Protein oxidation parameters in type 2 diabetic patients with good and poor glycaemic control

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SUMMARY
Aim: In order to examine the influence of oxidative stress on protein oxidation, type 2 diabetic patients without clinical evidence of complications, either in good or poor glycaemic control, were studied.

Methods: Plasma protein carbonyl (PCO), total thiol (T-SH), and advanced oxidation protein products (AOPP) levels as markers of protein oxidation, and lipid hydroperoxide (LHP) levels as markers of lipid peroxidation were determined. Glycated haemoglobin (HbA1c) levels were used as an index of glycaemic control. The subjects were divided into two groups according to their HbA1c level at inclusion as follows: good HbA1c ≤ 7%, and poor HbA1c > 7%.

Results: Plasma PCO and AOPP levels of diabetic patients with poor glycaemic control were increased significantly compared with those of the diabetic patients with good glycaemic control. The decreased plasma T-SH level in the diabetic patients with poor glycaemic control was not statistically significant. On the other hand, plasma LHP levels were increased significantly in the diabetic patients with poor GC compared with those of the diabetic patients with good glycaemic control.

Conclusion: This study supports the hypothesis that poor glycaemic control is an important factor in generation of increased protein oxidation in type 2 diabetic patients clinically free of complications. Increase in plasma PCO, AOPP, and LHP levels in the diabetic patients with poor glycaemic control may contribute to the development of diabetic complications.

Key-words: Type 2 diabetes · Protein oxidation · Lipid peroxidation · Glycaemic control · Carbonyl groups.

RÉSUMÉ

Méthodes : Le dosage de marqueurs de l’oxydation des protéines (protéine carbonyle plasmatique [PCO], thiols totaux [T-SH] et produits de l’oxydation avancée des protéines [AOPP]) et de marqueurs de la peroxydation lipidique (hydroperoxydes lipidiques [LHP]) a été réalisé. L’équilibre glycémique a été évalué par le taux d’HbA1c, en isolant 2 groupes de patients, équilibre glycémique correct (HbA1c ≤ 7 %) et insuffisant (HbA1c > 7 %).

Résultats : Les concentrations de PCO, d’AOPP et de LHP, mais non celles des T-SH totaux plasmatiques des diabétiques de type 2 mal équilibrés étaient significativement plus basses que celles des diabétiques bien équilibrés.

Conclusion : Ces résultats sont en faveur de l’hypothèse que l’hyperglycémie chronique induit une augmentation de l’oxydation des protéines chez les diabétiques de type 2 indemnes de complications cliniques. Cette augmentation serait susceptible de contribuer au développement des complications vasculaires liées au diabète.

Mots-clés : Diabète de type 2 · Oxydation des protéines · Peroxydation lipidique · Contrôle glycémique · Groupes carbonyls.
Introduction

Considerable evidence indicates that the maintenance of protein redox status is of fundamental importance for cell function, therefore structural changes in proteins are considered to be among the molecular mechanisms leading to diabetic complications [1]. Alterations in protein conformations can lead to increased aggregation, fragmentation, distortion of secondary and tertiary structure, susceptibility to proteolysis, and decrease of normal function [2,3].

Many different types of protein oxidative modification can be induced directly by reactive oxygen species (ROS) or indirectly by reactions of secondary by-products of oxidative stress. Protein modifications elicited by direct oxidative attack on Lys, Arg, Pro or Thr, by secondary reaction of Cys, His or Lys residues with reactive carbonyl compounds can lead to the formation of protein carbonyl (PCO) derivatives. PCO content is the most general and well-used biomarker of severe oxidative protein damage [4-6]. Several reports have been made on glycation-induced structural and functional modification of haemoglobin [7-9]. Compared to nonglycated haemoglobin, haemoglobin A1c (HbA1c) is more rapidly autooxidized [10]. In poorly controlled diabetes mellitus, glucose oxidation through the pentose phosphate pathway leads to the excessive formation of NADPH, which in turn can promote lipid peroxidation in the presence of the cytochrome P-450 system. Alternatively, inactivation or inhibition of antioxidant enzymes by glycosylation in poorly controlled diabetes mellitus may give rise to increased lipid peroxidation [11]. Lipid hydroperoxides (LHPs) are a large family of the first by-products of oxidized lipids, and their quantification could become a useful biomarker [12]. Dalle-Donne et al. [13] have reported that protein carbonyl PCO groups may be introduced proteins by secondary reaction of the nucleophilic side chains of Cys, His or Lys residues with reactive carbonyl compounds can lead to increased aggregation, fragmentation, distortion of secondary and tertiary structure, susceptibility to proteolysis, and decrease of normal function [2,3].

Albumin is the most abundant plasma protein and is a powerful extracellular antioxidant [14,15]. The measurement of plasma total thiol (T-SH) is a good reflection of excess free radical generation, since the conformation of albumin is altered, allowing thiol (-SH) groups to be oxidized [14–16]. Recently, a new marker of protein oxidation, advanced protein oxidation products (AOPP), has begun to attract the attention of various investigators [17-19]. AOPP are defined as dityrosine-containing cross-linked protein products and are considered as reliable markers to estimate the degree of protein oxidation [20]. Biochemical characterization of AOPP in plasma revealed that both the high- and low-molecular-weight AOPP peaks contain oxidized albumin in aggregate-forming or monomeric form [21].

The aim of this study was to reveal PCO, T-SH, and AOPP as markers of protein oxidation, as well as LHP levels as a marker of lipid peroxidation, and relation of HbA1c levels with these markers in plasma of type 2 diabetic patients in good and poor glycaemic control.

Materials and methods

Subjects

Patients with type 2 diabetes according to the criteria of the Expert Committee were entered the study [22]. The study included 40 consecutive type 2 diabetic patients attending the Istanbul University, İstanbul Faculty of Medicine, Central Laboratory of Biochemistry over a one-year period. Type 2 diabetic patients were free from any clinical evidence of retinopathy, nephropathy, or neuropathy. Demographic (gender, age) and anthropometric data (weight, height), medical history (duration of disease), and type of treatment were recorded. Patients with coronary heart disease, or hypertension were excluded from the study. The sample groups consisted of 23 diabetic patients with good glycaemic control (GC) (HbA1c ≤ 7%), and 17 diabetic patients with poor GC (HbA1c > 7%). All subjects were non-smokers. None of the patients was known to suffer from any acute illness or chronic inflammatory condition at the time of study. Type 2 diabetic patients were not taking any medication that might have adverse effects on the tests performed, and they were advised for diabetes diet. A total of 7 subjects were treated with insulin therapy (after failure of oral hypoglycaemic agents), 22 subjects with oral hypoglycaemic agents (sulfonylureas or sulfonylureas plus biguanides), 5 with the combination of insulin and oral hypoglycaemic agents, and 6 with diet alone.

These patients continued their regular clinical and laboratory visits, including HbA1c measurement, every 3 months. Blood samples were drawn during periodic routine control analyses. All samples were taken in the morning to avoid the confounding effect of diurnal variation of oxidative stress parameters as reported previously [23]. Venous blood samples were drawn in the fasting state and processed within 1 h of collection [24]. Blood samples were collected in tubes containing lithium heparin, EDTA or no additive depending on the analysis. For protein oxidation parameters, plasma samples containing lithium heparin were stored at −70 °C until analysis, all other parameters were determined on the day of collection. The ethical procedures were performed according to the World Medical Association Declaration of Helsinki [25].

Analytical methods

Apparatus. PCO, T-SH, AOPP, and LHP levels were measured by a spectrophotometer (Ultraspex 4050 LKB). Serum glucose, albumin, uric acid, total bilirubin, conjugated bilirubin, unconjugated bilirubin, unsaturated iron binding
capacity, total cholesterol, high density lipoprotein (HDL)-cholesterol, low density lipoprotein (LDL)-cholesterol, very low density lipoprotein (VLDL)-cholesterol, and whole blood HbA1c levels were determined on the Modular Analytics DPP discrete autoanalyzer (Roche Diagnostics, Switzerland). VLDL-cholesterol concentration was calculated using the Modular analyser software and the following equation: $\text{VLDL-cholesterol} = \text{Total cholesterol} - (\text{HDL}-\text{cholesterol} + \text{LDL}-\text{cholesterol})$. HDL-cholesterol concentration was determined enzymatically by cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups.

Assay of plasma protein carbonyl levels

Plasma PCO levels were measured spectrophotometrically by using the method of Reznick et al. [26]. PCO groups react with 2, 4-dinitrophenylhydrazine (DNPH) to generate chromophoric dinitrophenylhydrazones. DNPH was dissolved in HCl, and after the DNPH reaction, proteins were precipitated with an equal volume of 20% (w/v) trichloroacetic acid and washed three times with 4 ml of an ethanol/ethyl acetate mixture (1:1). Washings were achieved by mechanical disruption of pellets in the washing solution using a small spatula, and re-pelleting by centrifugation at 6000 g for 5 min. Finally, the precipitates were dissolved in 6 M guanidine-HCl solution and the absorbances were measured at 360 nm, using the molar extinction coefficient of DNPH, $e = 2.2 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$. Protein contents were determined on the HCl blank pellets spectrophotometrically using a Folin kit (Sigma Diagnostics, St.Louis, MO, USA). The coefficients of intra- and inter-assay variations for carbonyl assay were 5.2% (n = 12) and 9.3% (n = 10), respectively.

Assay of plasma total thiol levels

Plasma T-SH concentration was determined by using 5, 5-dithio-bis(2-nitrobenzoic acid) (DTNB) as described by Hu [16]. The coefficients of intra- and inter-assay variations were 1.8% (n = 10), and 4.6% (n = 9), respectively.

Assay of plasma advanced protein oxidation products

Spectrophotometric determination of AOPP levels was performed by modification of Witko-Sarsat’s method [21]. Samples were prepared in the following way: 200 ml of plasma was diluted 1:3 in PBS, 100 ml of 1.16 M potassium iodide was then added to each tube, two minutes later followed by 200 ml acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm against a blank containing 1 200 ml of PBS, 100 ml of KI, and 200 ml of acetic acid. The coefficients of intra- and inter-assay variations were 1.6% (n = 10), and 2.4% (n = 10), respectively. AOPP concentrations are expressed as micromoles/L of chloramine-T equivalents.

Assay of plasma lipid hydroperoxide levels

Plasma LHP levels were determined spectrophotometrically according to the method of ferrous oxidation with xylene orange version 2 (FOX 2) [27]. Hydroperoxides oxidize ferrous to ferric ions selectively in dilute acid and the resultant ferric ions can be determined by using ferric-sensitive dyes as an indirect measure of hydroperoxide concentration. Xylene orange binds ferric ions with high selectivity to produce a coloured (blue-purple) complex with an extinction coefficient of $1.5 \times 10^4 \text{M}^{-1} \text{cm}^{-1}$. Ninety microliter aliquots of plasma were transferred into micro-centrifuge reaction vials. Triphenylphosphine (TPP) (10 mM in methanol; 10 ml) was added to vials to remove hydroperoxides. Methanol alone (10 ml) was added to the remaining vials. The LHP content in the plasma samples is determined as a function of the absorbance difference of samples with and without elimination of LHPs by TPP. The samples were vortexed and subsequently incubated at room temperature for 30 min. FOX2 reagent (900 ml) was added and the samples were vortexed. After incubation at room temperature for 30 min, the samples were centrifuged at 15,000 g at 20 °C for 10 min, the supernatant was carefully decanted into a cuvette, and absorbance was read at 560 nm. The coefficients of intra- and inter-assay variations were 2.4% (n = 12), and 2.3% (n = 11), respectively.

Statistics

The computer program InStat was used for data analysis. Descriptive statistics were given as mean ± S.D. All data were not normally distributed. The plasma PCO, AOPP, T-SH, LHP, and endogenous antioxidant parameters levels of diabetic patients were compared using the Mann-Whitney U-test. Spearman’s rank correlation coefficients were used to investigate various oxidative stress markers. When not specified, $P < 0.05$ was considered significant.

Results

The demographic data, medical history, anthropometric data, and biochemical characteristics of type 2 diabetic patients are summarized in Table I. Fasting blood glucose, HbA1c, and triglycerides levels were significantly different between diabetic subjects with good and poor GC (Table I). The levels of both of glucose and HbA1c were higher in diabetic patients with poor GC with respect to diabetic patients with good GC ($P < 0.0001$, for both parameters). Among the lipid profile parameters, the levels of triglycerides were higher in diabetic patients with poor GC ($P < 0.05$), while the levels of the other lipid profile parameters did not differ. Serum endogenous antioxidant levels such as albumin, uric acid, total bilirubin, conjugated bilirubin, unconjugated bilirubin, and unsaturated iron binding capacity in diabetic patient groups with good and poor GC are given in Table II.
Serum albumin and unconjugated bilirubin levels were significantly lower in diabetic patients with poor GC (P < 0.05, for both parameters; Table II).

Protein oxidation markers in type 2 diabetic patients with good and poor glycaemic control are given in Table III. Plasma PCO levels of diabetic patients with poor GC were higher significantly compared with those of the diabetic patients with good GC (P < 0.01). Plasma T-SH levels were lower in the diabetic patients with poor GC but this difference was not statistically significant (P > 0.05).

Tableau I
Clinical characteristics of type 2 diabetic patients with good and poor glycaemic control. Values are mean ± S.D.

<table>
<thead>
<tr>
<th></th>
<th>Good glycaemic control (n = 23)</th>
<th>Poor glycaemic control (n = 17)</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>51.8 ± 8.1</td>
<td>53.8 ± 6.5</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>12/11</td>
<td>6/11</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.7 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>79.7 ± 9.5</td>
<td>75.8 ± 13.6</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>6.3 ± 4.7</td>
<td>7.1 ± 5.1</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>7.3 ± 1.9</td>
<td>11.5 ± 2.1***</td>
</tr>
<tr>
<td>HbA1c (normal&lt; 6.5%)</td>
<td>6.2 ± 0.5</td>
<td>8.5 ± 0.8***</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.4 ± 0.6</td>
<td>1.9 ± 0.7*</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.0 ± 0.9</td>
<td>5.1 ± 0.6</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>1.1 ± 0.3</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>3.1 ± 0.8</td>
<td>3.1 ± 0.5</td>
</tr>
<tr>
<td>VLDL-cholesterol (mmol/L)</td>
<td>0.7 ± 0.2</td>
<td>0.8 ± 0.3</td>
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*P < 0.05. ***P<0.0001 vs good glycaemic control.

Serum AOPP level was significantly higher in the diabetic patient group with poor GC than in the diabetic patient group with good GC (P < 0.05). On the other hand, plasma LHP levels were significantly higher in the diabetic patients with poor GC compared with those of the diabetic patients with good GC (P < 0.05).

Spearman's rank correlation coefficients between HbA1c and oxidative stress markers in the whole series of type 2 diabetic patients are shown in Table IV. In type 2 diabetic patients with good GC, serum AOPP levels positively correlated with HbA1c (r = 0.39; P < 0.05), while in diabetic patients with poor GC serum albumin levels correlated negatively with HbA1c (r = -0.50; P < 0.05). No correlation was found between the other oxidative stress markers and HbA1c levels in any of the groups of patients.

Discussion

In the last 8 years, attention has focused on the role of protein oxidation in diabetes [18,19,24,28-31]. There are reports of increased plasma PCO levels in diabetic patients [24,30,31], while Odetti et al. [29] could not find any significant change in plasma PCO levels between type 2 diabetic patients and controls.

Increased plasma lipid peroxidation parameters, measured by different methods, have been observed in type 2 diabetic patients [30,32,33]. It has been reported that in type 2 diabetic patients, plasma LHP did not correlate with HbA1c [33] and this result concurs with this study. An association between raised lipid peroxidation products and PCO formation has been reported in various studies [5,6,13,18,30,31]. The coexistence of high LHP levels and increased plasma
Protein oxidation and glycaemic control

PCO in the diabetic patients with poor GC in this study indicates that an increase in the total amount of LHP may play an additional role in the enhanced protein oxidation. Protein thiols may preemptively scavenge oxidants that initiate peroxidation, thus sparing vitamin E and/or lipids from attack [34]. The thiol (–SH) moiety of cysteine is highly prone to oxidative attack by several mechanisms, leading to the formation of disulfide bonds and thyl radicals [35]. There were no significant differences plasma T-SH levels in this study. This finding may be explained by the strict maintenance of redox homeostasis for thiol groups to be able to continue their critical functions mentioned above.

Mildly increased serum bilirubin has recently been suggested as a protective factor, possibly reducing the risk of coronary artery disease by acting as an antioxidant [36]. Unconjugated bilirubin is converted to biliverdin on oxidation, quenching 2 mol of peroxyl radicals for each mole of bilirubin consumed [37]. Bilirubin and more especially unconjugated bilirubin were found to be cytoprotective to rat hepatocytes, human erythrocytes, and human myocytes when these cells were exposed to oxyradicals [38-40]. Importantly, the ability of bilirubin at physiological concentration to effectively prevent oxidation of LDL lipids was recently demonstrated [41]. In a previous study, unconjugated bilirubin [42] and serum albumin [43] levels have been shown to be decreased in plasma of type 2 diabetics. In this work, serum unconjugated bilirubin and albumin levels of type 2 diabetic patients with poor GC were decreased significantly compared with those of the diabetic patients with good GC. AOPP is considered to be an oxidized albumin index [18,21]. In the present study, the occurrence of protein oxidation in diabetic patients with poor GC was also confirmed by a novel marker (AOPP assay) that provides information on the degree of oxidative damage to proteins, and the data obtained support those found with detection of PCO with 2, 4-DNPH. There was a significant positive correlation between the serum AOPP and HbA1c levels in diabetic patients with good GC, while there was no correlation between these parameters in diabetic patients with poor GC. There is no correlation analysis in the literature between the serum AOPP and HbA1c levels in type 2 diabetic patients with good and poor GC. An explanation for the lack of correlation may be altered redox homeostasis. On the other hand, a clear negative correlation was observed between serum albumin and HbA1c concentrations in type 2 diabetic patients with poor GC. The correlation between serum albumin and HbA1c levels is the indicator of a close relation between protein oxidation and glycation in type 2 diabetic patients with poor GC. There is evidence that the protein oxidation induced by glucose may cause inactivation of antioxidant defense enzymes and damage in the structure and function of plasma proteins [18,24,30,31,43]. Increase in plasma PCO and serum AOPP levels have been partly attributed to a decrease in indirect bilirubin and serum albumin levels in type 2 diabetic patients with poor GC.

Our previous findings of increased oxidative protein damage in type 1 and type 2 diabetic patients with no complications suggest that the increased protein oxidation may not be due to the complications, but rather contribute to their development [24,30,31]. This study has provided evidence which demonstrates that GC is associated with plasma PCO, AOPP, and LHP levels in type 2 diabetic patients free of clinical complications. Although the primary pathophysiological mechanisms by which diabetic

Tableau IV
Spearman's rank correlation coefficients between HbA1c and oxidative stress markers in the whole series of type 2 diabetic patients

<table>
<thead>
<tr>
<th>HbA1c</th>
<th>PCO</th>
<th>T-SH</th>
<th>AOPP</th>
<th>LHP</th>
<th>Albumin</th>
<th>Uric acid</th>
<th>Total Bilirubin</th>
<th>Conjugated Bilirubin</th>
<th>Unconjugated Bilirubin</th>
<th>Unsaturated iron binding capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.72</td>
<td>-0.21</td>
<td>0.45**</td>
<td>0.34*</td>
<td>-0.37*</td>
<td>-0.02</td>
<td>-0.29</td>
<td>-0.16</td>
<td>-0.21</td>
<td>-0.03</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05
complications develop remain to be elucidated, these results show that the increased oxidative protein damage and reduced antioxidative defenses were greater in diabetic patients with poor GC than good GC. According to the data obtained by this study, it is not possible to conclude whether increased protein oxidation is a cause or a secondary process in diabetes. These results also support the hypothesis that oxidative attack to proteins could be an important early event in the pathogenesis of complications secondary to diabetes, and may indicate that underlying subclinical pathology (oxidative stress and vascular dysfunction) may be present despite the good GC. Even if protein oxidation is not primary in diabetic complications it certainly contributes to their progression. The accumulation of oxidized protein reflects not only the rate of protein oxidation but also the rate of oxidized protein degradation, which is also dependent upon many variables, including the concentrations of proteases that preferentially degrade oxidized proteins and numerous factors (metal ions, inhibitors, activators, and regulatory proteins) that affect their proteolytic activities [5]. However, in this study, there was no relationship found between GC and oxidized protein degradation.

Until the present study, the clinical significance of GC on protein oxidation in type 2 diabetic patients had not been determined. This study revealed that better GC was associated with decreased protein oxidation in type 2 diabetic patients free from clinical complications. Beyond the classic treatments for diabetes, further development of protective agents involving antioxidants or antiadvanced glycation end-product molecules, acting early to prevent protein oxidation chain reactions, may offer interesting therapeutic options aimed at reducing long term vascular damage that occurs in complications secondary to diabetes, and therefore improving the evolution of diabetic patients.

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