Differential effects of vitamin D receptor activators on vascular calcification in uremic rats

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Vascular calcification is associated with cardiovascular disease, the most common cause of death in chronic kidney disease (CKD). Patients with CKD are treated with vitamin D receptor activators (VDRAs); therefore, we determined if this treatment affects vascular calcification. Uremic rats were given vehicle, calcitriol, paricalcitol, or doxercalciferol three times a week for 1 month. Calcitriol significantly increased the serum calcium–phosphate product and aortic calcium content. Paricalcitol had no effect but the same dose of doxercalciferol significantly increased the calcium–phosphate product and the aortic calcium content, the latter being confirmed by von Kossa staining. To see if the increased aortic calcium was due to an increased serum calcium–phosphate product or to a differential effect of the two VDRAs, we lowered the dose of doxercalciferol and increased the dose of paricalcitol. A lower doxercalciferol did not increase the calcium–phosphate product but increased the aortic calcium content. A higher dose of paricalcitol still had no effect. Doxercalciferol treatment increased the mRNA and protein expression of the bone-related markers Runx2 and osteocalcin in the aorta, whereas paricalcitol did not. Hence, different VDRAs have different effects on vascular calcification in uremic rats. The effects are independent of the serum calcium–phosphate product suggesting independent mechanisms.

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Cardiovascular complications are the leading cause of death in patients with chronic kidney disease (CKD).¹ Traditional risk factors, such as aging, hypertension, diabetes, and smoking do not fully explain the high rate of morbidity from the cardiovascular disease seen in these patients and other specific factors have been suggested which might contribute.²,³ Vascular calcification is common in patients with CKD and the progression of vascular calcification is associated with subsequent cardiovascular-related mortality.⁴–⁸ The precise mechanism(s) responsible for the development and progression of vascular calcification in patients with CKD is not fully understood. However, abnormalities in calcium (Ca) and phosphate (P) metabolism are considered to be particularly important in the development of vascular calcification.

For a long time, vascular calcification was thought to be a passive process resulting from increased serum P and Ca × P product. However, recent studies have shown that vascular calcification is also an active process as evidenced by the presence of many proteins in arterial tissues which are associated with bone metabolism.⁹–¹³ These proteins reflect a phenotypic change of vascular smooth muscle cells (VSMCs) into osteoblast-like cells.⁹,¹⁴ Exposing human VSMCs to high inorganic P levels can induce a concentration-dependent increase in the mineralization in VSMCs in association with the upregulation of markers of osteoblastic differentiation.¹⁵ Increased extracellular Ca concentrations also have a similar effect.¹⁶ These observations suggest an important role for increased extracellular P and Ca in the progression of vascular calcification.

Vitamin D receptor activators (VDRAs), such as 19-nor-1α,25(OH)₂D₃ (paricalcitol) or 1α-hydroxyvitamin-D₂ (doxercalciferol), which are less calcemic than calcitriol, are now commonly used to manage secondary hyperparathyroidism associated with CKD because they effectively suppress parathyroid hormone (PTH) but are less calcemic and phosphatemic than calcitriol. Animal studies demonstrate that extremely high doses of calcitriol can induce vascular calcification.¹⁷–²⁰ It is possible that supraphysiologic doses of calcitriol may indirectly contribute to the induction of vascular calcification in animal models of CKD by causing the oversuppression of PTH, which then leads to low-turnover bone disease and/or increased serum Ca, P, or Ca × P levels. Recent clinical observations demonstrate that
Each drug was given intraperitoneally three times a week for 1 month. Data are mean ± s.e.m. ANOVA, analysis of variance; Cr, creatinine; i-Ca, ionized calcium; Ca, serum calcium; P, serum phosphorus; PTH, parathyroid hormone.

Table 1 | Blood chemistries in normal and uremic rats

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Cr</th>
<th>i-Ca</th>
<th>Ca</th>
<th>P</th>
<th>Ca × P</th>
<th>PTH</th>
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<tr>
<td></td>
<td></td>
<td>mg/dl</td>
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<td>(mg²/dl)²</td>
<td>µg/ml</td>
</tr>
<tr>
<td>NC</td>
<td>6</td>
<td>0.7 ± 0.0</td>
<td>4.87 ± 0.06</td>
<td>9.5</td>
<td>4.5 ± 0.2</td>
<td>42.5 ± 2.3</td>
<td>15 ± 6</td>
</tr>
<tr>
<td>UC</td>
<td>6</td>
<td>1.3 ± 0.1</td>
<td>4.41 ± 0.08</td>
<td>8.8</td>
<td>7.0 ± 1.0</td>
<td>61.9 ± 8.7</td>
<td>438 ± 75</td>
</tr>
<tr>
<td>1,25D₃</td>
<td>6</td>
<td>1.4 ± 0.8</td>
<td>5.51 ± 0.14</td>
<td>11.2</td>
<td>11.0 ± 0.6</td>
<td>123.1 ± 6.8</td>
<td>52 ± 12</td>
</tr>
<tr>
<td>1αD₂</td>
<td>7</td>
<td>1.4 ± 0.1</td>
<td>5.02 ± 0.15</td>
<td>11.2</td>
<td>9.2 ± 0.7</td>
<td>103.6 ± 7.8</td>
<td>49 ± 21</td>
</tr>
<tr>
<td>19-nor</td>
<td>6</td>
<td>1.1 ± 0.1</td>
<td>5.39 ± 0.13</td>
<td>10.0</td>
<td>7.6 ± 1.1</td>
<td>76.3 ± 11.4</td>
<td>72 ± 15</td>
</tr>
<tr>
<td>ANOVA</td>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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</table>

Normal (NC) rats received vehicle and uremic rats were treated with vehicle (UC), 0.04 µg/kg of calcitriol (1,25D₃), or 0.16 µg/kg of doxercalciferol (1αD₂) or paricalcitol (19-nor). Each drug was given intraperitoneally three times a week for 1 month. Data are mean ± s.e.m.

VDRAs also provide a survival benefit for patients with CKD independent of serum Ca, P, and PTH levels.⁵¹⁻²⁵ This survival benefit is associated with a decrease in cardiovascular-related mortality. Additionally, there is no clear evidence that treatment with calcitriol or its analogs is directly responsible for the induction of vascular calcification in these patients. Taken together these facts suggest that newer VDRAs, which have less calcemic and phosphatemic activity, appear to be less likely to promote vascular calcification.

The aim of this study is to compare the effect of calcitriol and two VDRAs, paricalcitol and doxercalciferol, on serum Ca, P, and Ca × P product, to determine their effect on vascular calcification and to explore possible mechanisms involved.

RESULTS

Effect of calcitriol and VDRAs on serum Ca, P, Ca × P product, and PTH levels in uremic rats

In the first experiment, 5/6 nephrectomized rats were treated with 0.04 µg/kg of calcitriol or 0.16 µg/kg of doxercalciferol or paricalcitol intraperitoneally three times a week for 1 month. Body weights were similar in all groups (data not shown). As shown in Table 1, serum creatinine (Cr) levels were significantly increased in the UC group. Treatment with calcitriol or its analogs had no effect on serum Cr levels. Although ionized Ca was significantly higher in all of the treatment groups compared with the UC rats, the increase in Ca was much milder with paricalcitol treatment. Calcitriol significantly increased serum P levels compared to the UC group, whereas, doxercalciferol and paricalcitol did not. The serum Ca × P product was significantly higher in both the calcitriol and doxercalciferol group, whereas paricalcitol again had no effect. Serum PTH levels were significantly suppressed in all treatment groups compared with the UC rats. There were no statistical differences in serum PTH levels among the three treatment groups (Table 1).

Effect of calcitriol and VDRAs on the vascular calcification in uremic rats

As shown in Figure 1, there was no difference in the aortic Ca content between UC (2.0 ± 0.4 mg/g wet wt of tissue) and paricalcitol-treated rats (2.3 ± 0.6 mg/g wet wt of tissue), whereas treatment with calcitriol (31.4 ± 10.6 mg/g wet wt of tissue) and doxercalciferol (31.4 ± 6.7 mg/g wet wt of tissue) resulted in a marked elevation in the aortic Ca content. Aortic calcification was determined by von Kossa staining. Figure 2 shows that both calcitriol and doxercalciferol induced severe calcification in the medial layer of aorta, whereas paricalcitol did not.

To determine if this increase in aortic Ca was due to the increase in Ca × P product or to a differential effect of these two VDRAs on vascular calcification, we repeated the study using a lower dose of doxercalciferol (0.10 µg/kg) and a higher dose of paricalcitol (0.24 µg/kg). The calcemic action of 0.10 µg/kg of doxercalciferol was stronger than that of 0.24 µg/kg of paricalcitol. Although this lower dose of doxercalciferol did not significantly increase serum P levels (8.2 ± 0.9 mg/dl) or the Ca × P product (93.8 ± 11.2 mg²/dl²) (Figure 3), it still produced significant Ca deposition in the
aorta (16.2 ± 4.3 mg/g wet wt of tissue), whereas the higher dose of paricalcitol, (P: 8.1 ± 1.2 mg/dl, Ca × P product: 81.7 ± 13.5 mg²/dl²), did not increase aortic Ca deposition (2.5 ± 0.9 mg/g wet wt of tissue) (Figure 4). In addition, no statistical difference was observed in serum PTH levels between these two groups (doxercalciferol: 63 ± 55 pg/ml and paricalcitol: 77 ± 17 pg/ml).

**Differential effects of VDRAs on the process of vascular calcification**

To investigate further the different effects of these VDRAs on aortic calcification, we examined the mRNA expression of Runx2 and osteocalcin in aortic tissue from normal or uremic rats treated with vehicle, 0.04 µg/kg of calcitriol, 0.10 µg/kg of doxercalciferol, or 0.16 µg/kg of paricalcitol. Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analyses revealed that Runx2 mRNA expression in both calcitriol- and doxercalciferol-treated groups were significantly increased compared with that of the paricalcitol-treated group (Figure 5). There was no significant difference in osteocalcin mRNA expression between the uremic control and paricalcitol-treated groups, whereas treatment with either calcitriol or doxercalciferol resulted in a marked increase in osteocalcin mRNA expression (Figure 6). We then investigated the protein expression of Runx2 and osteocalcin in the doxercalciferol- (0.10 µg/kg) and paricalcitol-treated groups (0.16 µg/kg) by immunohistochemical analysis. Both Runx2 and osteocalcin protein were expressed in the calcified areas of aortic tissue in the doxercalciferol-treated group, but not in paricalcitol-treated
animals (Figure 7a and b). Runx2 and osteocalcin protein expression was similar to that seen for their mRNA expression. This strongly implicates both Runx2 and osteocalcin in the development of vascular calcification in this model.

DISCUSSION
Vascular calcification is the leading cause of morbidity and mortality in patients with CKD, and disturbances in mineral metabolism in these patients are closely linked to its pathogenesis. Calcitriol and VDRAs are effective in suppressing serum PTH levels in patients with secondary hyperparathyroidism. However, in vitro and in vivo studies have demonstrated that high concentrations of calcitriol can induce vascular calcification.17–20,26 This is probably due to the fact that the concentrations of calcitriol used in these studies were clearly toxic and resulted in hypercalcemia and hyperphosphatemia. Newer VDRAs, however, are less calcemic and phosphatemic and therefore, appear to be less likely to induce vascular calcification. Because there is no clear evidence that treatment with calcitriol or VDRAs directly contributes to vascular calcification in patients with CKD, it is important to understand the effect of these drugs when given at doses that suppress PTH but are not calcemic and phosphatemic. To address this issue, we examined the

![Graph](image1)

**Figure 6** Effects of 0.04 μg/kg of calcitriol (1,25D₃), 0.10 μg/kg of doxercalciferol (1αD₂), or 0.16 μg/kg of paricalcitol (19-nor) on mRNA expression levels in aorta from uremic rats. Osteocalcin mRNA levels were analyzed by real-time RT-PCR technique. Each drug was given intraperitoneally three times a week for 1 month. Values are mean ± s.e.m. (n = 6). *P < 0.01 by analysis of variance. **P < 0.01 and *P < 0.05 versus UC; KK *P < 0.01 and K P < 0.05 versus 19-nor by post hoc, Scheffe test.

![Images](image2)

**Figure 7** Representative microphotograph of immunohistochemistry for Runx2 or osteocalcin in aorta from uremic rats treated with 0.10 μg/kg of doxercalciferol (1αD₂) or 0.16 μg/kg of paricalcitol (19-nor). Each drug was given intraperitoneally three times a week for 1 month. Original magnification at (a) × 100 and (b) × 200.
effect of calcitriol and lower doses of two VDRAs on vascular calcification in uremic rats.

Because the vitamin D receptor (VDR) is found in VSMCs, it is reasonable to assume that calcitriol, the main ligand for the VDR has an effect on VSMCs. Calcitriol at concentrations as low as $10^{-10} M$ has been shown to decrease VSMC proliferation by inhibiting the activation of epidermal growth factor. In addition, exposing cultures of rat or rabbit VSMCs to calcitriol produces an upregulation of VDR expression. Jono et al. have also demonstrated that calcitriol at concentrations from $10^{-7}$ to $10^{-9} M$ induces calcification of bovine VSMCs in a dose-dependent manner. This induction of calcification by calcitriol is accompanied by the downregulation of PTH-related peptide. Thus, calcitriol has a number of effects on VSMCs including the promotion of calcification. It is important to point out, however, that these in vitro studies utilized supraphysiological concentrations of calcitriol which would be toxic in vivo and promote vascular calcification through the induction of hypercalcemia and hyperphosphatemia. To evaluate the effect of calcitriol at lower doses, we administered 0.04 μg/kg of calcitriol intraperitoneally three times a week for 1 month. However, even this dose induced aortic medial calcification with a marked increase in serum Ca, P, and the Ca × P product. Thus, it is difficult to determine if calcitriol directly induced vascular calcification or if it was the result of the hypercalcemia, hyperphosphatemia, and increased Ca × P product seen in these rats.

An increased Ca × P product has also been associated with high morbidity and mortality in patients undergoing dialysis. Accordingly, control of serum Ca and P is vital in controlling and avoiding the development of vascular calcification. To eliminate the effect of an increased Ca × P product on vascular calcification, we treated uremic rats with two different VDRAs, paricalcitol and doxercalciferol, which have lower calcemic and phosphatemic actions. When given at the same dose, 0.16 μg/kg intraperitoneally, three times a week for 1 month, doxercalciferol significantly increased the Ca × P product compared to paricalcitol. This would explain why substantial vascular calcification was seen in rats treated with doxercalciferol. We repeated the study using a lower dose of doxercalciferol (0.10 μg/kg) and a higher dose of paricalcitol (0.24 μg/kg) to compare similar Ca × P products. This lower dose of doxercalciferol suppressed serum PTH levels but still induced severe vascular calcification, whereas paricalcitol did not, suggesting a different mechanism(s) (e.g., selective effects on VDR pathways) between these two VDRAs on vascular calcification, which is independent of the Ca × P product.

Recent studies have shown that a number of proteins associated with bone metabolism can be expressed in arterial tissues, and reflect the transformation of VSMCs into osteoblast-like cells. This ectopic osteogenic process has been considered an important mechanism in the pathogenesis of vascular calcification. We found that both of the VDRAs had a unique effect on this process. Runx2 is an osteoblast-specific transcription factor and osteocalcin is one of its transcriptional target genes. Both have a crucial role in the development of vascular calcification. Doxercalciferol highly induced the expression of Runx2 and osteocalcin mRNA levels, whereas paricalcitol did not. In parallel with these higher mRNA levels, immunohistochemical analyses showed that the calcified areas of the doxercalciferol-treated group also had an increased protein expression of both Runx2 and osteocalcin. This strongly suggests that both Runx2 and osteocalcin are principal factors in the development of vascular calcification. Paredes et al. reported that Runx2 interacts with the VDR to upregulate the osteocalcin gene in osteoblastic cells. In response to calcitriol-dependent stimulation of the osteocalcin promoter, Runx2 stabilizes the interaction of the VDR with the vitamin D-response element. The half-life of doxercalciferol in patients with CKD is much longer than that of paricalcitol, 45 versus 15-30 h, respectively. Thus, in this animal model, doxercalciferol, but not paricalcitol, most probably induces vascular calcification by not only the increasing Ca × P product, but also by stabilizing the transcription of osteocalcin in aortic tissue. It is also possible that doxercalciferol, but not paricalcitol, could accelerate the transformation of VSMCs into osteoblast-like cells.

Serum PTH levels may also be an important risk factor in the development of vascular calcification. Some studies in which PTH was evaluated as a risk factor for vascular calcification failed to find an association between serum PTH levels and vascular calcification, although conflicting results also have been observed. In patients undergoing dialysis, low-turnover bone disease is also associated with vascular calcification. Low-turnover bone disease results from the oversuppression of PTH and leads to the reduction of Ca and P influx into the bone possibly promoting vascular calcification. Because there was no statistical difference in serum PTH levels between the doxercalciferol- and paricalcitol-treated groups in this study, the different effects of these VDRAs on vascular calcification are probably independent of serum PTH levels.

In conclusion, this study demonstrates that control of the Ca × P product is crucial in treatment with VDRAs to avoid the progression of vascular calcification. VDRAs can have very different effects on vascular calcification, which are independent of the Ca × P product and serum PTH levels.

Materials and Methods

The Effect of Vitamin D and Its Analogs on Vascular Calcification

All studies were approved by The Washington University Animal Studies Committee in accordance with federal regulations.
was induced in a group of female Sprague–Dawley rats (200–225 g) by 5/6 nephrectomy. This procedure involves the ligation of branches of the left renal artery and excision of the right kidney. The rats were then randomly divided into four groups as follows: control – vehicle, 100 μl propylene glycol (UC, n = 6), calcitriol – 0.04 μg/kg (1,25D3, n = 6), doxercalciferol – 0.16 μg/kg (1αD2, n = 7), or paricalcitol – 0.16 μg/kg (19-nor, n = 6). The rats were fed a high P diet containing 1.2% P and 0.8% Ca. Each drug was given intraperitoneally three times a week for 1 month. An additional group of normal rats were fed a standard rodent chow and served as the normal control (NC group, n = 6). After 1 month, rats were killed and blood was taken for the measurement of serum chemistries. The section of aorta from the thoracic to the renal branch was dissected out and rinsed in phosphate-buffered saline. The upper part was snap-frozen in liquid nitrogen and stored at −80°C for real-time RT-PCR analysis. From the remainder, two additional pieces (7 mm) were taken. One was weighed on a microbalance (CAHN-31, Orion Instruments, Inc., Boston, MA, USA) and retained for measurement of Ca content. The other was fixed in 10% formalin for histological examination. This study was then repeated with uremic rats receiving either 0.10 μg/kg of doxercalciferol or 0.24 μg/kg of paricalcitol given as described before three times a week for 1 month.

Analytical determinations
Serum levels of P and Cr were measured by autoanalyzer (COBAS-MIRA Plus, Branchburg, NJ, USA). Total Ca was measured by atomic absorption spectrophotometry (1100B, Perkin-Elmer, Norwalk, CT, USA). Ionized Ca was measured using a Nova 8 electrolyte analyzer (Nova Biomedical, Waltham, MA, USA). Intact PTH was measured by an immunoradiometric assay specific for intact rat PTH (Immunotopics, San Clemente, CA, USA).

Chemical and morphologic assessment of calcification
Aortic Ca content was measured after acid digestion as described previously.39,40 Tissue was weighed and hydrolyzed in 0.5 ml of 6 N HCl for 24 h. Ca content was then determined by atomic absorption spectrophotometry. Results were corrected by wet tissue weight and expressed as mg/g wet wt of tissue.

After being fixed in 10% formalin, specimens of aorta were embedded in paraffin blocks. Five-micrometer sections were deparaffinized and von Kossa staining41 or immunohistochemistry for Runx2 and osteocalcin was performed.

Quantitative RT-PCR analyses
Total RNA from each sample of aortic tissue was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instruction. For complementary DNA synthesis, RT-PCR was performed using Super Script II® (Invitrogen). Synthesized complementary DNA was amplified by a standard PCR protocol using SYBR Green Jump Start Taq Ready Mix® (Sigma, St Louis, MO, USA) and rat-specific primers for Runx2 or osteocalcin. Parallel amplifications with primers for glyceraldehyde-3-phosphate dehydrogenase were performed. The primer set for Runx2 was purchased from SuperArray (Frederick, MD, USA; assay ID: PPR53039A-200). Osteocalcin and glyceraldehyde-3-phosphate dehydrogenase primers were purchased from Qiagen (Germantown, MD, USA; assay ID of osteocalcin: Rn_Rgap2_2_SG, and glyceraldehyde-3-phosphate dehydrogenase: Rn_Gapd_2_SG). Cycling conditions were: 10 min preincubation at 95°C, 15 s denaturation at 95°C, 1 min annealing at 56°C for 40 cycles using GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Reaction kinetics were determined using a standard curve. PCR products from each group were separated on a 4% agarose gel and confirmed to have a single band. To correct for the amount of mRNA, the ratios of Runx2 to osteocalcin to glyceraldehyde-3-phosphate dehydrogenase mRNAs were calculated for each sample. All measurements were performed in duplicate.

Immunohistochemistry
Immunohistochemical staining for Runx2 and osteocalcin was performed using a commercially available kit (Histostain-Plus, rabbit; Zymed Laboratories Inc., South San Francisco, CA, USA). The negative control was obtained by substituting rabbit pre-immune IgG for the primary antibody. The sections were deparaffinized, rehydrated, and microwaved in 0.01 mol/l citrate buffer (pH 6.0) for 10 min to retrieve the antigens. The sections were subsequently blocked with 10% pre-immune goat serum for 30 min at room temperature. Primary antibodies for Runx2 (rabbit anti-human Runx2 antibody; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or osteocalcin (rabbit anti-bovine osteocalcin antibody; Biomedical Technologies, Stoughton, MA, USA), or pre-immune IgGs were added followed by an overnight incubation at 4°C. Biotinylated secondary antibody was applied, followed by a streptavidin–horseradish peroxidase conjugate. The immune complexes were visualized with 3-amin-9-ethylcarbazole substrate – chromagen.

Statistical analyses
All results were expressed as mean ± s.e.m. One-way analysis of variance was used to assess the statistical differences between groups. A post hoc Scheffe test, was employed to assess the statistical significance between all possible two-group comparisons. Non-paired t-test was employed when only two groups were compared. P < 0.05 was considered significant.

CONFLICT OF INTEREST
ES is a consultant/speaker for Genzyme and Abbott.

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REFERENCES


