Vitamin D Down-Regulates TRPC6 Expression in Podocyte Injury and Proteinuric Glomerular Disease

Ramon Sonneveld,* Silvia Ferrè,† Joost G.J. Hoenderop,† Henry B. Dijkman,† Jo H.M. Berden,* René J.M. Bindels,† Jack F.M. Wetzels,* Johan van der Vlag,* and Tom Nijenhuis*

From the Departments of Nephrology,* Physiology,† and Pathology,‡ Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

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Address correspondence to Tom Nijenhuis, M.D., Ph.D., Department of Nephrology 464, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB, Nijmegen, The Netherlands. E-mail: T.Nijenhuis@nier.umcn.nl.

The transient receptor potential cation channel C6 (TRPC6) is a slit diaphragm protein expressed by podocytes. TRPC6 gain-of-function mutations cause autosomal dominant focal segmental glomerulosclerosis. In acquired proteinuric renal disease, glomerular TRPC6 expression is increased. We previously demonstrated that acquired increased TRPC6 expression is ameliorated by antiproteinuric angiotensin receptor blockers and angiotensin-converting enzyme inhibitors. Vitamin D also has an antiproteinuric effect. We hypothesized that vitamin D reduces proteinuria by affecting TRPC6 expression in podocytes.

Adriamycin-induced nephropathy increased TRPC6 mRNA and protein expression and induced proteinuria in rats. Treatment with 1,25-dihydroxyvitamin D3 (1,25-D3) normalized TRPC6 expression and reduced proteinuria.

In vitro, podocyte injury induced by adriamycin exposure in cultured podocytes increased TRPC6 expression. Treatment of injured podocytes with 1,25-D3 dose dependently reduced adriamycin-induced TRPC6 expression. Chromatin immunoprecipitation analysis demonstrated that the vitamin D receptor directly binds to the TRPC6 promoter. Moreover, 1,25-D3 reduced TRPC6 promoter activity in a luciferase reporter assay. In 1,25-D3−deficient 25-hydroxy-1α-hydroxylase knockout mice, TRPC6 expression was increased, accompanied by podocyte foot process effacement and proteinuria. In vivo, podocyte injury induced by adriamycin exposure in cultured podocytes increased TRPC6 expression. Treatment of injured podocytes with 1,25-D3 dose dependently reduced adriamycin-induced TRPC6 expression. Chromatin immunoprecipitation analysis demonstrated that the vitamin D receptor directly binds to the TRPC6 promoter. Moreover, 1,25-D3 reduced TRPC6 promoter activity in a luciferase reporter assay. In 1,25-D3−deficient 25-hydroxy-1α-hydroxylase knockout mice, TRPC6 expression was increased, accompanied by podocyte foot process effacement and proteinuria. In vivo and in vitro podocyte injury, possibly through a direct effect on TRPC6 promoter activity. This TRPC6 down-regulation could contribute to the antiproteinuric effect of vitamin D.

TRPC6 gain-of-function mutations are associated with a hereditary form of FSGS. Moreover, in several acquired proteinuric diseases, increased glomerular TRPC6 expression was demonstrated. Therefore, it seems that TRPC6 plays a role in the pathogenesis of podocyte injury in hereditary and acquired proteinuric diseases. Previously, we described a correlation between TRPC6 expression and the severity of the focal glomerular sclerosis

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score in an animal model for acquired FSGS. Furthermore, we demonstrated that angiotensin II activates TRPC6 and increases TRPC6 expression via a calcineurin/nuclear factor of activated T cells—mediated positive feedback signaling pathway, which contributes to podocyte injury. Moreover, we demonstrated that angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, pivotal therapies to reduce proteinuria, decrease TRPC6 expression in injured podocytes and in animal models for proteinuric disease. Thus, enhanced TRPC6 activity and/or expression seems to mediate podocyte and glomerular injury, whereas decreasing TRPC6 expression is associated with reduced injury and amelioration of proteinuria.

In addition to angiotensin-converting enzyme inhibitors and angiotensin receptor blockers, new antiproteinuric therapies have recently emerged, including treatment with vitamin D analogues. Cholecalciferol (vitamin D3) is taken up in the gastrointestinal tract or is synthesized by the effect of sunlight in the skin, after which it can be converted by the liver into 25-hydroxyvitamin D3 and, subsequently, to 1,25-dihydroxyvitamin D3 (1,25-D3) in the proximal tubule of the kidney. 1,25-D3 is classically defined as the active form of vitamin D, which plays a central role in Ca2+ and PO43- metabolism. With the progression of renal insufficiency, deficiency of 1,25-D3 ensues. However, 1,25-D3 deficiency may not be only a consequence of renal injury but also, as recent studies indicated, the cause of renal injury. Clinical and preclinical studies demonstrated that treatment with vitamin D analogues reduces proteinuria and podocyte loss. It was demonstrated that podocytes express the vitamin D receptor (VDR) and undergo ultrastructural changes when exposed to 1,25-D3. More recently, it was shown that 1,25-D3 regulates the expression of several key podocyte proteins, such as nephrin and podocin. Other TRP channel family members, such as TRPV5 and TRPV6, are also regulated by 1,25-D3.

Taken together, these data suggest a role for 1,25-D3 in the regulation of TRPC6 expression in podocyte injury and proteinuric disease. Therefore, we investigated whether vitamin D regulates TRPC6 expression in cultured podocytes and in animal models for FSGS or 1,25-D3 deficiency.

**Materials and Methods**

**AN Rats**

The adriamycin nephropathy (AN) model for human FSGS was induced in 8-week-old Wistar rats (Charles River Laboratories, Wilmington, MA) by a single tail vein injection with 5 mg/kg body weight of adriamycin (Sigma-Aldrich, St. Louis, MO). Thereafter, rats were treated with daily i.p. injections of 2.5 μg/kg bodyweight of 1,25-D3 or vehicle for 6 weeks. At the end of the experiment, the rats were housed in metabolic cages to collect 24-hour urine samples. Subsequently, the animals were sacrificed and kidneys and blood samples were collected.

All the animals were kept at the Central Animal Facility of Radboud University Nijmegen, Nijmegen, The Netherlands, in a standard room at 21°C and controlled humidity. The animals were exposed to a 12-hour light/dark cycle with ad libitum access to food and water. All the procedures involving animals were approved by the Animal Ethics Committee of Radboud University Nijmegen in accordance with the guidelines of the Dutch Council for Animal Care and the European Communities Council Directive (86/609/EEC).

**Podocyte Cell Culture**

Conditionally immortalized mouse podocytes (MPC-5) were cultured at 33°C with 5% CO2 and were differentiated at 37°C in RPMI Dutch-modified medium (Invitrogen, Carlsbad, CA) supplemented with 10% v/v fetal calf serum, 1% v/v penicillin/streptomycin, 10 μg/mL of interferon gamma, and 1% penicillin/streptomycin as described previously. Depending on the exact experimental setup, differentiated podocytes were treated with 0.25 μg/mL of adriamycin and different concentrations of 1,25-D3 or vehicle for 24 hours (Sigma-Aldrich). In these experiments, four or five separate podocyte cultures were used per experimental condition per experiment, and all the experiments were repeated at least twice for confirmation.

**Construction of TRPC6 Promoter Luciferase Reporter Construct and Luciferase Activity Assay**

The 5′-promoter region of the mouse TRPC6 gene (−1500/+32; +1 designates the transcription start site, NM_013838.2) was obtained by amplification of genomic DNA using primers 5′-GACGCTCGAGTGTTGCTTCTGCAGCCCG-AGTG-3′ and 5′-GATCAAGCTTAGCCGGAAGGA-ACCTTGACC-3′. The PCR product was cloned into the pGL3-basic luciferase reporter vector, and the cloned promoter sequence was verified by sequencing analysis. The pRL-CMV vector encoding Renilla luciferase under the control of a CMV promoter was used as control for transfection efficiency (Promega Corp., Fitchburg, WI).

Opossum kidney (OK) cells were cultured in Dulbecco’s modified Eagle’s medium/F12 (1:1) medium supplemented with 10% fetal calf serum, 15 mmol/L HEPES, 2.5 mmol/L l-glutamine, and 1% penicillin/streptomycin at 37°C in a humidity-controlled incubator with 5% (v/v) CO2. Briefly, cells were seeded in a 12-well plate and were transfected the following day in serum-free medium. The transfection mixture was prepared in 60 μL of Opti-MEM medium (Invitrogen) and consisted of 1 μg of either TRPC6 promoter construct or empty pGL3-basic vector, 50 ng of pRL-CMV, and 1.25 μL of Lipofectamine 2000 reagent (Invitrogen). Four hours after transfection, cells were washed with PBS and were incubated with culture medium containing 1% fetal calf serum in the presence of 100 mmol/L 1,25-D3 or vehicle. Cells were harvested 48 hours after transfection, and luciferase activity was determined using a Dual-Luciferase reporter assay system (Promega). In these experiments, four to five
separate cultures were used per experimental condition per experiment, and all the experiments were repeated at least twice for confirmation.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) analysis was performed using OK cells transfected with the TRPC6 promoter luciferase reporter construct or the empty pGL3-basic vector without promoter. Cells were treated with 100 nmol/L 1,25-D3 as described previously herein. Cells were harvested 4 hours after transfection, and a Magna ChIP A assay (Merck Millipore, Billerica, MA) was performed according to the manufacturer’s protocol. Briefly, proteins were cross-linked to the DNA with formaldehyde, cells were lysed, and samples were sonicated twice for 30 seconds on ice at a 22-μm amplitude using a Soniprep 150 (MSE, London, UK). Samples were incubated with 5.0 μg of rabbit polyclonal antiVDR antibody (ab3508; Abcam Inc., Cambridge, MA) or with rabbit IgG isotype antibodies as control. Immunoprecipitates were enriched with protein A magnetic beads. Subsequently, chromatin complexes were eluted, the cross-links were reversed, and the DNA was isolated. The presence of TRPC6 promoter DNA was evaluated using real-time PCR with specific primers corresponding to the TRPC6 promoter region (5′-CTCAACGCCATGTCGCCCATAC-3′ and 5′-GTAACACCAAGGGAGGCG-3′). Subsequently, samples were loaded on a 2% agarose gel and visualized using ProXima C16 software version 3.0 (Isogen Life Science, De Meern, The Netherlands).

1,25-D3—Deficient 25-Hydroxy-1α-Hydroxylase KO Mice

The 1,25-D3—deficient 25-hydroxy-1α-hydroxylase KO mice were previously generated by targeted ablation of exon 8 encoding the heme binding domain of the enzyme. Mice were genotyped using PCR and Southern blot analysis, as described previously.51 Five-week-old wild-type (WT) and KO mice were given daily i.p. injections with 500 pg of 1,25-D3 (Sigma-Aldrich) or vehicle for 6 weeks. At the end of the experiment, the mice were housed in metabolic cages to collect 24-hour urine samples. Subsequently, the animals were sacrificed; kidneys and blood samples were collected.

Analytical Procedures

Urinary albumin and creatinine levels were determined by radial immunodiffusion and enzymatic colorimetry, respectively. Serum Ca2⁺ levels were measured by spectrophotometry.32

Real-Time PCR Analysis

RNA was isolated from cultured podocytes or kidney cortex and were reverse transcribed (Transcriptor Kit; Roche Diagnostics, Mannheim, Germany). Real-time quantitative PCR was performed using SYBR Green SuperMix (Roche Diagnostics) on a MyiQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA) as described previously.14 TRPC6 expression was quantified by the ΔΔCT method using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the housekeeping gene. In these experiments, four to five separate cultures were used per experimental condition per experiment, and all the experiments were repeated at least twice for confirmation.

Immunohistochemical Analysis

Glomerular expression of TRPC6 and desmin was determined by semiquantitative scoring of immunofluorescence staining in 2-μm cryosections as described previously.14 In rat kidneys, TRPC6 was probed using a rabbit polyclonal antibody against the C-terminal tail of rat TRPC6 (Abcam Inc.). TRPC6 expression in mice kidneys was detected by a rabbit polyclonal antibody against the N-terminal tail of mouse TRPC6 (Alomone Labs, Jerusalem, Israel). Desmin expression was detected using a goat polyclonal antibody against the C-terminus of mouse and rat desmin (Santa Cruz Biotechnology, Santa Cruz, CA). Alexa-conjugated secondary antibodies were used subsequently. Glomerular TRPC6 and desmin expression was scored semiquantitatively on a scale from 0 to 5 based on the extent of TRPC6 immunofluorescence staining and on a scale from 0 to 10 for desmin staining in the glomerulus as described previously.14 Scoring was performed independently by two investigators, who scored 35 to 50 glomeruli per animal on blinded sections (R.S. and T.N.).

Transmission Electron Microscopy

For electron microscopy, we used immersion fixation. Small fragments of cortex were fixed in 2.5% glutaraldehyde dissolved in 0.1 mol/L sodium cacodylate buffer, pH 7.4, overnight at 4°C and were washed in the same buffer. The tissue fragments were postfixed in Palade-buffered 2% OsO4 for 1 hour, dehydrated, and embedded in Epon 812, Luft’s procedure (Merck, Darmstadt, Germany). Ultrathin sections were contrasted with 4% uranyl acetate for 45 minutes and subsequently with lead citrate for 5 minutes at room temperature. Podocyte effacement was analyzed by blinded scoring of the glomeruli in a 1200 EX2 electron microscope (JEOL, Tokyo, Japan).

Statistical Analysis

All the results are depicted as means ± SEM. All the statistical analyses were conducted by two-tailed Student’s t-test when comparing two treatment groups or experimental conditions and by analysis of variance when comparing three or more treatment groups or conditions using SPSS software version 15.0 (IBM, New York, NY). A P < 0.05 was considered significant.
Results

Effect of 1,25-D₃ on TRPC6 Expression in an in Vivo FSGS Model

To study the in vivo effects of vitamin D on TRPC6 expression and proteinuria in an animal model for FSGS, control and adriamycin-exposed rats (AN) were treated with 1,25-D₃ or vehicle. Vehicle-treated AN rats exhibited an increased albumin/creatinine ratio compared with vehicle-treated control rats, which was significantly ameliorated by 1,25-D₃ treatment (Figure 1A). 1,25-D₃ treatment did not alter the urinary albumin/creatinine ratio in control rats. AN rats showed increased TRPC6 mRNA (Figure 1B) and glomerular TRPC6 protein expression (Figure 1C). By co-staining for TRPC6 and nephrin, we demonstrated that the enhanced TRPC6 expression occurs primarily in podocytes (data not shown). 1,25-D₃ treatment significantly reduced adriamycin-induced TRPC6 mRNA and protein expression. Furthermore, TRPC6 expression was not significantly altered by 1,25-D₃ in control animals. In addition, glomerular desmin protein expression, as a measure of podocyte damage, was increased in AN rats but was significantly reduced on treatment with 1,25-D₃ (Figure 1D).

Effect of 1,25-D₃ on TRPC6 Expression in Podocyte Injury in Vitro

In the adriamycin-induced podocyte injury model, TRPC6 expression was significantly increased compared with that in vehicle-treated control cells (Figure 2A). When injured podocytes were treated for 24 hours with 100 nmol/L 1,25-D₃, TRPC6 expression was reduced. Furthermore, a dose-dependent reduction of adriamycin-induced TRPC6 expression was observed when adriamycin-injured podocytes were treated with increasing concentrations of 1,25-D₃ (Figure 2B). In contrast, no effect of 1,25-D₃ on TRPC6 expression was seen in uninjured control podocytes.

Effect of 1,25-D₃ on TRPC6 Promoter Activity

To evaluate whether 1,25-D₃ directly regulates TRPC6 transcription, possibly through vitamin D–responsive elements (VDREs) in the TRPC6 promoter, the 1500 bp upstream of the mouse TRPC6 transcription start site was cloned upstream of the luciferase reporter gene. OK cells were subsequently transfected with either the mouse TRPC6 promoter luciferase reporter construct or an empty vector that does not contain transcriptionally active elements and were treated for 48 hours with 100 nmol/L 1,25-D₃ or vehicle. Treatment with 1,25-D₃ significantly reduced the activity of the TRPC6 promoter by approximately 25% compared with the vehicle-treated cells (Figure 3). Luciferase activity was not altered by 1,25-D₃ in cells expressing the empty vector.

ChIP Analyses of VDR Binding to the TRPC6 Promoter Region

To determine whether the previously described effect of 1,25-D₃ on TRPC6 promoter activity is mediated by direct binding of the VDR to the TRPC6 promoter, we performed a ChIP assay. OK cells were transfected with the mouse TRPC6 promoter luciferase construct (TRPC6) or empty vector and were treated with 100 nmol/L 1,25-D₃ for 4 hours. After the ChIP assay, we performed a real-time PCR analysis, which showed a 16-fold enrichment of the TRPC6 promoter when precipitated with the anti-VDR antibody compared with the rabbit IgG isotype control (Figure 4). Two percent of the chromatin used for immunoprecipitation was included as a control (Figure 4). No significant difference was seen between both antibodies using the empty vector (data not shown).

TRPC6 Expression and Glomerular Injury in 1,25-D₃-Deficient Mice

To study the in vivo effect of 1,25-D₃ deficiency, we used 25-hydroxy-1a-hydroxylase knockout (KO) mice, which
are unable to synthesize 1,25-D$_3$. These KO mice showed a significantly increased albumin/creatinine ratio compared with their WT littermates (Figure 5A). Subsequently, 1,25-D$_3$ supplementation in 25-hydroxy-1$\alpha$-hydroxylase KO mice reduced the albumin/creatinine ratio to WT levels without restoring the hypocalcemia in these mice. At sacrifice, means ± SEM serum Ca$^{2+}$ concentrations were 2.09 ± 0.01, 1.37 ± 0.03, and 1.39 ± 0.04 mmol/L for WT, KO, and KO supplemented with 1,25-D$_3$, respectively. 25-hydroxy-1$\alpha$-hydroxylase KO mice demonstrated increased TRPC6 mRNA (Figure 5B) and glomerular TRPC6 protein (Figure 5C) expression. By co-staining for TRPC6 and podocin, we could demonstrate that the enhanced TRPC6 expression occurs in podocytes (data not shown). Furthermore, 1,25-D$_3$ treatment normalized TRPC6 mRNA and protein expression in these mice. Expression of desmin was significantly increased in 25-hydroxy-1$\alpha$-hydroxylase KO mice, which was restored to normal levels by 1,25-D$_3$ treatment (Figure 5D). Electron microscopy analysis clearly demonstrated more, and in some segments total, podocyte foot process effacement in proteinuric 1,25-D$_3$-deficient mice. Hardly any effacement could be detected in their WT nonproteinuric littermates (Figure 5E). The extent of podocyte effacement was quantified as a means ± SEM of 1.9% ± 0.1% in WT animals versus 15.3% ± 2.9% in 25-hydroxy-1$\alpha$-hydroxylase KO animals. When treated with 1,25-D$_3$, the podocyte foot process effacement in 25-hydroxy-1$\alpha$-hydroxylase KO animals completely recovered to a means ± SEM of 2.2% ± 0.7%, and the glomerular ultrastructure was not distinguishable from that of WT mice.

Figure 2  Dose-dependent effect of 1,25-D$_3$ on TRPC6 expression in podocyte injury. Cultured podocytes were exposed to adriamycin (ADRIA) or vehicle (VEH) in the absence or presence of 100 nmol/L 1,25-D$_3$ for 24 hours (A). Cultured podocytes were treated with ADRIA or VEH and subsequently were treated with different concentrations of 1,25-D$_3$ (100 nmol/L to 1$\mu$mol/L) or VEH for 24 hours (B). TRPC6 mRNA levels were determined by real-time quantitative PCR and quantified by the ΔΔC$_T$ method using GAPDH as the housekeeping gene. Results are shown as means ± SEM percentages compared with VEH-treated control podocytes. *P < 0.05 versus VEH/VEH treated; †P < 0.05 versus ADRIA.

Figure 3  Effect of 1,25-D$_3$ on TRPC6 promoter activity. A luciferase assay was performed in OK cells transiently transfected with a Firefly luciferase TRPC6 promoter construct or empty vector after 48 hours of treatment with 100 nmol/L 1,25-D$_3$, or vehicle (VEH). A Renilla luciferase construct was cotransfected to correct for transfection efficiency. Firefly/Renilla luciferase ratios were determined as a measure of promoter activity. Results are depicted as means ± SEM percentages compared with VEH-treated cells transfected with the TRPC6 promoter construct. *P < 0.05 versus VEH-treated empty vector; †P < 0.05 versus VEH-treated TRPC6 construct.

Figure 4  VDR binds to the TRPC6 promoter region. To determine whether the VDR directly binds to the TRPC6 promoter region, we performed a ChIP analysis. OK cells were transfected with the mouse TRPC6 promoter luciferase construct or the empty vector without promoter and were treated with 100 nmol/L 1,25-D$_3$. After ChIP using an anti-VDR antibody or a rabbit IgG isotype control antiserum, DNA was isolated and a real-time PCR with specific primers designed for the TRPC6 promoter was performed. Real-time PCR showed a 16-fold enrichment of signal using the anti-VDR antibody (anti-VDR lane) compared with the isotype control (IgG lane) in the presence of the TRPC6 promoter construct, whereas this enrichment was absent using the empty vector (data not shown). As a control, 2% of the chromatin used for the immunoprecipitation is shown (input lane).
process effacement. In line with the aforementioned results, 1,25-D₃ supplementation reversed the increased TRPC6 expression and proteinuria and normalized the podocyte morphology. Taken together, we demonstrated that vitamin D down-regulates the enhanced TRPC6 expression in in vitro and in vivo podocyte injury, possibly through a direct effect on TRPC6 promoter activity.

The present study is the first to show that TRPC6 promoter activity and expression is regulated by means of a nuclear hormone receptor, the VDR. TRPC6 was generally regarded to be a primarily receptor-operated channel, regulated by cell surface receptors such as the angiotensin II type 1 receptor. The TRP channel family members TRPV5 and TRPV6 are also transcriptionally regulated by vitamin D. However, these channels are not regarded as primarily receptor-mediated signaling proteins but rather are involved in transcellular Ca²⁺ transport across the gastrointestinal and renal epithelia, processes known to be governed by vitamin D.

Although the TRPC6 promoter activity can be inhibited by 1,25-D₃ in uninjured OK cells, in cultured podocytes and in vivo in the rat kidney, 1,25-D₃ seems to reduce TRPC6 expression in injured podocytes but not in uninjured podocytes. One explanation could be the length (~1500 bp) of the cloned TRPC6 promoter region for the luciferase reporter construct. In this cloned region, there could be fewer transcription sites than in the native promoter in its chromatin context. Therefore, the native TRPC6 gene in the cultured or in vivo podocytes might be regulated more strictly by other (transcription) factors, which are unable to affect the 1500-bp promoter construct, or are not even present, such as higher-order chromatin structures. A second explanation could be that the kinetics of the turnover and the relative expression of the luciferase protein are different from those of the TRPC6 mRNA and protein. A third explanation could be that the basal TRPC6 promoter activity is regulated by another transcriptional complex in a specific chromatin context in uninjured cultured or in vivo podocytes compared with the TRPC6 promoter activity in uninjured podocytes and/or OK cells.

In general, transcriptional regulation of vitamin D–responsive genes occurs through interaction of the nuclear VDR complex with VDRE in the promoter region of these target genes. In this study, we demonstrated that the VDR indeed binds to the TRPC6 promoter region. A VDRE consensus sequence is available for positively regulated genes. Genes that are negatively regulated by vitamin D are sparse and, next to TRPC6, include the genes encoding parathyroid hormone and renin. When the negative VDRE promoter sequences in these genes were identified, they did not comply with the consensus sequence.

Similarly, the TRPC6 promoter region did not contain sequences complying with the VDRE consensus sequence (data not shown). Thus, TRPC6 seems to be part of a select group of genes that are negatively regulated by vitamin D.

The relationship between proteinuria and increased TRPC6 activity and/or expression was demonstrated in
several acquired human proteinuric diseases and animal models.

In addition, down-regulation of TRPC6 expression by angiotensin receptor blockers, angiotensin-converting enzyme inhibitors, or calcineurin inhibitors correlated with reduced proteinuria in proteinuric animal models. Recently, Eckel et al. illustrated the potential beneficial antiproteinuric effect of down-regulating TRPC6 expression by showing reduced angiotensin II–mediated albuminuria in TRPC6 KO mice. In the present study, we demonstrated that 1,25-D₃ treatment significantly reduces glomerular TRPC6 expression, proteinuria and expression of the podocyte injury marker desmin. Electron microscopy showed podocyte foot process effacement in the KO mice. To confirm that the enhanced TRPC6 expression was 1,25-D₃–mediated and to test whether damage could be prevented, mice were supplemented with 1,25-D₃, which normalized glomerular TRPC6 expression and proteinuria. Theoretically, the observed effects could also result from, eg, the striking hypocalcemia interfering with TRPC6 function in the podocytes of 25-hydroxy-1α-hydroxylase KO model, which displays undetectable levels of 1,25-D₃. Glomeruli showed increased TRPC6 expression, which was paralleled by similarly increased expression of the podocyte damage marker desmin. Electron microscopy showed podocyte foot process effacement in the KO mice. To confirm that the enhanced TRPC6 expression was 1,25-D₃–mediated and to test whether damage could be prevented, mice were supplemented with 1,25-D₃, which normalized glomerular TRPC6 expression and proteinuria. The present study is the first, to our knowledge, to describe the glomerular and proteinuric phenotype in this animal model of 1,25-D₃ deficiency. However, these results by no means prove a causal relationship between increased TRPC6 expression and the glomerular phenotype in this particular model. Taken together though, these data demonstrate that 1,25-D₃ down-regulates the enhanced TRPC6 expression in FSGS and that 1,25-D₃ deficiency results in increased TRPC6 expression, which was proved to be 1,25-D₃ sensitive.

Although the TRPC6 promoter luciferase reporter and ChIP analyses suggested that TRPC6 promoter activity is a specific target of vitamin D, it does not rule out that in vivo other mechanisms could also contribute to the down-regulation of TRPC6 expression by vitamin D. The gene encoding renin, important in angiotensin II biosynthesis, is negatively regulated by vitamin D. As we previously showed that angiotensin II enhances TRPC6 expression by stimulating a calcineurin/nuclear factor of activated T cells–mediated feed-forward pathway in adriamycin-induced podocyte injury, this could certainly be an additional mechanism reducing TRPC6 expression. Recent studies in mice with cardiomyocyte-specific deletion of the VDR demonstrated that vitamin D inhibits the calcineurin/nuclear factor of activated T cells signaling pathway in the cardiomyocyte, which also includes TRPC6. Thus, the demonstrated vitamin D–mediated inhibition of TRPC6 promoter activity could serve to counteract the effect of the transcription factor nuclear factor of activated T cells on the TRPC6 promoter. Furthermore, TRPC6 will certainly not be the only mediator of the effect of vitamin D on glomerular injury and proteinuria. Several studies described the protective role of 1,25-D₃ in different proteinuric disorders, such as FSGS and diabetic nephropathy, in which the authors hypothesized that 1,25-D₃ acts on various pathways. For example, 1,25-D₃ up-regulates expression of various structural podocyte proteins, such as podocin and nephrin. Nephrin was shown to inhibit TRPC6–phospholipase C complex formation, surface expression, and activation. Therefore, in addition to inhibiting TRPC6 expression directly, 1,25-D₃ could also affect TRPC6 indirectly via nephrin.

Altogether, the present results add important new data to our understanding of the regulation of TRPC6 expression in podocytes. The transcriptional regulation of TRPC6 by 1,25-D₃ demonstrated in cultured podocytes and illustrated in a rat FSGS model and in 1,25-D₃–deficient mice suggests that TRPC6 down-regulation could, indeed, contribute to the antiproteinuric effect of vitamin D.

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