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Relationship Between Advanced Oxidation Protein Product Plasma Level and Cathepsin B Activity in Plasma and Polymorphonuclear Cells in Patients with Type 2 Diabetes Mellitus

Związek między stężeniem zaawansowanych produktów oksydacji białek w osoczu a aktywnością Katepsyny B w osoczu i granulocytach obojętnochłonnych chorych na cukrzycę typu 2

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Abstract

Background. Hyperglycemia accelerates glycation and oxidative stress (OS) in diabetic patients, resulting in increased levels of advanced glycation end products and advanced oxidation protein products (AOPPs). Changes in the activities of some leukocytic enzymes, for example cathepsin B (CB), derived from polymorphonuclear cells (PMNs) can be observed.

Objectives. The relationship between AOPP plasma level and cathepsin B activity in plasma and PMN extracts in patients with type 2 diabetes mellitus (T2DM) was investigated.

Material and Methods. Fifty-three patients with T2DM and 27 healthy subjects were examined. CB activity in plasma and PMN extracts was measured with a fluorescent substrate (CBZ-Arg-Arg-NMec) and plasma AOPP concentration using a spectrophotometric method with a potassium iodide solution in an acidic environment.

Results. AOPP concentration and CB activity were higher in the diabetic patients than in the control subjects, but significant ($p < 0.001$) differences between the groups were observed only for plasma AOPP concentration and CB activity in PMN extracts. There was also a medium ($r = 0.41$) but significant ($p < 0.05$) correlation between these parameters. In tertiles of AOPP, only plasma CB activities increased progressively, but there were no significant differences between these subgroups. In groups with normal and above-normal values of ESR, plasma AOPP concentration and CB activity in PMN extracts were significantly different ($p = 0.0072$ and $p = 0.0394$, respectively).

Conclusions. These results indicate that OS and inflammation affect CB activity, especially in PMN extracts. Identifying the contributions of individual PMN proteases in the context of existing OS is important in the search for biomarkers to monitor diabetic late complications and for therapeutic targets (*Adv Clin Exp Med* 2009, 18, 4, 345–351).

Key words: advanced oxidation protein products, cathepsin B, type 2 diabetes mellitus.

Streszczenie

Wprowadzenie. U chorych na cukrzycę hiperglikemia nasila procesy glikacji oraz stres oksydacyjny (OS), co powoduje wzrost stężenia zaawansowanych produktów glikacji białek i zaawansowanych produktów oksydacji białek (AOPP). Prowadzi również do zmian aktywności niektórych enzymów leukocytarnych, np. Katepsyny B (CB) pochodzącej z granulocytów obojętnochłonnych (GO).

Cel pracy. Zbadanie, czy istnieje związek między stężeniem AOPP w osoczu a aktywnością CB w osoczu i w ekstraktach GO u chorych na cukrzycę typu 2 (T2DM).

Materiał i metody. W osoczu i ekstraktach GO pochodzących od 53 pacjentów z T2DM i 27 osób zdrowych oznaczono aktywność CB z użyciem substratu fluorescencyjnego (CBZ-Arg-Arg-NMec). Stężenie AOPP w osoczu mierzono metodą spektrofotometryczną po reakcji z roztworem jodku potasu w środowisku kwaśnym.

Wyniki. Zarówno stężenie AOPP, jak i aktywność CB były wyższe u chorych na cukrzycę niż w grupie kontrolnej, ale różnice istotne statystycznie ($p < 0.001$) zaobserwowano tylko dla stężenia AOPP w osoczu i aktywności CB w ekstraktach GO. Stwierdzono również przeciętną ($r = 0,41$), ale istotną statystycznie ($p < 0,05$), korelację między tymi parametrami. W tertylach wzrastającego stężenia AOPP jedynie aktywność CB w osoczu wzrastała progresywnie, ale nie wykazano zmian o charakterze istotnym statystycznie między podgrupami. W grupach chorych z prawidłową oraz zwiększoną wartością OB stężenie AOPP w osoczu oraz aktywność CB w ekstraktach GO były istotnie różne (odpowiednio $p = 0,0072$ i $p = 0,0394$).

Wnioski. Wyniki badań własnych wskazują, że OS i stan zapalny mogą wpływać na aktywność CB, szczególnie w ekstraktach GO. Określenie udziału poszczególnych proteaz granulocytów obojętnochołonnych w kontekście OS jest istotne ze względu na poszukiwanie biomarkerów do monitorowania późnych powikłań cukrzycy i zastosowania odpowiedniej terapii (*Adv Clin Exp Med* 2009, 18, 4, 345–351).

Słowa kluczowe: zaawansowane produkty oksydacji białek, kathepsyna B, cukrzyca typu 2.

Oxidative stress (OS) is defined as an imbalance between the cellular generation of reactive oxygen species (ROS) and the capacity of antioxidants to prevent oxidative damage. ROS are formed in the energy/respiratory pathways of the body, as inflammatory mediators in the immune system, and in other biochemical pathways. It is well known that OS in diabetic patients impairs their defense mechanisms, enhances their susceptibility to infections, and increases oxidative damage to plasma and tissue molecules [1, 2].

Neutrophils (PMNs – polymorphonuclear cells) are considered the major effector cells involved in pathogen destruction and the modulation of inflammatory processes. Intracellular granules of PMNs contain a number of multifunctional enzymes (e.g. myeloperoxidase, elastase, cathepsins B and G, lysozyme, proteinase 3) that can be released in response to different stimulating agents. They are also able to synthesize large amounts of ROS, especially free radicals and hypochlorous acid, which are intensively produced in the respiratory burst of these cells. PMNs are constantly stimulated in diabetes, which can impair a wide range of their functions and are associated with the development of late diabetic vascular complications [3, 4].

Cathepsin B (CB) is one of the most abundant lysosomal cysteine proteases and is considered necessary for housekeeping functions and protein turnover in cells. In pathological processes (e.g. atherosclerosis, tumor invasion and metastasis, rheumatoid arthritis, inflammation), overexpression of CB, its displacement to cell membranes, and its secretion to the extracellular matrix are observed [5–7]. Cathepsin B is also present in the primary granules of PMNs, which are a special type of lysosomes. Moreover, it was suggested that CB activity can be modulated by hyperglycemia and oxidative stress. Higher plasma CB activity has been shown in patients and animals with diabetes mellitus. In contrast, inhibition of CB activity by reactive carbonyls in macrophage cell lysate and after H_2O_2 exposure in PC12 cells culture has been reported [5, 8, 9].

Because of the chronic hyperglycemia and intensified OS in diabetes, particularly type 2 diabetes mellitus (T2DM), glycation and oxidation processes lead to the formation of irreversible advanced glycation end products (AGEs) and advanced oxidation protein products (AOPPs), which might be considered a family of compounds. These modifications may affect the structure, function, and stability of different proteins. These proteins are not only long-living ones, such as collagen and crystallin, but also those with shorter half-lives, such as hemoglobin and albumin as well as enzymes and their inhibitors [8, 10, 11]. AOPPs are present only in trace amounts in plasma under physiological conditions. Increased plasma concentration of AOPPs in diabetic patients reflects the intensity of oxidative stress and can be used as a marker for monitoring diabetic complications [1, 12].

In previous investigations, the present authors observed increased activity of CB in plasma and cell extracts from patients with T2DM compared with healthy people. However, the differences were significant only for the PMN extracts [13, 14]. Witko-Sarsar et al. [15] showed that AOPPs extracted from uremic plasma and generated *in vitro* are capable of triggering PMNs' respiratory burst. On the other hand it was postulated that OS may cause imbalance of the proteolytic-antiproteolytic system [8, 9].

The present study searched for relationships between OS, reflected by plasma AOPP concentration, and cathepsin B activity in plasma and in extracts of PMNs isolated from the blood of T2DM patients.

Material and Methods

Fifty-three patients with type 2 diabetes attending the Clinic of Angiology, Hypertension, and Diabetology of Wrocław Medical University were studied. The patients were informed about the aim of the investigation and gave their permis-

Table 1. Clinical and biochemical characteristics of the diabetic patients and healthy subjects**Tabela 1.** Kliniczna i biochemiczna charakterystyka pacjentów chorych na cukrzycę oraz osób zdrowych

	Diabetic patients (Chorzy na cukrzycę) n = 53	Healthy subjects (Osoby zdrowe) n = 27
Sex: F/M (Płeć: K/M)	39/14	16/11
Age – years (Wiek – lata)	65.00 (36.00–86.00)	62.00 (38.00–82.00)
Disease duration – years (Czas trwania choroby – lata)	11.00 (0.50–35.50)	– –
Glucose – mmol/l (Glukoza – mmol/l)	10.50* (4.50–24.20)	5.20 (3.90–6.10)
Glycated hemoglobin – % (Hemoglobina glikowana – %)	9.68* (5.63–13.40)	4.81 (3.55–5.90)
Erythrocyte sedimentation rate – mm/h (Szybkość opadania krwinek czerwonych – mm/h)	39.00 ^r (4.00–89.00)	10.00 (2.00–17.00)
White cell count – $n \times 10^9/l$ (Liczba białych krwinek – $n \times 10^9/l$)	7.20 (2.50–14.90)	6.40 (4.00–8.40)
PMN count – $n \times 10^9/l$ (Liczba granulocytów obojętnochłonnych – $n \times 10^9/l$)	6.00 (2.50–12.50)	5.10 (3.15–8.20)
Cholesterol – mg/dl (Cholesterol – mg/dl)	224.16 (158.89–320.70)	209.50 (178.50–242.10)
Triglycerides – mg/dl (Triglicerydy – mg/dl)	187.30 (36.50–295.35)	126.80 (40.52–192.00)

Values are given as the median and range (min–max).

Statistical significance: * $p < 0.001$; ^r $p = 0.0244$; ^ψ $p = 0.0471$.

Wartości podano jako mediany i zakres (min–max).

Istotność statystyczna: * $p < 0,001$; ^r $p = 0,0244$; ^ψ $p = 0,0471$.

sion to enter this study. The use of human blood was approved by the Bioethics Committee of Wrocław Medical University. The control group consisted of 27 healthy subjects without acute inflammatory states or abnormalities in lipid or carbohydrates metabolism, which was confirmed by laboratory parameters. These were employees of Wrocław Medical University. The clinical and biochemical characteristics of the studied populations are shown in Table 1.

Venous blood was collected after overnight fasting into standard vacuum tubes with heparin (16 IU/ml). Neutrophils were isolated by the procedure devised by Zemmann et al. [16] with the use of gradient density centrifugation ($254 \times g$ for 35 minutes) of whole blood over Gradisol G ($d = 1.119 \text{ g/cm}^3$) in a ratio of 3 : 2, respectively. The PMN fraction was collected and washed twice in phosphate-buffered saline (PBS). The purity of the isolated fractions was $> 93\%$ (as shown by histochemical staining). The cells were suspended in an equal volume of PBS and counted with the use of a Bürker chamber. A triple freezing-thawing cycle was applied to disrupt the biological membrane continuity and release intracellular cathepsin B.

Immediately after centrifugation ($379 \times g$ for 20 minutes), the activity of cathepsin B in the granulocyte extracts was measured. Plasma was obtained by centrifugation ($314 \times g$ for 10 minutes) of the remaining blood and stored at -85°C until use (no longer than 3 months).

The activity of cathepsin B in both the PMN extracts and plasma was measured according to Barrett [17] with the use of the fluorescent substrate CBZ-Arg-Arg-NMec. The results were expressed in mU/l for plasma and mU/mg of protein (estimated by Lowry method [18]) for the PMN extracts. One milliunit (mU) of enzyme activity was defined as 1 μmol of the reaction product released per minute. AOPP concentration in plasma was measured by a spectrophotometric assay according to Witko-Sarsat et al. [15] and expressed in $\mu\text{mol/l}$ using a calibration curve made for chloramine T, which absorbs at 340 nm in the presence of potassium iodide. Plasma glucose, blood glycated hemoglobin, cholesterol, and triglycerides were determined using routine clinical assays.

The results are expressed as the median (*Me*) and range (minimum and maximum values).

Statistical evaluation was performed with the Statistica 6.0 program using the Mann-Whitney *U* and/or Kolmogorov-Smirnov tests. Spearman's correlation was used to assess the relationships between parameters. A *p* value of less than 0.05 was considered statistically significant.

Results

The plasma levels of AOPPs and the activities of cathepsin B in plasma and the PMN extracts are presented in Table 2. The AOPP concentration in the diabetic patients was about 34% higher than in the control subjects ($p = 0.0035$). The activities of cathepsin B in plasma and PMN extracts from the patients were also higher than in the control group. Only the rise of over 50% in the CB activity in the PMN extracts was significant ($p < 0.001$), while the ca. 36% higher CB activity in plasma was statistically negligible. There was medium but significant correlation between CB activity in the PMN extracts and plasma AOPP level in the diabetic patients ($r = 0.41$, $p < 0.05$).

The levels of CB activity in plasma and PMN extracts in the subgroups of diabetic patients clustered according to tertiles (T1, T2, T3) of plasma AOPP level are shown in Figure 1. Only the CB activity in plasma increased progressively with growing tertiles of AOPP levels, but the differences between the subgroups were not statistically significant.

The results of groups of patients clustered on the basis of erythrocyte sedimentation rate (ESR)

were also analyzed and are shown in Figure 2. Statistically significant differences between the groups with normal (< 20 mm/h) and above-normal (> 20 mm/h) ESRs were observed for plasma AOPP level ($p = 0.0072$) and CB activity in PMN extracts ($p = 0.0394$).

Discussion

This study revealed a connection between oxidative stress, measured as plasma AOPP concentration, and CB activity in polymorphonuclear cell extracts in T2DM patients. The significant elevation of plasma AOPP level in the diabetic patients compared with the healthy controls confirms the existence of OS [1, 15, 19, 20]. In previous investigations [13] carried out some years ago concerning CB activity in T2DM patients, the present authors observed higher enzyme activities in both plasma and PMN extracts, but only the increase in the PMN extracts was significant ($p < 0.001$). Changes in CB activity have been shown to be associated with the development of late vascular complications and with poor glycemic control [14]. However, at that time relatively little attention was given to the influence of OS on cysteine cathepsin activities [21, 22]. There are no papers in the literature about AOPPs' contribution to changes in CB activity in T2DM. The present study noted a moderate but significant correlation between CB activity in PMN extracts and plasma AOPP level ($r = 0.41$, $p < 0.05$).

In a previous study [23], however, the present

Table 2. Levels of the examined parameters in the diabetic patients and healthy subjects and the values of the correlation coefficients

Tabela 2. Stężenia badanych wskaźników u pacjentów chorych na cukrzycę w porównaniu z osobami zdrowymi, wraz z wartościami współczynników korelacji

Parameters (Wskaźniki)	Diabetic patients (Chorzy na cukrzycę) n = 53	Healthy subjects (Osoby zdrowe) n = 27	Correlation coefficient ¹ <i>r</i> (Współczynnik korelacji <i>r</i>)
AOPP (plasma) – μmol/l (AOPP (osocze) – μmol/l)	135.64 * (61.50–279.70)	101.05 (55.90–172.01)	–
CB (plasma) – mU/l (CB (osocze) – mU/l)	8.95 (2.27–39.10)	6.97 (1.40–14.65)	0.17
CB (PMN extracts) – mU/mg of protein (CB w ekstraktach GO – mU/mg białka)	53.98 ** (38.67–155.50)	26.86 (11.75–88.70)	0.41 [#]

Values are given as the median and range (min–max).

Statistical significance: * $p = 0.0035$; ** $p < 0.001$.

¹ Correlation coefficients between AOPP concentration and CB activity in plasma and PMN extracts of patients (statistical significance: [#] $p < 0.05$).

Wartości podano jako mediany i zakres (min.–maks.).

Istotność statystyczna: * $p = 0,0035$; ** $p < 0,001$.

¹ Współczynnik korelacji między stężeniem AOPP a aktywnością CB w osoczu oraz ekstraktach GO pacjentów (istotność statystyczna: [#] $p < 0,05$).

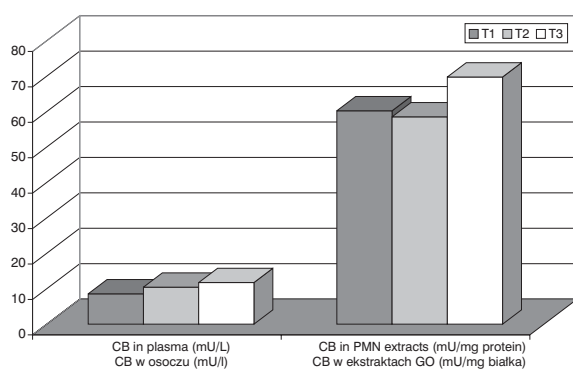


Fig. 1. CB activities in plasma and PMN extracts in tertiles of increasing concentration of plasma AOPPs

Ryc. 1. Aktywność CB w osoczu i ekstraktach GO w tertylach wzrastającego stężenia AOPP w osoczu

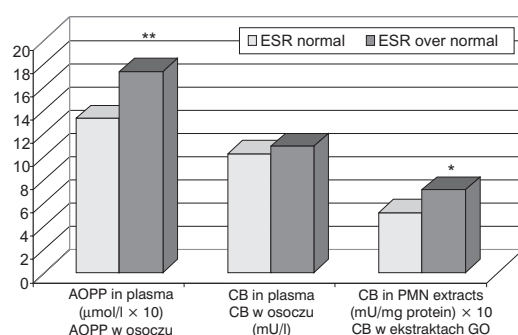


Fig. 2. Plasma concentration of AOPPs and CB activities in plasma and in PMN extracts in groups of diabetic patients with normal and above-normal erythrocyte sedimentation rate (statistical significance:

* $p = 0.0394$; ** $p = 0.0072$)

Ryc. 2. Stężenie AOPP w osoczu oraz aktywność CB w osoczu i ekstraktach GO w grupach chorych na cukrzycę z prawidłową i zwiększoną wartością OB (istotność statystyczna różnic: * $p = 0,0394$; ** $p = 0,0072$)

authors did not observe significant correlation between CB activity in plasma and PMN extracts, although there was such a dependence for other hydrolases, for example leukocyte elastase. The lack of association between neutrophil and plasma CB activity suggests that multiple systems might be involved in the inflammatory reaction during the development of late diabetic vascular complications. Plasma CB activity may be not only of leukocytic origin, but may also come from the lysosomes of other cells. Converging evidence shows an association between OS and lysosomal activation [9, 24, 25]. Moreover, one cannot rule out disturbances in neutrophil degranulation or resistance to enzyme release. It is known that CB is complexed by endogenous inhibitors in plasma, such as cystatins, stefins, kininogen, and α_2 -macroglobulin, which partially neutralize extracel-

lular proteolytic enzymes and are known to be elevated in diabetic patients [5, 26, 27].

Although cysteine cathepsins, including CB, are sensitive to oxidation, proteolytically active forms are found at inflammatory sites during *in vitro* experiments. It is also believed that subclinical inflammation is a part of T2DM [28]. During inflammation, oxidant-antioxidant and protease-antiprotease systems are altered and participate together in remodeling extracellular matrix (ECM) compounds. However, relatively little attention has been given to the role of oxidants in the enzymatic activities controlling cathepsins, for example CB. It has been demonstrated in an *in vitro* study that protein peroxides may contribute to the inactivation of cysteine proteases within the cell through the reaction with thiol groups, which results in the formation of sulfenic acid intermediates [29, 30]. There are also powerful arguments that the reactive glucose-derived compounds methylglyoxal, glyoxal, and glycoaldehyde may inactivate some cellular enzymes via the adduction of reactive carbonyls to Cys residues [8]. On the other hand, it was shown that catalase may directly participate in the prevention of extracellular cathepsin activities against peroxidation in a cell system [31].

Oxidative stress and microinflammation are typical of diabetes mellitus. In this case the elevation of different markers (e.g. CRP, cytokins, AGEs, malonyldialdehyde) was observed. Plasma AOPP concentrations, as markers of oxidative stress, show close relationships with inflammation and acute-phase products [10]. The present study did not measure any marker of the acute-phase reaction, such as CRP. Therefore the results were analyzed according to ESR. In the group with higher (> 20 mm/h) ESR, higher AOPP plasma concentration and CB activity in PMN extracts were observed. This is not surprising as it was recently reported that AOPPs react with RAGE, a central player in the inflammatory response [32].

In conclusion, it can be said that not only hyperglycemia, but also OS and inflammation have profound effects on the internal environment of leukocytes, which may disturb the CB-cysteine proteinase inhibitor balance which plays a significant role in the development of late vascular complications. The attachment of the enzymes (CB, LE) to plasma membrane allows them to remain active in an environment replete with inhibitors and may therefore be responsible for neutrophil- or neutrophil-debris-mediated tissue damage [33, 34]. The identification of the exact role of CB in this processes and studying the participation of different forms of CB (free, membrane-bound, and complexed with inhibitors) would be of special interest.

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