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Alterations of urinary metabolite profile in model diabetic nephropathy

Donald F. Stec [a], Suwan Wang [b], Cody Stothers [b], Josh Avance [c], Deon Denson [d], Raymond Harris [b], Paul Voziyan [b,*]

[a] Vanderbilt Institute of Chemical Biology, Vanderbilt University Medical Center, Nashville, TN 37232, United States
[b] Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232, United States
[c] Berea College, 1916 CPO, Berea, KY 40404, United States
[d] Choctaw Central High School, Philadelphia, MS 39350, United States

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ABSTRACT

Countering the diabetes pandemic and consequent complications, such as nephropathy, will require better understanding of disease mechanisms and development of new diagnostic methods. Animal models can be versatile tools in studies of diabetic renal disease when model pathology is relevant to human diabetic nephropathy (DN). Diabetic models using endothelial nitric oxide synthase (eNOS) knock-out mice develop major renal lesions characteristic of human disease. However, it is unknown whether they can also reproduce changes in urinary metabolites found in human DN. We employed Type 1 and Type 2 diabetic mouse models of DN, i.e. STZ-eNOS$^{-/-}$ C57BLKS and eNOS$^{-/-}$ C57BLKS *db*/*db*, with the goal of determining changes in urinary metabolite profile using proton nuclear magnetic resonance (NMR). Six urinary metabolites with significantly lower levels in diabetic compared to control mice have been identified. Specifically, major changes were found in metabolites from tricarboxylic acid (TCA) cycle and aromatic amino acid catabolism including 3-indoxyl sulfate, *cis*-aconitate, 2-oxoisocaproate, N-phenylacetylglycine, 4-hydroxyphenyl acetate, and hippurate. Levels of 4-hydroxyphenyl acetic acid and hippuric acid showed the strongest reverse correlation to albumin-to-creatinine ratio (ACR), which is an indicator of renal damage. Importantly, similar changes in urinary hydroxyphenyl acetate and hippurate were previously reported in human renal disease. We demonstrated that STZ-eNOS$^{-/-}$ C57BLKS and eNOS$^{-/-}$ C57BLKS *db*/*db* mouse models can recapitulate changes in urinary metabolome found in human DN and therefore can be useful new tools in metabolomic studies relevant to human pathology.

1. Introduction

A growing rate of diabetic kidney disease is one of the major pathologic consequences of diabetes pandemic [1]. Progression to end stage renal disease and accompanying cardiovascular complications are the primary causes of mortality in diabetic patients [2]. Introduction of new biomarkers to supplement microalbuminuria, which is currently used as the main indicator of kidney disease progression, should improve disease diagnosis and treatment. Active search for these biomarkers is underway involving genomic, transcriptomic, and proteomic technologies [3–5].

Several recent reports have demonstrated that metabolomics can be an important tool in identifying biomarkers of kidney disease and providing insights into pathogenic mechanisms [6–9]. In particular, urine metabolomics allows investigation of small biomolecules closely associated with kidney function or even originating from the kidney. In addition, urine is easier to collect and analyze compared to plasma, which is an important factor for potential clinical biomarker(s). Although metabolomic studies of diabetic kidney disease have been performed using mass spectrometry technology [6–9], proton NMR offers a number of advantages including minimal sample processing, high throughput, complete sample recovery, and direct structural identification of metabolites.

The results of several studies in animal models and human patients identified a number of urine metabolite biomarker candidates [6–8]. However, more studies utilizing different technologies

Abbreviations: DN, diabetic nephropathy; eNOS, endothelial nitric oxide synthase; NMR, nuclear magnetic resonance; TCA, tricarboxylic acid; ACR, albumin-to-creatinine ratio; STZ, streptozotocin; NOE, nuclear Overhauser effect; PCA, principal component analysis.

* Corresponding author at: Division of Nephrology, Vanderbilt University Medical Center, S-3223 MCN, 1161 21st Avenue South, Nashville, TN 37232-2372, United States. Fax: +1 615 343 7156.
E-mail address: paul.voziyan@vanderbilt.edu (P. Voziyan).

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are needed to identify metabolite changes common to diabetic kidney disease. In particular, finding disease-related commonalities in metabolite profiles from human patients and animal models would facilitate use of versatile model tools to help searching for clinically relevant biomarkers. In this study, we utilized robust mouse models of Type 1 and Type 2 diabetic nephropathy (DN), which most fully reproduce pathologic lesions characteristic of human DN [10,11]. We employed ^{1}H NMR spectroscopy, which has previously been applied to studies of metabolites in diabetic plasma [12,13], to determine changes in urinary metabolite profiles in diabetic kidney disease.

We identified six urinary metabolites with significantly decreased levels in diabetic compared to control samples. These same six metabolites were showing similar changes in either Type 1 or Type 2 diabetic animal models. Levels of 4-hydroxyphenyl acetic acid and hippuric acid showed the strongest reverse correlation with the decline in renal function. Based on our findings in this study and on previous human studies [6,7], we conclude that STZ-eNOS$^{-/-}$ C57BLKS and eNOS$^{-/-}$ C57BLKS *db*/*db* mouse models can recapitulate changes in urinary metabolome found in human DN. We propose that these models can be important new experimental tools in metabolomic studies relevant to human pathology.

2. Materials and methods

2.1. Animal studies

In this study urine samples from three groups of mice were investigated: a control group and Type 1 diabetic and Type 2 diabetic groups (11 mice per group). Animal experiments were performed at the AAALAC-accredited animal facilities at Vanderbilt University Medical Center according to institutional guidelines and IACUC-approved experimental protocol. Mice were housed in an approved facility and given standard chow (Lab Diet 5015; PMI Nutrition International, Richmond, IN) and water *ad libitum*. For Type 1 diabetic model, 8 week old eNOS$^{-/-}$ C57BLKS were injected with low doses of STZ (50 mg/kg of BW) for 5 consecutive days to minimize potential toxic effects as previously described [11]. For the Type 2 diabetic model, we used eNOS$^{-/-}$ C57BLKS *db*/*db* mice [10]. Both models develop robust diabetic renal disease with pathologic lesions closely approximating those found in human DN [10,11]. Diabetic mice and age-matched wild type control mice were sacrificed at 20 weeks of age. Blood and spot urine were collected before sacrifice. Serum and urine samples were stored at −80 °C until analysis.

2.2. Determination of blood glucose and urinary albumin excretion

Glucose levels were measured in blood collected from the tail vein using OneTouch glucometer and Ultra test strips (LifeScan, Milpitas, CA) as previously described [10,11]. Albumin and creatinine excretion was determined in spot urine collected from individually caged mice using Albuwell-M kits (Exocell Inc., Philadelphia, PA) as previously described [10,11].

2.3. NMR experiments

NMR spectra were acquired using a 14.0 T Bruker magnet equipped with a Bruker AV-III console operating at 600.13 MHz. All spectra were acquired in 3-mm NMR tubes using a Bruker 5-mm TCI cryogenically cooled NMR probe operating at 298°K. Samples were prepared as 200 μL solutions that included 100 μL of urine, 41 μL of combination of 70 mM sodium phosphate buffer, TSP, and NaN_3, and 59 μL of 90/10% H_2O/D_2O which served as the ^{2}H lock solvent. TSP (3-(trimethylsilyl)propionic-2,2,3,3-d4 acid) in the buffer solution served as the zero ppm chemical shift reference.

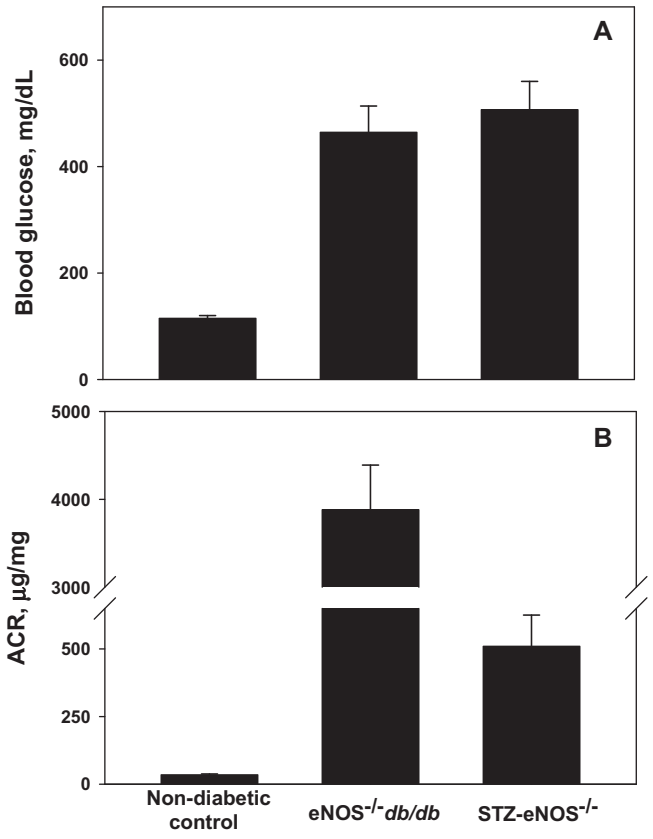

Fig. 1. Levels of blood glucose and albumin excretion in non-diabetic C57BLKS, eNOS$^{-/-}$ C57BLKS *db*/*db*, and STZ-eNOS$^{-/-}$ C57BLKS mice. Blood glucose (A) and urinary albumin-to-creatinine ratio (B) were determined in mice at 20 weeks of age as described under Section 2. Shown are means ± SEM; (n = 11).

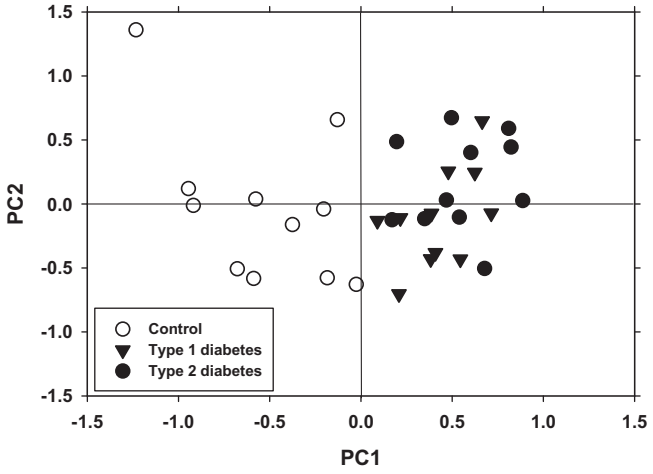

Fig. 2. Principal component analysis of the ^{1}H NMR data from control and diabetic urine samples. PCA scores were calculated using the data from control (*open circles*), diabetic Type 1 (*black triangles*) and diabetic Type 2 (*black circles*) urine samples as described under Section 2.

For 1D ^{1}H NMR, experiments were acquired using a one-dimensional nuclear Overhauser (1D-NOE) pulse sequence with presaturation solvent suppression to suppress the signal associated with water that is typically present in high concentration in mouse