Role of Nox2 in diabetic kidney disease

Young-Hyun You,1 Shinichi Okada,1 San Ly,1 Karin Jandeleit-Dahm,2 David Barit,2 Tamehachi Namikoshi,2 and Kumar Sharma1

1Center for Renal Translational Medicine, Division of Nephrology-Hypertension, Department of Medicine, University of California, San Diego, and Veterans Affairs San Diego Healthcare System, Veterans Medical Research Foundation, La Jolla, California; and 2Diabetes Complications Division, Baker IDI Heart and Diabetes Institute, Melbourne, Victoria, Australia

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The NADPH oxidase (Nox) system is considered a key contributor to generation of reactive oxygen species (ROS) in many cell types and tissues (29). The first Nox isoform to be identified was Nox2 (i.e., gp91phox) in neutrophils and macrophages (18). However, recent studies have identified Nox2 at varying expression levels in numerous cell types, including endothelial, vascular smooth muscle, mesangial, and tubular epithelial cells (4, 13, 16). There are presently five identified isoforms of Nox, plus the related Duox1 and Duox2 isoforms (5, 8, 11). Nox1 is primarily found in endothelial cells and is stimulated by angiotensin II to generate ROS. Nox3 is fetal with only low-level expression in adult tissues. Nox4 was initially identified in the kidney, but is now found to be ubiquitous. Nox5 is expressed in human endothelial cells, but not by rodent cells. Several studies have implicated the Nox system in diabetic microvascular complications, such as nephropathy (7, 10, 12, 15, 27).

The functional role of the Nox system in mediating diabetic microvascular complications has been deduced primarily using inhibitors such as apocynin and diphenylene iodonium in both cell and animal models. Apocynin inhibited albuminuria and renovascular dysfunction of diabetes, protects against glucose-induced ROS production in podocytes, and reduced podocyte apoptosis in the db/db mouse model of type 2 diabetes (32). As apocynin primarily appears to inhibit assembly of Nox2 and potentially also Nox1 and Nox3, the effects of apocynin may be independent of Nox4. The role of Nox4 in diabetic kidney disease was also examined using injection of anti-sense oligonucleotides to Nox4, and a protective effect was demonstrated in the streptozotocin (STZ) rat model of type 1 diabetes (15). Although these prior studies demonstrate a potential role for Nox, the specific contribution of Nox2 has not been determined. We, therefore, investigated the role of Nox2 in onset of diabetic kidney using wild-type (WT) and Nox2 knockout (KO) mice.

MATERIALS AND METHODS

Animal studies. Male C57BL/6J WT and age-matched Nox2 KO mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were given standard rodent chow and water ad libitum. A cohort of 2-month-old male WT and Nox2 KO mice were made diabetic with a multiple low-dose STZ protocol (60 mg·kg−1·day−1 × 5 days) using guidelines proposed by the Animal Models of Diabetic Complications Consortium (2, 35). Kidney structure and function were studied after 8 and 20 wk of diabetes. Blood glucose was measured using the Accu-Chek meter system (Roche). Portions of liver, heart, and kidney were snap-frozen in liquid nitrogen for RNA and protein isolation. An aliquot of kidney was frozen in OCT for immunostaining. All animal procedures were approved by the Institutional Animal Care and Use Committee of University of California, San Diego, and the Animal Ethics committee of the Alfred Hospital Medical Research and Education Precinct and Baker IDI Heart and Diabetes Institute.

Urine albumin and hydrogen peroxide measurements. Twenty-four-hour urine samples were collected from mice maintained in Nalgene metabolic cages after 8 wk of diabetes. Urine albumin and creatinine were measured using commercial kits (Albuwell and Creatinine companion kit, Exocell, Philadelphia, PA). Urine and serum creatinine were measured by HPLC, as previously described (3), according to the Animal Models of Diabetic Complications Consortium guideline. Creatinine clearance was estimated as the ratio of daily urinary creatinine excretion to plasma creatinine concentration, and expressed as milliliters per minute.
per square meter surface area. Urinary hydrogen peroxide was measured as an index of oxidative stress using the Amplex red assay, according to the manufacturer’s instructions (Invitrogen, Eugene, OR) and as previously described (31).

Immunofluorescent staining. Frozen kidney was cut into 4- to 6-μm sections, fixed using 4% paraformaldehyde and blocked with 5% fetal bovine serum for 1 h at room temperature. Sections were incubated overnight at 4°C with anti-Nox4 (Novus Biological), anti-collagen type IV (Abcam), anti-transforming growth factor (TGF)-β 1/2/3 (Santa Cruz Biotechnology), anti-fibronectin (FN) (Sigma-Aldrich), anti-F4/80 (Sigma-Aldrich) or anti-Nox2 (Santa Cruz Biotechnology,) antibodies, washed with ice-cold PBS, and then incubated with a rabbit or goat secondary antibody conjugated to Alexa Fluor dye (Molecular Probes) for 1 h in a humidified chamber in the dark. Sections were visualized by a confocal LSM 510 microscope (Zeiss).

Western blotting. Kidney cortex was homogenized by sonication in lysis buffer (120 mM NaCl, 1 mM EGTA in PBS, pH 7.4), lysed for 20 min on ice, and centrifuged (15,000 rpm for 30 min). Proteins were separated by electrophoresis on polyacrylamide gels and transferred to nitrocellulose membranes. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline with Tween for 1 h at room temperature. Sections were incubated overnight at 4°C with anti-Nox4 (Novus) and anti-β-actin (Sigma) antibodies, followed by application of secondary antibodies and visualization by chemiluminescence. Results were normalized to β-actin levels in the same sample.

RNA isolation and quantitative real-time PCR. Total RNA was extracted from kidney cortex using the TRIzol reagent (Invitrogen). One microgram of total RNA was reverse-transcribed, and RNA expression levels were quantified by real-time RT-PCR using a sequence detection system (Prism 7700; Applied Biosystems, Foster City, CA), as previously described (36). PCR was carried out using the following primers: Nox1, Nox2, Nox3, Nox4, p22phox, p47phox, monocyte chemoattractant protein-1 (MCP-1), α1-type IV collagen, FN, and TGF-β1. For quantitative analysis, the samples were normalized to 18S rRNA and β-actin expression using the ΔΔCT value method.

Morphological analysis. The left kidney was fixed in 4% buffered formalin and then embedded in paraffin, cut into 4-μm sections, and stained with periodic-acid-Schiff (PAS) reagent. For evaluating glomerular size and mesangial matrix area, 25 randomly selected glomeruli in the outer cortex of each kidney section were evaluated by point-counting techniques, as described previously (30). Briefly, the microscopic image of the kidney was overlaid with grids. The intersections of grids were counted and grouped as follows: capillary lumen, PAS-positive area, or nucleus. The number of all of the grid intersections was calculated for glomerular size. The PAS-positive area was defined as the mesangial matrix area. Tubulointerstitial area (TIA) was estimated at the corticomedullary junction using a point-counting system (14). For each field, 100 points were assessed on a 1-cm² eyepiece graticule with 10 equidistant gridlines. Six fields were analyzed per kidney at ×200. Results are expressed as the percentage of tubulointerstitial space within the area assessed. TIA was calculated according to the following equation: TIA = number of tubulointerstitial grid intersections/total number of intersections. Measurements were performed and analyzed by an observer blind to the experimental treatment and group.

Statistical analysis. Data are expressed as means ± SE. Differences between groups were evaluated for significance using independent t-test or one-way ANOVA with the Student-Newman-Keuls post hoc test. P < 0.05 was considered significant.

RESULTS

Metabolic analysis and expression of Nox isoforms/regulatory subunits in Nox2 KO and WT mice. Nox isoforms and subunit expression were evaluated in the WT and the Nox2 KO mouse kidney cortex (Fig. 1A). In WT kidneys, Nox2 and Nox4 were the most abundantly expressed Nox isoforms. In the Nox2 KO mouse kidney, Nox2 was predictably absent, and there was no change in other Nox isoforms.

Diabetes was induced in male 8-wk-old Nox2 KO and WT mice by intraperitoneal injection of 60 mg/kg body wt of STZ over 5 consecutive days. Mice displayed a significant increase of blood glucose levels 2 wk after STZ treatment. There was no difference in the rate of conversion to diabetes, with 97% (29/30) in the WT group and 95% (36/38) in the Nox2 KO group becoming hyperglycemic (>300 mg/dl). The degree of hyperglycemia and weight change was similar in both WT and Nox2 KO diabetic groups. Body weight was lower in both groups of diabetic mice compared with nondiabetic mice. There is a tendency for an increase in kidney weight in both diabetic groups after 8 wk of diabetes. A significant increase in the kidney-to-body weight ratio, a measure of kidney hyper-
Physiological characteristics of WT and Nox2 KO mice after diabetes in both WT and Nox2 KO diabetic mice (Fig. 3, merular FN and type IV collagen were significantly increased induced increase in gene expression of the extracellular matrix and WT mice. Between WT and Nox2 KO diabetic mice (Fig. 2), there was no significant difference in TIA in both WT and Nox2 KO diabetic mice (Fig. 2B). Hydrogen peroxide in urine was measured as an index of renal oxidative stress. After 8 wk of diabetes, there was a 15-fold increase of urinary hydrogen peroxide excretion in both the WT mice and the Nox2 KO mice (Fig. 2C).

Glomerular and tubulointerstitial pathology in both diabetic Nox2 KO and WT mice. Glomerular histology was normal in both WT and Nox2 KO diabetic mice. Diabetes-induced glomerular enlargement was of similar magnitude in both WT and Nox2 KO diabetic mice after 8 wk of diabetes (Fig. 2D). Glomerular mesangial matrix, quantified by PAS staining, was increased to a similar extent in WT and Nox2 KO diabetic mice after 8 wk of diabetes (Fig. 2E). To determine whether chronic features of diabetic kidney disease could be affected by Nox2, mice were also studied at 20 wk of diabetes. There was a modest but significant increase in TIA in both WT and Nox2 KO diabetic mice after 20 wk of diabetes, but no significant difference between WT and Nox2 KO diabetic mice (Fig. 2F).

Expression of FN and type IV collagen in diabetic Nox2 KO and WT mice. Real-time PCR showed a similar diabetes-induced increase in gene expression of the extracellular matrix proteins FN and type IV collagen in glomeruli from both WT and Nox2 KO control mice (Fig. 3, A and B). Localization of FN and type IV collagen by immunofluorescence indicated that glomerular FN and type IV collagen were significantly increased in both WT and Nox2 KO diabetic mice (Fig. 3, C–F).

Regulation of TGF-β, phospho-p38 MAPK, macrophages, and MCP-1 in diabetic Nox2 KO and WT mice. TGF-β, phospho-p38 MAPK, MCP-1, and macrophage infiltration were examined to determine whether specific mediators of fibrosis and inflammation were affected by Nox2 deletion. There was a significant and similar induction of glomerular TGF-β and phospho-p38 MAPK expression under diabetic conditions in both WT and Nox2 KO mice (Fig. 4, A–D). The numbers of macrophages were significantly increased only in WT diabetic mice (Fig. 4 E and F). Real-time PCR showed that MCP1 gene expression was stimulated in WT diabetic kidney but significantly less in the Nox2 KO diabetic mice (Fig. 4G).

Blood pressure and the heart weight-to-body weight ratio in WT and Nox2 KO mice. Systolic blood pressure was increased in Nox2 KO mice compared with WT mice when measured by either tail-cuff or radiotelemetry (Fig. 5A and Table 2). Diabetes (8 wk) increased systolic blood pressure in both WT and Nox2 KO mice when measured by the tail-cuff method. However, this finding was not confirmed by radiotelemetry, which recorded a significant reduction in systolic blood pressure in both diabetic groups (Table 2). The expected reduction in blood pressure during sleep was found in all groups, although there was a blunting of the somnolent drop in the Nox2 KO mice without diabetes. The heart rate was significantly lower in both groups of diabetic mice. Heart weight-to-body weight ratio was significantly greater in the KO mice and was reduced in the diabetic mice vs. WT control mice (Fig. 5B).

Nox4 expression in the glomeruli of Nox2 KO diabetic mice. To address the lack of renal protection in Nox2 KO diabetic mice, we investigated compensation by other components of the NAPDH oxidase system. There was no significant increase in gene expression by real-time PCR of any of the Nox isoforms or Nox subunits in the Nox2 KO diabetic kidney after 8 wk of diabetes (data not shown). However, by immunostaining, there was a marked upregulation of glomerular Nox4 (Fig. 6, A and B). Overlay studies using a podocyte-specific antibody indicated that the increase in glomerular Nox4 was primarily in podocytes. In addition, immunoblot analysis of renal cortical protein was performed. There was a marked increase in cortical Nox4 in the Nox2 KO diabetic kidney cortex (Fig. 6, C and D). Real-time PCR revealed a significant induction in Nox4 expression in 20-wk diabetic Nox2 KO mice compared with diabetic WT mice (Fig. 6E). There was a significant increase in Nox4 protein in the Nox2 KO mouse kidney by immunoblotting and immunofluorescence staining. However, there was no increase by real-time PCR for Nox4 gene expression in 8 wk (Fig. 1A) and 20 wk of nondiabetic kidney (Fig. 6E).

DISCUSSION

Our results show that albuminuria, H2O2 production, renal and glomerular hypertrophy, induction of TGF-β, phospho-p38 MAPK, and extracellular matrix expression in glomeruli are similarly increased in WT and Nox2 KO diabetic mice.

Table 1. Physiological characteristics of WT and Nox2 KO mice after diabetes

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Duration of DM, wk</th>
<th>Body Weight, g</th>
<th>Blood Glucose, mg/dl</th>
<th>Kidney Weight, g</th>
<th>Kidney/Body Weight, %</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>30</td>
<td>8</td>
<td>28.4 ± 0.7</td>
<td>194.0 ± 13.6</td>
<td>0.31 ± 0.06</td>
<td>0.54 ± 0.02</td>
</tr>
<tr>
<td>WTD M</td>
<td>20</td>
<td>8</td>
<td>24.6 ± 0.4*</td>
<td>517.8 ± 18.7*</td>
<td>0.34 ± 0.04</td>
<td>0.69 ± 0.02*</td>
</tr>
<tr>
<td>KO</td>
<td>24</td>
<td>8</td>
<td>28.8 ± 0.4</td>
<td>207.5 ± 15.4</td>
<td>0.35 ± 0.05</td>
<td>0.60 ± 0.03</td>
</tr>
<tr>
<td>KOD M</td>
<td>38</td>
<td>8</td>
<td>23.8 ± 0.4*†</td>
<td>571.8 ± 12.8*†</td>
<td>0.40 ± 0.07</td>
<td>0.84 ± 0.03†</td>
</tr>
<tr>
<td>WT</td>
<td>10</td>
<td>20</td>
<td>34.1 ± 0.91</td>
<td>219.6 ± 7.38</td>
<td>0.377 ± 0.025</td>
<td>0.55 ± 0.06</td>
</tr>
<tr>
<td>WTD M</td>
<td>10</td>
<td>20</td>
<td>26.6 ± 0.61*</td>
<td>388.8 ± 11.8*</td>
<td>0.487 ± 0.013*</td>
<td>0.92 ± 0.07*</td>
</tr>
<tr>
<td>KO</td>
<td>10</td>
<td>20</td>
<td>32.1 ± 0.72</td>
<td>176.4 ± 13.86</td>
<td>0.459 ± 0.055</td>
<td>0.71 ± 0.01</td>
</tr>
<tr>
<td>KOD M</td>
<td>10</td>
<td>20</td>
<td>24.4 ± 1.41†</td>
<td>419.4 ± 32.4†</td>
<td>0.431 ± 0.015</td>
<td>0.88 ± 0.04†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of mice. WT, wild-type mice; WTD M, streptozotocin (STZ)-induced diabetic wild-type mice; KO, Nox2 knockout mice; KOD M, STZ-induced diabetic Nox2 knockout mice; DM, diabetes mellitus. *P < 0.05 vs. WT. †P < 0.05 vs. KO.
Diabetic glomerular and tubulointerstitial disease were also not affected by deletion of Nox2.

Nox2 KO mice exhibited a relatively normal phenotype, other than showing mild hypertension, as detected by both tail-cuff plethysmography and radiotelemetry. Generation of ROS by endothelial Nox2 has been associated with hypertension (22), so deletion of Nox2 would not be predicted to promote hypertension, unless compensatory mechanisms are activated to result in hypertension. Deletion of Nox2 also did not alter the general manifestations of type 1 diabetes, such as hyperglycemia and weight loss. It was notable that diabetic mice, both WT and Nox2 KO, were hypotensive by radiotelemetry, but hypertensive by tail-cuff plethysmography. The hypotensive consequences of STZ-induced diabetes in rodents are well documented in conscious animals (17, 34), highlighting concerns that tail-cuff plethysmography can be inaccurate in diabetic rodents due to a reliance on assumptions of consistent mechanical properties of the skin and collagen in the tail.
Nox2 KO mice have been reported to exhibit reduced superoxide production in organs, such as the heart, lungs, and brain, and were protected from various pathophysiological conditions, such as cardiac hypertrophy, hypoxic pulmonary hypertension, and surgically induced brain injury (9, 20, 21). However, as Nox2 KO diabetic mice had no reduction in functional and structural features of diabetic renal disease, our results suggest that Nox2 is not critical for disease pathogen-

Fig. 3. The deposition of matrix proteins in glomeruli of diabetic mice. The expression levels of fibronectin (A) and type IV collagen (B) mRNAs in kidney cortex were determined by quantitative RT-PCR from mice after 8 wk of diabetes. Data were normalized to 18S. n = 4. Immunofluorescence image and quantification with anti-fibronectin (C and D) and anti-type IV collagen (E and F) antibodies was performed on frozen sections from mice after 8 wk of diabetes. Quantification of glomerular protein (green) pixel density divided by glomerular area is shown. Original magnification, ×63. Data are expressed as fold induction (4 images per mouse, 3 male mice/group). Values are group means ± SE. *P < 0.05 vs WT.
Fig. 4. Expression of transforming growth factor (TGF)-β, phospho-p38 MAPK, F4/80, and monocyte chemoattractant protein (MCP)-1. Immunofluorescence images with quantitative analysis of TGF-β (A and B), phospho-p38 MAPK (C and D) in the glomeruli, and F4/80 (E and F) of kidney cortex were performed on frozen sections from mice after 8 wk of diabetes. Sections were visualized by confocal microscopy. Original magnification, ×63. Quantification of glomerular protein (green) pixel density divided by glomerular area is shown. Data are expressed as fold induction (4 images per mouse, 3 mice/group).

G: MCP-1 mRNA expression showed in kidney cortex by quantitative RT-PCR. Data, normalized to 18S, are presented as the fold-increase relative to control. Values are group means ± SE; n = 4 for each group. *P < 0.05 vs. WT. **P < 0.05 vs. all other groups.
from type 2 diabetic db/db mice, while knockdown of Nox4 by siRNA in vitro decreased TGF-β and FN production in mouse proximal tubules (6, 27). At the time of the preparation of this paper, the role of Nox4 in diabetic kidney disease was recently examined using pharmacological therapy and with Nox4 KO mice. GKT136901, a Nox1/4 inhibitor, attenuates the development of nephropathy in db/db mice (28). A renoprotective effect of the Nox1/4 inhibitor may be beneficial through reduced oxidative damage and decreased ERK1/2 activation (28). Surprisingly, a separate study found no protective kidney benefit in the Nox4-deficient mouse in models of unilateral ureteral obstruction or STZ-induced diabetes (1), and an additional study found that Nox4-deficient mice had enhanced fibrosis after unilateral ureteral obstruction, perhaps due to lack of nuclear factor-erythroid 2-related factor 2 and hypoxia-inducible factor-1α upregulation (23). Thus it appears that Nox4 above baseline does contribute to diabetic kidney disease; however, baseline levels of Nox4 may also provide protective effects.

The mechanism that drives upregulation of Nox4 expression in the diabetic mouse kidney after deletion of Nox4 has yet to be defined. It was recently shown that Nox2 and Nox4 each compensate for the deficiency of each other in endothelial cells of the lung (24). It will be of interest to determine whether deletion or overexpression of Nox4 alters Nox2 levels and affects the overall pattern of diabetic kidney disease. Further studies are in progress to unravel the role of Nox4 and related signaling pathways activated by hyperglycemia in regulating Nox4 expression and ROS production in kidney.

The diabetes-induced infiltration of macrophages into the kidney was reduced in diabetic Nox2 KO mice. This may reflect a reduction in macrophage recruitment, as gene expression of the chemotactic factor MCP-1 was significantly lower in kidneys of diabetic Nox2 KO mice compared with diabetic WT mice. As MCP-1 production can occur in other cells, including podocytes in glomeruli of diabetic kidney (33) and activation of MCP-1 gene expression is mediated by ROS (19, 26), Nox2-derived ROS may be involved in pathways that enhance production of MCP-1 from macrophages, podocytes, and other cells in the diabetic kidney. The lack of Nox2-derived ROS has the potential to reduce the overall numbers of infiltrating inflammatory cells into the kidney. However, while reduced macrophage infiltration may be predicted to attenuate renal pathology in diabetes, the degree of glomerular and tubulointerstitial disease was not affected in the Nox2 KO diabetic mice.

In summary, we have demonstrated that glomerular mesangial matrix expansion, tubulointerstitial disease, and albuminuria were not ameliorated in diabetic Nox2 KO mice. These results suggest that ablating Nox2 activity does not protect against the major features of diabetic kidney disease.

Table 2. Systolic BP, heart rate, and body weight for WT and Nox2 KO mice after 8-wk diabetes

<table>
<thead>
<tr>
<th></th>
<th>Systolic BP by Tail-cuff, mmHg</th>
<th>Systolic BP by Radiotelemetry, mmHg</th>
<th>Heart Rate, beats/min</th>
<th>Body Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>116.32 ± 1.65</td>
<td>118.05 ± 1.174</td>
<td>505.50 ± 8.47</td>
<td>26.82 ± 2.38</td>
</tr>
<tr>
<td>WTDm</td>
<td>135.32 ± 3.36*</td>
<td>104.98 ± 1.588*</td>
<td>412.10 ± 7.290*</td>
<td>24.46 ± 2.39*</td>
</tr>
<tr>
<td>KO</td>
<td>136.21 ± 4.29*</td>
<td>136.06 ± 2.834*</td>
<td>484.60 ± 9.708*</td>
<td>24.01 ± 3.31</td>
</tr>
<tr>
<td>KODM</td>
<td>141.41 ± 3.18*</td>
<td>101.35 ± 1.25*†</td>
<td>408.86 ± 7.246*†</td>
<td>24.31 ± 2.37</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 10 for all groups. BP, blood pressure. *P < 0.05 vs. WT. †P < 0.05 vs. KO.
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No conflicts of interest, financial or otherwise, are declared by the author(s).

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


Fig. 6. Expression of Nox4 was increased in diabetic Nox2 KO mice. A: immunofluorescence imaging with anti-Nox4 antibody was performed on frozen sections from mice after 8 wk of diabetes. Sections were visualized by confocal microscopy. Original magnification, ×63. B: quantification of glomerular protein (green) pixel density divided by glomerular area is shown. Data are expressed as fold induction (4 images per mouse, 3 mice/group). The protein levels of Nox4 were analyzed by Western blot analysis (C) and quantitative analysis (D) of Nox4 protein levels and Nox4 gene expression (E) by quantitative RT-PCR in the kidney cortex after 20 wk of diabetes. Values are group means ± SE; n = 3 for each group. *P < 0.05 vs. WT. ***P < 0.05 vs. all other groups.
REFERENCES


