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Role of phosphorus and vitamin D analogs in the pathogenesis of vascular calcification

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Abstract

Vascular calcification is a mortality risk factor for Stage 5 CKD patients. We investigated the role of phosphorus (P) and vitamin D analogs in the pathogenesis of vascular calcification using *in vivo*, *ex vivo* and *in vitro* models. Our results demonstrate that uremic rats on a hyperphosphatemia-inducing diet did not exhibit aortic calcification despite elevated levels of serum P and CaxP product. Vitamin D analog 1α-hydroxyvitamin-D_2 (1α(OH)D_2) at 0.17 μg/kg raised serum Ca, P, CaxP product and aortic calcification in the uremic rats, but 19-nor-1α,25$(OH)_2D_2$ (19-nor) at the same dose had no significant effect. At 0.67 μg/kg, both 1α(OH)D_2 and 19-nor had similar effects on serum Ca, P, and CaxP product, but only 1α(OH)D_2 induced significant aortic calcification. Only aortic rings from 1α(OH)D_2-treated uremic rats exhibited a significant increase in ^{45}Ca uptake *ex vivo*. When aortic rings from normal rats or primary culture of human coronary artery smooth muscle cells were treated with P or vitamin D analogs *in vitro*, high P induced Ca accumulation and/or ^{45}Ca uptake in a dose/time-dependent manner, while vitamin D analogs including 1α(OH)D_2 up to 100 nM had no significant effect despite the presence of functional VDR. However, serum from 1α(OH)D_2-treated uremic rats induced ^{45}Ca uptake into smooth muscle cells cultured in high P. These results suggest that the regulation of vascular calcification *in vivo* cannot be easily replicated in the *ex vivo* or *in vitro* models, and high P and some vitamin D analogs such as 1α(OH)D_2 exert interactive effects on modulating vascular calcification.

Introduction

Chronic kidney disease (CKD) patients experience a high mortality rate from cardiovascular (CV) diseases (Zebrack et al., 2003; Weiner et al., 2004). Vascular calcification, the deposition of calcium phosphate mineral in cardiovascular tissues, contributes to CV morbidity and mortality in CKD patients (Blacher et al., 2001; London et al., 2003; Moe et al., 2004). Two distinct forms of calcification have been reported in the vessels (Jeziorska et al., 1998; Shanahan et al., 1999). Intimal calcification is often associated with inflammation and atherosclerosis (Jeziorska et al., 1998), whereas medial calcification occurs in smooth muscle cells (Shanahan et al., 1999). Stage 5 CKD patients experience 2 to 5-fold more coronary artery calcification than age-and gender-matched individuals (Braun et al., 1996). Moreover, histologic analysis shows that coronary plaques in patients with Stage 5 CKD are characterized by increased media thickness and marked calcification, suggesting that smooth muscle cell calcification plays a critical role (Schwarz et al., 2000; Moe et al., 2005).

The mechanisms involved in vascular calcification are still largely unknown. Recently it has been postulated that vascular calcification is a regulated process involving a variety of factors including hyperphosphatemia, loss of calcification inhibition, induction of bone formation in smooth muscle cells, cell death, and circulating nucleational complexes derived from bone (Raggi et al., 2002; Giachelli, 2004; Nishizawa et al., 2005; Ritz and Gross, 2005). Many proteins such as matrix Gla protein (MGP), fetuin-A, osteoprotegerin, pyrophosphatase (ENPP1), etc. have been shown to be involved in this process (Giachelli, 2004). While it is generally recognized that elevated serum phosphorus levels contribute to vascular calcification, medial calcification is also seen in diabetics and with aging in the presence of normal serum phosphorus (Everhart et al., 1988). Therefore, the role of phosphorus on calcification requires more studies.

1,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$, calcitriol) and its analogs such as 19-nor-1α,25$(OH)_2D_2$ (paricalcitol) or 1α-hydroxyvitamin-D_2 (1$\alpha(OH)D_2$) that activate vitamin D receptor (VDR) *in vivo* are commonly used to manage secondary hyperparathyroidism associated with CKD (Martin and Gonzalez,

2004). Recent retrospective clinical observations show that vitamin D analogs provide a survival benefit for Stage 5 CKD patients with an effectiveness order of: paricalcitol > calcitriol > no vitamin D analog therapy, independent of the PTH and calcium levels (Teng et al., 2003; Nakai et al., 2004; Teng et al., 2005); the survival benefit of vitamin D analogs is associated with a decrease in cardiovascular-related mortality (Shoji et al., 2004). More recently, results from the 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).

The observation that vitamin D analogs provide survival benefit in CKD seems contradictory to the general perception that these drugs, due to their potential impact on serum phosphorus (P) and calcium (Ca), may cause medial layer calcification in vessels (Rostand and Drueke, 1999). Information from the literature regarding how different vitamin D analogs affect vascular calcification has not been forthcoming. Furthermore, it is not known how vitamin D analogs interact with P on modulating calcification. In the present study, we used the sub-totally nephrectomized uremic rat model to examine the effect of P and vitamin D analogs on aortic calcification *in vivo*. We also prepared aorta tissue from normal or uremic rats to study the interactive effects of P and vitamin D analogs on calcification *ex vivo*. To investigate further the involvement of P and vitamin D analogs in calcification, we tested the effects of P and 3 different vitamin D analogs on Ca accumulation and ^{45}Ca uptake in human vascular smooth muscle cells. We went on to collect serum from uremic rats treated with vitamin D analogs and examined whether these serum samples affected smooth muscle cell calcification *in vitro*. Our results demonstrate that P and some vitamin D analogs are involved interactively in modulating calcification.

Methods

Materials: 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$, calcitriol, Calcijex®), 1α,25-dihydroxyvitamin D_2 (1α,25$(OH)_2D_2$, the active form of doxercalciferol), 1α-hydroxyvitamin D_2 (1α$(OH)D_2$, doxercalciferol, Hectorol®) and 19-nor-1α, 25-dihydroxyvitamin D_2 (19-nor-1α, 25$(OH)_2D_2$, 19-nor, paricalcitol, Zemplar®) were from Abbott Laboratories. Other reagents were of analytical grade.

Sub-totally nephrectomized rats: The 5/6 nephrectomized (SNX) uremic rats were obtained from Charles River. The nephrectomy was performed on male, Sprague–Dawley rats with a standard two-step surgical ablation procedure. About two weeks after the surgery, the rats were put on a hyperphosphatemia-inducing diet containing 0.9% phosphorous and 0.6% calcium (Slatopolsky, et al., 2003) for 4 weeks, followed by treatment with vehicle (5% ethanol + 95% propylene glycol, 0.4 ml/kg), 19-nor or 1α$(OH)D_2$ at indicated doses intraperitoneally (i.p.) at 3 times/week for 12 (n=4-8 per group) or 40 days (n=4-5 per group). Twenty-four hours after the last dose, animals were anesthetized with ketamine and blood and aortic tissue were collected.

Measurement of blood chemistry: Serum total calcium (Ca), blood ionized calcium (iCa), serum phosphorus (P), creatinine and BUN concentrations were measured using an Abbott Aeroset. Serum PTH was measured using a rat intact parathyroid hormone (PTH) ELISA kit obtained from ALPCO/Immutopics, Inc. (Windham, NH).

Tissue preparation and von Kossa staining: Aorta was collected into liquid nitrogen for quick freezing. Aorta was post fixed in 10% formalin, processed overnight, and followed by embedding into paraffin. Sections were stained by von Kossa method, and counterstained with hematoxylin.

Measurement of Ca and P contents in aorta from 5/6 nephrectomized rats: Aorta tissues were dried at 90°C for 4 h and weighed. Samples were incinerated to ash at 900°C for 8 h using an electric muffle furnace (type F62700, Barnstead International, Dubuque, Iowa), and then were dissolved in 6 N HCl with

Lanthanum chloride (0.1%). The solutions were diluted with distilled water, and Ca and P concentrations were measured using an autoanalyser (Aeroset, Abbott, Abbott Park, IL).

Measurement of ^{45}Ca uptake into aorta isolated from 5/6 nephrectomized rats: Aorta from uremic rats treated with drugs was denuded and cut open into 2-3-mm ring strips and placed in DMEM (with 0.9 mM P) containing 0.2 µCi/ml ^{45}Ca for 3 days at 37°C in a 5% CO_2 atmosphere. Afterwards, the aortic strips were washed three times in PBS. The tissues were dried in an oven, weighed, and then dissolved in a solution containing 6% H_2O_2 and 56% $HClO_4$ (final concentrations). Radioactivity was measured by liquid scintillation.

Measurement of ^{45}Ca uptake into aorta isolated from normal rats: Aorta was cut open, denuded and then cut into 2-3-mm ring strips and placed in DMEM containing different concentrations of test agents for 6 days at 37°C in a 5% CO_2 atmosphere. Medium containing the appropriate test agents were changed every three days. Then, 0.2 µCi/ml ^{45}Ca was added and the incubation continued for another 3 days. Afterward, the tissues were processed as described above, and radioactivity was measured by liquid scintillation.

Cell culture: Primary culture of human coronary artery smooth muscle cells (Cambrex, Walkersville, MD) were grown in SMGM-2 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. Primary culture of human coronary artery endothelial cells (Cambrex) were grown to confluence in EGM-2 Bullet Kit medium containing 2% FBS (Cambrex) at 37°C and used within four passages.

Measurement of ^{45}Ca uptake into cultured smooth muscle or endothelial cells: Smooth muscle cells cultured in DMEM containing different concentrations of test agents for 5 days at 37°C in a 5% CO_2 atmosphere. Medium containing the appropriate test agents were changed every three days. Then, 0.2 µCi/ml ^{45}Ca was added and the incubation continued for another day. Cells were then washed with PBS and solubilized in 0.1 N NaOH. Radioactivity was measured by liquid scintillation. In the co-culture

studies, endothelial and smooth muscle cells were initially cultured separately with endothelial cells in inserts. Cells were then put together in DMEM medium containing test agents and ^{45}Ca uptake determined as described above.

Measurement of Ca content in cultured smooth muscle cells: Smooth muscle cells cultured in DMEM containing different concentrations of test agents for different periods of time (as indicated) at 37°C in a 5% CO_2 atmosphere. Medium containing the appropriate test agents were changed every three days. Cells were then washed with PBS 2x, extracted with 0.6 N HCl for 24 hr at RT, and the Ca content in the extract determined using the Calcium CPC LiquiColor test kit (StanBio, Boerne, TX). The remaining cells were washed with PBS, solubilized in 0.1 N NaOH containing 0.1% SDS. The protein content was determined using the BCA protein assay kit (Pierce, Rockford, IL).

Real-time Reverse Transcription-PCR: Real-time reverse Transcription-PCR was performed with a iCycler (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 minutes at 95°C, followed by 40 cycles of 60°C for 1 minute and 95°C for 15 seconds. 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 ($1x10^6$ 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-VDR (Active Motif, Carsbad, CA) polyclonal antibody (500-fold dilution) in PBS-T overnight at 4°C. The membrane was washed with PBS-T and incubated with a horseradish peroxidase-labeled anti-rabbit antibody for 1 h at 25°C. The

membrane was then incubated with detection reagent (SuperSignal WestPico, Pierce, Rockford, IL). Specific bands were visualized by exposing the paper to Kodak BioMax films. Band intensity was quantified by Quantity One (Bio-Rad).

Data analysis: One way ANOVA Dunnett test with 95% confidence intervals of difference was used to determine a p-value among treatment groups. Statistical comparisons between two treatment groups were performed by unpaired t-test with 95% confidence intervals of difference.

Results

In the first study, uremic rats with hyperphosphatemia were treated with 0.17 μg/kg of 1α(OH)D_2 (the precursor of 1α,25$(OH)_2D_2$, which is activated by conversion to 1α,25$(OH)_2D_2$ in the liver) and 19-nor (19-nor-1α,25$(OH)_2D_2$, an analog of 1α,25$(OH)_2D_2$) at 3 times/week, i.p., for 40 days. As shown in Figure 1, the serum creatinine and BUN levels were significantly elevated in the 5/6 nephrectomized (SNX) rats. Both 19-nor and 1α(OH)D_2 at 0.17 μg/kg had no significant effect on creatinine or BUN (*vs.* own group-Day 0). Figure 2 shows that 19-nor and 1α(OH)D_2 effectively suppressed serum PTH throughout the treatment period. Serum Ca trended lower in the SNX (uremic)-Vehicle group on Day 41, while serum P tended to be higher. As shown in Figure 3, there was no significant difference in the aortic Ca and P contents among Sham, SNX-Vehicle, and 19-nor-treated groups, while 1α(OH)D_2 treatment resulted in a significant elevation in the aortic Ca (36-fold) and P (36.2-fold *vs.* vehicle) content. Figure 4 shows representative sections of aorta stained by von Kossa staining. No staining was detected in either vehicle or 19-nor-treated groups. In contrast, extensive staining was observed in the medial layer of aorta in the 1α(OH)D_2-treated group, indicating the presence of calcification. These results suggest that 1α(OH)D_2, but not 19-nor, exhibited significant effects on raising serum Ca, P and the CaxP product, which is consistent with a previous report by Slatopolsky et al. (Slatopolsky et al., 2002). Furthermore, only 1α(OH)D_2 induced aortic calcification.

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Although 19-nor has been shown to suppress PTH in uremic rats at doses that did not affect serum Ca or P (Slatopolsky et al., 1995), 19-nor could be hypercalcemic at very high doses (Takahashi et al., 1997). To investigate whether the difference in aortic calcification was due to the differential effect of 19-nor and 1α(OH)D_2 on serum Ca, P and CaxP product, we tested a substantially higher dose (0.67 μg/kg) of 19-nor and 1α(OH)D_2 in the hyperphosphatemic uremic rats using the same dosing paradigm (3x/week) and a 12-day treatment period. The studies were not carried out to 40 days due to the fact that animals did not survive well after prolonged treatment with vitamin D analogs at this high dose, especially in the 1α(OH)D_2–treated group. The blood chemistries are summarized in Table 1. Serum creatinine and BUN levels were significantly elevated in SNX-Vehicle, which was not affected by either 19-nor or 1α(OH)D_2 (*vs.* SNX-Vehicle). A significant decrease in serum Ca and an elevation in serum P were observed in the SNX-Vehicle group (*vs.* Sham). The serum CaxP product trended upward without achieving statistical significance. As expected, 19-nor and 1α(OH)D_2 at 0.67 μg/kg produced similar elevations in serum Ca, P and CaxP product (*vs*. Sham). Both compounds effectively suppressed PTH. Figure 5 shows that the Ca and P contents in aorta were not different for Sham, SNX-Vehicle, and the 19-nor-treated groups. In contrast, 1α(OH)D_2 produced a significant increase in the aortic Ca (10.4-fold) and P (15.2-fold *vs.* SNX-Vehicle) contents. As a control, we have also treated uremic rats on normal diet (0.9% phosphorous and 1.13% calcium) with 19-nor and 1α(OH)D_2 at 0.34 μg/kg for 12 days. No aortic calcification was observed in either SNX-Vehicle or drug-treated animals (data not shown). These results suggest that an increase in serum P and Ca do not necessarily produce aortic calcification in uremic rats, and 19-nor and 1α(OH)D_2 exert differential effects on aortic calcification independent of serum P and Ca levels.

To further investigate the different effects of P and vitamin D analogs on aortic calcification, we examined ^{45}Ca uptake into aortic ring strips prepared from the hyperphosphatemic uremic rats treated with vehicle, 19-nor or 1α(OH)D_2 at 0.67 μg/kg (i.p. for 12 days, 3x/week). In these studies, the aortic tissue was denuded of the endothelial layer to minimize potential variability among samples. Consistent with the *in vivo* observations shown above, a significantly increase in ^{45}Ca uptake (40-fold *vs.* Sham) was observed in aorta prepared from the 1α(OH)D_2-treated uremic rats, while minimal ^{45}Ca uptake occurred in

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aorta prepared from the Sham, SNX-Vehicle, or 19-nor-treated uremic rats (Figure 6A). In contrast, when aorta was prepared from normal rats, and then treated with P and/or vitamin D analogs *ex vivo*, the results were strikingly different. Figure 6B shows that an increase in P in the culture medium significantly increased ^{45}Ca uptake into aortic rings from normal rats in a dose-dependent manner. However, 1α,25(OH)$_2$D, 19-nor, or 1α,25(OH)$_2$D$_3$ had no effect on ^{45}Ca uptake in the presence of either 0.9 mM (Figure 6C) or 2.06 mM P (Figure 6D).

Taken together, the above results suggest that P and vitamin D analogs exert contradictory effects on aortic calcification *in vivo vs. ex vivo*. While high P does not necessarily cause aortic calcification in the *in vivo* situation, it profoundly induces calcification in aorta from normal rats *ex vivo.* As a comparison, vitamin D analogs have no effect on calcification *ex vivo*, but display differential effects on aortic calcification in the hyperphosphatemic uremic rat.

In order to further understand these observations, we tested P and vitamin D analogs in primary culture of human coronary artery smooth muscle cells (SMC). As shown in Figure 7A, increasing the P concentration from 0.9 to 2.64 mM induced a dose-dependent increase in the cellular Ca content in SMC. Figure 7B shows that the effect of P on Ca content in the cells was time-dependent. Consistently, Figure 7C shows that an increase in the P concentration from 0.9 to 3.22 mM dose-dependently increased ^{45}Ca uptake into these cells, reaching a plateau at 2.06 mM P. In contrast to the effects of P, as shown in Figure 8A, 1α,25(OH)$_2$D$_2$, 19-nor, or 1α,25(OH)$_2$D$_3$ at 100 nM had no effect on the cellular Ca content when cells were cultured in medium containing P ranging from 0.9 to 2.64 mM. Also, 1α,25(OH)$_2$D$_2$, 19-nor, or 1α,25(OH)$_2$D$_3$ at 1-100 nM had no effect on ^{45}Ca uptake at either 0.9 (Figure 8B) or 2.06 mM P (Figure 8C).

To investigate whether the lack of effect of vitamin D analogs on calcification *in vitro* was due to the absence of VDR in smooth muscle cells and in aorta tissues, VDR expression was examined. In Figure 9A, the expression of VDR mRNA was detected in SMC cultured at 0.9 or 2.06 mM P; the expression of VDR mRNA was higher at 2.06 mM P. Consistently, Figure 9B shows that, analyzed by

Western blotting, the expression of VDR at 2.06 mM P was about 1.8-fold of that at 0.9 mM P. As expected, 19-nor profoundly stimulated the expression of CYP24A1 mRNA in a dose-dependent manner in SMC at 0.9 or 2.06 mM P with EC_{50} at 87 and 157 nM, respectively (Fig. 9C). Fig. 9D shows that VDR mRNA was detected in aorta prepared from Sham or SNX-Vehicle rats. These results confirm that VDR was expressed under either normal or high P condition, suggesting that the lack of effects of vitamin D analogs on calcification *in vitro* is not due to the absence of VDR.

One key difference between the *in vivo* and *in vitro* models is the presence or absence of the endothelial cells. To further investigate whether endothelial cells play a role, we tested P and vitamin D analogs in the co-culture of human coronary artery smooth muscle and endothelial cells. Figure 10A shows that no significant ^{45}Ca uptake was observed in endothelial cells. As a comparison, Figure 10B shows that increasing the medium P concentration from 0.9 to 2.06 mM induced a 15-fold increase in ^{45}Ca uptake into smooth muscle cells. However, $1\alpha,25(OH)_2D_2$ or 19-nor at 100 nM exhibited no significant effect when compared to control (no drug). These results suggest that the contradictory effects of P and vitamin D analogs on calcification *in vivo vs. in vitro* are not likely due to the presence or absence of endothelial cells.

We then determined ^{45}Ca uptake into smooth muscle cells treated with serum prepared from hyperphosphatemic uremic rats. Figure 11 shows that serum from $1\alpha(OH)D_2$ -treated uremic rats induced a significant increase in ^{45}Ca uptake into smooth muscle cells cultured at 2.06 mM P (2.7 and 3.0-fold *vs.* Sham for 0.17 and 0.33 μg/kg of $1\alpha(OH)D_2$, respectively). As a comparison, serum prepared from uremic rats treated with 19-nor resulted in a 40% decrease in ^{45}Ca uptake into smooth muscle cells. Interestingly, none of the serum samples exhibited any significant effect on ^{45}Ca uptake when cells were cultured at 0.9 mM P. Also, 2.06 mM P in this study only induced Ca uptake by ~5-fold (*vs*. 0.9 mM P). These results suggest that (1) the uremic milieu from the $1\alpha(OH)D_2$-treated animals induced an increase in Ca uptake, and (2) the effect was only evident under a high P environment.

Discussion

Vascular calcification has become an important area of research during the past decade largely because it is often associated with cardiovascular morbidity and mortality, especially for CKD patients (Blacher et al., 2001; London et al., 2003; Moe et al., 2004). However, so far the available data remain controversial regarding how factors such as vitamin D analogs and phosphorus are involved in the calcification process. Moreover, the perception that vitamin D analog usage may be associated with a higher risk for vascular calcification contradicts recent retrospective clinical observations that vitamin D analogs provide survival benefit for dialysis patients. The goal of the present study was to investigate the interactive effects of vitamin D analogs and phosphorus on calcification. While contradictory results were obtained from *in vivo* and *in vitro* models, it is perhaps not surprising since vascular calcification is a complex process involving a delicate balance between various inhibitory and inducing factors.

First, we found that an increase in P and CaxP product *in vivo* not necessarily results in aortic calcification but high P consistently induces calcification *in vitro*. This observation suggests that high P may be an inducing factor for calcification, but a counter-regulatory mechanism exists *in vivo* to balance out the calcification promoting effects of hyperphosphatemia. Previously it has been suggested that some factors in serum may act as potent inhibitors of smooth muscle cell calcification (Shanahan, 2005). Thus, one possible explanation for the aortic calcification observed in the $1\alpha(OH)D_2$-treated hyperphosphatemic uremic rats is that the endogenous protective mechanism crucial for suppression of high P-induced vascular calcification is disturbed. Exactly what are altered by $1\alpha(OH)D_2$ treatment will require more studies.

Secondly, no aortic calcification was observed in uremic rats on normal diet treated with $1\alpha(OH)D_2$. Also, serum from uremic rats treated with $1\alpha(OH)D_2$ induced Ca uptake into cultured smooth muscle cells only at 2.06 mM P, but not at 0.9 mM P. Taken together, these observations suggest that (1) vascular calcification may not occur in uremic rats in the absence of permissive conditions such as

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hyperphosphatemia, and (2) some vitamin D analogs such as 1α(OH)D_2 interact with high P to undermine the endogenous protective mechanism against vascular calcification.

Thirdly, results from von Kossa staining of aorta sections prepared from the 1α(OH)D_2-treated uremic rats suggest that calcification occurred in the aortic medial layer (Figure 4). Consistent with this observation, denuded aortic rings prepared from 1α(OH)D_2-treated uremic rats exhibited a significant increase in ^{45}Ca uptake *ex vivo*. The results suggest that calcification under uremic conditions predominantly occurs in smooth muscle cells. Also, endothelial cells do not seem to be involved. However, these data do not explain why 1α,25$(OH)_2D_2$ and 1α,25$(OH)_2D_3$ failed to induce calcification in smooth muscle cells *in vitro*. Viewed collectively, these observations demonstrate that (1) the complex regulation of vascular calcification *in vivo* cannot be easily replicated in the *ex vivo* or *in vitro* models, and (2) it is important to employ a relevant disease state model such as the hyperphosphatemic uremic rat for vascular calcification studies.

Finally, we think it is necessary to compare and discuss findings from the present study *vs.* reports in the literature. Previously Hirata et al. (2003) showed that 1α,25$(OH)_2D_3$ induced vascular calcification in the 5/6 nephrectomized uremic rats, while 1,25$(OH)_2$-22-oxa-calcitriol (OCT), an analog of 1α,25$(OH)_2D_3$, didn't show any effect. They also showed that OCT at a high dose (6.25 μg/kg) raised serum Ca, P and CaxP levels to the same level as 1α,25$(OH)_2D_3$ at 0.125 μg/kg, but only 1α,25$(OH)_2D_3$ induced aortic calcification. Our observations confirm that different vitamin D analogs exert differential effects on vascular calcification independent of the serum Ca, P and CaxP levels. To the best of our knowledge, the differential effect of 1α(OH)D_2 and 19-nor on vascular calcification has not been reported previously. While our observation that high P induces an increase in Ca content and ^{45}Ca uptake into smooth muscle cells is consistent with previous reports by others (Wada et al., 1999; Steitz et al., 2001), the lack of effect of vitamin D analogs on inducing Ca uptake into cultured smooth muscle cells is in conflict with that reported by Jono et al. (1998). It is worth mentioning that, although Jono et al. (1998) showed calcification induced by calcitriol in cultured bovine aortic smooth muscle cells, a recent report by Wolisi and Moe (2005) indicated that neither 1α,25$(OH)_2D_3$ nor 19-nor show any effect on calcification in

bovine vascular smooth muscle cells. At present, these disparate results from studying cultured vascular cells cannot be readily reconciled although it is possible that different preparations of smooth muscle cells may differ in their functional response to vitamin D analogs.

In summary, we demonstrate that high P stimulates ^{45}Ca uptake and induces an accumulation of Ca in smooth muscle cells *in vitro* and *ex vivo*, but does not induce aortic calcification *in vivo*. In contrast, vitamin D analogs exhibit no effects on calcification *in vitro* and *ex vivo*, but display differential effects on aortic calcification *in vivo*. Furthermore, serum from uremic rats treated with 1α(OH)D_2 induced Ca uptake into cultured smooth muscle cells only under high P conditions. These results suggest that the complex regulation of vascular calcification *in vivo* cannot be easily replicated in the *ex vivo* or *in vitro* models, and some vitamin D analogs such as 1α(OH)D_2 may induce aortic calcification in uremic rats when a permissive condition such as hyperphosphatemia is present.

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Legends for Figures

Fig. 1. The serum creatinine and BUN levels in 5/6 nephrectomized (SNX) uremic rats on the hyperphosphatemia-inducing diet. Rats were dosed with vehicle, 1α(OH)D_2 or 19-nor at 0.17 μg/kg, i.p., 3 times/week, for 40 days. Handling of animals was as described in "Materials and Methods". On Day 0, 13 and 41 after the drug treatment, blood samples were collected for the measurement of serum creatinine (A) and BUN (B). Mean ± standard error was calculated for each group (n=4-5). One way ANOVA Dunnett test with 95% confidence intervals of difference was performed for statistical comparisons. *p<0.05, **p<0.01 *vs.* Sham - Day 0; #p<0.05 *vs.* own group - Day 0.

Fig. 2. The serum Ca, P, CaxP product and PTH levels in uremic rats. Rats were dosed with vehicle, 1α(OH)D_2 or 19-nor at 0.17 μg/kg, i.p., 3 times/week, for 40 days as in Figure 1. On Day 0, 13 and 41 after the drug treatment, blood samples were collected for the measurement of serum PTH (A), Ca (B), P (C), and CaxP product (D). Mean ± standard error was calculated for each group (n=4-5). One way ANOVA Dunnett test with 95% confidence intervals of difference was performed for statistical comparisons. *p<0.05, **p<0.01 *vs.* Sham - Day 0; #p<0.05, ##p<0.05 *vs.* own group - Day 0.

Fig. 3. The Ca and P content in the aorta of uremic rats. Rats were dosed with vehicle, 1α(OH)D_2 or 19-nor at 0.17 μg/kg, i.p., 3 times/week, for 40 days as in Figure 1. Twenty-four hours after the last dose, aorta was collected and processed for the determination of Ca (A) and P (B) as described in "Materials and Methods". Mean ± standard error was calculated for each group (n=4-5). One way ANOVA Dunnett test with 95% confidence intervals of difference was performed for statistical comparisons. **p<0.01 *vs.* Sham.

Fig. 4. The von Kossa staining in aorta. Uremic rats were dosed with vehicle, 1α(OH)D_2 or 19-nor at 0.17 μg/kg, i.p., 3 times/week, for 40 days as in Figure 1. Twenty-four hours after the last dose, aorta was collected and processed for von Kossa staining.

Fig. 5. The Ca and P content in the aorta of uremic rats dosed with 1α(OH)D_2 or 19-nor at 0.67 μg/kg. Rats were dosed with vehicle or drugs, i.p., 3 times/week, for 12 days as in Table 1. Twenty-four hours after the last dose, aorta was collected and processed for the determination of Ca (A) and P (B) as described in "Materials and Methods". Mean ± standard error was calculated for each group (n=4-8 as in Table 1). One way ANOVA Dunnett test with 95% confidence intervals of difference was performed for statistical comparisons. **p<0.01 *vs.* Sham.

Fig. 6. Uptake of ^{45}Ca into denuded aortic rings . (A) Aortic rings were prepared from uremic rats dosed with vehicle, 1α(OH)D_2 or 19-nor at 0.67 μg/kg, i.p., 3 times/week, for 12 days as in Table 1. ^{45}Ca uptake was determined as described in "Materials and Methods". Mean ± standard deviation was calculated for each group (n = 4-8 animals/group as in Table 1). One way ANOVA Dunnett test with 95% confidence intervals of difference was performed for statistical comparisons. **p<0.01 *vs.* Sham. For (B – D), aortic rings were prepared from normal rats, processed, and ^{45}Ca uptake determined as described in "Materials and Methods". (B) Aortic rings from normal rats (n=4) were cultured in DMEM containing different concentrations of P plus 3 U/ml of alkaline phosphatase. (C) Aortic rings from normal rats (n=6) were cultured in DMEM containing 0.9 mM P with or without 100 nM 1α,25$(OH)_2D_3$, 19-nor or 1α,25$(OH)_2D_2$. (D) Aortic rings from normal rats (n=3) were cultured in DMEM containing 2.06 mM P and 3 U/ml of alkaline phosphatase with or without 100 nM 1α,25$(OH)_2D_3$, 19-nor or 1α,25$(OH)_2D_2$. Mean ± standard deviation was calculated for each group. One way ANOVA Dunnett test with 95% confidence intervals of difference was performed for statistical comparisons. *p<0.05, ***p<0.001 *vs.* 0.9 mM P (B) or Control (C & D).

Fig. 7. Effect of P on Ca content and ^{45}Ca uptake in cultured human coronary artery smooth muscle cells. (A) Human coronary artery smooth muscle cells were cultured in medium containing different concentrations of P plus 3 U/ml of alkaline phosphatase for 9 days. (B) Cells were cultured in medium containing 0.9 or 2.06 mM P plus 3 U/ml of alkaline phosphatase for different periods of time. (C) Cells were cultured in medium containing different concentrations of P plus 3 U/ml of alkaline phosphatase for a total of 6 days. For (A) and (B), Ca content was determined. For (C), ^{45}Ca uptake was determined as

described in “Materials and Methods”. Mean ± standard deviation was calculated for each group (n = 6). (A) & (C) One way ANOVA Dunnett test with 95% confidence intervals of difference was performed for statistical comparisons. ***p<0.001 *vs.* 0.9 mM P. (B) Statistical comparisons were performed by unpaired t-test. ***p<0.001 *vs.* 0.9 mM P on corresponding days.

Fig. 8. Effect of P and vitamin D analogs on Ca content and ^{45}Ca uptake in cultured human coronary artery smooth muscle cells. (A) Cells were cultured in medium containing P ranging from 0.9 – 2.64 mM plus 3 U/ml of alkaline phosphatase with control, 100 nM 1α,25(OH)$_2$D$_3$, 19-nor or 1α,25(OH)$_2$D$_2$ for 6 days. Ca content was determined as described in “Materials and Methods”. (B) Cells were cultured in medium containing 0.9 mM P with or without different concentrations of 1α,25(OH)$_2$D$_3$, 19-nor or 1α,25(OH)$_2$D$_2$ for 6 days. (C) Cells were cultured in medium containing 2.06 mM P plus 3 U/ml of alkaline phosphatase with or without different concentrations of 1α,25(OH)$_2$D$_3$, 19-nor or 1α,25(OH)$_2$D$_2$ for 6 days. In (B) & (C), uptake of ^{45}Ca was determined as described in “Materials and Methods”. Mean ± standard deviation was calculated for each group (n=4). One way ANOVA Dunnett test with 95% confidence intervals of difference was performed for statistical comparisons. None of the values was statistically significant *vs.* control (no drug).

Fig. 9. VDR expression and effect of 19-nor on CYP24A1 mRNA. (A)-(C) SMC were cultured in medium containing 0.9 or 2.06 mM P plus 3 U/ml of alkaline phosphatase for 5 days. Real-time RT-PCR and Western blotting were performed as described in “Materials and Methods”. (A) The expression level of VDR mRNA was normalized to the GAPDH mRNA level. Values shown are mean ± the standard deviation (n = 4). Statistical comparisons were performed by unpaired t-test. **P<0.01. (B) The band intensity from Western blotting was normalized to the protein level in each sample. Results shown are representative of two independent experiments. (C) SMC were treated with increasing concentrations of 19-nor at 0.9 or 2.06 mM P, and RNA was isolated for real-time RT-PCR. The expression level of CYP24A1 mRNA was normalized to the GAPDH mRNA level. Values shown are mean ± the standard deviation (n = 4). (D) Aorta tissues were isolated from Sham or SNX (uremic) rats and RNA isolated for real-time RT-PCR. Values shown are mean ± the standard deviation (n = 6 per group).

Fig. 10. Effect of vitamin D analogs and P on ^{45}Ca uptake to cells in the co-culture of human coronary artery endothelial and smooth muscle cells. Endothelial (A) and smooth muscle (B) cells were initially cultured separately with endothelial cells in inserts. Cells were then put together in medium containing P at 0.9 or 2.06 mM plus 3 U/ml of alkaline phosphatase with control, 19-nor or $1\alpha,25(OH)_2D_2$ for 6 days. Uptake of ^{45}Ca was determined as described in "Materials and Methods". Mean ± standard deviation was calculated for each group (n=3). One way ANOVA Dunnett test with 95% confidence intervals of difference was performed for statistical comparisons. *P<0.05 *vs.* control (no drug) at 0.9 mM P.

Fig. 11. Effect of serum from uremic rats on ^{45}Ca uptake into human coronary artery smooth muscle cells. Cells in 0.5 ml of medium containing P at 0.9 or 2.06 mM plus 3 U/ml of alkaline phosphatase were treated with 10% serum from Sham (n=5) or uremic rats that were dosed with vehicle (SNX-Vehicle, n=3), 19-nor at 0.33 μg/kg (n=3) or $1\alpha(OH)D_2$ at 0.17 (n=4) or 0.33 μg/kg (n=3), i.p., 3 times/week, for 40 days. Serum from each treatment group was pooled. Uptake of ^{45}Ca was determined as described in "Materials and Methods". Mean ± standard deviation was calculated for each group (n=3). One way ANOVA Dunnett test with 95% confidence intervals of difference was performed for statistical comparisons. **P<0.01 *vs.* Sham at 2.06 mM P.

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Table 1. Blood chemistries in 5/6 nephrectomized uremic rats

Parameters	Sham	SNX-Vehicle	1α(OH)D_2	19-nor
iCa (mmol/dl)	1.33 ± 0.02	1.06 ± 0.07[b]	1.43 ± 0.04[d]	1.37 ± 0.02[d]
Ca (mg/dl)	9.98 ± 0.09	8.32 ± 0.55[b]	10.28 ± 0.09[d]	11.43 ± 0.17[b,d]
P (mg/dl)	6.50 ± 0.38	11.65 ± 1.03[a]	9.93 ± 0.82[a]	9.90 ± 1.28[a]
Ca x P $(mg/dl)^2$	64.8 ± 3.5	95.1 ± 7.6	102.2 ± 8.8	113.0 ± 5.4[a]
PTH (pg/ml)	168.5 ± 31.8	2833 ± 1437[b]	306 ± 86[d]	152 ± 41[d]
Creatinine (mg/dl)	0.53 ± 0.05	1.47 ± 0.21[b]	1.31 ± 0.10[b]	1.10 ± 0.07[a]
BUN (mg/dl)	12.00 ± 0.41	64.50 ± 6.44[b]	58.63 ± 6.79[b]	54.83 ± 4.96[b]

Note 1: 5/6 nephrectomized uremic rats on a hyperphosphatemia-inducing diet were treated with vehicle , 1α(OH)D_2 or 19-nor at 0.67 μg/kg, i.p., 3 times/week, for 12 days. Ca, serum calcium; iCa, ionized serum calcium; P, serum phosphorus; PTH, serum parathyroid hormone; BUN, blood urea nitrogen; SNX: 5/6 nephrectomy

Note 2: Data are mean ± SE (n= 4 in Sham, 6 in SNX-Vehicle, 8 in the 1α(OH)D_2 treated group, and 6 in the 19-nor treated group). One way ANOVA Dunnett test with 95% confidence intervals of difference was performed for statistical comparisons. a: $p<0.05$, b: $p<0.01$ vs. sham; c: $p<0.05$, d: $p<0.01$ vs. SNX-control

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Figure 1

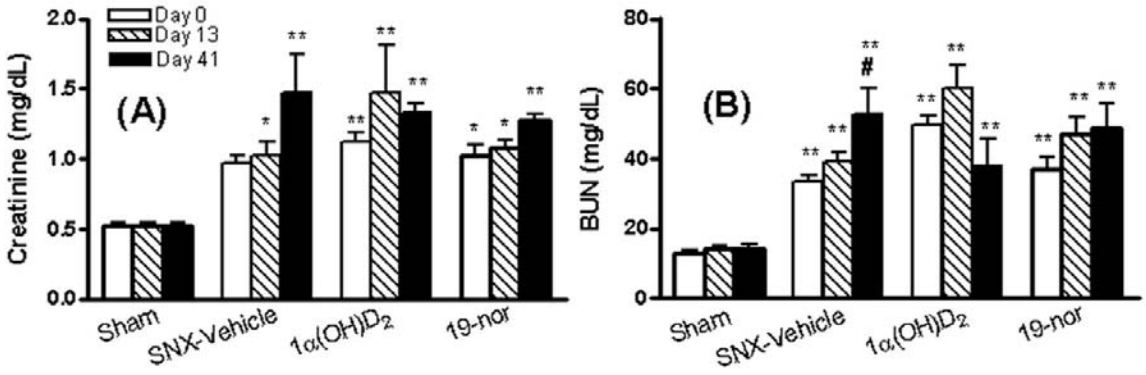

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Figure 2

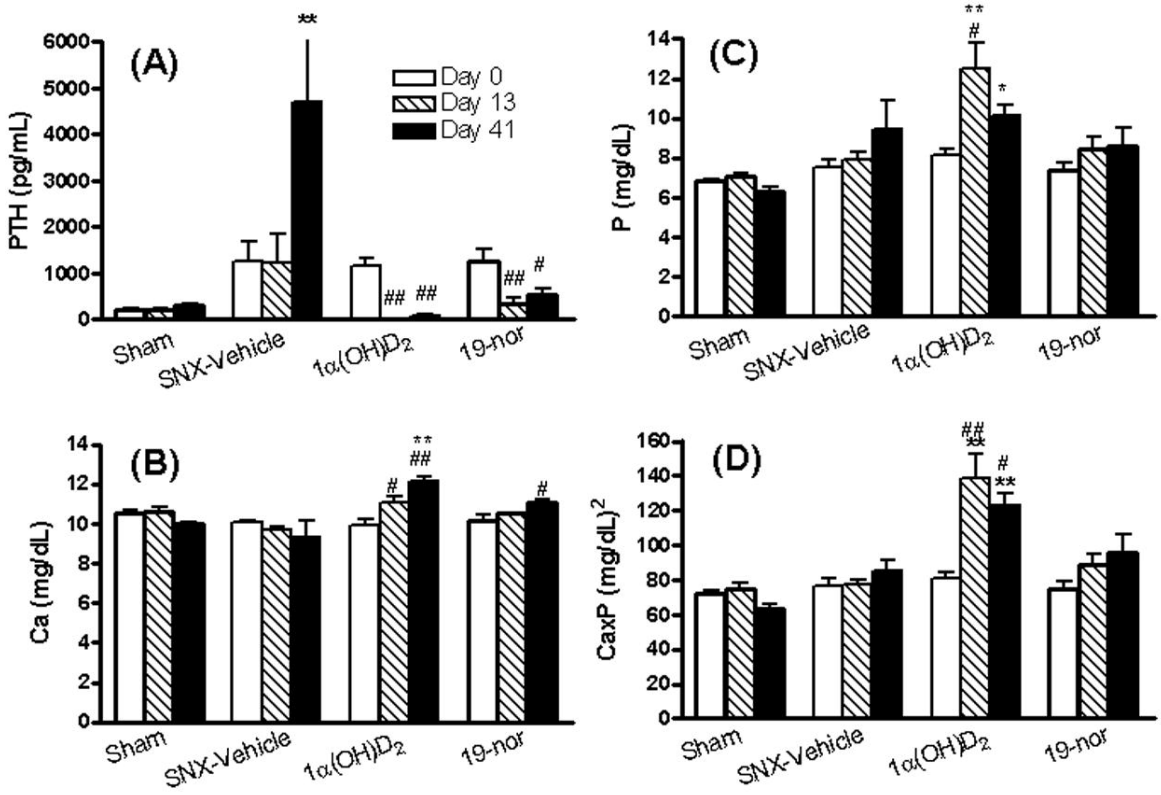

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Figure 3

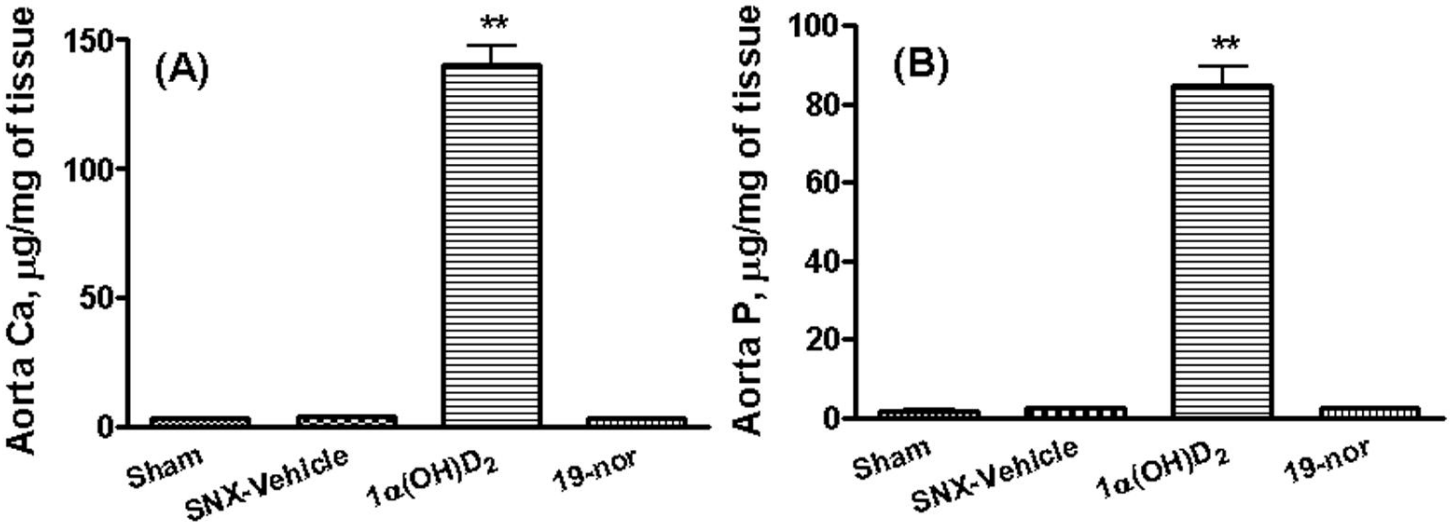

Figure 4

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Figure 5

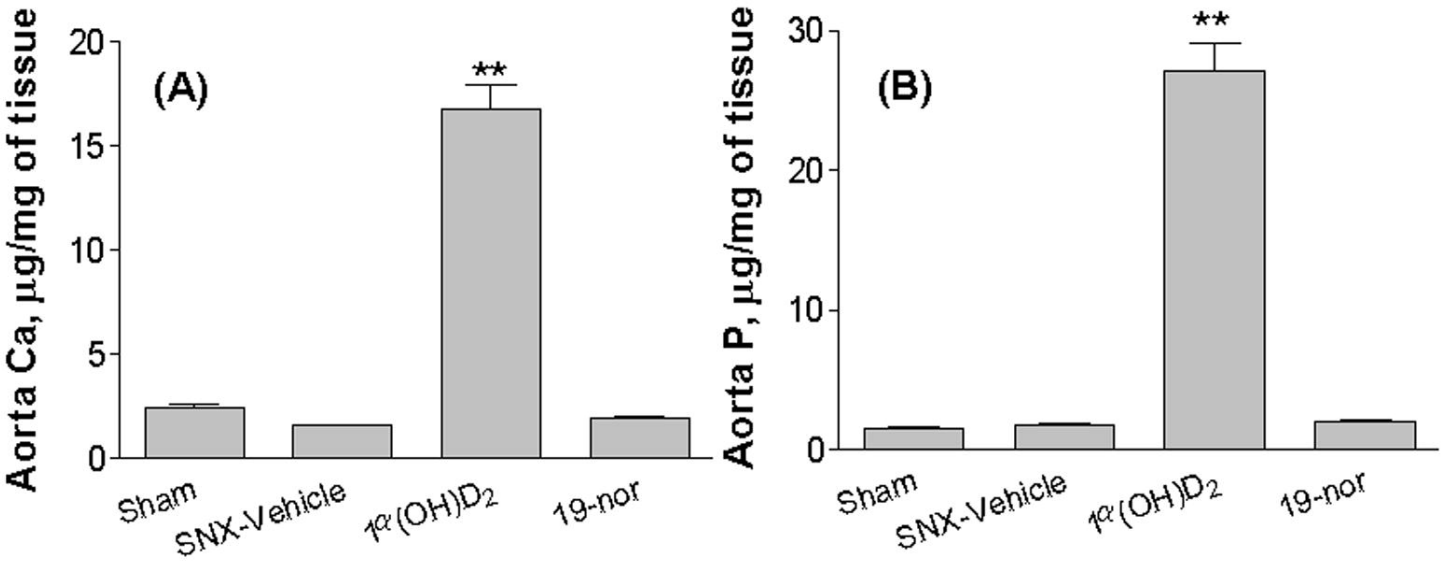

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Figure 6

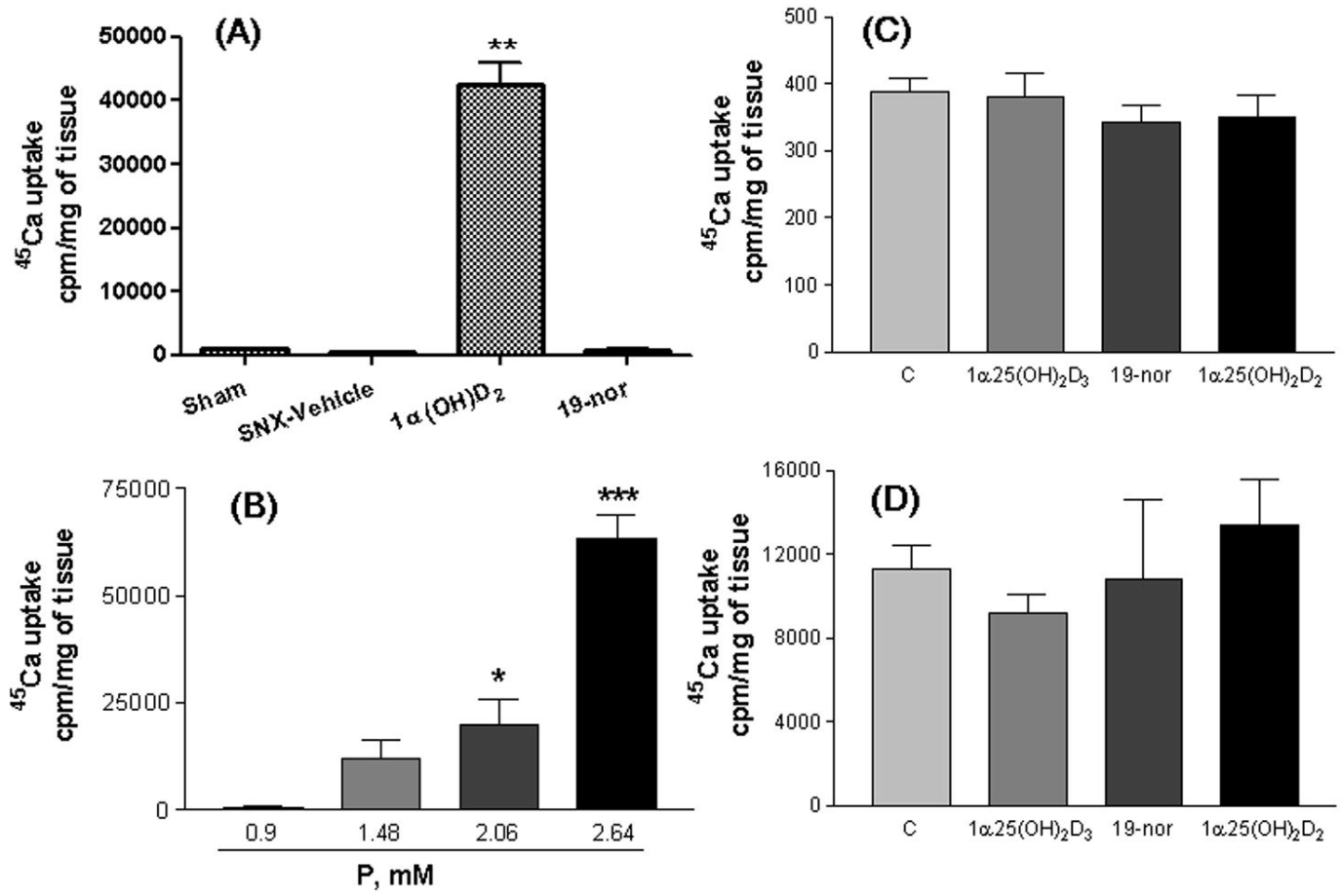

Figure 7

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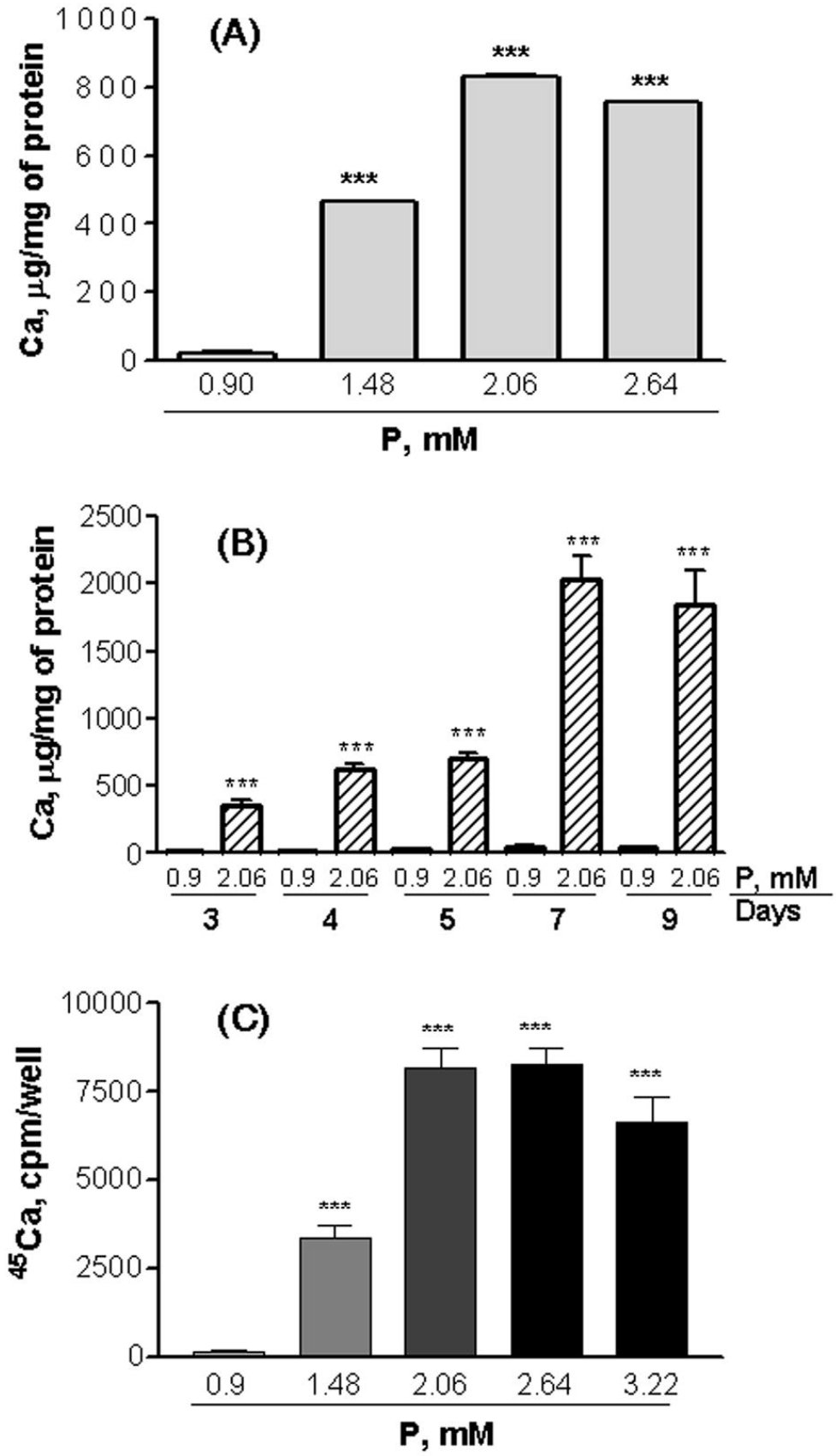

Figure 8

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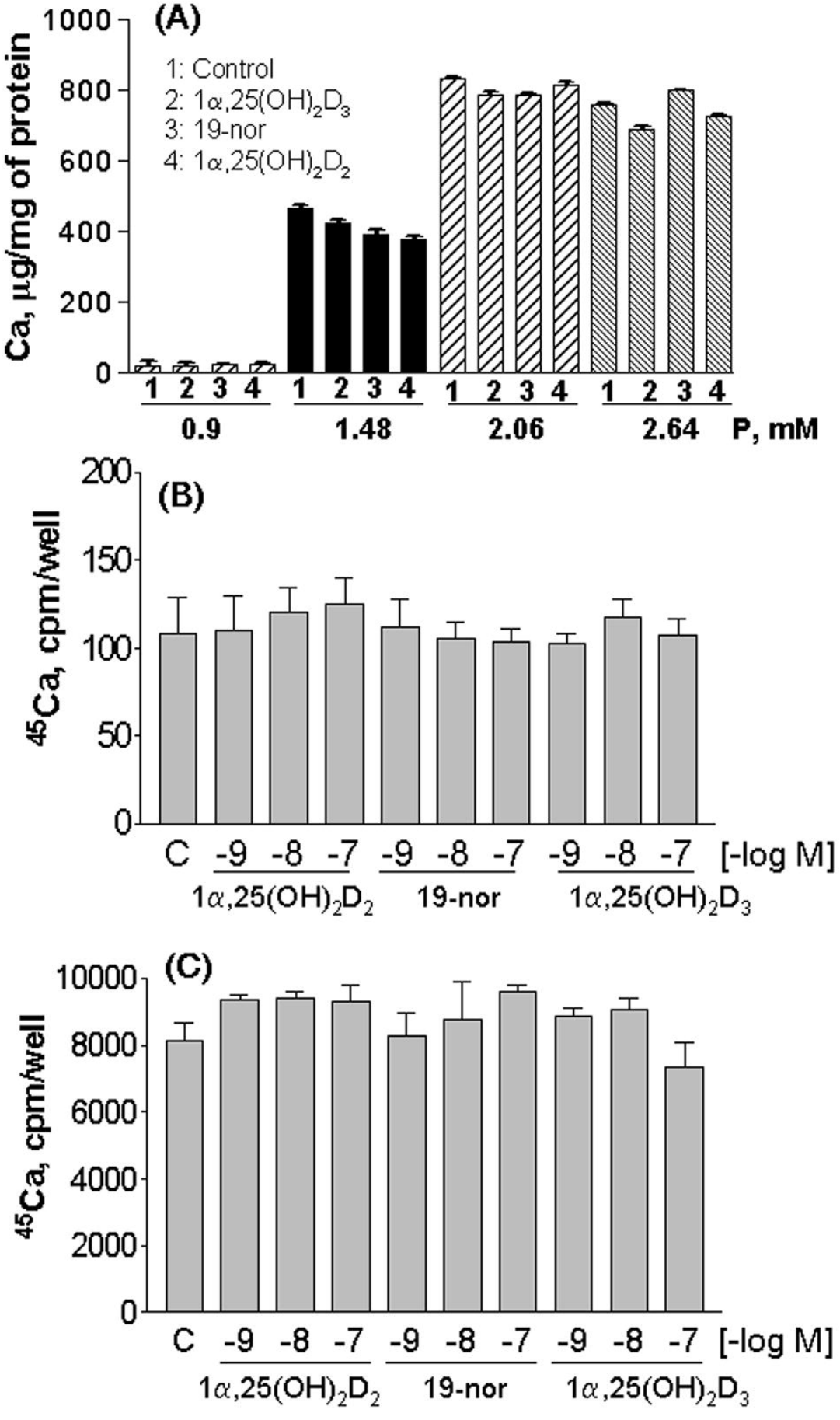

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Figure 9

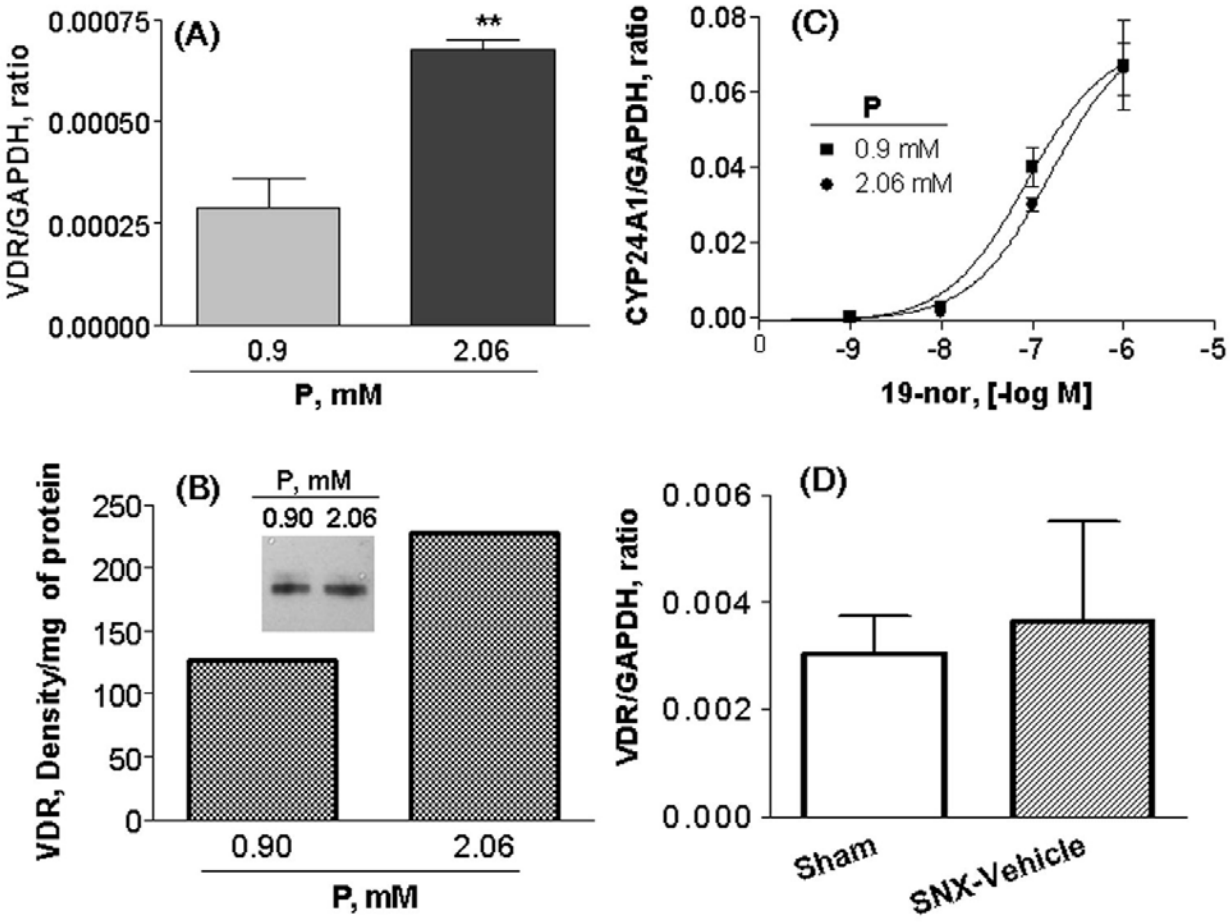

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Figure 10

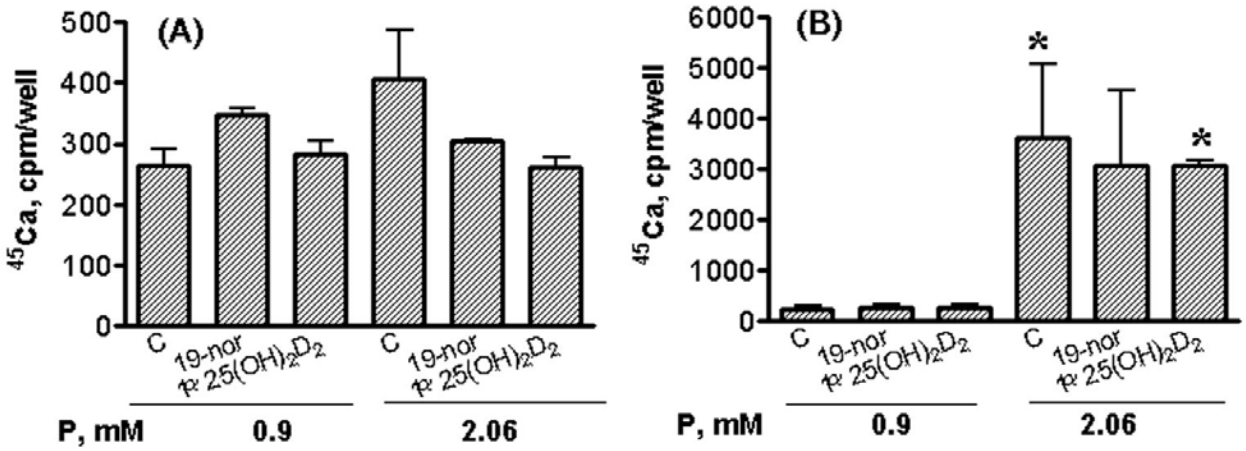

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Figure 11