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Fasting serum α-hydroxybutyrate and pyroglutamic acid as important metabolites for detecting isolated post-challenge diabetes based on organic acid profiles

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ABSTRACT

The aim of this study was to develop a method to detect serum organic acid profiles in patients with isolated post-challenge diabetes (IPD) and to compare the metabolites between IPD patients, type 2 diabetes mellitus (T2DM) and healthy controls. We developed a gas chromatography–mass spectrometry method to detect serum organic acids and validated it using serum from 40 patients with IPD, 47 with newly diagnosed T2DM, and 48 healthy controls. We then analyzed the organic acid profiles by multivariate analysis to identify potential metabolites. This method allowed the fast and accurate measurement of 27 organic acids in serum. Serum organic acid profiles differed significantly among IPD patients, T2DM patients, and healthy controls. IPD samples had significantly higher concentrations of α-hydroxybutyrate and β-hydroxybutyrate ($P < 0.05$) and lower pyroglutamic acid concentration ($P < 0.05$) compared with the healthy controls, and the area under the curve for the combination of α-hydroxybutyrate and pyroglutamic acid was 0.863 for the IPD group. These results provide useful information regarding the changes in organic acid metabolism associated with IPD. Measurement of these metabolites in fasting serum from IPD patients may provide useful diagnostic and/or prognostic biomarkers, as well as helpful markers for the therapeutic monitoring of IPD patients.

1. Introduction

Isolated post-challenge diabetes (IPD), characterized by 2-h postprandial plasma glucose (2-h PG) ≥ 11.1 mmol/L and fasting plasma glucose (FPG) ≤ 6.0 mmol/L, is a subtype of type 2 diabetes mellitus (T2DM). The earlier Diabetes Epidemiology: Collaborative Analysis of Diagnostic Criteria in Europe (DECODE) study reported that 50% of patients with newly diagnosed T2DM with 2-h PG ≥ 11.1 mmol/L had FPG < 6.0 mmol/L [1]. Similarly, in the Third National Health and Nutrition Examination Survey, 41% of patients with previously undiagnosed T2DM were identified as having IPD [2]. Several long-term population studies have shown that subjects with IPD have higher risks for cardiovascular events and mortality, and IPD has been reported to be a significant predictor of cardiovascular mortality and incidence [3,4]. FPG is the most commonly used diagnostic indicator of T2DM, due to its low cost, wide availability, and easy reproducibility. FPG is generally detected during routine physical examinations or large-scale screening for diabetes. However, FPG alone fails to detect IPD, often leading to a missed diagnosis, especially during large-scale screening for diabetes. We previously found that the combination of linoleic acid, oleic acid, and dehydroepiandrosterone sulfate was useful for detecting IPD [5,6]; however, some aqueous metabolites, such as short-chain organic acids, were not considered because of the limitations of the detection method. We therefore focused on the organic acid profiles of IPD patients using targeted metabolomics.

Gas chromatography coupled with mass spectrometry (GC–MS) has frequently been used to analyze low-molecular-weight metabolites in metabolomics studies, because of the high equipment stability and availability of user-friendly tools for data analysis [7,8]. Earlier research recognized organic acid profiling of urine by GC–MS as an invaluable tool for diagnosing the numerous inborn errors of metabolism known as organic acidurias [9,10]. GC–MS is currently used to determine blood tricarboxylic acid cycle (TCA) metabolites in some diseases [11,12] and alterations in the TCA cycle have been correlated with numerous pathologies such as cardiovascular diseases [13], metabolic syndrome [14], and diabetes [15]. Furthermore, organic acids

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in serum have been reported as potential biomarkers for T2DM [15–17]. Zhang et al. identified citrate as a promising early predictive and diagnostic marker for the development of T2DM and insulin resistance [16], while Yuan et al. suggested maleic acid, oxyacetic acid, and 2,5-dihydrocarbylphenylacetic acid as potential biomarkers for T2DM [17]. It is therefore necessary to develop an accurate method for studying the organic acid profiles and identifying biomarkers that can distinguish among healthy controls, and individuals with IPD or T2DM.

To this end, we designed a simple and stable method for detecting organic acid profiles using GC–MS. The method provided sensitive, accurate, and simultaneous measurement of 27 organic acids in human serum. We applied this method to analyze organic acid profiles in fasting serum from IPD and T2DM patients and from healthy controls, with the aim of identifying metabolites that could be used specifically to distinguish IPD. Such metabolites could provide helpful diagnostic and/or prognostic biomarkers for IPD in the clinic.

2. Materials and methods

2.1. Subjects

Forty-seven patients with newly diagnosed T2DM and 40 patients with IPD were selected according to FPG and 2-h-PG levels from a population-survey study to investigate the prevalence and risk factors of diabetes in Harbin. All subjects were recruited from communities of Harbin, Heilongjiang Province, in the North of China. T2DM patients were diagnosed according to the 1997 American Diabetes Association (ADA) criteria (Report of the expert committee on the diagnosis and classification of diabetes mellitus 1997). The cutoff value for FPG was 7.0 mmol/L and for 2-h-PG was 11.1 mmol/L. The 48 healthy adults in the healthy control group were from the same population-survey study of patients and were not related to the patients.

There were no significant differences among the three groups in age, sex, smoking and alcohol consumption (Table 1). In addition, body mass index (BMI), triglyceride (TG) level, total cholesterol (TC) level, systolic blood pressure (SBP), diastolic blood pressure (DBP) and insulin were not significantly different between IPD and T2DM individuals (Table 1). The study was approved by the Ethics Committee of Harbin Medical University, and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from each participant.

Table 1
Demographic and clinical chemistry characteristics of 135 subjects (Mean ± SD).

Parameter	Control (n = 48)	IPD (n = 40)	T2DM (n = 47)
Age, years	52.5 ± 6.7	54.1 ± 7.2	53.1 ± 6.4
Sex, F/M	20/28	24/16	24/23
Smoker (%)	31.25	22.5	36.17
Alcohol consumption (%)	47.92	32.5	38.3
BMI, kg/m^2	22 ± 1.1	26.9 ± 3.4**	26.9 ± 6.1**
DBP, mm Hg	114.2 ± 10	142.5 ± 27.8**	139.7 ± 23.1**
SBP, mm Hg	69.4 ± 5.7	85.1 ± 14.6**	84.0 ± 11.1**
FPG, mmol/L	4.5 ± 0.6	5.2 ± 0.5**	10.8 ± 2.7**
2 h-PG, mmol/L	4.4 ± 0.9	14.1 ± 3.2**	19 ± 5**
TG, mmol/L	0.9 ± 0.3	2 ± 1.3**	2.3 ± 1.1**
TC, mmol/L	4.3 ± 0.7	5.3 ± 1.5**	5.2 ± 0.9**
Insulin, mU/L	5.09 ± 1.18	8.79 ± 2.77**	9.60 ± 3.39**
Hb A1c, %	5.2 ± 0.8	6.5 ± 0.6**	7.7 ± 1.4**
HOMA-IR	1.01 ± 0.26	2.05 ± 1.66**	4.63 ± 2.00**

SBP: systolic blood pressure; DBP: diastolic blood pressure; BMI: body mass index; TG: triglycerides; TC: total cholesterol. FPG: fasting plasma glucose; 2 h-PG: 2 h-postprandial plasma glucose.

** $P < 0.001$, compared with healthy control.

2.2. Materials, reagents and chemicals

Organic acid standards: α-hydroxybutyrate, β-hydroxybutyrate, 2-hydroxyisocaproic acid, *cis*-Aconitic acid, citrate, fumarate, glutaric acid, glycolic acid, lactate, malate, malonic acid, ethylmalonic acid, oxalic acid, oxaloacetate, pimelic acid, pyroglutamic acid, pyruvate, sebacic acid, suberic aicd, succinate, α-ketoglutarate acid, caprylic acid, capric acid, methylmalonic acid, orotic acid, isocitrate, phosphoenol pyruvate and D4-succinate, methoxylamine hydrochloride, pyridine, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) were purchased from Sigma (St Louis, MO, USA, ≥99% purity). Acetone, acetonitrile and methanol (chromatographic grade) were purchased from Thermo Fisher Scientific Company (Waltham, MA, USA). Deionized water (18 MΩ cm) from a Millipore Milli-Q water purification system was used to prepare the solutions.

2.3. Preparation of standard solutions and serum sample collection

Stock solutions of the 25 organic acids including D4-succinate were prepared at 1 mg/mL in methanol. Stock solutions of 3 organic acids (isocitrate, orotic acid, phosphoenol pyruvate) were prepared at 1 mg/mL in deionized water. Working solutions with different were prepared (Table 2). All standard solutions were stored at −20 °C until required.

A pool of human serum sample was used for the optimization and validation of this method. Pooled sample was prepared by mixing equal volumes of different individual fasting serum samples (one healthy male and four healthy female). All fasting blood sample were collected and immediately centrifuged at 3000 ×*g* for 10 min and serum was transferred into a clean Eppendorf tube. The serum samples were stored at −80 °C until analysis.

2.4. Method validation

The linear correlations between organic acid standard concentrations (x) and peak area ratios (y) of each organic acid standard to the internal standard were calculated using linear regression analysis. Limit of detection (LOD) was defined as lowest concentrations with signal-to-noise (S/N) ratios of 3. The recoveries were determined in triplicate by spiking low, medium and high concentrations of the analytes into the serum samples. Intra-day and inter-day reproducibility was evaluated by analyzing three replicates. The precision (% CV) at each organic acid from the same pool sample was expected to be not > 15% and the accuracy was ranged from 80–120% of each organic acid.

Stability tests were conducted for the pool sample of organic acid. The derivative samples were stored at room temperature, 4 °C and −20 °C for three consecutive days. All stability results for spiked samples were evaluated by using relative standard deviation (RSD). The acceptance criterion was ± 15% deviation for all storage conditions.

2.5. Sample preparation

The human samples of three groups were prepared according to the following protocol. Aliquots (100 μL) of serum were spiked with internal standard (IS) working solution (9 μL MeOH-D4 Succinate 100 μg/mL), and 300 μL acetone was then added to deposit protein at −20 °C for 2 h. The solution was centrifuged at 15,000 rpm for 15 min at 4 °C to collect the supernatant. The supernatant were dried under nitrogen. And then, we added 30 μL of methoxylamine hydrochloride dissolved in pyridine (33 mg/mL) to the residues in order to protect ketone groups [18]. The solution was incubated for 1.5 h at 37 °C with agitation. Then, 45 μL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA)/acetonitrile (1:2) was added and samples were agitated for 10 min. After the agitation, samples were placed in the dark for 70 min at 50 °C and transferred into a vial before immediate analysis. In addition, the reacted optimization of serum sample preparation was shown in Supplementary method.