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ARTICLE

Deletion of platelet-derived growth factor receptor-β improves diabetic nephropathy in Ca^{2+}/calmodulin-dependent protein kinase IIα (Thr286Asp) transgenic mice

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Abstract
Aims/hypothesis The activation of platelet-derived growth factor receptor-β (PDGFR-β) signalling is increased in the glomeruli and tubules of diabetic animals. In this study, we examined the role of PDGFR-β signalling during the development of diabetic nephropathy.
Methods We recently generated pancreatic beta cell-specific Ca^{2+}/calmodulin-dependent protein kinase IIα (Thr286Asp) transgenic mice (CaMKIIα mice), which show very high plasma glucose levels up to 55.5 mmol/l and exhibit the features of diabetic nephropathy. These mice were crossed with conditional knockout mice in which *Pdgfr-β* (also known as *Pdgfrb*) was deleted postnatally. The effect of the deletion of the *Pdgfr-β* gene on diabetic nephropathy in CaMKIIα mice was evaluated at 10 and 16 weeks of age.
Results The plasma glucose concentrations and HbA_{1c} levels were elevated in the CaMKIIα mice from 4 weeks of age. Variables indicative of diabetic nephropathy, such as an increased urinary albumin/creatinine ratio, kidney weight/body weight ratio and mesangial area/glomerular area ratio, were observed at 16 weeks of age. The postnatal deletion of the *Pdgfr-β* gene significantly decreased the urinary albumin/creatinine ratio and mesangial area/glomerular area ratio without affecting the plasma glucose concentration. Furthermore, the increased oxidative stress in the kidneys of the CaMKIIα mice as shown by the increased urinary 8-hydroxydeoxyguanosine (8-OHdG) excretion and the increased expression of NAD(P)H oxidase 4 (NOX4), glutathione peroxidase 1 (GPX1) and manganese superoxide dismutase (MnSOD) was decreased by *Pdgfr-β* gene deletion.
Conclusions/interpretation The activation of PDGFR-β signalling contributes to the progress of diabetic nephropathy, with an increase in oxidative stress and mesangial expansion in CaMKIIα mice.

Keywords Knockout mice · Mouse model · Nephropathy · Oxidative stress

Abbreviations

BUN	Blood urea nitrogen
CaMKIIα	Ca^{2+}/calmodulin-dependent protein kinase IIα
CD-1	Caesarean derived-1
Cre-ER	Cre recombinase-oestrogen receptor
Cre-loxP	Cre recombinase-loxP
GPX1	Glutathione peroxidase 1
MnSOD	Manganese superoxide dismutase

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NOX4	NAD(P)H oxidase 4
8-OHdG	8-Hydroxydeoxyguanosine
PAS	Periodic acid–Schiff's reagent
PDGF	Platelet-derived growth factor
PDGFR	PDGF receptor
PFA	Paraformaldehyde

Introduction

Diabetic nephropathy is one of the most serious microvascular complications of diabetes mellitus [1]. Mesangial cell proliferation and production of excessive extracellular matrix in the kidney glomerulus are characteristic in diabetic nephropathy as well as other chronic renal diseases [2, 3]. These mesangial cell responses in renal diseases are of clinical interest for the prevention of glomerular sclerosis and improvement of functional recovery after renal insults [4, 5].

Platelet-derived growth factor (PDGF) is a potent mitogen that stimulates extracellular matrix accumulation in mesangial cells [6]. PDGF family members, PDGF-A, PDGF-B, PDGF-C and PDGF-D, are assembled as disulphide-linked homo- or heterodimers [7]. These signals are mediated by two types of PDGF receptors (PDGFRs), PDGFR-α and PDGFR-β. In the diabetic kidney, upregulation of the PDGF pathway has been shown in experimental diabetic nephropathy [8–10] and in the kidneys of patients with diabetes [11]. Among such patients, the production of PDGF-B and PDGFR-β is specifically increased and correlates to the progress of glomerular lesions such as diabetic nephropathy [12, 13]. The in vitro exposure to high glucose also induces PDGF-B production in human proximal tubular epithelial cells and mesangial cells, and PDGFR-β production in mesangial cells [14–16].

A number of specific interventions aimed at neutralising PDGF-B or -D or blocking PDGFR-β have been shown to reduce mesangial cell proliferation and matrix accumulation and to ameliorate renal dysfunction in experimentally induced glomerulonephritis [17–20]. In contrast, the effects of anti-PDGF therapy on diabetic nephropathy have not been well characterised so far [21]. To our best knowledge, intervention studies involving PDGF are limited to the work showing that tyrosine kinase inhibition with imatinib, a chemical tyrosine kinase inhibitor, retards the development of diabetic nephropathy in diabetic mice [22]. However, it is not clear whether the beneficial effects of imatinib are related to the inhibition of PDGFR tyrosine kinase or to the inhibition of other kinases [23]. Although they are not specific, growing clinical and experimental data are accumulating to suggest that the tyrosine kinase inhibitors are potential medications for diabetes patients [24, 25]. Thus, it is of urgent clinical necessity to determine whether specific antagonism of PDGF signalling pathways could be useful in the treatment of diabetic nephropathy.

Recently, we generated a novel mouse model for insulin-deficient diabetes mellitus by genetically overexpressing a mutant form (Thr286Asp) of Ca^{2+}/calmodulin-dependent protein kinase IIα (CaMKIIα) specifically in pancreatic beta cells [26, 27]. Cellular proliferation of the pancreatic beta cells was severely impaired in these transgenic mice, and the mice developed severe hypoinsulinaemic diabetes by 4 weeks of age. The urinary albumin/creatinine ratio was increased by 10 weeks of age, and several morphological changes typical of diabetic nephropathy were observed by 20 weeks, such as the proliferation of mesangial cells, the expansion of the mesangial area and an enlarged glomerular size. Furthermore, CaMKIIα (Thr286Asp) mice developed diabetic retinopathy, suggesting that these mice may be an ideal mouse model for diabetic nephropathy compared with other conventional models that do not necessarily show the functional and morphological features of diabetic nephropathy observed in human diabetic individuals [26, 27]. In parallel to these mice, we developed a conditional *Pdgfr-β* (also known as *Pdgfrb*) knockout mouse line following the Cre recombinase-loxP (Cre-loxP) system [28]. A substantial decrease in *Pdgfr-β* expression was systemically induced by giving tamoxifen to these mice at 4 weeks of age [29]. Afterwards, the mice with decreased PDGFR-β survived without apparent abnormalities in physiological variables for more than 1 year, which allowed us to investigate the contribution of PDGFR-β signalling in pathological conditions.

In the current study, we crossed diabetic CaMKIIα (Thr286Asp) mice with conditional *Pdgfr-β* knockout mice to examine the specific in vivo role of PDGFR-β signalling during the development of diabetic nephropathy. Furthermore, because oxidative stress has emerged as an important pathogenic factor in the development of diabetic vascular complications [30–34], we also examined the effect of PDGFR-β inhibition on oxidative stress.

Methods

Generation of CaMKIIα (Thr286Asp) transgenic and Pdgfr-β conditional knockout mice using Cre recombinase expressed under an actin promoter Pancreatic beta cell-specific CaMKIIα (Thr286Asp) transgenic mice (CaMKII$\alpha^{Tg/+}$ mice) with a Caesarean derived-1 (CD-1) (Charles River Laboratories Japan, Kanagawa, Japan) background were generated as described by Kato et al. [26]. Conditional *Pdgfr-β* knockout mice (actin-Cre recombinase-oestrogen receptor [Cre-ER]$^{TM+/-}$−*Pdgfr-β*$^{flox/flox}$ mice), which express a fusion protein consisting of Cre recombinase

and a mutated form of the mouse oestrogen receptor ligand-binding domain under the control of the actin promoter, were generated as described previously by Tokunaga et al. [28]. The genetic background of conditional *Pdgfr-β* knockout mice was originally that of C57BL/6 mice (Japan SLC, Shizuoka, Japan) [28]. These were backcrossed to CD-1 more than two times to generate diabetic strains. By crossing the CaMKIIα$^{Tg/+}$ mice and the actin-Cre-ER$^{TM+/-}$–*Pdgfr-β*$^{flox/flox}$ mice, CaMKIIα$^{Tg/+}$–actin-Cre-ER$^{TM+/-}$ *Pdgfr-β*$^{flox/flox}$ mice were generated. As shown in electronic supplementary material (ESM) Fig. 1, we finally crossed male CaMKIIα$^{Tg/+}$–actin-Cre-ER$^{TM+/-}$ *Pdgfr-β*$^{flox/flox}$ mice and female *Pdgfr-β*$^{flox/flox}$ (CaMKIIα$^{+/+}$–actin-Cre-ER$^{TM-/-}$) mice to generate the following four strains of mice: CaMKIIα$^{Tg/+}$–actin-Cre-ER$^{TM+/-}$–*Pdgfr-β*$^{flox/flox}$ mice (termed DM-KO); CaMKIIα$^{Tg/+}$–actin-Cre-ER$^{TM-/-}$–*Pdgfr-β*$^{flox/flox}$ mice (termed DM); CaMKIIα$^{+/+}$–actin-Cre-ER$^{TM+/-}$–*Pdgfr-β*$^{flox/flox}$ mice (termed KO); and CaMKIIα$^{+/+}$–actin-Cre-ER$^{TM-/-}$–*Pdgfr-β*$^{flox/flox}$ mice (wild type [WT]). Only male mice were used for the following experiments. All the mice were orally treated with 9 mg/40 g body weight per day of tamoxifen (Sigma-Aldrich, St Louis, MO, USA) for five successive days at 4 weeks of age to delete the *Pdgfr-β* gene. Genotyping for the deletion of the *Pdgfr-β* gene was performed at 10 weeks of age, as described by Ishii et al. [35]. The mice were given free access to laboratory pellet chow and water and were exposed to a 12 h light–dark cycle. All the procedures were performed according to the Institutional Animal Care and Use Committee guidelines of the University of Toyama, which follows 'Principles of laboratory animal care' (NIH publication no. 85-23).

PCR genotyping of renal cortex Genomic DNA was prepared from mouse kidney using DNeasy Mini Kit (Qiagen, Tokyo, Japan), and the genotyping of the recombinant allele of *Pdgfr-β* was performed using a PCR-based analysis, as described by Ishii et al. [35]. The primer sequences for genomic PCR of the *Pdgfr-β*$^{flox/flox}$ allele are shown in ESM Table 1 as primers 1–3. The PCR products represent the floxed allele (329 bp) and the Cre-mediated recombined allele (deleted allele; 410 bp).

Quantitative RT-PCR Total RNA (500 ng) extracted from the renal cortex was reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). The primers for *Pdgfr-β* and β-actin are shown in ESM Table 1 as *Pdgfr-β* (forward), *Pdgfr-β* (reverse), β-actin (forward) and β-actin (reverse). The reactions were performed in an Mx3000P system using a SYBR Premix Ex Taq (Perfect Real Time) (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. The expression of each mRNA sample was normalised to the values for β-actin mRNA.

Characterisation of chemical variables The levels of blood glucose and HbA_{1c} were measured using Accu-Chek Compact (Roche Diagnostics, Basel, Switzerland) and a DCA2000 analyser (Siemens Healthcare Diagnostics, Deerfield, IL, USA), respectively. Urinary albumin and creatinine levels were measured using an ELISA kit (Albuwell M; Exocell, Philadelphia, PA, USA) and a creatinine assay kit (Creatinine Companion; Exocell). Blood urea nitrogen (BUN) concentrations and serum creatinine levels were measured using BUN and creatinine assay kits (BUN Kainos and CRE Kainos; Kainos Laboratory, Tokyo, Japan), respectively. Urinary 8-hydroxydeoxyguanosine (8-OHdG) levels were determined using a commercial assay kit (New 8-OHdG Check; Nikken Seil, Shizuoka, Japan).

Blood pressure measurement Mice were placed in plastic restrainers. A cuff with a pneumatic pulse sensor was attached to the tail. Blood pressure was recorded on a Model MK-2000 (Muromachi Kikai, Tokyo, Japan) without heating. Data were averaged from at least three consecutive readings obtained from each mouse.

Preparation of kidney samples Mice were deeply anaesthetised using an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). After perfusion with ice-cold PBS solution, the left kidney was excised, decapsulated and weighed. For the isolation of mRNA and genomic DNA, the cortex of the kidney was cut into 3 mm cubes and immediately frozen in liquid nitrogen. The right kidney was perfused with 4% paraformaldehyde (PFA), excised, decapsulated and immersed in 4% PFA. For the immunohistochemical study and morphometric analysis, the kidneys were embedded in paraffin and sliced into 4 μm thick sections. The sections were then routinely stained with haematoxylin and eosin and periodic acid–Schiff's reagent (PAS).

Immunohistochemistry for PDGFR-β and manganese superoxide dismutase After deparaffinisation and rehydration of the glass-mounted tissue sections, antigen retrieval was performed using heat treatment with a target retrieval solution (pH 9.0; Dako, Carpinteria, CA, USA) for PDGFR-β staining. We handled the sections with Dako Pascal in 10 mmol/l of EDTA (pH 8.0) in an incubation container for 4 min. The sections were treated with 3% H_2O_2 containing 0.1% sodium azide and with Protein Block, Serum-Free (Dako). After washing in Tris-buffered NaCl containing 0.05% Tween 20, the sections were incubated with a goat polyclonal anti-PDGFR-β antibody (diluted 1:1,000; R&D Systems, Minneapolis, MN, USA) or a rabbit polyclonal manganese superoxide dismutase (MnSOD) antibody (diluted 1:500; Stressgen, Victoria, BC,

Canada) overnight at 4°C. After washing, the sections were incubated with Histofine Simple Stain MAX-PO (G) or Histofine Simple Stain MAX-PO (R) (Nichirei Bioscience, Tokyo, Japan) for 10 min at room temperature. Diaminobenzidine was used as a chromogen for the visualisation of the antigen localisation. The amount of PDGFR-β in glomeruli was quantified using BZ-9000 and BZ-H1C system (Keyence, Osaka, Japan).

Morphometric analysis of the glomeruli Sections were stained with haematoxylin and eosin or PAS using conventional methods. For the morphometric analysis of the glomeruli, sections stained with PAS were used as described by Nakagawa et al. [29]. In each animal, 20 glomeruli cut at the vascular pole were analysed. The glomerular area was traced along with the outline of a capillary loop using a computer-assisted colour image analyser (VH analyser; Keyence). The mesangial areas were also colour extracted and calculated using the VH analyser.

Western blotting Western blotting was performed as described by Yamazaki et al. [36]. Briefly, renal cortex was removed and homogenised in lysis buffer. Lysates were centrifuged to remove the insoluble materials. Western blotting of the lysates was performed using anti-NAD(P)H oxidase 4 (NOX4) antibody (Santa Cruz, Santa Cruz, CA, USA), anti-glutathione peroxidase 1 (GPX1) antibody (Santa Cruz), anti-MnSOD antibody (Santa Cruz) and anti-β-actin antibody (Cell Signaling, Danvers, MA, USA).

Statistical analyses Statistical analyses were performed using an ANOVA followed by the Tukey test. Data were expressed as the means±SE. $p<0.05$ was considered statistically significant.

Results

Pdgfr-β gene was deleted efficiently in the renal cortex of Pdgfr-β conditional knockout mice In the present study, we examined the following four mouse strains with a CD-1 background: CaMKIIα$^{+/+}$–actin-Cre-ER$^{TM-/-}$–*Pdgfr-β*$^{flox/flox}$ mice (WT); CaMKIIα$^{+/+}$–actin-Cre-ER$^{TM+/-}$–*Pdgfr-β*$^{flox/flox}$ mice (KO); CaMKIIα$^{Tg/+}$–actin-Cre-ER$^{TM-/-}$–*Pdgfr-β*$^{flox/flox}$

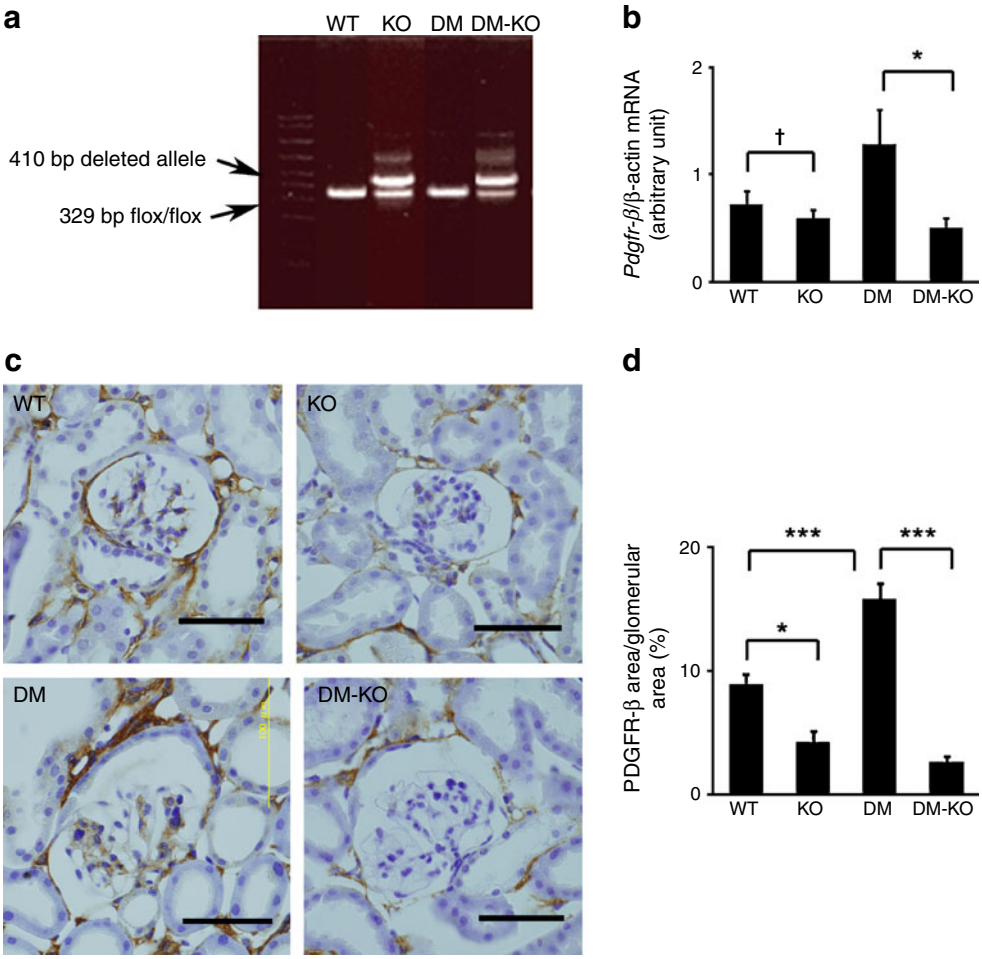

Fig. 1 The *Pdgfr-β* gene was efficiently deleted in the renal cortex of conditional *Pdgfr-β* knockout mice. The expression of *Pdgfr-β* is shown in WT, KO, DM and DM-KO mice at 10 weeks of age (**a**) or at 16 weeks of age (**b**–**d**). **a** Genomic DNA expression of *Pdgfr-β* in the renal cortex of mice. **b** mRNA expression of *Pdgfr-β* in the renal cortex of mice. The mRNA levels are normalised by β-actin. †$p=0.89$ for WT vs KO mice. **c** Immunohistochemistry of kidneys with anti-PDGFR-β antibody. Scale bars, 50 μm. **d** Amount of PDGFR-β protein in glomeruli. Data shown are the means±SE of eight mice per group. *$p<0.05$; ***$p<0.001$

mice (DM); and CaMKIIα$^{Tg/+}$–actin-Cre-ER$^{TM+/-}$–*Pdgfr-β*$^{flox/flox}$ mice (DM-KO). All four strains of mice were similarly treated with tamoxifen at 4 weeks of age. At 10 weeks of age, genomic PCR for *Pdgfr-β* was performed (Fig. 1a). The Cre-mediated recombined allele of *Pdgfr-β* (deleted allele) was detected in KO and DM-KO mice but not in WT or DM mice. A single 329 bp band corresponding to the floxed allele was observed in the renal cortex of both WT and DM mice. The same PCR generated predominantly 410 bp bands corresponding to the deleted allele, which was not 100% but ~90% of the total, and faint 329 bp bands in the renal cortex of KO and DM-KO mice. At 16 weeks of age, the *Pdgfr-β* mRNA in the kidney cortex was increased by 1.8-fold in the DM mice compared with the WT mice. The level was significantly lower in the DM-KO mice than in the DM mice. The mRNA level in the KO mice tended to be lower than in the WT, but the difference was not statistically significant (Fig. 1b). The amount of PDGFR-β protein was evaluated by immunostaining of the kidney using anti-PDGFR-β antibody (Fig. 1c, d). Immunostaining of the kidneys demonstrated that the production of PDGFR-β proteins in the mesangium of the glomeruli and tubules (Fig. 1c), which was increased in the DM mice compared with the WT mice, was also significantly reduced in the kidneys of the KO and DM-KO mice (Fig. 1c, d). The deletion of the *Pdgfr-β* gene and PDGFR-β protein was also observed at 10 weeks of age, similar to the results observed at 16 weeks of age (data not shown). The following experiments were performed at 10 and 16 weeks of age. Some histological evaluation and experiments on oxidative stress were only performed at 16 weeks of age.

Deletion of Pdgfr-β did not affect body weight, blood glucose levels or blood pressure in either normoglycaemic control mice or diabetic CaMKIIα (Thr286Asp) transgenic mice Before studying the roles of PDGFR-β signalling in the pathogenesis of diabetic nephropathy, the effects of its deletion on body weight changes, blood glucose levels and blood pressure were examined. The body weight of DM mice was lower than that of WT mice from 4 weeks of age. Postnatal deletion of *Pdgfr-β* decreased body weight at several time points in normoglycaemic mice, but did not affect it in diabetic mice (Fig. 2a). The blood glucose levels of the DM mice increased markedly from 3 weeks of age, and reached a level higher than 44.4 mmol/l thereafter. The *Pdgfr-β* deletion did not affect the blood glucose levels in either the normoglycaemic or diabetic mice (Fig. 2b). The HbA_{1c} levels were also significantly higher in the DM and DM-KO mice than in the WT and KO mice at 10 weeks (data not shown) and 16 weeks of age. No significant difference in the HbA_{1c} levels was observed between the mice with or without *Pdgfr-β* deletion (Fig. 2c). Systolic

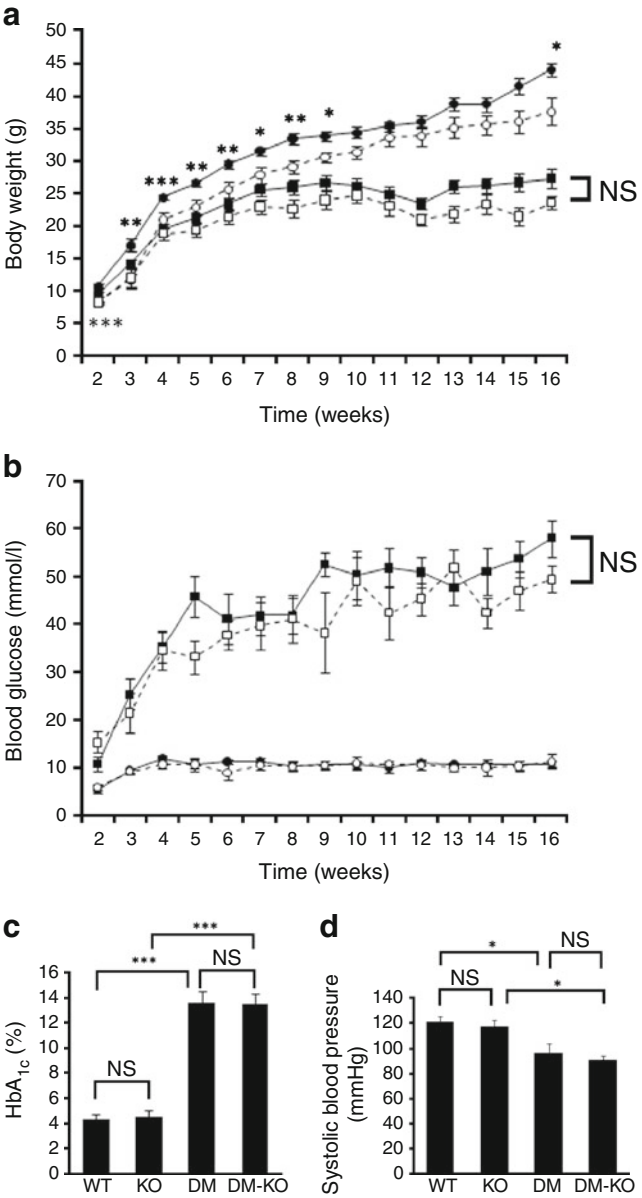

Fig. 2 Deletion of *Pdgfr-β* did not affect body weight, glucose levels or blood pressure in normoglycaemic or diabetic mice. Graphs show body weight (**a**), blood glucose levels (**b**), HbA_{1c} levels (**c**) and blood pressure (**d**) at 16 weeks of age. Black circles, white circles, black squares and white squares are WT, KO, DM and DM-KO mice, respectively. Data shown are the means±SE of six to eight mice per group. *$p<0.05$; **$p<0.01$; ***$p<0.001$. The comparison in (**a**) is WT vs KO. To convert values for HbA_{1c} in per cent to millimoles per mole, subtract 2.15 and multiply by 10.929

blood pressure at 16 weeks of age was significantly lower in DM and DM-KO mice than in WT and KO mice, respectively. The *Pdgfr-β* deletion did not affect the blood pressure in those mice (Fig. 2d).

Deletion of Pdgfr-β decreased the urinary albumin/creatinine ratio in diabetic CaMKIIα (Thr286Asp) transgenic mice To clarify the roles of PDGFR-β signalling in

the development of diabetic nephropathy, we next examined the effects of *Pdgfr-β* deletion on urinary albumin excretion and the serum BUN or creatinine levels. The urinary albumin to creatinine ratio was higher in the DM mice than in the WT mice at 10 and 16 weeks of age. The deletion of *Pdgfr-β* did not affect the ratio at 10 weeks of age but, interestingly, it decreased the ratio significantly at 16 weeks of age (Fig. 3a). The serum BUN and creatinine levels were not altered between the WT and DM mice at either 10 weeks (data not shown) or 16 weeks of age, suggesting that DM mice up to 16 weeks of age can be used as a model of early-stage diabetic nephropathy (Fig. 3b, c). No significant differences in the serum BUN and creatinine levels were observed between the WT and KO mice or the DM and DM-KO mice at 10 weeks (data not shown) and 16 weeks of age (Fig. 3b, c). These results suggest that the activation of PDGFR-β signalling is involved in the development of early-stage diabetic nephropathy.

Deletion of Pdgfr-β improved the pathological changes in glomeruli observed in diabetic CaMKIIα (Thr286Asp) transgenic mice We next examined the morphological changes in the kidneys of 16-week-old mice. The kidney weight/body weight ratio in DM and DM-KO mice was significantly higher than that in age-matched WT and KO mice (Fig. 4a). The deletion of *Pdgfr-β* did not affect the ratio in either the normoglycaemic or the diabetic mice. Microscopic examinations demonstrated that the glomeruli of DM mice were significantly larger than those of WT mice. They frequently showed the sclerotic changes of glomeruli, such as segmental changes (arrows in Fig. 4b), at 16 weeks of age. The glomerular sclerosis was not apparent in WT and KO mice, and nor was it apparent in DM-KO mice (Fig. 4b). Glomerular area and the mesangial area to glomerular area ratio were small in WT and KO mice at 16 weeks, and the difference was not significant between the two strains (Fig. 4c, d). These variables were significantly higher in DM than in WT and KO mice. The glomerular area in DM-KO mice was slightly lower than in DM mice, but the difference was not significant (Fig. 4c). The mesangial area to glomerular area ratio in DM-KO mice was significantly lower than in DM mice (Fig. 4d).

Deletion of Pdgfr-β decreased oxidative stress markers in diabetic CaMKIIα (Thr286Asp) transgenic mice We finally examined the effects of *Pdgfr-β* deletion on the levels of oxidative stress at 16 weeks of age. The urinary 8-OHdG level was significantly higher in DM mice than in WT mice, and was significantly lower in DM-KO mice than in DM mice (Fig. 5a). Western blot analysis revealed that the production of NOX4, a renal homologue of NAD(P)H oxidase, in the renal cortex tended to be upregulated in DM mice compared with WT mice, and was significantly downregulated in DM-KO mice compared with DM mice (Fig. 5b). Similarly, the production of GPX1 and MnSOD, antioxidant enzymes, in the renal cortex was upregulated in DM mice and downregulated in DM-KO mice (Fig. 5b). Finally, immunohistochemistry of MnSOD demonstrated the positive immunoreaction products that were distributed in the outer medulla in the kidney of DM mice, but significant staining was not detected in the kidneys of the other three strains of mice (Fig. 5c).

Discussion

PDGFR-β and its ligand the PDGF-B chain are reportedly increased in diabetic nephropathy, and data are accumulat-

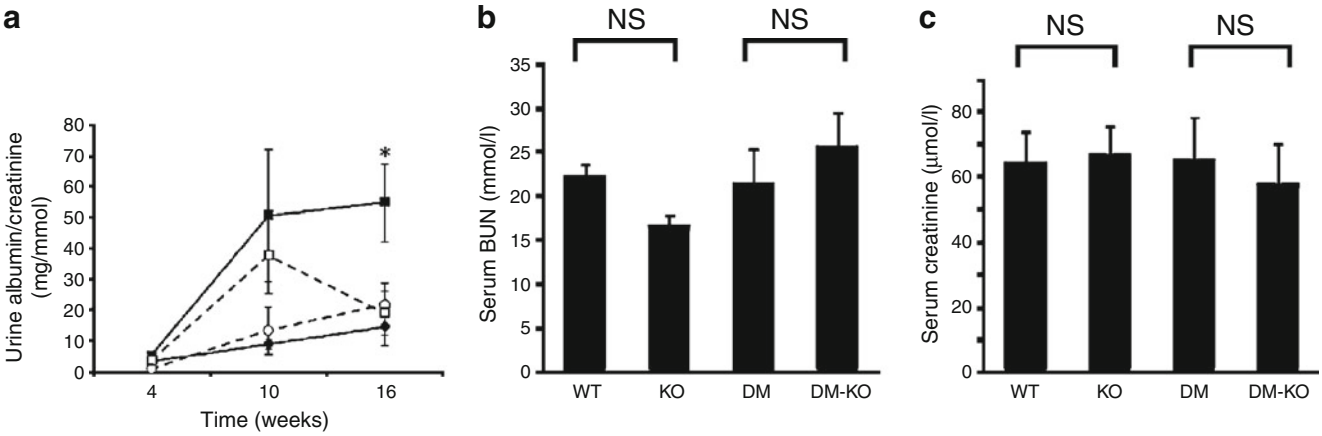

Fig. 3 Deletion of *Pdgfr-β* decreased urinary albumin/creatinine ratio in diabetic mice. **a** Urinary albumin/creatinine ratio at the indicated weeks of age. Black circles, white circles, black squares and white squares are WT, KO, DM and DM-KO mice, respectively. **b** Serum BUN levels at 16 weeks of age. **c** Serum creatinine levels at 16 weeks of age. Data shown are the means±SE of six to nine mice per group. *$p<0.05$, WT vs KO

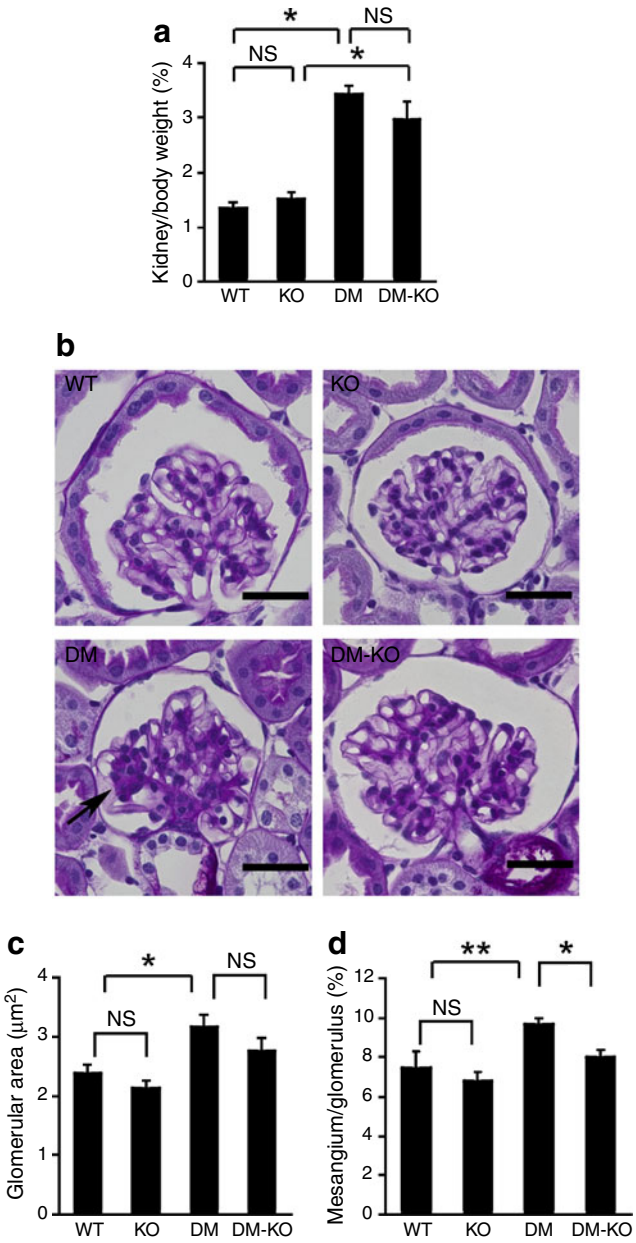

Fig. 4 Deletion of *Pdgfr-β* improved the pathological changes in the glomeruli observed in diabetic mice. **a** Kidney weight/body weight ratio. **b** Representative PAS staining of glomeruli. The arrow indicates a segmental lesion of the glomerulus; scale bars, 20 μm. **c** Glomerular area. **d** Mesangial area/glomerular area ratio at 16 weeks of age. Data shown are the means±SE of six to eight mice per group. *$p<0.05$; **$p<0.01$

ing to support their roles in the pathogenesis of the disease. However, the role of PDGFR-β signalling remains hypothetical because of the lack of specific inhibitor and/or the chronic nature of the disease. To investigate the role of PDGFR-β signalling in diabetic nephropathy, we induced *Pdgfr-β* gene deletion postnatally in diabetic CaMKIIα transgenic mice. Our present study suggests that enhanced PDGFR-β signalling plays important roles in the development of diabetic nephropathy in vivo and that increased oxidative stress is involved in this process.

In this study, we administered tamoxifen at 4 weeks of age to delete the *Pdgfr-β* gene. Blood glucose levels started to increase at just before 4 weeks of age, but no signs of diabetic nephropathy were observed at 4 weeks of age. Accordingly, the preventive effects observed in the current study may be due to the inhibition of PDGFR-β signalling during the early phase of diabetic nephropathy. These results are consistent with those of several recent studies, such as the reported increase in PDGFR-β during the early phase of diabetic nephropathy [10, 37]. Several chemical inhibitors of PDGF signalling, such as imatinib, have been shown to be effective for preventing diabetic nephropathy only when administered during the early phase of diabetic nephropathy development [22]. It is of note that albuminuria was improved at 16 weeks but not at 10 weeks of age in DM-KO mice (Fig. 3a). This may imply that PDGFR-β signalling is involved in the progress as well as initiation of the disease.

Deletion of the *Pdgfr-β* gene partially decreased the urinary albumin excretion and improved the pathological changes in the glomeruli. We speculated that the reasons for the partial, but not total, improvement in the diabetic changes were as follows. First, *Pdgfr-β* knockout efficiency was not 100% in our Cre-loxP system (e.g. PDGFR-β protein was deleted by 80–90%, as shown in Fig. 1c, d). This is not unusual in the system because the knockout efficiency could be affected by many factors including local concentration of tamoxifen, promoter usage for the Cre expression and so on. Those cells that escaped from gene deletion might have partly compensated for the expected phenotype. Second, the expression of the PDGF-α receptor gene remains intact even after the induction of *Pdgfr-β* deletion in our conditional knockout model. PDGF-C, which is a specific ligand for PDGFR-α, is reportedly upregulated in kidney glomeruli in a number of renal diseases and experimental glomerulonephritis [38, 39]. Thus, PDGFR-α signalling might also be one of the candidate systems compensating for *Pdgfr-β* deletion in diabetic nephropathy. Third, numerous complex mechanisms explaining how hyperglycaemia induces diabetic nephropathy have been reported other than the enhancement of PDGF signalling [40, 41]. Thus, even if the *Pdgfr-β* gene was totally deleted, the development of diabetic nephropathy may not be completely prevented.

In this study, the systemic *Pdgfr-β* gene deletion did not affect blood glucose (Fig. 2b, c) or blood pressure levels (Fig. 2d). These results indicate that *Pdgfr-β* deletion did not ameliorate the diabetic nephropathy through the indirect mechanisms of alterations in blood glucose or blood pressure. In contrast, *Pdgfr-β* deletion ameliorated the augmented urinary 8-OHdG levels, an oxidative stress marker of the kidney in diabetic mice (Fig. 5a). Recent

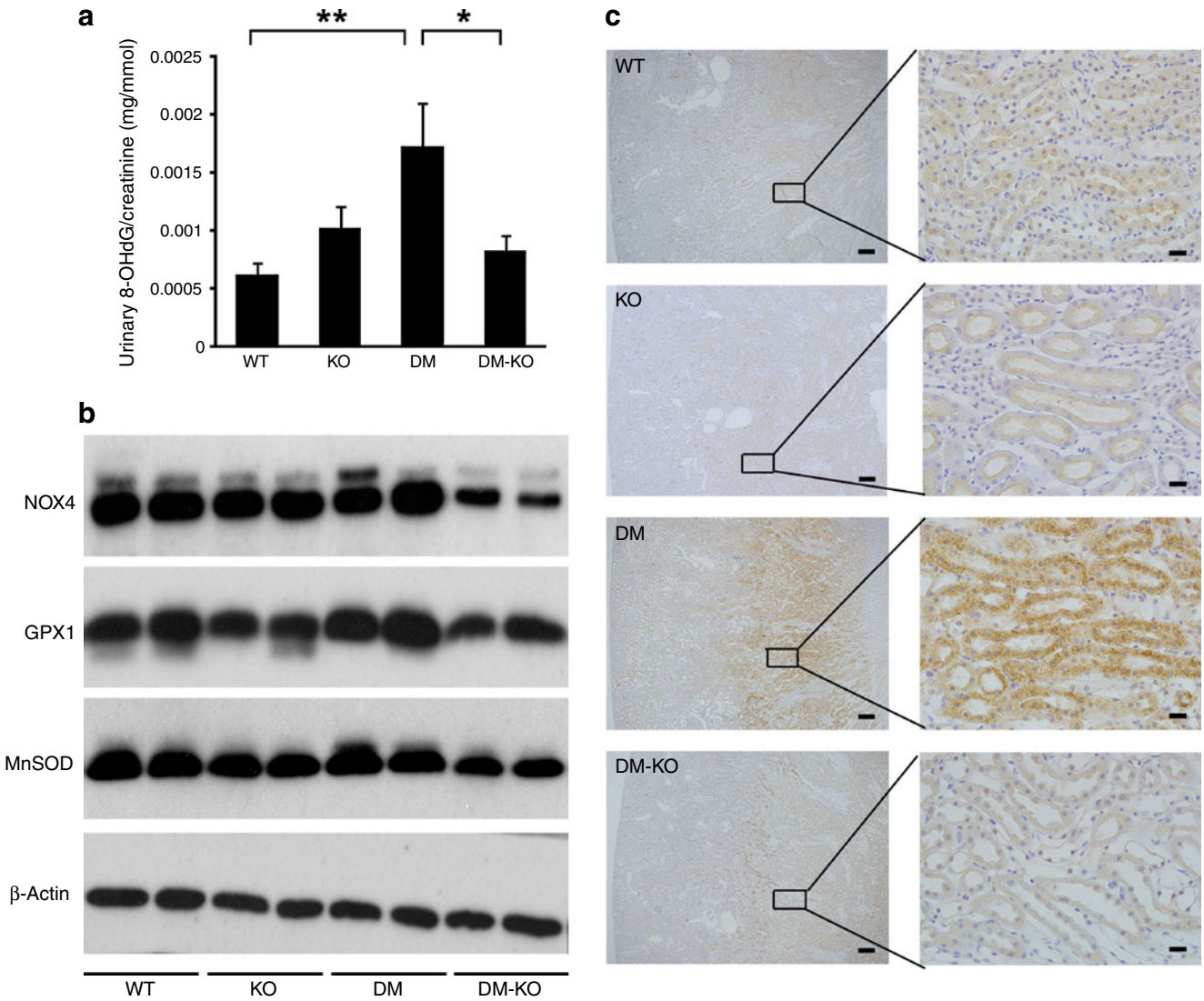

Fig. 5 Deletion of *Pdgfr-β* decreased oxidative stress markers in diabetic mice. **a** Urinary 8-OHdG levels. **b** Representative images of Western blotting of renal cortex with anti-NOX4, anti-GPX1, anti-MnSOD and anti-β-actin antibodies. **c** Representative immunostaining of the kidney with anti-MnSOD antibody at 16 weeks of age. Data shown are the means±SE of six mice per group. *$p<0.05$; **$p<0.01$. Scale bars, 200 μm or 20 μm (inset)

clinical and experimental data have shown that albuminuria and urinary excretion of 8-OHdG are well correlated, and oxidative stress on the podocytes is assumed to induce albuminuria [42, 43]. Thus, the decreased oxidative stress in the kidney may be related to the ameliorated albuminuria in the *Pdgfr-β*-deleted diabetic mice. The production of antioxidant enzymes, including GPX1 and MnSOD, was also upregulated in the renal cortex of DM mice and downregulated in DM-KO mice in accordance with the level of urinary 8-OHdG (Fig. 5b, c). These results suggest that inhibition of PDGFR-β signalling did not decrease oxidative stress through the upregulation of such antioxidant enzymes. Importantly, the production of NOX4, a renal homologue of NAD(P)H oxidase, tended to be upregulated in DM mice and significantly downregulated in DM-KO mice (Fig. 5b, d). It is reported that NOX4 is activated by PDGF-B followed by the generation of reactive oxygen species [44, 45]. Inhibition of NOX4 ameliorates diabetic nephropathy with decreasing NAD(P)H-dependent ROS generation [46, 47]. These reports suggest that downregulation of NOX4 may be involved, at least partly, in the improvement of diabetic nephropathy observed in DM-KO mice. It will be of future interest to clarify how PDGF signalling is involved in the production and activation of this enzyme and how it contributes to the progress of diabetic nephropathy in our mouse model. It is also very important to investigate whether the beneficial effects of *Pdgfr-β* deletion are also observed in different animal models of diabetes, especially in

the obese and type 2 animal models of diabetes, such as *db/db* mice.

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