Different Effects of Calcidiol and Calcitriol on Regulating Vitamin D Receptor Target Gene Expression in Human Vascular Smooth Muscle Cells

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Abstract—Vitamin D receptor (VDR) activation is associated with cardiovascular and survival benefits in chronic kidney disease patients. Whether VDR’s hormone, calcitriol, and prehormone, calcidiol, exhibit similar effects in human coronary artery smooth muscle cells (HCASMC) are not known. HCASMC were treated with calcitriol or calcidiol in the presence of 0.9 or 2.06 mM (elevated) phosphate (Pi). The expression of VDR target genes were determined by real-time PCR and Western blotting. HCASMC expressed CYP27B1 mRNA and protein, which was not significantly affected by elevated Pi. HCASMC converted calcidiol to calcitriol at a low rate (~10% in 24 h). Treating cells with high Pi for 48 hr significantly elevated the VDR mRNA (233%) and protein levels (181%). Calcitriol and calcidiol induced CYP24A1 expression with EC50 at 70 and 662 nM, respectively; the effects were not affected by high Pi. Calcitriol and calcidiol stimulated the expression of thrombomodulin with EC50 at 10-100 nM at either 0.9 or 2.06 mM Pi. Calcitriol suppressed the expression of thrombospondin-1 with IC50 at 10-100 nM, whereas calcidiol had no significant effect. Confocal microscopy revealed that calcidiol at 0.1 - 10 µM induced VDR translocation into the nucleus dose-dependently; the VDR localization pattern was similar in cells treated with calcitriol (0.01 µM). In conclusion, calcitriol exhibits a consistent effect on regulating VDR target gene expression, while calcidiol seems to have differential effects on the expression of different genes.

Keywords — calcidiol; calcitriol; smooth muscle cells; vitamin D receptor.


I. INTRODUCTION

Although the synthesis of vitamin D3 occurs naturally in the skin in response to adequate sunlight exposure, vitamin D3 is not active and needs to be converted to 25-hydroxyvitamin D3 (25(OH)D3, calcidiol), and then further hydroxylated by CYP27B1 (25-hydroxyvitamin D 1α-hydroxylase) to form the active metabolite, calcitriol (1,25-dihydroxyvitamin D3 or 1,25(OH)2D3). Calcitriol is a secosteroid hormone that binds to the vitamin D receptor (VDR), a nuclear receptor, to activate multiple signaling pathways in various cells and tissues1,2. According to the National Kidney Foundation, 26 million people in America have chronic kidney disease (CKD). CKD progresses through 5 stages; Stage 5 CKD requires renal replacement therapy (dialysis or transplantation). Deficient production of calcitriol is an early complication of CKD3. CKD patients experience an extremely high rate of cardiovascular disease and mortality4,5. In addition to using vitamin D analogs that activate VDR directly (VDR agonists) to manage PTH (parathyroid hormone) levels in CKD patients, the prehormones, ergocalciferol, cholecalciferol and calcidiol are also frequently used in CKD. Clinical observations demonstrate that treatment with VDR agonists including calcitriol confers cardiovascular and survival benefit in CKD, independent of PTH levels6-21. It is not well studied whether the prehormones have effects similar to VDR agonists in providing cardiovascular benefits and/or improving survival in CKD6,22.

As an attempt to investigate and compare the cardiovascular effects of the VDR hormone, calcitriol, and its prehormone, calcidiol, we have compared the effects of calcitriol and calcidiol on modulating the expression of VDR target genes (CYP24A1, thrombomodulin and thrombospondin-1) in primary culture of human coronary artery smooth muscle cells. Since hyperphosphatemia is associated with adverse cardiovascular outcomes in CKD23 and elevated phosphate has been shown previously to affect the properties of smooth muscle cells24,25, the effects of VDR agonists and the prehormone were studied in the presence of normal (0.9 mM) or elevated (2.06 mM) phosphate (Pi).
II. METHODS

Materials: Calcitriol was provided by Abbott Laboratories. \(^{3}H\)-25(OH)D\(_{3}\) (155 Ci/mmol, NET349025UC) was from PerkinElmer (Waltham, MA, USA). All other reagents were of analytical grade.

Cell culture: Primary culture of human coronary artery smooth muscle cells (Cell Applications, San Diego, CA) were grown in Smooth Muscle Cell Growth Medium (Cell Applications Catalog # 311-500) containing 5% FBS at 37°C in a humidified 5% CO\(_2\)-95% air atmosphere. Cells were grown to >80% confluence and used within five passages.

Real-time RT-PCR: Real-time reverse transcription-PCR was performed with an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Each sample had a final volume of 25 µL containing 200 ng of mRNA, 100 nM (final concentration) each of the forward and reverse PCR primers and 250 nM (final concentration) of the TaqMan™ probe (Applied Biosystems). Temperature conditions consisted of a step of 10 minutes at 95°C, followed by 45 cycles of 60°C for 1 minute and 95°C for 15 seconds. Data were collected during each extension phase of the PCR reaction and analyzed with the software package (Applied Biosystems). Threshold cycles were determined for each gene.

Preparation of anti-human CYP27B1 serum: The antiserum was produced as reported previously.\(^{35}\)

SDS-PAGE and Western Blot Analysis: Cells (1 x 10\(^6\) cells per sample) pretreated with or without test agents were solubilized in 100 µL of SDS-PAGE sample buffer (Invitrogen, Carlsbad, CA), and the protein content in each sample was determined by the Pierce (Rockford, IL) BCA protein assay. Samples were resolved by SDS-PAGE using a 4-12% NuPAGE gel (Invitrogen), and proteins were electrophoretically transferred to PVDF membrane for Western blotting. The membrane was blocked for 1 h at 25°C with 5% nonfat dry milk in PBS-T and then incubated with a rabbit anti-CYP27B1 polyclonal antibody (500-fold dilution), a mouse anti-VDR monoclonal antibody (200-fold dilution, Santa Cruz Biotechnology), a mouse anti-THBS1 (thrombospondin-1) monoclonal antibody (2000-fold dilution, Calbiochem, La Jolla, CA), or a mouse anti-TM (thrombomodulin) monoclonal antibody (2000-fold dilution, Santa Cruz Biotechnology) in PBS-T overnight at 4°C. The membrane was washed with PBS-T and incubated with a horseradish peroxidase-labeled anti-mouse or -rabbit antibody for 1 h at 25°C. The membrane was then incubated with Amersham ECL Plus Western Blotting Detection Reagents. Specific bands were visualized by the Multimage II of Alpha Innotech (San Leandro, California) imaging system. Band intensity was quantified using Spot Denso (Alpha Innotech). The density of each band was measured and normalized to the protein content in each sample.

Sample Extraction and High Performance Liquid Chromatography (HPLC) Analysis: Cells in 10 mL medium were incubated with 25(OH)D\(_{3}\) (100 nM) + [\(^{3}H\)-25(OH)D\(_{3}\) (~200,000 dpm) for 24 hrs. Cells were then mixed with an equal volume of acetonitrile. The mixture was vortexed for 30 seconds, and centrifuged at 3000 rpm for 10 min. The supernatant was applied to a SepPak C18 cartridge (Waters Associates, Milford, MA) that was pre-washed with 5 mL hexane, 5 mL chloroform, 5 mL methanol, followed by 5 mL water. After the sample application, the column was washed with 5 mL water, and then eluted with 4 mL of acetonitrile. The sample was dried under nitrogen and resuspended in 100 µL of solvent containing 50% acetonitrile and 50% Mobile Phase A (see below). Sample analysis was performed with an Agilent 1100 HPLC System equipped with a Packard Radiomatic 525TR radiodetector using a Phenomenex Luna 5 µm Phenyl-Hexyl column (4.6 × 250 mm) and a Phenomenex Octadecyl guard column (4 × 3 mm). The mobile phase consisted of (A) 95% of 10 mM ammonium acetate and 5% acetonitrile (pH 3.99), and (B) 100% acetonitrile. The 30 min gradient (1 ml/min) consisted of 0-3 min at 55% B and 3-30 min at 55-85% B linear, followed by 30 min of column re-equilibration prior to the next run.

**Fig. 1. Expression of CYP27B1 in HCASMC.** Cells were cultured at 0.9 or 2.06 mM Pi for 48 hr. (A) RNA was isolated and the CYP27B1 mRNA level analyzed by real-time RT-PCR. GAPDH was used for normalization. Statistical analysis was performed by unpaired t-test; n=3 per condition. (B) Samples were solubilized, resolved by SDS-PAGE, and subjected to Western blot analysis as described in “Materials and Methods”. Results were representative of 2 independent experiments.

**Fig. 2. Expression of VDR in HCASMC.** (A) Cells were cultured in medium containing 0.9 or 2.06 mM Pi for 48 hrs. The VDR mRNA level was analyzed by real-time RT-PCR. GAPDH was used for normalization. Statistical analysis was performed by unpaired t-test. ***p<0.001 compared with 0.9 nM Pi; n=3 per condition. (B) SMCs were cultured in medium containing 0.9 or 2.06 mM Pi for 5 days. Cells were solubilized and Western blotting performed as described in “Materials and Methods”. The specific bands were visualized and the density of each band was measured and normalized to the protein level in each sample.

Confocal Microscopy: Cells grown in 0.1% gelatin coated eight-chamber slides (Lab Tek II, Nunc, Rochester, NY) were treated with test agent (as indicated) for 60 min. Cells were washed with D-PBS for 30 sec, fixed with 4% paraformaldehyde in D-PBS for 20 min at room temperature,
The expression and activity of CYP27B1 in HCASMC were examined. Fig. 1A shows the results from real-time PCR that a low level of CYP27B1 mRNA was detected. Western blotting results indicated that the CYP27B1 protein was present in the cells (Fig. 1B). Increasing Pi from 0.9 to 2.06 mM slightly elevated the expression of CYP27B1, but the difference did not reach statistic significance. Calcitriol did not affect CYP27B1 expression in SMC after 48 hr of incubation (data not shown). Fig. 2 shows the results from real-time PCR and Western blotting that VDR expression was detected in HCASMC. Increasing the phosphate concentration from 0.9 to 2.06 mM in the culture medium up-regulated the VDR mRNA (233%) and protein (181%).

The activity of CYP27B1 was measured by the conversion of calcidiol into calcitriol. The HPLC profile of the [3H]-calcitriol standard is shown in Fig. 3A. Fig. 3B shows that, after [3H]-calcidiol (200,000 dpm of [3H]-calcidiol plus 100 nM unlabeled calcidiol) was incubated with HCASMC for 24 hr, a radioactive peak at the [3H]-calcitriol position (8.50 min) was detected, along with some small peaks at various time points. From the integrated area, the formation of calcitriol from calcidiol was 10% of total area during the 24 hr incubation period. When [3H]-calcidiol was incubated with culture medium without cells, no formation of [3H]-calcitriol was detected (Fig. 3C).

CYP24A1, a VDR target gene, encodes 25-hydroxyvitamin D-24-hydroxylase, the enzyme responsible for catabolizing vitamin D analogs\(^\text{7}\). The effects of calcitriol and calcidiol on the expression of CYP24A1 were compared. Fig. 4A shows that the basal expression level of CYP24A1 mRNA (in the absence of vitamin D analogs) was low. Calcitriol induced CYP24A1 mRNA expression in these cells with an EC\(50\) value at 70 nM, whereas calcidiol was less potent with an EC\(50\) at 662 nM. Fig. 4B shows that the different potency between calcitriol and calcidiol was observed at either 0.9 mM or 2.06 mM Pi. The results demonstrate that calcitriol was more potent than calcidiol in inducing CYP24A1 expression.

We have previously reported that vitamin D analogs such as calcitriol and paricalcitol modulate the expression of thrombospondin-1 (THBS1) and thrombomodulin (TM) in human coronary artery and aortic smooth muscle cells\(^\text{28,29}\). The effects of calcitriol and calcidiol in inducing the express of THBS1 and TM were compared. Fig. 5A shows that calcitriol suppressed the expression of THBS1 with an IC\(50\) at \(\sim\)10 nM,
and inducing TM with an EC50 between 10-100 nM. Interestingly, calcidiol did not show a significant effect on modulating the expression of THBS1, whereas it induced TM expression with a potency similar to calcitriol. The study was repeated at an elevated Pi concentration at 2.06 mM. As shown in Fig. 5B, similar results were obtained that calcidiol was similar to calcitriol in inducing TM, but did not have a significant effect on THBS1 expression.

Fig. 5. Effects of calcitriol and calcidiol on the expression of THBS1 and TM in HCASMC. HCASMC were treated with calcitriol or calcidiol (concentrations as indicated) in the presence of (A) 0.9 mM or (B) 2.06 mM Pi for 48 hrs. Samples were solubilized, resolved by SDS-PAGE, and subjected to Western blot analysis. The density of the THBS1 or TM band was measured and the density of each band was normalized to the protein level in each sample. Results are representative of two independent experiments.

To investigate the mechanism for the different effects of calcitriol and calcidiol on gene expression, we then treated HCASMC with calcitriol or calcidiol for 60 min, and then fixed and stained cells with an anti-VDR antibody to examine the effect of these two drugs on the subcellular localization of VDR. Fig. 6 shows representative fields from confocal microscopy. VDR was stained diffusely and evenly in cells without drug treatment. Both calcitriol at 0.1 µM and calcidiol at 10 µM induced more VDR translocation into the nuclei (Fig. 6A). Fig. 6B shows that the effect of calcidiol was dose-dependent and appeared to be maxed out at 1 µM.

IV. DISCUSSION

In this study, we aimed at comparing the effects of calcitriol and calcidiol on regulating gene expression in primary culture of human coronary artery smooth muscle cells (HCASMC). We were surprised to find that calcidiol, the prehormone of calcitriol, exhibited differential effects on modulating the expression of thrombospondin-1 and thrombomodulin. The results are significantly different from our previous observations in neonatal rat cardiomyocytes that VDR hormone calcitriol and its analog paricalcitol consistently exhibit more potent effects than the prehormone calcidiol in regulating the expression of various genes such as VDR, CYP24A1 and NPPB (the gene that encodes brain natriuretic peptide)25.

Fig. 6. Localization of VDR in HCASMC. (A) Cells were incubated without or with drugs (as indicated) for 60 min, and then stained for VDR (green), nucleus (blue) and cytoplasm (red, not shown) as described in “Materials and Methods”. (B) Cells were incubated with calcidiol at 0.1, 1 and 10 µM and stained as in (A). Dimensional bar: 50 µm.

Thrombospondin-1 (THBS1) and thrombomodulin (TM) are known to be involved in fibrinolysis and thrombogenicity. Up-regulation of THBS1 is associated with the development of thrombosis, atherosclerosis and vascular injury30,31, whereas an early loss of TM expression resulted in an enhanced thrombin generation32,33 and a local overexpression of TM prevented atherothrombosis34. Previously we have demonstrated that,
using Western blotting analysis, calcitriol suppressed the expression of THBS1 in HCASMC and human aortic smooth muscle cells with IC₅₀ in the range of 10-100 nM. Our current study shows similar results for calcitriol on regulating THBS1 expression, which is not affected by elevated phosphate (Pi). Results from this study on TM are also consistent with our previous reports that calcitriol induces the expression of TM in human smooth muscle cells with EC₅₀ in the range of 10-100 nM. However, while calcitriol is similar in potency to calcidiol in inducing the expression of TM, it has minimal effects in suppressing THBS1, and it is also about 100-fold less potent than calcitriol in inducing CYP24A1 expression. The same observation was made at normal (0.9 mM) or elevated (2.06 mM) Pi.

Our results clearly demonstrated that HCASMC expressed CYP27B1 and were capable of converting calcidiol to calcitriol. However, the conversion rate is relatively low with only ~10% of calcidiol converted into calcitriol during a 24 hr incubation period. The low conversion rate may explain why calcitriol is much less potent than calcitriol in regulating the expression of THBS1 and CYP24A1, but it cannot explain why calcidiol is similar in potency to calcitriol in inducing TM expression in HCASMC. Previously Kemmis et al. reported that in human mammary epithelial cells calcitriol and calcidiol exhibited similar potencies in inhibiting cell proliferation. However, Kemmis et al. did not measure CYP27B1 activity in their study. A study by Somjen et al. reported that vascular smooth muscle cells from human umbilical artery converted calcidiol to calcitriol dose-dependently with a maximal conversion rate at ~0.02% per hour. Somjen et al. also demonstrated that calcitriol inhibited the proliferation of vascular smooth muscle cells from human umbilical artery in a dose-dependent manner, which is similar to the observation made by Carthy et al. in rat vascular smooth muscle cells, and is also consistent with our previous report in HCASMC. However, Somjen et al. and Carthy et al. did not compare the functional effect of calcidiol vs. calcitriol. One limitation of the current study is the lack of data on other genes that may be involved in VDR's cardiovascular effects. Gene chip microarray analysis will be needed as a follow-up study to obtain a broad profile of the modulatory effects of calcitriol vs. calcidiol in these cells.

As a way to investigate whether calcitriol and calcidiol activate VDR differently, leading to differential effects on THBS1 and TM, VDR localization in the presence of calcitriol and calcidiol was studied. The confocal microscopy results show that calcitriol treatment for 60 min increases VDR staining in the nucleus. The observation is consistent with our previous studies in the HL-60 promyelocytic leukemia cells and the pig parathyroid cells that VDR agonists induce VDR translocation into the nucleus. However, unlike in the HL-60 and pig parathyroid cells that VDR resides predominantly in the cytoplasm in the absence of VDR agonists, the VDR staining in HCASMC without drug treatment exhibits a more diffused distribution throughout the cells. Our results suggest that calcitriol also induces VDR translocation into the nucleus in a dose-dependent manner, although it is at least 100-fold less potent than calcitriol. These results are consistent with the observations that calcidiol is less potent in regulating THBS1 and CYP24A1, but did not shed light on calcidiol’s effect on inducing the expression of TM.

The calcidiol (25(OH)D₃) level is routinely measured in the clinical setting to determine vitamin D deficiency. However, whether the calcidiol level is indicative of the VDR activation status remains a question. VDR agonists such as calcitriol and paricalcitol are associated with cardiovascular and survival benefits in CKD. Clinical Data demonstrate that the prehormones are also effective in controlling PTH (parathyroid hormone) in CKD, albeit the data are not as robust as those observed for calcitriol and its analogs that directly activate VDR. Nevertheless, correction of 25(OH)D₃ deficiency with ergocalciferol, cholecalciferol or calcidiol has constantly been advocated in CKD patients. Data from the current study show that, unlike calcitriol that has a consistent effect on regulating VDR target genes, calcidiol exhibits different effects on different genes in HCASMC. The results affirm the notion that there is a need for clinical studies to investigate and compare whether prehormones and VDR agonists provide similar cardiovascular and/or survival benefits in CKD patients or not.

V. CONCLUSIONS

In conclusion, our results demonstrate for the first time that, although calcitriol exhibits a consistent effect on regulating VDR target gene expression, calcidiol appears to have differential effects on the expression of different genes in primary culture of human coronary artery smooth muscle cells.

REFERENCES


J. Ruth Wu-Wong obtained her Ph.D. in biochemistry from Ohio State University and her M.B.A. in finance and management from University of Illinois. She joined Vidasym as CSO in June, 2009. Before Vidasym, she was at Abbott for 20+ years where she played a key role in the development of endothelin receptor antagonists, including atrasentan which is now in Phase III clinical studies for diabetic nephropathy. She was the lead scientist on several novel-target projects at Abbott including the vitamin D receptor modulator project. Her last position at Abbott was as a Senior Commercial Development Manager in Renal Care Marketing. She was also a Research Fellow in Abbott’s Volwiler Society, an inventor of 6 patent applications, and has published more than 100 peer-reviewed papers.