1,25-Dihydroxyvitamin D₃ Increases In Vitro Vascular Calcification by Modulating Secretion of Endogenous Parathyroid Hormone–Related Peptide

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Background—A significant association between vascular calcification and osteoporosis has been noted, suggesting that calcium homeostasis is important in vascular calcification as well as in osteoporosis. Moreover, results of our previous studies suggest that calcium-regulating hormones such as parathyroid hormone–related peptide (PTHrP) may modulate vascular calcification. Therefore, we hypothesized that 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] may have a direct impact on the calcium-regulating system of vascular smooth muscle cells, resulting in deposition of calcium in vascular wall.

Methods and Results—We investigated the effect of 1,25(OH)₂D₃ on in vitro calcification by bovine vascular smooth muscle cells (BVSMCs). 1,25(OH)₂D₃ dose dependently increased BVSMC calcification and alkaline phosphatase activity. 1,25(OH)₂D₃ also decreased secretion of PTHrP by BVSMCs in a dose-dependent manner and depressed its gene expression. Furthermore, exogenous PTHrP (fragment 1-34) antagonized the stimulatory effect of 1,25(OH)₂D₃ on BVSMCs. Finally, 1,25(OH)₂D₃ dose dependently increased the expression of the osteopontin gene, one of the bone matrix proteins in BVSMCs, contributing to its stimulatory action on BVSMC calcification.

Conclusions—These data suggest that 1,25(OH)₂D₃ exerts a stimulatory effect on vascular calcification through direct inhibition of the expression of PTHrP in BVSMCs as an endogenous inhibitor of vascular calcification. Moreover, the stimulatory effects of 1,25(OH)₂D₃ on alkaline phosphatase activity and osteopontin expression may contribute to its promoting action in vascular calcification. (Circulation. 1998;98:1302-1306.)

Key Words: calcification ■ vitamin D ■ muscle, smooth ■ peptides ■ osteoporosis

Calcification is almost invariably associated with atherosclerotic plaque formation. Recently, it was hypothesized that plaque calcification is an active, regulated process similar to osteogenesis. Bone morphogenetic proteins (BMPs), including BMP-2, and bone matrix proteins, such as osteopontin (OPN), oстеонектин, and osteocalcin, have been demonstrated in atherosclerotic plaques, especially calcified lesions, through immunohistochemistry and in situ hybridization. However, details of the mechanism by which vascular calcification is induced remain unclear. Using an in vitro model of vascular calcification, we demonstrated that the expression of alkaline phosphatase (ALP) is functionally important in the calcification of bovine vascular smooth muscle cells (BVSMCs) and that OPN mRNA is expressed exclusively in calcified BVSMCs. Therefore, it is likely that vascular smooth muscle cells (VSMCs) acquire calcifying capacity under certain conditions.

Vascular calcification often occurs in women with osteoporosis. Moreover, there is an inverse relationship between the degree of vascular calcification and bone mineral content, suggesting that calcium homeostasis is important in atherosclerotic calcification as well as in osteoporosis. Calcium-regulating hormones such as parathyroid hormone (PTH), PTH-related peptide (PTHrP), and vitamin D thus may modulate atherosclerotic calcification. We have demonstrated that PTH and PTHrP inhibit BVSMC calcification through depression of ALP activity and that PTHrP secreted from BVSMCs acts as an endogenous inhibitor of vascular calcification, suggesting that VSMCs are equipped with an autocrine and/or a paracrine system that regulates calcium metabolism. 1α,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃], an active metabolite of vitamin D, can exert a direct effect on VSMCs, which express vitamin D receptors. 1,25(OH)₂D₃ stimulates acute calcium influx into VSMCs and inhibits proliferation of VSMCs. Excess vitamin D induces vascular calcification in both humans and experimental animals; therefore, it is important to clarify whether the therapeutic use of vitamin D for osteoporosis promotes atherosclerosis and vascular calcification. However, the detailed mechanisms of the actions of vitamin D on VSMCs remain unclear.

We hypothesized that 1,25(OH)₂D₃ may have a direct impact on the calcium-regulating system of VSMCs, resulting in deposition of calcium in vascular wall.
in deposition of calcium in vascular wall. In this study, we investigated the effect of 1,25(OH)\(_2\)D\(_3\) on in vitro calcification by BVSMCs and demonstrated that 1,25(OH)\(_2\)D\(_3\) increases calcium deposition by depressing endogenous PTHrP expression.

**Methods**

**Cell Culture and In Vitro Calcification**

BVSMCs were obtained by explantation as previously described.\(^{10}\) Cells that had migrated from the explants were collected and maintained in DMEM (high glucose, 4.5 g/L glucose) containing 15% FBS and 10 mmol/L sodium pyruvate supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (growing medium) at 37°C in a humidified atmosphere containing 5% CO\(_2\). Cells up to passage 8 were used for the experiments. BVSMC calcification was induced as previously described.\(^{10}\) Briefly, BVSMCs were cultured in the growing medium. After confluence, the cells were inoculated in DMEM (high glucose, 4.5 g/L) containing 15% FBS and 10 mmol/L sodium pyruvate in the presence of 10 mmol/L β-GP supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (calcification medium) for 72 hours.

**Quantification of Calcium Deposition**

Cells were decalcified with 0.6N HCl for 24 hours. The calcium content of HCl supernatant was determined colorimetrically by the o-cresolphthalein complexone method (calcium C-test Wako; Wako Pure Chemical Industries).\(^{16}\) After decalcification, cells were washed 3 times with PBS and solubilized with 0.1N NaOH/0.1% SDS. Protein content was measured with a BCA protein assay kit (Pierce). Calcium content of the cell layer was normalized by protein content.

**Measurement of PTHrP**

We assessed secretion of PTHrP by BVSMCs by measuring the PTHrP content of the culture supernatant with an immunoradiometric assay kit (PTHrP IRMA kit; Mitsubishi Kagaku).\(^{16}\) The supernatant was collected in the presence of 10 μg/mL aprotinin and 1 mmol/L EDTA after the fresh medium containing 15% FBS was incubated for 72 hours with BVSMCs on a six-well plate. The content of PTHrP in the medium containing 15% FBS incubated for 72 hours without the cells was estimated as the background. The net quantity of PTHrP secreted from BVSMCs was estimated by subtracting the PTHrP content of the background from that in the cell culture supernatant. Finally, the data were normalized by the protein content of the cell layer.

**ALP Assay**

After the cells were washed twice with PBS, the cellular proteins were solubilized with 1% Triton X-100 in 0.9% NaCl and centrifuged, and the supernatants were assayed for ALP activity as described previously.\(^{10}\) One unit was defined as the activity producing 1 nmol of p-nitrophenol for 30 minutes. Protein concentrations were determined with a BCA protein assay kit (Pierce).

**RNA Isolation and Northern Blot Analysis**

Total RNA was isolated from BVSMCs by extraction with acid guanidinium thiocyanate–phenol–chloroform. Twenty micrograms of total RNA was electrophoresed onto 1% agarose gels containing formaldehyde and transferred to a nylon filter (Hybond N; Amersham International). Blots were prehybridized at 37°C for 24 hours in a buffer containing 50% formamide, 3× SSC (1× SSC, 0.15 mol/L NaCl, and 15 mmol/L sodium citrate, pH 7.4), 50 mmol/L Tris-HCl, pH 7.5, 0.1% SDS, 20 μg/mL denatured salmon sperm DNA, and 1× Denhardt’s solution and then hybridized at 37°C for 48 hours with cDNA probes for human PTHrP or bovine OPN that were labeled with [α-\(^{32}\)P]dCTP (3000 Ci/mL; New England Nuclear Research Products) by use of a random priming method (Megaprime cDNA labeling system, Amersham). Blots were washed and autoradiographed with x-ray film at −70°C. The amounts of RNA were quantified by densitometric scanning and normalized by comparison with GAPDH.

**Statistics**

In certain experiments, data were analyzed for statistical significance by ANOVA with posthoc analysis (Fisher’s protected least significant difference [PLSD]). These analyses were performed with the assistance of a computer program (StatView Version 4.1; Abacus Concepts).

**Results**

We first examined the effect of 1,25(OH)\(_2\)D\(_3\) on BVSMC calcification. 1,25(OH)\(_2\)D\(_3\) promoted this calcification in a dose-dependent manner, and the calcium content of the cell layer increased to 470% of the calcified control at 10\(^{-7}\) mol/L (Figure 1). Because ALP plays an important role in BVSMC calcification, as previously reported,\(^{10}\) we next examined the effect of 1,25(OH)\(_2\)D\(_3\) on ALP activity in BVSMCs. As we previously reported, ALP activities were increased in each group in the presence of β-GP (calcified condition) compared with its absence (uncalcified condition)\(^{10}\) (Figure 2). 1,25(OH)\(_2\)D\(_3\) dose-dependently increased ALP activities in both uncultured and calcified BVSMCs, and at 10\(^{-7}\) mol/L, ALP activities increased to 168% and 167% of the control, respectively (Figure 2). These data suggest that the stimulatory effect of 1,25(OH)\(_2\)D\(_3\) on calcification may be due to increased ALP activity.

Because we have demonstrated that PTHrP secreted from BVSMCs acts as an endogenous inhibitor of vascular calcification,\(^{16}\) we next examined the effect of 1,25(OH)\(_2\)D\(_3\) on
PTHrP secretion by BVSMCs. PTHrP secretion was decreased in each group in the presence of β-GP (calcified condition) compared with its absence (uncalcified condition) (Figure 3), as previously reported. $^{16}$ 1,25(OH)$_2$D$_3$ dose dependently decreased PTHrP secretion by BVSMCs under both uncalcified and calcified conditions, and at $10^{-7}$ mol/L, PTHrP levels decreased to 34% and 43% of each control, respectively (Figure 3). Moreover, 1,25(OH)$_2$D$_3$ inhibited the expression of PTHrP mRNA in BVSMCs ranging from $10^{-10}$ to $10^{-7}$ mol/L, and at $10^{-7}$ mol/L, the mRNA level decreased to 71.4% of control (Figure 4a). These data suggest that 1,25(OH)$_2$D$_3$ directly modulates PTHrP expression in BVSMCs. To prove this hypothesis, we performed an add-back experiment to examine whether exogenous PTHrP antagonizes the stimulatory effect of 1,25(OH)$_2$D$_3$ on BVSMC calcification. Human PTHrP (fragment 1-34) dose dependently antagonized the effect of 1,25(OH)$_2$D$_3$ on this calcification and almost completely blocked its effect at $10^{-7}$ mol/L (Figure 5), suggesting that 1,25(OH)$_2$D$_3$ exerts its stimulatory effect on BVSMC calcification through inhibition of endogenous PTHrP secretion.

Finally, we examined the effect of 1,25(OH)$_2$D$_3$ on the expression of OPN mRNA because we showed that OPN

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**Figure 2.** Effect of 1,25(OH)$_2$D$_3$ on ALP activity. Cells were cultured in calcification medium for 72 hours in the presence of the indicated concentrations of 1,25(OH)$_2$D$_3$. β-GP (+) and (−) indicate presence and absence of β-GP, respectively. ALP activities were measured and normalized by cellular protein content and are presented as mean±SEM. Differences compared with each control (CTL) were statistically significant ($^{*}$P<0.05, Fisher’s PLSD).

**Figure 3.** Effect of 1,25(OH)$_2$D$_3$ on PTHrP secretion by BVSMCs. Cells were cultured in calcification medium for 72 hours in the presence of the indicated concentrations of 1,25(OH)$_2$D$_3$. β-GP (+) and (−) indicate presence and absence of β-GP, respectively. After a 72-hour culture in the presence of the indicated concentrations of 1,25(OH)$_2$D$_3$, PTHrP contents were measured by immunoradiometric assay, corrected, and normalized. Data are presented as mean±SEM. Differences compared with each control (CTL) were statistically significant ($^{*}$P<0.05, Fisher’s PLSD).

**Figure 4.** Effects of 1,25(OH)$_2$D$_3$ on gene expressions of PTHrP (a) and OPN (b). Cells were cultured in calcification medium for 24 hours in the presence of the indicated concentrations of 1,25(OH)$_2$D$_3$. After a 24-hour culture in the presence of the indicated concentrations of 1,25(OH)$_2$D$_3$, cells were harvested for isolation of total RNA. Twenty micrograms of total RNA from BVSMCs was electrophoresed, blotted, and probed with cDNA of human PTHrP. a, Autoradiograph of Northern analysis of PTHrP. b, Autoradiograph of Northern analysis of OPN. CTL indicates control.

**Figure 5.** Effect of human PTHrP (fragment 1-34) on 1,25(OH)$_2$D$_3$-stimulated BVSMC calcification. Cells were cultured in calcification medium for 72 hours in the presence of the indicated reagent or reagents. Calcium contents were measured by the o-cresolphthalein complexone method and normalized by cellular protein content and are presented as mean±SEM. Differences compared with the 1,25(OH)$_2$D$_3$-treated control were statistically significant ($^{*}$P<0.05, Fisher’s PLSD). β-GP indicates 10 mmol/L β-GP; 1,25(OH)$_2$D$_3$, $10^{-7}$ mol/L 1,25(OH)$_2$D$_3$; and (+) and (−), presence and absence of the indicated reagent, respectively.
mRNA is expressed exclusively in calcified BVSMCs and that recombinant OPN peptide dose dependently increases this calcification (Jono et al, unpublished observation, 1997). 1,25(OH)2D3 dose dependently increased the expression of OPN gene in BVSMCs, and at 10−7 mol/L, the mRNA level reached 143.7% of control (Figure 4b). Therefore, upregulation of OPN gene by 1,25(OH)2D3 may contribute to its stimulatory action on BVSMC calcification.

**Discussion**

In this study, we demonstrated a possible mechanism by which 1,25(OH)2D3 stimulates vascular calcification through a direct action on VSMCs. Although it has been well documented that a high dose of vitamin D induces vascular calcification in humans and experimental animals,21–23 the mechanism of its stimulatory action in vivo on vascular calcification remains to be clarified. We recently demonstrated that PTHrP secreted by VSMCs acts as an endogenous inhibitor of vascular calcification.16 Therefore, we hypothesized that PTHrP is one of the molecular targets of the action of 1,25(OH)2D3 on VSMCs. As shown in the present study, 1,25(OH)2D3 directly modulates the expression of PTHrP in BVSMCs at both protein and gene levels, contributing to its action on vascular calcification (Figures 3, 4a, and 5). Therefore, it is likely that VSMCs are equipped with an autocrine/paracrine system that regulates calcium metabolism and that 1,25(OH)2D3 affects this local system to exert its stimulatory action on vascular calcification.

It has been demonstrated that 1,25(OH)2D3 suppresses PTHrP gene transcription in various types of cells, such as rat osteosarcoma cell line (ROS 17/2.8), human squamous cell line (NCI H520), and human keratinocytes.24–26 The PTHrP gene has an inhibitory vitamin D response element within its promoter region, which can interact with a vitamin D receptor/retinoid X-receptor heterodimer.24 Therefore, the inhibitory action of 1,25(OH)2D3 on PTHrP gene expression in BVSMCs, as shown in this study, may be exerted by the same mechanism. However, the precise mechanism of its action should be clarified.

As shown in this study, 1,25(OH)2D3 increased expression of the OPN gene (Figure 4b) as well as in vitro calcification by BVSMCs. We have previously demonstrated that expression of the OPN gene is dramatically increased in calcified BVSMCs.10 In our preliminary experiments, recombinant OPN dose dependently increased this calcification (Jono et al, unpublished observation, 1997). Therefore, upregulation of the OPN gene by 1,25(OH)2D3 may contribute to the promotion of BVSMC calcification. The mechanism of upregulation of this gene by 1,25(OH)2D3 has been investigated extensively in osteoblastic cells.27–30 1,25(OH)2D3 increases transcriptional activity of the gene, which has a vitamin D response element composed of 2 direct repeats within its promoter region.30 It is likely that 1,25(OH)2D3 exerts its effect on OPN expression in BVSMCs by the same mechanism.

1,25(OH)2D3 is well known to be a potent stimulator of osteoblastic differentiation and to increase gene expression of differentiation markers such as OPN and osteocalcin.31 Vascular calcification has several features similar to those of bone mineralization, including expression of BMP-2, ALP, OPN, osteonectin, and osteocalcin and hydroxyapatite crystal formation in calcified atherosclerotic lesions.5–10,32,33 Therefore, it is hypothesized that in the development of vascular calcification associated with atherosclerosis, VSMCs may differentiate into osteoblastic cells via several factors, including BMPs, and that this process may be promoted by the action of 1,25(OH)2D3. These hypotheses should be proved through further investigations.

An association between osteoporosis and vascular calcification with aging has been well documented in several studies.11–14 Vitamin D deficiency is common in the elderly and may contribute to bone loss by causing increased levels of PTH.15,16 Therefore, it is presumed that vitamin D deficiency may be involved in the pathogenesis of vascular calcification. Recently, it was reported that active serum vitamin D levels are inversely correlated with coronary calcification.18 This finding seems to suggest the protective roles of vitamin D in vascular calcification and to support the above-mentioned hypothesis. However, the vast majority of its values presented in the study were within normal range,19 and serum levels of 25-hydroxyvitamin D (25OHD), a serum marker for nutritional status of vitamin D, were not documented. Therefore, it is not clear whether vitamin D deficiency may contribute to such inverse correlation. Further studies are necessary to confirm the hypothesis.

Because vitamin D and calcium supplementation are widely used for the treatment of osteoporosis, especially in the elderly,36 it is important to determine whether long-term supplementation of vitamin D for osteoporosis exacerbates vascular calcification. It is suggested from the present study that pharmacological doses of 1,25(OH)2D3 may stimulate vascular calcification through a direct action on VSMCs. However, high doses of oral vitamin D, which induces vascular calcification in experimental animals, do not always increase serum levels of 1,25(OH)2D3 but 25OHD is increased,15,37,38 because the serum level of 1,25(OH)2D3 is strictly regulated within the narrow range by PTH, regardless of the nutritional status of vitamin D. The mechanism by which increased levels of serum 25OHD induce vascular calcification remains to be clarified. Local production of 1,25(OH)2D3 by 1α-hydroxylase expressed in macrophages accumulated in atherosclerotic lesions may be involved in this process.39 Therefore, it is important to monitor serum levels of 25OHD during vitamin D supplementation. If serum levels of 25OHD are increased beyond the normal range, vascular calcification may develop even within normal levels of 1,25(OH)2D3.

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