Long-term blockade of vascular endothelial growth factor receptor-2 aggravates the diabetic renal dysfunction associated with inactivation of the Akt/eNOS-NO axis

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Abstract

Background. Diabetic nephropathy is characterized by abnormal angiogenesis, and this is driven by several factors, including hyperglycaemia and ischaemia. We investigated the role of vascular endothelial growth factor receptor-2 (VEGFR-2) blockade and its effects on diabetic nephropathy.

Methods. Male db/db and db/m mice received long-term treatment with dRK6, an arginine-rich anti-VEGF hexapeptide, for 12 weeks or short-term treatment for only the first 4 weeks, starting from 8 weeks of age.

Results. The urinary albuminuria and VEGF excretion varied according to the duration of diabetes, and the urinary VEGF levels were strongly correlated with the levels of albuminuria. Diabetes increased the VEGFR-2 expression in the kidneys. At the end of the 12-week study, compared with the db/db control mice, the db/db mice with long-term dRK6 treatment, which selectively inhibited VEGFR-2, had more albuminuria, related to weak nephrin signalling and advanced renal phenotypes, which were associated with hypoxia-oxidative stress, and an increased number of apoptotic endothelial cells. Interestingly, these changes were related to a decrease in phospho-Akt/eNOS-NO bioavailability. On the in vitro study, dRK6 increased the number of apoptotic human umbilical vein endothelial cells (HUVECs) in the high glucose media by blocking phospho-Akt/eNOS-NO signalling, and this was related to the increased oxidative stress. The short-term inhibition of VEGFR-2 neither improved the albuminuria nor the renal phenotype induced by diabetes.

Conclusions. Long-term selective blockade of VEGFR-2 by dRK6 had deleterious renal effects, and this was associated with downregulation of the Akt/eNOS-NO axis in db/db mice. Short-term VEGFR-2 blockade did not improve the renal phenotypes and the albuminuria. These findings suggest that VEGF-A-VEGFR-2 inhibition, regardless of how long it may be, does not ameliorate diabetic nephropathy in type 2 diabetes.

Keywords: Akt/protein kinase B; diabetic nephropathy; eNOS; nitric oxide; VEGF-A-VEGFR-2

Introduction

Various growth factors have been recognized as having important roles in the pathogenesis of diabetic nephropathy [1–6]. Among these factors, vascular endothelial growth factor (VEGF or VEGF-A) belongs to a family of multipotent cytokines that include VEGF-B, -C, -D, -E and placenta growth factor. The receptors for VEGF-A are VEGFR-1, VEGFR-2 and VEGFR-3 along with high-affinity transmembrane tyrosine kinase receptors. In the kidney, VEGF production by the podocytes plays an important role in renal cell survival, regeneration and repair within the glomeruli, and it helps maintain the integrity of the glomerular filtration barrier with VEGFR-2 [7–15]. Recently, it was reported that glomerular structure and function require paracrine, not autocrine, VEGF-VEGFR-2 signalling [15].

The VEGFRs are expressed in the endothelium of the glomeruli, the peritubular capillaries and, to a lesser extent, the mesangial and tubular cells [16,17]. In the glomerular endothelial cell, VEGF-A stimulates the VEGFR-2/Akt axis to regulate endothelial nitric oxide synthase (eNOS) phosphorylation. eNOS is activated by the phosphorylation of serine (Ser1177) of the protein kinase Akt/PKB and is also known to regulate the glomerular haemodynamics by generation of NO. Several rodent type 1 and 2 diabetes models have shown that the abnormal angiogenesis and immature vessels induced by glomerular hypertension and low NO bioavailability along with a high VEGF-A expression (uncoupling of VEGF-A with NO) cause glomerular hypertrophy, albuminuria, the expression of profibrotic growth factor and inflammatory cell infiltration in the kidneys [18]. Blocking the increased VEGF-A with anti-pan-VEGF antibodies,
which block all of the VEGFRs at the level of the tyrosine kinase, improves the diabetes-related early renal dysfunction, and especially the hyperfiltration [17–24]. However, in human diabetic nephropathy, the VEGF-A expression is reduced in the glomeruli and the tubulointerstitium [25–27]. These findings suggest that when the VEGF level is too low it can be just as damaging as when the VEGF level is too high. Thus, the diverse biological effects of VEGF in diabetic nephropathy are due in part to “uncoupling of VEGF-A with NO” [28]. The protective role of VEGF in renal disease could be predominantly dependent on its ability to stimulate NO production in endothelial cells. In contrast, a potential adverse effect of VEGF-A inhibitors could be endothelial injury because endothelial cells require VEGF-A in the normal physiological condition. However, the effect of selective VEGF-A-VEGFR-2 blockade remains unanswered, especially during the evolution of diabetic nephropathy. The db/db mouse model has been shown to develop frank hyperglycaemia by 8 weeks of age and increased albuminuria excretion from 8 weeks, and the latter reaches peak levels by the age of 16 weeks [29–31].

dRK6 is an arginine-rich anti-VEGF hexapeptide, and it is a D-amino acid derivative of RK6 (Arg-Arg-Lys-Arg-Arg-Arg). The dRK6 binds with VEGF-A, and thereby, it can block the interaction between VEGF-A (mainly VEGF165 and VEGF121) and the VEGFRs. In a previous study, dRK6 showed significant inhibition of VEGF-induced angiogenesis, and dRK6 also retarded the growth and metastasis of colon carcinoma cells without direct cytotoxicity [32,33]. We recently demonstrated that subcutaneous injection of dRK6 every other day for 12 weeks had deleterious effects on the heart in db/db mice. In that study, systemic blockade of VEGFR-2 by dRK6 induced downregulation of VEGFR-2 and Akt/eNOS axis and enhanced oxidative stress in the heart [34]. Therefore, we evaluated whether dRK6 treatment would have beneficial effects in the kidney of db/db mice from the short- and long-term inhibition of the VEGF-A-VEGFR-2 axis in diabetic nephropathy.

Materials and methods

Experimental animals

All the experiments were performed according to our institution’s animal care guidelines. Six-week-old male C57BLKS/J db/db and db/m mice were purchased from Jackson Laboratories (Bar Harbor, ME). Non-diabetic db/m mice and diabetic db/db mice (n = 6, respectively) were used as controls. As for the dRK6 treatment groups, the db/db mice were divided into short-term (db/db+S) and long-term (db/db+L) treatment groups (n = 8, respectively). The db/db+L mice were treated with dRK6 from 8 weeks of age for 12 weeks of the whole study period. We also used db/m mice as a long-term treatment group (db/m+L). The db/db+S mice were the first four weeks starting from 8 weeks of age (at the beginning of overt hyperglycaemia). All the treatment groups received, three times per week, subcutaneous injection of 50 μg dRK6 that was dissolved in phosphate buffer saline (PBS) [32,33]. The control groups were treated with subcutaneous injection of PBS (vehicle).

Table 1. Primer sequences used for quantification of gene expression by semi-quantitative PCR and the resulting product size

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Size (bp)</th>
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<tbody>
<tr>
<td>VEGFR-1 (Flt-1)</td>
<td>5′-ACATGGGACAGTATGGGAGA-3′</td>
<td>425</td>
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<tr>
<td></td>
<td>5′-ACGGAGTTGTAAGAGACG-3′</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5′-GACCTGGACTGGTTGGA-3′</td>
<td>342</td>
</tr>
<tr>
<td></td>
<td>5′-TCTCTTTTCTGGATACCT-3′</td>
<td></td>
</tr>
<tr>
<td>Akt1</td>
<td>5′-GGGGAGTTGATGGCTGGC-3′</td>
<td>191</td>
</tr>
<tr>
<td></td>
<td>5′-TTACGCAGTGCAGCCAGCA-3′</td>
<td></td>
</tr>
<tr>
<td>eNOS</td>
<td>5′-CTGTTGCTCATCTGGGGAACACAGCA-3′</td>
<td>427</td>
</tr>
<tr>
<td></td>
<td>5′-GAATGGTTGCGTTCAAGCTGCGGCAAT-3′</td>
<td></td>
</tr>
<tr>
<td>18s rRNA</td>
<td>5′-CGCGTGTCTATTTTTGTTGGG-3′</td>
<td>219</td>
</tr>
</tbody>
</table>

Assessment of albuminuria and renal function

At weeks 0, 4 and 8, the mice were housed in metabolic cages (Nalgene, Rochester, NY) for 24 h to collect urine for the subsequent measurements of the albumin concentration by performing an immunoassay (Bayer, Elkhart, IN). At week 12, the plasma and urinary creatinine concentrations were measured using HPLC. The creatinine clearance was calculated by (urine [Cr] × urine volume) / (plasma [Cr] × time).

Light microscopic study

At week 12, the mouse kidneys were removed, fixed in 10% buffered formalin and embedded in paraffin. The histology was assessed after periodic acid-Schiff (PAS) staining of the 4-μm paraffin-embedded sections. More than 30 glomeruli that were cut through the vascular pole were observed, and images were taken using a digital camera (Olympus DP11; Olympus America Melville, NY) and analysed by using colour image analyser (TDI Scope Eye Version 3.0 for Windows, Olympus, Japan) to determine the mean mesangial matrix index and glomerular tuft areas. The mesangial matrix index was defined as the proportion of the glomerular tuft that was occupied by the mesangial matrix area (excluding nuclei). Mean glomerular tuft volume was determined from the mean glomerular cross-sectional area by means of TUNEL staining.

Immunohistochemistry for type IV collagen, nephrin, F4/80, connective tissue growth factor, platelet-endothelial cell adhesion molecule, TUNEL, thrombospondin-1 and 8-hydroxy-deoxyguanosine

We performed immunohistochemistry for type IV collagen, nephrin, F4/80, connective tissue growth factor (CTGF), platelet-endothelial cell adhesion molecule (PECAM-1) (CD31), terminal uridine deoxynucleo-
tidyl transferase dUTP nick end labeling (TUNEL), thrombospondin-1 and 8-hydroxy-deoxyguanosine (8-OH-dG). Four-micrometre-thick sections were deparaffinized, washed with PBS and then incubated with 1.5% H2O2 in methanol to block the endogenous peroxidase activity. The sections were incubated overnight in a humidified room at 4°C with anti-type IV collagen (1:150 in blocking solution; Biodesign Int., Saco, ME), anti-nephrin (1:50; United States Biological, Swampscott, MA, USA), anti-F4/80 (1:100; Serotek, Oxford, UK), anti-CTGF (1:250 in blocking solution; Abcam, Cambridge, UK), anti-PECAM-1 (1:50; BD Bioscience, San Diego, CA), anti-thrombospondin-1 (1:100; Thermo Scientific, Fremont, CA) and anti-8-OH-dG (1:100; JalCA, Shizuoka, Japan). The antibodies were localized with the ABC technique (Vector Laboratories, Burlingame, CA). We performed immunohistochemistry for nephrin using frozen tissue sections. For the quantification of the immunoreactivity for type IV collagen, nephrin, PECAM-1 and thrombospondin-1, ~20 views (×400 magnification) were randomly observed in the renal cortex and the corticomedullary junction of each slide, and images were taken and analysed to determine density × positive area / glomerular total area by using a computer image analysis programme (Scion Image Beta 4.0.2, Frederick, MA). For the quantification of glomerular accumulation of monocyte/macrophage, glomerulosclerosis, apoptosis and oxidative stress, the number of F4/80, CTGF, TUNEL and 8-OH-dG positive cells per glomerulus was determined by observing 20 glomeruli from each section. For the quantification of apoptotic endothelial cells, double staining for TUNEL and PECAM-1 was performed. The endothelial cells that were positive for apoptosis exhibited an intense brown nuclear (TUNEL-positive) and a red cytoplasmic colorimetric (PECAM-1-positive) reaction product. The number of apoptotic endothelial cells per glomerulus was determined by observing 20 glomeruli from each section under ×400 magnification.

Immunofluorescent staining for VEGFR-2 and hypoxic inducible factor-1α
We performed immunohistochemistry for VEGFR-2 (1:100 in blocking solution; Santa Cruz Biotechnology, Santa Cruz, CA) in the paraffin-embedded kidney sections. We also performed hypoxic-inducible factor-1α (HIF-1α) (1:50 in blocking solution; Novus Biologicals, Littleton, CO) immunofluorescent staining in the paraffin-embedded kidney sections by using tyramide signal amplification system (PerkinElmer Life Science, Boston, MA, USA). For quantification of the proportion of the area stained, ~20 views (×400 magnification) were randomly observed in the renal cortex of each slide, and images were taken and analysed to determine density (or intensity of fluorescence) × positive area / glomerular total area by using computer image analysis programme (Scion Image Beta 4.0.2, Frederick, MA).

24-Hour urinary nitrate + nitrite and 8-OH-dG
The total urinary NO3− + NO2− excretion was quantified using the Nitric Oxide Assay Kit (BioVision, Mountain View, CA, USA). To determine the oxidative DNA damage in the renal cortex, we also determined the 24-h urinary 8-OH-dG concentrations by performing competitive ELISA (Institute for the Control of Aging, Shizuoka, Japan).
Real-time PCR for VEGF-R1 (Flt-1), VEGF-R2 (Flk-1), Akt1, eNOS and 18s rRNA

We also performed real-time PCR (RT-PCR) to assess the gene expression levels of VEGF-R1 and R2. We used 18s rRNA as an internal control. The primers for amplification of VEGFR-1 and -2 and 18s rRNA are listed in Table 1.

Western blot for total eNOS and phospho-eNOS (Ser1177), total Akt and phospho-Akt (Ser473) and HIF-1α

Western blot analysis in the renal cortex was performed using the following antibodies: total Akt, phospho-Akt (Ser473), total eNOS, phospho-eNOS (Ser1177), (Cell Signaling Technology, Danvers, MA, USA), HIF-1α and β-actin (Abcam, Cambridge, UK). HIF-1α protein from the nuclear fractions of the renal cortex was isolated using a Nuclear Extraction Kit (Cayman Chemical, Ann Arbor, MI, USA) and following the manufacturer’s protocol.

HUVEC culture and TUNEL assay

HUVECs were obtained from collagenase type II (Biochrom, KG, Berlin, Germany)-digested umbilical cords, and these cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 15 mg/500 mL endothelial cell growth supplement (Sigma, St. Louis, MO, USA) at 37°C in a humidified 5% CO2/95% air atmosphere. Apoptosis was quantified using the in situ cell death detection kit, a TUNEL assay (CHEMICON International, Temecula, CA, USA). After treatment with different concentrations of d-glucose in the media (5 mM/L and 30 mM/L D-glucose and 5 mM/L D-glucose + 25 mM/L D-mannitol) and 0, 10−5, 10−6 and 10−7 mmol/L dRK6 for 48 h, the number of TUNEL-positive cells was counted in 10 randomly chosen fields at a magnification of ×400. We also measured the concentrations of NOx and 8-iso-PGF2α for the quantification of NO production and the free radical-induced oxidative stress in the cell culture media.

Table 2. Effects of dRK6 on the metabolic and physiologic parameters of the db/m and db/db mice at 12 weeks

<table>
<thead>
<tr>
<th>Parameters</th>
<th>db/m</th>
<th>db/m+L</th>
<th>db/db</th>
<th>db/db+S</th>
<th>db/db+L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt (g)</td>
<td>31.8 ± 0.5</td>
<td>29.9 ± 0.2</td>
<td>51.3 ± 2.3**</td>
<td>53.5 ± 4.1**</td>
<td>52.4 ± 2.1**</td>
</tr>
<tr>
<td>Food intake (g/24 h)</td>
<td>2.9 ± 0.4</td>
<td>1.2 ± 0.3*</td>
<td>7.3 ± 0.6**</td>
<td>7.4 ± 0.6**</td>
<td>8.8 ± 0.5**</td>
</tr>
<tr>
<td>Mean systolic pressure (mm Hg)</td>
<td>102 ± 5</td>
<td>105 ± 5</td>
<td>106 ± 6</td>
<td>97 ± 8</td>
<td>99 ± 5</td>
</tr>
<tr>
<td>Serum insulin (μg/mL)</td>
<td>0.19 ± 0.12</td>
<td>0.20 ± 0.15</td>
<td>0.46 ± 0.18*</td>
<td>0.47 ± 0.15*</td>
<td>0.41 ± 0.10*</td>
</tr>
<tr>
<td>HbA1c</td>
<td>4.3 ± 0.1</td>
<td>4.2 ± 0.1</td>
<td>10.0 ± 0.6**</td>
<td>10.5 ± 0.7**</td>
<td>10.1 ± 0.6**</td>
</tr>
</tbody>
</table>

Abbreviations: db/m, db/m control; db/m+L, long-term treated db/m; db/db, db/db control; db/db+S, short-term treated db/db; db/db+L, long-term treated db/db; wt, weight. Systolic pressure was measured at 12 weeks of study. *P < 0.05 vs the db/m and the db/m+L. **P < 0.001 vs db/m and db/m+L.

Table 3. Effects of dRK6 on the renal functional and structural parameters of the db/m and db/db mice at 12 weeks

<table>
<thead>
<tr>
<th>Parameters</th>
<th>db/m</th>
<th>db/m+L</th>
<th>db/db</th>
<th>db/db+S</th>
<th>db/db+L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney wt (g/100 g Bwt)</td>
<td>0.50 ± 0.01</td>
<td>0.53 ± 0.04</td>
<td>0.47 ± 0.06</td>
<td>0.44 ± 0.04</td>
<td>0.72 ± 0.03*</td>
</tr>
<tr>
<td>Urine volume (ml/24 h)</td>
<td>0.4 ± 0.2</td>
<td>0.7 ± 0.4</td>
<td>20.5 ± 2.4***</td>
<td>8.4 ± 2.6**</td>
<td>8.5 ± 2.8**</td>
</tr>
<tr>
<td>Serum Cr (mg/ml)</td>
<td>0.079 ± 0.009</td>
<td>0.070 ± 0.012</td>
<td>0.073 ± 0.008</td>
<td>0.081 ± 0.011</td>
<td>0.077 ± 0.011</td>
</tr>
<tr>
<td>Ccr (ml/min)</td>
<td>0.33 ± 0.28</td>
<td>0.29 ± 0.55</td>
<td>0.63 ± 0.30**</td>
<td>0.28 ± 0.07****</td>
<td>0.31 ± 0.06****</td>
</tr>
<tr>
<td>Glomerular volume (107 × μm3)</td>
<td>3.18 ± 0.46</td>
<td>3.33 ± 0.55</td>
<td>6.26 ± 0.38**</td>
<td>6.28 ± 0.50**</td>
<td>7.09 ± 0.65*</td>
</tr>
<tr>
<td>Mesangial matrix index (%)</td>
<td>7.1 ± 0.7</td>
<td>7.3 ± 1.0</td>
<td>13.7 ± 1.1**</td>
<td>13.6 ± 1.1**</td>
<td>16.9 ± 2.1*</td>
</tr>
</tbody>
</table>

Abbreviations: Bwt, body weight; Ccr, creatinine clearance; Cr, creatinine. *P < 0.05 vs db/db and db/db+S. **P < 0.05 vs db/m and db/m+L. ***P < 0.001 vs db/m and db/m+L. ****P < 0.05 vs db/db.

Statistical analyses

The data are expressed as means ± SDs. The statistical significance of differences between the groups was assessed by using two-way ANOVA with Bonferroni’s correction (SPSS 11.0, Chicago, IL). A P-value <0.05 was considered a statistically significant difference.

Results

Relationships between 24-h albuminuria and the VEGF concentrations during the study period

To determine the relationship between the urinary VEGF concentrations and albuminuria, we measured the 24-h albuminuria and urinary VEGF concentrations in the db/m and db/db control mice during the study period. As shown in Figure 1A, the albuminuria and urinary VEGF excretion peaked at 16 weeks of age, and then they began to decrease. In addition, there was a strong correlation between albuminuria and the urinary VEGF concentrations at 12 weeks of the study (Figure 2C, r = 0.543, P = 0.005).

Effects of dRK6 on the metabolic and physiologic parameters

The physical data are summarized in Table 2. There was no difference in systolic blood pressure in all the study animal groups. The HbA1c levels, serum insulin levels and food intake in the diabetic db/db groups were significantly higher than those in the non-diabetic db/m groups (P < 0.05). There were no differences in HbA1c, serum insulin levels
and food intake among the db/db study groups (Table 2). Administration of dRK6 did not influence the glucose levels in the diabetic mice.

Effects of dRK6 on the kidney weight, albuminuria and creatinine clearance

The urine volume, glomerular volume and mesangial matrix index in the db/db study groups were all significantly greater than those in the db/m groups (P < 0.05). In the db/db+S mice, the kidney weight was heavier and the albuminuria was more prominent at 8 and 12 weeks of the study than that in the db/db control mice and db/db+S mice (P < 0.05) (Table 3, Figure 2B). The creatinine clearance in the db/db control mice was increased compared with that in the db/m control mice. However, dRK6 treatment decreased creatinine clearance in the db/db+S and db/db+L mice compared with that of the db/db control mice (P < 0.05).

Fig. 3. The expression of VEGFR-1 and VEGFR-2 in the renal cortex. Representative photomicrographs of the RT-PCR for VEGFR-1, VEGFR-2 and 18s rRNA (A). Semi-quantitative assessment of the RT-PCR ratio of VEGFR-1 (B) and VEGFR-2 (C) in the kidney of the db/m, db/m+L, db/db, db/db+S and db/db+L groups. Representative photographs of the immunohistochemical staining for VEGFR-2 (D) in the glomerulus (original magnification, ×400). Quantitative assessment of VEGFR-2 (E) in the glomerulus. *P < 0.05 compared with the db/m control and db/m+L, **P < 0.05 compared with the db/m control, ***P < 0.01 compared with the db/db control, ###P < 0.05 compared with the db/db+S.
In contrast, there were no differences in kidney weight, albuminuria, glomerular volume and creatinine clearance between the db/m control and db/m+L mice.

**Effects of dRK6 on the VEGFR-1 and VEGFR-2 expressions**

As shown in Figure 3, there was a decreased mRNA expression of VEGFR-1 and an increased mRNA expression of VEGFR-2 in the db/db control mice compared with that of the db/m control mice. As expected, in the db/db+S mice, dRK6 treatment decreased the VEGFR-2 mRNA expression (P < 0.01), but not the VEGFR-1 mRNA expression. Consistent with the mRNA expression of VEGFR-2, the db/db+S mice showed decreased immunostaining of VEGFR-2 in the glomerulus compared with that of the db/db control mice (P < 0.01). Intriguingly, the VEGFR-2 mRNA and protein expressions were almost completely abolished in the db/db+L mice. In contrast, the VEGFR-1 and VEGFR-2 mRNA and protein expressions were increased in the db/m+L mice compared with that of the db/m control mice (P < 0.05).

**Effects of dRK6 on the Akt and eNOS expressions in the kidney and on the urinary NOx**

The real-time PCR showed a markedly decreased Akt1 mRNA expression among the db/db control mice, the
Consistent with the mRNA expression, the Ser-1177 phospho-Akt/total Akt protein ratio was significantly decreased in these three groups. Moreover, the eNOS mRNA and Ser-473 phospho-eNOS/total eNOS protein expressions were more decreased in the db/db+S mice compared to those in the db/db+S mice (P < 0.05). As shown in Figure 3, the 24-h urinary NOx concentrations were significantly decreased in the db/db groups. In the db/db+S mice, there was a much more decreased eNOS mRNA expression and Ser-473 phospho-eNOS/total eNOS expression and a decreased urinary NOx expression compared with that in the db/db control mice (P < 0.05). In contrast, there were increased mRNA and protein expressions of phospho-eNOS and an increased urinary NOx concentration in the db/m+L mice as compared with that of the db/m control mice (P < 0.05).

**Effects of dRK6 on the renal phenotypes**

All of the db/db groups showed increased type IV collagen accumulation and CTGF-positive cells in the glomeruli compared with that of the db/m groups (P < 0.05; Figure 5A–F). These db/db groups also showed a weak and fragmented nephrin expression in the glomeruli compared with that of the db/m groups (P < 0.05; Figure 5G–L). A greater F4/80-positive macrophage infiltration in the glomerulus was noted in the db/db groups compared with that in the db/m groups (P < 0.05; Figure 5Z–AE). In the db/db+S mice, there was more prominent type IV collagen (Figure 5A–F) and thrombospondin-1 (Figure 5M–R) expression, more CTGF-positive cells (Figure 5S–Y) and a decreased and fragmented nephrin expression in the glomeruli as compared with that of the db/db control mice and the db/db+S mice (P < 0.05). In contrast, these changes were not observed in the db/m+L mice. Thus, there were no differences in these renal phenotypes between the db/m and db/m+L mice.

**Effects of dRK6 on the glomerular vasculature, oxidative stress and hypoxic stress**

The density of the PECAM-1 expression in the db/db groups was decreased compared with that of the db/m groups (P < 0.05; Figure 6A, C). The number of apoptotic endothelial cells (both TUNEL- and PECAM-1 positive...
cells, Figure 6B, D) in the db/db+S mice and the db/db+L mice was increased compared with that of the db/m groups (P < 0.05). All of the db/db groups showed a much greater expression of HIF-1α, which is a marker of hypoxia in the glomeruli, than that in the db/m groups, as determined by immunohistochemistry and western blotting of HIF-1α (P < 0.05; Figure 6E–G). All of the db/db groups also had increased 24-h urinary 8-OH-dG levels, which is a marker of oxidative DNA damage, and an increased expression of 8-OH-dG in the glomeruli compared with that of the db/m groups (P < 0.05; Figure 6H–J). These changes were more pronounced in the db/db+L mice as compared with that of the db/db control mice and the db/db+S mice (P < 0.05; Figure 6A–J). In contrast with the db/db+L mice, dRK6 administration in the db/m+L mice had no effects on the glomerular vasculature and hypoxia-induced oxidative stress.

Effects of dRK6 on the HUVECs

We investigated the dRK6-induced apoptosis of HUVECs with the use of different doses of dRK6 and various con-
centrations of glucose in the media (5 and 30 mmol/L of 
D-glucose, 5 mmol/L D-glucose + 25 mmol/L mannitol) 
for 48 h. At a concentration of 30 mmol/L of D-glucose 
(high glucose media), the TUNEL-positive HUVECs 
were increased after treatment with dRK6 (P < 0.05, 
Figure 7A). Furthermore, the phospho-Akt/total Akt ratio 
and the phospho-eNOS/total eNOS ratio were significantly 
decreased in the high glucose media with dRK6 treatment, 
compared with that in the samples in the low and high 
glucose media without dRK6 (Figure 7B–D, P < 0.05). 
The NOx concentrations from the supernatant were de-
creased with high glucose media and dRK6 treatment 
(Figure 7E). In contrast, the 8-iso-PGF$_2$α concentrations 
were increased with high glucose media, and these con-
centrations were more increased by the dRK6 treatment 
(Figure 7F). However, these changes were not observed 
at concentrations of 5 mM D-glucose (low glucose) or 
5 mM D-glucose + 25 mM mannitol. Instead, there was 
a significant increase in the NOx concentration when 
using low glucose media and treatment with dRK6.

Discussion

The current VEGF-A inhibitors are classified into four 
groups: the inhibitors of VEGF secretion, the drugs that 
sequester VEGF, the drugs that block the binding of VEGF 
to VEGFRs and the inhibitors of receptor tyrosine kinase 
[18]. Importantly, the efficacy of these individual com-
pounds is not identical. Therefore, the present study inves-
tigated the role of VEGF-VEGFR-2 blockade in diabetic 
nephropathy. Consistent with the previous study [24,29], 
we found that the urinary albuminuria and VEGF excretion 
varied according to the evolution of diabetic nephropathy. 
Consistent with the previous study [24,29], we found that the urinary albuminuria and VEGF excretion both 
reached their peak at 16 weeks of age, and the urinary 
VEGF excretion was strongly correlated with the levels 
of albuminuria.

The present study also demonstrated that dRK6 treat-
ment decreased the VEGFR-2 mRNA expression and pro-
tein expressions and also downregulated Akt/eNOS-NO, 
downstream mediator of VEGF-2 receptor in db/db mice,
but not the VEGFR-1 mRNA expression. The inhibitory effect of VEGF-2 receptor and downstream mediator by dRK6 was not found in db/m mice. Taken together, we could cautiously conclude that dRK6 could selectively block VEGFR-2/Akt axis only in diabetic db/db mice but not in non-diabetic db/m mice. Long-term dRK6 treatment also aggravated the albuminuria, the renal hypertrophy with mesangial expansion, the podocyte injury and the macrophage infiltration in db/db mice, which were associated with increases in the number of apoptotic glomerular endothelial cells and the hypoxia-oxidative stress. Short-term dRK6 treatment in the early stage of diabetes did not achieve beneficial effects on the kidney. Intriguingly, these deteriorated effects were not observed in the non-diabetic db/m mice, suggesting diabetes per se might be the determinant of dRK6-induced renal effects.

Much recently accumulated evidence has clearly demonstrated that physiological NO is required to maintain the normal endothelial function and that NO levels that are either too high or too low may lead to endothelial dysfunction, especially in the diabetes milieu [18,28,34]. In this study, phosphorylation of Akt/eNOS was significantly inhibited in the kidney of the diabetic mice. Consistent with phosphorylation of Akt/eNOS in the kidneys, the urinary NOx concentrations were significantly decreased in the diabetic mice. In the in vitro study using HUVECs, high glucose also lowered the Akt/eNOS expression and the NOx levels, and this supported our animal study results.

Fig. 6. Immunohistochemical staining for PECAM-1 (CD31), double staining for PECAM-1 and TUNEL, immunohistochemical staining and western blotting for HIF-1α and immunohistochemical staining for 8-OH-dG in the renal cortical glomeruli and the 24-h urinary 8-OH-dG. Representative immunostaining for PECAM-1 (A) and double-label immunostains for PECAM-1 and TUNEL (B, the PECAM-1 and TUNEL cells are depicted by arrowheads). Quantitative assessment of PECAM-1 positive (C) and the number of apoptotic endothelial cells (both the TUNEL and the PECAM-1 positive cells, D) in the renal cortical glomerulus of the db/m, db/m+L, db/db, db/db+S and db/db+L groups. Representative immunostaining for HIF-1α (E) and quantitative assessment of HIF-1α (F) in the renal cortical glomerulus. Western blot analysis of HIF-1α in the renal cortex (G). Representative immunostaining for 8-OH-dG (H) and the quantitative assessment of 8-OH-dG (I) in the renal cortical glomerulus (original magnification, ×400). Twenty-four-hour urinary 8-OH-dG (J) in each group. *P < 0.05 compared with the db/m control and db/m+L. **P < 0.05 compared with the db/db control and db/db+S.
In addition, long-term dRK6 administration further lowered the phosphorylation of Akt/eNOS and the urinary NOx concentrations in diabetic mice. On the contrary, no such changes were found in the non-diabetic mice. Consistent with this observation, dRK6 blocked the phospho-Akt/eNOS and it lowered the NOx concentrations in the HU-VECs that were only exposed to high glucose, suggesting that the renal pathological phenotypes might be tightly linked to the activity of phospho-Akt/eNOS-NO signalling in the diabetic state. It is well known that NO can negatively regulate the VEGF-A-induced endothelial changes [36]. A potential explanation for the effects of dRK6 on the kidney might be related to the reduced NO bioavailability. An important functional consequence of decreased NO was the association with renal dysfunction that favours inflammation, glomerulosclerosis and apoptosis of glomerular endothelial cells. Paradoxically, it is likely that physiological levels of NO (like VEGF-A) are required to maintain adequate endothelial function and that NO levels that are too low, as induced by dRK6, may lead to endothelial damage.

The increased VEGF expression in early diabetes is associated with glomerular hyperfiltration and abnormal angiogenesis with endothelial cell proliferation [7,17,19,23]. The glomerular capillary function is also under the strict control of the VEGF system. It is well known that administering a pan-VEGF receptor tyrosine kinase inhibitor in db/db mice and also anti-angiogenesis agents, which block VEGFR-1, -2 and -3, ameliorates the glomerular hyperfiltration in streptozotocin-induced diabetic rats [37,38]. In this study, all the short- and long-term dRK6-treated db/db groups showed significantly reduced creatinine clearance compared with that of the db/db control group, indicating that diabetes-induced hyperfiltration might be mainly mediated by VEGFR-2 at the early stage of diabetes. However, it is not clear whether dRK6 can decrease glomerular filtration rate (GFR) in db/db mice because the serum creatinine does not really change, and the
Fig. 6. (continued).
Recent finding that endogenous creatinine clearance exceeds the inulin clearance by a factor of approximately two and about half of creatinine excretion is derived from tubular secretion in C57BL/6J mice [39]. A study using inulin clearance will be needed to confirm clearly whether dRK6 can decrease GFR.

Recent studies have shown that VEGF withdrawal results in regression of the vasculature, glomerulosclerosis and interstitial fibrosis in various animal models, including the db/db mouse [10,40–46]. Another study with mostly type 2 patients concluded that the VEGF expression, at the transcript and protein levels, was decreased in diabetic nephropathy, and even in the early stages, and that this VEGF deficiency was associated with worsening proteinuria, glomerular capillary and peritubular capillary rarefaction and ischaemic interstitial fibrosis [26]. In this study, we also demonstrated that the inhibition of VEGFR-2 by short-term dRK6 treatment in db/db mice at the early stage of diabetes did not ameliorate the albuminuria and the renal structural changes. Despite the beneficial renal effect of anti-VEGF-A antibodies, pan-VEGFR kinase inhibitor and 2-(8-OH-6-methoxy-1-oxo-1H-2-benzopyran-3-yl) propionic acid (an inhibitor of angiogenesis) [21,37,47], our study showed that the selective VEGFR-2 inhibition by dRK6 had no beneficial effect on diabetic renal alterations. We and others have demonstrated that VEGFR-2 inhibition exacerbated the diabetic cardiomyopathy associated with endothelial damage in db/db mice and the hypertension and renal disease in the hypertensive rat [34,48]. Gnudi et al. recently succeeded in treating diabetic nephropathy using the podocyte-specific overexpression of soluble VEGFR-1 [49]. In this study, neither the VEGF-A expression nor VEGFR-2 phosphorylation was significantly blocked by the overexpression of soluble VEGFR-1, suggesting that the VEGF-A function was partially inhibited. Thus, the partial inhibition of VEGF might be a means to treat diabetic nephropathy without any adverse effects [18]. We still need more data to extrapolate the results of this study, in regard the complex role of the VEGFR-2-Akt/eNOS-NO axis according to the stages of diabetic nephropathy, to human diabetic nephropathy.

**Fig. 7.** The effect of dRK6 on apoptosis, the Akt and eNOS protein, nitrate/nitrite (NO\textsubscript{x}) and 8-iso-PGF\textsubscript{2α} concentrations in the HUVECs. The apoptosis of HUVECs as determined by in situ TUNEL assay and induced by treatment with different doses of dRK6 at 10\textsuperscript{−6}, 10\textsuperscript{−8} and 10\textsuperscript{−10} mmol/L and different concentrations of glucose in the media (5 mmol/L of D-glucose; LG, 30 mmol/L of D-glucose; HG, 5 mmol/L D-glucose + 25 mmol/L mannitol; MN, the osmolality control) for 48 h (A). *P < 0.05 and **P < 0.01 compared with LG and MN. Western blot analysis of the total Akt and phospho-Akt and the total eNOS and phospho-eNOS (B) and quantitative assessment of the phospho-Akt/total Akt ratio (C) and the phospho-eNOS/total eNOS ratio (D) from different concentrations of D-glucose in the media with dRK6 (LG+dRK6 or HG+dRK6) or without dRK6 (LG or HG) and the osmolality control (MN or MN+dRK6). The nitrate/nitrite (NO\textsubscript{x}) (E) and 8-iso-PGF\textsubscript{2α} (F) concentrations in the cell culture media of each group. *P < 0.05 and **P < 0.01 compared with LG and MN, #P < 0.05 compared with HG. The images are representative of \( n = 4 \) independent experiments.
With regard to the renal histology, mesangial matrix expansion with type IV collagen accumulation, which is associated with vascular rarefaction in the glomerulus, was significantly increased in the db/db+L mice. These changes were accompanied by increases of thrombospondin-1 as well as CTGF. Thrombospondin-1, which also causes inhibition of endothelial cell proliferation and survival, may contribute to the vascular rarefaction. CTGF, which is known to produce extracellular matrix via the TGF-β-dependent or -independent pathways in the setting of diabetes, may have a big role in glomerulosclerosis. A recent human study has demonstrated that a reduction of Akt-eNOS activity was correlated with the extent of glomerulosclerosis and interstitial fibrosis and a reduction of podocytes, and this was all accompanied by a reduction in these molecules [25].

Another principal mechanism of diabetic nephropathy is the stimulation of excessive production of the reactive oxygen species (ROS) in the kidney. The ROS in turn can upregulate the expression of profibrotic growth factors such as CTGF and TGF-β and so increases the glomerular matrix deposition [50,51]. Alternatively, NO induced by Akt-eNOS activation is one of the most important survival factors for endothelial cells that are exposed to hypoxia-oxidative injury. Collectively, an uncoupling of VEGF with NO has been associated with the deterioration of hypoxia-oxidative injury in the kidneys. Higgins et al. recently found that activation of HIF-1α signalling in the renal epithelial cells directly promoted fibrogenesis by increasing the expression of the extracellular matrix and infiltration of inflammatory cells [52]. All together, our results suggest that the VEGF-A-VEGFR-2 blockade by dRK6 seems to have a harmful effect on the kidneys owing to the hypoxia-induced oxidative stress.

In conclusion, our results suggest that the long-term selective blockade of VEGF-A-VEGFR-2 by dRK6 was associated with deteriorating effects on the kidney in type
VEGFR-2 inhibition in diabetic nephropathy

2 diabetes mice. These effects were associated with the downregulation of the phospho-Akt-eNOS-NO axis and the up-regulation of hypoxia-induced oxidative stress. Although the beneficial renal effect of anti-VEGF-A antibodies has been shown in diabetic animal models [19–26], we can postulate that a potential adverse effect with VEGF-A-VEGFR-2 blockade could be endothelial injury because endothelial cells require adequate VEGF-A activation in a physiologic condition and even in the diabetic state. Therefore, we need to be cautious before using VEGF-A inhibitors in the diseased kidney. New specific strategies to modulate the VEGF-A-phospho-Akt-eNOS-NO axis will be needed based on the evolution of type 2 diabetes as well.

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Conflict of interest statement. None declared.

References

Renoprotective effects of green tea extract on renin-angiotensin-aldosterone system in chronic cyclosporine-treated rats

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Abstract

Background. Renin-angiotensin-aldosterone system (RAAS) activation plays an important role in cyclosporine (CsA)-induced nephropathy. The main aim of this study was to test whether the administration of green tea extract (GTE) prevents the development of CsA-induced nephrotoxicity.

Methods. The rats were treated for 21 days and divided into four groups (n = 6/group): control group (0.9% saline injection), CsA group (30 mg/kg/day by intraperitoneal injection), CsA–GTE group (CsA plus GTE 100 mg/kg/day subcutaneous injection) and GTE group (GTE alone).

Results. There were significant increased levels of serum blood urea nitrogen and creatinine in the CsA group compared with that of the control group and significantly improved in the CsA–GTE group. Biochemical analysis showed that the plasma renin activity (PRA) and serum concentration of aldosterone were significantly increased in the CsA group compared with the control group and sig-

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