Overexpression of Cu\(^{2+}/\text{Zn}^{2+}\) Superoxide Dismutase Protects Against Early Diabetic Glomerular Injury in Transgenic Mice

Patricia A. Craven, Mona F. Melhem, Stephen L. Phillips, and Frederick R. DeRubertis

Ex vivo and in vitro observations implicate superoxide as a mediator of cell injury in diabetes, but in vivo evidence is lacking. In the current studies, parameters of glomerular injury were examined in hemizygous non-diabetic transgenic mice (SOD) and streptozotocin-diabetic (D) transgenic mice (D-SOD), which overexpress human cytoplasmic Cu\(^{2+}/\text{Zn}^{2+}\) superoxide dismutase (SOD-1), and in corresponding wild-type littermates (WT, D-WT) after 4 months of diabetes. In both SOD and D-SOD mice, renal cortical SOD-1 activity was twofold higher than values in the WT mice; blood glucose and glycosylated hemoglobin (GHB) levels did not differ in the two diabetic groups. Urinary albumin excretion, fractional albumin clearance, urinary transforming growth factor-β (TGF-β) excretion, glomerular volume, glomerular content of immunoreactive TGF-β, and collagen α1 (IV) and renal cortical malondialdehyde (MDA) levels were significantly higher in D-WT mice compared with corresponding values in D-SOD mice. Glomerular volume, glomerular content of TGF-β and collagen IV, renal cortical MDA, and urinary excretion of TGF-β in D-SOD mice did not differ significantly from corresponding values in either the non-diabetic SOD or WT mice. In separate groups of mice studied after 8 months of diabetes, mesangial matrix area, calculated as a fraction of total glomerular tuft area, and plasma creatinine were significantly higher in D-WT but not in D-SOD mice, compared with corresponding values in the non-diabetic mice. In vitro infection of mesangial cells (MC) with a recombinant adenovirus encoding human SOD-1 increased SOD-1 activity threefold over control cells and prevented the reduction of aconitase activity, an index of cellular superoxide, and the increase in collagen synthesis that otherwise occurred in control MC in response to culture with 300 or 500 mg/dl glucose. Thus, increases in cellular SOD-1 activity attenuate diabetic renal injury in vivo and also prevent stimulation of MC matrix protein synthesis induced in vitro by high glucose. Diabetes 50: 2114–2125, 2001

Increased oxidative and glyco-oxidative stress has been implicated in the pathogenesis of diabetic complications, including nephropathy (1–16). Increases in oxidized lipoproteins, red cell membrane lipid peroxidation, advanced glycation end products, enhanced production of free radicals by circulating granulocytes and macrophages, and other markers of oxidative stress have been documented in blood and tissues of human and experimental diabetic subjects (1–7). Although multiple oxidant moieties may participate, there is in vitro and ex vivo evidence to support a role for superoxide anion in the pathogenesis of vascular dysfunction, lipid peroxidation, and formation of glyco-oxidation products in diabetes (7, 17–28). Superoxide is the first product of the univalent reduction of oxygen (29). It is converted to hydrogen peroxide and oxygen by the superoxide dismutase system, which constitutes the first line of defense against the deleterious effects of reactive oxygen species (29). In vitro or ex vivo studies also have shown that exogenous superoxide dismutase attenuates increases in endothelial albumin permeability, vascular dysfunction, lipid peroxidation, and formation of glyco-oxidation products induced by diabetes and/or high concentrations of glucose (18–23). With regard to kidney, recent studies demonstrated enhanced superoxide production ex vivo by renal cortex from streptozotocin (STZ)-diabetic rats (24). Increases in superoxide production also have been demonstrated in vitro in vascular endothelial and smooth muscle cells and glomerular mesangial cells (MC) in response to high concentrations of glucose and/or angiotensin II (25–28), two factors clearly implicated in the pathogenesis of diabetic nephropathy (30, 31). Although these and other observations implicate superoxide in the mediation of renal and vascular injury in diabetes, direct in vivo evidence for such a role is lacking. In the present studies, we examined the effects on parameters of early glomerular injury of overexpression of cytosolic Cu\(^{2+}/\text{Zn}^{2+}\) superoxide dismutase (SOD-1) in a transgenic mouse model in which diabetes was induced with STZ. We also conducted in vitro studies to assess 1) whether the stimulation of matrix protein synthesis that occurs in MC in response to culture with a high concentration of glucose is associated with an increase in cellular superoxide and 2) whether these glucose responses can be suppressed by overexpression of SOD-1.
RESEARCH DESIGN AND METHODS

Treatment of mice. Hemizygous SOD-1 transgenic mice (SOD) on a C57BL/J6 background, which express the human SOD-1 transgene driven by the human SOD-1 promoter, and their nontransgenic littermates (WT) were obtained from Jackson Laboratories (Bar Harbor, ME). At 6–8 weeks of age, the mice received an intraperitoneal injection of either STZ (100 mg/kg in 100 µl of 0.1 M citrate buffer, pH 4.0) or the citrate vehicle at 0 and 2 days. Glucose was determined on blood obtained from the retro-orbital venous plexus 2 days after the second injection and after each subsequent injection using a glucometer (Diastasc-S; Home Diagnostics, Eatontown, NJ). Mice with blood glucose levels of ≥300 mg/dl were entered into the study protocol as diabetics. When blood glucose was <300 mg/dl in mice after the second injection of STZ, the injection was repeated. On average, three injections of STZ were required to induce a blood glucose of ≥300 mg/dl in both WT and SOD mice. Mice were placed into one of four study groups: 1) nondiabetic WT, 2) diabetic (DT-WT), 3) nondiabetic SOD, and 4) D-SOD. Mice were maintained on ad libitum intake of water and standard mouse diet. Blood glucose levels were obtained at monthly intervals. During the week before the mice were killed, when urine was collected, mice were housed in metabolic cages and given free access to a liquid diet (Ensure; Abbott Laboratories, Columbus, OH) and water but no solid food for 48 h. The liquid diet increased urine volume and reduced contamination of the sample with feces and food. Urine was collected for 24 h during the final 2 days before the mice were killed, and an aliquot was frozen for subsequent determination of albumin and [14C]inulin. Inulin clearance (Cin) was determined during this period in conscious unrestrained mice from the clearance of [14C]inulin given by osmotic minipump implanted subcutaneously as described previously (32). Blood was collected in heparinized tubes. Plasma from plasma, albumin, and transforming growth factor-β (TGF-β) at the conclusion of the urine collection. Erythrocyte fraction was used for subsequent determination of GHB. Mice were killed by exsanguination 4 months after entry into the study protocol. Determination of creatinine, albumin, TGF-β, and renal cortical malondialdehyde. Plasma creatinine was determined with a kit obtained from Sigma Chemical (St. Louis, MO). Total (active plus latent) plasma and urinary TGF-β levels were determined after acid activation with the use of a sandwich enzyme-linked immunosorbent assay (ELISA) kit obtained from Genzyme (Boston, MA). TGF-β was not detectable in plasma or urine samples before acid activation. GHB was determined on erythrocyte hemolyzates with the use of a kit obtained from Sigma Chemical. The method uses an affinity resin column that separates GHB from non-GHB. GHB and non-GHB are quantified by absorbance at 415 nm, and GHB is expressed as a percentage of total hemoglobin.

Renal cortical malondialdehyde (MDA) was determined in quick-frozen samples of renal cortex after extraction in 1.15% KCl as described previously (14). Samples were reacted with thiobarbituric acid and heated at 95°C for 60 min. After extraction with n-butanol and pyridine (15:1 vol/vol), absorbance of the organic layer was measured at 532 nm; 1,2,3,9-tetramethoxyxipropene (MDA) was used as a standard.

Albumin was determined by an ELISA as described previously (32). Rabbit anti-rat albumin IgG fraction and peroxidase-conjugated rabbit anti-rat albumin were obtained from ICN Pharmaceuticals (Aurora, OH). Heat-inactivated normal rabbit serum (10%) was used as a blocker. Standard curves were linear between 40 and 0.5 ng of mouse albumin per well. Inulin and albumin clearance were determined by conventional methods. Fractional albumin clearance was calculated as the ratio of albumin to inulin clearance.

Immunohistochemical staining for TGF-β and collagen α1 (IV). Renal cortical sections (5 µm) for TGF-β staining were fixed in buffered formalin and blocked for 60 min. They then were incubated overnight at 4°C with 50 µg/ml affinity-purified polyclonal rabbit anti-TGF-β antibody (B & D Systems, Minneapolis, MN). This antibody reacts with TGF-β1, -β2, -β1/2, -β3, and -β5. Of these, TGF-β1, -β2, and -β3 have been reported to be produced by rat MC (33). Frozen renal cortical sections (5 µm) were used for assessment of collagen α1 (IV). Rabbit anti-mouse collagen α1 (IV) antibodies were obtained from Chemicon International (Temecula, CA). Nonspecific staining was assessed by replacing the primary antibody with affinity-purified, nonimmune, rabbit IgG (R & D Systems, Minneapolis, MN). Sections were washed and dehydrated through the standard protocol and stained with the diaminobenzidine tetrahydrochloride (DAB) and hematoxylin-eosin methods. The area and intensity of positive renal cortical immunohistochemical staining for TGF-β and collagen α1 (IV) was assessed quantitatively with a SAMBA 4000 image analyzer (Image Products International, Chantilly, VA) using specialized computer software (Immunohistochemistry Analysis, version 1.4; Microsoft, Richmond, WA), a color video camera, and a Compag computer. The image analyzer is an integrated system of Windows-based software (Microsoft, Redmond, WA) for densitometric, morphometric, and image analysis that is capable of quantitative image analysis of cells and tissues. Software designed for immunostaining analysis enabled the operator to set a density threshold value by averaging several fields on the negative control tissues in which the primary antibody was replaced by isotopic control IgG. Background subtraction then was performed automatically on every tissue. The slides were examined at 400× magnification. Twenty glomeruli per mouse were selected randomly and assessed for the area and intensity of staining. Using the manual mode and outlining the glomerular tuft, the instrument assessed the size of the glomerulus and the amount of immunostaining within the glomerular tuft. Current results are reported as a labeling index, which represents the percentage of total glomerular tuft area examined that stained positively. Staining intensity of positive areas (mean optical density) also was assessed by quantitative image analysis. A quick score then was calculated for each glomerulus examined (labeling index × mean optical density). Quick scores and labeling indexes gave analogous results with respect to differences observed among the study groups.

Glomerular volume and mesangial matrix fraction. Formalin-fixed outer-cortical sections were used for determination of glomerular volume (VG). The cross-sectional area of the glomerular tuft was determined on glomeruli that had been cut randomly at the vascular pole, as reported previously (15,32,34). The glomerular tuft area was measured with a digital planimeter using a SAMBA 4000 image analyzer. VG was calculated from glomerular tuft area using the formula $V_G = \frac{B}{k(AG)^{3/2}}$, where AG is cross-sectional areas of the glomerular tuft, B = 1.38 is the shape coefficient for spheres, and k = 1.1 is a size distribution coefficient. Mesangial matrix fraction was determined on the same formalin-fixed sections used to assess total glomerular tuft volume. Periodic acid–Schiff–positive material was measured with a digital planimeter and red-green-blue to hue-saturation-intensity colorimetric analysis of cells. Mesangial matrix fraction was expressed as a function of total glomerular tuft area.

Immunohistochemical staining for SOD-1. A mouse monoclonal anti-human SOD-1 antibody was obtained from Sigma Chemical. The antibody did not react with mouse SOD-1. Renal cortical segments were fixed in buffered formalin, and sections were prepared and blocked for 60 min. They were then incubated with the SOD-1 antibody (1,000-fold dilution) for 20 min. Sections were washed and developed further with a Dako LSAB2 kit. To assess human SOD-1 protein content of cultured MC (see below), cells were grown to 80% confluence on an eight-well chamber slide. Medium was aspirated and cells were fixed by immersion for 5 min in cold acetone. Cells were preincubated with 0.3% H2O2 to block endogenous peroxidase activity. The slides then were rinsed with phosphate-buffered saline, blocked, and incubated with a 2,000× dilution of the primary antibody anti-human SOD-1 for 20 min. Cells were washed and developed further with a Dako LSAB2 kit. Renal cortical tissue was used to determine the sensitivity of the SOD-1 antibody in mesangial matrix fraction and immunohistochemical staining were examined in a coded manner so that the pathologist did not know the study group from which a section came.

Culture of SV-40–transformed mouse MC. SV-40–transformed mouse MC (MES-13) cells were obtained from the American Type Culture Collection and maintained in RPMI 1640, containing 5% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 100 mg/dl glucose at 37°C in an atmosphere of 5% CO2, 95% air. Cells were passed three times per week. Where indicated in the text, the concentration of glucose in the medium was raised to 500 mg/dl for 2 weeks before study.

Adenovirus infections. Recombinant adenovirus containing a cytomegalovirus (CMV) promoter–driven gene encoding human SOD-1 (SOD-1-Ad) were obtained from Gene Transfer Vector Core (University of Iowa, Iowa City, IA). The adenovirus containing a CMV promoter–driven gene encoding green fluorescent protein (GFP-Ad) was a gift from the laboratory of Dr. Paul Robbins, Department of Medicine, University of Pittsburgh (Pittsburgh, PA). Virus preparations contained 1012 particles/ml. Infectivity of each of the preparations was estimated to be 1%. The virus preparations were diluted 10× in 10 mmol/l Tris-Cl (pH 7.5), 0.15 mol/l NaCl, 10 mmol/l MgCl2, 3% sucrose just before use. Cells were seeded in six-well plates in complete medium and allowed to reach 50% confluence. Medium then was changed to serum-free, and 40 µl of the diluted virus preparations was added to each well. Cells were diluted to achieve a multiplicity of infection of 80–100 infectious particles/cell. After 1.5 h, the medium was replaced with virus-free medium containing serum and the incubations were continued for 3 days. As assessed by either green fluorescence or positive immunohistochemical staining for human SOD-1 protein, >95% of cells were infected.
Preparation of homogenates for determination of enzyme activities in renal cortex or MES-13 cells. Quick-frozen renal cortex (50 mg) was homogenized on ice in 1 ml of 0.05 mol/l sodium phosphate buffer (pH 7.4) containing 1 × 10−4 mol/l EDTA using a Tissumizer (Tekmar, Cincinnati, OH). For preparing homogenates of MES-13 cells, culture medium was aspirated and cells were scraped into 0.5 ml/well of 0.005 mol/l sodium phosphate buffer (pH 7.4) containing 10−4 mol/l EDTA and sonicated with a Fisher Model 300 probe sonicator at the highest setting for 3-s bursts on ice. Homogenates of renal cortex or MES-13 cells were centrifuged at 700g for 10 min at 4°C, and supernatants were used for assays described below.

Assay of SOD-1 activity. Total SOD-1 activity was determined by its ability to inhibit the initial rate of 6-hydroxydopamine auto-oxidation as described previously (35). A standard curve was generated in the range of 25–200 ng/ml bovine erythrocyte SOD-1, and enzyme activity in the cortical homogenates was calculated by comparison with the standard curve. The proportion of total activity that could be inhibited by 5 mmol/l cyanide was taken to be SOD-1 activity. Mitochondrial Mn2+ superoxide dismutase (SOD-2) activity is not cyanide-sensitive (35).

Assays of glutathione peroxidase and catalase activity. Glutathione peroxidase activity of renal cortical homogenates was determined as described previously (36) from the absorbance change at 340 nm as a result of the oxidation of reduced glutathione by glutathione peroxidase, coupled to the disappearance of NADPH by glutathione reductase. Glutathione peroxidase activity is defined as the amount of enzyme that results in oxidation of 1 nmol of NADPH/min. Catalase activity was assayed spectrophotometrically as described previously (37). A unit of catalase activity is defined as the amount of enzyme that consumes 1 nmol of H2O2/min.

Assay of aconitase activity. The linear absorbance increase as a result of reduction of NADP+ was followed for 60 min at 340 nm at 25°C in a 1.0-ml reaction mixture containing 50 mmol/l Tris-Cl (pH 7.4), 5 mmol/l sodium citrate, 0.6 mmol/l MgCl2, 0.2 mmol/l NADP+, 1–2 units of isocitrate dehydrogenase, and 25–75 µg of extract protein (38). A unit of enzyme activity is defined as the amount of enzyme that generates 1 nmol of NADPH/min.

Collagen synthesis. Collagen synthesis was determined as described previously (39). Briefly, cells were seeded in six-well plates and harvested 4 days later. Additions were made for the times indicated in the text. 1-Ascorbic acid (50 µmol/ml) was added to the cells 24 h before harvest. Media were changed to fresh complete media containing 50 µmol/ml 1-ascorbic acid, 80 µg/ml β-aminopropionitrile, and 1 µmol/well [3H]proline for the final 6 h of incubation. At the end of the incubation, media were removed and cells were lysed by addition of 1 ml of H2O. Cells then were scraped and sonicated three times for 1 s each. Proteins were precipitated on ice at a final concentration of 10% trichloroacetic acid, and the supernatants were centrifuged at 1,250 g for 10 min at 4°C, and the pellets were washed with cold 10% trichloroacetic acid until the wash was free of radioactivity. They were then dissolved in 1.5 ml of 0.1N NaOH. The pH of the samples was adjusted to 7.4 with 1 mol/l HEPES. The resuspended pellet was then mixed with an equal volume of 0.1 mol/l Tris, 15 mmol/l CaCl2, 4 mmol/l Na-ethylmaleimide and incubated in the presence and absence of 40 units/ml collagenase VII for 90 min at 37°C. At the end of the incubation, carrier albumin was added to a final concentration of 0.5%. proteins were precipitated on ice with 5% trichloroacetic acid, samples were centrifuged, and the supernatants were counted. Counts released into the supernatant in the absence of collagenase were subtracted from the counts released by collagenase digestion. Results shown represent labeled proline incorporation into collagenase-sensitive protein as a function of total cellular protein.

Statistical analysis. Significance of differences was determined by analysis of variance followed by the Fisher’s multiple comparison test using Statview software. Data for male and female mice within each group were first analyzed separately. No significant differences were observed as a function of sex; accordingly, data from all mice within each study group were pooled for purposes of final statistical analyses.

RESULTS

As illustrated in Fig. 1, renal cortical SOD-1 specific activity of SOD mice was twofold higher than activity in WT mice. The diabetic state had no effect on renal cortical SOD-1 activity of either SOD or WT mice. Renal cortical SOD-2 specific activity did not differ among the four study groups (mean ± SE, 0.14 ± 0.02 µg/mg protein in WT mice). In WT and D-WT mice, SOD-1 and SOD-2 constituted ~89 and 11% of total renal cortical superoxide dismutase activity, respectively. In SOD and SOD-D mice, renal cortical SOD-1 and SOD-2 constituted ~95 and ~4% of total superoxide dismutase activity, respectively. As also illustrated in Fig. 1, renal cortical glutathione peroxidase specific activity was not different in SOD and D-SOD mice. Renal cortical glutathione peroxidase activity was slightly (25%) but significantly higher in D-WT, compared with values in the WT mice. Renal cortical catalase specific activity did not differ significantly among the four study groups (mean ± SE, 238 ± 36 units/mg protein in WT mice).

As illustrated in Fig. 2, immunohistochemical staining of renal cortex for human SOD-1 detected protein in glomerular endothelial and tubular epithelial cells of SOD mice. No renal cortical staining was observed in sections from WT or D-WT mice or when control rabbit IgG replaced the primary anti–SOD-1 antibody in assessment of renal cortex from SOD mice (Fig. 2). The renal cortical pattern of immunohistochemical staining for human SOD-1 protein was analogous in SOD and D-SOD mice.

Table 1 shows values for blood glucose, GHb, body and kidney weights, CIN, plasma creatinine, and renal cortical MDA in the four study groups. Mean blood glucose and GHb values were higher in two diabetic groups compared with either control group. However, neither the mean blood glucose nor GHb level of D-WT differed significantly from the corresponding value in the D-SOD group. Body weight did not differ significantly among the study groups. Kidney weight was higher in all diabetic groups compared with nondiabetic groups but did not differ between the D-SOD and D-WT study groups. As illustrated in Table 1, CIN did not differ between the WT and D-WT mice. By contrast, CIN was higher in D-SOD mice when compared with values either for SOD mice or for WT or D-WT mice. Plasma creatinine did not differ significantly among the four study groups but was modestly lower in D-SOD mice compared with values for nondiabetic WT or SOD or those of D-WT mice, consistent with the higher CIN in SOD-D mice. As also shown in Table 1, renal cortical MDA content was higher in D-WT versus WT mice. By contrast, values for MDA in renal cortex of D-SOD mice were lower than those of D-WT and did not differ significantly from those in nondiabetic WT or SOD mice.
As shown in Fig. 3, top, 24-h urinary albumin excretion (UAE) in SOD mice was significantly lower than values in the age-matched WT littermates at ~6 months of age. By contrast, UAE in D-SOD mice did not differ from values in either of the nondiabetic groups and was significantly smaller than $V_G$ of D-WT mice. Mesangial matrix fraction, expressed as a function of total glomerular tuft area, did not differ significantly among the four study groups (WT $0.08 \pm 0.01$; D-WT $0.12 \pm 0.02$; SOD $0.09 \pm 0.01$; D-SOD $0.10 \pm 0.01$). Thus, the increase in $V_G$ observed in D-WT reflected predominately glomerular cell hypertrophy that is characteristic of early diabetes, rather than the mesangial matrix expansion seen later in the course of the disorder.

Figure 5 illustrates glomerular staining for immunoreactive TGF-$\beta$ in representative sections of renal cortex obtained from the four study groups of mice, whereas Fig. 6 compares glomerular staining for TGF-$\beta$ as assessed by quantitative image analysis. Only faint glomerular staining for TGF-$\beta$ was observed by visual inspection in sections from either of the nondiabetic groups or those from D-SOD mice when compared with tissue sections that had been incubated with control IgG rather than primary anti–TGF-$\beta$ antibody (Fig. 5). However, obvious immunostaining for TGF-$\beta$ was observed in the mesangium and in epithelial cells of glomeruli from D-WT mice in cortical sections incubated with anti–TGF-$\beta$ antibody (Fig. 5). As assessed by quantitative image analysis (Fig. 6), the labeling index for TGF-$\beta$ staining was markedly higher in glomeruli of the D-WT mouse compared with that of any of the other study groups, including that of D-SOD mice. The labeling index for TGF-$\beta$ staining of glomeruli of D-SOD mice was not different from that of the SOD or WT mice.

As shown in Table 2, total TGF-$\beta$ concentration in plasma, as assessed by ELISA assay after acid activation, did not differ among the four study groups. Consistent with results obtained with immunohistochemical assessment of the TGF-$\beta$ content of renal cortex, urinary TGF-$\beta$ excretion was markedly higher in D-WT than in any of the other study groups. Urinary TGF-$\beta$ in D-SOD did not differ significantly from values in either nondiabetic group (Table 2).

Figure 7 illustrates the distribution of immunoreactive collagen $\alpha_1$ (IV) in representative sections of renal cortex obtained from the four groups of mice, whereas assessment of collagen IV staining in the four groups by quantitative image analysis is shown in Fig. 8. Predominant basement membrane staining for collagen IV was observed in sections from WT or SOD mice (Fig. 7). No differences were noted between the last two groups by visual inspection. D-WT mice had increased staining for collagen IV in basement membrane and mesangium compared with that of all other study groups. The staining of glomeruli from the D-SOD mice was not distinguishable from that of SOD or WT mice and was markedly reduced compared with values in the D-WT mice, as assessed by visual inspection (Fig. 7). Quantitative image analysis confirmed that glomerular collagen IV staining area was significantly greater in glomeruli of D-WT compared with the values in the D-SOD mice; the latter value was not different from staining in either of the two nondiabetic groups.
As noted above, mesangial matrix expansion was not apparent after 4 months of diabetes in either D-WT or D-SOD. Accordingly, this parameter was assessed after 8 months of diabetes in these two groups and in the corresponding age-matched nondiabetic control groups. Mesangial matrix fraction in D-WT mice at 8 months was significantly greater than corresponding values in either age-matched nondiabetic WT or SOD (Fig. 9). By contrast in D-SOD, mesangial matrix fraction did not differ from the values in the nondiabetic mice (Fig. 9). Similarly, plasma creatinine in D-WT (mean ± SEM, 0.71 ± 0.1 mg/dl) was significantly increased (P < 0.05; n = 9 mice per group) compared with values in D-SOD (0.47 ± 0.08 mg/dl), nondiabetic WT (0.50 ± 0.09 mg/dl), or SOD (0.43 ± 0.07 mg/dl) groups. The increases in mesangial matrix fraction and plasma creatinine observed in D-WT compared with D-SOD were not attributable to differences in the magnitude of hyperglycemia between these two groups after 8 months of diabetes. Thus, percent GHb in D-WT (8.5 ± 1.0) and D-SOD (8.8 ± 1.0) at 8 months did not differ. Percent GHb in each diabetic group at this time was significantly higher than corresponding values in either nondiabetic WT (5.2 ± 0.7) or SOD (4.9 ± 0.6).

Figure 10 shows SOD-1 immunoreactivity in MES-13 cells that had been infected with SOD-1-Ad or GFP-Ad and cultured in media containing 100 mg/dl glucose. As illustrated in Fig. 10A, diffuse staining for SOD-1 is seen in the cytoplasm of cells that had been infected with the SOD-1-Ad and reacted with SOD-1 antibody. No staining is seen in cells that had been infected with GFP-Ad and exposed to the SOD-1 antibody (Fig. 10B) or in cells that had been infected with the SOD-1-Ad and incubated with control IgG (Fig. 10C). Results of staining were analogous in cells that had been cultured in media containing 500 mg/dl glucose.

Figure 11 illustrates SOD-1 activity in MES-13 cells that had been infected with SOD-1-Ad and cultured in 100 or 500 mg/dl glucose. Infection with SOD-1-Ad increased cellular SOD-1 activity threefold compared with that observed in either cells that had been infected with GFP-Ad (Fig. 11) or uninfected cells (not shown). As also illustrated in Fig. 11, culture in high-glucose media for 2 weeks had no effect on SOD-1 activity. SOD-2 activity did not differ significantly in MES-13 cells that had been infected with either GFP-Ad or SOD-1-Ad and also was not altered by changes in media glucose concentrations (not shown). In cells that had been infected with GFP-Ad and in uninfected cells, SOD-1 and SOD-2 represented 93 and 7% of total SOD activity.

### TABLE 1

<table>
<thead>
<tr>
<th>Blood glucose (mg/dl)</th>
<th>GHb (%)</th>
<th>Body wt (g)</th>
<th>Kidney wt (g/kg body wt)</th>
<th>C_{IN} (ml/min)</th>
<th>Plasma creatinine (mg/dl)</th>
<th>MDA (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nontransgenic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>168 ± 27</td>
<td>4.4 ± 0.5</td>
<td>24 ± 1</td>
<td>7.1 ± 0.2</td>
<td>0.21 ± 0.05</td>
<td>0.41 ± 0.07</td>
</tr>
<tr>
<td>Diabetic</td>
<td>335 ± 52*</td>
<td>7.3 ± 0.8*</td>
<td>23 ± 1</td>
<td>9.2 ± 0.8*</td>
<td>0.21 ± 0.02</td>
<td>0.43 ± 0.1</td>
</tr>
<tr>
<td><strong>SOD-1 transgenic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>159 ± 25*</td>
<td>4.6 ± 0.6</td>
<td>27 ± 1</td>
<td>6.3 ± 0.4</td>
<td>0.19 ± 0.02</td>
<td>0.39 ± 0.09</td>
</tr>
<tr>
<td>Diabetic</td>
<td>372 ± 49*</td>
<td>7.5 ± 0.8*</td>
<td>23 ± 1</td>
<td>9.6 ± 1*</td>
<td>0.32 ± 0.1†</td>
<td>0.55 ± 0.01</td>
</tr>
</tbody>
</table>

Data are means ± SE of determination from 14 mice in each group. Blood glucose values were determined in the fed state; five sequential, monthly measurements were obtained in each rat. GHb are expressed as % total hemoglobin. Inulin clearance was determined during the week before the mice were killed as described in RESEARCH DESIGN AND METHODS. *P < 0.05 vs. corresponding control group. †P < 0.05 vs. corresponding value in nontransgenic mice.
of total cellular SOD activity, respectively. In cells that had been infected with SOD-1-Ad, SOD-1 and SOD-2 represented 98 and 2% of total cellular SOD, respectively.

Figure 12 illustrates aconitase activity in MES-13 cells that had been infected with SOD-1-Ad or GFP-Ad and cultured in 100 versus 500 mg/dl glucose media. Culture in high-glucose media significantly reduced aconitase activity in cells that had been infected with GFP-Ad (Fig. 12) and in noninfected control cells (not shown). When cultured in low-glucose media, cells that had been infected with SOD-1-Ad had significantly higher aconitase activity than that observed in cells that had been infected with GFP-Ad. When cultured in high-glucose media, cells that had been infected with SOD-1-Ad did not demonstrate the decrease in aconitase activity observed in cells that had been infected with GFP-Ad (Fig. 12) or in noninfected cells. Cells that were cultured in 300 mg/dl glucose had values for aconitase activity that were significantly lower than those in cells that were cultured in 100 mg/dl glucose but not different from those in cells that were cultured in 500 mg/dl glucose (100 mg/dl glucose 7.6 ± 0.9 nmol · min⁻¹ · mg protein⁻¹; 300 mg/dl glucose 5.9 ± 1 nmol · min⁻¹ · mg
compared with value in corresponding controls. Although UAE and fractional albumin clearance were higher in D-SOD compared with SOD, these parameters were also significantly lower in D-SOD compared with corresponding values in D-WT and did not differ from those in age-matched nondiabetic WT. The lower UAE observed at 6 months of age in SOD mice compared with WT reflected a suppression in superoxide dismutase of the age-related increases in UAE known to occur in rodents (32,40). At 6–8 weeks of age, UAE was lower than the 6-month values in both of the nondiabetic groups and did not differ in WT versus SOD mice. Thus, amelioration of albuminuria by overexpression of SOD-1 was not specific to diabetes but also affected age-related increases. Perhaps, as suggested by earlier observations (13–16,40), this reflects involvement of oxidative mechanisms in the mediation of glomerular injury in both instances. Thus, as with diabetic glomerulopathy (13–16), antioxidant treatment attenuates injury and the associated glomerular accumulation of MDA and F₂ isoprostanes that occur with age in the rat (40).

Overexpression of SOD-1 did not prevent the increase in renal mass associated with diabetes. Glomerular enlargement per se, which was prevented in D-SOD, has been implicated as a pathogenetic factor in diabetic nephropathy (41). However, at least in STZ diabetes, attenuation of glomerular injury despite an overall increase in kidney weight, of which glomerular size is a minor determinant, was demonstrated previously (13,15,32). Similarly, C₁₂ was higher in D-SOD than in any other study group. The mechanism responsible for increased GFR in the D-SOD is not known. Of note, prevention of albuminuria and glomerulosclerosis in association with persistent glomerular hyperfiltration is well documented in STZ diabetes, including in earlier studies that used dietary antioxidant supplementation (14,15) or angiotensin-converting enzyme (ACE) inhibition (42). Increases in glomerular capillary pressure rather than hyperfiltration per se may be the key hemodynamic determinant of glomerular injury, as suggested by the studies of Zatz et al. (42). It is possible that in D-SOD, as with ACE inhibition (42), intraglomerular capillary pressure remained normal despite hyperfiltration.

Differences in plasma creatinine and mesangial matrix fraction between diabetic and nondiabetic mice were not evident after 4 months of diabetes. However, after 8 months of diabetes, plasma creatinine and mesangial matrix fraction were significantly higher in the D-WT but not in the D-SOD mice than the corresponding values in the age-matched nondiabetic WT and SOD groups. The mesangial expansion and decline in renal function observed in D-WT were also significant.

**TABLE 2**

<table>
<thead>
<tr>
<th></th>
<th>Plasma TGF-β (ng/ml)</th>
<th>Urinary TGF-β (ng/mg creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nontransgenic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>35 ± 4</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Diabetic</td>
<td>31 ± 5</td>
<td>31 ± 6*</td>
</tr>
<tr>
<td><strong>SOD-1 transgenic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>32 ± 3</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>Diabetic</td>
<td>37 ± 5</td>
<td>7 ± 1†</td>
</tr>
</tbody>
</table>

Data are mean ± SE of determination from 14 mice in each group. *P < 0.05 vs. corresponding control group; †P < 0.05 vs. corresponding value in nontransgenic mice.

**FIG. 6.** Assessment of the positive glomerular immunohistochemical staining for TGF-β by quantitative image analysis in control (□) and diabetic (■) mice. Twelve randomly selected glomeruli from the 14 mice in each study group were assessed with a SAMBA 4000 image analyzer. Results are means ± SE of the labeling index. *P < 0.05 compared with value in corresponding controls.

**DISCUSSION**

These results demonstrate that 4-month STZ-diabetic mice that overexpress SOD-1 are protected from early renal injury compared with their nontransgenic diabetic littermates. Thus, in D-SOD mice, V₆₅, glomerular content of TGF-β and collagen IV, urinary excretion of TGF-β, and renal cortical MDA were not different from corresponding values for these parameters in nondiabetic WT or SOD mice and were significantly lower than those in D-WT. Although UAE and fractional albumin clearance were higher in D-SOD compared with SOD, these parameters...
mice compared with D-SOD were not attributable to differences in the magnitude of hyperglycemia between the two diabetic groups, as reflected by their similar GHb levels at 8 months. The decline in renal function observed in D-WT mice in the present study was delayed compared with the non–insulin-treated STZ-diabetic rat model in which impairment of renal function has been reported as early as 2 months after induction of diabetes (43,44). Whether the more gradual decline in renal function and preservation of glomerular structure reflect a genetically determined relative resistance of the mouse strain used (C57BL/6J) to rapid progression of diabetic glomerular injury or to other factors is uncertain. Nevertheless, the modest increases in both creatinine and matrix fraction induced by 8 months of diabetes in WT mice clearly were attenuated in diabetic mice that overexpressed SOD-1, consistent with an effect of the latter to protect from progressive as well as early diabetic renal injury.

High concentrations of glucose and/or angiotensin II increase superoxide production by vascular endothelial and smooth muscle cells and by glomerular MC in vitro (25–28). Conversely, exogenous superoxide dismutase has

FIG. 7. Immunohistochemical staining for collagen α1 (IV) of representative cortical sections from nontransgenic control mice (A), nontransgenic diabetic mice (B), SOD-1–transgenic control mice (C), and SOD-1–transgenic diabetic mice (D). E: Nontransgenic diabetic mice with control rabbit IgG substituted for collagen IV antibody.
been shown to prevent increases in regional blood flow and endothelial permeability induced by diabetes and to decrease lipid peroxidation and the formation of glycoxidation products in response to high glucose in vitro (18–23). Recent studies reported increased ex vivo production of superoxide by renal cortex of STZ-diabetic rats (24). These findings are consistent with a possible role for increased superoxide production in the mediation of vascular and renal injury in diabetes. The superoxide dismutase system represents the first line of defense against cellular damage from superoxide (29). Eukaryotic cells synthesize three superoxide dismutase isoforms. Cytoplasmic Cu^2+ /Zn^2+ SOD-1 constitutes 85% or more of the total cellular superoxide dismutase activity of most mammalian cells (29,45). Mitochondrial Mn^2+ SOD-2 constitutes only 10–15% of total cellular superoxide dismutase activity (29,45) but plays a key role in protection of mitochondria from oxidative injury during the aerobic metabolism of glucose and other substrates (46). Moreover, recent studies in endothelial cells that were cultured in high-glucose media implicated superoxide derived from mitochondrial glucose oxidation as a mediator of several metabolic responses linked to cell injury in diabetes (47,48). Overexpression of SOD-2 prevented these metabolic responses (47,48). Of note, analogous to the effects of SOD-1 (Fig. 12), overexpression of SOD-2 also prevents increases in superoxide and collagen synthesis induced by high glucose in MC (49). Extracellular Cu^2+/Zn^2+ superoxide dismutase (EC-SOD) is genetically distinct from SOD-1.
and constitutes 1% or less of total superoxide dismutase activity in most tissues (29,45). However, it is the dominant form of superoxide dismutase in large blood vessels and may play a unique role in the protection of the microvasculature from oxidative injury (29). In the current study, renal cortical SOD-1 activity in the transgenic mice was twofold higher than values in renal cortex from WT mice, whereas renal cortical SOD-2 activities of these groups did not differ. Renal cortical SOD-1 was not specifically separated from EC-SOD activity in our studies, and the assay method used will detect both of these Cu²⁺/Zn²⁺-dependent activities (35). Nevertheless, it is unlikely that EC-SOD contributed significantly to the total renal cortical Cu²⁺/Zn²⁺ superoxide dismutase values reported, because the former constitutes 1% or less of total superoxide dismutase activity in mouse kidney (45).

It also is notable that the diabetic state did not detectably reduce renal cortical SOD-1 activity in either the D-SOD or the D-WT mice compared with values in the corresponding nondiabetic groups. Nonenzymatic glycation of SOD-1 in vitro has been demonstrated to inactivate SOD-1 (50,51), but the effects of diabetes on endogenous SOD-1 activities have been variable. SOD-1 activity is reduced in erythrocytes and granulocytes of patients with diabetes (52,53). However, in experimental diabetes, SOD-1 activity has been reported to be increased, decreased, or unchanged in different tissues between 8 days and 8 months after induction of diabetes (54–59). In homogenates of whole kidney from STZ-diabetic rats, a transient increase in SOD-1 activity was reported between 2 and 6 weeks after induction of diabetes (55) but was not different from that of age-matched nondiabetic rats after 10 weeks (59). The latter observation is consistent with the comparable renal cortical SOD-1 activities found in WT versus D-WT mice after 4 months of diabetes. Thus, the susceptibility of SOD-1 to inactivation by glycation or other processes in diabetes may be tissue- or species-specific.

The mechanisms by which overexpression of SOD-1 attenuates diabetic glomerular injury in the transgenic mice are uncertain. However, the observation that overexpression of SOD-1 prevented the increase in renal cortical MDA observed in the D-WT mice is consistent with both reduced renal oxidative stress in D-SOD and an antioxidant mechanism of renal protection. Because SOD-1 activity is overexpressed in multiple tissues (60), the protection observed against diabetic renal injury may reflect the influence of increases in renal SOD-1, extrarenal SOD-1, or both. Clearly, an increase in SOD-1 activity in multiple extrarenal sites, including those documented to occur in circulating erythrocytes and macrophages in SOD mice (60,61), could enhance overall antioxidant activity as well as contribute to local renal antioxidant activity and lead to reductions in both circulating and local renal oxidants in the D-SOD mouse. However, our studies with cultured MC demonstrate that an increase in cellular SOD-1 activity but not the addition of extracellular SOD suppresses both MC superoxide production and stimulation of collagen synthesis induced by a high concentration of glucose. These and analogous observations in endothelial cells cultured in high glucose (47,48) implicate changes in intracellular oxidative stress in the pathogenesis of diabetic complications.

Glomerular TGF-β content and urinary excretion of TGF-β are increased in both human and experimental...
diabetes (62,63). In the present study, these changes were evident in D-WT but not in D-SOD mice and occurred in the absence of an increase in serum TGF-β in D-WT. The results indicate that overexpression of SOD-1 suppresses the increase in renal TGF-β production that is otherwise seen in diabetes. This prosclerotic cytokine has been implicated as a major mediator of collagen deposition and glomerular mesangial expansion in this disorder (62,63). Earlier studies in cultured MC demonstrated that several structurally distinct antioxidants prevent increases in both TGF-β and matrix protein synthesis induced not only by high concentrations of glucose but also by angiotensin II and thromboxane (64,65). These data suggest further that the suppression of both glomerular TGF-β and collagen IV accumulation in D-SOD mice may be linked to enhanced renal and/or systemic antioxidant activity.

Whatever the precise mechanisms involved, the current results demonstrate that overexpression of SOD-1 protects the glomerulus from both early diabetic injury and progression of glomerulopathy to the stage of at least modest mesangial expansion. They thereby provide the first in vivo evidence that superoxide may be involved in the pathogenesis of this injury. Direct assessment of the effects of overexpression of SOD-1 on diabetic renal injury in vivo is essential, because the consequences of enhancement of activity of this enzyme on oxidative cell injury are not predictable and may be dependent on the magnitude of the increase, cell type, genetic background, and other factors. A marked increase in SOD-1 can lead to generation of hydrogen peroxide and hydroxyl radicals from superoxide (29) at rates sufficient to mediate rather than protect from oxidative injury (66). In this regard, some strains of mice that are homozygous for the SOD-1 transgene demonstrate evidence of presumed oxidative tissue damage (66). By contrast, the hemizygous SOD-1 model used in the present study is protected from oxidative injury induced by several different processes (60,66).

ACKNOWLEDGMENTS

This work was supported by funds from the American Diabetes Association and the Department of Veterans Affairs.

The authors gratefully acknowledge the technical support of Mark Barsic, Julia Liachenko, and Diane George.

REFERENCES

32. Sod-1 protects against glomerular injury. Diabetes 50, SEPTEMBER 2001


