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Increased Vitamin D Receptor Level Enhances 1,25-Dihydroxyvitamin D_3–Mediated Gene Expression and Calcium Transport in Caco-2 Cells*†

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ABSTRACT

Altered vitamin D receptor (VDR) level has been proposed to explain differences in intestinal responsiveness to 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$]. We tested whether the enterocyte VDR level influences 1,25$(OH)_2D_3$-mediated gene expression and transepithelial calcium (Ca) transport in the human intestinal cell line Caco-2. Cells were stably transfected with a human metallothionein (hMT) IIA promoter–human VDR (hVDR) complementary DNA (cDNA) transgene that overexpressed hVDR in response to heavy metals. In MTVDR clones, induction of 25-hyroxyvitamin D_3–24-hydroxylase (24-OHase) messenger RNA (mRNA) expression by 1,25$(OH)_2D_3$ (10^{-9} M, 4 h) was correlated to metal-induced changes in nuclear VDR level (r^2 = 0.99). In MTVDR clones, basal VDR level was 2-fold greater and 1,25$(OH)_2D_3$-mediated Ca transport (10^{-7} M, 24 h) was 43% higher than in parental Caco-2 cells. Treatment of MTVDR clones with Cd (1 μM, 28 h) increased VDR level by 68%, significantly enhanced 1,25$(OH)_2D_3$-mediated Ca transport by 24%, and increased accumulation of calbindin D_{9K} mRNA by 76% relative to 1,25$(OH)_2D_3$ alone. These observations support the hypothesis that the enterocyte VDR level is an important modulator of intestinal responsiveness to 1,25$(OH)_2D_3$. (J Bone Miner Res 2001;16:615–624)

Key words: vitamin D receptor, 24-hydroxylase, calbindin D_{9k}, calcium transport, enterocyte, 1,25-dihydroxyvitamin D_3

INTRODUCTION

THE ACTIVE form of vitamin D, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], is a steroid hormone that affects the expression of a wide variety of genes through the action of a nuclear vitamin D receptor (VDR).[1] 1,25$(OH)_2D_3$ also can stimulate the active, transcellular component of intestinal calcium (Ca) absorption that is essential for the maintenance of Ca homeostasis.[2] Aberrations in Ca homeostasis can lead to low serum Ca, decreased bone mineral density (BMD), and increased fracture risk.[3–5] For example, age-associated intestinal resistance to vitamin D[6–8] may contribute to Ca malabsorption in the elderly.[9]

An altered intestinal VDR level has been proposed as a mechanism to explain differences in intestinal responsiveness to 1,25$(OH)_2D_3$. In vivo, high intestinal VDR levels may be responsible for hyperresponsiveness to 1,25$(OH)_2D_3$ in the genetic hypercalciuric rat,[10] whereas low VDR concentration has been associated with reduced Ca absorption in aging and estrogen deficiency.[11,12] In rats, aging reduces

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both the intestinal VDR level and the Ca absorption.[13,14] Age-related decreases in the intestinal calbindin D_{9k} messenger RNA (mRNA) level in rat intestine may be explained by a decrease in intestinal VDR levels.[15,16] A similar trend toward a declining intestinal VDR level with aging has been reported in some[17] but not all[18] human studies. Changes in VDR level also have been proposed as the biological mechanism to explain the effects of several restriction fragment length polymorphisms in the VDR gene on bone and Ca metabolism.[19–21]

Cell culture–based studies have shown an association between elevated VDR levels and increased $1,25(OH)_2D_3$-mediated gene expression.[22–24] An association also was shown to exist between the VDR level and the activity of the $1,25(OH)_2D_3$-inducible enzyme 25-hydroxyvitamin D_3-24-hydroxylase (24-OHase) in cultured mouse osteoblast-like cells after treatment with varying concentrations of $1,25(OH)_2D_3$.[25] These associations suggest that alterations in the level of VDR protein in vitamin D–responsive tissues such as bone or intestine may have profound influences on Ca metabolism. However, although these studies are highly suggestive, they are associative, and there is little direct evidence that more moderate differences in the VDR level, for example, as may occur with aging, have an important influence on physiological functions such as Ca transport. In this study, we show that moderate increases in VDR level enhance cellular responsiveness to $1,25(OH)_2D_3$ in the human Caco-2 intestinal cell line as measured by the induction of 24-OHase and calbindin D_{9k} mRNA expression and transepithelial Ca transport.

MATERIALS AND METHODS

Maintenance of Caco-2 cells in culture

Caco-2 cells (American Type Tissue Collection, Rockville, MD, USA) were cultured in 75-cm^2 flasks (1×10^6 cells per flask), 6-well dishes (2.0×10^5 cells per well), or permeable membrane filter supports (2.5×10^5 cells per well) as described previously.[26]

Construction of the metallothionein-hVDR-pCR3 expression vector

To create a cell culture model in which the VDR level could be manipulated**,** Caco-2 cells were stably transfected with an expression vector containing the human VDR (hVDR) complementary DNA (cDNA) downstream from the heavy metal–responsive metallothionein-IIA (MT) promoter.[27] The full-length hVDR cDNA was obtained by reverse-transcription polymerase chain reaction (RT-PCR) on RNA from Caco-2 cells using primers specific for the hVDR coding region (−14–1281).[28] The MT promoter (−736 to −47) was amplified by PCR from a plasmid containing the MT gene (American Type Culture Collection [ATCC], Manassas, VA, USA).[29] A *Hin*dIII site was incorporated into the 3′ end of the PCR product to facilitate the removal of the MT promoter from the expression vector pCR3.1 (Invitrogen, Carlsbad, CA, USA). The VDR cDNA PCR product and MT promoter were ligated into separate pCR3.1 expression vectors to create the plasmids pCR3.1MTIIA and pCR3.1hVDR. The plasmids were restriction enzyme–digested to verify the orientation of the inserts and PCR inserts in the correct orientation were sequenced by a PCR-based method to verify the sequence. Sequencing was conducted by the DNA Sequencing Center at Tufts University and New England Medical Center, Boston, MA, USA. The MT promoter was then removed from pCR3.1MTIIA by digesting with *Hin*dIII and subcloned into pCR3.1hVDR at a *Hin*dIII site 75 bases upstream from the hVDR cDNA, yielding the metal-inducible transgene construct pCR3.1MTIIA-hVDR**.**

Transfection of Caco-2 cells and selection of stable clones

Caco-2 cells were seeded at 1.5×10^5 cells in 35-mm culture plates and grown overnight. Cells were transfected with pCR3.1MTIIA-hVDR using a cationic lipid (pFx-2; Invitrogen) along with 4 μg of plasmid at a lipid to plasmid ratio of 3:1 in serum-free medium for 4 h. After an overnight incubation in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS), cells were grown in medium containing 0.5 mg/ml G-418 sulfate (Geneticin; Gibco-BRL, Grand Island, NY, USA). Those continuing to grow in the G-418 medium through at least five passages were considered to be stable transfects. Individual clones were isolated from very low density cultures (2000 cells/100 mm^2 dish) using cloning rings. Inducibility of the transgene and VDR overexpression were confirmed by treating clones with increasing amounts of Zn (as Zn acetate) or Cd (as $CdCl_2$) for 8 h. Transgene mRNA level was assessed by RT-PCR and VDR protein level was assessed by Western blot analysis. Clones were characterized further to assess their appropriateness as a model for intestinal Ca absorption.

Experimental protocols

Experiment 1. Characterization of the MTVDR clones: Transgene induction: MTVDR clones and parental Caco-2 cells were seeded in 6-well plastic dishes, grown to confluence and then treated with increasing concentrations of Zn (0–500 μM) or Cd (0–10 μM) for 8 h. For analysis of transgene-driven expression of VDR, total cellular RNA was isolated and subjected to RT-PCR. For VDR protein levels, cells were harvested and nuclear extracts were isolated and subjected to Western blot analysis for VDR.

Experiment 1. Characterization of the MTVDR clones: MTVDR clones as an in vitro model for absorptive epithelium: To determine whether the MTVDRA3 clone was comparable with the parent Caco-2 cell line as a model for vitamin D–mediated intestinal Ca transport, several parameters of cellular differentiation were assessed. First, the growth rates of the two cell lines were compared using a commercially available assay (the Cell Titer 96 Aqueous Assay, Promega, Madison, WI, USA). Briefly, cells were seeded in 96-well plates at 1000 cells per well, and growth was assayed at days 1, 2, and 3 postseeding. At each time point, a solution containing 50 μl of tetrazolium reagent

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethylphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) per 1 ml phenazinemethosulfate (PMS) reagent was warmed to 37°C. Twenty microliters of the solution was added to each well and incubated for 4 h at 37°C (5% CO_2, humidified). The reaction was stopped by adding 25 μl 10% sodium dodecyl sulfate (SDS) per well, and the production of a water-soluble formazan compound was measured by absorbance at 490 nm. The absorbance at 490 nm is correlated directly to the number of living cells in each well.

Next, the mRNA levels for two markers of intestinal cell differentiation sucrase and calbindin D_{9k}[30,31] were analyzed in cells cultured for up to 21 days. MTVDRA3 clones and parental Caco-2 cells were seeded in 6-well plastic dishes at 1.5×10^5 cells per well. Cells were then harvested on day 2 (50% confluent) confluence and days 4, 11, and 17 postconfluence. Total RNA was isolated and analyzed for sucrase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and calbindin D_{9k} mRNA levels by RT-PCR.

Finally, the ability of $1,25(OH)_2D_3$ to stimulate a net increase in apical-to-basolateral Ca transport was assessed in the MTVDRA3 clone. Clones were seeded at 2.5×10^5 cells per well onto permeable membrane filter inserts (24.5-mm diameter, 0.4-μm pore size; Costar, Cambridge, MA, USA) and grown for 13 days. On day 14, cells were treated with 10^{-7} M $1,25(OH)_2D_3$ for 48 h. Net Ca transport was assessed as described previously.[26]

Experiment 1. Characterization of the MTVDR clones: Effect of heavy metal treatment on cell viability and function: Cell viability after heavy metal exposure was assessed by measuring protein synthesis using a [H^3]-leucine incorporation assay.[32] MTVDR clones were seeded in 6-well plastic dishes (2.0×10^5 cells per well) or permeable membrane inserts (2.5×10^5 cells per well) and maintained in culture for 13 days. Cells were then exposed to varying doses of Zn (50–500 μM) or Cd (1–10 μM) for up to 24 h. A 30-minute treatment with 20 μg/ml cycloheximide was used to inhibit protein synthesis and this served as a positive control. Cells were washed three times with serum-free, leucine-free minimal essential medium and incubated for 1 h with [H^3]-leucine–containing MEM (2 μCi/ml; NEN-Life Science, Boston, MA, USA) at 37°C. Cells were harvested into ice-cold phosphate-buffered saline (PBS) and pelleted by centrifugation. Cell pellets were then mixed with 0.1 mg/ml bovine serum albumin (BSA) solution containing 0.02% NaN_3 along with 20% trichloroacetic acid (TCA) and incubated for 30 minutes. The resulting cell homogenate was subjected to vacuum filtration on 2.5-cm microfiber disks (Whatman Paper, Maidstone, UK), followed by two washes with 10% TCA and two washes with 100% ethanol. Disks were dried, transferred to vials containing scintillation fluid, and counted. [H^3]-leucine incorporation is expressed as percentage of the nontreated control group. Samples were analyzed in triplicate and each experiment was repeated three times.

An additional parameter used to assess the effects of metals on monolayer integrity and cell viability was the rate of phenol red transport (a marker of paracellular transport) during Ca transport studies. MTVDR clones and parental Caco-2 cells were grown on permeable membrane supports and treated with various doses of Zn (0–500 μM) or Cd (0–10 μM). The percent movement of 500 μM phenol red per hour across the monolayer was assessed as described below.

Experiment 2: Effect of increased VDR on the regulation of 24-OHase mRNA level by $1,25(OH)_2D_3$: MTVDRA3 clones were seeded in 6-well dishes (2.0×10^5 cells per well) and grown for 15 days. Cells were then pretreated with control medium or medium containing 500 μM Zn for 8 h to induce VDR expression. Cells were washed with Hanks' balanced salt solution (HBSS) and treated with increasing concentrations of $1,25(OH)_2D_3$ (ethanol, 10^{-9}, 10^{-8}, or 10^{-7} M) for 2 h or 8 h. Ethanol treatments were diluted to the same extent as the 10^{-7} M $1,25(OH)_2D_3$ solution. An additional experiment was conducted to show that the results from MTVDRA3 were not unique to that clone. Three clonal lines MTVDRA3, MTVDRA5, and MTVDRA7 were seeded into 6-well dishes as described previously and grown 15 days in culture. At this point, cells were pretreated with 500 μM Zn for 8 h, washed with HBSS, and then treated with 1 nM $1,25(OH)_2D_3$ or ethanol for an additional 8 h. After each experiment, total RNA was isolated and 24-OHase the mRNA level was assessed by RT-PCR.

Experiment 2: Correlation between nuclear VDR level and regulation of 24-OHase mRNA level by $1,25(OH)_2D_3$: MTVDRA3 clones and parental Caco-2 cells grown on plastic 6-well dishes were pretreated with increasing concentrations of Zn (0–500 μM, 8 h) to induce increasing levels of VDR expression. Cells were washed and then treated with 10^{-9} M $1,25(OH)_2D_3$ for 4 h. Cells were harvested and analyzed for VDR level by Western blot or for 24-OHase mRNA by RT-PCR as described below.

Experiment 3: Effect of increased VDR on regulation of Ca transport and calbindin D_{9k} mRNA level by $1,25(OH)_2D_3$: Parental Caco-2 cells and MTVDRA3 clones were seeded onto permeable membrane filter inserts (2.5×10^5 cells per well, 24.5-mm diameter, 0.4-μm pore size; Costar) and grown for 14 days. On day 14, cells were pretreated with 0 μM or 1 μM Cd for 4 h (basolateral side of the membrane only) and then treated with fresh medium containing 0 μM or 1 μM Cd (basolateral side only) and ethanol or 10^{-7} M $1,25(OH)_2D_3$ (both apical and basolateral sides) for 24 h. In one experiment, unidirectional apical-to-basolateral calcium transport was assessed in parental Caco-2 cells and the MTVDRA3 clone under the treatment conditions described previously; in another experiment net calcium transport was determined in the MTVDRA3 clone only. Apical-to-basolateral, basolateral-to-apical, and net calcium transport were assessed as described below.

Two additional, parallel experiments also were conducted using the same protocol as described previously. In the first, nuclear extracts were isolated from parental Caco-2 cells and MTVDRA3 clones grown on permeable membrane inserts and treated as described previously. These extracts were analyzed for VDR level by Western blotting. In the second, MTVDRA3 clones were seeded in 6-well plastic dishes (2.0×10^5 cells per well) and treated as described previously. Total RNA was isolated and calbindin D_{9k} and 24-OHase mRNA levels were analyzed by RT-PCR.

TABLE 1. PCR PRIMER INFORMATION

Gene	*Reference citation*	*Primer*	*Sequence*	*cDNA location*
Calbindin D_{9k}	Jeung et al.[57]	Forward	5′-ATGAGTACTAAAAAGTCTCCT-3′	1-21
		Reverse	5′-CTGGGATATCTTTTTTACTAA-3′	216-237
24-OHase	Chen et al.[43]	Forward	5′-CTCATGCTAAATACCCAGCTG-3′	1633-1653
		Reverse	5′-TCGCTGGCAAAACGCGATGGG-3′	1912-1932
VDR	Baker et al.[28]	Forward	5′-CCCGGCGGCCAGCGGCGGAAC-3′	536-556
		Reverse	5′-GTCTAGCAGAGTCCACGCTCC-3′	1053-1073
Sucrase	Lorentz et al.[30]	Forward	5′-GGTGGTCACATCCTACCATGTCAAG-3′	5121-5145
		Reverse	5′-CCAGTTGATTTCTATTGGTTCTTCT-3′	5516-5540
GAPDH	Fort et al.[58]	Forward	5′-CCATGGAGAAGGCTGGGG-3′	386-403
		Reverse	5′-CAAAGTTGTCATGGATGACC-3′	561-580

mRNA analysis

The expression of VDR, 24-OHase, calbindin D_{9k}, and sucrase mRNA levels was assessed by RT-PCR as previously described.[33–35] Briefly, cells were harvested into Tri Reagent and total cellular RNA was isolated as per the manufacturers directions (Molecular Research Center, Cincinnati, OH, USA). cDNA libraries were prepared from isolated total cellular RNA using an oligo dt primer (Gibco BRL). Full-length cDNAs were prepared using an oligo dt primer, and 0.1 μg RNA equivalent of cDNA was analyzed by PCR for VDR (30 cycles), 24-OHase (22 cycles), calbindin D_{9k} (25 cycles), sucrase (27 cycles), and GAPDH (18 cycles) using primers designed from previously published sequences (Table 1). The GAPDH mRNA level was used as a constitutively expressed control gene. Cycle numbers were previously determined to fall within the linear range of amplification efficiency for each of the primer sets (data not shown). Each target message was detected in a separate PCR reaction. The resulting PCR products were subjected to electrophoresis on a 2.5% agarose gel containing ethidium bromide and the PCR products were visualized under UV light. A photo negative of each gel was subjected to laser scanning densitometry. Results were normalized for the expression of GAPDH.

VDR analysis

Western blotting: The relative increase in VDR from Caco-2 clones containing the MT-hVDR transgene was determined by Western blot analysis using the rat anti-VDR monoclonal antibody 9A7 (Affinity Bioreagents, Golden, CO, USA). Cell monolayers were rinsed in HBSS, scraped, and resuspended in ice-cold homogenization buffer containing 10 mM Tris-HCl, 10 mM $NaMoO_4$, 1.5 mM EDTA, and 1.6 mM dithiothreitol (DTT), along with 200 μg/ml soybean trypsin inhibitor, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), and 500 trypsin inhibitory unit (TIU)/ml aprotinin. Cells were sonicated on ice and centrifuged at 8500*g* for 10 minutes at 4°C, and the supernatant was discarded. The resulting crude nuclear pellets were washed three times with detergent buffer (homogenization buffer containing 0.5% Triton X-100). Total protein level of the nuclear extracts was assessed by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA). Samples were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% Tris-glycine gel (NOVEX, San Diego, CA, USA). Proteins were transferred electrophoretically to a nitrocellulose membrane, and VDR was detected using the enhanced chemiluminescence (ECL)–based detection procedure (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Transcellular Ca transport studies

Transcellular Ca transport in the apical-to-basolateral direction was assessed as previously described.[26,33,36] Transport buffer contained 500 μM Ca as $CaCl_2$, 5 μCi/ml ^{45}Ca, and 500 μM phenol red. The quantitation of phenol red movement was used as an assessment of paracellular (diffusional) Ca transport through the monolayer.[37] Saturable Ca transport was determined by calculating the percentage of phenol red transported per minute and subtracting the equivalent amount of Ca from the value for total Ca transport, as previously described.[33] Experiments had either 3 or 6 wells per treatment and were conducted three times.

Experiments requiring determination of both apical-to-basolateral and basolateral-to-apical transport (e.g., for assessment of net transport) were conducted as described by Fleet et al.[26] Before the assay, an equal volume of transport buffer containing an equimolar concentration of Ca (500 μM) was placed on both sides of the monolayer. When apical-to-basolateral transport was assessed, 1 μCi/ml ^{45}Ca was placed in the apical compartment and movement was determined by sampling of the basolateral side. When basolateral-to-apical transport was assessed, the ^{45}Ca was placed on the basolateral side and movement was determined by sampling the apical compartment. Net Ca transport was calculated as the difference between apical-to-basolateral and basolateral-to-apical calcium flux rates from paired wells. Each treatment was done in 3 wells and each experiment was conducted three times.

Statistical analysis

Treatment effects were compared by analysis of variance (ANOVA) using the SYSTAT statistical software package

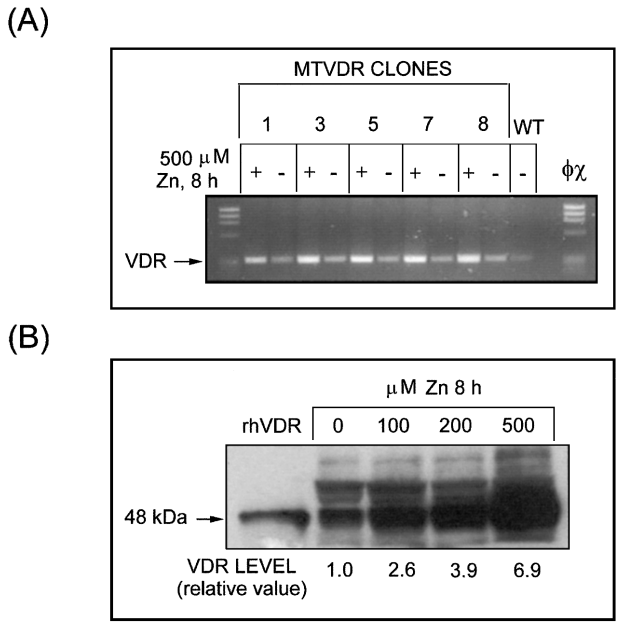

FIG. 1. Zn induction of the MT-hVDR transgene in transfected clones. MTVDR clones were seeded in 6-well plastic dishes and grown to confluence. Cells were then treated with up to 500 μM Zn for 8 h. (A) The VDR mRNA level was assessed by RT-PCR. WT refers to basal VDR expression in nontransfected parental Caco-2 cells. (B) Nuclear extracts from the MTVDRA3 clones were analyzed for the VDR protein level by Western blot analysis using antibody 9A7 and blots were quantified by laser scanning densitometry. Values are expressed relative to 0 μM Zn control = 1.0; 10 μg nuclear protein used per lane (rhVDR, recombinant hVDR).

(SYSTAT 8.0; Systat, Inc., Chicago, IL, USA). Comparisons of multiple group means were performed using Fisher's protected least significant difference (LSD). Differences between treatment means with $p < 0.05$ were considered statistically significant. Data are expressed in the text and figures as mean ± SEM.

RESULTS

Characterization of stably transfected Caco-2 cells

Figure 1A shows that pretreatment of five different MTVDR clones with 500 μM Zn for 8 h increased VDR mRNA between 5- and 10-fold depending on the clone. In addition, because the MT promoter is leaky, basal VDR mRNA and protein levels were higher in all MTVDR clones than in parental cells (2- to 5-fold for mRNA and 2-fold for protein). MTVDR clone A3 (MTVDRA3) was selected for further characterization. Figure 1B shows that treatment of MTVDRA3 with increasing amounts of Zn for 8 h resulted in progressively higher levels of VDR protein production. Similar responses were seen when cells were treated with Cd, although significantly less Cd (<10 μM) was needed to see such responses (data not shown). There was no effect of either Cd (<10 μM) or Zn (<500 μM) treatment on VDR mRNA or protein in the parental Caco-2 cells (data not shown).

Because not all clones from the parental Caco-2 cells differentiate sufficiently to justify their use as a model for transcellular calcium transport, we examined MTVDRA3 for various features related to its ability to differentiate in culture. First, MTVDRA3 clones grew at a similar rate relative to the parent cells (doubling time = 16 h), and expression of sucrase and calbindin D_{9k} mRNA increased comparably in both cell lines with increasing culture time (approximately 20-fold for sucrase mRNA and 300-fold for calbindin D_{9k} from proliferating cells to 21 days in culture). In addition, the clones showed positive net Ca transport that was increased from 0.094 ± 0.021 nmol/well per minute in the absence of $1,25(OH)_2D_3$ to 0.403 ± 0.017 nmol/well per minute after pretreatment with $1,25(OH)_2D_3$ (10^{-7} M, 48 h). Furthermore, phenol red transport was comparable (0.55 ± 0.07% per hour) to that observed in the parent Caco-2 cells and was unaffected by $1,25(OH)_2D_3$ treatment. These features show that the MTVDRA3 clone has retained the characteristics of the parental strain that make this cell line a good model system to study the mechanisms of vitamin D–mediated intestinal Ca absorption.

Effect of heavy metal treatment on cell viability

The heavy metals that induce the MT promoter can be toxic to cells. Initial characterization was done to determine the time and dose of heavy metal exposure that was detrimental to the cells. Exposure to high doses of Zn (≥500 μM) and Cd (≥10 μM) for 24 h or more inhibited protein synthesis by 50% (30 minutes of cycloheximide treatment resulted in 70–80% inhibition), increased phenol red transport by 600%, and caused significant cell detachment. To determine the proper level of heavy metal pretreatment, we titrated down the dose of metal to a point where cells did not detach from the surface of the dishes or filter inserts, phenol red transport was not increased (for transport studies only), and protein synthesis was not affected. For studies using cells seeded on plastic, doses of Cd < 10 μM and Zn < 500 μM did not markedly reduce protein synthesis or cause cell detachment. For studies using cells seeded on Transwell filter inserts, we found simultaneous metal treatment on both the mucosal and the basolateral sides of the filter insert to be extremely toxic to the cells. However, treatment with either 200 μM of Zn or 1 μM of Cd for up to 36 h on only the basolateral side of monolayers had no effect on protein synthesis or phenol red transport, suggesting a lack of chronic toxicity of metals at these doses.

Increased VDR level enhances 24-OHase mRNA induction by $1,25(OH)_2D_3$

Without $1,25(OH)_2D_3$ treatment, 24-OHase mRNA was not detected in either the parental Caco-2 cells or any of the MTVDR clones. In MTVDRA3, 24-OHase mRNA level increased in response to $1,25(OH)_2D_3$ treatment in both a time- and a dose-dependent manner (Table 2). Pretreatment of MTVDRA3 with Zn (500 μM, 8 h) increased the nuclear VDR level 7-fold (Fig. 1B) and enhanced accumulation of

TABLE 2. EFFECT OF INCREASED NUCLEAR VDR LEVEL ON $1,25(OH)_2D_3$-MEDIATED INDUCTION OF 24-OHASE MRNA

1,25(OH)$_2$D$_3$ Time Dose	*2 h*		*8 h*	
	Control[a]	*7X ↑ VDR*	*Control*[a]	*7X ↑ VDR*
None	ND	ND	ND	ND
10^{-9} M	ND	1.0 ± 0.0	1.1 ± 1.0	12.0 ± 1.3
10^{-8} M	1.8 ± 0.7	4.0 ± 0.9	6.6 ± 2.9	29.0 ± 4.5
10^{-7} M	4.1 ± 0.9	7.6 ± 1.5	11.2 ± 4.7	21.0 ± 2.3

VDR overexpression (7-fold) was induced in MTVDRA3 clones by treatment with 500 μM Zn for 8 h. Cells were washed and then treated with ethanol, 10^{-9}, 10^{-8}, or 10^{-7} M $1,25(OH)_2D_3$ for 2 h or 8 h. Without $1,25(OH)_2D_3$ treatment, Caco-2 cells do not have detectable 24-OHase mRNA levels. Thus, values are expressed relative to 24-OHase mRNA induction from high VDR + 10^{-9} M $1,25(OH)_2D_3$ at 2 h = 1. Values represent mean (± SEM) derived from three independent experiments (n = 3 per observation).

[a] Control, basal VDR in uninduced clone; 7X ↑ VDR, MTVDRA3 cells pretreated with 500 μM Zn for 8 h; ND, not detectable. Significant main effects were observed by ANOVA for time of $1,25-(OH)_2D_3$ treatment, $1,25-(OH)_2D_3$ dose, and VDR level ($p < 0.001$).

24-OHase mRNA in response to $1,25(OH)_2D_3$ at all concentrations and time points examined (Table 2). For example, after 2 h of treatment with 10^{-9} M $1,25(OH)_2D_3$, 24-OHase mRNA level went from undetectable to detectable (relative value of 1X). At 8 h, the largest relative effect of increased VDR was observed with 10^{-9} M of $1,25(OH)_2D_3$; vitamin D–induced 24-OHase mRNA level was increased by 10-fold (1.12 ± 1.04 to 11.95 ± 1.32, Table 2). The effect of the VDR level on 24-OHase mRNA accumulation was diminished as the $1,25(OH)_2D_3$ dose was increased, that is, 4-fold enhancement at 10^{-8} M and 2-fold enhancement at 10^{-7} M $1,25(OH)_2D_3$ (Table 2). The effect seen in MTVDRA3 also was observed in the two other clones we examined. Although increasing VDR content with 500 μM Zn for 8 h increased the response to $1,25(OH)_2D_3$ (1 nM, 8 h) by 12-fold in MTVDRA3, the same conditions resulted in a 9-fold increase in MTVDRA5 and a 5-fold increase in MTVDRA7. Neither Zn (≤500 μM) nor Cd (≤10 μM) pretreatment had any effect on induction of 24-OHase mRNA by $1,25(OH)_2D_3$ in parental Caco-2 cells (data not shown).

In a subsequent experiment, varying the amount of Zn in an 8-h pretreatment from 0 to 500 μM produced MTVDRA3 cells with VDR levels up to 5-fold higher than untreated MTVDRA3 clones. Treatment of the clones containing varying levels of VDR with 10^{-9} M $1,25(OH)_2D_3$ for 4 h showed that the accumulation of 24-OHase mRNA in response to hormone was directly and positively correlated with the nuclear VDR level ($r^2 = 0.99$, slope = 2.03; Fig. 2).

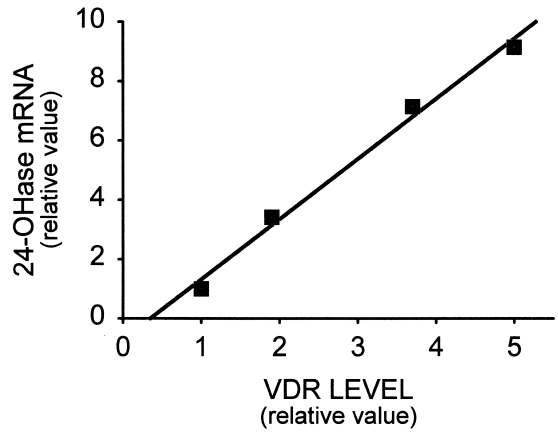

FIG. 2. Correlation between VDR level and induction of 24-OHase mRNA by $1,25(OH)_2D_3$. VDR expression was induced in MTVDRA3 clones by pretreating cells with 0–500 μM Zn for 8 h. Cells were then treated with ethanol or 10^{-9} M $1,25(OH)_2D_3$ for 4 h or harvested for VDR analysis. 24-OHase mRNA level was assessed by RT-PCR and normalized to GAPDH expression. The VDR level was assessed by Western blot using antibody 9A7. Values for 24-OHase mRNA are expressed relative to induction from 10^{-9} M at baseline VDR level = 1 (slope = 2.03; $r^2 = 0.99$).

Increased VDR enhances 1,25(OH)$_2$D$_3$-mediated induction of transcellular Ca transport and calbindin D$_{9k}$ mRNA

Because of the high basal activity of the MT promoter, the VDR level was 2.2-fold greater in MTVDRA3 compared with the parental Caco-2 line (Fig. 3B). Although basal apical-to-basolateral Ca transport was not different between MTVDRA3 and the parental Caco-2 cells (0.26 ± 0.02 nmol/well per minute vs. 0.24 ± 0.01 nmol/well per minute, respectively), the higher VDR level in MTVDRA3 was associated with enhanced $1,25(OH)_2D_3$-mediated Ca transport compared with the parental Caco-2 cells (43% higher, Fig. 3A). It takes 12–16 h for $1,25(OH)_2D_3$ to enhance Ca transport and increase calbindin D_{9k} mRNA levels in Caco-2 cells.[33] In addition, VDR has a short biological half-life (2 h[38]). Thus, we altered the induction protocol to permit a constant, low-level stimulation of the MTVDR transgene with 1 μM Cd to investigate the possible effects of the VDR level on calbindin D_{9k} mRNA level and Ca transport. Twenty-eight hours of treatment with 1 μM Cd in the basolateral compartment was sufficient to increase the VDR level of MTVDRA3 by 68% (to 3.7-fold above the level found in parental Caco-2 cells, Fig. 3B). Cd treatment (and thus higher VDR content) did not effect

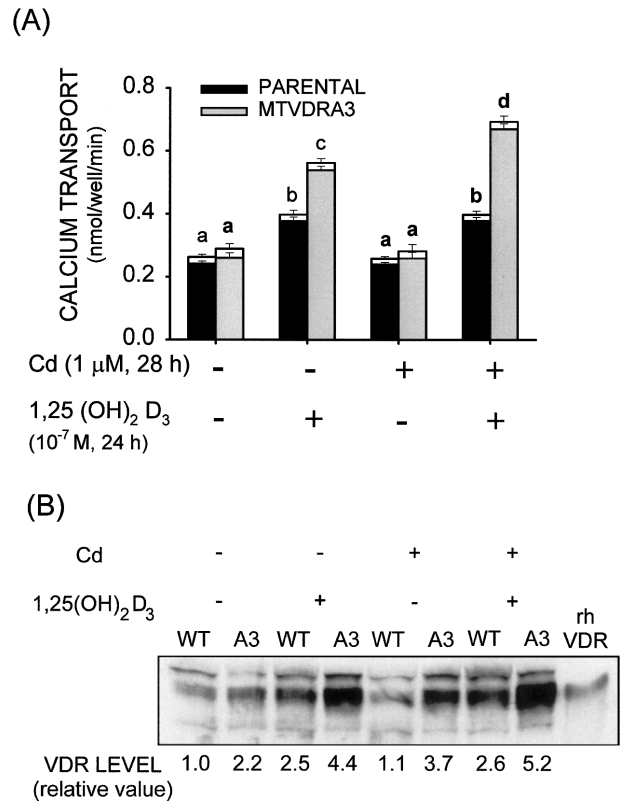

FIG. 3. Enhancement of $1,25(OH)_2D_3$-mediated induction of Ca transport by an increased nuclear VDR level. Caco-2 cells (WT) and MTVDRA3 (A3) clones were seeded in parallel on permeable membrane filter inserts and grown for 14 days. Cells were pretreated with 1 μM Cd (basolateral side only) for 4 h to induce VDR expression. Cells were then treated with fresh media containing 1 μM Cd (basolateral side only) combined with ethanol or 10^{-7} M $1,25(OH)_2D_3$ (both apical and basolateral exposure) for 24 h and the VDR content and Ca transport were assessed. (A) Bars represent total apical-to-basolateral calcium transport. Total transport can be divided into diffusional transport, represented by the open areas on top of each bar (derived from phenol red movement), and saturable transport, represented by the filled bars (the portion of total transport remaining after the correction for diffusion). Values represent the mean ($\pm$ SEM) for data from three independent studies, with each treatment performed in triplicate ($n = 9$ per observation). Bars with the same letter superscripts have saturable calcium transport levels that are not significantly different from one another. (B) Representative blot of the VDR level measured by Western analysis on nuclear extracts from cells treated as described previously. Values are expressed relative to nontreated WT Caco-2 cells = 1.0; 10 μg nuclear protein used per lane (rhVDR, recombinant hVDR).

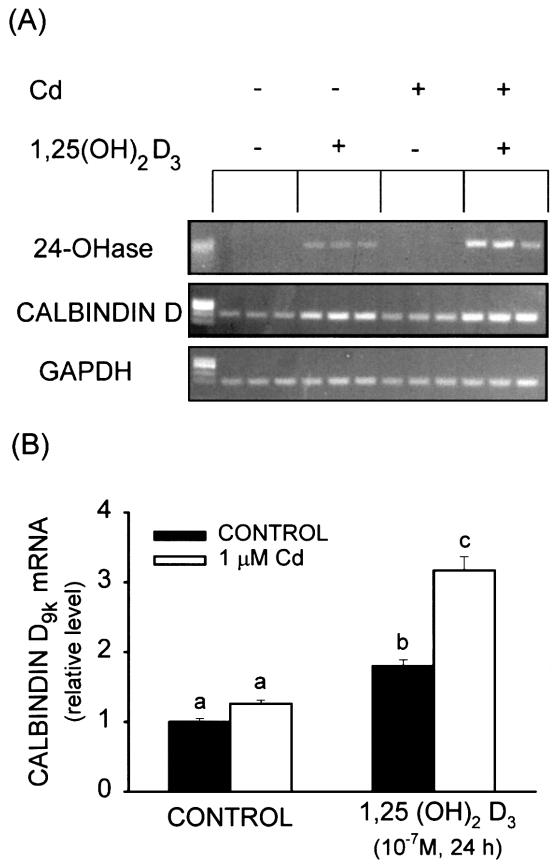

FIG. 4. Enhancement of $1,25(OH)_2D_3$-mediated induction of calbindin D_{9k} mRNA by increased nuclear VDR level. MTVDRA3 clones grown on plastic 6-well dishes were pretreated with 0 μM or 1 μM Cd for 4 h followed by 0 μM or 1 μM Cd combined with ethanol or 10^{-7} M $1,25(OH)_2D_3$ for 24 h. 24-OHase and Calbindin D_{9k} mRNA levels were then assessed by RT-PCR and normalized to GAPDH expression. (A) Shown is a representative gel from one experiment ($n = 3$). (B) Relative expression of calbindin D_{9k} mRNA level. Values represent the mean ($\pm$ SEM) from three independent studies, with each treatment performed in triplicate ($n = 9$ per observation). Bars with the same letter superscripts are not significantly different from one another.

basal apical-to-basolateral Ca transport in the absence of $1,25(OH)_2D_3$ (0.24 ± 0.01 nmol/well per minute; Fig. 3A). However, Cd treatment/higher VDR level enhanced $1,25(OH)_2D_3$-mediated Ca transport in MTVDRA3 by an additional 24% (Fig. 3A). This level of Cd (1 μM) had no effect on phenol red transport in either cell line and did not alter either VDR level, basal Ca transport, or vitamin D-induced Ca transport in the parental Caco-2 cells (Fig. 3).

We also examined how the treatment protocol influenced net calcium transport using MTVDRA3, that is, the difference between apical-to-basolateral and basolateral-to-apical movement of calcium across the monolayer. Compared with untreated control cells (0.073 ± 0.012 nmol/well per minute), net apical-to-basolateral Ca transport was not increased by 28 h of treatment with 1 μM Cd alone. As expected, $1,25(OH)_2D_3$ treatment (100 nM, 24 h) caused a significant 2.5-fold increase in net calcium transport (to 0.184 ± 0.039 nmol/well per minute; $p < 0.05$). Similar to the effect on apical-to-basolateral calcium transport, the combined treatment of Cd and $1,25(OH)_2D_3$ increased net calcium transport an additional 23% above $1,25(OH)_2D_3$ treatment alone ($p < 0.05$). Thus, the net calcium transport data are consistent with the unidirectional apical-to-basolateral calcium transport data.

Figure 4 shows that treatment of MTVDRA3 with 10^{-7} M $1,25(OH)_2D_3$ for 24 h increased calbindin D_{9k} mRNA levels by 80%. As we noted previously, 1 μM Cd pretreatment increased VDR levels by 68%. This was associated with a 76% increase in $1,25(OH)_2D_3$-induced calbindin D_{9k}

mRNA levels (Figs. 4A and 4B) and a doubling of 24-OHase mRNA levels (2.06 ± 0.21 relative increase; Fig. 4A) in MTVDRA3. In parental Caco-2 cells, Cd treatment had no impact on basal or vitamin D-induced increases in 24-OHase or calbindin D_{9k} mRNA levels (data not shown).

DISCUSSION

Our findings directly show that moderate, graded increases in VDR level enhanced three distinct $1,25(OH)_2D_3$-mediated events in Caco-2 cells: expression of 24-OHase mRNA (a transcriptional response), accumulation of calbindin D_{9k} mRNA (a posttranscriptional event[26]), and the physiological process of transcellular calcium transport. These data support the hypothesis that the VDR level is an important factor controlling the intestinal responsiveness to $1,25(OH)_2D_3$. Previously, others have observed higher duodenal VDR mRNA[39] or protein levels[10,40] in genetic hypercalciuric Ca stone-forming rats and they have suggested that higher VDR levels may drive intestinal hyperresponsiveness to $1,25(OH)_2D_3$ in these animals. In addition, estrogen increases both intestinal Ca absorption[12] and intestinal VDR levels in ovariectomized rats.[11] Conversely, studies performed in both rats and humans have shown the existence of age-associated Ca malabsorption and intestinal resistance to vitamin D that could be caused by a decreased VDR level. For example, Horst et al. found that the intestinal unoccupied VDR level drops 75% between 1 and 18 months of age in rats[13] whereas Takamoto et al. showed that the total VDR level falls from 580 to 450 fmol/mg protein between 6 and 24 months of age in rats.[14] A similar age-associated trend toward a reduced intestinal VDR level has been reported in humans.[17] This fall in VDR coincides with a period of reducing efficiency of Ca absorption[8,9,41]; yet it was not clear from these in vivo association studies whether alteration in intestinal VDR level alone was responsible for the observed changes in Ca absorption or intestinal responsiveness to vitamin D. However, recent observations in the VDR knockout mouse suggest that the primary function of the VDR in young growing mice is to maintain high levels of intestinal Ca absorption.[42] Collectively, these in vivo studies and our study using stably transfected Caco-2 cells, a human intestinal cell culture model, strongly support the hypothesis that the VDR level is an important regulator of vitamin D responsiveness in the intestine.

First, we showed that 24-OHase mRNA induction is strongly influenced by the cellular VDR level (Table 2 and Fig. 2). 24-OHase is a vitamin D–dependent gene regulated at the transcriptional level,[43] and it has been shown to be expressed in Caco-2 cells in response to $1,25(OH)_2D_3$[44] (unpublished data). Our current studies show that high levels of VDR increase both the sensitivity and the maximal response of Caco-2 cells to $1,25(OH)_2D_3$ (Table 2) and that similar effects are seen over a wide range of cellular VDR levels and $1,25(OH)_2D_3$ treatments (Table 2; Fig. 2). A similar relationship has been observed in rat osteoblast-like cells treated with either dexamethasone or parathyroid hormone (PTH) to induce the VDR level; 2- to 3-fold higher VDR level is associated with enhanced $1,25(OH)_2D_3$-induced 24-OHase activity[22] and mRNA level.[45] Our data correlating the Caco-2 cell VDR level with induction of the 24-OHase mRNA level (Fig. 2) is consistent with data from Chen et al. who observed that the VDR level was related to gene expression during the various growth phases of mouse osteoblast-like cells.[25]

With the use of stably transfected prostate and leukemic cell lines overexpressing the VDR, others have shown that an increased VDR level enhances the antiproliferative effect of $1,25(OH)_2D_3$.[46,47] Those data suggest that important physiological processes can be influenced by the VDR level. We used a similar approach with an inducible transgene to show that moderate increases in the VDR level would enhance $1,25(OH)_2D_3$-mediated Ca transport in Caco-2 cells. Caco-2 cells possess many characteristics of small intestinal epithelial cells[48–50] and have been shown to be a useful model for $1,25(OH)_2D_3$-mediated intestinal Ca absorption.[26,33,36] Our findings show that in the absence of $1,25(OH)_2D_3$ stimulation, the VDR level has no effect on transepithelial Ca transport across Caco-2 cell monolayers, but after vitamin D treatment, Ca transport is enhanced markedly. First, both the VDR level and the vitamin D–stimulated apical-to-basolateral Ca transport are greater in MTVDRA3 clones than the parental Caco-2 cells (2-fold and 43%, respectively). Second, further increasing the VDR level in MTVDRA3 with Cd pretreatment (by 68%) enhances $1,25(OH)_2D_3$-mediated apical-to-basolateral (24%, Fig. 3) and net (23%) Ca transport. These observations are consistent with the hypothesis that the enterocyte VDR level could be an important determinant of intestinal resistance to $1,25(OH)_2D_3$ associated with aging[6–8] or estrogen deficiency.[11,12] The effect of increased VDR on $1,25(OH)_2D_3$-mediated Ca transport probably is caused by the enhanced level of mRNA for the Ca-binding protein calbindin D_{9k} that we observed (Figs. 4A and 4B). Calbindin D_{9k} has been proposed as the rate-limiting determinant controlling transepithelial Ca transport in the intestine.[51] Our data are consistent with observations showing a correlation between $1,25(OH)_2D_3$-mediated transcellular Ca transport and either calbindin D_{9k} protein[7,52] or mRNA level in the intestine.[33]

We acknowledge several potential limitations to our experimental approach. First, supraphysiological doses of $1,25(OH)_2D_3$ ($\geq 10^{-9}$ M) were used in the experiments reported here. Also, the observations in Caco-2 cells may be different from what occurs in vivo. For example, Caco-2 cells have very low VDR levels (40 fmol/mg protein[50]) even after maximal stimulation of the transgene (250 fmol/mg protein). In contrast, intestinal VDR levels are high in humans and rats (between 500 and 1000 fmol/mg protein[13,18]) and aging is proposed to reduce these levels by only 50%.[13,14,17] Thus, the Caco-2 cells are a VDR-limited system in which we have enhanced capacity, while in vivo, the VDR level may not be limiting in the intestine even after it has fallen by 50%. Additional research using an animal model such as the VDR knockout mouse and comparing wild-type (100% VDR level), heterozygous (50% VDR level) and VDR null mice will be instructive as to whether the cell culture findings in Caco-2 cells we report here apply

in vivo. Finally, although we have shown a direct role for VDR level in the modulation of responsiveness to $1,25(OH)_2D_3$ in Caco-2 cells, other factors also can play an important role in determining cellular vitamin D responsiveness. For example, VDR-mediated transcription is now known to require a large number of coactivators and cofactors (summarized by Freedman[53]). Alterations in the level of p300, SRC1, or specific components of the vitamin D receptor interacting protein (DRIP) complex could theoretically influence VDR function and cellular vitamin D action. In addition, nongenomic actions of $1,25(OH)_2D_3$ have been identified[54] and these may modulate vitamin D action through phosphorylation of the VDR.[55] Because age-associated reductions in nongenomic actions of vitamin D have been reported in the intestine of rats,[56] the importance of this pathway in intestinal resistance to vitamin D cannot be discounted. Thus, further research will be necessary to determine whether additional factors contribute to the altered tissue responsiveness to $1,25(OH)_2D_3$ observed under various physiologically relevant states (e.g., aging and estrogen deficiency).

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