Diabetic Nephropathy (DN) is one of the lethal manifestations of diabetic systemic microvascular disease (16). Clinically, DN is characterized by glomerular hypertrophy, thickened glomerular basement membrane, and expanded extracellular matrix, which is a common cause of death in diabetes. A number of crucial factors have been identified to be involved in the pathogenesis of DN. In general, the cellular events include activation of the sorbitol-aldose reductase pathway, generation of advanced glycation end products, increased activity of protein kinase C, altered expression of cyclin kinases, and generation of reactive oxygen species, various growth factors, and cytokines which are all involved in the pathogenesis of DN (2, 13, 16). However, the DN causing crucial metabolic abnormalities, which may be associated with the progression of the disease, are still unclear so far.

As a novel approach for rapidly identifying global metabolic changes in biological systems, metabolomic analysis has been extensively applied for diagnosis and evaluation of diabetic patients, identification of potential biomarkers, and offering of global and crucial insights into the pathogenesis of the disease due to its advantages in evaluating systemic responses to any subtle metabolic perturbation (14, 15). Some studies about DN by the metabolomic approach have been reported recently (19, 20, 35). However, these previous works were primarily focused on the disease diagnosis. Few studies have been conducted to address the pathogenesis of DN. Understanding the pathogenetic process of DN holds the key to designing therapies to arrest its development and prevent the latter manifestations. In this study, streptozotocin (STZ)-induced DN rats were extensively used for creating the rodent model which develops renal injury similar to human DN (28). It is expected that holistic metabolic analysis of the model could provide a systemic approach for exploring metabolic profiles associated with DN and elucidating the underlying molecular mechanism of the disease.

In the present work, we performed nuclear magnetic resonance (NMR)-based metabolomic analysis of urine and kidney extracts obtained from STZ-induced diabetic 2-wk and 8-wk rats and identified the metabolic changes related to the pathogenetic process of DN. Our results may be helpful for understanding the pathogenesis of DN.

**Materials and Methods**

**Animals.** Male Sprague-Dawley rats (60 days, 194.0 ± 5.8 g) were kept in a barrier system, with regulated temperature and humidity and on a 12:12-h light-dark cycle. Rats were acclimated for 1 wk before conducting of the experiments. During the whole experimental process, rats were fed with certified standard rat chow and tap water ad libitum. All animal treatments were strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Shanghai Institute of Materia Medica, Chinese Academy of Sciences, has the license to use experimental animals. The studies were approved and overseen by the Department of Science and Technology, which is in charge of the Center for Laboratory Animals.

**Experimental design and sample collection.** Three groups of rats were used (n = 6/group), including diabetic 2-wk, diabetic 8-wk, and a control group. After a 12-h fast, rats were randomly selected and injected intraperitoneally with STZ (Sigma) freshly prepared in citrate buffer (0.1 M, pH 4.5) at a single dosage of 70 mg/kg body wt, whereas control rats were injected with the same volume of vehicle. Two days after STZ administration, the body weight of the rats was measured daily and the blood glucose concentration was measured using a tail nick and glucometer (One Touch Ultra, Lifescan). Rats with a fasting blood glucose level of >16.70 mM were defined as diabetic. After 2 wk, the diabetic 2-wk rats were euthanized. Then 8 wk later after STZ treatment, the diabetic 8-wk and the control rats were also euthanized. It was reported that STZ treatment for 8 wk was confirmed to induce a model similar to DN (22, 28).
Before euthanasia, urine samples of all the rats were collected for 24 h in individual urine collection cages into 0.1 ml of 1% sodium azide solution. After measurement of the urine volume for each rat, the urine samples were stored at −80°C. After blood was collected, each rat was decapitated and the cortical parts of right kidney tissues were weighted and immediately immersed in liquid nitrogen for metabolic analysis. The left kidney tissues were immersed in 10% (wt/vol) neutral buffered formalin solution for histological analysis. Clotted blood was centrifuged, and serum was collected and then stored along with the right kidney tissues at −80°C until further measurement.

Clinical chemistry and histopathology. The clinical chemistry analysis of serum samples was carried out for the measurement of serum parameters including triglyceride (TG), cholesterol (TC), and urine protein. The extraction of biochemical parameters was performed using an automatic biochemistry analyzer (Beckman 2000).

Histopathological changes in kidney tissues were assessed in at least 25 randomly selected tissue sections from each group under study. Sections were stained with Mayer’s hematoxylin and eosin for light microscopic observation.

Preparation of samples and acquisition of 1H-NMR spectra. Before NMR analysis, urine samples were thawed and 500 μl of the aliquot was mixed with 50 μl of phosphate buffer consisting of K2HPO4 and NaH2PO4 (pH 7.4, 1.5 M) to minimize variations in pH (36). The mixture was centrifuged to remove precipitates, and then 500 μl of supernatant were transferred to 5-mm NMR tubes containing 60 μl solution of 2.2,3,3-deuterotrimethylsilylpropionic acid (TSP; 1.5 mM) in D2O. D2O provided a field-frequency lock, and TSP was used as the chemical shift reference. All NMR spectra were recorded at 25°C on a Varian Unity INOVA 600-MHz NMR spectrometer equipped with a triple-resonance probe and z-axis pulsed field gradient. For urine samples, one-dimensional NOESY spectra were acquired with water suppression during the relaxation delay of 2 s and a mixing time of 150 ms. A total of 64 transients and 32 K data points were collected with a spectral width of 10,006 Hz, an acquisition time of 1.64 s, and a total pulse recycle delay of 5.76 s. The free induction decay was zero-filled to 64 K and multiplied by an exponential line-broadening function of 0.3 Hz before Fourier transformation.

The frozen kidney tissue was weighed and ground in a mortar under liquid nitrogen, and then 12% ice-cold perchloric acid (3 ml/g tissue) was added to fully mix the samples. The mixture was thawed in the mortar. After centrifugation (12,000 g, 4°C, 10 min), the supernatant was removed and neutralized with 10% KOH to pH 7.0. The precipitate of potassium perchlorate was removed by centrifugation, and then the supernatant was lyophilized and stored at −80°C until NMR analysis. Before recording of the NMR spectra, the tissue extracts were resuspended in 600 μl of phosphate buffer in which 60 μl of TSP solution in D2O was added, and then after centrifugation 500 μl of supernatant was transferred to 5-mm NMR tubes. 1H-NMR spectra were acquired at 25°C on a Varian Unity INOVA 600-MHz NMR spectrometer. A one-dimensional PRESAT pulse sequence was used to achieve satisfactory water suppression in aqueous extracts. For each sample, 128 transients were collected into 64 K data points with a spectral width of 9,612 Hz and a relaxation delay of 2 s.

Data reduction and multivariate pattern recognition analysis. All NMR spectra were phased and baseline corrected using the VNMR 6.1C software package (Varian). Then, data were reduced to 3,000 integrated regions with a width of 0.003 ppm, corresponding to the region of δ 9.5–0.5. The region of about δ 6.2–4.6 in the urine spectra was excluded from pattern recognition analysis (PCA) to remove the uncertainty of the residual water resonance and urea resonances. For NMR spectra recorded in kidney tissue extracts, the region of about δ 5.2–4.6 was removed to eliminate artifacts related to the residual water resonance. Following a preliminary PCA analysis, some spectral regions of urine and kidney samples including about δ 5.2, δ 4.6, δ 3.4–3.9, and δ 3.2, were also eliminated due to the presence of conspicuous glucose metabolite resonances (26, 37). Each data point was normalized to the sum of its row to compensate for the variation in total sample volumes, and the normalized integral values were then subjected to multivariate PCA using the SIMCA-P+ V12.0 software package (Umetrics, Umeå, Sweden).

Orthogonal-projection to latent structure-discriminant analysis (OPLS-DA) of the NMR spectral data was performed to differentiate among the groups of samples (18, 29). Data were visualized with the scores plot of the two principal components (PC1 and PC2), in which each point represented an individual spectrum of a sample. The metabolites associated with the group separations were indicated by the corresponding S-plots, in which each point stood for a single NMR spectral region segment (34). The scores and S-plots complemented each other. The position of each point along an axis in the scores plot was influenced by variables in an axis in the S-plot. Variables with large weights were scattered far away from the origin (on the positive or negative side) on the S-plot. OPLS-DA is a supervised method, where information on class affiliation is included in the model, which maximizes the separation according to classes (e.g., treatment group). This facilitates the identification of variables separating the classes. The quality of the models was described by R2 and Q2 values. R2 was defined as the proportion of variance in the data explained by the models and indicated goodness of fit, while Q2 was defined as the proportion of variance in the data predicted by the model and indicated predictability (34). Individual metabolite quantities were then calculated by spectral integration of the full resolution data and the internal standard, TSP. The metabolic concentrations of urine and kidney extracts were shown as micromoles per 24-h urine and millimoles per kilogram tissue, respectively. R values were generated by an unpaired t-test, and a P value of <0.05 was considered statistically significant.

Determination of ATP, ADP, and AMP in kidney extracts. The kidney extracts were prepared as described above. Adenine nucleotides (ATP, ADP, and AMP) were analyzed in the aliquots using HPLC-UV as described with minor modification (27). Chromatographic separation was achieved on a 150 × 4.6-mm-inner diameter Eclipse XDB-C18 column bonded to a 5-μm spherical silica packing (Agilent), preceded by a C18 reverse-phase cartridge guard column. The mobile phase was 100 mM K2HPO4-KH2PO4 buffer solution at pH 6.0. The HPLC-UV system was operated at room temperature, with a flow rate of 0.6 ml/min. The wavelength was set at 254 nm for detection and quantification. The identification of different adenine nucleotides was based on the retention times of known amounts of standards. The adenine nucleotides were quantified using a calibration line for each standard in the chromatogram. The results were shown as millimoles adenine nucleotides per gram tissue.

Statistical analysis. Statistical analyses were performed using SPSS software (version 11.0, SPSS). Data were first analyzed by ANOVA, and post hoc comparisons between group means were analyzed using a two-tailed unpaired t-test. A P value of <0.05 was considered statistically significant. Data are presented as means ± SE.

RESULTS

Clinical chemistry analysis and histopathology. Morphometric and body fluid parameters determined by biochemical analysis are depicted in Table 1. Two and 8 wk after STZ injection, the concentrations of blood glucose, TG, and TC were distinctly higher than those of control rats. Urinalysis showed that the urine proteins were only markedly increased in diabetic 8-wk rats. In addition, the kidney weights of these rats were significantly increased. Furthermore, it was observed that DN rats excreted more urine volume, even reaching an output of >15 times that of control rats by the end of the experiment (data not shown).

Microscopic examination showed the representative Mayer’s hematoxylin- and eosin-stained sections of kidney from control and diabetic 8-wk rats (Fig. 1). In diabetic 2-wk rats (data not shown) and control rats, minimal tubular injuries
were clearly observed, whereas diabetic 8-wk rats showed a dramatic increase in tubule injury. These results imply that manifest tissue damage and functional change were only shown in diabetic 8-wk rats.

\(^{1}H\)-NMR spectra and PCA of urine. Representative \(^{1}H\)-NMR spectra of urine samples obtained from diabetic 8-wk and control rats are shown in Fig. 2, respectively. Resonance assignments were achieved based on both two-dimensional NMR spectra (data not shown) and literature (33). The OPLS-DA score plots of the first and second principal components (PC1 and PC2) show that diabetic 2- and 8-wk groups are clearly separated from the control group along the PC1 direction (Fig. 3, \(R^2 = 0.98, Q^2 = 0.905\)), indicating that the metabolic characteristics of the groups are markedly different. Also the diabetic 2- and 8-wk rats are clearly separated along the PC2 direction, which indicates that the metabolic changes in the diabetic rats after STZ administration are time dependent. The S-plot for the first two principal components displays the decreased levels of creatine and dimethylamine in diabetic 8-wk rats, as well as increased levels of acetate, ascorbate, succinate, lactate, citrate, allantoin, 2-ketoglutarate, 3-hydroxybutyrate (3-HB), and hippurate compared with control rats. The potential biomarkers indicated by the plot have been summarized in Table 2.

\(^{1}H\)-NMR spectroscopic and PCA of kidney extracts. Representative \(^{1}H\)-NMR spectra of the kidney tissue extracts obtained from diabetic 8-wk and control rats are shown in Fig. 4, respectively, along with the resonance assignments of the metabolites achieved based on previous work (11). Similar to the case of urine results, the OPLS-DA score plot of kidney extracts shows that the diabetic 2- and 8-wk and control groups are also separated from each other (Fig. 5, \(R^2 = 0.991, Q^2 = 0.962\)). The S-plot for the first two principal components displays increased levels of 3-HB and taurine in diabetic 8-wk rats but decreased levels of succinate, creatine, myo-inositol, alanine, glycerophosphocholine (GPC), and lactate compared

Table 1. **Morphometric and metabolic parameters**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>Kidney Weight/Body Weight, %</th>
<th>Blood Glucose, mmol/l</th>
<th>TC, mmol/l</th>
<th>TG, mmol/l</th>
<th>Urine Protein, mg/24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rats</td>
<td>503.5 ± 9.6</td>
<td>0.83 ± 0.1</td>
<td>6.6 ± 0.4</td>
<td>1.2 ± 0.1</td>
<td>12.2 ± 0.6</td>
<td>8.1 ± 0.7</td>
</tr>
<tr>
<td>Diabetic 2-wk rats</td>
<td>366.9 ± 8.1*</td>
<td>0.87 ± 0.1</td>
<td>29.3 ± 2.1*</td>
<td>1.65 ± 0.2*</td>
<td>19.8 ± 1.9*</td>
<td>9.1 ± 2.2</td>
</tr>
<tr>
<td>Diabetic 8-wk rats</td>
<td>212.7 ± 9.9*</td>
<td>2.2 ± 0.2*</td>
<td>30.1 ± 2.5*</td>
<td>2.9 ± 0.3*</td>
<td>20.2 ± 1.8*</td>
<td>24.9 ± 4.4*</td>
</tr>
</tbody>
</table>

Values are mean ± SE (n = 6). TC, total cholesterol; TG, triglycerides. \(*P < 0.05\) compared with control rats.
with the control. The potential biomarkers indicated by the plot have also been summarized in Table 3.

**Quantitative analysis of metabolites in urine and kidney extracts.** Box plots for concentrations of typical metabolites are shown in Fig. 6, which indicate the diversity of individual metabolites among different groups. These plots illustrate that most of the metabolites in diabetic rats show manifest diversity compared with the control, indicating the differences in metabolic alteration in the diabetic state in vivo. Comparisons of the metabolite concentrations show that most metabolites display increased levels in diabetic 2- and 8-wk rats compared with control rats (except for creatine and alanine). Interestingly, the concentration of dimethylamine increased at the point of 2 wk of STZ diabetes, but decreased at the time of 8 wk of STZ diabetes. In addition, some organic acids related to glucose metabolism, such as lactate, citrate, 2-ketoglutarate, and succinate show significant increased trend with time after STZ administration.

**Fig. 3.** A: orthogonal-projection to latent structure-discriminant analysis (OPLS-DA) score plot based on 1H-NMR spectra of urine samples from diabetic 2 w (∆), diabetic 8 w (*) and control rats (○). B: S-plot of OPLS-DA from diabetic 8-wk and control rats. Data were visualized with the scores plot of the 2 principal components (PC1 and PC2), in which each point represented an individual spectrum of a sample. The axes plotted in the S-plot from the predictive component are p[1] vs. p(corr)[1], representing the magnitude and reliability, respectively. Variables labeled with squares (□) could be exploited as potential biomarkers.

**Table 2. Summary of potential biomarkers in urine samples from diabetic 8-wk and control groups of rats**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>∆H</th>
<th>Control</th>
<th>Diabetic 8 wk</th>
<th>P Value</th>
<th>Metabolism Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>1.33, 4.14</td>
<td>39.82 ± 1.27</td>
<td>111.66 ± 5.94</td>
<td>6.14 × 10^{-8}</td>
<td>Glycolysis</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.46</td>
<td>15.91 ± 0.73</td>
<td>11.14 ± 1.11</td>
<td>3.60 × 10^{-3}</td>
<td>Glucose metabolism</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.43</td>
<td>59.13 ± 2.93</td>
<td>102.05 ± 5.08</td>
<td>1.06 × 10^{-4}</td>
<td>TCA cycle</td>
</tr>
<tr>
<td>Citrate</td>
<td>2.52, 2.67</td>
<td>80.98 ± 6.83</td>
<td>218.50 ± 34.01</td>
<td>1.29 × 10^{-4}</td>
<td>TCA cycle</td>
</tr>
<tr>
<td>DMA</td>
<td>2.72</td>
<td>9.03 ± 0.53</td>
<td>5.91 ± 0.58</td>
<td>2.22 × 10^{-4}</td>
<td>Creatine metabolism</td>
</tr>
<tr>
<td>Creatine</td>
<td>3.04, 4.02</td>
<td>68.92 ± 1.86</td>
<td>31.73 ± 5.94</td>
<td>5.15 × 10^{-5}</td>
<td>Creatine metabolism</td>
</tr>
<tr>
<td>Allantoin</td>
<td>5.40</td>
<td>57.60 ± 22.11</td>
<td>107.58 ± 13.94</td>
<td>8.73 × 10^{-3}</td>
<td>Uric acid metabolism</td>
</tr>
<tr>
<td>Hippurate</td>
<td>7.55, 7.65, 7.83</td>
<td>63.50 ± 3.57</td>
<td>92.92 ± 7.66</td>
<td>3.75 × 10^{-3}</td>
<td>Amino metabolism</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 6). DMA, dimethylamine; TCA, tricarboxylic acid.
**Summary of the potential biomarkers in kidney extracts from diabetic 8-wk and control groups of rats**

Table 3.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>δ^1H Control</th>
<th>Diabetic 8 wk</th>
<th>P Value</th>
<th>Metabolism Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-HB</td>
<td>1.20</td>
<td>2.91 ± 0.11</td>
<td>4.29 ± 0.28</td>
<td>3.06 × 10^{-4}</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.33, 4.14</td>
<td>16.51 ± 1.03</td>
<td>21.39 ± 1.44</td>
<td>1.51 × 10^{-4}</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.46</td>
<td>1.70 ± 0.14</td>
<td>0.73 ± 0.03</td>
<td>9.46 × 10^{-9}</td>
</tr>
<tr>
<td>Glutamate</td>
<td>2.15, 2.38, 3.78</td>
<td>15.64 ± 0.33</td>
<td>9.19 ± 0.40</td>
<td>3.11 × 10^{-8}</td>
</tr>
<tr>
<td>GPC</td>
<td>3.21</td>
<td>1.94 ± 0.07</td>
<td>1.19 ± 0.05</td>
<td>3.57 × 10^{-6}</td>
</tr>
<tr>
<td>Succinate</td>
<td>2.43</td>
<td>2.21 ± 0.05</td>
<td>1.62 ± 0.11</td>
<td>7.84 × 10^{-4}</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>3.52, 3.62, 4.06</td>
<td>17.69 ± 0.76</td>
<td>12.20 ± 0.49</td>
<td>1.19 × 10^{-4}</td>
</tr>
<tr>
<td>Creatine</td>
<td>3.04, 4.02</td>
<td>2.12 ± 0.05</td>
<td>1.25 ± 0.07</td>
<td>2.09 × 10^{-3}</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The major microvascular complications of diabetes include DN, diabetic encephalopathy, and cardiomyopathy. As one of the most lethal diabetic complications, DN occurs in 30–40% of type 1 diabetes cases and in 15–20% of type 2 diabetes cases (21). NMR-based metabonomics is a novel approach for rapidly identifying the changes in global metabolite profiles of biological samples and is widely used in disease studies (23). Mäkinen et al. (20) have demonstrated that the NMR-based metabonomic analysis of sera could distinguish DN patients from healthy people. Furthermore, they established an effective metabonomic protocol for identifying the “polymetabolic metabolite manifold of type 1 diabetes,” which allowed the translation of metabolic disturbances tangible to clinical presentations (19). By analyzing the plasma samples from DN patients, Xia et al. (35) have developed a MS-based method for simultaneously identifying 21 compounds related to DN and finally found 5 potential biomarkers. To analyze the pathobiologic process of DN, the urine and kidney extract samples from STZ-induced diabetic 2- and 8-wk rats were investigated using NMR spectroscopy combined with multivariate statistical analysis in the present study. Changes in the concentrations of urinary metabolites are usually related to global changes in metabolism. Thereby, direct analysis of kidney tissues could exploit the possibility of correlating these characteristic metabolic alterations with the pathogenetic process of DN.

**Clinical chemistry and histopathology.** Serum and urine samples from diabetic 2- and 8-wk and control rats were examined using some conventional techniques, including clinical chemical analysis and histopathology. The level of blood glucose in diabetic rats was nearly three times higher than that in control rats. Moreover, other tissue fluid components, such as in concentration. These results imply that some metabolic changes in the kidney could occur for the case of high blood glucose, which is supported by those of clinical chemistry and histopathology.

**Fig. 5.** A: OPLS-DA score plot based on δ^1H-NMR spectra of renal extracts from diabetic 2 (△) and 8-wk (*) rats and control rats (●). B: S-plot of OPLS-DA from the diabetic 8-wk and control rats. Data were visualized with the score plot of the 2 principal components (PC1 and PC2), in which each point represented an individual spectrum of a sample. The axes plotted in the S-plot from the predictive component are p[1] vs. p(corr)[1], representing the magnitude and reliability, respectively. Variables labeled with squares (□) could be exploited as potential biomarkers.

**Table 3.** Summary of the potential biomarkers in kidney extracts from diabetic 8-wk and control groups of rats

Values are means ± SE (n = 6). 3-HB, 3-hydrobutyrate; GPC, glycerophosphocholine.
as lipids (TG and TC), also showed enhanced levels in diabetic 2- and 8-wk rats. These results are consistent with previous work (6). However, urine proteins which reflect kidney functional change, as well as kidney weight, displayed significantly increased levels only in diabetic 8-wk rats. Furthermore, changes indicative of renal tubular damage were also observed in diabetic 8-wk rats in microscopic examination. All the results confirm the formation of the DN rat model after STZ treatment for 8 wk. Despite the prominence of glomerular changes in DN, it has been reported that renal function correlates better with tubular and interstitial changes than with glomerular changes (12). Furthermore, tubular epithelial cells are direct targets for enhanced glucose levels present in diabetes (38). Thus it is suggested that the tubular injuries rather than glomerular injuries are associated with an earlier stage of DN. The metabolic alterations in diabetic 8-wk rats could offer invaluable information for understanding the pathogenetic process of DN.

Kidney function in diabetic rats. The reduced level of creatine was detected in diabetic 8-wk rats, which might be due to a variety of factors including creatine reabsorption, cell leakage, and changes in both muscle mass and caloric intake (26). The creatine depletion was also observed in Zucker rats with altered renal tubular function and morphology (30). Furthermore, as allantoin was not reabsorbed across the proximal tubule, the elevation of urinary allantoin excretion was indicative of increased glomerular filtration rate (5). These results are consistent with the clinical chemistry analysis, as the enhanced level of urinary proteins in diabetic 8-wk rats also indicates glomerular hyperfiltration, which might be implicated in dysfunction of the kidney in DN rats.

Lipid metabolism in diabetic rats. Both urine and kidney extracts showed elevated levels of acetoacetate and 3-HB. It is well known that both acetoacetate and 3-HB comprise ketone bodies, which are the metabolic products of fatty acids in liver mitochondria. The elevated levels of TG and TC revealed by clinical chemistry assay are also indicative of the lipid metabolic alteration in diabetic rats. The phenomenon was also observed in other diabetic patients and in the animal models of type 2 diabetes (26). Except for ketone bodies, acetate is another important product of fatty acid β-oxidation, and thus its elevation in urine samples of diabetic rats established an enhanced pathway of fatty acid β-oxidation in DN rats. Previous work also shows that acetate accumulation has a
negative effect on cellular metabolism in diabetic patients (31). These results are indicative of lipid metabolic excess in DN rats, which might be implicated in the pathogenesis of DN. Furthermore, the altered pathway of fatty acid β-oxidation suggests changes in energy metabolism in DN rats.

Energy metabolism in diabetic rats. Other metabolites involved in energy metabolism were also disturbed in DN rats. The urine samples from DN rats displayed increased levels of the intermediates in the tricarboxylic acid (TCA) cycle (citrate, succinate, and 2-ketoglutarate), similarly to those observed in

![Box plots of concentration of target metabolites in kidney extracts from different groups of rats.](image)

![HPLC chromatograms of standard substances of adenine nucleotides](image)

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*Fig. 7. Box plots of concentration of target metabolites in kidney extracts from different groups of rats.*

*Fig. 8. HPLC chromatograms of standard substances of adenine nucleotides (A) in kidney extracts from control (B) and the diabetic 8-wk rats (C). In A, the retention times are as follows: ATP, 6.3 min; ADP, 7.7 min; AMP, 14.9 min.*
urine of other type 2 diabetic models including Zucker fa/fa rats and db/db mice (26). As glycosuria was observed both in the diabetic model and in the DN model, the increases in urine concentrations of TCA cycle intermediates in DN rats might reflect the systemic stress resulting from the hyperglycemia effect. Furthermore, we have detected more metabolites related to glucose metabolism excretion in urine samples of diabetic 8-wk rats than those of diabetic 2-wk rats, suggesting the aggravation of glucose metabolism alteration as the diabetic process progressed. However, the kidney extracts from DN rats showed a decreased level of a TCA cycle intermediate (succinate), implying the inhibited glucose oxidation pathway in the kidney tissue. Furthermore, the decreased concentration of lactate in kidney extracts was also indicative of the somewhat inhibited glycolysis pathway. Belfiore et al. (3) has pointed out that the kidney is a special organ in the diabetic state. In diabetic rats, the liver and muscle tissues were associated with a catabolic process; however, the kidney showed an “anabolic response” to diabetes, with enhanced activity of enzymes involved in glucose utilization and protein synthesis (3). This might also account for the increased weights of kidneys observed in DN rats (Table 1).

Kidney extracts from diabetic 8-wk rats showed significantly decreased ATP concentration and a distinctly reduced ATP/AMP ratio compared with those from control rats. These results were supported by the previous work which showed decreased ATP levels in the brain (8), heart (9), and testis (1) of diabetic rats. Decreased levels of ATP and TCA intermediates probably imply impairment of mitochondrial function in kidney tissues of DN rats. We thereby hypothesize that the changes in energy metabolism in the kidney tissue might be an important event in the pathogenic process of DN (Fig. 10). Once diabetes occurs, the mitochondrial function is impaired and the produced ATP is not enough for the requirement of the kidney, which might consequently result in the deterioration of kidney function. However, the underlying mechanism of metabolic changes in DN rats remains to be addressed in detail.

Osmolyte metabolism in diabetic rats. The observed decrease in the myo-inositol concentration in kidney extracts from DN rats indicates the tubular dysfunction and renal cells stress under the hyperglycemia state, perhaps due to the up-regulated expression of the myo-inositol oxygenase in diabetic rats (16). Supplementation of myo-inositol could allow the right levels of glucose-induced proliferation and collagen synthesis in tubular cells (39).

As another osmolyte, taurine plays important physiological and pathological roles in vivo (10). Due to significant resonance overlapping of taurine with glucose in NMR spectra, the concentration of the metabolite in DN rats could not be determined readily. It has been reported that taurine could influence blood glucose and insulin levels and play a crucial role in the function and integrity of pancreatic β cells, which are the sites of insulin secretion (17). Previous work also suggests that taurine is a potent endogenous antioxidant, showing protective effects against kidney injury in STZ-induced
diabetic rats (32). These studies imply the possibility of an oxidative stress state in DN rats.

**Oxidative stress in diabetic rats.** Oxidative stress is implicated in the pathogenesis of DN (7). As a powerful antioxidant, ascorbate plays a crucial role in the antioxidant defense system and helps to relieve the oxidative stress associated with diabetic complications (25). A higher level of ascorbate was detected in urine samples from diabetic 8-wk rats, but not diabetic 2-wk rats. In addition, the elevation of this metabolite has not been reported in other diseases of nephrotoxicity. Therefore, as a potential indicator of the enhanced pathway of oxidative stress, ascorbate might be a specific metabolite of DN. However, more data are required to support this hypothesis.

**Other metabolic changes in DN rats.** Hippurate is usually detected in urine and its concentration is related to the microbial activity and composition of the gut (24). An increased level of hippurate in urine samples from diabetic rats indicates an alteration in gut microbiota in the diabetic state. However, the relationship between gut microbiota and diabetes remains unclear so far, although urinary hippurate has been reported to be an early biomarker of nephrotoxicity as a result of nephrotoxin administration in rats (4).

In summary, in the present work we used R-based metabonomic analysis to identify metabolic changes in urine and kidney extracts from STZ-induced diabetic 2- and 8-wk rats. We have observed changes in energy metabolism in the kidney tissue of DN rats, including an enhanced pathway of lipid or intermediary and glycolysis. It is expected that a reduction in ketone body synthesis and decreased levels of TCA cycle intermediates and glycolysis. It is expected that a reduction in ketone body synthesis and decreased levels of TCA cycle

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