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# Acceleration of diabetic renal injury in the superoxide dismutase knockout mouse: effects of tempol

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**Abstract**

Indices of renal injury and oxidative stress were examined in mice with deficiency of cytosolic $Cu^{2+}/Zn^{2+}$ superoxide dismutase (SOD1−/−, KO) and their wild-type (WT) littermates with streptozotocin-induced diabetes. After 5 weeks of diabetes, KO diabetic (D) but not WT-D mice developed marked albuminuria, increases in glomerular content of transforming growth factor $\beta$, collagen $\alpha$1(IV), and nitrotyrosine, and higher glomerular superoxide compared with corresponding values in nondiabetics. After 5 months of diabetes, increases in these parameters, mesangial matrix expansion, renal cortical malondialdehyde content, and severity of tubulointerstitial injury were all significantly greater, whereas cortical glutathione was lower, in KO-D than in WT-D. In contrast to WT-D, after 4 weeks of diabetes, KO-D mice did not develop the increase in inulin clearance ($C_{In}$) characteristic of early diabetes. The nitric oxide synthase inhibitor $N^{\omega}$-nitro-L-arginine methylester suppressed $C_{In}$ in WT-D, but had no effect on $C_{In}$ in KO-D. Treatment of KO-D with the SOD mimetic tempol for 4 weeks suppressed albuminuria, increases in glomerular transforming growth factor $\beta$, collagen $\alpha$1(IV), nitrotyrosine, and glomerular superoxide, and concurrently increased $C_{In}$. The latter action of tempol in KO-D was blocked by the $N^{\omega}$-nitro-L-arginine methylester. The findings provide support for a role for superoxide and its metabolism by SOD1 in the pathogenesis of renal injury in diabetes in vivo, and implicate increased interaction of superoxide with nitric oxide as a pathogenetic factor.
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## 1. Introduction

There is substantial evidence to support a role for oxidative and glycooxidative stress in the pathogenesis of diabetic nephropathy and other diabetic complications [1-10]. Activation of multiple metabolic pathways in diabetes leads to increased generation of superoxide and derivative reactive oxygen species (ROS). These include increased mitochondrial electron transport activity induced by hyperglycemia and fatty acids [7]; activation of the reduced forms of nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate oxidase systems of nonphagocytic cells [5,8]; increased expression with "uncoupling" of endothelial nitric oxide (NO) synthase (eNOS) leading to greater production of superoxide relative to NO [11,12]; enhanced glucose autooxidation [13]; and others [1,14,15].

The level of superoxide and other ROS in kidney, as in other tissues, in diabetes is determined by both their rate of formation and their rate of removal. The superoxide dismutase (SOD) enzyme system is a primary determinant of superoxide removal [16]. This enzyme system consists of 3 isoforms of SOD each encoded by a separate gene and localized within different cellular compartments or secreted extracellularly [16-19]. A $Cu^{2+}/Zn^{2+}$-containing SOD (SOD1) is located primarily within the cytosol [16,17], but is also found in the nucleus and the intermembrane space of mitochondria [18]. It is the predominant isoform of SOD in the renal cortex and glomeruli, accounting for more than 90% of total SOD activity in these tissues [16,17,19,20]. SOD1 is also the dominant SOD isoform in endothelial cells [21] where one of its key functions may be protection of NO from quenching by superoxide within these cells [21-23]. Residual SOD activity in the renal cortex and glomeruli is composed of mitochondrial $Mn^{2+}$ SOD (SOD2) and

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extracellular SOD (SOD3), also a $Cu^{2+}/Zn^{2+}$-containing enzyme [16-20]. In previous studies, genetic overexpression of SOD1 was found to attenuate glomerular injury and oxidative stress in mouse models of type 1 (streptozotocin [STZ] diabetic mouse) and type 2 (*db/db* mouse) diabetes mellitus [19,20]. These findings supported the participation of superoxide in the pathogenesis of diabetic renal injury in vivo and were consistent with an important role for SOD1 in the regulation of renal superoxide in diabetes [20]. In the present study, we examined the effects of SOD1 deficiency on functional, biochemical, and structural renal changes in a SOD1 knockout mouse in which diabetes was induced with STZ.

## 2. Research design and methods

The study was conducted in conformance with guidelines for use and care of animals at the Veteran Affairs Pittsburgh Healthcare System (VAPHS), whose animal facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Breeding pairs of heterozygous SOD1−/+ mice on a C57BL/6 genetic background were generously supplied by Dr Charles J. Epstein (University of California, San Francisco) and Dr Ting-Ting Huang (Stanford University). The procedures used to generate mice in which part of the SOD1 gene was deleted by gene targeting to render the mutant allele nonfunctional has been described in detail by Huang et al [24]. Mice homozygous for deficiency of the SOD1 gene (SOD1−/− or KO) and their wild-type (SOD1+/+ or WT) littermates were generated from matings of the SOD1−/+ mice in the animal research facility of the VAPHS. The genotype of each mouse was determined by reverse transcription-polymerase chain reaction of DNA from tail biopsies as previously described [18]. The primers used were GGC GGA TGA AGA GAG GTG AGC (forward) and ATT GGC CAC ACC GTC CTT TCC (reverse). WT and KO mice aged 6 to 8 weeks were given either an intraperitoneal injection of STZ (100 mg/kg in 100 $\mu$L sterile citrate buffer, pH 4) or an injection of the STZ vehicle every other day × 4 injections. Mice with blood glucose (BG) values of 350 mg/dL or greater 2 days after the fourth injection of STZ were entered into the study as diabetics. Other mice in the STZ group with BG less than 350 mg/dL received 1 additional injection of STZ and were entered as diabetics if BG was 350 mg/dL or greater 2 days later. KO and WT mice both typically required 4 injections of STZ to achieve the target BG. Mice were assigned to 1 of 4 study groups with 14 mice in each group: WT- (WT-ND): nondiabetic; WT-D; KO-ND or KO-D. These mice were assessed sequentially over a 5-month period of observation after completion of the serial injections of STZ or vehicle.

All mice were housed in a temperature- (22°C) and light- (12 hours) controlled facility throughout and allowed ad libitum access to water and standard mouse chow. Samples for BG were obtained every 2 weeks from the retroorbital venous plexus. BG was determined with strips (VET-660, CAA, Newburg, WI) using a glucometer (Home Diagnostics, Ft Lauderdale, FL). Systolic blood pressure (BP) was measured in conscious mice at entry and then monthly with a tail cuff, as previously reported [7]. During periods of urine collections, mice were housed in metabolic cages, and for 48 hours before collection, they were given free access to a liquid diet (Ensure, Abbott Laboratories, Columbus, OH) and water, but no solid food [20]. $C_{In}$ was determined in conscious unrestrained mice from the clearance of [$^{14}$C] inulin given by osmotic minipump as previously described [19,20]. Fractional clearance of albumin ($FC_{Alb}$) was calculated as previously reported [19,20]. In the studies above, all mice were weighed and then killed by exsanguination under isoflurane anesthesia. Blood was collected in heparinized tubes; kidneys were then processed for biochemical, histologic, and immunochemical determinations as described below or previously reported [19,20].

### 2.1. Assessment of the effects of tempol and $N^{\omega}$-nitro-L-arginine methylester

In a second study, the effects of the membrane-permeable, metal-free SOD mimetic 4-hydroxy-2,2,6,6-tetramethylpiperdine-*N*-oxyl (tempol, Sigma Chemical, St Louis, MO), the NOS inhibitor $N^{\omega}$-nitro-L-arginine methylester (L-NAME, BIOMOL, Plymouth Meeting, PA), or the combination of these 2 agents were examined in groups of 18 WT-ND and D, and KO-ND and D mice. Nine mice in each study group received tempol in the drinking water at a dosage of approximately 80 mg/kg body weight per day for the entire 35 days of the study, and 9 served as untreated controls. On day 25, an initial 24-hour urine collection for albumin, $NO_2/NO_3$ ($U_{NOX}$), guanosine 3′,5′-cyclic monophosphate ($U_{cGMP}$), and inulin clearance ($C_{In}$) was obtained in mice receiving no treatment or tempol alone. All mice then received L-NAME in the drinking water at a dosage of approximately 20 mg/kg body weight per day for 3 days (days 25-28) using a protocol and dose previously reported [18]. A second 24-hour urine sample was collected on day 3 of L-NAME administration. Mice were killed 1 week after discontinuation of L-NAME, with half of mice on continuous tempol treatment until sacrifice.

## 3. Biochemical determinations

Plasma and urinary albumin, BG, glycated hemoglobin ($HbA_{1c}$), NOX, cGMP, $Cu^{2+}/Zn^{2+}$ and $Mn^{2+}$ SOD activities in the renal cortex and isolated glomeruli, and renal cortical content of malondialdehyde (MDA) and reduced glutathione (GSH) were determined by methods previously reported [19,20]. SOD1 protein expression in the renal cortex was examined by Western blotting with sheep polyclonal IgG anti-$Cu^{2+}/Zn^{2+}$ SOD primary antibody (Upstate USA, Charlottesville, VA), using techniques previously reported [22].

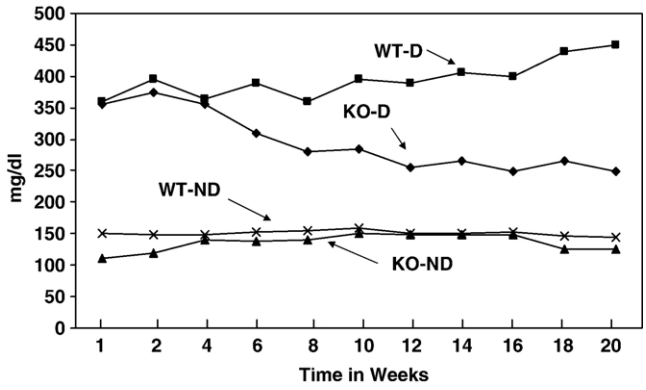


Fig. 1. Sequential changes in mean BG levels in nondiabetic (ND) and diabetic (D) WT and SOD1 KO mice obtained over 5 months after completion of serial injections of STZ (D) or its vehicle (ND); n = 14 mice per study group.

### 3.1. Determination of superoxide

Superoxide bioactivity was determined ex vivo from relative increases in fluorescence due to oxidation of dihydroethidine (DHE) [25] in isolated glomeruli. Glomeruli were isolated by graded sieving as previously reported [20]. Three pools of isolated glomeruli were prepared from each study group, using 6 kidneys to derive each glomerular pool. After isolation, glomeruli were suspended in HEPES-buffered saline solution at pH 7.4 and loaded with 20 $\mu$mol/L DHE (Molecular Probes, San Diego, CA) for 30 minutes at 37°C. Glomeruli were then washed with HEPES-buffered saline and aliquoted into a 96-well plate. DHE is oxidized by superoxide intracellularly, whose binding to cellular DNA produces a strong red fluorescence. Fluorescence in glomeruli was measured in a Spectra MAX2 fluorescence plate reader (Molecular Devices, Sunnyvale, CA) using excitation and emission wavelengths of 480 and 567 nm, respectively. Glomerular protein was extracted with sodium hydroxide and determined as previously reported [20]. Results are expressed as arbitrary fluorescent units normalized for the protein content of each well. Calibration curves were constructed by adding xanthine (100 $\mu$mol/L) and varying concentrations of xanthine oxidase (0-15 mU/mL) to DNA (50 $\mu$g/mL) in HEPES-buffered saline to verify the proportionality of superoxide bioactivity with fluorescence changes.

### 3.2. Glomerular morphometrics and tubulointerstitial injury

The total area of the glomerular tuft and the fraction occupied by mesangium were determined in cortical sections from 10 mice (30 glomeruli) randomly selected from each group; sections were stained with periodic acid-Schiff (PAS) and examined by light microscopy [20]. Tubulointerstitial injury was evaluated semiquantitatively in renal cortical sections from the same 10 mice from each group, using Masson Trichrome staining, as previously reported [26]. The following 0 to 4+ grading system was used to assess for the presence of (*a*) tubular atrophy, (*b*) tubular dilatation, (*c*) interstitial inflammatory cell infiltrates; and (*d*) interstitial fibrosis [26]. A grade of 0 indicated no lesions; +1, a single small focal lesion with minimal tubular alterations and inflammatory cell infiltration; 2+ to 4+, increasing severity of tubular lesions, inflammatory cell infiltrate, and/or fibrosis, +4, 50% or more of the tubules and interstitium involved.

### 3.3. Immunohistochemical staining

Immunohistochemical staining of cortical sections for transforming growth factor $\beta$ (TGF-$\beta$), collagen $\alpha$1(IV), and human SOD1 were performed using techniques and reagents previously described in detail [19,20]. Renal cortical content of SOD3 was also assessed by immunohistochemical staining using commercial anti–EC-SOD antibodies (Assay Designs, Ann Arbor, MI). Glomerular TGF-$\beta$, collagen $\alpha$1 (IV), and nitrotyrosine immunostaining were assessed with a SAMBA 4000 image analyzer (Image Products International, Chantilly, VA) using designed software (Microsoft, Richmond, VA) as previously described [19,20]. Thirty glomeruli from 10 mice randomly selected from each group were examined. Nonspecific staining of renal cortical sections for nitrotyrosine was assessed by prior exposure of the primary antinitrotyrosine antibody to 10 nmol/L nitrotyrosine for 1 hour at room temperature and application of this pretreated antibody to the renal cortex. Results are expressed as the percent area of the glomerular tuft staining positively (labeling index). Glomerular morphometrics including mesangial matrix area, severity of tubulointerstitial injury, and immunohistochemical staining were assessed by a pathologist (M.M.) blinded to the identity of the mouse study group from which the renal sections were derived.

### 3.4. Statistical analysis

Significance of differences was determined by analysis of variance followed by Fisher multiple comparison test using Statview software. Differences in the tubulointerstitial injury

Table 1
Blood glucose, $HbA_{1c}$, systolic BP, body and kidney weight, and renal cortical $Cu^{2+}/Zn^{2+}$ and $Mn^{2+}$ SOD activities of WT and KO mice

| | Nondiabetic | | Diabetic | |
|---|---|---|---|---|
| | WT | KO | WT | KO |
| Blood glucose (mg/dL) | 151 ± 30 | 134 ± 26 | 398 ± 57 * | 273 ± 61 *,† |
| $HbA_{1c}$ (%) | 4.5 ± 0.7 | 4.1 ± 0.6 | 8.9 ± 0.9 * | 7.0 ± 0.8 *,† |
| BP (mm/Hg) | 119 ± 6 | 112 ± 5 | 114 ± 4 | 121 ± 6 |
| Body weight (g) | 28 ± 1 | 26 ± 1 | 23 ± 2 * | 24 ± 1 |
| Kidney weight (g/kg body weight) | 7.1 ± 0.3 | 6.8 ± 0.4 | 10.8 ± 0.6 * | 7.9 ± 0.5 *,† |
| $Cu^{2+}/Zn^{2+}$SOD ($\mu$g/mg protein) | 3.1 ± 0.5 | 0.46 ± 0.07 | 2.6 ± 0.4 | 0.33 ± 0.07 † |
| $Mn^{2+}$SOD ($\mu$g/mg protein) | 0.35 ± 0.06 | 0.29 ± 0.05 | 0.25 ± 0.04 | 0.18 ± 0.03 * |

Values represent means ± SE (n = 14).

* $P < .05$ compared with corresponding value in the ND group.

† $P < .05$ compared with corresponding value in the WT-D group.

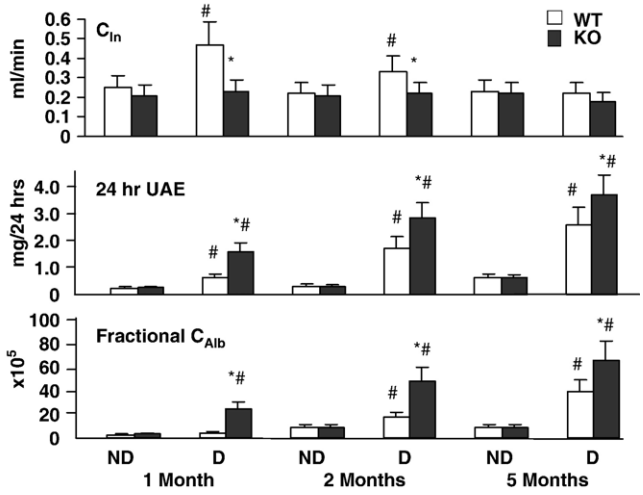


Fig. 2. Sequential changes in $C_{In}$, UAE, and $FC_{Alb}$ in WT (□) and KO (■) mice. Values represent means ± SE of determinations from 10 mice randomly selected from each group. *$P < .05$, comparing KO-ND or D to corresponding WT-ND or D at the same time point; #$P < .05$, comparing WT or KO-D to corresponding ND group at the same time point.

scores among the groups were assessed by Kruskal-Wallis analysis. Differences with a $P < .05$ were considered significant [19,20].

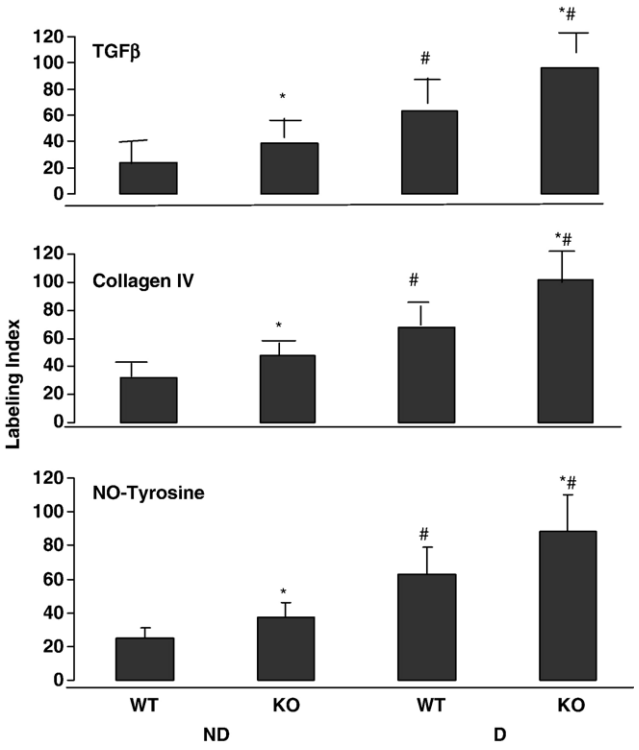


Fig. 3. Quantitative image analysis of immunohistochemical staining for glomerular content of TGF-$\beta$, collagen $\alpha$1(IV), and NO tyrosine at 5 months, expressed as labeling indices (percent area of the glomerular tuft that stained positively). Data represent means ± SE of values from 10 mice randomly selected from each group. *$P < .05$, comparing KO-ND or D with corresponding WT group; #$P < .05$, comparing WT or KO-D with value in corresponding ND group.

## 4. Results

Fig. 1 shows sequential changes in BG levels in the 4 study groups after completion of serial injections of STZ or vehicle. During the first month after STZ, BG levels were comparable in the 2 diabetic groups and markedly higher than values in the ND groups. However, after the first month, BG values in KO-D were lower than those in WT-D, although clearly higher than in the ND groups. The differences in BG between the 2 diabetic groups were not explained by either the cumulative dose of STZ given (467 ± 64 mg/kg body weight [mean ± SE] in WT-D and 421 ± 56 in KO-D) to achieve initial hyperglycemia, or daily food consumption, both of which were comparable. As shown in Table 1, mean BG over 5 months and $HbA_{1c}$ values at 5 months in WT-D were higher than in KO-D. However, in both diabetic groups, mean BG and $HbA_{1c}$ were significantly greater than corresponding values in the ND groups; the latter 2 groups had comparable mean BG and $HbA_{1c}$ values.

Body weight of WT-D was lower than of WT-ND, but not different from that of KO-D. Kidney weight, expressed as a function of body weight, was higher in WT-D than KO-D. BP did not differ significantly among the study groups. Renal cortical $Cu^{2+}/Zn^{2+}$ SOD activity in both KO groups was less than 15% of corresponding values in the WT mice (Table 1). Western blotting and immunostaining confirmed that SOD1 protein was absent in the renal cortex of both KO mouse groups (not shown).The residual $Cu^{2+}/Zn^{2+}$ SOD activity detected in the renal cortex of the SOD1 KO mice

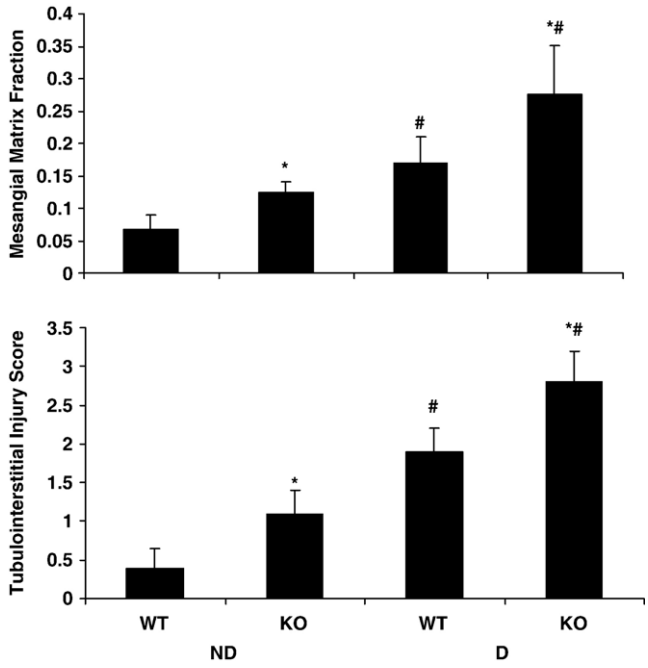


Fig. 4. Fractional area of the glomerular tuft occupied by mesangium (top) and score of severity of tubulointerstitial injury (bottom). Data represent means ± SE of determinations from 10 mice randomly selected from each group at 5 months. *$P < .05$, comparing KO-ND or D to corresponding WT group; #$P < .05$ comparing WT or KO-D to the corresponding ND group.

most likely reflected extracellular SOD (SOD3), which also is $Cu^{2+}/Zn^{2+}$ dependent [16-18] and would be detected by the assay method used in the current study [19]. SOD3 was detected by immunohistochemical staining of the renal cortex of all 4 mouse study groups. Glomerular content of SOD3 did not differ significantly among the groups as assessed by automated image analysis (not shown). There was no detectable alteration in $Cu^{2+}/Zn^{2+}$ SOD activity in either diabetic WT or KO mice, compared to values in the corresponding nondiabetic group. $Mn^{2+}$ SOD activity did not differ significantly among WT-ND, KO-ND, and WT-D. However, renal cortical $Mn^{2+}$ SOD activity was lower in KO-D than in the other groups (Table 1). Analogous differences in $Cu^{2+}/Zn^{2+}$ and $Mn^{2+}$ SOD activities among the study groups were found when measurements were made in isolated glomeruli (not shown).

As shown in Fig. 2, after 1 and 2 months of diabetes, $C_{In}$ was higher in WT-D than in the other study groups; $C_{In}$ of KO-D did not differ significantly from those of the ND groups at any time point. After 5 months of diabetes, $C_{In}$ had declined in WT-D to levels not different from those in KO-D or the 2 ND groups. Urinary albumin excretion (UAE) was higher in both D groups than in the ND groups at 1-, 2-, and 5-month time points (Fig. 2). However, after 1 month of diabetes, $FC_{Alb}$ was significantly and markedly increased over ND values only in the KO-D. UAE and $FC_{Alb}$ were higher in KO-D than in WT-D at all 3 time points examined (Fig. 2), whereas these same parameters did not differ significantly at any time point in KO-ND and WT-ND.

There were no significant differences in mean total areas of the glomerular tuft among the study groups (not shown). Glomerular content of immunoreactive TGF-$\beta$, collagen 1$\alpha$(IV), and nitrotyrosine (Fig. 3) and fractional mesangial matrix area (Fig. 4, top), all expressed as a proportion of total glomerular tuft area, were modestly but significantly higher in KO-ND compared with WT-ND. Values for these parameters in the KO-ND groups were, however, lower than those observed in either diabetic group. Moreover, values for these same parameters in KO-D were significantly higher than those in WT-D. Indices of tubulointerstitial injury, most notably fibrosis, were significantly greater in KO-D compared with WT-D. Similarly, the nondiabetic KO mice were found to have mild tubulointerstitial changes not observed in WT-ND (Fig. 4, bottom). Representative renal cortical sections from

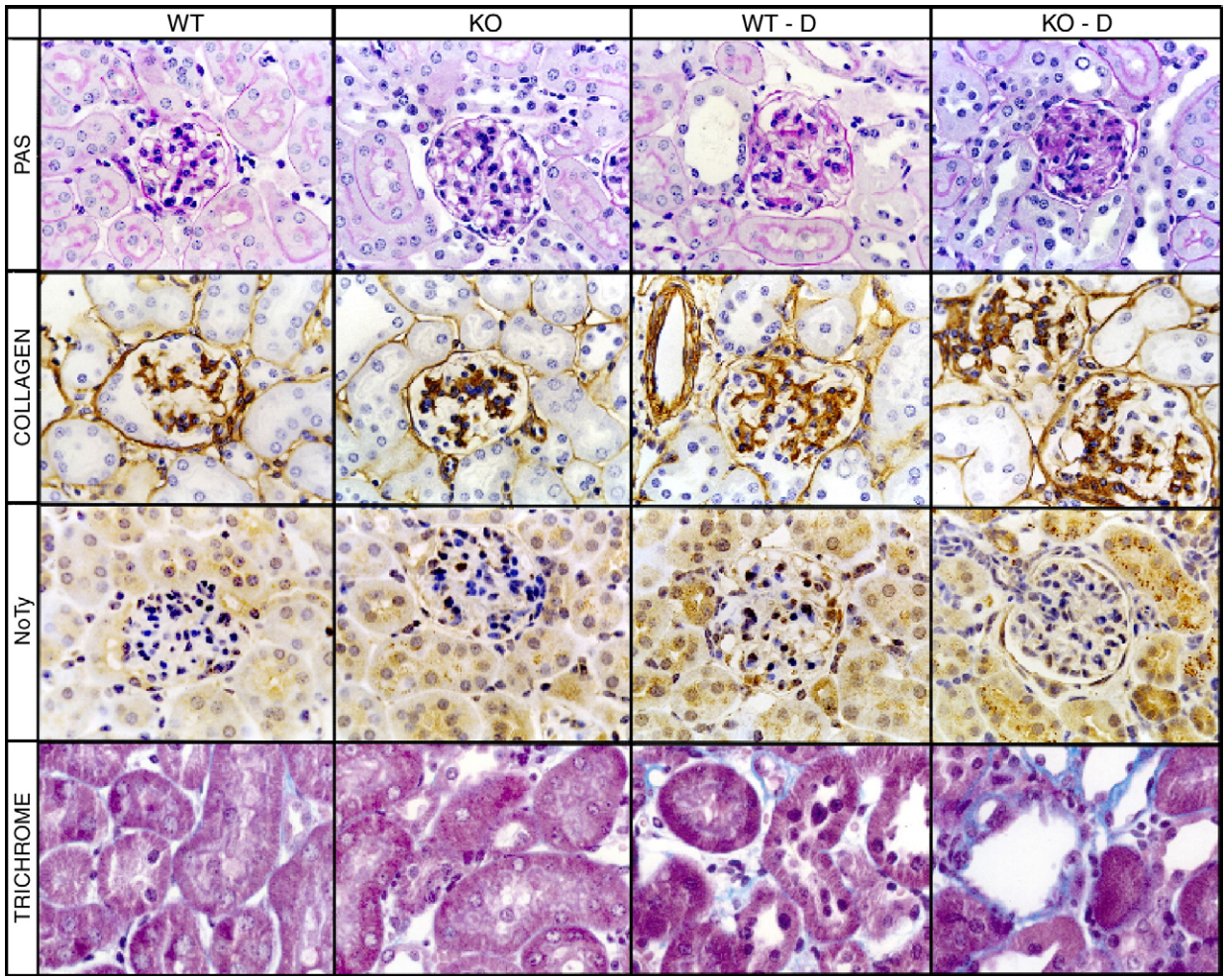


Fig. 5. Representative renal cortical section from the 4 mouse study groups stained with PAS or Trichrome or immunostained for collagen $\alpha$1(IV) or NO tyrosine.

each study group immunostained for collagen α1(IV) and nitrotyrosine or stained with PAS or Trichrome are shown in Fig. 5. Tubulointerstitial immunostaining for TGF-β, collagen α1(IV) nitrotyrosine (Fig. 5) and TGF-β (not shown) was also qualitatively most prominent in KO-D; however, differences among the study groups were not quantitated.

As shown in Fig. 6, renal cortical content of MDA and ex vivo glomerular superoxide bioactivity were significantly higher, and GSH lower, in both the KO-ND and KO-D compared with corresponding values in the WT groups. MDA and glomerular superoxide in both D groups were clearly higher, and GSH lower, than values for these same parameters in the corresponding ND groups; the highest values for renal cortical MDA and superoxide and lowest values for GSH were observed in the KO-D mice.

As shown in Fig. 7, treatment with tempol for 28 days after induction of diabetes significantly increased $C_{In}$, $U_{cGMP}$, and $U_{NOX}$ in KO-D. By contrast, tempol did not alter these same parameters in any of the other study groups, including WT-D. In WT-D, in the presence or absence of tempol, $C_{In}$, $U_{cGMP}$, and $U_{NOX}$ were markedly higher than in either nondiabetic or KO-D group. Values for these parameters in tempol-treated KO-D after 28 days of diabetes still remained lower than those observed in WT-D with or without tempol treatment. L-NAME suppressed $C_{In}$, $U_{cGMP}$, and $U_{NOX}$ in WT-D in the presence or absence of tempol, and also suppressed tempol-induced increases in these parameters in the KO-D mice. L-NAME did not alter $C_{In}$, $U_{cGMP}$, and $U_{NOX}$ in the ND groups either in the presence or in the absence of tempol treatment (Fig. 7).

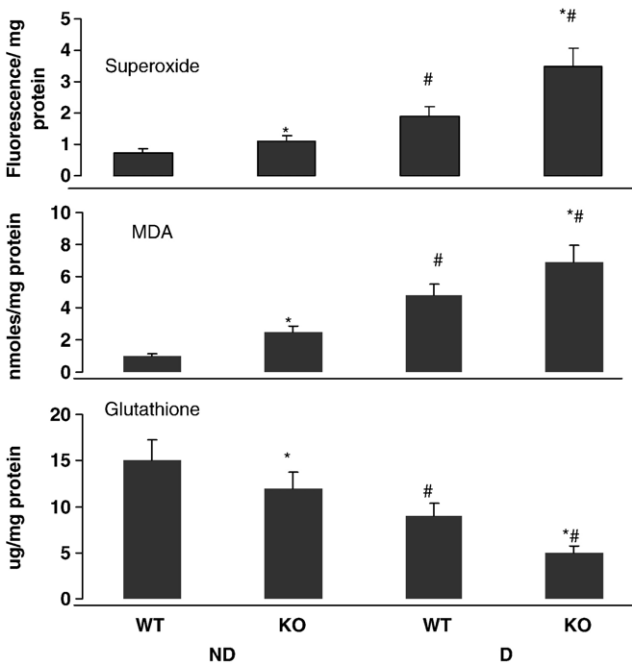


Fig. 6. Renal cortical MDA and GSH content and ex vivo superoxide bioactivity in isolated glomeruli. Data represent means ± SE of values from cortical samples or glomeruli from 10 to 14 mice in each study group at 5 months. Superoxide values are expressed as arbitrary fluorescence units per milligram glomerular protein in each well. *$P < .05$, comparing KO-ND with D to the corresponding WT group; #$P < .05$, comparing WT or KO-D with the corresponding ND group.

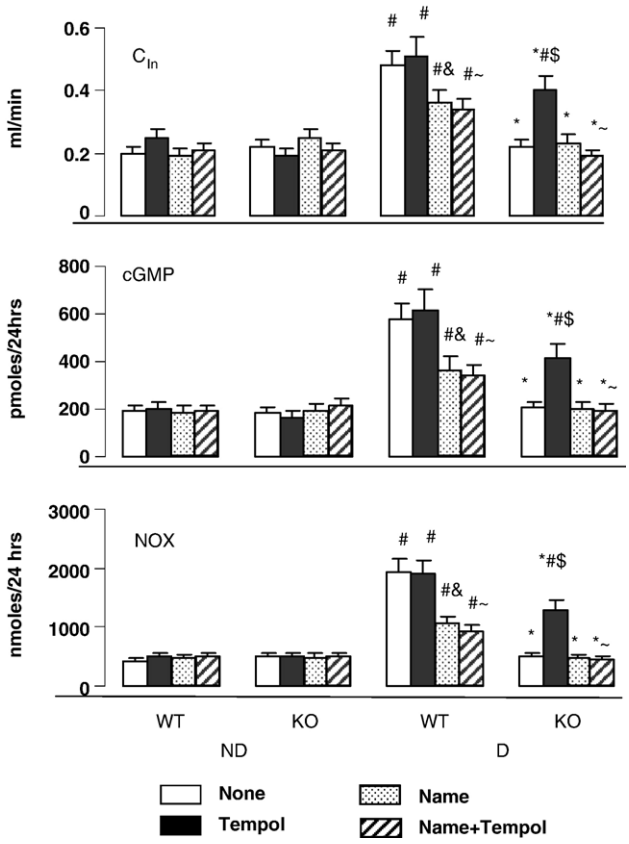


Fig. 7. $C_{In}$, $U_{NOX}$, and $U_{cGMP}$ were determined in WT and KO ND and D groups that received no treatment (None, □), tempol alone in the drinking water for 28 days (■), L-NAME alone in the drinking water for 25 to 28 days (▩), or tempol for 28 days with L-NAME for 3 days (▨). Data represent means ± SE of determinations from 9 mice per group. *$P < .05$, comparing KO-ND or D with the corresponding WT group; #$P < .05$, comparing WT or KO-D with the corresponding ND group; \$$P < .05$, comparing tempol alone with no treatment in the same group; &$P < .05$, comparing L-NAME alone with no treatment in the same group; ^$P < .05$ comparing tempol + L-NAME with tempol alone in the same group.

Table 2
Effects of tempol on $FC_{Alb}$ and glomerular content of immunoreactive TGF-β, collagen α1(IV), and NO tyrosine in ND and D KO mice

| | KO-ND | KO-D | KO-D + tempol |
|---|---|---|---|
| $FC_{Alb}$ (× $10^5$) | 5.3 ± 1.0 | 24.7 ± 5.6 * | 13.1 ± 2.9 *, † |
| TGF-β | 19.4 ± 3.4 | 55.0 ± 11.2 | 29.8 ± 5.1 *, † |
| Collagen | 21.8 ± 3.9 | 43.2 ± 8.5 * | 23 ± 4.0 † |
| NO tyrosine | 25.4 ± 6.1 | 59.6 ± 9.7 * | 35.3 ± 7.2 † |
| Superoxide (fluorescence/ mg protein) | 1.3 ± 0.3 | 3.5 ± 0.6 * | 1.9 ± 0.4 † |

Values represent means ± SE (n = 9). Values for glomerular immunoreactive content of TGF-β, collagen, and NO tyrosine are expressed as percent area of glomerular tuft staining positively.

* $P < .05$ compared with corresponding value in KO-ND group.

† $P < .05$ compared with untreated KO-D group.

As shown in Table 2, after 35 days of diabetes, $FC_{Alb}$, glomerular content of immunoreactive TGF-$\beta$, collagen $\alpha$1 (IV), and nitrotyrosine, and ex vivo superoxide bioactivity in isolated glomeruli were all higher in the untreated KO-D compared with KO-ND mice. At this early time point, values for these same parameters in KO-ND (Table 2) did not differ significantly from values in WT-ND or WT-D (not shown). Treatment of KO-D with tempol significantly suppressed $FC_{Alb}$ and glomerular content of TGF-$\beta$, collagen, and nitrotyrosine compared with corresponding values in untreated KO-D. Values for $FC_{Alb}$ and glomerular content of TGF-$\beta$ in tempol-treated KO-D remained higher than corresponding values in untreated KO-ND (Table 2) with the dose of tempol used. No significant difference in fractional mesangial area was found among the groups at this early time point. Tempol treatment also significantly suppressed ex vivo superoxide bioactivity in isolated glomeruli in both the KO-D (Table 2) and KO-ND ($0.6 \pm 0.1$). With the dose used, superoxide in tempol-treated KO-D remained modestly higher than in untreated KO-ND (Table 2) and considerably higher than in tempol-treated KO-ND ($0.6 \pm 0.1$).

## 5. Discussion

The current studies demonstrate acceleration of diabetic renal injury in an SOD1-deficient mouse model, with more pronounced UAE and $FC_{Alb}$ evident after only 1 month of diabetes in the latter group compared with WT-D. After 5 months of diabetes, renal injury and oxidative stress were more severe in KO-D, although mean BG was lower in this diabetic group compared with WT-D during the final 4 months of the study (Fig. 1). The mechanisms responsible for the apparent secondary attenuation of hyperglycemia in KO-D were not assessed. However, differences were not attributable to the total dose of STZ used to induce initial hyperglycemia or to food intake, both of which were comparable in KO and WT mice. In view of the direct vasoconstrictive action of superoxide and its capacity to inactivate NO [9,11,27,28], hypertension might be anticipated in SOD1 knockout mice, analogous to findings in the eNOS knockout model [29]. In the current study, BP did not differ significantly among the study groups. An earlier study reported lower systolic BP in nondiabetic SOD1 knockout mice compared with their age-matched WT littermates [22]. Whatever the basis for the apparent lack of a hypertensive effect of SOD1 deficiency, the current findings indicate that the acceleration of diabetic renal injury observed in this model is not attributable to systemic hypertension. It is of note that the KO-ND also demonstrated modest increases in glomerular content of TGF-$\beta$, collagen, nitrotyrosine, and mesangial matrix, and renal oxidative stress by approximately 7 months of age compared with these same parameters in age-matched WT-ND (Figs. 3-5). Previously reported studies of nondiabetic SOD1 knockout mice found evidence for increases in superoxide and endothelial dysfunction due to quenching of NO by superoxide in aorta [22], and for nitrotyrosine accumulation in mesenteric arteries [23]. In the present studies, the biochemical and histologic renal changes found in KO-ND were not associated with detectable differences in $C_{In}$ or $FC_{Alb}$ compared with WT-ND (Fig. 2). However, it is possible that differences in renal function between KO-ND and WT-ND would have emerged with a longer period of observation.

Conversely, there were notable sequential differences in $C_{In}$ between KO-D and WT-D. Thus, after 1 month of diabetes, $C_{In}$ in WT-D, but not KO-D, was clearly increased compared with ND mice (Figs. 2 and 7). The absence in KO-D of glomerular hyperfiltration that is characteristic of early diabetes may be at least in part attributable to increased superoxide. Thus, the SOD mimetic tempol enhanced $C_{In}$, in association with increases in $U_{cGMP}$ and $U_{NOX}$, and suppression of glomerular superoxide in KO-D. Conversely, both the early hyperfiltration observed in WT-D and the enhancement of $C_{In}$ induced by tempol in KO-D were at least in part dependent on NO. This is supported by the action of the NOS inhibitor L-NAME to suppress $C_{In}$ in both instances, and concurrently to reduce $U_{cGMP}$ and $U_{NOX}$. These data combined with the high content of glomerular nitrotyrosine in KO-D, likely a reflection at least in part of enhanced peroxynitrite formation [30], suggest that increased renal superoxide may have decreased renal bioactivity of NO in early diabetes in KO-D. Although not specifically assessed, quenching of NO by higher renal superoxide may also have contributed to the decline in $C_{In}$ in WT-D that occurred between 1 and 5 months.

The mechanisms by which tempol increased $U_{NOX}$ in KO-D (Fig. 7) in the present study were not delineated. As noted above, "uncoupling" of eNOS has been observed in diabetes with a resultant decreased production of NO and increased superoxide generation by this enzyme [11]. This change may be due to the direct oxidation of the zinc-thiolate complex of the enzyme by peroxynitrite [31], oxidative degradation of the eNOS cofactor tetrahydrobiopterin [11], or both. Tempol may have prevented uncoupling of eNOS and increased NO production by eNOS in KO-D via reduced peroxinitrite formation and/or stabilization of tetrahydrobiopterin [32]. In this regard, tempol has previously been shown to ameliorate glomerular injury and oxidative stress in hypertensive rats [33] and attenuate endothelial dysfunction in STZ diabetic rats [34]. The action of L-NAME to prevent tempol-induced increases in $U_{NOX}$ in KO-D (Fig. 7) is consistent with but does not prove a role for uncoupled eNOS in the changes in $U_{NOX}$ observed.

Deficiency of SOD1 would be expected to lead to increases in superoxide arising predominantly from cytosolic sources of production, in view of the known subcellular distribution of this SOD isoform [16,17]. However, SOD1 is also localized to the intermembrane space of mitochondria and degrades superoxide of mitochondrial origin that enters this space [18]. Thus, SOD1 deficiency in diabetes may also potentiate actions of superoxide derived from mitochondria

[6]. Mitochondria from the renal cortex of diabetic rats produce increased superoxide ex vivo, a change that is associated with glycooxidative damage to mitochondrial proteins [35]. Moreover, in contrast to the findings in WT-D and ND mice in which renal cortical and glomerular SOD2 activity did not differ, the latter activity was lower in the renal cortex and isolated glomeruli of KO-D than in either ND group. The explanation for this difference is uncertain. However, nitrosylation of tyrosine 34 of SOD2 has been shown to reduce the activity of this enzyme [36], and among the study groups, accumulation of NO tyrosine was greatest in glomeruli of KO-D mice (Fig. 3). Thus, it is possible that a secondary reduction in SOD2 activity occurred in KO-D by this mechanism and that this change contributed to renal oxidative stress and injury in this model.

Numerous examples of both positive and negative interplay among systems involved in formation and degradation of ROS within cells have been described [21], including an action of superoxide of mitochondrial origin to activate, and thus enhance, production of cytosolic superoxide by the nicotinamide adenine dinucleotide phosphate oxidase system of endothelial cells [37]. The latter in turn may lead to "uncoupling" of eNOS by oxidative degradation of tetrahydrobiopterin and consequent increased superoxide production from this source [21]. Thus, deficiency of SOD1 in the diabetic mouse may have had multiple secondary effects that amplified increases in superoxide and led to accelerated renal injury.

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