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Chemical and hormonal determinants of vascular calcification *in vitro*

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Vascular calcification is a complex process that is dependent not only on the physicochemical effects of Ca, PO_4, and pH, but also on smooth muscle factors that may be regulated by these ions as well as by 1,25-dihydroxyvitamin D_3 (calcitriol) and parathyroid hormone (PTH). These minerals and hormones were tested in a model of medial calcification in rat aorta maintained in culture for 9 days. Calcification was quantitated as incorporation of ^{45}Ca, alkaline phosphatase activity was measured in aortic homogenates, and osteopontin production was measured from immunoblots of culture medium. At 1.8 mM Ca (1.46 mM free), calcification occurred at or above 2.8 mM PO_4. At 3.8 mM PO_4, calcification occurred at or above 1.10 mM free [Ca]. At a constant [Ca] × [PO_4], calcification varied directly with [Ca] and inversely with [PO_4]. Calcification was directly related to pH between 7.19 and 7.50 but not altered by PTH or calcitriol. Alkaline phosphatase activity and osteopontin production were increased by Ca, PO_4, calcitriol, and PTH. We conclude that calcification of rat aorta *in vitro* requires elevation of both [Ca] and [PO_4], and that [Ca] rather than [PO_4] or the product of the two is the dominant determinant. The induction of alkaline phosphatase and osteopontin indicates that Ca and PO_4 have effects in addition to simple physicochemical actions. Although PTH and calcitriol did not increase calcification *in vivo*, they have effects on smooth muscle that could influence calcification *in vivo*. Calcification is enhanced by alkalinity within the range produced during hemodialysis.

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Vascular calcification is extremely common in patients with advanced renal failure or end-stage renal disease,[1] and may contribute to their elevated risk of cardiovascular disease. Although calcification can be associated with atherosclerosis in these patients, the deposition of hydroxyapatite is primarily medial rather than intimal, and is not associated with inflammation.[2,3] The pathophysiology of medial calcification is complex, involving not only physicochemical factors but also biologic actions in smooth muscle. Physicochemical considerations are the basis for the clinical use of the calcium–phosphorus product (Ca × P). Although the serum Ca × P is associated with soft tissue calcification,[4] coronary artery calcification,[1] and cardiovascular mortality[5] in hemodialysis patients, there are no data showing a causative role in vascular calcification. The principal biologic effect of smooth muscle is the production of inhibitors of calcification. Humans lacking an enzyme that produces extracellular pyrophosphate, a known inhibitor of hydroxyapatite formation,[6–8] develop severe medial vascular calcification in childhood,[9,10] indicating that calcification can occur at normal calcium and phosphate levels in the absence of inhibition. We have recently confirmed the inhibitory role of smooth muscle pyrophosphate in rat aortas *in vitro*.[11] Protein inhibitors have been described as well. Mice lacking matrix Gla protein and rats treated with warfarin to prevent its γ-carboxylation develop severe medial calcification,[12,13] although humans lacking this protein (Keutel syndrome) do not develop prominent calcification.[14] Mice deficient in osteoprotegerin also develop medial calcification,[15] and osteopontin inhibits calcification in cultured smooth muscle cells.[16,17] Although deficiency of osteopontin does not lead to vascular calcification in mice, it does worsen calcification in mice lacking matrix Gla protein.[18] An additional biologic action that may promote calcification is osteogenic differentiation of smooth muscle.[19]

Recent data suggest that biologic effects of smooth muscle on calcification may be governed by systemic mineral metabolism. In cultured vascular smooth muscle cells, both Ca and PO_4 increase alkaline phosphatase activity (which hydrolyzes pyrophosphate) and osteopontin production,[16,20] and phosphate induces osteoblastic differentiation factors such as osteocalcin and Cbfa-1.[21] High doses of vitamin D_3 induce medial vascular calcification in rats,[22] and calcitriol enhances calcium deposition in cultured smooth muscle

cells.[23] Parathyroid hormone (PTH) at high concentrations inhibits calcification of vascular smooth muscle cells in culture.[24]

In vitro studies of vascular calcification have been performed almost exclusively in cultured smooth muscle cells, but this model is limited by the substantial phenotypic changes these cells undergo in culture and the fact that they lack elastin, the site of medial calcification *in vivo*.[25] Thus, it is not clear that these cells accurately reflect the pathophysiology of medial vascular calcification and there are no data showing a direct role for Ca, PO_4, calcitriol, or PTH in the calcification of intact vessels. To address this, we recently developed an *in vitro* model of vascular calcification in rat aortas during long-term culture under conditions that maintain viability and normal histology and prevent apoptosis.[11] In the presence of high calcium and phosphate concentrations along with alkaline phosphatase to remove inhibitory pyrophosphate, medial deposits of hydroxyapatite develop along elastic lamina in a pattern similar to that observed in vessels from patients with chronic renal failure.[11] Using this model, we examined the effect of Ca, PO_4, pH, calcitriol, and PTH on the rate of calcification and the induction of proteins related to vascular calcification.

RESULTS

Figure 1 shows a von Kossa stain of an aortic ring cultured for 9 days in the presence of alkaline phosphatase and a high phosphate concentration. This degree of calcification is observed in cultured aortas when the calcium content is roughly 500 nmol/mg dry weight or greater, and contents below 50 nmol/mg are usually not visible on Von Kossa staining. To determine the dependence of calcification on the concentration of phosphate, incorporation of ^{45}Ca was measured at different phosphate concentrations with the calcium concentration maintained constant at 1.8 mM, the concentration normally present in Dulbecco's modified Eagle's medium (DMEM) (Figure 2). There was a very small incorporation of 45Ca into aortas cultured in 0.9 mM PO_4, the concentration normally present in DMEM. We have previously shown that this incorporation occurs in the first day and represents equilibration with Ca normally present in the vessel wall.[11] This incorporation began to increase when $[PO_4]$ reached 2.7 mM and increased substantially thereafter. The measured concentrations of ionized Ca at the lowest and highest $[PO_4]$ were 1.46 and 1.21 mM. The dependence of calcification on calcium concentration was assessed by varying the concentration of calcium at a constant phosphate concentration of 3.8 mM (Figure 3). Because the concentration of Ca is already high in DMEM, a medium similar to DMEM was reformulated from components to yield a reduced [Ca] of 1.33 mM. No calcification occurred at a [Ca] of 1.33 or 1.49 mM but calcification increased substantially starting at 1.65 mM (1.10 mM free [Ca]). Calcification did not occur at physiologic concentrations of PO_4 (0.9 mM) or Ca (0.99 mM free) despite maximal concentrations of the other ion. To determine whether the effects of Ca and PO_4 were governed by the product of their concentrations, the concentrations were varied inversely to maintain a constant product of 6.84 $mmol^2/l^2$. As shown in Figure 4, calcification varied directly with [Ca] and inversely with $[PO_4]$, with only basal calcium incorporation at the highest $[PO_4]$ and lowest [Ca] and extensive calcification at the highest [Ca] and lowest $[PO_4]$.

Because vascular calcification in uremia may be dependent on vessel injury and circulating factors other than Ca and PO_4, these experiments were repeated in the presence of uremic plasma or after vessel injury. The same dependence

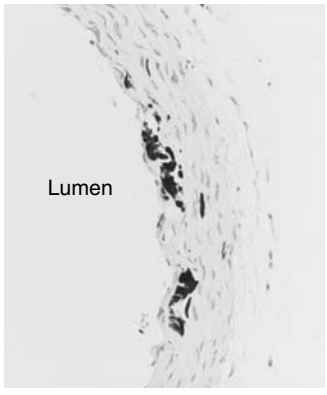

Figure 1 | Calcification of rat aorta cultured for 9 days in DMEM containing 3.8 mM PO_4 and alkaline phosphatase. Von Kossa stain shows dark staining of calcium phosphate in the media.

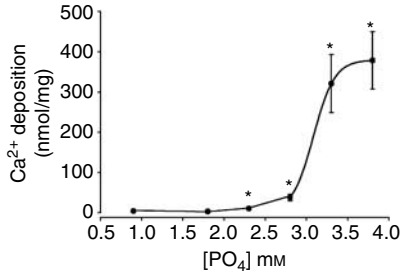

Figure 2 | Effect of phosphate concentration on calcification of rat aortas in culture. Calcium concentration was 1.8 mM. Values are the means of 3–8 individual aortic segments. Error bars: s.e. *$P < 0.001$ vs 0.9 mM phosphate.

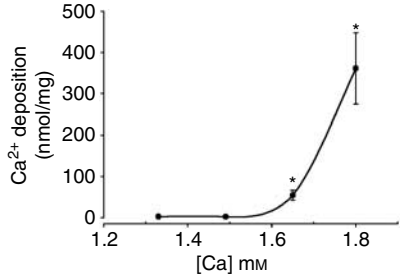

Figure 3 | Effect of calcium concentration on calcification of rat aortas in culture. Phosphate concentration was 3.8 mM. Values are the means of 4–6 individual aortic segments. Error bars: s.e. *$P < 0.001$ vs 1.33 mM calcium.