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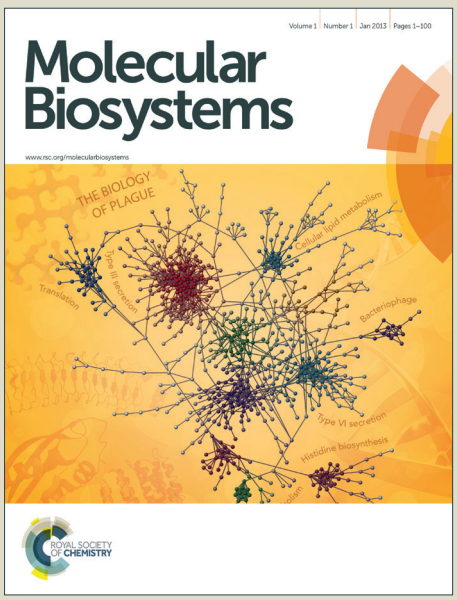

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ARTICLE TYPE

Systemic and characteristic metabolites in the serum of streptozotocin-induced diabetic rats at different stages as revealed by a ^{1}H-NMR based metabonomic approach

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Diabetes mellitus is a typical heterogeneous metabolic disorder characterized by abnormal metabolism of carbohydrates, lipids, and proteins. Investigating the changes in metabolic pathways during the evolution of diabetes mellitus may contribute to the understanding of its metabolic features and pathogenesis. In this study, serum samples were collected from diabetic rats and age-matched controls at different time points: 1 and 9 weeks after streptozotocin (STZ) treatement. ^{1}H nuclear magnetic resonance (^{1}H NMR)-based metabonomics with quantitative analysis was performed to study the metabolic changes. The serum samples were also subjected to clinical chemistry analysis to verify the metabolic changes observed by metabonomics. Partial least squares discriminant analysis (PLS-DA) demonstrated that the levels of serum metabolites in diabetic rats are different from those in control rats. These findings indicate that the metabolic characteristics of the two groups are markedly different at 1 and 9 wk. Quantitative analysis showed that the levels of some metabolites, such as pyruvate, lactate, citrate, acetone, acetoacetate, acetate, glycerol, and valine, varied in a time-dependent manner in diabetic rats. These results suggest that serum metabolites related to glycolysis, the tricarboxylic acid cycle, gluconeogenesis, fatty acid β-oxidation, branched-chain amino acid metabolism, and the tyrosine metabolic pathways are involved in the evolution of diabetes. The metabolic changes represent potential features and promote a better understanding of the mechanisms involved in the development of diabetes mellitus. This work further suggests that ^{1}H NMR metabonomics is a valuable approach for providing novel insights into the pathogenesis of diabetes mellitus and its complications.

Introduction

Diabetes mellitus is the most common metabolic disease in the world and one of the major public health concerns. The World Health Organization expects the number of diabetic patients to increase to 366 million by 2030.[1] The disease is associated with multiple defects in various tissues, such as cardiomyopathy, encephalopathy, retinopathy, and nephropathy, and affects patients for a lifetime.[2] Therefore, addressing the pathological process of diabetes and its underlying pathogenesis is an urgent need.

As a systemic metabolic disease, diabetes mellitus is firstly characterized by a chronic hyperglycemia, which have overwhelming and long-lasting injurious effects on target cells and organs and thereby contributing to diabetic complications.[3] Impairment of insulin function, or insulin deficiency exacerbates lipolysis and the release of free fatty acids from adipose tissue.[4] Alterations in glucose metabolism are known to facilitate profound disturbances in the metabolism of lipids, amino acids, and energy. However, the systemic and characteristic metabolic alterations involved in the pathological processes of diabetes remain unclear.

Metabonomic analysis, a novel approach used to rapidly identify global metabolic changes in biological systems, has been extensively applied in the diagnosis and evaluation of diabetic patients, identification of potential biomarkers, and provision of global and crucial insights into the pathogenesis of the disease because it can evaluate systemic responses to any subtle metabolic perturbation.[5,6] By NMR-based metabonomics, we observed significant differences in the metabolic profiles of serum and liver tissues between Zucker (*fa/fa*) obese and lean rats.[7] Then, to further investigate pathogenesis and pathogenic process of type 2 diabetes mellitus, urinary metabolic profiling of Zucker obese and Goto-kakizaki (GK) rats was compared in our study.[8] We also studied the metabolic levels of the kidney tissue of male Sprague-Dawley diabetic rats, and found hyperglycemia caused metabolic disorders, perturbed the metabolic pathway in local renal levels, and induced metabolic imbalance.[9] Nevertheless, a comprehensive and holistically understanding of metabolic characteristics at short term hyperglycemia and long term hyperglycemia remains to be achieved.

The serum metabonomics study can be a minimally invasive approach that is able to collect continuous data from numerous targets simultaneously, avoiding many limitations of current research methods. To extend our previous studies, we report here

Table 1 Clinical chemistry and haematology assays

Indicators	1-wk groups		9-wk groups	
	Control	Diabetes	Control	Diabetes
Total cholesterol (mM)	1.38 ± 0.22	1.90 ± 0.29*	1.44 ± 0.18	1.68 ± 0.30
Triglyceride (mM)	0.56 ± 0.10	1.14 ± 0.22**	0.57 ± 0.22	1.50 ± 0.62*
Aspartate aminotransferase (U/L)	123.28 ± 34.98	147.34 ± 11.35	106.97 ± 7.84	146.82 ± 30.71*
Alanine transarninase (U/L)	64.32 ± 11.62	138.64 ± 20.00**	65.59 ± 5.45	152.77 ± 46.69**
Total protein (g/L)	65.80 ± 5.05	54.69 ± 6.98*	73.27 ± 2.97	65.71 ± 5.70*

* $p<0.05$ and ** $p<0.01$, compared with the control groups as indicated by a two-tailed unpaired *t*-test.

the results from serum ^{1}H NMR spectra of diabetic 1- and 9-wk rats, in comparison with their age-matched controls. Both multivariate data and quantitative analyses of metabolites were employed to characterize the metabolic profiles and the systematic alterations of serum metabolites in diabetic rats, and thereby exploring the mechanisms of diabetes development.

Results

Clinical chemistry and hematology

Serum biochemical parameters determined by clinical chemistry analysis are depicted in Table 1. As expected, diabetic rats showed increased consumption of water and food but a decrease in body weight over the entire experiment period (data not shown). After STZ injection, the concentrations of triglyceride (TG) and total cholesterol (TC) in diabetic rats were distinctly higher than those in control rats, although the variation of TC levels among the 9 wk group was not significant. Higher levels of alanine transarninase (ALT) and aspartate aminotransferase (AST) in diabetic rats compared with control rats revealed liver dysfunction. In addition, the total protein (TP) concentrations in diabetic rats were significantly lower at the two studied time points. These results suggested that the metabolic pathways including lipids and amino acids were markedly altered in the diabetic state, and the metabolic characteristics might be time-dependent.

^{1}H NMR spectral analysis of serum samples

Figure 1 illustrates the typical 600 MHz ^{1}H NMR spectra of serum samples obtained from the control, diabetic 1 wk and diabetic 9 wk rats, respectively. The spectral resonances of the metabolites were assigned according to our previous work[10] and the 600 MHz library of the Chenomx NMR suite 7.0 (Chenomx Inc., Edmonton, Canada). To confirm the assignments made from 1D ^{1}H NMR spectra, some samples were also examined using 2D ^{1}H–^{1}H COSY and TOCSY spectra with solvent suppression.

The ^{1}H CPMG spectra of the serum permit the simultaneous measurement of numbers of endogenous metabolites, such as low-density lipoproteins (LDL, δ0.85), leucine/isoleucine (Leu/Ileu, δ0.97), valine (δ1.03), 3-hydroxybutyrate (3-HB, δ1.19), lactate (δ1.33), alanine (δ1.47), acetate (δ1.91), acetone (δ2.22), acetoacetate (AA, δ2.27), glutamate (δ2.30), pyruvate

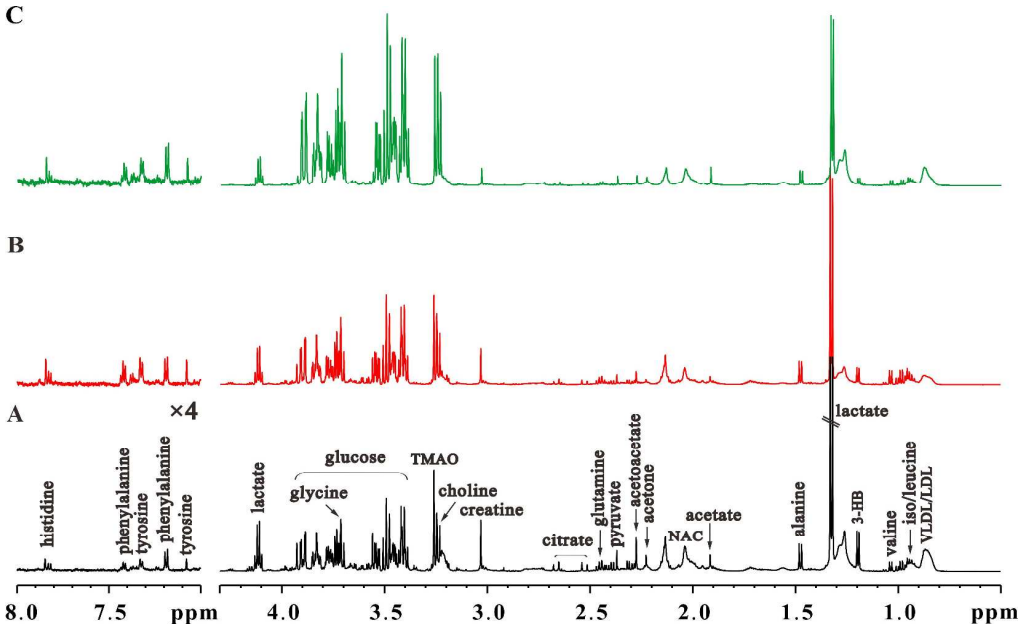

Fig. 1 Representative ^{1}H NMR spectra of serum samples obtained from one control 1-wk rat (A), one diabetic 1-wk rat (B) and one diabetic 9-wk rat (C), respectively.

(δ2.36), glutamine (δ2.46), citrate (δ2.52), creatine (δ3.03), choline (δ3.22), trimethylamine-*N*-oxide (TMAO, δ3.27), glycine (δ3.55), glucose (δ3.40–3.90), betaine (δ3.92), phenylalanine (δ7.45), tyrosine (δ7.15), and histidine (δ7.74). Visual comparison of these spectra showed that diabetic groups had higher levels of glucose and lower levels of lactate and pyruvate compared with the controls.

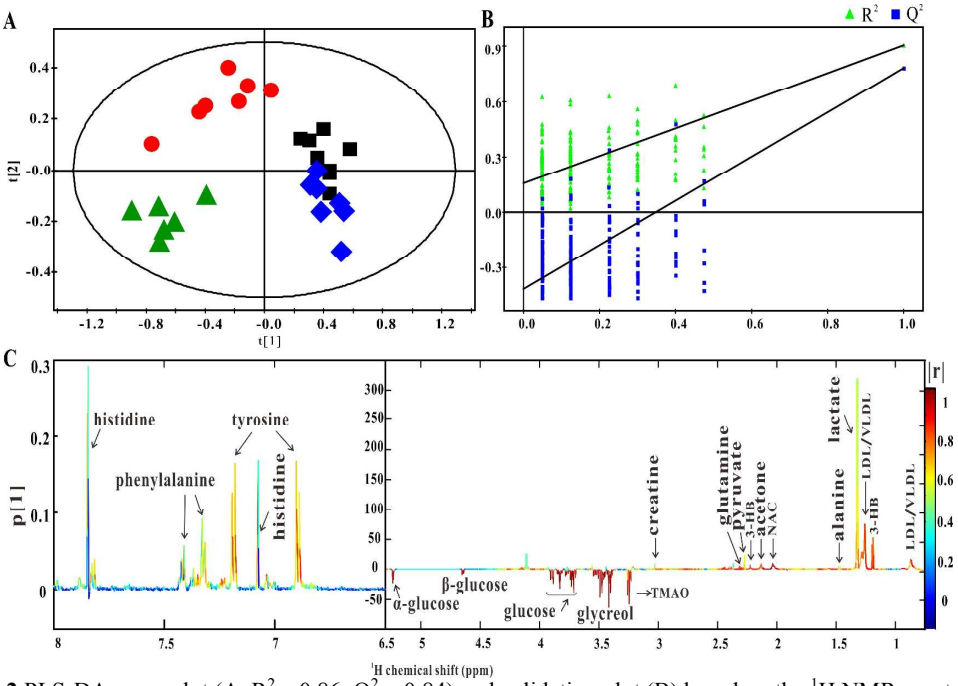

Fig. 2 PLS-DA score plot (A, $R^2 = 0.86$, $Q^2 = 0.84$) and validation plot (B) based on the ^{1}H NMR spectra of serum samples obtained from rats of diabetic 1-wk (●), control 1-wk (■), and diabetic 9-wk (▲), control 9-wk (◆) rats. The coefficient-coded loading plot (C) corresponding to PLS-DA revealing the metabolites with large intensities responsible for the discrimination of the corresponding score plots.

Pattern recognition analysis of serum samples

To extract more details about diabetes-induced changes in the various metabolic systems and identify the potential metabolic pathways associated with the disease, serum NMR spectra were segmented and subjected to multivariate data analysis (Figure 2). First, PLS-DA was performed for all of the samples (Figure 2A, $R^2 = 0.86$, $Q^2 = 0.84$), with findings showing that the diabetic samples were scattered, whereas the controls were clustered together. The diabetic 9 wk group is clearly separated from the diabetic 1 wk group along the PC2 direction, while the age-matched control rats mixed together almostly, which shows that the cluster of diabetic groups features diverse compared with the control groups. This finding indicates that the metabolic alterations in the diabetic state are more obvious than in the control state, so age-related factors may be less important compared with diabetes-related causes in this study. The validation plot of permutation tests showed that the PLS-DA model built for diabetic and control groups is robust and credible (Figure 2B).

Figure 2C illustrates the corresponding loading plot with color-coded correlation coefficients (|r|) of metabolites between diabetic and control groups at the two time points and shows the variables responsible for the separation of different groups. The weight of a variable in the discrimination is given by the square of its correlation coefficient, which is color-coded from zero in blue to high values in red. The negative regions in the loading plot corresponded to metabolites that increased in quantity in the serum of diabetic rats, whereas positive regions corresponded to metabolites that decreased in quantity in the serum of diabetic rats. And thus, Figure 2C illustrates that diabetic rats have higher serum concentrations of VLDL/LDL, glucose, glycerol, and TMAO but lower serum concentrations of lactate, 3-HB, acetone, glutamine, histidine, tyrosine, and other metabolites compared with the controls.

To further explore the metabolic profiles of diabetic rats at different diabetic stages, PLS-DA was performed based on the serum ^{1}H NMR spectra of diabetic and age-matched control groups at 1 and 9 wk, respectively (Figure 3). The clear separation of the diabetic 1 wk group from the age-matched control suggests that metabolic perturbation occurs early in the

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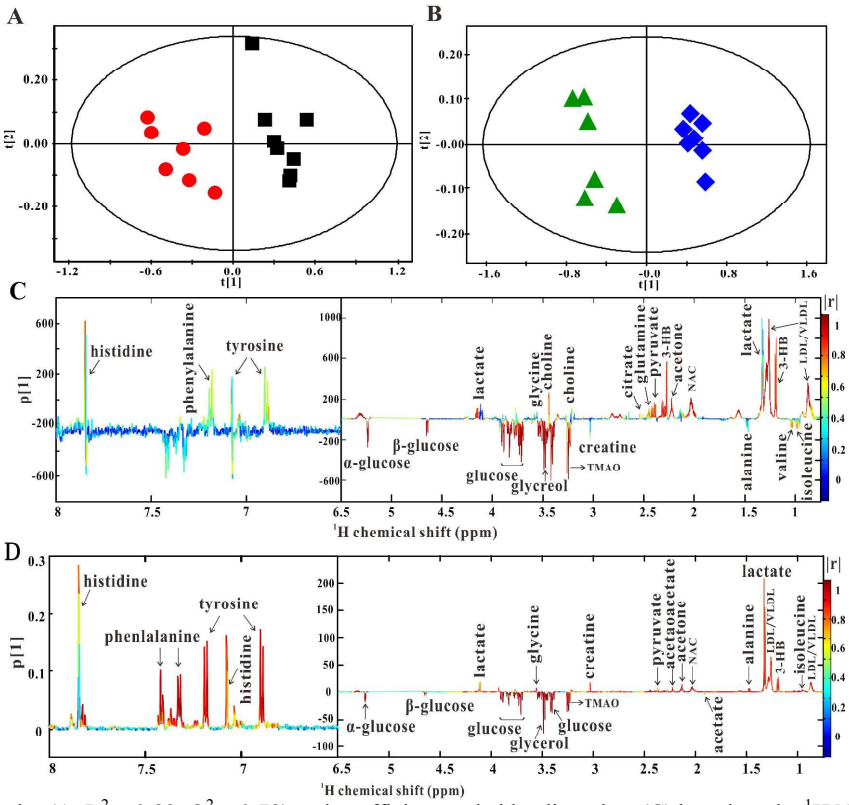

Fig. 3 PLS-DA score plot (A, $R^2 = 0.89$, $Q^2 = 0.78$) and coefficient-coded loading plots (C) based on the ^{1}H NMR spectra of serum samples obtained from diabetic 1-wk (●) and control 1-wk (■) rats. PLS-DA score plot (B, $R^2 = 0.90$, $Q^2 = 0.92$) and coefficient-coded loading plots (D) based on the ^{1}H NMR spectra of serum samples obtained from diabetic 9-wk (▲) and control 9-wk (◆) rats.

diabetic rats (Figure 3A, R^2 = 0.89, Q^2 = 0.78). The corresponding loading plot (Figure 3C) illustrates that the separation observed is due to the variables, including high levels of creatine, TMAO, valine, glucose, and glycerol and low levels of acetone, lactate, NAC, lipids, 3-HB, and acetoacetate. Similarly, the PLS-DA scores plot of 9 wk diabetic and age-matched control rats showed clear discrimination along the PC1 direction (Figure 3B, R^2 = 0.90, Q^2 = 0.92). The corresponding loading plot illustrates that the diabetic serum at 9 wk has lower contents of Leu/Ileu, valine, alanine, AA, choline, 3-HB, and acetone but higher contents of VLDL/LDL, TMAO, and glucose (Figure 3D).

Quantitative analysis of metabolite levels

Table 2 shows the relative integral levels of metabolites in serum samples from diabetic and control rats. The trend of the metabolic changes obtained by quantitative statistical analyses is in agreement with those indicated by the PLS-DA loading plots shown in Figure 3. The results showed that at both studied time points, the diabetic rats contains more LDL/VLDL, glycerol, but less tyrosine, citrate, pyruvate, glutamine, AA, acetone and 3-HB, compared with age-matched control rats. The almost constant levels of lactate, alanine, glycine, phenylanine and batanine observed at 1 wk, showed significantly decreased at 9 wk. To further assess changes during the diabetic evolution, diabetic 9-wk/1-wk ratios were also shown in Table 2, which shows sustained growth of glycerol level, and a concomitant decrease in the levels of citrate, glutamine, pyruvate and 3-HB. These features highlight the characteristic changes of metabolite levels during the development of diabetes. In addition, the levels of Leu/Ileu and valine were increased during the onset of diabetes (1 wk) and decreased during the diabetes evolution (9 wks).

Discussion

Diabetes mellitus is a metabolic disease that results from the destruction of insulin-producing cells in the pancreas or an insulin-resistance condition in the organism.[11] STZ-induced diabetic rat models exhibit metabolic characteristics similar to those in human beings with diabetes mellitus.[12] Systemic abnormalities in several metabolites of biological fluids occured in diabetic rats can lead to pathological changes in the organism. In this study, the ^{1}H NMR spectra of serum

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Table 2 Comparison of integral levels of metabolites in diabetic and age-matched rat

δ ^{1}H	Metabolites	Control 1-wk	Diabetes 1-wk	Control 9-wk	Diabetes 9-wk	Ratio (diabetes 9-wk/1-wk)
7.74	histidine	0.44 ± 0.06	0.42 ± 0.07	0.40 ± 0.04	0.16 ± 0.04**	0.38$^{\#}$
7.45	phenylalanine	2.61 ± 0.26	2.92 ± 0.48	2.33 ± 0.23	1.11 ± 0.24**	0.38$^{\#}$
7.15	tyrosine	1.31 ± 0.10	1.06 ± 0.26*	0.99 ± 0.09	0.51 ± 0.10**	0.48$^{\#}$
3.92	betanine	4.77 ± 0.55	5.55 ± 0.34	5.01 ± 0.56	2.70 ± 0.93**	0.49$^{\#}$
3.66	glycerol	95.33 ± 14.38	282.91 ± 25.55**	86.79 ± 15.00	342.25 ± 30.48**	1.21$^{\#}$
3.55	glycine	5.80 ± 0.38	5.34 ± 0.68	4.47 ± 0.43	2.49 ± 0.35**	0.47$^{\#}$
3.22	choline	7.13 ± 1.17	6.79 ± 1.52	6.09 ± 0.50	3.83 ± 0.67**	0.56$^{\#}$
3.03	creatine	7.11 ± 0.97	8.86 ± 1.78*	7.62 ± 0.94	3.09 ± 0.84**	0.35$^{\#}$
2.52	citrate	3.03 ± 0.26	2.42 ± 0.21**	2.31 ± 0.38	1.69 ± 0.28**	0.70$^{\#}$
2.46	glutamine	10.15 ± 1.18	7.52 ± 1.18**	8.53 ± 0.63	3.86 ± 0.76**	0.51$^{\#}$
3.27	TMAO	15.30 ± 1.24	20.40 ± 1.40**	13.61 ± 1.19	20.54 ± 2.23**	1.01
2.36	pyruvate	20.63 ± 1.99	13.96 ± 1.22**	16.59 ± 1.82	7.71 ± 1.66**	0.55$^{\#}$
2.32	glutamate	5.08 ± 0.59	2.49 ± 0.59**	3.94 ± 0.67	1.77 ± 0.56**	0.71$^{\#}$
2.27	AA	7.80 ± 1.21	3.12 ± 0.61**	2.98 ± 0.58	2.03 ± 0.58**	0.65
2.22	acetone	4.08 ± 0.72	1.37 ± 0.35**	3.47 ± 0.68	1.63 ± 0.24**	1.19
1.91	acetate	3.50 ± 0.33	3.35 ± 1.40	2.50 ± 0.46	3.30 ± 0.57**	0.99
1.47	alanine	10.18 ± 0.89	11.45 ± 2.14	10.72 ± 0.68	5.73 ± 0.56**	0.50$^{\#}$
1.33	lactate	87.67 ± 17.26	80.27 ± 11.83	100.96 ± 10.87	64.70 ± 18.64**	0.81
1.19	3-HB	9.29 ± 1.67	4.75 ± 0.91**	6.92 ± 1.07	2.91 ± 0.59**	0.61$^{\#}$
1.03	valine	4.02 ± 0.57	6.01 ± 1.12**	3.54 ± 0.42	1.81 ± 0.31**	0.30$^{\#}$
0.97	Leu/Ile	2.50 ± 0.34	3.07 ± 0.50*	2.09 ± 0.25	1.01 ± 0.17**	0.33$^{\#}$
0.85	LDL/VLDL	32.17 ± 5.28	55.56 ± 2.18**	31.47 ± 3.81	54.11 ± 7.03**	0.97

*p<0.05 and ** p<0.01, compared with the age-matched control groups, respectively,

$^{\#}$p<0.05 and $^{\#\#}$p<0.01, diabetes 9-wk compared with diabetes 1-wk, as indicated by a two-tailed unpaired *t*-test.

samples provide biochemical information that may be determined from the overall pattern of metabolic alteration. Then the variations of metabolites allowed us to explore some important information about the mechanisms involved in the evolution of diabetes. Figure 4 illustrates the key metabolic pathways during the onset and development of diabetes based on the KEGG database (http://www.genome.jp/kegg/pathway.html) involved in the evolution of diabetes.

Glucose metabolism

Apparently, the metabolites related to glucose metabolism pathways, such as lactate, pyruvate, citrate, are all involved in the development of diabetes. Following STZ injection, degressive glucose metabolism and augmented glucose production resulted in hyperglycemia in the organism. In the study, decreased levels of pyruvate, lactate, and citrate related to the tricarboxylic acid (TCA) cycle, were observed in serum samples of diabetic 1 and 9 wk rats. Our results were similar to those of studies on Goto-Kakizaki rats and STZ-induced 1-month diabetic rats.[8,13] Moreover, compared with diabetic 1 wk rats, we have detected persistent reduction of the metabolites in diabetic 9-wk rats. This suggests that pathways of glycolysis and aerobic metabolism are impaired in a time-dependent manner during the evolution of diabetes. It is known that the metabolites are substrates of energy

synthesis, as a result, decreases in the metabolites may cause less production of ATP. We thereby hypothesize that reduction in bioenergy metabolism in the organism might be an important event in the pathogenic process of diabetes, in which the impaired glycolysis and aerobic metabolism pathways are involved.

In addition, pyruvate can be converted into alanine via alanine aminotransferase, or transferred into glucose in the liver, which is

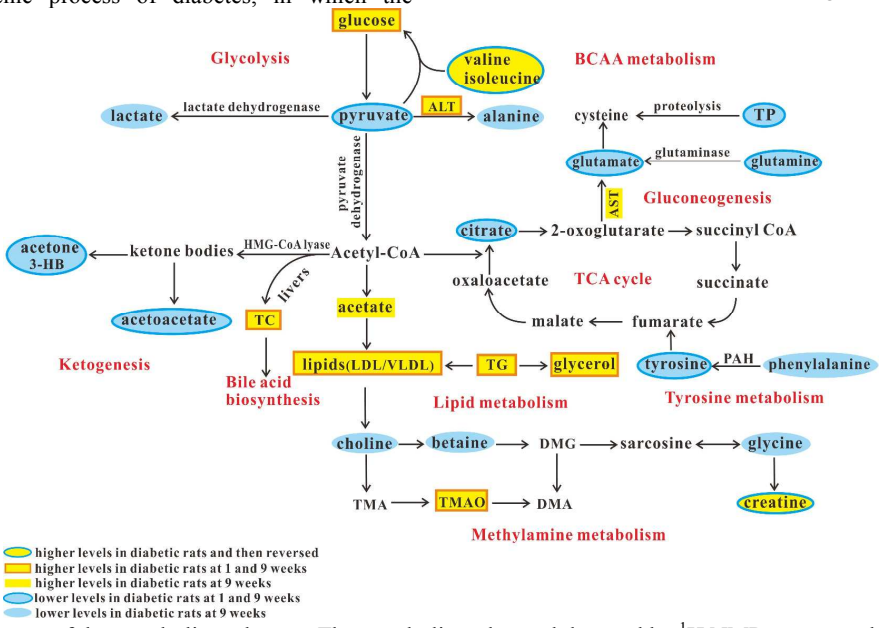

Fig. 4 Schematic diagram of the metabolic pathways. The metabolites changed detected by ^{1}H NMR serum analysis, and the pathway refered to KEGG database, showing the interrelationship of the identified metabolic pathways involved in the development of diabetes mellitus. Metabolites in yellow and blue represent increase and decrease in levels, respectively, compared with control.

called pyruvate-glucose cycle.[14] As a result, pyruvate and alanine can be regarded as glycogenic amino acids precursor[15], decreased levels of alanine and pyruvate observed in diabetic 9 wk rats suggest enhanced gluconeogenesis in the later hyperglycemic state, which will discussed with other metabolites later.

Lipids metabolism

In this study, increased levels of VLDL/LDL as well as saturated and unsaturated lipids in diabetic rats are in agreement with the serum clinical chemistry assays showing significantly increased levels of lipids (triglycerides and cholesterol). The results was corroborated in type 2 diabetic patients in previous study.[16] And, as the end-products of lipid oxidation, elevated levels of acetate and glycerol indicate enhancements in the β-oxidation pathway of fatty acids in the hyperglycemic state.

Increases in the intracellular concentrations of lipids, such as fatty-aceyl CoA, diacylglycerol, and ceramides, activate a serine/threonine kinase cascade and downstream signaling pathway, resulting in decreased insulin-stimulated activation of glucose transport,[17] consequently, tissue sensitivity to insulin is declined.[18] Also it was demonstrated that enhanced lipid synthesis was associated with decreased rates of mitochondrial dysfunction and reduced mitochondrial density in the pathogenesis of type 2 diabetes.[18] Meanwhile, acetone, AA, and 3-HB are all substances of ketone bodies metabolism, and among the metabolic products of lipids in liver mitochondria.[19] The decreased levels of ketone bodies in diabetic rats confirm that the ketogenesis pathway is reduced and that mitochondrial dysfunction occurs in the diabetic rats. Interestingly, Marta reported increased 3-HB content in STZ-induced diabetic 20-wk rats,[20] except dietary and pathological factors, time-dependent metabolic changes may also contribute to the differences.

Choline is one of products of lipids metabolism, and an important marker of renal papillary lesions.[19] Methylamine metabolites can be derived from lipids, or dietary choline, which break down to monoamine, such as betaine, dimethylamine. The decreased serum concentration of methylamine metabolism observed in the current study may indicate perturbations in renal function early in the development of diabetes. To sum up, diabetic animals have limited glucose utilization and primarily rely on other fuels, such as lipids, free fatty acids, and ketone bodies, for energy supply, which results in the accumulation of triglycerides, cholesterol, and other lipids. Our results also indicate the presence of mitochondrial dysfunction associated with lipid accumulation in the early stage of diabetes.

Amino acids metabolism

Compared with age-matched control rats, clinical chemistry assays displayed that diabetic rats at 1 and 9 wk show persistently lower total protein concentrations, suggesting altered levels of protein and amino acids metabolism in the diabetes. Previous studies proved an obvious protein catabolism in the liver and muscle tissues of diabetic rats.[21] The attenuation of protein synthesis may account for the decreased body weight of diabetic rats (data not shown), in which is accompanied by alterations in different amino acid metabolism pathways.

Levels of branched-chain amino acids (BCAAs), such as valine, leucine, and isoleucine, markedly increased in the serum of diabetic 1 wk rats but decreased in diabetic 9 wk rats compared with age-matched control rats. This suggests that the

pathway of BCAA metabolism is different with diabetes stages. Leucine can stimulate insulin release by pancreatic β-cells in vitro.[22] As important insulin secretatogues, BCAAs exert a regulatory effect on proteolysis and participate in building body organs.[23] Increased levels of BCAAs in diabetic 1 wk rats may result from enhanced muscle proteolysis during the onset of diabetes, which may be attenuated during the diabetes development as revealed by the decreased contents of BCAAs in diabetic 9 wk rats. Altered BCAA metabolism in a time-dependent manner is one of the characteristics of diabetes.

As the most abundant amino acid in the serum, glutamine is the most important amino acid gluconeogenic precursor for adding new carbon to the glucose pool.[24] Reduced concentrations of glutamate, glutamine and alanine were observed in diabetic rats, which illustrate the enhancement of glucogeogenesis in the diabetic state. Our results are consistent with previous studies that indicate that the conversion of glutamine and alanine is high in type 1 diabetic patients.[15] Alterations in glutamine and alanine metabolism are important in providing carbon to maintain fasting hyperglycemia in diabetic patients. In this study, increased gluconeogenesis ultimately results in elevated concentrations of glucose and decreased levels of gluconeogenic amino acids in diabetic rats.

Tyrosine and phenyalanine, the main substances involved in the tyrosine metabolism pathway, are precursors of a variety of biologically important substances, such as catecholamines, thyroid hormones, melanin pigments, and protein.[25] In this study, the attenuated pathway of tyrosine metabolism in diabetic rats was consistent with that reported in previous studies,[26] in which it is confirmed that insulin responses is correlated with the plasma amino acids. Furtermore, mixtures of wheat protein hydrolysate, including tyrosine, phenylalanine, and carbohydrate, could be applied as a nutritional supplement to quickly elevate insulin concentrations in healthy male volunteers.[27] These results provide a useful therapeutic tool with which to elevate blood insulin concentrations in diabetic patients.

Methods

Animals

Male Sprague-Dawley rats with 160–180 g body weight were purchased from the Shanghai SLAC Laboratory Animal Co. Ltd., and kept in a SPF colony of Laboratory Animal Center of Wenzhou Medical University with regulated temperature and humidity and a 12/12 h light–dark cycle with lights on at 08:00 am. During the entire experiment, rats were fed with standard rat chow and tap water. 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

After a 12 h fasting, some rats were randomly selected and injected intraperitoneally with streptozotocin (STZ, Sigma-Aldrich) freshly prepared in citrate buffer (0.1 M, pH 4.5) at a single dosage of 60 mg/kg body weight to prepare the diabetic model. And others (n=8) were injected with the same volume of sodium citrate as the control. Two days after the injection, the blood glucose concentration was measured using a tail nick and glucometer (One Touch Ultra, Lifescan), by which the rats of blood glucose level higher than 16.70 mmol/L were defined as diabetic rats (n = 7). Exactly 1 and 9 wks after STZ injection, 1.5 mL blood samples were drawn from the retro-orbital plexus of diabetic rats as well as from the controls under 10% chloral hydrate induced anesthesia. Blood samples were subsequently centrifuged at 3000 × *g* for 15 min at 4 °C and the serum was obtained. Part of the serum was used for biochemical parameter analyses immediately, and the remainder was frozen at -80 °C until NMR analysis.

Clinical chemistry and hematology measurements

Serum alanine transarninase (ALT), aspartate aminotransferase (AST), triglyceride (TG), total protein (TP), and total cholesterol (TC) were measured using an automatic biochemical analyzer (Mindray BS-300). Values were expressed as the mean ± SD.

Preparation of serum samples and acquisition of ^{1}H NMR spectra

Serum samples were thawed and 200 μL were diluted with 400 μL of phosphate buffer (0.2 mM Na_2HPO_4/NaH_2PO_4, pH 7.4) to minimize pH variations and with 60 μL of D_2O for field frequency locking prior to NMR analysis. The mixed serum was centrifuged at 12000 × *g* for 10 min at 4 °C to separate the precipitate. Supernatant aliquots of 500 μL were transferred into 5 mm NMR tubes. All NMR spectra were recorded at 298 K on a Bruker AVANCE III 600 NMR spectrometer operating at 600.13 MHz ^{1}H frequency and equipped with a triple resonance probe. The Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was used to attenuate broad NMR signals from slowly tumbling molecules, such as proteins, and retain those from low-molecular weight compounds and some lipid components with a fixed relaxation delay 2nτ of 120 ms.[28] Typically, 128 scans were collected into 64 K data points over a spectral width of 12000 Hz with a relaxation delay of 6 s and an acquisition time of 2.66 s. An exponential line-broadening of 0.3 Hz was applied to the free induction decay prior to Fourier transformation. All spectra were carefully phased, and the baseline was corrected and referenced to the methyl peak of lactate (CH_3, δ 1.33).[29]

Data reduction and multivariate pattern recognition analysis

Following phase and baseline correction, each spectrum was segmented into chemical shift regions with equal widths of 0.01 ppm (buckets) corresponding to the δ 10.0–0.4 region using the Bruker Topspin 2.1 software package. The δ5.20–4.40 region (containing the residual peak from the suppressed water resonance) was set to zero during analysis. The remaining spectral segments were normalized to the total sum of the spectral intensity to compensate for variations in total sample volume. The normalized integral values were then subjected to multivariate pattern recognition analysis using the SIMCA-P+ V12.0 software package (Umetrics, Umeå, Sweden).

Projection to latent structure discriminant analysis (PLS-DA) were carried out for class discrimination and biomarker identification.[30] Data were visualized by plotting the scores of the first two principal components (PC1 and PC2) to provide the most efficient 2D representation of the information, where the position of each point along a given axis in the scores plot was

influenced by variables in the same axis in the loading plot. PLS-DA revealed differences in the serum composition of different groups, which were necessary to eliminate outliers and enhance the quality of the PCA model. Differences in the metabolites between groups were shown as coefficient of variation plots, which facilitate interpretation because the loadings resemble NMR spectra.[31] The scores and loading plots complemented each other. The parameters R^2 and Q^2 were computed to test the goodness of fit and model validity, where R^2 is the fraction of the sum of square of the entire X's explained by the model and Q^2 represents the cross-validated explained variation with increasing reliability.[32] Leave-one-out cross validation and permutation tests (200 cycles) were conducted to measure the robustness of the model obtained because of the small number of samples. If the plot shows that the Q^2 regression line has a negative intercept, and all permuted R^2 values on the left are lower than the original point on the right, then, the PLS-DA model built for different groups is robust and credible.[31] PLS-DA loading plots were used to identify the metabolites responsible for the separation of groups. Significant differences among metabolites in PLS-DA were assessed by the absolute value of correlation coefficient, |r|, which was calculated in a Java environment.

Statistical analysis

To determine significant differences between metabolic changes, SPSS 13.0 software was used for statistical analysis of the normalized integral values. Data were analyzed using a two-tailed unpaired *t*-test. A calculated P-value of less than 0.05 was considered statistically significant. Data were presented as mean ± SD.

Conclusions

In summary, an NMR-based metabonomic approach was used to elucidate metabolic changes in serum samples from STZ-induced diabetic 1 wk rats, diabetic 9 wk rats, and reveal metabolic features related to the onset and development of diabetes. Time-dependent changes in metabolic pathways, including those of glucose (enhancement of gluconeogenesis, attenuation of glycolysis and the TCA cycle), lipids (enhancement of fatty acid β-oxidation, attenuation of ketogenesis and methylamine metabolism), and amino acids (alteration of BCAA metabolism, attenuation of tyrosine metabolism), were observed during the experimental period. Our studies improve the current understanding on the pathogenic processes of diabetic mellitus and its complications and offer important information for the diagnosis and treatment of the disease.

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