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ARTICLE

Podocyte-specific *Nox4* deletion affords renoprotection in a mouse model of diabetic nephropathy

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Abstract
Aims/hypothesis Changes in podocyte morphology and function are associated with albuminuria and progression of diabetic nephropathy. NADPH oxidase 4 (NOX4) is the main source of reactive oxygen species (ROS) in the kidney and *Nox4* is upregulated in podocytes in response to high glucose. We assessed the role of NOX4-derived ROS in podocytes in vivo in a model of diabetic nephropathy using a podocyte-specific NOX4-deficient mouse, with a major focus on the development of albuminuria and ultra-glomerular structural damage.
Methods Streptozotocin-induced diabetes-associated changes in renal structure and function were studied in male floxed*Nox4* and podocyte-specific, NOX4 knockout (pod*Nox4*KO) mice. We assessed albuminuria, glomerular extracellular matrix accumulation and glomerulosclerosis, and markers of ROS and inflammation, as well as glomerular basement membrane thickness, effacement of podocytes and expression of the podocyte-specific protein nephrin.
Results Podocyte-specific *Nox4* deletion in streptozotocin-induced diabetic mice attenuated albuminuria in association with reduced vascular endothelial growth factor (VEGF) expression and prevention of the diabetes-induced reduction in nephrin expression. In addition, podocyte-specific *Nox4* deletion reduced glomerular accumulation of collagen IV and fibronectin, glomerulosclerosis and mesangial expansion, as well as glomerular basement membrane thickness. Furthermore, diabetes-induced increases in renal ROS, glomerular monocyte chemoattractant protein-1 (MCP-1) and protein kinase C alpha (PKC-α) were attenuated in podocyte-specific NOX4-deficient mice.
Conclusions/interpretation Collectively, this study shows the deleterious effect of *Nox4* expression in podocytes by promoting podocytopathy in association with albuminuria and extracellular matrix accumulation in experimental diabetes, emphasising the role of NOX4 as a target for new renoprotective agents.

Keywords Albuminuria · Diabetic nephropathy · Glomerular basement membrane · NADPH oxidase 4 · Podocyte · Reactive oxygen species

Harald H. H. W. Schmidt and Karin A. Jandeleit-Dahm contributed equally as senior authors.

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Abbreviations

ACR	Albumin/creatinine ratio
DN	Diabetic nephropathy
ECM	Extracellular matrix
GBM	Glomerular basement membrane
8-OHdG	8-Hydroxy-2′-deoxyguanosine

MCP	Monocyte chemoattractant protein
NOX	NADPH oxidase
PKC	Protein kinase C
ROS	Reactive oxygen species
VEGF	Vascular endothelial growth factor

Introduction

Diabetic nephropathy (DN) is the leading cause of renal failure in the world, with patients often requiring dialysis or kidney transplantation [1, 2]. Podocyte damage and loss have been suggested to play pivotal roles in the pathogenesis of DN including albuminuria, which is a key feature of this condition [3, 4]. Podocytes are glomerular visceral epithelial cells connected by the slit diaphragm, which functions as a size-selective filtration barrier [5]. A number of studies have identified 'oxidative stress' as a crucial mediator in the development and progression of DN [6–8]. It has been postulated that this enhanced oxidative stress via the pro-oxidant enzyme NADPH oxidase (NOX), particularly NOX4 [7, 9, 10] and more recently NOX5 [11], in the diabetic kidney causes injury to podocytes and is associated with deleterious changes in renal function and structure [4, 12–14].

In a recent study, we found that global genetic deletion or pharmacological inhibition of NOX4 significantly attenuated diabetes-induced increases in albuminuria, glomerulosclerosis and accumulation of extracellular matrix (ECM) proteins via a reduction in reactive oxygen species (ROS) production in an experimental model of DN [9]. It has been suggested that stimuli such as high glucose, TGF-β and angiotensin II increase ROS production and lead to injury and apoptosis in podocytes, and are associated with increased excretion of albumin into the urine [4, 15–17]. Another study has reported that systemic administration of a nonspecific NOX inhibitor, apocynin, ameliorated urinary albumin excretion in a diabetic mouse model [18, 19]. Recently, we found that silencing NOX4 using short hairpin RNA or inhibiting NOX4 using a novel, specific NOX1/NOX4 inhibitor (GKT137831) in human podocytes significantly reduced the high glucose-induced increase in ROS production, as well as gene expression of profibrotic and proinflammatory markers [9]. In the present study, we aimed to examine whether the diabetes-induced activation of NOX4-derived ROS specifically in podocytes affects glomerular structure and function in vivo in an animal model of DN, using mice with specific deletion of *Nox4* within podocytes.

Methods

Animals Homozygous floxed*Nox4* (floxed NADPH oxidase 4) mice (provided by H. H. H. W. Schmidt, CARIM, Maastricht, the Netherlands) and heterozygous podocyte-specific *Cre* recombinase, podocin-*Cre* (NPHS2-*Cre* (pod*Cre*)) mice (provided by S. E. Quaggin, Robert H. Lurie Medical Research Center, Chicago, IL, USA) were crossed to generate podocyte-specific, NOX4 knockout (pod*Nox4*KO) mice. *Cre*-mediated recombination resulted in deletion of the *Nox4* flanked sequence in the podocyte. We confirmed *Nox4* deletion in the podocytes by genotyping (see electronic supplementary material [ESM] Fig. 1a) and qPCR (ESM Fig. 1b), as well as by immunostaining (ESM Fig. 1c). Detailed methods for the isolation of podocytes and glomerular and tubular fractions, as well as confirmation of *Nox4* deletion, are provided in ESM Methods. All of the mouse lines were maintained on a C57BL/6 background. Control mice were littermates of floxed*Nox4* and pod*Cre* mouse crosses.

All animal studies were approved by the Alfred Medical Research & Education Precinct Animal Ethics Committee under guidelines laid down by the National Health and Medical Research Council of Australia. All animals were housed at the Precinct Animal Centre of the Baker IDI Heart & Diabetes Institute. During the study, animals had unrestricted access to water and food and were maintained on a 12 h light/dark cycle in a pathogen-free environment on standard mouse chow (Specialty Feeds, Glen Forest, Perth, WA, Australia).

Induction of diabetes Diabetes was induced in 6-week-old male floxed*Nox4*, pod*Nox4*KO and pod*Cre* mice by five daily i.p. injections of streptozotocin (Sigma-Aldrich, St Louis, MO, USA), at a dose of 55 mg/kg body weight in citrate buffer, with control mice receiving citrate buffer alone. Only mice with blood glucose ≥15 mmol/l after injection of streptozotocin have been included in experiments; mice with blood glucose <15 mmol/l were excluded from the study (<10% of the total number of mice). After 10 and 20 weeks, the animals were anaesthetised using sodium pentobarbitone i.p. (100 mg/kg body weight; Euthatal, Sigma-Aldrich, Castle Hill, NSW, Australia). The kidneys were rapidly dissected, weighed and snap-frozen or processed in paraffin for subsequent analysis.

Measurement of metabolic variables At 10 and 20 weeks after induction of diabetes, mice were individually placed into metabolic cages (Iffa Credo, L'Arbresele, France) for 24 h. Urine was collected for subsequent analysis. Blood glucose and glycated haemoglobin were measured, as previously described [9, 20]. Systolic BP was assessed using a computerised non-invasive tail-cuff method [21]. Urinary albumin concentration was measured at 10 and 20 weeks after the induction of diabetes, using a mouse albumin ELISA quantification kit (Bethyl Laboratories, Montgomery, TX, USA). Urinary creatinine was determined using a commercially available creatinine assay kit (Abcam, Cambridge, UK). The urinary albumin/creatinine ratio (ACR) was

calculated. A mouse cystatin C ELISA kit (BioVendor, Brno, Czech Republic) was used to determine serum cystatin C according to the manufacturer's instructions.

Histological assessment Kidney sections (3 μm) were stained with periodic acid–Schiff for the measurement of glomerulosclerotic injury and mesangial expansion, as well as with Masson's trichrome for the assessment of glomerular ECM accumulation [22]. Mesangial area and ECM accumulation were analysed (percentage of glomerular area) from digital pictures of glomeruli (20 glomeruli per kidney per animal) using Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA), as previously described [9, 20]. Glomerulosclerotic injury was graded based on the severity of glomerular damage, as previously described [23]. Twenty glomeruli per kidney were assessed in a masked fashion.

In vivo transmission electron microscopy Kidney sections were fixed, embedded, cut and visualised using a Hitachi 7500 transmission electron microscope (Hitachi, Tokyo, Japan). Electron micrographs were used to determine the glomerular basement membrane (GBM) thickness, and the number of filtration slit pores was counted as previously described [24, 25]. For further details, see the ESM Methods.

Immunohistochemistry Immunostaining for collagen IV, fibronectin, nitrotyrosine, nephrin and protein kinase C (PKC)-α was performed and the proportional area of staining was quantified as previously described [9, 20]. For further details, see the ESM Methods.

Western blot The glomerular fraction was obtained from the frozen renal cortex of the respective control and diabetic mice, as described in ESM Methods. Protein extracts (5 μg) from each sample were electrophoresed on 7.5% acrylamide gels under non-reducing conditions, as previously described [26]. Western blot analysis was then performed with a primary antibody to collagen IV (1:1000, Abcam, Cambridge, MA, USA) and assessed with goat anti-rabbit (Dako, Carpinteria, CA, USA) secondary antibody. Membranes were subsequently probed for α-tubulin (Sigma-Aldrich) for determination of equal loading of samples. Blots were detected using the ECL detection kit (Sigma-Aldrich) and densitometry was performed using Quantity One software (Bio-Rad, Richmond, CA, USA).

In vivo glomerular gene expression analysis Total RNA was extracted from isolated glomeruli (Polytron PT-MR2100; Kinematica, Littau/Lucerne, Switzerland) in TRIzol reagent (Invitrogen Australia, Mt Waverely, VIC, Australia), as previously described [9, 20]. Probes and primer sequences for the RT-PCR of nephrin, vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein (MCP)-1 are described in ESM Table 1. Expression of the genes encoding nephrin, VEGF and MCP-1 was quantified and determined relative to expression of the housekeeping gene 18S (18S ribosomal RNA TaqMan Control Reagent kit) using the TaqMan system (ABI Prism 7500; Perkin-Elmer, Poster City, CA, USA). Results are expressed relative to non-diabetic floxed*Nox4* mice, which were arbitrarily assigned a value of 1.

Urinary VEGF ELISA The Quantikine Mouse ELISA kit (R&D Systems, Minneapolis, MN, USA) was used to measure VEGF in the urine, as per the kit instructions. Urinary VEGF is expressed as picograms per 24 h.

Urinary 8-isoprostanes ELISA An 8-isoprostanes enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA) was used to measure 8-isoprostanes in urine, as described by the manufacturer. Urinary 8-isoprostanes is expressed as picograms per 24 h.

Urinary 8-hydroxy-2′-deoxyguanosine ELISA A urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) enzyme immunoassay kit (StressMarq Biosciences Victoria, BC, Canada) was used to measure 8-OHdG in urine, as described by the manufacturer. Urinary 8-OHdG is expressed as nanograms per 24 h.

Table 1 General and metabolic variables after 10 and 20 weeks of study in control and diabetic floxed*Nox4* and pod*Nox4*KO mice (n=8–15 per group)

Variable	10 weeks				20 weeks			
	floxed*Nox4*		pod*Nox4*KO		floxed*Nox4*		pod*Nox4*KO	
	Control	Diabetes	Control	Diabetes	Control	Diabetes	Control	Diabetes
Body weight (g)	26±0.8	23±0.9*	29±1.1	25±0.7*	33±0.7	26±0.9*	34±1.1	27±1.1*
Kidney weight/body weight (%)	0.54±0.02	0.70±0.06*	0.57±0.01	0.70±0.03*	0.56±0.01	0.90±0.04*	0.57±0.01	0.83±0.03*
Systolic BP (mmHg)	97±2	99±1	103±2	107±1	98±3	99±2	104±2	105±2
Plasma glucose (mmol/l)	9.3±0.7	26.5±3.6*	11.6±0.7	27.2±3.4*	9.4±0.7	28.7±2.2*	11.3±0.6	24.8±2.3*
Total glycated haemoglobin (%)	6.2±0.1	10.6±1.3*	4.5±0.1	9.1±0.3*	5.9±0.8	9.9±1.1*	6.1±0.9	10.6±1.5*

Data are as means ± SEM

*p<0.05 vs the respective control

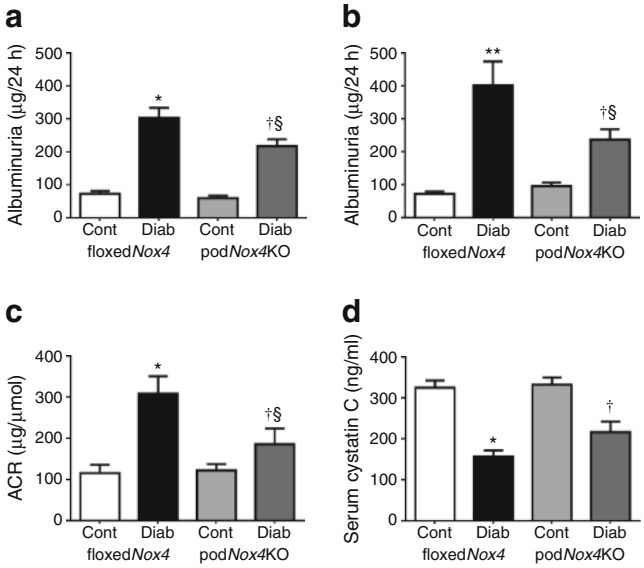

Fig. 1 Podocyte-specific *Nox4* deletion attenuated albuminuria in diabetic mice. Urinary albumin excretion at 10 (**a**) and 20 (**b**) weeks of diabetes, and ACR (**c**) and serum cystatin C (**d**) at 20 weeks of diabetes in floxed*Nox4* and pod*Nox4*KO mice (n=10–15 per group). Data are means ± SEM. *p<0.05, **p<0.01 vs control (Cont) floxed*Nox4* mice; $^{\dagger}p$<0.05 vs control (Cont) pod*Nox4*KO mice; $^{\S}p$<0.05 vs diabetic (Diab) floxed*Nox4* mice

Lucigenin assays Glomerular fractions obtained from the frozen renal cortices of the different experimental groups were harvested in 100 μl ice-cold phosphate buffer (50 mmol/l KH_2PO_4, 1 mmol/l EGTA and 150 mmol/l sucrose; pH 7.4) with protease inhibitors, as previously described [11]. Baseline activity was measured by adding 50 μl glomerular extract to 175 μl buffer and 2.5 μl 1 mmol/l lucigenin (Sigma-Aldrich). NADPH-dependent superoxide was measured by the addition of 25 μl 1 mmol/l NADPH (Sigma-Aldrich). Baseline activity was subtracted and normalised to the protein concentration.

Statistical analysis All variables were analysed by one-way ANOVA using GraphPad Prism 6 (GraphPad, San Diego, CA, USA) for multiple comparison of the means or by the two-tailed unpaired Mann–Whitney U test, when required. p<0.05 was considered to be statistically significant. Results are expressed as mean ± SEM unless otherwise specified.

Results

Metabolic variables Induction of diabetes was associated with reduced body weight, elevated blood glucose and increased glycated haemoglobin in both groups when compared with their respective non-diabetic controls (Table 1). Furthermore, no differences in metabolic variables were seen in diabetic pod*Nox4*KO mice compared with diabetic floxed*Nox4* mice (Table 1). In addition, systolic blood

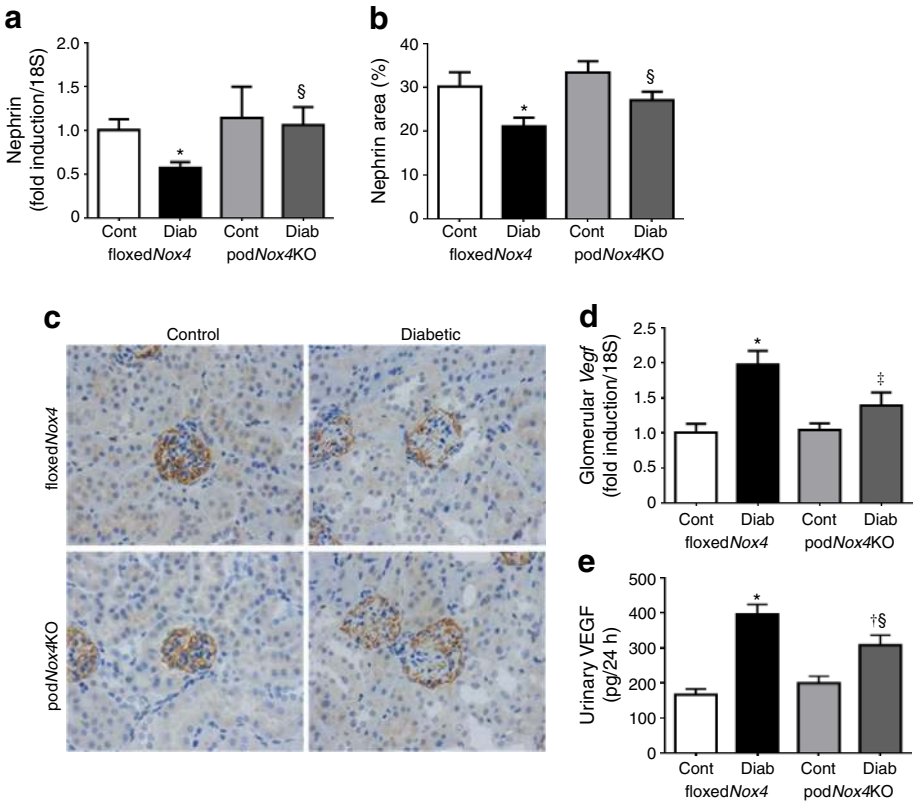

Fig. 2 Podocyte-specific *Nox4* deletion attenuated increased VEGF expression and preserved nephrin expression in diabetic mice. Glomerular gene (**a**) (n=5–6 per group) and protein (**b**, **c**) (n=7–8 per group) expression of nephrin (magnification ×40) in respective control (Cont) and diabetic (Diab) floxed*Nox4* and pod*Nox4*KO mice after 20 weeks of diabetes. Glomerular *Vegf* gene expression (**d**) (n=5–6 per group) and urinary VEGF excretion (**e**) (n=9–10 per group) in respective control and diabetic floxed*Nox4* and pod*Nox4*KO mice after 20 weeks of diabetes. Data are means ± SEM. *p<0.05 vs control floxed*Nox4* mice; $^{\ddagger}p$=0.05 vs diabetic floxed*Nox4* mice; $^{\dagger}p$<0.05 vs control pod*Nox4*KO; $^{\S}p$<0.05 vs diabetic floxed*Nox4* mice

pressure was similar in all groups. The kidney weight/body weight ratio was significantly increased in diabetic mice, with similar ratios in diabetic floxed*Nox4* and pod*Nox4*KO mice. Furthermore, metabolic variables were unchanged in pod*Cre* mice when compared with floxed*Nox4* mice after 20 weeks of diabetes (ESM Table 2).

Renal functional variables Albuminuria was significantly attenuated after 10 and 20 weeks of diabetes in pod*Nox4*KO mice when compared with diabetic floxed*Nox4* mice (Fig. 1a, b). Similar effects were observed when the data were expressed as urinary ACR after 20 weeks of diabetes (Fig. 1c). Furthermore, no difference in albuminuria was found in pod*Cre* mice when compared with floxed*Nox4* mice after 20 weeks of diabetes (ESM Table 2). The serum cystatin C level was reduced in both diabetic floxed*Nox4* and pod*Nox4*KO mice when compared with their non-diabetic counterparts (Fig. 1d), suggesting that the glomerular filtration rate was not reduced by *Nox4* deletion in podocytes and that both groups of diabetic mice exhibited hyperfiltration.

Gene and protein expression of nephrin As previously demonstrated in another model of DN [27], we found decreased gene and protein expression of glomerular nephrin in diabetic floxed*Nox4* mice (Fig. 2a–c). Interestingly, the diabetes-induced decrease in nephrin expression was preserved in diabetic pod*Nox4*KO mice (Fig. 2a–c).

Gene expression and urinary excretion of VEGF We observed that glomerular *Vegf* (also known as *Vegfa*) gene expression and urinary excretion of VEGF were higher in diabetic floxed*Nox4* mice when compared with non-diabetic controls, and these variables were attenuated in diabetic pod*Nox4*KO mice (Fig. 2d, e).

Glomerular structural assessment Glomerulosclerosis and mesangial area (Fig. 3a–c) as well as ECM accumulation (Fig. 3d, e) were increased in floxed*Nox4* mice after 20 weeks of diabetes when compared with non-diabetic floxed*Nox4* mice. Increased glomerulosclerosis and mesangial area ($p<0.05$) as well as ECM accumulation ($p=0.05$) were attenuated in diabetic pod*Nox4*KO mice (Fig. 3a–e).

GBM thickness and podocyte foot process effacement Using quantifying histomorphometric techniques, both thickening of GBM as well as irregularity in podocyte foot process

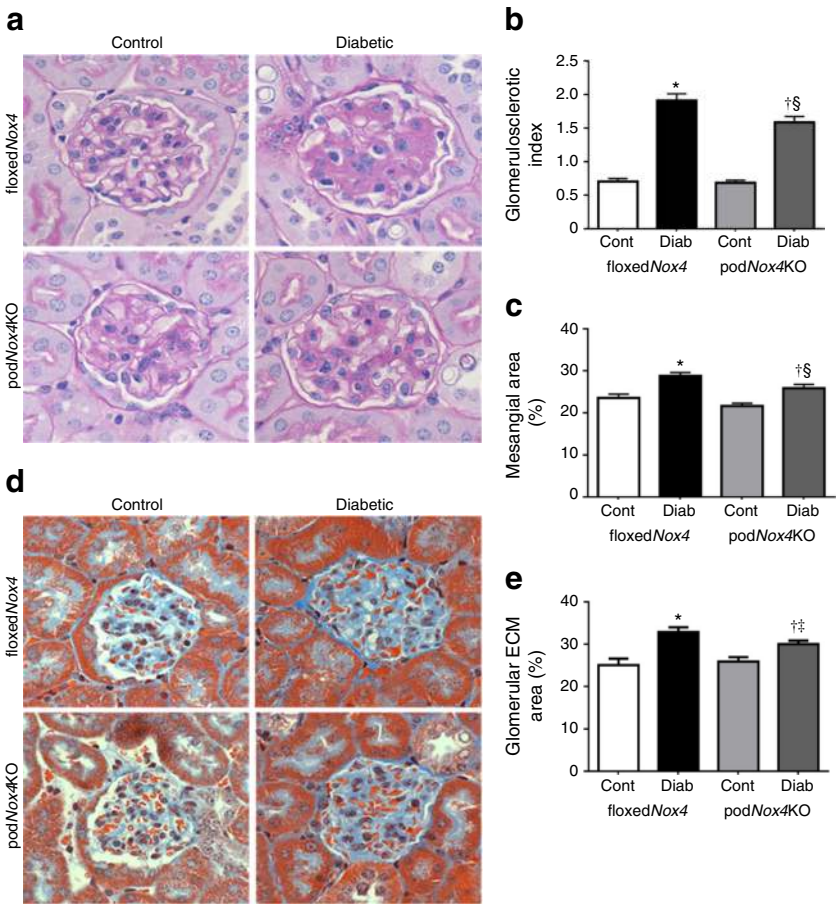

Fig. 3 Podocyte-specific *Nox4* deletion attenuated glomerular injury in diabetic mice. Periodic acid–Schiff staining (magnification ×40) (**a**), glomerulosclerotic index (**b**) and mesangial area expansion (**c**), as well as Masson's trichrome staining (magnification ×40) (**d**) and glomerular ECM accumulation (**e**) in control (Cont) and diabetic (Diab) floxed*Nox4* and pod*Nox4*KO mice ($n=7$–10 per group) after 20 weeks of diabetes. Data are means ± SEM. *$p<0.05$ vs control floxed*Nox4* mice; ‡$p=0.05$ vs diabetic floxed*Nox4* mice; †$p<0.05$ vs control pod*Nox4*KO; §$p<0.05$ vs diabetic floxed*Nox4* mice

dimensions and unevenly spread filtration slit pores suggestive of foot process effacement were seen in diabetic floxed*Nox4* mice when compared with control floxed*Nox4* mice (Fig. 4a, b). Furthermore, diminution in the number of filtration slit pores per 100 μm of GBM ($p<0.05$) was seen in diabetic floxed*Nox4* mice, indicative of foot process broadening (Fig. 4c). Interestingly, podocyte-specific NOX4-deficient diabetic mice appeared to have decreased GBM thickness, less podocyte foot process effacement and a high number of filtration slit pores when compared with diabetic floxed*Nox4* mice (Fig. 4a–c).

Glomerular ECM proteins We examined glomerular collagen IV and fibronectin accumulation in diabetic mice. Consistent with the findings on glomerulosclerosis and mesangial expansion, collagen IV protein production was significantly increased in the glomeruli after 20 weeks of diabetes in floxed*Nox4* mice when compared with non-diabetic floxed*Nox4* controls, as assessed by immunohistochemistry (Fig. 5a, b) or western blot (Fig. 5c, d). Importantly, the increase in collagen IV production was attenuated in pod*Nox4*KO mice (Fig. 5a–d). Similarly, fibronectin protein accumulation was increased in the glomeruli of floxed*Nox4* mice after 20 weeks of diabetes and this variable was attenuated in podocyte-specific NOX4-deficient mice (Fig. 5e, f).

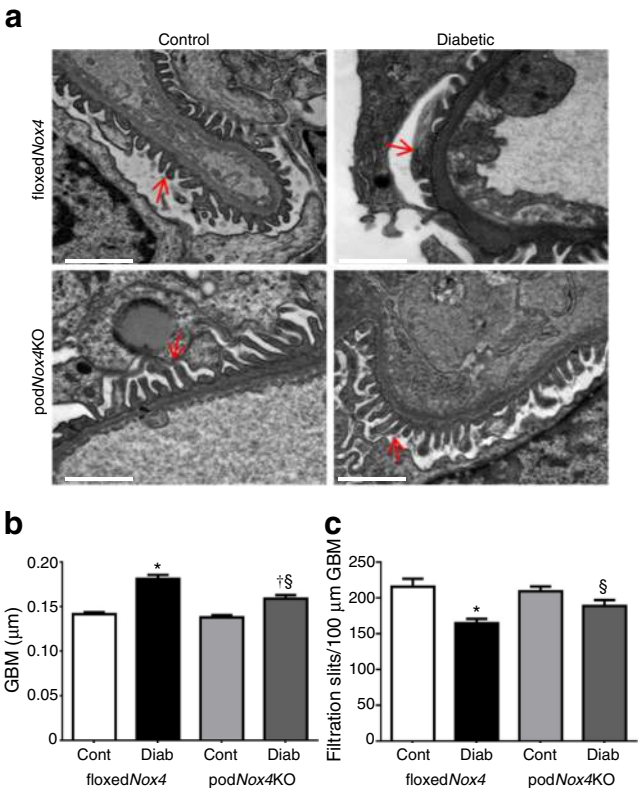

Fig. 4 Podocyte-specific *Nox4* deletion attenuated GBM thickness and filtration slit pores in diabetic mice. Transmission electron micrographs for GBM thickness (**a**, **b**), podocyte foot process effacement (**a**) and the number of slits per 100 μm of GBM (**c**) in respective control (Cont) and diabetic (Diab) floxed*Nox4* and pod*Nox4*KO mice after 20 weeks of diabetes. Red arrows indicate podocyte foot processes. Images were taken at ×30,000 magnification. Scale bar, 0.5 μm. Data are means ± SEM. *$p<0.05$ vs control floxed*Nox4* mice; †$p<0.05$ vs control pod*Nox4*KO; §$p<0.05$ vs diabetic floxed*Nox4* mice

Oxidative stress markers Urinary excretion of 8-isoprostane and 8-OHdG were increased in diabetic floxed*Nox4* mice compared with non-diabetic controls (Fig. 6a, b). The diabetes-induced increases in urinary level of 8-isoprostane and 8-OHdG were attenuated in podocyte-specific NOX4-deficient diabetic mice (Fig. 6a, b). To strengthen the findings on renal ROS levels, we next examined NADPH-dependent glomerular superoxide generation, as well as immunostaining of glomerular nitrotyrosine. Glomerular superoxide and nitrotyrosine accumulation were increased in floxed*Nox4* mice after 20 weeks of diabetes and attenuated in diabetic pod*Nox4*KO mice (Fig. 6c–e). To study the effect of NOX4 on H_2O_2 production, we examined podocytes with and without *Nox4* expression, and found that silencing of *Nox4* using *Nox4* shRNAin human podocytes resulted in attenuation of increased levels of H_2O_2 generation in response to high glucose (ESM Fig. 2).

Glomerular PKC-α and MCP-1 We examined glomerular PKC-α expression and found increased production of this PKC isoform in diabetic floxed*Nox4* mice when compared with non-diabetic controls (Fig. 7a, b). Furthermore, glomerular PKC-α production was attenuated in diabetic pod*Nox4*KO mice (Fig. 7a, b). Diabetes is associated with increased inflammation [28]. Indeed, we observed increased glomerular *Mcp-1* (also known as *Ccl2*) expression in diabetic floxed*Nox4* mice when compared with non-diabetic controls (Fig. 7c). This inflammatory variable was attenuated in diabetic pod*Nox4*KO mice ($p=0.05$) (Fig. 7c).

Discussion

This study provides, for the first time, clear evidence that podocyte-specific *Nox4* deletion affords renoprotection in vivo in a mouse model of DN. Previously it has been shown by our group that global genetic deletion of *Nox4* prevented the development of albuminuria and glomerular injury via a reduction in renal ROS in diabetic mice [9]. To further address this issue and to specifically determine the potential mechanisms of renoprotection at a cellular level, an in vitro study involving silencing of *Nox4* in human podocytes was performed. Indeed, knockdown of NOX4 in human podocytes reduced high-glucose-induced ROS production, as well as various markers of fibrosis and inflammation. These findings, albeit in vitro, suggested a deleterious effect of NOX4 within podocytes, potentially attenuating key pathways implicated in

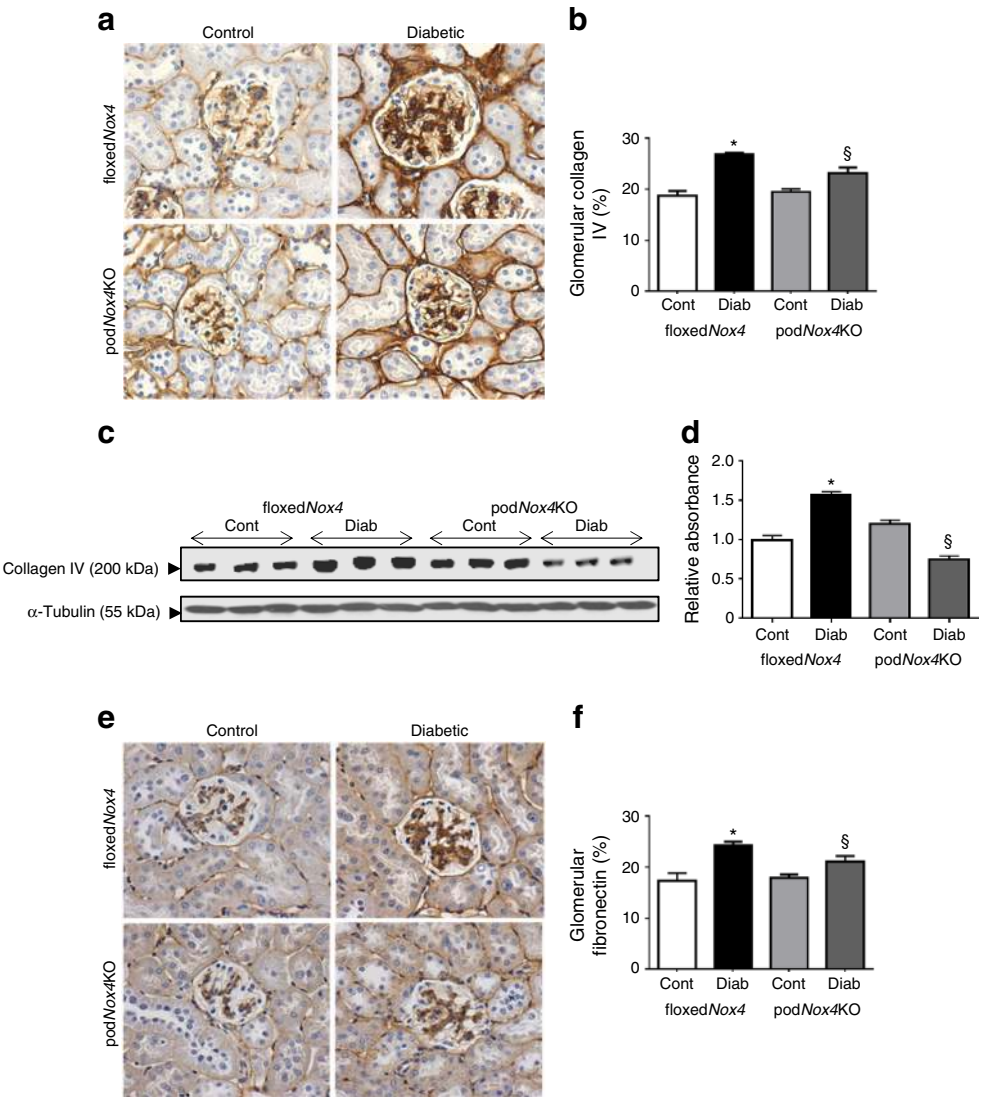

Fig. 5 Podocyte-specific *Nox4* deletion attenuated glomerular collagen IV and fibronectin expression in diabetic mice. Immunostaining of collagen IV (**a**, **b**) and fibronectin (**e**, **f**) (n=7–8 per group) (magnification ×40) as well as western blotting for collagen IV (**c**, **d**) (n=6 per group) in the glomeruli of respective control (Cont) and diabetic (Diab) floxed*Nox4* and pod*Nox4*KO mice after 20 weeks of diabetes. Data are means ± SEM. *p<0.05 vs control floxed*Nox4* mice; $^{§}p$<0.05 vs diabetic floxed*Nox4* mice

the development of albuminuria and renal injury in diabetes [9]. To translate these findings in podocytes to an in vivo context, the present study addressed and indeed demonstrated that podocyte-specific *Nox4* deletion attenuates the diabetes-induced increase in albuminuria by approximately 50% in diabetic pod*Nox4*KO mice. This finding strongly supports the view that NOX4-derived ROS in the podocyte plays a crucial role in the regulation of albuminuria in diabetes. With identification of the prevention of the diabetes-induced decrease in nephrin expression and reduced podocyte foot process effacement in the pod*Nox4*KO diabetic mice, this would be consistent with a deleterious effect of NOX4 in podocytes, consistent with the previous in vitro studies [9]. Indeed, changes in the expression of the podocyte-specific marker nephrin and podocyte damage, including podocyte foot process effacement, have been shown to correlate with albuminuria in diabetes [24, 27, 29].

To further explore potential mechanisms linking NOX4 to renal injury, renal and urinary expression of VEGF were examined, this growth factor having previously been reported to be linked to the development of albuminuria, particularly in the setting of diabetes [30–33]. We have previously reported a decrease in VEGF expression in the glomeruli of diabetic global NOX4 knockout mice, as well as in *Nox4* silenced human podocytes in response to high glucose [9]. Indeed, in this study, we observed a reduction in urinary VEGF excretion as well decreased glomerular VEGF expression in diabetic pod*Nox4*KO mice. These findings strengthen the hypothesis that NOX4-derived ROS play a crucial role in modulating and regulating VEGF expression in podocytes. Interestingly, podocyte-specific *Nox4* deletion also attenuated various glomerular ultra-structural changes, including glomerulosclerosis, mesangial expansion and accumulation of ECM proteins, including collagen IV and fibronectin, in diabetic pod*Nox4*KO mice. Abrogation of glomerular

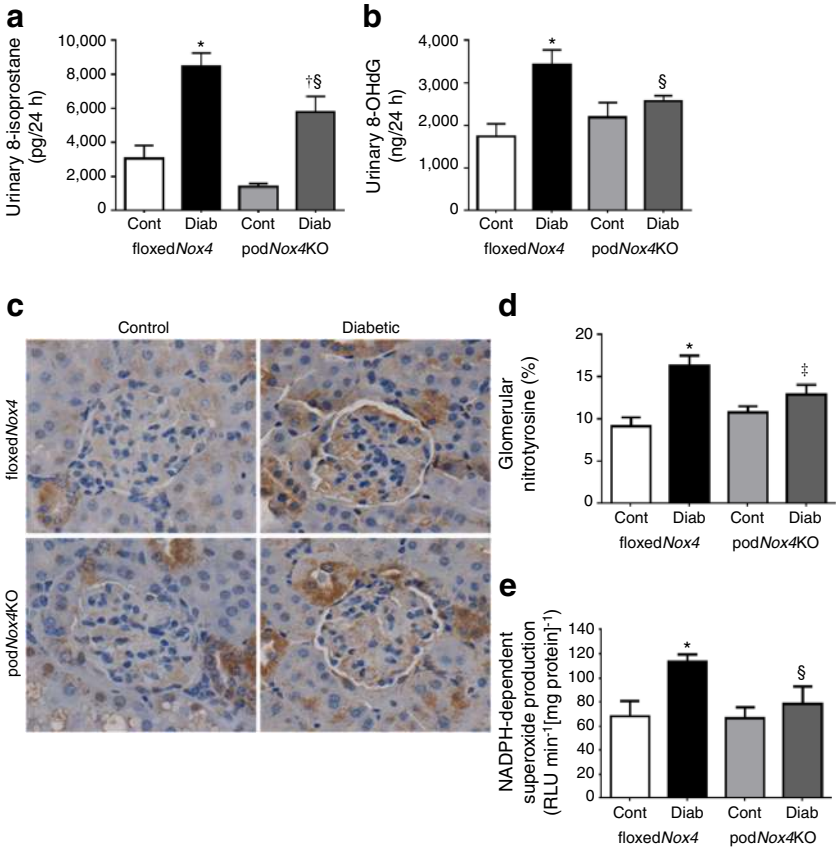

Fig. 6 Podocyte-specific *Nox4* deletion attenuated renal ROS in diabetic mice. Urinary 8-isoprostane (**a**) and 8-OHdG excretion (**b**) (n=8–10 per group) and immunostaining for glomerular nitrotyrosine (**c**, **d**) (magnification ×40) (n=6–8 per group) and NADPH-dependent lucigenin assay (**e**) (n=6) in respective control (Cont) and diabetic (Diab) floxed*Nox4* and pod*Nox4*KO mice after 20 weeks of diabetes. Data are means ± SEM. *p<0.05 vs control floxed*Nox4* mice; $^{\dagger}p$<0.05 vs control pod*Nox4*KO; $^{\ddagger}p$=0.05 vs diabetic floxed*Nox4* mice; $^{\S}p$<0.05 vs diabetic floxed*Nox4* mice

structural damage in diabetic pod*Nox4*KO mice would indicate potential cross-talk between podocytes and other glomerular cells that are involved in the process of glomerulosclerosis. A recent study, albeit in the heart, supports our findings that NOX4 plays a role in fibrosis and hypertrophy [34]. In that study, NOX4 was shown to induce cardiac fibrosis and hypertrophy via activation of the Akt/mTOR and nuclear factor-κB signalling pathways [34].

A correlation between albuminuria and GBM thickness has been reported previously [35, 36]. Thickening of the GBM is considered to be one of the characteristic lesions in diabetic patients with albuminuria [35, 37]. It has been shown that GBM thickening in diabetes occurs as a consequence of accumulation of increased ECM components [38, 39]. The accumulation of ECM proteins results from both increased production and decreased degradation of these proteins. Indeed,

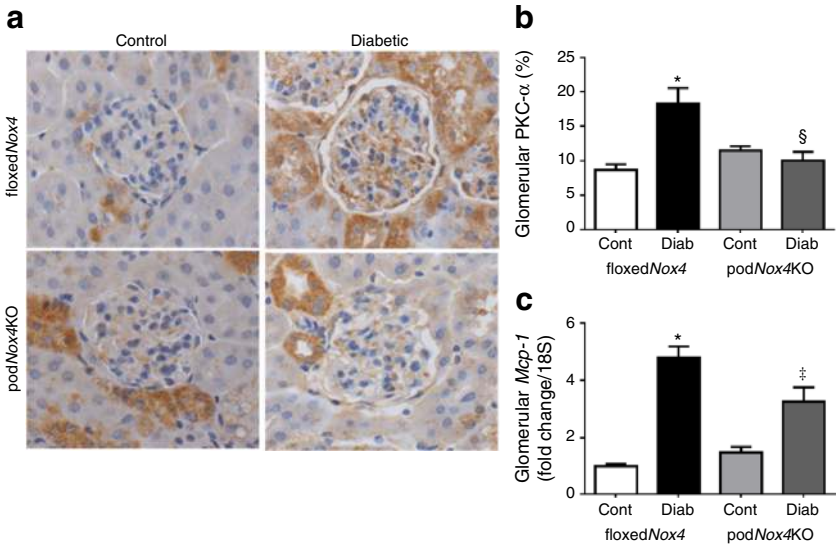

Fig. 7 Podocyte-specific *Nox4* deletion attenuated glomerular PKC-α and *Mcp-1* expression in diabetic mice. Immunostaining for PKC-α in glomeruli (n=8–10 per group) (**a**, **b**) (magnification ×40) and gene expression of glomerular *Mcp-1* (**c**) in respective control (Cont) and diabetic (Diab) floxed*Nox4* and pod*Nox4*KO mice after 20 weeks of diabetes. Data are means ± SEM. *p<0.05 vs control floxed*Nox4* mice; $^{\ddagger}p$=0.05 vs diabetic floxed*Nox4* mice; $^{\S}p$<0.01 vs diabetic floxed*Nox4* mice

increased GBM thickness and accumulation of ECM proteins (collagen IV and fibronectin) were seen in diabetic mice and these changes were attenuated in diabetic pod*Nox4*KO mice, consistent with NOX4 in podocytes playing a central role in regulating the production of ECM proteins. Another key morphological feature of DN, identified using electron microscopy, is a reduction in the number of slit pores per unit length of GBM, which reflects foot process broadening, as has been shown in diabetic rodents [24]. This diabetes-associated reduction in filtration slit pores was attenuated in diabetic pod*Nox4*KO mice, further emphasising the importance of NOX4 in promoting podocytopathy, as seen in the diabetic kidney.

Since it is considered that NOX4, also known as 'renox', is a key source of renal ROS, this study used various approaches to evaluate renal ROS generation. Markers of DNA oxidation (8-OHdG) and lipid peroxidation (8-isoprostane) have been shown to be increased in the urine in experimental models of diabetes [32, 40, 41]. In addition, increased urinary levels of 8-OHdG have been reported to be associated with loss of podocytes in diabetic rodents [40]. Thus, attenuation of urinary excretion of the oxidative stress markers 8-isoprostane and 8-OHdG, as well as glomerular superoxide and nitrotyrosine, in diabetic pod*Nox4*KO mice, as seen in this study, supports the view that NOX4-derived ROS mediate podocyte damage and ultimately other markers of renal injury. The ability of *Nox4* deletion only in podocytes to significantly reduce a range of markers of glomerular and renal ROS emphasises the importance of podocyte NOX4 per se as a source of ROS in the diabetic kidney.

There is a close interrelationship between ROS and certain PKC isoforms in the development and progression of DN [32, 42]. Recently, it has been shown that NOX4-derived ROS can activate certain PKC isoforms, including PKC-α, within the kidney, thereby promoting renal injury in experimental diabetes [32]. Thus, we examined the association of podocyte-specific NOX4 with PKC-α, which has been the most closely identified isoform with the development of albuminuria, nephrin depletion and upregulation of intrarenal VEGF in diabetes [43, 44]. Indeed, we found that the diabetes-induced increase in glomerular PKC-α expression was attenuated in diabetic pod*Nox4*KO mice. Quack et al have demonstrated that acute hyperglycaemia increases nephrin endocytosis in a PKC-α-dependent manner [45] and this effect is considered to promote albuminuria. In this study, we found similar changes in VEGF and nephrin expression as well as in albuminuria, in diabetic pod*Nox4*KO mice to those seen in diabetic *Pkcα*KO mice, and also noted a reduction in glomerular PKC-α expression in pod*Nox4*KO mice. Thus, we postulate that NOX4 is a key upstream regulator of PKC-α expression in the setting of diabetes. Indeed, the renoprotective effects of *Nox4* deletion could represent a new approach to target the deleterious effects of PKC-α in the diabetic kidney. In addition, the diabetes-induced increase in glomerular *Mcp-1* expression was found to be attenuated in podocyte-specific NOX4-deficient diabetic mice. This would indicate that targeting NOX4 in the podocytes not only reduces podocytopathy and glomerular ECM accumulation, but might also play a key role in attenuating intrarenal inflammation.

The findings of this study build on an increasing amount of data implicating NOX4 as a potential target for renoprotection. The advent of new agents that target NOX4, such as GKT137831, provide an opportunity to test whether pharmacological inhibition of NOX4 can be renoprotective. Although GKT137831 is not specific for the NOX4 isoform, several groups, including our own, have demonstrated renal benefits with this agent in various experimental models of DN [9, 46].

In conclusion, NOX4 within the podocyte appears to be a major contributor to renal ROS generation, activation of PKC-α, enhanced intrarenal fibrosis and inflammation, as well as increasing VEGF and decreasing nephrin expression. Furthermore, NOX4 plays a pivotal role in influencing podocyte ultrafiltration, with a subsequent impact on glomerular structure and albuminuria, hallmarks of DN. Hence, targeting NOX4 specifically in the podocyte might provide a new therapeutic approach in preventing the progression of diabetic kidney disease.

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Compliance with ethical standards

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