Original Article

Role of Local Versus Systemic Vitamin D Receptors in Vascular Calcification

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Objective—Calcitriol and various analogs are commonly used to suppress secondary hyperparathyroidism in chronic kidney disease but may also exacerbate vascular calcification. Although this could be because of increased intestinal calcium and phosphate absorption, direct effects through vitamin D receptors (VDRs) on vascular smooth muscle have also been proposed.

Approach and Results—The role of these receptors was investigated by examining gene regulation in rat aortas treated with calcitriol ex vivo and in vivo and by transplanting aortas from VDR-null (VDR−/−) mice into wild-type mice before induction of uremia and treatment with calcitriol. In cultured rat aortas, calcitriol increased the expression of mRNA for CYP24A1 but not mRNA for any bone-related or calcification-related genes. Gene expression in aortas in vivo was not altered by doses of calcitriol that promote calcification. Calcitriol markedly increased aortic calcification in uremic mice and this did not differ between VDR−/− aortic allografts and VDR+/+ recipient aortas.

Conclusions—Calcitriol promotes vascular calcification through a systemic action rather than through a direct vascular action. (Arterioscler Thromb Vasc Biol. 2014;34:00-00.)

Key Words: transplantation | vascular calcification | vitamin D

Calcitriol or other compounds that bind and activate the vitamin D receptor (VDR) are commonly used to control the secondary hyperparathyroidism that occurs in chronic kidney failure. However, there is concern that they may contribute to the medial vascular calcification (also known as Monkeberg arteriosclerosis) that commonly occurs in these patients and is associated with poorer outcomes.1−3 This concern stems from numerous studies showing that VDR agonists (VDRAs) increase vascular calcification in uremic animals4−9 and even normal animals at high doses.10 The possibility of accelerated vascular calcification has limited the use of VDRAs in chronic kidney disease, despite their potential benefits and the fact that VDRA use does not correlate with vascular calcification in cross-sectional studies of patients.11−13

The mechanism by which VDRAs promote vascular calcification is unclear and has not been explained by increased circulating calcium or phosphate levels in many studies.1,6−8 Vascular smooth muscle cells contain high-affinity binding sites for calcitriol,11 suggesting a direct action and, consistent with this, calcitriol has been reported to increase calcification of smooth muscle cells in culture15 and expression of bone-related genes in aortas in vivo.6 However, calcitriol does not induce calcification of intact aortas cultured under calcifying conditions,16 suggesting that vascular VDRs may not be responsible for the promotion of vascular calcification by VDR agonists.

To clarify the role of VDRs in vascular calcification, this study examined the effect of calcitriol on aortic gene expression ex vivo and in vivo and on medial calcification of aortas from VDR-deficient mice transplanted into wild-type mice.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Effect of Calcitriol on Gene Expression in Rat Aortas

The response of aortic smooth muscle to calcitriol was examined both ex vivo and in vivo by measuring the expression of several genes potentially involved in vascular calcification. These included the osteoblastic transcription factors runx2 and osterix,17,18 tissue-nonspecific alkaline phosphatase and ectonucleotide phosphodiesterase pyrophosphorylase I, ectoenzymes that determine extracellular levels of pyrophosphate through hydrolysis and synthesis, respectively,19,20 and the matrix-binding proteins osteopontin and matrix gla protein, which also inhibit calcification.21−23

VDR mRNA was also measured but the expression was too low to be accurately quantified. None of these genes showed changes in expression in rat aortas that were cultured for 6 days with calcitriol (Figure 1A). No calcification occurs under these conditions.16 However, there was a...
6-fold increase in mRNA for CYP24A1, an enzyme that is induced by and metabolizes calcitriol and used as a positive control. None of the genes, including CYP24A1, were upregulated in aortas from normal rats treated with calcitriol (100 ng/kg every 48 hours) in vivo (Figure 1B). There was no aortic calcification. Although there was increased expression of some of these genes in uremic rats, there was no change with calcitriol treatment (Figure 1C). The rats were fed a normal phosphate diet (to avoid any calcification that could have influenced the results) and received 4 injections of calcitriol 1, 3, 5, and 7 days before euthanization. No aortic calcification occurred in vivo.

Adenine-Induced Renal Failure in Mice
Initial studies were performed to determine the optimal dosing of adenine, which differed with age. Mice were like rats in that weight loss is a reliable method to follow the degree of uremia. All animals have an initial weight loss as a result of decreased food intake, presumably because of palatability. Animals that do not become uremic regain this weight within 2 weeks, whereas subsequent weight loss is dependent on the degree of uremia. The adenine content is reduced as necessary to keep the weight >75% of baseline, the threshold for euthanasia required by the Institutional Animal Care and Use Committee. A dietary content of 0.75%, which is routinely used in rats, resulted in 100% mortality in mice within 2 weeks, regardless of age. A reduction to 0.6% still resulted in 100% mortality in the younger mice, whereas most of the older mice survived but required a dose reduction in >50%. At 0.45% adenine, 70% to 80% of the younger mice survived and only 20% required a dose reduction; older mice did not develop uremia. At contents of ≤0.3%, mice did not develop uremia and did not develop aortic calcification, despite a high-phosphorus diet (2%) and high doses of calcitriol (1000 ng/kg).

Effect of Calcitriol on Vascular Calcification in Uremic Mice
Preliminary studies revealed that a 2% phosphorus diet was required to induce aortic calcification in uremic C57/B6 mice and, similar to rats, the calcification was variable. As shown in Figure 2, uremia resulted in a 32-fold increase in abdominal aortic calcium content compared with nonuremic mice fed a 2% phosphorus diet (P=0.0054). Treatment with 100 ng/kg calcitriol produced a further 8.5-fold increase in calcium content in uremic mice (P<0.027), whereas doses ≤1000 ng/kg did not increase abdominal aortic calcium content in nonuremic mice. The calcium content of the thoracic aorta of uremic mice was slightly less than half that in the abdominal aorta but similarly affected by calcitriol (not shown). Calcitriol increased plasma calcium but not phosphate or urea levels in uremic mice (Table).
survived the subsequent 2 or 3 months of renal failure. This did not differ between transplanted and nontransplanted mice. As shown in Figure 3, calcification in the VDR−/− allografts and adjacent VDR+/+ aorta did not differ visually after 3 months of uremia, a high-phosphorus diet, and calcitriol. Calcification was reduced at the anastamoses and these regions were omitted from quantitative analyses. Although calcium contents varied substantially between animals, there was no difference in calcium content between VDR−/− allografts and adjacent VDR+/+ aortas by paired analysis or between VDR−/− and VDR+/+ allografts (Figure 4C). Based on the results from the VDR+/+ to VDR−/− transplants, there was >90% chance of detecting a 3-fold or greater decrease in calcification in VDR−/− allografts. There was no difference between VDR+/+ allografts and adjacent VDR+/+ aorta, indicating that transplantation by itself did not alter calcification.

Histology was evaluated by hematoxylin and eosin staining, Verhoeff–Van Gieson staining (for elastin), and trichrome staining (for collagen) and showed no apparent differences between allografts and recipient aorta (Figure 4). Specifically, no cellular infiltrates, duplication or disruption of the elastic lamina, or excess deposition of collagen was observed in the allografts. The intima consisted of a single layer of endothelial cells in all cases and the cross-sectional area of the media was 0.0345±0.0022 mm² in the allografts compared with 0.0365±0.0006 mm² in the recipient aorta.

To be certain that calcitriol does not directly promote vascular calcification, these studies were repeated with a 10-fold higher dose of calcitriol (Figure 5). Aortic calcification was much greater at this dose but still did not differ between VDR−/− and VDR+/+ aortas either histologically or quantitatively. Histology is shown in Figure 6 but, because of the disruption by the calcification, assessment of any differences is difficult. Transplantation of VDR+/+ aortas into VDR−/− mice could not be performed because the VDR−/− mice are chronically ill and do not survive the surgery and subsequent uremia.

**Discussion**

This study demonstrates that the action of calcitriol to promote vascular calcification is independent of vascular VDRs and probably occurs through an indirect action on the vasculature. Although vascular smooth muscle contains VDRs, they do not play a role in promoting medial vascular calcification. Specifically, calcitriol did not increase expression of
genes related to calcification in aortas, either ex vivo or in vivo. Although not affected by calcitriol, expression of several of the genes was increased in aortas from uremic rats. The increased expression of tissue-nonspecific alkaline phosphatase and matrix gla protein has been reported previously from this laboratory and upregulation of Runx2 has been observed in other uremic rodent models. Despite the failure of calcitriol to alter expression of these genes in aorta, there was induction of CYP24A1, an enzyme that metabolizes calcitriol and is known to be regulated through the VDR. This is consistent with the previous demonstration of VDRs in vascular smooth muscle and indicates that calcitriol binds and activates VDRs in cultured aortas. The fact that calcitriol did not upregulate CYP24A1 in aorta in vivo can be explained by the much higher free levels of calcitriol obtained in the serum-free culture medium and suggests that clinically relevant doses of calcitriol do not activate responses downstream of the VDR in vascular smooth muscle. Treatment with calcitriol was reported previously to increase aortic expression of runx2 and osteocalcin in uremic rats, but this could be secondary to the concomitant calcification, which can directly upregulate runx2 in vascular smooth muscle cells. Cultured aortas do not calcify in response to calcitriol and, at the doses and duration used, calcitriol did not produce any calcification in vivo.

Calcification was not reduced in VDR−/− aortas that were transplanted into VDR+/+ mice subsequently rendered uremic and treated with calcitriol. This cannot be explained by an effect of the transplantation because VDR+/+ allografts showed the same degree of calcification as recipient VDR+/+ aorta. Furthermore, the allografts were histologically indistinguishable from recipient aorta with no overt evidence of rejection as indicated by the absence of cellular infiltrates or intimal changes even after 6 months. The absence of rejection is likely explained by transplantation between littermates of a highly inbred mouse strain. However, because of the limited histological analysis performed, a more subtle degree of rejection cannot be ruled out. The location and pattern of calcification also did not differ between allografts and recipient aorta. Because of the heterogeneity of aortic calcification, a small, partial effect of aortic VDRs cannot be excluded. Based on the effect of calcitriol in uremic VDR+/+ mice, an 8.5-fold decrease in calcification would be expected in VDR−/− allografts. Despite the heterogeneity, however, there was <10% chance of not detecting a 3-fold decrease in VDR−/− allografts. Thus, the results in aortic allografts conclusively demonstrate that VDRs in vascular smooth muscle do not have a major role in promoting vascular calcification.

Although the results do not exclude the possibility of a direct vascular action of calcitriol independent of VDRs, the lack of an effect on osteogenic gene expression in cultured rat aortas and the lack of effect on calcification of cultured aortas previously reported would argue against this. Calcitriol has been reported to increase calcification in vascular smooth muscle cells in culture, suggesting a direct effect on vascular calcification.

Figure 5. Calcification of vitamin D receptors-null (VDR−/−) and VDR+/+ aortas transplanted into uremic VDR+/+ mice treated with 1000 ng/kg calcitriol. Recipient mice were treated as described in Figure 4 but for 2 months. A, von Kossa stain of VDR−/− allograft. B, von Kossa stain of adjacent VDR+/+ aorta. C, von Kossa stain of VDR+/+ allograft. D, Quantification of calcium in allografts and adjacent recipient abdominal aortas. Lines connect corresponding allografts and recipient aortas. There was no difference between allografts and recipient aortas by paired analysis. Normal aortic calcium content is <10 nmol/mg.

Figure 4. Histology of recipient aorta (lefthand column) and allograft (righthand column) from a uremic mouse with mild aortic calcification. Duration of uremia was 3 months and the dose of calcitriol was 100 ng/kg. A, Hematoxylin and eosin stain. B, Verhoeff–van Gieson stain for elastin (black). C, Masson trichrome stain (collagen stains blue).
calcification. However, the results may not be relevant to medial calcification in vivo because these cells undergo substantial phenotypic changes in culture and lack a normal matrix, which is the site of calcification.

The wide variability in aortic calcification is a frequent finding in animal models, including the adenine model, and is a limitation to this study. However, vascular calcification is also highly variable in humans. Although this can obscure differences between experimental groups, the paired analysis in the transplantation studies obviates this. The fact that the variability in calcification was not observed between donor and recipient aorta and only existed between animals indicates that the variability is not innate to the vasculature but must be systemic in nature.

The findings in this study are limited to medial arterial calcification and do not address the separate process of neointimal calcification associated with atherosclerosis, which is also promoted by supraphysiological VDR activators in uremic mice. Thus, a direct role for VDRs in neointimal calcification cannot be ruled out. Although neointimal calcification is also increased in CKD, the increase in medial calcification is greater and, although medial calcification is clearly associated with poor outcomes, the significance of neointimal calcification, other than being a marker of atherosclerosis, is unclear because calcification may stabilize plaques. The results indicate an indirect action of calcitriol in promoting vascular calcification, unrelated to activation of VDRs in the smooth muscle. The mechanism by which this occurs is unclear but would seem to involve a systemic action. An increase in circulating calcium levels is a potential explanation because VDR agonists increase calcium absorption, and small increases in calcium concentration can markedly increase calcification of aortas in culture. Increases in plasma calcium occurred in this study but were not always statistically significant. The fact that plasma was obtained 48 hours after calcitriol administration and that total rather than ionized calcium was measured may have obscured an increase in plasma calcium. Calcitriol could also promote calcification by increasing circulating phosphate levels. Although this was observed in normal mice, there was no further increase in the hyperphosphatemia of uremic mice. An increase in circulating phosphate levels is not always observed with calcitriol and does not correlate well with vascular calcification in animal models. Further studies will be needed to determine whether medial vascular calcification is promoted by actions on systemic mineral metabolism or on other circulating factors.

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**References**

Calcitriol is known to promote vascular calcification but it is not clear whether this is mediated by its calcemic and phosphatemic actions or by direct effects on the vasculature. This study rules out a direct action on arteries to promote calcification, indicating that calcitriol and other vitamin D receptor agonists promote vascular calcification indirectly through a systemic action.
Materials and Methods

Aortic culture. Aortas were perfused with sterile saline and removed from normal rats under sterile conditions. After removal of the adventitia, the aortas were opened longitudinally, bisected, and placed in DMEM medium with 0.2 % bovine serum albumin with 100 nM calcitriol or vehicle alone. Medium was changed after 3 days and aortas were harvested after 6 days. A stock solution of calcitriol (1 ug/ml) was prepared by dissolving 10 ug of calcitriol (Sigma-Aldrich, St. Louis, MO) in ethanol and adding vehicle to a final volume of 10 ml. The vehicle contained 0.4 % polysorbate 20, 0.15 % NaCl, 1 % sodium ascorbate, 0.76 % Na₂HPO₄, 0.18 % NaH₂PO₄ and 0.11 % Na₂EDTA, pH 7.2.

Measurement of mRNA. Aortas were immediately frozen in liquid nitrogen and stored at -80 °C until processing. Total RNA was isolated from rats aorta using RNAzol® RT reagent (Molecular Research Center, INC; Cincinnati, OH). Following DNase treatment, reverse transcription was performed on 1 ug of RNA using Thermoscript RT-PCR kit (Invitrogen Carlsbad, CA) after preparation with a PCR/UV work station (Coy Laboratory Products, Grass Lake, MI). Primers were designed to cross intron-exon boundaries using Clone Manager Suite 7 software (Sci Ed Software, Durham, NC). The primer sequences of each mRNA are listed in Table 1.

Quantitative polymerase chain reaction (qPCR) was performed with IQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA) and with the following cycle parameters: 94°C for 2 minutes and 40 cycles at 94°C for 15 seconds, 55°C for 30 seconds, 72°C for 30 seconds with final extension at 72°C for 10 minutes. The Ct (threshold cycle) was defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. Expression of individual mRNAs was standardized to the rats 18S rRNA and calculated as the difference between the
threshold values of the two genes (2-Δct). Melting curve analyses were performed during real-time qPCR to verify the specificity of the reaction.

**Aortic transplantation.** Mice heterozygous for an inactivating mutation of the vitamin D receptor (VDR-/-) on a C57/B6 background were purchased from Jackson Laboratories (Bar Harbor, ME) and bred to obtain homozygous VDR-/- and VDR+/+ mice. Segments (4-5 mm) of infrarenal aortas were carefully dissected from donor animals aged 2-4 months and transplanted orthotopically into VDR+/+ littermates of the same sex after removal of a similar segment of aorta using 11-0 monofilament sutures for end-to-end anastomoses. Allografts and ends of recipient aortas were treated with heparin (200 units/ml) prior to anastomosis to prevent thrombosis. After 1-2 weeks, uremia was induced by adding 0.45% adenine to a powdered standard rodent diet for 2 months (1000 ng/kg calcitriol) or 3 months (100 ng/kg calcitriol). Adenine was decreased or discontinued prior to 2 months in some mice with rapid weight loss in order to reduce mortality; these. All mice that survived at least 2 months with or without adenine reduction were included in the analyses. NaCl (0.22 %) was added to the drinking water to counteract possible salt-wasting. Normal chow contains 0.4% available (non-phytate) phosphorus and this was increased to 2% by the addition of neutral phosphate to induce vascular calcification. For studies with high-dose calcitriol, mice were sacrificed after 2 months. For studies with low-dose calcitriol, mice were then fed standard chow with 2% phosphorus for another month prior to sacrifice to allow for sufficient aortic calcification. Calcitriol was diluted in phosphate-buffered saline and given subcutaneously three times per week beginning 2 weeks after initiation of the adenine diet and continued until sacrifice. Aortas were removed and separated into allografts and surrounding donor abdominal aorta after discarding aorta within 0.5mm of the suture lines. All animal protocols were approved by the Emory University
Biochemical assays. Aortas were dried, weighed, and extracted overnight in 1 M HCl, and calcium in the extract was measured colorimetrically by the cresolphthalein method \(^1\). Plasma urea was measured colorimetrically by the urease-glutamate dehydrogenase method (Sigma-Aldrich, St. Louis, MO), plasma phosphate was measured colorimetrically by the molybdate method \(^2\), and plasma calcium was measured by the cresolphthalein method. Genotyping in mice. DNA was extracted from tail clippings by standard methods and polymerase chain reaction was performed using the following primer sequences: CACGAGACTAGTGAGACGTG (VDR-/−), CTCCATCCCCCATGTGTCTTT (VDR+/+), and TTCTTCAGTGGCCAGCTCTT (common).

Histological analysis. Aortas were fixed with 10% formalin for 24 hours and embedded in OCT, and 5 micron sections were prepared. Staining was performed using standard clinical protocols.

Statistics. Data are presented as means ± standard errors. Differences between treatment groups were determined by t-testing or the Mann-Whitney U test for non-parametric data (aortic calcium content).

References


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