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ARTICLE

Deletion of *p47^{phox}* attenuates the progression of diabetic nephropathy and reduces the severity of diabetes in the Akita mouse

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Abstract
Aims/hypothesis Reactive oxygen species (ROS) contribute to diabetes-induced glomerular injury and endoplasmic reticulum (ER) stress-induced beta cell dysfunction, but the source of ROS has not been fully elucidated. Our aim was to determine whether p47^{phox}-dependent activation of NADPH oxidase is responsible for hyperglycaemia-induced glomerular injury in the Akita mouse, a model of type 1 diabetes mellitus resulting from ER stress-induced beta cell dysfunction.
Methods We examined the effect of deleting *p47^{phox}* (also known as *Ncf1*), the gene for the NADPH oxidase subunit, on diabetic nephropathy in the Akita mouse (*Ins2*$^{WT/C96Y}$) by studying four groups of mice: (1) non-diabetic mice (*Ins2*$^{WT/WT}$/*p47$^{phox+/+}$*); (2) non-diabetic *p47^{phox}*-null mice (*Ins2*$^{WT/WT}$/*p47$^{phox-/-}$*); (3) diabetic mice: (*Ins2*$^{WT/C96Y}$/*p47$^{phox+/+}$*); and (4) diabetic *p47^{phox}*-null mice (*Ins2*$^{WT/C96Y}$/*p47$^{phox-/-}$*). We measured the urinary albumin excretion rate, oxidative stress, mesangial matrix expansion, and plasma and pancreatic insulin concentrations in 16-week-old mice; we also measured glucose tolerance and insulin sensitivity, islet and glomerular NADPH oxidase activity and subunit expression, and pro-fibrotic gene expression in 8-week-old mice. In addition, we measured NADPH oxidase activity, subunit expression and pro-fibrotic gene expression in high glucose-treated murine mesangial cells.
Results Deletion of *p47^{phox}* reduced kidney hypertrophy, oxidative stress and mesangial matrix expansion, and also reduced hyperglycaemia by increasing pancreatic and circulating insulin concentrations. *p47$^{phox-/-}$* mice exhibited improved glucose tolerance, but modestly decreased insulin sensitivity. Deletion of *p47^{phox}* attenuated high glucose-induced activation of NADPH oxidase and pro-fibrotic gene expression in glomeruli and mesangial cells.
Conclusions/interpretation Deletion of *p47^{phox}* attenuates diabetes-induced glomerular injury and beta cell dysfunction in the Akita mouse.

Keywords Beta cell dysfunction · Diabetes · Diabetic nephropathy · Endoplasmic reticulum (ER) stress · NADPH oxidase · Oxidative stress · p47^{phox} · Reactive oxygen species

A. M. Herzenberg passed away suddenly during these studies and this manuscript is dedicated to him.

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Abbreviations

ER	Endoplasmic reticulum
KW:BW	Kidney:body weight ratio
MM	Mesangial matrix
NOX	NADPH oxidase
PAI-1	Plasminogen activator inhibitor 1
PAS	Periodic acid–Schiff's reagent
ROS	Reactive oxygen species
WT-1	Wilms tumour 1

Introduction

High glucose-induced generation of reactive oxygen species (ROS) is an important contributor to the pathogenesis of diabetic nephropathy [1–8]. Although superoxide can be generated from mitochondrial electron transport, uncoupled endothelial nitric oxide synthase activity or activation of the family of NADPH oxidase (NOX) enzymes [9–12], the contribution of each of these pathways to kidney injury has not been fully elucidated.

Several isoforms of NOX have been identified in the kidney, including NOX1, NOX2 and NOX4 [9]. Activation of the membrane-bound NOX isoforms, NOX1 and NOX2, is dependent on recruitment and phosphorylation of several cytosolic subunits, including $p47^{phox}$ [13–15], which has been implicated in the generation of superoxide in rat mesangial cells under high glucose conditions [16]. Apocynin, a non-specific antioxidant that targets $p47^{phox}$, reduces albuminuria and mesangial expansion in a mouse model of diabetes [17–20]. $p47^{phox}$-independent NOX4 has also been implicated in diabetes-induced oxidative stress in the kidney [21–24]. Therefore, although NOX-induced ROS may play a role in the progression of diabetic nephropathy, the role of specific NOX isoforms and the cytosolic subunits that regulate their activation remains uncertain.

Accordingly, we examined the effect of deleting $p47^{phox}$ (also known as *Ncf1*), the gene that encodes the NOX subunit, on diabetic kidney injury in the Akita mouse model of type 1 diabetes mellitus. Our aim was to test the hypothesis that $p47^{phox}$-dependent activation of NOX is an important determinant of experimental diabetic nephropathy.

Methods

Animals Four groups of male mice were studied: (1) non-diabetic mice ($Ins2^{WT/WT}/p47^{phox+/+}$); (2) non-diabetic $p47^{phox}$-null mice ($Ins2^{WT/WT}/p47^{phox-/-}$); (3) diabetic Akita mice ($Ins2^{WT/C96Y}/p47^{phox+/+}$); and (4) diabetic $p47^{phox}$-null mice ($Ins2^{WT/C96Y}/p47^{phox-/-}$). All mice were on a C57BL/6 background and had free access to water and standard 18% (wt/wt) protein rodent chow. Blood glucose levels and body weights were measured weekly from 4 to 16 weeks of age. Albumin excretion rates were determined from 24 h urine samples in 8- and 16-week-old mice using kits (Albuwell M ELISA; Exocell, Philadelphia, PA, USA). Systolic blood pressure was measured as previously described [25, 26]. At 8 and 16 weeks, mice were killed and kidneys removed and fixed in 10% (vol./vol.) formalin or snap-frozen. Pancreases were placed in 10 ml acid–ethanol (1.5% [vol./vol.] hydrochloric acid in 70% [vol./vol.] ethanol) for 18 h at −20°C. The tissue was homogenised, incubated overnight at −20°C and centrifuged for 15 min at 200 *g* and 4°C. The aqueous layer was transferred into a 15 ml tube. Acid–ethanol extract (100 μl) was neutralised with 100 μl of 1 mol/l TRIS at pH 7.5. Insulin content was measured with an RIA kit (Linco Research, St Charles, MO, USA). Total protein was measured by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). NOX was measured as previously described [25]. All procedures were conducted in accordance with the guidelines of the University of Toronto Animal Care Committee.

Primary mouse mesangial cell culture Mesangial cells were isolated as previously described [25]. Cells were maintained in serum-free medium for 18 h then treated with 5.6 mmol/l or 30 mmol/l D-glucose for 16 h.

Glomeruli isolation Mice were anaesthetised at 8 weeks of age by isoflurane and perfused through the left ventricle with 10 ml PBS containing 200 μl Dynabeads M-450 (Invitrogen Dynal, Oslo, Norway). Both kidneys were removed, minced with a razor blade and incubated at 37°C for 30 min with 10 ml HBSS containing 0.01 g collagenase A (Roche Diagnostics, Indianapolis, IN, USA) and 7.5 μl deoxyribonuclease I (Invitrogen, Carlsbad, CA, USA). After incubation, samples were passed through a 100 μm cell strainer (BD Falcon, Bedford, MA, USA), washed with 10 ml ice-cold HBSS and centrifuged for 5 min at 200 *g* and 4°C. Glomeruli were collected with a magnetic particle concentrator and stored at −80°C until use.

Renal histology Frozen kidney tissue sections (10 μm thick) were incubated for 1 h with dihydroethidium (2 μmol/l; Invitrogen Canada, Burlington, ON, Canada) at 37°C and scanned with a confocal laser-scanning microscope (LSM510; Carl Zeiss Canada, Toronto, ON, Canada). The image colour intensity of dihydroethidium-stained kidney sections was scored blindly on a scale of 0 to 4 (0 for dark, 4 for the strongest intensity). Formalin-fixed paraffin-embedded kidneys were sectioned and stained with periodic acid–Schiff's reagent (PAS), Picrosirius red and Masson's trichrome reagents as previously described [25, 26]. Mouse glomeruli (approximately 60 to 100) were scored blindly by a nephropathologist for severity of diabetic glomerulosclerosis in PAS-stained

sections. Each glomerulus was given a score of 0 (normal), 1 (mild, mesangial matrix [MM] increase approximately two times the width of a mesangial cell nucleus), 2 (moderate, MM increase approximately three to four times the width of a mesangial cell nucleus) or 3 (severe, MM increase more than four 4 times the width of a mesangial cell nucleus). The mean glomerular MM score was then calculated for each animal. Glomerular volume was calculated from scanned PAS slides using ImageScope software (Aperio, Vista, CA, USA). Formalin-fixed paraffin-embedded sections were used for immunohistochemical analysis. Anti-collagen I primary antibody was from Cedarlane (Cedarlane, Burlington, ON, Canada). All slides were scanned digitally at the Advanced Optical Microscope Facility (Princess Margaret Hospital, Toronto, ON, Canada) and ImageScope software was used to quantify collagen I immunostaining.

Wilms tumour 1 staining and podocyte quantification Deparaffinised mouse kidney slides were incubated with Wilms tumour 1 (WT-1) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then with anti-rabbit IgG antibody (Vectastain ABC Kit; Vector Laboratories, Burlington, ON, Canada). All slides were scanned digitally at the Advanced Optical Microscope Facility (Princess Margaret Hospital) and ImageScope software was used to count WT-1-positive nuclei in the glomerular profiles.

Quantitative real-time PCR RNA was extracted from isolated glomeruli and cultured primary mouse mesangial cells using a kit (RNeasy Mini; Qiagen Canada, Mississauga, ON, Canada). mRNA expression levels for *Nox2* (also known as *Cybb*) *Nox4*, $p47^{phox}$, $p22^{phox}$ (also known as *Cyba*), $p67^{phox}$ (also known as *Ncf2*), $p40^{phox}$ (also known as *Ncf4*), collagen type I α1, collagen type I α2, fibronectin, *Pai1* (also known as *Serpine1*), *Tgfb1* and nephrin were quantified by real-time PCR (TaqMan) using a sequence detection system (ABI Prism 7900; Applied Biosystems, Foster City, CA, USA) as previously described [25]. Specific mouse primer sets were purchased from Applied Biosystems (Foster City, CA, USA).

Western blot Western blot analysis of protein lysates from isolated glomeruli or mesangial cells was performed as previously described [25] using primary antibodies for $p47^{phox}$, NOX2 and β-actin (Santa Cruz). β-Actin was used as loading control and densitometry was measured using Scion Image software (Scion, Frederick, MD, USA).

Mouse islet isolation Under isoflurane anaesthesia, the common bile duct was clamped at the point where it enters the duodenum. A collagenase A solution (2 ml, 2 mg/ml; Roche Diagnostics) was injected into the common bile duct. The pancreas was removed and placed in a 50 ml tube, then incubated at 37°C in a water bath for 17 min. Ice-cold HBSS/HEPES solution (20 ml) was added to stop collagenase digestion, prior to centrifugation for 1 min at 450 *g*. The pellet was washed with ice-cold HBSS/HEPES, filtered through gauze and centrifuged for 1 min at 450 *g*. The pellet was then re-suspended in ice-cold HBSS/HEPES. Islets were isolated under a dissecting microscope, transferred into a 35 mm culture dish with medium (RPMI-1640, 10% [wt/vol.] FBS, 1 mmol/l sodium pyruvate, 11 mmol/l glucose, 2 mmol/l L-glutamate and 50 U/ml penicillin/streptomycin), and incubated overnight at 37°C to allow recovery.

Intraperitoneal glucose tolerance test and insulin sensitivity test Mice ($p47^{phox}$ wild-type and $p47^{phox}$-null) were fasted from 07:00 hours for 6 h. An intraperitoneal glucose tolerance test (IPGTT) was performed in accordance with the American Diabetic Complications Consortium protocol [27]. D-Glucose (100 mg/ml; BDH Chemicals, Toronto, ON, Canada) was injected intraperitoneally (10 μl/g body weight). For the insulin sensitivity test, insulin (Humulin R; Eli Lilly, Indianapolis, IN, USA) was injected intraperitoneally (0.75 U/kg body weight). Blood glucose values were measured at 0, 5, 15, 30, 60 and 120 min using tail venous blood and a device (Contour Meter; Bayer, Toronto, ON, Canada).

Statistical analysis Results are expressed as mean±SEM unless otherwise specified. Comparisons between multiple groups were performed by one-way ANOVA followed by Bonferroni's post hoc test. The two-tailed Student's *t* test was used for comparison between two groups. The 24 h urinary albumin excretion rates are presented as median with interquartile range. Comparisons between multiple groups were performed by the non-parametric Kruskal–Wallis test followed by Dunn's multiple comparison test. GraphPad Prism software was used for statistical tests (GraphPad, La Jolla, CA, USA).

Results

Kidney studies in 16-week-old mice Four groups of mice were studied: non-diabetic $p47^{phox}$ wild-type ($Ins2^{WT/WT}$/$p47^{phox+/+}$), non-diabetic $p47^{phox}$-null ($Ins2^{WT/WT}$/$p47^{phox-/-}$), diabetic $p47^{phox}$ wild-type ($Ins2^{WT/C96Y}$/$p47^{phox+/+}$) and diabetic $p47^{phox}$-null ($Ins2^{WT/C96Y}$/$p47^{phox-/-}$) mice. The onset of hyperglycaemia occurred between 4 and 6 weeks of age in both diabetic groups (Fig. 1a). Mean values for blood glucose diverged after 10 weeks of age and at 16 weeks were approximately 10 mmol/l greater in $Ins2^{WT/C96Y}$/$p47^{phox+/+}$ than in $Ins2^{WT/C96Y}$/$p47^{phox-/-}$ mice (electronic supplementary material [ESM] Table 1). All groups

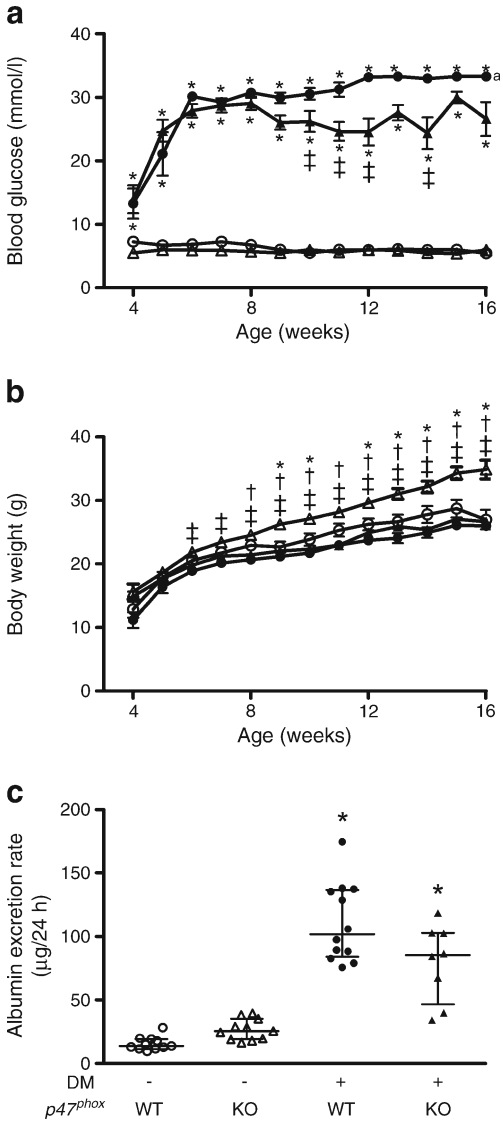

Fig. 1 (**a**) Blood glucose and (**b**) body weight in four groups of mice followed to 16 weeks of age. (**c**) 24 h urinary albumin excretion in 16-week-old mice. [a]Some values exceeded the measurable range of the glucometer (33.3 mmol/l) and were therefore recorded as 33.3 mmol/l on the graph. *$p<0.05$ vs non-diabetic groups; †$p<0.05$ vs the diabetic $p47^{phox}$-null group; ‡$p<0.05$ vs the diabetic $p47^{phox}$ wild-type group. DM, diabetic; WT, $p47^{phox+/+}$; KO, $p47^{phox-/-}$; white circles, non-diabetic $p47^{phox}$ wild-type mice; white triangles, non-diabetic $p47^{phox}$-null mice; black circles, diabetic $p47^{phox}$ wild-type mice; black triangles, diabetic $p47^{phox}$-null mice

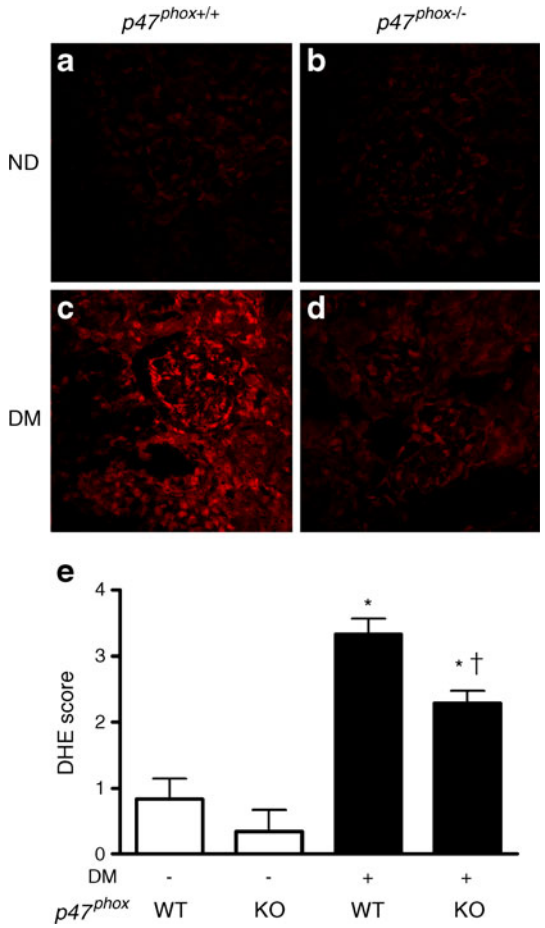

Fig. 2 Sections of kidneys from 16-week-old, non-diabetic $p47^{phox+/+}$ (**a**) and $p47^{phox-/-}$ (**b**), and diabetic $p47^{phox+/+}$ (**c**) and $p47^{phox-/-}$ (**d**) mice were stained with dihydroethidium to detect superoxide levels. Representative images from each group are shown (magnification: ×630). (**e**) The intensity of emission from dihydroethidium (DHE)-stained sections was scored for each of the four groups of mice. *$p<0.05$ vs the non-diabetic groups and †$p<0.05$ vs the diabetic $p47^{phox}$ wild-type group. DM, diabetic; WT, $p47^{phox+/+}$; KO, $p47^{phox-/-}$

of mice gained weight over the 16 weeks, although values for $Ins2^{WT/WT}/p47^{phox-/-}$ mice tended to be higher (Fig. 2a, ESM Table 1). Both groups of diabetic mice had an increased urinary albumin excretion rate. Deletion of $p47^{phox}$ in the $Ins2^{WT/C96Y}/p47^{phox-/-}$ diabetic mice did not reduce albuminuria (Fig. 1c, ESM Table 1).

At 16 weeks of age, both diabetic groups exhibited greater kidney:body weight ratios (KW:BW) than their non-diabetic littermates (ESM Table 1). Kidney hypertrophy and KW:BW were reduced by deletion of $p47^{phox}$ in diabetic mice ($Ins2^{WT/C96Y}/p47^{phox-/-}$). Deletion of $p47^{phox}$ also attenuated diabetic glomerular hypertrophy (ESM Table 1).

Oxidative stress in glomeruli was assessed in the four groups of mice using dihydroethidium staining to detect superoxide (Fig. 2a–d). Dihydroethidium staining was increased threefold in the glomeruli of $Ins2^{WT/C96Y}/p47^{phox+/+}$ compared with $Ins2^{WT/WT}/p47^{phox+/+}$ mice, this increase being attenuated by deletion of $p47^{phox}$ (Fig. 2e). Increased oxidative stress was associated with a significant increase in the MM score in the glomeruli of $Ins2^{WT/C96Y}/p47^{phox-/-}$ compared with $Ins2^{WT/C96Y}/p47^{phox+/+}$ mice (Fig. 3, ESM Table 1). In parallel with the MM score, glomerular collagen I immunostaining was increased in $Ins2^{WT/C96Y}/p47^{phox+/+}$ mice compared with $Ins2^{WT/WT}/p47^{phox+/+}$ mice and reduced by deletion of $p47^{phox}$ (ESM Fig. 1e). There were no differences in the number of glomerular WT-1-positive

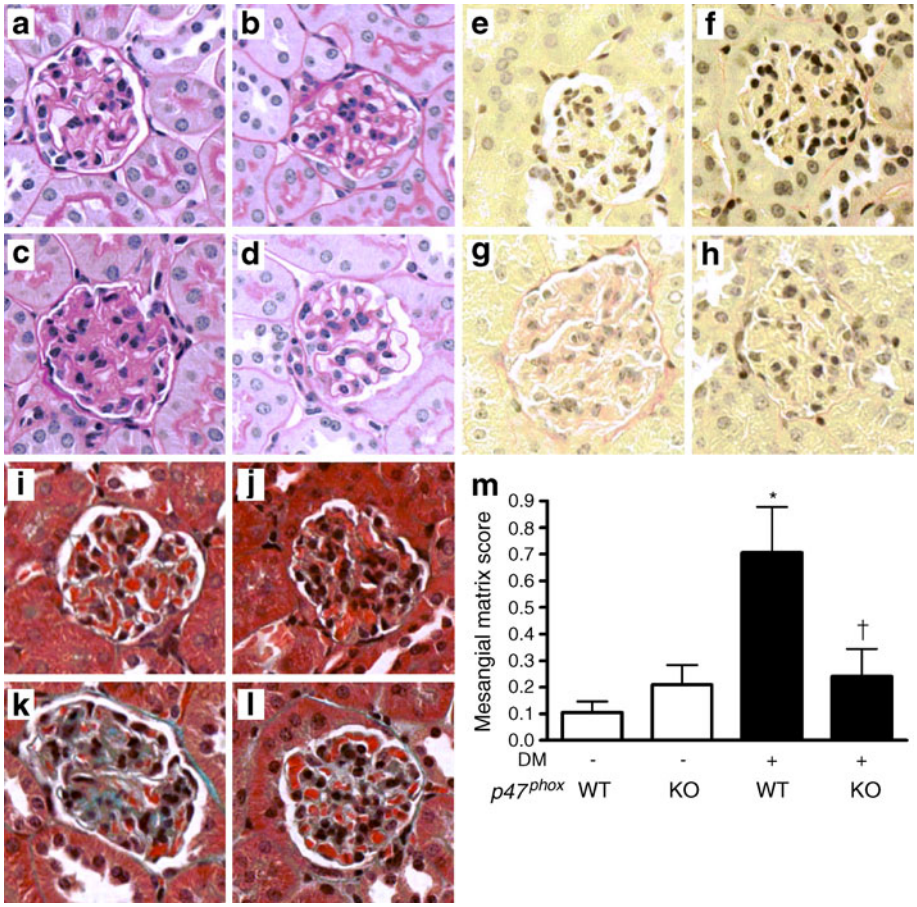

Fig. 3 Sections of 16-week-old mouse kidneys were stained with PAS (**a**–**d**), Picrosirius red (**e**–**h**) and Masson's trichrome (**i**–**l**) reagents. Representative images show glomeruli from each group, i.e. (**a**, **e**, **i**) non-diabetic $p47^{phox}$ wild-type, (**b**, **f**, **j**) non-diabetic $p47^{phox}$-null, (**c**, **g**, **k**) diabetic $p47^{phox}$ wild-type and (**d**, **h**, **l**) diabetic $p47^{phox}$-null mice (magnification: ×600). (**m**) The MM score was derived from four groups of 16-week-old mice. *$p<0.05$ vs the non-diabetic groups and †$p<0.05$ vs the diabetic $p47^{phox}$ wild-type group. DM, diabetic; WT, $p47^{phox+/+}$; KO, $p47^{phox-/-}$

cells in the four groups of mice (ESM Fig. 1j) and no significant differences in the glomerular basement membrane thickness across the four groups (data not shown). Renal cortical nephrin mRNA expression was similar in all four groups (ESM Fig. 2).

The effect of deletion of $p47^{phox}$ on plasma insulin concentration and pancreatic insulin content in 16-week-old diabetic mice Blood glucose levels were significantly lower in diabetic mice with a deletion of $p47^{phox}$ than in $Ins2^{WT/C96Y}/p47^{phox+/+}$ diabetic mice (Fig. 4a, ESM Table 1), a difference that emerged at 10 weeks of age (Fig. 1a). Plasma insulin levels and pancreatic insulin content were significantly greater in the diabetic mice with a deletion of $p47^{phox}$, suggesting that beta cell function was better preserved in these mice at 16 weeks of age (Fig. 4b, c).

The effect of deletion of $p47^{phox}$ on beta cell function and insulin sensitivity in non-diabetic mice To test the hypothesis that deletion of $p47^{phox}$ might improve beta cell function, we studied 8-week-old non-diabetic $Ins2^{WT/WT}/p47^{phox+/+}$ and $Ins2^{WT/WT}/p47^{phox-/-}$ mice. Mice with deletion of $p47^{phox}$ exhibited significantly better glucose tolerance than their wild-type littermates (Fig. 4e), albeit with slightly lower insulin sensitivity than in wild-type mice (Fig. 4f). We then isolated pancreatic islets from the four groups of mice and measured NOX activity. The $Ins2^{WT/C96Y}$ mutation increased NOX activity three- to fourfold in isolated islets of $Ins2^{WT/C96Y}/p47^{phox+/+}$ mice. Deletion of $p47^{phox}$ markedly attenuated this response (Fig. 4g).

Kidney studies in 8-week-old mice To determine the effect of deletion of $p47^{phox}$ on the kidney response to hyperglycaemia, we studied another four groups of mice at 8 weeks of age when no differences in blood glucose levels were evident (ESM Fig. 3a, ESM Table 2). Urinary albumin excretion rates were increased to a similar extent in both diabetic groups; mean values for systolic blood pressure were also similar in the four groups (ESM Table 2). There were no differences in body weight between the groups, although both diabetic groups exhibited an increase in kidney weight and KW:BW (ESM Table 2). Mean values for glomerular volume tended to be greater in the diabetic groups and there were early (but not significant) increases in MM scores in the diabetic compared with the non-diabetic groups (ESM Table 2). NOX activity was increased twofold in isolated glomeruli from $Ins2^{WT/C96Y}/p47^{phox+/+}$ mice and attenuated by deletion of $p47^{phox}$ (Fig. 5a). Dihydroethidium staining was also increased in $Ins2^{WT/C96Y}/p47^{phox+/+}$ compared with $Ins2^{WT/C96Y}/p47^{phox-/-}$ mice (Fig. 5b–e).

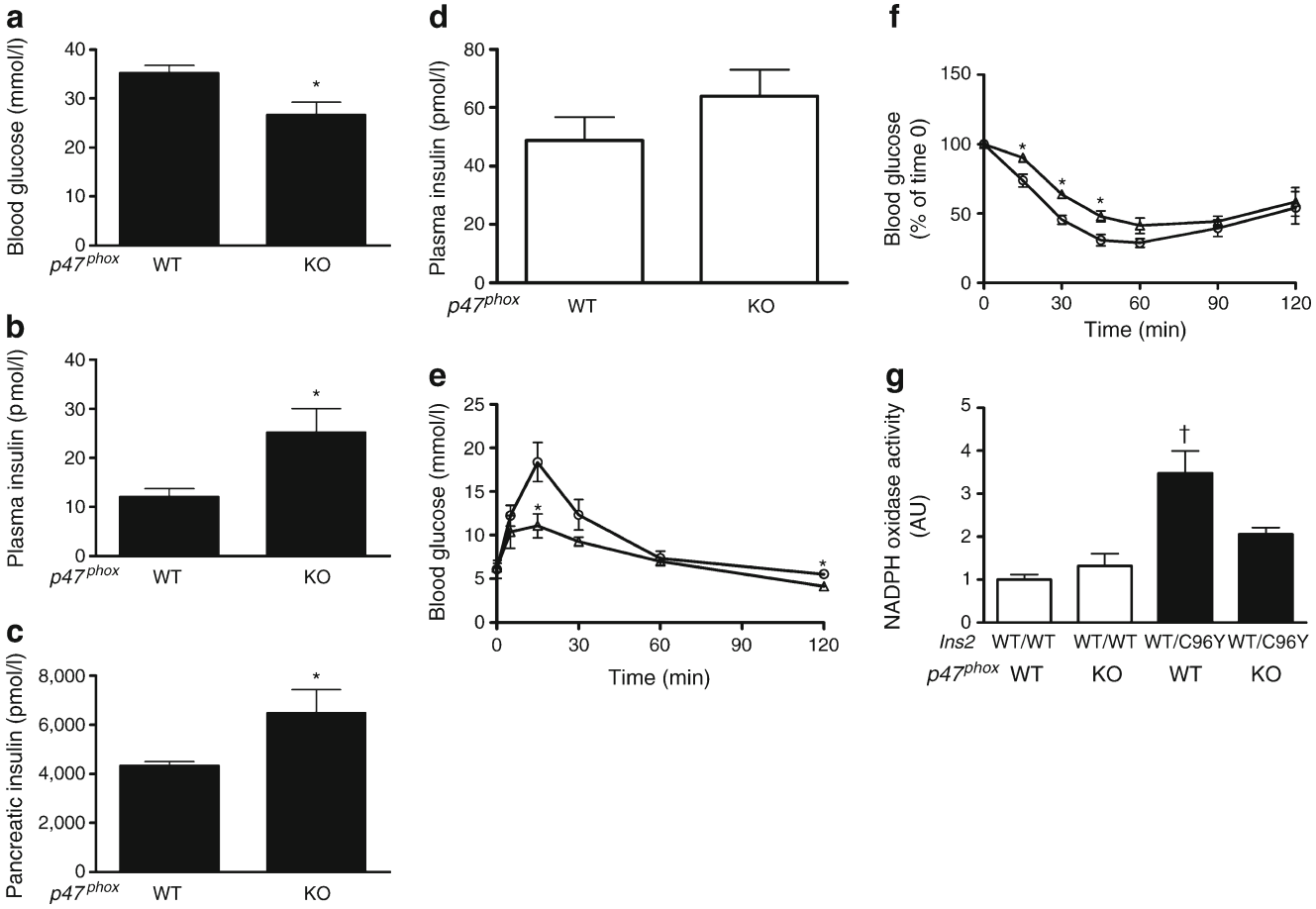

Fig. 4 (**a**) Blood glucose, (**b**) plasma insulin and (**c**) pancreatic insulin content in two groups of diabetic mice at 16 weeks of age. (**d**) Plasma insulin, (**e**) intraperitoneal glucose tolerance test and (**f**) insulin sensitivity test in 8-week-old non-diabetic $p47^{phox}$ wild-type (white bars/circles) and $p47^{phox}$-null (white bars/triangles) mice. (**g**) Islet NOX activity. Islets were isolated from all four groups. AU, arbitrary units. *$p<0.05$ vs the $p47^{phox}$ wild-type group and †$p<0.05$ vs all other groups. WT, $p47^{phox+/+}$; KO, $p47^{phox-/-}$

NOX subunit expression in isolated glomeruli NOX is a multiunit complex, so we looked at mRNA expression of the NOX subunits in isolated glomeruli from the four groups of mice (ESM Table 3). Hyperglycaemia-induced activation of NOX was associated with significant increases in expression of $p47^{phox}$, *Nox2*, $p22^{phox}$, $p40^{phox}$ and $p67^{phox}$ in the glomeruli of $Ins2^{WT/C96Y}/p47^{phox+/+}$ compared with those of $Ins2^{WT/WT}/p47^{phox+/+}$ mice. Mean values for *Nox4* mRNA expression tended to increase, but the difference did not reach statistical significance. Interestingly, deletion of $p47^{phox}$ attenuated the increase in expression of *Nox2*, $p22^{phox}$, $p40^{phox}$ and $p67^{phox}$ that was induced by hyperglycaemia, but did not affect *Nox4* mRNA expression. We then related changes in mRNA expression to protein levels of $p47^{phox}$ and NOX2 in isolated glomeruli from the four groups of mice. Western blot analysis showed that $p47^{phox}$ and NOX2 abundance paralleled the changes in mRNA expression (Fig. 6a–c).

Pro-fibrotic gene expression in isolated glomeruli To relate these early effects of deletion of $p47^{phox}$ on NOX activation to pro-fibrotic gene expression, we measured mRNA expression of collagen Iα1, collagen Iα2, fibronectin, *Tgfb1* and *Pai1* in isolated glomeruli. Mean values for these pro-fibrotic genes were increased in the glomeruli of $Ins2^{WT/C96Y}/p47^{phox+/+}$ compared with those of $Ins2^{WT/WT}/p47^{phox+/+}$ and $Ins2^{WT/C96Y}/p47^{phox-/-}$ mice (ESM Table 3).

Studies of NOX activation in primary mouse mesangial cells Our in vivo data showed that diabetic nephropathy was attenuated by deletion of $p47^{phox}$. To relate this finding to a cellular response to high glucose, we studied primary mesangial cells derived from $Ins2^{WT/WT}/p47^{phox+/+}$ and $Ins2^{WT/WT}/p47^{phox-/-}$ mice. Primary mesangial cells were exposed to 5.6 mmol/l or 30 mmol/l D-glucose. At 30 mmol/l, D-glucose increased NOX activity two- to threefold in wild-type mesangial cells from $Ins2^{WT/WT}/p47^{phox+/+}$ mice (Fig. 7a). This effect was not due to an osmotic stimulus (Fig. 7b) and the high glucose-induced increase in NOX was attenuated in mesangial cells from $Ins2^{WT/WT}/p47^{phox-/-}$ mice.

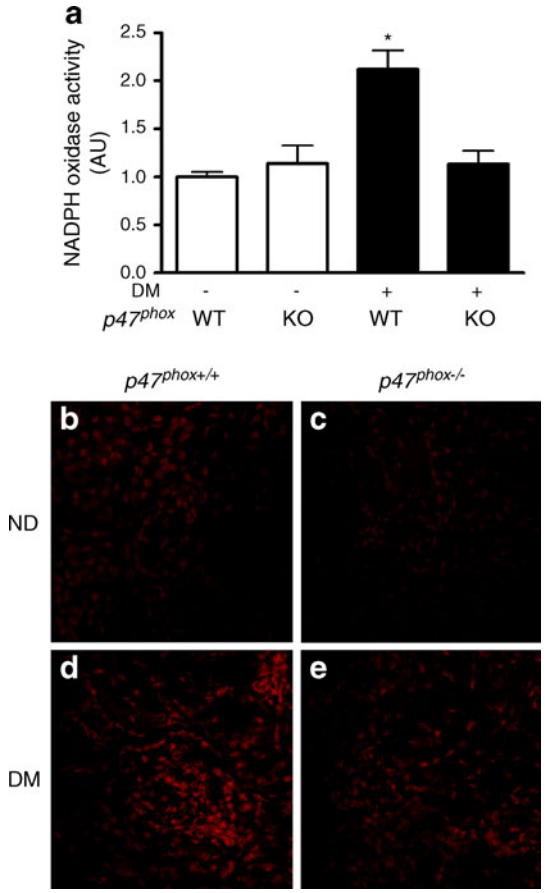

Fig. 5 NOX activity and superoxide concentration of isolated glomeruli from 8-week-old mice. (**a**) NOX activity of isolated glomeruli measured by a lucigenin chemiluminescence method. *$p<0.05$ vs all other groups. (**b**) Sections of kidneys from 8-week-old, non-diabetic $p47^{phox+/+}$ and (**c**) $p47^{phox-/-}$, and (**d**) diabetic $p47^{phox+/+}$ and (**e**) $p47^{phox-/-}$ mice were stained with dihydroethidium to detect superoxide levels. Representative images from each group are shown (magnification: ×630). DM, diabetic; WT, $p47^{phox+/+}$; KO, $p47^{phox-/-}$

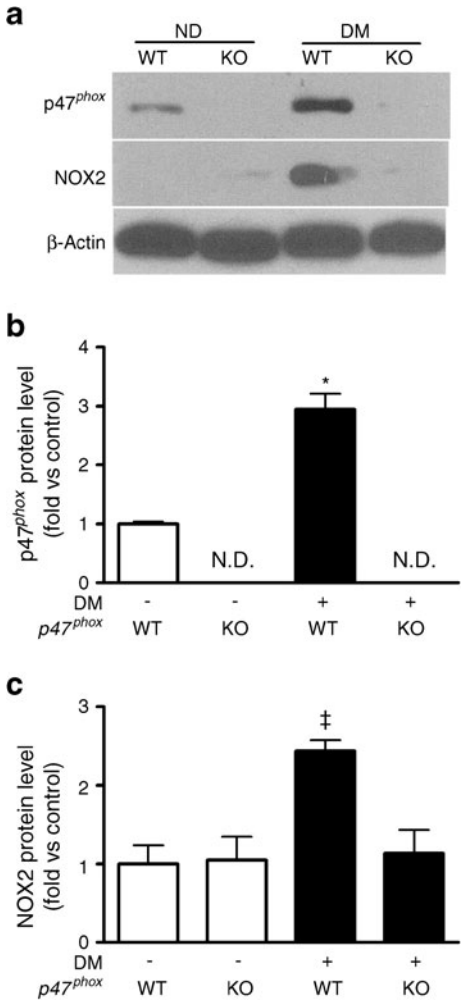

Fig. 6 Glomeruli were isolated from 8-week-old mice and protein levels of $p47^{phox}$ and NOX2 determined by western blot. (**a**) Representative western blot of $p47^{phox}$, NOX2 and β-actin. (**b**) Quantitative densitometry analysis of western blot for $p47^{phox}$ and (**c**) NOX2. N. D., non-detectable. *$p<0.05$ vs the non-diabetic $p47^{phox}$ wild-type group; †$p<0.05$ vs all other groups. ND, non-diabetic; DM, diabetic; WT, $p47^{phox+/+}$; KO, $p47^{phox-/-}$

NOX subunit expression in primary mesangial cells High glucose-induced activation of NOX was associated with an increase in mRNA expression of the NOX subunits $p47^{phox}$, *Nox2*, *Nox4*, $p22^{phox}$, $p67^{phox}$ and $p40^{phox}$ in primary mesangial cells, consistent with our in vivo data. Deletion of $p47^{phox}$ attenuated, but did not normalise the increases in *Nox2*, $p22^{phox}$, $p67^{phox}$ and $p40^{phox}$, while there was no effect on *Nox4* expression (ESM Table 4). Western blot analysis showed that $p47^{phox}$ and NOX2 protein levels paralleled the changes in mRNA expression (Fig. 8a–c).

Pro-fibrotic gene expression in mouse mesangial cells To relate the effect of deletion of $p47^{phox}$ on high glucose-induced pro-fibrotic gene expression, we measured mRNA expression of collagen Iα1, collagen Iα2, fibronectin, *Tgfb1* and *Pai1* in primary mesangial cells. Mean values for these pro-fibrotic genes were increased by 30 mmol/l glucose in mesangial cells from $Ins2^{WT/WT}/p47^{phox+/+}$ and $Ins2^{WT/WT}/p47^{phox-/-}$ mice, but the magnitude of the increase was significantly reduced in cells from $Ins2^{WT/WT}/p47^{phox-/-}$ mice (ESM Table 4).

Discussion

Oxidative stress is postulated to play a central role in the pathogenesis of diabetic nephropathy [1–6]. In this report, we focussed on the role of NOX, and specifically the cytosolic subunit $p47^{phox}$ in the generation of superoxide in a high glucose environment. The rationale for this approach is based on in vitro studies of rat mesangial cells exposed to high glucose [16, 20] and on in vivo studies of a mouse model of diabetes by Ohshiro and co-workers [15].

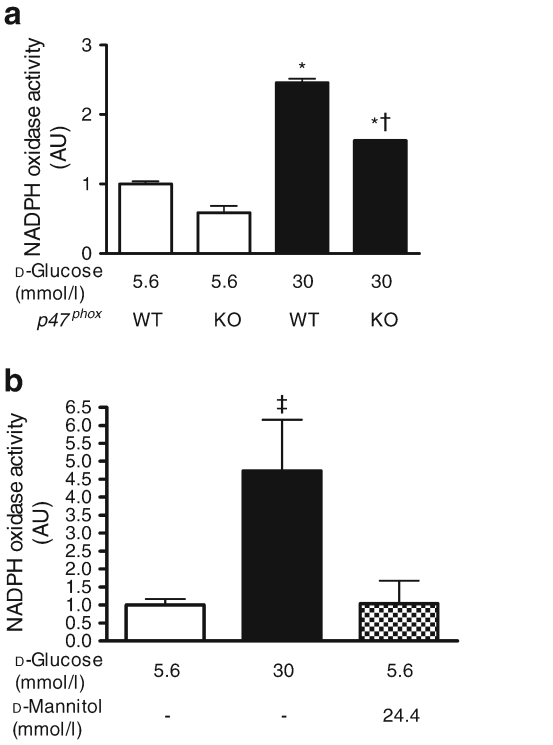

Fig. 7 (**a**) NOX activity of $p47^{phox}$ wild-type (WT) and $p47^{phox}$-null (KO) mouse mesangial cells treated with D-glucose as indicated. (**b**) NOX activity of $p47^{phox}$ wild-type mouse mesangial cells treated with D-glucose or D-glucose with D-mannitol as indicated. Results are expressed as arbitrary units (AU); *$p<0.05$ vs non-diabetic groups; †$p<0.05$ vs the diabetic $p47^{phox}$ wild-type group; ‡$p<0.05$ vs all other groups

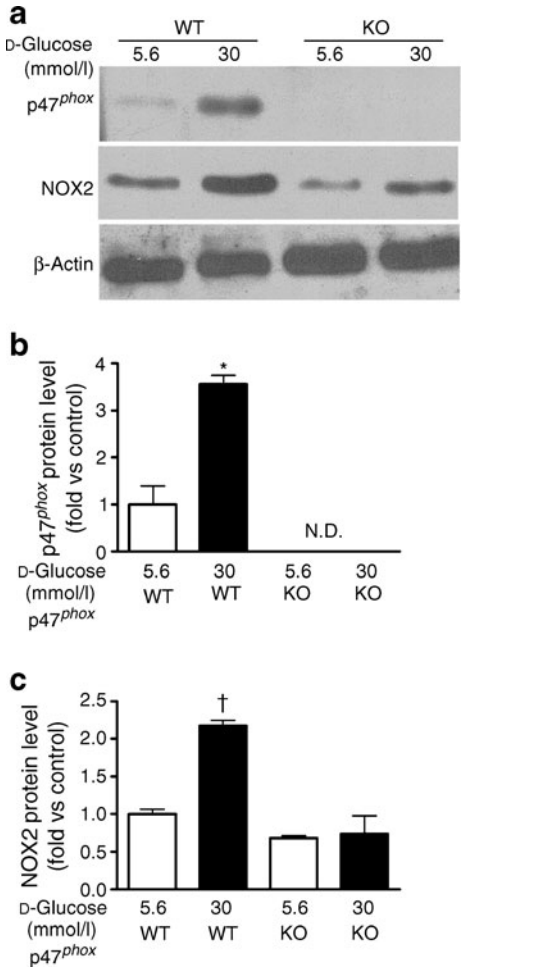

Fig. 8 $p47^{phox}$ wild-type (WT) and $p47^{phox}$-null (KO) mouse mesangial cells were treated with D-glucose as indicated and protein levels of $p47^{phox}$ and NOX2 determined by western blot. (**a**) Representative western blot of $p47^{phox}$, NOX2 and β-actin. (**b**) Quantitative densitometry analysis of western blot for $p47^{phox}$ and (**c**) NOX2. N. D., non-detectable. *$p<0.05$ vs 5.6 mmol/l D-glucose-treated $p47^{phox}$ wild-type group; †$p<0.05$ vs all other groups

Our first major observation was that deletion of the $p47^{phox}$ gene attenuated diabetic nephropathy in the Akita mouse. Hyperglycaemia was associated with increased urinary albumin excretion rates, kidney and glomerular hypertrophy, and MM expansion [25, 26, 28, 29]. These diabetes-induced changes were associated with increased renal oxidative stress. Deletion of $p47^{phox}$ lessened oxidative stress, kidney and glomerular hypertrophy, and MM expansion. The protective effect on diabetic kidney injury of the deletion of $p47^{phox}$ observed by us was at least partially dependent on improved glycaemic control.

Our second major observation was that deletion of $p47^{phox}$ also lessened the severity of diabetes, even though the onset and early phase of hyperglycaemia were similar in both groups of diabetic mice. This effect of $p47^{phox}$ deletion emerged and was significant after 10 weeks of age, with the difference persisting through to 16 weeks of age. The Akita mouse ($Ins2^{WT/C96Y}$) harbours a mutation of *Ins2* (Cys96Tyr), which disrupts a disulfide bond between A and B chains of the insulin molecule [30]. This mutation leads to C/EBP homologous protein (CHOP)-dependent ER stress in the beta cell, with the subsequent apoptosis leading to insulin deficiency and hyperglycaemia [20–32]. $p47^{phox}$ and NOX-generated ROS may play a role in ER stress-induced beta cell apoptosis [32, 33]. Accumulation of ROS has also been shown to be an initiation factor and a consequence of ER stress; it is also an important cellular response, linking protein misfolding in the ER to beta cell apoptosis [34]. Our data showing attenuation of the severity of diabetes in the Akita mouse suggest that the deletion of $p47^{phox}$ may have partially protected the beta cell from ER stress-induced injury, thus sustaining beta cell function over time.

To further explore the effect of deletion of $p47^{phox}$ on beta cell function, we measured plasma insulin concentrations and pancreatic insulin content in 16-week-old diabetic mice. The difference in blood glucose levels was associated with significant increases in pancreatic insulin content and circulating insulin levels in Akita diabetic mice with a deletion of $p47^{phox}$. In non-diabetic mice, the deletion of

p47^{phox} was also associated with improved glucose tolerance compared with wild-type littermates, a finding that was independent of an effect on insulin sensitivity, suggesting that beta cell function was enhanced in non-diabetic mice by deletion of *p47*^{phox}. Finally we examined NOX activity in isolated islets from the four groups of mice at 8 weeks of age when blood glucose levels were similar in the two diabetic groups. Hyperglycaemia was associated with increased NOX activity in isolated islets from *Ins2*$^{WT/C96Y}$/*p47*$^{phox+/+}$ mice, while this response was attenuated in *Ins2*$^{WT/C96Y}$/*p47*$^{phox-/-}$ mice, supporting our hypothesis that deletion of *p47*^{phox} protects pancreatic beta cells from injury by attenuating oxidative stress.

The 10 mmol/l difference in blood glucose levels between the two diabetic mouse groups could have contributed, at least in part, to the protective effect of *p47*^{phox} deletion on glomerular injury in *Ins2*$^{WT/C96Y}$/*p47*$^{phox-/-}$ mice, so our next series of studies was designed to more directly test the hypothesis that *p47*^{phox} deletion attenuated the glomerular response to hyperglycaemia. We studied mice at 8 weeks of age when the blood glucose levels were similar in the two groups of diabetic mice. Our third major observation was that the deletion of *p47*^{phox} attenuated NOX activity in isolated glomeruli of diabetic mice independently of blood glucose levels.

NOX is a protein complex consisting of two membrane subunits: NOX and p22^{phox}. There are several NOX isoforms, including NOX1, NOX2 and NOX4. The activation of NOX1 or NOX2 is dependent on recruitment of four cytosolic proteins: p40^{phox}, p47^{phox}, p67^{phox} and rac GTPase to the cell membrane [9]. The phosphorylation of p47^{phox} is a critical event in this recruitment [9]. In contrast to NOX1 and NOX2, NOX4 is constitutively active and does not require the cytosolic subunits for activation [35–37]. All of these subunits are produced in the kidney [13, 14], and our findings suggest that p47^{phox}-dependent NOXs are important sources of superoxide in the diabetic glomerulus. We also measured the mRNA expression of NOX subunits in isolated glomeruli from the four groups of mice. It has been reported that diabetes is associated with increased abundance of NOX2 [17, 21, 38, 39] and NOX4 [21–24, 40] in kidney cortex. We found that increased NOX activity was associated with significant increases in the mRNA expression of the cytosolic subunits *p47*^{phox}, *p40*^{phox} and *p67*^{phox}, and of the membrane subunits *p22*^{phox} and *NOX2* in the glomeruli of diabetic mice. NOX2 and p47^{phox} protein levels in isolated glomeruli paralleled the changes in mRNA levels. These effects were attenuated by deletion of *p47*^{phox}, suggesting that NOX-mediated oxidative stress may exert a positive feedback loop on subunit levels in the diabetic glomerulus. Although we did not address the mechanism responsible for this effect, studies by Bondi et al [41] provide a possible explanation that ROS-induced TGF-β1 may function in an autocrine manor to increase NOX subunit levels.

Our fourth major finding was that the early increases in NOX subunit abundance and activity were associated with increased levels of the extracellular matrix proteins collagen I and fibronectin, and of the pro-fibrotic factors TGF-β1 and plasminogen activator inhibitor 1 (PAI-1) [42–46] in isolated glomeruli. These early changes preceded significant differences in the MM scores. These findings are consistent with in vivo studies of the effect of apocynin on the severity of diabetic kidney injury, although apocynin functions as a general antioxidant rather than a specific antagonist of p47^{phox} in non-phagocytic cells [17–20]. Previous studies of protein kinase C-beta and diabetic nephropathy have also suggested a role for p47^{phox} in the development of kidney injury [15].

One limitation of our study is that we did not measure blood pressure at 16 weeks of age. Although blood pressure values were similar at 8 weeks of age, it remains possible that potential later differences could have contributed to the attenuation of diabetic nephropathy. Fujita and co-workers showed that an antioxidant, apocynin, reduced albuminuria and MM expansion in the Akita mouse at 14 weeks of age, but did not lower blood pressure. They also found that a blood pressure reduction of 13 mmHg with a calcium channel blocker did not attenuate albuminuria or MM expansion in the Akita mouse, unless the agents blocked the renin–angiotensin system [47].

Glomerular injury in the Akita mouse has been related to effects on podocytes and mesangial cells [18, 25, 26, 28, 48]. Although no difference in the number of podocytes was detected in our four groups of mice, we sought to link our in vivo observations to a direct cellular effect of *p47*^{phox} deletion in mesangial cells. High glucose-induced activation of NOX was attenuated, but not normalised in primary mesangial cells derived from *p47*^{phox}-null mice. We observed similar changes in abundance of the NOX subunits in the in vitro and in vivo studies. Changes in extracellular matrix protein abundance and pro-fibrotic factors like TGF-β1 and PAI-1 were attenuated, but not normalised in mesangial cells from *p47*^{phox}-null mice. These findings confirm previous work in rat mesangial cells [16, 20], and together support the hypothesis that deletion of *p47*^{phox} attenuates the mesangial cell response to high glucose.

Although our studies focused on p47^{phox}-dependent NOX activation, other pathways may also contribute to oxidative stress in diabetic glomeruli in vivo. More recently, Chacko and co-workers reported that treatment with a mitochondria-targeted ubiquinone reduced glomerular injury in diabetic Akita mice, suggesting a role for mitochondrial-derived superoxide [49]. In addition, Gorin et al delivered NOX4 antisense in vivo using osmotic mini-pumps, and showed that less oxidative stress and reduced glomerular

injury occurred in a streptozotocin-induced rat model of diabetes [21]. Experimental studies of diabetic mice with deletion of *Nox4* have not been reported; however, recent studies of the heart suggest that NOX4 may play a protective role [50].

In summary, our studies support the hypothesis that $p47^{phox}$-dependent activation of NOX is an important determinant of glomerular injury and beta cell dysfunction in the Akita mouse model of type 1 diabetes mellitus. The renoprotective effect of $p47^{phox}$ deletion on the kidney is dependent in part on improved glycaemic control.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement GCL, FF, HNR, RJ, AMH, AG, GYO and JWS were responsible for the conception and design of this research. GCL, FF, JZ, KK, SY, LL and AMH performed the experiments and analysed data. GCL, GYO and JWS interpreted the results of the experiments. GCL prepared the figures. GCL, AMH and JWS drafted the manuscript. GCL, FF, JZ, KK, SY, LL, HNR, RJ, AG, GYO and JWS edited and revised the manuscript. GCL, FF, JZ, KK, SY, LL, HNR, RJ, AG, GYO and JWS approved the final version of manuscript. AMH sadly passed away during these studies and was unable to edit and revise the manuscript, and approve the final version.

References

1. Suzuki D, Miyata T, Saotome N et al (1999) Immunohistochemical evidence for an increased oxidative stress and carbonyl modification of proteins in diabetic glomerular lesions. J Am Soc Nephrol 10:822–832
2. Brownlee M (2001) Biochemistry and molecular cell biology of diabetic complications. Nature 414:813–820
3. Ha H, Kim KH (1999) Pathogenesis of diabetic nephropathy: the role of oxidative stress and protein kinase C. Diabetes Res Clin Pract 45:147–151
4. Horie K, Miyata T, Maeda K et al (1997) Immunohistochemical colocalization of glycoxidation products and lipid peroxidation products in diabetic renal glomerular lesions. Implication for glycoxidative stress in the pathogenesis of diabetic nephropathy. J Clin Invest 100:2995–3004
5. Koya D, Hayashi K, Kitada M, Kashiwagi A, Kikkawa R, Haneda M (2003) Effects of antioxidants in diabetes-induced oxidative stress in the glomeruli of diabetic rats. J Am Soc Nephrol 14: S250–S253
6. Prabhakar S, Starnes J, Shi S, Lonis B, Tran R (2007) Diabetic nephropathy is associated with oxidative stress and decreased renal nitric oxide production. J Am Soc Nephrol 18:2945–2952
7. DeRubertis FR, Craven PA, Melhem MF, Salah EM (2004) Attenuation of renal injury in db/db mice overexpressing superoxide dismutase: evidence for reduced superoxide-nitric oxide interaction. Diabetes 53:762–768
8. DeRubertis FR, Craven PA, Melhem MF (2007) Acceleration of diabetic renal injury in the superoxide dismutase knockout mouse: effects of tempol. Metabolism 56:1256–1264
9. Bedard K, Krause K (2007) The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. Physiol Rev 87:245–313
10. Han HJ, Lee YJ, Park SH, Lee JH, Taub M (2005) High glucose induced oxidative stress inhibits Na^+/glucose cotransporter activity in renal proximal tubule cells. Am J Physiol Renal Physiol 288: F988–F996
11. Lee HB, Yu MR, Yang Y, Jiang Z, Ha H (2003) Reactive oxygen species regulated signaling pathways in diabetic nephropathy. J Am Soc Nephrol 14:S241–S245
12. Li JM, Shah AM (2003) ROS generation by nonphagocytic NADPH oxidase: potential relevance in diabetic nephropathy. J Am Soc Nephrol 14:S221–S226
13. Chabrashvili T, Tojo A, Onozato ML et al (2002) Expression and cellular localization of classic NADPH oxidase subunits in the spontaneously hypertensive rat kidney. Hypertension 39:269–274
14. Cheng G, Cao Z, Xu X, Meir EG, Lambeth JD (2001) Homologs of gp91*phox*: cloning and tissue expression of Nox3, Nox4, Nox5. Gene 269:131–140
15. Ohshiro Y, Ma RC, Yasuda Y et al (2006) Reduction of diabetes-induced oxidative stress, fibrotic cytokine expression, and renal dysfunction in protein kinase C-beta-null mice. Diabetes 55:3112–3120
16. Hua H, Munk S, Goldberg H, Fantus IG, Whiteside CI (2003) High glucose-suppressed endothelin-1 Ca^{2+} signaling via NADPH oxidase and diacylglycerol-sensitive protein kinase C isozymes in mesangial cells. J Biol Chem 278:33951–33962
17. Asaba K, Tojo A, Onozato ML et al (2005) Effects of NADPH oxidase inhibitor in diabetic nephropathy. Kidney Int 67:1890–1898
18. Susztak K, Raff AC, Schiffer M, Bottinger EP (2006) Glucose-induced reactive oxygen species cause apoptosis of podocytes and podocyte depletion at the onset of diabetic nephropathy. Diabetes 55:225–233
19. Heumuller S, Wind S, Barbosa-Sicard E et al (2008) Apocynin is not an inhibitor of vascular NADPH oxidases but an antioxidant. Hypertension 51:211–217
20. Thallas-Bonke V, Thorpe SR, Coughlan MT et al (2008) Inhibition of NADPH oxidase prevents advanced glycation end product-mediated damage in diabetic nephropathy through a protein kinase C-alpha-dependent pathway. Diabetes 57:460–469
21. Gorin Y, Block K, Hernandez J et al (2005) Nox4 NAD(P)H oxidase mediates hypertrophy and fibronectin expression in the diabetic kidney. J Biol Chem 280:39616–39626
22. Sharma K, Ramachandrarao S, Qiu G et al (2008) Adiponectin regulates albuminuria and podocyte function in mice. J Clin Invest 118:1645–1656
23. Eid AA, Ford BM, Block K et al (2010) AMP-activated protein kinase (AMPK) negatively regulates Nox4-dependent activation of p53 and epithelial cell apoptosis in diabetes. J Biol Chem 285:37503–37512
24. Sedeek M, Callera G, Montezano A et al (2010) 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
25. Oudit GY, Liu GC, Zhong J et al (2010) Human recombinant ACE2 reduces the progression of diabetic nephropathy. Diabetes 59:529–538
26. Wong DW, Oudit GY, Reich H et al (2007) Loss of angiotensin-converting enzyme-2 (Ace2) accelerates diabetic kidney injury. Am J Pathol 171:438–451
27. McClain D (2002) Intraperitoneal glucose tolerance testing (IPGTT). Animal models of diabetic complications consortium. www.diacomp.org/shared/showFile.aspx?doctypeid=3&docid=11, accessed 12 Oct 2011
28. Kakoki M, Sullivan KA, Backus C et al (2010) Lack of both bradykinin B1 and B2 receptors enhances nephropathy, neuropathy, and bone mineral loss in Akita diabetic mice. Proc Natl Acad Sci U S A 107:10190–10195
29. Brosius FC, Khoury CC, Buller CL et al (2010) Abnormalities in signaling pathways in diabetic nephropathy. Expert Rev Endocrinol Metabol 5:51–64
30. Wang J, Takeuchi T, Tanaka S et al (1999) A mutation in the insulin 2 gene induces diabetes with severe pancreatic β-cell dysfunction in the Mody mouse. J Clin Invest 103:27–37
31. Oyadomari S, Koizumi A, Takeda K et al (2002) Targeted disruption of the *Chop* gene delays endoplasmic reticulum stress-mediated diabetes. J Clin Invest 109:525–532
32. Song B, Scheuner D, Ron D, Pennathur S, Kaufman RJ (2008) Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes. J Clin Invest 118:3378–3389
33. Morgan D, Oliveira-Emilio HR, Keane D et al (2007) Glucose, palmitate and pro-inflammatory cytokines modulate production and activity of a phagocyte-like NADPH oxidase in rat pancreatic islets and a clonal beta cell line. Diabetologia 50:359–369
34. Malhotra JD, Kaufman RJ (2007) Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? Antioxid Redox Signal 9:2277–2294
35. Geiszt M, Kopp JB, Varnai P, Leto TL (2000) Identification of renox, an NAD(P)H oxidase in kidney. Proc Natl Acad Sci U S A 97:8010–8014
36. Martyn KD, Frederick LM, von Loehneysen K, Dinauer MC, Knaus UG (2005) Functional analysis of Nox4 reveals unique characteristics compared to other NADPH oxidases. Cell Signal 18:69–82
37. Shiose A, Kuroda J, Tsuruya K et al (2001) A novel superoxide-producing NAD(P)H oxidase in kidney. J Biol Chem 276:1417–1423
38. Satoh M, Fujimoto S, Haruna Y et al (2005) NAD(P)H oxidase and uncoupled nitric oxide synthase are major sources of glomerular superoxide in rats with experimental diabetic nephropathy. Am J Physiol Renal Physiol 288:F1144–F1152
39. Forbes JM, Cooper ME, Thallas V et al (2002) Reduction of the accumulation of advanced glycation end products by ACE inhibition in experimental diabetic nephropathy. Diabetes 51:3274–3282
40. Etoh T, Inoguchi T, Kakimoto M et al (2003) Increased expression of NAD(P)H oxidase subunits, NOX4 and p22phox, in the kidney of streptozotocin-induced diabetic rats and its reversibility by interventive insulin treatment. Diabetologia 46:1428–1437
41. Bondi CD, Manickam N, Lee DY et al (2010) NAD(P)H oxidase mediates TGF-beta1-induced activation of kidney myofibroblasts. J Am Soc Nephrol 21:93–102
42. Reeves WB, Andreoli TE (2000) Transforming growth factor beta contributes to progressive diabetic nephropathy. Proc Natl Acad Sci U S A 97:7667–7669
43. Ha H, Oh E, Lee HB (2009) The role of plasminogen activator inhibitor1 in renal and cardiovascular diseases. Nat Rev Nephrol 5:203–211
44. Lee EA, Seo JY, Jiang Z et al (2005) Reactive oxygen species mediates HG-induced plasminogen activator inhibitor-1 up-regulation in mesangial cells and in diabetic kidney. Kidney Int 67:1762–1771
45. Lassila M, Fukami K, Jandeleit-Dahm K et al (2007) Plasminogen activator inhibitor-1 production is pathogenetic in experimental murine diabetic renal disease. Diabetologia 50:1315–1326
46. Huang Y, Border WA, Yu L, Zhang J, Lawrence DA, Noble NA (2008) A PAI-1 mutant, PAI-1R, slows progression of diabetic nephropathy. J Am Soc Nephrol 19:329–338
47. Fujita H, Fujishima H, Morii T et al (2012) Modulation of renal superoxide dismutase by telmisartan therapy in C57BL/6-*Ins2*^{Akita} diabetic mice. Hypertens Res 35:213–220
48. Faulhaber-Walter R, Chen L, Oppermann M et al (2008) Lack of A1 adenosine receptors augments diabetic hyperfiltration and glomerular injury. J Am Soc Nephrol 19:722–730
49. Chacko BK, Reily C, Srivastava A et al (2010) Prevention of diabetic nephropathy in Akita mice by the mitochondria-targeted therapy MitoQ. Biochem J 432:9–19
50. Zhang M, Brewer AC, Schroder K et al (2010) NADPH oxidase-4 mediates protection against chronic load-induced stress in mouse hearts by enhancing angiogenesis. Proc Natl Acad Sci 107:18121–18126