

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

Andrea D. Hodgkinson,¹ Tracey Bartlett,¹ Peter J. Oates,² Beverley A. Millward,¹ and Andrew G. Demaine¹

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 oxidant damage may accentuate the oxidative injury. The expression of catalase (CAT), CuZn superoxide-dismutase (CuZnSOD), 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 ($P < 0.00001$). 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, expo-

sure 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

From the ¹Department of Molecular Medicine, Peninsula Medical School, Plymouth, U.K.; and ²Pfizer Global Development, Groton, Connecticut.

Address correspondence and reprint requests to Dr. A. Demaine, Room N32, ITTC Bldg., Tamar Science Park, Plymouth PL6 8BX, U.K. E-mail: andy.demaine@pms.ac.uk.

Received for publication 28 May 2002 and accepted in revised form 19 November 2002.

P.J.O. is employed by Pfizer Inc. Pfizer Inc. provides funds to the laboratory of A.G.D.

ARI, aldose reductase inhibitor; CAT, catalase; CuZnSOD, CuZn superoxide-dismutase; GPX, glutathione peroxidase; MnSOD, Mn superoxide-dismutase; PBMC, peripheral blood mononuclear cell; ROS, reactive oxygen species; RPA, ribonuclease protection assay.

TABLE 1
Subject characteristics

	Diabetic patients with nephropathy	Diabetic patients without complications	Normal control subjects
<i>n</i>	26	15	10
Age (years)	39.8 ± 10.5 (19–69)	46.6 ± 12.2 (27–64)	36.4 ± 8.04 (25–50)
Age at onset of diabetes (years)	11.3 ± 8.0 (2–33)	18.2 ± 9.2 (4–42)	—
Duration of diabetes (years)	28.6 ± 8.1 (15–43)	29.4 ± 8.2 (21–51)	—
HbA _{1c} (%)	9.1 ± 1.2 (8.1–11.8)	9.2 ± 1.3 (7.2–11.2)	—
Insulin dosage (units)	43.8 ± 15.7 (25–94)	58.2 ± 27.5 (20–118)	—
M:F	12:14	6:9	5:5

Data are means ± SD (ranges). All patients had type 1 diabetes as defined by the National Diabetes Group. The HbA_{1c} values were not significantly different between the patient groups.

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 according to their microvascular complications, as previously described (23).

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 Albustix 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 >10 years and had proteinuria (urine Albustix 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 blots per eye; 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 L-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 RNase-free Eppendorf; 200 µl chloroform (Sigma Chemicals) was added; the cells were vortexed and centrifuged at 13,000*g* 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, CAT, and GPX were amplified using three separate primers for each gene. The first round of amplification reaction was performed in 30 µl volumes, containing the amplimers for CuZnSOD (GenBank accession number K00065): sense 5'-GAG TTT GGA GAT AAT ACG GCA GGC-3' and antisense 5'-GAG GCC CCT TAA CTC ATC TGT TAT C-3'; MnSOD (GenBank accession number S77127): sense 5'-AGC CCA GCC TGC GTA GAC GGT C-3' and antisense 5'-CCA AGG GTA GGT TCC AGG CTG AGC-3'; CAT (GenBank accession number K02400): sense 5'-GCC TCA CAA GGA CTA CCC TCT CAT C-3' and antisense 5'-GAG AAG TGC GGA GAT TCA ACA CTG CC-3'; GPX (GenBank accession number XM003289): sense 5'-GCA ACC AGT TTG GGC ATC AGG AGA AC-3' and antisense 5'-GAC CAT TGA CAT CGA GCC TGA 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 amplimer 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 TAG GGA GGC CAA GGG TAG GTT CCA GGC TGA GC-3'; CAT: antisense +T7-5'-TAA TAC GAC TCA CTA TAG GGA GGG AGA AGT GCG GAG ATT CAA CAC TGC C-3'; GPX: antisense +T7-5'-TAA TAC GAG TCA CTA TAG GGA GGG ACC ATT GAC ATC GAG CCT GAC ATC-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 [³²P]UTP (Amersham Bioscience, Buckinghamshire, U.K.) using the MAXiscript 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 95% 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

	Diabetic patients with nephropathy (n = 26)		Diabetic patients without complications (n = 15)		Normal control subjects (n = 10)	
	Glucose		Glucose		Glucose	
	11 mmol/l	31 mmol/l	11 mmol/l	31 mmol/l	11 mmol/l	31 mmol/l
CAT	3.1 ± 0.24	1.9 ± 0.25*	1.0 ± 0.32	2.6 ± 0.35	2.9 ± 0.32	4.5 ± 0.27
CuZnSOD	1.6 ± 0.17	0.9 ± 0.07†	0.8 ± 0.15	3.0 ± 0.35	1.4 ± 0.26	2.9 ± 0.48
GPX	3.2 ± 0.27#	1.7 ± 0.19‡	1.2 ± 0.07	2.7 ± 0.26	2.6 ± 0.44	4.5 ± 0.37
MnSOD	4.5 ± 0.48	4.1 ± 0.5§	3.7 ± 0.4	4.1 ± 0.44	5.9 ± 0.27	6.2 ± 0.17

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, P = 0.002, and vs. control, P < 0.001. §versus control, P = 0.01. ||versus uncomplicated 11 mmol/l D-glucose, P = 0.00001. #versus uncomplicated 11 mmol/l D-glucose, P = 0.00001. Diabetic patients with nephropathy: 11 mmol/l D-glucose vs. 31 mmol/l D-glucose: CAT mRNA, P = 0.002; CuZnSOD mRNA, P = 0.0002; GPX mRNA, P = 0.0001.

overnight. Analysis of the bands used a phospho-imager (Biorad, Hertfordshire, U.K.). 18S RNA was set up as an internal control to ascertain any variations in the amount of RNA used in the assays.

Statistical analysis. The results are expressed as means ± SE. Statistical significance was assessed by Student's t tests after ANOVA or Mann-Whitney U test. A P value of <0.05 was considered to be significant. The changes in mRNA expression in the subjects were compared to their 5'ALR2 genotype using the χ² test.

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 samples of PBMCs stimulated with different concentrations of D-glucose (fold increase in the samples supplemented with D-glucose was up to 1.0 compared with normal samples) (an example is shown in Fig. 1). This demonstrates that equal amounts of study RNA (10 µg) were loaded and that variations in antioxidant enzyme mRNA expression were not due loading errors. The results are shown as fold difference in mRNA in relation to the level of mRNA expression obtained under hyperglycemic compared with normoglycemic conditions. Under normal glyceemic conditions, CuZnSOD and MnSOD mRNA expression was not significantly different between any of the study groups (Table 2). Patients with nephropathy had a higher level of CAT and GPX mRNA under normal glyceemic conditions versus the patients without complications

(CAT 3.1 ± 0.24 vs. 1.0 ± 3.2, P = 0.00001; GPX 3.2 ± 0.27 vs. 1.2 ± 0.07, P = 0.00001) (Table 2). Under hyperglycemic conditions, the groups were found to be significantly different for CAT, CuZnSOD, and GPX mRNA expression (P < 0.001). In the patients without complications and the control subjects, there was an increase in CAT mRNA expression (ranging from 2 to 3.5 ± 0.18) versus the patients with nephropathy 0.5 ± 0.03 (P < 0.001). Similar results were also observed in CuZnSOD and GPX levels (2.0 to 2.5 ± 0.1 vs. 0.7 ± 0.1, P < 0.001, and 2.0 ± 0.12 vs. 0.5 ± 0.03, P < 0.001, respectively) (Table 2; Fig. 2). In contrast, there was a decrease in the levels of CAT, CuZnSOD, and GPX mRNA in the patients with nephropathy under hyperglycemic conditions versus normoglycemic conditions (P < 0.05). There was no difference in the MnSOD mRNA under hyperglycemic conditions in any of 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/X (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/X 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/X where compared to those with the Z+2/Y and Z-2/Z+2

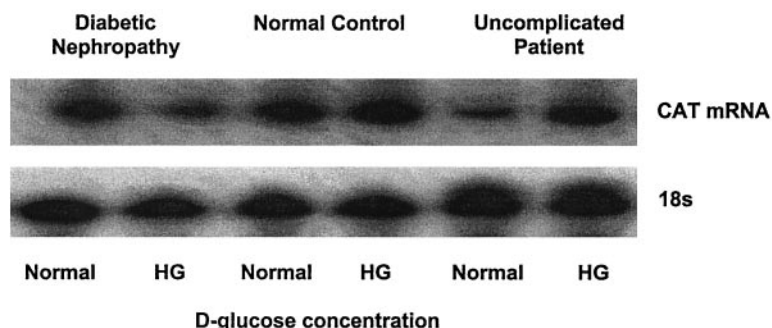


FIG. 1. An example of catalase (CAT) mRNA and 18S RNA expression in a patient with diabetic nephropathy, a normal control subject, and a patient without complications under normal (11 mmol/l D-glucose) and high glucose (HG) (31 mmol/l D-glucose) conditions. There was a significant decrease in CAT mRNA expression in the diabetic patients with nephropathy under HG conditions (P = 0.002). In the patients without complications and the control subjects, there was an increase in CAT mRNA expression under HG conditions (ranging from 2.0 to 3.5 ± 0.18) versus the patients with nephropathy (0.5 ± 0.3; P < 0.001). The level of 18S RNA expression was similar in the diabetic patient with nephropathy, the control subject, and the patient without complications under normal and HG conditions. The fold increase in the samples supplemented with D-glucose was 1.1 to 1.3.

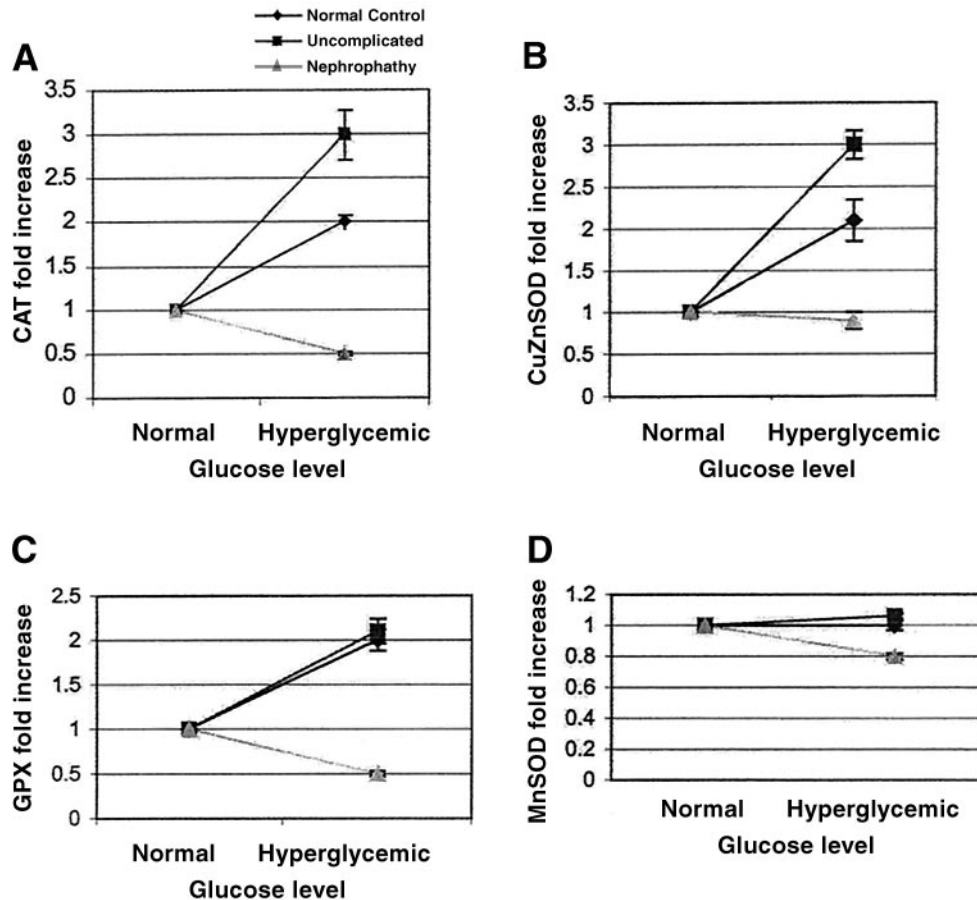


FIG. 2. The mean fold increase of CAT, CuZnSOD, GPX, and MnSOD mRNA in PBMCs from patients with nephropathy, patients without complications, and control subjects, cultured under normal and hyperglycemic conditions. CAT, CuZnSOD, and GPX mRNA expression was significantly different between the study groups ($P < 0.001$). A: The mean fold increase of CAT mRNA in PBMCs from patients with nephropathy, patients without complications, and control subjects under normal and hyperglycemic conditions. Patients with nephropathy versus control subjects, $P < 0.001$. B: The mean fold increase of CuZnSOD mRNA in PBMCs from patients with nephropathy, patients without complications, and control subjects under normal and hyperglycemic conditions. Patients with nephropathy versus patients without complications, $P < 0.001$; versus control subjects, $P < 0.001$. C: The mean fold increase of GPX mRNA in PBMCs from patients with nephropathy, patients without complications, and control subjects under normal and hyperglycemic conditions. Patients with nephropathy versus patients without complications, $P = 0.002$; versus control subjects, $P < 0.001$. D: The mean fold increase of MnSOD mRNA in PBMCs from patients with nephropathy, patients without complications, and control subjects under normal and hyperglycemic conditions. Patients with nephropathy versus control subjects, $P = 0.01$.

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 $10 \mu\text{mol/l}$ 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 ($P < 0.05$): 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

TABLE 3

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

	CAT	CuZnSOD	GPX	MnSOD	ALR2
Z - 2/X ($n = 15$)	$0.4 \pm 0.04^*$	$0.3 \pm 0.02^{\ddagger}$	$0.4 \pm 0.03^{\S}$	0.8 ± 0.02	4.7 ± 0.8
Z + 2/Y ($n = 3$)	0.6 ± 0.08	1.7 ± 0.47	0.6 ± 0.05	0.8 ± 0	2.0 ± 0.5
Z - 2/Z + 2 ($n = 3$)	0.5 ± 0.05	1.3 ± 1.05	0.4 ± 0	0.6 ± 0.2	4.4 ± 3.6
X/Y ($n = 5$)	0.6 ± 0.05	0.5 ± 0.07	0.7 ± 0.08	0.9 ± 0.02	3.4 ± 1.1

Data are mean fold increases \pm 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, $P = 0.02$. \dagger versus CuZnSOD Z + 2/Y, $P < 0.001$. \ddagger versus CuZnSOD Z - 2/Z + 2, $P = 0.009$. \S 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 insult 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-2 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

1. The Diabetes Control and Complications Trial Research Group: The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 329:977-986, 1993
2. U.K. Prospective Diabetes Study: Intensive blood glucose control with sulphonylurea or insulin compared with conventional treatment and the risk of complications in patients with type 2 diabetes. *Lancet* 352:837-853, 1998
3. Baynes JW: Role of oxidative stress in development of complications in diabetes. *Diabetes* 40:405-412, 1991
4. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813-820, 2001
5. Salahudeen AK, Kanji V, Reckelhoff JF, Schmidt AM: Pathogenesis of diabetic nephropathy: a radical approach. *Nephrol Dial Transpl* 12:664-668, 1997
6. Guigliano D, Ceriello A, Paolisso G: Oxidative stress and diabetic vascular complications. *Diabetes Care* 19:257-267, 1996
7. Mohanty P, Hamouda W, Garg R, Aljada A, Ghanim H, Dandona P: Glucose challenge stimulates reactive oxygen species (ROS) generation by leukocytes. *J Clin Endo Metab* 85:2970-2973, 2000
8. Trachtman H: Vitamin E prevents glucose-induced lipid peroxidation and increased collagen production in cultured rat mesangial cells. *Microvasc Res* 47:232-239, 1994
9. Ceriello A, Russo P, Amstad P, Cerutti P: High glucose induces antioxidant enzymes in human endothelial cells in culture. *Diabetes* 45:471-477, 1996
10. Ha H, Lee HB: Reactive oxygen species as glucose signaling molecules in mesangial cells cultured under high glucose. *Kidney Int* 58 (Suppl. 77):S19-S25, 2000
11. Trachtman H, Futterweit S, Maesaka J, Valderrama E, Fuchs A: Taurine ameliorates chronic streptozotocin-induced diabetic nephropathy in rats. *Am J Physiol* 269:F429-F438, 1995
12. Craven PA, Derubertis FR, Kagan VE, Melhem M, Studer RK: Effects of supplementation with vitamin C or E on albuminuria, glomerular TGF- β , and glomerular size in diabetes. *J Am Soc Nephrol* 8:1405-1411, 1997
13. Koya D, Lee I-K, Ishiichi H, Hanoh H, King GL: Prevention of glomerular dysfunction in diabetic rats by treatment of d- α -tocopherol. *J Am Soc Nephrol* 8:426-435, 1997
14. Mullerkey CJ, Edelstein D, Brownlee M: Free radical generation by early glycation products: a mechanism for accelerated atherogenesis in diabetes. *Biochem Biophys Res Commun* 173:932-939, 1990
15. Ceriello A, Quatraro A, Guigliano D: New insights on non-enzymatic glycosylation may lead to therapeutic approaches for the prevention of diabetic complications. *Diabet Med* 9:297-299, 1992
16. Hamada Y, Araki N, Koh N, Nakamura J, Horiuchi S, Hotta N: Rapid formation of advanced glycation end products by intermediate metabolites of glycolytic pathway and polyol pathway. *Biochem Biophys Res Commun* 228:539-543, 1996
17. Derubertis FR, Craven PA: Activation of protein kinase C in glomerular cells in diabetes: mechanisms and potential links to the pathogenesis of diabetic glomerulopathy. *Diabetes* 43:1-8, 1994
18. Williamson JR, Chang K, Frangos M, Hasan KS, Ido Y, Kawamura T, Nyengaard JR, Van den Enden M, Kilo C, Tilton RG: Hyperglycaemic pseudohypoxia and diabetic complications. *Diabetes* 42:801-813, 1993
19. Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, Wu J, Brownlee M: Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc Natl Acad Sci U S A* 97:12222-12226, 2000
20. Michiels C, Raes M, Toussaint O, Remacle J: Importance of Se-glutathione

- peroxidase, catalase and Cu/Zn SOD for cell survival against oxidative stress. *Free Radic Biol Med* 17:235–248, 1994
21. Ceriello A, Morocutti A, Mercuri F, Quagliaro L, Moro M, Damante G, Viberti GC: Defective intracellular antioxidant enzyme production in type 1 diabetic patients with nephropathy. *Diabetes* 49:2170–2177, 2000
 22. Ko BCB, Lam KSL, Wat NMS, Chung SSM: An AC (n): dinucleotide repeat polymorphic marker at the 5' end of the aldose reductase gene is associated with early onset diabetic retinopathy in NIDDM patients. *Diabetes* 44:727–732, 1995
 23. Heesom AE, Hibberd ML, Millward BA, Demaine AG: A polymorphism at the 5' end of the aldose reductase gene is strongly associated with the development of diabetic nephropathy in type 1 diabetes. *Diabetes* 46:287–291, 1997
 24. Moczulski DK, Scott L, Antonellis A, Rogus JJ, Rich SS, Warram JH, Krolewski AS: Aldose reductase gene polymorphisms and susceptibility to diabetic nephropathy in type 1 diabetes mellitus. *Diabet Med* 17:111–118, 2000
 25. Demaine AG, Cross DF, Millward A: Polymorphisms of the aldose reductase gene and susceptibility to retinopathy in type 1 diabetes mellitus. *Invest Ophthalmol Vis Sci* 41:4064–4068, 2000
 26. Heesom AE, Millward BA, Demaine AG: Susceptibility to diabetic neuropathy in patients with insulin-dependent diabetes mellitus is associated with a polymorphism at the 5' end of the aldose reductase gene. *J Neurol Neurosurg Psychiatry* 64:213–216, 1998
 27. Kao Y-L, Donaghue K, Chan A, Knight J, Silink M: A novel polymorphism in the aldose reductase gene promoter region is strongly associated with diabetic retinopathy in adolescents with type 1 diabetes. *Diabetes* 48:1338–1340, 1999
 27. Shah VO, Scavini M, Nikolic J, Sun Y, Vai S, Griffith JK, Dorin RI, Stidley C, Yacoub M, Vander Jagt DL, Eaton RP, Zager PG: Z-2 microsatellite allele is linked to increased expression of the aldose reductase gene in diabetic nephropathy. *J Clin Endocrinol Metab* 83:2886–2891, 1998
 28. Oates PJ, Mylari BL: Aldose reductase inhibitors: therapeutic implications for diabetic complications. *Exp Opin Invest Drugs* 8:2095–2119, 1999
 29. Ikegishi Y, Tawata M, Aida K, Onaya T: Z-4 allele upstream of the aldose reductase gene is associated with proliferative retinopathy in Japanese patients with NIDDM and elevated luciferase gene transcription in vitro. *Life Sci* 65:2061–2070, 1999
 30. Hodgkinson AD, Sondergaard KL, Yang B, Cross DF, Millward BA, Demaine AG: Aldose reductase expression is induced by hyperglycaemia in diabetic nephropathy. *Kidney Int* 60:211–218, 2001
 31. Lee AY, Chung SS: Contributions of polyol pathway to oxidative stress in diabetic cataract. *FASEB J* 13:23–30, 1999
 32. Gupta S, Chough E, Daley J, Oates PJ, Tornheim K, Ruderman NB, Keaney JF Jr: Hyperglycaemia increases superoxide anion production in rabbit endothelium leading to decreased Na⁺K⁺-ATPase activity. *Am J Physiol* 282:C560–C566, 2001
 33. Frayn KN, Langin D, Holm C, Belfrage P: Hormone-sensitive lipase: quantitation of enzyme activity and mRNA level in small biopsies of human adipose tissue. *Clin Chim Acta* 216:183–189, 1993
 34. Nishikawa T, Edelstein D, Du XL, Yamagishi S, Matsumura T, Kaneda Y, Yorek MA, Beebe D, Oates PJ, Hammes HP, Giardino I, Brownlee M: Normalising mitochondrial superoxide production blocks three pathways of hyperglycaemia damage. *Nature* 404:787–790, 2000
 35. Zou X, Lu J, Pan C: Effect of polymorphism of (AC)_n in the 5'-end of aldose reductase gene on erythrocyte aldose reductase activity in patients with type 2 diabetes mellitus. *Clin J Endocrinol Metab* 16:346–349, 2000
 36. Oates PJ: The polyol pathway and diabetes peripheral neuropathy. In *International Review of Neurobiology*. Vol. 50. Tomlinson DR, Ed. New York, Elsevier, 2002, p. 325–392
 37. Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R, Channon KM: Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation* 105:1656–1662, 2002
 38. Craven PA, Melhem MF, Phillips SL, DeRubertis FR: Overexpression of Cu²⁺/Zn²⁺ superoxide dismutase protects against early diabetes glomerular injury in transgenic mice. *Diabetes* 50:2114–2125, 2001
 39. Iso K, Tada H, Kuboki K, Inokuchi T: Long-term effect of epalrestat, an aldose reductase inhibitor, on the development of incipient diabetic nephropathy in type 2 diabetic patients. *J Diabetes Complications* 15:218–223, 2001