Human duodenum responses to vitamin D metabolites of TRPV6 and other genes involved in calcium absorption

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Balesaria S, Sangha S, Walters JR. Human duodenum responses to vitamin D metabolites of TRPV6 and other genes involved in calcium absorption. Am J Physiol Gastrointest Liver Physiol 297: G1193–G1197, 2009. First published September 24, 2009; doi:10.1152/ajpgi.00237.2009.—Calcium absorption by the intestine is necessary for bone mineralization. Much has been learned about this process and the role of vitamin D metabolites in gene transcription from animal studies, but the molecular mechanisms in humans are less well understood. We have used samples of normal human duodenal mucosa, obtained at endoscopy, to investigate the effects of the vitamin D metabolites, 1α,25-dihydroxycholecalciferol [1,25(OH)2D3] and 25-hydroxycholecalciferol (25OHD), on transcripts on genes involved in calcium absorption and vitamin D metabolism. TRPV6 transcripts were significantly higher after incubation for 6 h with 1,25(OH)2D3 (10−9 mol/l) than after control incubations (median difference 3.1-fold, P < 0.001). Unexpectedly, TRPV6 expression was also higher (2.4-fold, P < 0.02) after incubation with 25OHD (10−7 mol/l). Transcripts for the calcium-ATPase, PMCA1, were significantly higher with 1,25(OH)2D3; CYP24 transcripts were reliably detected after incubation with either metabolite, but calbindin-D9k transcripts were unaffected. The response of TRPV6 to 25OHD and the expression of transcripts for CYP27B1, the 25OHD-1α-hydroxylase, were significantly correlated (r = 0.82, P < 0.02). Basal duodenal expression of TRPV6 and CYP27B1 were significantly associated (r = 0.72, P < 0.001) in a separate previously reported series of subjects. Multiple regression analysis of the associations with basal duodenal TRPV6 expression identified CYP27B1 expression and serum 1,25(OH)2D3 as major factors. Expression of the CYP27B1 protein was demonstrated immunohistochemically in duodenal mucosa. This study has shown that human duodenal TRPV6, PMCA1, and CYP24 transcripts respond rapidly to 1,25(OH)2D3 and provides evidence suggesting that local duodenal production of 1,25(OH)2D3 by 25OHD-1α-hydroxylase may have a role in human calcium absorption.

cholcalciferol; calcium transporter; intestine; organ culture

THE ABSORPTION OF DIETARY CALCIUM by the intestine is necessary for bone mineralization and therefore is a factor that is important in the determination of peak bone mass and in the prevention of osteoporosis, a major public health concern. Calcium absorption varies greatly between individuals and is well recognized to involve vitamin D (8). Vitamin D3, cholecalciferol, is synthesized in the skin through UV-light exposure. The metabolism of vitamin D to the hormonally active form, 1α,25-dihydroxycholecalciferol [1,25(OH)2D3], and the physiological effects of gene expression have been the subject of much study in animal models though, in comparison, the mechanisms of its actions in humans are incompletely understood (25).

Several studies have shown the relationship of human intestinal fractional calcium absorption with blood levels of 1,25(OH)2D3 (3, 10, 12, 28). The proportion of the variability accounted for by 1,25(OH)2D3 is often quite low, around 25%. Other studies have shown significant effects on human fractional calcium absorption of 25-hydroxyvitamin D (25OHD), the main storage metabolite of vitamin D and the precursor of 1,25(OH)2D3 (3, 12, 13). 25OHD is converted to 1,25(OH)2D3 by the enzyme 25-hydroxyvitamin D 1α-hydroxylase (1αOHase), gene name CYP27B1. This enzyme is highly expressed in kidney, and has also been shown to be in other vitamin D target tissues, including colon, but not previously in small intestine (30). 1,25(OH)2D3 and 25OHD are able to bind the vitamin D receptor (VDR) with differing affinities and are metabolically inactivated through 24-hydroxylation by the cytochrome P450 enzyme, CYP24A1.

Transport of calcium across the intestinal cell involves three major steps, and the probable molecular mechanisms have been determined in animal models involving rats and mice, with confirmation in humans. Our previous studies have defined the human brush-border membrane calcium channel to be TRPV6, previously known as CAT1 or ECAC2 (4, 26). Calcium is buffered in the cytoplasm by calbindin-D9k (15) and extruded from the cell by the basolateral membrane Ca2+ ATPase, PMCA1 (16).

The effect of vitamin D on transcription of these genes in the intestine has been established in animals. Data in humans are restricted to certain cell lines, with only limited direct study of intestine. TRPV6 mRNA expression in mice is clearly regulated by 1,25(OH)2D3, and this has been confirmed in Caco-2 cells (9, 22, 24, 29). One paper has shown in Caco-2 cells that TRPV6 can also be increased by 25OHD (23). We have shown in a study of endoscopic duodenal biopsies that basal, fasting, unstimulated levels of TRPV6 transcripts have a strong relationship with 1,25(OH)2D3 in men, but this is not clear in women, where effects of age and the menopause were more significant (26). Basal duodenal levels of calbindin-D9k transcript levels are also correlated with 1,25(OH)2D3, but PMCA1 is not (27). In human cells including those derived from colon, CYP24A1 has been shown to be vitamin D dependent (9, 18).

There has previously been no confirmation that human duodenal gene expression is directly responsive to vitamin D metabolites; we aimed to demonstrate this using short-term organ culture of endoscopic biopsies of duodenal mucosa. Additionally, because there have been no previous studies of 1αOHase in small intestine, we investigated whether this could be a factor relevant for calcium absorption in humans.
MATERIALS AND METHODS

Subjects. Duodenal biopsies were obtained with informed, written consent from stable outpatients aged 24–80 yr undergoing diagnostic endoscopy for dyspepsia. The study was approved by the Hammer smith and Queen Charlotte’s Research Ethics Committee. Exclusion criteria included gastric or duodenal pathology (such as celiac disease, duodenal ulcers, or cancer), pregnancy, and treatment for osteoporosis or other mineral disorders. Biopsies were taken from the second part of the duodenum for routine histological examination, which was confirmed to be normal. Up to nine additional biopsies were obtained for study from each individual subject. In the baseline expression studies (33 subjects), reported before (26), biopsies were frozen in liquid nitrogen or placed in RNA later and stored at −80°C. Alternatively, in the explant culture experiments (26 subjects), they were placed in oxygenated tissue culture medium (as below) and transported to the laboratory within 5 min. Blood samples were obtained from most subjects for assay of vitamin D metabolites. Serum was extracted and separated by HPLC. 25OHD (5) and 1,25(OH)2D (19) were quantified as described before.

Intestinal explant culture. To demonstrate responsiveness of genes in short-term culture, explants from an individual subject were placed in groups of 3 or 4 with the villous surface up on microporous sintered with 10% heat-inactivated fetal calf serum, with penicillin, streptomycin, leupeptin, PMSF, and soybean trypsin inhibitor. Cultures were incubated at 37°C in a sealed container and gassed hourly with 5% CO2. They were maintained in 1.8-ml culture medium (DMEM, supplemented with 10% heat-inactivated fetal calf serum, with penicillin, streptomycin, leupeptin, PMSF, and soybean trypsin inhibitor). Cultures were incubated at 37°C in a sealed container and gassed hourly with 95% O2-5% CO2. This system broadly followed that described by Armbrecht et al. (2) for rat duodenum.

Intestinal responsiveness to vitamin D metabolites. The intestinal explants from each individual were incubated for 6 h using two or three varying conditions in parallel, including with the addition of ethanol as a control, with 1,25(OH)2D3 at 10−9 M, and in some cases with 25OHD at 10−7 M. The vitamin D metabolites were >99% pure (Sigma-Aldrich) and stored in ethanol; in the tissue incubations, the ethanol concentrations did not exceed 0.1%, and an identical concentration of ethanol was present in the control incubations. After the incubations, the explants were placed in RNA later (Ambion) at 4°C overnight and then stored at −80°C before RNA extraction.

RNA and cDNA preparation. RNA extractions were performed using the SV Total RNA Isolation System (Promega) according to manufacturer’s instructions. Following RNA extraction, RNA (1 μg) was treated with DNase I (1 U) to remove all contaminating genomic DNA. DNase-treated RNA was subsequently used for cDNA synthesis using Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega). Briefly, 0.5 μg of random primer was added to 1 μg of RNA, along with 1× MMLV Reaction Buffer, 1 mM each dNTP, 1 U RNasin Ribonuclease Inhibitor, and MMLV reverse transcriptase (100 U). cDNA was then stored at −20°C.

Quantitative RT-PCR. Real-time quantitative PCR analyses of various genes were performed with an ABI PRISM 7700 Sequence Detection System instrument and software (Applied Biosystems). All TaqMan assays were performed with inventoried primers and probes (Applied Biosystems) (26). All PCR reactions were carried out in a final volume of 25 μl in triplicate according to the manufacturer’s protocol. For each assay, a standard curve was obtained by analyzing a dilution series of pooled cDNA samples for the relevant gene. Data were analyzed with Sequence Detector 1.7 software. GAPDH was used as the internal standard to control for variability, and results are expressed as a ratio of the gene/GAPDH.

Immunohistochemistry. Immunohistochemical studies were performed with duodenal tissue obtained at surgery for pancreatic tumors. Antibody to 25-OH vitamin D-1α-hydroxylase was obtained from Dr. Martin Hewison and used as previously described (30). Paraffin-embedded sections were incubated with 1:150 dilution of antiserum and then with a second peroxidase-conjugated antibody and counterstained with hematoxylin. Standard control studies were performed, which included the omission of the primary antibody.

RESULTS

Effects of 1,25(OH)2D3 on duodenal transcript expression. Incubation of duodenal biopsy explants for 6 h with 1,25(OH)2D3 produced significant changes in the expression of some transcripts involved in Ca2+- absorption (Fig. 1). TRPV6 transcript expression was consistently higher with 1,25(OH)2D3 incubation in all subjects studied. TRPV6/GAPDH expression ratios of treated vs. untreated values were highly significantly different (P < 0.001, Wilcoxon signed rank test) with a median 3.1-fold difference in the overall group of 26 subjects. The mean expression ratio ± SD was 3.83 ± 0.55, but this was not normally distributed (P < 0.01, Kolmogorov-Smirnov test); therefore, nonparametric comparisons were used. Although the median TRPV6 expression with ethanol control incubation was lower in older, postmenopausal women than in younger, premenopausal women (1.0 vs. 2.1), the numbers were too small for this to be significant. All groups had significantly higher median values with 1,25(OH)2D3 incubation: in men, n = 12, 3.2-fold change, P = 0.002; younger women, n = 5, 2.2-fold, P = 0.04; and older women, n = 9, 3.1-fold, P = 0.007.

![Fig. 1. Expression ratios of transcripts of genes expressed in human duodenal mucosa after 6-h incubation with either the addition of 1α,25-dihydroxycholecalciferol [1,25(OH)2D3] at 10−9 mol/l or 0.1% ethanol control. Median and quartiles are shown for the change in expression induced by 1,25(OH)2D3. All expression values were normalized to GAPDH. A twofold change indicates that the expression value with 1,25(OH)2D3 was twice that seen with the ethanol control.](http://ajpgi.physiology.org/)
A smaller increase was found with PMCA1 (mean treated vs. untreated PMCA1/GAPDH expression ratios 1.81 ± 0.28, median 1.34-fold), which nevertheless was significant \( (P = 0.02) \). The other main transport protein in the pathway for \( \text{Ca}^{2+} \) absorption, calbindin-D9k, showed no significant change in transcript expression. Sucrase, studied as a control gene unrelated to \( \text{Ca}^{2+} \) absorption, was also unchanged.

Transcripts of the 24-hydroxylase, CYP24A1, were at the limits of detection in the control specimens but were reliably measured after incubation with 1,25(OH)\(_2\)D\(_3\). Values appeared similar in all groups, but the low levels in the control sample mean presentation of fold changes are uninformative. Transcripts of CYP27B1, the 1αOHase, were easily detected and were unchanged following incubation with 1,25(OH)\(_2\)D\(_3\). VDR transcripts were also unaffected by 1,25(OH)\(_2\)D\(_3\). Effects of 25OHD. Some sets of biopsies \( (n = 6) \) were also incubated with 25OHD\(_3\) at a 100-fold higher concentration \( (10^{-7} \text{ M}) \). Changes in the expression of TRPV6 and other transcripts were found to be similar with 25OHD\(_3\) as with 1,25(OH)\(_2\)D\(_3\) (Fig. 2). TRPV6 transcript ratios were significantly higher (mean ± SD 3.33 ± 0.96, median 2.41, \( P < 0.02 \)). Other differences included the 1.5-fold higher median PMCA1, which, however, in this smaller group did not reach significance. CYP24A1 transcripts were also detectable after incubation with 25OHD\(_3\).

Duodenal CYP27B1 expression. The increase in TRPV6 expression produced by incubation with 25OHD\(_3\) was shown to be significantly related to the expression level of CYP27B1 \( (n = 6, r = 0.82, P = 0.02, \text{Spearman rank correlation}) \). This suggested that 1αOHase enzymatic activity might be responsible for converting 25OHD\(_3\) into 1,25(OH)\(_2\)D\(_3\). Basal, unstimulated expression of TRPV6 and CYP27B1 were significantly associated in this group \( (n = 24, r = 0.42, P = 0.02) \).

Duodenal CYP27B1 expression was further analyzed in the group of patients reported previously \( (26) \) where baseline, fasting expression of TRPV6, and other genes for calcium transporters had been studied and related to vitamin D metabolites, age, and sex. Expression levels of TRPV6 and CYP27B1 were again strongly associated \( (\text{Fig. 3}; n = 33, r = 0.72, P < 0.001) \).

In this group, 24 subjects had a full set of data including TRPV6, CYP27B1, and VDR transcripts, with blood 1,25(OH)\(_2\)D and 25OHD measurements. The relationship of TRPV6 and CYP27B1 was shown in both men and women. In men \( (r = 0.92) \), it was similar in magnitude to that of TRPV6 with plasma 1,25(OH)\(_2\)D \( (r = 0.87) \) (26), and, in women \( (r = 0.64) \), it was similar to the negative association with age \( (r = -0.69) \) and much stronger than the nonsignificant association with 1,25(OH)\(_2\)D. Stepwise multivariate analysis was performed (Table 1) and indicated that CYP27B1 gene expression and 1,25(OH)\(_2\)D (both \( P < 0.002 \)) were the principal associations with TRPV6 expression in duodenum.

The protein product of the CYP27B1 gene, the 25OHD 1αOHase, was demonstrated by immunohistochemistry in duodenum (Fig. 4). Immunoreactivity was found in villus and crypt cells; the crypts appeared to express protein more strongly. Brunner’s glands stained negative for 25OHD 1αOHase.

**DISCUSSION**

These studies have been performed using endoscopic samples of duodenal mucosa to investigate aspects of the molecular mechanisms that determine calcium absorption in humans. They are the most direct means to show the relevance of findings from studies of cell lines or from animal models to those of calcium absorption or balance in groups of men and women.

We have confirmed that TRPV6 gene expression is responsive to 1,25(OH)\(_2\)D\(_3\) in human duodenum. The concentration \( (10^{-9} \text{ M}) \) and time point (6 h) were chosen to be most likely to show a change in mRNA levels before problems with tissue viability developed and show results similar to those found in rat intestine organ culture by Armbrecht and colleagues (2) and by Wood, Fleet, and colleagues (23, 29) in human colonic-
derived Caco-2 cells. Further studies of different 1,25(OH)2D3 concentrations and time points would be of interest to establish the speed of onset and duration of the 1,25(OH)2D3 actions. Although we have not previously shown any relationship of baseline, unstimulated TRPV6 expression with 1,25(OH)2D3 levels in women (26), it is important to note that stimulation of expression by 1,25(OH)2D3 was found in both men and women. This is clearly relevant in the treatment and prevention of osteoporosis in women, and it would be of interest to use this experimental setup to investigate the effect of estrogens on TRPV6 expression.

The human TRPV6 gene has been shown to have VDR response elements (VDRE) (20). These particular regions in TRPV6 and other genes are not well conserved between species and often have varying binding affinities for the VDR, indicating that findings in animals do not necessarily translate to humans. The role of VDR and 1,25(OH)2D3 on TRPV6 expression has been clearly established in mice (22, 24) although some recent work has challenged how critical TRPV6 is to overall calcium absorption (17). To be sure of the relevance of these findings to human calcium absorption, studies such as ours with human duodenum are vital.

These studies provide some evidence for 1,25(OH)2D3 activation of expression of the PMCA1 calcium pump. A VDRE has not been demonstrated in this gene in any species although there is evidence from animals of increased expression of PMCA1 in rats treated with 1,25(OH)2D3 (31). Our studies show the activation of CYP24 expression by 1,25(OH)2D3 in human duodenum, which was not detected in the unstimulated state. This confirms findings in Caco-2 cells (9), which have recently been shown to involve MAPK signaling (7). Similar findings of activation of expression of this enzyme for vitamin D metabolism have been shown in many vitamin D target tissues.

The response of TRPV6 expression in the tissue explants to 25OHD, together with the findings of CYP27B1 expression at RNA and protein levels, indicates a major area of vitamin D action that has not hitherto been studied in relation to calcium intestinal absorption. In Caco-2 cells, Taparia et al. (23) have also shown activation of TRPV6 (and CYP24) expression by 25OHD. Caco-2 cells are derived from colon, and, although they are a useful model for small intestinal enterocytes, their findings may not always reflect small intestinal function; therefore, these present findings are important. It is possible that 25OHD at the higher concentrations found in the body and used in these experiments may have direct effects on the VDR (12), but the associations with CYP27B1 make the activation of 25OHD to 1,25(OH)2D3 a likely mechanism. Local production of 1,25(OH)2D3 has been shown in a variety of human tissues, including large intestine and in immunologic cells (14, 30), and is increasingly thought to be important in the actions of vitamin D in cancer prevention and inflammatory diseases (1, 6, 21). A role in calcium absorption has not been suggested before but clearly needs further exploration.

TRPV6 and CYP27B1 transcript expressions were closely associated in both parts of this study, tissues studied in organ culture and the baseline expression reported previously. This

### Table 1. Multiple-regression analysis of factors influencing basal TRPV6 expression

| Steps | P   | r²       | Corrected |
|-------|-----|----------|-----------|
| 1αOHase (+) | 2.98 E-06 | 0.637 | 0.620 |
| 1,25(OH)2D3 (+) | 0.002 | 0.774 | 0.753 |

| Summary | n  | r   | r²  | SE  | P   |
|---------|----|-----|-----|-----|-----|
| Normal  | 24 | 0.880 | 0.774 | 0.117 |
| Corrected | 0.868 | 0.753 |

| Equation       | Coefficient | 95% CI (±) | SE   | T     | P     |
|----------------|-------------|------------|------|-------|-------|
| Constant       | -0.215      | 0.213      | 0.102 | -2.097 | 0.048 |
| 1,25(OH)2D3   | 0.009       | 0.005      | 0.003 | 3.5757 | 0.002 |
| 1αOHase       | 0.459       | 0.133      | 0.064 | 7.201  | 4.26 E-07 |

TRPV6 expression was analyzed as the y-variable in a stepwise multiple regression using sex, age, 25-hydroxycholecalciferol, 1α-dihydroxycholecalciferol [1,25(OH)2D3], CYP27B1 (25-hydroxyvitamin D 1α-hydroxylase, 1αOHase), and vitamin D receptor expression as x-variables. All gene expression was corrected for GAPDH expression; n = 24 subjects with all measurements. SE, standard error; CI, confidence intervals.

Fig. 4. Representative histology of human duodenum after immunochemical staining with an antibody to 25OHD-1αOHase (30). The solid arrow shows strong staining of the crypt, and the open arrow indicates the villi. B shows the Brunner’s glands. Magnification ×100.
association was as strong as that with blood 1,25(OH)2D. Although we could not show that the blood levels of 25OHd were an additional factor related to duodenal TRPV6 transcript expression, local production of 1,25(OH)2D from 25OHd may have important effects on human calcium absorption. Possibly, oral vitamin D, as ergocalciferol (D2) or cholecalciferol (D3) supplements, may be converted to 1,25(OH)2D in the duodenum and therefore increase transcriptional activation of TRPV6, CYP24, and other vitamin D-responsive genes. Vitamin D metabolites secreted in the bile may also need to be considered in producing local effects in the duodenum after activation (11).

These studies with human duodenal tissue have confirmed the 1,25(OH)2D3 responsiveness of the transcripts of TRPV6, PMCA1, and CYP24, as predicted from animal experiments. They have also produced unexpected findings related to expression of CYP27B1 that suggest that local activation of 25OHd to 1,25(OH)2D3 may be important in human duodenal calcium absorption. Further studies, including the use of inhibitors and quantitation of the protein products, are needed to confirm these findings. Clarifications of these mechanisms may help explain some of the unanswered questions relating to the physiology of human calcium absorption.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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