Advanced glycation end-products and cathepsin cysteine protease in type 2 diabetic patients

Ewa Grzebyk¹, Maria Knapik-Kordecka², Agnieszka Piwowar¹

¹ Department of Pharmaceutical Biochemistry, Wroclaw Medical University, Wroclaw, Poland
² Department and Clinic of Angiology, Hypertension and Diabetology, Wroclaw Medical University, Wroclaw, Poland

ABSTRACT

INTRODUCTION In type 2 diabetes, chronic hyperglycemia induces multi-faceted disturbances and contributes to late diabetic complications. Nonenzymatic glycation, leading to formation of advanced glycation end-products (AGEs), is one of the most important consequences of hyperglycemia. Alterations in the function of some proteolytic enzymes are also observed in diabetes.

OBJECTIVES The aim of the study was to assess the changes in and correlations between the plasma levels of AGEs and the activity of a proteolytic enzyme – cysteine cathepsin B – in plasma and neutrophils derived from patients with type 2 diabetes.

PATIENTS AND METHODS In 102 patients with type 2 diabetes and 55 healthy adults, the plasma levels of total AGEs, low-molecular-weight AGEs (LWM-AGEs), and high-molecular-weight AGEs (HWM-AGEs) as well as cathepsin B activity in plasma and neutrophils were measured by fluorescence methods. Diabetic complications in patients were also evaluated.

RESULTS Diabetic patients had significantly higher levels and activities of all the parameters compared with the control group. Moreover, in these patients, HWM-AGEs correlated negatively with plasma cathepsin B and LMW-AGEs with neutrophil cathepsin B. In the quartiles of the increasing levels of HWM-AGEs and LMW-AGEs, a successive decrease of cathepsin B in plasma and neutrophils, respectively, was observed. In patients with different late diabetic complications only the plasma level of LMW-AGEs was significantly different.

CONCLUSIONS Our study showed a significant increase of all forms of AGEs and corresponding changes in the activity of cathepsin B, both in plasma and neutrophils. A significant correlation between AGEs and cathepsin B as well as the ambiguous character of their alterations in patients with late diabetic complications indicate that they exert a complex effect on the course of diabetes.
Advanced glycation end-products and cathepsin cysteine proteases...

Moreover, it participates in numerous physiological functions and the turnover of proteins. It is also present in neutrophil primary granules (a particular class of lysosomes). Moreover, it participates in numerous physiological (e.g., cell growth and apoptosis, antigen presentation) and pathological (e.g., tumor invasion and metastasis) processes, mainly due to extracellular matrix degradation.11,12

**PATIENTS AND METHODS** The study involved 102 patients with type 2 diabetes (25 men and 77 women), treated at the Department of Angiology, Hypertension, and Diabetology of the Wroclaw Medical University. Microangiopathies were reported in 24 patients, macroangiopathies in 32, and 40 patients had both micro- and macroangiopathies. Only 6 subjects did not have any diabetic complications. The control group comprised 55 healthy adults (21 men and 34 women), who were recruited among individuals undergoing routine medical check-ups. The clinical and laboratory characteristics of diabetic patients and the control group are presented in **TABLE 1**. All subjects were informed about the aim of the study and provided written consent to participate in the study. The study protocol was approved by the Bioethics Committee of the Wroclaw Medical University.

---

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Characteristics of patients with type 2 diabetes and controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Diabetic patients</td>
</tr>
<tr>
<td>sex, female/male</td>
<td>77/25</td>
</tr>
<tr>
<td>age, y</td>
<td>58.20 ±11.60</td>
</tr>
<tr>
<td>disease duration, y</td>
<td>12.72 ±6.36</td>
</tr>
<tr>
<td>diabetic treatment: diet/oral/insulin/combined therapy, n</td>
<td>13/29/15/45</td>
</tr>
<tr>
<td>fasting plasma glucose, mg/dl</td>
<td>158.10 ±41.07</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>7.91 ±1.82</td>
</tr>
<tr>
<td>body mass index, kg/m²</td>
<td>28.07 ±4.57</td>
</tr>
<tr>
<td>total cholesterol, mg/dl</td>
<td>204.87 ±32.17</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>47.37 ±12.78</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dl</td>
<td>116.64 ±26.38</td>
</tr>
<tr>
<td>triglycerides, mg/dl</td>
<td>188.26 ±32.81</td>
</tr>
<tr>
<td>systolic/diastolic blood pressure, mmHg</td>
<td>131/78 ±16/10</td>
</tr>
<tr>
<td>white blood cell count, 10⁹/l</td>
<td>7.41 ±2.04</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation.

Conversions factors to SI units are as follows: for glucose – 0.05551, cholesterol – 0.02586, and triglycerides – 0.0114.

Abbreviations: HbA1c – hemoglobin A1c, LDL – low-density lipoprotein, HDL – low-density lipoprotein, NS – nonsignificant.
Venous blood samples were drawn in the fasting state into tubes containing heparin (16 IU/ml); neutrophil fraction was immediately isolated and plasma was obtained (stored at ~85°C until the assay). Neutrophils were isolated according to Zeman et al.,17 with our modification, with the use of Gradiisol G density (d = 1.115 g/ml) by centrifugation of overlayered whole blood on the Gradiisol G (in a ratio of 3:2, respectively). After isolation, neutrophils were suspended in 1 ml of phosphate-buffered saline and counted with the use of the Bürker chamber, and their high purity (over 96%) was confirmed by histochemical staining. Neutrophil extracts were obtained by a triple freezing–thawing cycle (at ~85°C) to disruption of biological membrane continuity and liberation of intracellular protease. Cathepsin B activity was measured by the fluorometric method in plasma and neutrophil extracts according to Barret18 with synthetic substrate (Z-Arg-Arg-NMec). All samples were made in triplicate, and the fluorescence of a fluorescent product (7-amino-4-methylcoumarin) liberated by the action of cathepsin B was recorded on the spectrofluorometer (Perkin-Elmer LS 50B) at excitation and emission wavelengths of 370 nm and 460 nm, respectively. The activity of cathepsin B in plasma was expressed as mU/l and in neutrophils as mU/mg of protein (determined by Lowry et al.).19

In plasma, the levels of total AGES, LMW-AGES, and HMW-AGES were measured according to Münch et al.20 and Wrobel et al.,21 as modified by ourselves. AGES exhibit characteristic fluorescence, which is the basis of their measurement in biological material. The levels of total AGES were determined in adequately diluted plasma samples by 0.9% NaCl. After plasma deproteination by 10% solution of trichloroacetic acid, the LMW-AGE level was measured in supernatant, whereas that of HMW-AGES in redissolved precipitate, obtained by centrifugation. All samples were made in triplicate. Characteristic fluorescence in a spectrophotometer Perkin-Elmer LS 50B was measured at excitation and emission wavelengths of 370 nm and 440 nm, respectively. The results were expressed in arbitrary fluorescence units (AFU) and presented as AFU × 10³.

The baseline biochemical parameters, presented in Table 1, were determined using commercially available assays with an automatic analyzer.

The results were presented as mean values and standard deviations. The statistical analysis was done using Statistica PL for Windows version 9.1. The parametric t test and nonparametric Mann–Whitney test were performed. The analysis of variance was also used. Associations between the parameters were determined by the Spearman correlation coefficient (r). A P-value of less than 0.05 was considered statistically significant.

### RESULTS

The plasma levels of total AGES, LMW-AGES, and HMW-AGES as well as the activity of cathepsin B in plasma and neutrophils in patients with type 2 diabetes and healthy individuals are presented in Table 2. The levels of all parameters were significantly higher in diabetic patients compared with the control group. The most significant differences were observed in the levels of total AGES and LMW-AGES and the activity of cathepsin B in neutrophil extracts (P <0.001). The differences in the levels of HMW-AGES and the activity of cathepsin B in plasma were borderline significant (P <0.05). The levels of total AGES, LMW-AGES, and HMW-AGES were higher by almost 39%, 24%, and 35%, respectively, in diabetic patients compared with healthy individuals. The activity of cathepsin B in plasma was almost 38% higher in diabetic patients compared

### Table 2

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Diabetic patients</th>
<th>Controls</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>total AGES, AFU × 10³</td>
<td>214.14 ± 77.25</td>
<td>154.18 ± 46.88</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LMW-AGES, AFU × 10³</td>
<td>7.22 ± 3.25</td>
<td>5.04 ± 2.18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HMW-AGES, AFU × 10³</td>
<td>37.07 ± 16.01</td>
<td>27.52 ± 10.13</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>cathepsin B in plasma, mU/l</td>
<td>13.29 ± 6.56</td>
<td>9.68 ± 4.36</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>cathepsin B in neutrophils, mU/mg</td>
<td>65.34 ± 31.93</td>
<td>25.98 ± 12.18</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation.

Abbreviations: AFU – arbitrary fluorescence units, AGES – advanced glycation end-products, HMW – high molecular weight, LMW – low molecular weight

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>Cathepsin B in plasma</th>
<th></th>
<th>Cathepsin B in neutrophils</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>total AGES</td>
<td>−0.19</td>
<td>NS</td>
<td>−0.14</td>
<td>NS</td>
</tr>
<tr>
<td>LMW-AGES</td>
<td>−0.17</td>
<td>NS</td>
<td>−0.31</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>HMW-AGES</td>
<td>−0.48</td>
<td>&lt;0.05</td>
<td>−0.12</td>
<td>NS</td>
</tr>
</tbody>
</table>

Abbreviations: see Tables 1 and 2
Advanced glycation end-products and cathepsin cysteine protease...

Moreover, there was a significant correlation between cathepsin B activity in neutrophils and the LMW-AGE level \((r = -0.31, P < 0.05)\). No significant differences were observed between Q4 and all the remaining quartiles (all, \(P > 0.05\)). The activity of plasma cathepsin B in Q1, Q2, and Q3 was about 32.0%, 21.6%, and 17.6% higher, respectively, than that in Q4. No significant differences between the remaining quartiles were observed. As shown in Figure 2, the highest activity of cathepsin B in neutrophils was also observed in the first quartile (Q1) in the group with the lowest level of HMW-AGEs. However, the lowest activity of cathepsin B was present in Q4 in patients with the highest HMW-AGE level. Significant differences were observed between Q4 and all the remaining quartiles (all, \(P < 0.05\)). As shown in Table 3, the activity of cathepsin B in neutrophils and plasma are presented in the context of the late diabetic vascular complications (Table 4). Patients were divided into 3 groups: with microangiopathy, macroangiopathy, and both types of angiopathies (micro- and macroangiopathy). Of the examined parameters, only the highest plasma level of HMW-AGEs, observed in the microangiopathy group, was significantly different compared with those with macrovascular complications \((P < 0.05)\). However, the lowest activity of cathepsin B in neutrophils, observed in patients with microangiopathies, was not significantly different compared with the remaining groups. The plasma HMW-AGE level was similar in all groups. The activity of plasma cathepsin B progressively decreased from the microangiopathy group, through the macroangiopathy group, to that with both types of angiopathies, but the differences were not significant.

**Figure 1** Activities of plasma cathepsin B in the quartiles of increasing plasma levels of high-molecular-weight advanced glycation end-products (HMW-AGEs) in patients with type 2 diabetes

**Figure 2** Activity of cathepsin B in neutrophils in the quartiles of increasing low-molecular-weight advanced glycation end-products (LMW-AGEs) in patients with type 2 diabetes

with the control group, and it was about 2.5-fold higher in neutrophil extracts.

Correlations between the levels of AGEs and the activity of cathepsin B in neutrophils and plasma are presented in Table 3. The magnitude of correlations between the examined parameters varied and as few as 2 associations were statistically significant. A negative correlation was observed between cathepsin B activity in plasma and the HMW-AGE level \((r = -0.48, P < 0.05)\). Moreover, there was a significant correlation between cathepsin B activity in neutrophils and the LMW-AGE level \((r = -0.31, P < 0.05)\).

Based on the above results, these 4 parameters (namely, HMW-AGEs, LMW-AGEs, plasma cathepsin B, and neutrophil cathepsin B) were included in the further analysis.
**TABLE 4** Plasma levels of low- and high-molecular-weight advanced glycation end-products as well as activities of cathepsin B in plasma and neutrophils in patients with type 2 diabetes and late diabetic vascular complications

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Diabetic patients with:</th>
<th>microangiopathy</th>
<th>macroangiopathy</th>
<th>micro- and macroangiopathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>cathepsin B in plasma, mU/l</td>
<td>18.14 ±5.56</td>
<td>17.55 ±5.48</td>
<td>16.58 ±3.19</td>
<td></td>
</tr>
<tr>
<td>HMW-AGEs, AFU × 10³</td>
<td>35.27 ±14.06</td>
<td>34.38 ±8.08</td>
<td>35.07 ±17.01</td>
<td></td>
</tr>
<tr>
<td>cathepsin B in neutrophils, mU/mgprot.</td>
<td>59.64 ±21.04</td>
<td>66.58 ±29.02</td>
<td>63.05 ±25.83</td>
<td></td>
</tr>
<tr>
<td>LMW-AGEs, AFU × 10³</td>
<td>9.67 ±3.05⁵</td>
<td>6.47 ±1.81</td>
<td>7.05 ±2.52</td>
<td></td>
</tr>
</tbody>
</table>

Data are presented as mean ± standard deviation.

a statistically significant difference between the groups with micro- and macroangiopathy

**DISCUSSION** The increased formation of AGEs under prolonged hyperglycemic conditions in diabetes and the role of AGEs in the development and progression of late diabetic complications have been well described but continue to be the subject of extensive research.¹ Recently, the participation of a number of enzymes, especially cathepsin cysteine proteases, have been indicated in various clinical disorders, including diabetes.⁴,¹³ AGEs have a wide range of chemical, cellular, and tissue effects implicated in the development and progression of late diabetic complications. They act, among others, on neutrophils and macrophages, enhanced free radical generation, as well as release of proteolytic enzymes.¹³,²² In the present study, we focused on their association with proteolytic enzymes.

To our knowledge, there have been no studies on the associations between the activity of cathepsin B and the levels of AGEs in type 2 diabetic patients. We showed significantly higher levels of all forms of AGEs in these patients compared with the control group, with the most significant differences reported for total AGEs and LMW-AGEs. The increased formation of AGEs in patients with type 2 diabetes was also reported previously.⁴,²³ Moreover, LMW-AGEs have been indicated by some authors as the best marker of diabetic disturbances, which is in line with our results. We demonstrated significant differences in the plasma levels of all forms of the examined AGEs (between diabetic patients and controls, in the groups of patients with late diabetic complications, and in the quartiles of increasing AGE levels). LMW-AGEs are considered to be a good marker of tissue AGE accumulation as well as late diabetic complications, especially nephropathy, which underlines their importance.²² HMW-AGEs are mainly associated with AGEs tendency to the formation of complexes and cross-links.²⁵

The regulation of the expression and activity of proteolytic enzymes in type 2 diabetes is altered, and the precise mechanism of these disturbances is complex. Prolonged exposure to chronically elevated glucose levels may result in glycation of enzymatic protein and its mRNA. Moreover, their excessive release from the cells has also been observed.²²,²³ It is well known that the function of neutrophils in type 2 diabetes is disturbed. The levels and activities of the majority of neutrophil enzymes, including cathepsin B, and their release to the extracellular matrix are changed.¹³ In diabetic conditions, cathepsin B may be either discharged from neutrophils in larger amounts and is probably overexpressed on their surface as membrane-bound cathepsin B. Its increased activity in diabetic neutrophils has been shown in our preliminary studies.²⁷

In the present paper, we showed significantly higher activity of cathepsin B in neutrophil extracts as well as in plasma of type 2 diabetic patients compared with healthy individuals. An increase in the activity of cysteine protease was higher in neutrophils than in plasma. This trend is in line with our previous studies,²⁸,²⁹ but the significant increase in the activity of cathepsin B reported in this paper is probably due to a larger study group. The involvement of AGEs in the development of late diabetic complications is well-known¹⁻²°; however, the role of cathepsin B in the course of type 2 diabetes remains unclear. A dual action has been indicated. On the one hand, its increase could be beneficial because of its participation in proteolytic digestion of AGEs and prevention of their accumulation. On the other hand, excessive cathepsin B activity may cause extracellular matrix degradation and tissue and vessel injury. We showed that cathepsin B activity in plasma was the highest in patients with microangiopathies, while that in neutrophils was the highest in the macroangiopathy group. However, no significant differences were observed between all the examined groups.

There is compelling evidence that reactive glucose-derived compounds (such as methylglyoxal, glyoxal, and glycoaldehyde) may inhibit cysteine proteases by modification and inactivation of the active site cysteine residue.⁷ However, Nagai et al.¹⁴ showed that the accumulation of AGEs in macrophages was present in atherosclerotic lesions. This leads to a suggestion that alterations in enzyme activities in neutrophils and endothelial cells, probably also cathepsin cysteine proteases, may contribute to the accumulation of modified protein in the tissue and may play a significant role in the development of late diabetic complications. Our results confirmed this hypothesis, providing novel findings. First of all,
we showed a significant negative correlation between the activity of cathepsin B in plasma and HMW-AGE levels as well as between its activity in neutrophils and LMW-AGE levels. Moreover, we observed a successive decrease of cathepsin B in plasma in the quartiles of increasing HMW-AGE levels as well as between its activity in neutrophils in the quartiles of increasing LMW-AGE levels. The most notable differences between protease activities were observed for cathepsin B derived from neutrophils. It indicates an interesting association between cathepsin B and AGEs and suggests its role in AGE transformation in type 2 diabetic patients.

A role of hypoglycemic agents in the modulation of protease activity has also been suggested. In a previous in-vitro study, we showed that metformin incubated with neutrophils isolated from type 2 diabetic patients and healthy people acts more effectively on the activities of cathepsin B compared with those of gliclazide. These novel findings may aid future research on diabetes.

Grimm et al. reported the involvement of cathepsin D in the removal of AGE-modified protein in vitro. The role of a number of cathepsin cysteine proteases (mainly D and L, less B) in the reduction of AGE toxicity has also been shown. Thus, a new question arises of whether the stimulation of these cathepsins can reduce AGE accumulation. Probably, it will be the basis for the development of new pharmaceutical products. We postulate that such research is also needed for cathepsin B and that it could provide new insight into its role in type 2 diabetes. Lutgens et al. indicated that serum levels of cathepsins L and S may be promising as biomarkers of atherosclerosis, whereas cathepsin B has the potential as an imaging tool.

In conclusion, we observed an increase in non-enzymatic glycation process in patients with type 2 diabetes, reflected by a significant increase of all forms of AGEs in plasma, which corresponds to the changes of cathepsin B activity both in plasma and neutrophil extracts. We revealed a significant relationship between cathepsin B activity in plasma and neutrophils and HMW-AGEs and LMW-AGEs, respectively; moreover, we showed changes in these parameters in the context of angiopathies. However, the nature of all those associations is complex and requires further research. In the light of our results and current evidence, cathepsin B may be useful in the management of numerous diseases in the future.

Acknowledgments We would like to thank Ewa Żuraw ska-Plaksej for language assistance.

REFERENCES


Zaawansowane końcowe produkty glikacji i proteaza cysteinowa katepsyna B u pacjentów z cukrzycą typu 2

Ewa Grzebyk¹, Maria Knapik-Kordecka², Agnieszka Piwowar¹

¹ Katedra i Zakład Biochemii Farmaceutycznej, Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu, Wrocław
² Katedra i Klinika Angiologii, Nacjonalnego Centrum Diabeteologii, Uniwersytet Medyczny im. Piastów Śląskich we Wrocławiu, Wrocław

STRESZCZENIE

Wprowadzenie
Występująca w cukrzycy typu 2 przewlekła hiperglykemia wywołuje wielorakie zaburzenia, które przyczynia się do rozwoju późnych powikłań cukrzycowych. Nieenzymatyczna glikacja, prowadząca do powstawania zaawansowanych produktów glikacji białek (advanced glycation end-products – AGE), jest jedną z najważniejszych konsekwencji hiperglykemii. W cukrzycy obserwuje się również zaburzenia w aktywnościach niektórych enzymów proteolitycznych.

Cele
Celem pracy była ocena zmian oraz zbadanie wzajemnej zależności między osoczowym poziomem AGE a aktywnością proteolitycznego enzymu – cysteinoową katepsyną B – w osoczu i neutrofilach chorych na cukrzycę typu 2.

Pacjenci i metody

Wyniki
U pacjentów z cukrzycą wartości wszystkich badanych parametrów były znacznie wyższe w stosunku do grupy kontrolnej. Ponadto u tych pacjentów, wykazano ujemną korelację między HMW-AGE a katepsyną B w osoczu oraz między HMW-AGE a katepsyną B pochodzącą z neutrofil. W kwartylach wzrastających wartości HMW-AGEs i LWM-AGEs zaobserwowano stopniowy spadek, odpowiednio, osoczowej i neutrofilowej aktywności katepsyny B. U chorych z różnymi późnymi powikłaniami cukrzycy istotnie różnił się tylko poziom HMW-AGE.

Wnioski
Nasze badanie wykazało istotny wzrost poziomu wszystkich postaci AGE, czemu towarzyszyły zmiany aktywności katepsyny B, zarówno w osoczu jak i w neutrofilach. Znamienna korelacja między AGE i katepsyną B u chorych na cukrzycę typu 2 oraz niejednoznaczne zmiany u pacjentów z przewlekłymi powikłaniami cukrzycy wskazuje na złożony charakter ich wpływu na przebieg cukrzycy.

SŁOWA KLUCZOWE
Cukrzycy typu 2, katepsyna B, proteazy cysteinywne, zaawansowane produkty glikacji białek