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Hypervitaminosis D Mediates Compensatory Ca²⁺ Hyperabsorption in TRPV5 Knockout Mice

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Vitamin D plays an important role in Ca²⁺ homeostasis by controlling Ca²⁺ (re)absorption in intestine, kidney, and bone. The epithelial Ca²⁺ channel TRPV5 mediates the Ca²⁺ entry step in active Ca²⁺ reabsorption. TRPV5 knockout (TRPV5^{-/-}) mice show impaired Ca²⁺ reabsorption, hypercalciuria, hypervitaminosis D, and intestinal hyperabsorption of Ca²⁺. Moreover, these mice demonstrate upregulation of intestinal TRPV6 and calbindin-D_{9K} expression compared with wild-type mice. For addressing the role of the observed hypervitaminosis D in the maintenance of Ca²⁺ homeostasis and the regulation of expression levels of the Ca²⁺ transport proteins in kidney and intestine, TRPV5/25-hydroxyvitamin-D₃-1 α -hydroxylase double knockout (TRPV5^{-/-}/1 α -OHase^{-/-}) mice, which show undetectable serum 1,25(OH)₂D₃ levels, were generated. TRPV5^{-/-}/1 α -OHase^{-/-} mice displayed a significant hypocalcemia compared with wild-type mice (1.10 \pm 0.02 and 2.54 \pm 0.01 mM, respectively; *P* < 0.05). mRNA levels of renal calbindin-D_{28K} (7 \pm 2%), calbindin-D_{9K} (32 \pm 4%), Na⁺/Ca²⁺ exchanger (12 \pm 2%), and intestinal TRPV6 (40 \pm 8%) and calbindin-D_{9K} (26 \pm 4%) expression levels were decreased compared with wild-type mice. Hyperparathyroidism and rickets were present in TRPV5^{-/-}/1 α -OHase^{-/-} mice, more pronounced than observed in single TRPV5 or 1 α -OHase knockout mice. It is interesting that a renal Ca²⁺ leak, as demonstrated in TRPV5^{-/-} mice, persisted in TRPV5^{-/-}/1 α -OHase^{-/-} mice, but a compensatory upregulation of intestinal Ca²⁺ transporters was abolished. In conclusion, the elevation of serum 1,25(OH)₂D₃ levels in TRPV5^{-/-} mice is responsible for the upregulation of intestinal Ca²⁺ transporters and Ca²⁺ hyperabsorption. Hypervitaminosis D, therefore, is of crucial importance to maintain normocalcemia in impaired Ca²⁺ reabsorption in TRPV5^{-/-} mice.

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Ca²⁺ is one of the most important cations in the human body as it is essential for many physiologic functions, including neuronal excitation, cardiac muscle contraction, blood clotting, and bone mineralization. Therefore, Ca²⁺ balance is tightly controlled by the concerted action of intestinal Ca²⁺ absorption, exchange of Ca²⁺ from bone, and Ca²⁺ reabsorption in the kidney (1). The active form of vitamin D, 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), and parathyroid hormone (PTH) and calcitonin are the calciotropic hormones that are known to be involved in the maintenance of plasma Ca²⁺ concentration (2,3). Ca²⁺ absorption from the intestinal lumen and Ca²⁺ reabsorption in the renal tubules is promoted by 1,25(OH)₂D₃ (4–6). Synthesis of 1,25(OH)₂D₃ requires the renal cytochrome P450 enzyme 25-hydroxyvitamin D₃-1 α -hydroxylase (1 α -OHase) (7). In addition, PTH stimulates 1,25(OH)₂D₃ production by 1 α -

OHase activation and the release of Ca²⁺ from bone to maintain plasma Ca²⁺ levels, whereas calcitonin lowers the plasma Ca²⁺ concentration by inhibition of bone resorption (2).

1,25(OH)₂D₃-stimulated transepithelial Ca²⁺ transport in kidney and intestine involves Ca²⁺ entry across the apical membrane *via* the epithelial Ca²⁺ channels TRPV5 and TRPV6, respectively. TRPV5 is localized at the luminal membrane of the late distal convoluted tubule (DCT) and connecting tubule (CNT) in kidney (8,9). TRPV6 is the homologous Ca²⁺ channel localized in the brush border membrane of duodenum (1,10–13). After entry of Ca²⁺ through TRPV5 and TRPV6, Ca²⁺ bound to Ca²⁺-binding proteins (calbindins) diffuses to the basolateral membrane of the cell. Ca²⁺ is finally extruded to the extracellular compartment by the Na⁺/Ca²⁺ exchanger (NCX1) in the kidney and the plasma membrane Ca²⁺-ATPase (PMCA1b) in intestine and kidney (9).

Recently, several animal models were designed to investigate Ca²⁺ absorption mechanisms and maintenance of Ca²⁺ balance. TRPV5 knockout (TRPV5^{-/-}) mice were generated by targeted ablation of the TRPV5 gene. These mice displayed robust renal Ca²⁺ wasting as a result of impaired Ca²⁺ reabsorption in DCT and CNT (14). Furthermore, elevated

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1,25(OH)₂D₃ serum levels, intestinal Ca²⁺ hyperabsorption, and reduced bone thickness and mineralization were demonstrated. In addition, Dardenne *et al.* (15) created an animal model for vitamin D deficiency rickets type 1 by genetic ablation of the 1 α -OHase gene. These 1 α -OHase knockout (1 α -OHase^{-/-}) mice showed a phenotype characterized by undetectable serum levels of 1,25(OH)₂D₃, severe hypocalcemia, hyperparathyroidism, bone abnormalities, and retarded growth (15–17). Until now, data on detailed urine analysis of the 1 α -OHase^{-/-} mice were not available. Previous studies demonstrated that 1,25(OH)₂D₃ supplementation rescues the severe phenotype of 1 α -OHase^{-/-} mice, which is accompanied by an upregulation of TRPV5, TRPV6, calbindins, NCX1, and PMCA1b expression, resulting in the normalization of serum Ca²⁺ levels. It is interesting that dietary Ca²⁺ supplementation in 1 α -OHase^{-/-} mice resulted in a similar rescue in the absence of 1,25(OH)₂D₃. Taken together, these studies suggest a pivotal role of the Ca²⁺ transport proteins in maintaining Ca²⁺ balance, partly independent of 1,25(OH)₂D₃ (18).

To investigate the contribution of elevated 1,25(OH)₂D₃ levels to the phenotype of TRPV5^{-/-} mice and to elucidate the involvement of 1,25(OH)₂D₃ in the compensatory Ca²⁺ hyperabsorption, we generated a mouse model in which both TRPV5 and 1 α -OHase were inactivated. In this study, we describe the phenotype of TRPV5/1 α -OHase double knockout (TRPV5^{-/-}/1 α -OHase^{-/-}) mice, including bone analysis and semiquantification of mRNA and protein expression levels of Ca²⁺ transport proteins and provide an extensive comparison with TRPV5 and 1 α -OHase single knockout mice.

Materials and Methods

Animal Experiments

TRPV5^{-/-} mice were generated as described previously (14). 1 α -OHase^{-/-} mice were provided by René St-Arnaud (Shriners Hospital for Children Montreal, QU, Canada) (15). Cross-breeding of 1 α -OHase^{-/-} and TRPV5^{-/-} mice resulted in offspring that were heterozygous for both TRPV5 and 1 α -OHase (TRPV5^{+/-}/1 α -OHase^{+/-}). This offspring was subsequently intercrossed to obtain TRPV5^{-/-}/1 α -OHase^{-/-} mice. Genotypes were determined by PCR analysis, using specific primers as described previously (14,15). For obtaining 24-h urine samples of all mouse genotypes, 8-wk-old mice were kept in a light- and temperature-controlled room in metabolic cages that enabled 24-h urine analyses. Standard pelleted chow (0.25% [wt/vol] Na, 1.1% [wt/vol] Ca, 0.2% [wt/vol] Mg, 0.7% [wt/vol] P, and 0.9% [wt/vol] K) and drinking water were available *ad libitum*. After the experiment, blood was collected and mice were killed. Kidney, duodenum, tibia, and fibula were sampled. The animal ethics board of the Radboud University Nijmegen approved all animal experimental procedures.

Urine and Serum Analyses

Urine and serum Ca²⁺ concentrations were analyzed using a colorimetric assay kit (Roche, Mannheim, Germany). An electronic ion analyzer was used to determine urinary pH. Serum vitamin D levels were determined with a [¹²⁵I]1,25(OH)₂D₃ RIA assay (IDS Inc., Fountain Hills, AZ). Mouse serum PTH was measured using an immunoradiometric assay (Immutopics Inc., San Clemente, CA).

Real-Time Quantitative PCR Analysis

For investigating mRNA expression levels of renal and duodenal Ca²⁺ transport proteins, total RNA from kidney and duodenum was

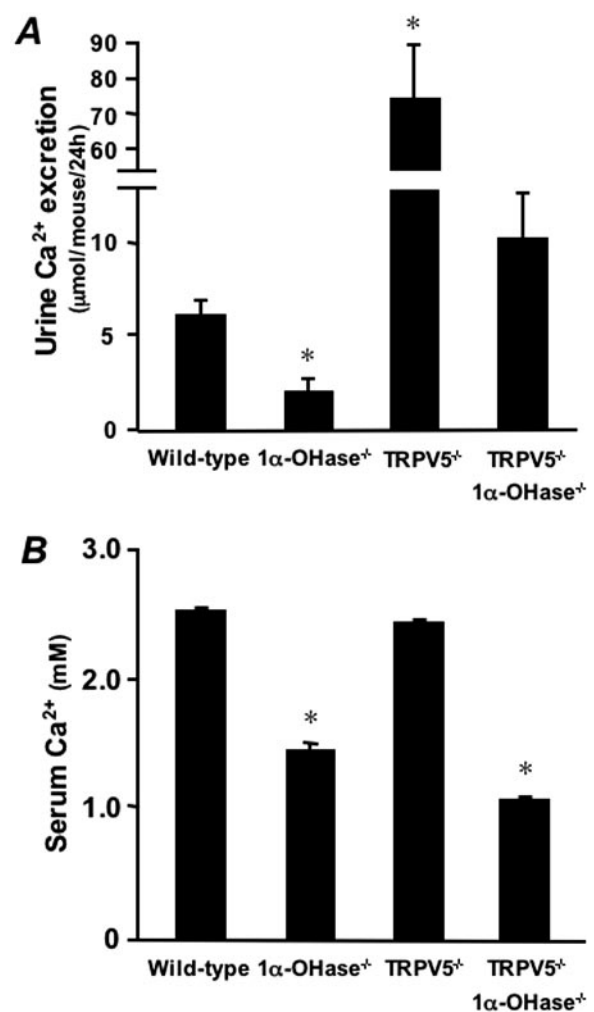


Figure 1. Urinary Ca²⁺ excretion and serum Ca²⁺ concentration. Urinary Ca²⁺ excretion (A) and serum Ca²⁺ levels (B) were determined in wild-type, 1 α -OHase^{-/-}, TRPV5^{-/-}, and TRPV5^{-/-}1 α -OHase^{-/-} mice. Data are presented as means \pm SEM. **P* < 0.05, significant difference from all groups.

isolated using TriZol Reagent (Life Technologies BRL, Life Technologies, Breda, The Netherlands) according to the manufacturer's protocol. RNA was treated with DNase (Promega, Madison, WI) to prevent contamination with genomic DNA. Total RNA was subjected to reverse transcription using Moloney-murine leukemia virus–reverse transcriptase (Life Technologies BRL, Life Technologies, Breda, The Netherlands) (11). Renal mRNA expression levels of calbindin-D_{28k}; calbindin-D_{9k}; NCX1; and duodenal TRPV6, calbindin-D_{9k}, and PMCA1b mRNA levels were quantified by real-time quantitative PCR as described previously (19,20), using the ABI Prism 7700 Sequence Detection System (PE Biosystems, Rotkreuz, Switzerland). The expression level of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase was used as an endogenous control.

Immunoblotting

Total kidney lysates of TRPV5^{-/-} and TRPV5^{-/-}/1 α -OHase^{-/-} mice were prepared as described previously (21). The protein concentration of the homogenates was determined with the Bio-Rad protein assay (Bio-Rad, München, Germany). Samples were submitted to 16.5% (wt/vol) SDS-PAGE and blotted to polyvinylidene difluoride-nitrocellulose

membranes (Immobilon-P, Millipore Corp., Bedford, MA). Blots were incubated with a rabbit anti-calbindin-D_{28K} polyclonal antibody (1:10,000) (22) or a rabbit anti-calbindin-D_{9K} polyclonal antibody (1:5000; Swant, Bellinzona, Switzerland) at 4°C for 16 h. Subsequently, blots were incubated with a goat anti-rabbit peroxidase-labeled secondary antibody (1:10,000; Sigma, St. Louis, MO). Immunoreactive protein was detected by the chemiluminescence method (Pierce, Rockford, IL). Immunopositive bands were scanned using an imaging densitometer (Bio-Rad Gs-690) to determine pixel density (Molecular Analyst Software; BioRad Laboratories, Hercules, CA).

Bone Analyses

For evaluating the effects of TRPV5 and 1 α -OHase-ablation on bone homeostasis, bone thickness, mineralization, and epiphyseal growth plate development were determined in tibiae from wild-type, TRPV5^{-/-}, 1 α -OHase^{-/-}, and TRPV5^{-/-}/1 α -OHase^{-/-} mice using microcomputed tomography (Skyscan 1072; SkyScan, Antwerp, Belgium). This technique allows for high-resolution, noninvasive computed tomographic imaging in small animals and small animal organs. Tibiae and fibulae were fixed in 4% (vol/vol) PBS-buffered formaldehyde and routinely processed for plastic embedding (23). The bones were scanned using the SkyScan 1072 microtomograph, and digital cross-sections were made at the site of the diaphysis and epiphysis. X-ray imaging of tibiae and fibulae was performed to compare bone lengths and mineralization in all mouse genotypes.

Statistical Analyses

Values are expressed as means \pm SEM. Statistical significance ($P < 0.05$) was determined by one-way ANOVA. All analyses were performed using the Statview Statistical Package software (Power PC version 4.51, Berkeley, CA) on an Apple iMac computer.

Results

Animal Experiments

TRPV5^{-/-} mice showed hypercalciuria along with normal serum Ca²⁺ levels, a significant increase of serum 1,25(OH)₂D₃ levels, and normal serum PTH levels. Moreover, TRPV5^{-/-} mice displayed an increased diuresis and a decrease of urine pH (Figure 1 and Table 1). 1 α -OHase^{-/-} mice displayed hypocalciuria, hypocalcemia, serum 1,25(OH)₂D₃ levels below the detection level (<6 pmol/L), and a significant hyperparathyroidism. In comparison with wild-type mice, diuresis and urine pH were normal in 1 α -OHase^{-/-} mice. The analysis of the

TRPV5^{-/-}/1 α -OHase^{-/-} mice phenotype showed that net urine Ca²⁺ excretion does not differ from wild-type mice, although inappropriately high given the severe hypocalcemia. Serum 1,25(OH)₂D₃ levels were below detectable levels, and serum PTH levels were further elevated in TRPV5^{-/-}/1 α -OHase^{-/-} mice compared with 1 α -OHase^{-/-} mice. In addition, diuresis and urine pH were normalized in TRPV5^{-/-}/1 α -OHase^{-/-} mice. Body weight was reduced in 1 α -OHase^{-/-} and TRPV5^{-/-}/1 α -OHase^{-/-} mice compared with litter-matched wild-type mice. 1 α -OHase^{-/-} and TRPV5^{-/-}/1 α -OHase^{-/-} mice had a decreased lifespan in comparison with wild-type and TRPV5^{-/-} mice.

Renal mRNA and Protein Expression of Ca²⁺ Transporters

To investigate the specific regulation of renal Ca²⁺ transporters by 1,25(OH)₂D₃, we determined the effects of TRPV5 and 1 α -OHase single or combined gene ablation on mRNA and protein expression levels by real-time PCR analysis and semi-quantitative immunoblotting. Calbindin-D_{28K}, calbindin-D_{9K}, and NCX1 mRNA expression levels were decreased in kidneys of TRPV5^{-/-} and 1 α -OHase^{-/-} mice compared with wild-type mice (Figure 2). It is interesting that a further significant decrease in the expression of these Ca²⁺ transporters was detected in TRPV5^{-/-}/1 α -OHase^{-/-} mice. Calbindin-D_{28K} and calbindin-D_{9K} protein abundance was significantly decreased in TRPV5^{-/-}/1 α -OHase^{-/-} mice compared with TRPV5^{-/-} mice as detected by immunoblot (Figure 3).

Duodenal mRNA Expression of Ca²⁺ Transporters

To evaluate the contribution of 1,25(OH)₂D₃ to expression of the duodenal Ca²⁺ transporters, we determined mRNA levels of TRPV6, calbindin-D_{9K}, and PMCA1b. TRPV5^{-/-} mice showed a significant increase in duodenal TRPV6 and calbindin-D_{9K} mRNA levels compared with wild-type mice (Figure 4). Additional 1 α -OHase ablation resulted in a significant decrease in TRPV6 and calbindin-D_{9K} expression in duodenum. Furthermore, 1 α -OHase^{-/-} mice demonstrated a downregulation of TRPV6 and calbindin-D_{9K} mRNA expression. PMCA1b expression levels did not differ significantly between the studied mouse genotypes.

Table 1. Urine and serum analyses and body weight^a

	Wild-Type	1 α -OHase ^{-/-}	TRPV5 ^{-/-}	TRPV5 ^{-/-} /1 α -OHase ^{-/-}
Urine				
Diuresis (ml/24 h)	1.8 \pm 0.2	2.3 \pm 0.3	4.4 \pm 0.6 ^b	1.8 \pm 0.5
pH	7.6 \pm 0.2	7.9 \pm 0.1	6.1 \pm 0.1 ^b	7.8 \pm 0.2
Serum				
1,25(OH) ₂ D ₃ (pmol/L)	193 \pm 27	<6	632 \pm 63 ^b	<6
PTH (pg/ml)	44 \pm 10	1444 \pm 123 ^b	29 \pm 6	2328 \pm 212 ^b
Body weight (g)	24.6 \pm 1.7	14.1 \pm 0.4 ^b	21.7 \pm 0.8	12.8 \pm 1.1 ^b

^aData are presented as means \pm SEM. 1 α -OHase^{-/-}, 1 α -OHase knockout mice; TRPV5^{-/-}, TRPV5 knockout mice; TRPV5^{-/-}/1 α -OHase^{-/-}, TRPV5/1 α -OHase double knockout mice; PTH, parathyroid hormone. The minimum detection level of the 1,25(OH)₂D₃ assay was 6 pmol/L.

^b $P < 0.05$, significant difference from wild-type mice.

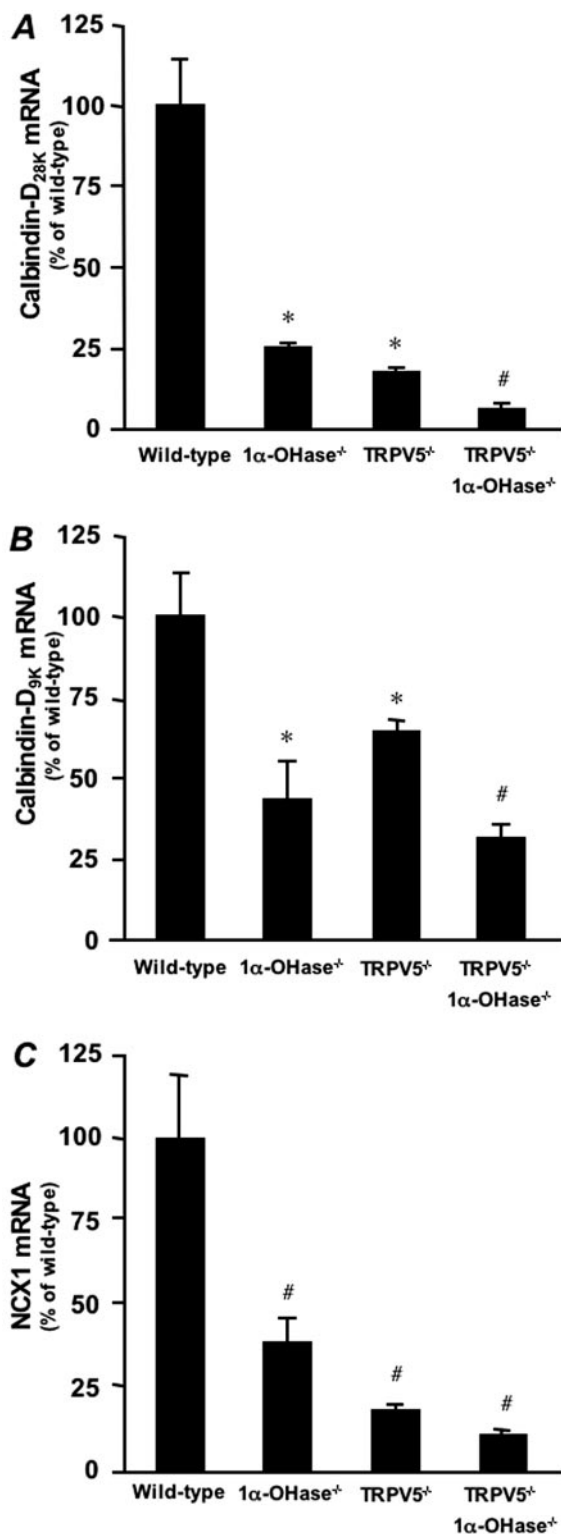


Figure 2. Renal mRNA expression of Ca²⁺ transport proteins. Renal mRNA expression levels of calbindin-D_{28K} (A), calbindin-D_{9K} (B), and NCX1 (C) in wild-type, 1 α -OHase^{-/-}, TRPV5^{-/-}, and TRPV5^{-/-}1 α -OHase^{-/-} mice, assessed by real-time PCR analysis as the ratio to hypoxanthine-guanine phosphoribosyl transferase (HPRT) mRNA levels. Expression levels are presented relative to wild-type mice. Data are expressed as means \pm SEM. **P* < 0.05 significant difference from wild-type mice; #*P* < 0.05 significant difference from all groups.

Bone Analyses

Bone analyses showed that TRPV5^{-/-} mice (Figure 5, J and N) and 1 α -OHase^{-/-} mice (Figure 5, K and O) demonstrated reduced bone thickness in both the trabecular and the cortical compartments compared with wild-type mice (Figure 5, I and M). This reduction was more severe in 1 α -OHase^{-/-} mice (Figure 5, K and O). In addition, the degree of bone mineralization was reduced in TRPV5^{-/-} mice (Figure 5, F, J, and N) and 1 α -OHase^{-/-} mice (Figure 5, G, K, and O) compared with wild-type mice (Figure 5, E, I, and M). Moreover, the epiphyseal growth plate was widened in 1 α -OHase^{-/-} mice (Figure 5, G versus E), which was not observed in TRPV5^{-/-} mice (Figure 5, F versus E). Tibial bone length was reduced in 1 α -OHase^{-/-} mice (Figure 5, C versus A and B). In TRPV5^{-/-}/1 α -OHase^{-/-} mice, the thickness of cortical bone and trabeculae was decreased and mineralization was diminished compared with all other mouse genotypes (Figure 5, H, L, and P). Moreover, tibial bone length was severely reduced and epiphyseal growth plate widening was more pronounced in TRPV5^{-/-}/1 α -OHase^{-/-} mice than demonstrated in 1 α -OHase^{-/-} mice (Figure 5, D and H versus C and G). Taken together, the TRPV5^{-/-}/1 α -OHase^{-/-} mice exhibited an aggravated skeletal phenotype in comparison with TRPV5^{-/-} and 1 α -OHase^{-/-} mice (Figure 5, D, H, L, and P).

Discussion

This study demonstrated that hypervitaminosis D in TRPV5^{-/-} mice is responsible for the upregulation of intestinal Ca²⁺ transport proteins and the resulting Ca²⁺ hyperabsorption. Hereby, TRPV5^{-/-} mice maintain normocalcemia, a feature absent in TRPV5^{-/-}/1 α -OHase^{-/-} mice, which exhibit a significant hypocalcemia. The urinary Ca²⁺ leak demonstrated in TRPV5^{-/-} mice persists in the TRPV5^{-/-}/1 α -OHase^{-/-} mice as an inappropriately high urinary Ca²⁺ excretion with respect to the hypocalcemia. Moreover, bone degradation is present in TRPV5^{-/-}/1 α -OHase^{-/-} mice, more severe than observed in TRPV5^{-/-} mice. Therefore, the absence of Ca²⁺ hyperabsorption results in a more aggravated phenotype than described for TRPV5 single-ablated mice. Thus, 1,25(OH)₂D₃ is of crucial importance to compensate the renal Ca²⁺ leak and to maintain normal serum Ca²⁺ levels in TRPV5^{-/-} mice.

1,25(OH)₂D₃ is an important regulatory hormone in Ca²⁺ and bone homeostasis (2). In duodenum, 1,25(OH)₂D₃ regulates the expression of Ca²⁺ transport proteins (24). Moreover, 1,25(OH)₂D₃ is synthesized from its precursor by the renal enzyme 1 α -OHase (7). 1 α -OHase^{-/-} mice were previously generated as a mouse model for vitamin D deficiency rickets type I (15,16). Inactivation of 1 α -OHase resulted in undetectable levels of serum 1,25(OH)₂D₃, a significant hypocalcemia, and reduced expression levels of duodenal Ca²⁺ transport proteins. In contrast, TRPV5^{-/-} mice demonstrated a normocalcemia with a significant hypervitaminosis D and increased duodenal Ca²⁺ transport protein expression leading to Ca²⁺ hyperabsorption (14). We hypothesized that the increased serum 1,25(OH)₂D₃ levels constitute a compensatory mechanism in an effort to correct for the significant renal Ca²⁺ leak. Additional gene inactivation of 1 α -OHase in TRPV5^{-/-} mice was per-

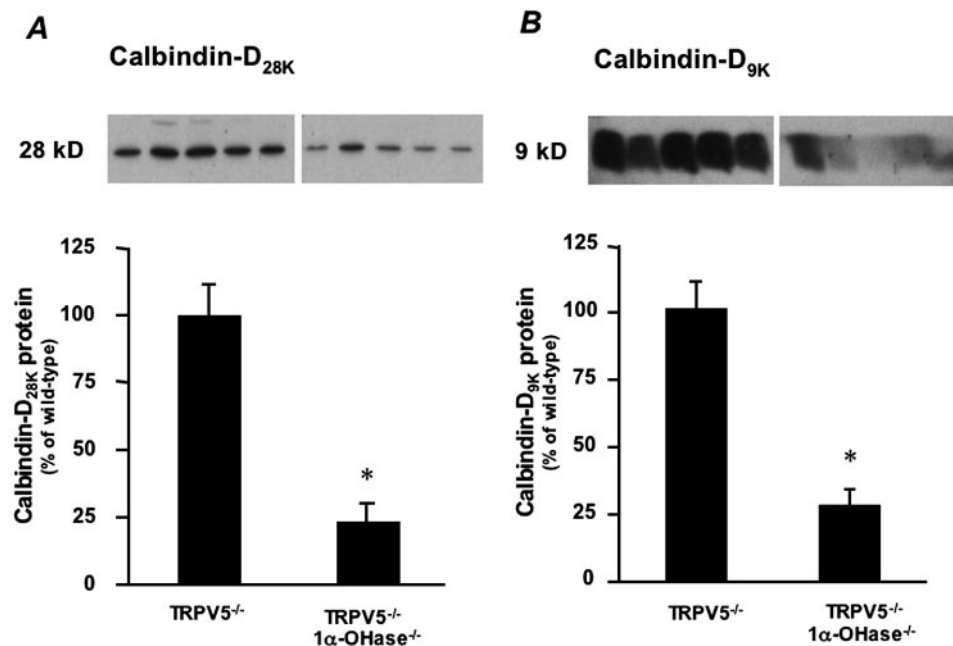


Figure 3. Renal calbindin-D_{28K} and calbindin-D_{9K} protein expression. Immunoblots of total kidney homogenates from TRPV5^{-/-} and TRPV5^{-/-}1α-OHase^{-/-} mice probed with anti-calbindin-D_{28K} (A) and anti-calbindin-D_{9K} (B). Intensities of immunopositive bands were quantified by densitometry. Calbindin-D_{9K} and calbindin-D_{28K} expression levels were depicted relative to TRPV5^{-/-} mice. Data are expressed as means ± SEM. **P* < 0.05 significant difference from TRPV5^{-/-} mice.

formed to investigate the effects of the elevated serum 1,25(OH)₂D₃ levels present in TRPV5^{-/-} mice. This double gene ablation resulted in undetectable serum 1,25(OH)₂D₃ levels and downregulation of duodenal Ca²⁺ transport protein expression. Moreover, a hypocalcemia developed in the TRPV5^{-/-}/1α-OHase^{-/-} mice. These data clearly showed that the increased expression of the intestinal Ca²⁺ transporters TRPV6 and calbindin-D_{9K} and the resulting Ca²⁺ hyperabsorption in TRPV5^{-/-} mice are due to a secondary hypervitaminosis D. These effects compensate for the renal Ca²⁺ leak to maintain normal serum Ca²⁺ levels in TRPV5^{-/-} mice.

TRPV5^{-/-} mice show a striking hypercalciuria. Previous micropuncture experiments demonstrated that Ca²⁺ reabsorption in DCT and CNT is abolished in these mice, illustrating a primary defect in renal active Ca²⁺ reabsorption (14). On the contrary, 1α-OHase^{-/-} mice demonstrated a significant hypocalciuria, a feature not described before, although explained by the downregulation of intestinal and renal Ca²⁺ transport proteins and, therefore, impairment of Ca²⁺ absorption and reabsorption. It is interesting that additional gene ablation of 1α-OHase in TRPV5^{-/-} mice normalized 24-h urinary Ca²⁺ excretion to values not significantly different from wild-type mice. Nevertheless, relative to the severe hypocalcemia, these double knockout mice still excreted inappropriately high amounts of Ca²⁺. Moreover, given the expression of the renal Ca²⁺ transporters in 1α-OHase^{-/-} mice, it can be concluded that active Ca²⁺ reabsorption is still present, although severely impaired despite the absence of 1,25(OH)₂D₃. Ca²⁺ reabsorption through TRPV5 therefore could be partly independent on 1,25(OH)₂D₃, which is in line with the results of previous

studies in which the rescue of 1α-OHase^{-/-} mice by dietary Ca²⁺ was described, showing the regulation of Ca²⁺ transport proteins independent of 1,25(OH)₂D₃ regulation (18,25).

Hypercalciuria increases the risk for the formation of Ca²⁺-containing urinary crystals (26,27). In addition to hypercalciuria, TRPV5^{-/-} mice displayed a significant polyuria and urine acidification. Polyuria facilitates the excretion of large quantities of Ca²⁺, and urine acidification creates an environment in which formation of crystals is restrained (28,29). It is interesting that in the double knockout mice, net calciuresis did not differ from wild-type mice and neither did urinary pH or diuresis. This indicated that the hypercalciuria in TRPV5^{-/-} mice, which originates from the DCT and CNT, could indeed influence diuresis and pH in more distal located nephron segments. Previously, thiazide treatment leading to a decrease in Ca²⁺ excretion in TRPV5^{-/-} mice was shown to normalize urinary pH and blunt polyuria (30). It was postulated that calcium-sensing receptor (CaSR) signaling in the apical membrane of the collecting duct functionally links renal Ca²⁺ and water metabolism (31). CaSR activation by high luminal Ca²⁺ concentrations might result in a decrease in water permeability of the collecting duct by downregulation of aquaporin-2 water channels and consequently increase the urine volume (32,33). We suggest that regulation of specific acid-base transport proteins, possibly as a result of CaSR activation, is involved in the observed urine acidification in TRPV5^{-/-} mice. The exact mechanisms that are responsible for the urine acidification and polyuria in TRPV5^{-/-} mice need further investigation.

The majority of the body Ca²⁺ content is stored in bone, where the balanced processes of bone formation and resorption

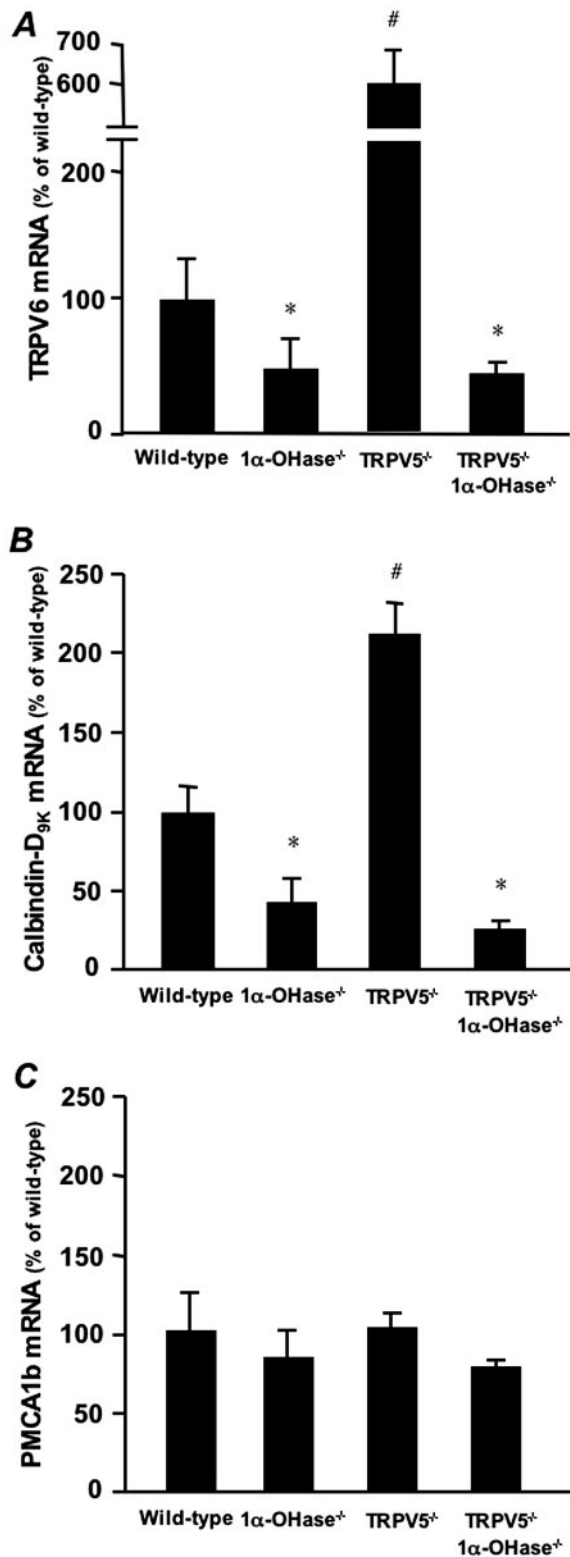


Figure 4. Duodenal mRNA expression of Ca²⁺ transport proteins. Duodenal mRNA expression levels of TRPV6 (A), calbindin-D_{9K} (B), and PMCA1b (C) in wild-type, 1 α -OHase^{-/-}, TRPV5^{-/-}, and TRPV5^{-/-}1 α -OHase^{-/-} mice, assessed by real-time PCR analysis and depicted as the ratio to HPRT mRNA levels. Expression levels are presented relative to wild-type mice. Data are expressed as means \pm SEM. **P* < 0.05 versus wild-type mice; #*P* < 0.05 significant difference from all groups.

maintain bone homeostasis (34). Detailed microcomputed tomography and x-ray analyses showed bone abnormalities in all three studied mouse models compared with wild-type mice. TRPV5^{-/-} mice displayed reduced trabecular and cortical bone thickness (14), which could be a consequence of the negative Ca²⁺ balance as a result of the renal Ca²⁺ leak. Moreover, it was previously suggested that high serum 1,25(OH)₂D₃ levels reduce cortical bone thickness, bone stiffness, and strength (35). However, 1,25(OH)₂D₃ levels were not detectable in TRPV5^{-/-}/1 α -OHase^{-/-} mice, which demonstrated a significant decrease in bone thickness, reduced bone mineralization, and rickets, noticeably more severe than the bone abnormalities observed in TRPV5^{-/-} and 1 α -OHase^{-/-} mice (14,15). This suggested that TRPV5 gene ablation might, at least in part, be directly responsible for the detected bone abnormalities, independent of the elevated serum 1,25(OH)₂D₃ levels. It is interesting that the expression of TRPV5 and TRPV6 in bone tissue was previously described and these epithelial Ca²⁺ channels therefore could serve as direct Ca²⁺ providers in bone (20). However, this hypothesis is hampered by the absence of comprehensive data on the exact actions of these Ca²⁺ channels in bone. Alternatively, the aggravated bone phenotype demonstrated in TRPV5^{-/-}/1 α -OHase^{-/-} mice could be explained by the secondary hyperparathyroidism leading to renal osteodystrophy.

Zheng *et al.* (36) recently studied the vitamin D receptor (VDR)/calbindin-D_{28K} double knockout mouse compared with VDR or calbindin-D_{28K} single gene-ablated mice. It is interesting that the VDR/calbindin-D_{28K} double knockout mice displayed downregulation of intestinal and renal Ca²⁺ transport proteins and a more aggravated phenotype, including hypercalciuria and hyperparathyroidism, a further decrease in bone mineral density and bone length, increased distortion of the growth plate, a decrease in body weight, and a decreased lifespan compared with the single gene-ablated mice. In line with our results, this study clearly revealed the critical role of vitamin D and Ca²⁺ transport proteins in Ca²⁺ homeostasis and, moreover, the crucial regulatory function of 1,25(OH)₂D₃ in the expression of intestinal and renal Ca²⁺ transport protein.

On the basis of the present data, we ascertained the important function of the renal Ca²⁺ transport protein TRPV5 in Ca²⁺ reabsorption and its possible involvement in bone Ca²⁺ transport, concomitant with a crucial role of 1,25(OH)₂D₃ in active Ca²⁺ (re)absorption by regulating epithelial Ca²⁺ transport and Ca²⁺ transport protein expression. The observed polyuria and urine acidification in TRPV5 knockout mice will need further investigation to obtain more insight into the responsible mechanisms.

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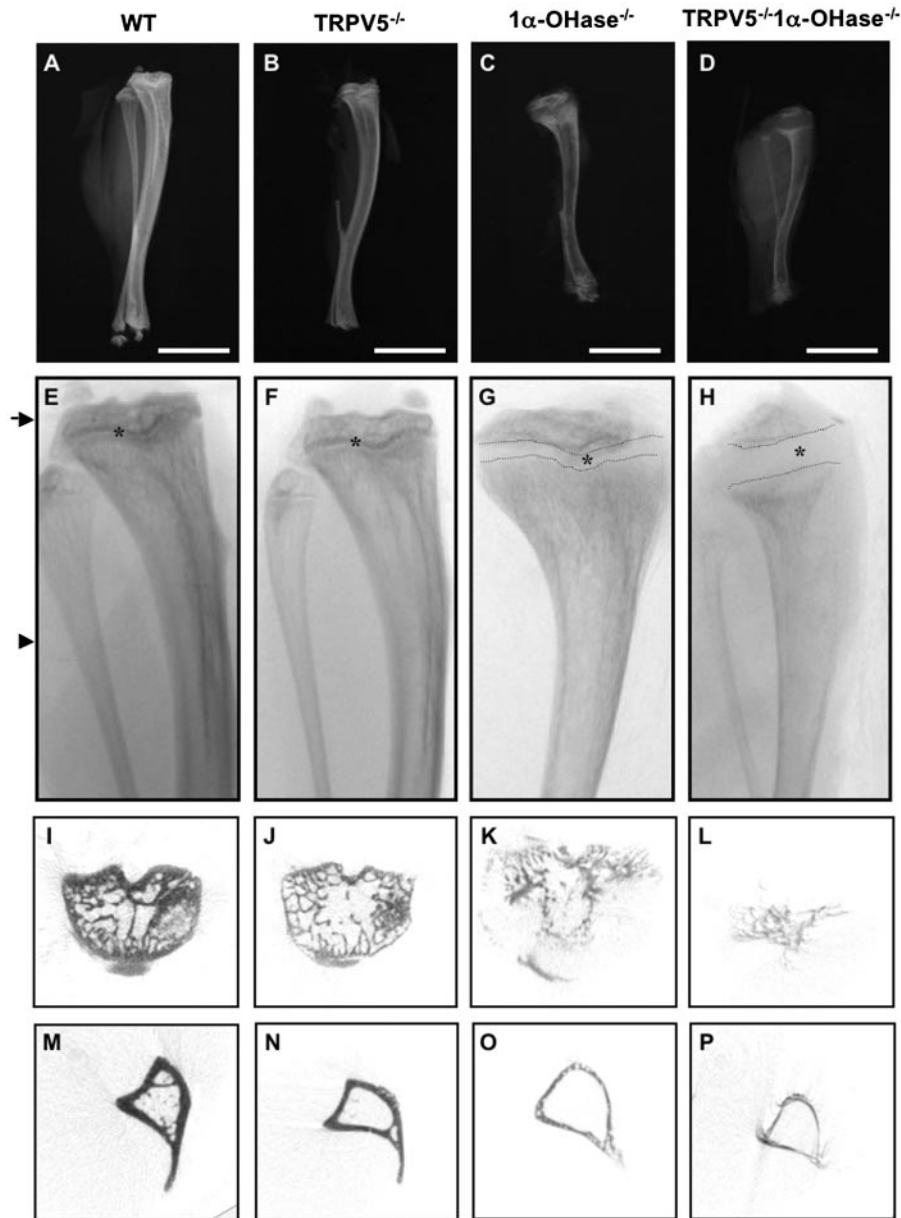


Figure 5. Bone phenotypes. Lower legs from 8-wk-old wild-type, $TRPV5^{-/-}$, $1\alpha\text{-OHase}^{-/-}$, and $TRPV5^{-/-}1\alpha\text{-OHase}^{-/-}$ mice were analyzed using x-ray imaging (A through D). Microcomputed tomography was used to generate tibial overviews (E through H), from which digital cross-sections were made at the site of the epiphysis (arrow; I through L) and the diaphysis (arrowhead; M through P). Representative images are shown for each genotype. Epiphyseal growth plates are indicated by asterisks (*) and outlined by dotted lines (G and H).

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