Malondialdehyde, glutathione, glutathione peroxidase and homocysteine levels in type 2 diabetic patients with and without microalbuminuria

Gülsen Ozdemir, Meltem Ozden, Hale Maral, Sevinc Kuskay, Pinar Celtnalp, Ilhan Tarkun

Abstract

Background High levels of homocysteine and oxidative stress are known to be associated with premature vascular disease in type 2 diabetes mellitus (DM). The aim of this study was to estimate homocysteine levels and oxidant-antioxidant status and to determine the relationship between them in type 2 diabetic patients with and without microalbuminuria.

Methods Fasting blood samples were obtained from 48 diabetic patients (17 with and 31 without microalbuminuria) and 20 healthy subjects. Serum total homocysteine (tHcy), plasma malondialdehyde (MDA) erythrocyte glutathione (GSH) and glutathione peroxidase (GPx) activity were measured in these patients and the results were compared with those of controls who were chosen among healthy subjects.

Results MDA levels were found to be significantly lower and GSH levels and GPx activities were found to be significantly higher in control subjects when compared with patients with and without microalbuminuria (MDA: P < 0.0001; GSH: P < 0.0001, P < 0.0001; GPx: P < 0.0001, P < 0.0001, respectively). MDA levels were found to be significantly higher in patients with microalbuminuria compared with patients without microalbuminuria (P < 0.0001); while similarly GSH levels were found to be significantly lower in patients with microalbuminuria (P < 0.0001). Although there were no significant differences with respect to tHcy levels and GPx activities between the microalbuminuric normal albuminuric patients (P > 0.05), there was a significant difference with respect to tHcy levels between healthy controls and patients with microalbuminuria (P < 0.05). The serum levels of tHcy correlated best with plasma MDA and erythrocyte GSH concentrations in all diabetic patients (r = 0.549, P < 0.0001; r = 0.385, P < 0.01).

Conclusion Decreased antioxidant levels, increased lipid peroxidation and increased tHcy levels were observed in patients with microalbuminuria. These changes may contribute to vascular disease, which is particularly prevalent in type 2 DM patients with microalbuminuria.

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Introduction

Type 2 diabetes mellitus (DM) is well known to be associated with an increased risk of all-cause and cardiovascular mortality. Lipid peroxidation of cellular polyunsaturated hydrocarbons is believed to be caused by endogenously generated toxic oxygen reduction metabolites and a considerable amount of research has been reported of studies on ageing, atherosclerosis and the complications of DM. Free radical reactions may play an important role in diabetic complications. DM has been shown to result in increased free radical formation. The most important free radicals that cause oxidative stress are superoxide (O2−), hydroxyl...
radical OH) and hydrogen peroxide (H₂O₂). In human erythrocytes there are antioxidant enzymes together with cytoplasmic radical scavengers directed against free radicals in order to protect erythrocytes. One of the cytoplasmic radical scavengers that can reduce free radicals is reduced glutathione (GSH). Glutathione peroxidase (GPx) catalyse the reduction of peroxide. It is generally believed that the protective effect of GSH against the oxidative breakdown of lipids is mediated through GPx by the reduction of endogenously formed hydroperoxides of unsaturated fatty acids to hydroxyl derivatives. Although the pathogenic mechanism of vascular complications in type 2 DM is very complex, free radical reactions induced by reactive oxygen species are thought to be one of the possible factors involved. The increased risk of cardiovascular disease in individuals with microalbuminuria is only partly due to a higher prevalence of established risk factors such as DM. The pathophysiological basis of the association between microalbuminuria and underlying generalized vascular injury may be endothelial dysfunction.

High plasma or serum total homocysteine (tHcy) concentration is a risk factor for atherothrombotic diseases which has recently come under increased scrutiny. A recent prospective study showed that the plasma tHcy concentration was a significant predictor of six-year all-cause mortality risk, both in patients with normoalbuminuria and those with microalbuminuria. It is possible that alterations in free radical activity and hyperhomocysteinemia may be important in the pathogenesis and high prevalence of cardiovascular disease in microalbuminuric type 2 DM. There has been a previous detailed study relating the plasma lipid peroxidation (i.e., malondialdehyde [MDA] production) and homocysteine in type 2 DM with and without microalbuminuria.

In view of these considerations, we determined the plasma MDA, GSH, GPx and tHcy levels, and examined the relationship between these parameters in type 2 DM patients with and without microalbuminuria.

**Patients and methods**

In all, 48 type 2 DM patients (24 women, 24 men; age range 30–70 years and 20 control subjects (10 women, 10 men; age range 44–75 years) were studied. Patients with hypertension were excluded and therefore no patient within the study received antihypertensive agents. The diagnosis of type 2 DM was established according to the Report of the Expert Committee on Diagnosis and Classification of Diabetes Mellitus. The patients did not have episodes of ketoacidosis, ketonuria or overt proteinuria.

Urine albumin was measured in 24-h urine collections by turbidimetric immunoassay using the Sigma microalbumin diagnostic kit (Sigma-Aldrich, Deisenhofen, Germany) on a Beckman CX-9 ALX autoanalyzer (Beckman Coulter Inc., Fullerton, CA, USA). Urinary albumin excretion (UAE) was expressed in milligrams per 24 h. The patients were divided into two subgroups according to UAE: (a) normal albuminuric with UAE less than 30 mg 24 h (n = 31) and (b) microalbuminuric with UAE from 30 to 300 mg 24 h (n = 17). Non-diabetic control subjects were recruited from among the clinical and laboratory staff and their families, and were selected to match for age and gender distribution of the diabetic group as a whole. None of the control subjects had a history of cardiovascular disease. Subjects characteristics are given in Table 1. It can be seen from the significant statistical differences under the table that there is incompleteness in the matching of the patients.

Two tubes of blood (whole blood for serum and di-sodium EDTA anticoagulated blood) were taken from the antecubital veins of each patient. In all, 200 μl of anticoagulated blood was taken for GSH measurement and the remaining blood samples (whole blood and anticoagulated blood) were centrifuged at 3000 rotations per minute (rpm) for 10 min at 4 °C to obtain serum and plasma. After removal of the plasma, the remaining erythrocyte mass was washed three times with 0.9% NaCl solution and chilled to 4 °C. Erythrocytes for GPx analysis were haemolysed by the addition of an equal volume of deinized water.

Serum glucose, triglycerides, total cholesterol, HDL-cholesterol and creatinine concentrations were determined with Technicon kits (Technicon, Bayer Diagnostics, New York, USA) on a Beckman CX-9 ALX autoanalyzer. The estimation of serum tHcy was carried out using the IMX analyzer (Abbott Diagnostics, Chicago, USA). The method measures the total concentration of thiol-, disulphide-, mixed disulphide- and protein-bound forms of homocysteine in the sample. Haemoglobin and haematocrit concentrations were estimated in the haemolyser using commercial reagent on Cell Dyn 3500 analyzer (Abbott diagnostics, Chicago, USA). Glycosylated haemoglobin (HbA1c) was determined with the ANSsym System (Abbott Diagnostics, Chicago, USA). Plasma lipid peroxide concentration, expressed in terms of MDA, was determined by the thiobarbituric acid method. Erythrocyte GPx activity was measured by the method of Paglia and Valentine and is expressed as μmol H₂O₂ consumed per minute. Plasma antioxidant activity was assayed using the method of Reutter et al. and expressed as mmol L⁻¹ red blood cells (RBC's).

The study was approved by the Ethics Committee of the Medical Faculty of the Kocaeli University.

All data are expressed as the mean ± standard deviation (SD) and median. Data were analysed using the Kruskall–Wallis and Mann–Whitney U test. Normalalbuminuric and microalbuminuric groups were
### Table 1. Characteristics of the control and type 2 diabetes mellitus patients with normoalbuminuria [Mic (-)] and microalbuminuria [Mic (+)]

<table>
<thead>
<tr>
<th></th>
<th>Control (n=20) (mean ± SD)</th>
<th>Type 2 diabetes mellitus (n=48) (mean ± SD)</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>50.4 ± 10.9</td>
<td>50.0 ± 8.0</td>
<td>56.9 ± 8.6</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.4 ± 4.7</td>
<td>29.3 ± 5.8</td>
<td>31.9 ± 4.4</td>
</tr>
<tr>
<td>Duration of diabetes (year)</td>
<td>8.1 ± 3.68</td>
<td>7.9 ± 5.1</td>
<td></td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>137 ± 16</td>
<td>141 ± 14</td>
<td>138 ± 16</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>38.9 ± 4.6</td>
<td>41.2 ± 4.0</td>
<td>40.7 ± 4.6</td>
</tr>
<tr>
<td>HbA1c (Hb fraction)</td>
<td>5.6 ± 0.6</td>
<td>7.5 ± 1.3*</td>
<td>7.9 ± 1.6*</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.30 ± 0.52</td>
<td>8.01 ± 2.07*</td>
<td>10.20 ± 0.44*</td>
</tr>
<tr>
<td>Urea (mmol/L)</td>
<td>5.01 ± 1.25</td>
<td>4.34 ± 1.09</td>
<td>5.57 ± 2.44</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>70.72 ± 8.84</td>
<td>79.56 ± 17.68**</td>
<td>88.4 ± 26.52</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.37 ± 0.60</td>
<td>5.59 ± 1.11*</td>
<td>4.81 ± 1.26*</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>0.51 ± 0.18</td>
<td>3.41 ± 0.86**</td>
<td>2.98 ± 0.84</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>1.11 ± 1.16</td>
<td>1.24 ± 0.34*</td>
<td>1.17 ± 0.29</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.17 ± 0.49</td>
<td>2.1 ± 1.4**</td>
<td>1.44 ± 1.11</td>
</tr>
<tr>
<td>UAE (mg/24 h)</td>
<td>9.51 ± 4.70</td>
<td>12.0 ± 7.4</td>
<td>103.6 ± 70.5*</td>
</tr>
</tbody>
</table>

UAE=urine albumin excretion; HbA1c=glycosylated haemoglobin; BMI=body mass index; Hb=haemoglobin; chol=cholesterol. Statistically significant differences: Control subjects and normoalbuminuric patients, *P<0.0001, **P<0.05. Control subjects and microalbuminuric patients, * P<0.0001, ** P<0.05. Normoalbuminuric and microalbuminuric patients, * P<0.0001, ** P<0.05.

### Table 2. Levels of malondialdehyde (MDA), glutathione (GSH), glutathione peroxidase (GPx) and homocysteine in control subjects and type 2 diabetes mellitus patients with normoalbuminuria [Mic (-)] and microalbuminuria [Mic (+)]

<table>
<thead>
<tr>
<th></th>
<th>Control mean ± SD (median)</th>
<th>Mic (-) mean ± SD (median)</th>
<th>Mic (+) mean ± SD (median)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (µmol/L)</td>
<td>0.07 ± 0.02 (0.07)</td>
<td>0.09 ± 0.01 (0.09)*</td>
<td>0.13 ± 0.02 (0.13)*</td>
</tr>
<tr>
<td>GSH (mmol/L RBC)</td>
<td>2.1 ± 0.49 (2.1)</td>
<td>0.99 ± 0.26 (0.95)*</td>
<td>0.64 ± 0.14 (0.63)*</td>
</tr>
<tr>
<td>GPx (MU/mol Hb)</td>
<td>3.11 ± 2.49 (2.17)</td>
<td>0.79 ± 0.29 (0.69)**</td>
<td>0.68 ± 0.08 (0.66)</td>
</tr>
<tr>
<td>Homocysteine (µmol/L)</td>
<td>9.5 ± 1.19 (9.8)</td>
<td>9.8 ± 4.8 (9.2)</td>
<td>12.0 ± 4.6 (10.7)</td>
</tr>
</tbody>
</table>

RBC=red blood cell; standard deviation (SD). Statistically Significant Differences: Control subjects and Normoalbuminuric patients, *P<0.0001, **P<0.001. Control subjects and Microalbuminuric patients, * P<0.0001, ** ** P<0.05. Normoalbuminuric and Microalbuminuric patients, * P<0.0001.

Results

MDA levels were found to be significantly lower in control subjects (0.07±0.03 µmol/L) compared with patients with (0.11±0.02 µmol/L) and without (0.09±0.01 µmol/L) microalbuminuria (<0.001 and 0.001, respectively; see Table 2). On the other hand, GSH levels and GPx activities were found to be significantly higher in control subjects (2.1±0.49 mmol/L, RBCs and 3.11±2.49 MU/mol Hb) when compared with patients with (0.64±0.14 mmol/L, RBCs and 0.68±0.08 MU/mol Hb) and without (0.09±0.01 mmol/L, RBCs and 0.79±0.29 MU/mol Hb) microalbuminuria (P<0.0001 and 0.001, respectively; see Table 2). MDA levels were found to be significantly higher in patients with microalbuminuria when compared to patients without microalbuminuria (P<0.0001), while similarly GSH levels were found to be significantly lower in patients with microalbuminuria (P<0.0001). Although there were no significant differences with respect to MDA levels and GPx activities between the microalbuminuric and normoalbuminuric patients (P>0.05), there was significant
difference with respect to tihcy levels between healthy controls (9.5 ± 1.9 mmol L⁻¹) and patients with microalbuminuria (P < 0.05; see Table 2). The serum levels of tihcy correlated best with plasma MDA, and erythrocyte GSH concentrations in all diabetic patients (r = 0.549, P < 0.0001; r = -0.385, P < 0.01; Figure 1).

Discussion

The significant increase in MDA and decrease in GSH and GPX levels of patients with type 2 DM compared with the control group suggests permanent structural membrane alterations in diabetes, as well as increased production of reactive oxygen species and decreased antioxidants in the circulation. It has been proposed that a diabetics' blood is more prone to lipid peroxidation due to the impaired antioxidant defence system. In fact, oxidative stress is an imbalance between free radical production and lipid peroxidation on one hand, and the antioxidant defence system on another. The pro-oxidant-antioxidant imbalance in diabetes may be due to either acceleration of cellular reactions leading to increased free radical production, such as non-enzymatic protein glycation, glucose oxidation and increased sorbitol pathway, or reduced antioxidant defence potential.

This study provides evidence that the imbalanced MDA and GSH levels are more pronounced in type 2 DM patients with microalbuminuria. Plasma MDA levels correlate with the duration of type 2 DM.
Excessive lipid peroxidation in the plasma can arise due to factors favouring the formation of reactive oxygen species. In poorly controlled DM, glucose oxidation through the pentose phosphate pathway leads to excessive formation of NADPH, which in turn can promote lipid peroxidation in the presence of the cytochrome P450 system. Oxhaemoglobin in erythrocytes could act like cytochrome P450 in the presence of NADPH and this could induce increased lipid peroxidation. Furthermore, inactivation or inhibition of antioxidant enzymes by glycosylation in poorly controlled DM may give rise to increased lipid peroxidation. Free radical reactions cause the oxidation and peroxidation of membrane lipids, denaturation of proteins, disturbed membrane permeability and increased inflammatory cell infiltration. In addition, it is suggested that the increase in free radical activity demonstrated in diabetes coexists with a reduction in the antioxidant state and could therefore potentially increase the deleterious effects of free radicals.

Microalbuminuria is a strong predictor of cardiovascular morbidity and mortality in type 2 DM. The increased risk of cardiovascular disease in individuals with microalbuminuria is only partly due to a higher prevalence of established risk factors such as diabetes, hypertension, smoking and dyslipidaemia. Hyperhomocysteinaemia is another recently recognized risk factor for cardiovascular disease. The present study shows that there is significant increase in homocysteine levels in type 2 DM patients with microalbuminuria compared with control patients. Microalbuminuria is thought to be caused by increased glomerular albumin filtration as a result of decreased glomerular charge selectivity and increased intraglomerular pressure, regulation of which is affected by renal endothelial and mesangial cell function. Mesangial cells have some properties in common with vascular smooth muscle cells. Hyperhomocysteinaemia may induce dysfunction of the vascular endothelium and increase proliferation of vascular smooth muscle cells, possibly by increasing oxidative stress.

The present study supports that hyperhomocysteinaemia is significantly related to increasing oxidative stress and decreasing GSH. For type 2 DM patients there was also significant positive correlation between MDA and homocysteine and a negative correlation between GSH and homocysteine levels. Increased homocysteine levels result in increased risk of atherosclerosis. Increased MDA levels are due to increased free radical production. Both homocysteine and free radicals oxidize LDL, which results in endothelial damage increasing the risk of atherosclerosis. Thus, increased levels of homocysteine and MDA are associated with increased risk of atherosclerosis.

In conclusion, decreased antioxidant levels and increased lipid peroxidation and homocysteine levels were observed in patients with microalbuminuria. These changes may contribute to vascular disease, which is particularly prevalent in type 2 DM patients with microalbuminuria.

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