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An Assay of Selected Serum Amino Acids in Patients with Type 2 Diabetes Mellitus

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. Amino acids are the building blocks of proteins. In case of insulin resistance, which is typical for type 2 diabetes mellitus (T2DM), proteolysis is increased and protein synthesis is decreased; therefore, we can observe changes in the levels of amino acids in diabetics vs. non-diabetics.
Objectives. The aim of this study was to find differences in the levels of selected amino acids between patients with diabetes (type 2) and a control group.
Material and Methods. Amino acids were derivatized with naphthalene-2,3-dicarboxaldehyde in the presence of potassium cyanide to form fluorescent 1-cyanobenz(f)isoindole product. Amino acids derivatives were measured using a high-performance liquid chromatography with fluorescence detection. The serum levels of glucose were determined using an automatic biochemistry analyzer, glycated hemoglobin HbA1c was measured by cation exchange chromatography.
Results. A total of 19 serum amino acids in T2DM patients and non-diabetics were measured. There were 9 amino acids, which were significantly different in these groups ($p < 0.05$). Significantly decreased levels of arginine, asparagine, glycine, serine, threonine and significantly increased levels of alanine, isoleucine, leucine, valine in diabetics were found.
Conclusions. Significant difference in metabolism of amino acids between diabetics and non-diabetics were observed. The altered levels of amino acids in diabetic patients could be a suitable predictor of diabetes (**Adv Clin Exp Med 2015, 24, 3, 447–451**).

Key words: insulin resistance, diabetes mellitus, amino acids.

Diabetes mellitus is one of the fastest growing public health problems. Currently, 347 million people worldwide have diabetes and the WHO projects that diabetes will be the 7th leading cause of death in 2030 [1]. Diabetes is a metabolic disease that is characterized by increased blood glucose, which may be due to the pancreatic β-cell dysfunction. This dysfunction leads to a lack of insulin production (type 1 diabetes, T1DM) or to development of insulin resistance (type 2 diabetes, T2DM). Insulin is the key hormone for metabolizing glucose; it facilitates glucose transport into cells, where glucose serves as an energy source. If cells do not get enough energy, there are other energy sources like lipids and proteins [2]. Deficiency of insulin contributes to increased gluconeogenesis, increased glycogenolysis and increased protein breakdown in skeletal muscle [3]. Therefore, the altered levels of amino acids can serve as potential biomarkers of diabetes.

The aim of this study was to find differences in the levels of selected amino acids between patients with diabetes (type 2) and a control group. For determination of amino acids, an HPLC method with fluorescence detection was used.

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Material and Methods

Serum samples were obtained from 50 patients with T2DM (18 women and 32 men; age median 63 years), which were defined according to the WHO diagnostic criteria [4] and 50 participants from a control group (24 women and 26 men; age median of 65 years). No participants of the control group had a serious or chronic disease. The Institutional Review Board of Regional Hospital Pardubice, Czech Republic, approved the study. Blood was collected by arm venipuncture into 8 mL serum collection tubes treated with clot activator (Greiner Bio-One, Kremsmünster, Austria, REF 455071) after an overnight fast. Serum was kept at –80°C until analysis.

To 100 µL of serum sample or mixed solution of amino acid standards, 20 µL of norleucine (500 µmol/L) and 900 µL of ethanol were added. After incubation (5 min, –20°C) and centrifugation (2910 × g, 10 min, 8°C), 100 µL of supernatant was pippeted into 1 mL vial. 300 µL of 0.1 mol/L borate buffer (pH 9.3), 60 µL of 40 mmol/L KCN and 20 µL of 5 mmol/L NDA were added to supernatant. After vortexing, the mixture was incubated in the dark (15 min, room temperature). The sample was then filtered through a nylon filter (pore size 0.20 µm, 4 mm diameter, Supelco, Bellefonte, PA, USA) and transferred into 1 mL amber vial.

Chromatographic analysis was performed with a liquid chromatograph (Shimadzu, Kyoto, Japan), LC-20AD solvent delivery, SIL-20AC autosampler, CTO-20AC column oven, DGU-20A degasser, RF-20A fluorescence detector and CBM-20A system controller. Data was collected digitally with LCSolution software (Shimadzu).

1-Cyanobenz(f)isoindole derivatives were separated in reversed phase mode (an analytical column LiChroCART® 250 × 4 mm, Purospher® Star RP-18e, 5 µm, fitted with a guard column LiChroCART® 4 × 4 mm, Purospher® Star RP-18e, 5 µm, Merck, Darmstadt, Germany) using a gradient elution. The mobile phase A consisted of 25 mmol/L sodium dihydrogen phosphate and ethanol (80 : 20, v/v), pH 7.2 and mobile phase B was 100% ethanol. The flow rate was kept constant at 0.5 mL/min and effluent was monitored with a fluorescence detector (excitation wavelength 420 nm and emission wavelength 480 nm).

The serum levels of glucose were determined by standard procedures using an automatic biochemistry analyzer (Dimension Vista 1500, Siemens Healthcare Diagnostics Inc., Newark, DE, USA). Glycated hemoglobin $HbA1_C$ was measured by cation exchange chromatography (Tosoh Bioscience G7 HPLC analyzer, Tosoh Corporation, San Francisco, CA, USA).

The data is presented as median ± IQR (interquartile range). Differences between patients with diabetes mellitus and a control group were analyzed using the Mann-Whitney Rank Sum Test and analysis of correlation was carried out using Spearman Rank-Order Correlation (software SigmaStat 3.5, Systat Software, Point-Richmond, CA, USA).

Results

In order to determine amino acids, an HPLC method with fluorescence detection was used. Amino acids were derivatized with NDA, an analogue of ortho-phthaldialdehyde (OPA). This reaction leads to the production of highly fluorescent 1-cyanobenz(f)isoindole derivatives which are more stable than derivatives with OPA. Derivatives of cystein and lysine have very low intensity of fluorescence. Proline has a secondary amino group in its molecule; therefore, it does not react with NDA [5, 6]. An unidentified substance co-eluted with aspartate. For this reason, we did not measure these amino acids. Analytical performances of this method were satisfactory: the coefficients of variation were below 10%, the spike recoveries ranged between 90–110%.

We measured a total of 19 amino acids, 16 proteinogenic and 3 non-proteinogenic amino acids (2-aminobutyric acid, taurine, and hypotaurine). Results are shown in Table 1 and 2. We found significantly increased levels of 4 amino acids (alanine, valine, leucine, and isoleucine) and significantly decreased levels of 5 amino acids (glycine, serine, threonine, arginine, and asparagine) in patients with T2DM, while the levels of the other amino acids were similar in both groups. We observed statistically significant correlations between levels of 7 amino acids (2-aminobutyric acid, glutamine, histidine, isoleucine, taurine, threonine, tyrosine) and levels of fasting glucose, and between levels of 3 amino acids (histidine, methionine, threonine) and levels of glycated hemoglobin. We used Spearman Rank-Order correlation test.

Discussion

Insulin is the main anabolic hormone. The effect of insulin on protein regulation is to decrease proteolysis and to enhance protein synthesis. Insulin resistance leads to alternations in metabolism of cells, and amino acids become an alternative energy source *via* gluconeogenesis. Gluconeogenesis takes place mainly in the liver; there is glucose synthetized from glucogenic amino acids; therefore,

Table 1. Comparison of selected amino acids between patients with diabetes mellitus (type 2) and a control group of blood donors

Amino acid	Control group	Merge cells	Patients with T2DM		p[b]
	median (μmol/L)	IQR[a] (μmol/L)	median (μmol/L)	IQR[a] (μmol/L)	
2-Aminobutyric acid	21.2	9.1	22.6	9.3	0.125
Alanine	462.1	190.0	537.2	155.3	0.019
Arginine	40.6	9.9	33.5	10.2	< 0.001
Asparagine	123.6	37.7	89.8	37.0	< 0.001
Glutamic acid	81.7	60.7	84.2	42.2	0.882
Glutamine	657.1	144.1	631.4	156.0	0.176
Glycine	290.6	116.5	262.4	90.1	0.007
Histidine	70.5	22.6	69.3	16.4	0.770
Hypotaurine	5.8	3.9	5.1	2.8	0.343
Isoleucine	67.5	19.9	85.0	38.3	< 0.001
Leucine	137.3	34.1	175.7	45.9	< 0.001
Methionine	25.7	8.9	24.2	7.2	0.844
Phenylalanine	75.8	23.8	69.4	19.6	0.137
Serine	131.2	70.0	95.3	55.5	< 0.001
Taurine	130.6	81.8	122.9	85.6	0.551
Threonine	114.6	54.4	104.3	41.6	0.024
Tryptophan	9.2	9.3	6.3	8.5	0.067
Tyrosine	69.6	23.2	66.1	16.6	0.189
Valine	260.2	76.3	306.1	88.8	< 0.001

[a] IQR, interquartile range is the difference between the upper quartile and the lower quartile.
[b] Mann-Whitney Rank Sum Test.

the levels of these amino acids can be reduced. This was also reported by Zhang et al. [3], but they observed decreased levels of both glucogenic and ketogenic amino acids. We found in patients with T2DM reduced levels of glucogenic amino acids with the exception of alanine, valine and glutamate. The metabolism of these amino acids is associated with other amino acids – leucine and isoleucine. Leucine, isoleucine and valine are referred to as branched-chain amino acids (BCAA). These amino acids have a different metabolism; unlike the other amino acids, they are degraded in muscles. Insulin resistance results in increased proteolysis and BCAA levels are elevated. The first step in the metabolism of BCAA is transamination with α-ketoglutarate to form branched-chain α-keto acids (BCKA) and glutamate. High accumulation of glutamate may lead to increased transamination of pyruvate to alanine. Similar results were found in obese subjects [7]. The same authors state that BCAA contribute to insulin resistance but it is independent of body weight. One study reported that BCAA and aromatic amino acids were elevated 12 years before the onset of diabetes and the risk of diabetes was fourfold higher. The authors assume that a combination of three amino acids (isoleucine, tyrosine and phenylalanine) could be a good predictor of diabetes [8]. We found decreased levels of phenylalanine, tyrosine and tryptophan, but there were no significant differences between diabetics and non-diabetics. Low levels of tryptophan were observed in patients with ketoacidosis; after treatment, the levels were normalized [9]. Glutamic acid is an intracellular amino acid which is co-secreted with glucagon by pancreatic α-cells. High concentration of glutamic acid is toxic for β-cells. Excessive dietary intake of glutamate may cause obesity and insulin resistance [10]. Low levels of glycine are the result of insulin resistance. Insulin resistance leads to expression of ALAS-H enzyme, which catalyzes a condensation of glycine and succinyl-CoA into 5-aminolevulinic

Table 2. Correlations between amino acids and glucose and between amino acids and glycated hemoglobin $HbA1_C$

Amino acid	Glucose		$HbA1_C$	
	R^a	p	R^a	p
2-Aminobutyric acid	0.235	0.010	–0.015	0.917
Alanine	0.118	0.411	–0.048	0.747
Arginine	–0.229	0.109	–0.217	0.137
Asparagine	0.114	0.428	0.097	0.511
Glutamic acid	–0.044	0.762	–0.010	0.499
Glutamine	–0.299	0.035	–0.278	0.056
Glycine	–0.240	0.094	–0.075	0.610
Histidine	–0.388	0.006	–0.420	0.003
Hypotaurine	–0.107	0.457	0.054	0.717
Isoleucine	0.335	0.018	0.207	0.156
Leucine	0.284	0.046	0.173	0.239
Methionine	–0.163	0.258	–0.311	0.032
Phenylalanine	0.033	0.817	–0.077	0.600
Serine	–0.141	0.327	–0.275	0.059
Taurine	–0.299	0.035	–0.202	0.167
Threonine	–0.300	0.034	–0.289	0.047
Tryptophan	–0.101	0.486	–0.097	0.510
Tyrosine	–0.286	0.044	–0.187	0.201
Valine	0.030	0.834	0.008	0.957

[a] Spearman's Rank Correlation Coefficient

acid [11, 12]. Low levels of glycine were detected in patients with diabetes and patients with impaired glucose tolerance. Wang-Sattler et al. [13] suggested that the glycine level may be a strong predictor of diabetes, even 7 years before disease onset.

We found a negative correlation between the levels of glucogenic amino acids glutamine, threonine, histidine and fasting glucose and a negative correlation between methionine, threonine, histidine and $HbA1_C$ in patients with T2DM. These results are in accordance with published data. Isoleucine and leucine correlated positively with fasting glucose, but there were no correlations between BCAA and $HbA1_C$, which were observed in another study [14]. We did not find a statistically significant difference between diabetic and non-diabetic participants in the levels of non-proteinogenic amino acids. There was only a negative correlation between taurine and fasting glucose.

There are only a few studies dealing with the determination of amino acids in patients with T1DM. A study conducted by la Marca et al. [15] monitored the levels of blood amino acids within newborn screening. They observed that children who developed T1DM during the first 6 years of life had levels of blood amino acids lower compared to children without diabetes, but statistically insignificant. Lanza et al. [16] measured the levels of amino acids in patients with T1DM. They found significantly increased levels of leucine and isoleucine, while the levels of the other amino acids were unchanged. Our results and the results of other studies dealing with the determination amino acids levels in patients with T2DM suggest that the levels of amino acids in both patients with T2DM are different from those in the control group and in patients with T1DM.

In conclusion, significant difference in metabolism of amino acids between diabetics and non-diabetics were observed. Our results are in agreement with other studies and support the statement that the altered levels of amino acids in diabetic patients could be a suitable predictor of diabetes in the future.

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