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Journal of Cellular Biochemistry 108:1175–1183 (2009)

Journal of Cellular Biochemistry

Compensatory Induction of the TRPV6 Channel in a Calbindin-D9k Knockout Mouse: Its Regulation by 1,25-Hydroxyvitamin D_3

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ABSTRACT

Active calcium transport is carried out by calcium channel proteins, cytosolic buffering or transfer proteins, and pump proteins. Several components of this transport system have recently been verified using gene knockout (KO) models. We previously generated *calbindin-D9k* (*CaBP-9k*) KO mice and reported that induction of expression of some calcium transport proteins can compensate for the *CaBP-9k* gene deficiency. In the current study, we have further clarified the compensatory regulation of calcium transport genes by two calcium regulating hormones, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and parathyroid hormone (PTH), in *CaBP-9k* KO mice, because the levels of these hormones differ between the KO and wild-type (WT) mice. The induction of *transient receptor potential cation channel, subfamily V, member 6* (*TRPV6*) in the duodenum was observed in adult KO male mice but induction was not modified by physiologic doses of $1,25(OH)_2D_3$. Duodenal *TRPV6* transcription in WT and female KO mice were modulated by $1,25(OH)_2D_3$ in a dose-dependent manner. This compensatory gene induction was not detected in the mice fed a vitamin D_3-deficient diet. Compensatory gene induction was not affected by PTH. Thus, the compensatory expression of duodenal *TRPV6* in the KO male mice may be tightly correlated with serum $1,25(OH)_2D_3$. *Vitamin D receptor* (*VDR*) transcription and protein levels were measured to examine whether *VDR* expression mediates differential regulation of duodenal *TRPV6* between WT and KO mice, but expression and levels of VDR were similar in both genotypes. The compensatory *TRPV6* transcripts in KO mice may be modulated by endogenous vitamin D_3 via other factors of VDR signaling complexes. J. Cell. Biochem. 108: 1175–1183, 2009.

KEY WORDS: TRPV6; CALBINDIN-D9k; ACTIVE CALCIUM TRANSPORT SYSTEM; KNOCKOUT MICE

In the duodenum and kidney, active calcium transport proteins play critical roles in calcium influx, transfer through the cytosol, and extrusion into the blood-stream. This trans-cellular calcium transport is facilitated by three types of proteins, calcium entry channels in the apical plasma membrane, cytosolic buffering or transfer proteins, and pump proteins in the basolateral plasma membrane [Hoenderop et al., 2002; Van Cromphaut et al., 2003; Diepens et al., 2004; Choi and Jeung, 2008]. Two highly selective calcium channels on the apical plasma membrane, the transient receptor potential cation channel subfamily V members 6 and 5 (TRPV6 and TRPV5), are the main calcium entry channels. Two calbindins, calbindin-D9k (CaBP-9k) and -D28k (CaBP-28k) are intracellular calcium-ion-binding proteins which participate in shuttling calcium ions from the apical to the basolateral membrane, where the Na^+/Ca^{2+} exchanger (NCX1) and plasma membrane Ca^{2+}-ATPase 1b (PMCA1b) are responsible for Ca^{2+} extrusion.

The proteins involved in trans-cellular calcium transport can vary depending on the tissues or organs. In the duodenum, epithelial calcium entry is mediated primarily by TRPV6 and is highly dependent on the intracellular free calcium concentration. CaBP-9k is the major intracellular calcium-binding protein in mammalian intestinal cells. The final extrusion of calcium across the plasma membrane requires an ATP-driven mechanism mediated by PMCA1b. In the kidney, trans-cellular calcium ion transport is carried out by cells lining the distal part of the nephron. Calcium entry is facilitated by apical TRPV5 channels. Subsequently, calcium binds to CaBP-9k or CaBP-28k and diffuses through the cytosol to the basolateral membrane where the calcium ions are pumped out of

Grant sponsor: BioGreen 21 program, Rural Development Administration; Grant number: 20070401034011.

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Received 6 February 2009; Accepted 12 August 2009 • DOI 10.1002/jcb.22347 • © 2009 Wiley-Liss, Inc.
Published online 23 September 2009 in Wiley InterScience (www.interscience.wiley.com).

the cell by NCX1 and PMCA1b [Hoenderop et al., 2002; Van Cromphaut et al., 2003; Diepens et al., 2004; Choi and Jeung, 2008]. A rapid hormonal stimulation of intestinal calcium tranport (transcaltachia) is another model for calcium transporting [Nemere et al., 1991]. The transcellular calcium transport is processed by the uptake of calcium ions into endocytic vesicles, fusion of these vesicles with lysosomes, and transfer of organellar calcium ions across the cell along microtubules for exocytosis at the basal–lateral membrane [Nemere et al., 1991].

The cytosolic calcium-binding protein CaBP-9k is expressed in the intestine, uterus, placenta, kidney, bone, and pituitary gland of mammals [Jeung et al., 1995; Reiswig et al., 1995; An et al., 2003; Lee et al., 2003; Hong et al., 2004; Yun et al., 2004; Choi et al., 2005; Jung et al., 2005; Nguyen et al., 2005]. Duodenal CaBP-9k is involved in intestinal calcium absorption and is up-regulated by the hormonal form of vitamin D and down-regulated by glucocorticoids [Roche et al., 1986; Wasserman and Fullmer, 1989; Darwish and DeLuca, 1992; Walters et al., 1999; Lee et al., 2006]. Renal CaBP-9k proteins expressed in the distal convoluted tubules facilitate calcium re-absorption [Lee et al., 2006]. Based on these observations, CaBP-9k appears to be a key factor in active calcium transport in the duodenum and kidney. Thus, we generated *CaBP-9k* null mice to elucidate the function of CaBP-9k. However, the mice showed no distinct phenotypes or abnormalities [Lee et al., 2007]. These knockout (KO) mice exhibited normal calcium absorption from the duodenum and normal calcium excretion into the urine. Therefore, a compensatory mechanism is activated in the *CaBP-9k* KO mice, suggesting that the expression of *TRPV6* gene in the duodenum and kidney is induced by the calcium restriction diet after wearing, and the duodenal *PMCA1b* gene is highly expressed following *CaBP-9k* depletion before the wearing period in order to compensate for loss of this protein in *CaBP-9k* null mice.

In the present study, we have examined the mechanism of compensatory *TRPV6* gene induction by the hormones 1,25-hydroxyvitamin D_3 ($1,25(OH)_2D_3$) and parathyroid hormone (PTH) in the matured *CaBP-9k* KO mice. The effect of these hormones on the calcium transport genes in the duodenum and kidney in these KO mice was tested. In addition, the levels of hormone receptors for PTH (PTHR) and $1,25(OH)_2D_3$ (vitamin D receptor; VDR) were measured and the effects of a vitamin D_3-deficient diet were examined.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS AND TREATMENTS

Wild-type (WT, C57BL/6) and *CaBP-9k* KO mice (9–10-week-old) were housed in polycarbonate cages and used after acclimation to environmentally controlled conditions (temperature, $23 \pm 2^{\circ}C$; relative humidity, $50 \pm 10\%$; frequent ventilation and 12 h light–dark cycle) [Lee et al., 2007].

To examine the effect of hormones ($1,25(OH)_2D_3$ and PTH) on the expression of duodenal and renal calcium-processing genes in WT and KO mice, the mice were divided into vehicle (ethanol) or treatment groups ($n = 5$ per each group, for each strain and gender). The mice were subcutaneously injected with $1,25(OH)_2D_3$ (1 μg/kg; Sigma–Aldrich, St. Louis, MO) or PTH (100 μg/kg, Sigma–Aldrich) daily for 3 days, according to previous studies [Brown et al., 1995; Song et al., 2003; Kim et al., 2005; Wang et al., 2006], or with ethanol as a negative control daily for 3 days, and then euthanized 24 h after the final injection. To examine the dose-dependency, mice of both genders and genotypes ($n = 5$ for each group) were treated with three dosages (0.1, 1, and 10 μg/kg) of $1,25(OH)_2D_3$ for 3 days and euthanized 24 h after final injection. For experiments comparing diets containing or deficient in vitamin D_3, WT, and KO male mice were fed with a normal diet (DYET #113295, AIN-76A purified rodent diet containing 0.8% phosphorus, 1.1% calcium, and 1 IU/g vitamin D_3, Dyets Inc., Bethlehem, PA), or vitamin D-deficient diet (D10373A, AIN-76A-based diets containing 0.8% strontium, 0.02% calcium, and 0.35% phosphorus, Research Diets, Inc., Brunswick, NJ) for 7 days [Song et al., 2003]. The contained strontium inhibits endogenous vitamin D_3 synthesis as previously described [Moon, 1994]. Some groups of mice were additionally administered 1 μg/kg of $1,25(OH)_2D_3$ for 3 days and euthanized 24 h after the final injection. All experimental procedures and animal use were approved by the Ethics Committee of the Chungbuk National University.

RNA EXTRACTION AND REAL-TIME PCR

Following euthanasia of the mice, the duodenum and kidney were rapidly excised and washed in cold sterile saline (0.9% NaCl). Total RNA was prepared using the Trizol reagent (Invitrogen, Carlsbad, CA) and the concentration of RNA was determined by the absorbance at 260 nm. Reverse transcription was performed as described previously [Lee et al., 2007]. Briefly, total RNA (1 μg) was reverse-transcribed to first-stand cDNA using the mMLV reverse transcriptase (Invitrogen) and random primers according to the manufacturer's protocol.

Real-time PCR was performed in 20 μl reactions containing 10 μl of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster, CA), 1 μl of 20× Assays-on-Demand™ Gene Expression Assay Mix (Applied Biosystems; *TRPV6*, Mm00499069_m1; *CaBP-9k*, Mm00486654_m1; *CaBP-28k*, Mm00486645_m1; *TRPV5*, Mm00486645_m1; *PMCA1b*, Mm00670208_m1; *NCX1*, Mm01232248_m1; *VDR*, Mm00437297_m1; *PTHR*, Mm00441046_m1; and *HPRT1*, Mm00446968_m1) and 5 μl of cDNA. PCR amplification was conducted using a 7300 Real-Time PCR System (Applied Biosystems), with an initial denaturation at 50°C for 2 min, followed by 90°C for 10 min. Each of the 40 amplification cycles consisted of denaturation at 95°C for 15 s, followed by annealing and extension at 60°C for 1 min. Relative expression levels for each sample were determined using the RQ software (Applied Biosystems). The expression of *TRPV6*, *TRPV5*, *CaBP-9k*, *CaBP-28k*, *NCX1*, *VDR*, *PTHR*, and *PMCA1b* were normalized relative to that of *HPRT1*.

WESTERN BLOT ANALYSIS

Protein was extracted using Proprep (iNtRON Bio. Inc., Sungnam, Kyungki-Do, Korea) according to the supplier's instructions. Total protein (50 μg per lane) was resolved using 10% SDS–PAGE and transferred to nitrocellulose membranes using a Trans-Blot Cell (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The membranes were blocked overnight with PBS containing 0.05% Tween-20 (PBS-T) and 5% skim milk. The membranes were then incubated with primary antibodies diluted in 1% BSA for 1 h at room temperature.

Primary antibodies to VDR (diluted 1:500) and β-actin (1:1,000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A horseradish peroxidase-conjugated anti-rabbit IgG (diluted 1:3,000 in 1% BSA, Santa Cruz Biotechnology) was used as the secondary antibody. The membranes were incubated with the Western Lighting Chemiluminescence reagent (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer's protocol. The signals were detected with Chemi Doc EQ (Bio-Rad) and analyzed using the Quantity One program.

DATA ANALYSIS

Data were analyzed by nonparametric one-way analysis-of-variance using the Kruskal–Wallis test, followed by Dunnett's test for multiple comparisons to vehicle. Data values were converted to ranks for these tests. All statistical analyses were performed with SPSS for Windows (SPSS Inco, Chicago, IL). $P < 0.05$ was considered statistically significant.

RESULTS

EFFECT OF 1,25(OH)$_2$D$_3$ ON THE EXPRESSION OF DUODENAL AND RENAL CALCIUM TRANSPORT GENES

The hormone 1,25(OH)$_2$D$_3$ plays a major role in calcium homeostasis. This hormone regulates calcium absorption in the intestine and kidney by altering gene transcription. Our previous study suggested that expression of several calcium transport genes is modified by *CaBP-9k* deficiency in the duodenum and kidney. Based on these previous results, we hypothesized that serum 1,25(OH)$_2$D$_3$ may mediate the compensatory regulation of calcium transport genes in the KO mice. In the present study, duodenal and renal induction of several calcium transport genes was observed after 1,25(OH)$_2$D$_3$ administration to *CaBP-9k* KO mice. In the duodenum, *TRPV6* mRNA was induced by 1,25(OH)$_2$D$_3$ in WT mice of both genders and in female KO mice; however, no difference in gene expression was observed in male KO mice (Fig. 1A). The *TRPV6* gene was up-regulated in vehicle-treated male KO mice relative to vehicle-treated WT mice, implying that expression of the duodenal *TRPV6* gene in male KO mice was induced by the *CaBP-9k* deficiency, and that additional 1,25(OH)$_2$D$_3$ could not further induce transcription of the gene. In WT mice, the duodenal *CaBP-9k* gene was significantly induced by 1,25(OH)$_2$D$_3$ treatment, with similar levels of expression in both males and females (Fig. 1A). Duodenal expression of *PMCA1b* and *NCX1* was not altered by 1,25(OH)$_2$D$_3$ treatment.

In the kidney, *TRPV6* expression was not altered by 1,25(OH)$_2$D$_3$. However, gender-dependent differential gene expression was detected (Fig. 1B), with female *TRPV6* mRNA levels significantly

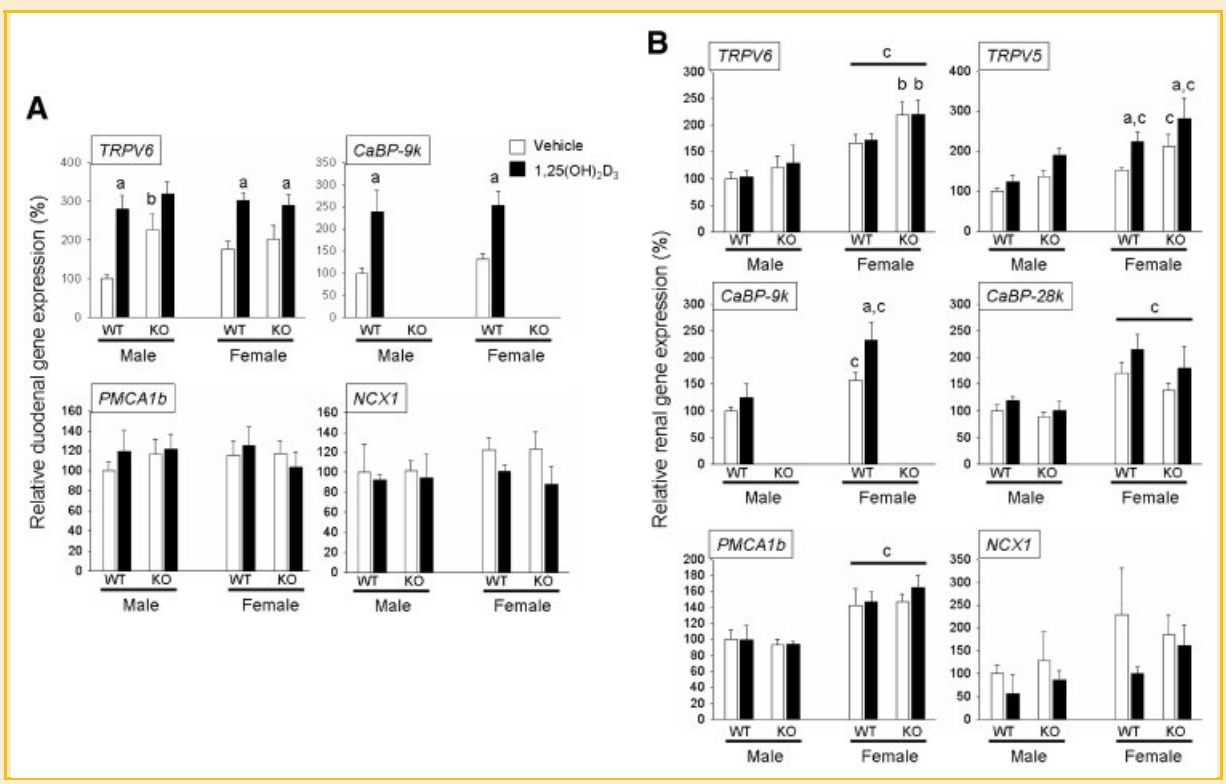

Fig. 1. Effect of 1,25(OH)$_2$D$_3$ on the expression of duodenal and renal calcium transport genes in male and female *CaBP-9k* KO mice. Both WT and KO mice (9–10-week-old, n = 5 per group) were euthanized 24 h after a 3-day treatment with 1 μg/kg of 1,25(OH)$_2$D$_3$, as described in the Materials and Methods Section. The relative duodenal (A) and renal (B) expressions of calcium transport genes were analyzed by real-time PCR. Gene expression was normalized using *HPRT1* as an internal control. The percentage of gene expression in WT male mice treated with ethanol (as the vehicle control) was set to 100%. Each value represents duplicate experimental analyses (means ± SD of all samples). [a]$P < 0.05$ versus the vehicle-treated mice of the same genotype and gender; [b]$P < 0.05$ versus WT mice of the same gender and hormone-treated mice; [c]$P < 0.05$ versus the same genotype and hormone-treated male mice.

higher than male, and compensatory induction of the *TRPV6* gene in the female KO mice. Expression of the renal *TRPV5* gene was induced by $1,25(OH)_2D_3$ treatment in female mice and *TRPV5* expression was significantly increased in both WT and KO female mice relative to male WT and KO mice, respectively. Renal *CaBP-9k* was up-regulated by $1,25(OH)_2D_3$ administration in female WT mice, but not in WT males. Renal expression of *CaBP-28k* and *PMCA1b* was higher in female mice than in male mice, but no differences in expression were observed following $1,25(OH)_2D_3$ treatment. The renal expression of *NCX1* gene was very variable between genders and treatments. In parallel with duodenal *PMCA1b*, *NCX1* level was not affected by $1,25(OH)_2D_3$ treatment. Thus, both compensatory and $1,25(OH)_2D_3$-mediated gene expression were detected in the renal active calcium transport genes.

EFFECT OF PTH ON THE DUODENAL AND RENAL EXPRESSION OF CALCIUM TRANSPORT GENES

PTH, one of the regulators of calcium transport genes, may be a mediator of compensatory expression of several active calcium transporting genes in the *CaBP-9k* null mice. Thus, in the current study, we examined whether PTH could regulate expression of calcium transport genes in the duodenum or kidney in WT and KO mice. In the duodenum, compensatory induction of the *TRPV6* gene by CaBP-9k deficiency in KO male mice was observed, but PTH treatment did not alter *TRPV6* gene expression (Fig. 2A). Duodenal expression of the *CaBP-9k*, *PMCA1b*, and *NCX1* genes was not altered by PTH administration in either KO or WT mice (Fig. 2A).

In contrast, renal *TRPV6* mRNA was induced in the both female WT and KO mice relative to male mice, and compensatory up-regulation was observed in female KO mice (Fig. 2B). However, PTH injection did not change renal *TRPV6* transcription in any treatment group. Similarly, renal *TRPV5* mRNA was up-regulated in the female KO mice, but no difference in its expression was observed by the hormone treatment. Renal *CaBP-9k* and *CaBP-28k* expression was not altered by PTH, but gender-specific differences in expression level were detected (Fig. 2B). No difference in renal *PMCA1b* or *NCX1* expression was detected between genotypes, but *PMCA1b* expression was significantly higher in female mice than in males.

EFFECT OF $1,25(OH)_2D_3$ AND PTH ON EXPRESSION OF THEIR COGNATE RECEPTORS

We further clarified whether the compensatory gene expressions are related to levels of the two hormone receptors, VDR and PTHR. No differences in duodenal *VDR* and *PTHR* mRNAs, or in renal *PTHR* transcripts, were observed among the genotypes and genders (Fig. 3). In addition, no difference in the protein levels of *VDR* between WT and KO male mice was detected (Fig. 4C). Expression of

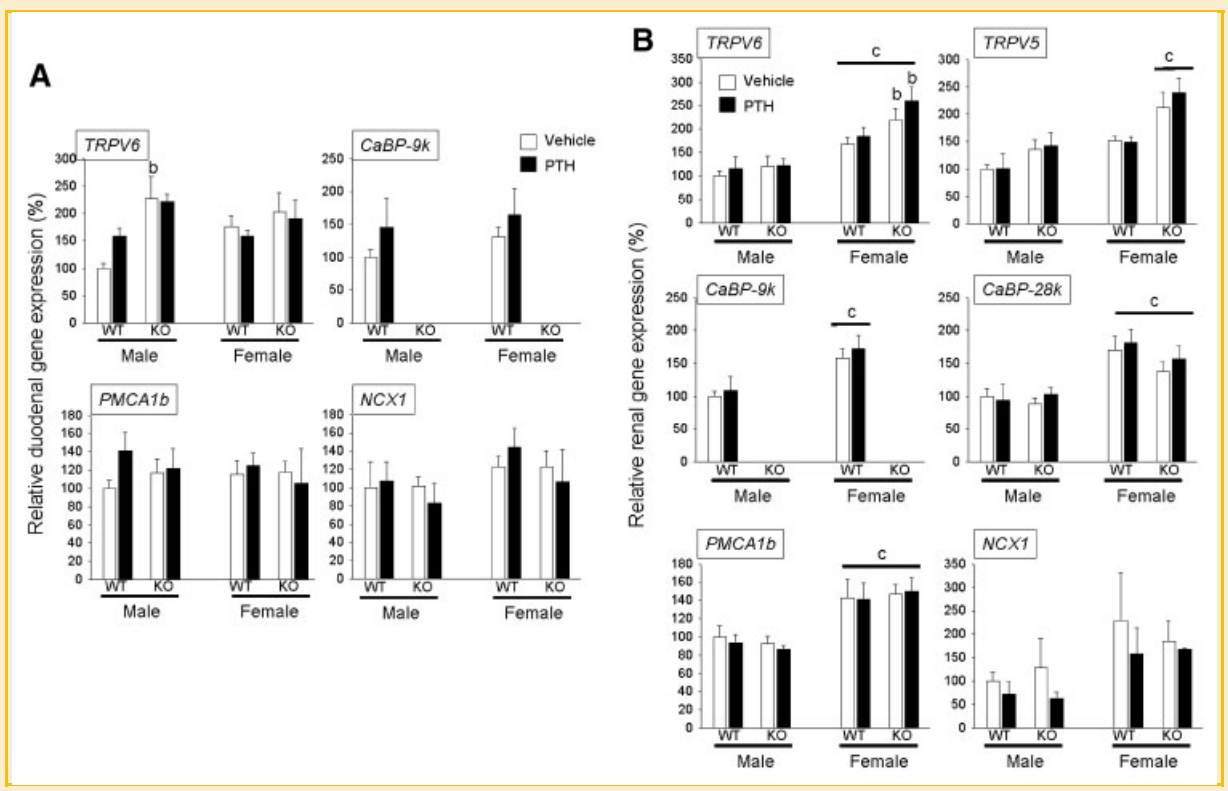

Fig. 2. Effect of PTH on the expression of duodenal and renal calcium transport genes in male and female *CaBP-9k* KO mice. Both WT and KO mice (9–10-week-old, n = 5 per group) were euthanized 24 h after a 3-day treatment with 100 μg/kg of PTH, as described in the Materials and Methods Section. The relative duodenal (A) and renal (B) expressions of calcium transport genes were analyzed by real-time PCR. Gene expression was normalized using *HPRT1* as an internal control. The percentage of gene expression in WT male mice treated with ethanol (as the vehicle control) was set to 100%. Each value represents duplicate experimental analyses (means ± SD of all samples). $^bP < 0.05$ versus WT mice of the same gender and hormone-treated mice; $^cP < 0.05$ versus the same genotype and hormone-treated male mice.

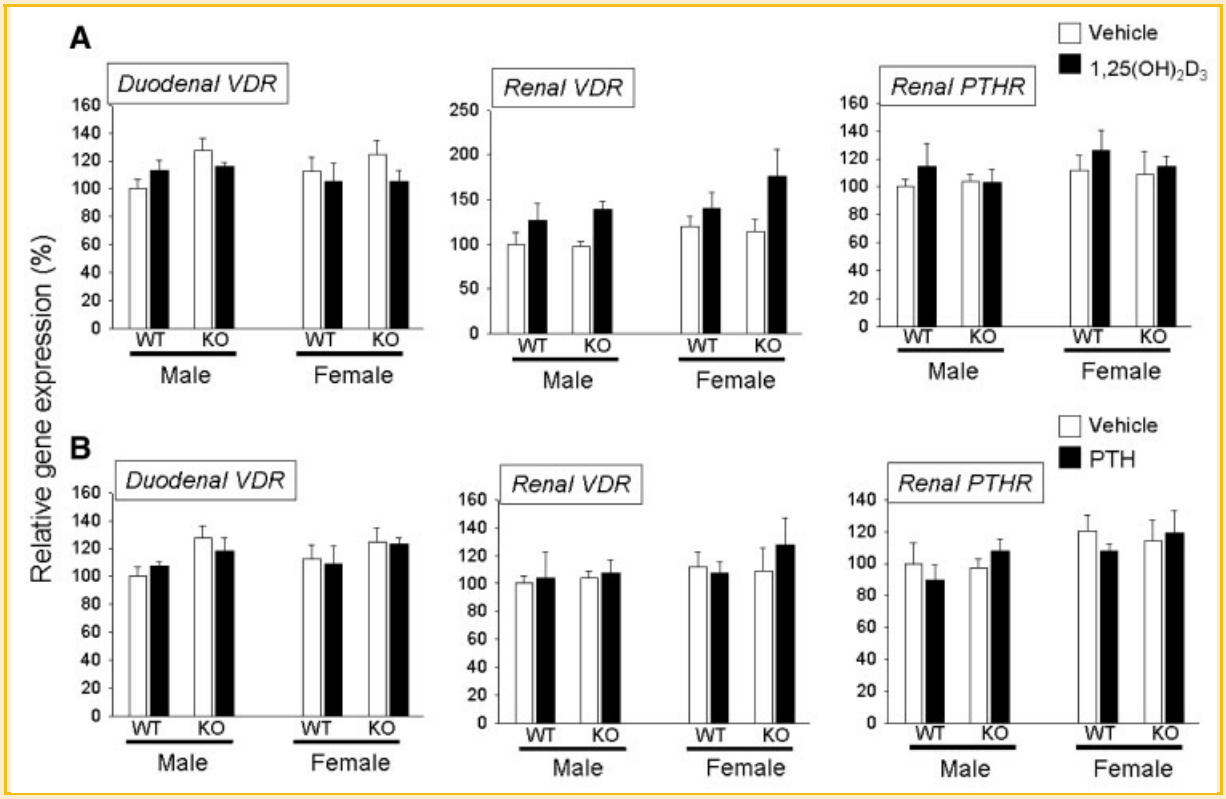

Fig. 3. Effect of $1,25(OH)_2D_3$ and PTH on the expression of duodenal and renal *VDR* and *PTHR* in male and female *CaBP-9k* KO mice. Both WT and KO mice (9–10-week-old, n = 5 per group) were euthanized 24 h after a 3-day treatment with 1 μg/kg of $1,25(OH)_2D_3$ (A) or 100 μg/kg of PTH (B), as described in the Materials and Methods Section. The relative duodenal and renal expression of *VDR* and *PTHR* was analyzed by real-time PCR. Gene expression was normalized using *HPRT1* as an internal control. The percentage of gene expression in WT male mice treated with ethanol (as the vehicle control) was set to 100%. Each value represents duplicate experimental analyses (means ± SD of all samples).

the duodenal *VDR* and renal *PTHR* genes was not affected by $1,25(OH)_2D_3$ treatment in any of the mice. Renal *VDR* transcription in KO mice was induced by $1,25(OH)_2D_3$ treatment (Fig. 3A), but PTH did not alter transcription of duodenal or renal *VDR*, or renal *PTHR* (Fig. 3B).

EFFECT OF INCREASING LEVELS OF $1,25(OH)_2D_3$ ON DUODENAL EXPRESSION OF TRPV6

In Figure 1A, duodenal *TRPV6* expression in male and female WT mice and female KO mice was induced by $1,25(OH)_2D_3$, but gene expression in male KO mice was not altered. The duodenal expression of *TRPV6* in male KO mice may have been induced as a compensatory mechanism and its mRNA levels were similar to that of the $1,25(OH)_2D_3$-treated groups. To precisely elucidate the effects of $1,25(OH)_2D_3$ on the duodenal levels of *TRPV6* mRNA in male KO mice, the mice were treated with increasing doses of $1,25(OH)_2D_3$. A low dosage (0.1 μg/kg) did not induce expression of duodenal *TRPV6* in the mice. An intermediate dosage (1 μg/kg) induced an increase in duodenal *TRPV6* expression in WT male and female mice and KO female mice, but did not alter expression levels in male KO mice, consistent with our earlier data. A high dosage (10 μg/kg) of $1,25(OH)_2D_3$ significantly increased gene expression in all groups (Fig. 4A). Taken together, these results indicate that the compensatory induction of *TRPV6* transcription may be mediated by endogenous vitamin D_3 in KO male mice.

EFFECT OF $1,25(OH)_2D_3$ ON DUODENAL EXPRESSION OF TRPV6 IN WT AND KO MALE MICE FED A VITAMIN D-DEFICIENT DIET

To eliminate endogenous vitamin D, WT and KO male mice were fed with a vitamin D_3-deficient diet containing strontium to inhibit endogenous vitamin D_3 synthesis [Moon, 1994; Song et al., 2003]. As seen in Figure 4B, the levels of duodenal *TRPV6* mRNA expression (about 40%) in both mice fed with vitamin D_3-deficient diet were significantly reduced when compared to those (set as 100%) of the WT male mice fed with normal diet. Interestingly, compensatory induction of the duodenal *TRPV6* gene was not observed in mice fed with a vitamin D_3-deficient diet. When WT and KO mice fed the vitamin D_3-deficient diet were subsequently treated with $1,25(OH)_2D_3$ (1 μg/kg) for 3 days, duodenal expression of *TRPV6* was induced (Fig. 4B). Thus, the compensatory expression of *TRPV6* in KO male mice was absent in mice fed a vitamin D_3-deficient diet.

In addition, the duodenal level of VDR was compared in male WT and KO mice fed a normal diet (Fig. 4C). Consistent with *VDR* transcript levels, VDR protein levels were not affected by gene

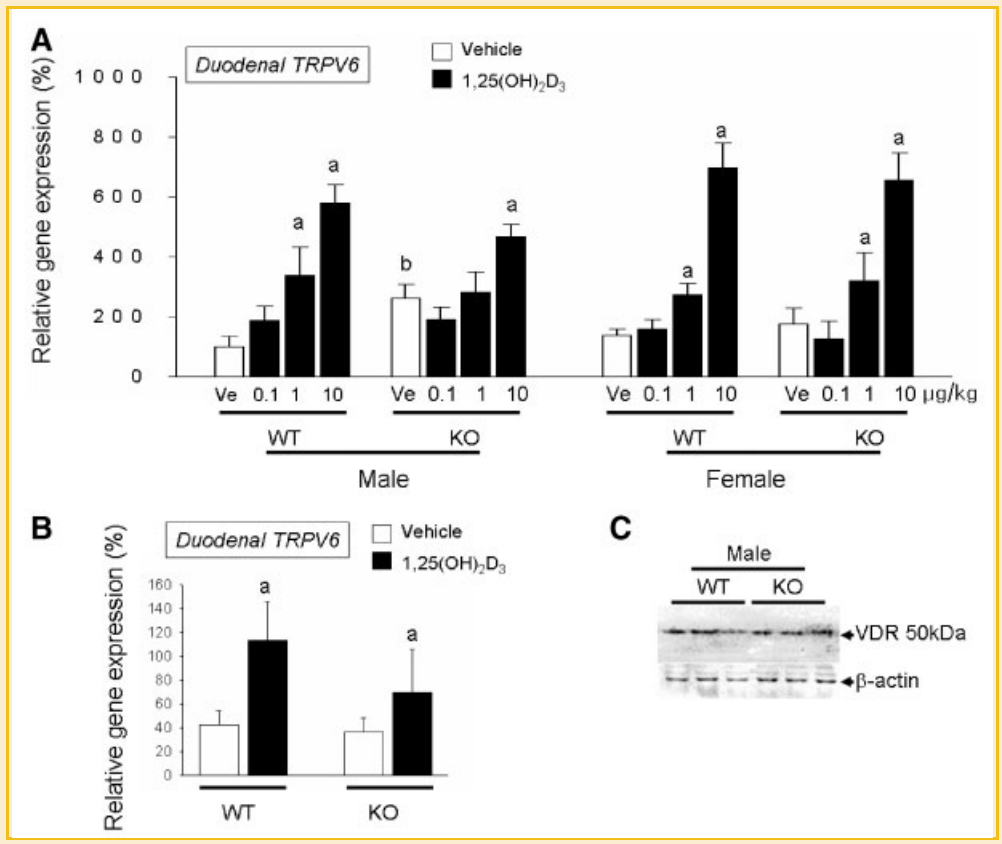

Fig. 4. Effect of $1,25(OH)_2D_3$ dosage or a vitamin D_3-deficient diet on the expression of duodenal *TRPV6*. A: WT and KO mice of both genders (9–10-week-old, n = 5 per group) were euthanized 24 h after a 3-day treatment with one of three doses (0.1, 1, and 10 μg/kg) of $1,25(OH)_2D_3$, as described in the Materials and Methods Section. B: WT and KO male mice (9–10-week-old, n = 5 per group) were fed a vitamin D_3-deficient diet for 7 days and treated with $1,25(OH)_2D_3$ (1 μg/kg) for 3 days, as described in the Materials and Methods Section. The relative duodenal and renal expression of *TRPV6* was analyzed by real-time PCR. *TRVP6* transcription was normalized using *HPRT1* as an internal control. The percentage of gene expression in WT male mice treated with ethanol (as the vehicle control, panel A) or the mice fed with the normal diet (panel B) was set to 100%. Each value represents duplicate experimental analyses (means ± SD of all samples). C: The effect of the absence of CaBP-9k on duodenal protein levels of VDR and β-actin (an internal control) by Western blot assay, as described in the Materials and Methods Section. [a] $P < 0.05$ versus vehicle-treated mice of the same genotype and gender. [b] $P < 0.05$ versus WT mice of the same gender and hormone-treated mice.

depletion, suggesting that other factors may govern the compensatory induction of *TRPV6* in the duodenum of *CaBP-9k* KO mice.

DISCUSSION

Active calcium transport has been studied for several decades in an effort to understand its role in calcium-related disorders, such as hypocalcaemia, rickets, and osteomalacia, using VDR null mice and 1α-hydoxylase deficient mice [Van Cromphaut et al., 2001; Hoenderop et al., 2002; Zheng et al., 2004]. Active calcium transport is hypothesized to be facilitated by several proteins, including TRPV5/6, CaBP-9k/28k, PMCA1b, and NCX1. The roles of these proteins have been recently verified using gene KO animals. Recently, two distinct strains of *CaBP-9k* KO mice were generated by Kutuzova et al. [2006] and by our laboratory [Lee et al., 2007]. These two *CaBP-9k* null mice appeared normal in viability, reproduction, and calcium homeostasis. We further investigated the phenotype of the *CaBP-9k* KO mice and found that some calcium processing genes were induced to compensate for depletion of CaBP-9k [Lee et al., 2007].

In the current study, the mechanism of compensatory gene expression in *CaBP-9k* KO mice was studied using the calcium homeostasis hormones $1,25(OH)_2D_3$ and PTH. Several calcium transport genes are tightly regulated by $1,25(OH)_2D_3$, and PTH may also be directly or indirectly involved in regulating active calcium transport genes [Pausova et al., 1994; Van Cromphaut et al., 2001; Cao et al., 2002; Akhter et al., 2007; Christakos et al., 2007]. Previously, these two hormones were examined in mice fed low-, normal-, or high-calcium diets [Lee et al., 2007]. The hormone concentrations in the mice fed low- and normal-calcium diets were significantly different between WT and *CaBP-9k* KO mice. Serum PTH levels in WT mice were much higher than those of KO mice and serum $1,25(OH)_2D_3$ in male WT and female KO mice was lower than in female WT and male KO mice. Based on these data, we focused on the effects of $1,25(OH)_2D_3$ and PTH in the regulation of calcium transport genes in *CaBP-9k* KO mice.

To examine the role of $1,25(OH)_2D_3$ in duodenal *TRPV6* expression in the male KO mice, WT and KO mice were fed a vitamin D-deficient diet for 1 week and some animals were then injected with physiologic doses of $1,25(OH)_2D_3$ daily for 3 days. The

vitamin D_3-deficient diet completely blocked the compensatory *TRPV6* induction in the duodenum of male KO mice. The injection of $1,25(OH)_2D_3$ in the KO mice induced the duodenal expression *TRPV6*, although the level of induction in the male KO mice was slightly less than that in WT mice injected with $1,25(OH)_2D_3$. We further tested whether differential VDR expression may play a role in the compensatory *TRPV6* transcription. Duodenal VDR transcript levels were similar in all mice and VDR protein levels were not different between WT and KO male mice, suggesting that other factors in the VDR signaling pathway may be involved in the insensitivity to $1,25(OH)_2D_3$ and compensatory gene induction.

The gene expression is modulated by $1,25(OH)_2D_3$ through a heterodimer between VDR and the retinoid X receptor (RXR) [Nagpal et al., 2005]. In the absence of ligand, most of the VDR is present in the cytoplasm [Barsony et al., 1990]; the presence of the ligand induces RXR–VDR heterodimerization and translocation into the nucleus [Cheskis and Freedman, 1994; Prufer et al., 2000]. The RXR–VDR heterodimer binds to vitamin D_3 response elements (VDREs) present in the promoter regions of responsive genes. VDR can positively regulate the expression of certain genes by binding to VDREs in the promoter region [Pinette et al., 2003; Sutton and MacDonald, 2003], negatively regulate the expression of genes by binding to negative VDREs [Liu et al., 1996; Dong et al., 2003], or inhibit the expression of genes by antagonizing the action of other transcription factors [Alroy et al., 1995; Harant et al., 1997; Takeuchi et al., 1998]. Vitamin D-dependent transcription requires the binding of ligand-occupied RXR–VDR heterodimers to VDREs present in the upstream regions of responsive genes. Ligand binding increases the affinity of VDR for various cofactor proteins that act as a bridge between the RXR–VDR heterodimer and the basal Pol II transcription machinery. Using genetic and biochemical approaches, a number of cofactors that interact with VDR and other nuclear receptors in a ligand-dependent manner have been identified. These cofactors do not show any DNA binding activity but possess the ability to modulate gene expression in transfected cells. Although the mechanism of VDR-mediated gene expression may appear simple, this modulation can become quite complex when several cofactors are participating. In the present study, the induction of *TRPV6* in the duodenum of the KO male mice may be affected by a VDR cofactor or other ortphan receptors. In male KO animals fed a normal diet, duodenal *TRPV6* transcription was up-regulated, potentially to provide normal calcium homeostasis in the absence of CaBP-9k. This compensatory induction of duodenal *TRPV6* in the KO male mice was not affected by physiologic doses of $1,25(OH)_2D_3$. The gene induction did not occur in KO male mice fed a vitamin D_3-deficient diet. Thus, the compensatory gene expression was observed in the presence of normal circulating levels of $1,25(OH)_2D_3$. Although differential expression of VDR cofactors and/or other ophan receptors were not examined in this study, the compensatory *TRPV6* transcription is consistent with the hypothesis that another cofactor in a liganded VDR signaling pathway may be involved in modulating gene expression in this system.

PTH is secreted by the parathyroid gland and acts principally to regulate calcium and phosphate metabolism by binding to its receptors (PTHR), which are expressed in kidney and bone [Abou-Samra et al., 1992; Pausova et al., 1994]. The PTHR is widely expressed in the osteoblast lineage of bone, and in the glomerulus and at several sites along the nephron in the kidney [Rouleau et al., 1988; McCuaig et al., 1995]. PTH regulates the conversion of 25-hydroxyvitamin D to the active metabolite $1,25(OH)_2D_3$. PTH also activates dihydropyridine-sensitive channels that mediate calcium entry [Bacskai and Friedman, 1990]. In addition, some evidence suggests that microtubule-dependent exocytosis, stimulated by PTH, is required for the activation of calcium channels and calcium influx. In the current study, we investigated whether differential regulation of calcium transport genes is apparent between WT and KO mice. Although no significant difference in the induction of these genes was observed by PTH treatment, we cannot exclude the possibility that other factors may be involved in compensatory gene induction.

In summary, we have further elucidated potential mediators of compensatory gene induction in *CaBP-9k* KO male mice. Previously generated *CaBP-9k* null mice showed normal calcium homeostasis and phenotypes, suggesting that some genes may be up-regulated to compensate for this gene deficiency. Compensatory gene induction of duodenal *TRPV6* was observed in adult KO male mice, however we were unable to determine the protein levels of duodenal TRPV6 using several commercial antibodies in Western blot assays. This compensatory *TRPV6* transcription in male KO mice is not altered by physiologic doses of $1,25(OH)_2D_3$, but *TRPV6* gene expression in other animals was up-regulated by $1,25(OH)_2D_3$. In addition, the compensatory gene induction was not affected by PTH administration, suggesting that the duodenal *TRPV6* gene in male KO mice may be insensitive to the hormonal form of vitamin D. However, the compensatory gene induction was not detected in the same mice fed a vitamin D_3-deficient diet. Thus, compensatory transcription of duodenal *TRPV6* in the male KO mice may be tightly regulated by serum $1,25(OH)_2D_3$. However, the current mRNA data is not sufficient to support this hypothesis, and further studies are required such as their protein expressions and intestinal calcium absorption. *VDR* transcription and translation were examined in WT and KO mice to determine the role of VDR in differential regulation of duodenal *TRPV6* expression, but the *VDR* mRNA and protein levels were similar in both WT and KO mice. Thus, the compensatory induction of *TRPV6* in *CaBP-9k* KO mice may be modulated by a factor linked with VDR signaling complex.

ACKNOWLEDGMENTS

This work was supported by a grant (20070401034011) from BioGreen 21 program, Rural Development Administration, Republic of Korea.

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