Basic Science Articles

Hypervitaminosis D Mediates Compensatory Ca^{2+} Hyperabsorption in TRPV5 Knockout Mice

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Vitamin D plays an important role in Ca^{2+} homeostasis by controlling Ca^{2+} (re)absorption in intestine, kidney, and bone. The epithelial Ca^{2+} channel TRPV5 mediates the Ca^{2+} entry step in active Ca^{2+} reabsorption. TRPV5 knockout (TRPV5$^{-/-}$) mice show impaired Ca^{2+} reabsorption, hypercalciuria, hypervitaminosis D, and intestinal hyperabsorption of Ca^{2+}. 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 Ca^{2+} homeostasis and the regulation of expression levels of the Ca^{2+} transport proteins in kidney and intestine, TRPV5/25-hydroxyvitamin-D$_3$-1α-hydroxylase double knockout (TRPV5$^{-/-}$/1α-OHase$^{-/-}$) mice, which show undetectable serum 1,25(OH)$_2$D$_3$ levels, were generated. TRPV5$^{-/-}$/1α-OHase$^{-/-}$ mice displayed a significant hypocalcemia compared with wild-type mice (1.10 ± 0.02 and 2.54 ± 0.01 mM, respectively; $P < 0.05$). mRNA levels of renal calbindin-D$_{28K}$ (7 ± 2%), calbindin-D$_{9K}$ (32 ± 4%), Na$^+$/Ca^{2+} exchanger (12 ± 2%), and intestinal TRPV6 (40 ± 8%) and calbindin-D$_{9K}$ (26 ± 4%) expression levels were decreased compared with wild-type mice. Hyperparathyroidism and rickets were present in TRPV5$^{-/-}$/1α-OHase$^{-/-}$ mice, more pronounced than observed in single TRPV5 or 1α-OHase knockout mice. It is interesting that a renal Ca^{2+} leak, as demonstrated in TRPV5$^{-/-}$ mice, persisted in TRPV5$^{-/-}$/1α-OHase$^{-/-}$ mice, but a compensatory upregulation of intestinal Ca^{2+} transporters was abolished. In conclusion, the elevation of serum 1,25(OH)$_2$D$_3$ levels in TRPV5$^{-/-}$ mice is responsible for the upregulation of intestinal Ca^{2+} transporters and Ca^{2+} hyperabsorption. Hypervitaminosis D, therefore, is of crucial importance to maintain normocalcemia in impaired Ca^{2+} reabsorption in TRPV5$^{-/-}$ mice.

Ca^{2+} 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, Ca^{2+} balance is tightly controlled by the concerted action of intestinal Ca^{2+} absorption, exchange of Ca^{2+} from bone, and Ca^{2+} reabsorption in the kidney (1). The active form of vitamin D, 1α,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), and parathyroid hormone (PTH) and calcitonin are the calciotropic hormones that are known to be involved in the maintenance of plasma Ca^{2+} concentration (2,3). Ca^{2+} absorption from the intestinal lumen and Ca^{2+} reabsorption in the renal tubules is promoted by 1,25(OH)$_2$D$_3$ (4–6). Synthesis of 1,25(OH)$_2$D$_3$ requires the renal cytochrome P450 enzyme 25-hydroxyvitamin D$_3$-1α-hydroxylase (1α-OHase) (7). In addition, PTH stimulates 1,25(OH)$_2$D$_3$ production by 1α-OHase activation and the release of Ca^{2+} from bone to maintain plasma Ca^{2+} levels, whereas calcitonin lowers the plasma Ca^{2+} concentration by inhibition of bone resorption (2).

1,25(OH)$_2$D$_3$-stimulated transepithelial Ca^{2+} transport in kidney and intestine involves Ca^{2+} entry across the apical membrane *via* the epithelial Ca^{2+} 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 homologues Ca^{2+} channel localized in the brush border membrane of duodenum (1,10–13). After entry of Ca^{2+} through TRPV5 and TRPV6, Ca^{2+} bound to Ca^{2+}-binding proteins (calbindins) diffuses to the basolateral membrane of the cell. Ca^{2+} is finally extruded to the extracellular compartment by the Na$^+$/Ca^{2+} exchanger (NCX1) in the kidney and the plasma membrane Ca^{2+}-ATPase (PMCA1b) in intestine and kidney (9).

Recently, several animal models were designed to investigate Ca^{2+} absorption mechanisms and maintenance of Ca^{2+} balance. TRPV5 knockout (TRPV5$^{-/-}$) mice were generated by targeted ablation of the TRPV5 gene. These mice displayed robust renal Ca^{2+} wasting as a result of impaired Ca^{2+} reabsorption in DCT and CNT (14). Furthermore, elevated

1,25(OH)$_2$D$_3$ serum levels, intestinal Ca^{2+} 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 1α-OHase gene. These 1α-OHase knockout (1α-OHase$^{-/-}$) mice showed a phenotype characterized by undetectable serum levels of 1,25(OH)$_2$D$_3$, severe hypocalcemia, hyperparathyroidism, bone abnormalities, and retarded growth (15–17). Until now, data on detailed urine analysis of the 1α-OHase$^{-/-}$ mice were not available. Previous studies demonstrated that 1,25(OH)$_2$D$_3$ supplementation rescues the severe phenotype of 1α-OHase$^{-/-}$ mice, which is accompanied by an upregulation of TRPV5, TRPV6, calbindins, NCX1, and PMCA1b expression, resulting in the normalization of serum Ca^{2+} levels. It is interesting that dietary Ca^{2+} supplementation in 1α-OHase$^{-/-}$ mice resulted in a similar rescue in the absence of 1,25(OH)$_2$D$_3$. Taken together, these studies suggest a pivotal role of the Ca^{2+} transport proteins in maintaining Ca^{2+} balance, partly independent of 1,25(OH$_2$)D$_3$ (18).

To investigate the contribution of elevated 1,25(OH)$_2$D$_3$ levels to the phenotype of TRPV5$^{-/-}$ mice and to elucidate the involvement of 1,25(OH)$_2$D$_3$ in the compensatory Ca^{2+} hyperabsorption, we generated a mouse model in which both TRPV5 and 1α-OHase were inactivated. In this study, we describe the phenotype of TRPV5/1α-OHase double knockout (TRPV5$^{-/-}$/1α-OHase$^{-/-}$) mice, including bone analysis and semiquantification of mRNA and protein expression levels of Ca^{2+} transport proteins and provide an extensive comparison with TRPV5 and 1α-OHase single knockout mice.

Materials and Methods
Animal Experiments
TRPV5$^{-/-}$ mice were generated as described previously (14). 1α-OHase$^{-/-}$ mice were provided by René St-Arnaud (Shriners Hospital for Children Montreal, QU, Canada) (15). Cross-breeding of 1α-OHase$^{-/-}$ and TRPV5$^{-/-}$ mice resulted in offspring that were heterozygous for both TRPV5 and 1α-OHase (TRPV5$^{+/-}$/1α-OHase$^{+/-}$). This offspring was subsequently intercrossed to obtain TRPV5$^{-/-}$/1α-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 Ca^{2+} 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 [^{125}I]1,25(OH)$_2$D$_3$ 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 Ca^{2+} transport proteins, total RNA from kidney and duodenum was
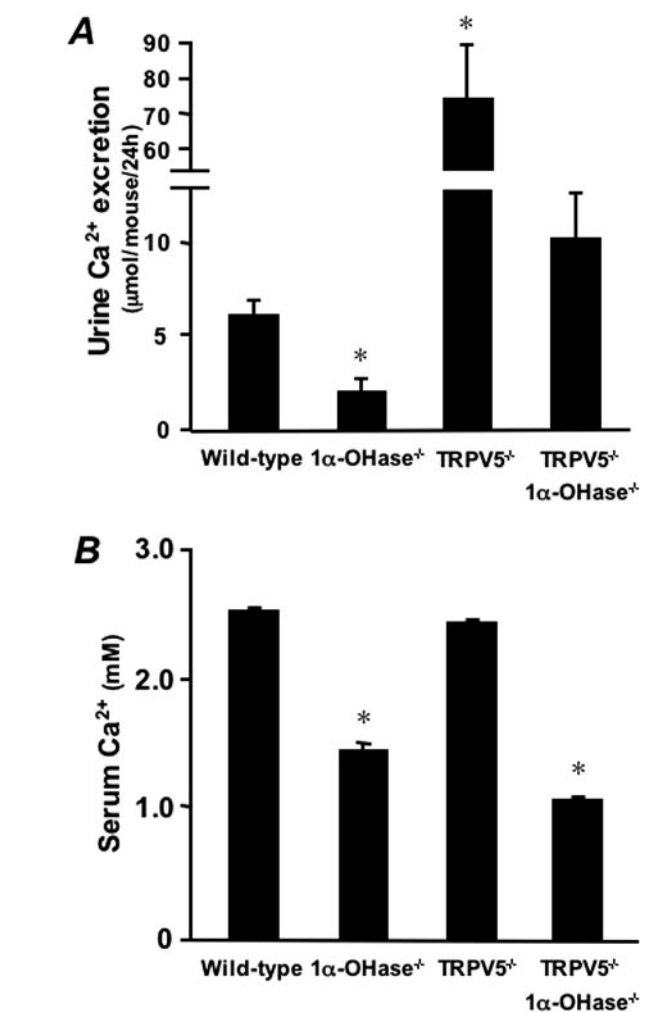

Figure 1. Urinary Ca^{2+} excretion and serum Ca^{2+} concentration. Urinary Ca^{2+} excretion (A) and serum Ca^{2+} levels (B) were determined in wild-type, 1α-OHase$^{-/-}$, TRPV5$^{-/-}$, and TRPV5$^{-/-}$1α-OHase$^{-/-}$ mice. Data are presented as means ± SEM. *P < 0.05, significant difference from 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$^{-/-}$/1α-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 polyvinyldifluoride-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 1α-OHase-ablation on bone homeostasis, bone thickness, mineralization, and epiphyseal growth plate development were determined in tibiae from wild-type, TRPV5$^{-/-}$, 1α-OHase$^{-/-}$, and TRPV5$^{-/-}$/1α-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 ± 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 Ca^{2+} levels, a significant increase of serum 1,25(OH)$_2$D$_3$ levels, and normal serum PTH levels. Moreover, TRPV5$^{-/-}$ mice displayed an increased diuresis and a decrease of urine pH (Figure 1 and Table 1). 1α-OHase$^{-/-}$ mice displayed hypocalciuria, hypocalcemia, serum 1,25(OH)$_2$D$_3$ 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 1α-OHase$^{-/-}$ mice. The analysis of the TRPV5$^{-/-}$/1α-OHase$^{-/-}$ mice phenotype showed that net urine Ca^{2+} excretion does not differ from wild-type mice, although inappropriately high given the severe hypocalcemia. Serum 1,25(OH)$_2$D$_3$ levels were below detectable levels, and serum PTH levels were further elevated in TRPV5$^{-/-}$/1α-OHase$^{-/-}$ mice compared with 1α-OHase$^{-/-}$ mice. In addition, diuresis and urine pH were normalized in TRPV5$^{-/-}$/1α-OHase$^{-/-}$ mice. Body weight was reduced in 1α-OHase$^{-/-}$ and TRPV5$^{-/-}$/1α-OHase$^{-/-}$ mice compared with litter-matched wild-type mice. 1α-OHase$^{-/-}$ and TRPV5$^{-/-}$/1α-OHase$^{-/-}$ mice had a decreased lifespan in comparison with wild-type and TRPV5$^{-/-}$ mice.

Renal mRNA and Protein Expression of Ca^{2+} Transporters

To investigate the specific regulation of renal Ca^{2+} transporters by 1,25(OH)$_2$D$_3$, we determined the effects of TRPV5 and 1α-OHase single or combined gene ablation on mRNA and protein expression levels by real-time PCR analysis and semiquantitative immunoblotting. Calbindin-D_{28K}, calbindin-D_{9K}, and NCX1 mRNA expression levels were decreased in kidneys of TRPV5$^{-/-}$ and 1α-OHase$^{-/-}$ mice compared with wild-type mice (Figure 2). It is interesting that a further significant decrease in the expression of these Ca^{2+} transporters was detected in TRPV5$^{-/-}$/1α-OHase$^{-/-}$ mice. Calbindin-D_{28K} and calbindin-D_{9K} protein abundance was significantly decreased in TRPV5$^{-/-}$/1α-OHase$^{-/-}$ mice compared with TRPV5$^{-/-}$ mice as detected by immunoblot (Figure 3).

Duodenal mRNA Expression of Ca^{2+} Transporters

To evaluate the contribution of 1,25(OH)$_2$D$_3$ to expression of the duodenal Ca^{2+} 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 1α-OHase ablation resulted in a significant decrease in TRPV6 and calbindin-D_{9K} expression in duodenum. Furthermore, 1α-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	1α-OHase$^{-/-}$	TRPV5$^{-/-}$	TRPV5$^{-/-}$/1α-OHase$^{-/-}$
Urine				
Diuresis (ml/24 h)	1.8 ± 0.2	2.3 ± 0.3	4.4 ± 0.6[b]	1.8 ± 0.5
pH	7.6 ± 0.2	7.9 ± 0.1	6.1 ± 0.1[b]	7.8 ± 0.2
Serum				
1,25(OH)$_2$D$_3$ (pmol/L)	193 ± 27	<6	632 ± 63[b]	<6
PTH (pg/ml)	44 ± 10	1444 ± 123[b]	29 ± 6	2328 ± 212[b]
Body weight (g)	24.6 ± 1.7	14.1 ± 0.4[b]	21.7 ± 0.8	12.8 ± 1.1[b]

[a]Data are presented as means ± SEM. 1α-OHase$^{-/-}$, 1α-OHase knockout mice; TRPV5$^{-/-}$, TRPV5 knockout mice; TRPV5$^{-/-}$/1α-OHase$^{-/-}$, TRPV5/1α-OHase double knockout mice; PTH, parathyroid hormone. The minimum detection level of the 1,25(OH)$_2$D$_3$ assay was 6 pmol/L.
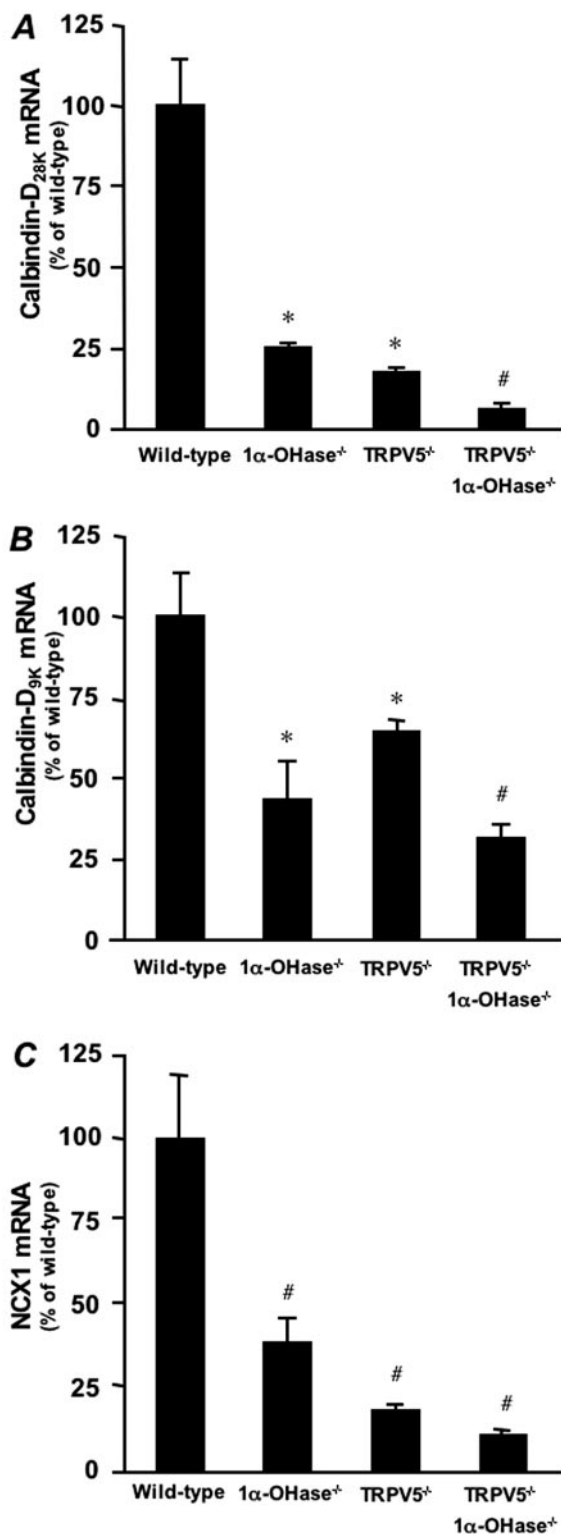[b]$P < 0.05$, significant difference from wild-type mice.

Figure 2. Renal mRNA expression of Ca^{2+} transport proteins. Renal mRNA expression levels of calbindin-D$_{28K}$ (A), calbindin-D$_{9K}$ (B), and NCX1 (C) in wild-type, 1α-OHase$^{-/-}$, TRPV5$^{-/-}$, and TRPV5$^{-/-}$1α-OHase$^{-/-}$ 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 ± 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 1α-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 1α-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 1α-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 1α-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 1α-OHase$^{-/-}$ mice (Figure 5, C *versus* A and B). In TRPV5$^{-/-}$/ 1α-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$^{-/-}$/1α-OHase$^{-/-}$ mice than demonstrated in 1α-OHase$^{-/-}$ mice (Figure 5, D and H *versus* C and G). Taken together, the TRPV5$^{-/-}$/1α-OHase$^{-/-}$ mice exhibited an aggravated skeletal phenotype in comparison with TRPV5$^{-/-}$ and 1α-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 Ca^{2+} transport proteins and the resulting Ca^{2+} hyperabsorption. Hereby, TRPV5$^{-/-}$ mice maintain normocalcemia, a feature absent in TRPV5$^{-/-}$/1α-OHase$^{-/-}$ mice, which exhibit a significant hypocalcemia. The urinary Ca^{2+} leak demonstrated in TRPV5$^{-/-}$ mice persists in the TRPV5$^{-/-}$/1α-OHase$^{-/-}$ mice as an inappropriately high urinary Ca^{2+} excretion with respect to the hypocalcemia. Moreover, bone degradation is present in TRPV5$^{-/-}$/1α-OHase$^{-/-}$ mice, more severe than observed in TRPV5$^{-/-}$ mice. Therefore, the absence of Ca^{2+} hyperabsorption results in a more aggravated phenotype than described for TRPV5 single-ablated mice. Thus, 1,25(OH)$_2$D$_3$ is of crucial importance to compensate the renal Ca^{2+} leak and to maintain normal serum Ca^{2+} levels in TRPV5$^{-/-}$ mice.

1,25(OH)$_2$D$_3$ is an important regulatory hormone in Ca^{2+} and bone homeostasis (2). In duodenum, 1,25(OH)$_2$D$_3$ regulates the expression of Ca^{2+} transport proteins (24). Moreover, 1,25(OH)$_2$D$_3$ is synthesized from its precursor by the renal enzyme 1α-OHase (7). 1α-OHase$^{-/-}$ mice were previously generated as a mouse model for vitamin D deficiency rickets type I (15,16). Inactivation of 1α-OHase resulted in undetectable levels of serum 1,25(OH)$_2$D$_3$, a significant hypocalcemia, and reduced expression levels of duodenal Ca^{2+} transport proteins. In contrast, TRPV5$^{-/-}$ mice demonstrated a normocalcemia with a significant hypervitaminosis D and increased duodenal Ca^{2+} transport protein expression leading to Ca^{2+} hyperabsorption (14). We hypothesized that the increased serum 1,25(OH)$_2$D$_3$ levels constitute a compensatory mechanism in an effort to correct for the significant renal Ca^{2+} leak. Additional gene inactivation of 1α-OHase in TRPV5$^{-/-}$ mice was per-
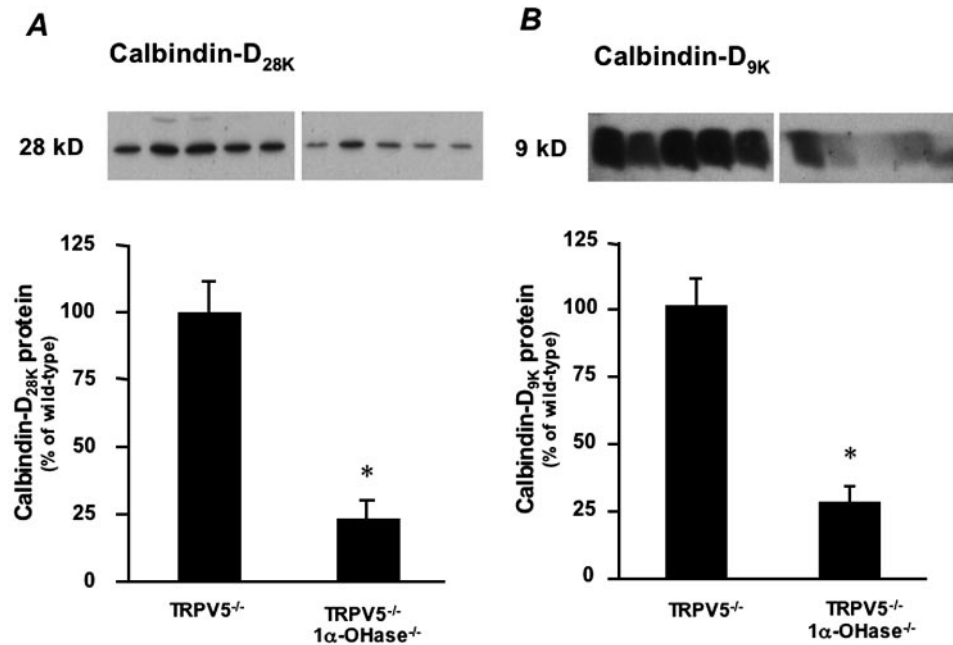
Figure 3. Renal calbindin-D_{28K} and calbindin-D_{9K} protein expression. Immunoblots of total kidney homogenates from TRPV5$^{-/-}$ and TRPV5$^{-/-}$1α-OHase$^{-/-}$ mice probed with anti–calbindin-D_{28K} (A) and anti–calbindin-D_{9K} (B). Intensities of immunopositive bands were quantified by densitometry. Calbindin-D_{9K} and calbindin-D_{28K} expression levels were depicted relative to TRPV5$^{-/-}$ mice. Data are expressed as means $\pm$ SEM. *$P < 0.05$ significant difference from TRPV5$^{-/-}$ mice.

formed to investigate the effects of the elevated serum 1,25(OH)$_2$D$_3$ levels present in TRPV5$^{-/-}$ mice. This double gene ablation resulted in undetectable serum 1,25(OH)$_2$D$_3$ levels and downregulation of duodenal Ca^{2+} transport protein expression. Moreover, a hypocalcemia developed in the TRPV5$^{-/-}$/1α-OHase$^{-/-}$ mice. These data clearly showed that the increased expression of the intestinal Ca^{2+} transporters TRPV6 and calbindin-D_{9K} and the resulting Ca^{2+} hyperabsorption in TRPV5$^{-/-}$ mice are due to a secondary hypervitaminosis D. These effects compensate for the renal Ca^{2+} leak to maintain normal serum Ca^{2+} levels in TRPV5$^{-/-}$ mice.

TRPV5$^{-/-}$ mice show a striking hypercalciuria. Previous micropuncture experiments demonstrated that Ca^{2+} reabsorption in DCT and CNT is abolished in these mice, illustrating a primary defect in renal active Ca^{2+} 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 Ca^{2+} transport proteins and, therefore, impairment of Ca^{2+} absorption and reabsorption. It is interesting that additional gene ablation of 1α-OHase in TRPV5$^{-/-}$ mice normalized 24-h urinary Ca^{2+} 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 Ca^{2+}. Moreover, given the expression of the renal Ca^{2+} transporters in 1α-OHase$^{-/-}$ mice, it can be concluded that active Ca^{2+} reabsorption is still present, although severely impaired despite the absence of 1,25(OH)$_2$D$_3$. Ca^{2+} reabsorption through TRPV5 therefore could be partly independent on 1,25(OH)$_2$D$_3$, which is in line with the results of previous

studies in which the rescue of 1α-OHase$^{-/-}$ mice by dietary Ca^{2+} was described, showing the regulation of Ca^{2+} transport proteins independent of 1,25(OH)$_2$D$_3$ regulation (18,25).

Hypercalciuria increases the risk for the formation of Ca^{2+}-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 Ca^{2+}, 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 Ca^{2+} 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 Ca^{2+} and water metabolism (31). CaSR activation by high luminal Ca^{2+} 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 Ca^{2+} content is stored in bone, where the balanced processes of bone formation and resorption
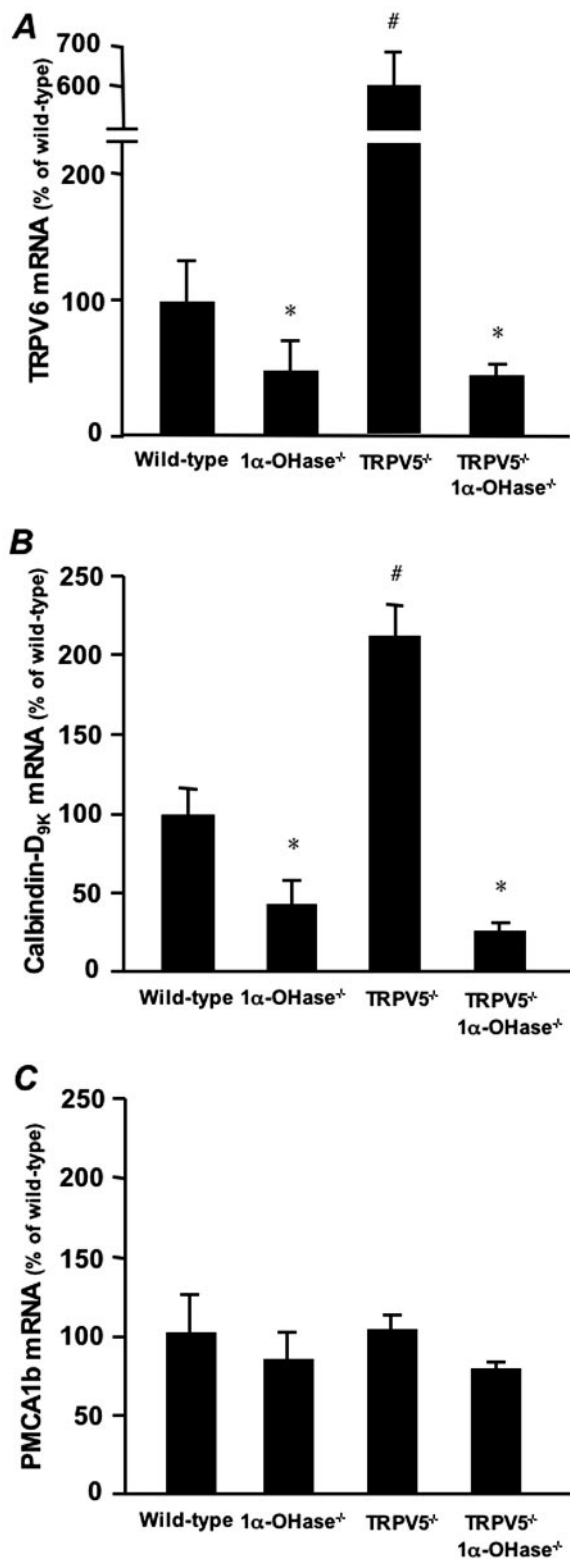
Figure 4. Duodenal mRNA expression of Ca^{2+} transport proteins. Duodenal mRNA expression levels of TRPV6 (A), calbindin-D$_{9K}$ (B), and PMCA1b (C) in wild-type, 1α-OHase$^{-/-}$, TRPV5$^{-/-}$, and TRPV5$^{-/-}$1α-OHase$^{-/-}$ mice, assessed by real-time PCR analysis and depicted as the ratio to HPRT mRNA levels. Expression levels are presented relative to wild-type mice. Data are expressed as means ± 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 Ca^{2+} balance as a result of the renal Ca^{2+} leak. Moreover, it was previously suggested that high serum 1,25(OH)$_2$D$_3$ levels reduce cortical bone thickness, bone stiffness, and strength (35). However, 1,25(OH)$_2$D$_3$ levels were not detectable in TRPV5$^{-/-}$/1α-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 1α-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)$_2$D$_3$ levels. It is interesting that the expression of TRPV5 and TRPV6 in bone tissue was previously described and these epithelial Ca^{2+} channels therefore could serve as direct Ca^{2+} providers in bone (20). However, this hypothesis is hampered by the absence of comprehensive data on the exact actions of these Ca^{2+} channels in bone. Alternatively, the aggravated bone phenotype demonstrated in TRPV5$^{-/-}$/1α-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 Ca^{2+} 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 Ca^{2+} transport proteins in Ca^{2+} homeostasis and, moreover, the crucial regulatory function of 1,25(OH)$_2$D$_3$ in the expression of intestinal and renal Ca^{2+} transport protein.

On the basis of the present data, we ascertained the important function of the renal Ca^{2+} transport protein TRPV5 in Ca^{2+} reabsorption and its possible involvement in bone Ca^{2+} transport, concomitant with a crucial role of 1,25(OH)$_2$D$_3$ in active Ca^{2+} (re)absorption by regulating epithelial Ca^{2+} transport and Ca^{2+} 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.
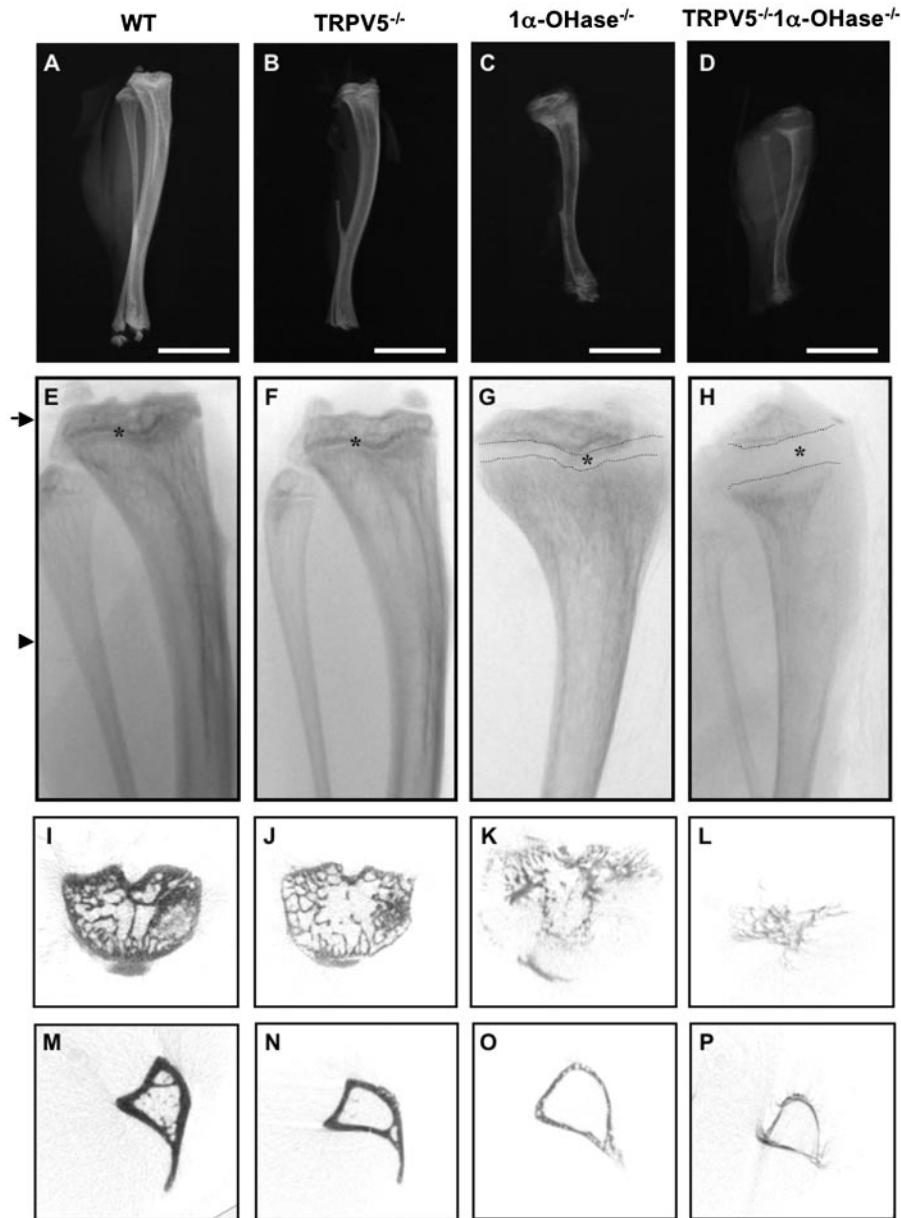
Figure 5. Bone phenotypes. Lower legs from 8-wk-old wild-type, TRPV5$^{-/-}$, 1α-OHase$^{-/-}$, and TRPV5$^{-/-}$1α-OHase$^{-/-}$ mice were analyzed using x-ray imaging (A through D). Microcomputed tomography was used to generate tibial overviews (E through H), from which digital cross-sections were 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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