Vascular Calcification Induced by Chronic Kidney Disease Is Mediated by an Increase of 1α-Hydroxylase Expression in Vascular Smooth Muscle Cells

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ABSTRACT

Vascular calcification (VC) is a complication of chronic kidney disease that predicts morbidity and mortality. Uremic serum promotes VC, but the mechanism involved is unknown. A role for 1,25(OH)2D3 in VC has been proposed, but the mechanism is unclear because both low and high levels have been shown to increase it. In this work we investigate the role of 1,25(OH)2D3 produced in vascular smooth muscle cells (VSMCs) in VC. Rats with subtotal nephrectomy and kidney recipient patients showed increased arterial expression of 1α-hydroxylase in vivo. VSMCs exposed in vitro to serum obtained from uremic rats also showed increased 1α-hydroxylase expression. Those increases were parallel to an increase in VC. After 6 days with high phosphate media, VSMCs overexpressing 1α-hydroxylase show significantly higher calcium content and RUNX2 expression than control cells. 1α-hydroxylase null mice (KO) with subtotal nephrectomy and treated with calcitriol (400 ng/kg) for 2 weeks showed significantly lower levels of vascular calcium content, Alizarin red staining, and RUNX2 expression than wild-type (WT) littermates. Serum calcium, phosphorus, blood urea nitrogen (BUN), PTH, and 1,25(OH)2D3 levels were similar in both calcitriol-treated groups. In vitro, WT VSMCs treated with uremic serum also showed a significant increase in 1α-hydroxylase expression and higher calcification that was not observed in KO cells. We conclude that local activation of 1α-hydroxylase in the artery mediates VC observed in uremia. © 2016 American Society for Bone and Mineral Research.

KEY WORDS: CARDIOVASCULAR DISEASE; CHRONIC KIDNEY DISEASE; VITAMIN D; MINERAL METABOLISM; UREMIC TOXINS

Introduction

Cardiovascular mortality is the leading cause of death in chronic kidney disease (CKD) patients. These patients develop extensive vascular calcification (VC), a condition that has been linked to higher cardiovascular morbidity and mortality.1,2 VC increases vascular stiffness and reduces vascular compliance, which in turn increase systolic blood pressure, pulse pressure, and pulse wave velocity. All of these complications can lead to hypertension, heart failure, altered coronary perfusion, and left ventricular hypertrophy.3 In the past, VC was regarded as a passive process in which increased calcium and phosphate levels over its solubility threshold would induce calcium mineral deposition in soft tissues. However, recent data show that VC is a regulated process similar to bone formation in which calcification inducers and inhibitors have an active role.4 On the one hand, an increase of inducers of VC such as RANKL,5 RUNX2,6 osteonectin,7 osteocalcin,8 or BMP2 promote the osteoblastic phenotype of vascular smooth muscle cells (VSMCs).9 These osteoblastic markers are found to be elevated in the vasculature of both uremic rats and CKD patients.10 On the other hand a decrease of inhibitors of VC, such as matrix Gla protein (MGP), osteopontin, fetuin,11 and osteoprotegerin, is also found in CKD12 and could also play a role in the increased VC levels observed in uremia. Treatments with 1,25(OH)2D3 or other vitamin D receptor (VDR) agonists are used in CKD patients to reduce secondary hyperparathyroidism and prevent excessive bone resorption. Both low and high 1,25(OH)2D3 levels have been proven to enhance VC.13 An excess of 1,25(OH)2D3 is arteriotoxic and induces VC in humans and experimental animals,14,15 whereas vitamin D deficiency reduces the levels of calcification inhibitors and increases the inflammatory response, contributing to increased VC.13,16 Consequently, tight regulation of active vitamin D levels is important in maintaining a healthy vessel wall. Vitamin D3 is either consumed in the diet or produced by UVB photoconversion of 7-dehydrocholesterol in skin. Vitamin D3 is first metabolized to 25 hydroxyvitamin D3 in the liver and
subsequently to the active form 1,25(OH)₂D₃ by the enzyme 1α-hydroxylase (cyp27b1). 1α-Hydroxylase action is the limiting step in active vitamin D biosynthesis and its activity in the kidney is tightly regulated by 1,25(OH)₂D₃ by a negative feedback loop, and by other factors related to mineral metabolism such as PTH and FGF23. In addition to a regulation of its activation, the levels of 1,25(OH)₂D₃ are also affected by the rate of degradation. The metabolism is mediated by the enzyme 24-hydroxylase, which is responsible for the hydroxylation of 25(OH)D₃ and 1,25(OH)₂D₃ to obtain 24,25(OH)₂D₃ and 1,24,25(OH)₃D₃, respectively. 1α-Hydroxylase is mainly located in the kidney but it can be found in a wide number of extrarenal tissues where it is regulated independently from the renal enzyme. Therefore, the expression of 1α-hydroxylase has been detected in endothelial cells, VSMC, the parathyroid gland, cells from the colon mucosa, macrophages, and keratinocytes, raising numerous questions about the physiological role of local endogenous 1,25(OH)₂D₃ production. Thus, in the vasculature, Zehnder and colleagues postulated that the synthesis of 1,25(OH)₂D₃ by endothelial cells has a paracrine/autocrine function and acts at a local level regulating leukocyte adhesion. Furthermore, its activity seems to be regulated in a different way than the renal isoform. For instance, production of active vitamin D is upregulated by proinflammatory cytokines in endothelial cells or in trophoblasts. In addition, some data point to a deregulation of extrarenal vitamin D metabolism in several conditions such as pancreatic disease, human granuloma-forming disease, cancer, or even uremia. Thus, in chronic renal failure, peripheral macrophages exhibited an enhanced 1α-hydroxylase activity and a decreased capacity to degrade 1,25(OH)₂D₃.

The fact that VSMCs express all the proteins involved in 1,25(OH)₂D₃ metabolism (VDR, 1α-hydroxylase, and 24-hydroxylase) and the U-shaped relationship of active vitamin D levels and VC led us to analyze the role of locally activated vitamin D in VC. In the present work, we analyze the role of the local synthesis of calcitriol on uremia-induced VC.

Materials and Methods

In vivo study

All the animal studies performed followed the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Ethics Committees in accordance with the guidelines of European Research Council for the care and use of laboratory animals. In all surgical procedures performed in animals, isoflurane was used as anesthetic and buprenorphine was used as analgesic after the surgery.

Studies involving human samples were performed in agreement with the Declaration of Helsinki and with local and national laws. The Human Ethics Committee of the Hospital Universitario Central de Asturias approved the study procedures, and all participants signed an informed consent before inclusion in the study, providing permission for their medical data to be anonymously used for research.

Human epigastric artery samples

Part of this study comprised 9 patients with CKD who showed clinical calcification and 10 kidney donors with normal renal function nonsmokers and free from diabetes and cardiovascular disease. Both arterial samples were obtained during the transplantation procedure (in the case of the CKD patients) or in the organ extraction process after signing informed consent.

Model of CKD in rats

Sprague-Dawley rats (200 to 225 g) underwent 5/6 nephrectomy as described. Rats were anesthetized and right nephrectomy was performed. After 1 week, animals were subjected to 2/3 nephrectomy of the left kidney by ligation of the renal parenchyma in both renal poles. Another group of sham-operated rats were used as control. At euthanasia, 8 weeks later, abdominal aortas were collected and processed to isolate RNA, protein, and to determine calcium content. Serum from sham and 5/6 nephrectomy rats was collected and processed to be used in the in vitro experiments.

Generation and characterization of CYP27B1⁻/⁻ mice

1α-Hydroxylase KO mice (CYP27B1⁻/⁻) were provided by Dr. David Goltzman (Montreal, Canada) and were generated by ablation of exon 6 to exon 9. These mice were bred to C57/BL6 wild-type (WT) animals and the heterozygous offspring were crossed to produce CYP27B1⁻/⁻ and CYP27B1⁺/⁻ animals. CYP27B1⁻/⁻ mice were fed a rescue diet; high Calcium diet (2% calcium, 1.25% phosphorus, 20% lactose, and 2.2 IU/g vitamin D3; TD.96348; Harlan Teklad, Cambridgeshire, United Kingdom) during growth and maintenance. Before starting the experimental process the diet was changed to a standard mouse chow (0.6% Ca²⁺, 0.8% phosphorus, and 0.6 IU/g vitamin D3; Harlan Teklad) to avoid interference of the diet with the different treatments.

Model of CKD in mice

Subtotal nephrectomy (STN) was induced in 10-week-old to 12-week-old mice after the two-step surgical procedure for 75% nephron reduction. Briefly, the parenchyma of the left kidney was reduced by 50%. The kidney was exposed, decapsulated, and carefully cauterized, reducing the parenchyma of the upper and the lower pole. After 1 week of recovery period, right-sided total nephrectomy was performed. Treatments started 1 week after nephrectomy to facilitate the recovery after the operation. A group of sham-operated mice was used as control in the present study (CYP27B1⁺/⁻, n = 7; CYP27B1⁻/⁻, n = 6).

STN CYP27B1⁺/⁻ and CYP27B1⁻/⁻ mice were treated daily with calcitriol (400 ng/kg) during 15 days (each group, n = 8). At euthanasia, blood was collected and aortas were divided in two parts, one frozen in liquid nitrogen for calcium content determination and the other one fixed in formalin solution followed by processing and paraffin embedding.

Serum biochemical analysis

Blood was collected by cardiac puncture and centrifuged at 700 g for 10 min at 4°C to obtain serum.

Ca²⁺ and P were analyzed by a standard colorimetric analysis in the Biochemistry service of the Arnau de Vilanova Hospital (HUAV) in Lleida. Blood urea nitrogen (BUN) was determined by colorimetric assay, using the QuantiChrom Urea assay kit (DIUR-500, Gentaur, San Jose, CA, USA). Immunoassays were used to determine 1,25-dihydroxy vitamin D (1,25-dihydroxy vitamin D EIA; Immunodiagnostic Systems, The Boldons, UK), and also PTH (PTH mouse ELISA kit; Immutopics, San Clemente, CA, USA).
Quantitative analysis of aortic calcium

Aortic tissue was desiccated for 20 to 24 hours at 60°C, crushed to a powder with a pestle and mortar, and decalciﬁed with HCl (1N) at 4°C, and then vortexed for 16 hours. After centrifugation, superna-
tant was collected and calcium content determined colorimetri-
cally using the o-cresolphthalein complexone method, whereas
protein content was determined by the Lowry method (Bio-Rad,
Hercules, CA, USA). Aortic calcium content was normalized by the
protein amount in the sample and expressed as nanograms of
calcium per milligrams of protein (ng Ca/mg of protein).

Histology and immunohistochemistry

Immunostaining for CYP27B1 (Cloud-Clone Corp, TX, USA) and RUNX2 (NBP1-01004; Novus Biologicals, Littleton, CO, USA) were carried out on 5-μm-thick tissue sections of human epigastric arteries and mice aortas, respectively. Tissue sections were deparaffinized through xylene and rehydrated through graded ethanol concentrations into distilled water, as described.[34] Shortly, antigen retrieval was done by boiling the slides in 10 mM citrate buffer (pH 6) for 10 min. Endogenous peroxidase
quenching (30 min incubation in 0.66% (vol/vol) H2O2/PBS) was
followed by blocking of nonspeciﬁc binding with normal horse
blocking serum (Vector Laboratories) for 30 min at room
temperature (RT). Anti-rabbit CYP27B1 (1/100) or RUNX2 antibody
(1/50) were incubated overnight at 4°C. After washing with PBS,
slides were treated with corresponding biotinylated secondary
antibody (30 min, RT), which was followed by the avidin-biotin-
peroxidase complex (30 min, RT) and 3,3′-diaminobenzidine
(DAB) as a chromogen (10 min, RT) (Vector Laboratories,
Burlingame, CA, USA). Sections were counterstained with Mayer’s
Hematoxylin to visualize the nuclei. Negative controls were
performed by incubation with nonimmune serum in place of a
speciﬁc antibody, which resulted in a complete absence of
staining. Stained tissue sections were examined using a Nikon
Eclipse 80i microscope with a Nikon automatic camera system.
Immunohistochemical results of CYP27B1 staining were evalu-
atated following the uniform pre-established criteria. Staining
intensity and percentage of positive cells were graded semiquan-
titatively. Histological scores were obtained from each sample as
follows: histoscore = 1 × (% light staining) + 2 × (% moderate
staining) + 3 × (% strong staining), which ranged from 0 (no
immunoreaction) to 300 (maximum immunoreactivity). The

Fig. 1. Uremia deregulates the expression of VDR, 1α-hydroxylase, and 24 hydroxylase, and induces calcification in rat artery in vivo. (A) Levels of
creatinine, calcium, phosphate, and PTH in serum from control and uremic rats. (B) Aortic calcium content in control and uremic rats. (C) Alizarin red
staining in arteries from control and uremic rats. (D) Expression of VDR, 1α-hydroxylase, and 24-hydroxylase determined by qPCR in artery from control
rats (black bars), and uremic rats (gray bars). (E) Protein expression of VDR, RUNX2, 24-hydroxylase, and 1α-hydroxylase determined by Western blot in
the artery from control and uremic rats. GAPDH was used as a loading control. (F) Quantitative analysis of Western blots. Data are mean ± SE (n = 6).
*p < 0.05 versus control (control rat). Scale bar = 100 μm.
reliability of such scores for interpretation of immunohistochemical staining of tissue sections has been shown. For calcium staining in aortic sections, samples were deparaffinized, rehydrated, and stained in 2% Alizarin red solution (Sigma A3757, Sigma Aldrich, Saint Louis, MO, USA) at pH between 4.1 and 4.3 for 5 min. After staining, samples were rehydrated with acetone, acetone-xylene (1:1), xylene and mounted in synthetic mounting medium (DPX; Sigma Aldrich).

**In vitro study**

**VSMC cultures from rat and mice (CYP27B1<sup>−/−</sup> and CYP27B1<sup>+/+</sup>)**

Primary rat and mouse aortic VSMCs were obtained as described and maintained in DMEM (GIBCO, Termo Fisher Scientific, Waltham, MA, USA) containing 10% FBS. Cells were plated (1 × 10<sup>5</sup> cells/plate) in 100-mm plates. When the cell confluence was about 80% VSMCs were shifted to treatment media, DMEM containing 15% serum from normal rats or 15% serum from 5/6 nephrectomized rats, 10 mmol/L sodium pyruvate, 10 mmol/L β-glycerophosphate (Sigma Aldrich). Final concentration of calcium was adjusted to 4 mM.

VSMCs between passages 2 and 8 were used in all the experiments. Every experiment was performed in triplicate, with three replicates per condition.

**Determination of VSMC calcification**

In all the calcification experiments, calcium levels were measured 6 days after the addition of the treatments (control serum and uremic serum). Cells were collected with PBS, centrifuged, and the pellet was incubated with 50 µL of HCL 0.6N in vortex at 4°C. After centrifugation, supernatant was used for calcium quantification by the o-cresolphthalein complexone method and the pellet was used to quantify the protein content.

For the Alizarin red staining, cells incubated in calcification media were washed with PBS two times and then fixed with formaldehyde at RT for 15 min. After fixation cells were washed again and incubated with 40 mM Alizarin red solution at pH between 4.1 and 4.3 for 20 min.

**Determination of calcitriol production (1α-hydroxylase activity)**

After treatments or 1α-hydroxylase overexpression, cell monolayers were washed six times with PBS and incubated with DMEM with or without 500 ng/mL of 25(OH)D<sub>3</sub>. After 1 hour, incubation media were collected and after sample delipidation with dextran sulfate and magnesium chloride, 100 µL of sample were run through a solid phase column coated with monoclonal antibodies against 1,25(OH)<sub>2</sub>D<sub>3</sub>. Afterward, the column was eluted with ethanol and the collected fraction was evaporated under nitrogen atmosphere and resuspended in assay buffer. Levels of 1,25(OH)<sub>2</sub>D<sub>3</sub> were then assessed by ELISA (Immuno-diagnostics Systems). The production was calculated as follows: 1,25(OH)<sub>2</sub>D<sub>3</sub> levels in the wells with 25(OH)D<sub>3</sub> minus 1,25(OH)<sub>2</sub>D<sub>3</sub> levels in wells without 25(OH)D<sub>3</sub>. Serum samples were processed in the same way. The percentage of recovery, as reported by the manufacturer, was 96%.

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**Fig. 2.** CKD patients with vascular calcification show an increase in arterial expression of 1α-hydroxylase. (A) Vascular calcium content form epigastric arteries obtained from CKD patients undergoing a renal transplant and from non-CKD kidney donors. (B) Immunohistochemistry for 1α-hydroxylase in the same arteries. (C) Quantification of the 1α-hydroxylase staining by histoscore. *p < 0.01.
Real-time PCR

Total cellular RNA from VSMCs and from rat aortic tissue samples was extracted with TRIzol reagent (Sigma Aldrich), following the manufacturer's instructions. The final RNA concentration was determined by Nanodrop (ND-100) spectrophotometer. Reverse transcription and real-time PCR were performed as described.\(^{(36)}\) Briefly, real-time PCR with gene-specific TaqMan probes for rat and mouse VDR, 1α-hydroxylase, 24-hydroxylase, RUNX2, and GAPDH as an endogenous control (Applied Biosystems, Foster City, CA, USA) was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using TaqMan Universal PCR Master Mix. Forty cycles at 95°C for 15 sec and 60°C for 1 min were performed. Every experiment was carried out three times and all samples were amplified in triplicate.

The relative RNA amount was calculated by standard formulas and expressed as fold induction over controls. Average and standard error from three experiments performed in triplicate were calculated.

Western blot analysis

Total cell lysates were obtained by washing the cell monolayer with cold PBS, scraping and suspending in lysis buffer (125 mM Tris, pH 6.8, 2% SDS, 2 mM phenylmethylsulfonyl fluoride [PMSF], and protease inhibitor cocktail). Samples were sonicated and the supernatant was collected after centrifugation for 10 min at 1100 g, 4°C. Aortic tissue was homogenized in lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 150 mM NaCl, 1 mM DTT, 1 μM PMSF, 1 mM Na₃VO₄, and protease inhibitor cocktail) using the TissueLyser LT (Qiagen, Hilden, Germany): four cycles of homogenization (50 Hz for 2 min each cycle) followed by three cycles in liquid nitrogen and thawing at 37°C. The supernatant was collected after centrifugation for 10 min at 1100 g, 4°C. Protein concentration was determined using a DC protein assay kit (Bio-Rad, Manassas, VA, USA). Twenty-five micrograms (25 μg) of proteins were electrophoresed on 10% SDS-PAGE gels, and transferred to PVDF membranes (Immobilon-P, Millipore, Billerica, MA, USA). Membranes were
probed with primary antibodies against VDR (1:1000; Santa Cruz, CA, USA), CYP24 (1:1000; Santa Cruz), CYP27B1 (1:1000; Santa Cruz), RUNX2 (1:1000; Novus Biologicals), anti-V5 epitope (1:1000; Sigma), GAPDH (1:5000; Sigma), α-tubulin (1:5000; Sigma), and α-actin (1:500; Santa Cruz) over night at 4°C. Corresponding peroxidase-conjugated secondary antibody: rabbit IgG+HRP (1:10000; Cell Signaling, Danvers, MA, USA), mouse IgG+HRP (1:10000; Jackson Immunoresearch, West Grove, PA, USA), or goat IgG+HRP (1:10000; Santa Cruz) were incubated for 1 hour at RT. The immunoreaction was visualized using chemiluminescent kits EZ ECL (Biological Industries, Kibbutz Beit Haemek, Israel) or ECL Advanced (Amersham Biosciences, Little Chalfont, UK). Images were digitally acquired by VersaDoc Imaging system Model 4000 (Bio-Rad). Positive immunoreactive bands were quantified by densitometry and compared with the expression of adequate loading control.

**Overexpression of CYP27B1 in rat VSMC**

The 1α-hydroxylase gene (CYP27B1) was subcloned via gateway technology into the lentiviral plasmid pDSL (ATCC, Manassas, VA, USA). Production of lentiviral particles was done as described. Empty FSV vector (EV) was used as a control of transfection. VSMCs from rat between passages 3 and 5 were used for the experiment.

**Statistical analysis**

Differences between groups were assessed by Student’s t test or by one-way ANOVA followed by Scheffe’s test. Values of p < 0.05 was considered statistically significant. All data examined are expressed as mean ± SE.

**Results**

Uremia deregulates the expression of VDR, 1α-hydroxylase, and 24-hydroxylase and induces calcification in rat artery in vivo

To study the effect of uremia in vivo, rats were subjected to 5/6 nephron reduction and euthanized at week 8. Uremic rats displayed an increase in creatinine, calcium and PTH serum levels (Fig. 1A) compared with controls. They also demonstrated
a decrease in phosphate levels, indicating they were in the early stages of CKD. The analysis of VC by calcium quantification showed higher levels of calcium content in the arteries of uremic rats compared with the control rats (Fig. 1B) and an increase in Alizarin red staining (Fig. 1C). Figure 1D–F shows the expression of VDR, 1α-hydroxylase, and 24-hydroxylase in the rat artery. Uremia significantly increased 1α-hydroxylase and 24-hydroxylase expression at mRNA (Fig. 1D) and protein level (Fig. 1E, F; data not shown for Runx2 qPCR).

Uremic patients with VC show increased immunostaining for 1α-hydroxylase in epigastric arteries

To confirm whether an increase in VC was associated with an increase in the expression of arterial 1α-hydroxylase in patients, epigastric arteries were obtained from uremic patients subjected to a kidney transplant and from kidney donors. VC was confirmed by an increase in the calcium levels in the epigastric arteries of uremic patients (Fig. 2A). In parallel, immunostaining for 1α-hydroxylase was higher in arteries obtained from CKD patients than in arteries obtained from non-CKD donors (Fig. 2B, C).

Uremic serum deregulates the expression of VDR, 1α-hydroxylase, and 24-hydroxylase, and induces calcification in rat VSMC

To examine the effect of uremia in vitro, rat VSMCs were incubated for 6 days in high-phosphate media supplemented with serum obtained from normal rats (control serum) or from rats subjected to 5/6 nephrectomy maintained on a normal diet for 4 months (uremic serum). Uremic serum significantly increased the expression of VDR and 1α-hydroxylase mRNA (Fig. 3A) in VSMCs, while 24-hydroxylase expression was not modified. Figure 3B shows the Western blot images of the same samples. The quantification in Fig. 3C showed that levels of Runx2 increased (although the increase was not significant) whereas levels of 1α-hydroxylase, 24-hydroxylase, and VDR increased significantly. The incubation of VSMCs with high P and uremic serum induced a dose-dependent increase in calcification as shown in Fig. 3E. Alizarin red staining was also increased.

![Figure 5](image-url) Subtotally nephrectomized (STN) CYP27B1+/+ and CYP27B1−/− mice treated with calcitriol have similar serum levels of Ca, P, PTH and 1,25(OH)2D3. Serum levels of (A) blood urea nitrogen (BUN), (B) calcium, (C) phosphate, (D) PTH, and (E) 1,25(OH)2D3 in STN CYP27B1+/+ and STN CYP27B1−/− mice (black bars) and STN mice treated with calcitriol (gray bars). Data are reported as means ± SE (n = 8). *p < 0.05 between STN and STN mice treated with calcitriol, #p < 0.05 between CYP27B1+/+ and CYP27B1−/− mice.
Furthermore, production of calcitriol was increased in cells treated with uremic serum compared with cells treated with control serum (Fig. 3F).

1α-Hydroxylase has a direct effect on calcification in rat VSMCs

To study the role of 1α-hydroxylase in VC, we stably overexpressed 1α-hydroxylase in rat VSMCs (pDSL 1α-hydroxylase). An empty vector (EV) served as a control. Figure 4 shows an evident increase of 1α-hydroxylase protein fused with the epitope V5 (Fig. 4A), as well as of 1α-hydroxylase mRNA (Fig. 4B) in rat VSMCs. VSMCs overexpressing 1α-hydroxylase showed significantly elevated production of 1,25(OH)2D3 (Fig. 4C), alongside markedly higher intracellular calcium content (Fig. 4D) and Alizarin red staining (Fig. 4E) compared with the control cells (EV). Furthermore, VSMCs overexpressing 1α-hydroxylase showed an evident upregulation of RUNX2 protein expression compared with control group (Fig. 4F).

STN CYP27B1+/+ and CYP27B1−/− mice treated with calcitriol have similar serum levels of Ca, P, PTH, and 1,25(OH)2D3

To further evaluate the role of locally produced calcitriol in vivo, we used a CYP27B1−/− mouse model with CKD. CYP27B1+/+ and CYP27B1−/− mice were subjected to subtotal nephrectomy and treated with calcitriol (400 ng/kg) to equilibrate serum Ca, P, PTH, and 1,25(OH)2D3, and to induce VC. Results showed similar levels of BUN in CYP27B1+/+ and CYP27B1−/− mice (Fig. 5A), suggesting a similar degree of renal impairment. Calcium levels (Fig. 5B), generally lower in CYP27B1−/− mice, increased with calcitriol treatment reaching similar levels as CYP27B1+/+ calcitriol-treated mice. Phosphate levels, which were comparable in both untreated mice groups, also increased to a similar degree when animals were treated with calcitriol (Fig. 5C). Levels of PTH, which are very high in CYP27B1−/− mice, decreased in both groups of animals treated with calcitriol reaching similar values (Fig. 5D). 1,25(OH)2D3 levels, generally undetectable in CYP27B1−/− mice, raised to similar levels in both treated groups (Fig. 5E).

STN CYP27B1+/+ mice treated with calcitriol present more severe VC than STN CYP27B1−/− mice treated with calcitriol

CYP27B1+/+ mice subjected to subtotal nephrectomy and treated with calcitriol showed significant increase of arterial calcium content compared with STN CYP27B1−/− mice that underwent the same treatment (Fig. 6A). Alizarin red staining (Fig. 6B) showed an increase in calcium deposits in the artery of
STN CYP27B1<sup>+/+</sup> mice treated with calcitriol mice, which was not observed in CYP27B1<sup>−/−</sup> animals. Furthermore, immunoreactivity for RUNX2, an essential regulator of VSMC calcification, was higher in the arteries of STN CYP27B1<sup>+/+</sup> mice treated with calcitriol mice (Fig. 6C) compared with STN CYP27B1<sup>−/−</sup> mice treated with calcitriol.

Uremic serum does not affect the expression of RUNX2, VDR, 1α-hydroxylase, and 24-hydroxylase and does not induce VC in CYP27B1<sup>−/−</sup> VSMCs.

Representative Western blots of RUNX2, VDR, 1α-hydroxylase, and 24-hydroxylase in VSMCs from CYP27B1<sup>+/+</sup> and CYP27B1<sup>−/−</sup> mice treated with high phosphate media and control or uremic serum are shown in Fig. 7A. Figure 7B shows quantification of the Western blots. CYP27B1<sup>+/+</sup> VSMCs treated with uremic serum showed an increased expression of RUNX2 (although the quantification did not reach statistical significance Fig. 7B), but was not modified in the VSMCs from CYP27B1<sup>−/−</sup> mice. VDR, 1α-hydroxylase, and 24-hydroxylase showed a significant increase in CYP27B1<sup>+/+</sup> VSMCs, whereas in CYP27B1<sup>−/−</sup> VSMCs the treatment with uremic serum did not affect the expression of these proteins. Figure 7C shows data of mRNA levels obtained from real-time PCR. Expression of RUNX2, 1α-hydroxylase, and 24-hydroxylase significantly increased, whereas the expression of VDR, in contrast to the protein levels, decreased in CYP27B1<sup>+/+</sup> VSMCs incubated with uremic serum (Fig. 7C). However, in VSMCs from CYP27B1<sup>−/−</sup> mice, only the expression of 24-hydroxylase was slightly but significantly upregulated. Calcium levels were significantly elevated in CYP27B1<sup>+/+</sup> VSMCs treated with uremic serum whereas calcification did not increase in VSMCs from CYP27B1<sup>−/−</sup> mice (Fig. 7D, E).

**Discussion**

In this study, we show that an increase of the local expression of 1α-hydroxylase in VSMCs is a mediator of the increase in VC observed in CKD. Thus, both in vitro and in vivo (in experimental animals and in patients), uremia-induced VC paralleled an
increase in 1α-hydroxylase expression. Furthermore, in the absence of 1α-hydroxylase, the increase of calcification induced by uremia was blunted and the overexpression of 1α-hydroxylase was enough to induce VC in VSMC.

1α-hydroxylase is expressed in the kidney and also in other tissues and cell types including VSMC, intestine (epithelial intestinal cells), lymphatic ganglia, prostate, spleen, monocyctic cells, macrophages, dendritic cells, parathyroid cells, β-pancreatic cells, and osteoblasts. In those other cell types, regulation of its expression seems to be independent of the conventional regulators that affect its expression in tubular cells. Thus, Adams and colleagues have shown that macrophage 1α-hydroxylase expression in sarcoidosis is driven by proinflammatory cytokines. In that condition the increased activity of 1α-hydroxylase would increase 1,25(OH)₂D₃ levels to supranormal concentrations. Furthermore, endothelial cells also show an upregulation of 1α-hydroxylase levels by proinflammatory cytokines. In placental tissue, the production of 1,25(OH)₂D₃ is upregulated by IGF-I and in many cancer types, the expression of 1α-hydroxylase is either upregulated or downregulated depending, in part, on the stage of progression of the cancer. Therefore, it seems clear that regulation of extrarenal 1α-hydroxylase expression is tissue-dependent and is modified in many different conditions.

CKD is a condition in which systemic production of 1,25(OH)₂D₃ is altered. Thus, there is a decrease in circulating levels of 1,25(OH)₂D₃ due to a reduction of renal 1α-hydroxylase activity, an increase of 1,25(OH)₂D₃ degradation, and impaired uptake in tubular cells of 25(OH)D₃, the substrate of 1α-hydroxylase. However, macrophages from uremic patients show an increased, rather than decreased, 1α-hydroxylase activity. Our results show that vascular smooth muscle cells show similar regulation. Thus both, in vitro and in vivo, the uremic milieu increases the levels of 1α-hydroxylase in VSMCs. The uremic factors that are involved in this upregulation of 1α-hydroxylase are unknown, but increased PTH levels or inflammatory cytokines could be playing a role.

Whether this increase of 1α-hydroxylase levels translate into an increase of intracellular 1,25(OH)₂D₃ in vivo is unknown, and current techniques to determine those levels lack sufficient sensitivity. However, our in vitro data show that VSMCs incubated in uremic media produce higher amounts of 1,25(OH)₂D₃ than cells incubated in normal media. In vivo data indirectly show an increase in VDR activation because both VDR and 24-hydroxylation protein levels are upregulated, suggesting that the increase in 1α-hydroxylase levels activates VDR locally. The increase in VDR activation should directly downregulate 1α-hydroxylase expression. However, in macrophages the synthesis of 1,25(OH)₂D₃ is not self-inhibited, and this appears to be the basis for the unregulated 1α-hydroxylase activity, showing that extra renal regulation of 1α-hydroxylase could be less sensitive to autoregulation by 1,25(OH)₂D₃. Therefore, the results point to an overactivation of VDR signaling in uremia, which has been shown to induce VC. Indeed, both in vivo and in vitro, supraphysiological levels of 1,25(OH)₂D₃ induce expression of osteoblastic genes such as RUNX2 and VC. In our experiments, uremic conditions induced increases in RUNX2 expression and VC in vivo and in vitro.

Our results also show that the increase in 1α-hydroxylase is necessary and sufficient to induce VC. Thus, overexpression of 1α-hydroxylase in VSMCs increased both RUNX2 expression and VC. Furthermore, the elimination of 1α-hydroxylase totally inhibited the VC in vitro. Indeed, VSMCs obtained from 1α-hydroxylase KO animals and incubated in calcification media showed no increase in RUNX2, calcium content, or Alizarin red staining, showing that the presence of 1α-hydroxylase is necessary for uremia-induced VC. The in vitro data also show some puzzling results. Thus, CYP27B1⁻/⁻ mice show basal protein levels of RunX2 lower than CYP27B1⁺/⁺ but similar mRNA expression levels. This could be explained by a regulation of RunX2 levels by activated VDR at the posttranscriptional or even the posttranslational level. This fact has been shown in the regulation of several proteins. Thus, absence of activated VDR in CYP27B1⁻/⁻ could affect RunX2 protein levels without altering gene expression. Furthermore, another discrepancy is observed in the effect of uremic in VDR levels in CYP27B1⁺/⁺ cells. In this case, uremic serum increases VDR protein levels but decreases mRNA at steady state. Uremic serum has been shown in our study to increase 1,25(OH)₂D₃ in our cells, therefore increasing the activation of VDR and, thus, inhibiting its degradation and explaining the higher levels of protein.

The decrease in mRNA levels could be explained by the existence of uremic toxins in serum that have been shown to inhibit the binding of activated VDR to certain vitamin D–responsive elements in the DNA, and therefore, decreasing the levels of transcription of some VDR-activated genes. In vivo results agree with the in vitro data. In our model of VC induced by STN, CYP27B1⁻/⁻ animals treated with toxic doses of calcitriol showed significantly lower levels of VC and RUNX2 expression, compared to CYP27B1⁺/⁺ littersmates, although calcium and phosphate levels were similar. The inhibition of calcification was not complete, because CYP27B1⁻/⁻ animals still showed an increase of VC after treatment with calcitriol and a tendency (although not statistically significant) was seen after 5/6 nephrectomy in the same animals. This can be explained by the multifactorial pathophysiology of VC. Thus, one significant pathophysiologic component seems to be related to the increases in 1α-hydroxylase activity and another part is probably related to the increases in blood Ca and P, which are also very high in the CYP27B1⁻/⁻ animals treated with calcitriol. Furthermore, the dose of calcitriol used was able to increase the blood concentrations of 1,25(OH)₂D₃ to similar levels in both groups, so differences in circulating levels of the hormone can be ruled out as the cause of this effect.

In conclusion, our results point to a paramount role of local expression of 1α-hydroxylase in VSMCs in regulating uremia-induced VC. These results could be of importance to identify new treatments to reduce VC in CKD patients.

**Disclosures**

All authors state that they have no conflicts of interest.

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