Inducible Overexpression of sFlt-1 in Podocytes Ameliorates Glomerulopathy in Diabetic Mice

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OBJECTIVE—Podocyte-specific, doxycycline (DOX)-inducible overexpression of soluble vascular endothelial growth factor (VEGF) receptor-1 (sFlt-1) in adult mice was used to investigate the role of the VEGF-A/VEGF receptor (VEGFR) system in diabetic glomerulopathy.

RESEARCH DESIGN AND METHODS—We studied nondiabetic and diabetic transgenic mice and wild-type controls treated with vehicle (VEH) or DOX for 10 weeks. Glycemia was measured by a glucose-oxidase method and blood pressure by a noninvasive technique. sFlt-1, VEGF-A, VEGFR2, and nephrin protein expression in renal cortex were determined by Western immunoblotting; urine sFlt-1, urine free VEGF-A, and albuminuria by enzyme-linked immunosorbent assay; glomerular ultrastructure by electron microscopy; and VEGFR1 and VEGFR2 cellular localization with Immunogold techniques.

RESULTS—Nondiabetic DOX-treated transgenic mice showed a twofold increase in cortex sFlt-1 expression and a fourfold increase in sFlt-1 urine excretion ($P < 0.001$). Urine free VEGF-A was decreased by 50%, and cortex VEGF-A expression was upregulated by 30% ($P < 0.04$). VEGFR2 expression was unchanged, whereas its activation was reduced in DOX-treated transgenic mice ($P < 0.02$). Albuminuria and glomerular morphology were similar among groups. DOX-treated transgenic diabetic mice showed a 60% increase in 24-h urine sFlt-1 excretion and an ~70% decrease in urine free VEGF-A compared with VEH-treated diabetic mice ($P < 0.04$) and had lower urine albumin excretion at 10 weeks than VEH-treated diabetic (D) mice: D-VEH vs. D-DOX, geometric mean (95% CI), 117.5 (69–199) vs. 43 (26.8–69) μg/24 h ($P = 0.003$). Diabetes-induced mesangial expansion, glomerular basement membrane thickening, podocyte foot-process fusion, and transforming growth factor-β1 expression were ameliorated in DOX-treated diabetic animals ($P < 0.05$). Diabetes-induced VEGF-A and nephrin expression were not affected in DOX-treated mice.

CONCLUSIONS—Podocyte-specific sFlt-1 overexpression ameliorates diabetic glomerular injury, implicating VEGF-A in the pathogenesis of this complication. ***Diabetes*** **57:2824–2833, 2008**

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Received 15 May 2008 and accepted 12 July 2008.

Published ahead of print at http://diabetes.diabetesjournals.org on 22 July 2008. DOI: 10.2337/db08-0647.

Vascular endothelial growth factor (VEGF)-A is constitutively expressed in glomerular visceral cells (podocytes). Paracrine VEGF-A signaling occurs between podocytes and adjacent endothelial and mesangial cells, which express VEGF receptors (VEGFRs) 1 and 2 (1–3), and both autocrine and paracrine signaling may occur in podocytes themselves (4).

VEGF-A has been implicated in the regulation of glomerular barrier properties to protein filtration. In normal animals (5,6) and in cancer patients (7), inhibition of VEGF-A results in proteinuria; while in proteinuric conditions, which are associated with glomerular VEGF-A upregulation such as diabetes, systemic inhibition of VEGF ameliorates albuminuria (8–10). This evidence suggests that a tight regulation of VEGF-A expression level is required to maintain the physiological permselective properties of the glomerular filter.

The results of previous studies conducted using either VEGF gene targeting techniques (5) or by administration of inhibitory agents of the VEGF/VEGFR system such as antibodies (6,8,9) or chemicals (10) may have been affected by potential interference with animal organ development and lack of tissue specificity in the mechanisms of action of systemic inhibitors. Soluble VEGF receptor-1 (sFlt-1), a splice variant of the VEGFR1, lacks the transmembrane and complete intracellular tyrosine kinase domain of VEGFR1 but binds to VEGF with the same affinity and specificity as that of the full-length receptor (11,12) and has potent and selective VEGF inhibitory action (11). sFlt-1 acts in two major ways: it can sequester VEGF competing for its binding to the VEGF receptors or can form heterodimers with the extracellular region of the membrane spanning VEGFR1 and VEGFR2, thus inhibiting the activation of downstream signaling pathways (11,12).

To target the action of the podocyte-expressed VEGF-A, we developed a transgenic mouse model to overexpress sFlt-1 specifically at the podocyte level with an inducible expression system that is induced only after complete development, in the adult animal, by the administration of doxycycline (*Tet-on*). The aim of this study was to investigate the role of VEGF-A upregulation in the pathogenesis of diabetic glomerulopathy by locally inhibiting podocyte-expressed VEGF-A activity.

RESEARCH DESIGN AND METHODS

All materials and chemicals were purchased from Sigma-Aldrich (Dorset, U.K.). Restriction endonucleases were obtained from Fermentas (St. Leon-Rot, Germany), and the DNA ligase kit was from Roche Applied Science (Sussex, U.K.).

Generation of transgenic animals. Construct generation (tetracycline reponsive element [TRE] construct): Human sFlt-1 cDNA was obtained from the plasmid pbb-sFlt-1 (gift from Dr. K.A. Thomas) (12) by *Bam*HI restriction endonuclease digestion. The obtained cDNA was then cloned into the plasmid

pBI-G Tet-Vector (GenBank accession no. U89933) (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France). The resulting plasmid, pTRE bidirectional LacZ/sFlt-1, was studied for the presence and orientation of the insert (sFlt-1 cDNA) in the final vector by restriction endonuclease digestion. The ~8-kb AsnI-AsnI DNA fragment was utilized for microinjection and generation of transgenic animals.

Generation of transgenic mice. Transgenic mice were generated in the Transgenic Facility of the Medical Research Council, Imperial College London.

Animals were kept according to the "Guidelines on the Use of Animals in Research," and the number of utilized animals was kept to a minimum. Mice were housed in a pathogen-free environment at 21°C, with a 12-h light-dark cycle, and all received a standard laboratory animal diet (Beekey Feeds) and water ad libitum.

Animal genotyping. Transgene genomic integration was initially studied using standard Southern blotting technique. Subsequently, mice were genotyped by PCR as described (13). For the "pTRE bidirectional LacZ/sFlt-1" plasmid, we used the following set of primers recognizing the 3′-sv40 poly-A DNA sequence: sense 5′-ACCTATAAAAATAGGCGTATCACGA-3′, antisense 5′-TGGCTGATTATGATCCTGCA-3′. The genotyping for the podocin-rtTA was also studied using PCR, as previously described (14).

Induction of podocyte sFlt-1 overexpression in transgenic mice. Experiments were conducted in mice obtained from the breeding of homozygous Podocin-rtTA mice (Pod/Pod) with heterozygous pTRE-LacZ/sFlt-1 (sFlt-1/+) mice in order to obtain litters containing animals heterozygous for both constructs (Pod/sFlt-1). Expression of sFlt-1 was induced in mice by the administration of doxycycline (DOX) (2 mg/ml) in the drinking water as previously shown (13). Water was supplemented with sucrose (5% wt/vol) (abbreviated as vehicle [VEH]) to enhance palatability. Doxycycline was replaced every 3rd day and protected from light at all time. Controls were given VEH alone.

X-gal staining. Standard histochemical detection of nuclear β-galactosidase activity was determined as previously described (13).

sFlt-1 expression. sFlt-1 protein levels were assessed by Western immunoblotting techniques in kidney cortex lysate and by enzyme-linked immunosorbent assay (ELISA) in 24-h urine collection.

Experimental design and determinations. Experiments were conducted in 8-week-old nondiabetic or diabetic control mice (Pod/+, only positive for the Podocin-rtTA transgene) and double heterozygote Pod/sFlt-1 mice either administered VEH or treated with DOX for up to 10 weeks.

Twenty four–hour urine collections for each animal (in individual metabolic cages) were made at baseline (8-week-old mice, before DOX administration), between 6 and 8 weeks later, and then 10 weeks thereafter. At the end of the study, mice were weighed and killed, and the cortex was isolated for protein molecular determinations and electron microscopy studies.

Streptozotocin-induced diabetes in transgenic mice. Diabetes was induced in ~5-week-old mice with daily intraperitoneal streptozotocin injections for 5 days as previously described (15). Control mice were injected with citrate buffer only. After 2 weeks, diabetes was verified by blood glucose determination with the glucose oxidase method. Mice with a glycemia <22 mmol/l were not included in the study.

Blood pressure. Systolic blood pressure was measured from the mouse tail with a CODA noninvasive pletismography blood pressure transducer (Kent Scientific).

Creatinine clearance. Blood samples were collected using heparinized tubes via cardiac puncture at the time of killing of the animals. Urine was collected as detailed above. Plasma and urine creatinine concentration was determined by isotope dilution electrospray mass spectrometry (16). Creatinine clearance ($\mu l \cdot min^{-1} \cdot g^{-1}$) was estimated as urinary creatinine × urine volume × 1,440 min^{-1} × plasma $creatinine^{-1}$ × body wt $(g)^{-1}$.

Urine albumin excretion. Urine volume was recorded and aliquots (1 ml) were stored at −80°C for subsequent analysis. Albumin concentration in urine was measured in triplicate by ELISA using an anti-mouse albumin antibody (Bethyl Laboratories, Montgomery, TX). Urine albumin excretion was expressed as the 24-h albumin excretion rate (in micrograms per 24 h).

Urine free VEGF-A and sFlt-1 levels. Urine samples were collected and immediately centrifuged at 13,000*g* for 5 min, then stored at −80°C. A commercial ELISA kit was used to measure free VEGF-A165 (R&D Systems Europe, Abingdon, U.K.). This assay specifically measures unbound VEGF-A and does not crossreact with VEGF-A bound to sFlt-1. Another ELISA was used to specifically measure sFlt-1 (RELIATech, Braunschweig, Germany) (17,18). For both determinations, assays were conducted in duplicate and results expressed as picograms per 24 h for VEGF-A165 and as nanograms per 24 h for sFlt-1.

Western blotting. The following antibodies (and dilutions) were used: rabbit monoclonal anti–sFlt-1 (1:500) (Sigma-Aldrich), rabbit polyclonal anti-nephrin intracellular domain (1:500) (donated by H. Holthofer, Helsinki University, Finland), goat anti-mouse VEGF-A (1:250) (R&D Systems), rat anti-VEGFR2 (1:250) (R&D Systems), rabbit anti-VEGFR2[pY (951)] (1:500) (BioSource, Nivelles, Belgium), rabbit polyclonal anti–transforming growth factor (TGF)β-receptor2 (1:400) (Santa Cruz Biotech, Santa Cruz, CA), rabbit polyclonal anti-TGFβ1 (1:400) (Santa Cruz Biotech), and anti–β-actin (1:5,000) (Sigma-Aldrich).

Pieces of renal cortex were lysed in modified RIPA buffer containing protease and phosphatase inhibitor cocktail (Sigma-Aldrich). Equal amounts of total protein lysate (40–200 μg) were run on 7.5 or 10% SDS-polyacrylamide denaturing gels and transferred to nitrocellulose membranes (Amersham Biosciences, Little Chalfont, U.K.). The membranes were incubated in 5% nonfat milk or 3% bovine serum albumin and 0.1% Tween in PBS for 3 h at room temperature for sFlt-1, VEGF-A, nephrin, VEGFR2, TGFβ-receptor2, TGFβ1, β-actin, and VEGFR2[pY (951)] antibodies. Membranes were subsequently probed with specific horseradish peroxidase–conjugated secondary antibodies (1:5,000–10,000) (Dako) for 1 h at room temperature. Bands were revealed using an enhanced chemiluminescence kit (Amersham Biosciences). Quantification of the immunoreactive bands was performed using densitometric scanning with image software (Image J; National Institute of Health, Bethesda, MD).

Glomerular morphology. Mesangial volume fraction, as an index of glomerular extracellular matrix deposition, glomerular basement membrane (GBM) thickening, podocyte foot process width (FPW), and glomerular capillaries endothelial fenestrae were studied with electron microscopy techniques as previously described (19).

Glomerular cell VEGF receptor expression. The Immunogold electron microscopy technique was used to study the presence of VEGF receptors on glomerular cells in both nondiabetic and diabetic mice. Pieces of cortical tissue 1 mm^3 in size were fixed in 2% paraformaldehyde for 4 h, dehydrated in alcohol, and embedded in LR-white resin (TAAB). Ultrathin sections were taken and mounted on carbon-coated nickel grids. The grids were first immersed in 80 mmol/l ammonium chloride in PBS (10 min), then in PBS containing 0.2 mol/l glycine and 0.5% BSA (10 min), followed by 10% normal rat serum for 30 min, all at room temperature. The grids were then incubated with primary antibody (rabbit anti-mouse VEGFR1 and VEGFR2; Abcam) diluted 1:100 in PBS/0.5% BSA overnight at 4°C. After washing in PBS/0.5% BSA, the grids were incubated with 10 nm gold-conjugated goat anti-rabbit IgG diluted 1:20 in PBS/0.5% BSA for 1 h at room temperature. The primary antibody was omitted in negative controls. After washing, the grids were stained with 2% aqueous uranyl acetate and examined by transmission electron microscopy.

Statistics. Results are expressed as the mean ± SEM unless otherwise stated. Data for albuminuria were log transformed before analysis. Among-group comparisons were performed by ANOVA followed by post hoc analysis for between-group differences using the least significant difference test. $P \leq 0.05$ was considered statistically significant.

RESULTS

Generation of transgenic mice. We obtained eight positive founders for the pTRE bidirectional LacZ/sFlt-1 construct. Seven of eight founders transmitted the transgene to the offspring. Mice derived from the F1 generation were then bred with FVB-N mice homozygous for the construct podocin-rtTA (gift from Dr. J. Kopp, Bethesda, MD) expressing the rtTA specifically in podocytes (14). First-generation offspring carrying either both transgenes or podocin rtTA only (mixed background BL6-CBA/FVB-N, abbreviated to Pod/sFlt1 and Pod/+ as controls) were studied for DOX-inducible transgene expression. Of these seven remaining lines, one showed a DOX-inducible LacZ (nuclear localization) expression in podocytes with no basal leaky expression of the transgene and was subsequently used in all experiments.

Role of podocyte sFlt-1 overexpression in adult control, nondiabetic mice. Eight-week-old adult double transgenic mice (Pod/sFlt-1) or single transgenic mice (Pod/+) were treated with DOX or administered VEH for up to 10 weeks (both sexes showed a similar phenotype and were analyzed together). X-gal staining revealed no signal in *Pod/+* DOX and VEH kidneys, confirming that the staining protocol did not detect endogenous galactosidase activity (not shown). Furthermore, *Pod/sFlt-1* mice admin-

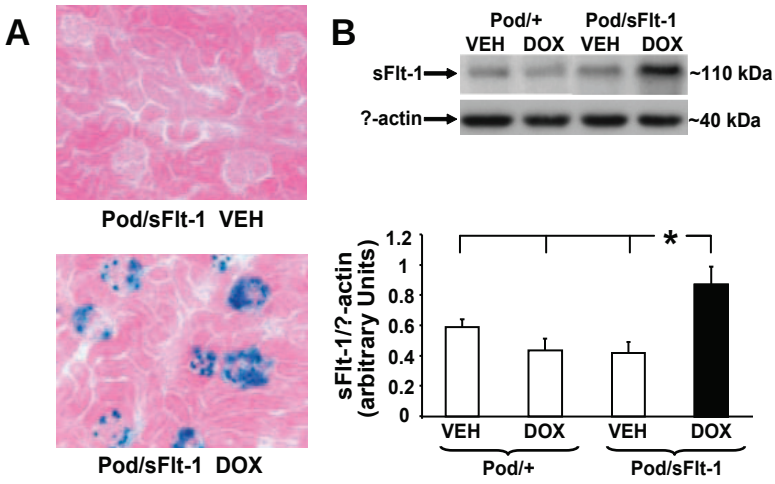

FIG. 1. Podocyte-inducible overexpression of LacZ/sFlt-1. *A*: Nuclear LacZ staining of glomerular podocytes was detected in DOX-treated but not in VEH-administered double transgenic mice (10 weeks of treatment). No staining was observed in Pod/+ administered VEH or DOX (not shown). *B*: Densitometry quantitative analysis of sFlt-1 expression (as a ratio with β-actin) in total kidney cortex lysate after 10 weeks of treatment with VEH or DOX. Data are expressed as mean ± SEM (P* < 0.01 for Pod/sFlt-1 DOX vs. all other groups, *P* = NS for Pod/+ VEH vs. Pod/+ DOX; *n* = 4–8/group). (Please see http://dx.doi.org/10.2337/db08-0647 for a high-quality digital representation of this figure.)**

istered with VEH also failed to give a positive signal (Fig. 1*A*), excluding "leakage" of *LacZ* expression in noninduced mice. In contrast, kidneys from adult *Pod/sFlt-1* mice exposed to DOX showed positive X-gal staining (Fig. 1*A*), consistent with podocyte expression of the transgene (13,14). X-gal staining was analyzed at different time points after DOX administration (10 days, 5–10 weeks), and the results were superimposable (not shown). After 10 weeks of treatment with DOX, Pod/sFlt-1 animals showed a significant inducible sFlt-1 overexpression (100% increase) when assessed in kidney cortex lysate (Fig. 1*B*). The upregulation of sFlt-1 was associated with an increase in 24-h urine sFlt-1 levels, which was four- to fivefold higher in DOX-treated Pod/sFlt-1 compared with Pod/sFlt-1 mice given VEH or with Pod/+ control mice ($P < 0.001$) (Fig. 2*A*). This was paralleled by a reduction in urine free VEGF-A

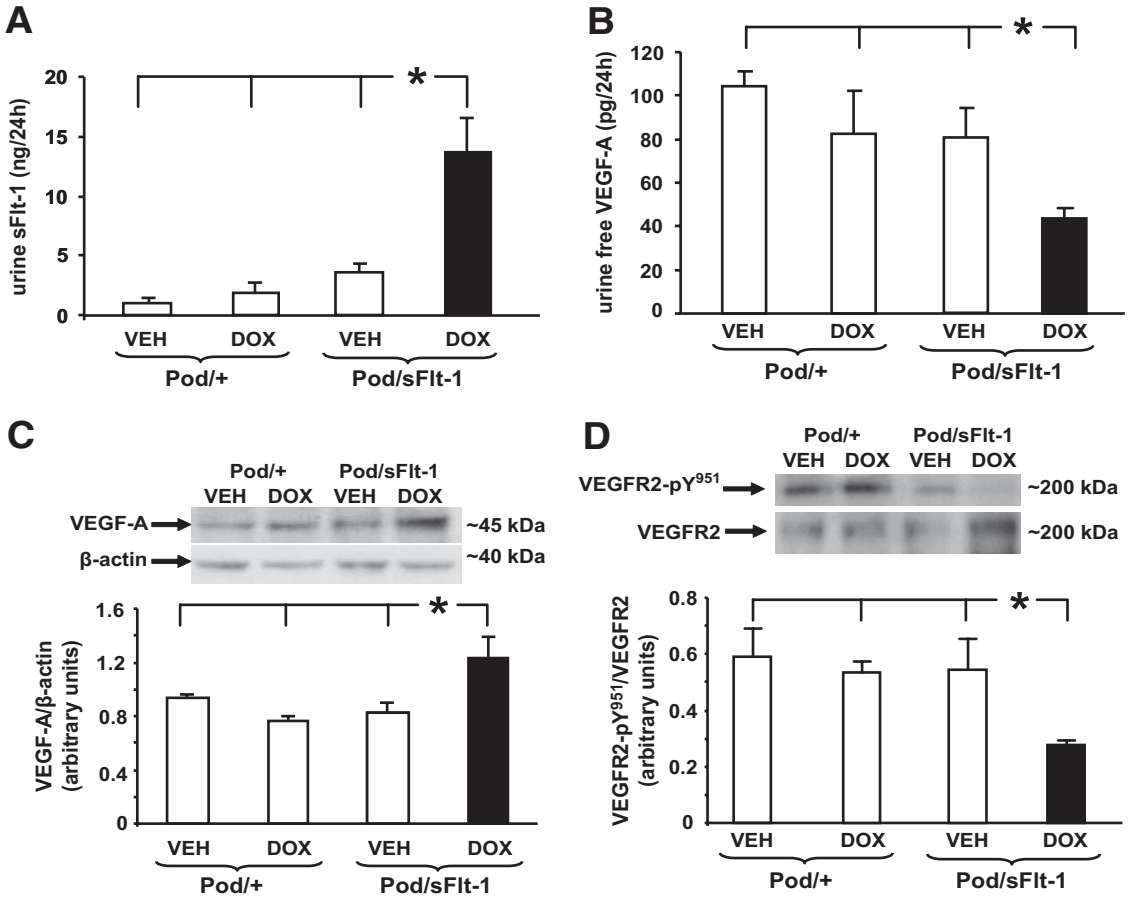

FIG. 2. sFlt-1 and VEGF-A 24-h urine excretion, VEGF-A expression, and VEGFR2 phosphorylation in renal cortex in podocyte-specific sFlt-1 overexpressing mice after 10 weeks of treatment with VEH or DOX. *A*: Urine sFlt-1 excretion was significantly increased in Pod/sFlt-1 DOX-treated mice (P* < 0.001 for Pod/sFlt-1 DOX vs. all other groups, *P* = NS for Pod/+ VEH vs. Pod/+ DOX; *n* = 6–10/group). *B*: 24-h urine free VEGF-A excretion was significantly reduced in Pod/sFlt-1 DOX-treated mice (**P* < 0.04 for Pod/sFlt-1 DOX vs. all other groups, *P* = NS for Pod/+ VEH vs. Pod/+ DOX; *n* = 5–10/group). *C*: By densitometry, quantitative analysis of renal cortex showing that VEGF-A protein expression was significantly upregulated in Pod/sFlt-1 DOX-treated mice (**P* < 0.03 for Pod/sFlt-1 DOX vs. all other groups, *P* = NS for Pod/+ VEH vs. Pod/+ DOX; *n* = 5–6/group). *D*: The ratio of phosphorylated [Tyr(951)] VEGFR2 over total VEGFR2 was downregulated in Pod/sFlt-1 DOX-treated mice (**P* < 0.01 for Pod/sFlt-1 DOX vs. all other groups, *P* = NS for Pod/+ VEH vs. Pod/+ DOX; *n* = 4–8/group).**

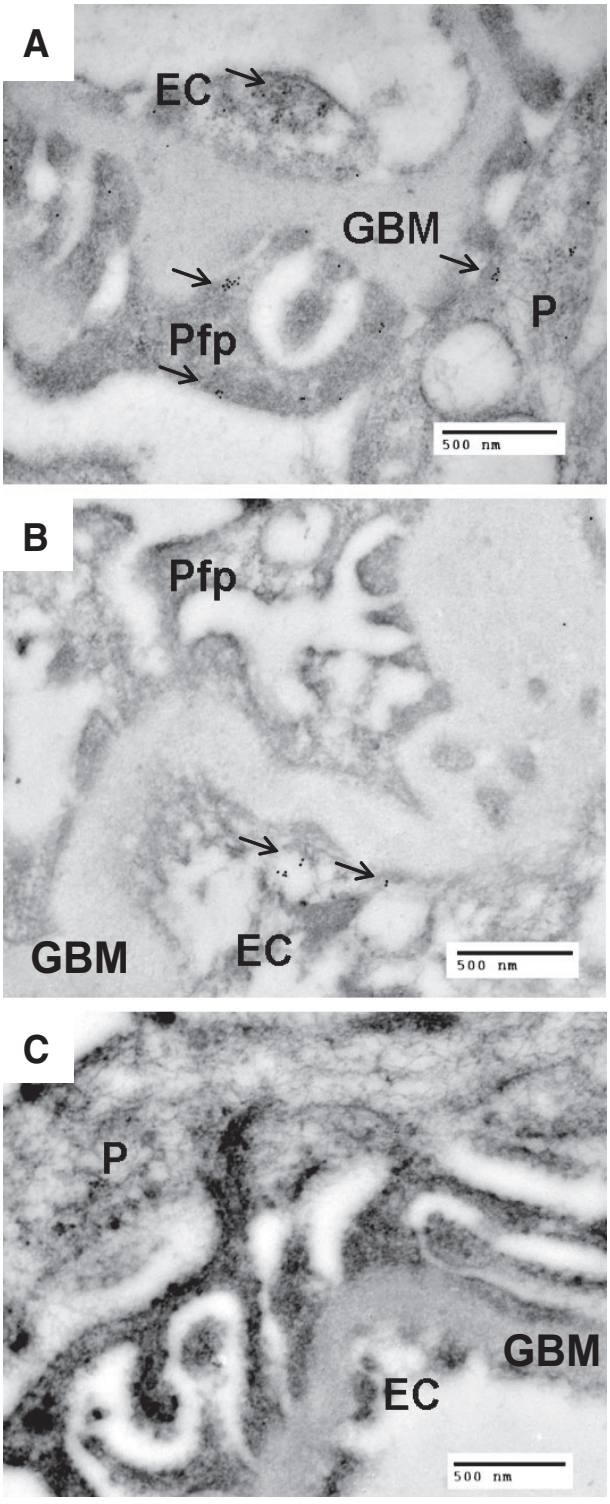

FIG. 3. Expression of VEGFR1 and VEGFR2 in mouse glomerular cells. Representative transmission electron microscopy images of VEGFR2 (*A*), VEGFR1 (*B*), and negative control (*C*). Immunogold staining (arrows on black dots) in mouse glomerular capillaries. Positive Immunogold staining is seen, as expected, in endothelial cells (EC) (*A* and *B*) and, for the first time, for VEGFR2 in podocyte cell body (P) and foot processes (Pfp) (*A*). Scale bars represent 0.5 μm.

in Pod/sFlt-1 DOX-treated mice, suggesting an sFlt-1–mediated "sequestration/binding" of VEGF-A ($P < 0.04$) (Fig. 2*B*). Renal cortex VEGF-A was upregulated in Pod/sFlt-1 DOX mice in comparison with the other groups ($P < 0.03$) (Fig. 2*C*). No difference was observed in the renal cortex expression levels of VEGFR2 among the groups of mice studied (*Pod*/+ VEH, 1.5 ± 0.3; *Pod*/+ DOX, 1.7 ± 0.3; *Pod/sFlt-1* VEH, 1.7 ± 0.3; *Pod/sFlt-1* DOX, 1.5 ± 0.2 VEGF-R2/β-actin arbitrary units, $n = 5$–6/group). VEGFR2 activation, determined by phosphorylation of VEGFR2 on Tyr (951) (20,21) was reduced by ~50%, but not abolished, in *Pod/sFlt-1* mice treated with DOX (Fig. 2*D*).

VEGFR expression in glomerular cells. Immunogold staining showed localization of VEGFR1 and VEGFR2 in endothelial cells as previously reported. We detected expression of VEGFR2, but not VEGFR1, in podocyte cell body and foot processes in vivo (Fig. 3).

Clinical and biochemical features. There was no difference in creatinine clearance at 10 weeks (*Pod*/+ VEH, 9.2 ± 1.5; *Pod*/+ DOX, 10.5 ± 2.2; *Pod/sFlt-1* VEH, 9.3 ± 1.6; *Pod/sFlt-1* DOX, 10.6 ± 1.7 $\mu l \cdot min^{-1} \cdot g$ body wt^{-1}, $n = 6$–9/group) and in albuminuria throughout the study ($n = 10$–12/group, geometric mean [95% CI] in μg/24 h, at baseline: *Pod*/+ VEH, 22.3 [11.9–41.9]; *Pod*/+ DOX, 23.9 [16.7–34.1]; *Pod/sFlt-1* VEH, 33.3 [17.1–65.0]; and *Pod/sFlt-1* DOX, 28.1 [18.8–42.1]; at 10 weeks: *Pod*/+ VEH, 20.6 [14–30.4]; *Pod*/+ DOX, 22.7 [15.7–32.9]; *Pod/sFlt-1* VEH, 28.4 [18.6–43]; and *Pod/sFlt-1* DOX, 22.2 [14.1–34.8]). Glomerular morphology was similar among the four groups of animals (not shown). DOX administration did not affect mice behaviors or body weight, which was similar in all groups (not shown).

Role of podocyte sFlt-1 overexpression in diabetic mice. In these experiments, only Pod/sFlt-1 mice were studied. Mice were made diabetic at 5 weeks of age. Treatment with VEH or DOX was started in 8-week-old adult nondiabetic and diabetic Pod/sFlt-1 mice and continued for up to 10 weeks (18 weeks of age). (Sexes were pooled for analysis because they showed a similar phenotype.)

Clinical and biochemical features. By the end of 10-week DOX treatment, diabetic animals were lighter, had raised blood pressure, and had higher creatinine clearance than control animals. DOX did not affect any of these variables in the nondiabetic and diabetic mice (Table 1).

Albumin excretion rate was similar at baseline in the two diabetic (D) groups: D-VEH vs. D-DOX, geometric mean (95% CI), in μg/24 h, 28 (21–38) vs. 23 (14–36). By week 10 it had risen to 117.5 (69–199) in D-VEH, and this rise was significantly blunted in the D-DOX group overexpressing sFlt-1: D-DOX, 43 (26.8–69) ($P = 0.003$).

To obtain insight in the role of primary podocytes' sFlt-1 overexpression in diabetes, we studied urine sFlt-1 and free VEGF-A excretion, VEGF-A protein expression, and VEGFR2 expression and phosphorylation in the renal cortex lysate.

sFlt-1 and free VEGF-A 24-h urine excretion. Urine sFlt-1 excretion was higher in VEH-administered diabetic mice compared with VEH-administered nondiabetic animals ($P = 0.02$). In the diabetic group, urine sFlt-1 excretion was significantly higher in DOX-treated Pod/sFlt-1 mice compared with VEH-administered animals (D-VEH vs. D-DOX, 19.9 ± 6.3 vs. 35 ± 4.9 ng/24 h, $P = 0.03$) (Fig. 4*A*), a pattern similar to that seen in control nondiabetic animals (Fig. 2*A*). Urine free VEGF-A 24 h excretion was significantly reduced after 10 weeks in DOX-treated diabetic mice compared with VEH-administered diabetic mice ($P = 0.04$) (Fig. 4*B*).

VEGF-A expression, VEGFR2 expression, and phosphorylation in renal cortex. Renal cortex VEGF-A protein expression was significantly upregulated as expected

TABLE 1
Clinical and biochemical features in Pod/sFlt-1 nondiabetic control and diabetic mice treated with VEH or DOX for 10 weeks

	Control VEH	Control DOX	Diabetic VEH	Diabetic DOX
Fed blood glucose (mmol/l)	7.1 ± 1.1	6.2 ± 1.0	26.08 ± 1.6	27.4 ± 1.8
Body weight (g)	28.4 ± 2.3	28.0 ± 1.8	21.1 ± 2.3	22.3 ± 0.7
Creatinine clearance ($\mu l \cdot min^{-1} \cdot g^{-1}$)	9.3 ± 1.6	10.6 ± 1.7	14.9 ± 2.9	14.5 ± 3.6
Systolic blood pressure (mmHg)	106.1 ± 5.0	107.1 ± 4.8	122.8 ± 4.9	124.0 ± 4.9

Data are means ± SEM. Diabetic mice were lighter than control mice. Systolic blood pressure and creatinine clearance were raised in the diabetic mice. None of these variables were affected by DOX administration within the nondiabetic and diabetic groups (n = 7–10/group).

in diabetic VEH-administered mice compared with nondiabetic VEH-administered mice (control [C]-VEH vs. D-VEH, P = 0.02); DOX treatment did not induce any change in VEGF-A expression in diabetic mice (Fig. 5*A*).

VEGFR2 expression was upregulated in diabetic mice by ~80% (C-VEH vs. D-VEH, 100 ± 16.7 vs. 178 ± 11.7 VEGF-R2/β-actin % change, n = 5–6, P = 0.01); DOX treatment did not affect VEGFR2 expression in diabetic mice (not shown). Similarly, VEGFR2 activation, determined as ratio of phosphorylation of VEGFR2 on Tyr (951) over total VEGFR2 was increased by ~60% in diabetic mice (C-VEH vs. D-VEH, 100 ± 15 vs. 158 ± 10 $VEGFR2^{Tyr951}/VEGFR2^{tot}$ % change, n = 5–6, P = 0.01); DOX treatment was accompanied by a nonsignificant decrease in VEGFR2 phosphorylation in diabetic mice (Fig. 5*B*).

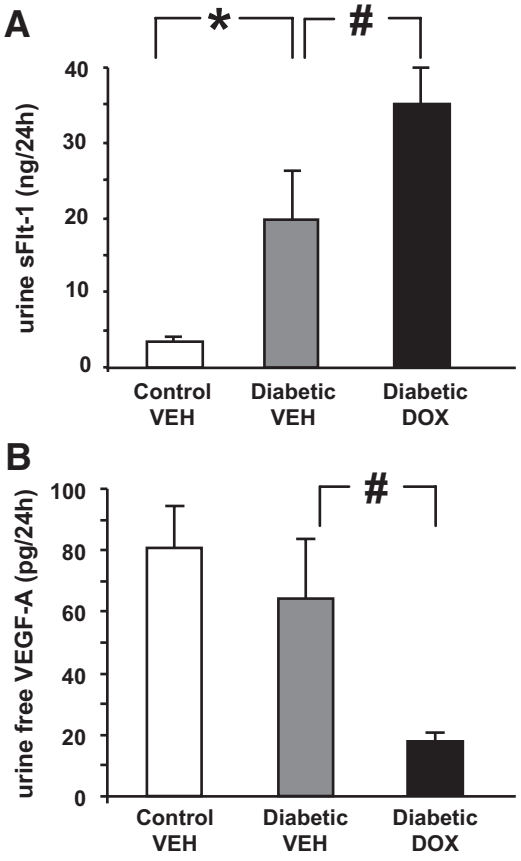

FIG. 4. Twenty four–hour urine sFlt-1 and free VEGF-A excretion in diabetic podocyte-specific sFlt-1 overexpressing mice after 10 weeks of treatment with VEH or DOX. *A*: VEH-treated diabetic mice had higher urine sFlt-1 excretion than VEH-treated nondiabetic control mice (P* = 0.02 for C-VEH vs. D-VEH, n = 6–10/group). Urine sFlt-1 excretion was significantly higher in diabetic DOX-treated mice than in diabetic VEH-treated animals (#*P* = 0.03 for D-VEH vs. D-DOX, n = 7–10/group). *B*: In diabetic mice, urine free VEGF-A excretion was significantly reduced in DOX- vs. VEH-treated animals (#*P* = 0.04 for D-VEH vs. D-DOX, n = 6–10/group).**

To further investigate the changes in albuminuria and to examine the interaction between the VEGF-A/VEGF receptor system and the TGFβ1 pathway in diabetic mice treated with DOX, we studied nephrin, TGFβ1, and TGFβ-receptor2 protein expression in renal cortex protein lysate with Western immunoblotting.

Nephrin, TGFβ1, and TGFβ-receptor2 protein expression in renal cortex. Analysis of nephrin protein expression in total renal cortex protein lysate resulted in two bands at 196 and 200 kDa representing different degrees of nephrin glycosylation (Fig. 6*A*) (22). Diabetic VEH-administered animals had a higher level of nephrin expression than nondiabetic control VEH-administered mice (P = 0.002). Treatment with DOX did not alter the expression of nephrin in the diabetic and the nondiabetic control mice.

TGFβ1 expression was upregulated in diabetic mice by 46% (C-VEH vs. D-VEH, 100 ± 5.4 vs. 146 ± 9.8 TGFβ1/β-actin % change, n = 4–5, P = 0.007). In diabetic mice, treatment with DOX significantly reduced TGFβ1 expression to virtually control levels (D-VEH vs. D-DOX, 146 ± 9.8 vs. 104.7 ± 4.5 TGFβ1/β-actin % change, n = 4–5, P = 0.001). DOX treatment did not change TGFβ1 expression in nondiabetic control mice (Fig. 6*B*).

TGFβ-receptor2 protein expression was slightly but not significantly increased in diabetic mice. DOX treatment did not affect its expression in nondiabetic and in diabetic animals (not shown).

Mesangial area expansion. Mesangial volume fraction (VvMes) was increased in VEH-administered diabetic mice compared with VEH-administered control animals (C-VEH vs. D-VEH, 0.16 ± 0.1 vs. 0.22 ± 0.01, P = 0.003). Administration of DOX significantly reduced VvMes in the diabetic mice (D-VEH vs. D-DOX, 0.22 ± 0.01 vs. 0.18 ± 0.01, P = 0.04) but had no effect in the control mice (C-VEH vs. C-DOX, 0.16 ± 0.01 vs. 0.17 ± 0.01) (Fig. 7*A* and *B*).

GBM thickening, FPW, and endothelial fenestrae. GBM thickness was significantly increased in diabetic mice compared with VEH-administered nondiabetic mice (C-VEH vs. D-VEH, 176 ± 8 vs. 219 ± 8 nm, P = 0.001). Treatment with DOX ameliorated GBM thickening in diabetic mice (D-VEH vs. D-DOX, 219 ± 8 vs. 197 ± 7, P = 0.05) but had no significant impact in nondiabetic control animals (Fig. 8*A* and *D*).

FPW was increased in VEH-administered diabetic mice compared with VEH-administered nondiabetic control animals (C-VEH vs. D-VEH, 197 ± 10 vs. 241 ± 11 nm, P = 0.005). Treatment with DOX inducing sFlt-1 overexpression was paralleled by a reduction in FPW in diabetic mice (D-VEH vs. D-DOX, 241 ± 11 vs. 210 ± 8, P = 0.03) but had no effect in nondiabetic control animals (Fig. 8*B* and *D*).

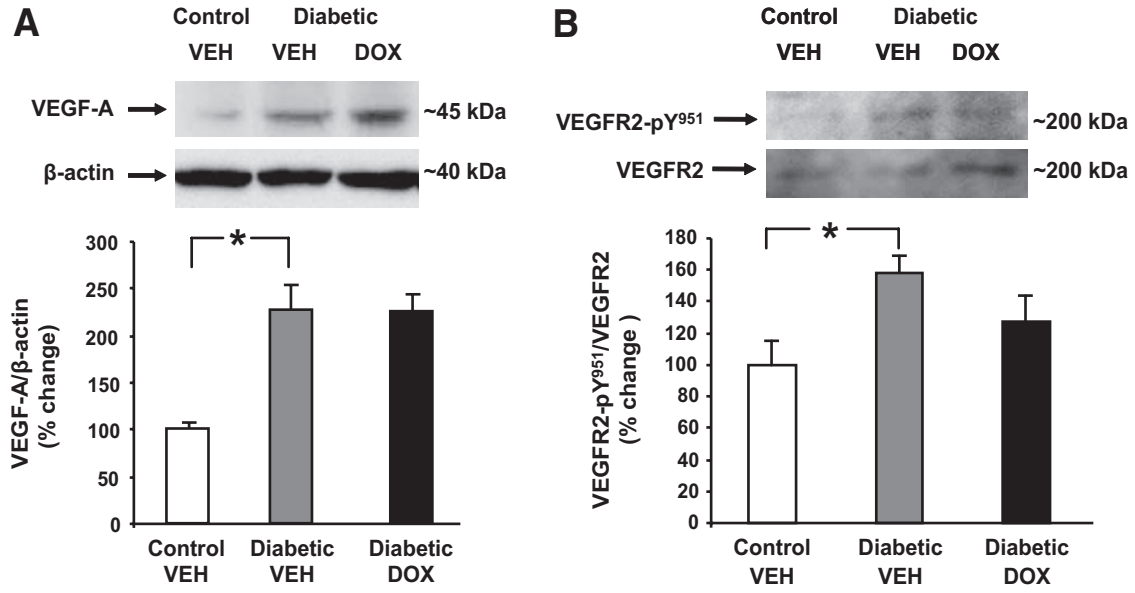

FIG. 5. Renal cortex VEGF-A protein expression and VEGFR2 phosphorylation in podocyte-specific sFlt-1 overexpressing diabetic mice after 10 weeks of treatment with VEH or DOX. Nondiabetic control mice treated with VEH are reported for comparison. *A*: Representative Western immunoblotting and densitometry quantitative analysis of renal cortex VEGF-A expression expressed as % change over nondiabetic VEH-treated mice. VEGF-A protein was significantly upregulated in diabetic VEH-treated mice by 30% over VEH-treated control animals (*$P = 0.02$ for C-VEH vs. D-VEH). No differences in VEGF-A expression were observed between VEH- or DOX-treated diabetic mice ($n = 5$–9/group). *B*: Densitometry quantitative analysis of phosphorylated [Tyr(951)] VEGFR2 over total VEGFR2 in kidney cortex lysate expressed as % change over nondiabetic VEH-treated mice. VEGFR2 phosphorylation was significantly raised in diabetic VEH-treated mice by 58% over VEH-treated control animals (*$P = 0.01$ for C-VEH vs. D-VEH). No differences were observed between VEH- or DOX-treated diabetic mice ($n = 5$–6/group).

No differences were observed in percentage of fenestrated endothelium occupying the endothelial surface, which was ~53–57% and similar in the four groups of mice studied (Fig. 8*C* and *D*).

DISCUSSION

VEGF-A appears to be critical for the maintenance of capillary integrity, particularly in the glomerulus. In normal animals, systemic blockade of VEGF-A action is

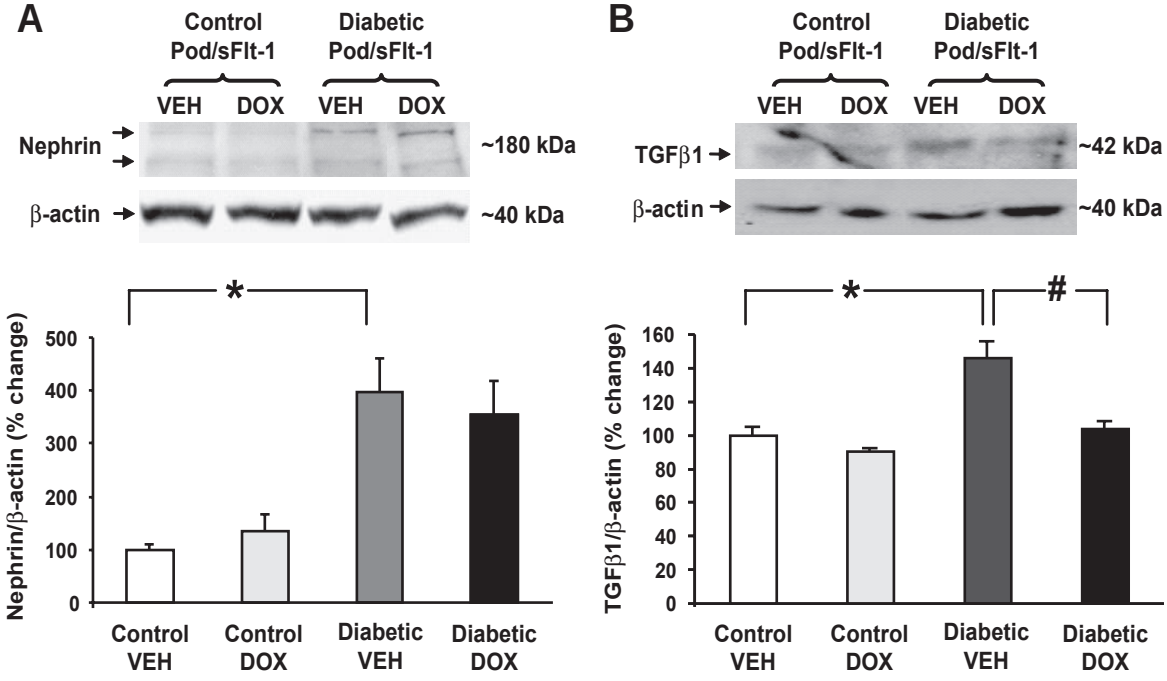

FIG. 6. Renal cortex nephrin and TGFβ1 expression in podocyte sFlt-1 overexpressing nondiabetic control and diabetic mice after 10 weeks of treatment with VEH or DOX. Renal cortex nephrin (*A*) and TGFβ1 (*B*) expression were upregulated, respectively, by ~4- and 1.5-fold in VEH-treated diabetic vs. VEH-treated nondiabetic control mice (*$P < 0.007$ for C-VEH vs. D-VEH). DOX administration did not affect nephrin expression in either diabetic or nondiabetic control mice. Conversely, DOX administration was paralleled by downregulation of TGFβ1 expression levels in diabetic mice (*B*) (#$P = 0.001$ for D-VEH vs. D-DOX). Representative Western immunoblotting and quantitation of multiple experiments expressed as % change over nondiabetic VEH-treated mice are shown for both nephrin and TGFβ1 ($n = 4$–8/group).

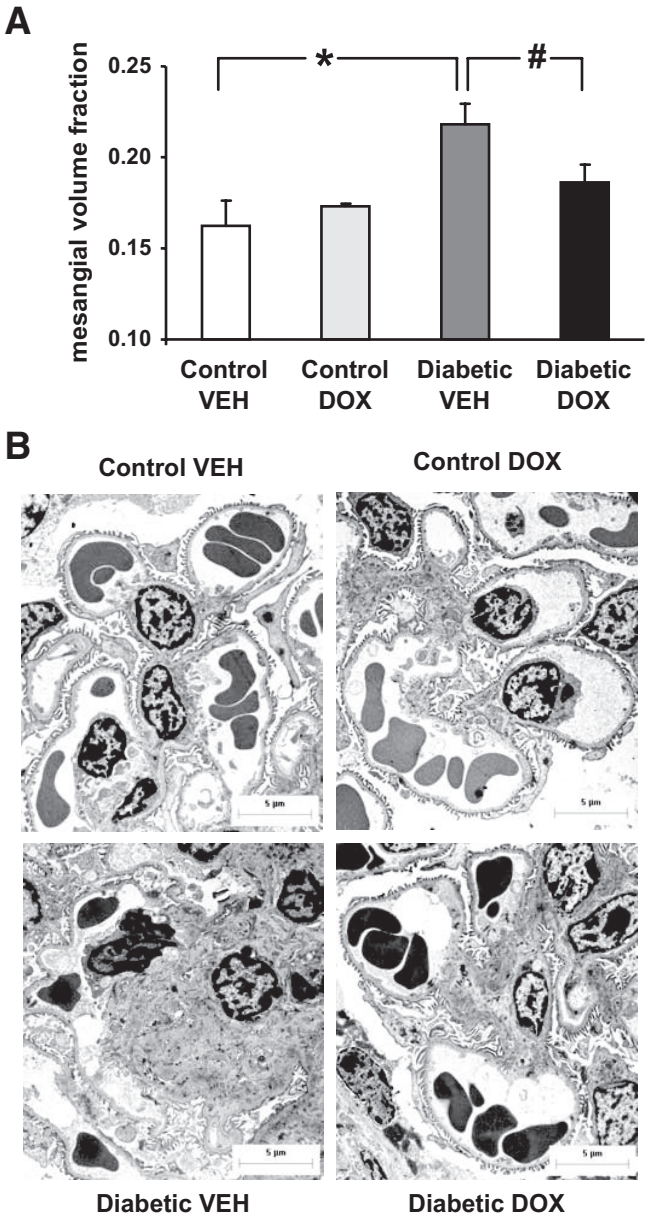

FIG. 7. Mesangial expansion in podocyte sFlt-1 overexpressing control nondiabetic and diabetic mice after 10 weeks of treatment with VEH or DOX. *A*: Quantitative electron microscopy of VvMes in control and diabetic mice treated with VEH or DOX (n = 6–8/group). VvMes was increased in VEH-treated diabetic mice (*P = 0.003 for C-VEH vs. D-VEH). Treatment with DOX significantly reduced VvMes in diabetic mice (#P = 0.04 for D-VEH vs. D-DOX) but had no effect in nondiabetic control animals. *B*: Representative images on transmission electron microscopy show amelioration of glomerular mesangial expansion in DOX-treated diabetic mice compared with VEH-treated diabetic animals. Scale bars represent 5 μm.

associated with endothelial cell damage, reduced nephrin expression, and proteinuria (6).

Moreover constitutive genetically mediated downregulation of VEGF-A expression in podocytes leads to severe impairment of glomerular function and glomerular endothelial damage (5). Recent elegant studies in mice using an inducible podocyte-specific system for site-specific VEGF ablation resulted in glomerular barrier disruption, proteinuria, and glomerular thrombotic microangiopathy mimicking the proteinuria and glomerular damage seen in patients receiving VEGF inhibitors, such as bevacizumav, for the treatment of neoplastic disease (23). Concordantly, in preeclampsia in humans, the greatly elevated circulating levels of sFlt-1 are associated with proteinuria and glomerular injury, suggesting that binding of sFlt-1 to VEGF may impede its vasoprotective action at the capillary level (18,24).

On the other hand, there are pathological conditions, such as diabetes, in which the development of proteinuria and glomerular pathology is associated with VEGF-A overexpression in the podocytes. In the experimental diabetic animal, systemic administration of VEGF inhibitors, such as antibodies or chemicals, ameliorates proteinuria and glomerular damage (8–10). These contrasting situations suggest that both deficiency and excess of VEGF may be detrimental to the physiological integrity of glomerular capillaries.

In this study, we used an inducible expression system that triggers VEGF-A inhibition after complete kidney development (in the adult mouse). To achieve this, we overexpressed, specifically in podocytes, the powerful VEGF inhibitor sFlt-1 (11), thus avoiding systemic elevation of sFlt-1 levels, which, as seen in preeclampsia, may disrupt the capillary wall (18). The system of podocyte-specific conditional inhibition of VEGF-A thus circumvents the potentially important confounding effects on renal glomerular development and aspecific VEGF inhibition in other tissues, which may have occurred with previous experimental approaches.

Our findings demonstrated that in diabetes, tissue-specific inhibition of VEGF-A by excess sFlt-1 led to amelioration of albuminuria and glomerulopathy, presumably by blocking the molecular events mediated by diabetes-induced VEGF overexpression.

sFlt-1 is a 110-kDa molecular weight protein that, under physiological conditions, is not filtered through the glomerular barrier. The podocyte-specific overexpression of sFlt-1, which was induced in our system by the administration of DOX (Fig. 1), would lead to a very localized action of this molecule that within the podocyte would sequester VEGF-A and inhibit its local autocrine and paracrine effect as well as its migration toward the endothelium and the mesangium.

The contention of autocrine and paracrine action of podocyte-secreted VEGF is supported by the presence of VEGF receptors in both endothelial and mesangial cells, as previously reported (3,25), and by the detection for the first time in this work of VEGFR2 in mouse podocytes in vivo. Both VEGFR1 And VEGFR2 are expressed in mouse podocyte in vitro (4), but we were unable to detect VEGFR1 in vivo; the discrepancy between the in vivo and in vitro observations is probably attributable to the artificial setting of the in vitro culture system, which can trigger the induction of molecules normally not expressed in vivo (3).

Supplementary proof of sFlt-1 podocyte overproduction was provided by its significantly increased 24-h urine excretion and clear, though indirect, evidence of sFlt-1 inhibitory action by the marked reduction in urine free VEGF-A excretion. This was true, though in different degrees, for both nondiabetic and diabetic mice treated with DOX, suggesting sequestration of VEGF-A.

In nondiabetic control mice, sFlt-1 overexpression–induced inhibition of VEGF-A resulted in no glomerular functional or morphological abnormalities. We believe that the absence of structural and functional alterations was probably explained by the upregulation of VEGF-A, which occurred in the DOX-treated animals. This upregu-

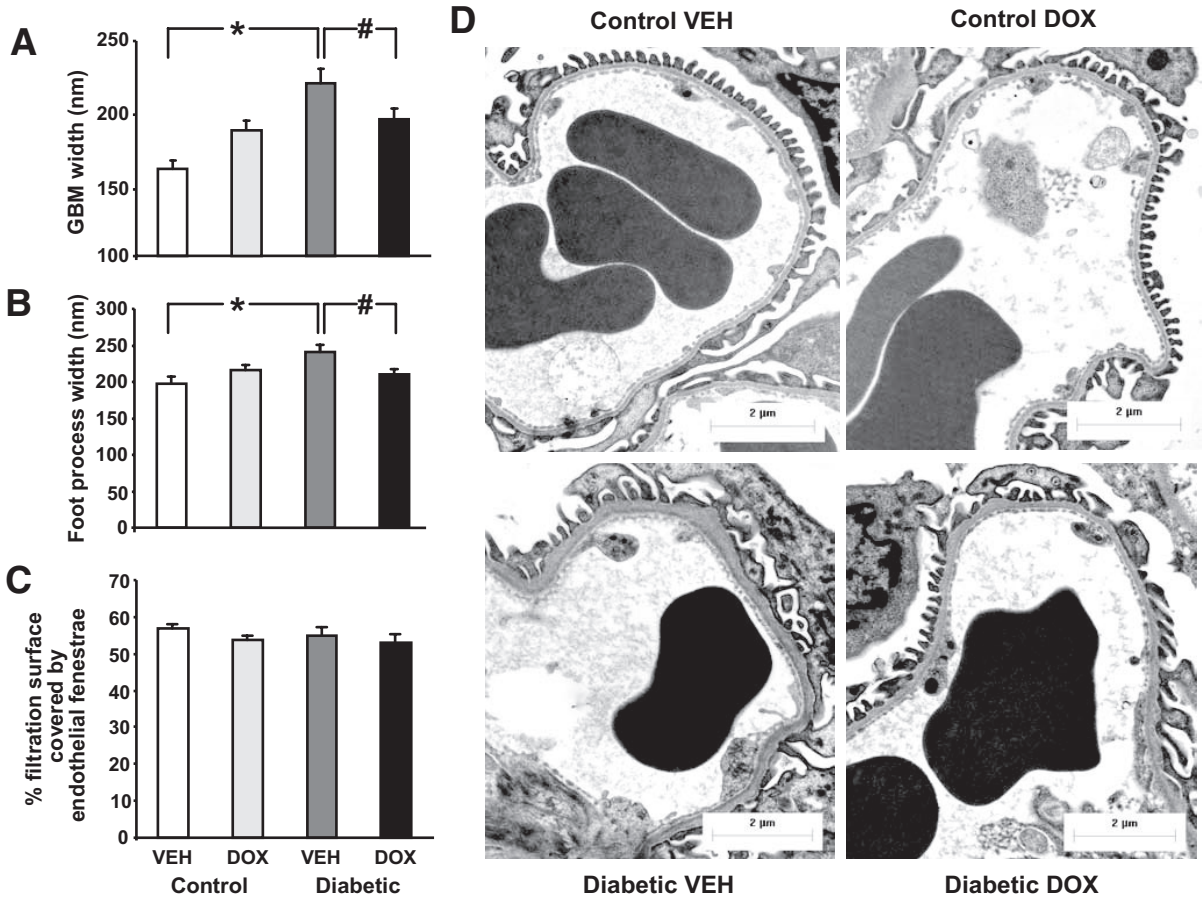

FIG. 8. GBM thickening, FPW, and endothelial fenestrae in podocyte sFlt-1 overexpressing control nondiabetic and diabetic mice after 10 weeks of treatment with VEH or DOX. Quantitative electron microscopy determination of GBM thickening (*A*), podocyte FPW (*B*), and endothelial fenestrae (*C*) in control and diabetic mice treated with VEH or DOX (n = 7–8/group). GBM thickness (*A*) was higher in VEH-treated diabetic mice vs. VEH-treated nondiabetic control animals (*P = 0.001 for C-VEH vs. D-VEH). Administration of DOX reduced GBM thickness in diabetic mice (#P = 0.05 for D-VEH vs. D-DOX) but had no effect in nondiabetic mice. FPW (*B*) was increased in VEH-treated diabetic mice vs. VEH-treated nondiabetic control animals (*P = 0.005 for C-VEH vs. D-VEH). DOX administration ameliorated foot process fusion in diabetic mice (#P = 0.03 for D-VEH vs. D-DOX), but no effect was seen in nondiabetic mice. The endothelial surface covered by endothelial fenestrae was not changed within the four groups of animal studied (*C*). Representative images on transmission electron microscopy show amelioration of GBM thickening and FPW in diabetic DOX-treated mice. Scale bars represent 2 μm.

lation may represent a compensatory response to interrupted VEGF-A signaling because of sFlt-1 overexpression and sequestration of VEGF-A. This might have mitigated the effect of sFlt-1–mediated inhibition, and indeed free VEGF-A in the urine, though reduced by ~50%, was still present in substantial amount and was paralleled by a still present although reduced VEGFR2 activation/phosphorylation. This interpretation is supported by recent evidence that suggests that in the adult mouse, full ablation of VEGF is required to induce definite glomerular pathological changes (23,26).

Diabetic mice developed the typical renal functional and structural abnormalities of proteinuria, mesangial expansion, GBM thickening, and podocyte foot process fusion. These changes were associated with upregulation of VEGF-A and VEGFR2 expression in renal cortex as previously described (25). Similar to nondiabetic mice, DOX-induced sFlt-1 overexpression resulted in raised 24-h urine excretion of sFlt-1 and significant reduction (though not abolition) of urine free VEGF-A excretion, suggesting again "sequestration" of functional VEGF-A. Of interest, 24-h urine sFlt-1 excretion was higher in the diabetic mice administered VEH compared with that in the control nondiabetic mice receiving VEH, mimicking similar observations described in diabetic patients (17).

DOX-treated diabetic mice showed a modest nonsignificant reduction in VEGFR2 phosphorylation compared with VEH-administered animals. The higher degree of glomerulosclerosis observed in VEH- versus DOX-treated diabetic mice might explain this observation, as it has been reported that VEGF receptor activation is inversely related to the degree of glomerular injury (27). We focused our work on VEGFR2 expression and phosphorylation because VEGFR2, rather than VEGFR1, is involved in the biological action of VEGF-A in adult animals (28,29) and is overexpressed in experimental diabetes (25).

Inhibition of VEGF-A at the podocyte level in diabetic mice improved the albuminuria and morphological changes of the glomerular filtration barrier by mitigating GBM thickening and preventing foot process fusion and also by markedly reducing mesangial matrix deposition. This suggests the presence of a podocyte-podocyte as well as a podocyte-mesangium cellular network, where podocyte-specific gene expression can modulate the biology of other subset of glomerular cells as previously suggested for other podocyte secreted cytokines (13,23,26). Indeed the sFlt-1 overexpression-induced amelioration of GBM thickening and of mesangial extracellular matrix volume is likely to result from an inhibition of the prosclerotic action of VEGF-A (30,31), the reduction of TGFβ1 expression

(32), or both. A potential relationship between VEGF-A and TGFβ1 is suggested by studies reporting a VEGF-A–mediated TGFβ1 expression in mouse glomerular endothelial cells (33).

Interestingly, in the diabetes sFlt-1 overexpressing mice, no further upregulation of the already overexpressed VEGF-A occurred, suggesting a "ceiling" for renal cortex VEGF-A upregulation in the context of diabetes. In this mouse model, neither diabetes nor the overexpression of sFlt-1 altered the number of endothelial fenestrae in the glomerular capillary.

Nephrin is a slit diaphragm protein whose deletion or expression downregulation has been implicated in the pathogenesis of proteinuria in diseases such as the congenital nephrotic syndrome of the Finnish type or in minimal change nephrotic syndrome (34–36). In experimental animal models of diabetes, nephrin expression has been reported to be either upregulated or downregulated (10,37–39). In our diabetic mice, nephrin was upregulated when compared with control nondiabetic animals, and the podocyte overexpression of sFlt-1 did not affect this upregulation. These findings, in line with previous reports (10), suggest that nephrin expression downregulation, though it may be sufficient, is not necessary for the development of proteinuria and concur with the concept that albuminuria may occur by a variety of mechanisms such as redistribution of nephrin at the ultrastructural level (10,40,41).

DOX, which was used in this study to induce sFlt-1 upregulation, is a nonselective inhibitor of metalloproteinases (42,43), enzymes that regulate extracellular matrix deposition, and may have confounded some of our findings. However, our present results in the nondiabetic control mice and previous observations in normal animals (13) and diabetic rodents (44) suggest no direct DOX-mediated effects on albuminuria and extracellular matrix at the dose used in this study. Moreover, DOX-induced inhibition of metalloproteinases would favor matrix accumulation and would result, if anything, in an underestimate of the improvement in glomerular extracellular matrix deposition in the podocyte sFlt-1 overexpressing diabetic mice.

Our findings provide insight into the direct, local (autocrine/paracrine) role of VEGF-A in diabetes-induced glomerular injury, although the experimental design of this work makes our results more relevant to the prevention rather than treatment of diabetic glomerular damage. Together with work by others (23), these findings support the notion that in order to preserve vascular integrity, a fine balance in the regulation of VEGF-A expression is required in as much as either too little or too much of this cytokine would result in capillary vascular pathology. These considerations highlight the potential pitfalls of using systemic therapies to tackle tissue-specific changes.

ACKNOWLEDGMENTS

This work was partly founded by a project grant from the Biotechnology and Biological Sciences Research Council (no. S13745), Diabetes UK (RD04/0002860), and the European Foundation for the Study of Diabetes/Servier.

We thank the Biomedical Electron Microscopy Unit (Newcastle University) for the electron microscopy study. We are grateful to Dr. J. Kopp for providing the Podocin-rtTA mice and to Dr. N. Dalton and Dr. C. Turner (Guy's and St. Thomas Hospital, London, U.K.) for the creatinine determination.

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