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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Running title: Local activation of vitamin D and vascular calcification

DISCLOSURE PAGE

Nothing to disclose

ABSTRACT

Vascular calcification (VC) is a complication of chronic kidney disease which predicts morbidity and mortality. Uremic serum promotes VC, but the mechanism involved is unknown. A role for $1,25(OH)_2D_3$ on VC has been proposed, but the mechanism is unclear because both, low and high levels have been shown to increase it. In this paper we investigate the role of $1,25(OH)_2D_3$ produced in vascular smooth muscle cells (VSMCs) on 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 two weeks showed significantly lower levels of vascular calcium content, alizarin red staining and RUNX2 expression than wild type (WT) littermates. Serum calcium, phosphorus, BUN, PTH and $1,25(OH)_2D_3$ 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.

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, a condition which has been linked to higher cardiovascular morbidity and mortality.[1,2] Vascular calcification (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, vascular calcification 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 demonstrate that VC is a regulated process similar to bone formation where 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 VSMC.[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 vascular calcification, such as MGP, osteopontin, fetuin[11] and osteoprotegerin, is also found in CKD[12] and could also play a role in the increased vascular calcification levels observed in uremia.

Treatments with $1,25(OH)_2D_3$ or other VDR agonists are used in CKD patients in order to reduce secondary hyperparathyroidism and prevent excessive bone resorption. Both low and high $1,25(OH)_2D_3$ levels have been proven to enhance VC.[13] An excess of $1,25(OH)_2D_3$ is arteriotoxic and induces vascular calcifications in humans and experimental animals,[14,15] while 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 D_3 is either consumed in the diet or produced by UVB photoconversion of 7-dehydrocholesterol in skin. Vitamin D_3 is first metabolized to 25 hydroxyvitaminD_3 in the liver and subsequently to the active form $1,25(OH)_2D_3$ by the enzyme 1α-hydroxylase (cyp27b1).[17] 1α-hydroxylase action is the limiting step in active vitamin D biosynthesis and its activity in the kidney is tightly regulated by $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ are also affected by the rate of degradation. The metabolism is mediated by the enzyme 24-hydroxylase, which is responsible for the hydroxylation of $25(OH)D_3$ and $1,25(OH)_2D_3$ to obtain $24,25(OH)_2D_3$ and $1,24,25(OH)_2D_3$, respectively.[18] 1α-hydroxylase is mainly located in the kidney but it can be found in a wide number of extra-renal tissues where it is regulated independently from the renal enzyme.[19] Thus, the expression of 1α-hydroxylase has been detected in endothelial cells,[20] VSMC,[21,22] parathyroid gland,[23] cells from the colon mucosa,[24] macrophages[25] and in keratinocytes[26] raising numerous questions about the physiological role of local endogenous $1,25(OH)_2D_3$ production. Thus, in the vasculature, Zehnder et al.[20] postulated that the synthesis of $1,25(OH)_2D_3$ 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[20] or in trophoblasts.[27] In addition, some data point to a deregulation of extrarenal vitamin D metabolism in several conditions such as pancreatic disease,[28] human granuloma-forming disease,[29] cancer[30] or even uremia. Thus, in chronic renal failure, peripheral macrophages exhibited an enhanced 1α-hydroxylase activity and a decreased capacity to degrade $1,25(OH)_2D_3$.[31]

The fact that VSMC express all the proteins involved in $1,25(OH)_2D_3$ metabolism (VDR, 1-αhydroxylase and 24-hydroxylase) and the U-shaped relationship of active vitamin D levels and vascular calcification led us to analyze the role of locally activated vitamin D in vascular calcification. In the present work, we analyze the role of the local synthesis of calcitriol on uremia-induced vascular calcification.

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 bruprenorphine 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 chronic kidney disease who showed clinical calcification and 10 kidney donors with normal renal function non-smokers 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 (200-225g) underwent 5/6 nephrectomy as previously described.[32] 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 (n=8). At sacrifice, 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.[33] These mice were bred to C57/BL6 wild type 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, Harlan Teklad, TD.96348) during growth and maintenance. Before starting the experimental process the diet was changed to a standard mouse chow (0.6% Ca $^{2+}$, 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 10-12-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 one 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).

Subtotally nephrectomized CYP27B1+/+ and CYP27B1-/- were treated daily with calcitriol (400ng/kg) during 15 days (each group, n=8). At sacrifice, 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 2500 rpm for 10 minutes at 4ºC to obtain serum.

Ca^{2+} and P were analyzed by a standard colorimetric analysis in the Biochemistry service of the Arnau de Vilanova Hospital (HUAV) in Lleida. BUN was determined by colorimetric assay, using the QuantiChrom Urea assay kit (DIUR-500). Immnunoassays were used to determine 1,25-DihydroxyvitaminD (1,25-dihydroxy VitaminD EIA, IDS), and also PTH (PTH mouse ELISA kit, Immunotopics).

Quantitative analysis of aortic calcium

Aortic tissue was desiccated for 20-24 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, supernatant was collected and calcium content determined

colorimetrically using the o-cresolphthalein complexone method, while protein content was determined by the Lowry method (Bio-Rad). Aortic calcium content was normalized by the protein amount in the sample and expressed as ng Ca/ mg of protein.

Histology and immunohistochemistry

Immunostaining for CYP27B1 (Cloud-Clone Corp, TX, USA) and RUNX2 (NBP1-01004, Novus Biologicals) were carried out on 5-µm thick tissue sections of human epigastric arteries or mice aortas respectively. Tissue sections were deparaffinized through xylene and rehydrated through graded ethanol concentrations into distilled water, as previously described.[34] Shortly, antigen retrieval was done by boiling the slides in 10 mM citrate buffer (pH 6) for 10 minutes. Endogenous peroxidase quenching (30 minutes incubation in 0.66% (v/v) H_2O_2/PBS)) was followed by blocking of non-specific binding with normal horse blocking serum (Vector Laboratories) for 30 min at RT. Anti-rabbit CYP27B1 (1/100) or RUNX2 antibody (1/50) were incubated overnight at 4ºC. After washing with PBS, slides were treated with corresponding biotinylated secondary antibody (30 minutes, RT), which was followed by the avidin-biotin-peroxidase complex (30 minutes, RT) and 3, 3'-diaminobenzidine (DAB) as a chromogen (10 minutes, RT) (Vector Laboratories). Sections were counterstained with Mayer´s Hematoxylin to visualize the nuclei. Negative controls were performed by incubation with non-immune 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 evaluated following the uniform pre-established criteria. Staining intensity and % positive cells were graded semiquantitatively. Histological scores were obtained from each sample as follows: histoscore = 1 x (% light staining) + 2 x (% moderate staining) + 3 x (% strong staining), which ranged from 0 (no immunoreaction) to 300 (maximum immunoreactivity). The reliability of such scores for interpretation of immunohistochemical staining of tissue sections has been shown previously.[35]

For calcium staining in aortic sections, samples were deparaffinized, rehydrated and stained in 2% alizarin red solution (Sigma A3757) at pH between 4,1-4,3 for 5 minutes. After staining, samples were rehydrated with acetone, acetone-xylene (1:1), xylene and mounted in synthetic mounting medium (DPX).

In Vitro Study

VSMC cultures from rat and mice (CYP27B1-/- and CYP27B1+/+)

Primary rat and mose aortic VSMCs were obtained as previously described[22] and maintained in DMEM (GIBCO) containing 10% FBS. Cells were plated (10^5 cells/plate) in 100mm 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 ß-glycerophosphate (Sigma). Final concentration of calcium was adjusted to 4mM.

VSMC between passage 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 50µl of HCL 0,6N in vortex at 4ºC. After centrifugation, supernatant was used for calcium quantification by the o-cresolphtalein 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 (Sigma A3757) at pH between 4,1-4,3 for 20 minutes.

Determination of calcitriol production (1α-hydroxylase activity)

After treatments or 1α-hydroxylase overexpression, cell monolayers were washed 6 times with PBS and incubated with DMEM with or without 500 ng/mL of 25(OH)D$_3$. After one hour, incubation media were collected and after sample delipidation with dextran sulphate and magnesium chloride, 100 ul of sample were run through a solid phase column coated with monoclonal antibodies against 1,25(OH)$_2$D$_3$. Afterwards, the column was eluted with ethanol and the collected fraction was evaporated under

nitrogen atmosphere and resuspended in assay buffer. Levels of $1,25(OH)_2D_3$ were then assessed by ELISA (Immunodiagnostics Systems). The production was calculated as follows: $1,25(OH)_2D_3$ levels in the wells with $25(OH)D_3$ minus $1,25(OH)_2D_3$ levels in wells without $25(OH)D_3$. Serum samples were processes in the same way. The percentage of recovery, as reported by the manufacturer, was 96%.

Real Time PCR

Total cellular RNA from VSMC and from rat aortic tissue samples was extracted with TRIzol reagent (Sigma Aldrich), following manufacturer's instructions. The final RNA concentration was determined by nanodrop (ND-100) spectrophotometer. Reverse transcription and real time PCR were performed as previously 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) was performed with an ABI Prism 7000 Sequence Detection System (Applied Biosystems) using TaqMan Universal PCR Master Mix. Forty cycles at 95ºC for 15 second and 60ºC for 1 minute were performed. Every experiment was carried out three times and all samples were amplified in triplicate

The relative RNA amount was calculated by standard formulae 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 PMSF and protease inhibitor cocktail). Samples were sonicated and the supernatant was collected after centrifugation for 10 minutes at 10000 rpm, 4ºC. 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 of Na_3VO_4 and protease inhibitor cocktail) using the TissueLyser LT (Qiagen): 4 cycles of homogenization (50 Hz for 2 min each cycle) followed by 3 cycles in liquid nitrogen and thawing at 37ºC. The supernatant was collected after centrifugation for 10 min at 10000 rpm, 4ºC. Protein concentration was determined using a DC protein assay kit (Bio-Rad). 25 μg of proteins were electrophoresed on 10% SDS-PAGE gels, and transferred to PVDF membranes

(Immobilon-P, Millipore). Membranes were probed with primary antibodies against VDR (1:1000; Santa Cruz), CYP24 (1:1000; Santa Cruz), CYP27B1 (1:1000; Santa Cruz), RUNX2 (1:1000; Novus Biologicals), anti-V5 epitop (1:1000, Sigma), GAPDH (1:5000, Sigma), α-tubulin (1:5000; Sigma) and α-actin (1:500, Santa Cruz) over night at 4ºC. Corresponding peroxidase-conjugated secondary antibody: rabbit IgG+HRP (1:10000; Cell Signaling), mouse IgG+HRP (1:10000; Jackson Immunoresearch) 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) or ECL Advanced (Amersham Biosciences). 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). Production of lentiviral particles was done as previously described.[35] Empty FSV vector (EV) was used as a control of transfection. VSMC 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. A $p < 0.05$ was considered statistically significant. All data examined are expressed as mean ± SEM.

RESULTS

Uremia deregulates the expression of VDR, 1α-hydroxylase, 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 sacrificed at week 8. Uremic rats displayed an increase in creatinine, calcium and PTH serum levels (Figure 1A) compared with controls. They also demonstrated a decrease in phosphate levels, indicating they were in the early stages of CKD. The analysis of vascular calcification by calcium quantification showed higher levels of calcium content

in the arteries of uremic rats compared with the control rats (Figure 1 B) and an increase in alizarin red staining (Figure 1C). Figure 1 (D, E, 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 (Figure 1D) and protein level (Figure 1E) in the rat artery. An increase of VDR and RUNX2 in the artery of uremic rats was only observed at the protein level (Figure 1 E, F; data not shown for Runx2 qPCR).

Uremic patients with vascular calcification show increased immunostaining for 1α-hydroxylase in epigastric arteries.

To confirm whether an increase in vascular calcification 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. Vascular calcification was confirmed by an increase in the calcium levels in the epigastric arteries of uremic patients (Figure 2A). In parallel, immunostaining for 1α-hydroxylase was higher in arteries obtained from CKD patients than in arteries obtained from non-CKD donors (Figure 2B and 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 (Figure 3A) in VSMCs, while 24-hydroxylase expression was not modified. Figure 3B shows the western blot images of the same samples. The quantification in Figure 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 Figure 3E. Alizarin red staining was also increased (Figure 3D). Furthermore, production of calcitriol was increased in cells treated with uremic serum compared with cells treated with control serum (Figure 3F).

1α-hydroxylase has a direct effect on calcification in rat VSMC

To study the role of 1α-hydroxylase in vascular calcification, 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 (Figure 4A), as well as of 1α-hydroxylase mRNA (Figure 4B) in rat VSMCs. VSMCs overexpressing 1α-hydroxylase showed significantly elevated production of $1,25(OH)_2D_3$ (Figure 4C), alongside markedly higher intracellular calcium content (Figure 4D) and alizarin red staining (Figure 4E) compared with the control cells (EV). Furthermore, VSMCs overexpressing 1α-hydroxylase showed an evident upregulation of RUNX2 protein expression compared with control group (Figure 4F).

STN CYP27B1$^{+/+}$ and CYP27B1$^{-/-}$ mice treated with calcitriol have similar serum levels of Ca, P, PTH and 1,25vitD.

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, $1,25(OH)_2D_3$ and to induce vascular calcification. Results showed similar levels of BUN in CYP27B1$^{+/+}$ and CYP27B1$^{-/-}$ mice (Figure 5A), suggesting a similar degree of renal impairment. Calcium levels (Figure 5B), generally lower in CYP27B1$^{-/-}$ mice, increased with calcitriol treatment reaching similar levels as CYP27B1$^{+/+}$ treated mice. Phosphate levels, which were comparable in both untreated mice groups, also increased to a similar degree when animals were treated with calcitriol (Figure 5C). Levels of PTH, which are very high in CYP27B1$^{-/-}$ mice, decreased in both groups of animals treated with calcitriol reaching similar values (Figure 5D). $1,25(OH)_2D_3$ levels, generally undetectable in CYP27B1$^{-/-}$ mice, raised to similar levels in both treated groups (Figure 5E).

STN CYP27B1$^{+/+}$ mice treated with calcitriol present more severe vascular calcification than STN CYP27B1$^{-/-}$ treated mice.

CYP27B1$^{+/+}$ mice subjected to subtotal nephrectomy and treated with calcitriol showed significant increase of arterial calcium content compared with STN CYP27B1$^{-/-}$

mice underwent the same treatment (Figure 6A). Alizarin red staining (Figure 6B) showed an increase in calcium deposits in the artery of STN CYP27B1$^{+/+}$ treated mice, which was not observed in CYP27B1$^{-/-}$ animals. Furthermore, immunoreactivity for RUNX2, an essential regulator of VSMC´s calcification, was higher in the arteries of STN CYP27B1$^{+/+}$ treated mice (Figure 6C) compared with STN CYP27B1$^{-/-}$ treated animals.

Uremic serum does not affect the expression of RUNX2, VDR, 1α-hydroxylase and 24-hydroxylase and does not induce vascular calcification in CYP27B1$^{-/-}$ VSMC.

Representative western blots of RUNX2, VDR, 1α-hydroxylase, 24-hydroxylase in VSMC from CYP27B1$^{+/+}$ and CYP27B1$^{-/-}$ treated with high phosphate media and control or uremic serum are shown in Figure 7A. Figure 7B shows quantification of the western blots. CYP27B1$^{+/+}$ VSMC treated with uremic serum showed an increased expression of RUNX2 (although the quantification did not reach statistical significance Figure 7B), but was not modified in the VSMCs from CYP27B1$^{-/-}$ mice. VDR, 1α-hydroxylase and 24-hydroxylase showed a significant increase in CYP27B1$^{+/+}$ VSMC, while in CYP27B1$^{-/-}$ VSMC 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, while the expression of VDR, in contrast to the protein levels, decreased in CYP27B1$^{+/+}$ VSMCs incubated with uremic serum (Figure 7C). However, in VSMCs from CYP27B1$^{-/-}$ mice, only the expression of 24-hydroxylase was slightly but significantly upregulated. Calcium levels were significantly elevated in CYP27B1$^{+/+}$ VSMC treated with uremic serum whereas calcification did not increase in VSMCs from CYP27B1$^{-/-}$ mice (Figures 7D and E).

DISCUSSION

In this study, we show that an increase of the local expression of 1α-hydroxylase in VSMC is a mediator of the increase in vascular calcification observed in CKD. Thus, both *in vitro* and *in vivo* (in experimental animals and in patients), uremia-induced vascular calcification 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 vascular calcification 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, monocytic cells, macrophages, dendritic cells, parathyroid cells, β-pancreatic cells and osteoblasts.[37] 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 *et al.* have shown that macrophage 1α-hydroxylase expression in sarcoidosis is driven by proinflammatory cytokines.[38] In that condition the increased activity of 1α-hydroxylase would increase $1,25(OH)_2D_3$ levels to supranormal concentrations.[39] Furthermore endothelial cells also show an upregulation of 1α-hydroxylase levels by proinflammatory cytokines.[20] In placental tissue, the production of $1,25(OH)_2D_3$ is upregulated by IGF-I[40] 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.[41] Therefore it seems clear that regulation of extrarenal 1α-hydroxylase expression is tissue dependent and is modified in many different conditions.

Chronic kidney disease is a condition in which systemic production of $1,25(OH)_2D_3$ is altered. Thus, there is a decrease in circulating levels of $1,25(OH)_2D_3$ due to a reduction of renal 1α-hydroxylase activity, an increase of $1,25(OH)_2D_3$ degradation, and impaired uptake in tubular cells of $25(OH)D_3$, the substrate of 1α-hydroxylase.[42] However, macrophages from uremic patients show an increased, rather than decreased, 1α-hydroxylase activity.[31] 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 VSMC. The uremic factors that are involved in this upregulation of 1α-hydroxylase are unknown, but increased PTH levels or in inflammatory cytokines could be playing a role.

Whether this increase of 1α-hydroxylase levels translate into an increase of intracellular $1,25(OH)_2D_3$ *in vivo* is unknown, and current techniques to determine those levels lack sufficient sensitivity. However, our *in vitro* data show that VSMC incubated in uremic media produce higher amounts of $1,25(OH)_2D_3$ than cells incubated in normal media. *In vivo* data indirectly show an increase in VDR activation because both VDR and 24-hydroxylase 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 $1,25(OH)_2D_3$ is not self-inhibited, and this appears to be the basis for the unregulated 1α-hydroxylase activity,[43] showing that extra renal regulation of 1α-hydroxylase could be less sensitive to autoregulation by $1,25(OH)_2D_3$.[19] Therefore, the results point to an overactivation of VDR signaling in uremia, which has been previously shown to induce vascular calcification. Indeed, both *in vivo* and *in vitro*, supraphysiological levels of $1,25(OH)_2D_3$ induce expression of osteoblastic genes like RUNX2 and vascular calcification. In our experiments, uremic conditions induced increases in RUNX2 expression and vascular calcification *in vivo* and *in vitro*.

Our results also demonstrate that the increase in 1α-hydroxylase is necessary and sufficient to induce vascular calcification. Thus, overexpression of 1α-hydroxylase in VSMC increased both RUNX2 expression and vascular calcification. Furthermore, the elimination of 1α-hydroxylase totally inhibited the vascular calcification *in vitro*. Indeed, VSMC obtained from 1α-hydroxylase KO animals and incubated in calcification media showed no increase in RUNX2, calcium content nor in alizarin red staining, demonstrating that the presence of 1α-hydroxylase is necessary for the uremia-induced vascular calcification. 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 post-transcriptional or even post-translational level. This fact has been already demonstrated in the regulation of several proteins.[44,45] 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 $1,25(OH)_2D_3$ in our cells, increasing therefore the activation of VDR

and, thus, inhibiting its degradation and explaining the higher levels of protein.[46] 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.[47] *In vivo* results agree with the *in vitro* data. In our model of vascular calcification induced by subtotal nephrectomy, CYP27B1^{-/-} animals treated with toxic doses of calcitriol showed significantly lower levels of vascular calcification and RUNX2 expression, compared to CYP27B1^{+/+} littermates, although calcium and phosphate levels were similar. The inhibition of calcification was not complete, as CYP27B1-/- animals still showed an increase of vascular calcification 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 vascular calcification. Thus, one significant pathophysiological 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 $1,25(OH)_2D_3$ 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 VSMC in regulating uremia-induced vascular calcification. These results could be of importance to identify new treatments to reduce vascular calcification in CKD patients.

ACKOWLEDGEMENTS

Authors' roles: Study design: NT, EF and JMV. Study conduct: NT, MB, SP, SB, ME, MVA. Data collection: NT, MB. Data analysis: NT, MB and JMV. Data interpretation: NT, JLF, DG, EF, and JMV. Drafting manuscript: NT and MB. Revising manuscript content: DG, EF and JMV. Approving final version of manuscript: All authors. JMV takes responsibility for the integrity of the data analysis.

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Figure Legends

Figure 1. Uremia deregulates the expression of VDR, 1α-hydroxylase, 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 (grey 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 ± sem (n = 6) *: p < 0.05 vs control (control rat). Scale bar represents 100 □m.

Figure 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

Figure 3. Uremia deregulates the expression of VDR, 1α-hydroxylase, 24-hydroxylase and induces calcification *in vitro*. VSMC treated with high phosphate media with 15% serum from healthy rat (control serum, black bars) or from 5/6 nephrectomized rat (uremic serum, grey bars). (A) Expression of VDR, 1α-hydroxylase and 24-hydroxylase determined by qPCR after 48h of treatment. (B) Representative western blot assessing protein expression of RUNX2, VDR, 1α-hydroxylase and 24-hydroxylase after 6 days of treatment. α-tubulin was used as a loading control. (C) Quantification of western blot. (D) Alizarin red staining (bright field pictures) in rat cells incubated with normal or uremic serum (15%). (E) Quantification of calcium content (micrograms of calcium per milligram of protein) in VSMC treated with high phosphate media and crescent concentrations of normal or uremic serum. (F) Production of 1,25(OH)$_2$D$_3$ in cells incubated with control or uremic serum. Data are mean ± sem (n = 6). *: p < 0.05 vs control serum. #: p<0.05 vs 10%. +: p<0.05 vs 15%. Scale bar represents 200 □m.

Figure 4. 1α-hydroxylase overexpression in rat VSMC increases calcification. (A) WB anti-V5-1α-hydroxylase to detect the overexpression in VSMC compared with the empty vector (EV). (B) Expression of 1α-hydroxylase in VSMC infected with the EV and PDSL-1α-hydroxylase determined by qPCR. (C) Production of 1,25(OH)$_2$D$_3$ in cells infected with the 1α-hydroxylase overexpression plasmid compared with cells infected with the empty vector. (D) Calcium content normalized to protein and (E) alizarin red staining in cells infected with the 1α-hydroxylase overexpression plasmid compared with cells infected with the empty vector. RUNX2 detection by western blot (F) in cells infected with the 1α-hydroxylase overexpression plasmid compared with cells infected with the empty vector. Data are reported as means ± sem (n = 12)*: p < 0.05 vs VSMC without 1α-hydroxylase overexpression (EV).

Figure 5. Subtotally nephrectomized (STN) CYP27B1$^{+/+}$ and CYP27B1$^{-/-}$ mice treated with calcitriol have similar serum levels of Ca, P, PTH and 1,25(OH)$_2$D$_3$. Serum levels of (A) Blood urea nitrogen (BUN), (B) Calcium, (C) Phosphate, (D) PTH, (E) and 1,25(OH)$_2$D$_3$ in STN CYP27B1$^{+/+}$ and STN CYP27B1$^{-/-}$ mice (black bars) and STN mice treated with calcitriol (grey bars). Data are reported as means ± sem (n = 8).

*: $p < 0.05$ between STN and STN-treated mice, # $p < 0.05$ between CYP27B1$^{+/+}$ and CYP27B1$^{-/-}$ mice.

Figure 6. Subtotally nephrectomized (STN) CYP27B1$^{+/+}$ mice treated with calcitriol show higher vascular calcification levels than STN-treated CYP27B1$^{-/-}$ mice. (A) Calcium content in abdominal aorta normalized by protein in STN CYP27B1$^{+/+}$ and STN CYP27B1$^{-/-}$ mice treated with calcitriol (400ng/kg) for two weeks, and in control animals. (B) Representative histochemical of alizarin red staining and (C) immunohistochemical staining of RUNX2. Results are showed as mean ± sem (n=8) *: P<0.05 between STN and STN-treated mice. # P<0.05 between CYP27B1$^{+/+}$ and CYP27B1$^{-/-}$ mice. Scale bar represents 100 □m.

Figure 7. Uremia does not affect calcification or the expression of RUNX2, VDR, 1α-hydroxylase and 24-hydroxylase in CYP27B1$^{-/-}$ VSMCs. (A) Representative Western blots assessing protein content for RUNX2, VDR, 1α-hydroxylase and 24-hydroxylase in CYP27B1$^{+/+}$ and CYP27B1$^{-/-}$ VSMCs treated with control or uremic serum. (B) Quantitative analysis of western blots. Data are expressed as fold change over wild type control levels. (C) Expression of RUNX2, VDR, 1α-hydroxylase and 24-hydroxylase in CYP27B1$^{+/+}$ and CYP27B1$^{-/-}$ VSMCs treated with control or uremic serum for 48 hours, determined by qPCR. Data are expressed as % over wild type control. (D) Quantification of calcium content normalized by mg of protein in VSMCs from CYP27B1$^{+/+}$ or CYP27B1$^{-/-}$ mice treated with control or uremic serum for 6 days. (E) Alizarin red staining in VSMCs from CYP27B1$^{+/+}$ or CYP27B1$^{-/-}$ mice treated with uremic serum for 6 days (bright field). Data are reported as means ± sem (n = 6) *: $p < 0.05$ between control and uremic serum. #: $p < 0.05$ between WT and CYP27B1$^{-/-}$ VSMCs.
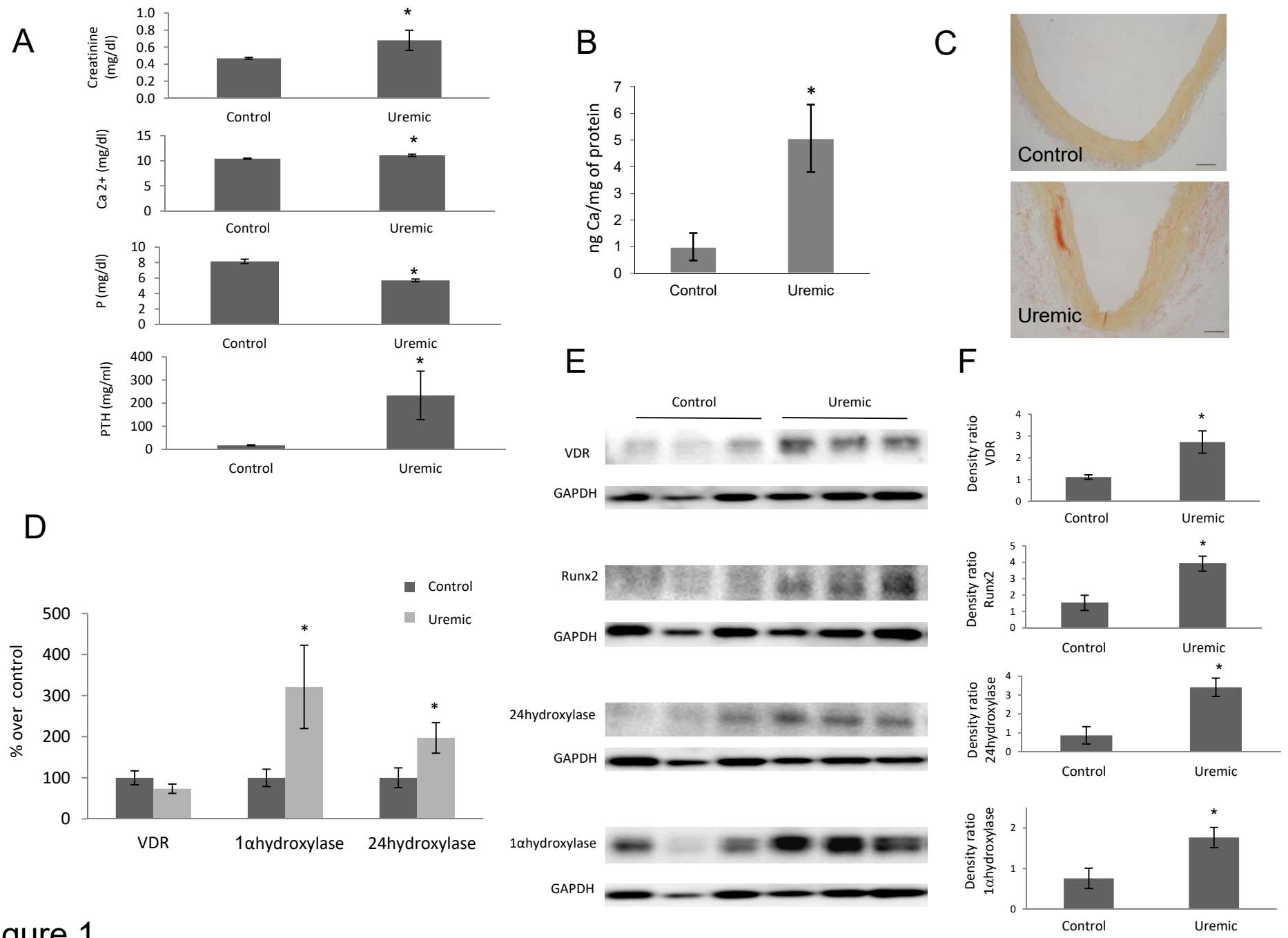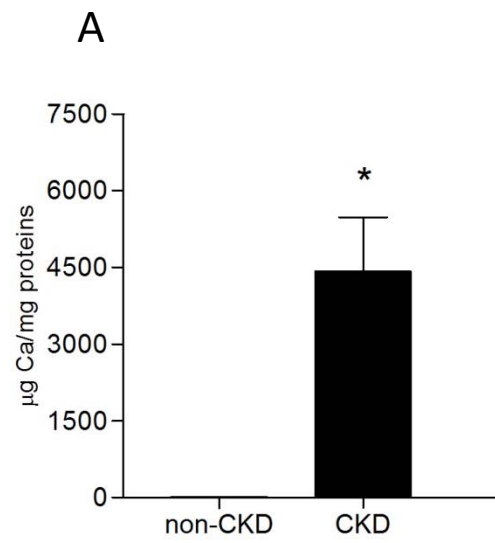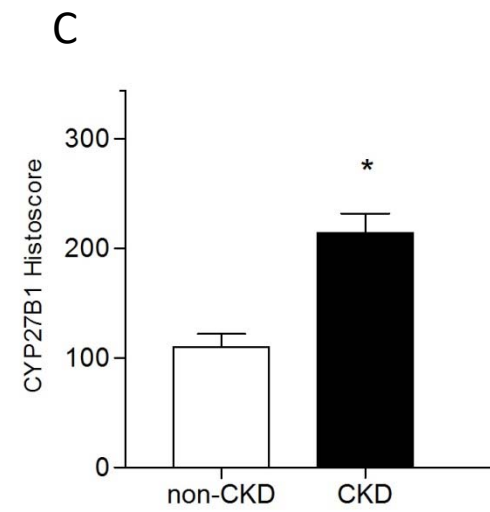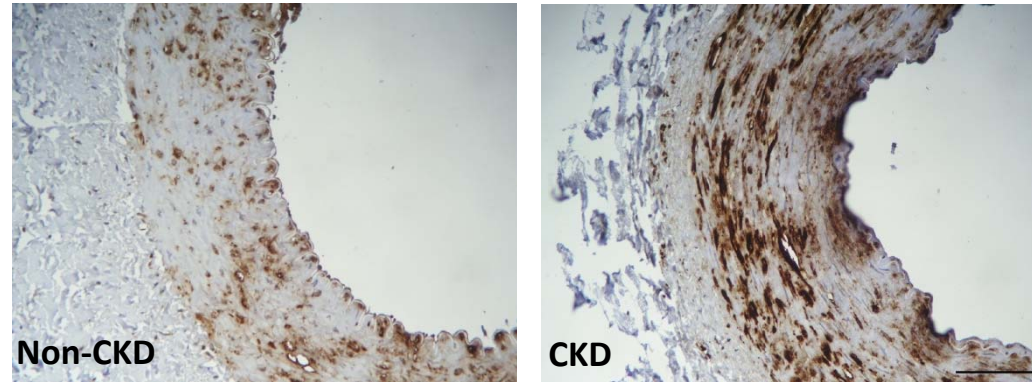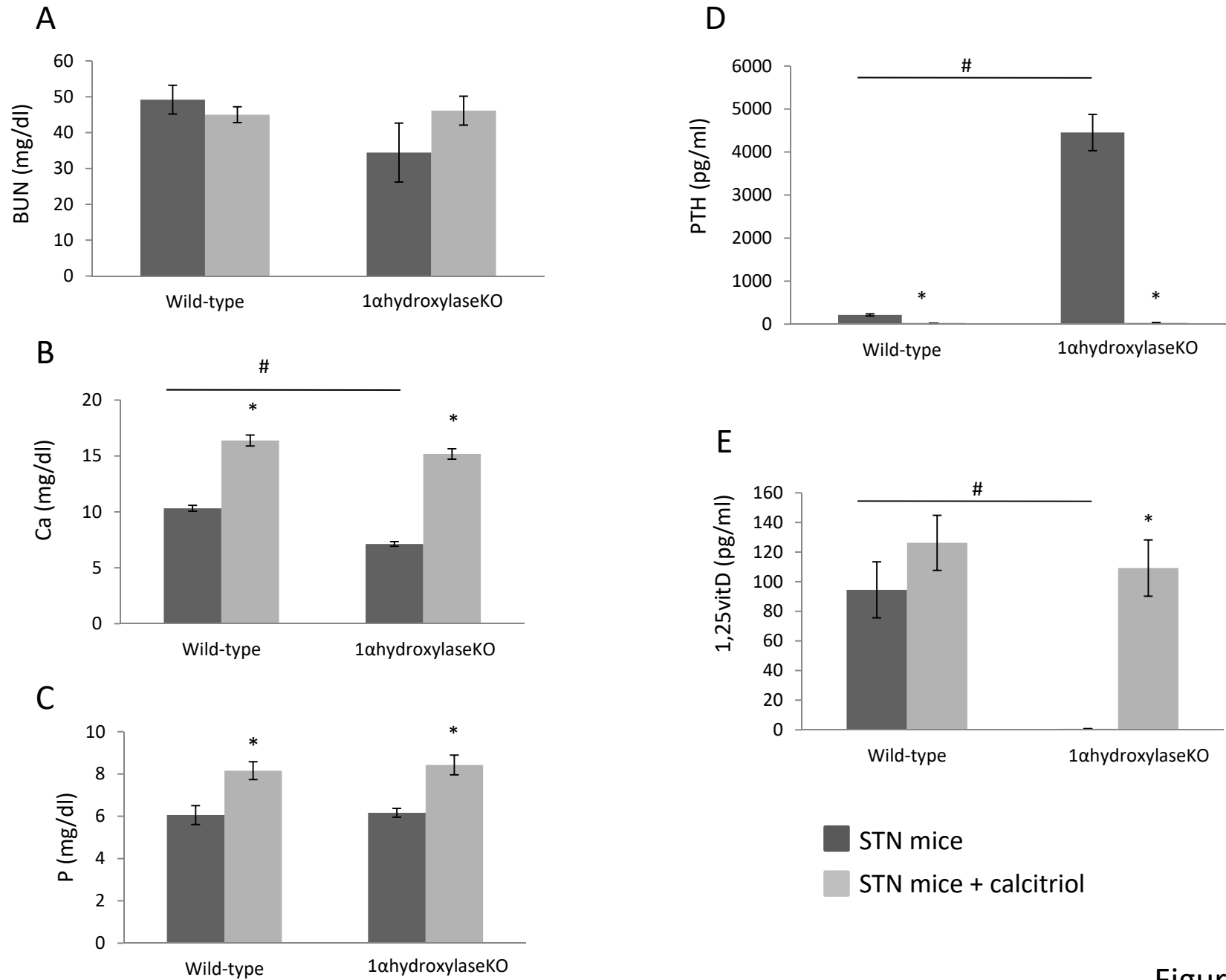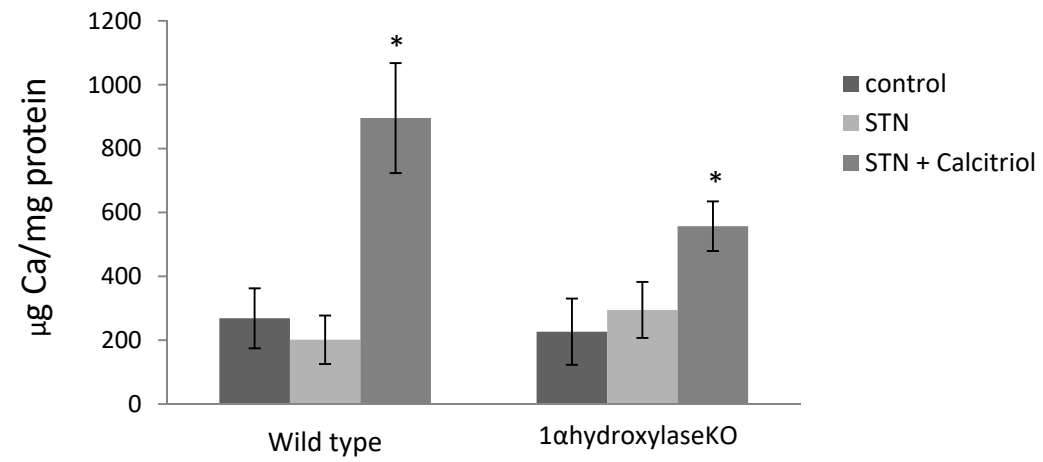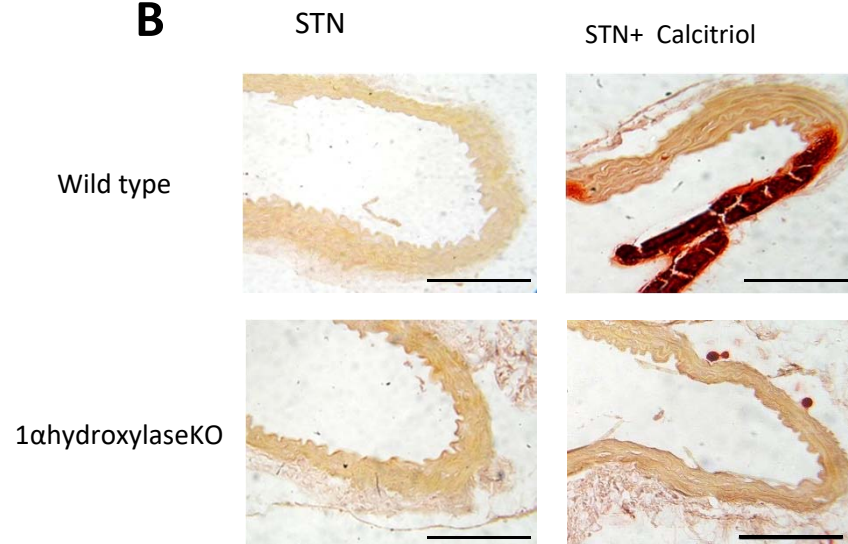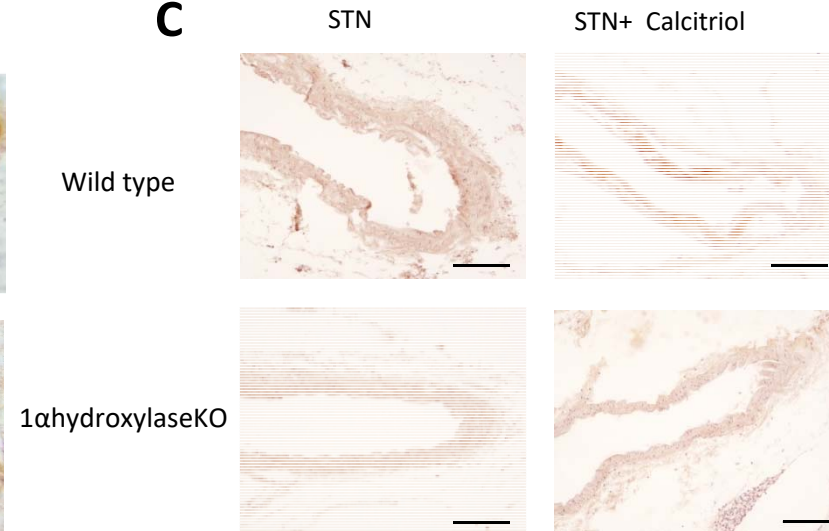
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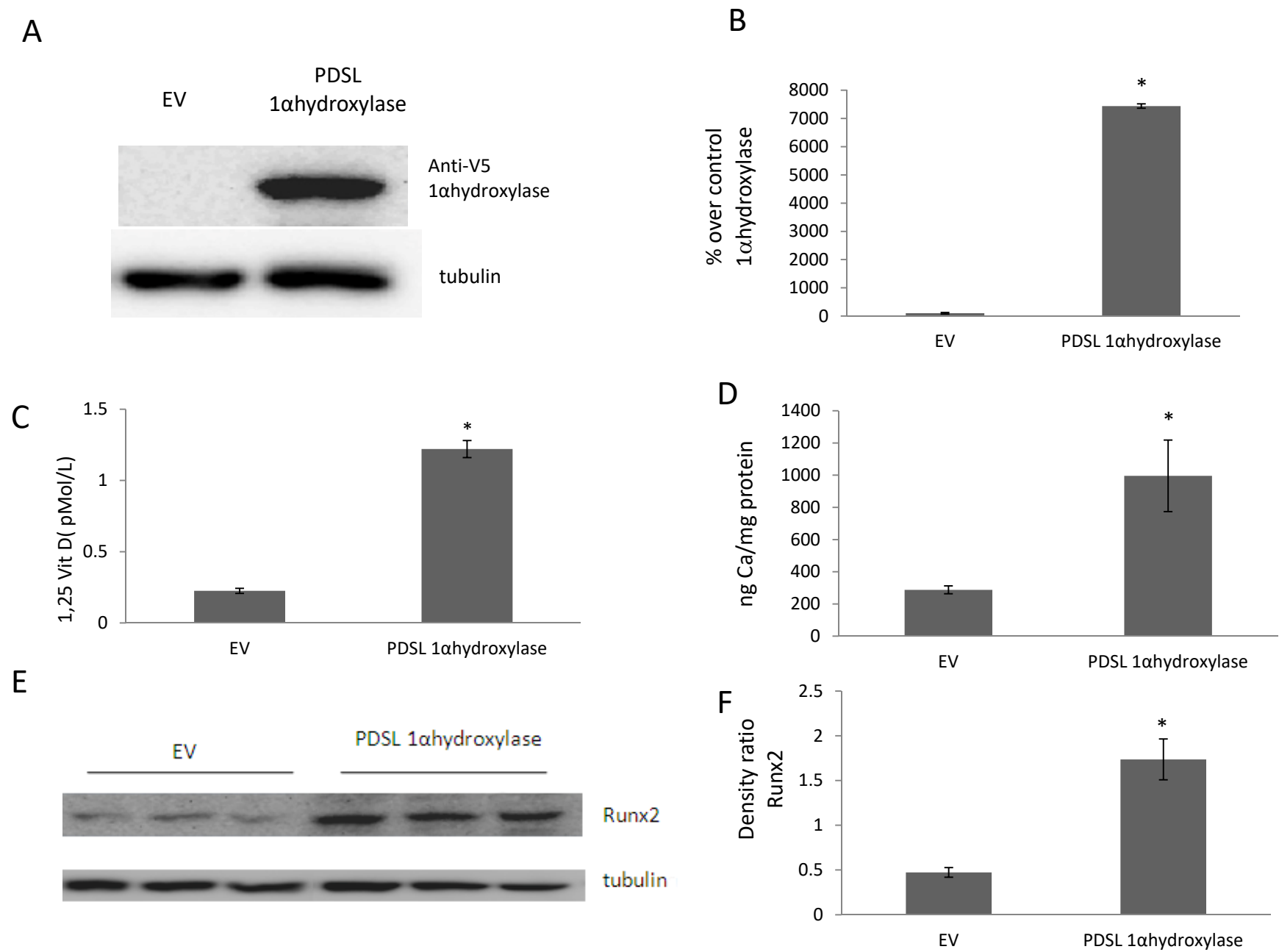
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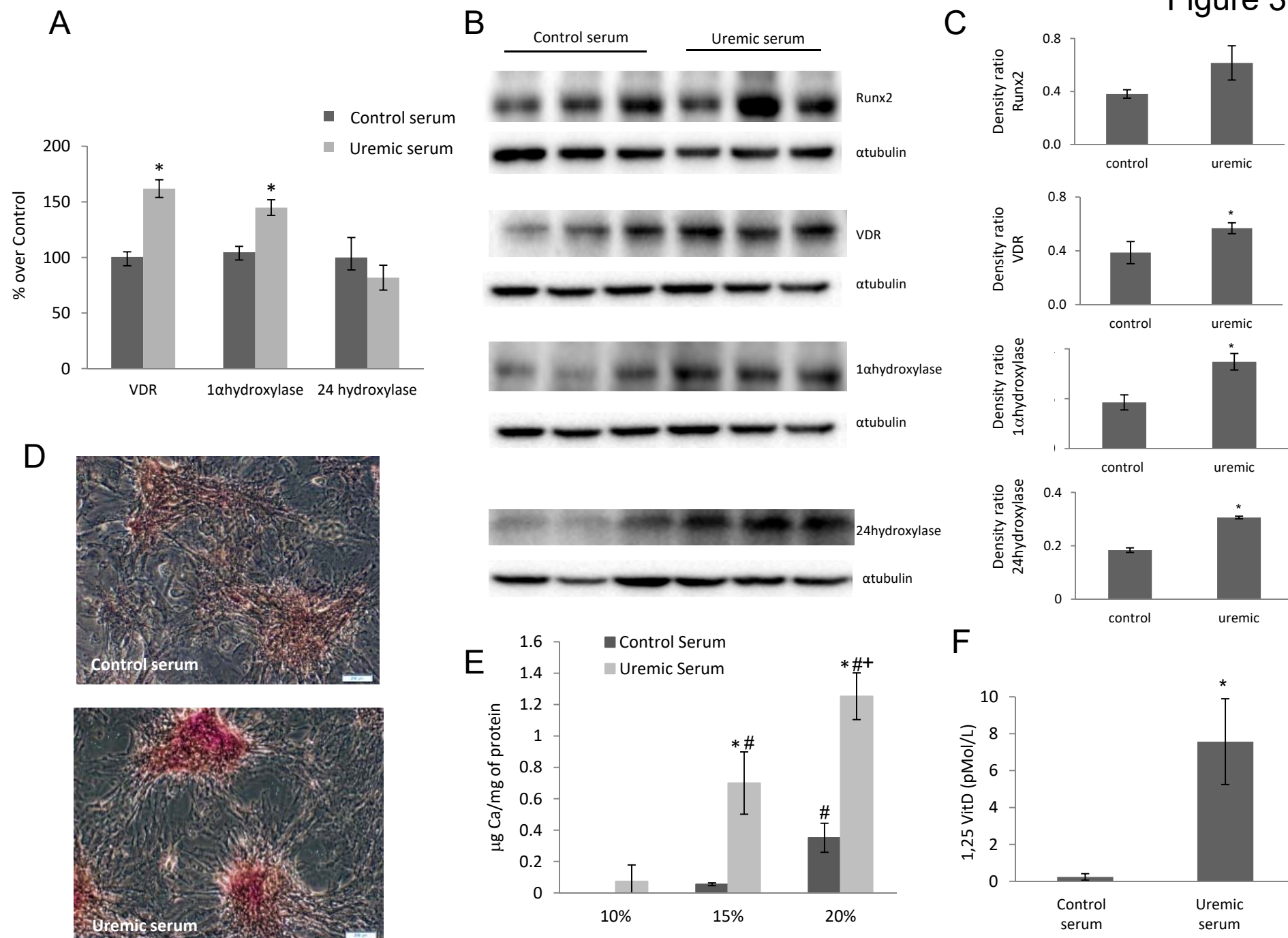
Figure 5

A

B Alizarin Red

C IHC for Runx2

Figure 6

Figure 7