Lipid Peroxidation and Trace Element Status in Diabetic Ketotic Patients: Influence of Insulin Therapy

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Lipid peroxidation is known to accelerate aging and microvascular lesions in diabetic patients. We studied the acute influence of improved glycemic control on the concentrations of plasma lipid peroxidation intermediates (malondialdehyde (MDA), organic hydroperoxides (OHP)) in ketotic insulin-dependent diabetic patients, as well as the interplay of enzymes such as glutathione peroxidase (GPX) and CuZn superoxide dismutase (CuZn-SOD), and trace elements (Zn, Se, Cu) postulated to be involved in free radical protection. These plasma components were measured on the first day of hospitalization (T0) and when the patient had attained a stable glycemic state after continuous insulin treatment (T1). Plasma MDA and OHP concentrations were high at the beginning of the study but approached reference values after glycemic equilibration. Plasma zinc concentrations were significantly (P < 0.05) decreased during the ketotic state, but also approached reference values with glycemic equilibration. Plasma selenium concentrations and GPX activity were relatively unchanged between T0 and T1. Erythrocyte GPX activity measured at T1 in six patients was below the reference values, whereas CuZn-SOD activity was not affected. Our results show that enhanced lipid peroxidation is associated with decreased zinc plasma concentrations in ketotic patients and underline the beneficial effects of continuous insulin infusion. The mechanisms involved are still unclear but may indicate the importance of extracellular zinc transfer secondary to glyceridic disorders.

Indexing Terms: malondialdehyde · glutathione peroxidase · superoxide dismutase · selenium · zinc · copper · erythrocytes · thiobarbituric acid

Lipid peroxidation has been implicated in the pathogenesis of many degenerative disorders, including naturally occurring or induced diabetes mellitus (1–3). Moreover, in insulin-dependent diabetes the activity of antioxidant enzymes and the micronutrient concentrations are significantly diminished (4, 5). More recently, peroxidative stress has been shown to increase the incidence of diabetic retinopathy, and zinc has been proposed as a therapeutic agent to protect the retina against free radical-induced destruction (6). However, specific studies on the zinc status of diabetic patients are controversial because results seem to vary with duration of the disease and associated complications. Nevertheless, most studies have found an increased urinary zinc loss in these patients (7).

We proposed to quantitatively describe the concentrations of the trace elements Zn, Cu, and Se and of the antioxidant enzymes superoxide dismutase (CuZn-SOD; EC 1.15.1.1) and glutathione peroxidase (GPX; EC 1.11.1.9), implicated in free radical protection, in hyperglycemic and moderately ketotic patients and to follow the evolution of these analytes—trace elements, antioxidant enzymes, and lipid peroxidation intermediates—in diabetic patients after continuous insulin treatment.4

Materials and Methods

Patients and Sample Collection

Sixteen adult insulin-dependent diabetics, 10 men and 6 women (mean age 42.3 ± 8 years), were hospitalized for a moderate ketotic episode in the Diabetologic Department of Grenoble Hospital. Their clinical and biological state did not necessitate intensive care (no dehydration, absence of neurological alterations, pH > 7.28). The main biological descriptions of the patients are given in Table 1. The mean duration of insulin therapy was 7 years (range 4–9 years).

In an attempt to reduce hemocencentration-related analytical errors, we regularly assessed the stability of the hematocrit and that of the total protein concentrations. There was no more than 5% change between measurements on the first day of hospitalization (T0) and at least on the fourth day after a stable clinical and biological state had been attained (T1).

The specific battery of laboratory tests was carried out with 10 mL of blood collected in heparanized polypropylene tubes free of trace elements. We also collected 24-h urines from six patients to measure urinary zinc loss. Samples were collected on two occasions: on T0, i.e., before intravenous insulin infusion with Actrapid HM 40 (Novo Nordisk, Paris, France; 50 to 60 IU/24 h), and T1. The mean duration between T0 and T1 was 14 ± 3 days. Stable clinical state was defined as an overnight fasting blood glucose of 4.9 to 5.7 mmol/L, measured by the glucose oxidase technique (Hitachi 717; Hitachi, Tokyo, Japan); the nondetection of urinary β-hydroxybutyrate by test strips (Lebistix; Ames Inc., Puteaux, France); and normal pH values.

4 Nonstandard abbreviations: CuZn-SOD, superoxide dismutase; GPX, glutathione peroxidase; Hb, hemoglobin; GSSG, glutathione; GSH, reduced glutathione; MDA, malondialdehyde; TBA, thiobarbituric acid; BHT, butylated hydroxytoluene; and OHP, organic hydroperoxides.
Trace Element Analysis

Trace element analysis was performed by using atomic absorption spectrophotometry. Before the trace element measurements, plasma was removed from the blood samples by centrifugation (3000 × g, 15 min).

Zinc concentrations were determined by flame atomic absorption spectrophotometry (Model 460; Perkin-Elmer, Norwalk, CT), as described previously (8). After careful mixing, the sample was diluted fivefold with 0.1 mol/L hydrochloric acid. The absorption signal was measured at 213.9 nm (slit width, 0.7 nm). Concentrations were calculated by using an exogenous calibration curve. The accuracy of the technique was checked against Seronorm Trace Element Control Serum, batch 112 (measured value 11.4 ± 0.4 μmol/L, n = 10; certified value 12.0 μmol/L).

Copper concentrations were also determined by flame atomic absorption spectrophotometry (Perkin-Elmer; Model 460) as described previously (9). Samples were diluted fivefold in 60 mL/L n-butanol. The absorption signal was measured at 324.7 nm (slit width, 0.7 nm). Concentrations were calculated by using an exogenous calibration curve. The accuracy of the technique was checked against Seronorm Trace Element Control Serum, batch 112 (measured value 17.1 ± 0.4 μmol/L, n = 15; certified value 17 μmol/L).

Selenium concentrations were determined by flameless atomic absorption spectroscopy after a sampling dilution procedure, with a Perkin-Elmer Model 5100 fitted with a HGA 600 graphite furnace, an AS 60 autosampler, and a transversal Zeeman background correction as described previously (10). An electrodeless discharge lamp and platform pyrolytically coated graphite tubes were used. Samples were diluted twofold with a solution containing 2 g/L Triton X-100 and 0.1 mol/L nitric acid. Absorbance was read at 196.0 nm (slit width 2 mm) after an injection of 10 μL of the diluted sample into the graphite furnace and an injection of 5 mL of a 2 g/L nickel solution as a matrix modifier. Serum selenium was determined by standard addition calibration curves. The accuracy of the technique was tested by analysis of Seronorm Trace Element Serum, batch 112 (measured value, 1.08 ± 0.04 μmol/L, n = 38; certified value, 1.1 μmol/L).

Analyses for Metalloenzymes

Preparation of erythrocyte lysates. We needed 5 mL of heparinized peripheral blood for metalloenzyme determination. We used 1 mL to determine the hemoglobin (Hb) concentration by the cyanomethemoglobin method. Erythrocyte pellets were obtained from blood by centrifuging at 500 × g for 15 min at room temperature; the plasma and buffy coat were then removed, and erythrocytes were washed twice in 5 mL of sterile 9 g/L NaCl solution. Plasma was kept for the determination of plasma GPX activity. Lysed erythrocytes were prepared by freezing and thawing twice and by the addition of nine volumes of ice-cold distilled water. Cell membranes were removed by centrifugation and the supernate was used for determining GPX activity in erythrocytes. Hb was removed from the erythrocyte lysate by adding cold ethanol (0.4 mL/mL lysate) and cold chloroform (0.4 mL/mL lysate). We determined CuZn-SOD activity in the resulting clear aqueous phase.

Enzymatic determination of CuZn-SOD. We determined the CuZn-SOD activity by monitoring the auto-oxidation of pyrogallol according to the method of Marklund and Marklund (11). One unit of CuZn-SOD activity is defined by the amount of the enzyme required to inhibit the rate of pyrogallol autooxidation by 50%, as determined from the standard curve generated with bovine SOD (Sigma Chemical Co, Paris, France).

Enzymatic determination of GPX. We measured GPX activity by the modified method of Gunzler et al. (12), using tert-butyl hydroperoxide as substrate. Hb was converted to cyanmethemoglobin by reaction with potassium cyanide and potassium ferricyanide, to minimize its pseudoperoxidase reactivity. Glutathione (GSSG) produced by the action of GPX and peroxide was reduced by glutathione reductase and NADPH, the decrease in concentration of NADPH being recorded at 340 nm. The reaction mixture, maintained at 24 °C, consisted of 3 mmol of reduced glutathione (GSH), 0.609 mmol of tert-butyl hydroperoxide, 5 U of yeast glutathione reductase (EC 1.6.4.2; Type III; Sigma), and 0.170 mmol of NADPH per liter of 50 mmol/L Tris buffer (pH 7.6).

The assay kinetics were calculated by using a molar absorptivity of NADPH of 6.22 × 10−3 L mol−1 cm−1 at 340 nm. Enzyme activity was expressed in terms of micromoles of NADPH oxidized per gram of Hb per minute (U/g Hb). For plasma measurements of GPX activity, the procedure was the one described previously (12), and the results were expressed as U/L.

Lipid Peroxidation Intermediates

Malondialdehyde (MDA). This measurement was performed with the Sobioda (Grenoble, France) MDA kit, as described by Richard et al. (13), in which the fluorescence of thiobarbituric acid (TBA) reactants in plasma is recorded. The reagents in the kit consisted of TBA, perchloric acid, and a calibration solution of 1,1,3,3-tetraethyloxypropane, 20 mmol/L, in ethanol. The TBA/acid working solution was prepared by combining two
volumes of TBA reagent with one volume of HClO₄ reagent. The stock standard solution was diluted with deionized water to a concentration of 10 μmol/L. The working solution and standard solution were prepared fresh daily. In addition, we also used n-butanol (fluorometric grade; Merck, Darmstadt, Germany) for extraction. Butylated hydroxytoluene (BHT; Sigma), 20 g/L, was prepared in absolute ethanol. FeSO₄·7 H₂O and ascorbate were purchased from Merck. The control serum was lyophilized ProbioCal AB 43 (BioMérieux, Lyon, France), reconstituted daily according to the manufacturer's instructions.

Each analytical run included the assay of reagent blank, tetraethoxypropane working standard solutions, plasma specimens, and a quality-control specimen. The continuation of lipid peroxidation was prevented by adding to each tube 10 μL of the 20 g/L BHT solution. We then combined in each tube 100 μL of plasma specimen and 750 μL of kit working solution, vortex-mixed, tightly capped the tubes, and placed them in a 95 °C water bath. After 60 min, the samples were chilled in an ice bath to stop the reaction. Reagent blank, standard blank, and assay blank were left at room temperature. We then added 2 mL of butanol to each tube, extracted the TBA–MDA complex by shaking, and separated the phases by centrifugation. For fluorometric determination of the TBA reactants complex in the n-butanol extracts, we used wavelengths of 532 nm (excitation) and 553 nm (emission).

**Organic hydroperoxides (OHP).** The method used was the enzymatic technique described by Heath and Tappel (14). The enzyme GPX can be used to measure OHP quantitatively. A timed reaction of GPX coupled with the oxidation of NADPH by glutathione reductase allows a direct spectrophotometric measurement of hydroperoxide. The sensitivity of the coupled assay is high because the absorbivity of NADPH is high. Adding catalase before the GPX permits the distinction between hydrogen peroxide and OHP.

The reagents for the assay were Tris HCl buffer, 124 mmol/L (Merck); EDTA, 0.2 mmol/L; catalase, 17,500 kU/L; NADPH, 2 mmol/L; GPX, 2500 kU/L; GSH, 4.25 mmol/L; and glutathione reductase, 100 kU/L. Catalase, NADPH, GPX, and glutathione reductase were purchased from Sigma.

Plasma (up to 0.9 mL), 0.7 mL of Tris HCl, and EDTA tetrazolium salt were added to a tube with 10 μL of catalase and were allowed to react at room temperature for at least 10 min before the next steps. Two tubes were used: one without catalase to measure total hydroperoxides and the other with catalase to measure OHP. To the reagents in each tube we added 50 μL of NADPH, 10 μL of GPX, and 0.1 mL of GSH. The timing of the test began at this point. Under normal circumstances, 1 min should be allowed for the handling of each tube. The solution was then incubated for at least 10 min. At the end of the incubation the absorbance of the solution was read at 340 nm, after which 5 μL of glutathione reductase was added. The absorbance was again read to determine the net amount of NADPH oxidized. For the conditions just described, the absorbance of the unreac-

Tabled NADPH was 0.336 ± 0.005. The values were expressed by comparison with a standard of tert-butyl hydroperoxide (0–600 μmol/L).

**Statistical Analysis**

All results concerning diabetic patients' measurements were expressed as mean ± SD. The data obtained at T0 and T1 were compared by using paired Student's t-test (PCSM software, Meylan, France). The sample values for diabetic patients obtained at T0 and T1 were compared with the range of values measured in our laboratory for nondiabetic subjects of the same age by using unpaired Student's t-test (PCSM software). The limit of statistical significance was set at P < 0.05.

**Results**

**Trace Element Status**

As Table 2 shows, plasma zinc concentrations, which were significantly decreased in the ketotic state, were normalized with glycemic control (P < 0.001). No significant sex-related differences in zinc concentrations were observed. Plasma zinc concentrations did not seem to be influenced by the presence or absence of microalbuminuria.

There were no significant differences observed for the T0 and T1 plasma concentrations of copper and selenium. We did not observe significant changes in the plasma concentrations of these two trace elements in diabetic patients when compared with the normal values of the laboratory.

Urinary zinc loss measured in six patients was significantly less at T1 than at T0 (25.0 ± 4.5 μmol/24 h vs 12.5 ± 3.2 μmol/24 h; P < 0.001). However, after restoration of glycemic control, the urinary loss of this trace element remained greater than the range of laboratory values established in 20 apparently healthy subjects of the same age (5.5 ± 1.2 μmol/24 h; P < 0.001).

**Antioxidant Enzymes and Lipid Peroxidation Intermediates**

No significant differences were observed in the concentrations of lipid peroxidation markers and antioxidant enzymes of male and female patients (Table 3). These markers also were not influenced by the extent of microalbuminuria.

Plasma MDA concentrations, initially high, decreased during the experimental period (T0 to T1), and

**Table 2. Concentrations of Plasma Trace Elements Measured before (T0) and after (T1) Establishment of Glycemic Control**

<table>
<thead>
<tr>
<th>Conc, μmol/L, mean ± SD</th>
<th>Reference values*</th>
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<tbody>
<tr>
<td>T0 (n = 16)</td>
<td>T1 (n = 16)</td>
</tr>
<tr>
<td>Plasma Zn</td>
<td></td>
</tr>
<tr>
<td>12.11 ± 2.02</td>
<td>16.62 ± 2.55</td>
</tr>
<tr>
<td>Plasma Cu</td>
<td>17.22 ± 3.91</td>
</tr>
<tr>
<td>Plasma Se</td>
<td>0.89 ± 0.18</td>
</tr>
<tr>
<td>15.24 ± 2.22</td>
<td>16.55 ± 3.51</td>
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<tr>
<td>1.08 ± 0.20</td>
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* Established in 94 women and 74 men, ages 40 to 50 years.

**P < 0.05 compared with reference values by unpaired Student's t-test.

**P < 0.001 compared with T0 by paired Student's t-test.
approached the reference values as glycemic control was established (P < 0.001). A similar trend was observed for OHP plasma concentrations, but, in this case, the decrease was not statistically significant.

GPX activity in plasma was relatively insensitive to intravenous insulin treatment. At both T0 and T1 measurements, GPX activity did not differ significantly from the laboratory’s reference values. In erythrocytes, GPX activity measured at T1 in six diabetic patients was significantly lower (P < 0.05) than the reference values. SOD activity in erythrocytes at T1 in six diabetic patients was not significantly different from the reference values.

Discussion

Diabetes mellitus is not simply a disorder of glucose homeostasis but is also accompanied by various degenerative manifestations such as accelerated aging, cardiovascular disease, and microvascular lesions leading to retinopathy and glomerulopathy (15). These events may be related to the hyperproduction of free radicals (16) and to a dysfunction of biological antioxidant systems such as low enzyme activity (4) or deficient micronutrient status (5). A relationship has been demonstrated between the frequency of ketotic periods and the severity of retinal lesions, secondary to enhanced peroxidation (17). In the present work, we evaluated the degree of lipid peroxidation and the antioxidant capacity of biological defense systems, before and after treatment by continuous insulin infusion.

On the first day of hospitalization (T0), the concentrations of lipid peroxide intermediates MDA and OHP were significantly increased above our laboratory’s reference values. After 3 days of glycemic homeostasis, we observed a significant decrease in plasma MDA and a tendency for plasma OHP to decrease. This observation suggests that lipid peroxidation is increased during a state of glycemic disequilibrium accompanied by overproduction of ketotic bodies, and that insulin treatment has a beneficial effect on peroxidative stress. Presumably this is because, during ketotic periods, the pentose shunt is blocked, leading to a decrease in cytoplasmic NADPHβ, a proton source implicated in the regeneration of glutathione synthetase. At the same time, MDA precursors such as free fatty acids are increased by mechanisms secondary to enhanced lipolysis (18).

Other factors such as micronutrients may be involved in lipid peroxidation. We observed an increase in plasma zinc concentrations under continuous insulin treatment. Interestingly, the normalization of the zinc status was accompanied by a decrease in lipid peroxidation intermediates—which could reflect the role of this trace element as a biological antioxidant (19). A normalization of plasma zinc concentrations could decrease lipid peroxidation because this metal can compete with iron, by the Fenton reaction, in production of free radicals (20). The decrease of lipolysis as a result of insulin infusion could be invoked to explain the lowering of lipid peroxidation, but not the normalization of plasma zinc concentrations, because the insulin solutions contained only 6 g of zinc per liter, corresponding to a total of 0.25 g of zinc after 14 days (daily absorption being 3 mg) (21). The cellular efflux of zinc ions in response to the increased glucose concentrations may also be involved in this phenomenon (22). We observed a persistent urinary zinc loss between T0 and T1. This observation coincides with those of other studies (23) and emphasizes an essential zinc transfer from tissues to plasma.

The CuZn-dependent SOD activity in erythrocytes was not modified in six of the patients. This can be attributed to the stabilizing influence of copper and the fact that plasma concentrations of zinc remain normal (24). Copper exerts its antioxidant effects by virtue of its presence in a prosthetic group of the active site of SOD, whereas zinc ions stabilize the apoprotein. Plasma selenium concentrations were unchanged by glycemic equilibration and remained within the reference range of values. Moreover, plasma selenium-dependent GPX activity was constant and remained within the norm, in contrast to erythrocyte GPX activity, which remained statistically below the reference values. Although the number of erythrocyte measurements was limited, the difference between selenium GPX activity in plasma and erythrocytes is not surprising considering the lifespan of erythrocytes and the absence of protein synthesis. Erythrocyte lipid peroxidation is increased in diabetic patients (25). In such conditions, toxic ligands such as MDA could partially inhibit erythrocyte GPX activity (26). The measurement of erythrocyte enzyme activity in larger populations and the monitoring of the selenium status of diabetic patients for a longer period after ketotic incidents are necessary to confirm these preliminary results.

In conclusion, this study demonstrates that peroxidative stress accompanies ketotic incidents in insulin-dependent diabetic patients and that insulin therapy has beneficial effects, normalizing the plasma concentrations of zinc and of lipid peroxidation markers. Ad-
ditional studies are necessary to more fully understand the consequences of zinc deficiency on lipid peroxidation, and to measure the effect of long-term zinc supplementation in diabetic patients.

References