Damage of tubule cells in diabetic nephropathy type 2: urinary N-acetyl-β-D-glucosaminidasis and γ-glutamil-transferasis

Oštećenje tubulskih ćelija u dijabetesnoj nefropatiji tip 2: urinarna N-acetil-β-D-glukozaminidaza i γ-glutamil-transferaza

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Abstract

Background/Aim. A damage of tubular epithelial cells is followed by the release of cell enzymes and production of proinflammatory compounds, which lead to the tubulointerstitial damage. The aim of this study was to examine the function of renal tubules in the patients with diabetes mellitus type 2 (DM type 2) and the various proteinuria degrees, to establish the damage of the proximal tubule cells caused by DM type 2 by determining urinary N-acetyl-β-D-glucosaminidasis (β-NAG) and γ-glutamil-transferase (γ-GT) activity in urine, as well as to compare the obtained results in the examined groups of patients with the values in the healthy examinees. Methods. A complete examination of renal function and selective enzymuria was performed in 37 patients with DM type 2, and 14 healthy examinees as the controls. The patients were divided in three groups according to the degree of proteinuria. The first group consisted of the patients with diabetes without microalbuminuria; the second one consisted of the patients with proteinuria of < 300 mg/24 h, and microalbuminuria of >20 mg/24 h, while the third one included the patients with proteinuria of >300 mg/24 h. Results. In the patients with DM type 2 and the preserved global renal function, fractional excretion of sodium, potassium and phosphates, as well as renal threshold of phosphates concentration, were not sensitive parameters for discovering the damage of the renal tubule function. The determination of β-NAG activity proved to be the most sensitive parameter for early discovering of tubule cells damages. The difference among the examined groups was statistically highly significant. Conclusion. The increased presence of β-NAG in the urine of DM type 2 patients, pointed out an early tubular disorder and damage of cells, while γ-GT was a less sensitive indicator of this damage.

Key words: diabetic nephropathies; kidney tubules; diabetes mellitus, type 2; enzymes; proteinuria; hexosaminidases.

Introduction

Low molecular weight proteins are filtered trough the kidney glomerular barier. Proximal tubular cells reabsorb proteins from glomerular ultrafiltrate. Depending on the quantity and conditions, these proteins and the products of their degradation may be harmful and may damage tubular cells. This is followed by the release of enzymes and pro-

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duction of proinflammatory compounds, which lead to the tubulointerstitial damage.

Over 50 various enzymes have been discovered in human urine, but only some can be used for the diagnosis of renal diseases.  

The urinary enzyme N-acetyl-β-D-glucosaminidasis (β-NAG) is found in the lysosomes of the proximal tubule epithelial cells. A high β-NAG activity in urine during the course of illness or toxic insult is a consequence of tubule cells damage in renal diseases or due to nephrotoxic effects (drugs, proteinuria, diabetes and pregnancy) and represents an early sign of tubule disorder. False positivity is rare. Thus, β-NAG is proposed to be an early sign of the presence of diabetic nephropathy.

Gamma glutamil transferasis (γ-GT) in urine originates from the surface of brushy border of epithelial cells membrane in the proximal tubules lumen. Gamma GT is a specific and sensitive indicator of these cells damages. A diagnostic value of γ-GT was confirmed also in glomerular diseases and chronic damage of the kidney caused with drugs and heavy metals. An increased urinary γ-GT was observed in the patients with diabetes without signs of renal function disorder, or even microalbuminuria. This enzyme excretion was connected with the duration of glycemia, degree of renal function damage and hemoglobin A1C level.

The aim of this study was to examine the function of kidney tubules in patients with diabetes mellitus type 2 (DM type 2) and various degrees of proteinuria, to establish the damage of proximal tubule cells caused by DM type 2 by determination of index of catalytic activities of the β-NAG and γ-GT enzymes in urine, and to compare the obtained results in the groups of patients with DM type 2 with the corresponding values in healthy persons.

### Methods

The patients with DM type 2 and clearance of endogenous creatinine above 80 ml/min, without clinically expressed hypovolemia and without taking diuretics during collecting of urine were examined. The examination was conducted in accordance with the Helsinki Declaration on Medical Research and with the consent of the patients. Microscopic and microbiological examinations of the urine sediment excluded the presence of infection. A total of 110 persons were examined, 14 healthy persons (the control group) and 96 patients with DM type 2. According to the order of appearance, they were separated into groups. A certain number of patients was not included into the study due to equality in the group size and comparability. In order to examine the influence of DM type 2 to kidney tubule cells, the patients were separated into three groups based on the degree of proteinuria. The first group consisted of the patients without findings of microalbuminuria. The second group encompassed the patients with proteinuria of less than 300 mg/24 h and microalbuminuria of over 20 mg/24 h, and the third group consisted of the patients with proteinuria over 300 mg/24 h. The complete examination encompassed 37 patients with DM type 2, the average age of 50.06±12.37 years. The control group included healthy examinees of average age of the 40.57±33.13 (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Groups</th>
<th>Significance</th>
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<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>N (m/l)</td>
<td>Mean ± SD</td>
<td>17 (5/12)</td>
</tr>
<tr>
<td>Age (yrs.)</td>
<td>Mean ± SD</td>
<td>54±9.87</td>
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<tr>
<td>Urea (mmol/l)</td>
<td>Mean ± SD</td>
<td>5.73±1.34</td>
</tr>
<tr>
<td>Cr (μmol/l)</td>
<td>Mean ± SD</td>
<td>79.24±16.86</td>
</tr>
<tr>
<td>CCr (ml/min)</td>
<td>Mean ± SD</td>
<td>94.43±37.07</td>
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<tr>
<td>Na⁺ (mmol/l)</td>
<td>Mean ± SD</td>
<td>141.76±6.64</td>
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<tr>
<td>K⁺ (mmol/l)</td>
<td>Mean ± SD</td>
<td>4.63±0.56</td>
</tr>
<tr>
<td>PO₄³⁻ (mmol/l)</td>
<td>Mean ± SD</td>
<td>1.43±0.38</td>
</tr>
<tr>
<td>UK⁺/Na⁺</td>
<td>Mean ± SD</td>
<td>0.27±0.16</td>
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<tr>
<td>UK⁺/(UK⁺+UNa⁺)</td>
<td>Mean ± SD</td>
<td>0.21±0.06</td>
</tr>
<tr>
<td>FEₚ₃⁻</td>
<td>Mean ± SD</td>
<td>1.06±0.61</td>
</tr>
<tr>
<td>FEₖ⁺</td>
<td>Mean ± SD</td>
<td>8.01±2.55</td>
</tr>
<tr>
<td>ß-D-glucosaminidasis (β-NAG) (U/mmol Cr)</td>
<td>Mean ± SD</td>
<td>0.88±0.87</td>
</tr>
<tr>
<td>γ-GT (U/mmol Cr)</td>
<td>Mean ± SD</td>
<td>2.53±1.67</td>
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Blood and urine samples and a 24 h collection of urine were taken in the morning. Creatinine (Cr) in the serum was determined by a colorimetric analysis on a Monarch plus IL, Milan, Italy, device. The values of urea were obtained on a device for Cr by the method of ureasis-glutamat-dehydrogenasis. Urea and Cr in a 24-hour urine sample were processed by the same method as in the serum, with a ten times greater solution. Microalbuminuria in a 24 h urine sample was determined nephelometrically on a BN 100 – Behring nephelometer. Concentrations of sodium (Na+) and potassium (K+) in the serum and a 24 h urine sample were determined colorimetrically on a Monarch plus IL, Milan, Italy, device. Proteinuria in a 24 h urine sample was determined colorimetrically with a Comassie Brilliant Blue (CBB). Urine osmolality was measured by the direct cryoscopic method with a Roebling osmometer. The clearance of a substance was calculated according to the formula: 

$$C_x = \frac{(U_x V_u)}{P_x}$$

where: 

- $C_x$ – clearance of the examined substance (ml/min), 
- $U_x$ – concentration of the examined substance in a 24 h urine sample (mmol/l), 
- $V_u$ – 24h urine sample volume (l), and 
- $P_x$ the examined substance plasma concentration (mmol/l).

The fractional excretion (FEx) of the substance was determined using the formula: 

$$FEx = \left(\frac{C_x}{CCr}\right) \times 100$$

where 

- $CCr$ represents the clearance of endogen creatinine (ml/min), 
- $C_x$ – clearance of examined substance (ml/min). The renal threshold of phosphates concentration represents the relation of tubule phosphate absorption maximum and glomerular filtration rate (TmPO4/GFR), interpreted by the nomogram for the assessment of the renal threshold of phosphates concentration (Bijovet and Walton). The index of $\beta$-NAG catalytic activity in a 24 h urine sample was determined spectrophotometrically by a commercial Boehringer Company test. The upper normal value of $\beta$-NAG in a 24 h urine sample calculated to mmol Cr was 0.56 U/mmol Cr. The index of $\gamma$-GT catalytic activity in a 24 h urine sample was determined by the enzyme method, commercial test, on a Monarch plus IL, Milan, Italy, device. The normal values ranged from 0.80 and 4.01 U/mmol Cr for a females and 1.02 to 3.35 U/mmol Cr for males. The 24 h urine sample proteinuria was determined colorimetrically with CBB.

Statistical analysis of the continuous variables were expressed as a mean with standard deviation (± SD). An analysis of variance (ANOVA) was used to compare the mean values between the groups. Correlation test was used to establish the connection of activity index of $\beta$-NAG and $\gamma$-GT enzyme. Statistical significance was considered when $p$-value was < 0.05. Microsoft EXCEL statistical package (a version for Microsoft 2000) was used to perform statistical analysis.

**Results**

Urea and Cr values in the serum, as well as CCr were used to assess functional status of the kidneys. These values were normal in all patients, without a significant difference among the groups. Serum concentrations of Na+, K+ and PO₄³⁻ were also within the normal values, without significant difference among the groups.

Hormone-depending activity of tubules was assessed indirectly by examining the relation of K⁺ and Na⁺ concentration in urine and the relation of the $K^+$/Na⁺ concentration in urine and the sum of K⁺ and Na⁺ concentration in urine. There was no difference between the examined groups, indicating that there was no compensatory influence of hormone to the transport processes in the distal tubule.

The functional status of proximal tubules was assessed by determining the values of fractional excretion of sodium (FENa⁺), potassium (FEK⁺), phosphate (FEPO₄³⁻), and renal threshold of phosphates concentration (TmPO₄³⁻/GFR). The obtained values of FEPO₄³⁻ and TmPO₄³⁻/GFR were within the reference ranges. The average values of FEPO₄³⁻ and TmPO₄³⁻/GFR did not show a significant difference among the examined groups (Table 1).

The functional status of Henle’s loop was assessed by determining urine osmolality (Uosm) and fractional excretion of urea (FEmeso). Urine osmolality and FEmeso values at all the examinees were within the reference values. No significant difference among the examined groups was found.

Indices of $\beta$-NAG and $\gamma$-GT activity in urine served for the assessment of damage to the tubule cells integrity. The activity of urinary $\beta$-NAG was normal only in the group of healthy examinees. In all other groups, this activity was significantly increased. The highest activity was observed in the group of patients with DM and microalbuminuria, where the average value was three times higher than the upper limit value. The patients with DM and proteinuria over 300 mg/24 h had $\beta$-NAG catalytic activity twice as high as the normal one. It was also increased in the first group of patients who had DM and no signs indicating the renal tubular damage. The difference between the first and the third group was statistically significant, ($p < 0.05$), and among the other groups highly statistically significant ($p < 0.001$) (Table 1).

The average values of the $\gamma$-GT activity index in urine were within the reference limits in all groups, but the third one consisted of the patients with proteinuria above 300 mg/24 h had an increased value of this index. The difference between the third group and all the other groups was statistically significant ($p < 0.05$), while no significant difference among other groups was found.

All the examinees had the normal $\beta$-NAG and $\gamma$-GT activity values in the serum.

The correlation between $\beta$-NAG and $\gamma$-GT activity in the examinees’ urine was checked by a correlation test within each of the groups by the individually paired enzyme activity values. In the first and the second group a positive correlation was found ($r = 0.46$ and $r = 0.48$) respectively, meaning that the increase in one enzyme activity follows the increase in the other. In the third group the correlation was even less expressed ($r = 0.28$), and in the group of healthy examinees it can be ignored ($r = -0.15$).

**Discussion**

Recent data suggest that the increased tubular metabolic activity and cell integrity changes could be the first signs of diabetic kidney disease. It was established that the kidney...
function in DM type 2 does not depend only on the duration of the disease, but also on other factors, such as: the presence of microalbuminuria, proteinuria and advanced glycation end products 2, 13–20.

In this study a hypothesis was established that the damage of renal tubules differs in relation to the presence or absence of proteins in a patient’s urine and that the quantity of proteins in glomerular filtrate reflects the severity of damage 14, 21–23.

In the selected groups of patients, the functional status of proximal tubules was assessed by establishing FE Na⁺ and K⁺. The differences among the groups were not significant. The highest value was found in the patients with proteinuria over 300mg/24 h. This can be explained by the fact that the increased quantity of protein in urine stimulates mRNA transciption for precursor of endotelin 1. Endotelin 1 influences activity of Na⁺/K⁺-ATP-asis, which participates in the regulation of primary active transport of Na⁺ through tubule membranes 24.

Establishing the relation of Na⁺ and K⁺ concentration in urine and the relation between K⁺ concentration and the sum of Na⁺ and K⁺ concentrations in urine assessed hormone-dependent activity of tubules, which is under the influence of aldosteron and hypovolemia. There was no significant difference among the examined groups, thus it can be concluded that there was not any important compensatory influence of hormones on transport processes in the distal tubule 25.

In the proximal tubule cells, the PO₄³⁻ transport functions due to the system of co-transport of PO₄³⁻ and Na⁺ (Na⁺/PO₄³⁻). Normally, 80–97% of filtered phosphates are reabsorbed. The surplus is excreted in urine. Parathormone does not influence phosphates reabsorption in the proximal part of tubules 26. When CcR is over 40 ml/min, TmPO₄³⁻/GFR values vary in accordance with GFR, but phosphatemia and this coefficient values are quite constant. Along with GFR, tubule reabsorbing of PO₄³⁻ is also influenced by parathormone, acid-base status, volume of extracellular liquid, Ca²⁺ in plasma, and PO₄³⁻ intake 26. The values obtained by calculation of renal threshold of the PO₄³⁻ and FEPO₄³⁻ concentration were within the normal ranges, suggesting that diabetic nephropathy type 2 does not significantly alter the capacity of tubules for phosphates reabsorbing, at least when CcR is within the normal limits.

The functional status of Henle’s loop was assessed by establishing urine osmolality and FE urea, which were within the normal ranges, implicating that the concentration capacity of tubules in this part of Henle’s loop was not damaged.

The discussed laboratory analyses were not reliable parameters for discovering early changes of the renal function in diabetes in this research.

In healthy subjects, enzymes from the tubule epithelial cells are excreted in urine in small quantities as a consequence of metabolic cell activity. Various harmful effects to cells (hypoxia, anoxia, ischemia and toxic effects of the very proteins), followed by the function damage, increase the quantity of these enzymes in urine 1, 6, 8, 27–29.

In diabetic patients, as a consequence of glycation, a change of energetic status of proximal tubules cell membrane leads to its damage and release of cytoplasma and organelles in the tubule lumen 1. The process commences with Ca²⁺ concentration increase in a cell, because Ca²⁺ with phospholipids and glycolipids in the membrane, reduces the membrane metabolic capacity 1, 30, 31. In this research, we expected increased γ-GT values in the group of diabetic patients with a lesser kidney damage (patients without microalbuminuria or proteinuria), since the enzymes from the brushy part of tubule cells are susceptible to changes at the very beginning. But, it was shown that only the patients with proteinuria over 0.3 g/24 h, had the increased γ-GT values, significantly higher when compared with the other groups. In the other three groups, the values γ-GT activity varied within the normal ranges, without significant differences among the groups. Still, it seems possible that the increased γ-GT activity in the group of patients with expressed proteinuria is a consequence of a harmful influence of proteinuria to metabolic active brushy part, rather than the influence of diabetic disease 32–35.

In lyzozomes of the proximal tubule epithelial cells there is β-NAG, which metabolized glycoproteins 1. As lyzozomes are metabolically very dynamic, small quantities of β-NAG are present in the healthy people’s urine 4. Many studies have highly evaluated the values of this enzyme in the assessment of renal function 4, 6, 8, 14, 28, 29, 36, 37. In this research, the catalytic activity of β-NAG in the urine of the patients with DM type 2 was the most sensitive parameter for early discovering of the tubule cells damage. This enzyme sensitivity is obvious. While in the control group β-NAG activity was within the normal ranges, in the groups of patients with DM type 2 without microalbuminuria and without proteinuria its activity was increased for 1.5 times over the upper reference limit. In the patients with microalbuminuria, catalytic activity of this enzyme was three times higher than the reference value, while in the third group, it was twice as high. These differences were statistically highly significant. This points out the great importance of this enzyme in discovering the renal tubule cells damage, especially at the early stage, since its increased value is found before the appearance of microalbuminuria.

We tried to determine to what extent the decrease or increase in the activity index of one enzyme is followed by changes in the activity of the other one. The obtained values showed that a slightly positive correlation existed in the first two groups. It was even weaker in the third group, while in the control group no correlation was found.

**Conclusion**

The β-NAG activity index proved to be the most sensitive parameter for early discovering the tubule cells damage. The values of β-NAG activity were increased before the microalbuminuria appearance, and the patients with microalbuminuria had the highest values. The difference among the examined groups was statistically highly significant. In this research, γ-GT was less sensitive biomarker of renal tubule damage in relation to β-NAG.
REFERENCES


