^{1}H NMR-based metabonomics study of urine and serum samples from diabetic *db/db* mice

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***Abstract*—A metabonomics approach based on high resolution ^{1}H NMR spectroscopy was applied to investigate the metabolite fingerprints in urine and serum samples from *db/db* mice of 8 weeks old, an animal model of type 2 diabetes mellitus (T2DM). Both NMR spectra and metabonomics results were discussed and the variations on related metabolic pathway were analyzed. The urinary excretions of diabetic mice have elevated levels of citrate, alanine, acetate, TMAO, hippurate, taurine, creatinine, succinate, pyruvate, glycine in addition to evident increase of glucose compared to the control ones. The metabolic variation in serum samples of *db/db* mice is marked by the increases of lactate, 3-hydroxybutyrate, glutamine, glutamate and choline and the decreases of leucine and valine. These results indicate that NMR-based metabonomics is an efficient approach for investigating the subtle metabolic alterations in urine and serum from diabetic mice and the findings of the characteristic metabolites would be helpful for early diagnosis and prevention of T2DM and its related complications.**

Keywords- NMR, metabonomics, urine, serum, T2DM

I. INTRODUCTION

Type 2 diabetes mellitus (T2DM) has been considered as one of the main threats to human health in the 21st century [1]. It is characterized by the resistance of body tissues to the action of insulin on glucose uptake, resulting in prolonged hyperglycemia after intake of carbohydrates. T2DM is not an isolated disease entity but is a more general metabolic disorder ("Metabolic Syndrome"). Therefore detections of the endogenous metabolic variations in the whole body with the development and progression of T2DM will be highly valuable.

Nuclear magnetic resonance (NMR) spectroscopy is one of the most powerful analytical tools that can provide a wealth of metabolite information of biological samples in relation to physiological and pathological disorders. The approach combining the NMR spectroscopic profiling of biological samples with multivariate statistical analysis is termed as NMR-based metabonomics [2]. It has been successfully applied in studying endogenous metabolic changes in biofluids such as urine, serum and saliva caused by diseases or drug toxicity [3]. Recently, a number of diabetes-related metabonomics studies have been reported on biofluids from animal models to human subjects [4,5]. Our preliminary results show that the ^{1}H NMR metabonomics approach is capable of accessing characteristic small metabolites in human serum from diabetic patients [6].

In this work, NMR-based metabonomics was applied to investigate the urinary and haemal metabolic fingerprints of an animal model for T2DM –C57BLKS/J (*db/db*) mice relative to its control C56BLKS/J (*db/m*) mice at an early insulin resistance stage. The differences in global metabolite profiles in urine and serum samples as a result of physiological variations were identified and the underlying factors for the alterations were analyzed. This study validates the feasibility of metabonomics as a tool in characterizing and understanding the biochemical signatures of T2DM and insulin resistance, which would be helpful for early diagnosis and prevention of T2DM and its related complications.

II. MATERIAL AND METHODS

A. Animal Handling and Samples collection

Five male *db/db* mice [7] were housed in plastic cages. Six male *db/m* mice were used as control. The animal room was under controlled condition (temperature, humidity, and a 12-hour light-dark cycle) and the animals were provided with food and water *at libitum*. Animal care and experimental procedures were in accordance with the guidelines of Xiamen University Institutional Animal Care and Use Committee. Animals were weighted and blood glucose levels were measured using blood glucose meter from 4 to 8 weeks of age and urine and serum sample were collected by 8 weeks old. The urine samples were then centrifuged (3000rpm, 5min at 4°C) to remove the particulate contaminants and were stored frozen at -80°C until measured, while the serum samples were centrifuged (3000rpm, 10min at 4°C) and stored frozen at -80°C until NMR experiments.

B. Sample preparation and ^{1}H NMR spectroscopy

For both urine and serum samples, a volume of 400μl was mixed with 200μl phosphate buffer solution (0.2M Na_2HPO_4/0.2M NaH_2PO_4, pH7.4, 100% D_2O) to minimize variations in pH. 0.3mM DSS (2,2-dimethyl-2-silapentane -5-sulfonic acid) was used as an internal reference standard at δ 0.0. The mixture was piped into 5 mm NMR tubes.

The ^{1}H NMR spectra of the urine and serum samples were acquired on Varian NMR system 500 MHz spectrometer equipped with triple resonance probe. The probe temperature was set to 298K and the 90° pulse lengths were calibrated

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individually for each sample. For the urine samples, a conventional presaturation pulse sequence for solvent suppression based on the 1D version of NOESY pulse sequence known as NOEPR (Nuclear Overhauser Effect spectroscopy with PResaturation; delay-90° -t_1-90°-t_m-90°-acquisition) was used [8], where the t_1 delay was 4 μs, the mixing time t_m was 100 ms, and a weak irradiation was applied to suppress water signal during recycle delay of 2 s and mixing time. For the serum samples, except for the same segment applied in urine, an additional Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse train was incorporated with the relaxation time (2nτ) 100 ms and the echo time (τ) 250 μs. A total of 256 scans with a spectral width of 5 kHz were collected for all NMR spectra. All the signals were zero filled to 16k before regular Fourier transformation (FT).

C. NMR data reduction and principal component analysis

The collected NMR data were phased and baseline-corrected manually using the software MestRe-C 4.8 (http://www.mestrec.com). The chemical shifts were referenced to the DSS at δ 0.0. Every spectrum was segmented into regions of 0.04 ppm width in the range of 0.2 ~ 9.8 ppm. The region of 4.6 ~ 6.0 ppm was excluded for urine spectra prior to principle component analysis (PCA) to remove the variations of both water and urea whereas only 4.6 ~5.0 ppm of water signal was excluded for serum spectra. The remaining spectral segments were scaled to total integrated area of each spectrum to account for the differences in concentration. PCA was carried out using the program written by ourselves.

III. RESULTS AND DISCUSSIONS

A. ^{1}H NMR spectroscopy and principal component analysis of urine samples

Two representative ^{1}H NMR spectra of urine from diabetic and control mice are shown in Fig. 1. The main urinary metabolites were assigned according to literature [9]. The spectroscopic profilings of these two groups reveal obvious alterations in metabolite concentrations. The intensities of glucose signals are much stronger for *db/db* mice compared to the control ones, which are evaluated as a function of glycosuria commonly in T2DM subject. The levels of other small metabolites are also found significantly different and will be further identified by pattern recognition analysis.

The score plot of PCA in Fig. 2(a) shows clear separation of the diabetic group from the control group, indicating the existence of discrepancy between these two classes. The data points of the diabetic group locate more dispersed than the control group owing to the larger differences in metabolic profiles of diabetes caused by complicated metabolic pathways of diabetes pathogenesis. The PC loading plot in Fig. 2(b) shows consistent characteristics with visual inspection of urinary ^{1}H NMR spectra. It suggests that the separation is attributed to the NMR signals at δ 1.21, 1.48, 1.93, 2.38, 2.45, 2.56, 3.06, 3.25, 3.30, 3.45, 3.57, 3.50-3.90 and 3.95 ppm, corresponding to butyrate, alanine, acetate, pyruvate, succinate, citrate, creatinine, arginine, TMAO, taurine, glycine, glucose and hippurate.

Each characteristic metabolite mentioned above was examined carefully in every spectrum and the variations of metabolite concentrations were identified with the help of loading plot. The higher urinary excretion of citrate in diabetic subjects may be caused by increased citrate production in tubular cells and/or reduced citrate re-absorption from the tubular fluid

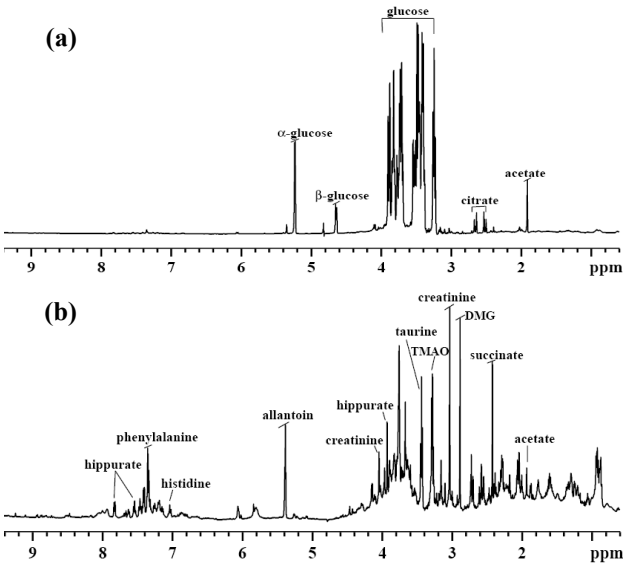

Figure 1. Representative ^{1}H NMR spectra of urine from *db/db* (a) and *db/m* (b) mice (8 weeks old). *Key:* DMG, dimethylglycine; TMAO, trimethylamine *N*-oxide.

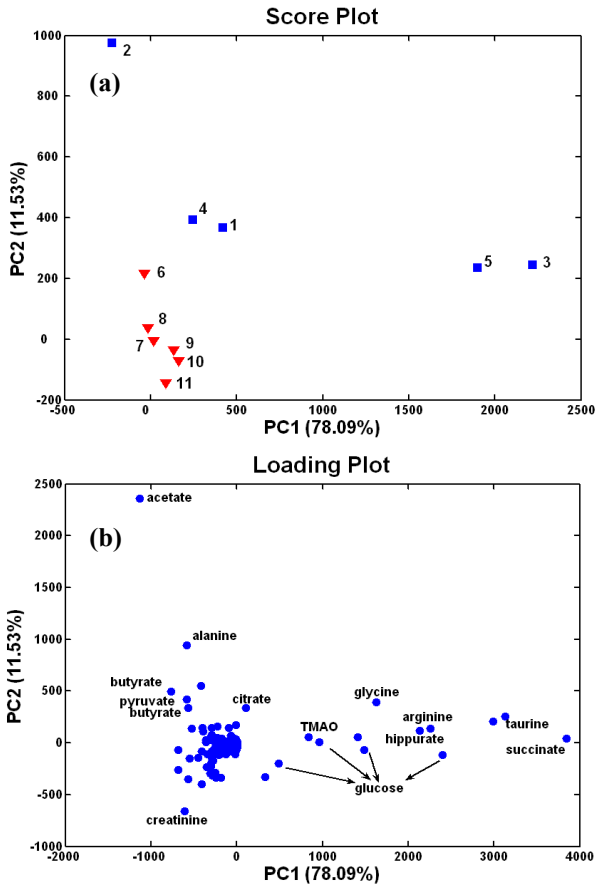

Figure 2. PC score plot (a) and loading plot (b) (PC1 vs PC2) of ^{1}H NMR spectra of urine from *db/db* (1-5,■) and *db/m* (6-11,▼) mice.

due to glucose flow. The increase of alanine in *db/db* mice may be associated with a reduced insulin-mediated suppression of plasma amino acid concentrations and also be indicative of initial tubular damage. Elevated acetate has been proved to be the expression of overt renal insufficiency. Over-excretion of TMAO in diabetes may be linked to the hyperosmotic effect of glucose, or it may be indicative of papillary dysfunction [10]. The increased hippurate in diabetic mice reflects difference in gut microfloral populations. The elevation of taurine level in urine is considered as the most important biomarker for liver damage caused by abnormal glucose metabolism. A slightly higher excretion of creatinine is observed and thought to be related to early stage of renal dysfunction. Besides, glucose overflow which stimulates some glycolytic enzymatic activities in tubular cells may also account for the concentration changes in succinate and pyruvate. The change of glycine is not considered because of the possible overlapping of the peak with part of the intense glucose signals.

B. ^{1}H NMR spectroscopy and principal component analysis of serum samples

Figure 3 shows the typical ^{1}H NMR spectra of serum from diabetic and control mice with the main metabolites assigned [8]. As indicated in Section II, the CPMG technique was used to suppress the resonances from proteins and lipoproteins which partially obscure the sharp peaks from small molecules and reveal subtle biochemical information of samples. Inspection of the spectra indicates that the intensities of glucose signals from diabetic mice are slightly higher than those from the control ones, which is consistent with the blood glucose measurements using a blood glucose meter (data not shown). This is mainly due to mild hyperinsulinemia in *db/db* mice observed as early as 10 days after birth and sustained at least up to 10 weeks old. However, insulin resistance gradually develops as a result of the defects in insulin signaling so that the concentrations of metabolites such as valine, 3-hydroxybutyrate and lactate etc. alter with the abnormal glucose and lipid metabolism.

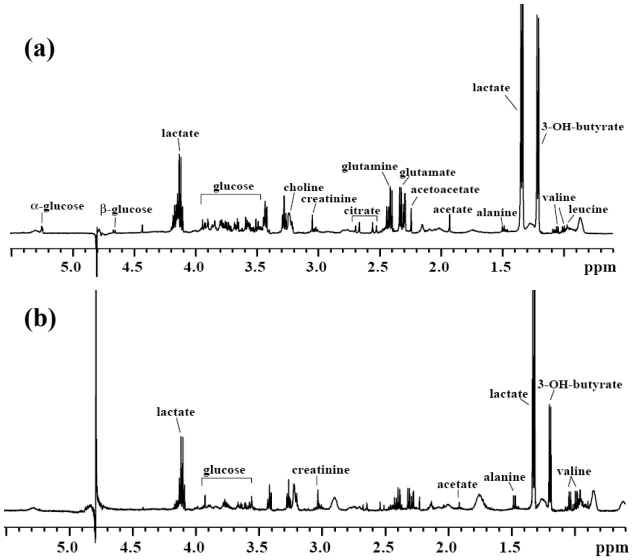

Figure 3. Representative ^{1}H NMR spectra of serum from *db/db* (a) and *db/m* (b) mice (8 weeks old).

The metabonomics differences of serum between the *db/db* and *db/m* groups are visible in Fig. 4(a), where the separation between two classes is obvious in spite of the individual differences in either class. The loading plot illuminates that this separation is attributed to the metabolites with δ 0.97 (leucine, valine), 1.20 (3-hydroxybutyrate), 1.33 (lactate), 2.36 (glutamate), 2.41 (glutamine) and 3.21 (choline) ppm.

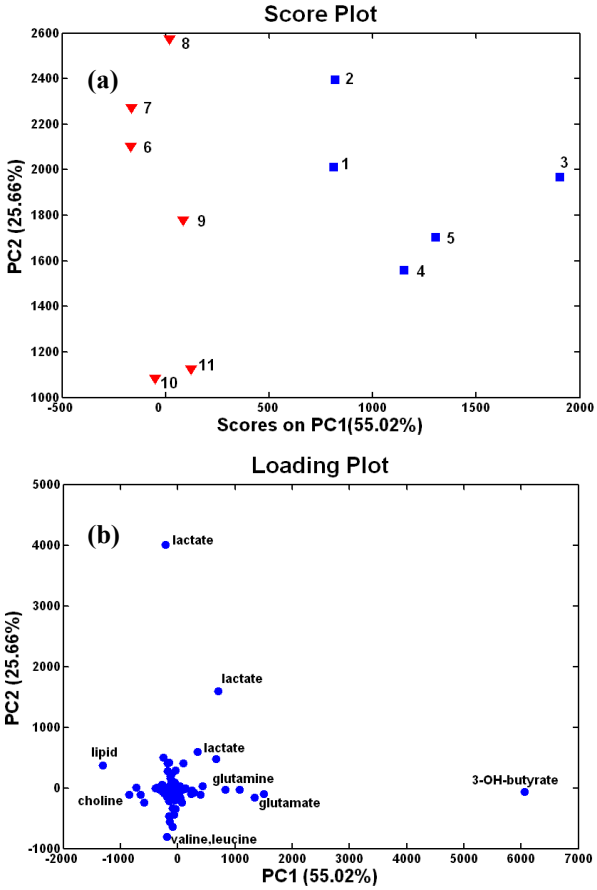

Figure 4. PC score plot (a) and loading plot (b) (PC1 vs PC2) of ^{1}H NMR spectra of serum from *db/db* (1-5,■) and *db/m* (6-11,▼) mice.

According to the ^{1}H NMR spectra of serum, leucine and valine, known as the branched chain amino acid (BCAA), are depressed evidently in diabetic mice compared to the control mice. This is analogous to the decreased plasma BCAA found in humans and taken as one of the biomarkers for T2DM. Elevated plasma lactate concentration was ever reported in high-fat diet induced insulin resistance model mice [11] and may reflect the effect of hyperglycaemia on enhanced muscle lactate production. Significantly increased level of 3-hydroxybutyrate which mainly came from elevated hepatic synthesis was found in the hyperketonemic diabetic animals. Glutamate and glutamine, intermediates and products of the tricarboxylic acid (TCA) cycle, may alter with the effect of 3-hydroxybutyrate derived acetyl-CoA. Variation of choline was also recognized in horse blood of insulin resistance subject [12].

IV. CONCLUSIONS

In this paper, high resolution ^{1}H NMR spectroscopy integrated with multivariate statistical analysis has been applied

to investigate the urine and serum of *db/db* mice – an animal model of type 2 diabetes mellitus. Clear differences in urine between 8-weeks-old diabetic (*db/db*) and control (*db/m*) mice were observed in both spectral and metabonomics traits. Except for the easily recognized sugar resonances, urine spectra from diabetic mice were highlighted by the increased level of citrate, alanine, acetate, TMAO, hippurate, taurine, creatinine, succinate, pyruvate, glycine. Furthermore, studies on the serum samples revealed the discrimination of T2DM mice from healthy controls in spite of the little variations in visual NMR spectral inspection. Compared to the control group, elevated serum lactate, 3-hydroxybutyrate, glutamine, glutamate and choline with declined leucine and valine were identified in the diabetic mice. These results indicate significant alterations in metabolic profiles of urine and serum at the insulin resistance stage of T2DM animals. Further investigations would be done to associate these results with the organ-specific metabolic information and clinical indicators of urine and blood tests, which may provide comprehensive knowledge of molecular biochemistry related to disease state.

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