—Full Paper—

Dietary Calcium and 1,25-Dihydroxyvitamin D₃ Regulate Transcription of Calcium Transporter Genes in Calbindin-D9k Knockout Mice

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Abstract. The effect(s) of oral calcium and vitamin D₃ were examined on the expression of duodenal and renal active calcium transport genes, i.e., calbindin-D9k (*CaBP-9k*) and calbindin-D28k (*CaBP-28k*), transient receptor potential cation channels (*TRPV5* and *TRPV6*), Na⁺/Ca²⁺ exchanger 1 (*NCX1*) and plasma membrane calcium ATPase 1b (*PMCA1b*), in *CaBP-9k* KO mice. Wild-type (WT) and KO mice were provided with calcium and vitamin D₃-deficient diets for 10 weeks. The deficient diet significantly decreased body weights compared with the normal diet groups. The serum calcium concentration of the WT mice was decreased by the deficient diet but was unchanged in the KO mice. The deficient diet significantly increased duodenal transcription of *CaBP-9k* and *TRPV6* in the WT mice, but no alteration was observed in the KO mice. In the kidney, the deficient diet significantly increased renal transcripts of *CaBP-9k*, *TRPV6*, *PMCA1b*, *CaBP-28k* and *TRPV5* in the WT mice but did not alter calcium-relating genes in the KO mice. Two potential mediators of calcium-processing genes, vitamin D receptor (VDR) and parathyroid hormone receptor (PTHr), have been suggested to be useful for elucidating these differential regulations in the calcium-related genes of the KO mice. Expression of VDR was not significantly affected by diet or the KO mutation. Renal *PTHr* mRNA levels were reduced by the diet, and reduced expression was also seen in the KO mice given the normal diet. Taken together, these results suggest that the active calcium transporting genes in KO mice may have resistance to the deficiency diet of calcium and vitamin D₃.

Key words: Calbindin-D9k (*CaBP-9k*), Calbindin-D28k (*CaBP-28k*), Knockout mouse, Na⁺/Ca²⁺ exchanger 1 (*NCX1*), Plasma membrane calcium ATPase 1b (*PMCA1b*), *TRPV5*, *TRPV6*

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Calcium is the most abundant ion in the body and is present in all tissues and organs, where it participates in vital processes such as cardiac and skeletal muscle contraction, neurotransmission, tissue differentiation and cell metabolism [1]. Therefore, calcium homeostasis is of crucial importance for many physiological functions, including neuronal excitation, muscle contraction, blood clotting and bone mineralization [2]. The calcium balance is tightly controlled through constant regulation of three processes: intestinal calcium absorption, renal calcium reabsorption, and bone calcium exchange [3].

The intestinal and renal calcium absorption response to 1, 25-hydroxyvitamin D₃ (1,25-(OH)₂D₃) involves the following three steps [4]: 1) calcium influx through two epithelial calcium channels belonging to the transient receptor potential channel vanilloid subfamily (*TRPV5* and *TRPV6*) [5], 2) intracellular calcium transfer by calbindin-D9k (*CaBP-9k*) and calbindin-D28k (*CaBP-28k*) [6, 7], and 3) calcium extrusion against an electrochemical gradient mediated by plasma membrane calcium ATPase 1b (*PMCA1b*) and Na⁺/Ca²⁺ exchanger 1 (*NCX1*) [8, 9]. Calcium ion influx occurs

through two highly Ca²⁺-selective members of the transient receptor potential cation channel, subfamily V (*TRPV5* and *TRPV6*), which act as gatekeepers for active Ca²⁺ (re)absorption in the kidney and intestine, respectively [10, 11]. In the regulation of calcium metabolism, CaBPs are important vitamin D target genes [12, 13], and two types of CaBPs have been identified: *CaBP-9k*, which is expressed primarily in the intestine and kidney, and *CaBP-28k*, which is expressed in the kidney and neurons [14]. In many species, PMCA isoforms are involved in calcium homeostasis [15, 16]. The isoform *PMCA1b* is located in cellular membranes, where it removes calcium ions from the cytosol to the extracellular compartment using an ATP-dependent pump [17]. *NCX1* is the primary Ca²⁺ extrusion mechanism in the kidney, but it extrudes only minor amounts of Ca²⁺ from the distal tubular cells [18]. *NCX1* appears to be of only minor importance in the small intestine [18].

The calcitropic hormone 1,25-(OH)₂D₃ is an active metabolite of vitamin D that contributes to the maintenance of serum calcium homeostasis by binding to the vitamin D receptor (VDR), thereby altering transcription of target genes [19]. Although treatment with 1,25-(OH)₂D₃ results in a significant increase in *VDR* mRNA levels in parathyroid glands *in vivo* [20, 21], conflicting reports indicate

both increasing levels in the intestine [22], and no significant changes [23, 24]; the response of renal *VDR* mRNA has not been reported [25]. Parathyroid hormone (PTH) is responsible for maintaining the serum level of calcium. It acts in concert with 1,25-(OH)₂D₃ to increase bone resorption and renal reabsorption of calcium [26, 27]. As a form of negative feedback regulation, 1,25-(OH)₂D₃ decreases PTH levels by negative regulation of the *PTH* gene [28].

In previous studies, two types of *CaBP-9k* knockout (KO) mice have been generated presenting with normal phenotypes [29, 30]. These reports show that KO mice have normal calcium homeostasis supported by compensatory mechanisms with normal and calcium deficient diets. Thus, the role of *CaBP-9k* has been extensively investigated for several decades, but its exact function remains to be unclear. In the present study, we fed calcium and vitamin D₃-deficient diets to adult mice for 10 weeks to maximally induce the active calcium transport systems of the duodenum and kidney and examined the expression of related genes to understand the role of *CaBP-9k* in this animal model. Moreover, we investigated the expression patterns of *VDR* and *PTH-receptor* (*PTHr*) to address the regulatory transcriptional mechanisms of calcium processing genes.

Materials and Methods

Experimental animals and treatments

The generation and characterization of *CaBP-9k* KO mice has been reported previously [30]. Male *CaBP-9k* KO and wild-type (WT, C57BL/6) mice were produced through breeding of heterozygous mice and were identified by PCR analysis of tail genomic DNA. All male mice (total n=20, n=5 per group) were housed in polycarbonate cages and acclimatized in an environmentally-controlled room (temperature of 23 ± 2 °C, relative humidity of 50 ± 10%, frequent ventilation) prior to experimentation. For experiments comparing the vitamin D₃- and calcium-deficient diets, KO and WT mice were fed a normal diet (DYET #113295; AIN-76A purified rodent diet containing 0.8% phosphorus and 1.1% calcium; Dyets, Bethlehem, PA, USA) or a low-calcium and vitamin D deficiency diet (D10373A; AIN-76A-based diets containing 0.8% strontium, 0.02% calcium and 0.35% phosphorus; Research Diets, Brunswick, NJ, USA) [31]. All fluorescent lighting was shielded, preventing the endogenous production of vitamin D₃. The mice were fed the experimental diets from 8 to 18 weeks of age. All experimental procedures were approved by the Ethics Committee of Chungbuk National University.

Serum collection and biochemical analysis

Blood was collected and centrifuged to obtain serum. Serum calcium levels were determined using ASAN Ca-Lq Reagents (Asanpharm, Seoul, Korea), according to the manufacturer's protocol.

Real-time PCR using TaqMan™

Total RNA was prepared from duodenal and kidney tissues using TRIzol Reagents (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. RNA was treated with

Ribonuclease Inhibitor (TaKaRa Bio, Ohtsu, Shiga, Japan), and then cDNA was generated by reverse transcription of 1 µg RNA using M-MLV Reverse Transcriptase (Invitrogen), as described previously [32]. Real-time PCR reactions (20 µl) contained 10 µl TaqMan™ Universal PCR Master Mix (Applied Biosystems), 1 µl 20 X Assays-on-Demand™ Gene Expression Assay Mix containing gene specific forward and reverse primers, and TaqMan probe (Applied Biosystems; gene specific IDs of the manufacture: *TRPV6*, Mm00499069_m1; *CaBP-9k*, Mm00486654_m1; *CaBP-28k*, Mm00486654_m1; *TRPV5*, Mm01166029_m1; *PMCA1b*, Mm00670208_m1; *NCX1*, Mm01232248_m1; *VDR*, Mm00437297_m1; *PTHr*, Mm00441046_m1; and *HPRT1*, Mm00446968_m1) and 2 µl 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 and then 40 amplification cycles comprising denaturation at 95 °C for 15 s followed by annealing and extension at 60 °C for 1 min. Relative expression levels were determined using the RQ software (Applied Biosystems). The expressions of *TRPV6*, *TRPV5*, *CaBP-9k*, *CaBP-28k*, *NCX1*, *PMCA1b*, *VDR* and *PTHr* were normalized relative to that of *HPRT1*. All real-time PCR experiments were repeated two or three times.

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. Data were converted to ranks, and all statistical analyses were performed using SPSS for Windows (SPSS, Chicago, IL, USA). P<0.05 was considered to be statistically significant.

Results

Effect of dietary calcium and vitamin D₃ on duodenal calcium-processing genes

To evaluate the effect of calcium and vitamin D₃ on the regulation of active calcium transport genes in *CaBP-9k* KO mice, the mice were fed for 10 weeks with calcium- and vitamin D₃-deficient diets. In the WT mice, duodenal *CaBP-9k* mRNA levels were induced significantly by the deficient diet, and as expected, no transcript was observed in the KO mice (Fig. 1A). The deficient diet enhanced duodenal *TRPV6* mRNA levels significantly in the WT mice, whereas it caused no significant change in the KO mice (Fig. 1B). However, oral calcium and vitamin D₃ intake did not affect the transcription levels of duodenal *PMCA1b* and *NCX1* in either the WT or KO mice (Fig. 1C and 1D).

Effect of dietary calcium and vitamin D₃ on renal calcium-processing genes

In the WT mice, the deficient diet significantly increased renal mRNA expression of *CaBP-9k* and *TRPV6*, whereas in the KO mice, the expression of these genes was absent or unaffected (Fig. 2A and 2B). However, there was no significant alteration in the *NCX1* transcription level following oral calcium and vitamin D₃ intake in either the WT or KO mice (Fig. 2C). In the WT mice, the deficient diet significantly increased the renal mRNA expressions

Fig. 1. Effect of dietary calcium and vitamin D₃ on duodenal calcium-transporter genes. The expressions of *CaBP-9k* (A), *TRPV6* (B), *PMCA1b* (C) and *NCX1* (D) in the duodenum of WT and *CaBP-9k* KO mice were analyzed by real-time PCR. The mice were fed a diet containing 1.1% calcium and 0.8% phosphorus (Normal diet, □) or 0.8% strontium, 0.02% calcium and 0.35% phosphorus without vitamin D₃ (Deficient diet, ■). Values are calculated as a percentage of the *hypoxanthine-guanine phosphoribosyl transferase* (*HPRT*) expression in WT mice on a normal diet. Data are presented as means ± SEM. a, $P < 0.05$ vs. WT on a normal diet.

of *PMCA1b*, *CaBP-28k* and *TRPV5*, while the expressions of these genes in the KO mice were decreased compared with the normal diet groups (Fig. 2D, 2E and 2F).

Expressions of *VDR* and *PTHr* mRNA in the duodenum and kidney

To clarify the molecular mechanisms underlying the effect of dietary calcium and vitamin D₃ on the expressions of calcium-processing genes, we used real-time PCR to examine expression of *VDR* and *PTHr* in duodenal or kidney tissues from WT and KO mice. The deficient diet did not alter the renal and duodenal *VDR* transcription levels of the WT and KO mice (Fig. 3A and B). However, the renal *PTHr* gene expressions of both types of mouse were differentially regulated by the deficient diet. The *PTHr* levels of the WT mice were reduced by the deficient diet, and the levels were also decreased by the normal diet in the KO mice; however no changes fed with deficient diets in *PTH* mRNA levels were observed in the *CaBP-9k* KO mice (Fig. 3C).

Fig. 2. Effect of dietary calcium and vitamin D₃ on renal calcium-transporter genes. The expressions of *CaBP-9k* (A), *TRPV6* (B), *NCX1* (C), *PMCA1b* (D), *CaBP-28k* (E), and *TRPV5* (F) in the kidneys of WT and *CaBP-9k* KO mice were analyzed by real-time PCR. The mice were fed a diet containing 1.1% calcium and 0.8% phosphorus (Normal diet; □) or 0.8% strontium, 0.02% calcium and 0.35% phosphorus without vitamin D₃ (Deficient diet; ■). Values are calculated as a percentage of the *HPRT* expression in the WT mice on a normal diet. Data are presented as means ± SEM. a, $P < 0.05$ vs. WT on a normal diet; b, $P < 0.05$ vs. *CaBP-9k* KO on a normal diet; c, $P < 0.05$ vs. a different genotype on the same diet.

Effect of the deficient diet on serum calcium levels and body weight

To understand the physiological statuses of the WT and KO mice fed the deficient diet for 10 weeks, we measured the serum calcium levels in the mice. The WT and KO mice fed the deficient diet appeared to show relatively low serum calcium levels; however, the levels in the WT mice were significantly different compared with the same strain fed normal food (Fig. 4). In addition, although we observed a normal range of body weights at 8 weeks of age in the WT and KO mice, significant reductions of body weights were observed in the WT and KO mice fed the deficient diet at 18 weeks, as shown in Fig. 5.

Fig. 3. Effect of deficient diets on renal and duodenal *VDR* and *PTHr* genes. The expressions of renal *VDR* (A), duodenal *VDR* (B) and renal *PTHr* (C) in WT and *CaBP-9k* KO mice were analyzed by real-time PCR. The mice were fed a diet containing 1.1% calcium and 0.8% phosphorus (Normal diet, □) or 0.8% strontium, 0.02% calcium and 0.35% phosphorus without vitamin D₃ (Deficient diet, ■). Values are calculated as a percentage of *HPRT* expression in the WT mice on a normal diet. Data are presented as means ± SEM. a, $P < 0.05$ vs. WT on a normal diet; b, $P < 0.05$ vs. *CaBP-9k* KO on a normal diet; c, $P < 0.05$ vs. different genotype on the same diet.

Discussion

Active calcium absorption plays a role in calcium homeostasis. Previous studies have elucidated the mechanism(s) underlying duodenal and renal calcium (re)absorption [8, 33]. The ionized calcium level in the blood is regulated primarily by PTH and 1,25(OH)₂D₃, which control calcium metabolism in the intestine, kidney and bone [34, 35]. In the present study, we examined the effect of dietary calcium and vitamin D₃ on the expression of calcium-regulating genes in *CaBP-9k* KO mice. The calcium-binding protein *CaBP-9k* is expressed in the intestine, uterus, placenta, kidney, bone and pituitary glands of mammals [36–41]. Duodenal *CaBP-9k* is involved in active calcium absorption and is upregulated by the hormonal form of vitamin D and downregulated by dexamethasone, a synthetic glucocorticoid [42, 43]. Renal *CaBP-9k* is expressed in the distal convoluted tubules, where it is believed to facilitate calcium re-absorption [43]. In a previous study, we examined the effect of a calcium deficient diet on the calcium transport system [30] and demonstrated that the *TRPV6* expression was greatly induced in the WT but not KO mice. It appears that

Fig. 4. Effects of dietary calcium and vitamin D₃ on serum calcium levels. Serum was collected from the WT and *CaBP-9k* KO mice on normal and deficient diets. The concentrations of serum calcium were examined using ASAN Ca-Lq Reagents (Asanpharm, Seoul, Korea), as described in the Materials and Methods. Data are presented as means ± SEM. a, $P < 0.05$ vs. WT on a normal diet.

Fig. 5. Effects of dietary calcium and vitamin D₃ on body weight. The body weights of 8- and 18-week-old WT or *CaBP-9k* KO mice were measured. Data are presented as means ± SEM of the body weights of all animals. a, $P < 0.05$ vs. different diet and the same genotype.

TRPV6 expression was induced in response to low dietary calcium in order to maximize absorption of this ion. It is of interest that this adaptation does not occur in *CaBP-9k* KO mice. Whether the absence of *CaBP-9k* plays in part in this response is unclear at present. In the kidney, low dietary calcium induced expressions of

several calcium transport genes, i.e., *CaBP-9k*, *CaBP-28k*, *PMCA1b*, *TRPV5* and *TRPV6*, but this was not seen in the KO mice. Expression of *NCX1* gene was not affected in either the WT or KO mice, suggesting that CaBP-9k does play a role in this adaptation in parallel to regulation of intestinal calcium transport genes. It is noteworthy that comparison of the WT and KO mice showed that the level of CaBP-28k was increased with the normal diet, pointing to a role of CaBP-28k in compensating for the absence of CaBP-9k in this tissue.

In contrast to the case of the WT and KO mice fed a normal diet, both genotypes exhibited reduced body weight and size following 10 weeks of treatment with the deficient diet. This result suggests that the mice fed the deficient diet suffered from severe malnutrition. A significant difference in serum calcium concentration was observed in the WT mice with the calcium-deficient diet, while the difference between the normal and deficient diets in the KO mice was not statistically significant. Both the WT and KO mice maintained serum calcium levels within the normal range. Our previous study indicated that *CaBP-9k* KO mice induce alternative calcium-related genes to maintain a normal serum calcium concentration when provided with a calcium-deficient diet [30]. The physiological data in the present study further supports the suggestion that a compensatory mechanism enables these mice to survive normally.

Calcium homeostasis is maintained primarily by the vitamin D₃ and PTH endocrine systems [44]. Expression of renal VDR is required for 1,25(OH)₂D₃-mediated renal calcium absorption and is regarded as an important mechanism for maintaining serum calcium levels. Therefore, since it is likely that VDR and PTHR are major regulators of active calcium transport, we examined their expression in the duodenum and kidney of both the WT and KO mice. Our results suggest that the calcium-processing genes are regulated by two distinct elements. For instance, we observed that duodenal and renal VDR expressions were not changed in both mice fed the deficient diet. These results appear to conflict with a previous study showing that both calcium and 1,25(OH)₂D₃ are required to activate VDR transcription [45]. The unchanged VDR transcription in the present study might have been caused by insufficient serum 1,25(OH)₂D₃ elimination due to use of a deficient diet containing strontium. However, the deficient diet has been shown to downregulate renal PTHR expression in the WT mice. However, the reduced PTHR levels in the KO mice increased to the levels of normal diet WT mice. Previously, it has been shown that a calcium deficient diet does not alter renal PTHR gene expressions [30]. However, vitamin D₃ deficiency did affect this gene in the present study. Regulation of renal PTHR transcriptions in the KO mice may support the differential gene expressions of the renal active calcium transport system, i.e., unchanged *TRPV6* gene and down-regulated *CaBP-28k* and *TRPV5*. Taken together, the different expression patterns of the calcium-processing genes observed between the genotypes and diets may be explained by distinct expression of duodenal and renal PTHR genes.

In the present study, the body weights of the deficient diet groups were significantly reduced after 10 weeks of exposure compared with those of the normal diet mice. Reduced body weight might be caused by insufficient bone calcification connected with calcium re-absorption from bone as a compensatory mechanism.

In regard to this sequence, the slight difference in the serum calcium levels of the normal and deficient diet groups probably results from a compensatory mechanism for animal survival. Calcium and vitamin D are core factors for bone calcification, but strontium may also have an impact on this process. In the present study, the deficient diet contained strontium, which may inhibit renal 1,25(OH)₂D₃; it was used to eliminate endogenous vitamin D synthesis and to enhance the long-term deficiency status [46]. Several studies have reported the toxicity of strontium, indicating that ingestion of strontium produces osteomalacia and rachitic changes in animal models [47], whereas its harmful effects on bone differ in young and adult rats [48]. When both young and adult rats of both genders were provided a diet containing 1.8% strontium for 7 months, the young rats exhibited a rachitic change, while the adult rats exhibited no changes after exposure. In addition, a similar experiment was performed using weaning and adult mice, and no significant impacts on bone formation parameters were found in the adult mice [49]. Thus, the strontium in the present deficient diet might not have affected bone mineralization because adult mice were employed and the total experimental period was completed within 10 weeks of exposure.

Although CaBP-9k is an important factor in active calcium transport, the KO mice survived normally without exhibiting any distinct phenotypes or abnormalities. To understand how a compensatory mechanism might function in *CaBP-9k* KO mice, these animals were fed a calcium and vitamin D₃-deficient diet, which induced the calcium transport system. Thus, the expression of duodenal and renal calcium-processing genes increased in the WT mice, but not in the KO mice, suggesting that the active calcium transport system containing CaBP-9k and other active calcium transport genes may be primarily involved in calcium resorption and absorption in the duodenum and kidney; however, one system may replace the other under calcium, vitamin D₃ or *CaBP-9k* gene deficiency conditions, which may be controlled by renal PTHR.

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