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Phosphate-Induced Vascular Calcification: Role of Pyrophosphate and Osteopontin

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Abstract. Hyperphosphatemia is thought to underlie medial vascular calcification in advanced renal failure, but calcification can occur in other conditions in the absence of hyperphosphatemia, indicating that additional factors are important. To identify these factors, a model of medial calcification in rat aorta *in vitro* was developed. Aortic rings from rats were incubated in serum-free medium for 9 d, and calcification was measured as incorporation of ^{45}Ca and confirmed by histology and x-ray diffraction. No calcification occurred in normal vessels despite elevated free Ca^{2+} and PO_4^{3-} concentrations of 1.8 mM and 3.8 mM, respectively, but mechanical injury resulted in extensive calcification in the media. Co-incubation studies revealed that normal aortas produced a soluble inhibitor of calcification in injured vessels that was destroyed by alkaline phosphatase. Culture of normal aortas with alkaline phosphatase resulted in calcification of the elastic lamina identified as hydroxyapatite by x-ray diffraction. This effect of alkaline phosphatase was not due to dephosphorylation of osteopontin (OPN), and calcification was not increased in aortas from OPN-deficient mice. The inhibitor was identified as pyrophosphate on the basis of the calcification induced in aortas cultured with inorganic pyrophosphatase, the inhibition of calcification in injured aortas by pyrophosphate, and the production of inhibitory levels of pyrophosphate by normal aortas. No calcification occurred under any conditions at a normal PO_4^{3-} concentration. It is concluded that elevated concentrations of Ca^{2+} and PO_4^{3-} are not sufficient for medial vascular calcification because of inhibition by pyrophosphate. Alkaline phosphatase can promote calcification by hydrolyzing pyrophosphate, but OPN is not an endogenous inhibitor of calcification in rat aorta.

Arterial calcification is common in patients with advanced renal failure and ESRD and is thought to contribute to their increased cardiovascular mortality (1). Two distinct forms of calcification are recognized (2,3). Intimal calcification occurs in atheromatous disease and is associated with inflammatory cells (3), whereas medial calcification occurs in the matrix between smooth muscle cells in the absence of atherosclerosis and inflammatory cells (2,4). Medial calcification commonly occurs in advanced renal failure (4,5), where it is thought to result from plasma concentrations of Ca^{2+} and PO_4^{3-} that exceed the solubility product for calcium phosphate. However, medial calcification is also seen in diabetes and with aging in the presence of normal serum Ca^{2+} and PO_4^{3-} concentrations (6), indicating that hyperphosphatemia is not required for medial calcification.

Considerable data suggest that vascular calcification is a spontaneous event, even at normal calcium and phosphate concentrations, that is prevented by inhibitory factors within the vessel wall. Several proteins have been implicated in this process. Mice deficient in matrix Gla protein (MGP) develop rapid and severe medial calcification (7), and a similar phenotype is seen in rats that are treated with warfarin to inhibit γ-carboxylation of MGP (8). Osteopontin (OPN), which is abundant at sites of medial calcification (2,9), inhibits hydroxyapatite crystallization *in vitro* (10) and calcification in cultured vascular smooth muscle cells (11,12). Although deficiency of OPN in mice does not lead to vascular calcification, it does accelerate calcification in MGP-deficient mice (13). Osteoprotegerin (14,15) and fetuin (16,17) are additional proteins implicated in the inhibition of ectopic calcification.

Elucidation of the pathophysiology of medial calcification has been hampered by the lack of an appropriate *in vitro* model. Cultures of vascular smooth muscle cells lack the architecture and matrix of a normal vessel. The rapid conversion of these cells to a proliferative, secretory phenotype and the use of growth factors are also problematic. To address this, we developed a model in intact vessels during long-term culture that allowed us to examine calcification both histologically and quantitatively. Using this model, we identified specific properties of vessels and medium that influence medial calcification.

Materials and Methods

Aortic Culture

Aortas (from the arch to the renal arteries) were removed in a sterile manner from male Sprague-Dawley rats that weighed 150 to 300 g. After most of the adventitia was gently removed by careful

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dissection, the vessels were cut into 2- to 3-mm rings and placed in culture medium for up to 9 d. We previously used this technique to demonstrate aldosterone-responsiveness of smooth muscle potassium fluxes for up to 7 d (18), indicating that viability is maintained in culture. Entire removal of adventitia before culture resulted in variable degrees of calcification, presumably as a result of smooth muscle injury. Some aortas were purposely injured by rubbing the abluminal surface 30 times with a cotton swab. In some vessels, selective removal of endothelium was accomplished by twirling a loop of 3-0 nylon monofilament suture in the lumen (19).

Culture Media

Aortic segments were placed into DMEM (Mediatech, Herndon, VA) with penicillin and streptomycin but no other additives and maintained at 37°C in a 5% CO_2 atmosphere with medium changes every 3 d. Serum was not added to culture medium because it caused outgrowth and proliferation of smooth muscle cells (not shown) and others have reported adverse effects of serum on cultured vessels (20). DMEM contains 1.8 mM Ca^{2+} and 0.9 mM PO_4^{3-}. The [PO_4^{3-}] was increased to 3.8 mM by adding 3 vol of 100 mM NaH_2PO_4 (unbuffered) to 100 vol of DMEM that had been equilibrated with 5% CO_2. The high-PO_4^{3-} medium was maintained in 5% CO_2 to prevent alkalinization and possible precipitation of calcium phosphate. The absence of precipitation was confirmed by measuring $^{45}Ca^{2+}$ concentration before and after centrifugation. Final free concentrations of Ca^{2+} in the low- and high-PO_4^{3-} media were 1.76 and 1.63 mM as calculated by CaBuffer software on the basis of the PO_4^{3-} concentrations and the concentration of Mg^{2+} (0.81 mM) and glycine (0.4 mM) in DMEM. Assuming that 43% of total Ca in human serum is ionized (21), these concentrations are equivalent to total calcium concentrations in human serum of 16.4 and 15.2 mg/dl. The serum phosphorus concentrations that correspond to the normal and high-phosphate DMEM are 2.7 and 11.4 mg/dl. Some media were supplemented with 7.5 U/ml calf intestinal alkaline phosphatase (Promega, Madison, WI) or 1.2 to 12 U/ml inorganic pyrophosphatase from Bakers' yeast (Sigma Diagnostics, St. Louis, MO). Conditioned medium was prepared by incubating four to five rings in 500 μl of normal DMEM for 3 d. Some conditioned medium was either filtered through a 10,000 molecular weight cut-off filter (Centricon, Amicon Corp.) or dialyzed against DMEM overnight at 100:1 with one change of the dialysate using a Spectra/Por membrane, 6000 to 8000 molecular weight cut-off (Spectrum Medical Industries, Los Angeles, CA) before use. Phosphate was added just before the use of the medium in culture.

Calcification Assay

Approximately 0.3 μCi/ml ^{45}Ca (DuPont-NEN, Boston, MA) was added to the culture medium, and at the end of the culture the aortic rings were washed five times in a HEPES-buffered (pH 7.4) physiologic salt solution that contained 1.8 mM Ca^{2+} and 0.9 mM PO_4^{3-}. The rings were then dried in an oven, weighed, and then dissolved in equal volumes of 70% H_2O_2 and 60% $HClO_4$, and radioactivity was measured by liquid scintillation. Results are expressed as nanomoles calcium per milligram of tissue.

Tissue Analyses

For histologic analysis, samples were placed in formalin and processed for paraffin embedding. Hematoxylin and eosin staining, von Kossa staining (silver nitrate plus nuclear fast red), and toluidine blue staining were according to standard protocols. For viability staining, aortic segments were incubated with 0.5 mg/ml methylthiazoletetrazolium (MTT) in DMEM for 3 h at 37°C and washed in physiologic saline three times and then embedded for frozen sectioning. For x-ray diffraction, a small piece of dried aorta was mounted on the end of a glass fiber, and various rotation frames were taken with a Bruker D8 x-ray diffractometer at 23°C using a SMART 1000 CCD detector and monochromatized CuKα radiation. The frames were processed, analyzed, and compared with standard samples by using GADDS-NT V4.0 software (Bruker AXS, Madison, WI).

Alkaline Phosphatase

Alkaline phosphatase was measured colorimetrically as the hydrolysis of p-nitrophenyl phosphate according to instructions from the supplier (Sigma Diagnostics). Aortas were homogenized with 1% Triton X-100 in 0.9% saline on ice and centrifuged in a microfuge at maximum speed for 5 min. Supernatant was removed for assay.

OPN

Immunoblots were performed after separation on a 10% SDS polyacrylamide gel and blotting onto PVDF membranes, using a mouse monoclonal antibody (MPIIIB10_1) created by M. Solursh and A. Franzen and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biologic Sciences (Iowa City, IA). Electrophoresis under nondenaturing conditions was performed on 6% polyacrylamide without SDS and mercaptoethanol. Rat recombinant OPN was produced as a hexahistidine fusion protein from cDNA (provided by Dr. Magnus Hook, Institute of Bioscience and Technology, Texas A&M University). For analysis of OPN mRNA, total RNA was extracted from aortas by the phenol-chloroform method, separated on a 1% agarose gel, and probed with rat OPN cDNA (obtained from Dr. Robert Taylor, Emory University). OPN-deficient mice were provided by Dr. Lucy Liaw (Maine Medical Center Research Institute).

Pyrophosphate

Measurement of pyrophosphate was performed enzymatically (22). Briefly, culture medium was incubated with [^{14}C]uridinediphosphoglucose (UDPG; DuPont-NEN) and UDPG pyrophosphorylase, phosphoglucomutase, glucose-6-phosphate dehydrogenase, and NADP. Production of [^{14}C]6-phosphogluconic acid was measured after removal of [^{14}C]UDPG with activated charcoal. Analysis of pyrophosphate hydrolysis was by thin layer chromatography using [^{32}P]pyrophosphate (23).

Statistical Analyses

Data are presented as means ± SE. Significance was determined by *t* test.

Results

The histology of aortic rings maintained in DMEM for 9 d is shown in Figure 1. Hematoxylin and eosin staining (Figure 1A) revealed normal-appearing smooth muscle with intact endothelium. For assessing cell viability, vessels were incubated with MTT, a technique commonly used to assess cell viability (24). This compound is converted by mitochondria to an insoluble compound that precipitates and thus stains viable cells. As shown in Figure 1B, all of the smooth muscle cells between the elastic lamina were stained, and this did not differ from fresh aortas (Figure 1C), indicating that all of the smooth

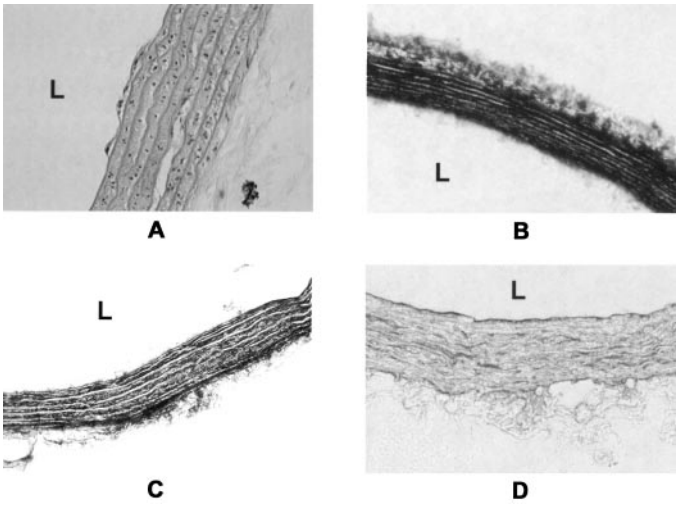

Figure 1. Histology of rat aortas after culture for 9 d in standard medium. (A) Hematoxylin and eosin stain. (B) Cultured aorta stained with methylthiazoletetrazolium (MTT; 10-μ section). (C) Freshly isolated aorta stained with MTT (10-μ section). (D) Aorta heated at 65°C for 10 min before staining with MTT (10-μ section). L, vessel lumen. Magnifications: ×400 in A, ×200 in B and C, ×300 in D.

muscle cells were viable after 9 d in culture. Identical results were obtained in vessels cultured in high-phosphate medium, and electrophoresis of DNA from 9-d cultures revealed no fragmentation of DNA indicative of apoptosis (not shown). By comparison, there was no staining of aortas that were heated at 65°C for 10 min (Figure 1D). Despite the high concentrations of calcium and phosphate in the medium, no calcification on was observed by von Kossa staining after 9 d (Figure 2A), and staining remained negative in aortas that were cultured for up to 21 d (not shown). However, extensive staining was visible after 9 d in aortas that were purposely injured by rubbing the abluminal surface before culture (Figure 2B). With this injury, ~20% of smooth muscle cells did not stain with MTT (not shown), and higher magnification revealed calcification of both cells and elastic laminae (Figure 2C). This was not due to loss of endothelium since selective removal of endothelium before culture did not result in calcification (not shown).

Calcification was quantitated as incorporation of ^{45}Ca from the medium (Figure 3). Because some calcification of residual adventitia occurred, it was removed before ^{45}Ca content was measured so that only medial calcification was measured. To ensure that all adventitia was removed, this was performed with a dissecting microscope and confirmed by histology (not shown). Consistent with the negative von Kossa stain, there was very little incorporation of ^{45}Ca into normal aortas cultured in high-phosphate DMEM. All of this was incorporated in the first day, and cultures up to 21 d showed no additional incorporation (not shown). In contrast, calcium incorporation into injured vessels showed a steady increase over time (Figure 3A). Basal incorporation into uninjured aortas decreased slightly but significantly when [PO_4^{3-}] was decreased to 0.9 mM (Figure 3B). Addition of 10 mM β-glycerophosphate instead of phosphate, which induces calcification in cultures of

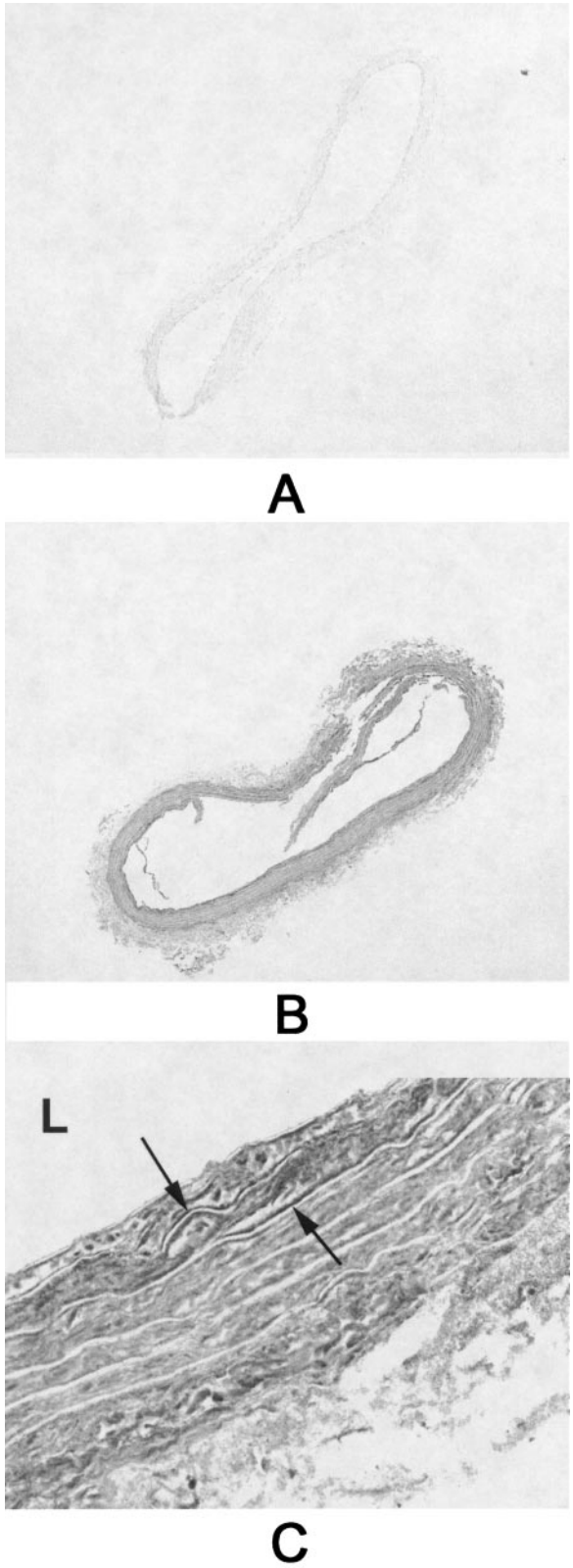

Figure 2. Calcification of cultured rat aortas detected by von Kossa staining. There was no staining of normal aortas after 9 d in culture (A) but diffuse staining of injured aortas (B). Higher magnification (C) revealed diffuse staining of the cells as well as linear staining of the elastic lamina between cells (arrows). Magnifications: ×40 in A and B, ×400 in C.

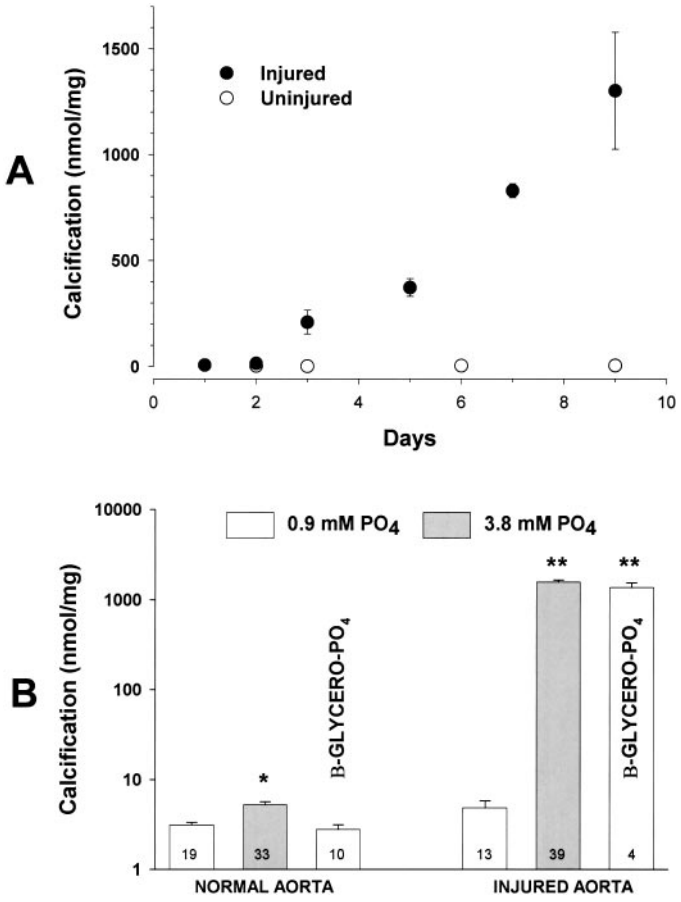

Figure 3. Incorporation of ^{45}Ca into aortic rings. (A) Time course in injured aortas (●) and uninjured aortas (○) in DMEM containing 1.8 mM Ca^{2+} and 3.8 mM PO_4^{3-}. Results are the means of at least five (injured) or four (uninjured) aortic rings. (B) Calcification under different conditions. Aortic rings were incubated for 9 d in DMEM containing 1.8 mM Ca^{2+} and either 0.9 or 3.8 mM PO_4^{3-} as indicated. The concentration of β-glycerophosphate was 10 mM. Aortas were injured by rubbing the abluminal surface. Numbers at bottom of bars indicate the number of aortic segments studied. $^{*}P < 0.001$ *versus* 0.9 mM PO_4^{3-}; $^{**}P < 0.001$ *versus* uninjured aorta in 3.8 mM PO_4^{3-}.

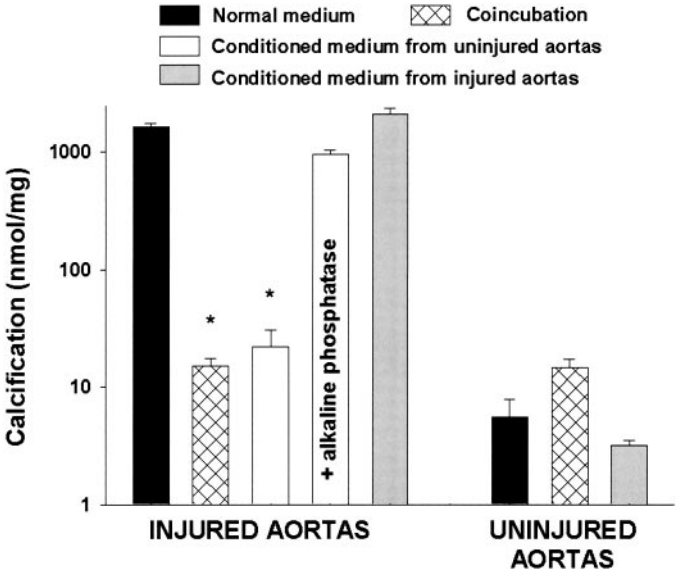

Figure 4. Effect of co-incubation and conditioned medium on aortic calcification in high-PO_4^{3-} medium. For control incubations of normal or injured aortas, single rings were incubated in 500 μl of medium. For co-incubations, single injured or uninjured rings were cultured with four uninjured or injured rings, respectively. Single rings were incubated in 500 μl of conditioned medium prepared by incubating four to five rings in 500 μl of normal DMEM for 3 d. Culture of injured aortas with uninjured aortas or medium from uninjured aortas prevented calcification, and this soluble factor was destroyed by addition of 7.5 U/ml alkaline phosphatase. Results are means of at least five aortic rings. $^{*}P < 0.001$ *versus* normal medium.

smooth muscle cells (25), did not increase Ca incorporation in normal aortas. Calcium incorporation increased >100-fold in injured aortas but only when the phosphate concentration was elevated or β-glycerophosphate was present. The slight increase in calcium incorporation in injured aortas compared with uninjured aortas in 0.9 mM [PO_4^{3-}] was NS.

Calcification of injured aortas was substantially reduced when they were co-incubated with uninjured aortas or incubated in conditioned medium from uninjured aortas (Figure 4), suggesting that a soluble inhibitor of calcification is released by normal aorta. There was no significant calcification of uninjured aortas when they were co-incubated with injured aortas or incubated with conditioned medium from injured aortas. Inhibition of calcification by conditioned medium was reversed by adding alkaline phosphatase, suggesting that the inhibitor was a phosphorylated compound. Addition of alkaline phosphatase during culture also induced calcium incorporation into uninjured aortas (Figure 5A), indicating that this inhibitor also prevents calcification under normal conditions. X-ray diffraction patterns from alkaline phosphatase–treated aorta and bone are shown in Figure 5B. The location and intensity of the rings, particularly the inner two, are identical and consistent with hydroxyapatite. However, these data do not rule out the presence of additional forms of calcium phosphate.

Staining of aortas cultured with alkaline phosphatase revealed medial calcification (Figure 6A), and under higher power, the von Kossa stain showed linear staining of the elastic lamina between the smooth muscle cells (Figure 6B). To identify the initial site of calcification, we examined earlier stages of calcification by culturing aortas for 6 d or in a lower phosphate concentration (2.8 mM). When silver nitrate staining was repeated after incubation of alkaline phosphatase–cultured aortas with MTT (Figure 6C), calcification was clearly visible in the elastic lamina between viable cells. Localization of the staining to elastic lamina is also apparent after staining with toluidine blue (Figure 6D).

OPN production was investigated to determine whether it was responsible for the inhibition of calcification and the effect of alkaline phosphatase. OPN mRNA was not detected in freshly isolated aortas but appeared during culture in normal DMEM (Figure 7A), suggesting transcriptional induction.

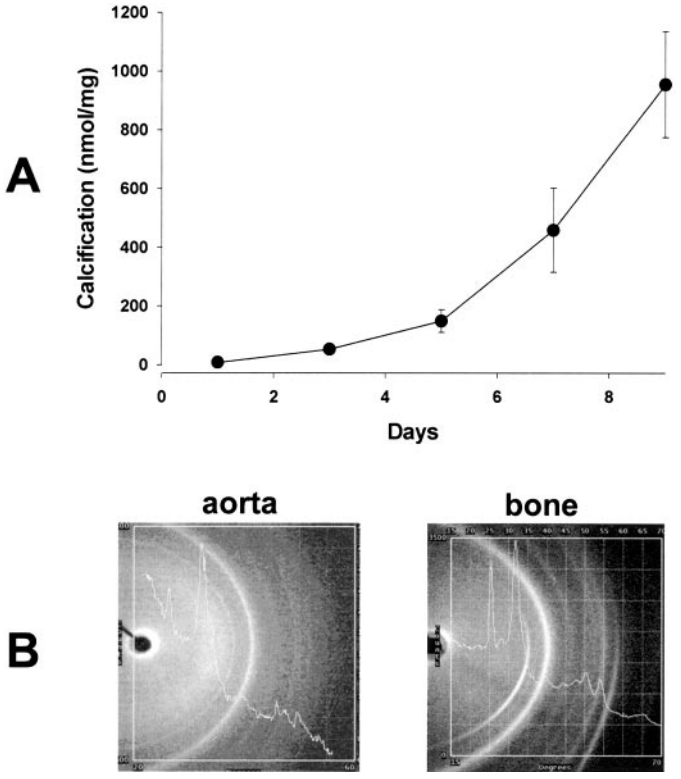

Figure 5. Induction of calcification by alkaline phosphatase. (A) Time course. Normal aortas were incubated in DMEM containing 3.8 mM PO_4^{3-} and 7.5 units/ml calf intestinal alkaline phosphatase with medium changes every 3 d. Adventitia was removed before measuring ^{45}Ca incorporation. Results are means of at least six aortic segments. (B) X-ray diffraction after 9 d.

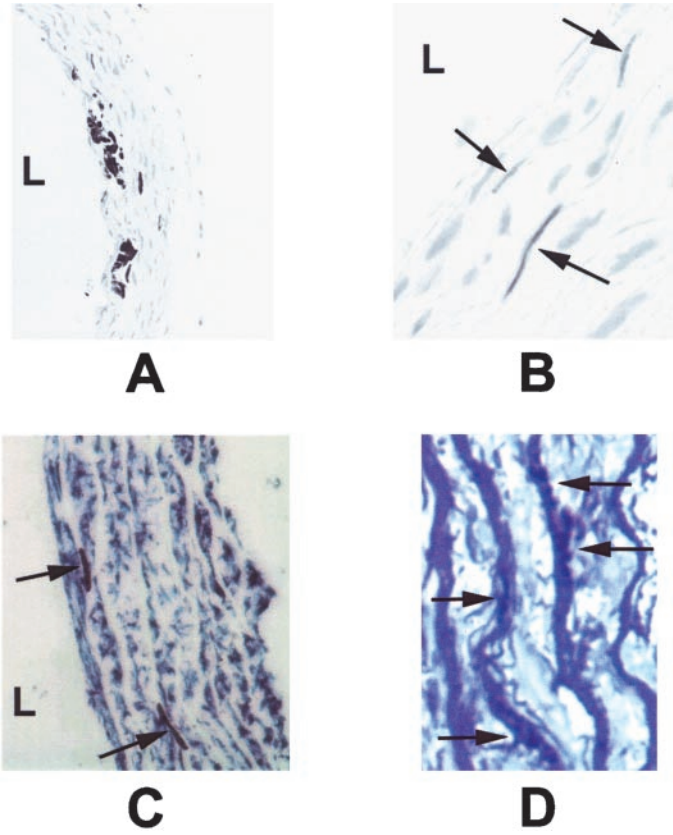

Figure 6. Histology of normal aortas cultured with alkaline phosphatase as described in the legend of Figure 5. (A) von Kossa stain showing scattered foci of calcification as well as aggregates. (B) Enlargement of a ×400 image of a von Kossa stain showing linear calcification (arrows) localized to the elastic laminae between cells. (C) Silver nitrate stain performed after incubation with MTT (9-d culture, 2.8 mM PO_4). The calcified elastic laminae (stained brown, arrows) are surrounded by viable cells (stained black). (D) Toluidine with MTT blue stain of elastic laminae shows areas of dark staining consistent with calcification in the laminae, without any calcification of the cells between the laminae (6-d culture, 3.8 mM PO_4). Magnifications: ×200 in A, ×400 in B through D.

Likewise, there was very little OPN production (measured in the medium) by freshly isolated aortas, but this increased substantially during culture (Figure 7B). OPN was also produced by injured aortas (Figure 7C), and the rate, as judged by densitometry, was similar to that for uninjured aortas (uninjured: 734 ± 104 ng/mg per d; injured: 741 ± 119 ng/mg per d). On nondenaturing polyacrylamide gels (Figure 7D), OPN in culture medium migrated faster than rat recombinant OPN, and this faster migration was eliminated by pretreatment with alkaline phosphatase, indicating that all of the OPN produced by normal and injured aorta was phosphorylated.

Inhibition of calcification by conditioned medium persisted after filtration to exclude proteins >10 kD in size and was removed by dialysis using a membrane with a size exclusion of 6 to 8 kD (Figure 8A). Immunoblotting revealed that OPN, which is 32 kD, was retained by the dialysis membrane and not present in the filtrate (Figure 8B). This was not consistent with OPN as the inhibitor of calcification in conditioned medium.

Pyrophosphate was also investigated as a possible inhibitor of calcification. It is not present in DMEM, but its concentration after 3 d of culture was 0.44 ± 0.03 μM (one ring in 500 μl of medium), indicating that it was produced by aortas. These measurements were made in normal DMEM to avoid sequestration of pyrophosphate in calcium phosphate deposits (26). Elimination of pyrophosphate by adding inorganic pyrophosphatase induced calcification of normal aortas (Figure 9A). This concentration of pyrophosphatase resulted in complete disappearance of [^{32}P]pyrophosphate from the culture medium (Figure 9B) but did not dephosphorylate OPN (Figure 9C). Von Kossa staining (Figure 9, D and E) revealed extensive calcification of the media, again with calcification of elastin fibers.

Addition of pyrophosphate prevented calcification in injured aortas (Figure 10), confirming that pyrophosphate inhibits medial calcification. There was no inhibition with 2.5 μM but almost complete inhibition with 10 μM pyrophosphate. On the basis of the rate of hydrolysis of [^{32}P]pyrophosphate in aortic cultures (not shown), the estimated concentrations 3 d after adding 5, 10, and 30 μM pyrophosphate were 1.8, 3.1, and 7.9 μM, respectively. Thus, pyrophosphate is actually a more potent inhibitor of calcification than indicated in the Figure 10.

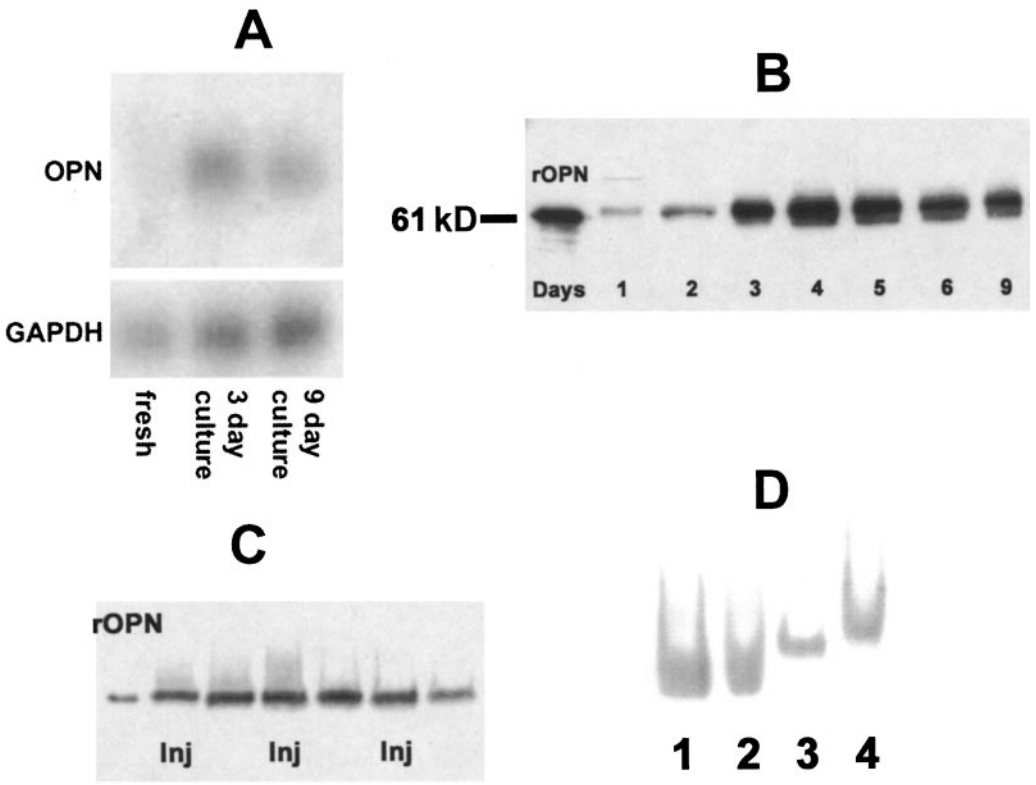

Figure 7. Osteopontin (OPN) production in cultured rat aorta. (A) Northern blot of mRNA from cultured rat aorta using probe against rat OPN probes and mouse glyceraldehyde-3-phosphate dehydrogenase. (B) Daily OPN production during culture of normal aortas. Culture medium was collected and replaced daily, and 20 μl of medium was analyzed by SDS-PAGE and immunoblotting. Numbers indicate the days on which the medium was collected. Left lane, 40 ng of recombinant rat osteopontin. (C) OPN production in injured and uninjured aorta during 3 d of culture. Left lane, 40 ng of rat recombinant OPN; inj, injured aortas; unlabeled lanes, uninjured aortas. By densitometry, OPN production per weight did not differ between injured and uninjured aortas. (D) Phosphorylation state of OPN in culture medium. PAGE was performed under nondenaturing conditions on normal DMEM from injured aortas (lane 1) or uninjured aortas (lane 2) cultured for 3 d. Lane 3, medium from uninjured aortas treated with 7.5 U/ml calf intestinal alkaline phosphatase for 2 h at 37°C; lane 4, recombinant rat OPN. All results are representative of at least three experiments. The results show that rat aorta produces phosphorylated OPN, and this is unchanged by injury.

The appearance rate of pyrophosphate in culture medium was substantially reduced in injured aortas (36 $\pm$ 4 pmol/mg per d, $n = 12$, *versus* 145 $\pm$ 8 pmol/mg per d in normal aortas, $n = 22$; $P < 0.001$). Alkaline phosphatase activity after 9 d of culture was significantly increased in injured aortas (1.16 $\pm$ 0.17 units/mg, $n = 15$ *versus* 0.43 + 0.04 units/mg, $n = 12$, in uninjured aorta; $P < 0.001$). The activity in cultured, uninjured aorta was identical to that in freshly isolated aorta (0.44 $\pm$ 0.05 units/mg, $n = 10$). No alkaline phosphatase activity was detected in the culture medium of normal or injured aortas.

The relative roles of pyrophosphate and OPN in inhibiting calcification were also examined in aortas from OPN-deficient mice. These aortas did not exhibit any greater calcification than aortas from wild-type mice after 9 d in high-PO_4^{3-} medium (Figure 11), and they calcified to the same extent in response to injury or culture with alkaline phosphatase or pyrophosphatase.

Discussion

This study demonstrates that medial calcification can be induced in intact rat aorta cultured with alkaline phosphatase or inorganic pyrophosphatase. The calcification is in the form of hydroxyapatite, requires a high PO_4^{3-} concentration, and is histologically similar to the calcification observed in vessels from uremic patients (5,27) and rats with chronic renal failure (28). That there was no histologic evidence of cell death and that the calcification occurred in the matrix between cells rather than within cells argue against dystrophic calcification. Rat aortas cultured without these enzymes and not subjected to injury exhibited no calcification in the high-PO_4^{3-} medium, even up to 21 d in culture. The small, initial incorporation of ^{45}Ca under normal conditions presumably represents equilibration with intracellular Ca and Ca normally bound to extracellular matrix because it did not increase over time. Concentrations of both Ca^{2+} and PO_4^{3-} are elevated in high-PO_4^{3-} medium compared with human serum and, on the basis of free concentrations, would be equivalent to a total calcium-phosphorus product in human serum of 180 mg^2/dl^2, which is well above generally accepted clinical thresholds. Thus, a supraphysiologic elevation of the calcium-phosphorus product is not sufficient to produce medial calcification *in vitro* in this model. Vascular calcification is a chronic process *in vivo*, and we

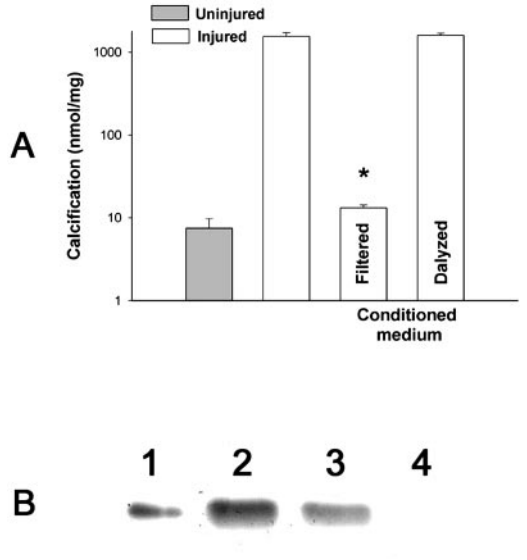

Figure 8. Role of OPN in rat aortic calcification. (A) Aortas were incubated in DMEM or conditioned medium from normal aortas, both containing 3.8 mM PO_4^{3-}, for 9 d as described in Figure 4. Conditioned medium was either filtered through a 10,000 molecular weight cut-off filter (Centricon, Amicon Corp.) or dialyzed against DMEM before use. Results are means of at least 10 aortic rings. $^*P < 0.001$ *versus* injured aorta in normal medium. (B) Immunoblot of OPN after filtration and dialysis. Lane 1, recombinant rat OPN; lane 2, conditioned medium. OPN is retained after dialysis (lane 3) and removed by filtration (lane 4). Thus, calcification is independent of OPN content in the medium.

cannot rule out the possibility that longer culture times are required to observe calcification of normal vessels *in vitro*. However, the absence of any increase in ^{45}Ca deposition over 3 wk argues against this. It is also possible that plasma factors not present in culture or mechanical effects such as blood flow and pulsatile vessel distension could contribute to calcification *in vivo*.

The absence of calcification was due to inhibitory activity in normal aortas, and this inhibition could be explained by the release of pyrophosphate from smooth muscle. Alkaline phosphatase and inorganic pyrophosphatase induced calcification of normal aortas, and pyrophosphate inhibited calcification of injured aortas. Pyrophosphate inhibits hydroxyapatite formation *in vitro* (26,29,30), and exogenous pyrophosphate inhibits aortic calcification in rats that are given large doses of vitamin D_3 (31,32). Diphosphonates, which are analogs of pyrophosphate, exhibit the same properties (33,34). It is likely that the inhibition by endogenous pyrophosphate demonstrated in cultured rat aortas also occurs *in vivo* because the concentration that maximally inhibited calcification in injured aortas (~3 μM) is similar to that reported for normal human plasma (35–38). Furthermore, deficiency of PC-1, an ecto-ATPase that produces pyrophosphate, results in reduced plasma pyrophosphate levels and extensive arterial calcification in humans (39), which can be prevented with diphosphonate therapy. Mice lacking ANK, a putative pyrophosphate transporter, exhibit reduced pyrophosphate production and extensive ectopic calcification (40), although not in vessels. Serum was not used in the aortic culture because it causes smooth muscle proliferation and outgrowth and can have detrimental effects on smooth muscle (20). Thus, it is possible that there are additional circulating inhibitors *in vivo*, such as fetuin (16,17), that would not be apparent in culture.

Whether alterations in pyrophosphate production or clearance play a role in the medial vascular calcification of advanced renal failure is unknown. Measurements performed more than two decades ago revealed slightly elevated plasma levels of pyrophosphate in patients who underwent hemodialysis (33), but dialytic clearance could be greater with current membranes and dialysis delivery. Hydrolysis of pyrophosphate by alkaline phosphatase may also play a role. The amount of this enzyme used to produce calcification in rat aorta was only severalfold higher than normal serum activity, and elevated serum activity of alkaline phosphatase is associated with calcific uremic arteriolopathy (41,42). That plasma pyrophosphate concentration is increased in hypophosphatasia, a genetic deficiency of alkaline phosphatase, indicates that this enzyme does influence pyrophosphate metabolism *in vivo* (35).

The calcification that occurred in injured aortas was associated with reduced levels of pyrophosphate in the medium and was prevented by exogenous pyrophosphate, suggesting a deficiency of pyrophosphate. This deficiency could be due to decreased production because there is loss of viable cells. However, this loss (as judged from the MTT staining) was far less than the reduction in medium pyrophosphate. Alternatively, the deficiency of pyrophosphate could have resulted from the increased alkaline phosphatase activity in injured aortas. Medial calcification occurs after balloon injury of rabbit aortas (43), indicating that injury induces calcification *in vivo* as well.

A surprising finding was that OPN did not correlate with calcification in rat aortas, which is in distinct contrast to its inhibition of calcification in cultures of rat aortic smooth muscle cells (11,12). Although inhibition of calcification was not tested directly by adding phosphorylated OPN to the cultures, phosphorylated OPN was produced by cultured aortas and levels present in culture medium (~4 μg/ml after 3 d) were equivalent to concentrations that fully inhibited calcification in cultured cells (12). No dephosphorylated OPN was detected, consistent with the low activity of alkaline phosphatase in aortas. Removal of OPN did not affect the ability of conditioned medium to inhibit calcification in injured aortas, and the degree of calcification produced by alkaline phosphatase, which dephosphorylates OPN but also destroys pyrophosphate, was no greater than that produced by inorganic pyrophosphatase, which does not dephosphorylate OPN. However, we cannot rule out the possibility that other unknown factors that influence calcification are also affected by these maneuvers. Last, calcification was not increased in aortas from OPN-deficient mice. It is important to note that although OPN was produced in culture, there was very little OPN mRNA or OPN production in freshly isolated aortas. This is consistent with previous studies (2,44) and indicates that OPN is not available

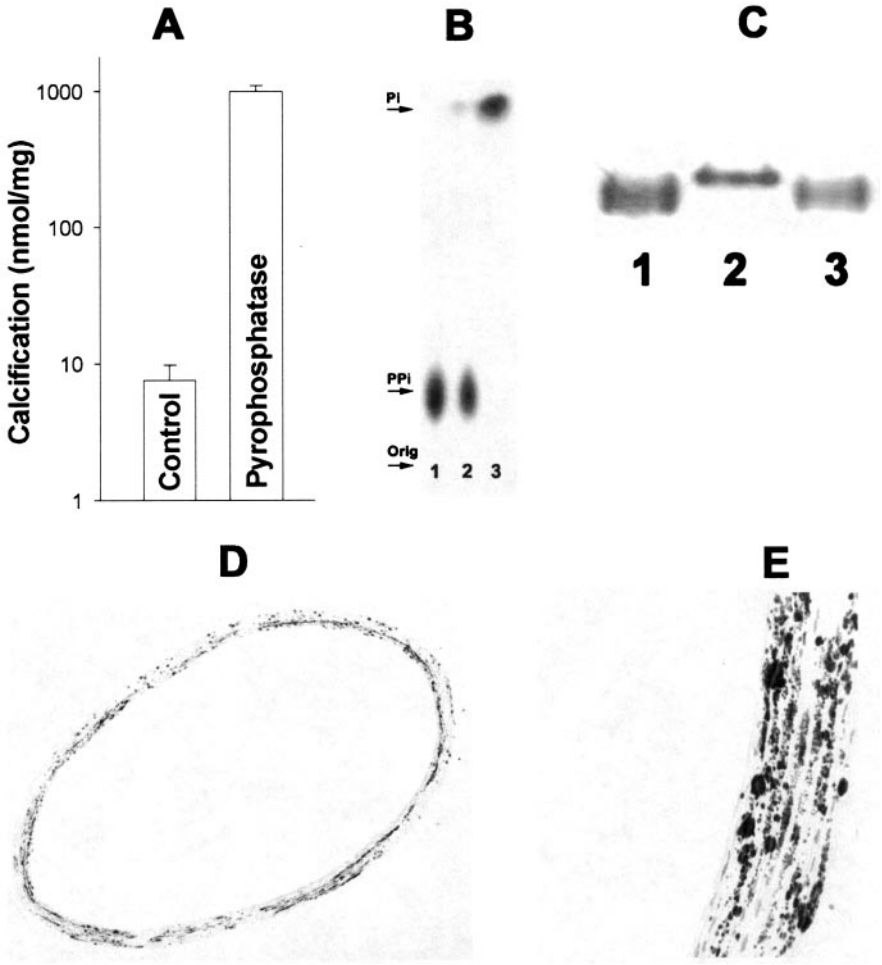

Figure 9. Inhibition of vascular calcification by pyrophosphate. (A) Incorporation of calcium in aortas incubated for 9 d in DMEM containing 3.8 mM PO_4^{3-} with or without 1.25 units/ml inorganic pyrophosphatase added daily. Results are means of at least 10 aortic rings. (B) Autoradiogram of thin-layer chromatography showing complete hydrolysis of pyrophosphate. Lane 1, [^{32}P]pyrophosphate; lane 2, medium from aortas cultured with [^{32}P]pyrophosphate for 24 h; lane 3, medium from aortas cultured with [^{32}P]pyrophosphate and 1.25 U/ml inorganic pyrophosphatase for 24 h; Pi, orthophosphate; Ppi, pyrophosphate; Orig, origin. (C) Immunoblot showing that pyrophosphatase does not dephosphorylate OPN. Lane 1, conditioned medium from normal aorta. Treatment with alkaline phosphatase (lane 2) retards migration, indicating dephosphorylation. This does not occur after treatment with pyrophosphatase (lane 3). (D) Von Kossa stain of aorta cultured with pyrophosphatase for 9 d. Higher magnification (E) shows both diffuse and focal calcification. 1.25 U/ml pyrophosphatase. Magnifications: ×20 in D, ×400 in E.

to inhibit calcification of normal vessels *in vivo*. These data suggest that OPN is not an endogenous inhibitor of calcification in normal vessels and are consistent with the lack of vascular calcification in OPN-deficient mice *in vivo* (45,46). OPN becomes abundant in calcified vessels (2,9), and OPN deficiency does accelerate vascular calcification in mice lacking matrix Gla protein (13), suggesting that OPN may modulate calcification at a more advanced stage.

Cultured aortas provide an opportunity to study calcification of intact vessels *in vitro*. The normal histology and the normal smooth muscle potassium fluxes and sensitivity to agonists (18) that we have previously demonstrated indicate that vessel structure and viability are maintained in culture. Furthermore, the calcification observed in rat aortas that were treated with alkaline phosphatase or pyrophosphatase was histologically similar to that observed in uremic human vessels (5) and occurred, at least initially, in the elastic laminae. Calcification of aortas in culture differed from that described in cultures of smooth muscle cells in several respects. β-Glycerophosphate, which induces calcification in cultured cells, was without effect in normal aorta. This is probably due to the 100-fold greater activity of alkaline phosphatase in cultured cells than in aorta (11), which would increase the availability of PO_4^{3-} from β-glycerophosphate and eliminate the inhibitory effect of pyrophosphate. OPN inhibits calcification in cultured cells (11,12), but in cultured aorta, the same concentration of OPN (present endogenously) does not prevent calcification. Because of these differences and because cultured cells lack the elastic laminae that are the initial site of calcification, we believe that

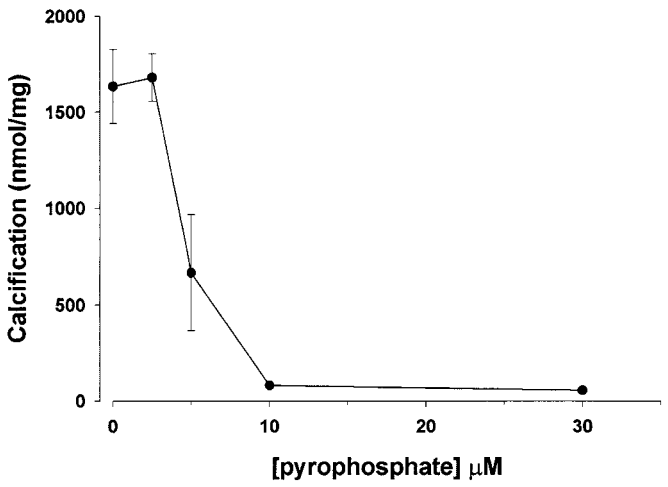

Figure 10. Suppression of calcification in injured aortas by pyrophosphate. Injured aortas were incubated for 6 d in DMEM containing 3.8 mM PO_4^{3-} and varying concentrations of pyrophosphate. Results are means of at least four aortic rings.

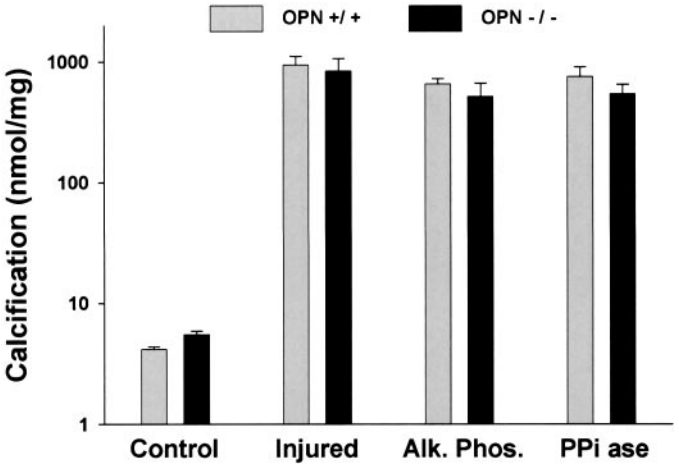

Figure 11. Calcification of aortas from OPN-deficient mice. Aortas were cultured for 9 d in high-PO_4^{3-} DMEM with or without 7.5 units/ml alkaline phosphatase 1.25 units/ml pyrophosphatase and medium changes every 3 d. Results are the means of at least six aortic rings.

aorta cultured with alkaline phosphatase or pyrophosphatase more accurately reflects medial vascular calcification *in vivo* and will be a useful model for future studies.

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