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Metabolic changes in urine and serum during progression of diabetic kidney disease in a mouse model

Nan Hee Kim[a,1], Jin Seong Hyeon[b,c,1], Nam Hoon Kim[a], Ahreum Cho[c], Gayoung Lee[c], Seo Young Jang[b,d], Mi-Kyung Kim[e], Eun Young Lee[f], Choon Hee Chung[g], Hunjoo Ha[c,d,*], Geum Sook Hwang[b,d,**]

[a] Division of Endocrinology and Metabolism, Department of Internal Medicine, Korea University College of Medicine, Seoul, South Korea
[b] Integrated Metabolomics Research Group, Western Seoul Center, Korea Basic Science Institute, Seoul, South Korea
[c] Graduate School of Pharmaceutical Sciences, Ewha Womans University, Seoul, South Korea
[d] Department of Chemistry and Nano Science, Ewha Womans University, Seoul, South Korea
[e] Division of Endocrinology and Metabolism, Department of Internal Medicine, Keimyung University Dongsan Medical Center, Daegu, South Korea
[f] Division of Nephrology, Department of Internal Medicine, Soonchunhyang University Cheonan Hospital, Cheonan, South Korea
[g] Division of Endocrinology and Metabolism, Department of Internal Medicine, Wonju College of Medicine, Yonsei University, Wonju, South Korea

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ABSTRACT

Diabetic kidney disease (DKD) involves various pathogenic processes during progression to end stage renal disease, and activated metabolic pathways might be changing based on major pathophysiologic mechanisms as DKD progresses. In this study, nuclear magnetic resonance spectroscopy (NMR)-based metabolic profiling was performed in *db/db* mice to suggest potential biomarkers for early detection and its progression. We compared concentrations of serum and urinary metabolites between *db/m* and *db/db* mice at 8 or 20 weeks of age and investigated whether changes between 8 and 20 weeks in each group were significant. The metabolic profiles demonstrated significantly increased urine levels of glucose and tricarboxylic acid cycle intermediates at both 8 and 20 weeks of age in *db/db* mice. These intermediates also exhibited strong positive associations with urinary albumin excretion, suggesting that they may be potential biomarkers for early diagnosis. On the contrary, branched chain amino acid and homocysteine-methionine metabolism were activated early in the disease, whereas ketone and fatty acid metabolism were significantly changed in the late phase of the disease. We demonstrated phase-specific alterations in metabolites during progression of DKD. This study provides insights into perturbed mechanisms during evolution of the disease and identifies potential novel biomarkers for DKD.

1. Introduction

Diabetic kidney disease (DKD) is a leading cause of end stage renal disease (ESRD), and the number of patients diagnosed with ESRD due to diabetes has been steadily increasing worldwide [1]. To combat this growing epidemic, appropriate biomarkers sensitive enough to detect early pathophysiologic changes of DKD are needed. Although albuminuria has been considered a useful diagnostic marker and major risk factor for DKD progression, the sensitivity and specificity of albuminuria has been challenged [2].

Several metabolomic studies have used various animal models of DKD and different methodologies [3–10]. Zhao et al. identified changes in energy metabolism in the kidney tissue of streptozotocin-induced type 1 diabetic rats with nephropathy, including enhancement of lipid or ketone body synthetic pathways and decreased levels of tricarboxylic acid (TCA) cycle intermediates and glycolysis [9]. These findings suggest that reduced bioenergy metabolism in kidney tissue may be associated with the pathogenic process of DKD. However, Guan et al. demonstrated enhanced beta-oxidation of fatty acids, as well as increased TCA cycle intermediates, in type 1 diabetic rats [4]. Relatively few studies have involved type 2 diabetic animal models. Recently, Wei et al. examined systemic metabolic changes in urine and kidney tissue of *db/db* mice during the development of DKD. They showed that elevated TCA cycle intermediates were evident at 9–17 weeks of age, hippurate and methylnicotinamide levels were increased in the later stages, and dimethylamine and ketogenesis were decreased during the

* Corresponding author. Graduate School of Pharmaceutical Sciences, Ewha Womans University, Seoul, South Korea.
** Corresponding author. Integrated Metabolomics Research Group, Western Seoul Center, Korea Basic Science Institute, Seoul, South Korea.
E-mail addresses: hha@ewha.ac.kr (H. Ha), gshwang@kbsi.re.kr (G.S. Hwang).
[1] These authors contributed equally to this work.

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entire course of DKD [6].

DKD involves various pathogenic processes during progression to ESRD, including increased vascular permeability, podocyte dysfunction, activated cytokines and growth factors, and increased extracellular matrix accumulation. From this viewpoint, activated metabolic pathways might be changing based on major pathophysiologic mechanisms as DKD progresses. However, few studies have investigated changing metabolite patterns from the early to late stages of DKD. Furthermore, the results from several metabolomic studies were highly variable because different animal models or different metabolic platforms were used [3–10].

The aim of this study was to investigate and contrast the expression patterns of urinary and serum metabolites in the early and late stages of DKD in *db/db* mice, a representative animal model for DKD. Our results will provide clinical insight into potential markers for early detection and disease progression, based on metabolic changes over the course of DKD identified through the use of high-resolution proton nuclear magnetic resonance (^{1}H NMR) spectroscopy.

2. Materials and methods

2.1. Animals

As models of DKD, 6-week-old C57BLKS/J m+/+ and *db/db* male mice were purchased from Charles River Laboratory (Shizuoka, Japan). They were housed in a room maintained at 22 ± 2 °C under a 12-h dark/12-h light cycle and fed a standard chow diet (PMI® Nutrition International, LLC Certified Rodent LabDiet® 5053, Purina Mills, USA) ad libitum until 20 weeks of age. We collected serum and 12-h urine from non-fasting mice housed in metabolic cages (both samples were obtained from the same mice). DKD in male *C57BLKS/J db/db* mice rapidly develop hyperglycemia (16 mmol/l at 6–10 wk), albuminuria (10–12 wk), and declining renal function (15–18 wk) [11,12]. The samples were collected at 8 weeks of age (*db/m* mice, n = 10; *db/db* mice, n = 8) and 20 weeks of age (*db/m* mice, n = 7; *db/db* mice, n = 9) as early and late stage, respectively. They were stored at −80 °C until NMR analysis. All animal experiments were approved by and conformed to the guidelines of the Institutional Animal Care and Use Committee of Ewha Womans University (No. 14–110).

2.2. Biochemistry analysis

Serum glucose levels were measured by the glucose oxidase-based method (OneTouch® Ultra, Johnson & Johnson Co., Milpitas, CA, USA), and urinary albumin was measured using competitive enzyme-linked immunosorbent assay (ALPCO, Westlake, OH, USA). Plasma hemoglobin A1c (HbA1c) levels were measured using a DCA2000 HbA1c reagent kit (Siemens Healthcare Diagnostics, Tarrytown, NY, USA). The blood was centrifuged at 900 g for 15 min at 4 °C, and the resulting supernatant was collected. Urine spectra were used to determine the urine creatinine concentration. Urine albumin levels were corrected for urine creatinine and presented as the urine albumin/creatinine ratio (UACR). Urinary Neutrophil gelatinase-associated lipocalin (NGAL) was measured by ELISA kits (Immunology Consultants Laboratory, Portland, OR, USA).

2.3. ^{1}H NMR experiment and data processing

Before NMR, the frozen serum and urine samples were thawed at room temperature and centrifuged at 12,000 rpm for 10 min at 25 °C to remove the pellet. For serum samples, 100 μL serum was mixed with 600 μL 0.9% w/v NaCl in D_2O. For software-assisted serum metabolite quantification (SASMeQ), valine was added as a reference molecule to the serum mixture for a final valine concentration of 2 mM. For urine samples, 100 μL urine supernatant was mixed with 0.2 M sodium phosphate buffer (pH 7.0) and 1 mM NaN_3 in D_2O. After adjusting the pH to 7.00, 540 μL sample was mixed with 60 μL 2 mM 3-(trimethylsilyl) propionic 2,2,3,3-acid (TSP) in D_2O. The final 600-μL samples were placed in 5-mm Bruker SampleJet NMR tubes (WG-1000-4-SJ, Wilmad-LabGlass, USA).

One-dimensional (1D) ^{1}H NMR spectra were acquired with an Ascend 800-MHz, AVANCE III HD Bruker spectrometer (Bruker BioSpin AG, Switzerland) using a triple-resonance 5-mm CPTIC cryogenic probe.

To acquire 1D ^{1}H spectra of the serum samples, Bruker standard 1D ^{1}H T2 filter (Car-Purcell-Meiboom-Gill [CPMG]) parameter was used: $RD - 90^\circ - [\tau - 180^\circ - \tau]_n - Acq$, with the relaxation delay (RD) = 4.0 s, CPMG echo delay (τ) = 0.2 ms, repetitions number (n) = 128, dummy scans = 16, loops = 160, and acquisition time (Acq) = 2.0 s. The water signal was suppressed at the water peak during RD. Free induction decay (FID) was acquired with a spectral width of 20 ppm for 65,536 data points.

To acquire 1D ^{1}H spectra of the urine samples, Bruker standard 1D nuclear Overhauser enhancement spectroscopy (NOESY)-presat (noesypr1d) pulse sequences were used: RD – 90° – short delay – 90° – mixing – 90°– Acq, with RD = 2.0 s, short delay = 13.9 us, n = 128, dummy scans = 16, Acq = 2.0 s, and mixing time (mixing) = 10 ms. The water signal was suppressed at the water peak during the RD and mixing time. FID was acquired at 65,536 data points with a spectral width of 20 ppm.

The NMR data were processed using TopSpin (ver. 3.1, Bruker BioSpin, Rheinstetten, Germany). All spectra were phase-corrected manually and baseline-corrected. The urine spectra were calibrated using TSP. The synthetic electronic reference signal (ERETIC, electronic reference to access in vivo concentration) was used instead of TSP in the serum sample. The serum spectra were calibrated using the chemical shift of formate at 8.445 ppm from the 800-MHz Chenomx library (ver. 7.1, Chenomx, Edmonton, AB, Canada). For SASMeQ, integrated the downfield doublet methyl peak from valine and defined the methyl peak as ERETIC peak, was added to the serum spectra. The ERETIC peak parameters were defined as follows: ERETIC peak position = 0.0 ppm, line width = 0.4 Hz, integral = $2.2e^{+008}$, and correction factor = 1.

The processed NMR spectra were imported into Chenomx for identification and quantification. The 800-MHz Chenomx library was used to identify individual compounds. The quantification results were normalized by quantifying urinary creatinine. The ERETIC peak of the serum samples and the intensities of identified metabolites were used to determine their relative concentrations. The assignment of ambiguous peaks due to peak overlap was confirmed by spiking with standard compounds. Signal assignment for representative samples was facilitated by the acquisition of two-dimensional (2D) correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), and heteronuclear single quantum correlation (HSQC).

2.4. Statistical analysis

The targeted profiling data were unit variance-scaled, which was performed for the multivariate statistical analyses using SIMCA-P+ (ver. 12.0, Umetrics, Umea, Sweden). PCA an unsupervised method, were performed to obtain an overview of the variation among the groups. PLS-DA were used as classification methods to model the discrimination by visualizing the score plots. Score plots and loading plots were obtained from the PLS-DA model. To validate the PLS-DA model, the permutation tests using 50 random permutations were performed. Statistical analyses were performed using the Statistical Package for the Social Sciences (ver. 21, Chicago, IL, USA). All results are expressed as mean ± standard error (SE). Non-parametric two-tailed Mann–Whitney U-tests were used to detect differences in serum and urinary metabolites between *db/m* and *db/db* mice at 8 or 20 weeks of age. The P-values from Mann–Whitney U-tests were adjusted using the Bonferroni correction for multiple comparisons. The Z-score was obtained using the equation: Z-score = [(observed value – 8 week *db/m*