

Human Vascular Smooth Muscle Cells Undergo Vesicle-Mediated Calcification in Response to Changes in Extracellular Calcium and Phosphate Concentrations: A Potential Mechanism for Accelerated Vascular Calcification in ESRD

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Abstract. Patients with ESRD have a high circulating calcium (Ca) × phosphate (P) product and develop extensive vascular calcification that may contribute to their high cardiovascular morbidity. However, the cellular mechanisms underlying vascular calcification in this context are poorly understood. In an *in vitro* model, elevated Ca or P induced human vascular smooth muscle cell (VSMC) calcification independently and synergistically, a process that was potently inhibited by serum. Calcification was initiated by release from living VSMC of membrane-bound matrix vesicles (MV) and also by apoptotic bodies from dying cells. Vesicles released by VSMC after prolonged exposure to Ca and P contained preformed basic calcium phosphate and calcified extensively. However, vesicles released in the presence of serum did not contain basic

calcium phosphate, co-purified with the mineralization inhibitor fetuin-A and calcified minimally. Importantly, MV released under normal physiologic conditions did not calcify, and VSMC were also able to inhibit the spontaneous precipitation of Ca and P in solution. The potent mineralization inhibitor matrix Gla protein was found to be present in MV, and pretreatment of VSMC with warfarin markedly enhanced vesicle calcification. These data suggest that in the context of raised Ca and P, vascular calcification is a modifiable, cell-mediated process regulated by vesicle release. These vesicles contain mineralization inhibitors derived from VSMC and serum, and perturbation of the production or function of these inhibitors would lead to accelerated vascular calcification.

Patients with ESRD develop extensive medial calcification, or Monckeberg's sclerosis, that causes increased arterial stiffness and contributes to the high cardiovascular mortality (1,2). Calciphylaxis is an increasingly common and life-threatening form of calcification characterized by destructive calcification in the media of subcutaneous arterioles, leading to occlusion and subsequent widespread tissue necrosis (2,3). The precise pathophysiology of vascular calcification in ESRD is unknown, but risk factors include age, hypertension, time on dialysis, and, most significant, abnormalities in calcium (Ca) and phosphate (P) metabolism (4,5). Normal serum concentra-

tions of Ca and inorganic P ions are metastable with respect to basic calcium phosphate (BCP; a mixture of octacalcium phosphate, dicalcium phosphate dihydrate, and apatite) precipitation but can support growth of nascent crystals. In ESRD, systemic Ca and inorganic P concentrations typically exceed 2.4 and 2.0 mM, respectively (4). Consequently, calcification in ESRD has traditionally been ascribed to supersaturation and subsequent precipitation of mineral ions. This has led to therapeutic measures to reduce the Ca/P product aimed mostly at reduction of P.

Recent studies have shown that vascular calcification is a regulated process similar to bone formation (6,7). VSMC in the normal artery wall constitutively express potent inhibitors of calcification, such as matrix Gla protein (MGP), whose absence results in spontaneous medial calcification (8). In atherosclerotic calcification and diabetic Monckeberg's sclerosis, expression of these endogenous inhibitors is reduced and VSMC express markers of both osteoblast and chondrocyte differentiation (7,9). Moreover, human VSMC in culture spontaneously convert to an osteo/chondrocytic phenotype and

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form calcified nodules (7,9). Calcification is initiated in nodules by release of apoptotic bodies (AB) and matrix vesicle (MV)-like structures from VSMC that act as a nidus for BCP nucleation (10). AB are calcium-enriched membrane-bound vesicles ~0.3 to 1.0 μm in diameter, released from apoptotic VSMC (10). MV are smaller (30 to 300 nm) and are released from the plasma membrane of viable cells (11). VSMC-derived MV have been associated with calcification *in vivo*, but their composition and function are poorly understood (12).

Vascular calcification in ESRD may also be actively regulated. Bone matrix proteins are deposited in medial artery calcifications of ESRD patients, suggesting osteogenic conversion of resident VSMC, while *in vitro* studies have shown increased calcification and osteogenic changes in VSMC in the presence of elevated P (13,14). More recently, it was suggested that the circulating serum protein fetuin-A, which is reduced in patients with ESRD, may play a role in regulating calcification. However, its mechanism of action is unclear (15).

In this study, we set out to determine the effects of increased extracellular Ca and P concentrations on *in vitro* calcification of human VSMC. Using this model, we demonstrate that VSMC calcification is a vesicle-mediated phenomenon and identify potent local and circulating inhibitors. Furthermore, we show that, in the presence of raised P, a small increase in Ca concentration substantially increases calcification.

Materials and Methods

In Vitro Model of VSMC Calcification: Media and Treatments

Explants of human aortic tissue (obtained with appropriate ethical approval) from organ donors aged 2 to 68 yr ($n = 10$) were established, and VSMC were cultured in M199 medium supplemented with 20% FCS and antibiotics (16). VSMC were grown to 80% confluence and washed in Earle's Balanced Salt Solution (EBSS) before treatment with control (1.8 mM Ca/1.0 mM P) or test media; high calcium (Ca media; 2.0 to 2.8, 3.6, or 5.4 mM Ca), high phosphate (Pi media; 1.5 to 5.0 mM P), or both (CaPi media; 2.0 to 2.8 mM Ca/2.0 to 3.0 mM P). The standard CaPi media used was 2.7 mM Ca/2.0 mM P. CaCl_2 and NaH_2PO_4 were used to supplement ionic calcium and phosphate in the medium. Media either were serum-free (SF) and supplemented with 0.5% BSA or contained 20% FCS or 1 to 10% human serum (HS). Experiments using the above control and test media were also performed in cell-free conditions.

For apoptosis experiments, 0.00025% DMSO (vehicle) or 100 μM of the caspase inhibitor ZVAD.fmk were added at the time of test media. VSMC were pretreated for 3 d with warfarin (10 μM) before addition of test media.

Calcification Assays

Calcification was visualized 24 h to 10 d after treatment with test media by alizarin red staining as described previously (10). Calcification was quantified by including ^{45}Ca (~50,000 cpm/ml) in test and control media (17). Briefly, VSMC were decalcified in 0.1 M HCL, neutralized with 0.1 M NaOH/0.1% SDS, scraped and ^{45}Ca incorporation was measured by liquid scintillation counting. In cell-free experiments, BCP precipitates were harvested by centrifugation and ^{45}Ca incorporation in the pellet measured as above. Cell viability was measured by trypan blue (0.5%) staining as standard.

Time-Lapse Videomicroscopy and Detection of Apoptosis

Time-lapse videomicroscopy was performed at 37°C over 24 h, and images were recorded as described previously (18). Apoptosis was measured by direct counting of morphologically apoptotic cells and verified using fluorescence transferase-mediated dUTP nick-end labeling (TUNEL) staining visualized by a rhodamine-labeled anti-digoxigenin antibody (10). Nuclei were detected with DAPI, and >1000 nuclei were quantified.

Preparation of VSMC Vesicles and ^{45}Ca Uptake

VSMC vesicles were harvested using a modified MV isolation protocol (19,20). Confluent VSMC were washed twice with EBSS and transferred to control or test media that contained 0.1% BSA. After 4 to 24 h, the medium was decanted and spun at 2500 rpm in a Sorvall RF7 centrifuge to remove AB. MV were then harvested from the supernatant by centrifugation at 35,000 rpm (100,000 $\times g$) for 30 min at 4°C in a Beckman Ultracentrifuge. The MV and AB pellets were resuspended, and protein content was determined using a Biorad Kit (21,22). An increase in protein was indicative of an increase in the number of vesicles isolated as confirmed by electron microscopy (EM) and FACS analysis (not shown). *In vitro* vesicle calcification assays were performed in triplicate over 24 h using a standard calcifying buffer and 4 to 15 μg of VSMC-derived MV or AB as described previously (10).

EM and Energy Dispersive X-Ray Analysis

VSMC for energy dispersive x-ray (EDX) analysis were grown on nucleopore filters and transferred to standard CaPi media for 24 h. Samples were quench-frozen in melting propane, cooled in liquid nitrogen, then freeze-substituted against pure tetrahydrofuran for 72 h at -85°C and embedded in Lowicryl HM20. Dry sections (300 nm) were mounted on 100 mesh nickel grids onto a Formvar film and coated with carbon before viewing in a Philips XL30 FEG-SEM with an Oxford Instruments GEM detector. Areas of calcification were identified using a solid-state, backscattered electron detector, and x-ray spectra were collected using an Oxford Instruments INCA EDX system. Consecutive 50-nm-thick sections were stained with uranyl acetate and lead citrate and viewed in a Philips CM100. Sections were decalcified by floating on 0.5% EDTA for 5 min before contrast staining to reveal any masked structures.

Vesicle fractions isolated by differential centrifugation (MV and AB) were resuspended at a concentration of 10 $\mu\text{g}/\mu\text{l}$ and adsorbed onto glow-discharged, carbon-coated Formvar film grids. They were negatively stained with neutral potassium phosphotungstate, and vesicle diameters were measured with a Phillips CM100 with an on-line Kinetic Imaging analysis system. EDX analysis was carried out on vesicle fractions adsorbed to grids, rinsed in deionized water, and air dried without staining.

Western Blot Analysis

Western blots for MGP and fetuin-A used 5 μg of vesicle protein. Polyacrylamide gels (12.5% [wt/vol]) were run under denaturing conditions in 8 M urea for MGP. After blotting, membranes were incubated at 4°C overnight with biotin-conjugated a3-15 MGP IgG or a rabbit polyclonal antibody raised against bovine fetuin (23,24). Visualization was *via* streptavidin–horseradish peroxidase (Amersham Pharmacia, Uppsala, Sweden) and development with ECL Western blotting detection reagents (Amersham Pharmacia). Recombinant MGP was used as a positive control (24).

Statistical Analyses

Data were analyzed using *t* test or for multiple comparisons ANOVA with *post hoc* Scheffe test.

Results

Ca and P Act Synergistically to Induce VSMC Calcification

VSMC were treated with increasing concentrations of Ca or P or both in the absence of serum. VSMC accumulated ⁴⁵Ca in response to elevated Ca or P. Ca was a more potent inducer of calcification than P at equivalent fold increases above normal physiologic levels (Figure 1A, top). Treatment of VSMC with Ca and P together had a synergistic effect on calcification in

that a small elevation in extracellular Ca markedly enhanced the effects of elevated phosphate (Figure 1A, bottom). In CaPi medium, extensive calcification of the VSMC matrix was usually observed within 24 to 72 h and appeared as a granular precipitate on alizarin red staining (Figure 1C, 2).

Serum Inhibits VSMC Calcification Induced by Ca and P

The above experiments were repeated in the presence of serum. Normal HS inhibited VSMC calcification in a concentration-dependent manner (data not shown) with maximal inhibition occurring at a concentration of 5%. Equivalent levels

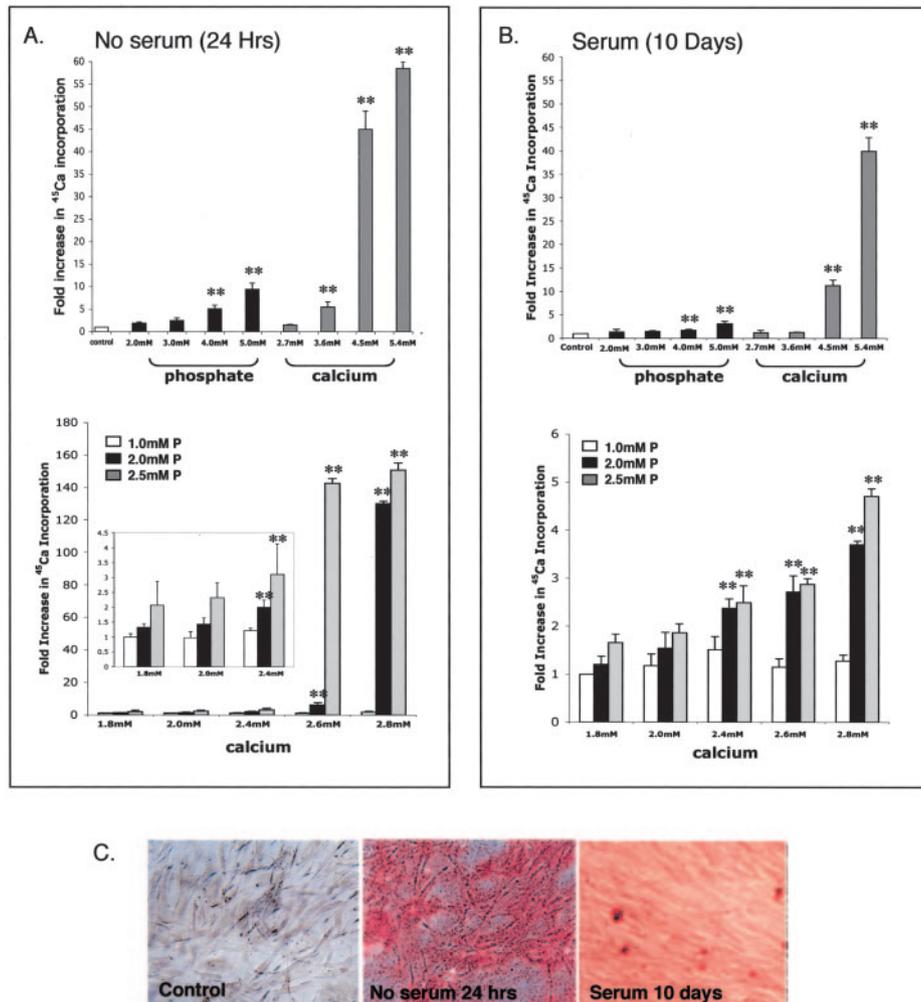


Figure 1. Calcium (Ca) and phosphate (P) induce vascular smooth muscle cell (VSMC) calcification in the presence and absence of serum. Graphs show ⁴⁵Ca accumulation in a VSMC isolate induced by Ca or P or both to calcify in serum-free (SF; 0.5% BSA) conditions for 24 h (A) or in the presence of serum (5% human serum [HS]) for 10 d (B). Note the synergistic effect on ⁴⁵Ca accumulation of CaPi media (2.8 mM Ca/2.0 mM P) compared with Ca and P alone. Significant increases in calcification were observed even at lower concentrations of Ca in the presence of P (A, inset). In serum, calcification was induced in the same dose-dependent manner as in SF conditions but at a slower rate, and Ca accumulation was significantly less (note y-axis range). Results are normalized and expressed as fold increase in ⁴⁵Ca accumulation compared with controls. The mean ± SD of a representative experiment performed in triplicate is shown. Similar results were obtained in five independent experiments performed on different VSMC isolates. ***P* < 0.05 versus controls analyzed by ANOVA. (C) Alizarin red staining demonstrating the punctate pattern of mineral accumulation occurring after treatment of VSMC with CaPi media (2.7 mM Ca/2.0 mM P) in the presence of serum after 10 d (right) and the granular pattern induced in the absence of serum after 24 h (middle). No calcification was observed in VSMC that were treated with control media (left).

of calcification to those achieved in SF media were not achieved even after 10 d in HS (Figure 1B). The calcification induced in the presence of serum was unevenly distributed in a punctate or nodular pattern throughout the VSMC matrix (Figure 1C, 3).

VSMC Inhibit Spontaneous BCP Precipitation

To assess the possibility that the calcification observed at higher Ca \times P concentrations was due to spontaneous precipitation of Ca and P, we repeated the above experiments in cell-free conditions. In the absence of cells, precipitation of Ca and P in SF media was observable by phase-contrast microscopy within 6 h at a Ca \times P of between 7.8 and 8.4 mM, resulting in high ^{45}Ca incorporation measured after 24 h (Figure 2B). However, in the presence of VSMC, this precipitation did not occur, and in some isolates, little calcification/ ^{45}Ca incorporation occurred over 24 h (Figure 2).

VSMC Vesicle Release Contributes to Calcification

Time-lapse videomicroscopy was used to observe the calcification process induced by extracellular Ca and/or P in the absence of serum. This showed that VSMC in culture continually released small vesicles from the plasma membrane (Figure 3). Vesicle release increased within 60 min of treatment with Ca and/or P, and apoptosis, characterized by membrane blebbing, was also observed (18). Apoptosis and AB and MV release continued until rapid mineralization of the matrix was observed. In this context, calcification appeared as alizarin red–positive small granules (data not shown) that encased trypan blue excluding VSMC (Figure 3A, box). When these cells were released from the calcified matrix by trypsinization, they proliferated in cell culture, confirming their viability (data not shown).

Time-lapse experiments in the presence of serum revealed spontaneous vesicle release that was also further stimulated in the presence of increased Ca and P. However, apoptosis was not induced, and there was little subsequent calcification.

EM sections and EDX analysis of calcified VSMC showed the presence of microspicules of crystalline mineral, predominantly composed of Ca and P, on the matrix, within vesicular structures and on the membrane surface of vesicles (Figure 3B). Most vesicles were extracellular, but occasional intracellular calcified vesicles were observed. It was not possible to determine whether these had formed *in situ* or had been phagocytosed. Removal of calcium from sections with EDTA revealed numerous vesicles underlying all areas of matrix calcification, suggesting that vesicles were the source of mineral nucleation (Figure 3B, iii). Calcified vesicles ranged in size from ~ 70 nm to 1.0 μm , consistent with their derivation from both apoptotic and viable cells (Figure 3B).

VSMC Vesicle Release is Increased by Ca and P

Differential centrifugation revealed two populations of vesicles with mean diameters of 220 ± 24 and 127 ± 7 nm (Figure 4A), consistent with their being derived predominantly from AB and MV, respectively. The time-lapse studies suggested that vesicle release was potentiated in the presence of increased

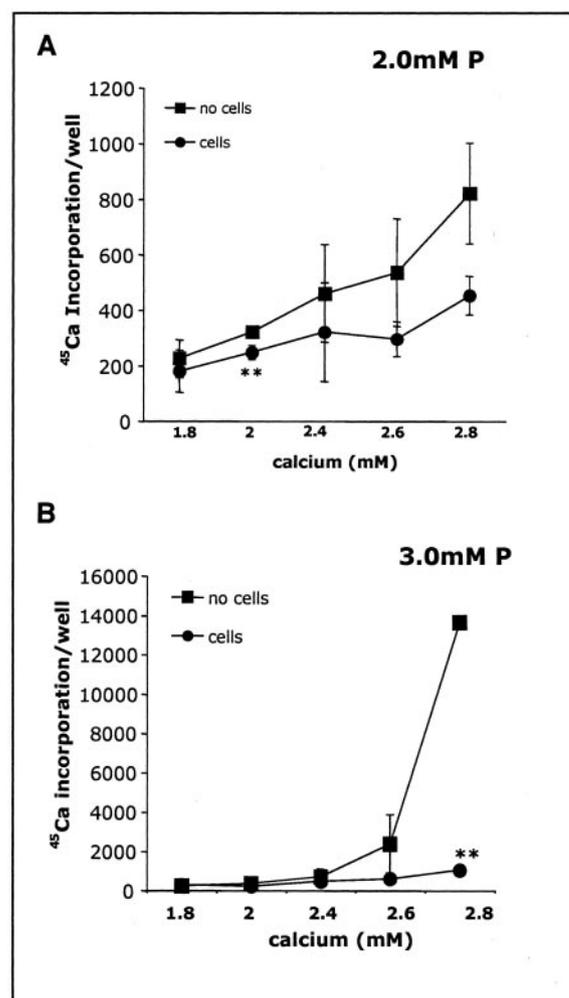


Figure 2. VSMC inhibit precipitation of Ca and P. ^{45}Ca accumulation in a VSMC primary culture (cells) compared with ^{45}Ca accumulation in cell-free conditions using media that contained either 2.0 mM P (A) or 3.0 mM P (B) and 1.8 to 2.8 mM Ca. VSMC (●) calcified minimally (monitored by alizarin red staining) and accumulated little ^{45}Ca and there was no evidence for mineral precipitation in solution over a 24-h period. In the absence of cells, extensive mineral precipitation was observed in the media (■), particularly at higher concentrations of Ca and P, resulting in ^{45}Ca accumulation. Mean \pm SD of a representative experiment (of $n = 4$ experiments using different VSMC isolates) performed in triplicate is shown. ** $P < 0.05$ (ANOVA).

extracellular Ca and P. Therefore, vesicle release was quantified from equivalent numbers of VSMC treated with Ca and/or P for 24 h. This demonstrated that vesicle release was stimulated more than twofold in the presence of extracellular Ca and Ca/P ions both in serum (not shown) and in SF conditions (Figure 4B). AB release was increased only in the absence of serum, consistent with the stimulation of apoptosis (Figure 4B).

Inhibition of Apoptosis Decreased Calcification

To test the role of apoptosis in VSMC calcification, we quantified apoptotic cells in test media in the absence of serum

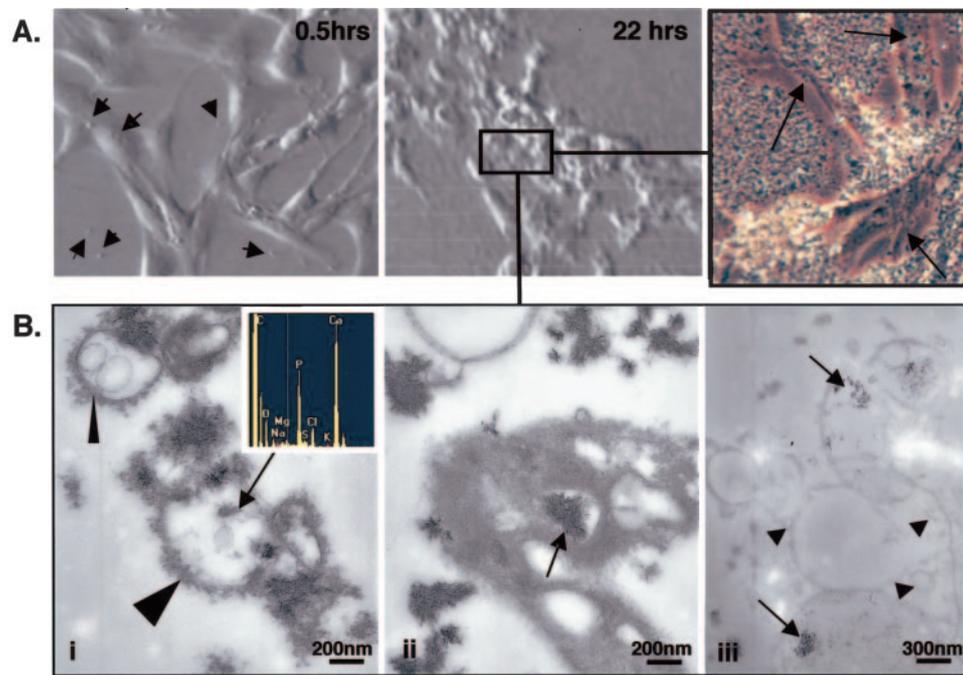


Figure 3. VSMC calcification is mediated by vesicle release. (A) VSMC in CaPi medium release vesicles (arrows, left) and calcification appears as a granular deposit on the VSMC and their surrounding matrix (middle). Trypan blue staining of calcified VSMC demonstrates that cells that are present within the calcified matrix remain viable and do not take up the dye (arrows in enlargement of boxed area in A). (B) Electron microscopy (EM) analysis of calcified VSMC showing calcification within (arrows) and on the surface of vesicles (arrowheads in i). Some calcified intracellular vesicles were observed (arrow in ii). Removal of Ca from the sections (iii) reveals “ghosts” of small (arrowheads) and larger underlying vesicles with some residual crystalline calcification (arrows), suggesting that vesicles act as the nidus for calcification. Energy dispersive X-ray analysis of vesicle content (inset) indicates basic calcium phosphate (note high Ca and P peaks), a basic Ca- and P-containing apatitic mineral.

using time-lapse videomicroscopy and TUNEL. VSMC apoptosis was markedly induced, particularly when Ca and P were added together (Figure 5A). TUNEL staining confirmed that 4 h after the addition of extracellular mineral ions, ~20% of cells were apoptotic (Figure 5B).

For measuring the contribution of apoptosis to calcification, VSMC in SF Cai or CaPi media were treated with ZVAD.fmk. ZVAD.fmk reduced incorporation of ^{45}Ca into VSMC by 20 to 30% (Figure 5C). However, the pattern of calcification that occurred was identical to that observed in the absence of ZVAD.fmk and appeared as a granular alizarin red-positive extracellular deposit (data not shown).

Vesicle Calcification Is Increased by Ca and P

EM analysis of calcified VSMC suggested that vesicles were responsible for initiating calcification. We therefore measured the ability of isolated VSMC-derived vesicles to accumulate calcium using an *in vitro* assay. Vesicles that were released by VSMC in the absence of serum after treatment with 2.7 mM Ca or 3.0 mM P incorporated two- to threefold more ^{45}Ca than vesicles that were released at physiologic concentrations of Ca and P. Vesicles that were released in CaPi medium (2.7 mM Ca/2 mM P) concentrated 50- to 200-fold more calcium (Figure 6A). However, consistent with our previous results, vesicles that were released in the presence of serum and CaPi were suppressed in their ability to calcify (Figure 6B). In experi-

ments in which the same VSMC isolate was treated with CaPi medium in the presence or absence of serum, SF-derived vesicles consistently exhibited a significantly greater capacity to calcify than those isolated from serum-treated VSMC (Figure 6C).

To determine why vesicles that were released in CaPi medium calcified so readily, we analyzed them by EDX. This revealed that MV that were released from VSMC in physiologic concentrations of Ca and P, both in the presence and the absence of serum, contained no BCP (Table 1). In contrast, MV that were released in SF CaPi medium contained BCP, whereas those that were released in the presence of serum did not. AB did not contain BCP, suggesting that there are differences in the properties of the two vesicle populations. However, when placed in the *in vitro* calcifying buffer for 24 h, both AB and MV that were released in SF CaPi medium were able to nucleate BCP (Table 1).

MV Contain Serum- and Cell-Derived Inhibitors of Mineralization

To determine whether the reduced calcification potential of vesicles isolated from VSMC in medium that contained serum was due to the presence of serum-derived calcification inhibitors, we performed Western blots for fetuin-A in isolated vesicle populations. Fetuin-A was present at low levels in

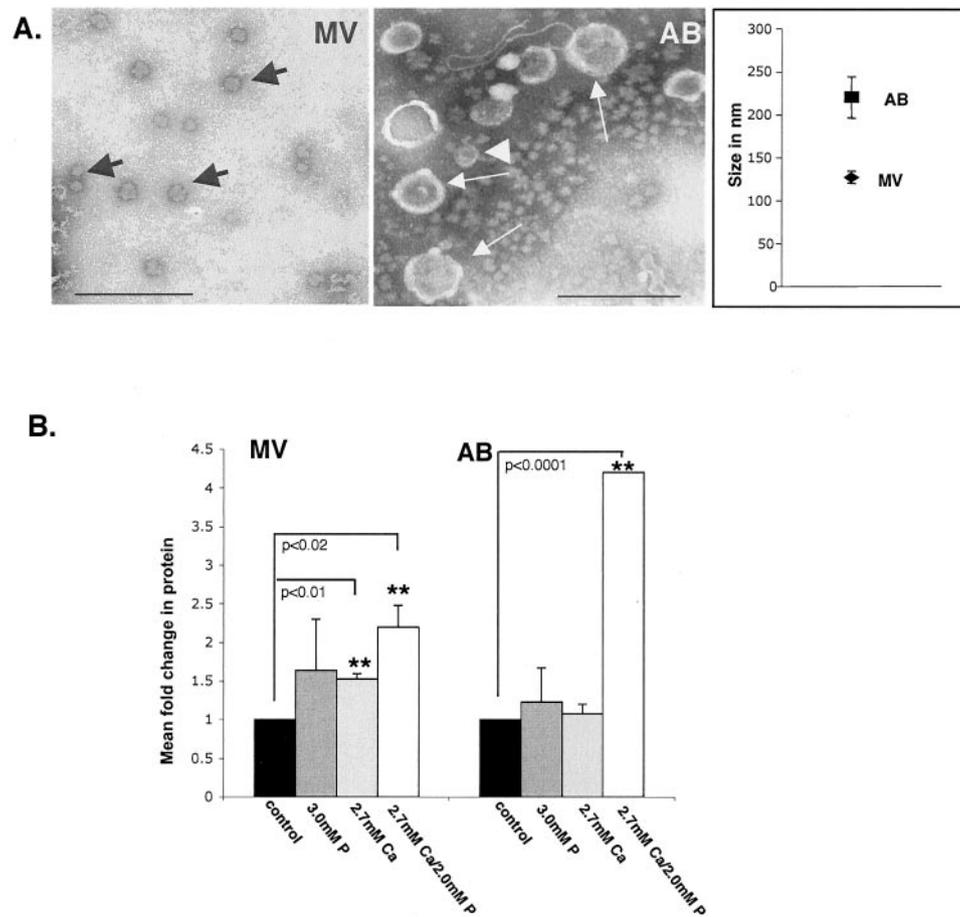


Figure 4. Ca and P induce VSMC vesicle release. (A) EM analysis of isolated vesicle populations. Differential centrifugation isolated two vesicle populations. The smaller population was uniform in size (arrows) and most likely represents matrix vesicles (MV). The population of larger vesicles were of a size consistent with being derived from apoptotic bodies (AB; white arrows). However, there was some contamination with smaller vesicle structures (arrowhead), probably isolated by “sticking” to the larger vesicles during centrifugation. Graph shows mean population sizes \pm SD ($n = 30$). Bar = 500 nm. (B) Slightly elevated extracellular Ca (2.7 mM Ca) induced increased MV release from VSMC but had no effect on AB release. CaPi media significantly increased MV release. AB release was also increased in CaPi medium but only in the absence of serum, consistent with the induction of apoptosis. Results are shown for SF conditions. Mean \pm SD shown ($n = 6$ independent experiments for each treatment analyzed by *t* test).

vesicles that were derived from VSMC in SF conditions but was highly concentrated in vesicles that were isolated in the presence of serum (Figure 7A). Thus, the association of serum-derived proteins with MV may inhibit their ability to nucleate BCP.

Our previous data showed that VSMC inhibit spontaneous BCP precipitation, and Western blot showed that the endogenous mineralization inhibitor MGP was also concentrated in VSMC-derived MV (Figure 7B). We hypothesized that inhibition of MGP γ -carboxylation by warfarin would potentiate VSMC calcification in response to increased Ca and P. Warfarin had no effect on MV release by VSMC (Figure 8B). However, it significantly increased calcification of VSMC in response to increased Ca and P at all concentrations tested (Figure 8A). MV that were released after warfarin treatment exhibited a threefold increase in their basal ability to calcify after incubation *in vitro* in calcifying buffer (Figure 8C).

Discussion

We have demonstrated that concentrations of extracellular Ca and P commonly found in serum of patients on dialysis induce VSMC calcification and that this effect is potentiated in the absence of serum. Our findings suggest that in the presence of increased P, even modest increases in Ca can substantially exacerbate calcification, which is induced by nucleation of BCP in vesicles that are released from both viable and apoptotic VSMC. Our results suggest that under normal physiologic conditions, MV do not calcify as a result of the presence of mineralization inhibitors derived from both cells and serum. However, in the presence of raised extracellular concentrations of Ca and P, if serum proteins are limiting or the action of endogenous inhibitors is compromised, then vascular damage is exacerbated, vesicle release is potentiated, and MV can nucleate BCP. Taken together, these studies suggest that “metastatic” calcification observed in patients with ESRD results

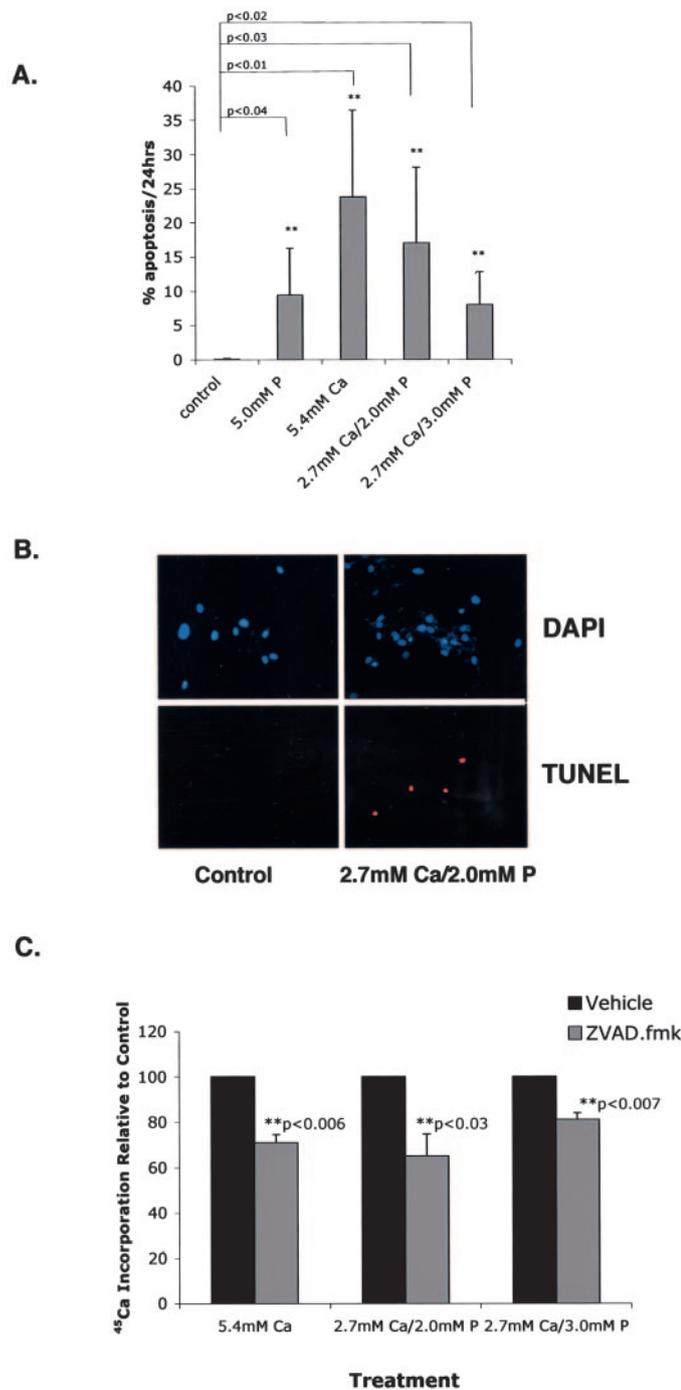


Figure 5. Ca and P induce VSMC apoptosis. (A) Ca and P alone at high concentrations and together at lower concentrations significantly induced VSMC apoptosis in SF conditions as measured by time-lapse over 24 h. The lower level of apoptosis observed in 2.7 mM Ca/3.0 mM P was due to calcification occurring before the end of the 24-h time period; therefore, apoptosis could no longer be measured. Mean \pm SD shown for $n = 3$ independent experiments for each treatment normalized to each control. $**P < 0.05$ (t test). (B) Apoptosis was further confirmed by transferase-mediated dUTP nick-end labeling (TUNEL) staining. Note the presence of TUNEL-labeled cells in standard CaPi media. (C) ZVAD.fmk significantly inhibited VSMC calcification in Cai and CaPi media in SF conditions at 24 h. Mean \pm SD of three independent experiments for each treatment shown. $**P < 0.05$ (t test).

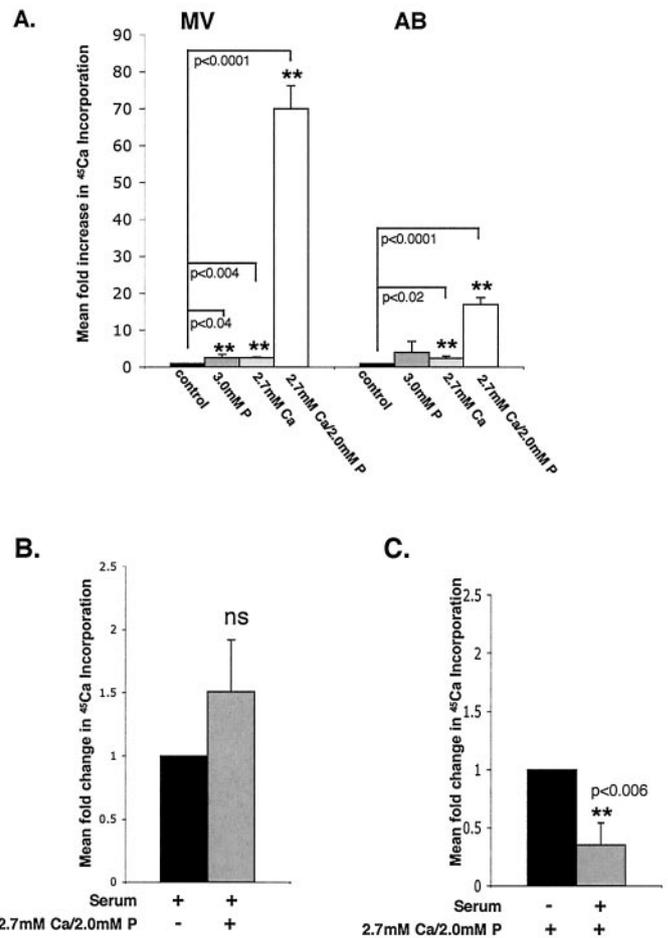


Figure 6. Ca and P increase vesicle calcification, and serum inhibits the calcification potential of MV. (A) *In vitro* calcification of both MV and AB isolated from SF media was significantly increased (2.5-fold) in response to mild Cai, whereas mild Pi increased only MV calcification by approximately the same amount. Strikingly, vesicles that were isolated from cells that were treated with CaPi media had a >70-fold increase in MV calcification and ~15-fold increase in AB calcification. Results show the mean \pm SD of $n = 5$ independent experiments. All controls were normalized to 1 to allow comparisons between treatments. $**P < 0.05$ (t test). (B) The calcification potential of MV that were isolated from cells that were treated with CaPi media in the presence of serum (5% HS) for 24 h was not significantly increased compared with untreated VSMC in normal medium (this compares to the 70-fold induction observed in SF conditions after 24 h). Mean \pm SD of $n = 4$ independent experiments. (C) The *in vitro* calcification potential of MV that were derived from VSMC that were treated with CaPi media in the presence of serum is significantly reduced compared with MV that were isolated from SF CaPi media. Mean \pm SD of $n = 3$ independent experiments shown.

from a reduced capacity of VSMC to inhibit mineralization *via* cell-mediated mechanisms.

Vesicle-Mediated VSMC Calcification

We have previously shown that apoptosis and AB release can initiate VSMC calcification *in vitro*, and abundant AB have been demonstrated in calcified atherosclerotic plaques

Table 1. EDX analysis of mineral content of VSMC-derived vesicle populations^a

Treatment	MV	AB
SF	–	–
SF + 2.7 mM Ca/2.0 mM P	+	–
5% Human serum	–	–
Serum + 2.7 mM Ca/2.0 mM P	–	–
SF + 2.7 mM Ca/2.0 mM P <i>in vitro</i>	+	+
Serum + 2.7 mM Ca/2.0 mM P <i>in vitro</i>	–	–

^a MV, matrix vesicles; AB, apoptotic bodies; SF, serum-free; Ca, calcium; P, phosphate. + indicates that BCP was present in the vesicle population isolated after treatment in the test media indicated. The energy dispersive x-ray profiles (not shown) were similar to that shown in Figure 3B and demonstrated high levels of Ca and P and some O, consistent with the presence of a calcium/phosphate-rich apatite. *In vitro* indicates the content of vesicles after 24 h of incubation in the calcifying buffer assay.

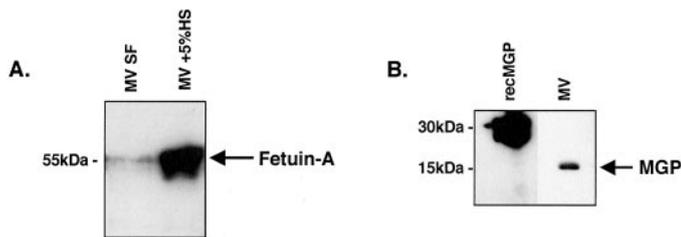


Figure 7. MV contain the calcification inhibitors fetuin-A and matrix Gla protein (MGP). (A) Western blot showing the presence of fetuin-A in VSMC-derived MV. Small amounts are seen in MV derived from SF VSMC, but the concentration is increased in MV derived from VSMC cultured in 5% HS. (B) Western blot analysis demonstrating that MGP protein is present in VSMC-derived MV. Recombinant MGP (recMGP) used as a control is fused to dihydrofolate reductase and therefore has a molecular weight of ~30 kD. MGP from vesicles is detected as a 15-kD band, slightly larger than that predicted by the amino acid sequence, *i.e.*, 12 kD, probably as a result of posttranslational modifications.

(10,25). Indeed, the concentration of Ca in atherosclerotic plaques may exceed 30 mM; therefore, the apoptosis observed in high Ca_i medium may model processes in atherosclerotic calcification (26). However, our present data clearly show that cell death is not the only mechanism responsible for VSMC calcification, because we were able to distinguish AB from MV and demonstrate that both are capable of initiating calcification, albeit by possibly different mechanisms. In our experiments, VSMC apoptosis was rare in the presence of serum, whereas MV were released by viable VSMC, particularly in the presence of elevated levels of extracellular Ca and P. Vesicle release by VSMC has been described *in vivo* in a number of conditions, including atherosclerosis, hypertension, and Ca overload induced by vitamin D₃ toxicity (27–29). It has been suggested that Ca-loaded vesicles are released from VSMC to protect against the cytotoxic effects of intracellular Ca overload (29). Our data showing that VSMC increased

vesicle release in response to extracellular Ca and remained viable within the calcified matrix supports this notion but suggests that if phagocytosis were limited, then the extracellular accumulation of these vesicles, particularly in the context of elevated Ca and P, would lead to calcification.

In chondrocytes, MV become “mineralization competent” during hypertrophic differentiation, when they are exposed to retinoic acid. A transient rise in intracellular Ca triggers the relocation of annexins II, V, and VI from the cytoplasm to the plasma membrane, where they act as Ca channels (11,22). Chondrocyte MV also contain sodium-dependent P channels and concentrate alkaline phosphatase, thereby providing an internal environment suitable for the nucleation of BCP (30). Our data suggest that in the presence of elevated Ca and P, VSMC can also release mineralization-competent MV; however, the mechanisms regulating this process in VSMC remain to be determined. There is evidence to suggest that VSMC-derived MV have similarities with those derived from chondrocytes. A previous study showed that in the presence of elevated P, VSMC calcification could be inhibited if Pit-1, a sodium-dependent phosphate transporter, were blocked, suggesting a vesicle-mediated process (14).

We demonstrated that VSMC could inhibit spontaneous precipitation of BCP. There are a number of mechanisms that may underlie this observation. Many of the bone-associated proteins expressed by osteo/chondrocytic VSMC are regulators or inhibitors, rather than nucleators of mineralization. In addition, VSMC possess a G protein–coupled cation–sensing mechanism that can induce expression of MGP in response to increased extracellular Ca (31). Results of this study suggest that MGP is produced and packaged, along with excess intracellular Ca, into vesicles that are released back into the extracellular environment. The increase in calcification seen after inhibiting the function of MGP with warfarin treatment is consistent with this mechanism, with the presence of undercarboxylated MGP in MV increasing their calcification potential (32). Furthermore, calcification progressed much faster once BCP had been nucleated, and MV produced in CaPi conditions already contained BCP. Thus, accelerated vascular matrix calcification will result if endogenous inhibitors of calcification are either impaired or absent from MV.

Serum Contains Inhibitors of VSMC Calcification

Previous *in vitro* studies showing accelerated calcification of bovine VSMC in uremic HS were interpreted as demonstrating the presence of potentiators of calcification (33). However, an alternative interpretation is that uremic serum lacks calcification inhibitors. We found that both VSMC calcification and isolated vesicle calcification were inhibited by normal HS and that MV contain the potent serum-derived calcification inhibitor α_2 -Heremans Schmid glycoprotein/fetuin-A. Fetuin-A is produced in the liver, binds Ca and P in extracellular fluids, and can occur within cells, and knockout mice develop extensive soft tissue calcification (15,34). Circulating levels of fetuin-A are substantially reduced in patients with ESRD and correlate inversely with cardiovascular mortality (3,15). Our study shows that fetuin-A was concentrated in MV derived

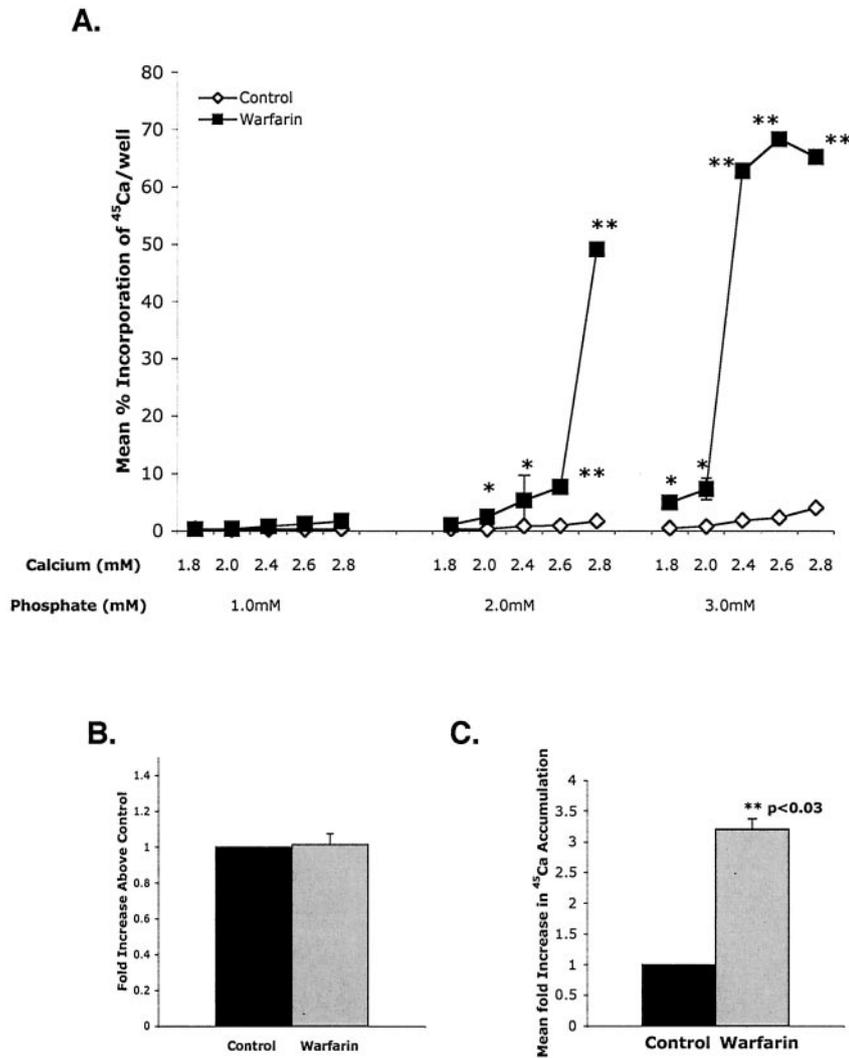


Figure 8. Warfarin accelerates VSMC calcification and increases the calcification potential of MV. (A) Pretreatment of VSMC with warfarin significantly increased ⁴⁵Ca incorporation into VSMC cultures in response to extracellular mineral ions. Mean ± SD of an experiment performed in quadruplicate shown. **P* < 0.05 and ***P* < 0.001 (ANOVA). (B) Treatment with warfarin did not increase MV release from VSMC. (C). MV released from warfarin-treated VSMC calcified *in vitro* three times more potently than control MV from untreated cells. Mean ± SD of *n* = 3 independent experiments shown (*t* test).

from VSMC in normal serum. Thus, calcification in ESRD may be due to the combined effect of an elevated Ca × P in the context of reduced levels of serum inhibitory proteins, leading to increased apoptosis and vesicle calcification. Investigation of the effects of individual serum proteins on VSMC calcification/phenotype are now required.

Clinical Implications

Our *in vitro* model suggests that, in the context of raised P, VSMC calcification is extremely sensitive to elevations in serum Ca and that even transient increases in Ca would lead to release of vesicles that contain high concentrations of both Ca and P from stressed or dying VSMC. In the context of reduced levels of circulating inhibitors such as fetuin-A, this would lead to matrix mineralization. Calcification would be further exacerbated if the activity of local inhibitors, such as MGP, were reduced. Our data therefore provide a cell biologic explanation for the observed effects of warfarin on the progression of calciphylaxis in some patients with ESRD (3,32,35). They may also explain some of the observed effects of Ca channel blockers and drugs that reduce serum Ca levels on calcification (36). Blockade of L-type Ca channels prevents VSMC Ca overload,

vesicle release, and calcification in animal models of hypertension and vitamin D₃ toxicity (29). It also inhibits the progression of vascular calcification in hypertensives and improves outcome in ESRD (37–39). Perhaps the most important observation is the pivotal role played by Ca in the context of raised P. However, it will be important to determine how raised Ca and P in this model system equates to a serum electrolyte imbalance in uremia. Thus, further experiments incorporating uremic serum into the model are warranted but beyond the scope of the present study (33). Despite these limitations, our findings do suggest that greater attention should be paid to preventing elevation in serum Ca than has traditionally been the case and that strategies aimed at enhancing endogenous and circulating inhibitors of calcification may have therapeutic benefits.

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