Vitamin D receptor activators inhibit vascular smooth muscle cell mineralization induced by phosphate and TNF-α

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Abstract

Background. Vascular calcification is a highly regulated process. Tumor necrosis factor-α (TNF-α) has been shown to accelerate the highly regulated osteogenic process in vascular smooth muscle cells (VSMCs). Vitamin D receptor activators (VDRAs) have been associated with beneficial cardiovascular outcomes in patients with chronic kidney disease. We examined whether maxacalcitol, a vitamin D3 analog, exhibits a suppressive effect on VSMC mineralization induced by phosphate and TNF-α.

Methods. Human VSMCs were treated with either vehicle, maxacalcitol (10−9 to 10−7 M), or calcitriol (10−9 to 10−7 M) in 2.5 mM of phosphate media with TNF-α (1 ng/mL) for 9 days. VSMC mineralization was determined and expression of genes associated with the osteogenic process was examined by real-time reverse transcription–polymerase chain reaction. Expression of
matrix metalloproteinase-2 (MMP-2) messenger RNA (mRNA) in VSMCs and MMP-2 protein in media was also analyzed.

**Results.** Vehicle-treated VSMCs exhibited massive mineralization, which was inhibited by maxacalcitol in a concentration-dependent manner. Calcitriol also inhibited the mineralization. While vehicle-treated VSMCs exhibited increased mRNA expression of genes associated with the osteogenic process (Cbfα1/Runx2 and osteocalcin) compared with VSMCs grown in normal media without TNF-α (control), maxacalcitol and calcitriol suppressed the increase in mRNA species. Furthermore, vehicle-treated VSMCs exhibited increased MMP-2 mRNA and protein in the media that were suppressed notably by maxacalcitol.

**Conclusions.** Both the VDRAs abrogated the acceleration of the osteogenic process induced by phosphate and TNF-α in VSMCs, which was linked to inhibition of mineralization in VSMCs. MMP-2 blockade by VDRAs may contribute to an inhibitory effect on vascular calcification.

**Keywords:** inflammation; MMP-2; vascular calcification; vitamin D receptor activator

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**Introduction**

Vascular calcification has been shown to be an active process that involves the transformation of vascular smooth muscle cells (VSMCs) into osteoblast-like cells [1]. Vascular calcification is based on a balance between inhibitors and inducers of this transformation. Numerous factors have been shown to induce the osteoblastic transformation of VSMCs. Inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), are among the inducers. TNF-α has been shown to play a crucial role in vascular pathophysiology, such as atherosclerosis and vascular calcification. TNF-α influences many aspects of atherogenesis, including promoting foam cell formation [2], inducing macrophage differentiation [3], increasing endothelial cell permeability [4] and promoting monocyte adhesion [5]. TNF-α not only accelerates the process of atherosclerosis but also that of vascular calcification, by directly activating Cbfα1/Runx2, transcription factors that are important for the transformation and in part via the cyclic AMP pathway in VSMCs [6].

VSMCs have been shown to express 25-hydroxyvitamin D$_3$-1α-hydroxylase [7] and the vitamin D receptor (VDR) [8], indicating that vitamin D exhibits both systemic and local roles in VSMCs. In addition to mineral metabolism, vitamin D possesses several properties that can regulate pathophysiological phenomena, including anti-inflammatory effects [9]. Several observational studies have demonstrated that calcitriol or its analogs improved the survival of patients with chronic kidney disease (CKD) [10]. Furthermore, low and high serum vitamin D levels are both associated with vascular calcification in these patients [10]. The inflammatory state is easily exacerbated in the uremic milieu, especially under dialysis, and an association has been made between inflammation and poor survival in this population [11]. Taking these facts into consideration, the contribution of VDR activators (VDRAs) to the longevity of CKD patients may be due in part to the anti-inflammatory properties via the appropriate activation of the VDR in cardiovascular tissues. In current clinical practice, vitamin D derivatives are commonly used in CKD patients since active vitamin D$_3$ calcitriol easily increases serum calcium and phosphate levels, which are major risk factors for vascular calcification in these patients. In addition, the ongoing development of newer vitamin D analogs [12, 13] suggests the importance of tissue-specific activation of VDR as well as systemic physiologic action of an active vitamin D$_3$, such as calcitriol. From these basic and clinical streams, it is imperative to study the role of vitamin D analogs in various disease situations, in addition to mineral disturbance.

In the present study, we investigated the effect of VDRAs, the calcitriol analog, maxacalcitol, and calcitriol, on the phosphate and TNF-α-induced mineralization of VSMC.

**Materials and methods**

**Cell culture**

Human VSMCs were purchased from Takara Bio Inc. (Tokyo, Japan) and were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and penicillin−streptomycin solution (culture medium) in a humidified incubator maintained at 37°C, with 5% CO$_2$. Cells were maintained at 70–80% confluence by passaging as needed. Cells at passage <10 were used for the present study. VSMCs were cultured with the culture medium in six-well plates until they were 70–80% confluent and were then cultured under the different treatment conditions for 9 days as follows: culture medium with a normal phosphate concentration (0.9 mM) with ethanol as the vehicle control (NP group); medium with a high-phosphate concentration (HP: 2.5 mM) and ethanol (HP group); medium with a normal phosphate concentration containing 1 ng/mL TNF-α (NP + TNF-α group) and medium with a high-phosphate concentration containing 1 ng/mL TNF-α (HP + TNF-α group). In addition, different concentrations of a vitamin D$_3$ analog, maxacalcitol (10$^{-7}$, 10$^{-8}$ and 10$^{-9}$ M), or calcitriol (10$^{-7}$, 10$^{-8}$ and 10$^{-9}$ M) were added to the HP + TNF-α medium. VSMCs were also cultured with the HP media in the presence or absence of either maxacalcitol (10$^{-7}$ M) or calcitriol (10$^{-7}$ M). Phosphate was added in the form of Na$_2$HPO$_4$ and Na$_2$HPO$_4$ (1:2) to achieve final concentrations of 0.9 or 2.5 mM. Media were replaced every 2 days.

**Mineralization analyses**

Von Kossa staining and the quantification of mineralization were performed as previously described [14]. Each well was fixed with 2% paraformaldehyde and then rehydrated with water, followed by a 1-h incubation with 5% silver nitrate solution. Wells were rinsed three times in distilled water, followed by a reductive reaction with thiosulfate solution for 5 min. Nuclear fast red solution was used as a counterstain (5 min), and the wells were then rinsed with tap water. For the quantification of mineralization, cells were decalcified in 0.6 N HCl for 24 h and the Ca concentration in the supernatant was determined by the appropriate activation of the VDR in VSMCs. The remaining cells were solubilized in cell lysis buffer (Cell Signaling Technology, Beverly, MA) and the cell protein content was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). The Ca content was normalized by correcting for the protein content and expressed as micrograms of calcium per milligram of protein. The Ca concentration and the protein content were both measured in duplicate.
Quantitative real-time reverse transcription–polymerase chain reaction (RT–PCR)

Gene expression of Cbfa1/Runx2, osteocalcin (OC) and matrix metalloproteinase-2 (MMP-2) was quantified by real-time reverse transcription–polymerase chain reaction (RT–PCR), as previously reported [14]. Total RNA was extracted from VSMCs using TRIzol according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Two micrograms of total RNA was reverse transcribed to first-strand complementary DNA (cDNA) using SuperScript II reverse transcriptase (Invitrogen). The synthesized cDNA was amplified using a standard polymerase chain reaction (PCR) protocol with SYBR Green Jump Start TaqReady Mix (Sigma) and human-specific primers for Cbfa1/Runx2, OC, MMP-2 and VDR. Parallel amplification was performed with primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The primer sets for Cbfa1/Runx2, OC, MMP-2 and GAPDH were purchased from Qiagen (Germantown, MD; assay ID for Cbfa1/Runx2: Hs_RUNX2_1_SG, OC Hs_BGLAP_1_SG; MMP-2: Hs_MMP2_1_SG, VDR; Hs_VDR_1_SG and GAPDH: Hs_GAPDH_2_SG). Cycling conditions were 10 min of pre-incubation at 95°C, 15 s of denaturation at 95°C and 1 min of annealing at 58°C for 40 cycles using an ABI PRISM 7700 (Applied Biosystems, Foster City, CA). The amounts of Cbfa1/Runx2, OC, MMP-2 and VDR were normalized to the amount of GAPDH mRNA in each sample. All measurements were performed in duplicate.

Supernatant MMP-2 determination

MMP-2 levels were determined in VSMC culture supernatants by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer’s instructions (R&D Systems, Inc., Minneapolis, MN). All supernatants were kept frozen at −80°C until analysis and ELISA assays were performed in batches. All experiments were performed in triplicate.

Statistical analyses

All results are expressed as the mean ± SEM. One-way analysis of variance was employed to assess the statistical differences between groups. Scheffe’s post hoc test was used to assess the differences between all possible two-group comparisons. P-values < 0.05 were considered significant.

Induction of VSMC mineralization by high phosphate and TNF-α

No signs of VSMC mineralization were observed with the normal phosphate media (0.9 mM). TNF-α (1 ng/mL) in the normal phosphate media slightly increased the degree of mineralization (2.6 ± 2.6 μg/mg protein). On the other hand, when VSMCs were cultured with the high-phosphate media (2.5 mM), VSMC mineralization was significantly augmented (39.4 ± 10.4 μg/mg protein) compared with that with the normal phosphate media. The mineralization was further augmented when TNF-α was added (62.1 ± 15.1 μg/mg protein; Figures 1 and 2).

The effect of the VDRAs on VSMC mineralization

The addition of maxacalcitol (10⁻⁹ to 10⁻⁷ M) to the high phosphate with TNF-α media resulted in a concentration-dependent inhibition of VSMC mineralization. The concentration-dependent inhibitory effect of maxacalcitol on VSMC mineralization (10⁻⁹ M, 28.3 ± 5.0 μg/mg protein; 10⁻⁸ M, 26.7 ± 1.3 μg/mg protein and 10⁻⁷ M, 14.0 ± 1.8 μg/mg protein) was similar to that of calcitriol (10⁻⁹ M, 31.8 ± 5.8 μg/mg protein; 10⁻⁸ M, 20.0 ± 2.0 μg/mg protein and 10⁻⁷ M, 10.8 ± 2.0 μg/mg protein; Figure 3). Both VDRAs at 10⁻⁷ M exhibited a significant inhibitory effect on the mineralization compared with HP + TNF-α (30.6 ± 3.2 μg/mg protein; Figure 3). The inhibitory effect of VDRAs (maxacalcitol at 10⁻⁷ M, 20.5 ± 1.3 μg/mg protein and calcitriol at 10⁻⁷ M, 21.8 ± 1.6 μg/mg protein) on the mineralization was abrogated when TNF-α

Fig. 1. Representative microphotographs of VSMC mineralization, as confirmed by von Kossa staining in the NP, NP + TNF-α, HP and HP + TNF-α groups. Magnification ×200.
was not added to the HP media (24.6 ± 6.4 μg/mg protein; Figure 4).

The effect of the VDRAs on the osteogenic process in VSMCs

Expression of genes associated with the osteogenic process, i.e. Cbfa1/Runx2 (1.12 ± 0.21) and OC (1.04 ± 0.06), was increased in HP + TNF-α compared with NP (Cbfa1/Runx2, 0.61 ± 0.05 and OC, 0.83 ± 0.01), and the increase in the gene expression was markedly suppressed after the addition of maxacalcitol (Cbfa1/Runx2, 0.32 ± 0.10 and OC, 0.56 ± 0.06; Figure 4). Similar results were obtained after the addition of calcitriol at 10⁻⁷ M (Cbfa1/Runx2, 0.65 ± 0.19 and OC, 0.54 ± 0.13; Figure 5A and B).

The effect of the VDRAs on MMP-2 expression in VSMCs

MMP-2 gene expression in VSMCs was significantly increased in HP + TNF-α (3.34 ± 0.60) compared with NP (0.01 ± 0.01), which was attenuated with the addition of 10⁻⁷ M of maxacalcitol (1.19 ± 0.67; Figure 6). Similar results were observed with the addition of 10⁻⁷ M of calcitriol (1.39 ± 0.39). MMP-2 protein concentrations in the media with HP + TNF-α were significantly suppressed by both maxacalcitol and calcitriol in a concentration-dependent manner (10⁻⁹ to 10⁻⁷ M). While calcitriol (10⁻⁷ M, 134 ± 3 pg/mL) significantly suppressed the MMP-2 concentration compared with HP + TNF-α group (184 ± 4 pg/mL), the same concentration of maxacalcitol (113 ± 1 pg/mL) had a greater inhibitory effect on MMP-2 (Figure 7). At each concentration, maxacalcitriol exhibited a significantly greater inhibitory effect on MMP-2 levels compared with calcitriol (Figure 7).

The effect of the VDRAs on VDR expression in VSMCs

No statistical difference in VDR gene expression was observed in VSMCs between the NP and HP + TNF-α treatment groups. Neither of the VDRAs exhibited any effect on VDR gene expression in VSMCs incubated with HP + TNF-α (Figure 8).

Discussion

Vascular calcification has been recognized as an active process in which several mechanisms, such as transformation of VSMCs into osteoblast-like cells (the osteogenic process), apoptosis and imbalance between calcification inhibitors and inducers, are implicated. Elastin degradation also plays an important role in the active process. In the in vitro study, VSMC mineralization was induced by a high-phosphate condition and further augmented with the
addition of an inflammatory cytokine, TNF-α. The VDRAs maxacalcitol and calcitriol inhibited the VSMC mineralization induced by TNF-α and phosphate in a concentration-dependent manner. This inhibitory effect was associated with the interaction between the blockade of osteogenic process and MMP-2 suppression.

Mineral disturbance and chronic inflammation are very common and are associated with cardiovascular disease in the uremic milieu. Both phosphate and TNF-α are major inducers of the osteogenic process in VSMCs [6, 15–19]. In the present study, when these two inducers were combined, the osteogenic process was accelerated, resulting in VSMC mineralization. Maxacalcitol and calcitriol suppressed the augmented osteogenic process, as indicated by the decrease in expression of Cbfa1/Runx2 and OC. We have previously shown that paricalcitol, a vitamin D3 analog, inhibited the progression of vascular calcification in 5/6 nephrectomized uremic rats that were fed a high-phosphate diet, while doxercalciferol did not exhibit any inhibitory effects, even under similar serum phosphate levels [20]. Although the precise mechanisms by which paricalcitol inhibits vascular calcification remain to be clarified, factors associated with the osteogenic process, i.e. Cbfa1/Runx2 and OC, were not expressed in the VSMCs, indicating that paricalcitol did not advance or stabilize the osteogenic process. Similar results have been obtained by other groups, suggesting the lesser calcific effects of paricalcitol [21]. Maxacalcitol, a vitamin D3 analog, has also been shown to exhibit less of a calcific effect on the vasculature in uremic rats compared with calcitriol [22]. Although this reduced calcific effect of maxacalcitol needs to be further investigated in detail, the effect was independent of serum calcium or phosphate levels. In the present study, expression of Cbfa1/Runx2 and OC mRNA was increased along with VSMC mineralization induced by phosphate and TNF-α.
and calcitriol suppressed these increases suggesting that the osteogenic process was directly or indirectly modulated by the VDRAs, even though the VDRAs did not affect the expression of VDR mRNA.

TNF-α has been shown to accelerate the osteogenic process in VSMCs, resulting in mineralization [6, 15]. In the present study, media with a high-phosphate concentration induced VSMC mineralization and the mineralization was further augmented when TNF-α was added, indicating the importance of inflammation in the progression of VSMC mineralization. On the other hand, VDRA has been shown to exhibit an anti-inflammatory effect, which is favorable for various disease conditions including kidney [23] and infectious diseases [24]. Serum vitamin D insufficiency is associated with vascular endothelial dysfunction in patients with CKD [25] as well as asymptomatic subjects [26]. In cultured vascular endothelial cells, calcitriol inhibits activation of the pro-inflammatory transcription factor, nuclear factor κB (NF-κB) [27], as well as release of the inflammatory cytokine interleukin-6 [28], a downstream target of NF-κB. Paricalcitol has been shown to have an inhibitory effect on renal inflammation via NF-κB signaling in a mouse model of obstructive nephropathy [29]. These anti-inflammatory properties of VDRAs in various tissues suggest that the acceleration of VSMC mineralization by TNF-α in the present study might be attenuated by the VDRAs, which is likely to be related to the anti-inflammatory effects. Furthermore, the VDRAs had no significant effect on phosphate-induced VSMC mineralization in the absence of TNF-α, suggesting the importance of the anti-inflammatory effects.

One noteworthy observation in the present study is that the VDRAs suppressed the release of MMP-2 from VSMCs stimulated by phosphate and TNF-α and that this effect of maxacalcitol was greater than that of the same concentration of calcitriol. Elastin degradation can be induced by either mechanical (hypertension, aging or injury) or chemical (inflammation) stress and has been observed in almost all types of vascular calcification [1, 30]. MMP-2, a major elastase that induces elastin degradation and is secreted by VSMCs, has been identified to be closely linked to elastin degradation, thus mediating the osteogenic process [31]. We have recently reported that the calcified area in the medial aortic layer of uremic rats exhibited fragmentation and thinning of the elastic lamellae and that MMP-2 expression was observed in the same area [32]. Furthermore, MMP-2 activity has been shown to be associated with elastin degradation and vascular calcification in CKD patients [33]. These findings suggest the involvement of elastin degradation, which is associated with MMP-2 activation, in the progression of vascular calcification in the uremic milieu. Furthermore, NF-κB-dependent MMP-2 activation has also been reported in other cell lines, suggesting a close relationship between MMP-2 activation and inflammation [34]. Taking these facts into consideration, the present study indicated that inflammatory stimulation by TNF-α augmented VSMC mineralization under high-phosphate conditions and that maxacalcitol notably inhibited the acceleration not only by attenuating the osteogenic process but also by suppressing MMP-2 activity in VSMCs. Since the present study investigated MMP-2 expression, but not MMP-2 activity, further studies are needed to elucidate the role of MMP-2 in the osteogenic process in VSMCs.

In conclusion, the VDRAs abrogated the acceleration of the osteogenic process(es) induced by phosphate and TNF-α in VSMCs, which was linked to inhibition of VSMC mineralization. Especially the distinguishing effect of maxacalcitol on MMP-2, which might be associated with its anti-inflammatory property, may exert an inhibitory effect on vascular calcification. We postulated that appropriate application of VDRAs would be beneficial for vascular calcification in which inflammation is involved and that its anti-inflammatory property may bring about a new therapeutic strategy to arrest the progression of vascular calcification.

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Conflict of interest statement. None declared.

(See related article by Drücke and Massy. Role of vitamin D in vascular calcification: bad guy or good guy? Nephrol Dial Transplant 2012; 27: 1704–1707.)

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