

# Attenuation of Renal Injury in *db/db* Mice Overexpressing Superoxide Dismutase

## Evidence for Reduced Superoxide–Nitric Oxide Interaction

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The effects of overexpression of  $\text{Cu}^{2+}/\text{Zn}^{2+}$  superoxide dismutase-1 (SOD-1) on indexes of renal injury were compared in 5-month-old nontransgenic (NTg) *db/db* mice and *db/db* mice hemizygous for the human SOD-1 transgene (SOD-Tg). Both diabetic groups exhibited similar hyperglycemia and weight gain. However, in NTg-*db/db* mice, albuminuria, glomerular accumulation of immunoreactive transforming growth factor- $\beta$ , collagen  $\alpha 1(\text{IV})$ , nitrotyrosine, and mesangial matrix were all significantly increased compared with either nondiabetic mice or SOD-Tg-*db/db*. SOD-1 activity and reduced glutathione levels were higher, whereas malondialdehyde content was lower, in the renal cortex of SOD-Tg-*db/db* compared with NTg-*db/db* mice, consistent with a renal antioxidant effect in the transgenic mice. Inulin clearance ( $C_{\text{IN}}$ ) and urinary excretion of guanosine 3',5'-cyclic monophosphate ( $U_{\text{cGMP}}$ ) were increased in SOD-Tg-*db/db* mice compared with corresponding values in nondiabetic mice or NTg-*db/db* mice.  $C_{\text{IN}}$  and  $U_{\text{cGMP}}$  were suppressed by *N* $\omega$ -nitro-L-arginine methyl ester in SOD-Tg-*db/db* but not in NTg-*db/db* mice, implying nitric oxide (NO) dependence of these increases and enhanced renal NO bioactivity in SOD-Tg-*db/db*. Studies of NO-responsive cGMP in isolated glomeruli supported greater quenching of NO in glomeruli from NTg-*db/db* compared with SOD-Tg-*db/db* mice. Evidence of increased NO responsiveness and the suppression of glomerular nitrotyrosine may both reflect reduced NO-superoxide interaction in SOD-Tg-*db/db* mice. The results implicate superoxide in the pathogenesis of diabetic nephropathy. *Diabetes* 53:762–768, 2004

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AGE, advanced glycation product; CCh, carbamylcholine;  $C_{\text{IN}}$ , inulin clearance; cGMP, guanosine 3',5'-cyclic monophosphate; eNOS, endothelial nitric oxide synthase;  $\text{FC}_{\text{Alb}}$ , fractional clearance of albumin; GPx, glutathione peroxidase; GSH, reduced glutathione; IL-2, interleukin-2; L-NAME, *N* $\omega$ -nitro-L-arginine methyl ester; NP, nitroprusside; ROS, reactive oxygen species; SOD, superoxide dismutase; STZ, streptozotocin; TBARS, renal cortical malondialdehyde; TGF- $\beta$ , transforming growth factor- $\beta$ ; UAE, urinary albumin excretion;  $U_{\text{cGMP}}$ , urinary excretion of cGMP;  $U_{\text{NO}_x}$ , urinary clearance of  $\text{NO}_2/\text{NO}_3$ ;  $V_{\text{G}}$ , glomerular volume.

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Oxidative and glyco-oxidative stress have been implicated in the pathogenesis of diabetic complications, including nephropathy (1–6). There is evidence for multiple pathways of increased generation of reactive oxygen species (ROS) in diabetes. These include enhanced glucose auto-oxidation; increased mitochondrial superoxide production; protein kinase C-dependent activation of NADPH oxidase by angiotensin-II, glucose, and other stimuli; uncoupled endothelial nitric oxide synthase (eNOS) activity; formation of advanced glycation products (AGEs); stimulation of cellular ROS production by extracellular AGEs via their receptors; and others (1–4). Markers of oxidative stress and reduced levels of antioxidants have been found in blood and/or tissues in both human and experimental diabetes, including kidney (1–10). In addition to the potential cytotoxic effects of increased ROS generation, there is increasing evidence that ROS function as physiological cellular second messengers via redox sensitive gene transcription factors and cell signaling pathways (1,2). Thus, increases in ROS in the diabetic environment may alter several redox-sensitive genes and/or cellular signaling pathways (1,2). With respect to diabetic nephropathy, studies in cultured mesangial cells support a role for increased superoxide production in signaling increases in matrix protein synthesis induced by either high ambient glucose concentrations or angiotensin-II (11,12), two factors strongly linked to renal injury in vivo in diabetes (13,14). Similarly, studies in cultured endothelial cells have demonstrated increased superoxide and eNOS expression in response to high glucose (15) and have implicated increased superoxide from mitochondria (2) and uncoupled eNOS (16) in the activation of signaling pathways linked to cell injury.

Consistent with a role for oxidative mechanisms in the pathogenesis of diabetic renal injury in vivo, dietary antioxidant supplementation with vitamin E, *N*-acetyl-cysteine, olerutin, taurine, or lipoic acid have all been found to attenuate nephropathy in experimental models of diabetes (5,8,16–18). Recently, we demonstrated that transgenic mice that overexpress human cytoplasmic  $\text{Cu}^{2+}/\text{Zn}^{2+}$  superoxide dismutase (SOD)-1 are protected from early diabetic renal injury compared with their nontransgenic diabetic littermates in a model of streptozotocin

(STZ)-induced type 1 diabetes (11). These observations provided the first in vivo evidence for the involvement of superoxide in the pathogenesis of diabetic nephropathy.

In the current studies, we assessed the effects of overexpression of SOD-1 on renal injury in an experimental model of type 2 diabetes and the possibility of altered superoxide interaction with nitric oxide (NO) in diabetic mice that overexpress SOD-1.

## RESEARCH DESIGN AND METHODS

This study was conducted in conformance with guidelines for the use and care of animals of the Veterans Affairs Pittsburgh Healthcare system, whose animal facility is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. To produce compound mutant mice with an SOD-1 +/- *db/db* genotype, hemizygous female SOD-1 transgenic mice (SOD-Tg), which express the human SOD-1 transgene driven by the human SOD-1 promoter (The Jackson Laboratory, Bar Harbor, ME), were bred to male mice heterozygous for the *db* mutation coupled to the misty (M) allele (C57BL/6J mice kindly supplied by Dr. Streamson Chua, Molecular Biology/Molecular Genetic Core, NY Obesity Research Center, New York, NY). Both the SOD-Tg and *db* heterozygous breeders were on a C57BL/6J genetic background. Offspring from the F1 generation, which were heterozygous for the *db* mutation and also expressed the SOD-1 transgene, were then bred to mice heterozygous for the *db* mutation. The F2 generation contained the desired compound mutant (SOD-Tg, *db/db*) and the other genotypes used. Genotypes were determined by RT-PCR amplification of tail DNA as described below. The following four genotypes were studied using 10 age-matched, male mice in each group: 1) wild-type nontransgenic (NTg)-nondiabetic (ND), 2) NTg-*db/db*, 3) SOD-Tg-ND, and 4) SOD-Tg-*db/db*.

Animals were maintained in a 22°C room with a 12-h light/dark cycle and given free access to food (Purina pellet mouse chow) and water. Blood glucose levels were determined at monthly intervals on blood obtained from the retro-orbital venous plexus. Urine samples were collected in metabolic cages at age 5 months and frozen for subsequent determination of albumin and [<sup>14</sup>C]inulin when inulin clearance ( $C_{IN}$ ) was examined.  $C_{IN}$  was determined in conscious unrestrained mice from the clearance of [<sup>14</sup>C]inulin given by osmotic minipumps (Durect, Cupertino, CA) implanted subcutaneously, as previously reported (5,11).  $C_{IN}$  and fractional clearance of albumin ( $FC_{Alb}$ ) were calculated as previously described (5,11). At age 5 months, mice were weighed and then killed by exsanguination under isoflurane anesthesia. Their blood was collected in heparinized tubes and their kidneys were weighed and then processed for biochemical, histological, and immunohistochemical determinations, as described below or previously reported (5,11).

In separate studies using four additional experimental mouse groups analogous to those described above,  $C_{IN}$  and 24-h urinary excretion of guanosine 3',5'-cyclic monophosphate ( $U_{cGMP}$ ) and  $NO_2/NO_3$  ( $U_{NOx}$ ) were determined before and on the 3rd day of oral administration of *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME), a competitive inhibitor of NOS (5,11,19). Preliminary studies indicated that ingestion of ~20 mg · kg body wt<sup>-1</sup> · day<sup>-1</sup> of L-NAME for 3 days yielded marked suppression of  $U_{NOx}$  in NTg-*db/db* mice, and therefore this dosage was used in the current experiments. Average daily consumption of L-NAME per mouse was ~23 mg/kg body wt and did not differ significantly among the four study groups. Mice were killed 1 week after completion of the L-NAME studies; their kidneys were removed and their glomeruli were harvested by differential sieving, as previously described (20). Glomeruli harvested from kidneys of five randomly selected mice from each study group were used for determination of SOD-1 activities.

**Glomerular incubations.** After being isolated, glomeruli were washed with and then resuspended in Krebs-Ringer bicarbonate buffer containing 5 mmol/l glucose and 2 mmol/l 1-methyl-3-isobutylmethylxanthine, an inhibitor of phosphodiesterase activity. The suspension was then aliquoted (0.5 ml/well in 24-well plate) and incubated at 37°C for 30 min with shaking in a Plexiglas chamber in an atmosphere of 95%/5% CO<sub>2</sub> in the presence or absence of carbamylcholine (CCh) or nitroprusside (NP), as indicated in RESULTS. Incubations were terminated by the addition of 0.5 ml sodium acetate (pH 6.0) at 95°C and cGMP extracted from glomeruli plus media.

cGMP was assayed in extracts of glomerular incubates and urine, as previously reported (20) using a commercial radioimmunoassay system (Amersham Pharmacia Biotech, Piscataway, NJ). The glomerular pellet was extracted in NaOH and used for determination of protein by the Bradford method (19).

**RT-PCR.** DNA was extracted from tail snips using an Aquapure Genomic DNA Isolation Kit (Bio-Rad, Hercules, CA). The PCR protocol for detection of the human SOD-1 transgene was obtained from The Jackson Laboratories (Bar

Harbor, ME). The primers used were CAT CAG CCC TAA TCC ATC TGA, forward, and CGC GAC TAA CAA TCA AAG TGA, reverse (21). Interleukin-2 (IL-2) was used as an internal control for human SOD-1. The primers used for IL-2 were CTA GGC CAC AGA ATT GAA AGA TCT, forward, and GTA GGT GGA AAT TCT AGC ATC ATC C, reverse. PCR amplification was performed with *Taq* polymerase (Invitrogen, Carlsbad, CA). The primers used amplified a 236-bp fragment of the human SOD-1 gene. Simultaneous amplification was performed with primers specific for a 324-bp fragment of the IL-2 gene as an internal control. A cycle number of 35 was chosen based on preliminary experiments to ensure that amplification was within the linear range.

The PCR protocol for detection of the *db* mutation has been previously described by Schreyer et al. (22). The primer sequences used were AGA ACG GAC ACT CTT TGA AGT CTC, forward, and CAT TCA AAC CAT AGT TTA GGT TTG TGT, reverse. The primers introduce a new *Rsa* I site into the mutant sequence. The PCR product is digested with *Rsa* for 1 h at 37°. The *db* mutant allele yields a 135-bp fragment that is not cut by *Rsa* I.

**Additional assays.** Plasma and urinary albumin, GHb,  $U_{NOx}$ , renal cortical activities of SOD-1, mitochondrial Mn<sup>2+</sup> SOD, catalase and glutathione peroxidase (GPx), renal cortical malondialdehyde (TBARS), and reduced glutathione (GSH) content were determined as previously reported (5,11,19).

**Immunohistochemical staining.** Immunohistochemical staining of renal cortical sections for transforming growth factor-β (TGF-β), collagen α1(IV), and human SOD-1 were performed using techniques and reagents previously described in detail (5,11). To assess nitrotyrosine content of glomeruli by immunohistochemistry, sections of formalin-fixed mouse renal cortex were pretreated for 30 min at 95° with Target Retrieval Solution (Dako, Carpinteria, CA). Sections were cooled and blocked with endogenous oxidation blocking solution (PBS plus 3% H<sub>2</sub>O<sub>2</sub>) for 10 min at room temperature. Sections were then blocked with Superblock (ScyTek, Logan, UT). The primary antibody, antinitrotyrosine (5 ug/ml, rabbit immunofluorescence purified IgG; Upstate Biotechnology, Lake Placid, NY) was then applied and the incubation was continued overnight at 0°C. Sections were incubated for 30 min with biotin-conjugated goat antirabbit IgG diluted 1/500 (The Jackson Laboratories) followed by a 10-min incubation with horseradish-conjugated streptavidin (Dako). Color was developed with diaminobenzidine. Nonspecific staining was assessed by prior exposure of the primary antinitrotyrosine antibody to 10 mmol/l nitrotyrosine for 1 h at room temperature and application of this pretreated antibody preparation to renal cortical sections.

Glomerular TGF-β, collagen α1(IV), and nitrotyrosine immunostaining were assessed with a SAMBA 4000 image analyzer (Image Products, Chantilly, VA) using designed software (Microsoft, Richmond, VA), as previously described (5,11). A total of 30 glomeruli per mouse were examined. Results are expressed as the proportional area of positive staining of the glomerular tuft (labeling index), as previously reported (5,11).

**Glomerular morphometrics.** Glomerular volume ( $V_G$ ) was calculated from the cross-sectional area of the glomerular tuft as determined by light microscopy (23). The fractional area of the glomerular tuft occupied by mesangial matrix or mesangial cells was determined by a modification of the procedure reported previously (23). Briefly, a 1 × 1 cm grid was placed over each photomicrograph of periodic acid-Schiff stains of renal cortical sections. The number of boxes or fraction of boxes that contained portions of extracellular mesangial matrix or mesangial cells were counted and expressed as a proportion of the total number of boxes containing glomerular tuft. Measurements were made on 30 glomeruli per mouse at a magnification of ×257. For assessment of both quantitative immunohistochemical staining and glomerular morphometrics, all samples were randomized and examined in a coded fashion by a pathologist blinded to the treatment group from which the sample came.

**Statistical analysis.** Significance of differences was determined by ANOVA followed by Fisher's multiple comparison test using Statview software. Differences at  $P < 0.05$  were considered significant (5,11).

## RESULTS

As shown in Table 1, blood glucose levels, GHb levels, and body weight, but not kidney weight, were all significantly increased in the NTg-*db/db* and SOD-Tg-*db/db* mice compared with nondiabetic mice from the same colony. Values for these parameters were not different in NTg-*db/db* compared with the SOD-Tg-*db/db* groups. The  $C_{IN}$  of NTg-ND, SOD-Tg-ND, and NTg-*db/db* did not differ significantly, although values were modestly increased in the latter group compared with nondiabetic mice. In contrast,  $C_{IN}$  in SOD-Tg-*db/db* mice was clearly higher than in nondiabetic

TABLE 1  
Characteristics of four study groups

	Blood glucose (mmol/l)	GHb (%)	Body weight (g)	Kidney weight (g)	C <sub>IN</sub> (ml/min)	UAE (μg/24 h)	FC <sub>Alb</sub> (×10 <sup>5</sup> )
NTg-ND	8.8 ± 1.7	3.9 ± 0.7	29 ± 2	0.21 ± 0.02	0.23 ± 0.05	574 ± 95	8.4 ± 1.9
NTg- <i>db/db</i>	17 ± 3*	6.8 ± 0.9*	53 ± 4*	0.25 ± 0.032	0.32 ± 0.07	1,689 ± 417*	16.3 ± 2.7*
SOD-Tg-ND	9.3 ± 2.1	4.2 ± 0.8	33 ± 3	0.23 ± 0.03	0.25 ± 0.06	323 ± 68†	4.1 ± 0.8†
SOD-Tg- <i>db/db</i>	18 ± 4*	7.0 ± 0.9*	56 ± 4*	0.26 ± 0.03	0.47 ± 0.10*†	506 ± 151†	3.4 ± 1.3†

Data are means ± SE of determinations from 10 mice in each group. Five sequential monthly determination of blood glucose values were obtained in the fed state. GHb is expressed as percent total hemoglobin. C<sub>IN</sub>, UAE, and FC<sub>Alb</sub> were determined at age 5 months during the week before the mice were killed. \**P* < 0.05 versus value in the corresponding nondiabetic group; †*P* < 0.05 versus value in corresponding nontransgenic group.

and NTg-*db/db* mice. Urinary albumin excretion (UAE) and FC<sub>Alb</sub> in the 5-month-old SOD-Tg-ND group were significantly lower than values in the NTg-ND mice. As previously reported (11), these differences may have been related to suppression of age-related increases in UAE in the SOD-Tg-ND compared with those of the NTg-ND mice. UAE and FC<sub>Alb</sub> in NTg-*db/db* mice were increased compared with values in either nondiabetic group. In SOD-Tg-*db/db* mice, UAE and FC<sub>Alb</sub> did not differ from values in SOD-Tg-ND mice and were significantly lower than those in the NTg-*db/db* mice.

Consistent with our earlier observations in SOD-Tg mice with or without STZ-induced diabetes (11), immunohistochemical staining of renal cortex for human SOD-1 was localized primarily to glomerular endothelial and tubular epithelial cells of both SOD-Tg-ND and SOD-Tg-*db/db* mice, with only faint staining of glomerular mesangial cells (not shown). No renal cortical staining for human SOD-1 protein was observed in either of the NTg groups.

As shown in Table 2, renal cortical SOD-1-specific activity was significantly higher in both the SOD-Tg-ND and the SOD-Tg-*db/db* mice compared with values in the corresponding nontransgenic groups. Renal cortical SOD-1 activity was comparable in the SOD-Tg-ND and SOD-Tg-*db/db* mice. Renal cortical SOD-1-specific activity of NTg-ND and NTg-*db/db* also did not differ significantly (Table 2).

Specific activities of renal cortical SOD-2 or catalase showed no significant differences among the four study groups (Table 2). However, renal cortical GPx activity was higher in both NTg-*db/db* and SOD-Tg-*db/db* mice compared with values in the corresponding nondiabetic mouse groups. Renal cortical TBARS content of NTg-*db/db* mice was significantly higher than values in either of the two nondiabetic mouse groups or the SOD-Tg-*db/db* mice. Renal cortical TBARS of the latter mice was somewhat higher than values in the SOD-Tg-ND mice, but this difference was not statistically significant. Conversely,

renal cortical GSH content was lower in NTg-*db/db* mice than values in the SOD-Tg-*db/db* and the nondiabetic groups, and the levels in SOD-Tg-*db/db* did not differ significantly from levels in nondiabetic mice (Table 2).

As shown in Fig. 1, glomerular content of immunoreactive TGF-β, collagen α1(IV), and nitrotyrosine, expressed as the percent of the area of the glomerular tuft that stained positively, was significantly increased in NTg-*db/db* mice compared with corresponding values in either of the nondiabetic groups or in the SOD-Tg-*db/db* mice. Glomerular TGF-β and nitrotyrosine of SOD-Tg-*db/db* mice did not differ from corresponding values in either of the nondiabetic mice groups. However, glomerular content of collagen α1(IV) of SOD-Tg-*db/db* mice was higher than the value in the SOD-Tg-ND group (Fig. 1).

As shown in Fig. 2, the fractional area of the glomerular tuft occupied by the mesangial matrix was significantly higher in the NTg-*db/db* mice than in any of the other study groups. In contrast, the mesangial matrix area in the SOD-Tg-*db/db* mice did not differ detectably from values in either of the two nondiabetic groups. There were no significant differences in V<sub>G</sub> or the fractional area of the glomerular tuft occupied by mesangial cells among the study groups. No significant tubulointerstitial histological changes were observed in either of the diabetic groups compared with the nondiabetic mice.

As shown in Fig. 3, C<sub>IN</sub> and U<sub>CGMP</sub> were both higher in SOD-Tg-*db/db* mice than in the other groups. The administration of L-NAME for 3 days significantly suppressed C<sub>IN</sub> (Fig. 3, upper panel) and U<sub>CGMP</sub> (lower panel) in SOD-Tg-*db/db* mice, but not in the other groups. In the presence of L-NAME, C<sub>IN</sub> and U<sub>CGMP</sub> in SOD-Tg-*db/db* did not differ from corresponding values in the other groups. As shown in Table 3, administration of L-NAME did not significantly alter U<sub>NOx</sub> excretion in the nondiabetic mice. U<sub>NOx</sub> was higher in both diabetic groups compared with values in nondiabetic mice. By contrast, L-NAME markedly suppressed U<sub>NOx</sub> to similar absolute levels in the diabetic

TABLE 2  
Renal cortical antioxidant enzyme activities, TBARS, and GSH content

	SOD-1 (μg/mg protein)	SOD-2 (μg/mg protein)	GP <sub>X</sub> (nmol · min <sup>-1</sup> mg protein <sup>-1</sup> )	Catalase (units/mg protein)	TBARS (nmol/mg protein)	GSH (μg/mg protein)
NTg-ND	2.7 ± 0.4	0.18 ± 0.03	201 ± 36	259 ± 49	0.61 ± 0.1	15 ± 2
NTg- <i>db/db</i>	2.0 ± 0.3	0.22 ± 0.04	308 ± 43*	275 ± 56	1.39 ± 0.3*	9 ± 1*
SOD-Tg-ND	4.9 ± 0.9†	0.23 ± 0.04	217 ± 41	231 ± 43	0.53 ± 0.2	19 ± 2
SOD-Tg- <i>db/db</i>	4.3 ± 0.8†	0.25 ± 0.05	334 ± 52*	268 ± 4	0.80 ± 0.2†	15 ± 2†

Data are means ± SE of determinations from 10 mice in each group. \**P* < 0.05 versus value in the corresponding nondiabetic group; †*P* < 0.05 versus value in corresponding nontransgenic group.

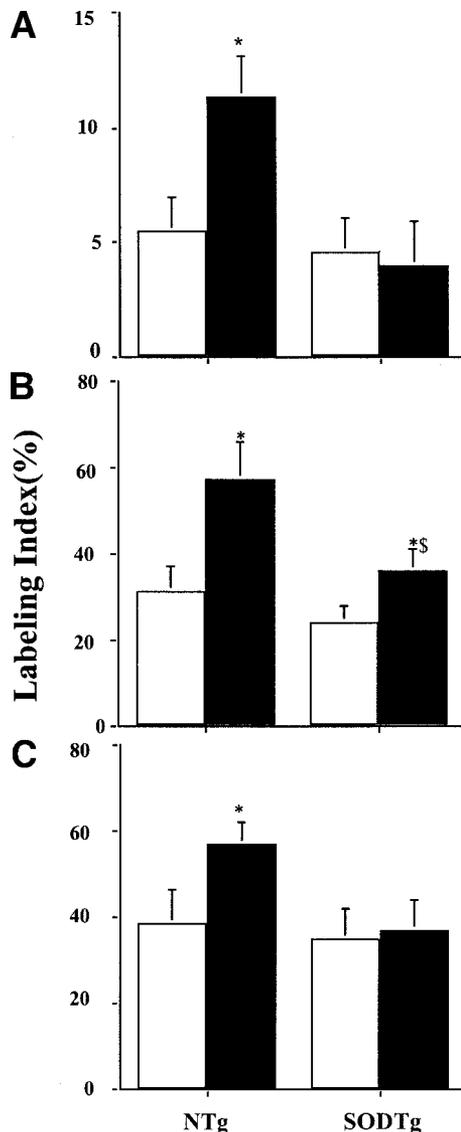


FIG. 1. Quantitative image analysis of immunohistochemical staining for glomerular TGF- $\beta$  content (A), collagen  $\alpha$ 1 (IV) (B), and NO-tyrosine (C). Results are expressed as the percent area of positive staining of the glomerular tuft (labeling index). Data represent means  $\pm$  SE of values from 10 mice in each group, 30 glomeruli per mouse. \* $P$  < 0.05 for *db/db* (■) versus corresponding nondiabetic mice (□); \$ $P$  < 0.05 for SOD-Tg mice vs. corresponding NTg.

mice. However, values for  $U_{NOx}$  in each diabetic group after L-NAME administration remained higher than the value in the corresponding nondiabetic group (Table 3).

As shown in Fig. 4, increases in cGMP accumulation in glomeruli from NTg-*db/db* mice in response to CCh or the lower concentration (0.1 mmol/l) of NP tested were significantly attenuated compared with corresponding responses of glomeruli from SOD-Tg-*db/db* or the nondiabetic groups. Conversely, the cGMP responses to CCh or 0.1 mmol/l NP of glomeruli from SOD-Tg-*db/db* were comparable to those of glomeruli from nondiabetic mice.  $N^G$ -mono-methyl-L-arginine, a competitive inhibitor of NOS, blocked cGMP responses to CCh (Fig. 4), but not the increases induced by the exogenous NO donor NP (not shown), consistent with an action of CCh on cGMP-mediated (via endogenous NO) generation. Basal cGMP accumulation of glomerular incubates (no added test

agent) and glomerular cGMP responses to a high (10 mmol/l) concentration of NP did not differ significantly among the study groups (Fig. 4). SOD-1-specific activity in glomeruli from SOD-Tg-*db/db* ( $2.2 \pm 0.5$   $\mu$ g/mg protein) was significantly higher than in glomeruli from NTg-*db/db* ( $0.9 \pm 0.2$ ;  $P$  < 0.05). There were similar differences in glomerular SOD-1 between SOD-Tg-ND ( $2.5 \pm 0.4$ ) and NTg-ND ( $1.3 \pm 0.3$ ;  $P$  < 0.05). By contrast, glomerular SOD-2 activity did not differ in either diabetic or nondiabetic transgenic versus nontransgenic mice (not shown).

## DISCUSSION

The mouse model of experimental diabetes used in our studies (*db/db* on a C57BL/6J background) is characterized by obesity, insulin resistance, sustained hyperinsulinemia, hyperleptinemia, and moderate hyperglycemia (24), characteristics that are analogous to those of many type 2 diabetic patients. Overexpression of SOD-1 activity in this model prevented or attenuated several indexes of diabetic renal injury, including albuminuria, glomerular accumulation of TGF- $\beta$ , collagen  $\alpha$ 1(IV), and mesangial matrix expansion. These changes in 5-month-old NTg-*db/db* mice were not accompanied by a decline in renal function, and thus represented an early stage of nephropathy. In this regard, it is known that *db/db* mice on the C57BL/6J genetic background develop less severe hyperglycemia and renal injury than *db/db* mice on a C57BL/KsJ background (25,26). Of note in the SOD-Tg-*db/db* mice, the attenuation of markers of renal injury occurred despite the presence of glomerular hyperfiltration. This finding is analogous to those of earlier studies in the STZ-diabetic mouse, which overexpresses SOD-1 (11) as well as in STZ-diabetic rat models treated with antioxidants or ACE inhibitors as interventions (27–29). It may be related to the fact that persistent increases in GFR in the presence of these agents are unassociated with glomerular capillary hypertension, as previously documented in the case of

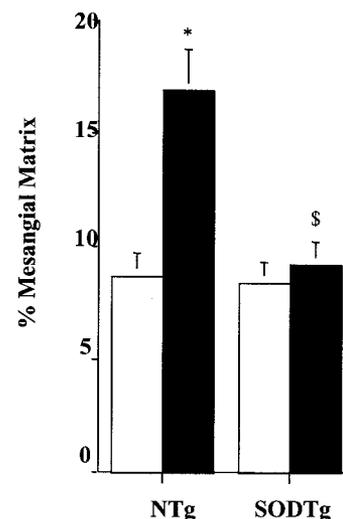


FIG. 2. The fractional area of the glomerular tuft occupied by mesangial matrix was expressed as the percent of the total area (mesangial matrix area/total glomerular tuft area  $\times$  100). Measurements were made using light microscopy and periodic acid-Schiff stains of renal cortical sections. Data represent means  $\pm$  SE of determinations from 10 mice each group, 30 glomeruli per mouse. \* $P$  < 0.05 for *db/db* (■) versus the corresponding nondiabetic group (□); \$ $P$  < 0.05 for SOD-Tg mice versus corresponding nontransgenic (NTg) group.

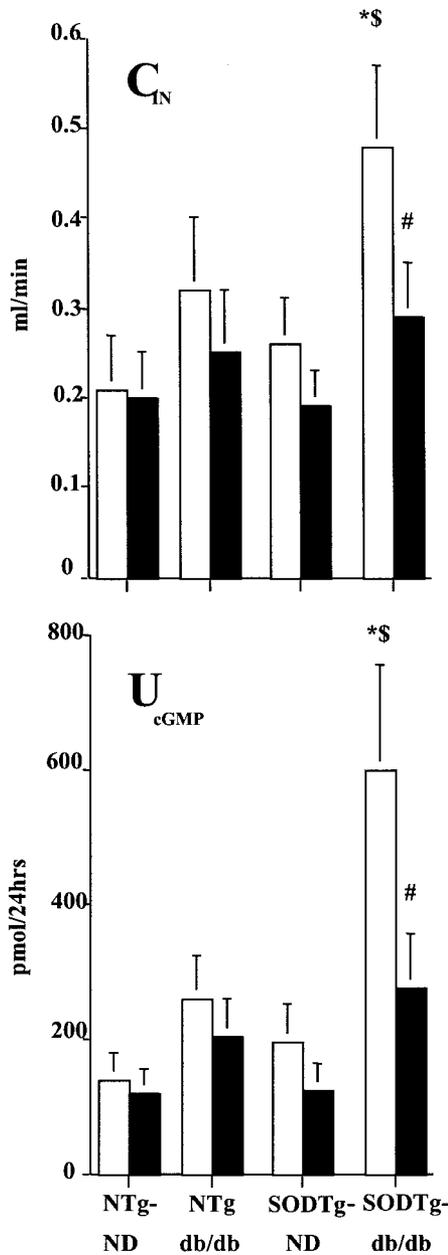


FIG. 3. Effects of treatment of 5 month-old-mice with L-NAME on  $C_{IN}$  and  $U_{cGMP}$ . Before and on the 3rd day of treatment, 24-h urine collections with mid-point plasma samples for [ $^{14}C$ ]inulin were obtained with oral L-NAME (average dosage  $19 \text{ mg} \cdot \text{kg body wt}^{-1} \cdot \text{day}^{-1}$ ). Data represent means  $\pm$  SE of determinations from 10 mice in each group. \* $P < 0.05$  for values before ( $\square$ ) or after L-NAME ( $\blacksquare$ ) in the *db/db* mouse groups versus values obtained under the same experimental conditions in the corresponding NTg or SOD-Tg nondiabetic groups; # $P < 0.05$  compared with value before L-NAME in the same group; \$ $P < 0.05$  for SOD-Tg-*db/db* (before or after L-NAME) versus corresponding value in NTg-*db/db*.

ACE inhibition (29). The renal protection observed in *db/db* mice that overexpressed SOD-1 was not attributable to effects on either the magnitude of hyperglycemia or weight gain (Table 1). It is also unlikely that the renal effects of SOD-1 overexpression in *db/db* mice were related to modification of the hyperinsulinemia that is characteristic of this model (24). Similar effects of SOD-1 overexpression were previously observed in the STZ mouse model of type 1 diabetes (11), which is characterized by weight loss and low plasma levels of insulin.

TABLE 3  
Effects of L-NAME on  $U_{NOx}$

	Before L-NAME	After L-NAME
NTg-ND	423 $\pm$ 97	298 $\pm$ 72
NTg- <i>db/db</i>	1,269 $\pm$ 325*	607 $\pm$ 101*†
SOD-Tg-ND	586 $\pm$ 113	419 $\pm$ 83
SOD-Tg- <i>db/db</i>	1,834 $\pm$ 389*	732 $\pm$ 130*†

Data are means  $\pm$  SE of determinations from 10 mice in each study group and represent nmol/24 h of  $U_{NOx}$ . \* $P < 0.05$  versus value in the corresponding non-diabetic group. † $P < 0.05$  versus corresponding value before treatment with L-NAME.

The renoprotective effect of SOD-1 overexpression in the *db/db* mice was associated with increased renal cortical SOD-1 enzymatic activity, lower TBARS, and higher GSH levels compared with corresponding values in renal cortex from NTg-*db/db* mice, findings consistent with a renal antioxidant effect. However, in the transgenic model used, SOD-1 activity is increased in multiple organs as well as in erythrocytes and leukocytes (30). Thus it is uncertain whether the changes in renal cortical TBARS and GSH reflect consequences of higher renal SOD-1, higher SOD-1 activity at one or more extrarenal sites, or a combination of these changes. It seems likely that superoxide generated in or entering the cell cytosol was the predominant target of SOD-1, given its subcellular distribution (31). However, an effect of SOD-1 on superoxide of mitochondrial origin cannot be completely excluded. Recent studies have demonstrated that SOD-1 is present not only in the cell cytosol but also in the intermembrane region of mitochondria, and may participate in the degradation of superoxide of mitochondrial origin that gain access to that region (32). The potential participation of SOD-1 in the

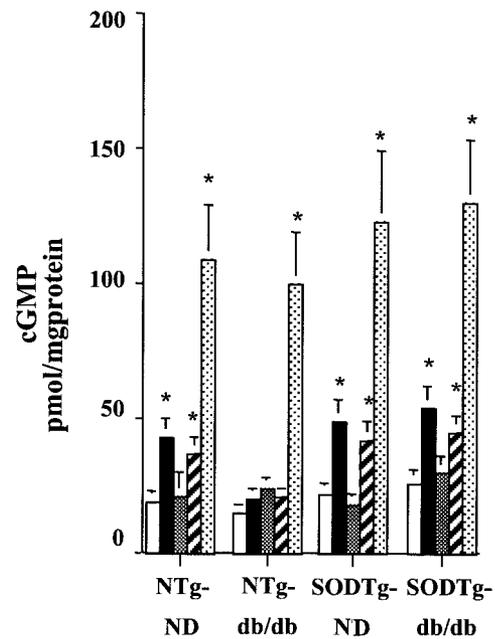


FIG. 4. Basal cGMP accumulation (no added test agent) and cGMP responses to 0.1 mmol/l CCh ( $\blacksquare$ ), CCh plus 0.5 mmol/l  $N^G$ -mono-methyl-L-arginine ( $\square$ ), 0.1 mmol/l NP ( $\square$ ), or 10 mmol/l NP ( $\square$ ) in incubates of glomeruli isolated from the four study groups of mice shown. All incubations were conducted for 20 min in the presence of 2 mmol/l 1-methyl-3-isobutylmethylxanthine at 37°C; cGMP was extracted from media plus glomeruli at the termination of the incubations. Data represent means  $\pm$  SE of determinations of glomerular cGMP from 10 mice in each group. \* $P < 0.05$  compared with basal cGMP.

dismutation of superoxide of mitochondrial origin is of interest in view of the work of Brownlee and colleagues (2,33). These studies implicate enhanced mitochondrial superoxide generation induced by high ambient concentrations of glucose, fatty acids, or leptin in vascular endothelial and other cells in the activation of multiple signaling pathways leading to cell injury (2,33). All of these potential mediators of mitochondrial superoxide generation are relevant to the *db/db* model (24) and may have contributed to renal oxidative stress.

SOD-1 converts superoxide to  $H_2O_2$ , a potent oxidizing agent. Thus, enhanced formation of  $H_2O_2$  might be expected in mice overexpressing SOD-1 and is a potential concern. Of interest, however, is that an increase in cellular SOD-1 activity has not been uniformly observed to increase  $H_2O_2$  levels. Indeed, overexpression of SOD-1 in some cells has been associated with a decrease in  $H_2O_2$  levels (34). As noted by Liochev and Fridovich (35), the net effects of SOD increases on cellular  $H_2O_2$  formation and levels depend on the cellular targets available to superoxide as well as the activities of antioxidant enzymes involved in  $H_2O_2$  metabolism, predominantly catalase and GPx (36,37). No differences were found in the activity of renal cortical catalase among the four mouse groups in the current study. However, consistent with prior assessments of kidney from rats with experimental diabetes (37), renal cortical GPx was higher in both diabetic groups compared with controls (Table 2). This enzymatic change may have attenuated any increase in the levels of renal  $H_2O_2$  in the diabetic mouse groups.

Overexpression of SOD-1 in *db/db* mice prevented accumulation of nitrotyrosine in glomeruli and was associated with both an NO-dependent increase in the glomerular filtration rate and preservation of glomerular cGMP responses to NO *ex vivo*. These findings suggest that enhanced dismutation of superoxide by SOD-1 may have suppressed the reaction of superoxide with NO with a resultant decrease in peroxynitrite generation and a concurrent increase in the bioavailability of NO. An increase in nitrotyrosine accumulation in kidney and other tissues has previously been described in experimental diabetes (6,38–40). Accumulation of this product, at least in part, reflects formation of peroxynitrite (6,38–40) from the reaction of NO with superoxide, and provides a stable footprint of peroxynitrite formation (6,38–41). Increased peroxynitrite generation may lead to modification of proteins, nucleic acids, and lipids, with consequent cell dysfunction or death (41). Of note, studies in cultured vascular endothelial cells have indicated that eNOS *per se* is a target for inactivation by peroxynitrite and that inhibition of eNOS in these cells is prevented by overexpression of SOD-1, but not SOD-2 (42). Thus, overexpression of SOD-1 in glomerular endothelial cells in *db/db* mice may have enhanced NO production by these cells.

Similarly, the SOD-Tg-*db/db* mice displayed increases in  $C_{IN}$ ,  $U_{cGMP}$ , and  $U_{NOx}$ , which were dependent on NO, as reflected by the suppression of these increases by administration of L-NAME. NO-dependent increases in  $C_{IN}$  and  $U_{cGMP}$  imply enhanced production and/or bioavailability of NO in SOD-Tg-*db/db* compared with NTg-*db/db* mice. In the latter group, basal  $C_{IN}$  and  $U_{cGMP}$  were modestly, albeit insignificantly, increased. However, these parame-

ters were not altered by L-NAME in NTg-*db/db* mice. In contrast, basal  $U_{NOx}$  was clearly elevated in NTg-*db/db* mice and suppressed by L-NAME.  $U_{NOx}$  has been used as an index of renal NO production (42–45). Previous studies in nontransgenic STZ diabetic rats have found increases in  $U_{NOx}$  and  $U_{cGMP}$  associated with glomerular hyperfiltration, and have suggested a role for NO in mediating the latter two changes (19,45). In the current studies, the dissociation between the actions of L-NAME on  $U_{NOx}$  versus  $C_{IN}$  and  $U_{cGMP}$  suggests that the increase in  $U_{NOx}$  in NTg-*db/db* mice was not reflective of a corresponding enhancement *in vivo* of the renal bioactivity of NO. To the extent that increases in  $U_{NOx}$  reflected changes in renal NO production in the diabetic mice, one possible explanation for the failure to observe concurrent NO-dependent increases in  $C_{IN}$  and  $U_{cGMP}$  in NTg-*db/db* mice is accelerated inactivation of NO in this group compared with the SOD-Tg-*db/db* mice. Consistent with this possibility, increased oxidant quenching of NO has been implicated in the impairment of endothelial dependent vasodilation that is characteristic of diabetes (46), an impairment that is reversed by antioxidants, including superoxide dismutase (46,47). Thus, in the SOD-Tg-*db/db* model used in the current study, inactivation of NO by superoxide may have been attenuated by enhanced SOD-1 activity.

Evidence for reduced bioactivity of NO in the NTg-*db/db* mice compared with either the SOD-Tg-*db/db* group or nondiabetic mice was also provided by *ex vivo* studies of the cGMP responses of isolated glomeruli to NO (Fig. 4). These observations suggest increased quenching of NO by glomeruli from the NTg-*db/db* mice, analogous to earlier findings in glomeruli from STZ-diabetic rats (19,48). Although the influence of an increase in SOD activity on NO depends on multiple variables and is complex (35), the current data imply that in glomeruli of SOD-Tg-*db/db* mice, the net effect of overexpression of SOD-1 was preservation of the biological action of NO relative to that observed in glomeruli from the nontransgenic diabetic mice. In addition to renal hemodynamic effects of NO (19,43–45), studies in cultured glomerular mesangial cells support direct actions of NO to suppress activation of protein kinase C, increases in TGF- $\beta$ , and accumulation of matrix protein induced by high concentrations of glucose, angiotensin II, or thromboxane- $A_2$  (49,50). Thus, enhanced expression of NO actions in glomeruli of SOD-Tg-*db/db* mice relative to the NTg-*db/db* mice may be linked to the suppression of glomerular TGF- $\beta_1$  and mesangial matrix accumulation observed in the transgenic group.

In summary, the current findings demonstrated that overexpression of SOD-1 prevents biochemical, functional, and structural changes induced in kidney in a model of type 2 diabetes. They imply a role for superoxide *in vivo* in the mediation of these changes, possibly in part via reduced superoxide interaction with NO in the SOD-Tg-*db/db* mouse.

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