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VDR-Mediated Gene Expression Patterns in Resting Human Coronary Artery Smooth Muscle Cells

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Abstract Vitamin D analogs such as paricalcitol and calcitriol that activate the vitamin D receptor (VDR) provide survival benefit for Stage 5 chronic kidney disease (CKD) patients, possibly associated with a decrease in cardiovascular (CV)-related incidents. Phenotypic changes of smooth muscle cells play an important role in CV disease. The role of vitamin D analogs in modulating gene expression in smooth muscle cells is still not well understood. In this study, DNA microarray analysis of ~22,000 different human genes was used to characterize the VDR-mediated gene expression profile in human coronary artery smooth muscle cells (CASMC) at rest. Cells in serum free medium were treated with 0.1 μM calcitriol (1α,25-dihydroxyvitamin D_3) or paricalcitol (19-nor-1α,25-$(OH)_2D_2$) for 30 h. A total of 181 target genes were identified, with 103 genes upregulated and 78 downregulated (>two fold changes in either drug treatment group with $P < 0.01$). No significant difference was observed between calcitriol and paricalcitol. Target genes fell into various categories with the top five in cellular process, cell communication, signal transduction, development, and morphogenesis. Twenty-two selected genes linked to the CV system were also impacted. Real-time RT-PCR and/or Western blotting analysis were employed to confirm the expression patterns of selected genes such as 25-hydroxyvitamin D-24-hydroxylase, Wilms' tumor gene 1, transforming growth factorβ3, plasminogen activator inhibitor-1, thrombospondin-1 (THBS1), and thrombomodulin (TM). This study provides insight into understanding the role of VDR in regulating gene expression in resting smooth muscle cells. J. Cell. Biochem. 100: 1395–1405, 2007.

Key words: vitamin D analogs; vitamin D receptor; human coronary artery smooth muscle cells; microarray

1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$, calcitriol) is a secosteroid hormone that activates multiple signaling pathways in various cells and tissues. Although the synthesis of vitamin D_3 occurs naturally in the skin with adequate sunlight exposure, vitamin D_3 is not active and needs to first be converted to 25-OH-D_3 by 25-hydroxylase in the liver. From the liver, 25-OH-D_3 is transported to the kidney and then hydroxylated by 25-hydroxyvitamin D 1α-hydroxylase to form the active hormone, calcitriol. Calcitriol is metabolized by 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) [Brown et al., 2002] to yield the biliary excretory product calcitroic acid. The binding of calcitriol or its analogs to the vitamin D receptor (VDR), a nuclear receptor, activates VDR to recruit cofactors to form a complex that binds to vitamin D response elements in the promoter region of target genes to regulate gene transcription [Carlberg et al., 2001].

Chronic kidney disease (CKD) patients experience a high mortality rate from CV diseases [Weiner et al., 2004; Coresh et al., 2005]. Calcitriol or its analogs such as paricalcitol (19-nor-1α,$25(OH)_2D_2$) are commonly used to manage secondary hyperparathyroidism associated with CKD [Martin and Gonzalez, 2004]. Recent retrospective clinical observations show that vitamin D analogs provide survival benefit for Stage 5 CKD patients in the effectiveness order of paricalcitol > calcitriol > no vitamin D analog therapy, independent of the PTH and calcium levels [Teng et al., 2003, 2005; Nakai et al., 2004]. Furthermore, the survival benefit is associated with a decrease in CV-related incidents [Shoji et al., 2004]. More recently, results from the

Abbreviation used: SMC, human coronary artery smooth muscle cells; VDR, vitamin D receptor.

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DOPPS time-dependent analyses suggest that the survival benefit is in the order of IV paricalcitol > IV calcitriol > oral vitamin D analogs = no vitamin D analogs [Young et al., 2005].

Although data from clinical studies demonstrate the positive impact of vitamin D analogs on the CV system, the mechanism of action is largely unknown. CKD patients are known to have advanced atherosclerotic disease [Raggi, 2005]. Atherosclerosis, the principal cause of myocardial infarction, stroke, and peripheral vascular disease, is a process that involves a complex interplay among different factors and cell types including smooth muscle cells [Kher and Marsh, 2004]. The role of vitamin D analogs on modulating gene expression in smooth muscle cells is not well studied. We have previously shown that, in proliferating human coronary artery smooth muscle cells (CASMC), VDR plays a key role in modulating a cluster of genes involved in cell differentiation/proliferation and that paricalcitol and calcitriol inhibit smooth muscle cell growth [Wu-Wong et al., 2006b]. But it remains unknown how vitamin D analogs modulate gene expression in smooth muscle cells at rest. In this study, DNA microarray analysis was used to characterize the VDR-mediated gene expression profile in human CASMC in serum free medium.

MATERIALS AND METHODS

Materials

1α,25-dihydroxyvitamin D_3 (1α, 25-$(OH)_2D_3$, calcitriol) and 19-nor-1α, 25-dihydroxyvitamin D_2 (19-nor-1α, 25-$(OH)_2D_2$, paricalcitol) were from Abbott Laboratories.

Cell Culture

Primary culture of human CASMC (Cambrex) were grown in SmGM-2 containing 5.5 mM glucose, 5% FBS, 50 μg/ml gentamicin, 50 ng/ml amphotericin-B, 5 μg/ml insulin, 2 ng/ml hFGF, and 0.5 ng/ml hEGF at 37°C in a humidified 5% CO_2 to 95% air. Cells were grown to >80% confluence and used within five passages.

Microarray

Total RNA was extracted from human CASMC that were treated with control (0.01% ethanol), 100 nM paricalcitol, or calcitriol in serum free media for 30 h. The yield of total RNA was generally low (~2.0 μg total), however the RNAs were intact as judged by Agilent 2100 analysis. One microgram of total RNA from each sample was used to prepare biotin-labeled cRNA target using standard Affymetrix protocols. Prepared cRNA targets were of good quality and quantity. The Affymetrix Human chip U133Av2 was used (22,000+ probe sets) and 10 μg cRNA target was applied to each array. After hybridization and chip scanning, the quality control data report (i.e., scaling factor, GAPDH 5′/3′ ratio, noise, background) demonstrated that every array passed all quality criteria. Scanned images were loaded into the Rosetta Resolver 4.0 database and processed using the Resolver Affymetrix error model. Within Resolver the vitamin D analog-treated sample replicates (n = 3) were informatically combined and ratios constructed relative to the combined vehicle control samples. A combination of hierarchical clustering, gene ontology analysis, and pathway mapping were used to assess the function of the regulated genes.

Real-Time Reverse Transcription-PCR

Real-time reverse transcription-PCR was performed with a MyiQ Real-Time PCR Detection System (BioRad, Hercules, CA). Each sample has a final volume of 25 μl containing 100 ng of cDNA, 0.4 mM each of the forward and reverse PCR primers, and 0.1 mM of the TaqMan™ probe for the gene of interest (Applied Biosystems). Temperature conditions consisted of a step of 5 min at 95°C, followed by 40 cycles of 60°C for 1 min, and 95°C for 15 s. Data was collected during each extension phase of the PCR reaction and analyzed with the software package (BioRad). Threshold cycles were determined for each gene.

SDS–PAGE and Western Blot Analysis

Cells (~1×10^6 cells per sample) pretreated with or without test agents were solubilized in 50 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 mouse anti-plasminogen activator inhibitor-1 (PAI-1) monoclonal antibody

(1,000-fold dilution, Santa Cruz Biotechnology, Santa Cruz, CA), a mouse anti-thrombospondin-1 (THBS1) monoclonal antibody (2,000-fold dilution, Calbiochem, La Jolla, CA), or a mouse anti-thrombomodulin (TM) monoclonal antibody (2,000-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 antibody for 1 h at 25°C. The membrane was then incubated with detection reagent (SuperSignal WestPico, Pierce). Specific bands were visualized by exposing the paper to Kodak BioMax films. Band intensity was quantified by Quantity One (Bio-Rad). For normalizing purposes, the membrane was stripped following the manufacturer's instruction (Pierce), and re-probed with a goat anti-actin antibody (500-fold dilution, Santa Cruz Biotechnology).

RESULTS

Effects of Paricalcitol Versus Calcitriol

Using a two fold change in average difference in either the paricalcitol- or calcitriol-treated group as cutoff with $P < 0.01$ for significantly modulated expression, a total of 181 target genes were identified with 103 and 78 genes up- and downregulated, respectively. 25-hydroxyvitamin D-24-hydroxylase (CYP24A1), a known target gene of VDR and a key enzyme involved in the metabolism of endogenous vitamin D, exhibited the largest change, with a 47.5 and 46.6-fold upregulation by paricalcitol and calcitriol, respectively. Figure 1 shows the hierarchical clustering of genes regulated by either paricalcitol or calcitriol. These results suggest that there is no significant difference between the effects of paricalcitol and calcitriol (at 0.1 μM after 30 h of incubation) on target gene expression in resting SMC.

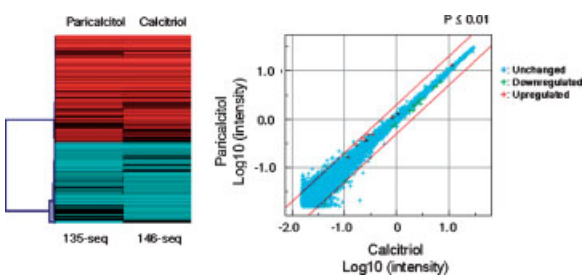

Fig. 1. The effects of paricalcitol and calcitriol. The genes regulated by either paricalcitol or calcitriol are shown in hierarchical clustering.

Functional Clustering of Modulated Genes: Gene Ontology Analysis With DAVID 2.1

Because there was no significant difference observed between paricalcitol and calcitriol, only the paricalcitol data set was analyzed using DAVID 2.1 (http://apps1.niaid.nih.gov/david/) to assess the general effects of drugs on intracellular signaling and metabolic pathways. A number of functionally related clusters were revealed. Gene ontology categories with at least three genes with $P < 0.01$ regulated by paricalcitol are shown in Table I. The top five clusters under "Biological Process" ranked by *P*-value were morphogenesis, cell communication, organ development, organogenesis, and development.

Selected Genes Linked to Cell Differentiation/Proliferation

Selected genes regulated by paricalcitol and calcitriol that have been previously linked to cell differentiation/proliferation are shown in Table II. When these results were compared to our previous study testing the effect of paricalcitol and calcitriol on proliferating human CASMC [Wu-Wong et al., 2006b], several genes previously identified in proliferating SMC were no longer observed. For example, tetraspan 1 (Affy ID# 209114_at), cyclin F (Affy ID# 204827_s_at), and cholecystokinin B receptor (Affy ID# 210381_s_at), which were downregulated in proliferating cells, were no longer detected in this study. Also, transforming growth factor beta 2 (Affy ID# 220407_s_at) and CKD5 regulatory subunit associated protein 2 (Affy ID# 220935_s_at), which were upregulated in proliferating cells, were not significantly changed in this study. Furthermore, seven genes that were not detected in proliferating cells were now identified.

Selected Genes Linked to Cardiovascular (CV) Functions

Selected genes regulated by paricalcitol and calcitriol that have been previously linked to CV functions are shown in Table III. When these results were compared to our previous study in proliferating human CASMC, 10 out of 16 genes that were previously identified in proliferating SMC exhibited similar changes (either up- or downregulation) in this study (Table III), but changes in natriuretic peptide prescursor B, coagulation factor II receptor-like 1, fibroblast

TABLE I. Gene Ontology Analysis Using DAVID 2.1 (http://apps1.niaid.nih.gov/david; Based on Paricalcitol Expression With at Least Three Genes and $P < 0.01$; Top Categories in Biological Process, Molecular Function, and Cellular Component Are Shown)

Cluster	# of genes	%	*P*-value
A: Biological process			
Morphogenesis	32	22	2.26E-10
Cell communication	57	39	1.42E-8
Organ development	26	18	1.98E-8
Organogenesis	26	18	2.06E-8
Development	36	25	5.32E-8
Response to wounding	15	10	5.42E-7
Signal transduction	46	32	1.96E-6
Neurogenesis	14	9	1.06E-5
Response to external stimulus	26	18	1.39E-5
Second-messenger-mediated signaling	9	6	6.37E-5
Response to stress	20	13	7.61E-5
Intracellular signaling cascade	20	13	1.51E-4
Growth	8	5	1.62E-4
Cell surface receptor linked signal transduction	27	18	2.03E-4
Chemotaxis	7	4	3.10E-4
Taxis	7	4	3.10E-4
Regulation of cellular process	17	11	3.29E-4
G-protein signaling, coupled to IP3 second messenger (phospholipase C activating)	6	4	3.47E-4
Phosphoinositide-mediated signaling	6	4	3.67E-4
Response to chemical substance	9	6	3.71E-4
Cell motility	8	5	6.24E-4
Inflammatory response	8	5	6.42E-4
Response to abiotic stimulus	11	7	6.43E-4
Cellular process	104	72	1.02E-3
Cell proliferation	19	13	1.87E-3
Regulation of cellular physiological process	12	8	4.82E-3
Wound healing	5	3	6.08E-3
Cellular morphogenesis	8	5	8.67E-3
Reproductive physiological process	4	2	8.81E-3
Cell organization and biogenesis	14	9	9.23E-3
B: Molecular function			
Protein binding	33	23	6.18e-5
Receptor binding	15	10	7.64E-5
Signal transducer activity	41	28	8.62E-4
Cytokine activity	8	5	1.23E-3
Enzyme inhibitor activity	8	5	1.34E-3
Growth factor activity	7	4	1.51E-3
Endopeptidase inhibitor activity	6	4	3.57E-3
Protease inhibitor activity	6	4	3.67E-3
Pattern binding	5	3	5.96E-3
Enzyme regulator activity	11	7	8.71E-3
Binding	83	58	8.85E-3
Calmodulin binding	5	3	9.29E-3
C: Cellular component			
Extracellular region	23	16	3.89E-4
Integral to plasma membrane	19	13	4.26E-3
Plasma membrane	23	16	6.75E-3

growth factor receptor 3, fibroblast growth factor 9, matrix metalloproteinase 14, and transforming growth factor beta 2 were no longer noted. Interestingly, 12 CV-related genes unique to this study were identified (Table III).

Confirmation of Gene Expression Patterns by Real-Time RT-PCR

Results from real-time RT-PCR analysis confirm that VDR was involved in modulating the expression of CYP24A1, Wilms' tumor gene 1 (WT1) and transforming growth factorβ3 (TGFβ3) (Fig. 2). WT1 encodes a transcription factor that regulates cell differentiation, and has been shown to modulate the expression of VDR [Maurer et al., 2001]. TGFβ signaling is well known to inhibit cell proliferation and promote differentiation; previously it has been shown that vitamin D analogs induced the expression of TGFβ in cancer cells [Yang et al., 2001]. The EC_{50} (concentration that achieves 50% of maximum effect) values of paricalcitol and calcitriol on CYP24A1 were 6.9 and 1.3 nM, on WT1, 9 and 14 nM, and on TGFβ3, 2.5 and 2.8 nM, respectively. The increase induced by

TABLE II. Effects of Paricalcitol and Calcitriol on Selected Genes That Are Linked to Cell Differentiation/Proliferation

Affy ID	Sequence name(s)	Sequence description	Paricalcitol		Calcitriol	
			FC	*P*-value	FC	*P*-value
209540_at	IGF1	*Insulin-like growth factor 1 (somatomedin C)*	4.1	0.000	4.2	0.000
209541_at	IGF1	*Insulin-like growth factor 1 (somatomedin C)*	2.6	0.000	2.8	0.000
209542_x_at	IGF1	*Insulin-like growth factor 1 (somatomedin C)*	2.7	0.000	2.9	0.000
211577_s_at	IGF1	*Insulin-like growth factor 1 (somatomedin C)*	2.5	0.000	2.8	0.000
210058_at	MAPK13	*Mitogen-activated protein kinase 13*	2.1	0.000	1.9	0.000
210059_s_at	MAPK13	*Mitogen-activated protein kinase 13*	2.9	0.000	2.6	0.000
213524_s_at	G0S2	*Putative lymphocyte G0/G1 switch gene*	3.1	0.000	3.0	0.000
203372_s_at	SOCS2	*Suppressor of cytokine signaling 2*	-2.2	0.000	−1.9	0.000
209747_at	TGFB3	*transforming growth factor, beta 3*	2.8	0.000	2.8	0.000
206954_at	WIT-1	*Wilms tumor associated protein*	2.7	0.000	2.7	0.000
206067_s_at	WT1	*Wilms tumor 1*	4.5	0.000	4.5	0.000
216953_s_at	WT1	*Wilms tumor 1*	7.4	0.000	7.6	0.000
202921_s_at	ANK2	Ankyrin 2, neuronal	−1.6	0.130	−2.0	0.003
202575_at	CRABP2	Cellular retinoic acid binding protein 2	2.1	0.000	2.1	0.000
203475_at	CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1	3.1	0.004	2.6	0.020
205659_at	HDAC9	Histone deacetylase 9	−1.9	0.000	−2.1	0.000
210511_s_at	INHBA	Inhibin, beta A (activin A, activin AB alpha polypeptide)	−2.2	0.000	−2.1	0.000
206391_at	RARRES1	Retinoic acid receptor responder (tazarotene induced) 1	1.8	0.003	2.1	0.000
209189_at	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	−4.4	0.005	−1.6	0.477

Genes in italic have been previously identified in proliferating SMCs [Wu-Wong et al., 2006b]. FC, fold of change.

TABLE III. Effects of Paricalcitol and Calcitriol on Selected Genes That Are Linked to Cardiovascular Function

Affy ID	Sequence name (s)	Sequence description	Paricalcitol		Calcitriol	
			FC	*P*-value	FC	*P*-value
205608_s_at	ANGPT1	*Angiopoietin 1*	2.7	0.000	2.1	0.000
204273_at	EDNRB	*Endothelin receptor type B*	3.3	0.000	3.1	0.000
209542_x_at	IGF1	*Insulin-like growth factor 1(somatomedin C)*	2.7	0.000	2.9	0.000
206825_at	OXTR	*Oxytocin receptor*	−2.3	0.000	−2.0	0.000
204939_s_at	PLN	*Phospholamban*	−1.4	0.212	−2.5	0.004
215813_s_at	PTGS1	*Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)*	3.5	0.000	3.4	0.000
203887_s_at	THBD (TM)	*Thrombomodulin*	4.6	0.000	4.3	0.000
201107_s_at	THBS1	*Thrombospondin 1*	−5.5	0.000	−2.6	0.002
209747_at	TGFB3	*Transforming growth factor, beta 3*	2.8	0.000	2.8	0.000
204933_s_at	TNFRSF11B	*Tumor necrosis factor receptor superfamily, member 11b(osteoprotegerin)*	−2.2	0.000	−2.1	0.000
209869_at	ADRA2A	Adrenergic, alpha-2A-, receptor	−2.0	0.000	−2.3	0.000
215199_at	CALD1	Caldesmon 1	−1.8	0.010	−2.2	0.002
205066_s_at	ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1	2.1	0.005	2.1	0.005
218083_at	PTGES2	Homo sapiens prostaglandin E synthase 2 (PTGES2), mRNA.	−4.5	0.001	−2.3	0.031
218730_s_at	OGN	Osteoglycin (osteoinductive factor, mimecan)	−2.4	0.000	−2.3	0.000
205907_s_at	OMD	Osteomodulin	−2.3	0.006	−2.1	0.010
209355_s_at	PPAP2B	Phosphatidic acid phosphatase type 2B	−1.8	0.000	−2.2	0.000
215894_at	PTGDR	Prostaglandin D2 receptor (DP)	1.8	0.008	2.2	0.000
200635_s_at	PTPRF	Protein tyrosine phosphatase, receptor type, F	2.0	0.000	2.0	0.000
202628_s_at	SERPINE1 (PAI-1)	Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	−3.6	0.000	−3.9	0.000
201149_s_at	TIMP3	Tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	2.1	0.000	2.4	0.000
212171_x_at	VEGF	Vascular endothelial growth factor	2.4	0.000	2.6	0.000

Genes in italic have been previously identified in proliferating SMCs [Wu-Wong et al., 2006b]. FC, fold of change.

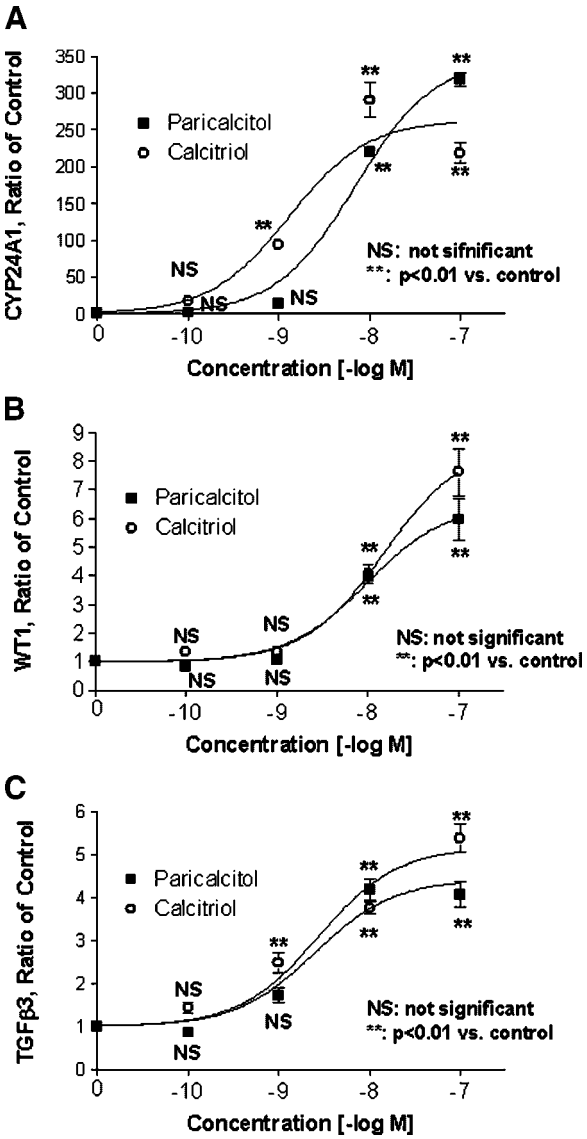

Fig. 2. Effects of paricalcitol and calcitriol on CYP24A1 (**A**), TGFβ3 (**B**), and WT1 (**C**) mRNA expression. Smooth muscle cells in serum free medium were treated with paricalcitol or calcitriol at indicated concentrations for 24 h. RNA was isolated and real-time RT-PCR was performed as described. The mRNA levels of target genes were first normalized to the GAPDH mRNA level, and then calculated as a ratio of control (untreated, ratio = 1). The basal levels of CYP24A1, TGFβ3, and WT1 were 0.014%, 0.007%, and 0.002% of the GAPDH level, respectively. Statistical analysis was performed by ANOVA (n = 4 per condition).

100 nM paricalcitol or calcitriol was ~250-fold, 6.5-fold, and 4.5-fold for CYP24A1, WT1, and TGFβ3, respectively. These results are consistent with the microarray data. Interestingly, although paricalcitol is about fivefold less potent than calcitriol in inducing CYP24A1, it is as potent as calcitriol in regulating the expression of WT1 and TGFβ3, suggesting that VDR agonists may have differential effects on different genes.

We also selected three genes from the list of CV function genes for follow-up. TM is a monomeric transmembrane protein that serves as a cell surface receptor for thrombin; downregulation of TM is associated with thrombosis and atherosclerosis. Thrombospondin-1 (THBS1) is a large glycoprotein that is released into the extracellular matrix by several cell types including smooth muscle. Plasminogen activator inhibitor-1 (PAI-1) is one of the risk markers for coronary heart disease; upregulation of PAI-1 is associated with the development of thrombosis, atherosclerosis, and vascular injury.

Results from real-time RT-PCR analysis show that the TM mRNA level was upregulated by both paricalcitol and calcitriol in a dose-dependent manner in SMC (Fig. 3A). The EC_{50} values of paricalcitol and calcitriol for upregulation of TM mRNA were 3.2 and 6.0 nM, respectively. At 0.1 μM, paricalcitol or calcitriol stimulated TM mRNA expression by ~3.5-fold, which was in line with the ~4.5-fold change shown in the microarray study (Table III). Figure 3B shows that, although the expression level of TM mRNA was reduced in proliferating SMC (25% of that in resting SMC), both paricalcitol and calcitriol at 100 nM still upregulated TM expression by 2.3- and 2.6-fold, respectively.

The microarray data show that paricalcitol at 100 nM suppressed THBS1 expression by 5.5-fold, while calcitriol did by 2.6-fold. However, Figure 4A shows that the THBS1 mRNA level was reduced by both paricalcitol and calcitriol in a dose-dependent manner with a ~2.5-fold decrease observed at 100 nM paricalcitol or calcitriol (Fig. 4A). The EC_{50} of paricalcitol and calcitriol on inhibiting THBS1 mRNA were 0.09 and 0.16 nM, respectively. Figure 4B shows that the expression of THBS1 mRNA was decreased in SMC cultured in growth medium (55% of that in resting SMC), and both paricalcitol and calcitriol at 100 nM suppressed THBS1 mRNA expression by 40% and 44%, respectively.

Similarly, the PAI-1 mRNA level was reduced by both paricalcitol and calcitriol in a dose-dependent manner (Fig. 5A). The maximal suppression of PAI-1 mRNA expression (~50%) was achieved at 0.1 μM of paricalcitol or calcitriol. The EC_{50} values of paricalcitol and

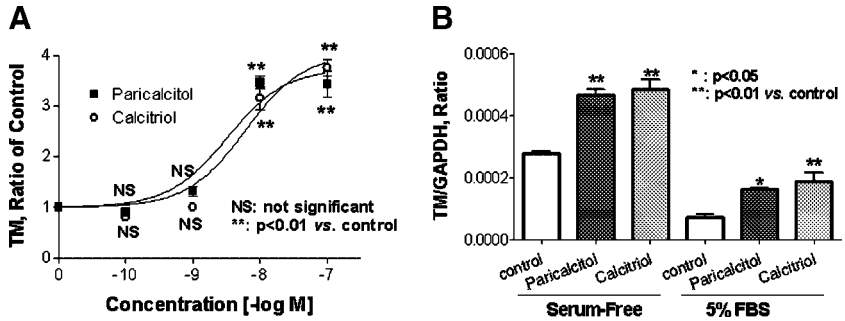

Fig. 3. Effect of paricalcitol and calcitriol on TM mRNA expression. **A**: Smooth muscle cells in serum free medium were treated with paricalcitol or calcitriol at indicated concentrations for 24 h. The expression level of TM mRNA were first normalized to the GAPDH mRNA level, and then calculated as a ratio of control (untreated, ratio = 1). **B**: Cells in serum free medium or growth medium containing 5% FBS were treated with paricalcitol or calcitriol at 100 nM for 24 h. RNA were isolated and the TM mRNA level analyzed by real-time RT-PCR. GADPH was used for normalization. The basal levels of TM at serum-free and 5% FBS conditions were 0.028% and 0.007% of the GAPDH level, respectively. Statistical analysis was performed by ANOVA (n = 4 per condition).

calcitriol on inhibiting PAI-1 mRNA were 0.1 and 1.5 nM, respectively. Figure 5B shows that, although the expression of PAI-1 mRNA was higher in SMC cultured in growth medium (160% of that in resting SMC), both paricalcitol and calcitriol at 100 nM still suppressed PAI-1 mRNA expression by 39% and 31%, respectively. These results are consistent with our previous observations that vitamin D analogs downregulated PAI-1 expression in SMC cultured in growth medium [Wu-Wong et al., 2006b].

Protein Expression Patterns of Selected Genes by Western Blotting Analysis

Consistent with the real-time RT-PCR results, Figure 6A shows the results from Western blotting analysis that paricalcitol and calcitriol increased the expression of TM protein in a dose-dependent manner. Figure 6B shows that paricalcitol and calcitriol downregulated the expression of THBS1 protein in a dose-dependent manner. Similarly, Figure 6C shows that paricalcitol and calcitriol downregulated the expression of PAI-1 protein in a dose-dependent manner. In general, the basal levels of these three proteins were in the rank order of PAI-1 > THBS1 > TM. After treatment with vitamin D analogs for 48 h, the expression levels of these three proteins changed and were in the rank order of TM > PAI-1 > THBS1. These results confirm that vitamin D analogs regulate the expression of these three genes at both the mRNA and protein levels.

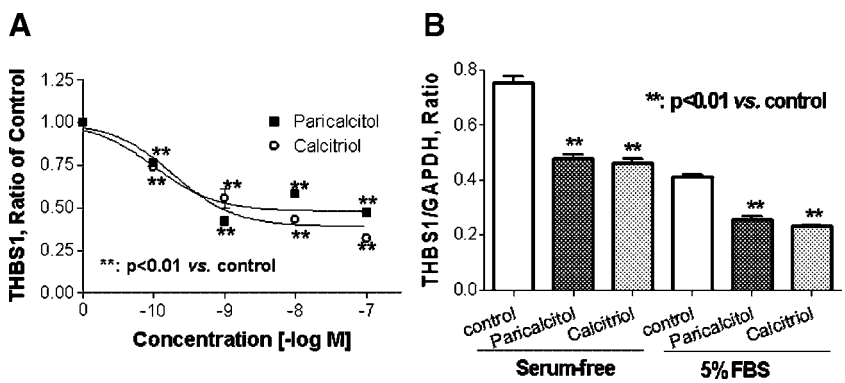

Fig. 4. Effects of paricalcitol and calcitriol on THBS1 mRNA expression. Smooth muscle cells were harvested, RNA isolated, and the THBS1 mRNA level analyzed by real-time RT-PCR. **A**: Smooth muscle cells in serum free medium treated with paricalcitol or calcitriol at indicated concentrations for 24 h. The expression level of THBS1 mRNA were first normalized to the GAPDH mRNA level, and then calculated as a ratio of control (untreated, ratio = 1). **B**: Cells in serum free medium or growth medium containing 5% FBS treated with paricalcitol or calcitriol at 100 nM for 24 h. GADPH was used for normalization. The basal levels of THBS1 at serum-free and 5% FBS conditions were 75% and 41% of the GAPDH level, respectively. Statistical analysis was performed by ANOVA (n = 4 per condition).

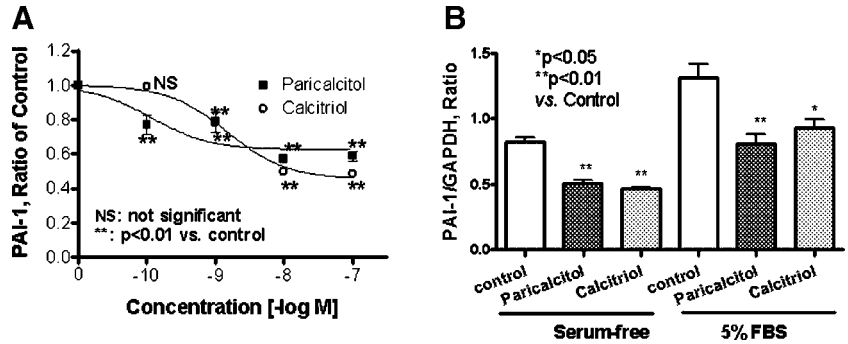

Fig. 5. Effects of paricalcitol and calcitriol on PAI-1 mRNA expression. **A**: Smooth muscle cells in serum free medium were treated with paricalcitol or calcitriol at indicated concentrations for 24 h. RNA were isolated and the PAI-1 mRNA level analyzed by real-time RT-PCR. The expression level of TM mRNA were first normalized to the GAPDH mRNA level, and then calculated as a ratio of control (untreated, ratio = 1). **B**: Cells in serum free medium or growth medium containing 5% FBS treated with paricalcitol or calcitriol at 100 nM for 24 h. The expression level of PAI-1 mRNA was normalized to the GAPDH mRNA level. The basal levels of PAI-1 at serum-free and 5% FBS conditions were 83% and 131% of the GAPDH level, respectively. Statistical analysis was performed by ANOVA (n = 4 per condition).

Involvement of Protein Synthesis in VDR-Mediated Regulation of Gene Expression

In order to further understand the mechanism for VDR-mediated gene regulation, we pretreated cells with 10 μg/ml cycloheximide for 4 h before addition of paricalcitol, and examined the expression of TM, THBS1, and PAI-1 in the presence or absence of paricalcitol. Figure 7A shows that upregulation of TM mRNA by paricalcitol was slightly affected by cycloheximide ($EC_{50} = 10$ and 28 nM in the presence or absence of cycloheximide, respectively). In contrast, Figure 7B,C shows that, in cells treated with cycloheximide, the suppression of PAI-1 and THBS1 by paricalcitol was no longer observed.

DISCUSSION

The goal of this study was to investigate how vitamin D analogs modulate gene expression in human CASMC cultured in serum-free medium. Cells were treated with a high concentration of paricalcitol or calcitriol for 30 h in order to detect the majority of genes that are likely regulated by vitamin D analogs. While the study does not differentiate genes that are directly regulated versus those that are indirectly regulated by VDR, it allows for the identification of many genes that were known targets of vitamin D analogs (e.g., CYP24A1), as well as a large number of additional genes that were previously not known to be affected by vitamin D analogs.

In our previous study testing the effect of paricalcitol and calcitriol on proliferating human CASMC, we observed that a large cluster of genes involved in cell differentiation/proliferation were affected by vitamin D analogs and that paricalcitol and calcitriol inhibit smooth muscle cell growth. In this study, some genes previously identified in proliferating SMC still exhibited similar vitamin D analog-modulated changes (either up- or downregulation), but there are also obvious differences that several genes previously identified in proliferating SMC were no longer observed and seven genes that were not detected in proliferating cells were now identified (Table II). Although it will require further investigations to fully understand the effect of vitamin D analogs on modulating the expression of these cell-cycle-related genes, the results are consistent with the current understanding that VDR may play a role in maintaining cellular differentiation.

Regarding vitamin D analog-modulated CV-related genes, it is perhaps of interest to note that PAI-1, TM, and THBS1, all known to be involved in fibrinolysis and thrombogenicity, were affected by vitamin D analogs. Upregulation of PAI-1 and THBS1 is associated with the development of thrombosis, atherosclerosis, and vascular injury [Raugi et al., 1990], while an early loss of TM expression resulted in an enhanced thrombin generation [Kim et al., 2002] and a local overexpression of TM prevented atherothrombosis [Waugh et al., 1999]. Previously it has been shown that TM mRNA expression is decreased in aorta, liver, and

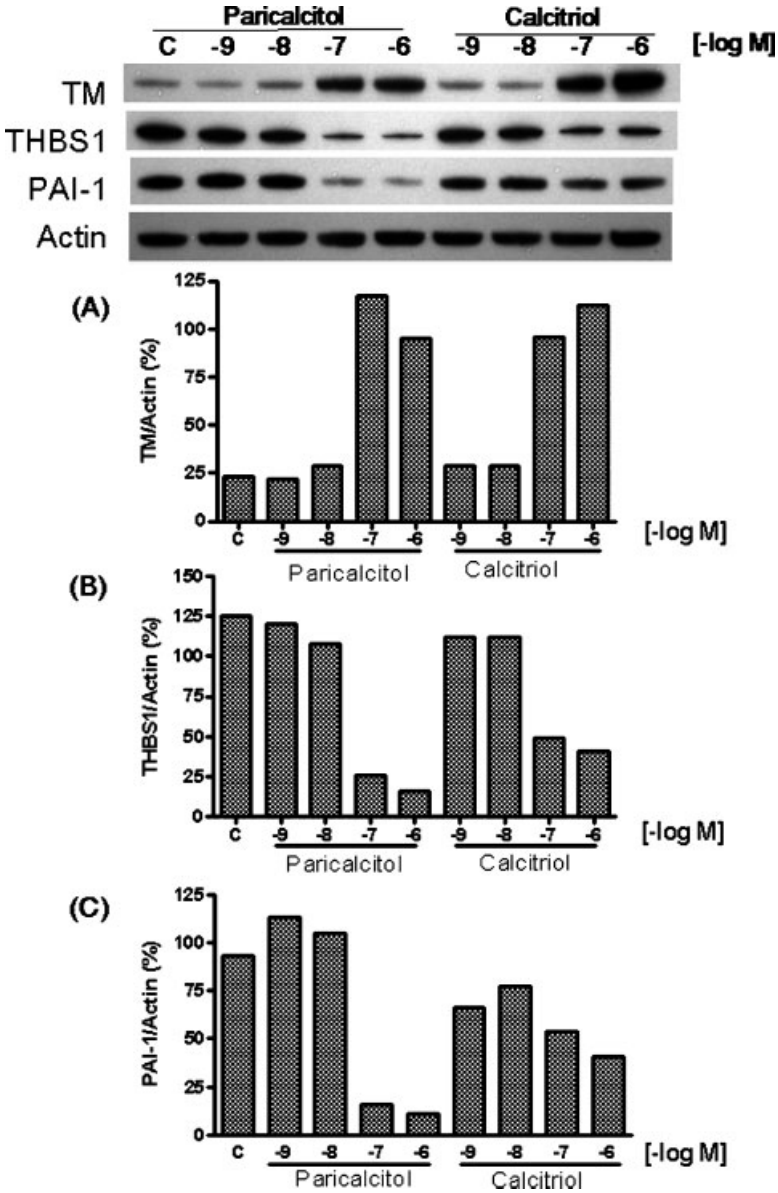

Fig. 6. Effect of paricalcitol and calcitriol on protein levels of selected genes. Smooth muscle cells in serum free medium were treated with paricalcitol or calcitriol at indicated concentrations for 48 h. Cells were then solubilized and Western blotting was performed as described in Materials and Methods. The specific bands were visualized and the density of each band was measured and normalized to the actin level in each sample. Results shown are representative of three independent experiments. **A**: TM, (**B**) THBS1, (**C**) PAI-1. C: control, no drug treatment.

kidney prepared from the VDR knockout mice [Aihara et al., 2004], and calcitriol upregulates TM expression in human leukemic cells and monocytes [Koyama et al., 1998; Ohsawa et al., 2000]. We have also previously reported that, in proliferating human CASMC, paricalcitol and calcitriol downregulated the expression of PAI-1 mRNA and protein [Wu-Wong et al., 2006b], which is replicated in the current study in resting SMC. Regarding THBS1, based on a recent literature search, the role of VDR in the regulation of THBS1 in human smooth muscle cells has not been reported previously.

Although whether VDR is involved in modulating thrombogenicity will certainly require more studies, it is worth mentioning that VDR-mediated changes in the expression patterns of these thrombogenicity-related genes in smooth muscle cells should not be overlooked. Under normal conditions, many of these factors

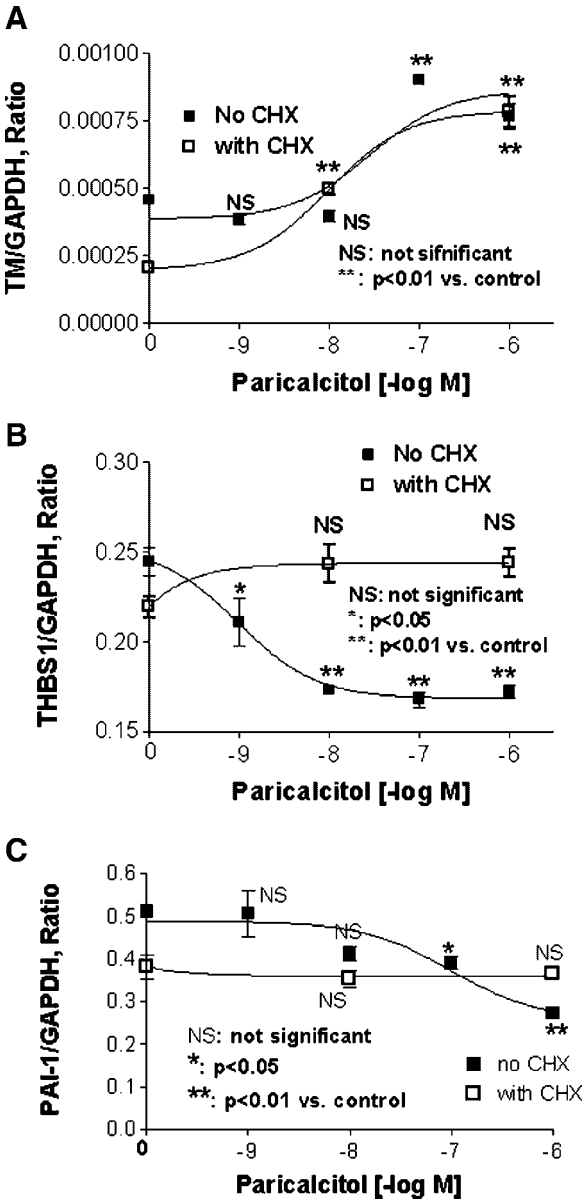

Fig. 7. Effect of paricalcitol on TM, THBS1, and PAI-1 expression differentially blocked by cycloheximide. SMC cells in growth medium containing 5% FBS were treated with 3 μg/ml cycloheximide (CHX) for 4 h, followed by addition of paricalcitol at indicated concentrations for 24 h. RNA was isolated and real-time RT-PCR was performed as described. The expression level of TM (**A**), THBS1 (**B**), or PAI-1 (**C**) mRNA was normalized to the GAPDH mRNA level. Values shown are mean ± standard deviation (n = 4). Statistical comparisons were performed by ANOVA (n = 4 per condition).

involved in thrombosis are predominantly localized on endothelial cells. However, vascular injury often results in altered expression patterns of these factors. For example, in both human and mouse, the expression of PAI-1 is prevalent in activated endothelial cells at the early stages of atherosclerosis, but its expression in advanced atherosclerotic lesions is predominantly in smooth muscle cells [Koyama et al., 1998; Laukkanen et al., 2002]. In atherosclerotic vessels, TM has been shown to be markedly downregulated in endothelial cells and SMC may become a relevant source of TM under pathological conditions such as advanced atherosclerosis [Tohda et al., 1998; Laszik et al., 2001; Yoshii et al., 2003]. THBS1 expression in the vascular wall is significantly increased in injured vessels and in stent-induced neointima [Sajid et al., 2001; Zohlnhofer et al., 2001]. Furthermore, increased expression of THBS1 has been detected in the adventitia of blood vessels from diabetic rats, and in cultured vascular smooth muscle cells in response to glucose stimulation [Stenina et al., 2003].

Our results also show that the effect of paricalcitol on suppressing PAI-1 and THBS1 is blocked by cycloheximide, while the upregulation of TM by paricalcitol is not, suggesting that the regulation of THBS1 and PAI-1 by VDR may be mediated through intermediate factors involving protein synthesis, but that TM is likely a direct target of VDR. Vitamin D analogs are known to have diverse effects on different genes. When VDR acts as a positive regulator, the activated VDR heterodimerizes with RXR and recruits co-activators, which then binds to VDR response elements in the promoter region of target genes to regulate gene expression. However, when VDR is involved as a negative regulator, the mechanism becomes more complicated and much less understood. Additional studies are required to elucidate the molecular mechanism of how VDR downregulates PAI-1 and THBS1 expression.

In conclusion, our data suggest that VDR modulates the expression of a variety of genes in human CASMC. While more studies are needed to fully understand VDR-mediated CV effects, our data suggest that modulation of human vascular smooth muscle cells via VDR activation may be one of the mechanisms contributing to the benefits of vitamin D analog therapy on reducing mortality and morbidity risk in CKD patients.

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