Phosphate Regulation of Vascular Smooth Muscle Cell Calcification

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Abstract—Vascular calcification is a common finding in atherosclerosis and a serious problem in diabetic and uremic patients. Because of the correlation of hyperphosphatemia and vascular calcification, the ability of extracellular inorganic phosphate levels to regulate human aortic smooth muscle cell (HSMC) culture mineralization in vitro was examined. HSMCs cultured in media containing normal physiological levels of inorganic phosphate (1.4 mmol/L) did not mineralize. In contrast, HSMCs cultured in media containing phosphate levels comparable to those seen in hyperphosphatemic individuals (>1.4 mmol/L) showed dose-dependent increases in mineral deposition. Mechanistic studies revealed that elevated phosphate treatment of HSMCs also enhanced the expression of the osteoblastic differentiation markers osteocalcin and Osf2/Cbfa-1. The effects of elevated phosphate on HSMCs were mediated by a sodium-dependent phosphate cotransporter (NPC), as indicated by the ability of the specific NPC inhibitor phosphonoformic acid, to dose dependently inhibit phosphate-induced calcium deposition as well as osteocalcin and Cbfa-1 gene expression. With the use of polymerase chain reaction and Northern blot analyses, the NPC in HSMCs was identified as Pit-1 (Glvr-1), a member of the novel type III NPCs. These data suggest that elevated phosphate may directly stimulate HSMCs to undergo phenotypic changes that predispose to calcification and offer a novel explanation of the phenomenon of vascular calcification under hyperphosphatemic conditions. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2000;87:e10-e17.)

Key Words: vascular calcification • hyperphosphatemia • inorganic phosphate • human smooth muscle cell • sodium-dependent phosphate transport • Pit-1 • Cbfa-1

Vascular calcification refers to the deposition of calcium phosphate mineral, most often hydroxyapatite, in cardiovascular tissues including arteries, heart valves, and cardiac muscle. Vascular calcification is often encountered in the development of atherosclerotic intimal lesions and is a common consequence of aging.1 In diabetic patients and individuals with renal failure, vascular calcification contributes to both the morbidity and mortality associated with these diseases.2 For example, vascular calcification is positively correlated with increased risk of myocardial infarction and increased risk of dissection after angioplasty.3 Moreover, calcification is a major cause of failure for both native and tissue prosthetic heart valves, affecting 1% to 2% of the aging population.4

Until recently, vascular calcification was considered to be a passive, degenerative, and end-stage process of vascular disease. However, the observation of matrix vesicles, bone morphogenetic proteins, and noncollagenous bone matrix proteins such as osteopontin, osteonectin, osteocalcin, and matrix Gla protein (MGP) in calcified vascular tissues has challenged this paradigm (see Giachelli5 for review). Similarly, vascular media–derived cell cultures have the capacity to express alkaline phosphatase, osteocalcin, and osteopontin and calcify their extracellular matrix under appropriate conditions.6–8 Perhaps the most compelling evidence for active regulation of vascular calcification, however, has come from recent genetic studies in mice. The MGP-null mouse shows extensive calcification of the arterial tree, indicating that MGP, which is constitutively expressed in arterial SMCs, is normally an important inhibitor of vascular calcification.9 In addition, several other mutant mouse strains including the KLOTHO mouse deficient in b-glucosidase,10 the carbonic anhydrase II mutant,11 desmin-null mouse,12 and osteoprotegerin-null mouse13 show enhanced susceptibility to vascular calcification. Finally, structures identical to bone and bone marrow are occasionally found in advanced atherosclerotic lesions, calcified cardiac valves, and Monckeberg’s sclerosis (see Parhami et al14 for review). These findings suggest that vascular calcification is in fact an actively regulated process in which vascular cells may acquire osteoblast-like functions.

Despite its clinical impact and evidence of genetic control, the molecular mechanisms regulating vascular calcification remain obscure. A clue to this process, however, is suggested by several observations linking serum phosphate levels with a tendency toward vascular calcification. First, a high serum phosphate level (hyperphosphatemia, ie, phosphate levels higher than the normal adult range of 1.0 to 1.5 mmol/L) is highly correlated with extent of vascular calcification and vascular disease. One of the most common causes of hyperphosphatemia is chronic renal failure and subsequent kidney dialysis, in which serum inorganic phosphate (Pi) levels can typically exceed 2 mmol/L. Vascular calcification observed in these patients is routinely referred to as metastatic calcification because it occurs in the presence of a systemic mineral imbalance. Second, in both experimental animals and children, prosthetic valve calcification is correlated with elevated phosphate levels. Third, as mentioned above, the KLOTHO mutant mouse develops extensive vascular medial calcification and has twice the serum phosphate levels found in wild-type mice. Finally, local disturbances of calcium and phosphate metabolism in atherosclerotic plaques have been suggested to contribute to the development of vascular calcification. Thus, it is hypothesized that an important regulator of vascular calcification is the level of Pi.

To test this hypothesis and further clarify the molecular mechanisms regulating vascular calcification, we have characterized the response of human aortic smooth muscle cell (HSMC) cultures to Pi levels. Our findings indicate that Pi directly regulates HSMC calcification through a sodium-dependent phosphate transporter–sensitive mechanism and implicate this mechanism in the development of ectopic calcification in vivo.

**Materials and Methods**

**Reagents**

DMEM (high glucose, 4.5 g/L of glucose) and FBS were purchased from Gibco. Platelet-derived growth factor-BB was obtained from Sigma. cDNAs encoding human osteocalcin and core-binding factor α subunit 1 (Cbfa-1) were gifts from Dr Shioi. H3PO4, L-[3H]alanine and [α-32P]dCTP were obtained from New England Nuclear. Unless otherwise mentioned, all other reagents were obtained from Sigma.

**Human Tissues and Cell Culture**

HSMCs were obtained by enzymatic digestion as previously described. Briefly, medial tissues were separated from segments of human adult or fetal aorta obtained at heart transplant surgery and autopsy, respectively. For plaque SMCs, coronary atherectomy–derived tissues were obtained at the time of surgery. Small pieces of tissue (1 to 2 mm²) were digested overnight in DMEM supplemented with 165 U/mL collagenase type I, 15 U/mL elastase type III, and 0.375 mg/mL soybean trypsin inhibitor at 37°C. Single cell suspensions were placed in 6-well plates and cultured for several weeks in DMEM supplemented with 20% FCS at 37°C in a humidified atmosphere containing 5% CO₂. Cultures that formed colonies were collected at confluence and maintained in growth medium (DMEM containing 15% FBS and 1 mmol/L sodium pyruvate supplemented with 100 U/mL of penicillin and 100 μg/mL of streptomycin; final P₀ concentration=1.4 mmol/L). Purity of cultures was assessed by immunostaining for α-SM actin and calponin and absence of von Willebrand factor staining as previously described. Human adult and fetal aortic medial and coronary plaque primary cells were used to passage 8. Fetal and adult HSMC cultures immortalized using the HPV-E6E7 as previously described were used between passages 20 and 30. Human fetal SMCs were used for most studies unless otherwise indicated.

**Induction of Calcification**

HSMCs were routinely subcultured in growth medium. At confluence, the cells were switched to calcification medium (DMEM containing 15% FBS and 1 mmol/L sodium pyruvate in the presence of 2 mmol/L Pi, unless otherwise stated) supplemented with 100 U/mL of penicillin and 100 μg/mL of streptomycin for up to 14 days. Both growth medium and calcification medium contained 1.8 mmol/L calcium. The medium was replaced with fresh medium every 2 days. For time-course experiments, the first day of culture in calcification medium was defined as day 0.

**Quantification of Calcium Deposition**

Cells were decalcified with 0.6 N HCl for 24 hours. The calcium content of HCl supernatants was determined colorimetrically by the o-cresolphthalein complexone method (Calcium kit; Sigma) as previously described. After decalcification, the cells were washed three times with PBS and solubilized with 0.1 N NaOH/0.1% SDS. The protein content was measured with a BCA protein assay kit (Pierce). The calcium content of the cell layer was normalized to protein content.

**Cytochemical and Ultrastructural Analysis**

Cell cultures were fixed either in pure ethanol for mineral analyses or in an aldehyde mixture for several hours followed by dehydration and embedding in LR White acrylic resin (Marivac). For morphological observations, sections were cut on a Reichert ultramicrotome and placed onto glass slides or electron microscopy grids. Mineral deposition was assessed at the light microscopic level by von Kossa staining (30 minutes, 5% silver nitrate) and sections were counterstained with toluidine blue. Transmission electron microscopy and electron diffraction were also performed on selected areas of the cultures.

**Phosphate and Alanine Transport Assays**

Transport studies were performed at 37°C in EBSS with 15 mmol/L HEPES adjusted to pH 7.4. The composition of this solution was (in mmol/L) sodium or choline 143, potassium 5.36, magnesium 0.8, calcium 1.8, and chloride 125. Transport was initiated by adding 1 mL of the above medium containing the labeled substrate H3PO₄ (1 μCi/mL) to HSMCs at confluence. For alanine transport, the EBSS solution contained 0.1 mmol/L L-[3H]alanine (1 μCi/mL). After various incubation times, the uptake was stopped by washing the cell monolayer three times with ice-cold stop solution (EBSS with 15 mmol/L HEPES adjusted to pH 7.4) at 4°C without Pi. The cells were solubilized with 1 mL of 0.1 N NaOH/0.1% SDS, and the radioactivity of 100-μL aliquots was counted by standard liquid scintillation techniques. Protein concentrations were determined with a BCA protein assay kit (Pierce), and the data were normalized by the protein content of the cell layer.

**Reverse Transcription–Polymerase Chain Reaction (RT-PCR) and Preparation of cDNA Probes**

RT-PCR was performed as previously described. Human type III sodium-dependent phosphate cotransporter (NPC) Pit-1 (Glivr-1) cDNA was amplified using the following primer sequences: forward: 5′-TACCATCTCCTATCCTGTTG-3′, reverse: 5′-TACGGCCTTGACTGAACTGG-3′. A 409-bp fragment was obtained by reverse transcription of an mRNA from human fetal SMCs, followed by the polymerase chain reaction and subcloning into the TA cloning vector (Invitrogen). The identity of the cDNA insert as Pit-1 corresponding to positions 1060 to 1469 of the coding region was confirmed by DNA sequence analysis (data not shown).
RNA Isolation and Northern Blot Analysis
Total RNA was isolated from HSMCs by extraction with Trizol as suggested by the manufacturer (Gibco). Total RNA was isolated from fetal aortic tissue by extraction with acid guanidium thiocyanate-phenol-chloroform. Total RNA was electrophoresed on 1% agarose gels containing formaldehyde and transferred to a nylon filter (Zeta-Probe GT, Bio-Rad). Blots were prehybridized at 42°C for 1 hour in a buffer containing 50% formamide, 0.75 mol/L NaCl, 50 mmol/L Tris-HCl (pH 7.5), 1% SDS, 10% dextran sulfate, 20 μg/mL denatured salmon sperm DNA, and 1× Denhardt’s solution and then hybridized at 42°C for 24 hours with cDNA probe for human Pit-1, human osteocalcin, and human Cbfa-1, which were labeled with [α-32P]dCTP (3000 Ci/mL; New England Nuclear) with use of a random priming method (Megaprime cDNA labeling system; Amersham). Blots were washed and autoradiographed with x-ray film at −70°C. The amounts of RNA were quantified by densitometric scanning and normalized by comparison with GAPDH.

Statistics
Data were analyzed for statistical significance by ANOVA with post hoc Scheffé’s F analysis, unless otherwise stated. These analyses were performed with the assistance of a computer program (StatView version 4.11, Abacus Concepts, Berkeley, Calif).

Results
P₅ Induces Calcium Deposition in HSMCs
Vascular calcification occurs frequently in individuals with hyperphosphatemia whose serum phosphate levels typically exceed 1.4 mmol/L.¹⁵⁻¹⁷ To develop an in vitro model for human vascular calcification, the effect of P₅ on calcification of HSMC cultures was examined. In media containing normal serum phosphate levels (P₅=1.4 mmol/L), HSMCs accumulated very little calcium mineral (Figure 1A). In contrast, in the presence of 2 mmol/L P₅, calcium deposition dramatically increased in a time-dependent manner (on day 9, calcified HSMC cultures versus uncalcified control: 210.3±2.4 versus 15.1±2.4 μg/mg protein, mean±SEM, n=3) (Figure 1A). Furthermore, the effect of P₅ was dose-dependent (on day 6, calcified HSMCs [in the presence of 2 mmol/L P₅] versus uncalcified HSMCs [in the presence of 1.4 mmol/L P₅]: 142.9±13.3 versus 37.1±1.2 μg/mg protein, mean±SEM, n=3) (Figure 1B). Induction of calcification by elevated P₅ appeared to be a general feature of HSMCs because primary cells derived from different sources (human adult and fetal aortic and coronary plaque), as well as immortalized derivates of these cells, showed similar behavior (Figure 1C). In addition, bovine aortic valve interstitial cells, but not NIH3T3 cells or bovine aortic endothelial cells, underwent matrix calcification under similar elevated phosphate conditions (data not shown). No spontaneous deposition of calcium mineral occurred in calcification media or in media supplemented with up to 10 mmol/L P₅, indicating that cells or cell-derived matrix was necessary for mineralization of HSMC cultures. These results indicate that HSMC cultures are susceptible to calcification when cultured in media containing P₅ concentrations typically observed in patients with hyperphosphatemia.

Morphology of HSMC Calcification
HSMC cultures grown in growth media showed areas of monolayer and multilayered growth typical of cultured smooth muscle (Figure 2A). After culturing HSMCs in calcification medium for 10 days, granular deposits developed throughout the cell culture, whereas in the control culture, no deposits were found during the culture period. The deposits were identified as phosphate-containing mineral by von Kossa staining and light microscopy (Figure 2B). Discrete, black-stained areas were diffusely scattered throughout the cell layer, predominantly in the extracellular regions, with greatest accumulation at sites of cell multilayering. Transmission electron microscopy and selected area electron diffraction of specific sites within the calcified cultures (data not shown) confirmed the presence of an apatitic mineral phase,
calcified collagen fibers, and matrix vesicles—observations essentially identical to those previously described for calcifying bovine SMC cultures. 8

A Functional NPC Is Required for HSMC Culture Calcification

To examine the mechanism by which phosphate levels might control matrix mineralization, we examined phosphate transport in HSMCs. In the presence of sodium chloride, phosphate uptake was increased in a time-dependent manner (Figure 3A). Transport was abolished when sodium ions were replaced with choline ions in the media. Maximal phosphate accumulation was achieved after \(120\) minutes (at \(120\) minutes, sodium chloride versus choline chloride was \(12.24 \pm 0.27\) versus \(0.36 \pm 0.06\) nmol/mg protein, mean \(\pm\) SEM, \(n = 3\)) (Figure 3A). We next examined the relationship between extracellular phosphate concentration and intracellular phosphate uptake in HSMC cultures. When the concentration of phosphate in the medium was increased, phosphate uptake was dose dependently elevated (in the presence of \(3.0\) mmol/L P, versus \(0.1\) mmol/L P; \(28.23 \pm 0.63\) versus \(0.64 \pm 0.20\) nmol/mg protein, mean \(\pm\) SEM, \(n = 3\)) (Figure 3B). Three features—time dependence, sodium dependence, and phosphate gradient dependence—are consistent with the presence of an NPC in HSMCs.

To determine whether NPC activity was required for HSMC culture calcification, phosphonoformic acid (PFA), a specific, competitive inhibitor of NPCs, was used. 26 As shown in Figure 4A, PFA time dependently inhibited sodium-dependent phosphate uptake in HSMCs (at \(90\) minutes, vehicle versus \(1.0\) mmol/L PFA: \(12.46 \pm 0.41\) versus \(3.31 \pm 0.33\) nmol/mg protein, mean \(\pm\) SEM, \(n = 3\)) (Figure 4A). Moreover, in the presence of increasing concentrations of PFA, phosphate uptake was dose dependently decreased (vehicle versus \(1.0\) mmol/L PFA: \(12.18 \pm 0.861\) versus \(3.07 \pm 0.15\) nmol/mg protein, mean \(\pm\) SEM, \(n = 3\)) (Figure 4B). The effect of PFA on HSMC calcification was then determined. Increasing concentrations of PFA, dose dependently inhibited HSMC calcium deposition (calcified control [vehicle-treated cells] versus \(2.0\) mmol/L PFA: \(165.4 \pm 2.8\) versus \(39.8 \pm 1.5\) \(\mu\)g/mg protein, mean \(\pm\) SEM, \(n = 3\)) (Figure 4C). In addition, arsenate, a second inhibitor of NPC function, also completely inhibited both P, uptake and mineralization in HSMC cultures (data not shown). Together, these data strongly suggest that an NPC transport system was necessary for mineral deposition in cultured HSMCs.

Phosphate Induces and PFA Blocks Bone Specific Gene Expression in HSMCs

To further address the mechanism by which phosphate induces HSMC calcification, we examined levels of

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**Figure 2.** Morphology of calcification of HSMC culture. HFSMCs were cultured in calcification medium containing 2.0 mmol/L P, or growth medium containing 1.4 mmol/L P, for 10 days. After incubation, calcified HFSMCs or uncultivated HFSMCs were fixed with ethanol. Mineral deposition was assessed at light microscopic level by von Kossa staining. The sections were counterstained with toluidine blue. A, No deposits were found in the control culture. B, Black deposits indicate deposits of P, containing mineral in predominantly the extracellular regions of the cultures. Magnification \(\times 200\).

**Figure 3.** Time course and dose response of P, transport by HFSMCs. A, P, uptake was determined in EBSS medium containing 0.1 mmol/L \(H_3^{32}PO_4\) (1 \(\mu\)Ci/mL) and either 143 mmol/L sodium chloride (\(O\)) or 143 mmol/L choline chloride (\(C\)) for the indicated times. P, uptake was normalized by cellular protein content and is presented as mean \(\pm\) SEM. The differences compared with choline chloride–treated cells at the same time point were statistically significant (*\(P < 0.01\), Scheffé’s test). B, P, uptake was determined in EBSS medium containing 0.1 to 3.0 mmol/L \(H_3^{32}PO_4\) and either 143 mmol/L sodium chloride (\(O\)) or 143 mmol/L choline chloride (\(C\)) for 20 minutes. P, uptake was normalized by cellular protein content and is presented as mean \(\pm\) SEM. The differences compared with choline chloride–treated cells at each point were statistically significant (*\(P < 0.01\), Scheffé’s test).

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osteoblast-specific genes that have previously been shown to both mark the osteoblast lineage and regulate bone formation.27 As shown in Figure 5, levels of osteocalcin and Cbfa-1 mRNA were strongly induced under elevated phosphate conditions. Furthermore, PFA dose dependently inhibited phosphate-induced expression of these genes (Figures 5A and 5B). PFA did not affect calcification or gene expression in HSMCs cultured in 1.4 mmol/L phosphate (data not shown).

HSMC Cultures and Human Arterial Tissues Contain the Type III NPC Pit-1 (Glvr-1)

Three families of NPCs have been reported to date. The type I family consists of a single species (NaP\(_1\)-1), which has thus far been found only in rabbit kidney.28 The type II family consists of six species homologues, NaP\(_i\) 2 to 7, that are expressed predominantly in renal epithelial tissues.29–33 The type III family is the most recently identified and consists of two members, Pit-1 (also called Glvr-1) and Pit-2 (also called Ram-1).34 To determine which NPC was potentially involved in HSMC phosphate uptake, we used RT-PCR with specific primers in an attempt to amplify NPC cDNAs that have previously been identified in human tissues. As shown in Figure 6A, a 409-bp band using specific primers of Pit-1 was obtained from mRNA derived from fetal HSMCs as well as fetal human aorta, nondiseased human adult coronary, and diseased human coronary. Sequence analysis of the 409-bp cDNA fragment amplified using the human Pit-1 primers confirmed the identity as human Pit-1 (data not shown). No bands were obtained when primers for NaPi-3 (the human type II NPC family homologue) or Pit-2 were used in RT-PCR from these same tissues (data not shown).

Using the 409-bp human Pit-1 fragment as a probe, we confirmed the expression of Pit-1 in the various HSMCs isolates used in our studies by Northern blot analysis (Figure 6B). An mRNA band of 3.7 kb was observed in all HSMCs.
tested, and this is consistent with the size reported for Pit-1 mRNA transcripts in osteoblasts. A similarly sized Pit-1 mRNA band was also observed in mRNA derived from human fetal aorta (Figure 6C), confirming the RT-PCR data in Figure 6A. Whereas Cbfa-1 and osteocalcin levels were upregulated after treatment of HSMCs with 2 mmol/L Pi, for 24 hours (Figures 5A and 5B), Pit-1 mRNA levels remained unchanged (data not shown). These data identify Pit-1 as a major NPC constitutively present in HSMCs in vitro and in vivo.

Discussion

The role of Pi in vascular SMC mineralization was investigated using an in vitro model system that was shown to mimic many of the features seen in human metastatic vascular calcification in vivo. We found that HSMCs cultured in media containing normal serum phosphate levels do not mineralize. In contrast, HSMCs treated with media containing phosphate levels comparable to those seen in patients with hyperphosphatemia accumulated significant levels of apatitic mineral in the their matrices. HSMCs were found to contain a functional sodium-dependent phosphate uptake system and mRNA for the type III NPC Pit-1 (Glvr-1).

Figure 6. Expression of Pit-1 in HSMCs and human aorta. A, Total RNA was isolated from HFSMCs and human fetal aortic tissues. Gene expression of Pit-1 was analyzed by RT-PCR as described in Materials and Methods. An expected 409-bp fragment was detected. The sequence of the PCR fragment was identical to human Pit-1. St. indicates DNA ladder standard; lane 1, HFSMCs; lane 2, 85-day-old; lane 3, 67-day-old, and lane 4, 78-day-old. B, Ten micrograms of total RNA obtained from various isolates of primary and immortalized HSMCs was analyzed by Northern blot analysis with a probe for human Pit-1. The membrane was stripped and rehybridized with a probe for GAPDH. A similarly sized Pit-1 mRNA band was also observed in mRNA from human fetal aortic tissues and were analyzed by Northern blot analysis with a probe for human Pit-1. The membrane was stripped and rehybridized with a probe for GAPDH. A similarly sized Pit-1 mRNA band was also observed in mRNA from human fetal aorta. Lane 1 indicates HFSMCs; lane 2, 81-day-old; lane 3, 87-day-old; lane 4, 72-day-old; and lane 5, 84-day-old.

Function of this transporter appeared to be critical for mineralization of HSMC cultures given that PFA significantly inhibited calcium deposition and gene expression of osteoblastic differentiation markers osteocalcin and Cbfa-1 in a dose-dependent manner. These data strongly support the hypothesis that SMCs are induced to secrete a matrix capable of mineralization in response to elevated phosphate levels, and that this process is dependent on NPC function.

In this study, P, levels regulated the propensity of HSMC cultures to calcify. P, increased HSMC calcification in a time- and dose-dependent manner, and mineralization induced by P, was similar to that observed in other culture systems and calcified vascular tissues in vivo. In patients with chronic renal failure treated by hemodialysis, hyperphosphatemia is commonly associated with widespread vascular calcification. In fact, recent studies indicate a striking association of serum phosphorous levels with mortality risk in chronic hemodialysis patients, probably a result of the increased calcinosis, calciphylaxis, and secondary hyperparathyroidism typically observed in these patients. When combined with the data from our studies, these observations support the concept that P, levels may directly regulate vascular calcification. Interestingly, P, has also been recently implicated in the direct regulation of the parathyroid gland secretion of parathyroid hormone, which is also elevated in individuals with uremia. Notably, P, has been implicated in the regulation of chondrocyte and osteoblast activity. However, elevated P, is clearly not the only stimulus for vascular cell calcification, because pericytes and calcifying vascular cells apparently do not require supplemental phosphate for mineralization in culture.

To determine how HSMCs might sense elevated phosphate levels, we examined phosphate uptake by these cells. Our data suggest that HSMCs sense P, levels, at least in part, through an NPC. Three types of NPCs have been identified and are grouped according to homology. Type I NPC includes NaPi-1, which is found exclusively in the kidney of rabbits. Six members comprise the type II NPC family, including the species homologues NaPi-2, -3, -4, -5, -6, and -7, and these also appear to be predominantly expressed in renal and gut epithelium in many species and are most likely important in the phosphate reabsorption function of these tissues. Most recently, the type III NPC system was identified and found to be expressed in heart, kidney, bone, and liver. This family includes NPCs that act as receptors for gibbon ape leukemia virus (Glvr-1; Pit-1) and amphotropic murine retrovirus (Ram-1; Pit-2). Of these known NPCs, only Pit-1 was identified in our HSMCs as well as in human fetal aorta. This is the first description of an NPC of any type in vascular SMCs. However, definitive proof of Pit-1 as the NPC involved in mediating matrix mineralization in response to elevated phosphate in HSMCs awaits development of a specific Pit-1 inhibitor.

How might elevated phosphate and NPC activity induce HSMC-mediated mineralization? We speculate that under conditions of high extracellular phosphate or enhanced cellular NPC levels, intracellular levels of P, are increased via the action of Pit-1. This may lead to mechanisms initiating promineralizing metabolic processes within the cell. One of these mechanisms may be increased elaboration of an extra-
cellular matrix that is prone to mineralize. In support of this idea, we found that elevated phosphate levels stimulate expression of both Cbfa-1 and its downstream transcriptional target, osteocalcin, in HSMCs. Similarly, elevated phosphate level was previously shown to induce osteopontin expression in bovine aortic SMCs. Osteocalcin and osteopontin are major noncollagenous proteins found in bone matrix and are believed to regulate mineralization (see Giachelli et al. for review). Cbfa-1 is an osteoblast-specific transcription factor required for osteoblast differentiation, bone matrix gene expression, and, consequently, bone mineralization. Cbfa-1 has been previously shown to directly regulate the expression of the major components of bone matrix including collagen type I, osteocalcin, and osteopontin. Thus, it is likely that phosphate-signaled increases in Cbfa-1 gene expression in HSMCs leads to enhanced transcription and secretion of an osteoid-like extracellular matrix that contributes to enhanced calcification under hyperphosphatemic conditions. Finally, it is also possible that P, loading of matrix vesicles released by the mineralizing HSMCs is involved in the calcification process, considering that NPCs have been found in these structures in chondrocytes. Accumulation of phosphate in these vesicles, together with the activity of membrane-associated alkaline phosphatase, is thought to play an important role in the initial mineralization of bones and teeth, and matrix vesicles have also been found in calcified vascular tissues in vivo.

In conclusion, these findings support the hypothesis that extracellular phosphate directly regulates the ability of vascular SMCs to initiate matrix mineralization. Phosphate uptake by an NPC, potentially Pit-1, leads to increased expression of Cbfa-1, a bone-specific transcription factor, and subsequent elaboration of a promineralizing matrix that contains osteopontin and osteocalcin. These findings offer a novel mechanism for explaining metastatic calcification and may lead to new therapeutics aimed at reducing or preventing ectopic calcification.

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