Critical role of Nox4-based NADPH oxidase in glucose-induced oxidative stress in the kidney: implications in type 2 diabetic nephropathy

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Sedeek M, Callera G, Montezano A, Gutsol A, Heitz F, Szymbalewicz C, Page P, Kennedy CR, Burns KD, Touyz RM, Hébert RL. Critical role of Nox4-based NADPH oxidase in glucose-induced oxidative stress in the kidney: implications in type 2 diabetic nephropathy. Am J Physiol Renal Physiol 299:F1348–F1358, 2010. First published July 14, 2010; doi:10.1152/ajprenal.00028.2010.—Molecular mechanisms underlying renal complications of diabetes remain unclear. We tested whether renal NADPH oxidase (Nox) 4 contributes to increased reactive oxygen species (ROS) generation and hyperactivation of redox-sensitive signaling pathways in diabetic nephropathy. Diabetic mice (db/db) (20 wk) and cultured mouse proximal tubule (MPT) cells exposed to high glucose (25 mmol/l, ð-glucose) were studied. Expression (gene and protein) of Nox4, p22phox, and p47phox, but not Nox1 or Nox2, was increased in kidney cortex, but not medulla, from db/db vs. control mice (db/m) (P < 0.05). ROS generation, p38 mitogen-activated protein (MAP) kinase phosphorylation, and content of fibronectin and transforming growth factor (TGF)-ß1/2 were increased in db/db vs. db/m (P < 0.01). High glucose increased expression of Nox4, but not other Noxes vs. normal glucose (P < 0.05). This was associated with increased NADPH oxidase activation and enhanced ROS production. Nox4 downregulation by small-interfering RNA and inhibition of Nox4 activity by GK-136901 (Nox1/4 inhibitor) attenuated ð-glucose-induced NADPH oxidase-derived ROS generation. High ð-glucose, but not l-glucose, stimulated phosphorylation of p38MAP kinase and increased expression of TGF-ß1/2 and fibronectin, effects that were inhibited by SB-203580 (p38MAP kinase inhibitor). GK-136901 inhibited ð-glucose-induced actions. Our data indicate that, in diabetic conditions: 1) renal Nox4 is upregulated in a cortex-specific manner, 2) MPT cells possess functionally active Nox4-based NADPH, 3) Nox4 is a major source of renal ROS, and 4) activation of profibrotic processes is mediated via Nox4-sensitive, p38MAP kinase-dependent pathways. These findings implicate Nox4-based NADPH oxidase in molecular mechanisms underlying fibrosis in type 2 diabetic nephropathy.

superoxide; hydrogen peroxide; hyperglycemia; nicotinamide adenine dinucleotide phosphate reduced form oxidase; diabetes

DIABETES MELLITUS IS A MAJOR cause of end-stage renal disease (ESRD) (36). Clinical hallmarks of diabetic nephropathy include progressive increase in urinary albumin excretion and decline in glomerular filtration rate, which occur in association with an increase in blood pressure, leading to ESRD (14, 24). These functional changes develop as a consequence of structural abnormalities, including thickening of the basement membrane of glomerular capillaries, arterioles, collecting tubules, glomerulosclerosis, and tubulointerstitial fibrosis (27, 43). Additionally, there is monocyte-macrophage invasion, mesangial cell hyperplasia, matrix expansion, and podocyte injury (9, 38).

Molecular mechanisms underlying nephropathy in diabetes involve hyperglycemia, impaired insulin signaling, and activation of the renin-angiotensin system (15, 25, 45). Common to these processes is increased bioavailability of reactive oxygen species (ROS) (oxidative stress), leading to inflammation, fibrosis, and endothelial dysfunction (3, 25, 46). Oxidative stress is increased and thiol reductive capacity is impaired in diabetic rats (1, 13). In diabetic mice overexpressing catalase to reduce oxidative stress, kidney function and structure were improved (8). Clinical and experimental studies show increased circulating levels of oxidative markers in diabetes (41, 47, 51).

Of the many enzymatic systems implicated in ROS generation in the kidney, including mitochondrial respiration, uncoupled eNOS, and nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase (Nox; see Refs. 32 and 39), Nox appears to be particularly important (18, 32, 39). The prototype NADPH oxidase is that found in phagocytes and comprises membrane-bound flavocytochrome b558, composed of gp91phox (Nox2) and p22phox, and three cytosolic regulators, p47phox, p67phox, and p40phox (4). In nonphagocytic cells, other Nox homologues have been described, including Nox1, Nox3, Nox4, Nox5, Duox-1, and Duox-2 (27). Renal cells differentially express NADPH oxidase subunits: p47phox, p67phox, and p22phox are expressed in mesangial cells, podocytes, endothelial cells, vascular smooth muscle cells, fibroblasts, thick ascending limb, distal convoluted tubules, including macula densa cells, and cortical collecting ducts (18, 32); Nox2 in podocytes, mesangial cells, and endothelium; Nox4 in glomerulus, proximal tubule, and distal convoluted tubule; and Nox3 in fetal kidney (4, 5, 18, 27, 32, 40). Of the renal Noxes, Nox4 is most abundantly expressed and hence was originally termed Renox (20, 42).

The exact physiological function of Nox4 has not yet been elucidated, but it may be an oxygen sensor that regulates erythropoietin production in the kidney, and it has been implicated as a major source of renal ROS (31, 32). In vitro studies showed that insulin-induced Õ2 production is Nox4-dependent (30) and that Nox4-derived ROS are involved in angiotensin II-induced hypertrophy in mesangial cells (48). In a rodent model of type 1 diabetes (streptozotocin induced), renal expression of Nox4 and p22phox was increased, and this was associated with ROS-induced renal damage (12, 13, 21). Whether similar processes occur in type 2 diabetes and whether Nox4 is the renal source of oxidative stress remain unclear. We tested the hypothesis that upregulation of renal Nox4, induced by high glucose, contributes to increased ROS generation and...
hyperactivation of redox-sensitive signaling pathways, which underlie oxidative damage in diabetic nephropathy. In particular, we questioned whether Nox4-based NADPH oxidase regulates growth and profibrotic processes in renal cells through redox-sensitive p38 mitogen-activated protein (MAP) kinase.

MATERIALS AND METHODS

Animals. Experiments in this study were approved by the University of Ottawa Animal Ethics Committee and were performed according to the recommendations of the Canadian Council for Animal Care. Seven male \textit{db/db} mice (20 wk), which carry a point mutation in the leptin receptor and which develop type 2 diabetes and obesity, and five age-matched control nondiabetic mice (\textit{db/m}) were studied. Mice on the C57BL/BLKS background were used because this model of type 2 diabetes recapitulates the glomerular and tubulointerstitial injury associated with progressive diabetic nephropathy in humans (10, 35).

Plasma and urine measurements. Blood samples (via cardiac puncture) and spot urines were collected immediately before death. Urine albumin-to-creatinine ratio was measured using a commercial kit (Albuwell and Creatinine companion kit; Exocell, Philadelphia, PA) as recommended by the Animal Models of Diabetic Complications Consortium (10). Levels of plasma glucose were determined using an automated analyzer (Synchron CX5 PRO; Beckman, Fullerton, CA).

Table 1. Sequence of primers for Nox1, Nox4, and GAPDH

<table>
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<tr>
<th>mRNA</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>Nox1</td>
<td>5’-CGCTCCAGCAGAAAGTGTATTACCAAGG-3’</td>
<td>5’-GGAGTGACCCCAATCTGGCCTGAAAACCA-3’</td>
</tr>
<tr>
<td>Nox4</td>
<td>5’-TGTGGGCGCTAGGATTGTTT-3’</td>
<td>5’-AAAGAGATGAGCCTGAGAGTGG-3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’-CCAAAGTGGAGATTGGCCTGACAT-3’</td>
<td>5’-CTTTGACTCTGGCCGTGAATTTC-3’</td>
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Nox, NADPH oxidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Fig. 1. Renal NADPH oxidase (Nox) 4 expression in control (\textit{db/m}) and diabetic (\textit{db/db}) mice. \textbf{A}: representative immunofluorescence images demonstrating NADPH oxidase (Nox) 4 expression in renal cortex of \textit{db/m} (n = 5) and \textit{db/db} (n = 7) mice. Fixed sections (6–8 μm) were probed with anti-Nox4 antibody (1:500) and secondary antibody conjugated to Alexa fluor. Nuclei were stained with DAPI (blue fluorescence). Arrows indicate proximal tubules. Sections were visualized by fluorescence microscopy. \times 63 Magnification. \textbf{B}: expression of Nox4 mRNA (left) and protein (right) in renal cortex as assessed by RT-PCR and Western blotting, respectively. \textbf{C}: Nox4 protein expression in renal medulla from \textit{db/m} and \textit{db/db} mice. \textit{Left}, expression by Western blotting. \textit{Right}, expression by immunofluorescence. \times 20 Magnification. Protein data are presented as representative immunoblots with corresponding bar graphs. Data are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Values are expressed as means ± SE of 5 \textit{db/m} and 7 \textit{db/db} mice. *P < 0.01 vs. \textit{db/m}.
Nox4 and fibronectin immunofluorescence in kidneys from db/db and db/db mice. Kidneys were perfused with ice-cold saline and immersed in cooled optimum-cutting temperature compound. Frozen tissues were cut into 6- to 8-μm sections, fixed using cold acetone (−20°C for 5 min), and blocked with 5% BSA for 1 h at room temperature. Sections were incubated overnight at 4°C with anti-Nox4 or anti-fibronectin antibodies (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA) or washed with ice-cold PBS, and then incubated with a rabbit or goat secondary antibody (conjugated to Alexa fluor 488 or FITC; Molecular Probes, Carlsbad, CA) for 1 h in a humidified chamber in the dark. Sections were visualized by fluorescence microscopy (Zeiss Axioskop 2 MOT; Zeiss).

Cell culture. An immortalized cell line from mouse proximal tubules (MPT) was studied (gift from E. Neilson, D. Pileth, Vanderbilt University, Nashville, TN). Cells were cultured in DMEM (GIBCO, Invitrogen, Carlsbad, CA) + F-12 (GIBCO, Invitrogen) containing 10% FBS and 1% penicillin/streptomycin. Subconfluent MPT cells were maintained in serum-free media for 24 h and then exposed to either normal n-glucose (5 mM), high b-glucose, or high l-glucose (25 mmol/l) for 2–24 h. Stimulation was stopped by removing the high-glucose media and adding ice-cold PBS.

Western blotting. Proteins were extracted from frozen kidney cortex and MPT cells as previously described (21, 48). Proteins were separated by electrophoresis on polyacrylamide gels (8 or 15%) and separated by electrophoresis on polyacrylamide gels (8 or 15%) and transferred on nitrocellulose membranes as we described (49). Non-specific 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 the following antibodies (1:1,000): anti-Nox4, anti-Nox1, anti-p47phox, anti-p22phox, and anti-transforming growth factor (TGF)-β1/2, anti-p22phox, anti-transforming growth factor (TGF)-β1/2, anti-proliferating cell nuclear antigen (PCNA), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti-p47phox (Santa Cruz Biotechnology), anti-p38MAP kinase, anti-phospho-p38MAP kinase (Thr180/Tyr182), anti-fibronectin (1:5,000) (Sigma-Aldrich, Oakville, Ontario, Canada), and anti-Nox2 (gift from M. Quinn, Bozeman University). After incubation with secondary antibodies, signals were revealed with chemiluminescence, visualized by autoradiography, and quantified densitometrically. In some experiments, antibodies to nonphosphoproteins were used as loading control and carried out on the same membranes for phosphorylated proteins. Results were normalized to GAPDH or β-actin.

Measurement of NADPH oxidase activity and O2·− production in kidney tissue and MPT cells. Serum-deprived MPT cells were exposed to normal (5 mmol/l) or high (25 mmol/l) glucose for 4 h. Cells were washed with PBS and scraped off in lysis buffer (20 mmol/l KH2PO4, 1 mmol/l EGTA, 100 mmol/l Na2HPO4, 100 mmol/l phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, and 1 mg/ml aprotonin). Kidney cortex was homogenized with a glass-to-glass homogenizer in Krebs buffer. The lucigenin-derived chemiluminescence assay was used to determine NADPH oxidase activity in total protein homogenates. The reaction was initiated by addition of NADPH (0.1 mmol/l) to the suspension containing sample (25 μl), lucigenin (5 μmol/l), and assay phosphate buffer (50 mmol/l KH2PO4, 1 mmol/l EGTA, and 150 mmol/l sucrose, pH 7.4). Luminescence was measured every 1.8 s for 3 min in a luminometer (Orion II mMicroplate luminometer; Montreal Biotech, Kirkland, QC, Canada), as we have previously detailed (48). Buffer blank was subtracted from each reading. Activity was expressed as arbitrary units per milligram protein.

Measurement of H2O2 in kidney cortex. Kidney cortex was homogenized as described above. H2O2 was measured using an Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (A22188) according to the manufacturer’s instructions (Molecular Probes). The Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit contains an assay that uses the Amplex Red reagent to detect H2O2 or peroxyxidase activity. In the presence of peroxidase, the Amplex Red reagent reacts with H2O2 in a 1:1 stoichiometry to produce a red-fluorescent oxidation product, which is assessed fluorometrically.

Measurement of intracellular H2O2 in MPT cells. Intracellular H2O2 was measured in cells after exposure to normal (5 mM) or high (25 mmol/l) glucose for 4 h. Cells were washed and incubated with 5-(and-6)-carboxy-2’,7’-dichlorodihydrofluorescein diacetate (DCFDA; Molecular Probes) (1 μmol/l) for 30 min as we previously described (49). Intracellular H2O2 production was measured by fluorescence

Fig. 2. Renal expression of NADPH oxidase subunits in db/m and db/db mice. A: expression of Nox2 mRNA (left) and protein (right) in renal cortex as assessed by RT-PCR and Western blotting, respectively. B: expression of p22phox protein in renal cortex from db/m (n = 5) and db/db (n = 7) mice. Top, representative immunoblots with corresponding bar graphs. C: expression of p47phox in renal cortex from db/m (n = 5) and db/db (n = 7) mice. Protein data are presented as immunoblots with corresponding bar graphs. The black lines in the immunoblots signify that blots have been spliced, from the same membrane, to indicate the most representative Western blots corresponding to the bar graphs. Values are expressed as means ± SE. *P < 0.01 vs. db/m.
spectrophotometry (excitation/emission, 485/535 nm). Fluorescence data were normalized to protein content.

RT-PCR analysis. Total RNA was extracted from kidney cortex and proximal tubules using the TRIzol method (Invitrogen, Carlsbad, CA). Quantitative real-time PCR (TaqMan; Applied Biosystems, Roche Molecular Diagnostics, Pleasanton, CA) was used to measure the expression of Nox1, Nox2, and Nox4 in kidney cortex and isolated proximal tubules. The expression of Noxes was interpolated from a standard curve (constructed from an independent sample of mouse kidney cDNA) and expressed relative to the housekeeping gene GAPDH. The primer sequences for Nox4 are depicted in Table 1. We used Nox2 predesigned primers to amplify Nox2 mRNA in kidney cortex, using the Applied Biosystems 7300 real-time PCR system (Foster City, CA).

Nox inhibition by GKT-136901 in cell-free assays. Membranes from polymorphonuclear cells (expressing high levels of Nox2) or from cells overexpressing Nox1 or Nox4 were prepared as previously described (29, 34). Briefly, cells were lysed by sonication in sonication buffer (11% sucrose, 120 mM NaCl, 1 mM EGTA in PBS, pH 7.4 for Nox4-expressing cells). Sonicates were centrifuged (200 g, 10 min), and the supernatant was layered on a 17/40% (wt/vol) discontinuous sucrose gradient and centrifuged (150,000 g for 30 min). Membrane fractions were collected from the 17/40% interface. Fractions were stored at −80°C. Protein concentration was determined with Bradford reagent. ROS (H$_2$O$_2$ production) measurements of membrane expressing different Nox subunits was determined using the Amplex Red method (Invitrogen).

p38 MAP kinase inhibition by SB-203580 in cells. In some experiments, cells were preexposed to 10$^{-5}$ mol/l 2-(2-chlorophenyl)-4-methyl-5-(pyridin-2-ylmethyl)-1H-pyrazolo[4,3-c]pyridine-3,6(2H,5H)-dione (GKT-136901), a selective Nox4 inhibitor, for 30 min before exposure to normal or high glucose. GKT-136901, provided by Genkyotex (Plan-Les-Ouates, Geneva, Switzerland), is a drug-like small molecule with high affinity and specificity for inhibition of Nox4 (33).

p38 MAP kinase inhibition by SB-203580 in cells. SB-203580 (Calbiochem, Darmstadt, Germany), a potent and selective p38 MAP kinase inhibitor, was used to evaluate the role of p38MAP kinase in
high-glucose-induced signaling. Cells were pretreated with 10^{-5} mol/l SB-203580 for 30 min before exposure to different glucose concentrations.

RNA interference and cell transfection. High-performance purity-grade small-interfering RNAs (siRNA) were generated against mouse Nox4 (Santa Cruz Biotechnology). A mix of three double-stranded siRNA was used. DNA target sequence of the annealed double-strand siRNA used was: sense strand A: 5’-CCAUGUAAGCAGUCUUAUA-3’ (1360–1378), sense strand B: 5’-CCAAGGUGUAUAUGUCUGUAU-3’ (1709–1727), and sense strand C: 5’-CAAGACCUCUCUUGCUGUAU-3’ (2587–2605). MPT cells were seeded at a density of 1 × 10^5 cells/well in six-well plates in normal glucose medium containing 7.5% FBS and transfected with siRNA using HiPerfect Transfection Reagent (Qiagen, Mississauga, ON, Canada) according to the manufacturer’s instructions. Briefly, Nox4 siRNA was diluted in 100 μl culture medium without serum (final concentration, 25 nmol/l). HiPerfect transfection reagent was added to the diluted siRNA and incubated (15 min, 24°C) to allow formation of transfection complexes. The transfection complex was added dropwise to the cells and 8 h later replaced by serum-free medium (16 h). After transfection, gene silencing was monitored at the protein level by Western blotting. MPT cells were also exposed to HiPerfect transfection reagent and a nonsilencing siRNA oligonucleotide sequence (scrambled RNA) that does not recognize any known homology to mammalian genes as a negative control.

Statistical analysis. Data are expressed as means ± SE. Nonparametric t-test or one-way ANOVA were used to analyze data. P < 0.05 was considered significant.

RESULTS

Metabolic characteristics of db/db and db/m mice. Levels of plasma glucose were significantly higher in db/db mice than in db/m mice (Fig. 4). Effects of glucose on Nox expression in mouse proximal tubule (MPT) cells. MPT cells were exposed to normal glucose conditions (5 mmol/l, control) and high concentrations of d-glucose and l-glucose (25 mmol/l) for 4 h. A: Nox4 expression in MPT cells. B: expression of Nox1 in MPT cells. C: Nox2 expression in MPT cells exposed to normal and high glucose concentrations. Protein data are presented as immunoblots with corresponding bar graphs. The black lines in the immunoblots signify that blots have been spliced, from the same membrane, to indicate the most representative Western blots corresponding to the bar graphs. Top, representative immunoblots. Bottom, corresponding bar graphs. Data are normalized to β-actin and are presented as the degree of increase relative to control, taken as 1.0. Values are presented as means ± SE of 6 experiments. *P < 0.01 vs. other groups.

Fig. 5. Effects of glucose on NADPH oxidase subunit expression in MPT cells. MPT cells were exposed to normal glucose conditions (5 mmol/l, control) and high concentrations of d-glucose and l-glucose (25 mmol/l) for 4 h. A: p22phox expression in MPT cells. B: expression of p47phox in MPT cells. The black lines in the immunoblots signify that blots have been spliced, from the same membrane, to indicate the most representative Western blots corresponding to the bar graphs. Data are normalized to GAPDH and are presented as the degree of increase relative to control, taken as 1.0. Values are presented as means ± SE of 6 experiments.
(62.3 ± 4 mmol/l) compared with db/m mice (17 ± 1.6 mmol/l, \( P < 0.001 \)). The urine albumin-to-creatinine ratio was greater in db/db mice vs. db/m mice (504 ± 106 vs. 38 ± 4 \( \mu \)g/mg, \( P < 0.001 \)).

Renal Nox4, NADPH oxidase activity, and ROS production in db/db mice. In db/db mice, expression of Nox4 was increased in renal cortex at the gene and protein levels compared with db/m mice (Fig. 1, A and B). As shown in Fig. 1A, Nox4 distribution is heterogeneous and is present in glomeruli and tubules, with greater abundance in db/db mice than db/m mice. Although Nox4 was expressed in renal medulla, there was no significant difference in Nox4 protein expression between db/db and db/m mice (data not shown). Expression of Nox2 gene and protein (Fig. 2A) was lower in the kidney cortex of db/db mice compared with db/m mice. Nox1 levels were undetectable in renal cortex in db/db and db/m mice as measured by RT-PCR and Western blotting (data not shown).

Production of \( \mathrm{H}_2\mathrm{O}_2 \), the major ROS generated by Nox4, was higher in kidney cortex from db/db mice compared with db/m (Fig. 3A). Fibronectin content, as assessed by immunofluorescence and by Western blotting, was greater in db/db mice compared with db/m mice (Fig. 3, B and C). Phosphorylation of \( \alpha \)-38MAP kinase and expression of TGF-\( \beta \)1/2 were greater in cortex from db/db mice compared with db/m mice (Fig. 3, D and E). Phosphorylation of other MAP kinases, ERK1/2 and JNK, was not different between db/db and db/m mice (data not shown).

Effect of high glucose on Nox protein expression in MPT cells. As shown in Fig. 4A, exposure of MPT cells for 4 h to high \( \alpha \)-glucose, but not to l-glucose, resulted in a significant increase in Nox4 expression. However, protein expression of Nox1 was not different between high \( \alpha \)-glucose and control conditions (data not shown). Phosphorylation of other MAP kinases, ERK1/2 and JNK, was not different between high \( \alpha \)-glucose and control conditions (data not shown).

Fig. 6. Inhibition of Nox-dependent reactive oxygen species (ROS) production by 2-(2-chlorophenyl)-4-methyl-5-(pyridin-2-ylmethyl)-1H-pyrazolo[4,3-c]pyridine-3,6(2H,5H)-dione (GKT-136901). A: chemical structure of the dual Nox4/Nox1 specific inhibitor GKT-136901. B: concentration-response curve of GKT-136901 on Nox4, Nox1, Nox2, and xanthine oxidase (XO). Values are presented as means ± SE of 3 experiments performed in triplicate. The IC_{50} values measured from the assays were converted to absolute inhibition constant (\( K_i \)) using the Cheng-Prusoff equation \( K_i = IC_{50}/[1 + (S)/K_m] \), where \( K_i \) is the binding affinity of the inhibitor, \( IC_{50} \) is the functional strength of the inhibitor, \( [S] \) is substrate concentration (NADPH concentration used in the assay), and \( K_m \) is the affinity of the substrate (NADPH) for the enzyme. B and C: effects of Nox4 inhibition on glucose-induced activation of NADPH oxidase and ROS production. B: effect of GKT-136901 (10^{-5} mol/l) on glucose-stimulated activation of NADPH oxidase in MPT cells as assessed by enhanced lucigenin (5 \( \mu \)mol/l) chemiluminescence. C: effect of GKT-136901 (10^{-3} mol/l) on glucose-induced generation of intracellular \( \mathrm{H}_2\mathrm{O}_2 \) as assessed by 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFDA) fluorescence. Data, normalized to protein content, are presented as the degree of increase relative to control, taken as 1.0. Values are presented as means ± SE of 6 experiments. * \( P < 0.05 \) vs. control. \& \( P < 0.05 \) vs. high \( \alpha \)-glucose.
**Table 2. Inhibitory effect of GKT-136901 on ROS-producing enzymes, redox-sensitive enzymes, and other proteins**

<table>
<thead>
<tr>
<th>Protein/Enzyme</th>
<th>Inhibition at 10 μM, %</th>
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<tr>
<td>Xanthine oxidase</td>
<td>5</td>
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<tr>
<td>NOS</td>
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</tr>
<tr>
<td>eNOS</td>
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<td>nNOS</td>
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<tr>
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<td>0</td>
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<tr>
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<td>MAO-B</td>
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</tr>
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<tr>
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<td>PTP1b</td>
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ROS, reactive oxygen species; iNOS, inducible nitric oxide synthase (NOS); eNOS, endothelial NOS; nNOS, neuronal NOS; MPO, myeloperoxidase; MAO-B, monoaminooxidase-B; PKC, protein kinase C; PI 3-kinase, phosphatidylinositol 3-kinase; MAPK2, mitogen-activated protein kinase 2; MAPKAP-K2, mitogen-activated protein kinase-activated protein kinase 2; MEK, mitogen/extracellular signal-regulated kinase; AMPK, AMP-activated protein kinase; ASK1, apoptosis signal-regulating kinase 1; GSK3, glycogen synthase kinase 3; Kv, voltage-gated K⁺ channel; ING-IR, insulin-like growth factor-I receptor; PTP1b, protein-tyrosine phosphatase 1B.

...increase in Nox4 protein expression compared with control glucose ($P < 0.05$). Exposure of cells to high glucose had no effect on expression of Nox1 or Nox2 (Fig. 4A, B, and C). High glucose had no effect on expression of NADPH oxidase subunits, p22⁶⁰⁰⁰ and p47²⁷⁰⁰⁰, and GKT-136901 did not influence expression of these subunits (Fig. 5).

**Pharmacological profile of GKT-136901, a dual Nox4/Nox1 inhibitor.** Using well-described methods to assess Nox activity (29, 34), we found that GKT-136901 is a specific NADPH oxidase inhibitor (33) with high potency on Nox4 [inhibitory constant ($K_i$) = 165 ± 5 nM] and Nox1 ($K_i$ = 160 ± 10 nM) and with a 10-fold selectivity over Nox2 ($K_i$ = 1530 ± 90 nM) (Fig. 6A). GKT-136901 inhibits both Nox4 and Nox1 (82 and 86%, respectively), with a partial effect on Nox2 (60% inhibition). The affinity of GKT-136901 for xanthine oxidase is very low ($K_i$ > 100 μM), and maximal inhibition at a concentration of 100 μM was only 40%. The irreversible flavoprotein inhibitor diphenylidionium (DPI) showed no selectivity in the same assays with $K_i$ in the range of 60–70 nM on Nox1, Nox2, Nox4, and $K_i$ of 10 nM for xanthine oxidase (100% inhibition with DPI was obtained on the 4 targets at 33 μM). Furthermore, GKT-136901 was evaluated in vitro at 10 μM in an extensive pharmacological profile including 135 different target proteins to establish its specificity for NADPH oxidases (screening assays performed at Cerep). GKT-136901 demonstrated excellent specificity with very low (~15% inhibition) or no inhibition of ROS-producing enzymes, redox-sensitive enzymes, and other proteins (Table 2).

**Effects of glucose on Nox4-mediated ROS production.** Exposure of MPT cells to high glucose for 4 h resulted in a significant increase in NADPH oxidase-derived O₂⁻ production (Fig. 6B) and in H₂O₂ generation (Fig. 6C) as assessed by lucigenin chemiluminescence and DCFDA fluorescence, respectively. Pretreatment of cells with GKT-136901 (Nox1/4 inhibitor) for 30 min significantly attenuated high-D-glucose-induced effects (Fig. 6). GKT-136901 alone or in combination with l-glucose had no significant effect on NADPH oxidase activity or ROS generation.

**Nox4 siRNA downregulates Nox4 protein expression and decreases D-glucose-induced ROS production in MPT cells.** To further demonstrate the importance of Nox4 in glucose-induced ROS formation, Nox4 expression was knocked down by siRNA. Significant reduction in Nox4 protein expression (~40%) was evident 24 h after siRNA transfection (Fig. 7A). RNA interference knockdown of Nox4 abrogated ROS generation induced by high D-glucose in MPT cells (Fig. 7B). There was a tendency for ROS production to be increased in the presence of scrambled siRNA and high D-glucose; however, significance was not achieved.

**Effect of Nox4 inhibition on high-glucose-induced activation of p38MAP kinase in mouse proximal tubular cells.** Stimulation of MPT cells with high D-glucose for 2 h induced significant phosphorylation of p38MAP kinase, as shown in Fig. 8. Pretreatment with GKT-136901 abolished the effect of high glucose on p38MAP kinase activity or ROS generation.

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**Fig. 7. Downregulation of Nox4 is associated with decreased activation of NADPH oxidase. A: effect of Nox4 small-interfering RNA (siRNA) and scrambled (Scr) siRNA (24 h) on Nox4 protein expression in MPT cells. Data, presented as the degree of change relative to control, taken as 1.0, are means ± SE of 3 experiments. *$P < 0.05$ vs. other groups. B: effect of Nox4 gene silencing on high-glucose-induced activation of NADPH oxidase in MPT cells. NADPH oxidase activity was assessed by enhanced lucigenin chemiluminescence. Data, normalized to $β$-actin, are presented as the degree of change relative to control, taken as 1.0. Values are presented as means ± SE of 3–9 experiments. *$P < 0.05$ and **$P < 0.01$ as indicated.**
High-glucose-induced profibrotic signaling pathways were ameliorated by statin therapy (16). Block et al. (7) previously showed that Nox4 localizes in mitochondria and that, in a rat model of diabetes, mitochondrial Nox4 expression is decreased in db/db mice. These findings, which were similarly demonstrated in isolated proximal tubules from db/db mice, appear to be cortex-specific because Nox4 content in medulla, although expressed, was not different between control and diabetic groups. Immunofluorescence studies indicated that Nox4 localized to glomeruli and tubules, indicating its heterogeneous distribution within the cortex. Others have demonstrated by in situ hybridization that Nox4 mRNA localizes in the renal cortex, specifically epithelial cells of proximal tubules, with lower expression in the medulla (32, 42). In human kidney, Nox4 mRNA is also expressed in medullary collecting ducts and in papillary epithelium (16, 32, 42). Nox4 gene upregulation has also been demonstrated in diabetic conditions, activation of renal Nox4-based NADPH oxidase is increased and that Nox4-sensitive ROS production and redox signaling are augmented. These findings are consistent with an important role for Nox4 in molecular mechanisms underlying renal injury in diabetes.

Fig. 8. GKT-136901 attenuates glucose-induced phosphorylation of p38MAP kinase in MPT cells, without effect on proliferating cell nuclear antigen (PCNA) expression. MPT cells were exposed to GKT-136901 (10⁻⁵ mol/l) for 30 min before normal (control) or high glucose exposure. A: representative immunoblots. B: corresponding bar graphs of glucose effects, with and without GKT-136901, on p38MAP kinase phosphorylation. Data, normalized to total p38MAP kinase content, are presented as the degree of change relative to control, taken as 1.0, and are means ± SE of 3 experiments. *P < 0.05 as indicated. A: top, representative immunoblots. Bottom, corresponding bar graphs of glucose effects, with and without GKT-136901, on PCNA expression. Data are normalized to β-actin and presented as the degree of increase relative to control, taken as 1.0, and are the means ± SE of 3 experiments.
suggest that upregulation of Nox4-based NADPH oxidase may be counterregulated by downregulation of Nox2/NADPH oxidase. Asaba et al. (2) reported that, in diabetic rats, expression of p47phox and Nox2 is increased and that apocynin normalizes these changes. Increased Nox4/p22phox/p47phox content was associated with enhanced ROS generation, implicating the potential importance of Nox4-based NADPH oxidase in oxidative damage associated with diabetic nephropathy. Increased ROS generation in the diabetic kidney is associated with inflammation, endothelial dysfunction, cell proliferation, migration and activation, extracellular matrix deposition, fibrosis, angiogenesis, and vascular remodeling (16, 21). These effects are mediated through redox-sensitive regulation of multiple signaling molecules, including MAP kinases, protein tyrosine phosphatases, tyrosine kinases, proinflammatory genes, ion channels, and Ca$^{2+}$ (16, 44). Of particular importance in the pathophysiology of diabetic nephropathy is activation of p38MAP kinase, which we previously demonstrated to be a redox-sensitive MAP kinase (11), was increased in db/db mice, an effect associated with increased expression of TGF-β1 and fibronectin.

Our data from whole kidneys from db/db mice suggest that increased activation of renal cortical Nox4-based NADPH oxidase is associated with oxidative stress and upregulation of redox-sensitive profibrotic and proinflammatory signaling pathways, which predispose to diabetic nephropathy. These findings are in line with those of Hecker et al. (23) demonstrating the importance of Nox4 in fibrogenic responses.

To investigate in greater detail whether Nox4 is an enzymatic source of ROS in kidney cells and whether dysregulation of Nox4 in diabetes contributes to oxidative stress and redox signaling, we examined cultured MPT cells in conditions that recapitulate diabetes, by exposing cells to high glucose. In rat mesangial cells, high glucose increased ROS production through activation of NADPH oxidase and mitochondrial electron transfer (48). In our study, 25 mmol/l D-glucose induced a significant increase in Nox4 expression with associated increased generation of O$_2^-$ and H$_2$O$_2$. These effects were not observed with 25 mmol/l L-glucose, indicating that effects are
due to high glucose actions and not to hyperosmotic effects. To confirm that glucose stimulates ROS generation through Nox4-based NADPH oxidase, Nox4 was downregulated using two strategies, by pharmacological inhibition of Nox4 activity using the small molecule GKT-136901 and by siRNA. GKT-136901 is a Nox-specific inhibitor (33, 34) that abrogated NADPH oxidase-derived generation of O$_2$^− and H$_2$O$_2$ in response to high glucose in MPT cells. Similar observations were made in cells in which Nox4 gene was knocked down by siRNA. Although knockdown by siRNA resulted in only a 30–40% decrease in Nox4 protein expression in MPT cells, this was sufficient to significantly decrease high-glucose-stimulated activation of NADPH oxidase and production of ROS.

In the presence of scrambled siRNA, glucose-stimulated activation of NADPH oxidase was slightly, but not significantly, increased. Reasons for this remain unclear but may suggest that the scrambled sequence had some effect on NADPH oxidase activity.

To confirm the functional significance of glucose in Nox4-sensing signaling, we showed that high glucose stimulated phosphorylation of p38MAP kinase and increased expression of TGF-β1/2 and fibronectin, indicating the importance of Nox4-based NADPH oxidase in renal ROS production and redox signaling. In line with our findings, Goettsch (19) demonstrated that, in endothelial cells, activation of p38MAP kinase is mediated via Nox4-derived ROS. To further investigate the relationship between glucose-stimulated redox-sensitive p38MAP kinase and profibrotic signaling, cells stimulated by high glucose were preexposed to SB-203580, which inhibited glucose-stimulated expression of TGF-β and fibronectin. These findings define a pathway in proximal tubule cells whereby a profibrotic milieu promotes fibrosis through Nox4-ROS-p38MAP kinase-TGF-β signaling. Previous studies showed that p38MAP kinase inhibition blocks de novo TGF-β1 protein synthesis and activation (50), confirming that p38MAP kinase is upstream of TGF-β1. However, others reported that TGF-β1 induces p38MAP kinase activation, indicating that p38MAP kinase is downstream of TGF-β1 (52). Taken together, these data suggest a feedforward system whereby p38MAP kinase influences TGF-β1, which in turn modulates p38MAP kinase. Such circuitous relationships between redox-sensitive signaling molecules have been well described (37). In our study, stimulation of signaling pathways by high glucose is not a generalized phenomenon, but seems to be highly specific, because molecular events associated with cell growth were unaffected by high glucose, whereas those associated with fibrosis were markedly activated by high glucose.

Taken together, our data highlight the importance of Nox4 as a source of ROS in the kidney. Moreover, we provide evidence that renal oxidative stress and profibrotic signaling pathways in prodiabetic conditions are mediated via increased expression/activation of cortex-specific Nox4-based NADPH oxidase. We have also defined an important link between Nox4-ROS-p38MAP kinase and TGF-β signaling in proximal tubule cells. Because changes in cellular function resulting in oxidative stress play an important role in the development and progression of diabetic nephropathy, interventions to reduce ROS production by targeting Nox4 may be attractive therapeutic strategies. Future studies examining Nox4-deficient mice as well as diabetic mice treated with GKT-136901 may provide further insights into this possibility.

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DISCLOSURES

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REFERENCES


33. Page P, Orchard M, Fioraso-Cartier L, Mottironi B.
34. Lassègue B, Clempus RE.
35. Jerums G, Panagiotopoulos S, Premaratne E, MacIsaac RJ.
41. Gurley SR, Clare SE, Snow KP, Hu A, Meyer TW, Coffman TM.
44. F1358 UPREGULATION OF Nox4 BY GLUCOSE IN KIDNEY CELLS