CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

1,25-Vitamin D₃ Deficiency Induces Albuminuria

Ramon Sonneveld,^{*,†} Joost G.J. Hoenderop,[†] Andrea W.D. Stavenuiter,[‡] Evelina Ferrantelli,[‡] Marijke P.A. Baltissen,^{*} Henry B. Dijkman,[§] Sandrine Florquin,[§] Angelique L. Rops,^{*} Jack F.M. Wetzels,^{*} Jo H.M. Berden,^{*} Johan van der Vlag,^{*} and Tom Nijenhuis^{*}

From the Departments of Nephrology,^{*} Physiology,[†] and Pathology,[§] Radboud University Medical Center, Nijmegen; and the Department of Cell Biology and Immunology,[‡] VU Medical Center, Amsterdam, the Netherlands

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Address correspondence to
Tom Nijenhuis, M.D., Ph.D.,
Department of Nephrology,
464, Radboud University Medical Center, PO Box 9101, 6500
HB Nijmegen, the Netherlands. E-mail: tom.nijenhuis@radboudumc.nl.

Vitamin D plays an important role in renal (patho)physiology. Patients with glomerular diseases have an injured renal filtration barrier, leading to proteinuria and reduced renal function. An impaired renal function also leads to 1,25-vitamin D₃ deficiency as a result of reduced renal 1 α -hydroxylase activity. Vitamin D treatment to reduce proteinuria remains controversial, although there is an inverse correlation between vitamin D levels and proteinuria. Herein, we showed that 1,25-vitamin D₃-deficient 25-hydroxy-vitamin-D₃-1 α -hydroxylase knockout mice and 1,25-vitamin D₃-deficient rats develop podocyte injury and renal dysfunction. Glomerular injury was characterized by proteinuria and partial podocyte foot process effacement. Expression of nephrin, podocin, desmin, and transient receptor potential channel C6 in the podocyte was significantly altered in 1,25-vitamin D₃-deficient animals. Supplementation with 1,25-vitamin D₃ or 1,25-vitamin D₂ prevented podocyte effacement or reversed glomerular and tubulointerstitial damage in 1,25-vitamin D₃-deficient animals, thereby preserving and restoring renal function, respectively. The effect of 1,25-vitamin D₃ deficiency and 1,25-vitamin D₃ and 1,25-vitamin D₂ repletion on proteinuria could not be explained by hypocalcemia, changes in parathyroid hormone, or fibroblast growth factor 23. This study demonstrates that 1,25-vitamin D₃ deficiency directly leads to renal injury in rodents. Translated to human subjects, this would underline the need for early vitamin D supplementation in patients with glomerular disease and chronic renal insufficiency, which might inhibit or potentially reverse renal injury. (*Am J Pathol* 2016, 186: 794–804; <http://dx.doi.org/10.1016/j.ajpath.2015.11.015>)

Vitamin D is a steroid hormone with important physiological roles in several organs and tissues.¹ 1,25-Vitamin D₃ (1,25-D₃) is the active form of vitamin D, and known for its role in calcium and phosphate homeostasis. However, the pluripotent 1,25-vitamin D₃ also influences processes in growth and development,^{2,3} cardiovascular disease,^{4,5} cancer,^{6,7} and the immune system.⁸ Treatment with vitamin D analogs seems to delay renal function decline and reduce proteinuria in animals and patients with chronic kidney disease.^{9–11} However, the therapeutic effect of vitamin D remains heavily debated.¹² Interestingly, scarce literature data describe an association between low serum vitamin D levels and morbidity, mortality, and renal damage as well as increased albuminuria.^{4,13–15} These studies do not prove causality, and the exact molecular mechanisms by which vitamin D deficiency would cause these effects are unknown.

Podocytes and the interconnecting slit diaphragm in the glomerular filtration barrier are injured in glomerular diseases, such as focal segmental glomerular sclerosis (FSGS)

and diabetic nephropathy, leading to renal dysfunction and proteinuria.^{16–19} Persisting renal injury can lead to chronic kidney disease and end-stage renal disease. In renal insufficiency, renal proximal tubular cells show reduced 1 α -hydroxylation capacity, resulting in 1,25-D₃ deficiency.²⁰ Patients with chronic kidney disease are treated with vitamin D analogs, but often only after secondary hyperparathyroidism has developed, with the goal to correct the disturbed calcium-phosphorus homeostasis. We

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Table 1 Primary Antibodies Used for Immunofluorescence Staining

Antigen	Antibody	Description	Dilution	Manufacturer
TRPC6	ACC-017	Rabbit—anti-mouse	1:1600	Alomone (Jerusalem, Israel)
TRPC6	AB62999	Rabbit—anti-rat	1:500	Abcam (Cambridge, UK)
Desmin	SC-7559	Goat—anti-mouse	1:200	Santa Cruz (Santa Cruz, CA)
Nephrin	AF-3159	Goat—anti-mouse	1:200	R&D (Minneapolis, MN)
Podocin	SC-22298	Goat—anti-mouse	1:50	Santa Cruz
Caspase 3	9661-S	Rabbit—anti-mouse	1:200	Cell Signaling Technology (Boston, MA)
Granulocytes	LY-6G	Rat—anti-mouse	1:100	eBioscience, Inc. (San Diego, CA)
NGAL		Rat—anti-mouse	1:50	R&D
Synaptopodin	65194	Mouse—anti-rat	1:5	Progen Pharmaceuticals Ltd (Toowong, QLD, Australia)
IgG	A11001	Goat—anti-mouse	1:200	Invitrogen (Carlsbad, CA)
IgM	A21044	Goat—anti-mouse	1:200	Life Technologies (Carlsbad, CA)
IgA	556978	Rat—anti-mouse	1:100	BD Pharmingen (San Diego, CA)
C3	14631	Goat—anti-mouse	1:40	Nordic (Tilburg, the Netherlands)
CD4	550278	Rat—anti-mouse	1:50	BD Pharmingen
CD8	550281	Rat—anti-mouse	1:50	BD Pharmingen
CD68	MCA1957	Rat—anti-mouse	1:1600	AbD (Serotec, UK)

NGAL, neutrophil gelatinase-associated lipocalin; TRPC6, transient receptor potential channel C6.

suggest that vitamin D deficiency itself induces or aggravates glomerular injury. Podocytes express the vitamin D receptor (VDR), and undergo ultrastructural changes when exposed to vitamin D.^{21,22} Vitamin D is known to regulate vital proteins in the slit diaphragm, such as nephrin and podocin, and we recently demonstrated the direct regulation of the transient receptor potential channel C6 (TRPC6) by vitamin D.^{23–27} Thus, in addition to being a consequence of renal insufficiency, we hypothesize that 1,25-D₃ deficiency itself can also cause renal injury.

In our study on regulation of TRPC6 by vitamin D, we demonstrated a previously unreported proteinuria in 1,25-D₃—deficient 25-hydroxy-vitamin-D₃-1 α -hydroxylase knockout mice (CYP27B1 or 1 α -OHase KO).²⁶ Herein, we assessed the renal phenotype of these animals by studying in detail the effects of this inherited 1,25-D₃ deficiency on the glomerulus, in particular the podocyte, and the tubulointerstitium. In addition, we studied glomerular, tubular, and podocyte injury in rats with acquired 1,25-D₃ deficiency. Furthermore, we excluded that the effects of 1,25-D₃ deficiency were secondary to the hypocalcemia or altered hormonal regulation of calcium-phosphorus balance in this situation. Elucidating the renal phenotype of these 1,25-D₃—deficient animal models provides new insights into the complex relationship between renal injury and 1,25-D₃ deficiency, and increases our understanding of the pluripotent effects of vitamin D.

Materials and Methods

Animal Studies

All animal procedures were approved by the Animal Ethics Committee of the Radboud University (Nijmegen, the Netherlands) or the VU Medical Center (Amsterdam, the Netherlands), in accordance with the guidelines of the Dutch Council for Animal Care and the European Communities Council Directive (86/609/EEC).

1 α -OHase (CYP27B1) KO mice with a C57BL/6 background were generated as previously described.²⁷ From an age of 5 weeks, mice received either a 2.0% calcium diet, as performed previously,²⁸ or daily i.p. injections with 1000 pg/g body weight 1,25-D₂, 500 (high dose) or 25 (low dose) pg/g body weight 1,25-D₃ (Sigma-Aldrich, St. Louis, MO), or vehicle for 6 weeks ($n = 8$ per group). These animals and untreated 5-week-old wild-type (WT) and KO mice were housed in metabolic cages for 24 hours to collect urine samples. Subsequently, animals were sacrificed and kidneys and blood were sampled for analysis.

Wistar rats (Charles River WU, Sulzfeld, Germany), weighing 275 to 300 g at the start of the experiment, were injected with 32 ng 1,25-D₂ at days 0, 2, 4, 7, 9, and 11 to deplete the endogenous pool of vitamin D₃. In addition, rats received a vitamin D deficient, but high calcium and phosphorus, diet (TD.120503 Brown C.C. Vitamin D Deficient Diet with Lactose; Harlan Laboratories, Inc. Madison, WI). From day 21, the animals were orally treated three times weekly with vehicle, 100 ng/kg 1,25-D₂, or 30 ng/kg 1,25-D₃, as described before ($n = 8$ animals per group).²⁹ The choice of particularly these two vitamin D analogs was on the basis of our *in vitro* experimental data (described in [Results](#)). After 8 weeks of treatment, rats were sacrificed, and urine, blood, and kidneys were harvested.

Serum and Urine Chemistry

Urinary neutrophil gelatinase-associated lipocalin (NGAL; R&D, Minneapolis, MN) and IgG (Roche, Basel, Switzerland for mouse; Antibodies-online, Aachen, Germany for rat) levels and serum fibroblast growth factor 23 C-terminal (Immunotopics, San Clemente, CA) and parathyroid hormone (PTH) 1-84 (Immunotopics) concentrations were measured in enzyme-linked immunosorbent assays. All enzyme-linked immunosorbent assays were performed according to the manufacturer's protocol. Calcium, albumin, urea, and

Figure 1 Renal injury in 25-hydroxy-vitamin-D₃-1 α -hydroxylase knockout (1 α -OHase KO) mice. Renal injury was assessed by urinary albumin/creatinine ratio (A), IgG/creatinine ratio (B), and neutrophil gelatinase-associated lipocalin (NGAL)/creatinine ratio excretion (C) in 4-week-old 1 α -OHase KO and wild-type (WT) mice. Five-week-old 1 α -OHase KO mice were given daily injections with a low-dose (25 pg/g) calcitriol (1,25-D₃) or vehicle for 6 weeks, after which mice were sacrificed. Glomerular and interstitial injury was assessed by urinary albumin/creatinine ratio (D), IgG/creatinine ratio (E), and NGAL/creatinine ratio excretion (F). Tubulointerstitial NGAL protein (G) and cleaved caspase 3 (H) expression. Protein and mRNA expression are depicted as percentage of vehicle-treated WT mice. Statistical tests used were *t*-test (A–C) and analysis of variance with Bonferroni post hoc analysis (D–H). **P* < 0.05 versus WT; †*P* < 0.05 versus KO. *N* = 8 mice per group (A–H). Original magnification, $\times 400$ (G). KO, knockout.

phosphate were determined on an Architect C16000 Clinical Chemistry Analyzer (Abbott Diagnostics, Chicago, IL). Serum vitamin D levels were assessed by radioimmunoassay after immunoextraction (IDS, Tyne and Wear, UK).

Transmission EM

Immersion fixation was used for electron microscopy (EM). Kidney cortex was fixed in 2.5% v/v glutaraldehyde dissolved in 0.1 mol/L sodium cacodylate buffer, pH 7.4, overnight at 4°C. The tissue was post-fixed in palade-buffered 2% w/v OsO₄, dehydrated, and embedded in Epon812, Luft's procedure (Merck, Darmstadt, Germany). Ultrathin sections were contrasted with 4% w/v uranyl acetate and subsequently with lead citrate. Podocyte effacement was analyzed using a Jeol 1200 EX2 electron microscope (JEOL, Tokyo, Japan).

Immunohistochemistry

Frozen kidney cortex of five to eight animals per group was cut into cryosections (2 μ m thick) and stained for several proteins (Table 1). Alexa-conjugated secondary antibodies

were used for detection, and sections were embedded in Vectashield (Vector Labs, Burlingame, CA). The antibody against granulocytes was directly labeled and, therefore, no secondary antibody was used. Analysis was performed on 30 glomeruli per animal by determining the fluorescent intensity and surface area using ImageJ software version 1.49i (NIH, Bethesda, MD). For the caspase-3 staining, we counted the amount of positive cells per microscopic field. We used a standard periodic acid–Schiff staining on paraffin sections to study glomerular and tubulointerstitial morphology by light microscopy.

Cell Culture Studies

Conditionally immortalized mouse podocytes (MPC-5) cells were cultured as described previously.³⁰ After 2 weeks of differentiation, cells were treated with 0.25 μ g/mL doxorubicin (Adriamycin) in combination with vehicle or a vitamin D analog [cholecalciferol (D₃), calcitriol (25-D₃), α -calcitriol (1 α -D₃), calcitriol (1,25-D₃), ergocalciferol (D₂), doxercalciferol (1 α -D₂), or paricalcitol

Table 2 Clinical Parameters of Wild-Type and 1 α -OHase KO Mice Treated with Vehicle or 1,25-D₃

Parameters	WT	KO	KO + Vitamin D
Serum PTH (pg/mL)	315 $\pm$ 68	1907 $\pm$ 353*	1540 $\pm$ 174*
PTH receptor expression (% vs WT)	100 $\pm$ 12.1	81.5 $\pm$ 5.6	89.0 $\pm$ 28.0
VDR expression (% vs WT)	100 $\pm$ 12.5	27.0 $\pm$ 4.6*	10.4 $\pm$ 2.8* [†]
Serum FGF23 (pg/mL)	225 $\pm$ 66	ND	ND
Body weight (g)	23.4 $\pm$ 1.7 M 18.5 $\pm$ 0.8 F	17.4 $\pm$ 0.6 M* 13.9 $\pm$ 0.7 F*	19.0 $\pm$ 1.3 M 14.3 $\pm$ 0.2 F
Serum calcium (mmol/L)	2.12 $\pm$ 0.08	1.41 $\pm$ 0.05*	1.45 $\pm$ 0.02*
Corrected serum calcium (mmol/L)	2.6 $\pm$ 0.04	1.86 $\pm$ 0.09*	1.90 $\pm$ 0.16*
Serum phosphate (mmol/L)	2.58 $\pm$ 0.09	2.02 $\pm$ 0.08	2.27 $\pm$ 0.05* [†]
Serum albumin (g/L)	16.2 $\pm$ 0.7	17.4 $\pm$ 0.6	17.5 $\pm$ 0.6
24-h albumin excretion (mg)	0.01 $\pm$ 0.00	0.32 $\pm$ 0.09*	0.07 $\pm$ 0.04 [†]
Blood urea nitrogen (mg/dL)	30.1 $\pm$ 5.0	29.1 $\pm$ 1.8	29.8 $\pm$ 3.4

Five-week-old 1 α -OHase KO mice were given daily i.p. injections with 1,25-D₃ or vehicle for 6 weeks. The weight of the mice was determined, and blood and urine samples were analyzed. Data are given as means $\pm$ SD.

* P < 0.05 vs WT.

[†] P < 0.05 vs KO.

F, female; M, male; FGF, fibroblast growth factor; KO, knockout; ND, not detectable; PTH, parathyroid hormone; VDR, vitamin D receptor; WT, wild type; 1 α -OHase, 25-hydroxy-vitamin-D₃-1 α -hydroxylase; 1,25-D₃, calcitriol.

(1,25-D₂) at a concentration of 0.1 or 1 μ mol/L. After 24 hours, cells were harvested.

The *TRPC6* promoter activity assay was performed as described previously.²⁶ Briefly, after transfection, cells were treated with vehicle or a vitamin D analog (D₃, 25-D₃, 1 α -D₃, 1,25-D₃, D₂, 1 α -D₂, or 1,25-D₂) at a concentration of 0.1 or 1 μ mol/L. After 24 hours, cells were harvested and a luciferase reporter assay (Promega, Madison, WI) was performed.

Real-Time Quantitative PCR Analysis

RNA was isolated from kidney cortex or cultured MPC-5 cells. cDNA synthesis and real-time quantitative PCR was performed as described previously.³¹

Statistical Analysis

Results in the text and figures are depicted as means $\pm$ SEM. The appropriate test was used to determine significance with SPSS software version 13 (IBM, New York, NY), as described in the figure legends. P < 0.05 was considered significant.

Results

Inherited 1,25-D₃ Deficiency Induces Glomerular and Tubulointerstitial Injury and Proteinuria

A significant proteinuria, determined as albumin/creatinine ratio and total 24-hour urine albumin excretion, was detected in 4-week-old 1 α -OHase KO mice (Figure 1A and Table 2), with increased urinary IgG excretion suggesting a glomerular origin (Figure 1B). Urinary NGAL excretion, a measure of tubular damage, was also increased in the KO mice (Figure 1C). Treating KO animals from week 5 onward with

a low dose (25 pg/g) of 1,25-D₃ for 6 weeks normalized the urinary albumin/creatinine ratio (Figure 1D) and total 24-hour albumin excretion (Table 2), and reduced urinary IgG (Figure 1E) excretion to WT levels, without affecting NGAL excretion (Figure 1F). Immunohistochemistry demonstrated increased tubular NGAL expression in the KO mice, which remained unchanged by supplementation with 1,25-D₃ (Figure 1G). Tubulointerstitial staining for cleaved caspase-3 was increased in KO mice, and was reduced, but not normalized, by 1,25-D₃ (Figure 1H).

Podocyte Injury in Inherited 1,25-D₃ Deficiency

Periodic acid–Schiff staining showed no visible abnormalities in the glomerulus, tubuli, or tubulointerstitium of 4- or 11-week-old 1 α -OHase KO mice (results not shown). EM analysis detected no podocyte foot process effacement in the 4-week-old KO mice (results not shown). However, glomerular desmin and TRPC6, as measures of podocyte injury, were increased in the KOs when compared with WT animals (Figure 2, A and B). Moreover, nephrin expression was down-regulated in the KO mice (Figure 2C). To determine the progression and reversibility of podocyte injury, we also examined KO mice treated with a low dose (25 pg/g) of 1,25-D₃ for 6 weeks. EM revealed partial foot process effacement in the untreated 11-week-old KO animals, which was reversed by 1,25-D₃ treatment (Figure 2D). This was accompanied by a down-regulation of desmin and TRPC6 expression in the 1,25-D₃ supplemented mice compared with the 11-week-old KO to WT levels (Figure 2, E and F). Nephrin (Figure 2G) and podocin (Figure 2H) expression levels were up-regulated to WT levels by 1,25-D₃ administration compared with the KO animals. No difference in the expression of the actin cytoskeleton scaffolding protein CD2AP was found (Figure 2I). The calcineurin/nuclear factor of activated

Figure 2 Podocyte injury and altered expression of key podocyte proteins in 25-hydroxy-vitamin-D₃-1 α -hydroxylase knockout (1 α -OHase KO) mice. Expression of podocyte damage markers desmin (**A**), transient receptor potential channel C6 (TRPC6; **B**), and nephrin (**C**) was determined in 4-week-old 1 α -OHase KO and wild-type (WT) mice. Five-week-old 1 α -OHase KO mice were given daily injections with a low dose (25 pg/g) of calcitriol (1,25-D₃) or vehicle for 6 weeks, after which mice were sacrificed. **D**: Renal ultrastructure was assessed by electron microscopy. Expression of desmin (**E**), TRPC6 (**F**), nephrin (**G**), podocin (**H**), CD2AP (**I**), myocyte-enriched calcineurin-interacting protein (MCIP; **J**), and synaptopodin (**K**). Results are shown as percentage of vehicle-treated WT mice. Statistical tests used were *t*-test (**A–C**) and analysis of variance with Bonferroni post hoc analysis (**E–K**). **P* < 0.05 versus WT; †*P* < 0.05 versus KO. *N* = 8 mice per group (**A–K**). Original magnification, $\times 400$. KO, knockout.

T-cell (NFAT) pathway was identified as a pathway enhancing podocyte injury. Next to TRPC6, myocyte-enriched calcineurin-interacting protein is an NFAT target.³² Myocyte-enriched calcineurin-interacting protein expression was increased in 1 α -OHase KO mice and restored to WT levels when mice were supplemented with 1,25-D₃ (Figure 2J). Calcineurin activation might induce breakdown of the podocyte cytoskeletal protein synaptopodin. However, no significant differences in synaptopodin expression between groups were found (Figure 2K).

GBM Composition Is Altered in 1,25-D₃ Deficiency

EM analysis showed some variation in glomerular basement membrane (GBM) width in 11-week-old 1 α -OHase KO mice, where within one glomerular capillary loop thinner sections were present, reminiscent of GBM changes sometimes encountered in FSGS lesions (Supplemental Figure S1A). However, there was no clear global thinning or thickening, heterogeneity, or multiplication of the GBM. Interestingly, 1 α -OHase KO mice showed a mildly, but significantly, reduced glomerular collagen IV expression, which was no longer significant in the 1,25-D₃ supplementation group (Supplemental Figure S1B).

Endocrine Alterations and Calcium-Phosphorus Balance in Inherited 1,25-D₃ Deficiency

Endocrine alterations secondary to 1,25-D₃ deficiency could be involved in the renal phenotype in these mice. Serum PTH level was significantly elevated in the 1 α -OHase KO mice, and remained high in the low-dosed vitamin D-supplemented mice (Table 2). No differences were observed in renal cortical PTH receptor expression. In contrast to PTH, serum levels of fibroblast growth factor 23 were not detectable in KO and vitamin D-supplemented KO mice. Renal VDR expression was strongly reduced in the KO mice, but was even lower in the 1,25-D₃-supplemented 1 α -OHase KO group. The male and female KO mice had a decreased body weight and reduced serum calcium, albumin-corrected serum calcium, and serum phosphate compared with their WT littermates. The serum albumin levels were comparable between groups, suggesting that the significant proteinuria in 1 α -OHase KO mice did not induce nephrotic syndrome. No difference was observed in blood urea nitrogen levels between groups.

1,25-D₃ Deficiency Does Not Lead to Immune-Mediated Glomerular Injury

Because vitamin D affects the immune system, immune-mediated renal injury was examined. However, no differences

Figure 3 *In vitro*, calcitriol (1,25-D₃) and paricalcitol (1,25-D₂) down-regulate the expression of the podocyte damage marker transient receptor potential channel C6 (TRPC6). **A:** Cultured podocytes were injured by doxorubicin (Adriamycin) application and treated with several vitamin D analogs at a concentration of 0.1 and 1 μ mol/L, and TRPC6 mRNA expression was measured. The effect of vitamin D analogs on TRPC6 promoter activity was examined using a luciferase promoter assay. Cells were treated with several vitamin D analogs at a concentration of 0.1 and 1 μ mol/L. **B:** The Firefly/Renilla ratio was calculated and normalized for vehicle-treated cells. Each experiment was repeated at least twice. The statistical test used was analysis of variance with Bonferroni post hoc analysis (**A** and **B**). * P < 0.05 versus vehicle-treated cells; $^{\dagger}P$ < 0.05 versus vehicle-treated doxorubicin injured cells. N = 4 to 8 podocyte cultures per group (**A** and **B**). D₂, ergocalciferol; D₃, cholecalciferol; 1 α -D₂, doxercalciferol; 1 α -D₃, α -calcidiol; 25-D₃, calcidiol.

in IgG (Supplemental Figure S2A), IgA (Supplemental Figure S2B), IgM (Supplemental Figure S2C), or complement C3 (Supplemental Figure S2D) deposition were apparent. In addition, neither granulocyte influx (Supplemental Figure S2E), nor CD4 (Supplemental Figure S2F), CD8 (Supplemental Figure S2G), or CD68 (Supplemental Figure S2H) staining differed between treatment groups. Quantification of the staining described in this paragraph is shown in Supplemental Table S1.

In Vitro 1,25-D₃ and 1,25-D₂ Down-Regulate Expression of Podocyte Damage Marker TRPC6

Cultured MPC5 podocytes were injured using doxorubicin and treated with a wide range of vitamin D analogs, and podocyte injury was assessed by TRPC6 expression. We previously showed that the classic activated 1,25-D₃ analog down-regulates TRPC6 expression.²⁶ Doxorubicin-injured podocytes showed increased TRPC6 expression when compared with control cells (Figure 3A). In doxorubicin-injured cells treated with 1,25-D₃ and 19-nor-1,25-dihydroxyvitamin-D₂ (1,25-D₂) at a concentration of 100 nmol/L, TRPC6 expression was down-regulated. At a concentration of 1.0 μ mol/L, all hydroxylated vitamin D₃ analogs and 1,25-D₂ were able to down-regulate TRPC6 expression (Figure 3A). Moreover, we measured TRPC6 promoter activity using a luciferase TRPC6 promoter reporter construct. TRPC6 promoter activity showed a similar pattern when compared with the TRPC6 expression data, with the exception that treatment with 0.1 μ mol/L 1,25-D₂ did not result in a decreased TRPC6 promoter activity (Figure 3B).

Both 1,25-D₃ and 1,25-D₂ Supplementations Reverse Glomerular Injury in 1 α -OHase KO Mice

Our *in vitro* data suggested that, in analogy to the classic role of vitamin D in mineral metabolism, the hydroxylation state of vitamin D analogs is also important for its protective effect in podocytes. Therefore, we treated 1 α -OHase KO mice with 1,25-D₃ and 1,25-D₂, which were in our *in vitro* experiments the most potent analogs down-regulating TRPC6 expression. We used a high-dose 1,25-D₃ and 1,25-D₂ that restored serum calcium to the WT level (WT, 2.24 $\pm$ 0.04 mmol/L; KO, 1.67 $\pm$ 0.12 mmol/L; KO + 1,25-D₂, 2.18 $\pm$ 0.13 mmol/L; KO + 1,25-D₃, 2.26 $\pm$ 0.02 mmol/L). KO mice showed albuminuria (Figure 4A) and increased IgG excretion (Figure 4B), which were completely corrected by both vitamin D analogs. Moreover, tubulointerstitial damage was partly and completely reversed by 1,25-D₂ and 1,25-D₃, respectively (Figure 4C). EM showed that the partial podocyte foot process effacement in the KO mice was absent after treatment with both vitamin D analogs (Figure 4F). Expression of podocyte injury markers desmin and TRPC6 was increased in KO animals and normalized by 1,25-D₂ and 1,25-D₃ supplementation (Figure 4, D and E). More important, treating 1 α -OHase KO mice with a high (2.0%) calcium diet versus a normal (0.8%) calcium diet normalized serum calcium levels (WT,

Figure 4 High-dose calcitriol (1,25-D₃) and paricalcitol (1,25-D₂) supplementation reverses glomerular injury in 25-hydroxy-vitamin-D₃-1 α -hydroxylase knockout (1 α -OHase KO) mice. Five-week-old 1 α -OHase KO mice were given daily i.p. injections with 1000 pg/g 1,25-D₂, a high-dose 1,25-D₃ (500 pg/g), or vehicle for 6 weeks. Glomerular and tubulointerstitial injury was assessed by urinary albumin/creatinine ratio (A), IgG/creatinine ratio (B), and neutrophil gelatinase-associated lipocalin (NGAL) ratio (C). Expression of podocyte damage markers desmin (D) and transient receptor potential channel C6 (TRPC6; E). Five-week-old 1 α -OHase KO mice received a 0.8% or 2.0% calcium diet for 6 weeks. F: Renal ultrastructure was assessed by electron microscopy. Glomerular and tubulointerstitial injury was assessed by urinary albumin/creatinine ratio (G), IgG (H), and NGAL excretion (I). Expression of podocyte damage markers desmin (J) and TRPC6 protein (K) was seen. Expression levels are depicted as percentage of vehicle-treated wild-type (WT) mice. Statistical tests used were analysis of variance with Bonferroni post hoc analysis (A–C, E, and F) and *t*-test (G–K). **P* < 0.05 versus WT; †*P* < 0.05 versus KO. *N* = 8 mice per group (A–K). Original magnification, $\times 3000$ (F). KO, knockout.

2.26 $\pm$ 0.05 mmol/L; KO with 0.8% calcium diet, 1.61 $\pm$ 0.10 mmol/L; KO with 2.0% calcium diet, 2.21 $\pm$ 0.09 mmol/L), but did not reverse renal injury in 1 α -OHase KO mice, as shown by albumin/creatinine ratio (Figure 4G), urinary IgG (Figure 4H), and NGAL (Figure 4I) excretion. Moreover, glomerular desmin (Figure 4J) and TRPC6 (Figure 4K) expression did not differ between diets. Accordingly, the normocalcemic 1 α -OHase KO on a high calcium diet still showed partial podocyte foot process effacement (Figure 4F), to a comparable extent as the untreated hypocalcemic KO mice.

Acquired 1,25-D₃ Deficiency Also Leads to Glomerular and Tubulointerstitial Injury and Proteinuria

In addition to genetic 1,25-D₃ deficiency, we also studied acquired 1,25-D₃ deficiency in rats. After

depleting the endogenous vitamin D pool, 1,25-D₃ serum levels were lower compared with control animals (control, 295 $\pm$ 58 pmol/L; deficient, 35 $\pm$ 9 pmol/L; *P* < 0.05), thus rendering these animals 1,25-vitamin D₃ deficient. After 5 weeks, a significant albuminuria (Figure 5A) and increased urinary IgG excretion (Figure 5B) were observed, which could be prevented by supplementation with 1,25-D₂ and 1,25-D₃. Urinary NGAL excretion was also increased in the 1,25-vitamin D₃-deficient animals and reduced with 1,25-D₂ and 1,25-D₃ supplementation (Figure 5C). EM revealed no ultrastructural alterations (results not shown). However, the expression of desmin (Figure 5D) and TRPC6 (Figure 5E) was up-regulated in vitamin D-deficient rats compared with either control or 1,25-D₂ and 1,25-D₃ supplemented groups.

Figure 5 Acquired 1,25-vitamin (Vit) D₃ deficiency leads to glomerular and tubulointerstitial injury and proteinuria. Wistar rats were made vitamin D deficient and, subsequently, treated with paricalcitol (1,25-D₂) or calcitriol (1,25-D₃) for 5 weeks. Glomerular and tubulointerstitial injury was assessed by urinary albumin/creatinine ratio (A), IgG/creatinine ratio (B), and neutrophil gelatinase-associated lipocalin (NGAL)/creatinine ratio excretion (C). Expression of podocyte damage markers desmin (D) and transient receptor potential channel C6 (TRPC6; E). Protein and mRNA expression levels are depicted as percentage of control. The statistical test used was analysis of variance with Bonferroni post hoc analysis. **P* < 0.05 versus control; †*P* < 0.05 versus untreated rats. *N* = 8 rats per group (A–E).

Endocrine Alterations and Calcium-Phosphorus Balance in Acquired 1,25-D₃ Deficiency

Endocrine alterations secondary to 1,25-D₃ deficiency could be involved in the renal phenotype in these rats. Serum PTH levels were significantly elevated in the 1,25-D₃—deficient rats, as well as in the vitamin D-supplemented rats (Table 3). No difference was observed in body weight, serum calcium, and corrected serum calcium. A high phosphorus diet caused serum phosphate to be elevated in the deficient groups. No differences were observed in serum albumin and blood urea nitrogen levels between groups.

Discussion

The present study demonstrated that hereditary and acquired 1,25-vitamin D₃ deficiency leads to 1,25-D₃— and 1,25-D₂—remediable glomerular injury and albuminuria, as well as tubulointerstitial damage in 1,25-D₃ deficient 1 α -OHase KO mice. Moreover, KO mice showed partial podocyte foot process effacement, altered expression of functionally relevant slit-diaphragm—associated proteins, and activation of

the deleterious calcineurin/NFAT pathway. Acquired 1,25-D₃ deficiency in rats also induced proteinuria, in combination with an increased expression of podocyte injury markers. *In vitro*, we determined that the fully hydroxylated 1,25-D₃ and 1,25-D₂ analogs were most potent in preventing the up-regulation of the podocyte injury marker TRPC6. *In vivo*, 1,25-D₃ and 1,25-D₂ supplementation largely reversed glomerular injury and the above described expressional changes. Thus, this study provides important evidence that vitamin D treatment is not only able to ameliorate, but also to reverse, podocyte injury, at least in 1,25-D₃—deficient animal models. Reversal of podocyte injury would be consistent with the debated antiproteinuric effect of vitamin D analogs in clinical practice.

Herein, we showed that loss of 25-hydroxy-vitamin-D₃-1 α -hydroxylation capacity, which also occurs in patients with renal insufficiency, and acquired 1,25-D₃ deficiency lead to renal injury. An association between low 25-hydroxyvitamin D levels and an increased prevalence of albuminuria was previously described in a population-based survey.¹³ A causal relationship between both could not be proved. However, vitamin D analogs have been suggested to ameliorate proteinuria in several glomerular diseases.^{9–11}

Table 3 Clinical Parameters in Acquired 1,25-D₃ Deficiency and Vitamin D Supplementation in Rats

Parameters	Control	1,25-D ₃ deficiency	1,25-D ₃ deficiency + 1,25-D ₂	1,25-D ₃ deficiency + 1,25-D ₃
Serum PTH (pg/mL)	29 ± 12	56 ± 14*	18 ± 2*†	28 ± 7†
Body weight (g)	463 ± 23	445 ± 30	446 ± 29	447 ± 34
Serum calcium (mmol/L)	3.29 ± 0.03	3.30 ± 0.07	3.36 ± 0.05	3.34 ± 0.05
Corrected serum calcium (mmol/L)	3.7 ± 0.03	3.7 ± 0.09	3.7 ± 0.07	3.7 ± 0.11
Serum phosphate (mmol/L)	2.51 ± 0.18	3.34 ± 0.37*	3.37 ± 0.28*	3.98 ± 0.42*
Serum albumin (g/L)	22.1 ± 2.1	21.6 ± 1.4	21.2 ± 2.4	23.8 ± 2.2
Blood urea nitrogen (mg/dL)	28.8 ± 3.8	30.1 ± 2.1	29.6 ± 6.0	28.9 ± 3.9

Vitamin D—deficient rats were treated with 1,25-D₂, 1,25-D₃, or vehicle. Deficient rats were treated with a high calcium and phosphate diet. The weight of the rats was determined, and blood samples were analyzed. Data are given as means ± SD.

**P* < 0.05 vs control.

†*P* < 0.05 vs 1,25-D₃—deficient rats.

PTH, parathyroid hormone; 1,25-D₂, paricalcitol; 1,25-D₃, calcitriol.

In the present study, two models for vitamin D deficiency in two different species were used to show that 1,25-D₃ deficiency results in podocyte injury and glomerular proteinuria.

Although overt podocyte effacement was not yet present in 4-week-old 1 α -OHase KO mice, proteinuria and glomerular injury markers were already detectable. Moreover, a significant progressive urinary albumin loss was found when 4- and 11-week-old 1,25-D₃-deficient KO mice are compared, the latter showing clear podocyte effacement. Interestingly, the urine IgG excretion is reduced after 4 weeks, which could be a consequence of weaning of the mice. IgG is transferred to offspring via the milk. Thus, after weaning, this IgG source is lost, which is probably reflected by the decreased IgG urine excretion in the presence of persistent glomerular injury. Previously, it was shown that the VDR is expressed in the glomerulus, especially in podocytes and parietal epithelial cells.²² The renoprotective effects of vitamin D might thus be mediated locally by directly affecting the podocyte, or theoretically other glomerular cells, like the glomerular endothelium, with which there is considerable cross talk. However, because vitamin D is a pluripotent hormone, also acting on hematopoietic cells, we cannot completely exclude that the effects found in this study are indirectly regulated through effects on other cell types or tissues.³³

In both the inherited and the acquired vitamin D-deficient models, we demonstrated down-regulation of the expression of structural slit diaphragm-associated proteins nephrin and podocin and up-regulation of TRPC6 expression. In proteinuric glomerular diseases, such as acquired FSGS, membranous nephropathy, and diabetic nephropathy, decreased expression of podocin and nephrin and TRPC6 overexpression were reported.^{34–36} Loss-of-function mutations in nephrin and podocin, as well as TRPC6 gain-of-function mutations, lead to (congenital) nephrotic syndrome and hereditary FSGS.^{37–40} Previous studies already suggested that vitamin D regulates nephrin and podocin expression.^{24,25} In accordance, diabetic VDR KO mice and rats that constitutively express CYP24A1, the enzyme breaking down 1,25-D₃, displayed proteinuria accompanied by podocyte effacement and decreased nephrin expression.^{41,42} More important, healthy VDR KO and animals overexpressing CYP24A1 also experienced increased urinary protein loss. Wang et al²⁵ demonstrated that overexpression of the VDR in podocytes protects the kidney from diabetic injury. We previously demonstrated that 1,25-D₃ specifically binds to the *TRPC6* promoter region, thereby down-regulating TRPC6 expression.²⁶ The combination of podocin expressional loss and *TRPC6* up-regulation in 1,25-D₃-deficient animals is particularly significant because podocin regulates TRPC6 channel gating in podocytes. It was recently demonstrated that podocin knockdown markedly increases TRPC6 mechanosensitivity and would lead to a profound TRPC6 overactivation in podocyte foot processes, leading to a situation of Ca²⁺ overload.⁴³ In addition, the channel appears to function in a deleterious angiotensin II-induced positive feedback TRPC6-calcineurin/NFAT-TRPC6 signaling loop in

the podocyte, which might be enhanced by 1,25-D₃ deficiency.^{12,31} Myocyte-enriched calcineurin-interacting protein expression, as a measure of calcineurin/NFAT activity, was increased in kidneys of 1 α -OHase KO animals. Calcineurin activation was also shown to lead to dephosphorylation of the podocyte-specific actin-binding protein synaptopodin, which is crucial for maintaining the podocyte actin cytoskeleton.^{44,45} However, enhanced synaptopodin degradation does not appear to have occurred in these mice. Although an attractive hypothesis, the proof for TRPC6-activated and subsequent calcineurin-mediated synaptopodin degradation has yet to be delivered. 1,25-D₃ deficiency in the KO mice also increased tubulointerstitial damage, as exemplified by detection of increased NGAL as a tubular injury marker and caspase-3 as a measure of apoptotic tubular cells. Although a low dose of vitamin D did not alter urinary NGAL excretion, a higher dose of 1,25-D₃ normalized NGAL excretion, which was also partially accomplished by 1,25-D₂. In contrast to NGAL, cleaved caspase 3 expression was already decreased by a low dose of vitamin D, indicating fewer apoptotic cells. Whether the GBM alterations and interstitial injury are a direct result of 1,25-D₃ deficiency, or are secondary to the proteinuria, is not evident from our data. Because most GBM constituents are produced by the podocytes, podocyte injury could be suggested to affect the GBM. Consistent with our results, VDR KO mice showed increased tubular atrophy and interstitial fibrosis when subjected to unilateral urethral obstruction.⁴⁶ Inoue et al⁴⁷ found that the vitamin D analog maxacalcitol prevented tubulointerstitial fibrosis in urethral obstructed rats. A beneficial effect of vitamin D on caspase 3 expression was shown in several renal injury models.^{11,48} The reversal of the interstitial damage in our study by 1,25-D₃ might need higher vitamin D supplementation doses, or more time to recover when it is secondary to the glomerular injury.

Taken together, regulation of nephrin, podocin, and TRPC6 expression by 1,25-D₃ in inherited and acquired 1,25-D₃ deficiency appears to be involved in the glomerular injury and proteinuria in 1,25-D₃-deficient animals. The reversal of these effects by 1,25-D₂ and 1,25-D₃ treatment demonstrated that vitamin D can reverse podocyte injury and prevent foot process effacement, and that the phenotype is vitamin D dependent. An important additional mediator that could be partly responsible for the described effects is the renin-angiotensin system, which is regulated by 1,25-D₃. It was previously shown that administration of 1,25-D₃ reduces blood pressure, plasma renin activity, and angiotensin II levels. Consistent with this, 1 α -OHase KO mice have a 1,25-D₃-dependent increased blood pressure, renin, and angiotensin II level, and particularly the latter is known to be an important pathogenic factor in glomerular injury.⁴⁹ However, as outlined, the direct transcriptional regulation of nephrin, podocin, and TRPC6 was previously demonstrated.^{23–27} Hypothetically, the calcium/phosphate homeostasis, or the hormonal regulation thereof, could be mediating the deleterious effect of 1,25-D₃ deficiency. However, we could not demonstrate sustained correlation between the proteinuric phenotype in both animal

models and serum levels of fibroblast growth factor 23, PTH, phosphate, and calcium. More important, Ca²⁺ supplementation itself, normalizing serum calcium, did not correct the phenotype. To further study the contribution of possible mechanistic contributors, the generation of kidney- or even podocyte-specific 1 α -OHase KO mice would represent valuable tools for future research.

In conclusion, we demonstrated that 1,25-D₃ deficiency leads to glomerular and tubulointerstitial damage. When translated to human subjects, this has clear implications for clinical practice. Patients with glomerular diseases and/or proteinuria should be tested for (1,25-)vitamin D₃ deficiency, and supplementation of 1,25-D₃ or 1,25-D₂ should be considered. Furthermore, our data add mechanistic evidence to the debate on the antiproteinuric and renoprotective effects of vitamin D, suggesting that vitamin D supplementation might be indicated in patients with renal function decline in an earlier phase, before the development of overt hyperparathyroidism. However, this strategy would have to be based on solid clinical trials showing that early vitamin D treatment reduces the rate of renal function decline in human patients, which are not available in the literature as yet. In general, these data provide new input into the debate on the pluripotent effects of vitamin D, at a moment when the initial excitement seems to be fading.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.11.015>.

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