Markers of oxidative protein damage in plasma and urine of type 2 diabetic patients

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Accepted: 25 August 2009

ABSTRACT

This study aims to measure selected markers of oxidative protein damage (OPD) in patients with type 2 diabetes mellitus (T2DM) in order to estimate their utility as indicators of oxidative stress (OS) and to search for possible associations between them. The concentrations of advanced oxidation protein products (AOPP) and total sulphydryl (TSH) and reactive carbonyl (RCO) groups are measured in the plasma (P) and urine (U) of 60 patients and 22 controls using spectrophotometric methods. Significantly higher plasma concentrations of AOPP ($P<0.001$), RCO groups ($P<0.01$) and their P/U indexes ($P<0.001$) as well as urinary levels of the RCO and TSH groups ($P<0.001$) were observed in the diabetic patients compared with the controls. In contrast, the plasma levels and P/U index of the TSH groups were significantly lower ($P<0.001$). A progressive increase in AOPP (plasma, urine and P/U index) in the course of albuminuria was noted, but significant differences among the subgroups of patients (with normoalbuminuria, microalbuminuria and macroalbuminuria) were found only in plasma. Plasma levels of all the measured parameters of OPD showed significant changes in T2DM patients compared with the control group. The largest increase was observed for AOPP. As the urinary AOPP concentration was not significantly different to that of the controls, it cannot be recommended as a marker of oxidative stress for monitoring the development of diabetic nephropathy. The P/U indexes did not provide any more information than the plasma concentrations of the studied markers.

KEY WORDS: Diabetes mellitus, type 2.
Nephropathy.
Oxidative damage.
Plasma.
Urine.

Introduction

The link between hyperglycaemia and oxidative stress (OS) in diabetic patients, especially those with type 2 diabetes mellitus (T2DM), and its participation in biochemical and clinical disturbances have been studied intensively.[1–4] Oxidative stress is a key player in the development of diabetic complications, but according to some authors there appears to be no direct link between acute hyperglycaemia and glycooxidative stress.[5] Proteins, lipids and nucleic acids are targets for OS, which leads to functional and structural modifications of these macromolecules. Intracellular disturbance, cell membrane dysfunction and damage to vascular endothelium and other tissues, among other things, are caused by the accumulation of these altered macromolecules in biological systems.[4,6,7] Intensification of these processes has deleterious effects on the function of the kidneys, retina, nerves, vascular tissues and, to a lesser extent, pancreatic islets in T2DM.[1,2,8]

Several techniques are available for OS assessment, but most are not applicable in a routine medical laboratory due to the complex methodology and/or financial constraints. Direct measurement of OS parameters, such as free radicals (e.g., hydrogen peroxide), is difficult because of their reactivity and short lifespans. The indirect parameters used include the ferric-reducing antioxidant power (FRAP) of serum, total antioxidant status (TAO) and the total radical-trapping antioxidant parameter (TRAP). Therefore, the measurement of markers of oxidative injury of different macromolecules, such as sulphydryl (SH) and carbonyl (CO) groups, and advanced oxidation protein products (AOPP) for proteins, malondialdehyde (MDA) and thiobarbituric acid reactive substances (TBARS) for lipids, and 8-hydroxydeoxyguanosin (8-OHdG) for nucleic acid, particularly in plasma, is widely applied.[9–11]

The present study is concerned with oxidative protein damage (OPD) in the plasma and urine of patients with T2DM. The data on OPD markers in the plasma of these patients, and their utility as diagnostic markers, are considerable,[6,12–14] but there are none on urine, although this is a more easily accessible biological material than plasma or serum. Generally, urinary metabolites act as good indicators of oxidation products and the intensity of oxidative stress, and are excreted over a broad timespan.[15]

It is well known that reducing OS in diabetic patients can prevent late vascular complications (e.g., diabetic nephropathy [DN]).[1] Thus, searching for biomarkers of OS that are helpful in monitoring its development, as well as in monitoring antioxidative treatment, is very important.

The aim of the present study is to measure selected parameters of OPD (i.e., AOPP, TSH and RCO groups) simultaneously in plasma and urine from patients with T2DM and to search for associations between them. Additionally, AOPP will be analysed in subgroups of patients with different degrees of albuminuria.

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Materials and methods

The study included 60 patients with T2DM with a mean disease duration of 12.8±8.6 years (range: 2–40 years) treated at the Clinic of Angiology, Hypertension and Diabetology, Wroclaw Medical University. Twenty patients were treated with oral hypoglycaemic agents, 19 with insulin alone, and 21 with a combination of insulin and oral hypoglycaemic agents. All the patients were in a stable clinical condition and without symptoms of acute infection. The control group consisted of 22 healthy adults who showed no evidence of inflammation, abnormalities in lipid or carbohydrate metabolism, or kidney disorders during routine medical examination. All participants were informed about the aim of the study and gave their informed consent. The clinical and biochemical characteristics of the studied populations are shown Table 1.

Venous blood samples were drawn after overnight fasting (minimum of 12 h) into tubes containing heparin (250 units/mL). Glycated haemoglobin (HbA1c) was determined in haemolysates of blood using a routine latex-enhanced turbidimetric immunoassay and measured at 550 nm on a cobas chemistry system (Roche). The remaining parameters were measured in plasma obtained by centrifuging whole blood.

First morning samples of urine (midstream) were taken from the same patients. Plasma and urine samples were frozen and stored at –86°C (no longer than two months). The urine samples were centrifuged (1500 rpm for 10 min) immediately before analysis. Use of human blood and urine for research was approved by the Bioethics Committee of Wroclaw Medical University.

The levels of the markers of OPD were determined in the samples of plasma and urine derived from the same individuals. The concentration of AOPP was measured by a spectrophotometric assay according to the method of Witko-Sarsat *et al*.[16] This method is based on the colour reaction of AOPP with a potassium iodide solution in an acidic environment. Briefly, 1 mL of biological material, diluted 10 times, was mixed with 50 μL potassium iodide (1.16 mol/L) and 100 μL 10% (v/v) acetic acids. Absorbance was measured immediately at 340 nm against blank samples,

Table 1. Clinical and biochemical characteristic of the healthy controls and the patients with type 2 diabetes mellitus.

Parameters/groups	Control group	Type 2 diabetes	Significance
Number (*n*)	22	60	
Gender (M/F)	7/15	12/48	
Age (years)	55.14±10.28 54.50 (35.00–76.00)	61.16±11.40 58.50 (33.00–77.00)	*P*=0.0508
Systolic blood pressure (mmHg)	125.18±8.60 128.00 (105.00–135.00)	136.13±18.32 140.00 (100.00–135.00)	*P*=0.0118
Diastolic blood pressure (mmHg)	75.86±5.52 77.50 (65.00–85.00)	85.64±21.12 81.00 (50.00–140.00)	*P*=0.0112
BMI (kg/m^2)	23.53±1.94 23.90 (19.00–26.70)	28.42±6.83 26.85 (20.00–49.00)	*P*=0.0015
FPG (mmol/L)	4.99±10.32 4.86 (4.27–5.83)	8.30±1.67 8.24 (4.77–12.27)	*P*<0.001
HbA1c (%)	5.14±0.78 5.20 (3.70–6.10)	8.67±1.76 8.45 (5.90–13.20)	*P*<0.001
Total cholesterol (mmol/L)	4.37±0.45 4.20 (3.91–5.31)	5.16±1.29 5.10 (3.29–9.07)	*P*=0.0201
HDL-cholesterol (mmol/L)	1.50±0.34 1.45 (0.96–2.07)	0.58±0.29 1.29 (0.78–2.00)	*P*=0.0678
LDL-cholesterol (mmol/L)	2.74±0.60 2.76 (1.35–3.50)	2.97±1.00 2.70 (1.65–6.00)	*P*=0.7374
Triglycerydes (mmol/L)	1.57±0.42 1.44 (0.62–2.26)	2.07±0.87 1.88 (0.66–4.63)	*P*=0.0244
Plasma creatinine (μmol/L)	91.13±0.20 87.96 (62.76–123.76)	115.38±33.58 110.50 (61.00–175.03)	*P*=0.0032

Results expressed as mean±SD, with the median and range in parentheses.

and the results were expressed in chloramine T units (a calibration curve was made for chloramine T concentrations of up to 100 μmol/L).

The level of total thiol groups (TSH) was measured using 5-5′-dithio-bis(2-nitrobenzoic acid) (DTNB) using Ellman's method.[17] Briefly, 100 μL plasma (diluted 10 times) or urine was mixed with 100 μL 10% sodium phosphate buffer and 800 μL iodide-phosphate buffer (10 mmol/L, pH 8.0). Absorbance was read at 412 nm against blank samples 1 h after adding 100 μL DTNB solution (50 nmol/L). The concentration of TSH was calculated using reduced glutathione for calibration (range: 0.1–1.0 mmol/L).

Reactive carbonyl groups were evaluated using 2,4-dinitrophenylhydrazine (DNPH) according to the method of Dalle-Donne *et al*.[18] for plasma, and the method of Cvetković *et al*.[19] for urine. Briefly, to 1 mL plasma, diluted 40 times, was added the same volume of trichloroacetic acid (20%). This was centrifuged and decanted and 1 mL 10 mmol/L DNPH solution in 2 mol/L HCl was added and incubated at room temperature in the dark for 1 h (vortex-mixed every 10 min). It was then precipitated, centrifuged, washed (x3) with 2 mL portions of ethanol/ethyl acetate mixture, then resuspended in guanidine hydrochloride (6 mol/L) and incubated at 37°C for 15 min (vortex-mixed).

After centrifugation, the absorbance was read at 365 nm against blank samples. A sample of urine (0.5 mL) precipitated by an equivalent volume of sulphosalicylic acid (20%) was also centrifuged and decanted, and DNPH solution was added. The samples were incubated at 37°C in the dark for 1 h (vortex-mixed every 10 min). The samples were then centrifuged and 2 mL NaOH solution (2 mol/L) was added. This was mixed and the absorbance was read against blank samples.

The plasma concentrations of creatinine, glucose, total cholesterol and its fractions (high-density lipoprotein-[HDL] and low-density lipoprotein [LDL]-cholesterol) and triglycerides were determined using routine clinical assays.

The concentration of albumin in urine was determined according to Shosinsky *et al*.,[20] based on the dye-binding properties of albumin with bromophenol blue. The absorbance of 100 μL urine mixed with 3 mL colour reagent (0.188 mmol/L) in glycine buffer (230 mmol/L, pH 3.0) was measured against blank reagent at 610 nm after 30 sec. The results were calculated using a standard human albumin solution and expressed in g/L. This permitted calculation of the albumin:creatinine ratio (mg/g) and division of the patients into three groups according to its value (i.e., those with normoalbuminuria [ratio <30, n=15], microalbuminuria [ratio: 30–300, n=28] and macroalbuminuria [ratio: >300, n=19]).

Furthermore, the plasma/urine (P/U) indexes of the examined markers were calculated by dividing the value in plasma by the respective value in urine of the same patients. The results of all the parameters in the urine samples were expressed in the respective concentration units per gram creatinine.

Table 2. Levels of plasma (P) and urine (U) markers of oxidative protein damage, and the plasma-to-urine index (P/U-index) in the control group and the patients with type 2 diabetes mellitus (all).

Parameters/groups	Control group	Type 2 diabetes mellitus	Statistical significance
P-AOPP (μmol/L)	99.85±38.12 89.50 (51.48–215.98)	211.83±84.51 184.51 (102.82–390.20)	P<0.001
U-AOPP (μmol/g creatinine)	131.12±46.57 128.40 (64.80–210.26)	114.39±56.42 94.26 (36.74–245.65)	P=0.1152
P/U-index of AOPP	0.98±0.38 1.13 (0.36–1.45)	3.62±1.89 3.32 (1.21–7.62)	P<0.001
P-RCO groups (nmol/L)	59.91±13.80 63.63 (34.59–79.99)	77.60±19.32 74.01 (52.81–120.12)	P=0.0017
U-RCO groups (μmol/g creatinine)	83.06±12.82 89.34 (58.20–97.48)	138.07±44.92 125.92 (79.21–254.76)	P<0.001
P/U-index of RCO	0.62±0.09 0.62 (0.50–0.82)	0.79±0.24 0.72 (0.43–1.42)	P=0.0025
P-TSH groups (μmol/L)	601.73±82.72 578.94 (490.00–769.25)	482.76±95.89 497.19 (310.24–693.44)	P<0.001
U-TSH groups (μmol/g creatinine)	43.56±12.31 41.67 (31.29–61.84)	131.43±69.82 112.09 (44.51–305.20)	P<0.001
P/U-index of TSH	13.49±3.74 14.70 (8.20–18.52)	7.97±2.44 7.61 (3.83–12.99)	P<0.001

Results expressed as mean±SD, with median and range of results in parentheses.

Table 3. Values of AOPP in plasma (P), urine (U) and the plasma-to-urine index (P/U index) in the subgroups of type 2 diabetes patients with different stages of kidney disease (normoalbuminuria, microalbuminuria and macroalbuminuria).

Parameters/subgroups	Normoalbuminuria	Microalbuminuria	Macroalbuminuria
P-AOPP (μmol/L)	113.27±36.39 118.17 (68.24–171.13)	174.16±39.71 157.39[*] (125.00–255.63)	244.74±48.44 235.69[†#] (192.78–308.45)
U-AOPP (μmol/g creatinine)	80.02±38.72 69.99 (26.74–149.69)	111.20±48.74 94.26 (42.27–188.73)	131.05±25.83 125.54[‡] (87.56–165.65)
P/U-index of AOPP	2.67±1.09 2.36 (1.22–4.39)	3.43±1.34 3.63 (1.21–5.04)	4.49±1.17 4.11[§] (3.32–6.72)

Results expressed as mean±SD, with the median and range of results in parentheses.

Comparisons between the microalbuminuria and normoalbuminuria groups ([*]P=0.0013), macroalbuminuria and normoalbuminuria groups ([†]P<0.001, [‡]P=0.0017, [§]P=0.0024) and macroalbuminuria and microalbuminuria groups ([#]P=0.0148).

The main parameters of the groups under investigation are shown in the Tables as mean value (+SD) as well as the median and minimum and maximum values. Statistical analysis was performed with Statistica PL for Windows (version 8.0). The normal distribution of all parameters was checked by the Shapiro-Wilk test. Comparisons of the groups were made using the non-parametric Mann-Whitney U test and differences among subgroups were evaluated using the Kruskal-Wallis non-parametric analysis of variance (ANOVA) test. P<0.05 was considered statistically significant.

Results

The concentrations of AOPP and the TSH and RCO groups in the plasma and urine of the healthy controls and the T2DM patients are shown in Table 2. The calculated values of the P/U indexes of these parameters are also given. The concentration of AOPP in plasma and the value of its P/U index were significantly higher (P<0.001) in the diabetic patients than in the control group. However, there was no significant difference between these two groups in AOPP level in urine (expressed in μmol/gram creatinine). The concentrations of the RCO groups in plasma and the values of its P/U index were higher in the diabetic patients than in the control group, but this increase was weaker (about 30% and 28%, respectively, P<0.01). Unlike AOPP, the urinary concentrations of the RCO groups were also significantly higher (P<0.001) in the diabetics than in the healthy individuals.

In contrast to the above parameters, the plasma levels of the TSH groups (and their P/U index values) were significantly lower (each P<0.001) in the patients with T2DM than in the healthy group, by almost 20% and 41%, respectively. The concentration of the TSH groups in urine was over three times higher in the diabetics than in the control group (P<0.001).

The levels of AOPP in plasma and urine (as well as the plasma/urine index values) in the patients grouped according to the degree of albuminuria are shown in Table 3. Progressive increases in AOPP were observed in plasma, urine and P/U index, together with the increase in albuminuria (Fig. 1). The highest levels of AOPP (i.e., those recorded in the macroalbuminuria group) were significantly different from those of patients with normoalbuminuria (P<0.001 [plasma], P<0.01 [urine and P/U index]). Only plasma levels of AOPP showed statistically significant differences in patients with macroalbuminuria versus microalbuminuria (P<0.05) and microalbuminuria versus normolabuminuria (P<0.01).

Discussion

Over the past years there has been substantial interest in oxidative stress and its potential role in diabetogenesis, the development of diabetic complications, atherosclerosis and associated cardiovascular disease, but effective knowledge remains limited. Microvascular and macrovascular angiopathy are currently the principal causes of morbidity and mortality in patients with diabetes, and nephropathy is one of the most frequent disturbances.[2,4,9]

Reactive oxygen species (ROS) react with all types of biological substrates, but protein is possibly the most immediate vehicle for inflicting oxidative damage. It is well established that the exposure of proteins to ROS can alter the physical and chemical structure of the target, causing consequent oxidation of side-chain groups, protein scission, backbone fragmentation, cross-linking, unfolding and the formation of new reactive groups. The last includes the oxidation of hydrophobic amino acyl residues to hydroxy and hydroperoxy derivatives, protein carbonylation, oxidation of thiol groups, dityrosine formation, and many others. Some of these are applied as markers of oxidative stress intensity and the degree of oxidative protein damage.[16,21–23]

Many studies have concentrated on biomarkers of oxidative stress such as thiol and carbonyl groups as well as AOPP, especially in plasma, in different disorders, including diabetes.[13,14,21,22,24,25] In contrast, only a few refer to its urinary levels,[19,26,27] and none have considered simultaneous measurement in the plasma and urine of T2DM patients.

In the present study, the levels of AOPP, RCO and TSH groups were measured simultaneously in the same experiment for the first time in the plasma and urine of patients with T2DM. The results obtained for the studied

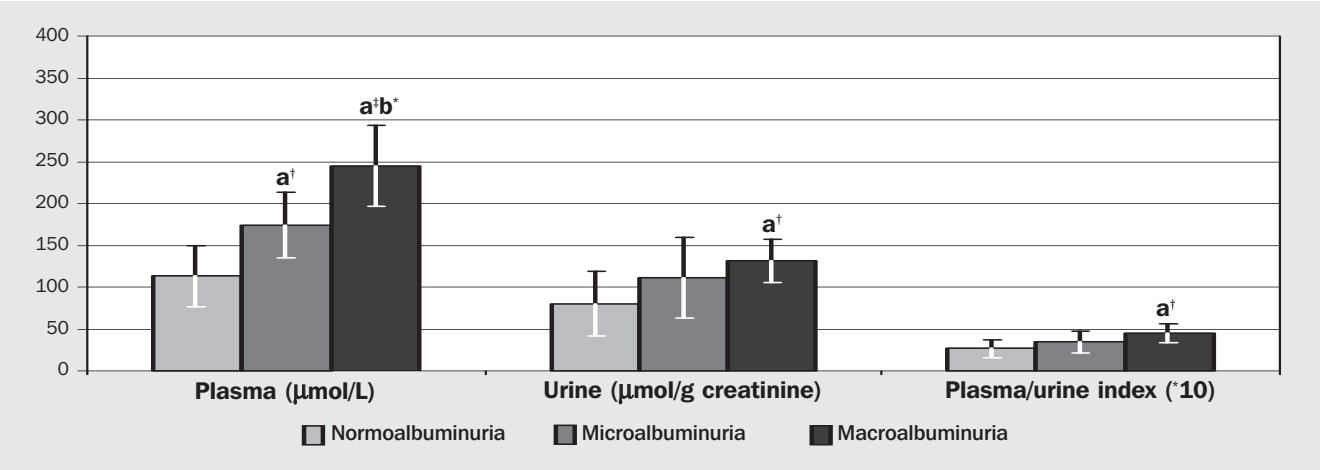

Fig. 1. Concentrations of AOPP in the plasma and urine, and plasma-to-urine index values in the subgroups of patients with type 2 diabetes with normoalbuminuria, microalbuminuria and macroalbuminuria compared with the normoalbuminuria group (a) and the microalbuminuria group (b). $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$. Results expressed as mean±SD

plasma OPD biomarkers are in accordance with previous observations that showed significantly higher levels of AOPP and carbonyl groups and lower thiol group levels in diabetics compared with healthy conrols.[13,25,28,29] The significant relationships observed between AOPP and the TSH and RCO groups in the plasma of diabetic patients were similar to those found previously,[29] but there were no significant relationships between these parameters in urine (data not shown). The increased plasma levels of AOPP in the patients may be partly explained by increased formation or diminished renal clearance. It has been shown *in vitro* that the liver and spleen are the main route of plasma clearance of oxidised albumin, and the kidney participates in these processes to a limited degree (9%).[30]

The results obtained for RCO and TSH groups are in accord with those of Cvetković *et al*.[19,31] who proposed measuring these parameters in urine as markers of oxidative kidney damage in type 1 and 2 diabetic nephropathy. The present study did not confirm significant differences between diabetic patients and controls in the urinary levels of AOPP. To date, only Chandramathi *et al*.[10] have recognised AOPP in urine as a useful non-invasive oxidative stress biomarker in patients with breast cancer, but they studied patients with normal kidney function as measured by the blood urea:creatinine ratio, whereas the present work studied patients with diabetic nephropathy.

In the second part of the study, AOPP levels were analysed in the course of nephropathy development. The diabetic patients were divided into groups according to the urinary albumin:creatinine ratio, a parameter frequently used in laboratory examinations.[32] Progressive increases in AOPP levels were found in both plasma and urine, as well as in the P/U index, together with increasing albuminuria. The most significant differences among these groups were in the plasma concentration of AOPP. Previous work showed that plasma levels of AOPP correlated with plasma creatinine concentration and rose progressively with the degree of albuminuria, This suggested that measurement should be applied to the monitoring of diabetic nephropathy.[33]

The mechanisms underlying the development of diabetic kidney disease are extremely complex and remain to be fully understood. Increased OS, related to the severity of microalbuminuria, has been shown in T2DM patients with nephropathy.[34] In an animal model, chronic accumulation of AOPP, which may promote an inflammatory response in the diabetic kidney, probably through the activation of renal NADPH oxidase, was observed. This was associated with structural and functional abnormalities of the kidney (e.g., glomerular hypertrophy, fibronectin accumulation and albuminuria). It was postulated recently that localised tissue OS is a key component in the development of diabetic nephropathy.[24,35,36]

In conclusion, all measured parameters of OPD were altered in the plasma of T2DM patients compared with controls, which is in accordance with previous work. The results indicate that the determination of urinary AOPP cannot be recommended as a biomarker of diabetic nephropathy. This is in contrast to the situation with plasma AOPP, which may also be useful in identifying and following up patients who need antioxidant therapy. □

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