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Vitamin D receptor is required for dietary calcium-induced repression of calbindin-D9k expression in mice

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Abstract

Calbindin (CaBP), the vitamin D-dependent calcium-binding protein, is believed to play an important role in intracellular calcium transport. The aim of this study was to investigate the effect of high dietary calcium on the expression of CaBP-D9k and CaBP-D28k in the presence and absence of a functional vitamin D receptor (VDR). Treatment with the HCa–Lac diet containing 2% calcium, 1.5% phosphorus and 20% lactose reversed the hypocalcemia seen in adult VDR-null mice in 3 weeks but did not significantly change the blood ionized calcium in wild-type mice. This dietary treatment dramatically suppressed both the duodenal and the renal CaBP-D9k expression in wild-type mice at both mRNA and protein levels but had little effect on the expression of the same gene in VDR-null mice. Removal of this diet gradually restored the expression of CaBP-D9k to the untreated level in wild-type mice. Only moderate or little change in CaBP-D28k expression was seen in wild-type and VDR-null mice fed with the HCa–Lac diet. The VDR content in the duodenum or kidney of wild-type mice was not altered by the dietary treatment. These results suggest that calcium regulates CaBP-D9k expression by modulating the circulating 1,25-dihydrxyvitamin D_3 level and that VDR is thus required for the dietary calcium-induced suppression of CaBP-D9k expression. Calcium regulation of the CaBP-D9k level may represent an important mechanism by which animals maintain their calcium balance.

Keywords: Calcium; Calbindin-D9k; Calbindin-D28k; 1,25-dihydroxyvitamin D_3; Vitamin D receptor; Hypocalcemia

1. Introduction

Dietary calcium is the sole external source of calcium intake for mammals and is thus crucial for the maintenance of calcium homeostasis. Ingested calcium is absorbed in the intestine and excreted primarily from the kidney; thus, a proper control of intestinal and renal tubular calcium transport is essential to maintain calcium balance. Calbindin (CaBP), the cytosolic vitamin D-dependent calcium-binding protein, has long been recognized as a key factor involved in transcellular calcium transport in the epithelia of the intestine and renal tubules. Two classes of CaBP have been identified in animals: CaBP-D9k, which is expressed mainly in the mammalian intestine and kidney, and CaBP-D28k, which is highly expressed in the mammalian kidney and the avian intestine [1]. It is well known that 1,25-dihydroxy vitamin D_3 [1,25$(OH)_2D_3$] is a major regulator of CaBP expression [1]. Calcium has also been found to affect the expression of CaBP. In duodenal organ cultures, calcium stimulates CaBP-D9k mRNA synthesis [2]; in chicks and rats, the intestinal expression of CaBP-D28k and CaBP-D9k is stimulated by a low-calcium diet and inhibited by a high-calcium diet [3,4]. Extracellular calcium has also been shown to modulate vitamin D-induced renal CaBP-D28k expression [5]. However, the relationship between dietary calcium and vitamin D in the regulation of CaBP in vivo remains elusive.

We have shown that mice lacking the vitamin D receptor (VDR) develop impaired mineral homeostasis [6], which may be largely due to reduced renal calcium reabsorption resulting from a dramatic decrease in renal CaBP-D9k level [7], suggesting that CaBP-D9k may play a key role in distal tubular calcium transport in the kidney. In this report, we studied the effect of high dietary calcium on the expression of CaBP-D9k and CaBP-D28k in VDR-null

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and wild-type mice. Our results demonstrate that VDR is required for the calcium-induced suppression of CaBP-D9k expression in vivo.

2. Materials and methods

2.1. Animal maintenance and treatment

Generation and characterization of VDR-null (−/−) mice have been reported elsewhere [6]. VDR(−/−) and wild-type mice were produced through breeding of heterozygous mice and identified by PCR analysis of tail genomic DNA. Mice were fed with an autoclaved regular rodent diet containing 1% calcium, 0.85% phosphorus and 4 IU/g vitamin D_3 (regular diet) and exposed to a 12-h light/12-h dark cycle. For dietary treatment, 6- to 8-week-old sex- and age-matched VDR(−/−) and wild-type mice were fed with a special diet (TD96348) that contains 2% calcium, 1.25% phosphorus, 4 IU/g vitamin D_3 and 20% lactose (HCa–Lac diet) (Teklad, Madison, WI, USA) for 1–7 weeks, and three to four pairs of mice were sacrificed weekly. In other experiments, mice were fed with the HCa–Lac diet for 3 weeks and then returned to the regular diet for 1 to 7 weeks. Three to four pairs of mice were sacrificed weekly in the postdietary treatment period. The use of animals in this study was approved by the Institutional Animal Care and Use Committee of the University of Chicago.

2.2. Measurement of blood ionized calcium

Blood was collected through tail snipping into ice-cold Eppendorf tubes containing heparin. The blood ionized calcium concentration was determined using a NOVA-2 pH/calcium analyzer (Nova Biomedical, Waltham, MA, USA) as described previously [7].

2.3. RNA extraction and Northern blot

Total RNA was isolated from duodena and kidneys using a TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. CaBP-D9k and CaBP-D28k mRNA levels were determined by Northern blot analyses as described previously [7]. The relative amount of mRNA was quantitated using a PhosphorImager (Molecular Dynamic, Sunnyvale, CA, USA). Variations in RNA loading were normalized with 36B4 RNA, the acidic ribosomal phosphoprotein PO [8].

2.4. Western blot

Duodenal and renal homogenates were prepared in a Laemmli sample buffer from freshly dissected duodena and kidneys. Western blot analyses of CaBP-D9k, CaBP-D28k and VDR proteins were performed as described previously [7]. Protein amounts were quantified by scanning the immunoblot bands with a Umax Power Look II scanner (Umax Data Systems, Taiwan) followed by image processing by an IP Lab Gel software, Version 2.0a (Signal Analytics, Vienna, VA, USA).

2.5. Statistical analysis

Data were presented as mean±S.D. Student's t test was used to identify significant differences. P values of .05 or lower were considered to be statistically significant.

3. Results

Fig. 1 shows the blood ionized calcium concentrations of VDR(−/−) and wild-type mice treated and posttreated with the HCa–Lac diet. VDR(−/−) mice remained normocalcemic before weaning (20 days old), and adult VDR(−/−) mice developed hypocalcemia, with the blood ionized calcium level decreased by about 30% as compared with wild-type littermates. Treatment with the HCa–Lac diet for 1 week normalized the blood ionized calcium of adult VDR(−/−) mice, and the normacalcemia was maintained on the HCa–Lac diet. This diet did not significantly alter the blood calcium level of wild-type mice (Fig. 1A). On the other hand, when the HCa–Lac diet was removed from the normocalcemic adult VDR(−/−) mice, their blood ionized calcium gradually dropped to the pretreatment level after 7 weeks (Fig. 1B).

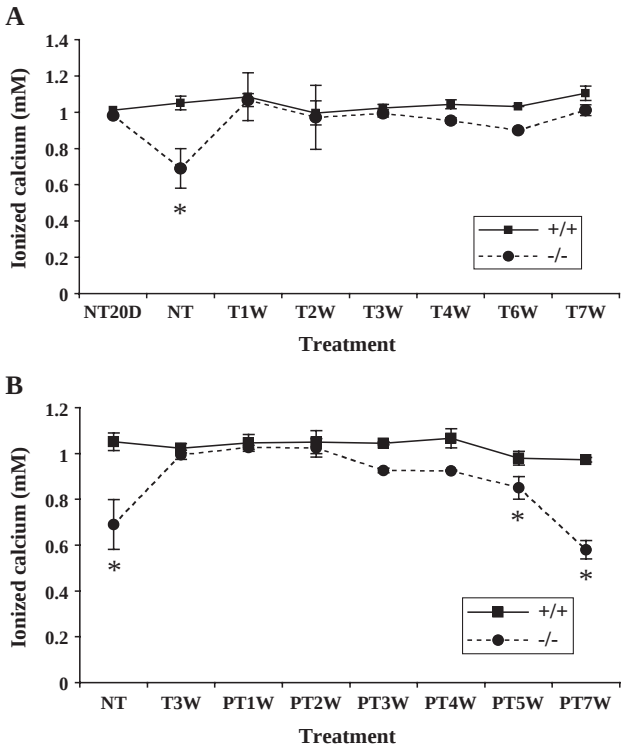

Fig. 1. Effect of dietary calcium on the blood ionized calcium levels in VDR(−/−) and wild-type (+/+) mice. The blood ionized calcium levels were determined using a NOVA-2 pH/calcium analyzer. (A) Ionized calcium levels during the treatment. (B) Ionized calcium levels in the posttreatment period. Values of the blood ionized calcium were determined weekly during and after the HCa–Lac dietary treatment as described in Materials and Methods. T1W–T7W indicate 1–7 weeks of dietary treatment; PT1W–PT7W, 1–7 weeks of posttreatment; NT, nontreated; NT20D, nontreated 20-day-old mice. *P <.05 as compared with corresponding wild-type values. $n \geq 3$ in each group.

We next determined the effect of the dietary treatment on the expression of duodenal and renal CaBP-D9k and CaBP-D28k in wild-type and VDR(−/−) mice by quantitative Western and Northern blot analyses. In wild-type mice, both the protein and mRNA levels of duodenal and renal CaBP-D9k were decreased with time during the HCa–Lac diet treatment (reduced by 70% and 50%, respectively, after 6–7 weeks of treatment) and then gradually returned to the untreated control level after the treatment was stopped at the third week (Fig. 2A–C). As we showed previously [7], CaBP-D9k expression in VDR(−/−) mice was about 50% and 10% of the wild-type level in the duodenum and kidney, respectively (Fig. 2A–C). No reduction in CaBP-D9k protein or mRNA was observed in VDR(−/−) mice in the dietary treatment and postdietary treatment periods; rather, there was a moderate increase in the posttreatment period (Fig. 2A–C). These results indicate that a functional VDR is required for the diet-induced suppression of CaBP-D9k expression. In contrast, both renal CaBP-D28k protein and mRNA levels were largely unaltered during and after the HCa–Lac dietary treatment in both wild-type and VDR(−/−) mice (only the protein content was moderately but significantly reduced after 6–7 weeks of treatment) (Fig. 3A and B), indicating that the expression of renal CaBP-D28k is not significantly affected by dietary calcium. No CaBP-D28k expression was detected in the duodena (data not shown).

Since changes in the VDR content can alter expression of many target genes [9–11], we then investigated whether changes in VDR expression were involved in the calcium-induced repression of CaBP-D9k in wild-type mice. As shown in Fig. 4, the HCa–Lac dietary treatment did not alter the VDR protein level in the duodenum or kidney of treated and posttreated wild-type mice. Thus, the reduction in VDR content was not involved in CaBP repression.

4. Discussion

As calcium is essential for multiple physiological functions such as bone formation, nerve pulse transmission and blood coagulation, animals maintain the extracellular calcium concentration at a narrow range. The blood ionized calcium level is mainly regulated by PTH and $1,25(OH)_2D_3$, which control calcium metabolism in the intestine, kidney and bone [12]. Under adequate dietary calcium conditions, the body maintains a zero calcium balance so that the amount of calcium absorbed in the intestine is largely equal to the amount of calcium excreted from the kidney. Thus, variations in calcium supply in the diet will inevitably

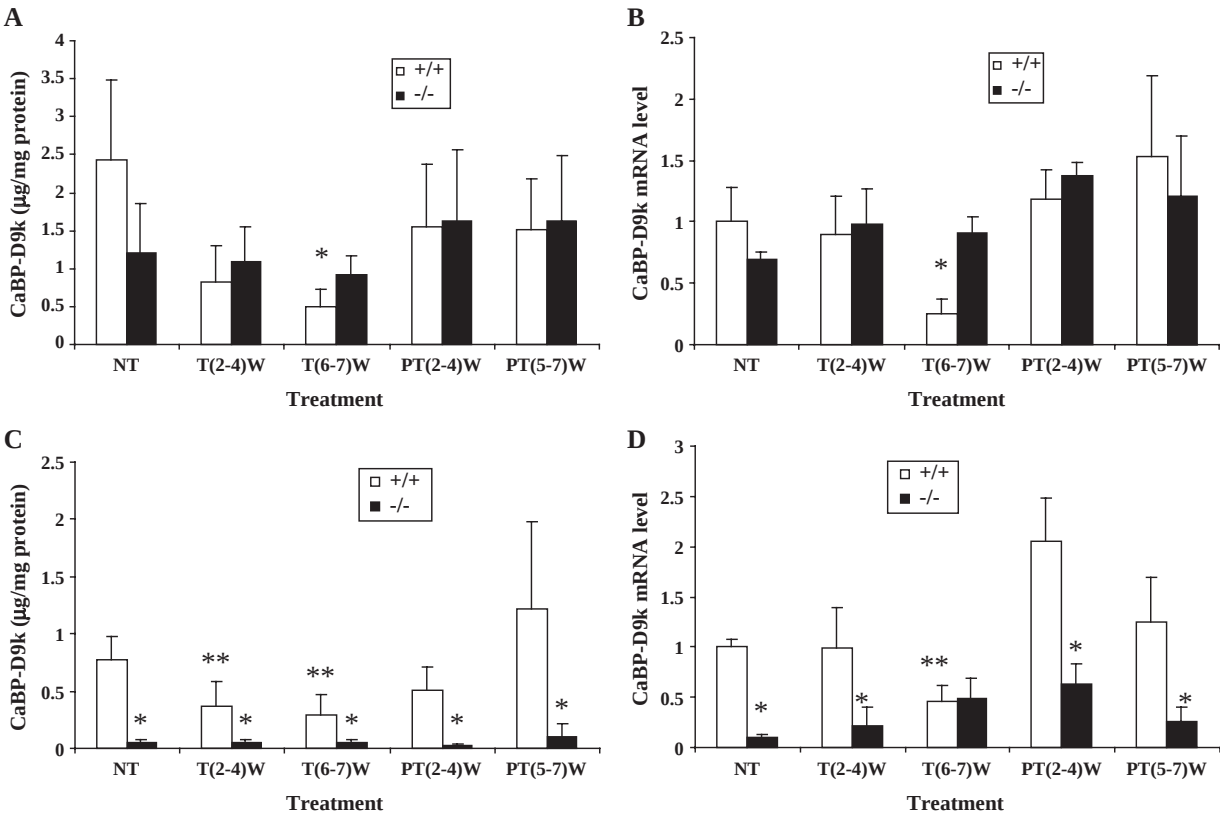

Fig. 2. Effects of dietary treatment on the expression of duodenal and renal CaBP-D9k in VDR(−/−) and wild-type (+/+) mice. (A) Duodenal CaBP-D9k protein levels quantified by Western blot. (B) Duodenal CaBP-D9k mRNA levels quantified by Northern blot. (C) Renal CaBP-D9k protein levels quantified by Western blot. (D) Renal CaBP-D9k mRNA levels quantified by Northern blot. NT indicates nontreated controls; T(2–4)W and T(6–7)W, 2–4 and 6–7 weeks of treatment; PT(2–4)W and PT(5–7)W, 2–4 and 5–7 weeks of posttreatment. *$P<.01$ as compared with corresponding +/+ values; **$P<.05$ as compared with NT +/+ control. $n \geq 3$ in each group.

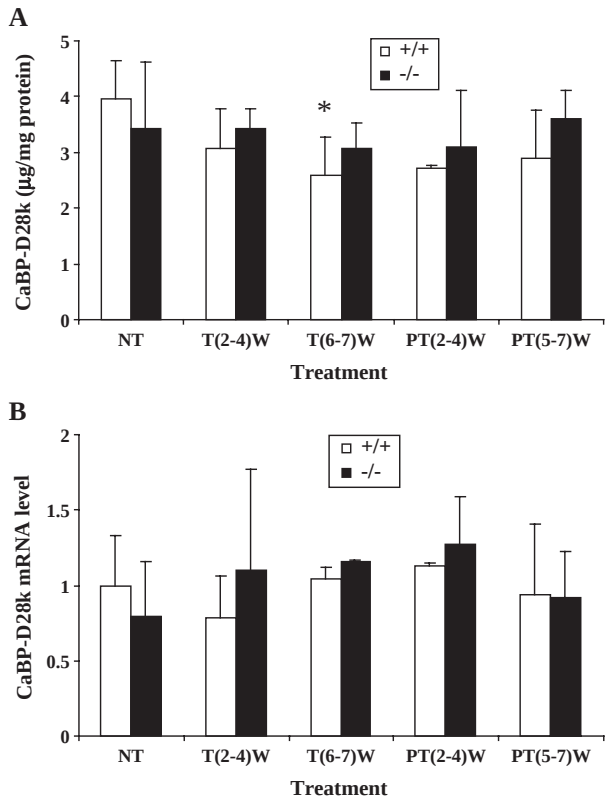

Fig. 3. Effects of dietary treatment on the expression of CaBP-D28k in the kidneys of VDR(−/−) and wild-type (+/+) mice. (A) Renal CaBP-D28k protein levels quantified by Western blot. (B) Renal CaBP-D28k mRNA levels quantified by Northern blot. NT indicates nontreated controls; T(2–4)W and T(6–7)W, 2–4 and 6–7 weeks of treatment; PT(2–4)W and PT(5–7)W, 2–4 and 5–7 weeks of posttreatment. *$P < .02$ as compared with corresponding +/+ values. $n \geq 3$ in each group.

influence calcium absorption and excretion. However, the mechanism by which dietary calcium affects intestinal and renal calcium transport remains elusive.

In the present study, we examined the chronic effect of dietary calcium on the expression of CaBP-D9k and CaBP-D28k, the cytosolic proteins believed to be important for calcium transport, in the presence and absence of VDR. We found that high dietary calcium suppresses CaBP-D9k expression in both the intestine and kidney in wild-type mice but not in VDR-null mice and that dietary calcium has little effect on CaBP-D28k. These observations indicate that a functional VDR is required to mediate the dietary suppression of CaBP-D9k. This is consistent with the finding in a recent study using 25-hydroxyvitamin D_3-1α-hydroxylase knockout mice that regulation of CaBP-D9k by dietary calcium requires $1,25(OH)_2D_3$ [13]. These results argue against a direct effect of calcium on the CaBP-D9k gene in vivo, although an up-regulation of intestinal CaBP-D9k expression by calcium was reported in fetal rat duodenal organ cultures in an early study [2]. As VDR content in wild-type mice is not altered by the dietary calcium, it is conceivable that calcium regulates CaBP-D9k expression by systemically modulating the level of circulating $1,25(OH)_2D_3$ through the PTH/vitamin D endocrine system, as outlined in Fig. 5. In fact, alteration of circulating $1,25(OH)_2D_3$ by dietary calcium has been well known in animals [14], and a most recent study confirms that this HCa–Lac diet indeed suppresses circulating PTH and $1,25(OH)_2D_3$ levels in wild-type mice [15]. Thus, consistent with our recent observation that CaBP-D9k is highly regulated by $1,25(OH)_2D_3$ [7], low circulating $1,25(OH)_2D_3$ resulting from high calcium intake should lead to decreased expression of CaBP-D9k and, apparently, a functional VDR is necessary to mediate the effect.

The fact that dietary calcium suppresses the expression of CaBP-D9k supports the notion that CaBP-D9k is critically involved in the regulation of calcium metabolism [7,16]. By suppressing CaBP-D9k expression in

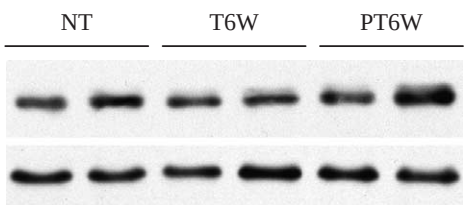

Fig. 4. Western blot analyses of VDR protein levels in the duodena and kidneys of wild-type mice under different dietary conditions. Duodenal and kidney homogenates (25 μg protein/lane) isolated from wild-type mice were separated by SDS-PAGE and subjected to Western blot analyses as described in Materials and Methods. NT indicates nontreated controls; T6W, 6 weeks on the HCa–Lac diet; PT6W, 6 weeks after the HCa–Lac dietary treatment.

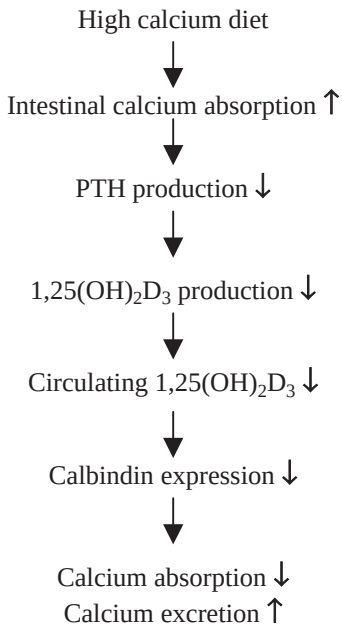

Fig. 5. Outline of dietary calcium-induced effect on calcium metabolism in mice. High-calcium diet increases intestinal calcium absorption, which leads to reduced PTH production from the parathyroid glands. The reduction in PTH then decreases renal $1,25(OH)_2D_3$ biosynthesis, which leads to a reduction in CaBP expression. As a consequence, intestinal calcium absorption is reduced, whereas renal calcium excretion is increased, and the homeostatic state of calcium is thus maintained.

response to a high-calcium intake, the wild-type mice are able to reduce intestinal absorption and increase renal excretion to maintain a normocalcemic status during the HCa–Lac diet treatment. This high-calcium diet, on the other hand, allows VDR-null mice to absorb more calcium, probably through the passive, paracellular transport in the intestine [17], as the level of CaBP-D9k is hardly changed by the diet in the absence of VDR. Most likely, it is a positive balance between the intestinal absorption and renal excretion that eventually normalizes the blood calcium level of VDR-null mice.

Recent studies demonstrate that the epithelial calcium channel and calcium transporter in the apical membrane also play an important role in intracellular calcium transport [13,18,19]. Dietary calcium may also influence calcium entry through the transporter to modulate its transport. Studies also suggest that increased dietary calcium may also have a vitamin D-independent effect on calcium absorption [15], the mechanism of which remains elusive. Our finding that dietary calcium has little effect on CaBP-D28k is consistent with our previous observation that CaBP-D28k expression is hardly regulated by $1,25(OH)_2D_3$ in mice [7] because calcium can not modulate CaBP-D28k via $1,25(OH)_2D_3$. However, the insensitive nature of CaBP-D28k to calcium or $1,25(OH)_2D_3$ status does not necessarily prove that CaBP-D28k is not important in calcium metabolism [16]. In fact, our recent studies of VDR/CaBP-D28k double knockout mice clearly demonstrate that CaBP-D28k also plays a critical role in renal calcium reabsorption and calcium homeostasis [20].

In summary, our study demonstrates that high dietary calcium suppresses CaBP-D9k expression by a VDR-dependent mechanism. This may be one important way that animals use to regulate their calcium balance.

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