In addition to the water in the food mixture, rats had free access to water intake. For the first 21 days, rats were housed individually in normal rat cages, and each rat received 35 g of the food mixture [food (15 g) + water (20 ml)]/day. Thereafter, for the last 2 days the rats were housed in metabolic cages and were given 15 g grinded rat chow (2018S, Harlan Teklad) mixed with 25 ml tap water containing 0.9 mmol LiCl/day. All rats had free access to water intake. In this protocol, three different kidney zones (COR, OM, and IM) were subjected to the metabonomic analysis.

Protocol 2. This protocol was identical to protocol 1 for the Li treatment. Vehicle-treated male Sprague-Dawley rats (control rats, n = 6) and Li-treated male Sprague-Dawley rats (Li-treated rats, n = 6) were studied. In contrast to protocol 1, whole kidney samples were used for the metabonomic analysis.

Daily water intake and urine output were monitored. Urine was analyzed for concentrations of Na⁺, K⁺, creatinine and osmolality and was kept at −80°C for the analysis of metabolites. During the entire experiment, there was a 12:12-h artificial light-dark cycle, a temperature of 20 ± 2°C, and humidity of 55 ± 2%. On day 24, the rats were anesthetized with enfurane inhalation and a large laparotomy was made. Blood was collected from the inferior vena cava into blood collection tubes containing sodium heparin (BD Vacutainer, BD, Franklin Lakes, NJ). The plasma was prepared to be analyzed for Na⁺, K⁺, Li⁺, creatinine, and osmolality. The right whole kidney was rapidly removed, rapidly frozen in liquid nitrogen, and kept at −80°C before the analysis of metabolites.

Analysis of Biochemistry

Blood was centrifuged for 15 min at 4,000 g in a tabletop centrifuge (Eppendorf 5810R, Hamburg, Germany), and plasma was transferred to Eppendorf tubes. Measurements of plasma or urine concentrations of Na⁺, K⁺, Li⁺, urea nitrogen, and creatinine were determined by Vitros 250 (Johnson & Johnson). Measurement of plasma and urine osmolality was carried out by freezing-point depression (Osmomat 030-D, Gonotec, Berlin, Germany). Creatinine clearance was calculated as $C_{cr} = \frac{\text{urine creatinine concentration} \times \text{urine volume}}{\text{plasma creatinine concentration}}$. Free water reabsorption was calculated as $T_{H_2O} = \text{urine volume} \times \left[\left(\frac{\text{Uosm}}{\text{Posm}}\right) - 1\right]$, where Uosm/Posm is the urine-to-plasma osmolality ratio.

Sample Preparation for Metabonomic Analysis

Urine samples were thawed, vortexed, and centrifuged at 13,000 rpm for 10 min. Each of the 400-μl urine supernatants was mixed with 200 μl buffer solution (0.2 M phosphate buffer containing 0.02% sodium azide, pH 7.0) to minimize variations in the pH of samples. For NMR experiments, each of 540-μl buffered samples was mixed with 60 μl of D₂O containing 5 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal chemical standard.

For kidney samples, right kidneys from control (n = 4) and Li-treated rats (n = 4) were divided into three different zones, i.e., COR, OM, and IM in protocol 1, whereas right whole kidneys from both control (n = 6) and Li-treated rats (n = 6) were used in protocol 2: I) tissues were homogenized with a Precellys 24 bead-based homogenizer (ceramic bead, Stretton Scientific, Stretton, UK) containing chloroform (6 ml/g), methanol (6 ml/g), and distilled water (4 ml/g); and 2) the homogenate was vortexed and left on ice for 10 min to partition, and then centrifuged for 10 min. The upper polar layer was transferred into separate vials and lyophilized. Each extract was dissolved in 400 μl distilled water and then prepared using the same protocol as for urine (e.g., buffer condition, pH correction, and internal standard content).

NMR Spectroscopic Measurement of Urine and Kidney Homogenates

1H-NMR spectra were acquired on a VNMRS-600-MHz NMR spectrometer (Varian, Palo Alto, CA) using a triple-resonance 5-mm HCN salt-tolerant cold probe. For urine and kidney extract, the NOESY-presat pulse sequence was applied to suppress the residual water signal. Free induction decays were collected with 1,024 transients into 67,568 data points using a spectral width of 8,445.9 Hz with a relaxation delay of 2 s, an acquisition time of 4.00 s, and a mixing time of 100 ms. All spectra were zero-filled to 128 K data points, and line-broadening of 1.5 Hz was applied.

NMR Spectral Data Reduction and Multivariate Statistical Analysis

Analysis of NMR spectral data was accomplished using targeted profiling through the use of Chenomx NMR Suite 5.1 (Chenomx, Edmonton, Canada), and concentrations were determined using the 600-MHz library from Chenomx NMR Suite 4.6, which compares the integral of a known reference signal (DSS-d6) with signals derived from a library of compounds containing chemical shifts and peak multiplicities. Each urinary metabolite concentration was normalized to creatinine (μM/μl creatinine) in each urine sample, and kidney metabolite concentration was normalized to tissue weight used for extraction in each tissue sample.

Multivariate data analysis, principal component analysis (PCA), and orthogonal partial least squares-discriminant analysis (OPLS-DA) were performed on metabolite concentrations that had been log₂ transformed and mean centered with unit variance scaling applied using SIMCA-P (version 12, Umetrics, Umeå, Sweden). PCA, an unsupervised pattern recognition (PR) method, was performed to examine the intrinsic variation in the data set, and OPLS-DA, a

<table>
<thead>
<tr>
<th>Table 1. Changes in renal function in Li-treated rats (protocol 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>BW, g</td>
</tr>
<tr>
<td>RK wt, g</td>
</tr>
<tr>
<td>P-Na, mmol/l</td>
</tr>
<tr>
<td>P-K, mmol/l</td>
</tr>
<tr>
<td>P-urea nitrogen, mg/dl</td>
</tr>
<tr>
<td>P-Cr, mg/dl</td>
</tr>
<tr>
<td>P-osm, mosmol/kgH₂O</td>
</tr>
<tr>
<td>P-Li, mmol/l</td>
</tr>
<tr>
<td>UO, m/d 72</td>
</tr>
<tr>
<td>U-urine, mosmol/kgH₂O</td>
</tr>
<tr>
<td>Ccr, mmol/l</td>
</tr>
<tr>
<td>U-Na  × Uvol, mmol</td>
</tr>
<tr>
<td>U-K  × Uvol, mmol</td>
</tr>
<tr>
<td>FE₅₀₆, %</td>
</tr>
<tr>
<td>FE₉₅, %</td>
</tr>
<tr>
<td>T⁵⁰₇H₂O, m/d 43</td>
</tr>
<tr>
<td>Ccr, ml/min</td>
</tr>
</tbody>
</table>

Values are means ± SE, n, No. of rats; BW, body weight; RK, right kidney weight; P-Na, plasma sodium; P-K, plasma potassium; P-urea nitrogen, plasma urea nitrogen; P-Cr, plasma creatinine; P-osm, plasma osmolality; P-Li, plasma lithium concentration; UO, urine output; U-osm, urine osmolality; U-PF₅₀₆, urine-to-plasma osmolality ratio; UA × Uvol, urinary excretion of sodium; UK × Uvol, urinary excretion of potassium; FE₅₀₆, fractional excretion of sodium; FE₉₅, fractional excretion of potassium; T⁵⁰₇H₂O, solute-free water reabsorption; Ccr, creatinine clearance. *P < 0.05 Li compared with control.
supervised PR method, was also employed to maximize the separation between the control and Li-treated samples. The quality of the models was described by $R^2$ and $Q^2$ values. $R^2$ is defined as the proportion of variance in the data explained by the models and indicates goodness of fit, and $Q^2$ is defined as the proportion of variance in the data predictable by the model and indicates predictability (42). P values were generated by an unpaired t-test, and $P < 0.05$ were considered significant.

RESULTS

Li-NDI was Associated with Increased Urine Output and Decreased Urine Osmolality

In protocol 1, the rats fed Li-containing food for 23 days demonstrated high plasma Li concentrations: $1.9 \pm 0.2$ mM in Li-treated rats (Table 1). The Li-treated group demonstrated significantly increased urine output compared with control rats ($P < 0.05$, Table 1). In parallel, increased urine output was accompanied by markedly decreased urine osmolality ($264 \pm 23$ mosmol/kgH$_2$O), Uosm/Posm, and $T^3$H$_2$O (Table 1). In particular, urine osmolality was less than that of plasma (Uosm/Posm: $0.9 \pm 0.1$, Table 1), indicating that chronic Li treatment was associated with a severe impairment of water reabsorption in the collecting duct and an inability to concentrate urine, as previously demonstrated (23, 27). In addition, plasma urea nitrogen levels were increased and glomerular filtration rate (measured by creatinine clearance) was decreased in Li-treated rats (Table 1). In protocol 2, Li-treated rats demonstrated plasma Li concentrations of $2.0 \pm 0.2$ mM (Table 2). Similar to the decreased urinary concentration observed in protocol 1, the Li-treated group in protocol 2 also demonstrated significantly increased urine output and decreased urine osmolality, Uosm/Posm, and $T^3$H$_2$O (Table 2), indicating a severe impairment of urinary concentration.

Metabonomic Analysis of Kidney Homogenates from Control and Li-Treated Rats

Figure 1 shows representative $^1$H-NMR spectra of homogenates of IM, OM, and COR, respectively, from control and Li-treated rats (protocol 1). The $^1$H-NMR spectra of whole kidney homogenates (protocol 2) was similar to that of COR in Li-treated rats (Table 1). The Li-treated group demonstrated significantly increased urine output compared with control rats ($P < 0.05$, Table 1). In parallel, increased urine output was accompanied by markedly decreased urine osmolality ($264 \pm 23$ mosmol/kgH$_2$O), Uosm/Posm, and $T^3$H$_2$O (Table 1). In particular, urine osmolality was less than that of plasma (Uosm/Posm: $0.9 \pm 0.1$, Table 1), indicating that chronic Li treatment was associated with a severe impairment of water reabsorption in the collecting duct and an inability to concentrate urine, as previously demonstrated (23, 27). In addition, plasma urea nitrogen levels were increased and glomerular filtration rate (measured by creatinine clearance) was decreased in Li-treated rats (Table 1). In protocol 2, Li-treated rats demonstrated plasma Li concentrations of $2.0 \pm 0.2$ mM (Table 2). Similar to the decreased urinary concentration observed in protocol 1, the Li-treated group in protocol 2 also demonstrated significantly increased urine output and decreased urine osmolality, Uosm/Posm, and $T^3$H$_2$O (Table 2), indicating a severe impairment of urinary concentration.

Table 2. Changes in renal function in Li-treated rats (protocol 2)

<table>
<thead>
<tr>
<th></th>
<th>Control ($n = 6$)</th>
<th>Li ($n = 6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW, g</td>
<td>285 ± 6</td>
<td>184 ± 8*</td>
</tr>
<tr>
<td>P-osm, mosmol/kgH$_2$O</td>
<td>301 ± 1</td>
<td>304 ± 3</td>
</tr>
<tr>
<td>P-Li, mmol/l</td>
<td></td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>UO, ml/day</td>
<td>18.0 ± 0.8</td>
<td>60.9 ± 6.7*</td>
</tr>
<tr>
<td>U-osm, mosmol/kgH$_2$O</td>
<td>890 ± 50</td>
<td>235 ± 26*</td>
</tr>
<tr>
<td>(U/P)osm</td>
<td>3.0 ± 0.2</td>
<td>0.8 ± 0.1*</td>
</tr>
<tr>
<td>$T^3$H$_2$O, ml/day</td>
<td>34.7 ± 1.7</td>
<td>-20.0 ± 7.9*</td>
</tr>
</tbody>
</table>

Values are means ± SE. $n$, No. of rats *$P < 0.05$ Li compared with control.

Fig. 1. Representative 600-MHz $^1$H-nuclear magnetic resonance (NMR) spectra of inner medulla (IM; A), outer medulla (OM; B), and cortex (Cor; C) homogenates obtained from control and Li-treated rats.
protocol 1 (not shown). Spectral resonances of metabolites were assigned according to the literature (21, 30, 47) and the 600-MHz library from Chenomx NMR suite 5.1. The ambiguous peaks due to the overlap or a slight shift, such as glycerophosphocholine (GPC), were confirmed by spiking samples with the respective standard compounds. For each kidney homogenate, nearly 40 metabolites were identified and quantified for control and Li-treated rats. Peak intensity signals of each NMR spectrum in tissue homogenates were different between the kidney zones because the amounts of kidney tissues obtained from the different kidney zones were not the same. However, since the amounts of tissue extracted from the same zone of the kidney were not highly different among the kidneys and measured metabolite concentrations were normalized to the

Fig. 2. Principal component analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) score plots of metabolite concentrations determined in the IM (A and B), OM (C and D), Cort (E and F) and whole kidney (G and H) homogenates from control and Li-treated rats. PCA models (A, C, E, and G) were generated with PCs, and OPLS-DA (B, D, F, H) models were generated with predictive component(T)s and orthogonal component(TO)s to discriminate between control and Li-treated rats. The statistics for PCA models differentiating metabolites from control and Li-treated rats revealed $R^2$ values of 0.94 (A), 0.71 (C), 0.92 (E), and 0.77 (G) and $Q^2$ values of 0.68 (A), 0.16 (C), 0.26 (E), and 0.46 (G). The statistics for OPLS-DA models revealed high $R^2$X values of 0.99 (B), 0.98 (D), 0.70 (F), and 0.74 (H), and $Q^2$ values of 0.99 (B), 0.98 (D), 0.88 (F), and 0.92 (H). The $R^2$ value represents the goodness of fit of the models, and the $Q^2$ value represents the predictability of the models.
weight of each kidney tissue used for homogenization, the metabolic pattern in the same zone of the kidney could be comparable between control and Li-treated rats. Major endogenous metabolites from kidney homogenates contained products of glycolysis (glucose, lactate) and amino acids, as well as organic osmolytes such as betaine, myo-inositol, taurine, and GPC (Fig. 1).

PCA was initially performed on the metabolite concentrations obtained from the analysis of kidney homogenates collected from control and Li-treated rats (Fig. 2, A, C, E, and G). PCA score plots showed fairly clear differences between control and Li-treated rats in all zones of the kidney as well as whole kidney, indicating the significant changes in kidney metabolism in Li-treated rats. $Q^2$ and $R^2$ values were calculated for the models to assess any significance of differentiation. The statistics for the PCA model showed high goodness of fit with $R^2$ values from 0.71 to 0.95 and quite poor predictive capability with $Q^2$ value from 0.16 to 0.68, depending on the zone of kidney. OPLS-DA was employed to maximize separation between the two groups (Fig. 2, B, D, F, and H) and showed a clear differentiation. Compared with the PCA model, these OPLS-DA models (Fig. 2, B, D, F, and H) showed improved predictability, with a $Q^2$ value from 0.88 to 0.99.

To identify the metabolites responsible for the differentiation in OPLS-DA score plots between the two compared groups, OPLS-DA loading plots were generated (Fig. 3, A–D). Since all the OPLS-DA score plots for tissue data are entirely dominant for T1, as shown in Fig. 2, B, D, F, and H, we showed the OPLS-DA loading plots for only T1. The loading plots represented which metabolites were quantitatively higher or lower in Li-treated rats compared with control rats. The changes of quantitative metabolite concentrations between control and Li-treated rats were different depending on the zone of the kidney (Fig. 3, A–C). Only creatine level among the observed metabolites in kidney homogenates was observed to be increased in all kidney zones from the Li-treated group. In contrast, levels of metabolites such as alanine, betaine, glucose, isoleucine, methionine, phenylalanine, succinate, taurine, and valine were decreased in all kidney zones in Li-treated groups.

Importantly, many metabolites revealed zonal differences in their levels (Fig. 3, A–C). Levels of allantoin, choline, GPC, and urea were decreased in the IM from Li-treated rats, whereas they were increased in the OM and the COR. In contrast, levels of fumarate and pyruvate were increased in the IM from Li-treated rats, whereas they were decreased in the OM and the COR. Levels of metabolites such as formate,
glutamine, leucine, and myo-inositol were decreased in the IM and OM from Li-treated groups, whereas they were unchanged or increased in the COR. The metabolite differences in the whole kidney between Li-treated and control rats (Fig. 3D) were mostly similar to those in the COR (Fig. 3C), illustrating that cortical tissue constitutes most of the kidney tissue. Thirty metabolites responsible for the observed difference between control and Li-treated rats are summarized in Table 3, with the relative concentration of metabolites in control and Li-treated rats for different kidney zones.

Metabolomic Analysis of Urinary Metabolites from Control and Li-Treated Rats

Typical $^1$H-NMR spectra of urine from control and Li-treated rats (protocol 1) are shown in Fig. 4, along with the metabolite assignments. Although urinary NMR spectra had similar peak patterns, significant metabolic differences were observed between the NMR spectra from the control and Li-treated rats. Comparison of the urinary metabolite profiles between control and Li-treated rats (protocol 1) revealed a significant increase in acetate and creatine but a decrease in hippurate in Li-treated rats.

PCA and OPLS-DA score plots were performed on the urinary metabolite concentrations from control and Li-treated rats (Fig. 5, A and B). As seen in the kidney tissues, both PCA and OPLS-DA score plots showed clear differences between control and Li-treated rats. This indicates that Li-treated rats have significantly altered renal metabolism, and hence the altered profiling of urinary metabolites could reflect the changes in renal metabolism affected by Li treatment. The statistics for PCA analysis of urinary metabolite concentrations showed high goodness of fit with an $R^2$ value of 0.95 and $Q^2$ value of 0.72. The OPLS-DA model showed an improved $R^2$ value of 0.98 and $Q^2$ value of 0.99, exhibiting a distinct differentiation between the two groups. To identify the urinary metabolites responsible for the differentiation in OPLS-DA score plots for two compared groups, an OPLS-DA loading plot was generated (Fig. 6). The loading plot represented which metabolites were quantitatively higher or lower in Li-treated groups compared with control groups. Levels of metabolites such as 1-MNA, 2-oxoglutarate, allantoin, benzotate, creatine, dimethylamine, lactate, niacinamide, propylene glycol, succinate, trimethylamine (TMA), urea, and trans-aconitate were significantly increased in Li-treated groups, whereas the level of hippurate was decreased. Urinary metabolites responsible for the observed difference between control and Li-treated rats were summarized in Table 4, with the relative metabolite concentration of control and Li-treated groups.

DISCUSSION

Metabolites are endogenous and exogenous molecules which play a role in cellular regulatory and biological systems. Meta-

Table 3. $^1$H chemical shift assignment of metabolites observed in kidney homogenates from control and Li-treated rats and their relative concentration (protocol 1)

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Proton Group</th>
<th>$\delta$-$^1$H, ppm</th>
<th>Inner medulla</th>
<th>Outer medulla</th>
<th>Cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>$\alpha$-CH, $\beta$-CH$_3$</td>
<td>3.81 (q), 1.48 (d)</td>
<td>0.67</td>
<td>0.87</td>
<td>0.84</td>
</tr>
<tr>
<td>Allantoin</td>
<td>CH$_1$</td>
<td>5.40 (s)</td>
<td>0.74</td>
<td>1.48</td>
<td>2.16</td>
</tr>
<tr>
<td>Asp</td>
<td>$\beta$-CH$_3$</td>
<td>2.68 (dd)</td>
<td>1.06</td>
<td>0.80</td>
<td>0.94</td>
</tr>
<tr>
<td>Betaine</td>
<td>CH$_1$, CH$_2$</td>
<td>3.26 (s), 3.89 (s)</td>
<td>0.33</td>
<td>0.97</td>
<td>0.69</td>
</tr>
<tr>
<td>Choline</td>
<td>N-CH$_2$, $\beta$-CH$_2$, $\alpha$-CH$_2$</td>
<td>3.19 (s), 3.50 (m), 4.07 (m)</td>
<td>0.39</td>
<td>1.19</td>
<td>1.42</td>
</tr>
<tr>
<td>Creatine</td>
<td>CH$_1$, CH$_2$</td>
<td>3.04 (s), 3.93 (s)</td>
<td>3.71</td>
<td>3.70</td>
<td>7.42</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>NH-CH$_2$, HO-CH$_2$</td>
<td>3.15 (t), 3.78 (t)</td>
<td>0.67</td>
<td>1.09</td>
<td>0.95</td>
</tr>
<tr>
<td>Formate</td>
<td>CH</td>
<td>8.45 (s)</td>
<td>0.35</td>
<td>0.69</td>
<td>1.30</td>
</tr>
<tr>
<td>Fumarate</td>
<td>CH</td>
<td>6.53 (s)</td>
<td>1.75</td>
<td>0.85</td>
<td>0.82</td>
</tr>
<tr>
<td>Glucose</td>
<td>I-CH</td>
<td>5.22 (d)</td>
<td>0.65</td>
<td>0.68</td>
<td>0.87</td>
</tr>
<tr>
<td>Glutamate</td>
<td>$\alpha$-CH, $\beta$-CH$_2$, $\gamma$-CH$_2$</td>
<td>3.76 (m), 2.06 (m), 2.36 (m)</td>
<td>0.96</td>
<td>0.76</td>
<td>0.96</td>
</tr>
<tr>
<td>Glutamine</td>
<td>$\alpha$-CH, $\beta$-CH$_2$, $\gamma$-CH$_2$</td>
<td>3.76 (m), 2.15 (m), 2.46 (m)</td>
<td>0.25</td>
<td>0.77</td>
<td>1.06</td>
</tr>
<tr>
<td>Glycine</td>
<td>CH$_2$</td>
<td>3.55 (s)</td>
<td>0.54</td>
<td>0.68</td>
<td>0.70</td>
</tr>
<tr>
<td>Inosine</td>
<td>N-CH$_2$, $\beta$-CH$_2$, $\gamma$-CH$_2$, $\beta$, $\alpha$-CH$_3$</td>
<td>6.09 (d), 8.22 (s), 8.36 (s), 4.43 (t)</td>
<td>0.83</td>
<td>1.05</td>
<td>1.17</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>$\alpha$-CH$_2$, $\beta$-CH$_3$, half $\gamma$-CH$_2$, half $\gamma$-CH$_2$, $\beta$, $\alpha$-CH$_3$</td>
<td>0.93 (t), 1.00 (d), 1.28 (m), 1.47 (m), 1.96 (m), 3.68 (d)</td>
<td>0.53</td>
<td>0.56</td>
<td>0.82</td>
</tr>
<tr>
<td>Lactate</td>
<td>CH$_1$, CH$_2$</td>
<td>4.12 (q), 1.33 (d)</td>
<td>0.96</td>
<td>1.32</td>
<td>0.94</td>
</tr>
<tr>
<td>Leucine</td>
<td>$\alpha$-CH, $\gamma$-CH, $\delta$-CH$_3$, $\delta$-CH$_2$</td>
<td>3.72 (t), 1.69 (m), 0.97 (d), 0.94 (d)</td>
<td>0.48</td>
<td>0.59</td>
<td>0.91</td>
</tr>
<tr>
<td>Lysine</td>
<td>$\alpha$-CH, $\beta$-CH$_2$, $\gamma$-CH$_2$, $\delta$-CH$_3$, $\epsilon$-CH$_2$</td>
<td>3.77 (t), 1.92 (m), 1.73 (m), 1.47 (m), 3.05 (t)</td>
<td>0.93</td>
<td>0.80</td>
<td>0.96</td>
</tr>
<tr>
<td>Methionine</td>
<td>$\alpha$-CH$_2$, $\gamma$-CH$_2$, $\epsilon$-CH$_2$, $\delta$-CH$_3$</td>
<td>3.87 (m), 2.16 (m), 2.65 (dd), 2.15 (s)</td>
<td>0.37</td>
<td>0.78</td>
<td>0.76</td>
</tr>
<tr>
<td>NA</td>
<td>N-CH$_2$, $\gamma$-CH$_2$, $\beta$, $\alpha$-CH$_3$(P)</td>
<td>8.92 (d)</td>
<td>1.13</td>
<td>0.97</td>
<td>1.62</td>
</tr>
<tr>
<td>GPC</td>
<td>N-CH$_2$, $\gamma$-CH$_2$, $\beta$, $\alpha$-CH$_3$(P)</td>
<td>3.24 (s), 3.67 (dd), 3.78 (m), 4.36 (m)</td>
<td>0.20</td>
<td>2.24</td>
<td>1.15</td>
</tr>
<tr>
<td>PA</td>
<td>3,5-CH, 4,CH, 2,6-CH, half $\beta$-CH$_2$</td>
<td>0.62</td>
<td>0.54</td>
<td>0.81</td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>CH$_1$</td>
<td>2.37 (s)</td>
<td>0.64</td>
<td>0.84</td>
<td>1.45</td>
</tr>
<tr>
<td>Succinate</td>
<td>2CH$_2$</td>
<td>2.41 (s)</td>
<td>0.64</td>
<td>0.54</td>
<td>0.49</td>
</tr>
<tr>
<td>Taurine</td>
<td>CH$_2$N, CH$_2$S</td>
<td>3.27 (t), 3.43 (t)</td>
<td>0.79</td>
<td>0.95</td>
<td>0.76</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>half $\beta$-CH$_2$, half $\beta$-CH$_2$, $\alpha$-CH, 3,5-CH, 2,6-CH</td>
<td>3.06 (dd), 3.16 (dd), 3.94 (dd), 6.87 (d), 7.20 (d)</td>
<td>0.74</td>
<td>0.55</td>
<td>0.77</td>
</tr>
<tr>
<td>Uracil</td>
<td>CH, CH</td>
<td>5.81 (d), 7.59 (d)</td>
<td>0.43</td>
<td>0.58</td>
<td>1.20</td>
</tr>
<tr>
<td>Urea</td>
<td>NH$_3$</td>
<td>5.8 (br)</td>
<td>0.77</td>
<td>1.75</td>
<td>3.09</td>
</tr>
<tr>
<td>Valine</td>
<td>$\alpha$-CH, $\beta$-CH$_2$, $\gamma$-CH$_2$, $\gamma$-CH$_3$</td>
<td>3.59 (d), 2.25 (m), 0.98 (d), 1.03 (d)</td>
<td>0.59</td>
<td>0.50</td>
<td>0.83</td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>1,3-CH, 2,CH, 5-CH, 4,6-CH</td>
<td>3.53 (dd), 4.06 (dd), 3.28 (t), 3.63 (t)</td>
<td>0.38</td>
<td>0.99</td>
<td>1.16</td>
</tr>
</tbody>
</table>

Relative concentrations were calculated by [Li]/[control]. Asp, aspartate; NA, niacinamide; GPC, glycerophosphocholine; PA, phenylalanine.
bolic profiling has recently been exploited for the studies of the pathophysiology of diseases (11, 24, 31, 34, 45). Metabonomics, especially based on NMR spectroscopy, has identified important biomarkers and prognostic factors improving clinical diagnosis through successful application in the study of human diseases (2, 3, 8, 20, 36, 41, 44). A relatively new approach of targeted profiling (38, 43), which identifies as many metabolites as possible in an NMR spectrum, was used in our study to understand the changes in metabolic responses of the kidney to chronic Li treatment at the biochemical level.

We controlled the potential factors that impact the metabolic profiles, e.g., different food intake, gender, age, and diurnal rhythm. Previously, Slupsky et al. (39) demonstrated the effects of gender, age, and diurnal variation on human urinary metabolomic profiles. In particular, metabolites related to mitochondrial energy metabolism were revealed to differentiate by gender and age. For example, TCA cycle intermediates (citrate and fumarate) and creatine concentrations in human urine were significantly higher in females than males, whereas metabolites related to fatty acid oxidation (carnitine, acetylcar

citrate, and acetone) and creatinine concentrations were higher in males than females. Creatine and creatinine concentrations have been shown to be associated with diet (28); however, the finding noted above demonstrating higher urinary levels of creatine in females suggests higher endogenous synthesis. Moreover, diet and diurnal rhythms impacted the urinary

Fig. 4. Representative 600-MHz 1H-NMR spectra of urine obtained from control and Li-treated rats.

Fig. 5. PCA and OPLS-DA score plots of metabolite concentrations determined in the urine from control and Li-treated rats. PCA model was generated with PCs, and OPLS-DA model was generated with predictive component(T)s and orthogonal component(TO)s to discriminate between control and Li-treated rats. The statistics for PCA models differentiating metabolites from control and Li-treated rats revealed $R^2$ value of 0.95 and $Q^2$ value of 0.72. The statistics for OPLS-DA models revealed $R^2$ value of 0.98 and $Q^2$ value of 0.99. $R^2$ and $Q^2$ are defined as in Fig. 2.

Fig. 6. OPLS-DA loading plots of urine from control and Li-treated rats. The loading plots represent which metabolites are quantitatively higher or lower in Li-treated groups compared with control groups. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
metabolic profiles (39). In this study, male Sprague-Dawley rats receiving the same amount of food in the morning (once a day) were examined, and 24-h urine collected in the morning before food was given was used for metabonomic analysis.

The accurate concentrations of metabolites in kidney tissue and urine were rapidly measured using the target-profiling procedure, and thereafter the differences in the levels of metabolites were compared using multivariate analysis such as PCA and OPLS-DA. The targeted-profiling and multivariate pattern recognition approach we have used herein permitted us to observe simultaneously a broad range of metabolites, the concentrations of which could be changed under biological stimuli or certain disease conditions. Since metabolites can be analyzed the whole feature of metabolites rather than several stimuli or certain disease conditions. Since metabolites can be measured of the renal medulla previously.

### Table 3. 1H chemical shift assignment of metabolites observed in urine from control and Li-treated rats and their relative concentration (protocol 1)

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Proton Group</th>
<th>δ - 1H ppm</th>
<th>Relative Concentration, Li/control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-MNA</td>
<td>CH3, 6-CH, 2-CH, 5-CH, 4-CH</td>
<td>4.48 (s), 8.97 (d), 9.28 (s), 8.19 (t), 8.90 (d)</td>
<td>4.41</td>
</tr>
<tr>
<td>2-OG</td>
<td>β-CH2, γ-CH2</td>
<td>3.02 (t), 2.50 (t)</td>
<td>2.77</td>
</tr>
<tr>
<td>3-IS</td>
<td>5-CH, 6-CH, 4-CH, 7-CH</td>
<td>7.20 (t), 7.27 (t), 7.51 (d), 7.70 (d)</td>
<td>1.32</td>
</tr>
<tr>
<td>Acetate</td>
<td>CH3</td>
<td>1.91 (s)</td>
<td>17.33</td>
</tr>
<tr>
<td>Alanine</td>
<td>α-CH, β-CH3</td>
<td>3.81 (q), 1.48 (d)</td>
<td>1.57</td>
</tr>
<tr>
<td>Allantoin</td>
<td>CH3</td>
<td>5.40 (s)</td>
<td>1.86</td>
</tr>
<tr>
<td>Citrate</td>
<td>1-CH2, 3-CH2</td>
<td>2.69 (d), 2.54 (d)</td>
<td>1.29</td>
</tr>
<tr>
<td>Creatine</td>
<td>CH3, CH2</td>
<td>3.04 (s), 3.93 (s)</td>
<td>58.09</td>
</tr>
<tr>
<td>Creatinine</td>
<td>CH3, CH3</td>
<td>3.05 (s), 4.06 (s)</td>
<td>—</td>
</tr>
<tr>
<td>DMA</td>
<td>2xCH3</td>
<td>2.71 (s)</td>
<td>1.87</td>
</tr>
<tr>
<td>Formate</td>
<td>CH</td>
<td>8.45 (s)</td>
<td>0.94</td>
</tr>
<tr>
<td>Fumarate</td>
<td>CH</td>
<td>6.53 (s)</td>
<td>0.76</td>
</tr>
<tr>
<td>Glycine</td>
<td>CH2</td>
<td>3.55 (s)</td>
<td>1.69</td>
</tr>
<tr>
<td>Hippurate</td>
<td>CH3, 2,6-CH, 4-CH, 7-CH</td>
<td>3.97 (d), 7.84 (d), 7.55 (t), 7.64 (t)</td>
<td>0.36</td>
</tr>
<tr>
<td>Lactate</td>
<td>CH, CH3</td>
<td>4.12 (q), 1.33 (d)</td>
<td>1.80</td>
</tr>
<tr>
<td>NA</td>
<td></td>
<td>8.92 (d)</td>
<td>4.33</td>
</tr>
<tr>
<td>PAG</td>
<td>2,6-CH, 3,5-CH, Ph-CH2, N-CH2</td>
<td>7.43 (m), 7.37 (m), 3.75 (d), 3.68 (s)</td>
<td>1.57</td>
</tr>
<tr>
<td>Succinate</td>
<td>2xCH2</td>
<td>2.41 (s)</td>
<td>1.66</td>
</tr>
<tr>
<td>Taurine</td>
<td>CH,N, CH3</td>
<td>3.27 (t), 3.43 (t)</td>
<td>1.94</td>
</tr>
<tr>
<td>Trigonelline</td>
<td></td>
<td>4.42 (s), 8.07 (t), 8.82 (q), 9.11 (s)</td>
<td>1.09</td>
</tr>
<tr>
<td>TMA</td>
<td>3xCH3</td>
<td>2.88 (s)</td>
<td>29.85</td>
</tr>
<tr>
<td>TMAO</td>
<td>3xCH3</td>
<td>3.27 (s)</td>
<td>1.03</td>
</tr>
<tr>
<td>Urea</td>
<td>NH2</td>
<td>5.8 (br)</td>
<td>1.82</td>
</tr>
<tr>
<td>cis-Aconitate</td>
<td>half CH2, half CH2</td>
<td>5.70 (s), 3.11 (s)</td>
<td>1.10</td>
</tr>
<tr>
<td>trans-Aconitate</td>
<td>half CH2, half CH2</td>
<td>6.57 (s), 3.44 (s)</td>
<td>2.74</td>
</tr>
</tbody>
</table>

Each metabolite concentration was normalized to creatinine (μM/nM creatinine) in each urine. Then, relative concentrations were calculated by [Li]/[control], 1-MNA, 1-methylnicotinamide; 2-OG, 2-oxoglutarate; 3-IS, 3-indoxylsulfate; DMA, dimethylamine; NA, niacinamide; PAG, phenylacetylglycin; TMA, trimethylamine; TMAO, trimethylamine-N-oxide.

Renal medullary cells are exposed to the stress of long-term hyperosmolarity and respond to this stress by accumulating compatible organic osmolytes (17). A previous study revealed that organic osmolytes except myo-inositol were increased along the corticopapillary axis in normal rat kidneys (35). In contrast, in rats with Li-induced impaired urinary concentration, HPLC measurement of the renal medulla previously demonstrated significantly decreased intracellular osmolytes (e.g., sorbitol, betaine, inositol, taurine, and GPC) (5). Amiloride administration, which inhibits the major Li-transporting proteins, however, resulted in a restoration in the amounts of intracellular organic osmolytes and urinary concentration (5). Consistent with this, vasopressin increased urinary concentration in vasopressin-deficient Brattleboro rats, associated with a rise in inner medullary osmolytes (e.g., urea, sodium, GPC, sorbitol, betaine, and inositol) (17). In the present study, we demonstrated altered levels of a number of metabolites in the kidney and urine from rats with Li-NDI by metabonomics using 1H-NMR spectroscopy. Importantly, we demonstrated the different levels of organic osmolytes in different kidney zones: betaine and taurine were decreased in all the kidney zones of Li-NDI, whereas GPC and urea were only decreased in IM and myo-inositol was decreased in both IM and OM. Thus the observed decreased levels of medullary organic osmolytes could reduce the medullary osmotic gradient, contributing, at least in part, to the impaired urine concentration in Li-NDI. Compatible with the observed decreased level of myo-inositol in the medulla, Li was demonstrated to inhibit two enzymes in the inositol pathway, namely, inositol poly-phosphate 1-phosphatase and inositol monophosphatase (26, 37), leading to the depletion of inositol levels in tissue (7).

Intracellular contents of nearly all the investigated amino acids (Ala, Asp, Glu, Gln, Gly, Ile, Leu, Lys, Met, and Val; Table 3) were unchanged or decreased in the kidney medulla of Li-NDI. In particular, the glutamine level was markedly decreased in IM, suggesting substantially increased metabolic demand in Li-NDI. The role of amino acids in renal energy metabolism and gene expression has not been well understood. Interestingly, a previous study revealed an important role of...
glutamine as a major nutrient regulator of cardiac gene expression PKA and mammalian target of rapamycin (mTOR) were involved (46). Thus it is of interest to study whether glutamine, the most abundant amino acid in extracellular fluid, or other amino acids play a role in the regulation of gene expression and signaling pathways particularly involved in urine concentration, e.g., transcriptional response for collecting duct AQPs and their regulatory proteins or the PKA-cAMP pathway.

NMR-based metabonomic studies previously revealed that renal ischemia and reperfusion (I/R) injury in animal models was associated with increased levels of citrate, dimethylamine, lactate, and acetate in urine as well as increased levels of allantoin and trimethylamine-N-oxide (TMAO) in blood (19, 36). Moreover, in human patients, renal I/R injury was associated with significantly increased serum levels of hypoxanthine and inosine (13). In this study, levels of a number of metabolites in urine were changed in Li-NDI rats, and, in particular, the observed elevated levels of acetate, lactate, allantoin, TMA, and creatine in urine could suggest the Li-induced renal cell stress or injury. N-acetylcysteine, which was known to be effective in the prevention of hypoperfusion and toxin-induced renal failure (12), attenuated Li-induced nephrotoxicity (15), further indicating the Li treatment could induce cell injury. However, it is debatable whether chronic Li treatment per se induces chronic interstitial nephritis and chronic renal failure (6). Moreover, the results supporting I/R injury as a main pathogenic mechanism in Li-induced renal cell injury are weak in the present study, and we do not readily discriminate between I/R injury and an imbalance between metabolic demand and supply. It is known that Li treatment is associated with damage to the mitochondria and to the endoplasmic reticulum in the kidneys of human patients (1) and rats (29). Thus it is more likely that Li-induced inhibition of the mitochondrial superoxide dismutase, increased oxidative stress, and reduced metabolic capacity might produce I/R injury-like processes. Further studies are needed to address this important issue.

In summary, the present study illustrates the application of metabolomics based on 1H-NMR spectroscopy of kidney tissues and urine for an understanding of the renal side effects of chronic Li treatment at the biochemical level. We demonstrate decreased levels of organic osmolytes and amino acids in the different zones of the kidney. Moreover, elevated levels of several urinary metabolites which were previously known as biomarkers for kidney cell injury are observed. These findings indicate that metabolomics based on 1H-NMR spectroscopy could provide insight into the effects of Li-induced renal effects and cell injury.

ACKNOWLEDGMENTS

The authors thank Hyo-Jung Choi and Jung-Suk Lim for expert technical assistance.

GRANTS

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (R01-2007-000-20441-0) and Converging Research Program (20090081952) funded by the Ministry of Education, Science and Technology (MEST), Korea; Korea Research Foundation Grants KRF-2008-005-J00701 and 2006-KRF-531-C00038 funded by the MEST, Korea; and the Korea Healthcare Technology R&D Project, Ministry of Health and Welfare, Korea (A080143).

DISCLOSURES

No conflicts of interest are declared by the author.

REFERENCES


19. Hauet T, Baumert H, Gibelin H, Hameury F, Goujon JM, Carreter M, Eugene M. Noninvasive monitoring of citrate, acetate, lactate, and...
Innovative Methodology


