Renoprotective effects of a novel Nox1/4 inhibitor in a mouse model of Type 2 diabetes


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Abstract
Nox (NADPH oxidase)-derived ROS (reactive oxygen species) have been implicated in the development of diabetic nephropathy. Of the Nox isoforms in the kidney, Nox4 is important because of its renal abundance. In the present study, we tested the hypothesis that GKT136901, a Nox1/4 inhibitor, prevents the development of nephropathy in db/db (diabetic) mice. Six groups of male mice (8-week-old) were studied: (i) untreated control db/m, (ii) low-dose GKT136901-treated db/m (30 mg/kg of body weight per day), (iii) high-dose GKT136901-treated db/m (90 mg/kg of body weight per day), (iv) untreated db/db; (v) low dose GKT136901-treated db/db; and (vi) high-dose GKT136901-treated db/db. GKT136901, in chow, was administered for 16 weeks. db/db mice developed diabetes and nephropathy as evidenced by hyperglycaemia, albuminuria and renal injury (mesangial expansion, tubular dystrophy and glomerulosclerosis). GKT136901 treatment had no effect on plasma glucose or BP (blood pressure) in any of the groups. Plasma and urine TBARSs (thiobarbituric acid-reacting substances) levels, markers of systemic and renal oxidative stress, respectively, were increased in diabetic mice. Renal mRNA expression of Nox4, but not of Nox2, increased, Nox1 was barely detectable in db/db. Expression of the antioxidant enzyme SOD-1 (superoxide dismutase 1) decreased in db/db mice. Renal content of fibronectin, pro-collagen, TGFβ (transforming growth factor β) and VCAM-1 (vascular cell adhesion molecule 1) and phosphorylation of ERK1/2 (extracellular-signal-regulated kinase 1/2) were augmented in db/db kidneys, with no change in p38 MAPK (mitogen-activated protein kinase) and JNK (c-Jun N-terminal kinase). Treatment reduced albuminuria, TBARS and renal ERK1/2 phosphorylation and preserved renal structure in diabetic mice. Our findings suggest a renoprotective effect of the Nox1/4 inhibitor, possibly through reduced oxidative damage and decreased ERK1/2 activation. These phenomena occur independently of improved glucose control, suggesting GKT136901-sensitive targets are involved in complications of diabetes rather than in the disease process.

Key words: db/db mouse, diabetes, kidney, NADPH oxidase, NOX inhibitor, oxidative stress

INTRODUCTION

Diabetes mellitus is the leading cause of ESRD (end-stage renal disease), with nephropathy accounting for >35% of incident cases of ESRD in developed countries [1,2]. Clinical features of diabetic nephropathy include albuminuria and reduced GFR (glomerular filtration rate), which occur in association with increased BP (blood pressure), ultimately leading to ESRD [3]. These changes develop as a consequence of glomerular, tubular, vascular and interstitial damage, characterized by thickening of the glomerular basement membrane, glomerulosclerosis and tubulointerstitial fibrosis. Associated with these structural changes is monocyte–macrophage invasion, mesangial cell hyperplasia, matrix expansion and podocytopenia [4,5].

Molecular mechanisms underlying diabetic nephropathy remain elusive, but hyperglycaemia and activation of the RAS (renin–angiotensin system) are important [6,7]. Common to these processes is oxidative stress, defined as damage...
to macromolecules caused by altered redox signalling, leading to inflammation, fibrosis and vascular dysfunction [8,9]. Oxidative stress mediated by high glucose-induced ROS (reactive oxygen species) generation contributes to the development and progression of diabetes and related complications. Hyperglycaemia promotes ROS generation and formation of AGEs (advanced glycation end-products) [10], which themselves induce ROS production. In animal models of diabetes, renal and vascular oxidative stress is increased and antioxidant treatment ameliorates renal, functional and morphological changes of diabetes [11,12]. Clinically, patients with diabetes exhibit increased levels of biomarkers of ROS formation [13,14] and recent trials demonstrated that bardoxolone methyl, an oral antioxidant inflammation modulator, improves renal function in patients with advanced CKD (chronic kidney disease) and Type 2 diabetes [15].

Among the ROS important in cardiovascular and renal biology are superoxide anion (O$_2^-$), H$_2$O$_2$, NO and ONOO$^-$ (peroxynitrite), which activate signalling molecules implicated in diabetes-associated renal injury, including PKC (protein kinase C), MAPK (mitogen-activated protein kinase), TGFβ1 (transforming growth factor β1) and JAK/STAT (Janus kinase/signal transducer and activator of transcription) [16,17]. High glucose and pro-diabetic conditions increase ROS production via activation of many enzymes, including Nox (NADPH oxidase) [18,19]. Multiple Nox homologues have been identified [20,21], of which Nox4 may be particularly important in renal pathobiology because of its abundance in the kidney (hence termed Renox) [22]. Nox4 is a major source of ROS in renal cells and in kidney tissue in diabetes [23]. We have demonstrated previously that high glucose-induced activation of Nox4 in mouse proximal tubular
NADPH oxidase and diabetic nephropathy cells is associated with oxidative stress and pro-fibrotic signalling, effects blocked by GKT136901, a Nox1/4 inhibitor [24]. These in vitro studies suggested potential protective actions of this Nox inhibitor in pro-diabetic conditions. Whether similar effects occur in vivo is unknown. In the present study, we sought to determine whether GKT136901 prevents the development and/or progression of nephropathy in a mouse model of Type 2 diabetes and investigated putative molecular mechanisms underlying these processes.

MATERIALS AND METHODS

An expanded Materials and methods section can be found at http://www.clinsci.org/cs/124/cs1240191add.htm.

Animals

Studies were approved by the University of Ottawa Animal Ethics Committee and performed according to recommendations of the Canadian Council for Animal Care. Male db/db mice (C57/BLKS background) with their control heterozygous (db/m) littermates were examined. Six groups of male mice (8-week-old, n = 10–13/group) were studied. Group 1, db/m untreated; group 2, db/m receiving low dose GKT136901 (30 mg/kg of body weight per day); group 3, db/m receiving high dose GKT136901 (90 mg/kg of body weight per day); group 4, db/db, untreated; group 5, db/db, low-dose GKT136901; and group 6, db/db, high-dose GKT136901. GKT136901 was mixed in mouse chow. Mice were treated for 16 weeks until 24 weeks of age. BP was measured by tail-cuff methodology every 2 weeks as we have described previously [25] and albuminuria (24 h urine) and plasma glucose levels were assessed every 4 weeks.

Measurement of plasma glucose levels

Blood samples were collected every 4 weeks from the tail vein and at the end of the study by cardiac puncture. Levels of plasma glucose were determined using an automated analyser (Synchron CX5 PRO; Beckman and Fullerton).

Measurement of urinary albumin

24-h urine was collected for the measurement of albumin (Bethyl Laboratories.).

Measurement of urinary and plasma TBARSs (thiobarbituric acid-reacting substances)

Urinary and plasma MDA (malondialdehyde) levels were measured using a colorimetric assay as we described [26]. TBARS concentration was obtained from a standard curve produced by serially diluting TBARS.

Histopathology

General pathological assessment and quantification were based on the standard validation criteria [27]. The glomerular surface area was measured in captured digital images by tracing the perimeter of the glomerulus using the polygram tool. GCSA (glomerular cross-sectional area, μm²) was counted as CCSA = GCSA–MCSA. Mesangial cell density was calculated as NMC/MCSA (cells/mm²). The degree of tubular dystrophy in each field was graded on a scale of 0–3: grade 0 = normal; grade 1 = number of dystrophic tubules up to 25% of visible tubules; grade 2 = number of dystrophic tubules 25–50% (moderate); and grade 3 = number of dystrophic tubules >50% (severe).

Immunofluorescence studies of renal Nox4

Immunohistochemical and immunofluorescence staining of kidney sections were carried out by standard protocol. The sections were incubated overnight at 4°C with anti-Nox4 antibody...
Immunostaining to evaluate renal inflammation
Specific antibodies against neutrophil marker myeloperoxidase and mature macrophages (F4/80 antigen) were used to evaluate renal inflammation.

Measurement of renal Nox-derived \( \text{O}_2^- \) generation
The lucigenin-enhanced chemiluminescence assay was used to determine Nox activity in total protein homogenates from the renal cortex [28]. Luminescence was measured as we have detailed previously [24,29].

Real-time PCR
Total RNA was extracted from the kidney cortex, subjected to DNase treatment and reverse transcribed. Real-time PCR primer sequences are shown in Supplementary Table S1 (http://www.clinsci.org/cs/124/cs1240191add.htm). mRNA levels were normalized to \( \beta \)-actin mRNA and the relative fold difference in expression was calculated using the \( 2^{-\Delta\Delta C_T} \) method. Real-time PCR was conducted using the Applied Biosystems 7300 real-time PCR system.

Western blotting
Total protein from the kidney cortex was separated by electrophoresis on a polyacrylamide gel, and transferred onto a nitrocellulose membrane. The membranes were incubated with specific antibodies: TGFβ1/2, pro-collagen-1, catalase, VCAM-1 (vascular cell adhesion molecule 1) (Santa-Cruz Biotechnology), Nox4 (from Professor Ajay M. Shah), p47\( \text{phox} \) p22\( \text{phox} \) (from Dr Mark Quinn, Montana State University, Bozeman, Montana, U.S.A.), fibronectin (Sigma–Aldrich), ERK1/2 (extracellular-signal-regulated kinase 1/2), JNK (c-Jun N-terminal kinase), p38MAPK (total and phospho-specific), SOD-1 (superoxide dismutase 1), catalase and \( \beta \)-actin (Cell Signaling Technology, Inc.).

Statistical analysis
Data are expressed as means ± S.E.M. The groups were compared by two-ways ANOVA or by Student’s \( t \) test where appropriate. \( P < 0.05 \) was considered significant.

RESULTS

Effect of GKT136901 on plasma glucose levels, systolic BP and body weight in diabetic mice.
BP was similar in \( \text{db}/\text{m} \) and \( \text{db}/\text{db} \) mice, without significant effect of GKT136901 treatment (Figures 1A and 1B). Plasma glucose levels were higher in \( \text{db}/\text{db} \) mice than in control \( \text{db}/\text{m} \) mice (Figures 1C and 1D). Plasma glucose increased progressively from 8 to 24 weeks of age in \( \text{db}/\text{db} \) mice. GKT136901, at low and high doses, did not influence plasma glucose levels in either \( \text{db}/\text{m} \) or \( \text{db}/\text{db} \) groups. \( \text{db}/\text{db} \) mice were heavier than \( \text{db}/\text{m} \) mice. Treatment did not influence body weight in either group (Figures 1E and 1F).

Reduced albuminuria in diabetic mice treated with GKT136901.
Diabetic mice exhibit significant albuminuria as early as 8 weeks of age (Figure 2A). Treatment with GKT136901 (low and high dose) for 8 weeks showed significant reduction in urinary albumin compared with untreated \( \text{db}/\text{db} \) mice (Figure 2B). After 16 weeks of study \( \text{db}/\text{db} \) mice exhibited significant albuminuria, with no differences between untreated and treated \( \text{db}/\text{db} \) groups.

Effect of GKT136901 on urinary and plasma TBARS levels in \( \text{db}/\text{db} \) mice.
Figure 3(A) shows increased urinary TBARS levels in \( \text{db}/\text{db} \) against \( \text{db}/\text{m} \) mice. GKT136901 significantly reduced urinary TBARS levels in \( \text{db}/\text{db} \) mice compared with control counterparts. Figure 3(B) demonstrates plasma TBARS levels in \( \text{db}/\text{m} \) and \( \text{db}/\text{db} \) mice. Plasma TBARS levels were significantly increased in untreated \( \text{db}/\text{db} \) mice compared with \( \text{db}/\text{m} \) mice (\( P < 0.01 \)). In high-dose GKT136901-treated \( \text{db}/\text{db} \) mice, the plasma TBARS levels were significantly reduced compared with untreated \( \text{db}/\text{db} \)
mice. GKT136901 had no effect on plasma TBARS in control groups.

**Morphometric analysis of kidneys**

MCSA was significantly increased in untreated \(db/db\) mice compared with control counterparts (Figure 4A). GKT136901 (low dose) reduced MCSA in \(db/db\) mice. Capillary area/mesangial area was significantly reduced in \(db/db\) mice (Figure 4B), an effect that was normalized by GKT136901. Associated with the increased MCSA was a significant increase in GCSA in \(db/db\) mice (Supplementary Figure S1 at http://www.clinsci.org/cs/124/cs1240191add.htm). GCSA in low-dose-treated mice was greater than that in \(db/db\) control counterparts, whereas in high-dose-treated \(db/db\) mice, there was no significant increase compared with control \(db/m\) mice.

Untreated diabetic mice exhibit severe tubular dystrophy (grade 3 >50% dystrophic tubules) (Figure 5A). This was prevented by low-dose, but not by high-dose, GKT136901. There was no evidence of tubular pathology in kidneys from \(db/m\) mice.

As demonstrated in Figure 5(B) (upper panels), some untreated \(db/db\) mice exhibited renal inflammation as evidenced by abundant neutrophils and macrophages. In the high-dose-treated \(db/db\) group, the renal parenchyma was well preserved with rare local, non-destructive inflammatory infiltrates. Some
macrophages are present in the interstitium and there are small foci of scanty neutrophils within the renal tubules (Figure 5B, lower panel).

**Effect of GKT136901 on renal Noxs and antioxidant enzymes**

Nox4 distribution in mouse kidneys is shown in Supplementary Figure S2 (http://www.clinsci.org/cs124/cs1240191add.htm). Renal distribution of Nox4 was similar in db/m and db/db mice. Expression of renal Nox4 mRNA was greater in db/db mice compared with control counterparts (P < 0.05) (Figure 6A). High-dose GKT136901 reduced expression of Nox4 mRNA. Nox4 protein expression was not different between groups (Figure 6B). mRNA expression of Nox2 was not different in db/db mice and treatment had no effect on Nox2 mRNA (Figure 6C). Nox1 expression in db/m and db/db mice was very low (results not shown). Content of renal SOD-1 was significantly reduced in untreated db/db mice compared with control counterparts.
NADPH oxidase and diabetic nephropathy

Figure 6 Renal expression of Nox isoforms and antioxidant SOD
Expression of Nox 4 mRNA (A), Nox4 protein (B), Nox 2 mRNA (C) and SOD-1 (D) in kidneys from untreated and GKT136901-treated db/m and db/db mice. The upper panels are representative immunoblots of Nox4 and SOD-1. *P < 0.01 compared with db/m counterparts. Data are means ± S.E.M., 5–10 mice/group. Proteins normalized to β-actin and results expressed as fold increase above control, taken as 1.0.

GKT136901 differentially influences renal MAPK in db/db mice
To evaluate the effects of GKT136901 on renal MAPK signalling, we assessed the levels of phosphorylated forms of ERK1/2, p38MAPK and JNK/SAPK (stress-activated protein kinase) in kidneys from untreated and treated mice. Levels of phosphorylated ERK1/2 significantly increased in untreated db/db mice compared with control db/m mice (Figure 7). This effect was normalized by high-dose GKT136901. Phosphorylation of p38MAPK and JNK was not altered in db/db mice and GKT136901 had no effect on these MAPK in db/m or db/db groups (Figures 7B and 7C).

Increased pro-fibrotic and pro-inflammatory responses in kidneys from diabetic mice
Pro-fibrotic (fibronectin, pro-collagen-1, TGFβ) and pro-inflammatory (VCAM-1) molecules were evaluated in kidneys from db/m and db/db mice. Expressions of fibronectin, pro-collagen 1, TGFβ and VCAM-1 significantly increased in untreated db/db mice compared with control db/m mice (Figure 8). In support of these molecular findings, histological analysis demonstrated increased interstitial fibrosis by Picosirius Red staining for collagen in db/db mice compared with db/m (Supplementary Figure S5 at http://www.clinsci.org/cs/124/cs1240191add.htm). GKT136901 treatment normalized expression of pro-collagen-1, TGFβ and VCAM-1 compared with db/m counterparts, but not compared with untreated db/db mice.

DISCUSSION
The experimental data suggest an important role for oxidative stress in the development of diabetic nephropathy [30–33].
Figure 7 Effect of GKT136901 on renal MAPK
Phosphorylation of ERK1/2 (A), p38MAPK (B) and JNK (C) in kidneys from untreated and GKT136901-treated db/m and db/db mice. The upper panels are representative immunoblots. The lower panels are the corresponding quantification. Data are means ± S.E.M., n = 6–12 mice/group. Data are normalized to β-actin and expressed as fold increase above control, taken as 1. *P < 0.05 against db/m counterparts.

Recent clinical studies support this as evidenced by the significant improvement in renal function in patients with Type 2 diabetes and CKD treated with bardoxolone, an antioxidant [15]. Noxs are increasingly implicated as major sources of ROS involved in cardiovascular and renal complications of diabetes. We previously demonstrated in in vitro studies, the role of Nox4, but not of Nox1 or Nox2, in high glucose-induced ROS generation in kidney cells [24]. In the present study, we extend our cell-based studies to whole animals by testing effects of a Nox1/4 inhibitor on the development of diabetes and associated kidney injury in db/db mice. Major findings demonstrate significant proteinuria and kidney damage (mesangial expansion, interstitial fibrosis, glomerulosclerosis and inflammation) in diabetic mice. Associated with renal damage in diabetes was increased lipid peroxidation (a marker of oxidative stress) and activation of mitogenic and pro-inflammatory signalling pathways, effects that were variably ameliorated by the Nox1/4 inhibitor.

Although the ideal murine model of human diabetic kidney disease remains to be identified [34–36], db/db mice on the C57/BLKS background studied here exhibit many features of diabetic nephropathy in humans, including significant hyperglycaemia, proteinuria (20-fold increase at 8 weeks of age), mesangial expansion, glomerulosclerosis, tubulointerstitial fibrosis and inflammation. Among the many molecular mechanisms implicated in the development of diabetic kidney disease are hyperglycaemia-associated oxidative stress derived in large part from AGEs, glucose autoxidation, the polyol pathway, NEFAs (non-esterified fatty acids), leptin and activation of Noxs [37–39]. Nox1, Nox2 and Nox4, all of which require p22phox for activation, are expressed in the kidney [40–42]. Increased renal expression/activity of Nox4 and up-regulation of p22phox have been demonstrated in experimental models of CKD and diabetic nephropathy [24,40–42]. Recent human studies identified a genetic variant in the promoter region of p22phox in patients with kidney diseases and Type 1 diabetes [43].

Untreated db/db mice demonstrated a progressive increase in plasma glucose, evident by 8 weeks of age. In our study, plasma glucose was measured by highly sensitive automated clinic-based methods, rather than by glucometer, which clamps glucose readings at ~30 mmol/l. Accordingly glucose levels in our db/db mice tended to be higher than those previously reported [34–36]. BP was normal in diabetic mice and did not change during the course of the study. Others have also shown normal BP in db/db mice [44]. Neither low- nor high-dose GKT136901 had any effect on plasma glucose, suggesting that the hyperglycaemia in db/db mice is probably independent of GKT136901 targets. Previous studies have shown that Nox inhibitors prevent complications of diabetes with variable effects on glucose control [45,46]. Although Noxs may not be directly involved in the pathophysiology of Type 2 diabetes, there is growing evidence indicating that pancreatic Noxs play a role in insulin production in Type 1 diabetes [47].

Despite GKT136901 having no effect on plasma glucose levels, it protected against diabetes-associated renal complications in db/db mice. Mechanisms for this may relate to direct actions of GKT136901, where oxidative damage in the kidney is reduced by treatment, as evidenced by decreased renal TBARS and sparing of the kidney structure. db/db mice exhibited marked albuminuria typical of early nephropathy in diabetes. After 8 weeks of treatment, albuminuria was significantly reduced. However, after 16 weeks this renoprotective effect was no longer present. Reasons for this partial functional effect are unclear, but it may be possible that GKT136901 doses were not high enough to completely prevent development of nephropathy. Associated with renal dysfunction was altered kidney structure.
NADPH oxidase and diabetic nephropathy

Figure 8 Effect of GKT136901 on molecular processes associated with renal fibrosis and inflammation

Expression of pro-fibrotic [fibronectin (FN), pro-collagen-1 (Pro coll 1) and TGFβ1/2 (A–C)] and pro-inflammatory proteins [VCAM-1 (D)] in kidneys from db/m and db/db mice treated with GKT136901. The upper panels are representative immunoblots. The lower panels are the corresponding quantification. Data are means ± S.E.M., n = 6–12 mice/group. Data are normalized to β-actin and expressed as fold increase above control, taken as 1. *P < 0.05 compared with db/m counterparts.

Bioavailability of ROS depends on the balance between systems that are pro-oxidant and anti-oxidant. In db/db mice, renal content of the antioxidant enzyme SOD-1 was reduced, an effect which was and is normalized by treatment with GKT136901. These data suggest that increased oxidative stress in diabetes may derive not only from increased production, but also from down-regulation of antioxidant systems, such as SOD-1. In support of our findings, DeRubertis et al. [50] reported that overexpression of SOD-1 attenuated renal injury in db/db mice. On the other hand, diabetic nephropathy was accelerated in SOD-1 knockout mice [51]. Mechanisms whereby GKT136901 normalized SOD-1 are unclear, but perhaps reduced Nox4-derived ROS production influences SOD-1 in a feedback manner to prevent further ROS accumulation. In vitro studies have demonstrated close interactions between Nox4, ROS and SOD-1 [52]. These SOD-1 changes are not generalized phenomena, since catalase, another antioxidant enzyme, was not influenced by diabetes or by treatment.

To assess renal oxidase status better we measured the activity of Nox by lucigenin chemiluminescence. Unlike the previous studies [33–36], we did not find significant changes in renal Nox activity in db/db mice. Reasons for this may relate to the fact that

in diabetic mice. Mesangial expansion was increased, capillary density was reduced, tubules exhibited significant dystrophy and inflammatory cells were abundant. Treatment prevented some of these changes in db/db mice.

As previously demonstrated in various experimental models of diabetes [30–33,45,46], biomarkers of oxidative stress (TBARS) were elevated, both systemically (plasma) and renal-derived (urine). Although the exact source of increased ROS in db/db mice is unclear, our results suggest that Nox1/4 may be important, since a high dose of GKT136901 normalized oxidative biomarkers. We cannot exclude the possibility that other enzymes are also involved, but in the kidney, where Nox4 mRNA expression was increased and reduced by treatment in db/db mice, this isoform may be particularly important, especially with respect to renal oxidative stress. Since Nox4 is constitutively active, unlike most of the other Noxs that are activated upon stimulation, changes in expression at the gene level probably reflect activation status [48,49]. Nox1 was barely detectable in the kidney and Nox2 mRNA expression was unchanged in db/db mice. Nox2 was assessed at the mRNA level, rather than at the protein level, due to lack of optimal Nox2 antibodies to accurately measure protein expression.
we studied whole tissue, comprising many cell types, in which Noxs may be variably expressed and activated. This may result in a dilution effect with no overall change in oxidase activity. Dissecting out regions of interest, for example, with laser capture microdissection, may facilitate the assessment of renal Nox activity in a cell-specific manner. Fundamental to renal injury in diabetes is fibrosis and inflammation. At the molecular level, expression of pro-fibrotic (fibronectin, pro-collagen 1 and TGFβ) and pro-inflammatory (VCAM-1) proteins in db/db mice was increased, similar to that previously demonstrated [36,53]. These molecular findings are in line with those observed histologically. Of the many upstream signalling pathways regulating fibrosis and inflammation are MAPK, particularly ERK1/2 [54]. Activation of renal ERK1/2, but not of p38MAPK or JNK, was increased in db/db mice, suggesting that in this model of diabetes, activation of MAPK is highly regulated and not a global phenomenon. GKT136901 normalized ERK1/2 phosphorylation, and, at a high dose, had a partial effect on the expression of pro-collagen-1, TGFβ and VCAM-1.

In summary, we demonstrate that GKT136901, a Nox1/4 inhibitor, has some renoprotective effects in db/db mice. This is evidenced by reduced oxidative stress, decreased albuminuria and preserved renal structure. These processes are associated with blunted activation of renal ERK1/2, an MAPK critically involved in molecular events underlying kidney cell injury. These phenomena occur independently of improved glucose control, and suggest that GKT136901-sensitive targets are involved in renal complications of diabetes rather than in the development of the disease.

CLINICAL PERSPECTIVES

- Increased Nox-derived ROS production has been implicated in experimental and human diabetic nephropathy. Of the Nox isoforms, Nox4 is abundant in the kidney and may be important in renal injury.
- Diabetic mice treated with an oral Nox1/4 inhibitor, GKT136901, demonstrated reduced systemic oxidative stress, decreased albuminuria and reduced kidney inflammation and fibrosis. These results indicate a potential renoprotective effect of the Nox1/4 inhibitor.
- Our findings suggest that targeting Noxs in an isoform-specific manner, particularly Nox1/4, may have therapeutic potential in the prevention and/or treatment of diabetic nephropathy.

AUTHOR CONTRIBUTION

The study was conceived and designed by Rhian Touyz, Chris Kennedy, Kevin Burns and Richard Hebert. Mona Sedeek conducted most of the studies with assistance from Alex Gutsol, Augusto Montezano, Dylan Burger and Aurelie Nguyen Dinh Cat. The paper was written by Mona Sedeek and Rhian Touyz, with contributions from Chris Kennedy, Kevin Burns, Mark Cooper, Karin Janedeit-Dahm, Patrick Page, Cedric Szondralewicz, Freddy Heitz and Richard Hebert.

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SUPPLEMENTARY ONLINE DATA

Renoprotective effects of a novel Nox1/4 inhibitor in a mouse model of Type 2 diabetes


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MATERIALS AND METHODS

Animals
Studies were approved by the University of Ottawa Animal Ethics Committee and performed according to recommendations of the Canadian Council for Animal Care. Male db/db mice (C57/BLKS background) with their control heterozygous (db/m) littermates were examined. Six groups of mice (8 week-old) were studied. Group 1, db/m untreated; group 2, db/m receiving low-dose GKT136901 (30 mg/kg of body weight per day); group 3, db/m receiving high dose GKT136901 (90 mg/kg of body weight per day); group 4, untreated db/db; group 5, db/db, receiving low-dose GKT136901; group 6, db/db receiving high-dose GKT136901. GKT136901 was mixed in the mouse chow (prepared commercially by Harlan (Harlan Laboratory). Mice were treated for 16 weeks until 24 weeks of age. BP was measured by tail cuff methodology every 2 weeks as we described [1] and albuminuria (24-h urine) and plasma glucose levels were assessed every 4 weeks. We have shown previously that tail cuff measurements of BP accurately reflect those measured by telemetry [1]. Considering the chronic nature of the present study, it was not feasible to measure BP by telemetry.

Mice were killed using CO2. Kidneys were collected and processed for immunofluorescence, histochemistry, protein and RNA isolation.

Measurement of plasma glucose levels
Blood samples were collected every 4 weeks from the tail vein and at the end of the study by cardiac puncture immediately prior to killing. Levels of plasma glucose were determined using an automated analyser (Synchron CX5 PRO; Beckman and Fullerton).

Measurement of urinary albumin
24-h urine was collected from mice (housed in metabolic cages) for albumin measurement. Albumin levels were quantified using a mouse albumin ELISA kit (Bethyl Laboratories), according to the manufacturer’s instructions.

Measurement of urinary and plasma TBARS levels
Urinary and plasma MDA (malondialdehyde) levels were measured using a colorimetric assay as we described [2]. Briefly samples were treated with Quantinella reagent (0.25 M HCl, 15% trichloroacetic acid and 26 mM thiobarbituric acid) and 2% BHT (butylated hydroxytoluene), heated, centrifuged and the absorbance read at 535 nm. TBARS concentration was obtained from a standard curve produced by serially diluting MDA.

Histopathology
Each kidney was divided sagitally into two equal parts. One half was dipped in OCT and snapfrozen in liquid nitrogen, and the other was immersion-fixed in 10% neutral buffered formalin and embedded in paraffin. After deparaffinization and hydration 5μm-thick sections were stained with H&E (haematoxylin and eosin), periodic acid–Schiff, trichrome Masson and Picrosirius Red.

General pathological assessment and quantification were based on standard validation criteria [3–6]. Slides were analysed in blinded fashion. Renal sections were visualized at ×400 and ×630 magnification (Axioskop 2, Imager A1; Zeiss) and images captured, stored as TIFF files and imported into ImagePro-Plus software (Media Cybernetics) using the threshold function. Images were processed so that positive staining was represented by pixels of the corresponding colour measured as a percentage of the area of interest or total image. Glomerular surface area was measured in captured digital images by tracing the perimeter of the glomerulus using the polygram tool. The analysing software was calibrated to an objective stage micrometer. GCSA (μm²), MCSA (μm²) and NMC were measured in 50 cortical glomeruli per group. Capillary cross sectional area (CCSA; μm²) was counted as CCSA = GCSA – MCSA. Mesangial cell density was calculated as NMC/MCSA (cell/mm²). The degree of tubular dystrophy in each field was graded on a scale of 0–3: grade 0 = normal; grade 1 = number of dystrophic tubules up to 25% of visible tubules; grade 2 = number of dystrophic tubules 25–50% (moderate); and grade 3 = number of dystrophic tubules > 50% (severe).

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Immunofluorescence studies of renal Nox4
Immunohistochemical and immunofluorescence staining of kidney sections were carried out by standard protocols. Frozen tissues were cut (6–8 μm sections), fixed (cold acetone, −20°C, 5 min) and blocked with 5 % FBS (fetal bovine serum) for 1 h at room temperature. Sections were incubated overnight at 4°C with anti-Nox4 antibody, with conjugated Alexa488 secondary antibodies (Invitrogen). Hoechst 33342 was used for nuclear counterstaining. Simultaneous incubation with appropriate dilutions of non-immune IgG was used as a substitute for the primary antibody as a negative control. Sections were visualized by fluorescence microscopy (Zeiss Axioskop 2 MOT).

Immunostaining to evaluate renal inflammation
After antigen retrieval by boiling in 10 mM sodium citrate buffer and blocking with 10% donkey serum, slides were incubated overnight with specific antibodies against neutrophil marker myeloperoxidase (rabbit polyclonal, Thermo Fisher Scientific), and mature macrophages, F4/80 antigen (rat monoclonal anti-mouse; eBioscience). Vectastain ABC peroxidase kit (Vector Laboratories) was used for visualization. Sections were then either counterstained with Mayer’s haematoxylin for analysis or left unstained for quantitative analysis.

Measurement of renal Nox-derived O2− generation
Kidney cortex was homogenized with a glass-to-glass homogenizer in Kreb’s buffer. The lucigenin-enhanced chemiluminescence assay was used to determine Nox activity in total protein homogenates [7,8]. 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, 150 mmol/l sucrose, pH 7.4). Luminescence was measured every 1.8 s for 3 min in a luminometer (Orion II microplate luminometer; Montreal Biotech), as we previously detailed in [8]. Buffer blank was subtracted from each reading. Activity was expressed as arbitrary units/mg of protein.

Real-time PCR
Total RNA was extracted from the kidney cortex using the TRIzol method (Invitrogen). RNA (10 μg) was subjected to DNase treatment (DNA-free kit; Invitrogen) and reverse transcription cDNA synthesis performed by the TaqMan methodology (Applied Biosystems). Real-time PCR primers were designed using primer 3 software. Sequences are shown in Supplementary Table 1. mRNA levels were normalized to β-actin mRNA and the relative fold difference in expression was calculated using the 2ΔΔCt method. Real-time PCR was conducted using the Applied Biosystems 7300 real-time PCR system.

Western blotting
Total protein from the kidney cortex was separated by electrophoresis on a polyacrylamide gel, and transferred onto a nitrocellulose membrane. Non-specific binding sites were blocked with 5% non-fat dried skimmed milk powder. Membranes were incubated with specific antibodies overnight at 4°C. After incubation with secondary antibodies, signals were revealed with chemiluminescence, visualized by autoradiography and quantified densitometrically (Image J, National Institute of Health). The following antibodies were used: TGF-β1/2, Nox1, pro-collagen-1, catalase, VCAM-1, (Santa-Cruz Biotechnology), Nox4, p47phox, p22phox, fibronectin (Sigma-Aldrich Inc), ERK1/2, JNK, p38MAPK (total and phospho-specific), SOD-1 and β-actin (Cell Signaling Technology).

Pharmacology of GKT136901
In our previous papers [8,9], we described in detail the pharmacological properties of GKT136901. In brief, we found that GKT136901 is a specific Nox inhibitor with high potency on Nox4 (Ki = 165 ± 5 nM) and Nox1 (Ki = 60 ± 10 nM) and with a 10-fold selectivity over Nox2 (Ki = 1530 ± 90 nM). GKT136901 inhibits both Nox4 and Nox1 (82 and 86 % respectively), with a partial effect on Nox2 (60 % inhibition). The affinity of GKT136901 for xanthine oxidase is very low (Ki > 100 μM) and maximal inhibition at a concentration of 100 μM was only 40 %. In an extensive pharmacological profile (including 135 different target proteins) in order to establish its specificity for Noxs (screening assays performed at Cerep), GKT136901 demonstrated excellent specificity with very low (<15 % inhibition) or no inhibition of ROS producing enzymes, redox-sensitive enzymes and other proteins [9].

Table S1 Sequences of the primers used in the present study

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession number</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>Nox4</td>
<td>NM_015760</td>
<td>Forward: 5′-TGTTGGGCTTAGATTGTGT-3′</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: 5′-CACTGCAGCTCATCTTAA-3′</td>
</tr>
<tr>
<td>Nox2</td>
<td>NM_007807.4</td>
<td>Forward: 5′-TGTTACCACTGGGACAGA-3′</td>
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<td></td>
<td></td>
<td>Reverse: 5′-GGGGTGTTGAAGGTCTCAAA-3′</td>
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<td>Nox1</td>
<td>NM_172203.1</td>
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<tr>
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<td></td>
<td>Reverse: 5′-CCAGCCAGTGAGGAAGGTC-3′</td>
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<tr>
<td>p22phox</td>
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<td>Forward: 5′-CGTTGCCTACTGGCGAGTT-3′</td>
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<td>Reverse: 5′-TGGACCCCTTTTCTCTTC-3′</td>
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<td>β-Actin</td>
<td>NM_007393.3</td>
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<tr>
<td></td>
<td></td>
<td>Reverse: 5′-GGGGTGTTGAAGGTCTCAAA-3′</td>
</tr>
</tbody>
</table>
Figure S1 GCSA in kidneys from untreated and GKT13690-treated db/m and db/db mice
Data are means ± S.E.M., n = 4–5 for db/m, 7–11 for db/db. *P < 0.05 compared with db/m counterpart.

Figure S2 Distribution of Nox4 in mouse (db/m) kidneys
Immunofluorescence microscopic assessment of Nox4 distribution in normal mouse (db/m) kidney. (A) Nox4 is abundant in the proximal tubules (S3 segments) within the outer medulla (yellow arrow, ×100). (B) Within the renal cortex, Nox4 localizes mainly in the cortical collecting ducts (yellow arrow) and also in the basal part of the parietal epithelium of Bowman’s capsule (white arrows ×200). (C) Nox4 is expressed in the basal zone of the epithelium of the inner medullary collecting ducts (arrow) but there is no signal in the interstitium (asterisks ×200). (D) Nox4 is abundant in the protective transitional epithelium (white arrow) covering the RP (renal pelvis). Adjacent artery and vein (asterisks) have little detectable Nox4 in their walls, whereas the neighbouring proximal tubules have abundant Nox4 (yellow arrow, ×200). Nox4 distribution was similar in kidneys from db/db mice.
Figure S3  Renal expression of Nox subunits and catalase
Expression of p22<sub>phox</sub> (A), p47<sub>phox</sub> (B) and catalase (C) in kidneys from the untreated and GKT136901-treated db/m and db/db mice. Upper panels are representative immunoblots. Data are means ± S.E.M., 5–10 mice/group. Proteins were normalized to β-actin and results are expressed as fold increase above control, taken as 1.0.

Figure S4 Activation of renal Nox in untreated and treated db/m and db/db mice
Nox-derived superoxide generation in kidneys from untreated and treated db/m and db/db mice. Activation of Nox was assessed by lucigenin (5 μmol/l) ECL® (enhanced chemiluminescence). Data are means ± S.E.M., n = 8–10 mice/group. AU, arbitrary units.
NADPH oxidase and diabetic nephropathy

Figure S5 Interstitial collagen content in untreated and GKT136901-treated db/m and db/db mice

(A) Percentage collagen relative to the total area of the renal cortex. Collagen was detected by the specific picrosirius red staining. Data are means ± S.E.M., n = 5–8 mice/group. * P < 0.05 compared with db/m counterparts. (B) Representative images of cortical sections from db/m and db/db mice stained with Picrosirius Red.

References


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