Reduction of Renal Superoxide Dismutase in Progressive Diabetic Nephropathy

Hiroki Fujita,*† Hiromi Fujishima,* Shinsuke Chida,‡ Keiko Takahashi,† Zhonghua Qi,† Yukiko Kanetsuna,§ Matthew D. Breyer,† Raymond C. Harris,‡ Yuichiro Yamada,* and Takamune Takahashi†

*Division of Endocrinology, Metabolism and Geriatric Medicine, and ‡Bioscience Education Research Center, Akita University School of Medicine, Akita, Japan; †Division of Nephrology and Hypertension, Vanderbilt University Medical Center, Nashville, Tennessee; and §Division of Pathology, Jikei University School of Medicine, Tokyo, Japan

ABSTRACT
Superoxide excess plays a central role in tissue damage that results from diabetes, but the mechanisms of superoxide overproduction in diabetic nephropathy (DN) are incompletely understood. In the present study, we investigated the enzyme superoxide dismutase (SOD), a major defender against superoxide, in the kidneys during the development of murine DN. We assessed SOD activity and the expression of SOD isoforms in the kidneys of two diabetic mouse models (C57BL/6-Akita and KK/Ta-Akita) that exhibit comparable levels of hyperglycemia but different susceptibility to DN. We observed down-regulation of cytosolic CuZn-SOD (SOD1) and extracellular CuZn-SOD (SOD3), but not mitochondrial Mn-SOD (SOD2), in the kidney of KK/Ta-Akita mice which exhibit progressive DN. In contrast, we did not detect a change in renal SOD expression in DN-resistant C57BL/6-Akita mice. Consistent with these findings, there was a significant reduction in total SOD activity in the kidney of KK/Ta-Akita mice compared with C57BL/6-Akita mice. Finally, treatment of KK/Ta-Akita mice with a SOD mimetic, tempol, ameliorated the nephropathic changes in KK/Ta-Akita mice without altering the level of hyperglycemia. Collectively, these results indicate that down-regulation of renal SOD1 and SOD3 may play a key role in the pathogenesis of DN.


Diabetic nephropathy (DN) is the leading cause of end-stage renal disease. Although hyperglycemia is clearly a prerequisite for the development of DN, alone it is insufficient for its development. Epidemiologic studies demonstrate only 10% to 40% of all diabetic patients get DN, despite comparable levels of glucose control in those subjects developing DN versus spared. In addition, sibling studies show a strong familial component for the risk of developing persistent proteinuria, suggesting a genetic basis for DN risk.1,2 However, the molecular or cellular mechanisms coupled with the genetic susceptibility to DN are incompletely understood.

There is compelling evidence that superoxide excess induced by diabetic hyperglycemia plays a central role in diabetic vascular cell damage.3 High glucose flux increases the production of superoxide anion (O$_2^\bullet^-$) by mitochondrial electron-transport chain, and the overproduced superoxide enhances the major pathways of hyperglycemic vascular cell
damage, including protein kinase C, advanced glycation end (AGE) products, and hexosamine pathways. In addition, superoxide is produced by multiple pathogenic pathways of diabetes. These include increased nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase activity, uncoupled endothelial nitric oxide synthase (eNOS), and enhanced signaling of AGES, angiotensin II, and oxidized-LDL receptors. Excessive production of superoxide anion results in the formation of secondary reactive oxygen species (ROS) including peroxynitrite and hydroxyl radicals, leading the damage of DNA, proteins, and lipids, and causes vascular cell injury. Thus, superoxide overproduction is considered as a major pathogenic pathway in diabetic vascular complication.

A net accumulation of superoxide anion is determined by a balance between superoxide production and antioxidant capacity. In this context, antioxidant defense system could play a critical role in diabetic vascular damage. Superoxide dismutase (SOD) is the major antioxidant enzyme for superoxide removal, which converts superoxide into hydrogen peroxide ($\text{H}_2\text{O}_2$) and molecular oxygen. The hydrogen peroxide is further detoxified to water ($\text{H}_2\text{O}$) by catalase or glutathione peroxidase. In mammals, three SOD isoforms exist: cytoplasmic CuZnSOD (SOD1), mitochondrial MnSOD (SOD2), and extracellular CuZnSOD (SOD3, ecSOD). Each SOD isoform is derived from distinct genes but catalyzes the same reaction, producing $\text{H}_2\text{O}_2$ from $\text{O}_2^-$. There is substantial evidence that SOD activity in peripheral blood cells is reduced in the diabetic patients with DN as compared with those without diabetic complication. In addition, recent studies have implicated SOD (SOD1 and SOD2) gene polymorphism in human DN risk. Furthermore, it was shown that the transgenic mice with SOD (SOD1 or SOD2) gene are resistant to diabetes-induced vascular injuries, including nephropathy. In aggregate, these findings suggest a pivotal role of SOD enzyme in the pathogenesis of DN. However, the changes in renal SOD enzymes in DN and their significance are poorly described.

Recent studies of streptozotocin (STZ)-induced diabetic mice have shown that genetic factors significantly affect the development and the severity of DN in mice as well as in human. Among the inbred strains of mice, KK/HJ and DBA/2 strains have been identified as DN-prone strains, whereas the widely used C57BL/6 strain is relatively resistant to DN. Compared with the STZ model, spontaneously diabetic mice offer a unique opportunity to assess the pathogenic pathways in DN without the potential nonspecific tissue toxicity of STZ. Ins2Akita mouse (Akita mouse) is a well-studied nonobese hypoinsulinemic diabetic mouse. This diabetic strain has a mutation in cysteine 96 to tyrosin in the insulin 2 gene (Akita mutation) and exhibits marked hyperglycemia as early as 4 wk of age. However, Akita mouse does not develop overt DN due to C57BL/6 background. To investigate renal alteration of SOD enzyme in advanced DN without the nonspecific tissue toxicity of STZ, we here generated a new congenic strain of the Akita mutation that exhibits progressive DN by backcrossing C57BL/6-strain Akita mouse (C57BL/6-Akita) to the nephropathy prone KK/Ta strain mouse. Our data demonstrate that renal expression of SOD1 and SOD3, but not SOD2, is prominently down-regulated in KK/Ta-strain Akita mouse (KK/Ta-Akita), which exhibits progressive DN, whereas renal SOD expression was not altered in the DN-resistant C57BL/6-Akita mouse. Furthermore, the present study demonstrates that treatment with tempol, a SOD mimetic, remarkably ameliorates the nephropathic changes in KK/Ta-Akita mice. Taken together, these results suggest that downregulation of renal SOD1 and SOD3 may play a key role in the pathogenesis of DN.

RESULTS

Development of the KK/Ta-Akita Mouse

To investigate the alterations of renal SOD enzyme in advanced DN, first we backcrossed C57BL/6-Akita mouse to KK/Ta strain, a DN-prone strain mouse, for 10 generations and developed an Akita mouse strain that accompanies progressive DN. The male mice were characterized and used for the study. As shown in Figure 1, A and B, KK/Ta-Akita males developed hyperglycemia (>300 mg/dl) at around 5 wk of age as did C57BL/6-Akita males, and both strains exhibited markedly elevated blood glucose and HbA$_1c$ levels after 10 wk of age. KK/Ta-Akita and C57BL/6-Akita mice showed comparable blood glucose levels, and there was no difference in the severity of hyperglycemia between these two groups of mice. KK/Ta and C57BL/6 wildtype (WT) mice exhibited normal blood glucose levels during the study period, although KK/Ta-WT mice showed higher blood glucose levels than C57BL/6-WT mice at 20 wk of age. As shown in Figure 1C, both KK/Ta-Akita and C57BL/6-Akita mice exhibited significantly lower body weight as compared with nondiabetic WT mice, and an increase in body weight was not observed in these mice after 10 wk of age. No difference was observed in body weight between C57BL/6-Akita and KK/Ta-Akita males. Table 1 shows systolic BP and blood parameters for each group of mice. KK/Ta-Akita mice showed increases in systolic BP, total cholesterol, triglyceride, blood urea nitrogen (BUN), and creatinine as compared with KK/Ta-WT mice. Significant differences were not observed in these parameters between C57BL/6-WT and C57BL/6-Akita mice, although C57BL/6-Akita mice showed higher level of systolic BP. KK/Ta-WT mice showed higher BP and plasma triglyceride levels than C57BL/6-WT mice.

Renal Phenotype in KK/Ta-Akita and C57BL/6-Akita Mice

Renal changes in KK/Ta-Akita and C57BL/6-Akita males were assessed by the measurements of urine albumin excretion, FITC-inulin clearance to estimate GFR, kidney-to-body weight ratio, and renal histopathology. Compared with KK/Ta-WT males, KK/Ta-Akita males showed significantly increased urine albumin excretion as early as 5 wk of age, and...
albuminuria became pronounced as the mice aged, exceeding 600 of albumin-to-creatinine ratio (ACR), whereas the increase in urine albumin in C57BL/6-Akita males was very small and insignificant even at 20 wk of age (Figure 2A). Mild albuminuria was also observed in KK/Ta-WT males at 20 wk of age. Figure 2B shows GFR in KK/Ta-Akita and C57BL/6-Akita males. Both KK/Ta-Akita and C57BL/6-Akita males exhibited increased GFR at 10 and 15 wk of age as compared with WT mice, indicating development of glomerular hyperfiltration in these mice. Notably, GFR in KK/Ta-Akita males declined at 20 wk of age, whereas reduction of GFR was not observed in C57BL/6-Akita males. Figure 2C shows left kidney weight-to-body weight ratio (LKW/BW) in these mice. KK/Ta-Akita showed significantly increased LKW/BW as early as 5 wk of age as compared with KK/Ta-WT males, and this became more evident at 15 wk of age, demonstrating prominent diabetic renal hypertrophy in KK/Ta-Akita mice (Figure 2C). Statistically, significant increase in LKW/BW was not observed in C57BL/6-Akita males at these time points.

Renal histopathology was assessed by light and electron microscopy. Figure 3A shows representative glomerular light micrographs from KK/Ta-Akita and C57BL/6-Akita males. Moderate mesangial expansion, as evidenced by increased accumulation of periodic acid-Schiff (PAS)-positive material in the mesangial area, was observed in 15-wk-old KK/Ta-Akita males (Figure 3Ad), whereas mesangial expansion was relatively mild in 15-wk-old C57BL/6-Akita males (Figure 3Ab). KK/Ta-WT and C57BL/6-WT mice showed normal glomerular histology. Semiquantitative analysis of PAS-stained kidney sections revealed significantly higher mesangial expansion score in 15-wk-old KK/Ta-Akita males as compared with KK/Ta-WT and C57BL/6-Akita mice (Figure 3C). Glomerular lesions in KK/Ta-Akita males progressed as the mice aged, and prominent mesangial expansion and glomerulosclerosis were observed in approximately 40% of glomeruli at 20 wk of age (Figure 3A, e and f). Arteriolar hyalinosis and focal tubulointerstitial fibrosis were also noted in 20-wk-old KK/Ta-Akita mice (data not shown). Figure 3B shows representative glomerular electron micrographs from the 15-wk-old KK/Ta-Akita mice. Mesangial matrix expansion and irregular thickening of the basement membrane were evident in 20-wk-old KK/Ta-Akita mice (Figure 3B).

Table 1. Physiological parameters of C57BL/6-Akita and KK/Ta-Akita mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>C57BL/6</th>
<th>Akita</th>
<th>KK/Ta</th>
<th>Akita</th>
</tr>
</thead>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>93 ± 2</td>
<td>109 ± 6</td>
<td>118 ± 2a</td>
<td>126 ± 4c</td>
</tr>
<tr>
<td>Plasma creatinine (mg/dl)a</td>
<td>0.152 ± 0.007</td>
<td>0.178 ± 0.008</td>
<td>0.125 ± 0.004</td>
<td>0.248 ± 0.030d</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>27.0 ± 3.6</td>
<td>41.8 ± 2.5</td>
<td>31.1 ± 0.7</td>
<td>45.1 ± 4.2e</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>81.0 ± 4.6</td>
<td>66.2 ± 3.4</td>
<td>83.3 ± 4.2</td>
<td>106.9 ± 15.7f</td>
</tr>
<tr>
<td>Triglyceride (mg/dl)</td>
<td>63.8 ± 7.3</td>
<td>62.0 ± 4.3</td>
<td>185.3 ± 18.3b</td>
<td>192.4 ± 23.1c</td>
</tr>
</tbody>
</table>

Values are means ± SEM. The data of 15-wk-old mice are shown. n = 5 to 8 per group.

aThe data indicates plasma creatinine levels at 20 wk of age.

bP < 0.01 versus C57BL/6-WT.

cP < 0.01 versus C57BL/6-Akita.

dP < 0.001 versus KK/Ta-WT.

eP < 0.05 versus KK/Ta-WT.

fP < 0.05 versus C57BL/6-Akita.
ing of glomerular basement membrane (GBM) were evident in KK/Ta-Akita mice (Figure 3B, a and b). Morphometric analysis revealed a significant increase in GBM thickness in KK/Ta-Akita males compared with KK/Ta-WT males (Figure 3D). In addition, subendothelial insudation, mesangiolysis, local foot process effacement, and macrophage infiltration were observed in the KK/Ta-Akita glomeruli. No electron dense deposits were observed in the KK/Ta-Akita glomeruli. Obvious ultrastructural changes were not observed in C57BL/6-Akita glomeruli other than mild mesangial matrix accumulation. KK/Ta-WT and C57BL/6-WT mice showed normal glomerular morphology.

Figure 2. Renal changes in male KK/Ta-Akita and C57BL/6-Akita mice. (A) Urinary albumin excretion was assessed by the determination of urine ACR (n = 8 per group). (B) GFR was measured by FITC-inulin clearance method in conscious mice (n = 8 per group). (C) LKW/BW was assessed at 5 and 15 wk of age (n = 6 per group). Values are means ± SEM. *P < 0.001 versus C57BL/6-Akita mice; +P < 0.01; ++P < 0.05 versus KK/Ta-WT mice; ***P < 0.001 versus C57BL/6-WT mice.

Figure 3. Morphology of KK/Ta-Akita glomeruli. (A) Representative glomerular histopathology in each group mice at 15 to 20 wk of age. (a) C57BL/6-WT (15 wk old), (b) C57BL/6-Akita (15 wk old), (c) KK/Ta-WT (15 wk old), (d) KK/Ta-Akita (15 wk old), (e and f) KK/Ta-Akita (20 wk old). PAS stain, original magnification: ×400. (B) Representative glomerular electron micrographs from 15-wk-old KK/Ta-Akita and KK/Ta-WT mice. (a and b) KK/Ta-Akita, (c) KK/Ta-WT. Arrows indicate irregular thickening of GBM. Asterisks indicate mesangial matrix expansion. CL, capillary lumen; En, endothelial cells; Mφ, macrophage; Mes, mesangial cells. Original magnification, ×5000 in a) ×15,000 in b and c. (C) Glomerular mesangial expansion scores of 15-wk-old mice. The scores were determined on perfusion-fixed PAS-stained kidney sections as described in the Concise Methods section. Data are presented as means ± SEM. #P < 0.001 versus C57BL/6-WT mice; *P < 0.001 versus C57BL/6-Akita mice; **P < 0.001 versus KK/Ta-WT mice; n = 6 per group. (D) GBM thickness in 15-wk-old KK/Ta-Akita and KK/Ta-WT mice. Data are presented as means ± SEM; +P < 0.0001 versus KK/Ta-WT mice; n = 6 per group.

Superoxide Production and SOD Activity in KK/Ta-Akita and C57BL/6-Akita Kidneys
To explore the mechanism by which KK/Ta-Akita mice show different susceptibility to DN, next we examined their renal superoxide production, which is thought a central pathogenic pathway in this disease. KK/Ta strain mouse is known to de-
velop obesity (exceeding 35 g of body weight) from 17 to 18 wk of age and become diabetic at around 20 wk of age (phenotypic data; CLEA Japan). Indeed, 20-wk-old KK/Ta-WT males showed higher blood glucose levels, body weight, and urine ACR, than C57BL/6-WT males (Figures 1 and 2). We therefore used 15-wk-old mouse kidneys for this investigation. Renal superoxide levels were assessed by water-soluble tetrazolium salt [2-(4-lodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium; WST-1] method and dihydroethidium (DHE) histochemistry. The specificity of the assay was confirmed by pretreating the samples with polyethylene glycol (PEG)-SOD protein. Consistent with previous reports, superoxide production was increased in the kidneys of C57BL/6-Akita and KK/Ta-Akita mice as compared with nondiabetic mice (Figure 4A). More importantly, the level of superoxide in KK/Ta-Akita kidney was significantly higher than that in C57BL/6-Akita kidney, despite comparable levels of hyperglycemia. Furthermore, glomeruli of KK/Ta-Akita mice showed stronger hydroethidium fluorescence than C57BL/6-Akita glomeruli, indicating increased glomerular superoxide in the mice. With the finding that KK/Ta-WT and C57BL/6-WT mice showed comparable levels of renal superoxide, these data suggested increased superoxide production and/or impaired antioxidant defense capacity in KK/Ta-Akita kidneys. Therefore, we next examined alteration of SOD antioxidant enzyme activity in KK/Ta-Akita and C57BL/6-Akita kidneys. SOD activity in kidney tissue extracts was determined by inhibition rate of WST-1 reduction reaction. Renal expression of SOD isoform was assessed by western blot analysis. As shown in Figure 4C, SOD activity was significantly reduced in the KK/Ta-Akita kidneys at 15 wk of age as compared with KK/Ta-WT or C57BL/6-Akita kidneys, while there was no difference in renal SOD activity among the four groups at 5 wk of age. Furthermore, Western blot analysis revealed remarkably reduced SOD1 and SOD3 proteins in the 15-wk-old KK/Ta-Akita kidneys, whereas SOD downregulation was not observed in C57BL/6-Akita kidneys (Figure 4D). Reduction of SOD2 protein was not observed in both KK/Ta-Akita and C57BL/6-Akita kidneys (Figure 4D). The downregulation of SOD1 and SOD3 proteins was not observed in 5-wk-old KK/Ta-Akita kidneys (data not shown).

To date, several intracellular pathways were implicated in superoxide overproduction in diabetic vasculature. These include NADPH oxidase, uncoupled eNOS, xanthine oxidase, and mitochondrial respiratory chain enzymes. Among these pathways, NADPH oxidase and uncoupled eNOS were shown to serve as a major source of glomerular superoxide in a diabetic animal. We therefore investigated alteration of these two enzymes in KK/Ta-Akita kidneys (data not shown). However, no difference was observed in renal NADPH oxidase activity between C57BL/6-Akita and KK/Ta-Akita kidneys, yet these kidneys exhibited significantly higher NADPH oxidase activity as compared with nondiabetic counterparts. Furthermore, the eNOS expression was downregulated in KK/Ta-Akita kidneys as compared with C57BL/6-Akita kidneys. Consequently, these findings suggest that the increase in ROS in this model may largely result from the disruption of SOD antioxidant defense system.

Figure 4. Renal superoxide production and SOD activity and expression in KK/Ta-Akita and C57BL/6-Akita mice. (A) Renal superoxide production in 15-wk-old mice. Data are presented as means ± SEM. SOD+, kidney tissue preincubated with SOD-PEG protein. SOD-, kidney tissue without SOD-PEG protein. *P < 0.05 versus SOD- C57BL/6-WT kidney; +P < 0.001 versus SOD- KK/Ta-WT kidney; #P < 0.01 versus SOD- C57BL/6-Akita kidney; n = 6 per group. (B) Representative glomerular DHE staining in 15-wk-old mice. (a) C57BL/6-WT, (b) C57BL/6-Akita, (c) KK/Ta-WT, (d) KK/Ta-Akita. Original magnification, ×400. (C) Renal SOD activity at 5 and 15 wk of age. Data are presented as means ± SEM. *P < 0.05 versus C57BL/6-Akita mice; +P < 0.001 versus KK/Ta-WT mice; n = 8 per group. (D) Western blot analysis of renal SOD isoform expression in 15-wk-old mice. The relative intensity of SOD-to-actin ratio to the C57BL/6-WT mice is also shown (lower panel). Data are presented as means ± SEM. +P < 0.001 versus KK/Ta-WT mice; *P < 0.001 versus C57BL/6-Akita mice; n = 4 per group.
Renal Expression of SOD Isoforms in KK/Ta-Akita and C57BL/6-Akita Mice

To determine the cell type responsible for the SOD downregulation in KK/Ta-Akita kidney, we further investigated the renal SOD isoform expression by immunofluorescence histochemistry. As shown in Figure 5, SOD1 and SOD2 were broadly immunolabeled in multiple renal cell types, including glomerular cells and tubular epithelium. Compared with SOD1, dominant SOD2 immunoreactivity was observed in proximal tubules, and SOD2 was low levels in glomerulus. In contrast, high level of SOD3 expression was observed in glomerular capillary and arterial/arteriolar wall (not shown), and its expression was highly restricted to these sites. There was no difference in the pattern of SOD isoform expression between C57BL/6-WT and KK/Ta-WT kidneys. Consistent with the results of western blot analysis, SOD1 and SOD3 immunoreactivity were reduced in the 15-wk-old KK/Ta-Akita kidney (Figure 5, D and L), whereas their expression were unaltered in C57BL/6-Akita kidney (Figure 5, B and J). SOD1 down-regulation was observed in tubular epithelium as well as in glomerulus in KK/Ta-Akita kidney. SOD2 immunoreactivity was not altered in both C57BL/6-Akita and KK/Ta-Akita kidneys.

Effects of Tempol on DN in KK/Ta-Akita Mice

To determine the significance of SOD downregulation in DN of KK/Ta-Akita mice, we next treated KK/Ta-Akita mice with the membrane-permeable, metal-free SOD mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxide (tempol) and asked whether supplementation of SOD activity ameliorates DN of the KK/Ta-Akita mice. Ten-week-old KK/Ta-Akita and KK/Ta-WT mice were treated either with tempol (1 mmol/L in drinking water) or vehicle for 4 wk (n = 6 for each group). Blood parameters, BP, urinary albumin excretion, and GFR were measured before and after 4 wk of tempol treatment. Renal superoxide production and LKW/BW were assessed at the end of treatment. Histologic analysis was performed on PAS-stained kidney sections after 4 wk of treatment. Table 2 shows the physiologic parameters in tempol-treated or vehicle-treated KK/Ta-WT and KK/Ta-Akita mice. Shown in the Table 2, 4-wk tempol treatment did not alter blood glucose, HbA1c,

Table 2. Physiological parameters after 4-wk tempol treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>KK/Ta-WT Vehicle</th>
<th>Tempol</th>
<th>KK/Ta-Akita Vehicle</th>
<th>Tempol</th>
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<tr>
<td>Body weight (g)</td>
<td>30.1 ± 0.5</td>
<td>31.2 ± 0.9</td>
<td>23.1 ± 0.9</td>
<td>21.6 ± 0.7</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>113 ± 4</td>
<td>116 ± 2</td>
<td>122 ± 3</td>
<td>117 ± 5</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>100 ± 19</td>
<td>100 ± 4</td>
<td>519 ± 34</td>
<td>486 ± 29</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>3.7 ± 0.1</td>
<td>3.7 ± 0.1</td>
<td>12.3 ± 0.4</td>
<td>11.5 ± 0.2</td>
</tr>
<tr>
<td>Plasma creatinine (mg/dl)</td>
<td>0.135 ± 0.003</td>
<td>0.149 ± 0.022</td>
<td>0.165 ± 0.014</td>
<td>0.176 ± 0.014</td>
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<tr>
<td>BUN (mg/dl)</td>
<td>23.0 ± 1.4</td>
<td>22.2 ± 1.0</td>
<td>44.1 ± 4.1</td>
<td>42.0 ± 5.8</td>
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<tr>
<td>Total cholesterol (mg/dl)</td>
<td>87.7 ± 6.6</td>
<td>83.7 ± 6.2</td>
<td>147.4 ± 10.4</td>
<td>139.8 ± 15.5</td>
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<tr>
<td>Triglyceride (mg/dl)</td>
<td>202.4 ± 10.5</td>
<td>195.9 ± 12.3</td>
<td>202.8 ± 10.6</td>
<td>112.0 ± 17.9</td>
</tr>
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</table>

Values are means ± SEM. n = 6 per group; n = 4 per group for plasma creatinine.

*P < 0.01 versus KK/Ta-Akita vehicle.

Figure 5. Immunofluorescence SOD isofrom staining of 15-wk-old mouse kidney sections. (A to D) SOD1, (E to H) SOD2, (I to L) SOD3, (A, E, and I) C57BL/6-WT mice, (B, F, and J) C57BL/6-Akita mice, (C, G, and K) KK/Ta-WT mice, (D, H, and L) KK/Ta-Akita mice. Arrows indicate glomeruli. Original magnification, ×200 in A through D, ×100 in E through H, and ×400 in I through L.
body weight, systolic BP, plasma creatinine, BUN, and total cholesterol levels in KK/Ta-Akita mice. However, the tempol treatment dramatically reduced albuminuria and suppressed elevation of GFR and renal hypertrophy close to the levels of KK/Ta-WT mice (Figure 6, A, B, and C). In agreement with the improvement in renal functions, glomerular DHE stain and renal superoxide levels were significantly reduced in the tempol-treated KK/Ta-Akita mice (Figure 6D). Finally, tempol-treated KK/Ta-Akita mice showed limited mesangial matrix expansion as compared with the vehicle-treated group (Figure 6, E and F). In aggregate, these findings suggest that reduction of renal SOD enzyme may play a key role in the development of overt DN.

**DISCUSSION**

Our data clearly indicate that two Akita diabetic mouse models, C57BL/6-Akita and KK/Ta-Akita, exhibit differential susceptibility to DN, despite comparable levels of hyperglycemia. KK/Ta-Akita mice develop overt albuminuria as the mice age, whereas C57BL/6-Akita mice are relatively resistant to the development of albuminuria. In addition, the time course of diabetic renal disease in KK/Ta-Akita mice resembles that in human DN. KK/Ta-Akita mice exhibit glomerular hyperfiltration early after the onset of diabetes followed by mesangial expansion, GBM thickness, and a progressive decline in GFR. Although an increase in urine albumin excretion was already observed in KK/Ta-Akita mice at 5 wk of age, this could be because approximately 50% of Akita mice develop hyperglycemia soon after weaning. It is known that KK/Ta mouse strain develops obesity and insulin resistance from 17 to 18 wk of age and becomes diabetic at around 20 wk of age. Therefore, a decline in GFR in KK/Ta-Akita mice at 20 wk of age may be caused by these factors. However, we think this is unlikely as there is no obesity in KK/Ta-Akita mice. Thus, C57BL/6-Akita and KK/Ta-Akita mice provided us a platform for comparative analysis of renal SOD enzyme in the development of overt DN without use of chemicals such as STZ or alloxan, which were documented to generate ROS. Furthermore, the involvement of a single gene, Ins2, in the induction of diabetes in this model minimizes a possible involvement of complex traits for DN.

![Figure 6](image_url)

**Figure 6.** Effects of tempol on DN in KK/Ta-Akita mice. The tempol treatment started at 10 wk of age (0 wk) and ended at 14 wk of age (4 wk). (A) Changes in urinary albumin excretion levels. Values are means ± SEM. *P < 0.01; #P < 0.0001; n = 6 per group. (B) Changes in GFR. Values are means ± SEM. **P < 0.05; n = 6 per group. (C) LKW/BW in KK/Ta-WT and KK/Ta-Akita mice after 4-wk treatment. Values are means ± SEM. **P < 0.05; n = 6 per group. (D) Representative glomerular DHE stain (upper panels) and renal superoxide production (lower panel) in KK/Ta-WT and KK/Ta-Akita mice after 4-wk treatment. Data are presented as means ± SEM. ##P < 0.001; n = 5 per group. SOD+, kidney tissue preincubated with SOD-PEG protein; SOD-, kidney tissue without SOD-PEG protein. Original magnification (upper panels), ×400. (E) Representative light microscopic images of the KK/Ta-Akita glomeruli after 4-wk treatment. (a and b) vehicle-treated; (c and d) tempol-treated. PAS stain, original magnification, ×400. (F) Glomerular mesangial expansion scores in KK/Ta-WT and KK/Ta-Akita mice after 4-wk treatment. Values are means ± SEM. *P < 0.01; n = 6 per group.
The present study demonstrates that renal SOD activity is reduced in the KK/Ta-Akita mice that develop overt DN, concomitant with remarkable down-regulation of renal SOD1 and SOD3 proteins. Furthermore, DN in KK/Ta-Akita mice was effectively suppressed by SOD mimetic, although severity of diabetes was not altered by the treatment. These findings emerged an important role of renal SOD enzyme in the development of overt DN. It is known that SOD enzyme is up-regulated in response to an increase in oxidative stress.\textsuperscript{30,31} This is a critical cellular defense mechanism against excessive oxidative stress. It is therefore conceivable that reduction of SOD enzyme in the diabetic condition causes cytotoxic levels of superoxide overproduction, leading to diabetic renal cell injury. Several studies have investigated renal SOD activity and expression in rodent models of diabetic nephropathy. These include db/db mouse,\textsuperscript{19,32} KKAy mouse,\textsuperscript{33} and STZ mouse or rat.\textsuperscript{33–36} However, these investigations showed up-regulated or unaltered renal SOD activity. With the fact that nephropathy in these rodent models is milder than that in KK/Ta-Akita mice, these findings suggest that SOD downregulation (disruption of an adaptive renal response to increased oxidative stress) may be a key pathogenic mechanism in the progression of DN. It is noteworthy that C57BL/6-Akita mice do not develop overt DN despite of increased renal superoxide levels; the finding suggests that the level of renal SOD activity may be a critical determinant for the development of overt DN. The decrease in renal SOD activity was not observed in KK/Ta-Akita mice at 5 wk of age, despite the fact that they were already diabetic at this age. Rather, both KK/Ta-Akita and C57BL/6-Akita mice showed higher values of SOD activity than nondiabetic mice. This finding suggests that initial response of renal SOD enzyme to hyperglycemia in KK/Ta-Akita mice does not differ from that in C57BL/6-Akita mice. It is therefore likely that reduction of SOD enzyme in KK/Ta-Akita mouse kidney is caused by the secondary factor of chronic hyperglycemia, such as hypertension or inflammation. In this context, it is of interest that TNF\textsubscript{a} or IL-1\textbeta was shown to down-regulate SOD1 or SOD3 protein in cell cultures.\textsuperscript{37,38} Further study would be required about this point.

The SOD family consists of three isoforms, SOD1, SOD2, and SOD3.\textsuperscript{8,10} SOD1 is expressed as a predominant isoform in all cells including blood vessels. SOD2 is considered the first line of defense against O\textsubscript{2}\textsuperscript{−}, because the mitochondria electron transport chain is a major source of O\textsubscript{2}\textsuperscript{−} under normal conditions. SOD3 is the only SOD isoform that is expressed extracellularly, binding to cell surfaces and extracellular matrix via its heparin-binding domain. SOD3 is produced by vascular smooth muscle cells (VSMC) and localized throughout the vessel walls, particularly between endothelium and VSMC.\textsuperscript{39} In normal mouse artery, SOD1 accounts for 50\% to 80\% of total SOD activity, SOD2 accounts for 2\% to 12\%, and SOD3 accounts for the remainder.\textsuperscript{10,40} Like artery, evident SOD3 immunoreactivity was observed in glomerular microvasculature; the finding implicates SOD3 as a major SOD isoform in glomerulus. It is thought that one major function of SOD3 is to protect nitric oxide (NO) activity as it diffuses from endothelium to VSMC. SOD3\textsuperscript{−/−} mice showed decreased basal NO activity and impaired endothelium-dependent relaxation.\textsuperscript{41} SOD3 deficiency was also shown to increase arterial pressure in two models of hypertension.\textsuperscript{41,42} Furthermore, gene transfer of SOD3 reduced vascular superoxide levels and increased NO bioactivity in the spontaneously hypertensive rat (SHR).\textsuperscript{43} It has been suggested that SOD1 and SOD3 are major determinants of NO bioavailability in blood vessels.\textsuperscript{39} Thus, it is conceivable that downregulation of both SOD1 and SOD3 enzymes may largely increase superoxide levels in the glomerular microvasculature and also reduce bioactivity of glomerular NO, resulting in advanced DN. Indeed, an important role of SOD1 in DN was suggested by a recent study demonstrating that the SOD1-deficient mouse exhibits accelerated renal injury with high-dose STZ model.\textsuperscript{44} It would be important to determine the precise role of SOD1 and SOD3 enzymes in DN using SOD isoform-deficient spontaneously diabetic mice.

Thus, the present study demonstrates a crucial role of renal SOD enzyme in the development of overt DN. Further efforts to elucidate the molecular mechanism by which chronic hyperglycemia disrupts the renal SOD antioxidant defense system would give a new insight into pathogenesis of DN and lead to better treatment protocol for this disease.

### CONCISE METHODS

#### Animals
C57BL/6 and KK/Ta mice were purchased from CLEA Japan (Tokyo, Japan). Ins\textsuperscript{2Akita} mice with C57BL/6 background were from SLC (Hamamatsu, Japan). Ins\textsuperscript{2Akita} mice with KK/Ta background were generated by backcrossing male C57BL/6-Akita mice to KK/Ta females for 10 generations. Genotyping was performed as described previously.\textsuperscript{22} The mice were allowed unrestricted access to standard rodent chow and water. All animals were treated in accordance with the Animal Welfare Guidelines of Akita University, and all procedures were approved by the Committee on Animal Experimentation of Akita University.

#### Blood Parameter Measurements
We measured blood glucose using Glucocard Diameter (Arkray, Tokyo, Japan) on samples obtained after a 6-h daytime fast. We determined Hba\textsubscript{1c} levels using a DCA 2000 Analyzer (Bayer, Elkhart, IN). We enzymatically measured BUN, plasma total cholesterol, and plasma triglycerides by an autoanalyzer (Fuji Dry-Chem 5500; Fuji Medical Systems, Tokyo, Japan) on samples obtained after a 6-h daytime fast. We enzymatically measured BUN, plasma total cholesterol, and plasma triglycerides by an autoanalyzer (Fuji Dry-Chem 5500; Fuji Medical Systems, Tokyo, Japan).

#### BP Measurement
We measured systolic BP in conscious trained mice at room temperature using a noninvasive tail cuff and pulse transducer system (BP-98A; Softron, Tokyo, Japan).
Urine Albumin and Creatinine
We assessed urinary albumin excretion by determination of ACR on morning spot urine as described previously.21 We measured urine albumin by Alburell-M Murine Microalbuminuria ELISA kit (Exocell, Philadelphia, PA), and we determined urine creatinine levels using the Creatinine Companion kit (Exocell).

Measurement of GFR
We measured GFR by a single-bolus FITC-inulin injection method as described previously.46

Histologic Analysis
We assessed renal histopathology at 15 and 20 wk of age. We anesthetized the mice by an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt), and we perfused the kidneys with PBS and then with 4% paraformaldehyde in PBS via left ventricle. The kidneys were removed, weighed, and fixed overnight in 4% paraformaldehyde in PBS at 4°C. We stained 2-μm-thick paraffin sections with PAS. We used a semiquantitative score to evaluate the degree and extent of glomerular mesangial expansion as described previously.21 We analyzed six mice per group and assessed more than 60 glomeruli in each mouse. For electron microscopic examination, we perfused the kidneys with PBS, removed them, cut the cortex into small tissue fragments (1 mm3), and then fixed them in 3% glutaraldehyde in 0.1 mol/L cacodylate buffer (pH 7.4) overnight at 4°C. The kidney fragments were embedded in Epon resin by standard methods. We observed the sections using a H-7650 transmission electron microscope (Hitachi, Tokyo, Japan) at 80 kV. The thickness of GBM was measured using the H-7650 software (Hitachi). The measurements were undertaken perpendicularly from the endothelial cytoplasmic membrane to the outer lining of the lamina rata externa underneath the cytoplasmic membrane of the epithelial foot process. We assessed at least five glomeruli (total of 75 intercepts) per mouse, and we determined the mean GBM thickness for each mouse. Six mice were analyzed for each group.

Renal Superoxide Production
We assessed the levels of renal superoxide by WST-1 reduction assay and DHE histochemistry as described previously.27,47 WST-1 is reduced by O2•− to WST-1 formazan, whose levels can be measured by the absorption at 450 nm. DHE is converted by O2•− to ethidium (Eth), which binds to DNA and produces the Eth-DNA red fluorescence. We used PBS-perfused and freshly removed kidneys for fluorescence at 480-nm excitation and 610-nm emission using a LSM510 confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany).

Renal SOD Activity
We determined SOD activity in whole kidney tissue lysate using a SOD assay kit-WST (Dojindo Molecular Technologies) as described previously.27 We measured the amount of protein using a bicinchoninic acid protein assay (Sigma-Aldrich). Enzymatic activity was expressed in units per mg protein.

Western Blot Analysis
PBS-perfused kidney cortex tissues were homogenized in lysis buffer (0.25 M sucrose, 1 mM EDTA, pH 7.4), centrifuged at 10,000 × g for 10 min at 4°C, and the cleared lysate was used for western blot analysis. We determined the amount of protein using a bicinchoninic acid protein assay. Twenty micrograms protein were separated by SDS-PAGE, and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). After blocking, the membranes were reacted with: (i) rabbit anti-Cu/Zn SOD (SOD1) (1:10,000; Stressgen, Ann Arbor, MI); (ii) rabbit anti-Mn SOD (SOD2) (1:10,000; Stressgen); and (iii) rabbit anti-EC SOD (SOD3) (1:200; Stressgen) polyclonal antibodies. After washing, we incubated the membranes with HRP-conjugated goat anti-rabbit IgG antibody (1:10,000; DakoCytomation, Glostrup, Denmark). We visualized the reactions using an enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Loading of lysate protein was evaluated by immunoblot using rabbit anti-actin antibody (1:1000; Sigma-Aldrich) and HRP-conjugated goat anti-rabbit IgG antibody (DakoCytomation). The intensity of the signals was semiquantified using Adobe Photoshop (version 5.5; Adobe Systems, San Jose, CA) and determined by subtracting background of the adjacent area.

Immunohistochemistry
Cryostat sections were prepared as described in DHE histochemistry. We blocked the sections and labeled them with rabbit anti-Cu/Zn SOD (SOD1) (1:100; Stressgen), rabbit anti-Mn SOD (SOD2) (1:100; Stressgen), or rabbit anti-EC SOD (SOD3) (1:50; Stressgen) polyclonal antibodies for 1 h at room temperature, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody (1:200; Molecular Probes, Eugene, OR) for 30 min at room temperature. We then counterstained the sections with ToPro-3 (Molecular Probes) and took images using the LSM510 confocal laser-scanning microscope.

Treatment with Tempol
Metal-free SOD mimetic tempol (Sigma-Aldrich) was added in the drinking water (1 mmol/L) and given to the mice ad libitum for 4 wk as described previously.44,48 Control group mice were given the same water alone as vehicle.

Statistical Analysis
We presented all data as means ± SEM. We performed statistical analysis of the data using GraphPad Prism software (GraphPad, San Diego, CA). For comparisons between two groups, we used an un-
paired t test to assess statistical significance. We determined differences between multiple groups by one-way ANOVA followed by Bonferroni multiple comparison test. \( P < 0.05 \) was considered statistically significant.

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DISCLOSURES

None

REFERENCES


