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ORIGINAL CONTRIBUTION

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1,25-Dihydroxyvitamin D and 25-hydroxyvitamin D – mediated regulation of TRPV6 (a putative epithelial calcium channel) mRNA expression in Caco-2 cells

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Summary *Background* TRPV6 is a member of the vanilloid subfamily of transient receptor potential (TRP) proteins and likely functions as an epithelial calcium channel in calcium-transporting organs, such as the intestine, kidney, and placenta. TRPV6 mRNA expression is strongly regulated by 1,25-dihydroxyvitamin D (1,25VD), the active hormonal form of vitamin D, in intestine and in Caco-2 cells, a human colon cancer cell line. *Aim of the study* The aim of the present study was to characterise the mode of regulation of the 1,25VD-mediated TRPV6 mRNA expression and to test the effect of the precursor of 1,25VD namely 25 hydroxyvitamin D (25 VD) on TRPV6 mRNA expression in Caco-2 cells. *Methods* Caco-2 cells were treated in a 2 x 2 format with 1,25VD and the transcriptional inhibitor actinomycin D (AD, 4 μg/ml), and also with translational inhibitor cycloheximide (CHX, 10 μg/ml) after 14 days in culture and TRPV6 mRNA levels were determined using reverse transcription-real time PCR. TRPV6 mRNA half life studies were performed by inhibiting transcription followed by sampling at various time points for TRPV6 mRNA. Varying concentrations of 25 VD were used to test their effect on TRPV6 mRNA in the presence of 5 % FBS and also in the absence of serum (but containing insulin-transferrin-selenium mixture) for 24 h. *Results* Treatment with 10^{-7} M 1,25VD for 8 h resulted in a 60-fold increase in TRPV6 mRNA and this increase could be completely blocked with AD. Treatment with CHX to inhibit *de novo* protein synthesis did not prevent the initiation of 1,25VD-induced TRPV6 expression, although it did reduce the extent of TRPV6 mRNA accumulation. We found that TRPV6 mRNA half-life was 8 h in Caco-2 cells and was not altered by 1,25VD treatment. Finally, we observed that treatment with 10^{-6} M of the pro-hormone 25 VD for 24 h resulted in a significant increase in TRPV6 expression in Caco-2 cells, which is consistent with the presence of 1α-hydroxylase (CYP27B1) expression in Caco-2 cells and a possible autocrine vitamin D signaling pathway in colon cells. *Conclusions* 1,25 dihydroxyvitamin D regulates *TRPV6* expression by a process that requires new mRNA and protein synthesis and the point of regulation lies likely at the transcriptional level especially since vitamin D did not increase the half life of TRPV6 mRNA. In addition, the prohormone form of 1,25 dihydroxyvitamin D, i. e. the 25 hydroxyvitamin D, induced TRPV6 mRNA expression in Caco-2 cells.

Key words CaT1 – TRPV6 – gene expression – vitamin D – intestine

Introduction

1,25-dihydroxyvitamin D is the primary hormonal regulator of intestinal calcium absorption by a process involving an increase in transcellular calcium transport [1]. However, the molecular mechanism of calcium transport across the enterocyte has not been fully described. Three important steps in transcellular calcium transport include: calcium influx across the apical brush border membrane, by an as yet unidentified transporter; transcytosis of absorbed calcium, likely by calbindin D, a vitamin D-dependent cytosolic mobile calcium buffer; and finally, calcium extrusion across the basolateral membrane by an energy-dependent process involving calcium ATPase [1, 2].

The transient receptor potential (TRP) family of proteins function as plasma membrane channels that mediate important cellular functions such as osmoregulation, sensory response to touch, hot and cold temperature [3]. TRPV6 (transient receptor potential channel, subfamily V, member 6) gene encodes an epithelial calcium channel namely CaT1 (calcium transporter-1) which was first identified by Peng and colleagues [4]. It is an integral membrane protein with six membrane-spanning domains and a pore region for ion transport [5] TRPV6 mRNA was first shown to be regulated by 1,25-dihydroxyvitamin D [6] in Caco-2 cells, a human intestinal cell line. 1,25-dihydroxyvitamin D is also known to increase transcellular calcium transport in Caco-2 cells [7, 8]. Thus, we have proposed that gene product of TRPV6 is a candidate molecular gatekeeper for vitamin D-dependent calcium entry into the enterocyte [6]. The dependence of TRPV6 mRNA expression on the VDR-mediated genomic actions of 1,25-dihydroxyvitamin D is supported by increased intestinal TRPV6 mRNA expression after injection of mice with 1,25-dihydroxyvitamin D [9, 10] and that TRPV6 expression was lower in vitamin D receptor knockout mice as compared to wild type mice [10]. However, the mode of 1,25-dihydroxyvitamin D-mediated regulation of TRPV6 expression is unknown. It is not known whether 1,25-dihydroxyvitamin D increases the abundance of TRPV6 transcripts merely by *de novo* transcription or also by decreasing the degradation rate of TRPV6 mRNA and whether or not new protein synthesis is required for 1,25-dihydroxyvitamin D to upregulate TRPV6 mRNA.

Transcriptional activation and repression of vitamin D-dependent genes is a primary mechanism of action of 1,25-dihydroxyvitamin D in the cell [11]. However, several studies have shown that 1,25-dihydroxyvitamin D is also involved in post-transcriptional regulation [12–18] by altering mRNA half-life of vitamin D-dependent genes. For example, 1,25-dihydroxyvitamin D increases the expression of epidermal growth factor receptor (EGFR) exclusively by increasing mRNA half life [12], while 1, 25-dihydroxyvitamin D increases both the transcription rate and mRNA half life of osteocalcin [14] and collagenase [15]. Therefore, we hypothesised that 1,25 dihydroxyvitamin D may also stabilise the TRPV6 transcript.

Plasma 1,25-dihydroxyvitamin D concentration is determined by the availability of the pro-hormone 25-hydroxyvitamin D and the activity of the renal 1α-hydroxylase. The conversion of 25-hydroxyvitamin D (the pro-hormone form of vitamin D) to the active 1,25-dihydroxyvitamin D hormone is tightly controlled by the renal 1α-hydroxylase enzyme. However, some non-renal cells, including those of the prostate [19], colon [20], pancreas [21], placenta [22], parathyroid gland [23] and cervix [24] also express 1α-hydroxylase, which may result in the local conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D, setting up the possibility of an autocrine or paracrine vitamin D signaling pathway in these tissues [20].

The objective of this study was to characterise the mode of regulation of 1,25-dihydroxyvitamin D -mediated TRPV6 mRNA expression and also to test the effect of 25-hydroxyvitamin D (a precursor of 1,25 vitamin D) on TRPV6 mRNA expression in human intestinal Caco-2 cells.

Materials and methods

Materials

Caco-2 cells were obtained from American Type Culture Collection (HTB37; American Type Culture Collection, Rockville, MD). 1,25-dihydroxyvitamin D and 25-hydroxyvitamin D were purchased from Biomol, Plymouth Meeting, PA. Actinomycin D and cycloheximide were from Sigma, St. Louis, MO. TRIreagent and bromochloropropane for RNA isolation were purchased from Molecular Research Center, Cincinnati, OH. Moloney-murine leukemia virus reverse transcriptase enzyme was bought from Invitrogen, Carlsbad, CA. SYBR Green master mix (2X) for real time PCR was from Applied Biosystems, Foster City, CA. Fetal bovine serum (FBS) was from Hyclone, Logan UT. Plastic six-well (35-mm diameter) cell culture dishes were from Costar, Cambridge, MA. Other cell culture materials were purchased from Invitrogen, Carlsbad, CA.

Cell culture

Caco-2 cells were cultured at 37 °C in a humid 5 % CO_2 and 95 % air atmosphere. Cells used in these studies were between passages 30 to 50. The maintenance medium for the cells consisted of high glucose (4.5 g/L glucose) Dulbecco's Modified Eagle Medium (DMEM),

supplemented with 1 mM sodium pyruvate, 100 µM non-essential amino acids, 50 µg/L gentamicin sulfate, 100 U/L penicillin/streptomycin, 2 mM glutamine and 10 % FBS. Routine passaging of the cells was done with cell culture medium containing 20 % FBS. For experiments, Caco-2 cells were seeded at 2 x 10^5 cells per well in six-well dishes and fed every other day with cell culture medium containing 10 % FBS for a period of two weeks to achieve a fully differentiated intestinal cell phenotype [25] prior to experimental treatment.

Experimental treatments

Starting on day 14 post-seeding, all cells were treated using cell culture medium containing 5 % FBS, unless indicated otherwise.

For studies evaluating the necessity of *de novo* mRNA synthesis in the 1,25-dihydroxyvitamin D-mediated increase in TRPV6 expression, Caco-2 cells were treated with either the transcriptional inhibitor actinomycin D (4 µg/ml x 1 h, 37 °C) or an equivalent volume of the ethanol vehicle. Then, the ethanol-treated group received either vehicle or 10^{-7} M 1,25-dihydroxyvitamin D, while the actinomycin D-treated group was treated with 4 µg/ml actinomycin D alone or in combination with 10^{-7} M 1,25-dihydroxyvitamin D. Total RNA was harvested after 8 h of incubation at 37 °C. Three independent experiments were performed with every treatment in triplicate.

For studies investigating the necessity of *de novo* protein synthesis for the 1,25-dihydroxyvitamin D-mediated increase in TRPV6 expression, Caco-2 cells were pre-treated with 10 µg/ml cycloheximide to inhibit protein synthesis or with an equivalent amount of ethanol for 1 h. These two groups of cells were then further treated with cycloheximide or 10^{-7} M 1,25-dihydroxyvitamin D, or a combination of the two, for 2,4,8 and 24 h. Two independent experiments were conducted with treatments in triplicate.

To determine the effects of 1,25-dihydroxyvitamin D on TRPV6 mRNA stability, Caco-2 cells were first treated with 1,25-dihydroxyvitamin D (10^{-7} M) or ethanol (equivalent volume) for 24 h and then with actinomycin D (5 µg/ml) to arrest transcription followed by RNA isolation after 0, 2, 4, 8, 10 and 12 h of actinomycin D treatment. TRPV6 mRNA expression was determined by real time PCR. The decay rate of TRPV6 mRNA and transcript half-life were determined, as described below, for control and 1,25-dihydroxyvitamin D-treated cells in two independent experiments with each treatment performed in triplicate.

For studies determining the effect of 25-hydroxyvitamin D on gene expression, two independent experiments with treatments in duplicate were conducted with various concentrations (10^{-8}, 10^{-7} and 10^{-6} M) of 25-hydroxyvitamin D or equivalent ethanol for 24 h in the presence of 5 % FBS. Another experiment was conducted to test for the effect of 25-hydroxyvitamin D in serum-free conditions, wherein a 1 % mixture of Insulin-Transferrin-Selenium (ITS, Invitrogen, Carlsbad, CA) was included in the 10^{-8}, 10^{-7} and 10^{-6} M 25-hydroxyvitamin D treatments or control (ethanol) for 24 h. As a positive control for TRPV6 mRNA expression in these experiments, some cells were treated with 10^{-7} M 1,25-dihydroxyvitamin D.

RNA isolation and reverse transcription

TRIreagent and bromochloropropane were used to isolate total RNA per the manufacturer's directions. RNA samples were quantified spectrophotometrically using their absorbance at $\lambda = 260$. A cDNA library was prepared for every sample by reverse transcription of total RNA using an oligodT primer (Invitrogen, Carlsbad, CA).

Real time PCR

For all experiments, except for the detection of 1α-hydroxylase, also called CYP27B1 (see below), mRNA expression was measured by real time PCR using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Reactions consisted of 1X SYBR Green dye, 0.3 µM forward and reverse primers each for TRPV6 and GAPDH. CYP24 primers (0.25 µM) used in PCR reactions and their sequences were as described [26]. The sequences of the forward and reverse primers for TRPV6 were 5'-TCATCAGTGCCAGCGGG-3' and 5'-GGTCGCCAAAAATCATCTTCTG-3' respectively, with the resulting amplicon being 138 bp with a Tm of 82 °C. The sequences of the forward and reverse primers for GAPDH were 5'-CATCAGCAATGCCTCCTGC-3' and 5'-CCATCCACAGTCTTCTGGGTG-3' respectively, with the resulting amplicon being 131 bp with a Tm of 82 °C. The specificity of all primers was confirmed by BLAST searches. Primer Express (Applied Biosystems, Foster City, CA) was used for designing primers for TRPV6 (accession number NM_018646) and GAPDH (accession number M33197). All primers were designed to cross exon-exon boundaries of the coding sequence such that any unwarranted genomic DNA amplifications could easily be identified. Primers were optimised and validated for the comparative Ct method, as described in the manufacturer's manual. A dissociation curve protocol was linked to every PCR assay to ensure the specificity of the PCR product. ABI Prism SDS software version 1 was used for the analysis of the amplification plots. The fold change ± SD in TRPV6 expression was normalised to GAPDH for a given treatment group and expressed

relative to the vehicle treated-control group, unless described otherwise.

CYP27 B1 mRNA detection

Caco-2 cell cDNA (100 ng) was amplified by 28 PCR cycles using ampliTaq (Applied Biosystems, CA, USA) and specific primers for 1α hydroxylase (CYP27B1), the primer sequences of which have described previously [27]. A PCR blank (PCR cocktail + sterile water) was included to ensure that there was no contamination. The PCR products were electrophoretically separated on a 2.5 % agarose gel in TAE (Tris acetic acid and ethylenediaminetetraacetic acid (EDTA)) buffer containing ethidium bromide and further visualised under UV light and the images were digitally captured (Gel Doc 2000, Biorad, Hercules, CA).

Determination of TRPV6 mRNA decay rate and half-life

Caco-2 cells were treated with ethanol vehicle (control group) or 10^{-7} M 1,25-dihydroxyvitamin D (vitamin D group) for 24 h. Then, all cells were treated with 5 μg/ml actinomycin D to arrest further new mRNA synthesis and cells were harvested 0, 4, 8, 10, and 12 h later. Total RNA was isolated to measure TRPV6 mRNA by real time PCR. The linear decay of TRPV6 mRNA was expressed relative to the amount of mRNA measured at zero time (100 %) normalised to GAPDH mRNA. The effect of 1,25-dihydroxyvitamin D on TRPV6 mRNA stability was assessed from the difference in the rate of mRNA decay determined from the slopes of the best fit linear regression lines (GraphPad version 4 for Windows, GraphPad Software, San Diego CA). The mRNA half-life was calculated from the decay curves as the time where 50 % of the initial mRNA at time '0' had disappeared.

Statistical analyses

SYSTAT (version 10) statistical software package (SPSS, Chicago, IL) was used for all statistical analyses, while GraphPad was used for linear regression analyses for half-life experiments, as described above. Treatment means were compared by analysis of variance followed by a Fisher's LSD method for post hoc comparisons of individual means. A P-value of < 0.05 was considered statistically significant.

Results

1,25-dihydroxyvitamin D-induced TRPV6 expression depends on *de novo* mRNA synthesis

To determine to what extent *de novo* mRNA synthesis was responsible for the observed 1,25-dihydroxyvitamin D-mediated increase in TRPV6 mRNA levels in Caco-2 cells, we treated cells with the transcription inhibitor actinomycin D. As illustrated in Fig. 1A, 1,25-dihydroxyvitamin D treatment for 8 h caused a marked 60-fold increase ($P < 0.001$) in TRPV6 mRNA that could be completely blocked by actinomycin D. Thus, the accumulation of TRPV6 mRNA following 1,25-dihydroxyvitamin D treatment of Caco-2 cells is dependent upon new mRNA synthesis.

De novo protein synthesis is necessary for maximal 1, 25-dihydroxyvitamin D-induced TRPV6 mRNA expression

To further characterise the response of TRPV6 to 1,25-dihydroxyvitamin D in Caco-2 cells, we determined to what extent *de novo* protein synthesis was necessary for 1,25-dihydroxyvitamin D-induced TRPV6 mRNA expression. We measured TRPV6 expression at 2, 4, 8, and 24 h after 1,25-dihydroxyvitamin D treatment (10^{-7} M) in the absence or presence of cycloheximide, a protein synthesis inhibitor. As shown in Fig. 1B, TRPV6 mRNA expression (normalised to GAPDH housekeeping gene) was significantly increased ($P < 0.001$) between 2 h and 24 h after 1,25-dihydroxyvitamin D treatment in both the absence and presence of cycloheximide. Cycloheximide had no significant effect on the 1,25-dihydroxyvitamin D-induced accumulation of TRPV6 mRNA at 2 h, but resulted in a significantly lower ($P < 0.001$) level of expression between 4 h and 24 h. At 24 h, TRPV6 mRNA was 85 % lower in the cycloheximide plus 1,25-dihydroxyvitamin D treatment group, as compared to 1,25-dihydroxyvitamin D treatment alone. Thus, although *de novo* protein synthesis is clearly not needed for triggering the 1,25-dihydroxyvitamin D-mediated increase in TRPV6 mRNA levels in Caco-2 cells, it is necessary to achieve maximal accumulation of TRPV6 transcripts.

1,25-Dihydroxyvitamin D does not change TRPV6 mRNA decay rate

Increasing the mRNA half-life is known to be one of the ways that 1,25-dihydroxyvitamin D can regulate the level of target mRNAs. We hypothesised that the observed marked increase in TRPV6 mRNA following 1,25-dihydroxyvitamin D treatment could be due in part to stabilisation of the TRPV6 transcript. To examine this

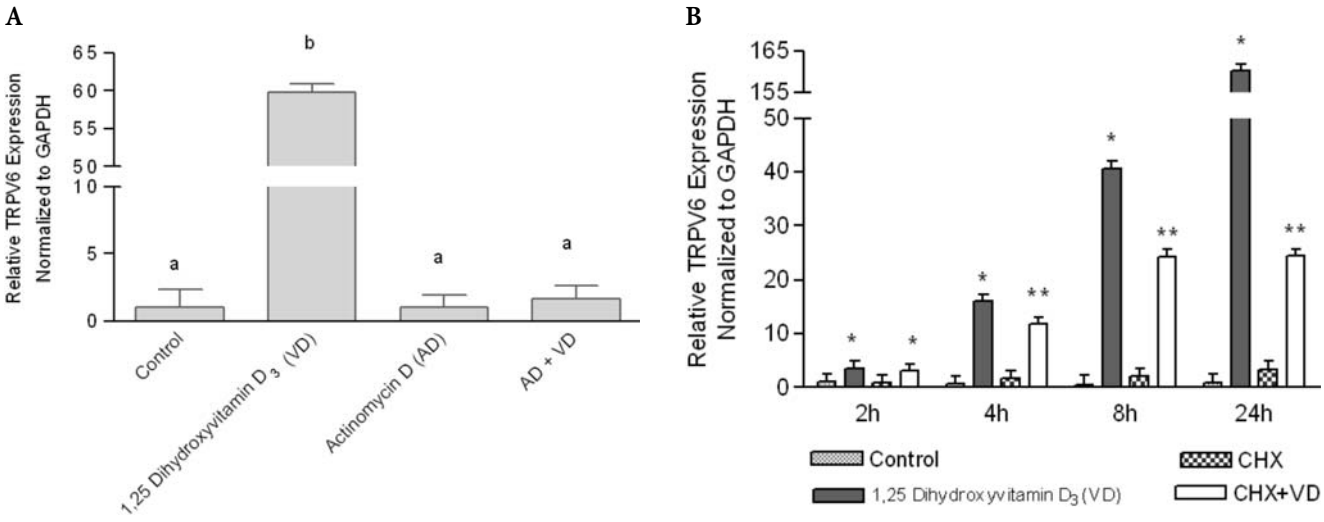

Fig. 1 Effect of actinomycin D (AD) and cycloheximide (CHX) on 1,25-dihydroxyvitamin D-mediated increase in TRPV6 mRNA. Caco-2 cells were treated in a 2 X 2 format with (**A**) actinomycin D and 1,25 dihydroxyvitamin D (10^{-7} M) for 8 h at 37 °C, subsequent to a one hour pre-treatment with 4 µg/ml actinomycin D, or (**B**) with 10 µg/ml cycloheximide (CHX) and 10^{-7} M 1,25-dihydroxyvitamin D for 2, 4, 8 and 24 hours at 37 °C, subsequent to a one hour pre-treatment with 10 µg/ml CHX. Total RNA was collected and analysed by reverse transcription-real time PCR for TRPV6 expression and normalised to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The bars, in the graph representing the actinomycin D experiment, are the mean ± SD fold-change in gene expression relative to control from three independent experiments with treatments done in triplicates. Bars within each figure with different letters are significantly different (Fisher's LSD). The combination of actinomycin D + 1,25-dihydroxyvitamin D blocked the 1,25-dihydroxyvitamin D-dependent increased expression of TRPV6 as compared to 1,25-dihydroxyvitamin D alone ($P < 0.001$, ANOVA, post-hoc test Fisher's LSD). **B** For the cycloheximide experiments, the bars are mean ± SD fold-change for gene expression relative to control at 2 h from two independent experiments with each treatment in triplicate. A single asterisk indicates that the TRPV6 mRNA expression in the treatment group was significantly higher as compared to the control group of that time point. A double asterisk indicates that the 1,25-dihydroxyvitamin D + cycloheximide treatment was significantly less than 1,25-dihydroxyvitamin D alone for that time point. 1,25-dihydroxyvitamin D significantly increased TRPV6 mRNA expression compared to the ethanol treated-control at each time point, starting at 2 h. CHX significantly reduced the 1,25-dihydroxyvitamin D-mediated TRPV6 expression at 4, 8 and 24 h ($p < 0.001$, ANOVA, post-hoc test Fisher's LSD) compared to the 1,25-dihydroxyvitamin D group alone

possibility, we determined the effect of 1,25-dihydroxyvitamin D treatment on half-life of the TRPV6 mRNA.

Expressing the relative TRPV6 mRNA (normalised for GAPDH mRNA) levels as a percent of the 0 h time point in each treatment group, we used the slope of the time-dependent linear loss of TRPV6 mRNA to estimate the fractional rate of TRPV6 mRNA decay in the control and 1,25-dihydroxyvitamin D-treated groups. The rate of TRPV6 mRNA decay in Caco-2 cells was 5.7 %/h (95 % CI: 4.4 %, 7 %) in controls and 6.9 %/h (95 % CI: 5.5 %, 8.3 %) in 1, 25-dihydroxyvitamin D-treated cells (Fig. 2). The corresponding calculated TRPV6 mRNA half-life was 8.0 h in controls and 6.7 h in 1,25-dihydroxyvitamin D-treated cells, but this difference was not statistically significant, based on a comparison of the slopes of the two linear regression lines ($P = 0.17$). Thus, we found no evidence of a 1,25-dihydroxyvitamin D-dependent stabilisation of TRPV6 mRNA that would be instrumental in the observed marked increase in TRPV6 mRNA following 1,25-dihydroxyvitamin D treatment.

The vitamin D pro-hormone 25-hydroxyvitamin D increases TRPV6 mRNA expression in Caco-2 cells

In light of recent reports [19, 21] that 25-hydroxyvitamin D, the pro-hormone form of 1,25-dihydroxyvitamin D, may have an effect on gene expression in prostate and pancreatic cells, we determined whether 25-hydroxyvitamin D treatment alone could affect TRPV6 mRNA expression in Caco-2 cells, a colon-derived human intestinal cell line. Cells were treated for 24 h with increasing doses of 25-hydroxyvitamin D (10^{-8} to 10^{-6} M) in both the absence and presence of 5 % fetal bovine serum (a source of vitamin D binding protein) in the cell culture medium. As illustrated in Fig. 3 A, in the absence of FBS (ITS containing medium), there was a significant marked increase ($P < 0.001$) in TRPV6 mRNA expression at all doses of the pro-hormone studied. TRPV6 mRNA was ~35-fold higher in Caco-2 cells treated with 10^{-8} M 25-hydroxyvitamin D compared to vehicle-treated controls. At a 10-fold higher concentration of 25-hydroxyvitamin D, TRPV6 mRNA expression was increased by ~75-fold, although an equivalent amount of the 1,25-dihydroxyvitamin D hormone caused a significantly greater increase in TRPV6 expression. A further increase in TRPV6 mRNA expression could be achieved

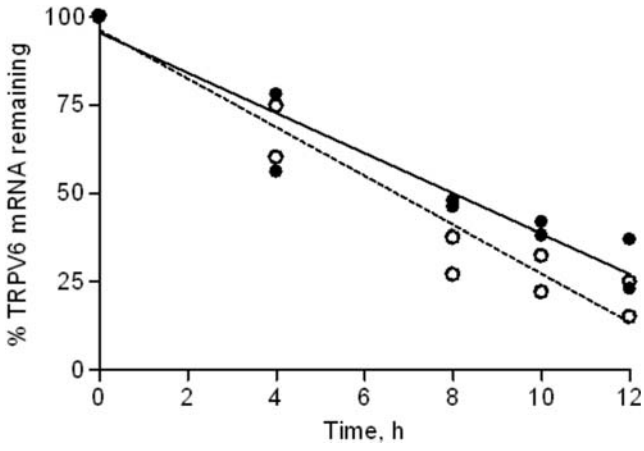

Fig. 2 Effect of 1,25-dihydroxyvitamin D on TRPV6 mRNA Decay Rate. Caco-2 cells were cultured for 14 d and treated with either 10^{-7} M 1,25-dihydroxyvitamin D or ethanol vehicle for 24 h, followed by addition of 5 µg/ml actinomycin D to arrest transcription. Cells were then incubated for 0, 4, 8, 10 or 12 h and total RNA was collected and subsequently analysed by real time PCR for TRPV6 mRNA expression and normalised to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The data are the mean percentage of triplicate samples of TRPV6 mRNA remaining relative to time zero (set to 100 %) from two independent experiments. Solid circles represent ethanol-treated control cells and open circles represent 1,25-dihydroxyvitamin D-treated cells. The best-fit linear regression line is represented by a solid line for controls and a dashed line for 1,25-dihydroxyvitamin D-treated cells. The slopes of the two lines were not statistically significantly different (P = 0.17). The half-lives calculated from the linear regression were 8.0 h and 6.7 h for control and 1,25-dihydroxyvitamin D-treated groups, respectively

in Caco-2 cells by increasing the 25-hydroxyvitamin D concentration to 10^{-6} M. However, as shown in Fig. 3B, TRPV6 gene expression in response to 25-hydroxyvitamin D treatment was markedly reduced in the presence of fetal bovine serum in the cell culture medium. In the presence of FBS, treatment with 25-hydroxyvitamin D at 10^{-8} and 10^{-7} M had no significant effect on TRPV6 mRNA expression, although TRPV6 expression was clearly (> 150-fold increase) evident at the highest dose of the pro-hormone (10^{-6} M) investigated and was not significantly different than the TRPV6 mRNA response to 10^{-7} M 1,25-dihydroxyvitamin D.

To determine whether or not the effect of 25-hydroxyvitamin D on gene expression in Caco-2 cells was specific to TRPV6, or more likely represented a general effect on vitamin D-mediated gene expression, we also measured 24-hydroxylase (CYP24) mRNA induction in cells treated with various doses of the 25-hydroxyvitamin D pro-hormone in the absence or presence of FBS in the cell culture medium. CYP24 is a well known vitamin D-dependent gene that is not detectable by PCR in Caco-2 cells in the absence of 1,25-dihydroxyvitamin D treatment [8, 26]. Consistent with the pattern of 25-hydroxyvitamin D-induced TRPV6 expression, a similar pro-hormone concentration-dependent response in CYP24 expression was observed in Caco-2 cells (data not shown).

Consistent with findings of Bareis and coworkers [27], we also confirmed by RT-PCR that Caco-2 cells express 1-α hydroxylase (CYP27B1; Fig. 3C). This enzyme converts 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D. Cross et al. [28] showed that radiolabelled 25-hydroxyvitamin D is converted to 1,25-dihydroxyvitamin D ($1\alpha, 25\ (OH)_2D_3$) in Caco-2 cells.

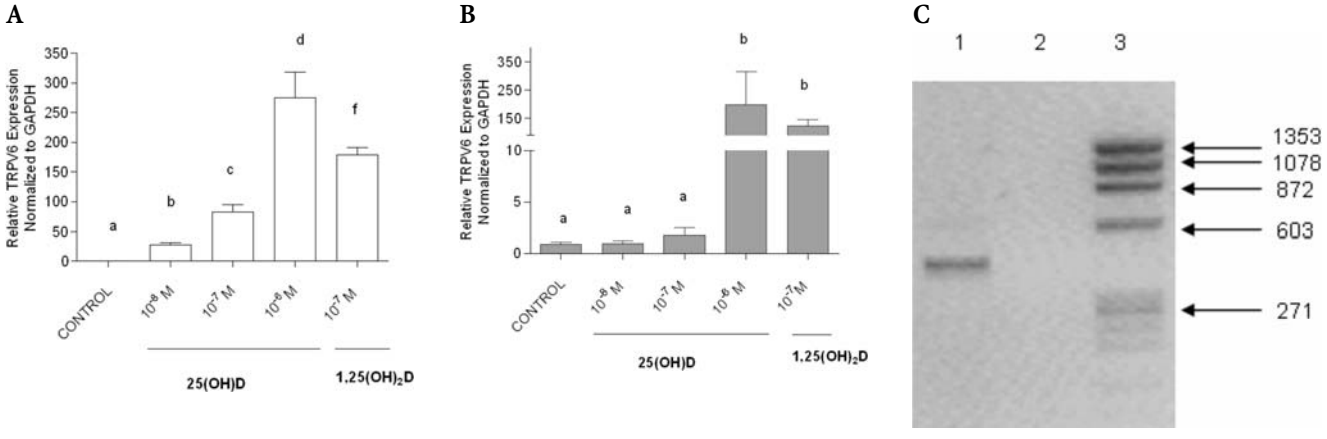

Fig. 3 Effect of 25-hydroxyvitamin D pro-hormone on TRPV6 mRNA expression in Caco-2 cells. Caco-2 cells cultured for 14 d were treated in triplicate for 24 h with 10^{-8} to 10^{-6} M 25-hydroxyvitamin D or 10^{-7} M 1,25-dihydroxyvitamin D either in the absence (**A**) or presence (**B**) of 5 % FBS in cell culture medium. Total RNA was isolated and subsequently analysed using real time PCR for TRPV6 mRNA and normalised to GAPDH mRNA. Bars in the graph represent mean ± SD fold-change in gene expression. Bars with different letters within each figure are statistically different ($p < 0.05$, ANOVA, post-hoc test Fisher's LSD) from each other. **A** In the absence of FBS, there was a dose-dependent 25-hydroxyvitamin D-mediated increase in TRPV6 expression. **B** In the presence of FBS, only the highest dose of 25-hydroxyvitamin D resulted in a significant increase in TRPV6 expression. **C** The presence of CYP27B1 mRNA (440 bp size amplicon) in Caco-2 cells was confirmed by RT-PCR and ethidium bromide stained gel based detection. Lane 1 of the gel shows the CYP27B1 RT-PCR product, lane 2 was the PCR blank to ensure the absence of contamination of PCR cocktail, while lane 3 shows the Phi X DNA digested by Hae III (Invitrogen, Carlsbad, CA)

Discussion

The strong vitamin D-mediated expression of TRPV6 mRNA in Caco-2 cells that we observed using real time PCR is consistent with our previous observations in this cell line [6, 29], as well as previous *in vivo* observations in mice [9] and in vitamin D receptor knockout mice [10]. The TRPV6 gene codes for an epithelial calcium channel protein [4, 5, 30] that is believed to play an important role in intestinal calcium absorption. Our observations in the current studies in an intestinal cell culture model support the idea that the upregulation of TRPV6 mRNA expression in the enterocyte may be an important mechanism of vitamin D-mediated calcium absorption.

Novel findings of the current study include the observations that the 1,25-dihydroxyvitamin D-dependent increase in TRPV6 mRNA is solely dependent upon *de novo* mRNA synthesis and that this response can occur in the absence of new protein synthesis, although clearly the extent of the increase in TRPV6 mRNA depends on the latter. Our findings of transcriptional control of TRPV6 gene expression in Caco-2 cells is in keeping with the presence of Vitamin D Receptor Elements (VDREs) in the promoter region of TRPV6 gene, experimentally verified by Wang et al. recently [31]. Moreover, our findings show that the average half-life of the TRPV6 transcript in Caco-2 cells is 8 h and TRPV6 transcript is clearly not stabilised by 1,25-dihydroxyvitamin D. Because the TRPV6 mRNA decay rate is not affected by 1,25-dihydroxyvitamin D, it seems likely that the need for *de novo* protein synthesis to achieve a maximal TRPV6 mRNA level is probably related to an effect on vitamin D-mediated transcriptional activity in this cell line, perhaps to replenish a rapidly turning over protein involved in the transcriptional complex. Identification of the limiting component mediating this effect will require further investigation.

A particularly interesting novel finding of the current study was that treatment of Caco-2 cells with the prohormone 25-hydroxyvitamin D resulted in a marked increase in TRPV6 expression. Given the purported role of TRPV6 in intestinal calcium absorption, this in vitro observation would be consistent with the suggestion that plasma 25-hydroxyvitamin D concentration is a determinant of intestinal calcium absorption in humans [32]. In this regard, we made two potentially noteworthy observations. First, given that the normal range for circulating 1,25-dihydroxyvitamin D is 60–108 picomolar, while 25 hydroxyvitamin D ranges from 24.9–169.5 nanomolar according to the clinical laboratory reference values established for Massachussetts General Hospital [33], we found that in the absence of FBS in the cell culture medium, the increase in TRPV6 mRNA expression with 100 nM or 10^{-7} M 25-hydroxyvitamin D treatment was about one-half that following an equivalent dose of 1,25-dihydroxyvitamin D_3 and a 10-fold higher dose i. e. 1000 nM or 10^{-6} M of 25-hydroxyvitamin D resulted in significantly greater increase in TRPV6 expression. This observation is of potential nutritional and public health interest because circulating 25-hydroxyvitamin D concentrations are readily manipulated by dietary vitamin D supplementation. *In vivo* plasma 25-hydroxyvitamin D concentrations, being normally three orders of magnitude higher than 1,25-dihydroxyvitamin D [34], suggest that usual circulating 25-hydroxyvitamin D concentrations may influence intestinal gene expression *in vivo* providing a possible mechanistic explanation of the known association between vitamin D status and the risk of developing colon cancer [35, 36] or an apparent effect of 25-hydroxyvitamin D on calcium absorption performance [32]. A second point to consider is our finding of the relative response of TRPV6 and CYP24 expression to vitamin D metabolites in the presence and absence of FBS, which may have some bearing on the possible biological importance of circulating 25-hydroxyvitamin D on the enterocyte *in vivo*. It has generally been considered that any effect of 25-hydroxyvitamin D on cell function is limited by reduced cellular bioavailability of 25-hydroxyvitamin D due to its high binding affinity for the plasma vitamin D binding protein (DBP) [37]. This would be consistent with our observation in this study that the effectiveness of 25-hydroxyvitamin D on TRPV6 (or CYP24) mRNA expression in Caco-2 cells was appreciably lower in the presence of FBS, a source of vitamin D binding protein. Heaney and colleagues [32] had previously suggested a molar ratio for physiological potency of approximately 100:1 for the pro-hormone and hormonal form of vitamin D in reference to intestinal calcium absorption. While we cannot rule out a direct effect of 25 hydroxyvitamin D on TRPV6 gene expression, the induction of TRPV6 mRNA and 24-hydroxylase (CYP24) mRNA by 25-hydroxyvitamin D treatment of Caco-2 cells may likely be due to it being converted into the active hormone 1,25-dihydroxyvitamin D. Caco-2 cells have been shown to convert $^{3H}25(OH)D_3$ to $1\alpha,25(OH)_2D_3$ as determined by HPLC and its binding affinity to VDR [28]. To our knowledge, this is the first report of the ability of 25-hydroxyvitamin D to induce TRPV6 expression in calcium transporting intestinal Caco-2 cells. Our current observation that a high concentration of 25-hydroxyvitamin D in Caco-2 cells can stimulate gene expression in the presence of FBS (Fig. 3B) suggests that further *in vivo* study of this 25-hydroxyvitamin D effect in the enterocyte is warranted.

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