

# Role of the Sodium-Dependent Phosphate Cotransporter, Pit-1, in Vascular Smooth Muscle Cell Calcification

Xianwu Li, Hsueh-Ying Yang, Cecilia M. Giachelli

**Abstract**—Vascular calcification is associated with cardiovascular morbidity and mortality. Hyperphosphatemia is an important contributor to vascular calcification. Our previous studies demonstrated that elevated phosphate induces calcification of smooth muscle cells (SMC) in vitro. Inhibition of phosphate transport by phosphonoformic acid blocked phosphate-induced calcification, implicating sodium-dependent phosphate cotransporters in this process. In the present study, we have investigated the role of the type III sodium-dependent phosphate cotransporter, Pit-1, in SMC calcification in vitro. Human SMC stably expressing Pit-1 small interfering double-stranded RNA (SMC-iRNA) were established using a retroviral system. SMC-iRNA had decreased Pit-1 mRNA and protein levels and sodium-dependent phosphate transport activity compared with the control transduced cells (SMC-CT) (2.9 versus 9.78 nmol/mg protein per 30 minutes, respectively). Furthermore, phosphate-induced SMC calcification was significantly inhibited in SMC-iRNA compared with SMC-CT at all time points examined. Overexpression of Pit-1 restored phosphate uptake and phosphate-induced calcification in Pit-1 deficient cells. Mechanistically, although Pit-1-mediated SMC calcification was not associated with apoptosis or cell-derived vesicles, inhibition of phosphate uptake in Pit-1 knockdown cells blocked the induction of the osteogenic markers Cbfa-1 and osteopontin. Our results indicate that phosphate uptake through Pit-1 is essential for SMC calcification and phenotypic modulation in response to elevated phosphate. (*Circ Res.* 2006;98:905-912.)

**Key Words:** sodium-dependent phosphate cotransporter ■ Pit-1 ■ vascular calcification ■ smooth muscle cell

Vascular calcification refers to calcium phosphate deposition in blood vessels, myocardium, and cardiac valves under pathological conditions. Vascular calcification is associated with increased risk of cardiovascular morbidity and mortality.<sup>1,2</sup> It is highly prevalent in patients with atherosclerosis, diabetes, and renal failure.<sup>3</sup> Approximately 50% of all deaths in end-stage renal disease (ESRD) patients are attributed to cardiovascular diseases.<sup>4</sup> Intimal, as well as medial, calcification contributes to the increased mortality in these patients.<sup>5</sup> Recent evidence indicates that vascular calcification is an actively regulated process, and molecules that inhibit or promote vascular calcification are currently being identified.<sup>6,7</sup> Mineralization inhibiting molecules appear to play a major role in preventing ectopic calcification under physiological conditions. However, processes that promote calcification may predominate under pathological conditions and are less well defined.

Although the mechanisms of vascular calcification are not fully understood, abnormalities in mineral metabolism are considered important risk factors. In particular, phosphate has emerged as an important regulator of vascular calcification as well as a risk factor for cardiovascular mortality in dialysis patients. In humans, normal levels of serum phosphate range from 1 to 1.5 mmol/L.<sup>8</sup> Hyperphosphatemia, typically ob-

served in patients with ESRD, is defined as serum phosphate levels exceeding 2 mmol/L. In these patients, hyperphosphatemia and elevated calcium $\times$ phosphate product are highly correlated with calcification of coronaries, peripheral arteries, and cardiac valves.<sup>9-12</sup> Importantly, several large epidemiological studies have found that elevated serum phosphate is a major, nontraditional risk factor for cardiovascular mortality in ESRD patients.<sup>12,13</sup>

The mechanisms that drive vascular calcification are currently under intense investigation. We and others have reported that elevated phosphate induces smooth muscle cell (SMC) calcification in vitro.<sup>14-17</sup> Concomitant with mineralization, elevated phosphate leads to SMC phenotypic change exemplified by upregulation of osteochondrogenic differentiation markers, such as Cbfa-1 and osteopontin (OPN).<sup>15,16,18</sup> Subsequent studies implicated sodium-dependent phosphate cotransporters in phosphate-induced vascular calcification. Inhibition of sodium-dependent phosphate cotransporters by phosphonoformic acid blocked both phosphate-induced phenotypic transition and calcification in vascular SMC.<sup>16,18</sup> These studies suggested that elevated phosphate, via a sodium-dependent phosphate cotransporter-mediated mechanism, might have direct pro-mineralizing effects on SMC independent of raising the calcium $\times$ phosphate product.

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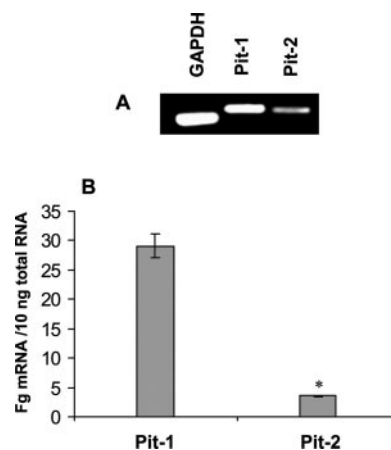
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The types and mechanisms of sodium-dependent phosphate cotransporter function in vascular calcification are not currently known. Sodium-dependent phosphate cotransporters are glycosylated proteins with 7 to 10 transmembrane-spanning regions. The common function of sodium-dependent phosphate cotransporters is transporting phosphate from the extracellular environment into the cell in a sodium-dependent manner. Three types of sodium-dependent phosphate cotransporters have been identified based on structure, tissue expression, and regulation. The type I and type II sodium-dependent phosphate cotransporters are expressed predominantly in kidney and intestinal epithelium. The type I family includes NaPi-1, RNaPi-1, NPT-1, and Npt-1 and have been identified from various species.<sup>19,20</sup> The physiological roles of this family remain to be elucidated. In contrast, the members of the sodium-dependent phosphate cotransporters type II family, including NaPi-2, NaPi-3, NaPi-4, NaPi-5, NaPi-6, and NaPi-7, are crucially involved in renal and intestinal phosphate absorption and play an important role in the maintenance of serum phosphate homeostasis.<sup>19,20</sup> The type III sodium-dependent phosphate cotransporters, Pit-1 and Pit-2, are the most recently discovered subtypes and were originally identified as cell surface receptors for the gibbon ape leukemia virus (Glv-1) and the amphotropic murine retrovirus (Ram-1), respectively.<sup>21–23</sup> Type III sodium-dependent phosphate cotransporters are expressed in many tissues and cell types including kidney, brain, heart, liver, lung, osteoblast, and SMC, but their requirement in crucial cellular processes has not yet been determined. In the present studies, we have examined the role of the predominant phosphate cotransporter found in SMC, Pit-1, in phosphate-induced SMC calcification using RNA interference. We provide novel evidence that phosphate transport through Pit-1 plays a critical role in SMC calcification *in vitro*. In addition, our results further confirm that the modulation of SMC phenotypic change could be one of the mechanisms by which phosphate, via Pit-1, mediates calcification of vascular SMC cultures.

### Materials and Methods

Human immortalized aortic SMC (human SMC) were used for experiments. SMC stably expressing Pit-1 small interfering double-stranded RNA (siRNA) were generated using the pSUPER RNA interference system (Oligoengine, Seattle, Wash). Total RNA was isolated from human SMC using the RNeasy kit from Qiagen (Chatsworth, Calif). Reverse transcription was performed using Omniscript Reverse Transcriptase from Qiagen. TaqMan PCR reagents kits and the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, Calif) were used to quantitatively determine levels of human Pit-1, Pit-2, Cbfa-1, and OPN mRNAs. Phosphate uptake assays were performed using  $H_3^{32}PO_4$ . Calcium content was determined using the *O*-cresolphthalein complexone method. Membrane-bound vesicles and apoptotic bodies were isolated from SMC cultures by differential centrifugation and identified by transmission electron microscopy and biochemical marker analysis.

An expanded Materials and Methods section can be found in the online data supplement available at <http://circres.ahajournals.org>.



**Figure 1.** Expression of Pit-1 and Pit-2 mRNA in human SMC. A, Total RNA was isolated from human SMC; identical amounts of RNA were used for RT-PCR reaction with primers for human Pit-1, Pit-2, or GAPDH, respectively. A 409-bp fragment of Pit-1 and a 384-bp fragment of Pit-2 were detected. B, Ten nanograms of total RNA were used for real-time PCRs for human Pit-1 or Pit-2, respectively. Data are expressed as mean ± SD from 3 individual PCR reactions.  $29.1 \pm 2.03$  and  $3.52 \pm 0.131$  for Pit-1 and Pit-2, respectively. \*Significant decrease ( $P=0.004$ ) compared with Pit-1.

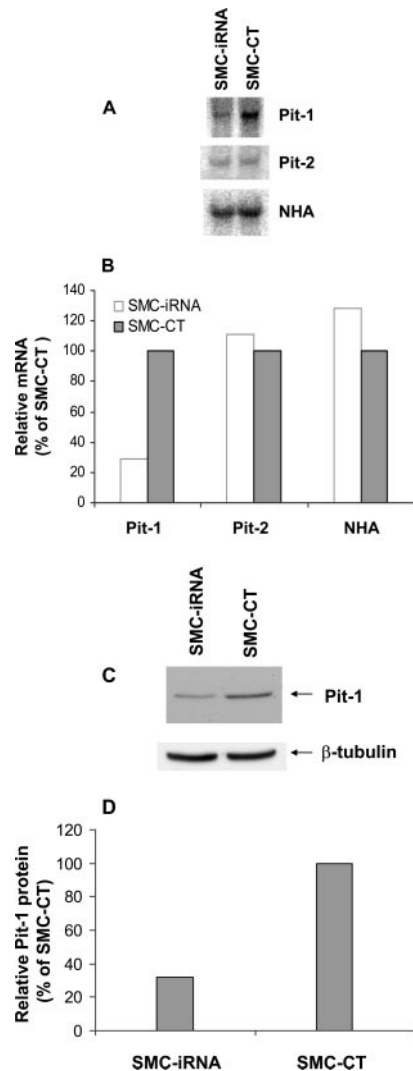
## Results

### Pit-1 Is the Predominant Sodium-Dependent Phosphate Cotransporter Expressed in Human SMC

Our previous studies indicated that sodium-dependent phosphate cotransporters might be involved in phosphate-induced SMC calcification.<sup>18</sup> To determine the expression profile of sodium-dependent phosphate cotransporters in human SMC, the abundance of type I, type II, and type III sodium-dependent phosphate cotransporters were determined initially using RT-PCR. Using this technique, no bands were obtained using primers for NPT1 (human type I family) and NaPi-3 (human type II family) (data not shown). However, a strong band at 409 bp was obtained using Pit-1 primers, and a weaker band at 384 bp was amplified using Pit-2 primers (Figure 1A), indicating that members of the human type III family were present. Sequence analysis of these amplified fragments confirmed their identities as human Pit-1 and Pit-2, respectively (data not shown). Similar results were obtained in primary human aortic SMC (data not shown). Real-time PCR was used to precisely quantitate the expression levels of Pit-1 and Pit-2 in the cells. As shown in Figure 1B, the Pit-1 mRNA levels were 8-fold higher than that of Pit-2 in human SMC. These results indicated that Pit-1 is the predominant form of sodium-dependent phosphate cotransporter expressed in human SMC. Therefore, subsequent experiments focused on determining the role of Pit-1 in human SMC calcification.

### Downregulation of Pit-1 mRNA Leads to Decreased Pit-1 Protein Levels in Human SMC

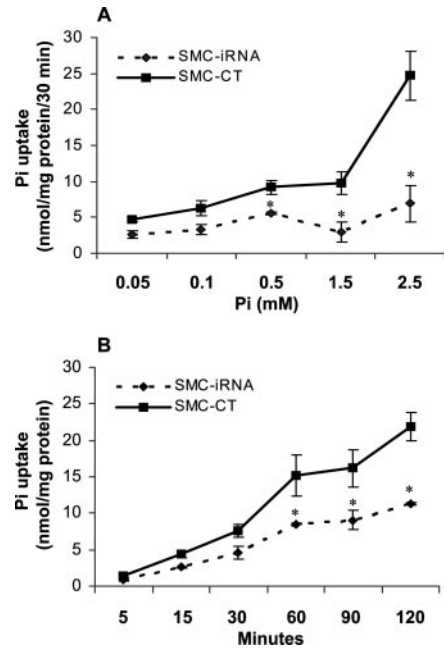
Previous studies using phosphonoformic acid, a generic sodium-dependent phosphate cotransporter inhibitor, implicated a crucial role for these cotransporters in phosphate-mediated SMC calcification.<sup>18</sup> To more specifically address the role of Pit-1 in this process, RNA interference was used to



**Figure 2.** Specific knockdown of Pit-1 mRNA and protein levels by Pit-1 siRNA. A, Northern blot analysis of RNA from SMC-iRNA and SMC-CT. The blots were hybridized with probes for human Pit-1, Pit-2, or NHA, respectively. mRNA levels were visualized by PhosphorImager (Amersham Biosciences). B, Quantitation of Pit-1, Pit-2, or NHA mRNA levels. The blots from A were stripped and hybridized with an 18S ribosomal RNA probe. mRNA levels were normalized to 18S rRNA and data are presented as the percentage of SMC-CT levels. C, Western blot analysis of Pit-1 protein. Protein lysates were prepared from SMC-iRNA and SMC-CT. Specific proteins were detected using anti-Pit-1 or anti- $\beta$ -tubulin antibodies. D, Pit-1 protein levels were quantified by densitometry and normalized to  $\beta$ -tubulin levels. Data are presented as the percentage of SMC-CT levels. Identical results were obtained in a replicate experiment.

suppress endogenous Pit-1 mRNA levels. SMC stably expressing Pit-1 siRNA (SMC-iRNA) or control construct (SMC-CT) were established using a retroviral system. Effects on Pit-1 expression levels were examined by Northern and Western blot analysis. As shown in Figure 2A and 2B, Pit-1 mRNA levels were dramatically reduced (80%) in SMC-iRNA compared with levels in SMC-CT. Western blotting confirmed a comparable decrease in Pit-1 protein levels in SMC-iRNA (Figure 2C and 2D).

To determine the specificity of the Pit-1 siRNA, mRNA levels of 2 other membrane transporters, Pit-2 and sodium



**Figure 3.** Effect of Pit-1 siRNA on sodium-dependent phosphate uptake in SMC. A, Phosphate uptake assay was performed by incubation of SMC with buffer containing various concentrations of phosphate for 30 minutes. Data shown are means  $\pm$  SD ( $n=3$ ). B, Phosphate uptake was determined by incubation of SMC with buffer containing 0.1 mmol/L phosphate for indicated times. Data shown are means  $\pm$  SD ( $n=3$ ). \*Significant decrease ( $P<0.05$ ) compared with SMC-CT.

hydrogen antiporter (NHA), were measured. Pit-2 is the other member of type III sodium-dependent phosphate cotransporter family and shares 58% homology at the amino acid level with Pit-1.<sup>22</sup> Despite this sequence similarity, Pit-1 siRNA had no effect on Pit-2 mRNA levels. Similarly, NHA mRNA was not inhibited by Pit-1 siRNA (Figure 2A and 2B). These results demonstrated that the Pit-1 siRNA specifically inhibited Pit-1 expression.

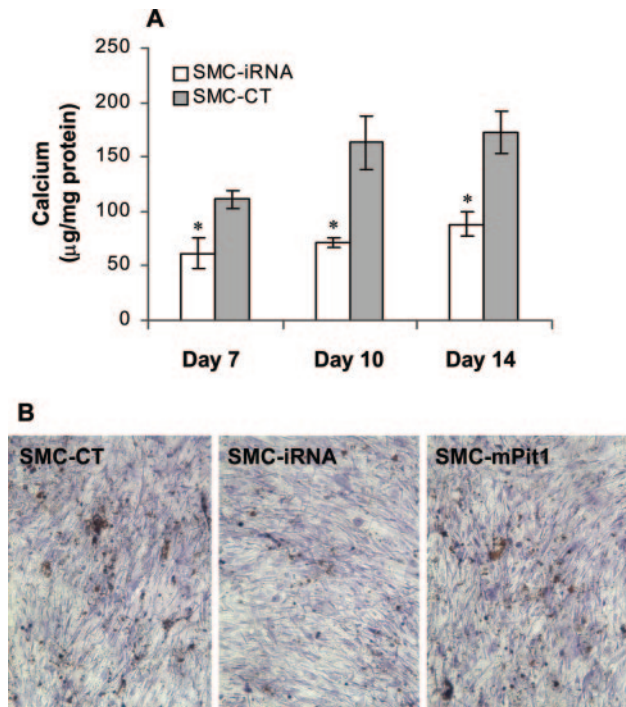
### Inhibition of Sodium-Dependent Phosphate Uptake by Pit-1 siRNA

To determine whether decreased Pit-1 mRNA and protein levels translated to decreased Pit-1 function, phosphate uptake assays were performed in SMC-iRNA and SMC-CT. As shown in Figure 3A and 3B, phosphate uptake was concentration and time-dependent in both cell types. However, overall phosphate uptake was substantially decreased in SMC-iRNA compared with SMC-CT at all concentrations (at 30 minutes in 1.5 mmol/L Pi, SMC-iRNA versus SMC-CT: 2.90 versus 9.78 nmol/mg protein, respectively). Likewise, phosphate uptake was substantially decreased in SMC-iRNA compared with SMC-CT at all time points (at 120 minutes in 0.1 mmol/L Pi, SMC-iRNA versus SMC-CT: 11.27 versus 21.90 nmol/mg protein, respectively). These results indicated that a decrease in cellular Pit-1 levels caused by the Pit-1 siRNA resulted in a comparable decrease in phosphate uptake. No effect on sodium-independent phosphate transport was observed (data not shown).

### Inhibition of SMC Calcification by Pit-1 siRNA

To determine whether Pit-1 knockdown affected the ability of human SMC to calcify, both SMC-iRNA and SMC-CT were



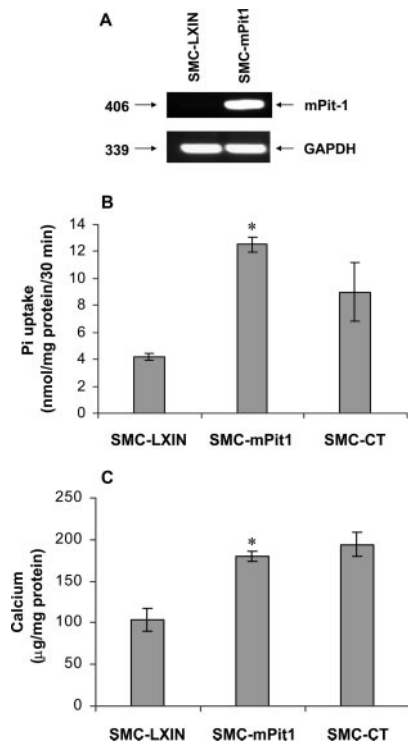


**Figure 4.** Effect of Pit-1 siRNA on phosphate-induced calcification in SMC. A, SMC-iRNA and SMC-CT were cultured in CM for the indicated times. The calcium content was measured by O-cresolphthalein complexone method and normalized by the protein content. Data shown are means $\pm$ SD (n=3). \*Significant decrease ( $P<0.05$ ) compared with SMC-CT. B, SMC-iRNA, SMC-CT, and SMC-mPit1 were cultured in CM for 10 days. After incubation, the cells were fixed with 4% paraformaldehyde. Mineral deposition was visualized by Von Kossa staining. The sections were counterstained with toluidine blue.

incubated with either growth media (GM) (1.4 mmol/L phosphate) or calcification media (CM) (2.6 mmol/L phosphate) for 7, 10, and 14 days, and calcification was determined biochemically and histologically. As shown in Figure 4A, SMC-iRNA showed significantly less calcium deposition than SMC-CT at all time points (at day 10, SMC-iRNA versus SMC-CT: 71.84 versus 163.09  $\mu\text{g}/\text{mg}$  protein, respectively). Mineral deposits were further identified morphologically by Von Kossa staining and light microscopy. As shown in Figure 4B, after culture in CM for 10 days, abundant mineral deposits associated with extracellular matrix could be detected throughout the SMC-CT culture. Comparable to biochemical data, much less mineral deposition was observed in SMC-iRNA compared with SMC-CT. No mineral deposits were observed in cultured SMC treated with GM at any time points (data not shown). These results suggested that Pit-1 was necessary for SMC calcification induced by elevated phosphate in vitro.

#### Restoration of Calcification by Overexpression of Mouse Pit-1 in Pit-1 Deficient Human SMC

To further determine the specific requirement of Pit-1 in SMC calcification and to rule out potential off-target effects of Pit-1 siRNA, we examined whether overexpression of Pit-1 could restore phosphate-induced calcification in SMC-iRNA. To do this, we took advantage of a 3-bp mismatch



**Figure 5.** Effect of Pit-1 overexpression on sodium-dependent phosphate uptake and calcification in SMC. A, RT-PCR analysis of mouse Pit-1 expression. Total RNA was isolated from SMC-mPit1 or SMC-LXIN. PCR was performed with primers for mouse Pit-1 or GAPDH as an internal control. An expected 406-bp fragment of mouse Pit-1 was detected. B, Phosphate uptake was determined by incubation of SMC with 0.1 mmol/L phosphate for 30 minutes as described in Materials and Methods. Data shown are means $\pm$ SD (n=3). C, Cells were cultured in CM for 10 days. The calcium content was measured by O-cresolphthalein complexone method and normalized by the protein content. Data shown are means $\pm$ SD (n=3). \*Significant increase ( $P<0.05$ ) compared with SMC-LXIN.

between human and mouse Pit-1 in the siRNA targeting region. Thus, the human Pit-1 siRNA is unable to target the mouse Pit-1 transcript for degradation. Retroviral constructs encoding mouse Pit-1 cDNA or empty vector pLXIN were, therefore, generated and used to infect SMC-iRNA. The transduced cells are referred to as SMC-mPit1 or SMC-LXIN, respectively. As shown in Figure 5A, a 406-bp fragment for mouse Pit-1 was amplified in SMC-mPit1. No amplified fragment was detected in SMC-LXIN. Next we examined the functionality of the expressed mouse Pit-1 by performing phosphate uptake assays. Figure 5B shows that phosphate uptake was increased 3-fold in SMC-mPit1 compared with SMC-LXIN (at 30 minutes in 0.1 mmol/L Pi, SMC-mPit1 versus SMC-LXIN: 12.51 versus 4.18 nmol/mg protein, respectively). The phosphate uptake in SMC-mPit1 was comparable to that of SMC-CT. These results demonstrated that the overexpressed mouse Pit-1 functioned properly as a phosphate transporter in SMC-iRNA.

We next determined whether overexpression of Pit-1 restored calcification induced by elevated phosphate. SMC-mPit1 or SMC-LXIN and SMC-CT were cultured in CM for 10 days, and calcification was determined biochemically and histologically. As shown in Figure 5C, calcium deposition

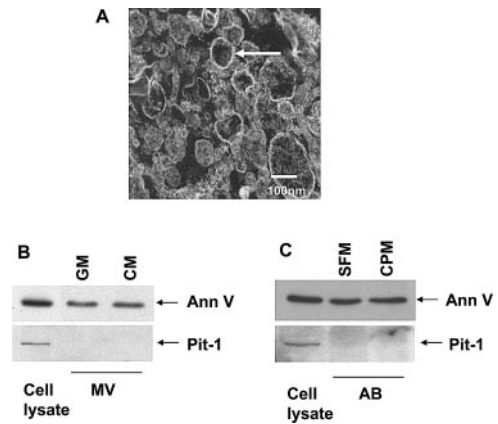
was almost twice as high in SMC-mPit1 compared with SMC-LXIN (SMC-mPit1 versus SMC-LXIN: 179.84 versus 103.44  $\mu\text{g}/\text{mg}$  protein, respectively). Furthermore, the calcium content in SMC-mPit1 was almost the same as SMC-CT (SMC-mPit1 versus SMC-CT: 179.84 versus 194.14  $\mu\text{g}/\text{mg}$  protein, respectively). Von Kossa staining revealed that overexpression of mPit-1 increased the formation of mineral deposits in the extracellular matrix of cultured SMC, and the levels of calcification in these cells were comparable to that of SMC with normal Pit-1 levels (Figure 4B). Taken together, these results suggest that Pit-1 is an important mediator of calcification in SMC.

### Pit-1 Is Not a Component of Matrix Vesicles or Apoptotic Bodies Derived From SMC

One possible mechanism for the requirement of Pit-1 in SMC calcification is in mediating phosphate loading of calcifying extracellular vesicles. Growing evidence suggests that membrane-bound vesicles derived from cells may be involved in both physiological and pathological calcification. In bone and cartilage, matrix vesicles have been identified as cell-derived, membrane-bound vesicles intimately associated with sites of active extracellular matrix mineralization.<sup>24</sup> In addition, Shanahan and colleagues have described calcifying membrane-bound vesicles and apoptotic bodies released from human aortic SMC cultured in serum-free media (SFM).<sup>25</sup> In both cases, it is postulated that these membrane-bound vesicles participate in mineralization by concentrating calcium and phosphate in a protected microenvironment, thereby promoting mineral nucleational events.

To determine whether Pit-1 was a component of SMC-derived vesicles, we used two different methods to isolate membrane-bound vesicles from cultured SMC. In the first approach, the classic collagenase method for isolation of matrix vesicles from bone and cartilage was used.<sup>26</sup> As shown in Figure 6A, transmission electron microscopy revealed that vesicles derived from the extracellular matrix of human SMC were membrane bound and heterogeneous in shape and size, with diameters ranging from 100 to 200 nm, identical to those described from chondrocyte cultures.<sup>27</sup> Equivalent amounts of matrix vesicles, as determined by protein content, were isolated from SMC cultured in CM compared with in GM (CM versus GM: 2.9 versus 3.0 mg/mL protein respectively). Western blot analysis demonstrated that these vesicles contained Annexin V (Figure 6B), a previously identified marker of matrix vesicles.<sup>24</sup> Annexin V levels were identical in matrix vesicles isolated from human SMC cultured in GM or CM, suggesting again that elevated phosphate treatment does not alter matrix vesicle formation or protein content. However, no Pit-1 protein was detected in these matrix vesicle preparations under either normal or elevated phosphate conditions (Figure 6B), suggesting that Pit-1 was not involved in phosphate loading by these structures.

In the second approach, membrane-bound vesicles and apoptotic bodies were isolated from apoptotic human SMC following serum starvation under normal (SFM) or elevated calcium and phosphate conditions (CPM), as previously described.<sup>25</sup> Identical to results obtained with matrix vesicles,



**Figure 6.** Characterization of membrane-bound vesicles derived from SMC. A, Matrix vesicles (MV) were isolated from SMC cultures by collagenase digestion and analyzed by transmission electron microscopy. Bar indicates 100 nm. B, SMC were cultured in GM or CM for 2 days; matrix vesicles were prepared by collagenase digestion and were subjected to immunoblot analysis for Annexin V or Pit-1. Unfractionated human SMC lysate was used as a positive control. C, SMC were cultured in SFM or elevated calcium and phosphate containing media (CPM) overnight. Apoptotic bodies (AB) released from cultured SMC were prepared by differentiated centrifugation and subjected to immunoblot analysis for Annexin V or Pit-1. A total of 40  $\mu\text{g}$  of protein was loaded for immunoblot analysis.

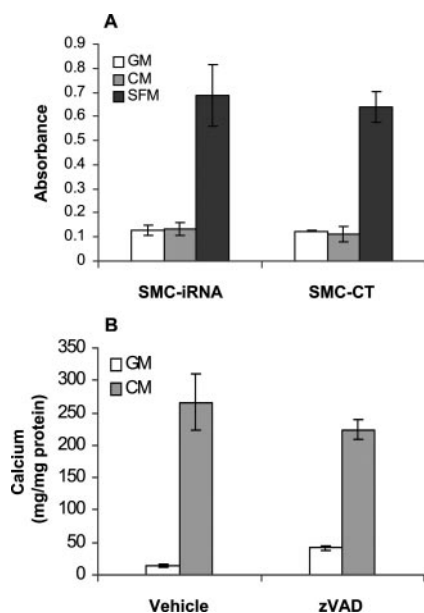
Pit-1 was not detected in these apoptotic vesicle preparations under any condition examined (Figure 6C).

### Pit-1 Does Not Promote SMC Apoptosis in Response to Elevated Phosphate

Previous studies have shown that apoptosis can accelerate mineralization in serum-deprived and long-term cultured human SMC.<sup>25,28</sup> Thus, another possible mechanism for elevated phosphate- and Pit-1-mediated SMC calcification might be through stimulation of apoptosis. To determine whether elevated phosphate induced SMC death via Pit-1, we examined apoptosis in SMC-iRNA and SMC-CT cells. Both cells were cultured in GM or CM and apoptosis was detected by specific ELISA to monitor mono- and oligonucleosomal chromatid formation. As shown in Figure 7A, very little apoptosis was detected in both cell types when cultured in GM. Importantly, treatment of the SMC-CT cells with CM did not increase apoptosis, and identical results were obtained in SMC-iRNA cells. As expected, treatment of cells with SFM overnight strongly induced apoptosis, and levels were similar in SMC-CT and SMC-iRNA cells (Figure 7A). Finally, treatment of human SMC with 100  $\mu\text{mol}/\text{L}$  zVAD, a potent caspase inhibitor, did not inhibit elevated phosphate-induced calcification (Figure 7B). These results indicate that the phosphate concentrations used in the present studies do not stimulate SMC apoptosis and that cells deficient in Pit-1 (SMC-iRNA) are not less susceptible to cell death than cells containing normal levels of Pit-1 (SMC-CT). Thus, apoptosis is unlikely the mechanism for Pit-1 mediated SMC calcification.

### Pit-1 Is Required for Elevated Phosphate-Induced Osteogenic Gene Expression in Human SMC

We and others have previously shown that elevated phosphate stimulates SMC calcification concomitant with pheno-

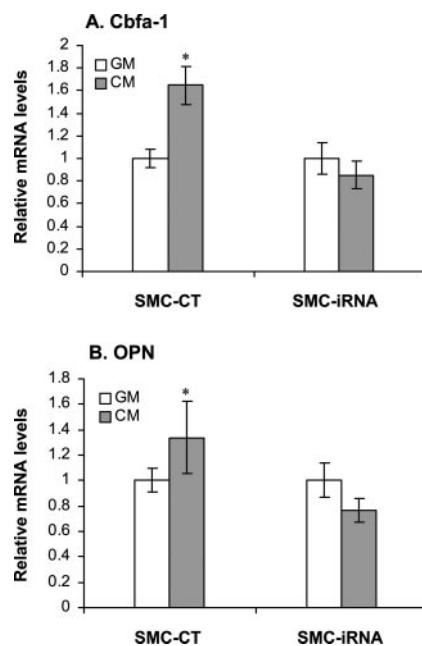


**Figure 7.** Assessment of apoptosis in cultured human SMC. A, SMC-iRNA and SMC-CT were cultured in GM or CM for 2 days. Apoptosis was determined by Cell Death Detection ELISA (Roche). As a positive control, SMC apoptosis was induced by treatment with SFM overnight. Data shown are means $\pm$ SD (n=3). B, SMC were cultured in GM or CM in the presence or absence of 100  $\mu$ mol/L of zVAD for 10 days. The calcium content was measured by *O*-cresolphthalein complexone method and normalized to protein content. Data shown are means $\pm$ SD (n=3).

typic change, characterized by expression of osteogenic genes such as *Cbfa-1* and *OPN*.<sup>15,16,18</sup> To determine whether *Pit-1* was required for elevated phosphate-induced SMC phenotypic transition, we examined *Cbfa-1* and *OPN* mRNA levels in SMC-CT and SMC-iRNA using real-time PCR. As expected, both *Cbfa-1* and *OPN* mRNA levels were significantly induced by elevated phosphate relative to normal phosphate conditions in SMC-CT at 7 days following treatment (Figure 8A and 8B). In contrast, *Cbfa-1* and *OPN* mRNA levels did not increase in response to elevated phosphate treatment compared with normal phosphate conditions in SMC-iRNA. Similar results were obtained when SMC-CT and SMC-iRNA were incubated with elevated phosphate for 2 days (data not shown). These results suggest that *Pit-1* was required for expression of *Cbfa-1* and *OPN* mRNA in human SMC in response to elevated phosphate conditions.

### Discussion

Previous studies indicated that elevated phosphate could induce SMC calcification as well as an osteochondrogenic phenotypic change in vitro.<sup>15,18</sup> Blocking the activity of sodium-dependent phosphate cotransporters with the nonspecific inhibitor phosphonoformic acid inhibited SMC calcification, suggesting an important role for phosphate uptake in SMC calcification. In the present study, we examined the requirement for the major sodium-dependent phosphate cotransporter in SMC, *Pit-1*, in calcification using RNA interference and overexpression approaches. We found that stable expression of *Pit-1* siRNA specifically inhibited *Pit-1* mRNA



**Figure 8.** Effect of *Pit-1* siRNA on induction of *Cbfa-1* and *OPN* by elevated phosphate in SMC. SMC-iRNA and SMC-CT were cultured in GM or CM for 7 days. Levels of *Cbfa-1* (A) or *OPN* (B) mRNA were determined by quantitative real-time PCR in triplicate and normalized to 18S rRNA levels. Data are expressed as means $\pm$ SD (n=4). \*Significant increase ( $P<0.05$ ) compared with GM.

and protein levels and led to a dramatic decrease in sodium-dependent phosphate uptake in SMC. Furthermore, calcification in response to elevated phosphate was substantially inhibited in SMC stably expressing *Pit-1* siRNA. Subsequent restoration of phosphate uptake by overexpression of *Pit-1* in *Pit-1*-deficient SMC rescued phosphate-induced calcification. Finally, we found that elevated phosphate-induced SMC phenotypic transition, as exemplified by upregulation of *Cbfa-1* and *OPN* mRNA levels, was *Pit-1* dependent. Our results are the first to demonstrate that *Pit-1*, via phosphate uptake, plays a critical role in human SMC calcification in response to elevated phosphate.

The present studies confirm the concept that vascular calcification is a highly cell-regulated process. This idea has been highlighted recently by the identification of several local and systemic, cell-derived inhibitors of calcification, such as osteopontin, pyrophosphate, and matrix gla protein, whose deficiency leads to inappropriate vascular calcification in vivo and in vitro.<sup>29–32</sup> In addition to negative regulators, a growing number of positive regulators of calcification, such as lipids, cytokines, and elevated phosphate levels, have also been identified.<sup>33</sup> Induction of SMC calcification by elevated phosphate is of particular interest, because hyperphosphatemia is a major risk factor for vascular calcification in dialysis patients. In the present studies, modulation of the levels of a cell membrane transporter, *Pit-1*, controlled the susceptibility of SMC to calcification in the presence of elevated phosphate. Despite equivalent calcium and phosphate levels in the culture media, cells that were deficient in *Pit-1* had substantially reduced extracellular matrix calcification compared with cells with normal levels of *Pit-1*. These



findings strongly suggest that cellular mechanisms, including phosphate uptake and/or signaling via Pit-1 is required for SMC calcification.

To begin to understand the mechanism(s) whereby Pit-1 mediates human SMC calcification in response to elevated phosphate, we considered several possibilities. First, we hypothesized that Pit-1 might be important for phosphate loading of calcifying membrane-bound vesicles, such as matrix vesicles and/or apoptotic bodies, that have been implicated in both physiological and pathological mineralization. There is considerable evidence suggesting the important role of matrix vesicles in the mineralization of bone and cartilage by concentrating calcium and phosphate and initiating mineral nucleation.<sup>24</sup> In addition, a recent study by Shanahan and colleagues identified membrane-bound vesicles containing apoptotic bodies from apoptotic human SMC that could calcify after prolonged exposure to elevated calcium or phosphate.<sup>25</sup> Although matrix vesicles as well as apoptotic bodies could be isolated from human SMC in the present study, we were unable to detect Pit-1 protein in either preparation, regardless of culture under normal or elevated phosphate conditions. Furthermore, equivalent numbers of matrix vesicles and apoptotic bodies were isolated in normal and elevated phosphate containing media. Thus, it is unlikely that Pit-1 mediates calcification of human SMC by facilitating synthesis, release, or phosphate loading of matrix vesicles or apoptotic bodies.

Next, we tested the hypothesis that Pit-1 might be required to induce apoptosis in human SMC in response to elevated phosphate treatment, thereby promoting calcification. Previous studies suggested that extremely high phosphate levels (4 to 10 mmol/L) induced apoptosis in chondrocytes as well as SMC and that mineralization was mediated, in part, through apoptosis-dependent mechanisms.<sup>25,28,34,35</sup> In the present studies, human SMC cultured in serum-containing media in the presence of 2.6 mmol/L phosphate did not show increased apoptosis compared with cells cultured in normal phosphate containing media (1.4mmol/L phosphate). Furthermore, cells deficient in Pit-1 had very low levels of apoptosis that were comparable to cells with normal Pit-1 levels, and the susceptibility of these cells to apoptosis was not altered by elevated phosphate treatment. Finally, the caspase inhibitor zVAD failed to prevent phosphate-induced calcification in our system. These results suggest that phosphate-induced apoptosis is not the mechanism by which elevated phosphate induces SMC calcification under our culture conditions. These findings are in contrast to Reynolds et al, who showed that elevated calcium (2.7 mmol/L) combined with elevated phosphate (2.0 mmol/L) induced apoptosis as well as calcification of human SMC cultured in SFM.<sup>25</sup> The difference in these findings is most likely explained by differences in culture conditions. Reynolds et al used SFM combined with elevated calcium and phosphate to induce SMC apoptosis, apoptotic body formation, and calcification, whereas the human SMC in our studies were cultured in serum-containing media, with calcium and phosphate levels kept well below concentrations that could result in spontaneous mineral precipitation in solution or cause apoptosis. Indeed, incubation of human SMC in SFM strongly induced apoptosis in our

study as well. Thus, the requirement of Pit-1 in human SMC calcification under the more physiological conditions of our system does not appear to be via a mechanism involving altered apoptosis.

Finally, we hypothesized that phosphate uptake through Pit-1 was required for the phenotypic modulation of human SMC to an osteochondrocytic phenotype, thereby regulating the mineralization capacity of these cells. In support of this hypothesis, human SMC deficient in Pit-1 failed to upregulate Cbfa-1 and OPN mRNA levels in response to elevated phosphate treatment. In contrast, both genes were induced in human SMC containing normal levels of Pit-1, consistent with previous findings.<sup>15,18</sup> Of interest, Pit-1 has also been implicated in bone cell differentiation and mineralization. Pit-1 mRNA increases during osteoblast differentiation and correlates with the time at which calcification is observed.<sup>36</sup> Likewise, studies by Palmer et al found that Pit-1 mRNA was expressed in mineralizing, hypertrophic chondrocytes from day 17 in embryonic murine metatarsals, whereas no Pit-1 mRNA was detected in fully differentiated, nonmineralized chondrocytes.<sup>37</sup> Thus, phosphate uptake via Pit-1 may be a major regulator of cellular differentiation programs involved in both normal and pathological calcification.

In summary, knockdown of Pit-1 expression by siRNA inhibited sodium-dependent phosphate uptake as well as phosphate-dependent SMC calcification. Restoration of phosphate uptake by overexpression of Pit-1 in Pit-1-deficient cells rescued calcification. Our results indicate that phosphate uptake is required for SMC calcification induced by elevated phosphate. Our studies also suggest that Pit-1-dependent SMC calcification is not mediated by enhanced apoptosis or membrane-bound vesicle phosphate loading. However, inhibition of phosphate uptake in Pit-1 knockdown cells did abrogate induction of osteogenic markers, such as Cbfa-1 and OPN, in human SMC. These findings demonstrate a requirement for phosphate uptake via the sodium-dependent phosphate cotransporter, Pit-1, for SMC phenotypic transition and calcification that is likely to be important in the development of ectopic calcification of blood vessels under hyperphosphatemic conditions. These studies also point to Pit-1 as a potential target for therapies aimed at reducing vascular calcification.

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