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The Effect of Differentiation on 1,25 dihydroxyvitamin D - Mediated Gene Expression in the Enterocyte-Like Cell Line, Caco-2

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Abstract

We examined 1,25 dihydroxyvitamin D (1,25(OH)₂D₃)-induced expression of 25-hydroxyvitamin D₃ 24-hydroxylase (CYP24) and apical calcium channel (TRPV6) mRNA levels in 2, 9 and 15 day cultures Caco-2 cells that model proliferating, post-proliferative, and differentiated enterocytes. 1,25(OH)₂D₃-induced (10 nM, 8 h) CYP24 and TRPV6 mRNA levels were significantly greater in differentiated and post-proliferative than proliferating Caco-2 cells (> 16X and > 3X, respectively). Neither CYP24 mRNA half-life nor induction of a -298 bp rat CYP24 promoter-luciferase reporter construct (10 nM 1,25(OH)₂D₃, 24 h) were different between proliferating and post-proliferating Caco-2 cells. We next tested whether the blunted response of natural genes to 1,25(OH)₂D₃ in proliferating Caco-2 cells is due to altered chromatin remodeling. VDR and coactivator protein levels do not increase with differentiation but the level of the co-repressor Alien falls by 50% with differentiation. Over-expression of Alien reduced 1,25(OH)₂D₃-induced activity of a minimal VDRE containing promoter-luciferase construct by more than 60% in differentiated Caco-2 cells while siRNA knockdown of Alien in proliferating Caco-2 cells increased 1,25(OH)₂D₃-induced CYP24 mRNA level by 40%. These observations suggest that Alien is a regulator of VDR-mediated gene transcription in Caco-2 cells. In addition, we found that 1,25(OH)₂D₃-induced association of VDR with chromatin and with the CYP24 promoter was lower in proliferating cells. This suggests that decreased recruitment of VDR to vitamin D response elements also contributes to the blunted transcriptional responsiveness to 1,25(OH)₂D₃ in proliferating Caco-2 cells.

Keywords

intestine; Caco-2; differentiation; gene expression; VDR; Alien

Introduction

The intestinal epithelium is a primary target tissue of the biologically active form of vitamin D, 1,25 dihydroxyvitamin D₃ (1,25(OH)₂D₃) (Fleet, 2006). In this tissue, 1,25(OH)₂D₃ stimulates calcium absorption, a critical step in the maintenance of whole body calcium homeostasis (Fleet, 2006). Intestinal calcium absorption occurs via paracellular and transcellular pathways (Bronner, 2003). According to the facilitated diffusion model proposed by Bronner (Bronner et al., 1986), 1,25(OH)₂D₃ increases the efficiency of the

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transcellular pathway for calcium absorption by increasing the abundance of proteins critical for this pathway: e.g. the apical membrane calcium channel transient receptor vanilloid family member 6 (TRPV6), the intracellular calcium binding protein calbindin D_{9k} (CaBPD $_{9k}$), and two basolateral membrane calcium transporters (i.e. the plasma membrane calcium ATPase 1b, PMCA1b, and a Na^+/Ca^{2+} exchanger). 1,25(OH) $_2$ D $_3$ also induces the expression and activity of 25-hydroxyvitamin D $_3$ 24-hydroxylase (CYP24), an enzyme critical for attenuation of 1,25(OH) $_2$ D $_3$ signaling (Tomon et al., 1990).

The actions of 1,25(OH) $_2$ D $_3$ are mediated through the vitamin D receptor (VDR), a ligand-inducible transcriptional factor that is a member of the nuclear receptor superfamily (Haussler et al., 1998). Deletion of VDR from enterocytes reduces expression of TRPV6, PMCA1b and CaBPD $_{9k}$ and causes a 70% reduction in the efficiency of transcellular calcium absorption (Van Cromphaut et al., 2001; Song et al., 2003a). In addition to the VDR, 1,25(OH) $_2$ D $_3$ -stimulated gene transcription requires the retinoid X receptor (RXR) (MacDonald et al., 1993) and transcriptional co-activators e.g. steroid receptor coactivators 1, 2, and 3 (SRC1, SRC2, SRC3) (Featherstone, 2002), histone acetyltransferases (p300 and CBP) (Chan and La Thangue, 2001) and mediator complex subunit 1 (MED1). (Rachez et al., 2000). VDR transcriptional activity can also be inhibited by co-repressors such as the silencing mediator for retinoid/thyroid hormone receptors (SMRT) (Chen et al., 1996), the nuclear hormone receptor-corepressor (NCoR) (Polly et al., 2000), hairless (Hsieh et al., 2003) and Alien (Polly et al., 2000).

Normally, the efficiency of 1,25(OH) $_2$ D $_3$ -regulated transcellular calcium absorption is increased as enterocytes differentiate (Walters and Weiser, 1987; Chan and Atkins, 1983; Bikle et al., 1984). This suggests that proliferating enterocytes may be less sensitive to 1,25(OH) $_2$ D $_3$, however, this has not been carefully studied. In this report we examined how the state of cellular differentiation affects 1,25(OH) $_2$ D $_3$ -stimulated transcriptional activity in Caco-2 cells, a model for absorptive epithelial cells of the small intestine (Pinto et al., 1983) that has been used to study 1,25(OH) $_2$ D $_3$ -stimulated events in enterocytes (Giuliano and Wood, 1991; Fleet and Wood, 1994; Fleet and Wood, 1999; Fleet et al., 2002; Tomon et al., 1990). We found that 1,25(OH) $_2$ D $_3$ -induced gene expression increases as Caco-2 cells differentiate. This observation cannot be explained by changes in the level of VDR, RXR α , or transcriptional co-activators. Instead, the blunted transcriptional responsiveness of proliferating Caco-2 cells is due to higher Alien corepressor levels and to impaired hormone-induced recruitment of VDR to chromatin and vitamin D response elements (VDRE).

Material and methods

Reagents

Unless otherwise noted, all chemicals were obtained from Sigma (St. Louis, MO), the cell culture reagents were obtained from Cambrex (Rockland, ME), and the cell culture plasticware was obtained from Corning-Costar (Cambridge, MA). 1,25(OH) $_2$ D $_3$ was purchased from Biomol International (Plymouth Meeting, PA).

Cell culture

The parental line (HTB-37) and the BBe clone (CRL-2102) of Caco-2 cells were purchased from American Type Cell Culture (ATCC, Rockville, MD) and cultured as described elsewhere (Fleet et al., 2002). The parental Caco-2 cells were studied between passages 24 and 50, whereas the BBe cells were studied between passages 52 and 77. We used Caco-2 cells at three different cell stages: proliferating/50% confluent (2 d cells), post-proliferating/5 days post-confluent (9 d cells), and differentiated/11 days post-confluent (15 d cells). The

treatment solutions containing $1,25(\text{OH})_2\text{D}_3$ or vehicle (ethanol at a final concentration of 0.01%) were prepared in a medium containing 5% FBS for all experiments with the exception of the reporter gene assay, in which the treatment solutions were prepared in serum-free OPTImem medium (Invitrogen, Carlsbad, CA).

RNA Isolation and real-time reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was extracted from cells using TriReagent according to manufacturer's directions (Molecular Research Center, Inc., Cincinnati, OH). Specific transcript levels were analyzed using real-time RT-PCR. The synthesis of cDNA was performed as described elsewhere (Fleet and Wood, 1999). Gene expression levels were determined using SYBR green supermix in a MyIQ real-time PCR system (Bio-Rad, Hercules, CA) following the manufacturer's recommendations. Each PCR reaction was carried out using 0.05 μg of cDNA and 5 pmol each of a forward and reverse primer for the gene of interest. The primer set specificity was verified by both the post-PCR melting curve and the examination of the PCR product by agarose gel electrophoresis (100 V, 30 minutes). Cycling conditions were designed according to MyIQ real-time PCR system manufacturer's guidelines. PCR primers used were: Alien, Forward primer (F) = 5' ATGTGGATTGGAATCAG^{3'}, Reverse (R) = 5' CTGCACTCCGAATATAGGTC^{3'}, 221 bp, $T_A = 54^\circ\text{C}$ (Condon et al., 2003); CBP, F = 5' AGTCCCAGCCTCCACATTC^{3'}, R = 5' CTACAAGCCCTCCACAACTTC^{3'}, 265 bp, $T_A = 58^\circ\text{C}$ (Linja et al., 2004); CYP24, F = 5' CTCATGCTAAATACCCAGGTG^{3'}, R = 5' TCGCTGGCAAAACGCGATGGG^{3'}, 299 bp, $T_A = 54^\circ\text{C}$; MED1, F = 5' AAACCATTCAAGCCGAC^{3'}, R = 5' CGTTCCTGTGATTGTC^{3'}, 259 bp, $T_A = 54^\circ\text{C}$ (Pineda, I et al., 2004); GAPDH, F = 5' TCACCATCTTCCAGGAGCG^{3'}, R = 5' CTGCTTACCACCTTCTTGA^{3'}, 571 bp, $T_A = 54^\circ\text{C}$ (Song et al., 2003b); NcoR, F = 5' GTGGTGAGACACGAAGAGAG^{3'}, R = 5' TGGAGTACTGCTGAGCATC^{3'}, 281 bp, $T_A = 54^\circ\text{C}$ (Dunlop et al., 2004); p300, F = 5' CCTGAGTAGGGCAACAAGA^{3'}, R = 5' GTGTCTCCACATGGTGCTTG^{3'}, 352 bp, $T_A = 57^\circ\text{C}$ (Linja et al., 2004); RXR α , F = 5' GAGGCCTACTGCAAGCACAAG^{3'}, R = 5' CAGGCGGAGCAAGAGCTTAG^{3'}, 65 bp, $T_A = 61^\circ\text{C}$; SMRT, F = 5' CGCAACTGGTCGGCCA^{3'}, R = 5' GCTGCAAGATCTCATCGAGGT^{3'}, 109 bp, $T_A = 54^\circ\text{C}$ (Keeton and Brown, 2005); SRC1, F = 5' ATGGTGAGCAGAGGCATGACA^{3'}, R = 5' AAACGGTGATGCTCATGTTG^{3'}, 348 bp, $T_A = 54^\circ\text{C}$ (Linja et al., 2004); SRC2, F = 5' TAATGCACAGATGCTGGCC^{3'}, R = 5' TCTGTGTATGTGCCATTCGG^{3'}, 313 bp, $T_A = 61^\circ\text{C}$ (Linja et al., 2004); SRC3, F = 5' CGTCCTCCATATAACCGAGC^{3'}, R = 5' TCATAGGTTCCATTCTGCCG^{3'}, 255 bp, $T_A = 57^\circ\text{C}$ (Linja et al., 2004); TRPV6, F = 5' TTCCTGCGGGTGAAGACAGGCA^{3'}, R = 5' ACGCAGGTCTCTCCTCAGGGTCCC^{3'}, 235 bp, $T_A = 63^\circ\text{C}$; VDR, F = 5' CCTTACCATTGGACGACATG^{3'}, R = 5' CGGCTTTGGTCACGTCAC^{3'}, 78 bp, $T_A = 57^\circ\text{C}$ (Lou et al., 2005). The relative expression of each target gene was determined using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). Expression of specific genes was normalized to the level of glyceraldehyde 3' phosphate dehydrogenase (GAPDH) mRNA levels in each sample. The GAPDH gene was not influenced by either Caco-2 cell differentiation status or $1,25(\text{OH})_2\text{D}_3$ treatment (data not shown). In the time-course experiment the fold change was calculated as a ratio between GAPDH-normalized levels found for a given hormone dose and the amount found in the vehicle-treated cells.

CYP24 mRNA half-life in proliferating (2 d) and differentiated (15 d) Caco-2 cells

Following a 16 h pretreatment with 10 nM $1,25(\text{OH})_2\text{D}_3$ to induce CYP24 mRNA, proliferating (2 d) and differentiated (15 d) Caco-2 cells were treated with actinomycin D (4 $\mu\text{g}/\text{ml}$) to block synthesis of new mRNA. Cells were collected at various time points after adding the actinomycin D (0, 1, 2, 3, 4, 5, 6, 7, 8, and 12 h). RNA was extracted and CYP24 and GAPDH mRNA were quantified using real time RT-PCR. The level of CYP24 mRNA

at the time the actinomycin D was added (t=0 h) was used as the reference point (=100%). The experiment was repeated twice using three replicates per time-point.

Western Blot Analysis

Nuclear extracts were prepared using a Nuclear Extract Kit (Active Motif, Carlsbad, CA) as per the manufacturer's instructions. The total protein content of samples was determined using Bio-Rad Protein Assay Dye Reagent Concentrate and bovine serum albumin as a protein standard. 50 µg nuclear extracts were separated by SDS-PAGE on 12.5 % or 7.5% Tris-Glycine gels (Bio-Rad, Hercules, CA) at 40 mA constant current for 90 minutes at 4°C. For medium size proteins (e.g. VDR, RXR, TFIIB, Alien) samples were transferred from the gels to Immobilon-P membranes (Millipore, Bedford, MA) using standard Towbin buffer (25 mM Tris-HCl pH 8.0, 192 mM Glycine and 20% (v/v) methanol) at 83 V for 2.5 h. For high molecular weight proteins (i.e. NCoR and SMRT) samples were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) using modified Towbin buffer (25 mM Tris, 192 mM glycine, 10% methanol (v/v), and 0.01% SDS) at 30 V overnight. Transfers were performed at 4°C with continuously mixing of the transfer solution. The following primary antibodies were used for immunodetection: rat anti-chick VDR (Ab9A7), rabbit-anti SRC1 (Affinity Bioreagents, Golden, CO), rabbit anti-human RXR α , rabbit-anti-mouse MED1 (TRAP220), mouse anti-human CBP, rabbit anti-human TFIIB (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-Alien (PEPAK, a gift of Dr. A. Baniahmad, University of Jena, Germany), mouse anti-human p300, rabbit anti-SMRT, rabbit anti-NCoR (Upstate, Charlottesville, VA).

With the exception of VDR, the immunodetection was conducted using the following protocol. After protein transfer, the membranes were incubated overnight in blocking solution (5% non-fat, dry milk, 0.1% Tween, 10 mM Tris HCl, 150 mM NaCl, pH 7.4) at 4°C with agitation. After three 5-minute washes with 50 ml TBS-T buffer (10 mM Tris HCl, 150 mM NaCl, 0.1% Tween, pH 7.4) the samples were incubated for 1 h with a primary antibody solution at room temperature. A 1:2,000 dilution of primary antibody prepared in blocking solution was used (1:6,000 dilution for anti-SMRT antibody). Next, membranes were washed and incubated with a 1:5,000 dilution of horseradish-peroxidase-conjugated IgG secondary antibody (Invitrogen, Carlsbad, CA). After three washes the specific binding was detected using the Pierce ECL Western blotting substrate (Pierce, Rockford, IL).

To minimize non-specific binding, VDR immunodetection was carried out using three antibodies: rat anti-chick VDR antibody Ab9A7 (1:2,000), rabbit anti-rat IgG (1:5,000 dilution, Chemicon, Temecula, CA), and horseradish peroxidase-conjugated anti-rabbit IgG, (1:20,000 dilution, Amersham Biosciences, Piscataway, NJ). The membrane was incubated with each antibody for 1 h at room temperature. Following the incubation of membranes with blocking solution and with each antibody solution, membranes were washed once for 15 minutes and twice for 5 minutes with TBS-T buffer. Specific binding was detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

Chemiluminescence was detected by exposure of the membranes to X-ray film. The band intensities were recorded by densitometry using a Bio-Rad imaging densitometer and quantified using Quantity One software (Bio-Rad, Hercules, CA). Specific protein levels were normalized within each sample using TFIIB protein levels.

Transient transfection and luciferase assays

Caco-2 cells seeded in 24-well plates were transfected with 0.33 µg rat CYP24 promoter (-298 to +74 bp) (Kerry et al., 1996) or VDRE₃TK-Luc promoter (three osteopontin VDRE-thymidine kinase-firefly luciferase reporter gene; a gift from Dr. S. Nagpal, Lilly Research

Labs, Indianapolis, IN). To correct for the transfection efficiency we used 3.3 ng of Renilla luciferase expressing control vectors (pRL-CMV renilla for CYP24-Luc promoter, and TK-Renilla for VDRE₃TK-Luc promoter). The cells were transfected using a 1:5:5 ratio of DNA:Plus:Lipofectamine reagent for proliferating cells and a 1:10:10 ratio of DNA:Plus:Lipofectamine reagent for post-proliferating cells (Invitrogen, Carlsbad, CA). After a 3-h incubation in transfection medium, cells were switched to OPTImem medium supplemented with 10% FBS. Treatments were initiated following a 12-h recovery. Firefly and renilla luciferase activity were measured using a TD20/20 Luminometer (Sunnyvale, CA) following the procedure outlined in the Dual Luciferase reporter assay manual (Promega, Madison, WI).

RXR α overexpression

Human RXR α expression vector (Solomon et al., 1999) was a gift from Dr. Kremer (McGill University, Montreal, Canada). Proliferating Caco-2 cells were transfected with 0.33 μ g -298 to +74 rat CYP24 promoter luciferase, 3.3 ng PRL-null renilla and 0.4 ng, 4 ng, 40 ng of RXR α or backbone plasmid per well. Triplicate wells were treated with vehicle or 10 nM 1,25(OH)₂D₃ for 24 h and harvested. CYP24 promoter activity was normalized for variations in renilla expression to control for transfection efficiency.

Alien overexpression and RNA interference

In experiment 1, proliferating and differentiated Caco-2 cells were transfected with 0.5 μ g pcDNA3-Alien plasmid (a gift from Dr. A. Baniahmad, University of Jena, Germany) or control plasmid with 0.33 μ g VDRE₃TK-Luc promoter and 3.3 ng TK-renilla per well. 24 h after transfection, cells were treated with vehicle or 0.5 nM, 5 nM 1,25(OH)₂D₃ for 24 hours and reporter gene analysis was conducted.

In experiment 2, small interfering RNA (siRNA) specific for Alien (ON-TARGET plus Duplex J-004126-10-0010) was purchased from Dharmacon, Inc. (Lafayette, CO). A non-targeting siRNA (siControl Non-Targeting siRNA #3, D-001210-03-20, Dharmacon, Inc., Lafayette, CO) was used as a control. Caco-2 cells seeded at 30-50% in antibiotics free media were transfected with Alien siRNA or control siRNA at a final concentration of 100 nM using oligofectamine (Invitrogen, Carlsbad, California). 48 h after siRNA transfection, cells were treated with 10 nM 1,25(OH)₂D₃ for 2 h and mRNA levels of Alien, CYP24 and GAPDH were examined by real time PCR after reverse transcription.

Crude chromatin assay

Proliferating (2 d) and the differentiated (15 d) Caco-2 cells treated with 10 nM 1,25(OH)₂D₃ or vehicle for 1 h were used to obtain soluble and chromatin fractions as described previously (Ismail et al., 2004). The volume of chromatin and soluble fraction obtained for each treatment condition was equalized and VDR and RXR α content are analyzed by Western blot analysis.

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as described (Meyer et al., 2006) with some modifications. Proliferating and differentiated Caco-2 cells were fixed with 1.5% formaldehyde at room temperature for 15 minutes. After neutralization with 0.125 M glycine, cells were washed with ice-cold PBS twice and scraped from dishes. Cell nuclei were extracted and then subjected to sonication to produce chromatin fragments from 200 bp to 1000 bp using a Fisher model 60 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA). The sonicated extract was diluted with buffer (16.7 mM Tris-HCl, pH 8.1; 150 mM NaCl; 0.01% SDS; 1.1% Triton X-100; 1.2 mM EDTA) and pre-cleared for 1 h with 50%

slurry of Fastflow protein G (Amersham Biosciences, Piscataway, NJ) at 4°C. Equal amounts of chromatin from proliferating and differentiated Caco-2 cells were used in immunoprecipitations. All immunoprecipitations were performed overnight with 2 µg rabbit IgG (Upstate, Charlottesville, VA), VDR antibody or RXR antibody (Santa Cruz Biotechnology, Santa Cruz, CA), at 4°C. Immune complexes were collected with 50% slurry of protein G for 1 h at 4°C. The immunocomplexes were washed and the cross-links were reversed overnight in 1% SDS, 0.1 M NaHCO₃, and 0.2 M NaCl at 65 °C. DNA fragments were purified using QIAGEN QIAquick Spin Kits (Valencia, CA) and then subjected to PCR using primers reported in the literature to amplify fragments of the proximal human CYP24A1 promoter region (-252 bp to -51 bp) (Meyer et al., 2006). The PCR products were resolved on 2.5% agarose gels and visualized using ethidium bromide. A small amount of DNA acquired after pre-clearing (2% of chromatin for an immunoprecipitation) was saved as input to assess the initial presence of the gene fragments.

Statistical analysis

Data is reported as the means ± SEM. The treatment effects in each experiment were compared by one- or two-way ANOVA using the SYSTAT statistical software package (SYSTAT 7.0, Chicago, IL). Pairwise comparisons were conducted when appropriate using Fisher's Protected LSD. Differences between means were considered significant at $p < 0.05$.

Results

1,25(OH)₂D₃-induced expression of TRPV6 and CYP24 increases with Caco-2 cell differentiation

We examined the dose-dependent changes in TRPV6 and CYP24 mRNA levels in proliferating (2 d) and post-proliferating (9 d and 15 d) parental Caco-2 cells treated with 0, 1, 10 or 100 nM 1,25(OH)₂D₃ for 8 h. Both CYP24 and TRPV6 gene expression were induced in Caco-2 cells treated with 1,25(OH)₂D₃ in a dose responsive manner regardless of the state of differentiation (Figure 1A, B). The lowest dose of 1,25(OH)₂D₃ that significantly induced both CYP24 and TRPV6 gene expression at 8 h was 10 nM. At both 10 and 100 nM 1,25(OH)₂D₃, the induction of TRPV6 and CYP24 mRNA was higher in post-proliferating (9 d and 15 d) than proliferating Caco-2 cells. For example, at 10 nM 1,25(OH)₂D₃ the induction of CYP24 mRNA was 16 ($p=0.03$) and 29 ($p=0.02$) times greater in 9 d and 15 d Caco-2 cells, respectively, compared to proliferating Caco-2 cells. Similarly, the induction of TRPV6 mRNA was 3.3 ($p=0.015$) and 5.6 ($p=0.015$) times greater for 9 d and 15 d cultures of Caco-2 cells than 2 d cultures. Similar patterns of CYP24 and TRPV6 mRNA induction in response to 10 nM 1,25(OH)₂D₃ treatment were also observed in the BBe clone of Caco-2 cells (data not shown).

The change in 1,25(OH)₂D₃-mediated CYP24 mRNA induction during Caco-2 differentiation is not due to altered mRNA half-life

There are two explanations for why 1,25(OH)₂D₃-induced expression of CYP24 mRNA is lower in proliferating cells: the efficiency of VDR-mediated transcription of CYP24 genes is reduced or the half-life of CYP24 message is shorter in proliferating cultures. To distinguish between these alternatives we determined CYP24 mRNA half-life in 2 d and 15 d cultures of Caco-2 cells (Figure 2). The rate of CYP24 mRNA decay was significantly slower in proliferating as compared to differentiated Caco-2 cells: 4.18% per h ($t_{1/2} = 9.3$ h) and 8.37% per h ($t_{1/2} = 5.9$ h), respectively. This demonstrates that destabilization of CYP24 mRNA messages does not account for the low level of 1,25(OH)₂D₃-induced CYP24 mRNA accumulation we observe in proliferating Caco-2 cells.

Changes in VDR and RXR levels during Caco-2 differentiation

Proliferating and post-proliferating Caco-2 cells have comparable levels of VDR mRNA and protein while the levels of RXR α mRNA and protein were significantly higher in 9 d and 15 d cells (Figure 3A, 3B). To evaluate whether lower RXR α protein levels contributes to reduced induction of CYP24 mRNA in proliferating Caco-2 cells, we co-transfected varying amounts of RXR α and -298 to +74 bp rat CYP24 promoter-luciferase construct into proliferating Caco-2 cells. As shown in Figure 3C, co-transfection of RXR α did not enhance 1,25(OH) $_2$ D $_3$ -induced CYP24 promoter activity.

1,25(OH) $_2$ D $_3$ -induced reporter gene activity in proliferating and post-proliferating Caco-2 cells is similar

2 day and 9 day Caco-2 cells were transfected with a rat -298 to +74 bp CYP24 promoter-luciferase vector and treated for 8 h with either 10 nM 1,25(OH) $_2$ D $_3$ or vehicle and reporter gene activity was assessed. As shown in Figure 4, activation of the CYP24 promoter was not significantly different at the two cells stages (induction = 5-fold for both proliferating and post-proliferative cells, data not shown). This suggests that all of the components necessary for adequate 1,25(OH) $_2$ D $_3$ -mediated transcription are present in the two cell stages.

Transcriptional cofactor expression in Caco-2 cells

We hypothesized that reduced induction of CYP24 in proliferating cells was due to events necessary for modulation of the genes in their natural context of chromatin (i.e. coactivators and corepressors). To test the hypothesis we examined mRNA levels for VDR, RXR α , coactivators (SRC1, SRC2, SRC3, p300, CBP, MED1) and corepressors (NcoR, SMRT, and Alien) in the RNA from 2 d, 9 d, and 15 d parental Caco-2 cells. We found the mRNA levels for all the coactivators involved in 1,25(OH) $_2$ D $_3$ -genomic actions were significantly lower (by 25-80%) in post-proliferating (9 d and 15 d) than proliferating Caco-2 cells (Figure 5A, SRC2 and SRC3 data not shown). The mRNA level of SMRT, NCoR and Alien corepressors in differentiated Caco-2 cells was reduced by 56%, 46%, and 30%, respectively, compared to proliferating Caco-2 cells (Figure 5B).

Western blot analysis of nuclear extracts indicates that abundance of coactivators SRC-1 and p300, and corepressors SMRT and NCoR were not different between 2 d and 15 d Caco-2 cells (Figure 5C). In contrast, MED1 and Alien protein levels decreased by over 50% with differentiation (Figure 5C). We further examined nuclear extracts from 2 day, 9 day and 15 day Caco-2 cells and found that both 9 day and 15 day Caco-2 cells have 50% lower Alien protein levels than 2 day cells (data not shown).

Alien plays a role in VDR-mediated gene expression

To determine whether higher Alien levels in proliferating Caco-2 cells could suppress 1,25(OH) $_2$ D $_3$ -mediated gene expression we conducted several experiments. First, we tested whether Alien over-expression influences basal or 1,25(OH) $_2$ D $_3$ -induced transcription in differentiated Caco-2 cells with lower Alien protein levels. As expected, 1,25(OH) $_2$ D $_3$ induced a dose-dependent increase in VDRE $_3$ TK promoter activity (2.2 and 4.3-fold higher with 0.5 nM and 5 nM 1,25(OH) $_2$ D $_3$, Figure 6A) Co-transfection with Alien expression vector decreased 1,25(OH) $_2$ D $_3$ regulated VDRE $_3$ TK promoter activity by 55% at the 0.5 nM dose and 70% at the 5 nM 1,25(OH) $_2$ D $_3$ dose (Figure 6A). In contrast, over-expression of Alien had no effect on 1,25(OH) $_2$ D $_3$ -induced promoter activity in proliferating Caco-2 cells that already express high levels of Alien (Figure 6B). Similar results were obtained using the -298 to +74 bp rat CYP24 promoter-luciferase construct (data not shown).

To further investigate role of Alien in basal and 1,25(OH) $_2$ D $_3$ regulated gene expression, we used siRNA to reduce Alien mRNA in proliferating Caco-2 cells. 48 h after siRNA

transfection Alien mRNA levels were reduced by 72% (data not shown). Alien siRNA transfection significantly increased 1,25(OH)₂D₃-regulated CYP24 mRNA by 43% (Figure 6C).

The state of cellular differentiation affects binding of VDR and RXR α to chromatin and the CYP24 promoter

Levels of VDR and RXR α were analyzed in the chromatin and soluble fractions isolated from 2 d and 15 d cultures of Caco-2 cells following 1h treatment with 10 nM 1,25(OH)₂D₃ or vehicle. Whereas VDR is evenly distributed between the chromatin and soluble fractions from vehicle-treated cells, VDR is redistributed to the chromatin fraction in cells treated with the hormone (Figure 7A). The stage of cell differentiation significantly influenced this effect; chromatin from 1,25(OH)₂D₃-treated 15 d cells had four-fold more VDR than chromatin isolated from 1,25(OH)₂D₃-treated 2 d cultures. In contrast, the distribution of RXR α between the chromatin and soluble fraction was not influenced by the presence of 1,25(OH)₂D₃.

To investigate whether differentiation changes binding of the VDR-RXR heterodimer to DNA at specific gene promoters, we performed chromatin immunoprecipitation assays with samples isolated from 1,25(OH)₂D₃ treated proliferating and differentiated Caco-2 cells. 1,25(OH)₂D₃-induced the association of both VDR and RXR with the CYP24 promoter after 1h in differentiated Caco-2 cells but not in proliferating Caco-2 cells (Figure 7B). These data indicate that the reduced hormone-induced binding of VDR to chromatin may contribute to the blunted response of proliferating Caco-2 cells to 1,25(OH)₂D₃.

Discussion

1,25(OH)₂D₃-regulated expression of CYP24 and TRPV6 mRNA has been previously characterized in mouse duodenum (Song et al., 2003b), in rat IEC cells (Armbrecht et al., 2001), in the colon tumor cell line LS180 (Meyer et al., 2006) and in differentiated Caco-2 cells (Tomon et al., 1990; Taparia et al., 2006; Fleet et al., 2002). The induction of these messages by 1,25(OH)₂D₃ is an actinomycin D-sensitive transcriptional event (Tomon et al., 1990; Taparia et al., 2006) that is VDR-dependent (Van Cromphaut et al., 2001; Song et al., 2003b; Meyer et al., 2006; Vaisanen et al., 2005). Here we extend these findings by showing that the state of cellular differentiation significantly influences 1,25(OH)₂D₃ action in the enterocyte-like cell line, Caco-2. Induction of both CYP24 and TRPV6 mRNA by 1,25(OH)₂D₃ was significantly blunted in proliferating Caco-2 cells as compared to cells that had completed the proliferative phase and had differentiated (i.e. 9 d and 15 d cells, Figure 1).

In our initial analysis we tested the simply hypothesis that differentiation-dependent changes in VDR or RXR protein levels account for the effect we observed. We have previously shown that 1,25(OH)₂D₃-stimulated CYP24 mRNA accumulation is sensitive to VDR level in Caco-2 cells over-expressing VDR (Shao et al., 2001) and in mice with only one active VDR allele that express 50% less VDR than normal mice (Song and Fleet, 2007). However, VDR levels were not lower in proliferating Caco-2 cells (Figure 3) nor are they different between proliferating crypt cells and differentiated villus enterocytes in the human intestine (Colston et al., 1994). Previous research shows that RXR α augments VDR-dependent activation of a VDRE-containing promoter in ROS 17/2.8 cells (MacDonald et al., 1993). However, although we observed that RXR α protein levels are 50% lower in proliferating Caco-2 cells, RXR α over-expression had no effect on 1,25(OH)₂D₃-induced reporter gene activity in Caco-2 cells.

Our data also do not support a role for reduced mRNA stability as the mechanism accounting for lower accumulation of CYP24 mRNA in 1,25(OH)₂D₃ treated proliferating Caco-2 cells. Zierold et al., (Zierold et al., 2003) had previously shown that the half-life for the CYP24 mRNA in the presence of 1,25(OH)₂D₃ was 5.5 h in AOK-B50 kidney cells while Koyama et al., (Koyama et al., 1994) recorded a half-life of less than 3 h in proliferating IEC-6 cells. We found that the CYP24 mRNA was actually more stable in proliferating Caco-2 cells (half-life=9.3 h) than differentiated cells (half-life = 5.9 h).

Previously Oda et al. (Oda et al., 2003) found that differences exist in the major co-activators associated with VDR in proliferating and differentiated keratinocytes. In addition, elevated levels of the corepressors NCoR (Banwell et al., 2006) and SMRT (Khanim et al., 2004) can attenuate 1,25(OH)₂D₃ responsiveness in breast and prostate cancer cells, respectively. Our data show that while mRNA levels for all of the co-activators we examined are down-regulated during Caco-2 cell differentiation (Figure 5A), none of these proteins were significantly reduced with differentiation. While NCoR or SMRT protein levels were not higher in proliferating Caco-2 cells, we found that Alien protein levels were more than 50% higher in proliferating cells than post-proliferative Caco-2 cells (Figure 5C). Polly et al. (Polly et al., 2000) previously showed that Alien over-expression repressed induction of a VDRE_{4x}-luciferase reporter gene construct by 1,25(OH)₂D₃. Consistent with a repressive role for Alien, we found that Alien over-expression significantly reduced 1,25(OH)₂D₃-induced VDRE-promoter activity in differentiated Caco-2 cells and that inhibition of Alien expression increased 1,25(OH)₂D₃-induced CYP24 expression in proliferating Caco-2 cells. Collectively, our data suggest that Alien is a co-repressor of VDR-mediated gene transcription in the Caco-2 cell and that it contributes to the blunted transcriptional responsiveness of proliferating Caco-2 cells to 1,25(OH)₂D₃. Since Alien also associates with other nuclear receptors, e.g. androgen receptor (Moehren et al., 2007) and thyroid receptor (Dressel et al., 1999), higher Alien levels in proliferating Caco-2 cells may also influence the transcriptional response induced by other hormones as well.

Although Alien suppresses 1,25(OH)₂D₃ action in proliferating enterocytes, the Alien effect is more modest than the impact we observed for cell stage on CYP24 and TRPV6 mRNA levels. Our last experiment suggests an additional mechanism to account for the reduced responsiveness of proliferating Caco-2 cells to 1,25(OH)₂D₃. We found that 1,25(OH)₂D₃-induced accumulation of VDR on chromatin and on the CYP24 gene promoter was significantly lower in these cells compared to differentiated Caco-2 cells (Figure 7). This suggests that either ligand-induced nuclear import of VDR or the movement of VDR within the nucleus to DNA response elements is reduced in proliferating Caco-2 cells. 1,25(OH)₂D₃-mediated transcriptional responses are initiated in the cytoplasm when ligand-induced heterodimerization of VDR with RXR leads to nuclear translocation (Racz and Barsony, 1999; Prufer and Barsony, 2002). However, our previous data shows that Caco-2 cell differentiation does not influence the ratio of cytoplasmic to nuclear VDR nor does it alter ligand-induced nuclear import of VDR (Klopot et al., 2006). This suggests that in proliferating Caco-2 cells, VDR is capable of entering the nucleus after binding ligand but that once there, it is less able to associate with DNA, i.e. intranuclear movement is impaired. Unfortunately, the regulation of intranuclear VDR movement has not been studied. In contrast, others have shown that the ligand-induced mobility of glucocorticoid receptors within the nucleus is dependent on a molecular chaperones including hsp90, hsp70, Ydj-1, Hop, and p23 (Elbi et al., 2004). A role for molecular chaperones in ligand-dependent VDR gene transcription was recently supported by Angelo et al., (Angelo et al., 2008) who showed that 1,25(OH)₂D₃-mediated gene expression is impaired by the hsp90 inhibitor geldanamycin as well as siRNA against hsp90β in proliferating Caco-2 cells. While this is consistent with the hypothesis that hsp90 regulates intranuclear trafficking of the VDR to VDREs, additional experiments are needed to fully test this hypothesis.

In summary, our studies provide strong evidence that the state of cellular differentiation influences the regulation of VDR target genes in Caco-2 cells. This is at least partly due to an inhibitory effect of Alien on the induction of target genes by 1,25(OH)₂D₃ in proliferating cells. In addition, our data also show that ligand-induced association of VDR with DNA is impaired in proliferating Caco-2 cells and we hypothesize that this is due to changes in intranuclear VDR movement.

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Abbreviations

1,25 (OH)₂D₃	1, 25 dihydroxyvitamin D ₃
CaBPD_{9k}	calbindin D _{9k}
CBP	CREB (cAMP response element binding protein)-binding protein
CYP24	25-hydroxyvitamin D ₃ 24-hydroxylase
DMEM	Dulbecco's Modified Eagle Medium
DRIP(MED1)	Vitamin D Receptor-Interacting Protein
EDTA	Ethylenediamine-tetraacetic acid disodium salt
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid tetrasodium salt
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3' phosphate dehydrogenase
HAT	histone acetyltransferase
HDAC	histone deacetylase
NCoR	nuclear hormone receptor-corepressor
p300	E1A binding protein p300
PIPES	Piperazine-1,4-bis(2-ethanesulfonic acid)
PMCA1b	plasma membrane calcium ATPase 1b
RT-PCR	reverse transcriptase polymerase chain reaction
RXR	retinoid X receptor
Sin3A	histone deacetylase complex subunit Sin3a
SMRT	silencing mediator for retinoid/thyroid hormone receptors
SRC1	2 and 3, steroid receptor coactivator 1, 2, and 3
TBP	TATA binding protein
TRPV6	transient receptor vanilloid family member 6
VDR	vitamin D receptor
VDRE	vitamin D responsive element

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Figure 1.

The induction of CYP24 and TRPV6 mRNA by 1,25(OH)₂D₃ in proliferating (2 d), post-proliferative (9 d), and differentiated (15 d) Caco-2 cells. Parental Caco-2 cells were treated with 0, 1, 10 and 100 nM 1,25(OH)₂D₃ for 8 h (A). Total RNA was collected and analyzed by real time RT-PCR for CYP24 mRNA (left graph) or TRPV6 mRNA (right graph). A representative gel showing the effects of differentiation on the ability of 1,25(OH)₂D₃ (10 nM, 8 h) to induce CYP24 and TRPV6 mRNA is shown in panel (B). TRPV6 and CYP24 mRNA levels were normalized to GAPDH expression within each sample. Data in graphs are expressed as mean±SEM of the fold change above non-treated levels. Values with an asterisk (*) are significantly different from vehicle-treated controls (p<0.05). The values with # are significantly different from 2 d value at the same time point or dose (p<0.05).

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Figure 2.

Effect of cell stage on CYP24 mRNA stability. 2 and 15 d Caco-2 cells were treated with 1,25(OH)₂D₃ (10 nM, 16 h) followed by treatment with 4 µg/ml of actinomycin D to arrest transcription. Cells were harvested hourly until 8 h and then again at 12 h. Total RNA was collected and analyzed by real time RT-PCR for CYP24 and GAPDH mRNA expression. The data was described by linear regression for the two groups. The half-lives calculated from the linear regression were 9.3 h and 5.9 h for 2 d and 15 d cells, respectively.

Figure 3.

Effect of cell stage on the VDR and RXR α levels in Caco-2 cells. **(A)** The changes in VDR and RXR α mRNA levels during Caco-2 cell differentiation. The mRNA levels of VDR and RXR α were analyzed in proliferating (2 d), post-proliferative (9 d) and differentiated (15 d) Caco-2 cells using real time RT-PCR analysis. The mRNA levels were normalized to GAPDH mRNA levels and expressed relative to the amount found in 2 d cells. The values with an asterisk (*) are significantly different from 2 d levels ($p < 0.05$). **(B)** Changes in VDR and RXR α protein levels during Caco-2 cell differentiation. The levels of VDR and RXR α were determined in nuclear extracts prepared from 2 d, 9 d, and 15 d cultures of parental Caco-2 cells using Western blot analysis. The figure is representative of data from three

independent experiments. (C) RXR α does not affect 1,25(OH) $_2$ D $_3$ dependent expression of VDRE containing reporter gene construct. Caco-2 cells were transfected with 0.33 μ g -298 to +74 rat CYP24 promoter luciferase, 3.3 ng PRL-null renilla and varying amounts of RXR α (0.4 ng, 4 ng, 40 ng) or backbone plasmid per well. Triplicate wells were treated with vehicle or 10 nM 1,25(OH) $_2$ D $_3$ for 24 h and harvested. CYP24 promoter activity was normalized for variations in renilla expression to control for transfection efficiency. Fold induction in comparison with vehicle-treated controls is calculated and presented as means $\pm$ SEM. (n=3)

Figure 4.

Effect of 1,25(OH)₂D₃ on induction of -298 to +74 rat CYP24 promoter-luciferase reporter gene activity in proliferating (2 d) and differentiating (9 d) Caco-2 cells. Cells were transfected with the reporter gene and a TK-renilla control vector, treated for 8 hours with 10 nM 1,25(OH)₂D₃ or vehicle and promoter activity was assessed by measuring luciferase activity. CYP24 promoter activity was normalized for variations in renilla expression to control for transfection efficiency. Reporter gene activity was set at 1 for vehicle treated cells at both cell stages. Bars represent the means $\pm$ SEM (n = 3). Bars with an asterisk (*) indicates significant difference from vehicle treatment (P < 0.05).

Figure 5.

Changes in co-activator mRNA (**A**), co-repressor (**B**) mRNA and protein (**C**) levels during Caco-2 cell differentiation. The mRNA levels of cofactors were analyzed in proliferating (2 d), post-proliferating (9 d), and differentiated (15 d) Caco-2 cells using real time RT-PCR analysis. The mRNA levels were normalized to GAPDH mRNA levels. The expression of each cofactor in 9 and 15 d cells was expressed relative to 2 d values (relative value = 1). Symbols represent the mean $\pm$ SEM (n=3). * Value is significantly different from 2 day mRNA level ($p < 0.05$). The protein levels of co-regulators were determined in nuclear extracts prepared from 2 d and 15 d cultures of parental Caco-2 cells using Western blot analysis.

Figure 6.

Impact of Alien on VDR-mediated gene transcription. **(A)** The effect of Alien overexpression on induction of VDRE_{3x}TK-Luc promoter activity in 15 day Caco-2 cells. Cells were co-transfected with 0.33 μ g VDRE_{3x}TK-Luc, 3.3 ng TK-renilla and 0.5 μ g Alien over-expressing vector (Alien pcDNA3) or control vector (pcDNA3) per well, reporter gene activity was examined in cells treated with 0, 0.5 nM or 5 nM 1,25(OH)₂D₃ for 24 h. **(B)** The effect of Alien overexpression on induction of VDRE_{3x}TK-Luc promoter activity in proliferating Caco-2 cells. Cells were co-transfected with 0.33 μ g VDRE_{3x}TK-Luc, 3.3 ng TK-renilla and 0.5 μ g Alien over-expressing vector (Alien pcDNA3) or control vector (pcDNA3) per well, reporter gene activity was examined in cells treated with 0, 0.5 nM or 5

nM $1,25(\text{OH})_2\text{D}_3$ for 24 h. (C) The effect of Alien knockdown on CYP24 mRNA induction in proliferating Caco-2 cells. Cells were transfected with control or Alien siRNA, after 48 h, cells were treated with vehicle or 10 nM $1,25(\text{OH})_2\text{D}_3$ for 2 h. CYP24 mRNA levels were normalized to GAPDH mRNA levels. Bars represent the mean $\pm$ SEM (n=3). Bars with different letter superscripts are significantly different ($P<0.05$).

Figure 7.

Association of VDR and RXR with chromatin (**A**) and the CYP24 promoter (**B**) in proliferating (2 d) and differentiated (15 d) Caco-2 cells. (**A**) Cells were treated for 1 h with 10 nM 1,25(OH)₂D₃ or vehicle after which soluble (S) and chromatin (Ch) fractions prepared and analyzed for VDR and RXRα levels by Western blot analysis. The data in the figure is representative of three independent experiments. (**B**) Chromatin immunoprecipitation experiments were performed with IgG, VDR, and RXR antibodies using an identical amount of chromatin from proliferating or differentiated Caco-2 cells that had previously been treated with 100 nM 1,25(OH)₂D₃ or vehicle for 1h. PCR was performed with primers amplifying the proximal promoter region of the CYP24 gene.