\textbf{1H NMR-based Metabonomic Analysis of Metabolic Changes in Streptozotocin-induced Diabetic Rats}

Liangcai ZHAO,† Xia LIU,‡ Liyun XIE,§ Hongchang GAO,** and Donghai LIN*,***†

*Shanghai Institute of Materia Medica, The Chinese Academy of Sciences, Shanghai 201203, P. R. China
**School of Pharmacy, Analysis and Testing Center, Wenzhou Medical College, Wenzhou 325035, P. R. China
***The Key Laboratory for Chemical Biology of Fujian Province, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, P. R. China

Diabetes mellitus is a complex metabolic disorder characterized by chronic hyperglycemia, hypoinsulinemia, and ketosis. To access the biochemical process of diabetes, we applied quantitative 1H NMR-based metabonomics to analyze urine, serum, and liver extracts from streptozotocin-induced diabetic rats. Principle component analysis (PCA) of 1H NMR spectra disclosed metabolic pattern differences between diabetic and control rats, and identified the related metabolic changes. The PCA scores plot demonstrated that the diabetic group could be distinguished from the control group, indicating that the metabolic characteristics of the two groups were markedly different. Our work reveals the accumulation of triglycerides, fatty acids and acetocacetate in diabetic rats, and may provide an efficient, convenient way for evaluating the pathological state and biochemical process of diabetes mellitus.

(Received July 20, 2010; Accepted August 26, 2010; Published December 10, 2010)
acclimated for 1 week prior to conducting 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.

Experimental design and sample collection

Three groups of rats were used (n = 6 per group): 1) normal control group, 2) STZ-treated diabetic group, and 3) insulin-treated group (STZ-INS). After 12 h fasting, rats were randomly selected and injected i.p. with STZ (Sigma, NY) freshly prepared in citrate buffer (0.10 mol L⁻¹, pH 4.5) at a single dosage of 70 mg kg⁻¹ of body weight, whereas the remaining animals were taken as a control group and injected with the same volume of vehicle. Two days after STZ administration, the body weight was measured daily and the blood glucose concentration was measured using a tail nick and glucometer (One Touch Ultra, Lifescan), by which the rats of fasting blood glucose level higher than 16.70 mmol L⁻¹ were defined as diabetic rats. To dissociate the direct effects of STZ from the effects of STZ-induced hyperglycemia, blood glucose levels in the STZ-INS group were maintained euglycemic by s.c. injections of insulin (Humulin-N and Humalog; Lilly). The STZ-INS rats received 2–3 injections of insulin per day in 48 h following STZ administration over the whole experimental period. After 14 days, STZ treatment was confirmed to induce in a model similar to type 1 diabetes mellitus.

Urine was collected, for 24 h each, before STZ injection (pre-dose) and after injection (0–24 h, 48–72 h, 13–14 d) for metabolic alteration analysis of the model group. In addition, urine samples from both the control and STZ-INS groups were analyzed using an appropriate kit for the measurement of the following parameters: alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol, triglycerides and FFA. Values were expressed as the mean ± SEM. Statistical comparisons were made using ANOVA followed by Newman-Keul’s multiple comparison test. A value of P < 0.05 was chosen as the limit of statistical significance.

Clinical chemistry and hematology

Clinical chemistry analysis of serum samples was carried out on UV-visible spectrophotometer (Shimadzu, Tokyo, Japan) using appropriate kits for the measurement of the following serum parameters: alanine aminotransferase (ALT), aspartate aminotransferase (AST), cholesterol, triglycerides and FFA. Values were expressed as the mean ± SEM. Statistical comparisons were made using ANOVA followed by Newman-Keul’s multiple comparison test. A value of P < 0.05 was chosen as the limit of statistical significance.

Preparation of samples and acquisition of ¹H NMR data

Prior to NMR analysis, serum and urine samples were thawed, and 300 μL aliquots were mixed with 300 μL of phosphate buffer (0.2 mol L⁻¹ NaH₂PO₄/NaH₂PO₃, pH 7.4) to minimize variations in pH. All NMR spectra were recorded at 25°C on a Varian Unity INOVA 600 MHz NMR spectrometer equipped with a triple resonance probe and a z-axis pulsed field gradient. For serum samples, Detailed protocol refer to our previous work.21

For urine samples, one dimensional spectra were acquired using a one-dimensional NOESY pulse sequence with water suppression during the relaxation delay of 2 s and a mixing time of 150 ms. Sixty-four free induction decays were collected into 32 K data points with a spectral width of 10006.3 Hz, an acquisition time of 1.64 s and a total pulse recycle delay of 5.76 s. FID was zero-filled to 64 K and multiplied by an exponential line-broadening function of 0.5 Hz prior to Fourier transformation.

Liver tissue extracts were prepared as reported.22 Each portion of frozen liver was weighted (100 ± 2 mg tissue) and grinded in a mortar under liquid nitrogen, then added 12% ice-cold perchloric acid (3 mL g⁻¹ tissue) and grinded to completely mix the sample. The mixture was thawed in the mortar. The supernatant was removed after centrifugation (12000g, 4°C, 10 min), and neutralized with 10% KOH to pH 7. The potassium perchlorate precipitate was removed by centrifugation; then, the supernatant was lyophilized and the sample was stored at −80°C until NMR analysis. Prior to NMR analysis, resuspended the tissue extracts in 600 μL of phosphate buffer in which 60 μL solution of TSP in D₂O was added, and after centrifugation 500 μL of supernatant was transferred to 5 mm NMR tubes. One-dimensional presat pulse sequence was used to achieve satisfactory water suppression in the aqueous extracts. For each sample, 128 transients were collected into 64 K data points with a spectral width of 9612 Hz and a relaxation delay of 2 s. Resonance assignments were performed based on the extant literatures23,24 and 2D ¹H-¹H COSY and TOCSY spectra (data not shown).

Data reduction and multivariate pattern recognition analysis

All NMR spectra were phased and baseline corrected, and then data-reduced to 225 integrated regions of 0.04 ppm width corresponding to the region of δ9.5 to 0.5 using the VNMR 6.1C software package (Varian, Inc.). The region of δ6.2 – 4.6 in the urine spectra was excluded from the recognition analysis to remove any uncertainty of the residual water and urea resonances. For NMR spectra of serum and liver tissue extracts, the region of δ5.2 – 4.6 was removed to eliminate artifacts related to the residual water resonance. Following a preliminary principal component analysis (PCA) analysis, regions including δ5.2, δ4.6, δ3.4 – 3.9, δ3.2 of all spectra were also removed due to the presence of conspicuous glucose metabolite resonances.

| Table 1 Effect of STZ treatment on various blood serum parameters as measured by clinical chemistry assaysa |
|-------------------------------------------------|----------------|----------------|
| Serum measurement                       | Control group | Diabetic group | STZ-INS group |
| Glucose/mmol L⁻¹         | 5.16 ± 0.18   | 28.80 ± 2.02** | 8.23 ± 0.88†† |
| Cholesterol/mmol L⁻¹     | 1.29 ± 0.047  | 1.64 ± 0.14*   | 1.62 ± 0.28*  |
| Triglycerides/mmol L⁻¹   | 0.26 ± 0.09   | 0.69 ± 0.09**  | 4.38 ± 1.24*** |
| ALT/μL L⁻¹             | 16.80 ± 5.47  | 43.10 ± 7.42*  | 37.85 ± 7.34* |
| AST/μL L⁻¹             | 103.0 ± 2.3   | 192.3 ± 1.7*   | 123.1 ± 4.3†  |
| FFA/mmol L⁻¹           | 0.53 ± 0.0036 | 0.48 ± 0.021** | 0.054 ± 0.0070†† |

a. Values are expressed as mean ± SEM. ALT, Alanine aminotransferase; AST, aspartate aminotransferase; FFA, free fatty acid. *p < 0.05, **p < 0.01 compared with the control group; †p < 0.05, ††p < 0.01 compared with the diabetic group.
Each data point was normalized to the sum of its row to compensate for any variation in total sample volumes, and the normalized integral values were then subjected to multivariate analysis as variables for the multivariate pattern recognition analysis using the SIMCA-P Ver. 10.5 software package (Umetrics, Umeå, Sweden). PCA was performed to differentiate among the groups of samples. Score plots of the first two PCs were used to visualize the separation of the groups, while the values of the loadings plots were used to identify which spectral variables significantly contribute to the separation of the samples on the score plot.

Results

Clinical chemistry and haematology

Compared with control rats, diabetic rats on 14th day from onset of STZ administration showed a significant elevation of glucose levels (Table 1). As expected, increased water and food intakes and decreased body weights in diabetic rats were also observed during a whole experiment period. Furthermore, STZ-INS rats demonstrated that administration of insulin tended to increase bodyweight and maintain euglycemia. Alterations of some serum metabolites and enzyme activity were also observed, e.g., the levels of ALT, TG, and FFA were increased in diabetic rats. These results indicate that the insulin treatment significantly enhances metabolite alterations, which was consistent with the previous report.25

1H NMR spectra and pattern recognition analysis of serum

1H NMR spectra of serum taken from the diabetic rats 14th day after STZ administration show marked changes in the levels of endogenous metabolites compared with the control rats (Fig. S1 (Supporting Information)). In order to illustrate the differences in the metabolic profile, NMR spectra were further segmented and subjected to PCA. The score plot (Fig. 1A) reveals distinct separation between the control group and the diabetic group, while the STZ-INS group is clustered closely to the control group, suggesting that during insulin treatment the metabolic profile of diabetic rats recovers towards that of the control group. The loading plot (Fig. 1B) indicates significant metabolic changes in the diabetic group compared with the control group, while the STZ-INS group is clustered closely to the control group, suggesting that during insulin treatment the metabolic profile of diabetic rats recovers towards that of the control group. The loading plot (Fig. 1B) indicates significant metabolic changes in the diabetic group compared with the control group, including (1) decreased levels of lactate ($\delta_{4.14}$, $\delta_{1.33}$), methionine ($\delta_{2.18}$), alanine ($\delta_{1.46}$), creatine/creatinine

Fig. 1 (A) PCA score plot (PC1/PC2) based on the 1H NMR spectra of serum samples from rats in the control (■), diabetic (▲), and STZ-INS (●) groups, respectively. (B) Loading plot ($p[1]/p[2]$) revealing the spectral regions (variables) responsible for the discrimination of diabetic animals from control animals.

Fig. 2 (A) PCA score plot (PC1/PC2) based on the 1H NMR spectra of urine samples from rats in the control (■), diabetic (▲), and STZ-INS (●) groups, respectively. (B) Loading plot ($p[1]/p[2]$) revealing the spectral regions (variables) responsible for the discrimination of diabetic animals from control animals.
(δ3.02), pyruvate (δ2.42), citrate (δ2.7), valine (δ1.02), tyrosine (δ6.94, δ7.22), leucine + isoleucine (δ0.98); (2) increased levels of VLDL/LDL (δ1.3), acetocetate (δ2.3), trimethylamine (δ2.78), lipid (δ0.9), NAC (δ2.1) and unsaturated lipid (δ5.34).

1H NMR spectra and pattern recognition analysis of urine

1H NMR urinalysis highlights perturbations in a number of endogenous species on 14th day from onset of STZ administration (Fig. S2 (Supporting Information)). Similarly to the case of serum samples, a score plot of the PCA analysis on urine samples (Fig. 2A) shows that the diabetic group is separated distinctly from the control group, while the STZ-INS group is clustered closely to the control group. With the loading plot (Fig. 2B), a number of metabolic alterations were identified in urine samples of diabetic rats: (1) decreased levels of acetate (δ1.94), succinate (δ2.42), citrate (δ2.58, δ2.66), α-ketoglutarate (δ2.46, δ3.02), creatine/creatinine (δ3.06, δ4.06), NAC (δ2.06), methionine (δ2.62), alanine (δ1.46), choline (δ3.21), lactate (δ1.33); (2) increased levels of hippurate (δ3.94, δ7.54, δ7.86) and dimethyamine (δ2.74).

A pairwise day-to-day comparison by PCA could provide a more detailed pathological process of the diabetic rats (Fig. 3).

The time trajectory of the loading plot shown in Fig. 3 indicates an apparent increase in the levels of citrate and α-ketoglutarate during the first 72 h after STZ treatment (data not shown). Meanwhile, a dramatic decrease in the levels of creatine/creatinine and allantoin was also observed. The shift of metabolic changes in the diabetic group reached a maximum at 72 h p.d., as compared with that in the pre-dose group. However, after 72 h p.d., the levels of citrate and α-ketoglutarate were decreased.

1H NMR spectroscopic and pattern recognition analysis of liver

As one of the main organ of glucose metabolism, the damage of the liver may influence normal glucose and other substance metabolism. Therefore, we performed an analysis on the metabolic alteration of livers from diabetic rats. 1H NMR spectra of liver extracts produce characteristic metabolite profiles (Fig. S3 (Supporting Information)). The PCA analysis reveals complete separation between the diabetic group and the control group in the score plot (Fig. 4A). With the loading plot (Fig. 4B), a number of metabolic alterations were identified in liver samples of diabetic rats: (1) elevated levels of valine (δ1.06), glutamate/glutamine (δ2.02, δ2.38), alanine (δ1.46), succinate (δ2.42), acetate (δ1.94), 3-hydroxybutyrate (δ1.98), creatine/creatinine (δ3.06); (2) decreased levels of lactate (δ1.33), TMAO (δ3.26) and choline (δ3.22). Unexpectedly, the INS-STZ group is also separated from two other groups in the score plot. It has been reported that insulin induced hypertrophy and hyperplasia in livers of diabetic rats28 and caused a concomitant increase in the activity of some enzymes.29 Probably for this reason the trend of the INS-STZ group in the score plot of liver tissue extract is not consistent with those of the urine and serum samples described above.

Discussion

Type 1 diabetes mellitus is an autoimmune disease that results in the permanent destruction of the insulin-producing cells of the pancreas, the β islets, and the subsequent disturbance of glucose and lipid metabolism. This persistent metabolic imbalance is linked to a high incidence of vascular complications, such as encephalopathy, nephropathy, cardiomyopathy and retinal disease. STZ, which selectively targets and destroys the insulin-secreting β cells of the pancreas, is an antibiotic synthesized by the bacterium Streptomyces achromogenes.28
However, it was reported that STZ could also directly damage some organs, e.g., kidney and liver.29,30 Therefore, the metabolic alterations observed in our studies might be due to the deleterious action of STZ directly on organs, rather than as a result of diabetes-mediated metabolic alterations in the STZ-treated rats. The PCA results of serum and urine samples indicate that the insulin treated diabetic rats are not distinctly separated from the control group, suggesting that insulin treatment results in a metabolic profile similar to non-diabetic rats. It seems reasonable to describe the holistic metabolic perturbations observed in diabetic rats as a diabetes-mediated metabolic disturbance rather than the generalized STZ toxicity.

The energy needed for the body to function requires ATP, which primarily comes from glycolysis, glucose oxidation and lipid oxidation. Diabetic animals either lack insulin or are unable to use insulin effectively, leading to impaired glucose uptake and utilization. Consequently, the body rapidly regulates its energy metabolism, resulting in a systemic augmentation of synthesis and metabolism.31 Under normal physiological conditions, multiple substrate can be utilized, such as glucose, amino acids, ketone bodies and fatty acids. Glucose and lipids are the major substrates impacted by diabetes.32 Following STZ injection, decreased glucose metabolism and augmented glucose production results in hyperglycemia.33 In our work, clinical chemistry assays indicate increased serum levels of triglycerides and free fatty acids, and NMR spectra show increased serum levels of VLDL/LDL, saturated and unsaturated lipids in diabetic rats. Our results are in agreement with previously reported work for evaluating diabetic patients.34 The enhanced levels of lipids and triglycerides are most likely attributed to a lower activity of the lipoprotein lipase.35 It has also been reported that, in the cardiac muscle, fatty acids were derived from lipolysis of triglycerides, and in the diabetic animals, the supply of fatty acids to the heart was enhanced.35 Furthermore, our work demonstrates that the concentrations of serum acetoacetate and 3-hydroxybutyrate in the liver of STZ-induced diabetic rats are significantly higher than those in the liver of control rats, which is consistent with previous reports.36,37 The enhanced levels of acetoacetate and 3-hydroxybutyrate may confirm the shift in energy metabolism towards the formation and utilization of ketone bodies. A number of metabolites involved in energy metabolism are perturbed due to hyperglycemia and hypoinsulinemia after STZ treatment. In urine samples of diabetic rats the decreased levels of citrate, α-ketoglutarate and succinate, which are intermediates of the tricarboxylic acid cycle, are indicative of alterations in energy metabolism, as well of impairment in mitochondrial function or activity in the presence of elevated blood glucose.38 Our results are consistent with the previous work which demonstrates that higher blood glucose levels can inhibit pyruvate kinase, glucokinase and phosphofructokinase.39,40 Furthermore, Shearer et al.41 revealed elevated plasma level of citrate in a mouse model of diet-induced insulin resistance, and suggested that inherent differences either between animal species or between the diabetic models used might contribute to the changes in the level of citrate. Interestingly, we also observed an elevation in citrate levels 72 h after STZ administration. The metabolic alterations observed at different time-points suggest that time-dependent in vivo effects occur during the earlier period after STZ injection, which was in accord with a previous study for examining the metabolic differences between acute (72 h) and chronic (14 d) STZ-induced diabetes.42 These time-dependent alterations might contribute to some differences between our observations and those of Zhang et al.20 Furthermore, being consistent with a previous study using the 11-week-old Zucker diabetic rat and PPAR-α null mouse model,43,44 the concentrations of several glucogenic amino acids were also decreased, indicative of higher rates of gluconeogenesis.

The variation in the concentration of metabolites is usually associated with the molecular mechanism mediating the disease. Our findings indicate that the increase of serum NAC in diabetic rats was comparable to that in control rats. NAC not only exists as a serum glycoprotein, where it binds basic drug molecules, but also plays additional roles, most notably, as a modulator of immune function.44 In some organs, such as kidney, a metabolic disorder causes an anabolic response, leading to organ hypertrophy and accumulation of glycoprotein.45 The increase in NAC might be relevant to the development of diabetic microangiopathy.

Hippurate is normally found in urine, and its concentration is related to the microbial activity and composition of the gut.46 An increased level of hippurate in the urine of diabetic rats indicates an alteration in gut microbiota. Lipid intermediary metabolites, such as choline, betaine and TMAO, are present in the urine, serum and multiple tissues of mice, rats and humans.47 One of the principle roles of methylamines is to act as non-perturbing renal osmolytes.48 Our study shows that diabetic rats excrete higher concentrations of trimethylamine in the serum as well as dimethylamine in the urine. The upregulation of the methylamine pathway in vivo may be linked to the hypertonic effect of glucose, or it might be indicative of a renal papillary dysfunction.49 Additionally, our work demonstrates the level of choline is decreased in the urine and liver tissues samples of diabetic rats, probably because choline is not the sole precursor of the methylamines.

It is well known that the liver plays a crucial role in glucose homeostasis, lipid and energy metabolism, and dysregulation of the homeostatic system is a major factor for development of diabetes mellitus.49 Published reports demonstrated that metabolic alterations in a variety of organs occurred earlier after functional deficits developed in diabetic rats.49 Thus, by analyzing the metabolic alterations of the liver of diabetic rats, we may gain additional biological information related to the mechanisms of diabetes. The loading plots of the liver tissue extracts show increased levels of several amino acids and their derivatives in diabetic rats, such as valine, glutamine/glutamate, alanine and creatine/creatinine, indicative of the degradation of proteins and glycoproteins in the liver. Creatine/creatinine is synthesized and metabolized in the liver, thus it is reasonable to owe the increase of the metabolite to the damage of the liver parenchyma which is indicative of hepatic necrosis. This is consistent with a reported increased activity of ALT and AST in serum clinical chemistry assays.48 Additionally, the observation that some amino acids were altered in the liver of diabetic rats is in agreement with the results reported by Bloxam et al. and Wijekoon et al.50 It is known that TMAO are readily formed from trimethylamine, which is generated from the activity of intestinal microflora on trimethylamine precursors, such as dietary aromatic compounds and choline.48 Decreased levels of amino acids in the livers of diabetic rats indicate a block in the microflora-induced N-oxidation pathway of choline or glycerophosphorylcholine.

In conclusion, 1H NMR-based metabonomic analysis of urine, serum and liver samples reveals some characteristic metabolic features in the pathological processes of diabetes. We thereby hypothesize that diabetic animals, which have limited glucose utilization, primarily rely on other fuels, such as lipids, free fatty acids and ketone bodies, and results in the accumulation of triglycerides, fatty acids and acetoacetate. Further NMR-based
metabonomic studies of various diabetic models could provide insights into the molecular mechanisms involved in the pathogenesis of diabetes mellitus.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (20705037, 30730026), the Medicine & Health Foundation of Zhejiang Province (2010QNA016) and the Chinese Academy of Sciences Knowledge Creative Program (KSCX2-YW-R-118).

Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analisci/.

References