PLASMA ENDOTHELIN-1 LEVELS AND ALBUMINURIA IN PATIENTS WITH TYPE 2 DIABETES MELLITUS

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Summary

Introduction. Microalbuminuria is a very important independent risk factor for the progression of renal diseases as well as diseases of the cardiovascular system. Pathophysiological mechanisms that lead to the development of microalbuminuria in patients with diabetes are complex and they are a result of numerous factors. In the past decade, endothelin-1, the most potent vasoconstrictor peptide, was identified as an important factor that significantly contributes to the functional and structural renal changes. The objective of this study was to investigate the relationship between plasma concentration of endothelin-1 and urinary albumin excretion in patients with type 2 diabetes mellitus. Material and Methods. There were 76 patients with type 2 diabetes who were divided into those having normoalbuminuria (n=33), microalbuminuria (n=29), and macroalbuminuria (n=14), and 30 healthy controls. Plasma levels of endothelin-1 were measured by enzyme-linked immunosorbent assay. Results. There were significant differences in plasma concentration of endothelin-1 among groups (p<0.01). The correlation between endothelin-1, albuminuria, proteinuria and glomerular filtration rate was significant. In multiple regression analyses the plasma concentration of endothelin-1 was independently and significantly associated with albuminuria (β=0.01, p=0.009), proteinuria (β=0.02, p=0.001) and glomerular filtration rate (β=0.01, p=0.0006). Conclusion. Higher plasma concentrations of endothelin-1 are independently associated with the levels of urinary excretion of albumin which may corroborate the hypothesis of a potential role of this peptide in the development of microalbuminuria in diabetic nephropathy. Key words: Diabetes Mellitus, Type 2; Albuminuria; Proteinuria; Glomerular Filtration Rate; Endothelin-1; Diabetic Nephropathies; Risk Factors; Enzyme-Linked Immunosorbent Assay

Sažetak

Uvod. Mikroalbuminurija predstavlja veoma značajan nezavisni faktor rizika za progresiju bubrežnih bolesti kao i bolesti kardiovaskularnog sistema. Patofiziološki mehanizmi koji dovode do razvoja mikroalbuminurije kod bolesnika sa dijabetesom kompleksni su i rezultat delovanja brojnih faktora. Poslednjih godina izdvojio se endotelin-1, kao najpotencijalniji vazokonstruktorni peptid koji značajno doprinosi funkcionalnim i strukturnim bubrežnim promenama. Cilj ove studije bio je da se ispišu odnos plazmatske koncentracije endotelina-1 i urinarne ekskrecije albumina kod pacijenata sa tipom 2 šećerne bolesti. Materijal i metode. Izmerene su plazmatske koncentracije endotelina-1 sendvič-imunometrijskom metodom (ELISA) kod ukupno 76 bolesnika sa tipom 2 šećerne bolesti, koji su podešeni u grupe na osnovu različitog stepena albuminurije: na grupu sa normoalbuminurijom (n = 33), mikroalbuminurijom (n = 29) i makroalbuminurijom (n = 14). Rezultati. Postojala je statistički značajna razlika u vrednostima plazmatske koncentracije endotelina-1 između ispitivanih grupa (p < 0,01). Takođe postojala je značajna korelacija između endotelina-1 i albuminurije, proteinurije kao i izmerene vrednosti jačine glomerularnoj filtracije. U množičnoj regresijskoj analizi, plazmatska koncentracija endotelina-1 nezavisno je povezana sa albuminurijom (β = 0,01, p = 0,009), proteinurijom (β = 0,02, P < 0,001) i jačinom glomerularnih filtracija (β = -0,01, p = 0,0006). Zaključak. Povišene vrednosti plazmatske koncentracije endotelina-1 u korelaciji su sa stepenom urinarne ekskrecije albumina na što može ukazati na potencijalnu ulogu ovog peptida u razvoju mikroalbuminurije kod dijabetesne nefropatije. Ključne reči: dijabetes mellitus tip 2; albuminurija; proteinurija; glomerularna filtracija; endotelin-1; dijabetesna nefropatija; rizik faktor; imunometrijska metoda

Introduction

Microalbuminuria occurs in about 25% of patients with type 2 diabetes and it is an important independent risk factor for the progression of kidney disease as well as diseases of the cardiovascular system [1]. Increased urinary excretion of protein in patients with type 1 diabetes is indicative of the development of diabetic nephropathy; whereas, microalbuminuria as well as hypertension is already present at the time of
diagnosis of diabetes in most patients with type 2 diabetes. This is most likely the result of a generalized disorder of vascular endothelium. The development of this complication of diabetes is accompanied with the progressive increase in proteinuria and blood pressure, which contributes to the progression of nephropathy [2, 3]. Pathophysiological mechanisms that lead to the development of microalbuminuria in diabetic patients are complex and they are a result of the action of a number of hemodynamic and metabolic factors. Nowadays, it is believed that hemodynamic factors, such as systemic hypertension, glomerular hypertension and glomerular hyperfiltration, as well as ultrastructural changes at the level of the glomeruli such as thickening of the glomerular basement membrane and loss of negatively charged proteoglycans, are important factors in the development of albuminuria in diabetic nephropathy [4–6].

In the past decade, endothelin-1 (ET-1) was identified as an important factor among vasoactive factors contributing to the onset and progression of proteinuria. ET-1 is a potent vasoconstrictor peptide, derived from the production of a vascular endothelium, from big ET-1 and its splitting under the action of endothelial-converting enzyme inhibitors [6, 7]. ET-1 is produced as a result of activation of the endothelin type A (ETA) receptors and endothelin type B (ETB) receptors [7, 8]. ETA receptors are predominantly localized in the vascular smooth muscle cells and mediate vasoconstriction of large and small blood vessels, while ETB receptors located on endothelial cells mediate vasodilation through the production of nitric oxide (NO) and prostacyclin [7–9].

Endothelin-1 is a vasoactive peptide which has an important role in the regulation of renal function and blood pressure. The pathophysiological processes, in which ET-1 participates, have been identified. These processes consist of the mechanisms of vasoconstriction, increased vascular permeability, and inflammatory and oxidative effects of ET-1 [9–12].

Previous studies have indicated the presence of elevated concentrations of plasma ET-1 levels in patients with diabetes; however, the connection between ET-1 and albuminuria is not yet entirely clear; therefore, the objective of this study was to examine the relationship between plasma levels of ET-1, a marker of endothelial dysfunction and potent vasoactive factors and urinary albumin excretion in patients with type 2 diabetes.

**Material and Methods**

This cross-sectional study was conducted in the Clinical Center of Vojvodina in the period from June 2012 to July 2013. Of 106 respondents included in the study, 76 were the patients with type 2 diabetes (secondary insulin-dependent), who were treated at the Department of Endocrinology, Diabetes and Metabolic Diseases, Clinical Center of Vojvodina in Novi Sad and 30 were healthy controls matched by sex and age. The excluding criteria were: glomerular filtration rate (GFR) below 60 ml/min/1.73 m², any cardiovascular incident having happened 6 months before the inclusion into the study, malignancy, liver disease, acute inflammatory or infectious processes, and no change in concomitant therapy and supplementation, levels of physical activity, body weight changes, diet and smoking habits in the last 6 months.

The clinical evaluation of patients consisted of a detailed history taken, physical examination, measurement of body weight and height, as well as the calculation of body mass index (BMI), and blood pressure measurement. Laboratory evaluation was performed in the morning, after 12-hour fasting and a 30-minute resting period. Blood samples and 24-hour urine were taken on the same day when the measurement of glomerular filtration rate (GFR) was done, with prior verbal explanation to the respondents along with the attached written instructions on the methodology of collecting 24-hour urine. All analysis were done immediately after sampling, with the exception of ET-1 (samples were frozen for no longer than one month prior to the determination of ET-1).

The diabetic patients were divided into groups according to their values of urinary albumin excretion (UAЕ): the first group included the respondents with diabetes mellitus (DM) and the normoalbuminuria (UAЕ less than 30 mg/24 h), the second group of diabetic patients with microalbuminuria (UAЕ between 30–299 mg/24 h) and the third group of diabetic patients with macroalbuminuria (UAЕ ≥300 mg/24 h).

**Determination of Urinary Albumin Excretion - Albuminuria**

Albuminuria was determined from a sample of 24-hour urine, by sandwich immunometric assay, with the use of commercial Nyco Card tests (Oslo, Norway) on Nyco Card reader, the upper reference limit being 30 mg/24 h. The diagnosis of persistent micro or macroalbuminuria could be made in case of positive finding in at least two of three urine samples at intervals of three to six months.

Proteinuria was determined from a sample of 24-hour urine, by modified staining method on biochemical analyzer ADVIA 1800 by means of commercial Siemens kits (Siemens Health Care Diagnostics, Tarrytown, USA), the upper reference limit being 140 mg/24 h.

**ET-1 measurements**

The level of plasma endothelin-1 was determined by ELISA method by means of a commercial company sets (R&D Systems, USA) on the biochemical analyzer RYTO. The assay is based on the direct sandwich technique with monoclonal antibodies directed to the ET-1. The results were expressed as pg/mL.
GFR measurement

Assessment of GFR was determined by using a single-spaced model with the isotopic clearance of $^{99m}$Tc-labeled diethylene-triamine-penta acetic acid ($^{99m}$Tc-DTPA), using a single injection at a dose of 37 MBq and measurements of two blood samples, taken after 180 and 240 minutes. The values were normalized to a standard body surface and adjusted according to the age of patients [13, 14].

Serum concentrations of creatinin, urea and uric acid were determined by standard biochemical methods on the biochemical analyzer Olympus AU400. Furthermore, in all respondents serum concentrations of glucose were measured by the glucose oxidase method, the reference range being 4.0 – 5.9 mmol/L and glycated hemoglobin A1c (HbA1c) by the immune-inhibitory test (Beckman-Coulter, Ireland), the reference range being 4.7–6.0%.

Statistical Analysis

A statistical analysis was performed using SPSS version 12.0 (StatSosftinc, Tulsa, OK, USA) for Windows. Descriptive statistics, including median, arithmetic mean and standard deviation (SD) were used to describe the studied parameters. The distribution of numeric variables was tested by means of Kolmogorov-Smirnov test. Differences in distributions of individual parameters between the study groups were analyzed with the parametric (t-test, ANOVA) and nonparametric tests (Mann-Whitney test, Kruskal–Wallis test, chi-square test). Pearson and Spearman coefficients of linear correlation were used to determine the correlation between the variables. Multiple regression analysis was performed to estimate the independent contribution of plasma ET-1 concentration to albuminuria. A difference was considered significant if the p-value was less than 0.05.

Results

A total of 76 patients with type 2 DM (43 female and 33 male) were evaluated according to the level of urinary albumin excretion and classified into the following groups: diabetic patients with normoalbuminuria (n=33), microalbuminuria (n=29) and macroalbuminuria (n=14).

The main characteristics of the study respondents are shown in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Normo/Grupa sa normo (n = 33)</th>
<th>Micro/Mikro (n = 29)</th>
<th>Macroalbuminurija Makroalbuminurija (n = 14)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (f/m)/Broj (ž/m)</td>
<td>18/15</td>
<td>12/17</td>
<td>8/6</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Age (year)/Starosno doba (godine)</td>
<td>59 (39-75)</td>
<td>60 (46-77)</td>
<td>59 (52-75)</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)/ITM (kg/m$^2$)</td>
<td>28.9 (±4.5)</td>
<td>27.4 (±4.2)</td>
<td>29.4 (±3.2)</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Duration of diabetes (year)/Trajanje dijabetesa (godine)</td>
<td>10 (3-27)</td>
<td>13 (3-26)</td>
<td>12.5 (9-30)</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Insulin therapy (year)/Trajanje insulinске terapije (godine)</td>
<td>4 (1-12)</td>
<td>5 (1-25)</td>
<td>6 (2-7)</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Fasting glucose (mmol/L)/Glikemija (mmol/L)</td>
<td>8.7 (4.5-13)</td>
<td>8.6 (4.6-16)</td>
<td>8.8 (5-17)</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>HbA1c/HbA1c (%)</td>
<td>7.7 (5.6-9.1)</td>
<td>7.7 (5.8-9)</td>
<td>8.0 (7.2-9)</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Systolic BP (mmHg)/Sistolni KP (mmHg)</td>
<td>140 (110-180)</td>
<td>130 (110-180)</td>
<td>140 (120-190)</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)/Dijastolni KP (mmHg)</td>
<td>83 (±11)</td>
<td>81 (±6)</td>
<td>84 (±10)</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>HT (n)/Prisustvo HT (n)</td>
<td>27/33</td>
<td>21/29</td>
<td>14/14</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Duration of HT (year)/Trajanje HT (godine)</td>
<td>5 (1-20)</td>
<td>5 (1-30)</td>
<td>6 (3-20)</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>ACE inhibition use (n)/Upotreba ACE inhibitora (n)</td>
<td>16/27</td>
<td>14/29</td>
<td>13/14</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>mGFR/mJGF (mL/min/1.73 m$^2$)</td>
<td>98 (±12)</td>
<td>94 (±18)</td>
<td>71 (±11)</td>
<td>Pab&lt;0.05</td>
</tr>
<tr>
<td>Creatinine/Kreatinin (µmol/L)</td>
<td>73 (±12)</td>
<td>74 (±13)</td>
<td>81 (±10)</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Urea/Urea (µmol/L)</td>
<td>5.4 (3.8-7)</td>
<td>5.6 (4.8-9)</td>
<td>6 (4.4-10)</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Uric Acid/Mokraćna kiselina (µmol/L)</td>
<td>291 (±77)</td>
<td>299 (±91)</td>
<td>360 (±65)</td>
<td>Pab&lt;0.05</td>
</tr>
<tr>
<td>UAE (mg/24 h)/UEA (mg/24 h)</td>
<td>10 (7.8-15)</td>
<td>50 (30-127)</td>
<td>340 (300-860)</td>
<td>P&lt;0.05</td>
</tr>
<tr>
<td>Proteinuria (g/24 h)/Proteinurija (g/24 h)</td>
<td>109 (45-149)</td>
<td>196 (40-501)</td>
<td>400 (235-800)</td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>

BP – blood pressure, ACE- angiotensin-converting enzyme, HT-hypertension, HbA1c - glycated hemoglobin, a - p<0.05 – compared macro to normoalbuminuria group; b - p<0.05 – compared macro to microalbuminuria group; ITM – indeks telesne mase, KP – krvni pritisak, inhibitori ACE – angiotenzin- konvertujućeg enzima, HT – hipertenzija, HbA1c – glikozilirani hemoglobin, UEA – urinarna ekskrecija albumina, a – p<0.05 – poredjenje grupe sa makro- u odnosu na grupu sa normoalbuminurijom, b – p < 0.05 – poredjenje makro u odnosu na grupu sa mikroalbuminurijom, JGB – jačina glomerulske filtracije
Endothelin-1 is a potent vasoactive factor with proliferative, profibrotic and proinflammatory properties with a high expression in the renal vasculature and parenchyma. In this study, there was an increase in the plasma concentration of ET-1 with increased levels of urinary albumin excretion in the patients with type 2 DM. The value of plasma ET-1 was significantly higher in the group of diabetic patients with macroalbuminuria than in the group of patients with micro and normoalbuminuria, and significantly higher plasma ET-1 in the group with microalbuminuria than in the patients with normoalbuminuria. There was also a significant correlation of plasma concentrations of ET-1 with the values of urinary albumin excretion and proteinuria in the patients with type 2 DM. Similar results were obtained in studies of Zannatta et al., who found a significant association of elevated plasmatic concentration of ET-1 with the degree of albuminuria in diabetic patients [15]. The study of Bruno CM revealed elevated levels of plasma ET-1 in the group of normotensive diabetic patients with microalbuminuria compared to the healthy population, as well as its significant correlation with the severity of urinary albumin excretion [15, 16].

One of the possible mechanisms by which ET-1 affects the glomerular damage and contributes to the development of proteinuria is podocyte damage. Podocytes are highly differentiated cells with complex morphology, which play an important role in maintaining the integrity of filtration membranes. It is believed that the influence of ET-1 leads to podocyte effacement and disruption of the podocyte actin cytoskeleton. Furthermore, it leads to a loss of protein such as nephrin, which plays an important role in maintaining intercellular conjunctions. In their in vitro experimental study Morigi et al. have found that podocytes are subject to phenotype changes, i.e. dedifferentiation influenced by autocrine and paracrine functioning of ET-1, which leads to damage of glomerular filtration membrane [16]. These data are consistent with the evidence of in vivo study in a mice model of “overload” proteinuria obtained by Benigni A. et al. which exhibit an enhanced renal production of ET-1 leading to the development of some structural damage to podocytes [17, 18]. Another possible mechanism is the hemodynamic effect of ET-1, which in conjunction with other vasoactive factors lead to an increase in intraglomerular capillary pressure which increases glomerular permeability and thus leads to increased filtration of proteins [18, 19]. Considering the role of ET-1 in the development of proteinuria, the results of a number of experimental studies have pointed to renoprotective action of endothelin receptor blockers [20, 21].

As for the presence of hypertension, 82% of the total number of patients with diabetes had hypertension. It is known that hypertension in newly diagnosed cases of type 2 DM is usually already present, and it had often existed before type 2 DM was diagnosed. The data indicate that the prevalence of hypertension in patients diagnosed with type 2 diabetes is about 50%, and with the development of microalbuminuria this percentage increases to about 80%. On the other hand, the prevalence of hyperten-
sion in patients diagnosed with type 2 DM with development of macroalbuminuria is about 90% [21, 22]. This corresponds to the findings of our study.

The findings of this study showed that diabetic patients with a higher degree of UAE or macroalbuminuria had lower GFR than the diabetic patients with microalbuminuria and normoalbuminuria. This was expected considering the fact that proteinuria itself is one of the most important independent factors of progression of renal disease. A significant correlation was also found between the values of plasma ET-1 and the GFR, i.e. the diabetic patients with higher values of plasma ET-1 had lower GFR. In addition to the already well-known fact that the value of plasma ET-1 in patients with DM is elevated compared to the healthy population, numerous studies have turned to examining its role in the development and progression of microvascular complications [21–23]. The experimental studies done on diabetic rats so far suggest a role of elevated levels of ET-1 in glomerular and interstitial renal damage, which were proportional to the plasma concentration of ET-1 [24–28]. However, previous clinical studies dealing with the relationship of ET-1 and GFR in diabetic patients yielded different findings, which could have resulted from the number of patients involved and the status of the functional kidney. Namely, no significant correlation was found between ET-1 and GFR in the group of patients with relatively preserved kidney function, while studies involving respondents with greater reduction of GFR revealed a significant correlation between ET-1 and the status of kidney function [29–34].

**Conclusion**

The findings from this study show that the higher values of plasma endothelin-1 concentrations are independently correlated with a higher degree of albuminuria and proteinuria, which supports the hypothesis of potential role of vasoactive peptides in the development of microalbuminuria in diabetic nephropathy. In addition, an independent correlation of plasma concentrations of endothelin-1 and glomerular filtration rate indicates a potentially harmful effect of elevated levels of endothelin-1 on kidney function, which contributes to the further progression of diabetic nephropathy. The above findings suggest a possible role of endothelin receptor antagonists in preventing the development and further progression of diabetic nephropathy.

**References**


