VEGF regulates TRPC6 channels in podocytes

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Abstract

Background. Both, increased plasma concentrations of vascular endothelial growth factor (VEGF) and increased expression of transient receptor potential canonical type 6 (TRPC6) channels in podocytes have been associated with proteinuric kidney diseases. Now, we investigated the hypothesis that VEGF regulates TRPC6 in podocytes.

Methods. TRPC6 messenger RNA (mRNA) and TRPC6 protein expression were analyzed in cultured podocytes after administration of VEGF165 using quantitative real-time reverse transcription–polymerase chain reaction and immunoblotting, respectively. YFP-tagged TRPC6 in podocytes was analyzed using confocal laser scanning microscopy. TRPC6-associated calcium influx was measured fluorometrically. Both, immunofluorescence and immunohistochemistry were performed in renal tissue from patients with diabetes mellitus and controls.

Results. Administration of VEGF165 to podocytes significantly increased TRPC6 mRNA expression and TRPC6 protein levels. The effects of VEGF165 were dose dependent and could be blocked by phosphoinositide-3-kinase inhibitors. In the presence of cycloheximide, an inhibitor of protein biosynthesis, we did not observe an effect of VEGF on TRPC6 protein levels, indicating the requirement of de novo protein synthesis. VEGF165 significantly increased TRPC6-mediated calcium influx in podocytes. Calcium influx was significantly lower in podocytes after gene knockdown using siRNA against TRPC6. Immunohistochemistry showed both increased TRPC6 channel protein and VEGF receptor type 2 (VEGFR-2) protein in podocytes from patients with diabetic nephropathy compared to control subjects. There was a significant association between VEGFR-2 mRNA and TRPC6 mRNA ($r^2 = 0.585; P < 0.0001$) in human renal cortex.

Conclusion. VEGF regulates TRPC6 in podocytes.

Keywords: podocytes; transient receptor potential canonical channel type 6; vascular endothelial growth factor

Introduction

Podocytes are of critical importance for the filter capacity of the glomerular capillary wall. Changes in their function and structure are of pathogenetic relevance to human proteinuric kidney diseases. The podocyte foot processes form a complex interdigitating pattern with slits that are bridged by extracellular protein–protein contacts to form the slit diaphragm [1]. Transient receptor potential channel canonical type 6 (TRPC6) channels, which belong to a family of non-selective cation channels are expressed in podocyte foot processes. TRPC6 are cation-permeable ion channels, which enable the influx of calcium and sodium ions into the cell, which consequently activate a cascade of signaling events [1, 2]. Alterations of TRPC6 in podocytes have recently been attributed to proteinuric kidney diseases [3–5]. A gain-of-function mutant of TRPC6 has been associated with familial focal and segmental glomerulosclerosis [3]. Möller et al. [4] described an increased TRPC6 expression in glomeruli from patients with membranous nephropathy. Liu et al. [5] observed a significant correlation between the ratio of TRPC3/TRPC6 transcripts in kidney cortex and urinary albumin excretion in Munich Wistar Frömter rats. However, the underlying cause of increased TRPC6 expression in proteinuric kidney disease is still unknown.

Increased vascular endothelial growth factor (VEGF) was observed in proteinuric kidney diseases [6–8]. Liu et al. [9] showed that transgenic rabbits expressing human VEGF165 in their kidneys exhibit progressive proteinuria with increased glomerular filtration rate at the early stage and decreased glomerular filtration rate at the later stage. Sung et al.
Materials and methods

Cell culture
Conditionally immortalized mouse podocytes (podocyte cell line E11), which have been cloned from the outgrowth of glomeruli isolated from H-2kb-tsA58 transgenic mice were purchased from Cell Lines Service (Eppelheim, Germany). In these cells, we determined markers of differentiated in vivo podocytes showing several unique podocyte proteins, including nephrin, podocin, Wilms tumor protein-1 (WT-1) and synaptopodin to verify podocyte integrity as reported for these cells previously [12, 13]. Cells were serum starved by incubation in a serum-free medium containing antibiotics 24 h before use. The cell viability was >95% as indicated by trypan blue exclusion. Podocytes were cultured in the absence or presence of the recombinant, human 165-amino acid isoform of VEGF (VEGFI65, final concentration, 10 ng/mL). PI3K was inhibited using wortmannin (final concentration 1 μmol/L) or LY294002 (final concentration, 10 μmol/L).

siRNA knockdown of TRPC6
Podocytes were transfected with siRNA specific for TRPC6 for 24 h using a silencer siRNA transfection kit (Ambion, Austin, TX). Briefly, podocytes were resuspended in RPMI medium containing 10% fetal bovine serum and incubated with siPORT amine (Ambion, Cambridgeshire, UK) and 1 μL chemically synthesized siRNA (final concentration 20 nmol/L; Ambion) specific for TRPC6. The target sequences were 5’-GGUUAAUGUUCGGAUUGUGGtt-3’ (sense) and 5’-CCACAAUCCGGAU-AACCGg-3’ (antisense) for TRPC6. In control experiments using the described transfection procedure, podocytes were transfected with siRNA containing a luciferase reporter including nephrin to verify podocyte integrity as reported for these cells previously [12, 13]. Cells were serum starved by incubation in a serum-free medium containing antibiotics 24 h before use. The cell viability was >95% as indicated by trypan blue exclusion. Podocytes were cultured in the absence or presence of the recombinant, human 165-amino acid isoform of VEGF (VEGFI65, final concentration, 10 ng/mL). PI3K was inhibited using wortmannin (final concentration 1 μmol/L) or LY294002 (final concentration, 10 μmol/L).

DNA isolation and quantitative real-time polymerase chain reaction
RNA isolation and quantitative real-time polymerase chain reaction (PCR) were performed as recently described by our group [14]. mRNA (PCR) were performed as recently described by our group [14]. mRNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and purified according to the manufacturer’s instructions. cDNA synthesis was performed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, CA). The primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3-0.4.0/) to amplify specific segments of the mouse TRPC6, GAPDH, VEGF165, and VEGF-R2 mRNAs. The primers were chosen to produce amplicons of 100 to 400 bp in length. The primer sequences are listed in Table 1. Real-time PCR was performed using the LightCycler-Fast Start DNA SYBR Green I mix (Roche Diagnostics) and 500 nmol/L of each primer were used in a final volume of 20 μL. PCR was started with denaturation at 95°C for 10 min. Then, 45 cycles were performed under the following conditions: denaturation at 95°C for 10 s, annealing at 57°C (hTRPC6, mTRPC6, hGAPDH), 58°C (hNephrin), 60°C (mGAPDH), 63°C (mNephrin) and 64°C (hVEGF, hVEGF-R2) for 10 s and extension at 72°C for 15 s. Fluorescence data were acquired at the end of each extension phase. After amplification, a melting curve analysis from 65 to 95°C with a heating rate of 0.1°C/s with a continuous fluorescence acquisition was made to assure correct PCR amplification. Data were recorded on a LightCycler 2.0 Instrument and cycle threshold values (crossing points, Cq) for each reaction were determined using LightCycler Software Version 4.0 (Roche Diagnostics). The median from triplicate determinations was used.

The TRPC6 expression was determined relative to the housekeeping gene GAPDH and normalized ratios of gene expression were calculated including efficiency correction and calibrator normalization. Normalized ratios were expressed according to the following equation: Normalized ratio = (E4  10^(-3C(T(4 mins))−C(Titurbo reverse sample))/E4  10^(-3C(T(minutes))−C(Tturbo reverse calibrator)) with E4 or E0, efficiency of target/reference amplification; C(t) or C(p), cycle number at target/reference detection threshold (crossing point); T, target; R, reference; S, unknown sample; C, calibrator.

Immunoblotting of TRPC6
In the first step, renal tissue was transferred to a tube containing 1 mL ice-cold phosphate-buffered saline (PBS) and homogenized on ice for 30 s using an Ultraturrax instrument (IKA-Werke, Germany), while podocytes and podocytes were centrifuged for 3 min at 3000 r.p.m. The supernatant was discarded. Then, 1 mL solution L2 containing 25 mmol/L Tris–HCl, pH 8; 1 mol/L NaCl; 200 mmol/L ethylenediaminetetraacetic acid (EDTA); 1 mol/L β-mercaptoethanol; 1 mol/L sodium fluoride and complete mini protease inhibitor cocktail (Roche Diagnostics) was added. The homogenate was sonicated three times for 20 s and centrifuged at 4°C for 10 min at 10500 r.p.m. The proteins in the supernatant were concentrated using a centrifugal filter device (Amicon ultra; Millipore), mixed with loading buffer, heated up to 100°C for 3 min, separated using a denaturing 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis at 150 V for 90 min and transferred to pure nitrocellulose membranes (Trans-Blot transfer medium; Bio-Rad Laboratories) at 14 V overnight. Membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences, Bad Homburg, Germany) for 1 h at room temperature. Membranes were incubated with primary rabbit anti-TRPC6 antibody (Alomone Labs, Jerusalem, Israel), anti-GFP antibody, or anti-β-actin (Santa Cruz Biotechnology) at a 1:10000 solution containing 0.1% Tween 20. Odyssey blocking buffer and PBS for 1 h, washed four times for 5 min. Membranes were then incubated with the secondary antibody [IRDye800 infrared fluorescent dye-conjugated sheep anti-rabbit antibody (Biomol, Hamburg, Germany)] at a 1:1000 solution containing 0.1% Tween 20, Odyssey blocking buffer and PBS for 1 h and washed four times for 5 min. Imaging was performed at a wavelength of 800 nm.
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The visualization of green fluorescent protein-tagged TRPC6 and yellow fluorescent protein-tagged TRPC6 in podocytes using laser scanning microscopy

The visualization of GFP-tagged TRPC6 or YFP-tagged TRPC6 in transfected cells was performed as described previously by our group [15, 16]. Transient transfection of podocytes was carried out using lipofectamine (Invitrogen, Carlsbad, CA) according to the supplier’s recommendations. GFP-tagged TRPC6 was kindly provided by Dr A. Dietrich (Marburg, Germany), and YFP-tagged TRPC6 was kindly provided by Dr M. Schaefer (Leipzig, Germany). The characteristics of GFP-tagged TRPC6 and YFP-tagged TRPC6 have been described previously [17, 18]. For characterization of the constructs, we performed sequencing of the cloning vector pcDNA3 harboring GFP-tagged TRPC6. Comparison with the Refseq database confirmed 100% identity of the sequence of cloned human TRPC6. Fluorescence signals were visualized at room temperature on a Zeiss LSM 510 META inverted confocal laser scanning microscope (objective lens, ×100/1.3 oil; optical section, <0.8 μm; multitrack mode; GFP, excitation 488 nm, argon laser; 500–530 nm band-pass filter; YFP, excitation 514 nm, argon laser; 500–530 nm band-pass filter; trypan blue, excitation 543 nm, helium–neon laser; 560 nm long-pass filter). The signals were computed using Zeiss LSM 510 acquisition software (Release 3.2 SP2). For quantification of fluorescence intensities, signal strength was calculated from a minimum of 50 separate cells and analyzed statistically.

Fluorescence measurements of cytosolic calcium

For the measurement of cytosolic calcium, podocytes were loaded with 2 μM of the calcium-sensitive, cell permeable, intracellular fluorescence dye fura2/AM. Fluorescence measurements were performed in a temperature-controlled 96-well fluorescent plate reader at 37°C (Fluoroskan Ascent Fluorometer; Thermo LabSystems Oy, Helsinki, Finland) at 510 nm emission with excitation wavelengths of 340 and 380 nm.

Immunofluorescence staining

For immunostaining, 2–3 μm sections were cut, de-paraffinized and subjected to a heat-induced epitope retrieval step before incubation with antibodies. Sections were immersed in sodium citrate buffer solutions at pH 6.0 and heated in a high-pressure cooker. The slides were rinsed in cool running water, washed in Tris-buffered saline (pH 7.4) and incubated with primary antibodies. For double immunofluorescence labeling, sections were incubated with rabbit anti-TRPC6 (dilution 1:50; Alomone Labs, Jerusalem, Israel) or rabbit anti-VEGF-R2 (1:20, Calbiochem #676488) followed by Alexa Fluor 555-conjugated anti-rabbit antibody (1:100; Invitrogen), washed three times in PBS and incubated with mouse anti-WT-1 (1:50; Dako, Glostrup, Denmark) followed by Alexa Fluor 488-conjugated anti-mouse antibody (1:100, Invitrogen). Nuclei were counterstained with DAPI (1:1500; Roche, Mannheim, Germany) and slides mounted in Fluormount-G (Southern Biotech, Birmingham, AL). Images were acquired using a fluorescence microscope (Axioskop2 Plus, Zeiss) equipped with a CCD camera (AxioCam MRm) and processed with Axiovision software (Carl Zeiss MicroImaging, Inc).

Immunohistochemistry

The expression of TRPC6 protein, VEGF-R2 and podocyte specific WT-1 protein in kidney biopsies was analyzed by immunohistochemistry using formalin-fixed paraffin-embedded tissue. The indication to perform a kidney biopsy was determined by each patient’s physician. Informed consent was obtained from each patient. All diabetic patients and controls were male. One patient had diabetes mellitus type 1 and three patients had diabetes mellitus type 2. Mean age was 59 ± 6 years. Mean body mass index was 32 ± 2 kg/m². Systolic blood pressure was 149 ± 12 mmHg. Diastolic blood pressure was 83 ± 5 mmHg. Compared to controls, diabetic patients had significantly increased glucose levels (180 ± 8 vs 88 ± 5 mg/dL) and an increased glycated hemoglobin HbA1c (7.7 ± 0.6 versus 5.7 ± 0.2%; each n = 4; each P < 0.05). On the other hand, cholesterol, high-density lipoprotein (HDL)-cholesterol, low-density lipoprotein (LDL)-cholesterol and creatinine levels were not significantly different between the two groups (cholesterol, 162 ± 22 versus 190 ± 15 mg/dL; HDL-cholesterol, 41 ± 6 versus 48 ± 9 mg/dL; LDL-cholesterol, 102 ± 21 versus 129 ± 17 mg/dL; creatinine, 3.1 ± 0.5 versus 1.9 ± 0.9 mg/dL; each P > 0.05 between the two groups). Four micrometer thick sections were cut, de-paraffinized and subjected to a heat-induced epitope retrieval step before incubation with antibodies. For this purpose, sections were immersed in sodium citrate buffer at pH 6.0 and heated in a high-pressure cooker. After cooking, the slides were rinsed in running water, washed with Tris-buffered saline, pH 7.4, and incubated with rabbit anti-TRPC6 antibodies (dilution 1:1000; Alomone Labs) diluted in 1% bovine serum albumin in PBS. The streptavidin AP kit (K5005; Dako) was used for detection and alkaline phosphatase was developed using Fast Red as the chromogen. After counterstaining with hematoxylin, the slides were dehydrated and mounted. Negative controls were performed by omitting the primary antibodies. Intensity of protein expression levels in human glomerula was scored as negative (−), weak (+), moderate (+++) or strong (+++). Immunohistochemistry was independently evaluated by two investigators that were unaware of the patients’ disease status and in the case of discrepancy slides were discussed over a multihead microscope until consensus was reached.

Podocyte migration assay

Podocyte migration was evaluated using the scratch assay as described [19]. Podocyte migration into the denuded area was evaluated by microscopy, documented by photographs and the number of cells was counted. Each experiment was performed in triplicate.

All substances were obtained from Sigma–Aldrich (Taufkirchen, Germany) or Merck Biosciences (Schwalbach, Germany) if not indicated otherwise.

Statistics

All data were expressed as mean ± SEM of at least four independent experiments and were compared using a two-tailed t-test or analysis of variance with Bonferroni’s multiple comparison post-test, as appropriate. The null hypothesis was rejected at P < 0.05. Relations between variables were investigated using linear regression analysis. Where error bars do not appear on the figure, error was within the symbol size. All data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA).

Results

VEGF increases TRPC6 mRNA in podocytes

TRPC6 mRNA was identified in podocytes using real-time RT–PCR (Figure 1A). Specificity of the amplification process by PCR was confirmed by a single band at 327 bp in the gel electrophoresis. Melting curve analysis confirmed specificity of transcripts of TRPC6 in podocytes. To test the hypothesis that VEGF165 may affect TRPC6 expression, cultured podocytes were exposed to physiological concentrations of VEGF165. 10 ng/mL VEGF165 significantly increased TRPC6 mRNA expression in podocytes by 3.03-fold (n = 8; P < 0.05). As indicated in Figure 1B, the effect of VEGF165 was dose dependent. Furthermore, the VEGF165-induced upregulation of TRPC6 mRNA was dependent on activation of the PI3K pathway. In the presence of the specific PI3K inhibitors, wortmannin and Ly294002, VEGF165 induced only a 1.20-fold or 0.81-fold change of TRPC6 mRNA expression, respectively (each n = 8; P < 0.05 compared to VEGF alone; Figure 1C).

VEGF increases TRPC6 protein in podocytes

We evaluated the effects of VEGF on both endogenous TRPC6 and GFP-tagged TRPC6 protein transfected into podocytes. An immunoblot of TRPC6 protein together with protein markers is depicted in Figure 2A. Immunoblotting with specific TRPC antibodies showed that the molecular weight was 106 kDa for TRPC6, confirming that these antibodies can be used to identify TRPC6 in podocytes. Immunoblotting showed that VEGF increased endogenous TRPC6 in podocytes. Compared to control conditions, VEGF increased endogenous TRPC6 protein to 1.86 ± 0.04-fold (P < 0.01; Figure 2B). Quantitative fluorescence

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VEGF increases calcium influx and migration in podocytes

Next, we investigated the functional changes after VEGF induced TRPC6 overexpression in podocytes. In line with recent literature, we used the TRPC6-specific agonist hyperforin to evaluate TRPC6-dependent calcium influx [21]. As indicated in Figure 3A–C, the administration of VEGF165 significantly increased the hyperforin-induced calcium influx by 1.23-fold compared to control conditions. To gain further evidence that the observed effects were due to increased TRPC6 function, the experiments were repeated with podocytes, which had been transfected with siRNA against TRPC6. Specific gene knockdown by administration of siRNA against TRPC6 reduced the increased hyperforin-induced calcium influx in response to VEGF165. In podocytes that had been transfected with siRNA against TRPC6, the hyperforin-induced calcium influx after administration of VEGF165 was significantly reduced (Figure 3C). These results indicate that, firstly, hyperforin induces TRPC6-dependent calcium influx; secondly, VEGF increases TRPC6 protein levels and thirdly, siRNA against TRPC6 reduces the VEGF-induced increase of TRPC6 protein.

Podocyte migration was quantified using the scratch assay under control conditions and after administration of VEGF165. Repopulation of the denuded area was followed by microscopy. Photographs are shown in Figure 3D. Administration of VEGF165 induced a 1.65-fold increase in podocyte migration (P < 0.01; Figure 3E).

Increased TRPC6 in diabetic nephropathy

In kidney biopsies, presence of podocytes was confirmed using podocyte specific WT-1 protein expression. Figure 4A shows co-staining of WT-1 and TRPC6, whereas Figure 4B shows co-staining of WT-1 and VEGFR-2, whereas Figure 4C shows co-staining of TRPC6 and VEGFR-2.

Now, we also compared the expression of glomerular TRPC6 protein in biopsies from four diabetic and four non-diabetic patients using immunohistochemistry. We observed an increased TRPC6 channel expression in podocytes from patients with diabetic nephropathy compared to control subjects. We observed a grade ++ or grade +++ expression of TRPC6 in four of four patients with diabetic nephrathy, whereas all control subjects showed a grade + expression. In addition, using immunohistochemistry, we observed a grade ++ or grade +++ expression of VEGFR-2 in four of four patients with diabetic nephropathy, whereas all four control subjects showed a grade + expression.

Lastly, we investigated VEGFR-2 and TRPC6 transcripts in human renal cortex from subjects that had been characterized previously [14]. In human renal cortex, we observed a significant association between VEGFR-2 mRNA and TRPC6 mRNA (n = 48; r² = 0.585; P < 0.0001; Figure 4D).

Discussion

In the present study, we were able to demonstrate that VEGF165 increases TRPC6 mRNA and TRPC6 protein in podocytes, which causes increased TRPC6-dependent calcium influx and increased podocyte migration. Furthermore,
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Fig. 2. VEGF increases TRPC6 protein in podocytes via PI3K pathway. 

(A) Immunoblotting of endogenous TRPC6 protein in podocytes. Protein markers are also shown. The predicted molecular weight for TRPC6 was 106 kDa (indicated by arrow). 

(B) Immunoblotting showing that VEGF increases endogenous TRPC6 in podocytes. **P < 0.01; each n = 4. 

(C) VEGF increases GFP-tagged TRPC6 protein transfected into podocytes. Fluorescence of GFP-tagged TRPC6 was quantified at 510 nm emission with excitation wavelengths from 400 to 480 nm. Loading control is depicted by β-actin expression, which quantifies the amount of cells loaded for fluorescence determinations. 

(D) Cycloheximide (CHX, 10 μmol/L), an inhibitor of protein biosynthesis, blocked the VEGF-induced GFP-TRPC6 protein increase in podocytes. Representative immunoblottings are illustrated. Summary data show results from four experiments. 

(E) Dose-dependent effect of VEGF165 on GFP-TRPC6 protein expression in podocytes. Each N = 5. **P < 0.01 compared to control conditions by ANOVA with Bonferroni’s multiple comparison post-test. 

(F) Inhibition of VEGF165-induced upregulation of GFP-TRPC6 protein expression in podocytes (n = 10) by PI3K inhibitors wortmannin (Wort; n = 5) and Ly294002 (Ly; n = 5). The final concentration of VEGF165 was 10 ng/mL. **P < 0.01 compared to control conditions, ++P < 0.01 compared to VEGF alone by ANOVA with Bonferroni’s multiple comparison post-test. 

(G) Representative fluorescence scans of transiently transfected podocytes with YFP-tagged TRPC6, which were cultured in the absence (Control) and presence of VEGF. All scans were recorded using the same setup parameters and are representative of two independent experiments. Scale bar = 5 μm. Summary data obtained in 50 cells showing that VEGF significantly increased total fluorescence from YFP-tagged TRPC6 in podocytes. **P < 0.01.
an increased TRPC6 protein expression was found in glomeruli from patients with diabetic nephropathy compared to controls.

Non-selective cation channels from the TRPC family have been associated with vascular and renal diseases [22]. As a rule of thumb, an increased TRPC expression appears to be associated with increased agonist-induced calcium influx and increased cell activity. In familial focal and segmental glomerulosclerosis, seven different mutations in TRPC6 channels have been identified in seven different families. Four of these seven mutations (R895C, E897K, P112Q and Q889K) allow significantly higher calcium currents compared to wild-type TRPC6 [23]. These gain-of-function mutants of TRPC6 in podocytes were associated with proteinuria and chronic kidney disease [3, 23]. However, mutations of TRPC6 channels appear to be a rare event. Santin et al. performed TRPC6 mutation analysis by direct sequencing in 130 Spanish patients from 115 unrelated families with focal and segmental glomerulosclerosis. They found only three cases of missense substitutions, indicating that in a cohort of proteinuric kidney disease, TRPC6 channel mutations are only observed in ~2% of affected patients [24]. In the present study, we observed increased wild-type TRPC6 protein expression in podocytes from patients with diabetic nephropathy.

Fig. 3. VEGF increases TRPC6-associated calcium influx and podocyte migration. (A) Representative tracings showing the hyperforin-induced increase of F340/F380 nm fluorescence ratio in fura2-loaded podocytes, which had been cultured in the absence (Control, open circles) and presence of VEGF (filled circles). Data are mean ± SEM from four experiments. (B) Representative tracings showing the hyperforin-induced increase of F340/F380 nm fluorescence ratio in fura2-loaded podocytes transfected with scramble siRNA (open circles) or transfected with siRNA against TRPC6 (filled circles) which had been cultured in the presence of VEGF. Data are mean ± SEM from four experiments. (C) Summary data of hyperforin-induced increase of F340/F380 nm in podocytes transfected with scrambled siRNA (Control) or transfected with siRNA against TRPC6 (hatched bar) which had been cultured in the absence (Control, open bar) and presence of VEGF (filled bar and hatched bar). **P < 0.01 by ANOVA with Bonferroni’s multiple comparison post-test. (D) and (E) Representative images and summary data of podocyte migration. Podocyte migration was quantified using the scratch assay under control conditions and after administration of VEGF. Repopulation of the denuded area was followed by microscopy and documented by photography after 24 h. The number of podocytes migrated into the denuded area indicated by dashed lines was counted after 24 h. **P < 0.01 by t-test.
In the literature, there are several findings linking VEGF and proteinuric kidney disease. Firstly, plasma VEGF levels are higher in patients with Type 2 diabetes mellitus compared to controls [25]. Secondly, during an 8-year-follow-up, the risk of developing microalbuminuria was significantly higher in patients with increased VEGF serum levels compared with those with normal levels at the beginning of the study even after adjustment for confounding variables [26]. Thirdly, renal and glomerular VEGF mRNA are increased in several experimental models of Type 2 diabetes mellitus [27, 28]. Fourthly, VEGF expression is increased in podocytes of patients with diabetes and proteinuria [8]. Now, we show that VEGF dose dependently increases TRPC6 mRNA and protein in podocytes. These findings may link VEGF and proteinuric kidney disease with TRPC6.

Ligation of VEGF to its receptor VEGFR-2 activates several signaling cascades including the PI3K pathway. In the present study, we confirmed these findings, showing that the VEGF165-induced TRPC6 expression was significantly blocked by PI3K inhibitors. Specific activation of TRPC6 channels by hyperforin has recently been identified [21]. In the present study, we observed that VEGF165 enhanced hyperforin-induced calcium influx in podocytes. Furthermore, administration of siRNA against TRPC6 significantly reduced the VEGF165-induced calcium influx. These findings indicate that VEGF enhances TRPC6-associated calcium influx in podocytes. The results are in line with previous findings on non-podocyte cells. Poock et al. [29] showed that VEGF increases vascular permeability via DAG-mediated calcium entry through TRPCs. Hamdollah et al. [30] showed that TRPC6 is essential in VEGF-mediated increase in cytosolic calcium and subsequent downstream signaling events in endothelial cells. Poteser et al. [31] observed VEGF-induced

Fig. 4. Increased TRPC6 protein in diabetic nephropathy. (A and B) Representative light micrographs and immunofluorescence of human kidney biopsies. Glomerula are shown from a control subject and a patient with diabetic nephropathy. PAS, periodic acid schiff staining; WT-1, podocyte-specific WT-1; DAPI, counterstaining of nuclei. Bar denotes 10 μm. VEGFR-2 could be observed both in ‘Control’ and ‘Diabetic nephropathy’. VEGFR-2 staining in the control glomerulus is light. However, VEGFR-2 is indicated by arrows in ‘Control’ and ‘Diabetic nephropathy’. (C) Representative immunofluorescence of human kidney biopsies. Glomerula are shown from a control subject and a patient with diabetic nephropathy. DAPI, counterstaining of nuclei. (D) VEGFR-2 mRNA and TRPC6 mRNA were determined in human renal cortex from 48 subjects. We observed a significant association between VEGFR-2 mRNA with TRPC6 mRNA (n = 48; linear regression, r² = 0.585; P < 0.0001).
calcium entry and TRPC3 expression in progenitor cells isolated from human adipose stroma.

We showed that compared to control conditions, VEGF increased endogenous TRPC6 protein as well as GFP-tagged TRPC6 and YFP-tagged TRPC6. GFP-TRPC6 and YFP-TRPC6 are under control of the CMV promoter and not the endogenous promoters. These results may indicate that either the amount of mRNA of the fusion proteins may be affected by the VEGF treatment of podocytes or the mRNA stability may be modified. The increase in GFP-TRPC6 and YFP-TRPC6 by VEGF may be interpreted as a positive effect of VEGF on GFP-TRPC6- and YFP-TRPC6-mRNA stability, as reported previously for other mRNA-species [32, 33]. A modification of the activity of the CMV promoter, while demonstrated for several factors [34–36], has not been reported for VEGF yet.

We observed a significant correlation in the expression of VEGFR-2 mRNA and TRPC6 mRNA in human renal cortex. Several experimental results indicate that the effects of VEGF are mainly mediated by VEGFR-2. Of note, increased glomerular expression of VEGF mRNA, VEGFR-2 mRNA and VEGFR-2 protein is observed in two models of proteinuric kidney diseases, namely in passive Heymann nephritis and puromycin aminonucleoside nephrosis [37]. In line with our present findings, Cheng et al. showed that VEGF is able to activate heterologously expressed TRPC6 channels through VEGFR-2 in endothelial cells. They showed that VEGF cannot activate TRPC6 channels in the absence of VEGFR-2 [38]. These findings indicate that several VEGF-mediated signaling pathways can regulate TRPC6 expression and function. Furthermore, the present study endorses a strong association between increased VEGF levels, increased TRPC6 expression and proteinuric diabetic kidney diseases. In summary, our present study shows that VEGF regulates TRPC6 in podocytes.

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