Defective Intracellular Antioxidant Enzyme Production in Type 1 Diabetic Patients With Nephropathy

Antonio Ceriello, Anna Morocutti, Franceschina Mercuri, Lisa Quagliaro, Marina Moro, Giuseppe Damante, and Gian Carlo Viberti

There is an individual susceptibility to diabetic nephropathy, and oxidative stress is believed to play an important role in the pathogenesis of diabetic complications. Active oxygen species induce antioxidant enzyme expression in tissues, an effect considered to be a defensive mechanism. To test whether altered intracellular antioxidant enzyme production might explain the predisposition to diabetic nephropathy, we studied the effect of long-term (12 weeks) exposure to normal (5 mmol/l) or high (22 mmol/l) glucose concentrations on fibroblast antioxidant enzyme gene expression and protein activity in type 1 diabetic patients with and without nephropathy, nondiabetic nephropathic patients, and nondiabetic control subjects. Under conditions of normal glucose concentration in the culture media, CuZnSuperoxide-dismutase, MnSuperoxide-dismutase, catalase, and glutathione-peroxidase activity and mRNA expression were not different among the four groups. Under high-glucose conditions, CuZnSuperoxide-dismutase mRNA and activity increased similarly in all groups (P < 0.001 vs. basal), whereas MnSuperoxide-dismutase catalase did not change. In contrast, catalase mRNA and activity as well as glutathione-peroxidase mRNA and activity increased in fibroblasts from type 1 diabetic patients without nephropathy (P < 0.001), in fibroblasts from nondiabetic nephropathic patients (P < 0.001), and in fibroblasts from nondiabetic control subjects (P < 0.001), but not in fibroblasts from type 1 diabetic patients with nephropathy. Exposure to high glucose concentrations significantly increased lipid peroxidation in cells, higher levels being found in cells from diabetic patients with nephropathy (P < 0.001). These data, while confirming that exposure to high glucose concentrations induces an antioxidant defense in skin fibroblasts from normal subjects, demonstrate a failure of this defensive mechanism in cells from type 1 diabetic patients with nephropathy, whereas skin fibroblasts from diabetic patients without complications of diabetes do not have an increased antioxidant response to glucose-induced oxidative stress. Diabetes 49:2170–2177, 2000

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Animal and human studies and in vitro experiments suggest a role for oxidative stress, via an increased formation of free radicals, in the pathophysiology of diabetic complications (1,2). Increased generation of reactive oxygen metabolites, such as superoxide anion and hydrogen peroxide, has been shown to occur in diabetes in response to hyperglycemia (3). Previous studies have demonstrated that exposure to high glucose concentrations increases the levels of oxygen radical–scavenging enzymes in cultured endothelial cells (4) and in the kidney of rats with streptozotocin-induced diabetes (5,6). High glucose concentrations can induce formation of free radicals and activation of oxidative stress through nonenzymatic glycation of protein (7,8), auto-oxidative glycation (9), activation of protein kinase C (10), and increased polyol pathway (11). Excessive generation of reactive oxygen metabolites may also play a role in the pathophysiology of a variety of renal diseases (12). In the kidney, as in other organs, endogenous antioxidant enzymes protect cells against the toxic effect of free radicals and are an essential defense system against oxidative injury (13). Indeed, oxygen-derived free radicals are associated in different tissues with an elevation of antioxidant enzyme activity, and an imbalance between antioxidant enzyme production during exposure to free radicals may lead to tissue injury (13,14).

There is an individual predisposition to diabetic nephropathy (15,16), whose development is only partially explained by the effects of high glucose concentration (17). To test whether defective antioxidant enzyme regulation in response to hyperglycemia may explain susceptibility to tissue injury in diabetic nephropathy, we studied expression and biological activity of the antioxidant enzymes CuZnSuperoxide dismutase (CuZnSOD), MnSuperoxide dismutase (MnSOD), catalase (CAT), and glutathione peroxidase (GPX) in skin fibroblasts obtained from type 1 diabetic patients with or without nephropathy, from nondiabetic nephropathic patients, and from normal healthy control subjects, cultured in normal or high glucose concentrations.

RESEARCH DESIGN AND METHODS

Skin fibroblasts obtained from 20 type 1 diabetic patients with nephropathy, defined as a persistent albumin excretion rate (AER) >200 µg/min in sterile
stopped by the addition of 7 ml of medium containing serum, after verification with phosphate-buffered saline (PBS) and detached by treatment with 2.5 ml trypsin-EDTA (Life Technologies) for 4–6 min at 37°C. Trypsin activity was with age and sex and the diabetic patients had similar durations of diabetes, and degrees of glycemic control, which was estimated by calculating the mean creatinine, AER, and glomerular filtration rate (GFR) values (Table 1).

Arterial blood pressure was measured, with a standard mercury sphygmomanometer, to the nearest 2 mm Hg, in each subject's dominant arm after at least 10 min of rest in the supine position. Mean blood pressure was calculated as diastolic blood pressure plus one-third pulse pressure. GFR was measured by 51Cr-EDTA clearance in the patients with albuminuria only (18). All diabetic patients with and without nephropathy were taking antihypertensive medication and their blood pressure was well controlled and similar to that of the two other groups. The diabetic patients with normoalbuminuria were taking no medication other than insulin. Three timed urine samples were obtained from each subject for measurement of urinary albumin (~19) and the median values were used for classification (Table 1).

All subjects gave their written informed consent to the study, which was approved by the ethics committees of Guy's Hospital and the University of Udine. Skin biopsies were taken by excision under local anesthetic from the anterior surface of the forearm, and fibroblasts were cultured in Dulbecco's modified Eagle's medium (ICN Biochemicals, Thame, U.K.) supplemented with 20% fetal calf serum (Life Technologies, Paisley, Scotland, U.K.), 2 mM glutamine (Sigma Chemical, Dorset, U.K.), 50 U/ml penicillin (Life Technologies, Paisley, Scotland, U.K.), and 2 mM glutamine (Sigma Chemical, Dorset, U.K.).

At the fourth passage, cells were frozen until used for the experiments. It is well recognized that even long-term cryopreservation does not affect fibroblasts' functional activities (20). Excluding cells from nephropathic patients without diabetes, all of the other cell lines had been used for previously published studies (21,22).

All experiments were conducted between the sixth and eighth passages, using the same batches of medium and fetal calf serum. The purchased medium contained 5 mM glucose, to which mannitol or glucose was added in order to obtain iso-osmolar experimental media. Cells were cultured in high (22 mM) glucose and in iso-osmolar normal (5 mM) glucose.

Each sample of cells was grown for 12 weeks, with renewal of the medium every second day. For each culture condition (normal or high glucose), twelve parallel experiments were performed. Isolated fibroblasts were prepared from 20 type 1 diabetic patients without renal disease (AER <20 µg/min), 20 nephropathic nondiabetic patients with diagnosis of membranous glomerulonephritis, and 20 nondiabetic healthy subjects. The four groups of subjects were matched for age and sex and the diabetic patients had similar durations of diabetes, and degrees of glycemic control, which was estimated by calculating the mean creatinine, AER, and glomerular filtration rate (GFR) values (Table 1).

Antioxidant enzyme activity. For the determination of CAT and GPX activities, the monolayers were rinsed twice with ice-cold PBS and the cells were harvested with a sterile rubber cell scraper. The cells were sedimented for 4 min at 1,600g and processed either for enzyme/protein or for mRNA analyses. For enzyme/protein lysates, cells were resuspended in 50 mM potassium phosphate buffer containing 0.5% Triton X-100 and sonicated (in an ice-water bath) for two 30-s bursts on a Branson sonicator B15 (position 2, continuous setting; Branson Ultrasonic, Danbury, CT) with a 20-s cooling interval. Total protein concentration was determined according to the procedure of Bradford (24).

For CAT and GPX activities, sonicates were first spun 5 min at 800g (4°C). The supernatants were assayed according to the procedure of Clairborne and Günzler and Flohé (26) for GPX activity. For superoxide dismutase (SOD) measurements, cells were suspended in 100 mM triethanolamine-diethanolamine buffer and homogenized with a teflon glass Dounce homogenizer. The homogenate was centrifuged at 105,000g for 1 h (4°C) and the supernatant was passed through a small Sephadex G25 (coarse) column to remove low-molecular-weight substances that interfere with the enzyme assay, according to the procedure of Paolletti et al. (27). An aliquot of the eluate was applied onto a 5.5% polyacrylamide gel to localize SOD activity according to the procedure of Beauchamp and Fridovich (28), with the exception that no tetramethyl-ethylenediamine was used for staining. MnSOD activity was determined in mitochondrial fractions prepared by differential centrifugation. Mitochondria were disrupted by freezing-thawing in a high ionic strength buffer (0.25 mM sucrose, 0.12 M KCl, and 10 mM Tris-HCl, pH 7.4). Mitochondrial membranes were removed by sedimentation at 105,000g for 1 h, and enzyme activity was measured in the supernatant.

Northern blot analysis. Total RNA was prepared according to the procedure of Chirgwin et al. (29). Briefly, 10 µg total RNA was electrophoresed on a 1.4% agarose-formaldehyde gel and then transferred to gene screen membranes. The filters were prehybridized in 50 mM Tris-HCl, pH 7.5, 0.1% sodium pyrophosphate, 1% SDS, 2.2% poly(vinylpyrrolidone), 0.2% ficol, 5 mM EDTA, 50% formamide, 0.2% bovine serum albumin, 1 × standard sodium citrate (SSC), and 150 µg/ml denatured salmon sperm DNA at 65°C for 6 h. Blots were hybridized with 32P-labeled probes for human CuZnSOD (30), human CAT (31), human MnSOD (30), and bovine GPX (32), to a specific activity of 1 × 106 cpm/ml in hybridization fluid at 65°C overnight. The filters were washed at 65°C twice for 15 min with 2 × SSC-0.1% and twice for 15 min with 0.1 × SSC-0.1% SDS and then subjected to autoradiography using an intensifying screen at ~85°C. Densitometry was performed on an LKB laser scanning densitometer. Hybridization to glyceraldehyde-3-phosphate dehydrogenase cDNA was used as internal control to correct for loading inequalities.

Lipid peroxidation. Cells were trypsinized and centrifuged at 250g for 10 min at 4°C. Cell pellets were resuspended in 1 ml cold PBS for assay of dihydroxybenzidine-reactive substances and conjugated dienes, as previously described (33).

Statistical analysis. Data did not show a significant departure from normal distribution. Analysis of variance (ANOVA) was used to test differences among the three groups. Paired t test was used to compare, for each group of fibroblasts, the results under conditions of normal versus high glucose concentration, while Fisher's least significant differences test was used to evaluate the difference between the three different groups either in normal or high

<table>
<thead>
<tr>
<th>TABLE 1 Clinical features of type 1 diabetic subjects with nephropathy, type 1 diabetic patients without nephropathy, nondiabetic nephropathic patients, and nondiabetic control subjects</th>
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<tbody>
<tr>
<td><strong>Diabetic subjects</strong></td>
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<tr>
<td>-----------------------</td>
</tr>
<tr>
<td>n (M/F)</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
</tr>
<tr>
<td>MBP (mmHg)</td>
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<tr>
<td>Plasma creatinine (µmol/l)</td>
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<tr>
<td>AER (µg/min)</td>
</tr>
<tr>
<td>GFR (ml · min⁻¹ · 1.73 m⁻²)</td>
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</tbody>
</table>

Data are means ± SD for HbA1c and MBP; medians (range) for age, duration of diabetes, and GFR; and range for AER. MBP, mean blood pressure.
glucose condition. *P < 0.001 vs. fibroblast from diabetic subjects with nephropathy cultured in high glucose.

**results**

In normal glucose concentration, CuZnSOD, MnSOD, CAT, and GPX activity and mRNA expression were not different among the four groups (Tables 2 and 3; Figs. 1 and 2). In high glucose conditions, CuZnSOD mRNA and activity increased similarly in all groups (*P = NS by ANOVA) (Tables 2 and 3; Figs. 1 and 2). CAT and GPX mRNA (*P < 0.001) and activity (*P < 0.001) were significantly different between the groups by ANOVA (Tables 2 and 3; Figs. 1 and 2). MnSOD did not change in any group (Tables 2 and 3; Fig. 1). Comparing the groups in high glucose conditions, CAT and GPX mRNA expression and CAT and GPX protein activity were significantly higher in control subjects, nephropathic nondiabetic subjects and diabetic subjects without nephropathy versus nephropathic diabetic subjects, without difference between diabetic subjects without nephropathy and control subjects (Tables 2 and 3; Figs. 1 and 2).

High glucose concentrations significantly increased lipid peroxidation in every group of cells (Fig. 3). Higher levels were found in cells of diabetic patients with nephropathy (*P < 0.001; Fig. 3).

**discussion**

The results of this study indicate that exposure to high glucose concentrations induces an increase in mRNA levels and biological activity of CuZnSOD, CAT, and GPX in fibroblasts from control subjects, nephropathic nondiabetic subjects, and diabetic subjects without nephropathy. By contrast, in fibroblasts from diabetic subjects with nephropathy, only CuZnSOD is increased. This finding may have important consequences concerning glucose-induced oxidative stress damage to the cell. Both CuZnSOD, which is located primarily in the cytoplasm, and MnSOD, a structurally distinct protein located in the mitochondria, catalyze the reaction \( \text{O}_2^- + \text{O}_2^- + 2\text{H}^+ = \text{O}_2 + \text{H}_2\text{O}_2 \) (33). \( \text{H}_2\text{O}_2 \) is converted to \( \text{H}_2\text{O} \) in peroxisomes by the antioxidant enzyme CAT and in the cytoplasm by GPX (35). These antioxidant enzymes protect the cell from oxidative stress, but the threshold of protection can vary dramatically as a function of their activity and balance (14). CAT and GPX are far more efficient than CuZnSOD in protecting fibroblasts against oxidative stress (14,36). Moreover, in several instances, cells with increased levels of CuZnSOD are hypersensitive to oxidative stress rather than protected from it (14). This happens because CuZnSOD increases the formation of \( \text{H}_2\text{O}_2 \), which, if not efficiently converted to \( \text{H}_2\text{O} \) by an adequate level of CAT and GPX, may be detrimental to the cell.

**table 2**

<table>
<thead>
<tr>
<th>Enzyme and mRNA</th>
<th>Diabetic subjects with nephropathy</th>
<th>Diabetic subjects without nephropathy</th>
<th>Nondiabetic nephropathic subjects</th>
<th>Normal control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mmol/l glucose</td>
<td>22 mmol/l glucose</td>
<td>5 mmol/l glucose</td>
<td>22 mmol/l glucose</td>
</tr>
<tr>
<td>CuZnSOD mRNA/GAPDH</td>
<td>4.1 ± 1.3</td>
<td>0.001</td>
<td>9.7 ± 3.2</td>
<td>5.4 ± 1.7</td>
</tr>
<tr>
<td>MnSOD mRNA/GAPDH</td>
<td>0.7 ± 0.3</td>
<td>NS</td>
<td>0.7 ± 0.2</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Catalase</td>
<td>4.2 ± 1.2</td>
<td>NS</td>
<td>4.4 ± 1.2</td>
<td>4.4 ± 1.1</td>
</tr>
<tr>
<td>GPX mRNA/GAPDH</td>
<td>2.0 ± 0.7</td>
<td>NS</td>
<td>2.0 ± 0.7</td>
<td>2.0 ± 0.8</td>
</tr>
</tbody>
</table>

Data are means ± SD, unless otherwise indicated. *P < 0.001 vs. fibroblast from diabetic subjects with nephropathy cultured in high glucose.

**table 3**

Antioxidant enzyme activity in skin fibroblasts from diabetic subjects with or without nephropathy, nondiabetic nephropathic subjects, and normal control subjects cultured in normal or high glucose concentrations

<table>
<thead>
<tr>
<th>Enzyme and U/mg protein</th>
<th>Diabetic subjects with nephropathy</th>
<th>Diabetic subjects without nephropathy</th>
<th>Nondiabetic nephropathic subjects</th>
<th>Normal control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 mmol/l glucose</td>
<td>22 mmol/l glucose</td>
<td>5 mmol/l glucose</td>
<td>22 mmol/l glucose</td>
</tr>
<tr>
<td>CuZnSOD U/mg protein</td>
<td>0.49 ± 0.20</td>
<td>0.001</td>
<td>0.95 ± 0.29</td>
<td>0.57 ± 0.27</td>
</tr>
<tr>
<td>MnSOD U/mg protein</td>
<td>0.26 ± 0.05</td>
<td>NS</td>
<td>0.26 ± 0.03</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>Catalase U/mg protein</td>
<td>0.31 ± 0.07</td>
<td>NS</td>
<td>0.31 ± 0.07</td>
<td>0.32 ± 0.06</td>
</tr>
<tr>
<td>GPX U/mg protein</td>
<td>0.54 ± 0.11</td>
<td>NS</td>
<td>0.54 ± 0.11</td>
<td>0.57 ± 0.14</td>
</tr>
</tbody>
</table>

Data are means ± SD. *P < 0.001 vs. fibroblast from diabetic subjects with nephropathy cultured in high glucose.
It is therefore not surprising that generally an increase in CuZnSOD is accompanied by a concomitant increase in CAT and GPX (14). In the presence of high glucose concentrations, we confirmed this phenomenon in the fibroblasts derived from control subjects, nephropathic nondiabetic subjects, and long-term diabetic patients without complications. In the fibroblasts of subjects with diabetes and nephropathy, however, high glucose induced an increase only in CuZnSOD, but no change in the activity of CAT and GPX. This result suggests that cells of type 1 diabetic subjects with nephropathy are not able to adjust their antioxidant defenses when high glucose concentration–induced oxidative stress is produced, so that they are more susceptible to oxidative stress. In our study, this point of view is supported by the evidence that cells from patients with nephropathy show higher levels of lipid peroxidation when exposed to high glucose concentrations.

High glucose concentrations in vitro and hyperglycemia in vivo are well-known stimuli for the production of free radicals and the generation of oxidative stress (1–6), with a consequent increase in the expression and activity of antioxidant enzymes (4–6), which act as a defense system against cell damage (12–14,36). Hyperglycemia is also a necessary factor for the development of the glomerular lesions of diabetes (17). The observation that, despite hyperglycemia, only a portion of the population of type 1 diabetic patients will progress to diabetic nephropathy indicates that there is individual diversity in cell response to high glucose concentrations. It is therefore of great relevance that a disturbance in the mechanisms of protection from oxidative stress was found only in the cells of patients with nephropathy. By contrast, in long-term type 1 diabetic patients with normoalbuminuria, a group that appears protected from renal complications, the defense mechanisms against high glucose–induced oxidative stress were intact, or similar to those of nondiabetic individuals.

Although we cannot categorically refute that the abnormalities described in this report may be secondary to renal failure, this scenario seems unlikely, because fibroblasts from nondiabetic subjects with nephropathy reveal an antioxidant enzyme response to hyperglycemia similar to that of both control subjects and diabetic subjects without nephropathy. Because culture in normal glucose concentrations did not reveal any difference among the groups, it would be surprising that nephropathy would spare the basal antioxidant status of skin fibroblasts while impairing their response to glucose. The hypothesis that renal impairment is secondary to oxidative damage and not the cause of an altered antioxidant enzyme response is also supported by a study in rats with acutely induced reduction in GFR and proteinuria, in which the animals with a better inducible tissue antioxidant enzyme response were more resistant to renal functional deterioration (37).

Also, we do not believe that our findings may be due to worse glycemic control or to a high glucose memory (38) in the group of diabetic subjects with nephropathy, because cells of all groups were exposed in vitro to the same glucose concentration, and, as far as we were able to assess, there had been no difference in glycemic control between the two groups of diabetic patients in the previous four years. It is conceivable that memory of high glucose may be retained differently by cells of patients with nephropathy compared with those without nephropathy, but this would imply a specific intrinsic difference between the two sets of cells in response to hyperglycemia. The most plausible explanation is that the observed deficiencies in free radical–scavenging capacities are...
FIG. 2. Effect of incubating fibroblasts from nondiabetic control subjects, nondiabetic nephropathic patients, and diabetic patients with or without nephropathy in medium containing 5 or 22 mmol/l glucose on CuZnSOD, catalase, and GPX mRNA expression and activity.
intrinsic to the cell and are uncovered only under extreme conditions of oxidative stress, such as those induced by high glucose concentrations.

In our study, fibroblasts from control subjects exhibit a mean increase of antioxidant enzymes, after exposure to high glucose concentrations, similar to that of fibroblasts from diabetic subjects without nephropathy. This finding raises another question: because diabetic nephropathy is related to exposure to high glucose concentrations, but occurs in only some diabetic patients—whose phenotypes may be defined as nephropathy-prone—fibroblasts from control subjects would be expected to comprise a certain number of such phenotypes and therefore exhibit a behavior of antioxidant enzymes intermediate between that of fibroblasts from diabetic patients with or without nephropathy.

The following two considerations can be made:
1) The difference in the tendency to have nephropathy defines two different populations of diabetic patients, whereas the sample from the normal population is chosen without regard to that characteristic.
2) The prevalence of nephropathy in the population of diabetic individuals with a 25-year history of disease in the 1990s in the U.K., such as that from which our sample was drawn, was ~10% (39). Assuming that in the nondiabetic population the prevalence of the characteristic “nephropathy-prone if diabetic” were the same, the mean would not be expected to change significantly in a sample as small as 20 subjects.

We cannot be certain that the same abnormalities we found in skin fibroblasts would be present in kidney resident cells as well, but there is substantial evidence that at least one cell-

FIG. 3. Thiobarbituric acid–reactive substances and conjugated dienes levels in fibroblasts from control subjects, diabetic patients with or without nephropathy, and nondiabetic nephropathic patients, cultured in high or normal glucose concentration. *P < 0.01 versus other groups.
lar feature associated with susceptibility to diabetic nephropathy, i.e., the sodium-hydrogen antiport activity, is shared by many different types of cells (40). The sodium-hydrogen antiport is an integral plasma membrane protein that catalyzes the electroneutral exchange of extracellular sodium for intracellular hydrogen and regulates major cellular events, such as intracellular pH, cell volume and stimulus-response coupling, and cell proliferation. Increased sodium-hydrogen antiport activity was reported in cell types as varied as erythrocytes (41), platelets (42), and lymphocytes (43) of type 1 diabetic patients with microalbuminuria or proteinuria. Recently, we were able to show that this abnormal phenotype was present also in cultured skin fibroblasts (21,22)—the same used in this study. Elevated sodium-hydrogen antiport activity has also been reported in immortalized lymphoblasts of type 1 diabetic subjects with nephropathy (44). Interestingly, high glucose levels magnify the difference in this activity, which is already present between cells from diabetic subjects with nephropathy and those from control subjects and diabetic subjects without nephropathy (45). Thus, it appears that the observations made in skin fibroblasts may be a generalized phenomenon affecting other tissues.

An intrinsic anomaly in function suggests that there may be a genetic basis for it, which would be consistent with those reports that support the notion of a genetic predisposition to nephropathy and presumably to the pathological processes responsible for it (46).

ACKNOWLEDGMENTS
This study has been supported by grants 115.25662, 115.31085, and 115.28640 from Consiglio Nazionale delle Ricerche, Italy.

REFERENCES


