The Response of Antioxidant Genes to Hyperglycemia Is Abnormal in Patients With Type 1 Diabetes and Diabetic Nephropathy

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Increased flux of glucose through the polyol pathway may cause generation of excess reactive oxygen species (ROS), leading to tissue damage. Abnormalities in expression of enzymes that protect against oxidative damage may accentuate the oxidative injury. The expression of catalase (CAT), CuZn superoxide-dismutase (CuZn-SOD), glutathione peroxidase (GPX), and Mn superoxide-dismutase (MnSOD) mRNA was quantified in peripheral blood mononuclear cells—obtained from 26 patients with type 1 diabetes and nephropathy, 15 with no microvascular complications after 20 years’ duration of diabetes, and 10 normal healthy control subjects—that were exposed in vitro to hyperglycemia (HG) (31 mmol/l D-glucose). Under HG, there was a twofold increase in the expression of CAT, CuZnSOD, and GPX mRNA in the patients without complications and the control subjects versus patients with nephropathy (P < 0.0001), and MnSOD did not change in any of the groups. The aldose reductase inhibitor zopolrestat partially restored the levels of CAT, CuZnSOD, and GPX mRNA in the patients with nephropathy (P < 0.05). There was a highly significant correlation between increased aldose reductase (ALR2) expression, CAT, CuZnSOD, and GPX mRNA levels under HG conditions and polymorphisms of ALR2 in the patients with nephropathy (P < 0.0001). In conclusion, these results suggest that high glucose flux through aldose reductase inhibits the expression of antioxidant enzymes. Diabetes 52:846–851, 2003

It is clear that hyperglycemia is an important determinant in the pathogenesis of diabetic microvascular complications in both type 1 and type 2 diabetes (1,2). Recent studies in clinical as well as experimental models of diabetes suggest that oxidative stress plays an important role in the pathogenesis of the long-term complications of diabetes (3,4). For instance, exposure of mesangial and other cell types to high glucose leads to an increase in reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion, and hydroxyl radicals (5–9). The generation of ROS is likely to lead to tissue damage through a number of mechanisms including increased synthesis of extracellular matrix proteins and expression of inflammatory mediators such as transforming growth factor-β (10–13). The precise biochemical events have still to be elucidated, although it is known that excess ROS production due to high glucose concentrations in cells will lead to oxidative stress by increasing the flux through the polyol and glucosamine pathways, formation and activation of protein kinase C, formation of advanced glycation end products, and glycation (14–19).

It is well known that a number of enzymatic systems exist to protect cells from the damage that would be caused by excessive production of ROS. These systems include superoxide dismutase (SOD) in the cytosol (CuZn-dependent [CuZnSOD]) and mitochondria (Mn-dependent [MnSOD]) that convert superoxide into hydrogen peroxide, together with the glutathione peroxidase (GPX) and catalase (CAT) enzymes in the cytosol and peroxisomes that convert hydrogen peroxide to water (20). It has recently been suggested that the production of intracellular antioxidant enzymes in response to high glucose may be defective in patients with type 1 diabetes and nephropathy (21). This defect may be part of the genetic predisposition to nephropathy, although there have been no substantive studies of polymorphisms of the genes coding for the antioxidant enzymes. There is growing, but not unanimous, evidence to suggest that polymorphisms in the promoter region of the aldose reductase gene (ALR2) are associated with susceptibility to nephropathy as well as retinopathy and neuropathy (22–28). Further, it has also been shown that this susceptibility region in the regulatory region of the gene is associated with elevated expression of ALR2 (27,29,30). Elevated tissue levels of ALR2 are associated with increased indexes of oxidative stress (31), and an inhibitor of aldose reductase, zopolrestat, has been reported to block glucose-stimulated production of superoxide in vascular tissue (32). Clearly, increased flux through the polyol pathway could have important consequences for the generation of ROS in individuals with diabetes. This, together with a possible defect in the ROS-protective enzymatic systems may, in part, underlie the pathogenesis of nephropathy. There have been no studies looking at both the function and expression of the
ALR2 gene together with those coding for antioxidant genes. In this study, we have investigated the expression of both antioxidant and ALR2 genes in human peripheral blood mononuclear cells (PBMCs) derived from patients with type 1 diabetes and either nephropathy or no microvascular complications after 20 years of diabetes. The PBMCs were exposed for 5 days in either normal or elevated glucose concentrations, as well as in the presence or absence of zopolrestat and aldose reductase inhibitor.

**RESEARCH DESIGN AND METHODS**

**Patient subjects.** Forty-one patients were recruited from the Diabetic Out-patient Clinic, Derriford Hospital (Plymouth, U.K.). Local ethics committee approval was obtained. The patients were classified as being without complications.

**Patients without complications.** These patients (n = 15) had type 1 diabetes for at least 20 years but remained free of retinopathy (fewer than five dots or bolts per fundus), proteinuria (negative on urine Albstix on at least three consecutive occasions over the previous 12 months), and overt neuropathy. Overt neuropathy was defined as any clinical evidence of peripheral or autonomic neuropathy.

**Patients with nephropathy.** These patients (n = 26) had type 1 diabetes for more than 10 years and had proteinuria (urine Albstix positive on at least three consecutive occasions over 12 months or three successive total urinary protein excretion rates of >0.5 g/24 h) in the absence of hematuria or infection on midstream urine samples. Diabetic nephropathy was always associated with retinopathy. Retinopathy was defined as more than five dots or blot or peep; hard or soft exudates, new vessels, or fluorescein angiographic evidence of maculopathy, or previous laser treatment for preproliferative or proliferative retinopathy; and maculopathy or vitreous hemorrhage. Fundoscopy was performed by both a diabetologist and ophthalmologist. The clinical characteristics of the subjects are shown in Table 1.

**Healthy control subjects.** Peripheral blood samples were obtained from 10 healthy volunteers (n = 10) with no family history of type 1 or type 2 diabetes.

**Isolation and culturing of PBMCs from whole blood.** From each subject, 20 ml peripheral blood was collected into 5% EDTA Vacutainers (Becton Dickinson, Oxford, U.K.) and diluted 1:1 in PBS. The PBMCs were harvested by density gradient centrifugation, washed four times in PBS, resuspended at a concentration of 0.5 × 10^6/ml in RPMI 1640 (11 mmol/l d-glucose) supplemented with penicillin/streptomycin (Invitrogen, Paisley, Scotland), 10% FCS (Invitrogen), and 1-glutamine (Sigma Chemicals, Poole, Dorset, U.K.), and separated into two flasks. The first flask was cultured for 5 days in the above medium with phytohemagglutinin (normoglycemia: 11 mmol/l D-glucose concentration within the medium), and the second flask was supplemented with 20 mmol/l d-glucose (hyperglycemia: 31 mmol/l d-glucose final concentration) before culture for the same incubation period.

**Extraction of RNA.** For the extraction of the RNA, the PBMCs were pelleted by centrifugation at the end of the incubation period, the supernatant was removed, and the PBMCs were resuspended and lysed in RNA Stat60 (Biogenesis, Poole, U.K.). The cells were then transferred to a RNA-free Eppendorf; 200 μl chloroform (Sigma Chemicals) was added; the cells were vortexed and centrifuged at 13,000g for 15 min at 4°C, and the upper phase was transferred to a clean tube before mixing with 500 μl isopropanol and centrifuging. The supernatant was discarded, and the pellet was washed in 75% ethanol and resuspended in 50 μl diethyl pyrocarbonate–treated water by vortexing. The approximate amount of total RNA extracted was determined using a Cecil 5500 spectrophotometer scanning 240–280 nm (Cecil Instruments, Nottingham, U.K.).

**Preparation, amplification, radiolabeling, and purification of antisense RNA probes.** Nucleon extraction kits were used to prepare high–molecular weight DNA from 10 ml peripheral blood (Scotlab, Paisley, Scotland). An aliquot of this DNA was amplified using PCR. Exons of CuZnSOD, MnSOD, and CAT, and GPX were amplified using three separate primers for each gene. The first round of amplification of the reaction was performed in 30 μl volumes, containing the amplifiers for CuZnSOD, MnSOD, and CAT (GenBank accession number K00065); sense 5′-GAG TTT GGG GTA GAT ACG GCA GGC-3′ and antisense 5′-GAG GCC CCT TAA CTC TAT CCT C-3′; MnSOD (GenBank accession number S71277); sense 5′-AGCC GCA GCC TGC GTA GAC GGT C-3′ and antisense 5′-CCA AGG GTA GTT ACC CTG AC-3′; and antisense 5′-GCC TCA CAA GGA GTC CCC TCT CAT C-3′ and antisense 5′-GAG AAG TGG GGA GTA TCA ACA CTG CC-3′; GPX (GenBank accession number XM003289); sense 5′-GCA ACC ATG TTG GGC AGG AGA AC-3′ and antisense 5′-GAC CAT TGA CAT CGA GCC TGC CAT C-3′; 10 mmol/l dNTPs (Invitrogen, Paisley, Scotland); 10× buffer solution, 10 mmol/l MgCl₂, and 1 unit Taq polymerase (Invitrogen). The samples for CuZnSOD and MnSOD were subjected to an initial cycle of denaturation for 2 min at 96°C, followed by 30 cycles of amplification (denaturation for 30 s at 94°C, annealing for 1 min at 65°C, and extension for 1 min at 72°C) in a PTC-200 Thermal Cycler (MJ Research, Essex, U.K.). Samples for GPX and CAT were as above except the annealing temperature was 55°C for GPX and 58°C for CAT. An antisense amplifier with a RNA polymerase T7 adapter and stuffer nucleotides was then employed for a second round of amplification: CuZnSOD: antisense +T7-5′-TAA TAC GAC TCA CTA TAG GGA GGG AGG CCC CTT AAC TCA TCT GTT ATC-3′; MnSOD: antisense +T7-5′-TAA TAC GAC TCA CTA TAG GGA GCC CAA GGG TAG GTT CCA GGC TGA GC-3′; CAT: antisense +T7-5′-TAA TAC GAC TCA CTA TAG GGA GGG AGG AGA GCG GAT CAA CAC TGC C-3′; GPX: antisense +T7-5′-TAA TAC GAC TCA CTA TAG GGA GCC CAA GGG TAG GTT CCA GGC TGA GC-3′; 10 mmol/l dNTPs, 10× buffer solution, 10 mmol/l MgCl₂, and 1 unit Taq polymerase. The samples for CuZnSOD, MnSOD, CAT, and GPX were then subjected to DNA amplification as described above. The radioactive RNA probes were generated by incorporating [α-32P]UTP (Amersham Bioscience, Buckinghamshire, U.K.) using the MaxScript system (Ambion, Abingdon, U.K.) into 1 μg of the PCR product. An equal volume of gel loading buffer (Ambion) was added to the reaction and heated to 85–95°C for 3–5 min and loaded onto a 5% acrylamide/8 mol/l urea gel and run at ~100–200 volts for 40 min to 1 h. The gel was wrapped in SaranWrap and exposed to Kodak X-Omat film (Scientific Imaging Systems, Cambridge, U.K.) for 10–30 s. The probe was excised, immersed in 350 μl elution buffer (Ambion), and incubated overnight at 37°C to obtain 90% recovery.

**Hybridization of ribonuclease protection assay (RPA) probe to RNA sample.** The RPA antisense probe and the RNA sample were mixed together and precipitated with ethanol and ammonium acetate (33). This mixture was pelleted by centrifugation, resuspended in 20 μl hybridization buffer (Ambion), incubated for 2–3 min at 92°C; vortexed, centrifuged, and incubated overnight at 42°C. For the Rnase digestion, 200 μl diluted RNase mix (Ambion) was added and incubated for 30 min at 37°C. Then 300 μl Rnase inactivation/precipitation solution (Ambion) was added, vortexed, and centrifuged. The reaction was transferred to –20°C for at least 15 min. The protected fragments were separated and detected by centrifugation of the sample for 15 min at 4°C, and the pellet was resuspended in gel loading buffer. The sample was loaded onto a 5% acrylamide gel after heating for 3–4 min at 94°C, electrophoresed for ~40 min to 1 h at 200 volts, transferred to filter paper (Whatman, Maidstone, U.K.), and exposed to Kodak X-Omat film at –80°C.
TABLE 2
Overall mean levels of CAT, CuZnSOD, GPX, and MnSOD mRNA expression in normal and hyperglycemic conditions in the study groups

<table>
<thead>
<tr>
<th></th>
<th>Diabetic patients with nephropathy (n = 26)</th>
<th>Diabetic patients without complications (n = 15)</th>
<th>Normal control subjects (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11 mmol/l</td>
<td>31 mmol/l</td>
<td>11 mmol/l</td>
</tr>
<tr>
<td>CAT</td>
<td>3.1 ± 0.24</td>
<td></td>
<td>1.9 ± 0.25*</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>1.6 ± 0.17</td>
<td>0.9 ± 0.07†</td>
<td>0.8 ± 0.15</td>
</tr>
<tr>
<td>GPX</td>
<td>3.2 ± 0.27#</td>
<td>1.7 ± 0.19†</td>
<td>1.2 ± 0.07</td>
</tr>
<tr>
<td>MnSOD</td>
<td>4.5 ± 0.48</td>
<td>4.1 ± 0.5§</td>
<td>3.7 ± 0.4</td>
</tr>
</tbody>
</table>

Data are means ± SE. P values are all vs. PBMCs from diabetic nephropathy cultured under hyperglycemic conditions. *versus uncomplicated, P = 0.09, and vs. control, P < 0.001. †versus uncomplicated, P < 0.001, and vs. control, P < 0.001. ‡versus uncomplicated 11 mmol/l D-glucose, P = 0.0001. §versus uncomplicated 11 mmol/l D-glucose, P = 0.0001. #versus uncomplicated 31 mmol/l D-glucose, P = 0.0001.

RESULTS
The mean levels of CAT, CuZnSOD, GPX, and MnSOD mRNA in PBMCs exposed to normal and hyperglycemic conditions in the patients with nephropathy, patients without complications, and normal control subjects are shown in Table 2. Figure 1 depicts an example RPA of CAT mRNA in a patient with nephropathy, a patient without complications, and a control subject. The level of 18S rRNA housekeeping gene was similar between all the study groups. The decrease in CAT, CuZnSOD, and GPX mRNA levels seen in the patients with nephropathy under hyperglycemic versus normoglycemic conditions was significantly correlated to the 5′ALR2 susceptibility genotype Z-2/Y (Table 3). Those patients with the susceptibility genotype had the greatest decrease in CAT, CuZnSOD, and GPX mRNA levels under hyperglycemic conditions, compared with patients with other genotypes (P < 0.001). The PBMCs from Z-2/Y genotype patients also had the greatest fold increase in ALR2 mRNA (4.7 ± 0.8), compared with patients with the Z+2/Y genotype (2.0 ± 0.5; P < 0.02), the latter being the group with the lowest fold increase in ALR2 mRNA (Table 3). The most significant relationship was seen with CuZnSOD when patients with the Z-2/Y where compared to those with the Z+2/Y and Z-2/Z+2
genotypes (0.3 ± 0.02, 1.7 ± 0.47 [P < 0.001], and 1.3 ± 1.05 [P = 0.009], respectively).

When the PBMCs from five patients with nephropathy, three of whom had the Z-2 allele, were cultured under hyperglycemic conditions with 10 μmol/l of the aldose reductase inhibitor (ARI) zopolrestat, the levels of CAT, CuZnSOD, and GPX were significantly increased, ranging from 1.5- to 3.0-fold (P < 0.001): CAT, 0.5 ± 0.08 vs. 1.8 ± 0.18 (P = 0.0002); CuZnSOD, 0.6 ± 0.14 vs. 2.2 ± 0.3 (P = 0.002); and GPX, 0.4 ± 0.008 vs. 2.0 ± 0.06 (P = 0.001). This is in stark contrast to the failure to respond (i.e., tendency to decrease) seen previously under hyperglycemic conditions in the diabetic patients with nephropathy (Table 2).

**DISCUSSION**

In this study, we have shown that exposure to high glucose induces the elevation of mRNA expression of CAT, CuZnSOD, and GPX genes in PBMCs from individuals with no microvascular complications after 20 years’ duration of diabetes as well as healthy control subjects. In marked contrast to the failure to respond seen previously under hyperglycemic conditions in the diabetic patients with nephropathy (Table 2).

**TABLE 3**

Antioxidant enzyme and aldose reductase mRNA levels with respect to 5′ALR2 genotype in patients with diabetic nephropathy

<table>
<thead>
<tr>
<th></th>
<th>CAT</th>
<th>CuZnSOD</th>
<th>GPX</th>
<th>MnSOD</th>
<th>ALR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z – 2/X  (n = 15)</td>
<td>0.4 ± 0.04*</td>
<td>0.3 ± 0.02†‡</td>
<td>0.4 ± 0.03§</td>
<td>0.8 ± 0.02</td>
<td>4.7 ± 0.8</td>
</tr>
<tr>
<td>Z + 2/Y (n = 3)</td>
<td>0.6 ± 0.08</td>
<td>1.7 ± 0.47</td>
<td>0.6 ± 0.05</td>
<td>0.8 ± 0</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>Z – 2/Z + 2 (n = 3)</td>
<td>0.5 ± 0.05</td>
<td>1.3 ± 1.05</td>
<td>0.4 ± 0</td>
<td>0.6 ± 0.2</td>
<td>4.4 ± 3.6</td>
</tr>
<tr>
<td>X/Y (n = 5)</td>
<td>0.6 ± 0.05</td>
<td>0.5 ± 0.07</td>
<td>0.7 ± 0.08</td>
<td>0.9 ± 0.02</td>
<td>3.4 ± 1.1</td>
</tr>
</tbody>
</table>

Data are mean fold increases ± SE of the PBMCs exposed to hyperglycemia versus those cultured under normal conditions. X, any allele other than Z + 2; Y, any allele other than Z – 2. *versus CAT X/Y, P = 0.02. †versus CuZnSOD Z + 2/Y, P < 0.001. ‡versus CuZnSOD Z – 2/Z + 2, P = 0.009. §versus GPX Z + 2/Y, P = 0.02.
contrast, there was a decrease in the expression of the antioxidant genes in the PBMCs from patients with nephropathy. The abnormal response of the antioxidant genes to hyperglycemia confirm those results recently published by Ceriello et al. (21), who found similar abnormalities in the response to hyperglycemia of CAT and GPX antioxidant genes of skin fibroblasts from patients with nephropathy. In our study, we found that CuZnSOD in PBMCs from patients with nephropathy also did not respond; in the previous report, this antioxidant gene was induced in the fibroblasts of patients with nephropathy under hyperglycemic conditions. This discrepancy may be a reflection of differences in the cell type and experimental conditions as well as the detection end points in the two studies. In this study, we used PBMCs and cultured the cells for 5 days at 11 vs. 31 mmol/l d-glucose; the previous study used skin fibroblasts that were cultured for 12 weeks at 5 vs. 22 mmol/l glucose. Further, we used a highly sensitive and quantitative RPA assay rather than conventional Northern blot analysis, although this is unlikely to account for the different set of results between the studies. More likely, the expression of these genes may also vary with the cell type and the degree of glucose-linked oxidative stress under the conditions studied.

Both the present study and that of Ceriello et al. (21) found that the mitochondrial MnSOD was not induced by hyperglycemia in cells from any of the subjects, including the control subjects. This is in seeming contrast to studies in bovine cells, where transgenic overexpression of MnSOD has been found to protect against excessive ROS production and associated biochemical changes (34), including increased hexosamine pathway flux (19). However, the mechanisms responsible for the increased expression of the antioxidant enzymes in response to hyperglycemia are clearly complex and are likely dependent on species, cell type, and experimental conditions.

This study suggests that the defect might be found in the cytoplasmic compartment of the cell. This is supported by the observation that the effect in human PBMCs from patients with nephropathy is linked to the genotypes that are associated strongly in humans with expression (30) and activity levels (35) of a soluble cytoplasmic enzyme, aldose reductase (Table 3), and that responsiveness to hyperglycemia insulin can be at least partially restored by use of an ARI. This same ARI at the same in vitro dose was recently reported to have no direct antioxidant activity, but to completely prevent glucose-induced superoxide production by vascular tissue (32). A likely mechanism of action of the ARI to reduce excessive polyol pathway–induced oxidative stress (31) is to tend to normalize NADPH and NADH coenzyme imbalances associated with excessive polyol pathway metabolism (18,36). Particularly important may be the reduction of excess free cytoplasmic NADH, which is a substrate for NAD(P)H oxidase, now shown to be elevated in diabetic human arteries and veins (37). Further studies in both the human and bovine systems will be needed to clarify these important issues.

In regard to the magnitude of induction of cytoplasmic antioxidant enzymes, it should be noted that a difference of two-fold in the renal expression level of CuZnSOD in transgenic mice has been shown to ameliorate experimental diabetic nephropathy (39). Therefore the magnitude of the changes described herein may be quite relevant to the progression rate of human diabetic nephropathy. This is also consistent with the finding that alleles such as Z-Z that are associated with high expression levels of aldose reductase (27,35) have been found in several reports (23,24,27) to also be associated with diabetic nephropathy. That an aldose reductase inhibitor can reverse the defective response of cytoplasmic and peroxisomal antioxidant enzyme induction to hyperglycemia in the cells of patients with nephropathy offers encouragement for the potential use of such inhibitors for treating or slowing the progression of diabetic nephropathy (39).

REFERENCES