Sodium-Dependent Phosphate Cotransporters and Phosphate-Induced Calcification of Vascular Smooth Muscle Cells

Redundant Roles for PiT-1 and PiT-2

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Objective—Elevated serum phosphate has emerged as a major risk factor for vascular calcification. The sodium-dependent phosphate cotransporter, PiT-1, was previously shown to be required for phosphate-induced osteogenic differentiation and calcification of cultured human vascular smooth muscle cells (VSMCs), but its importance in vascular calcification in vivo and the potential role of its homologue, PiT-2, have not been determined. We investigated the in vivo requirement for PiT-1 in vascular calcification using a mouse model of chronic kidney disease and the potential compensatory role of PiT-2 using in vitro knockdown and overexpression strategies.

Approach and Results—Mice with targeted deletion of PiT-1 in VSMCs were generated (PiT-1\(^{Δsm}\)). PiT-1 mRNA levels were undetectable, whereas PiT-2 mRNA levels were increased 2-fold in the vascular aortic media of PiT-1\(^{Δsm}\) compared with PiT-1\(^{fllox/fllox}\) control. When arterial medial calcification was induced in PiT-1\(^{Δsm}\) and PiT-1\(^{fllox/fllox}\) by chronic kidney disease followed by dietary phosphate loading, the degree of aortic calcification was not different between genotypes, suggesting compensation by PiT-2. Consistent with this possibility, VSMCs isolated from PiT-1\(^{Δsm}\) mice had no PiT-1 mRNA expression, increased PiT-2 mRNA levels, and no difference in sodium-dependent phosphate uptake or phosphate-induced matrix calcification compared with PiT-1\(^{fllox/fllox}\) VSMCs. Knockdown of PiT-2 decreased phosphate uptake and phosphate-induced calcification of PiT-1\(^{Δsm}\) VSMCs. Furthermore, overexpression of PiT-2 restored these parameters in human PiT-1-deficient VSMCs.

Conclusions—PiT-2 can mediate phosphate uptake and calcification of VSMCs in the absence of PiT-1. Mechanistically, PiT-1 and PiT-2 seem to serve redundant roles in phosphate-induced calcification of VSMCs. (Arterioscler Thromb Vasc Biol. 2013;33:2625-2632.)

Key Words: phosphate ■ PiT-1 ■ PiT-2 ■ vascular calcification ■ vascular smooth muscle cell

Cardiovascular mortality remains the leading cause of death in patients with chronic kidney disease (CKD). The extent of arterial calcification independently predicts cardiovascular disease and mortality in patients with CKD, and this ectopic calcification occurs decades earlier in patients with CKD than in the general population. The calcium phosphate mineral deposits occur in the form of hydroxyapatite or whitlockite and can contribute to disease progression in 2 main locations within arterial vessels: the intimal layer and the medial layer. Calcification associated with the intimal layer is a reflection of atherosclerotic burden and may result in arterial occlusion. Arterial medial calcification (AMC) contributes to vascular stiffening without occlusion and can lead to hypertension and heart failure. Studies have shown that AMC is a dominant form of vascular calcification (VC) in dialysis patients.

During the past decade, elevated serum phosphorus has emerged as a key risk factor for VC in patients with CKD, as well as the general population. High extracellular phosphate has been widely established to induce vascular smooth muscle cell (VSMC) matrix calcification in vitro. Indeed, elevated phosphate has been linked to several highly regulated processes in the vessel wall that promote AMC. These include VSMC osteogenic differentiation, VSMC apoptosis, and matrix degradation (reviewed in Lau et al and Shanahan et al). The phenotype change is particularly striking, whereby the VSMCs cease to express smooth muscle markers (smooth muscle-α actin, SM22) and instead express bone-forming markers.
PiT-1 in phosphate uptake and matrix mineralization in vitro

trol mice, prompting us to examine potential compensatory or inflammation. As global deletion of PiT-1 is lethal when subjected to partial renal ablation, develop robust more than HPO$_4^{2-}$ expressed in kidney and intestinal epithelium, are important for whole-body phosphate homeostasis. The type II (SLC34 family) cotransporters, predominantly expressed in kidney and intestinal epithelium, are important for whole-body phosphate homeostasis. The type III (SLC34 family) cotransporters, PiT-1 and PiT-2, are more generally expressed and have been identified as the predominant phosphate transporters in rat and human VSMCs. PiT-1 and PiT-2 are 62% identical at the amino acid level and both contain 12 transmembrane spanning domains with a single large intracellular domain. They exhibit a high affinity for phosphate and have a preference to transport HPO$_4^{2-}$ more than HPO$_4^{2-}$ with a 2:1 ratio of sodium:phosphate in both acidic and alkaline pH. The Michaelis–Menten affinity constant ($K_m$) for sodium-dependent phosphate uptake for PiT-1 and PiT-2 is similar and ranges from 25 to 300 μmol/L phosphate depending on the species. We have shown previously that PiT-1 was required for phosphate-induced osteogenic differentiation and calcification of human VSMCs in vitro. When PiT-1 was knocked down in human aortic VSMCs using short hairpin RNA interference (shRNA), phosphate-induced mineralization was suppressed, and VSMC phenotype was preserved. Subsequent overexpression of mouse PiT-1 restored phosphate transport, VSMC osteogenic phenotype change, and calcification. Given the compelling in vitro evidence, we hypothesized that PiT-1 would be required for VC in vivo. To test this, a well-characterized mouse model of phosphate-driven uremic AMC was used. Calcification-prone DBA/2J mice, when subjected to partial renal ablation, develop robust AMC on dietary phosphate loading without atherosclerosis or inflammation. As global deletion of PiT-1 is lethal during mouse embryogenesis, we generated VSMC-specific knockdown of PiT-1 using a SM22α promoter–driven Cre/loxP system. This resulting PiT-1$^{lox/lox}$;Sm22α-Cre$^{eOE}$ dual transgenic mouse line (abbreviated as PiT-1$^{lox/lox}$) lacked PiT-1 expression in VSMCs. Unexpectedly, sodium-dependent phosphate uptake and matrix mineralization in isolated VSMCs, as well as high-phosphate feeding–induced uremic AMC, did not differ between PiT-1$^{lox/lox}$ and PiT-1$^{lox/lox}$ control mice, prompting us to examine potential compensatory mechanisms. Our data indicate that PiT-2 compensates for PiT-1 in phosphate uptake and matrix mineralization in vitro and are the first to suggest redundant roles for PiT-1 and PiT-2 in phosphate-driven AMC.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

PiT-1 and PiT-2 Are the Only NaPi Cotransporters Expressed by Mouse VSMCs

Reverse transcription polymerase chain reaction (PCR) was used to determine the expression profile of NaPi cotransporters in cultured C57Bl/6 wild-type VSMCs. Using this technique, no specific bands were obtained using primers for type I transporters (SLC17A1, A4 and A7) or type II transporters (SLC34A1, A2 and A3), whereas both type III transporters were detected (Figure I in the online-only Data Supplement).

VSMC-Specific Knockdown of PiT-1

In contrast to the global PiT-1 knockout that resulted in embryonic lethality because of anemia, mice with smooth muscle–specific PiT-1 deletion were born at the expected Mendelian ratios and showed no gross abnormalities in health, weight, or lifespan (data not shown). Likewise, histological analyses of aortas from these mice were normal (data not shown). As shown in Figure II in the online-only Data Supplement, PCR amplification of genomic DNA isolated from VSMCs from PiT-1$^{lox/lox}$ mice showed a 500-bp band (compared with 950 bp band in the PiT-1$^{lox/lox}$ cells) consistent with deletion of PiT-1 protein in whole aortic tissue in PiT-1$^{lox/lox}$ mice (Figure 1A and 1B), and immunohistochemistry showed selective PiT-1 depletion in VSMCs but not endothelial cells (Figure 1C). Consistent with the immunostaining data, PiT-1 mRNA was undetectable in the aortic media isolated from healthy PiT-1$^{lox/lox}$ mice (Figure III in the online-only Data Supplement). Because a trend for decreased expression of PiT-1 mRNA in cardiac tissue from PiT-1$^{lox/lox}$ compared with PiT-1$^{lox/lox}$ was observed, and because SM22α is transiently expressed in the heart during mouse embryogenesis, there was a possibility that SM22α promoter–driven PiT-1 deletion could have affected cardiogenesis. However, resting echocardiography under minimal sedation on healthy PiT-1$^{lox/lox}$ mice showed no difference in ejection fraction, left ventricular wall thickness, and left ventricular end-diastolic volume, compared with wild-type controls (Table II in the online-only Data Supplement).

Deletion of PiT-1 From VSMCs Had No Effect on AMC Induced by Uremia and High-Phosphate Feeding

We previously developed and characterized a CKD mouse model of AMC using calcification-prone DBA/2J mice fed a high-phosphate diet. In this model, robust AMC in the absence of inflammation or atherosclerosis develops in CKD mice in response to high-phosphate feeding but does not occur in CKD mice fed a normal phosphate diet. Thus, this model is well suited to investigating the role of phosphate-dependent mechanisms in AMC. To determine whether the

Nonstandard Abbreviations and Acronyms

| AMC | arterial medial calcification |
| CKD | chronic kidney disease |
| PiT-1 | type III sodium-dependent phosphate cotransporter 1 (SLC20A1) |
| PiT-2 | type III sodium-dependent phosphate cotransporter 2 (SLC20A2) |
| shRNA | short hairpin RNA interference |
| VC | vascular calcification |
| VSMC | vascular smooth muscle cell |

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loss of PiT-1 expression in smooth muscle cell (SMC) would affect the extent of calcification in this animal model, partial renal ablation was used to generate CKD in PiT-1<sup>fl/fl</sup> and PiT-1<sup>∆sm</sup> mice (n=16 per group). After feeding with the high-phosphate diet for 4 weeks, calcium accumulated in the aorta as expected, but the extent of calcification did not differ significantly between the 2 groups (Figure 2A). Consistent with previous studies, no AMC was observed in CKD mice fed a normal phosphate diet (PiT-1<sup>fl/fl</sup>, n=8) and PiT-1<sup>∆sm</sup> mice normalized to the loading control and given in arbitrary units (AU). C, Immunohistochemistry using anti–PiT-1 antibody performed on wild-type (WT) and PiT-1<sup>∆sm</sup> (SM) paraffin-embedded aorta sections. No primary. Negative control that was not treated with the anti–PiT-1 antibody. Arrows, Positive staining in medial vascular smooth muscle cell (S) and endothelial cells (E) in WT, but only endothelial cells (E) in PiT-1<sup>∆sm</sup>. Scale bars, 50 μm.

**Aortas From PiT-1<sup>∆sm</sup> and PiT-1<sup>fl/fl</sup> Mice Showed Similar Histology and SMC Gene Expression**

Aortas from high-phosphate–fed PiT-1<sup>∆sm</sup> and PiT-1<sup>fl/fl</sup> CKD mice showed similar calcified medial wall lesions by von Kossa staining, characterized by elastocalcinosis in the absence of inflammatory cells (Figure 2B). Hematoxylin and eosin evaluation showed no differences in vascular wall structure between PiT-1<sup>∆sm</sup> and PiT-1<sup>fl/fl</sup> CKD mice (Figure 2C-i and 2C-ii), and elastin degradation was evident in both experimental groups under eosin fluorescence imaging (Figure 2C-iii and 2C-iv). VSMC phenotype change, as characterized by the loss of SM22α staining (Figure 2C-v and 2C-vi), was apparent in both groups. Furthermore, transcriptional profiling of aortic mRNA showed increased expression of osteochondrogenic markers Runx2, osteopontin, and osteoprotegerin in CKD versus healthy PiT-1<sup>fl/fl</sup> mice aortas, but no statistically significant differences were observed in these genes between CKD PiT-1<sup>∆sm</sup> and PiT-1<sup>fl/fl</sup> mice fed a high-phosphate diet (Table III in the online-only Data Supplement). Finally, because recent studies have implicated PiT-1 in cell proliferation and survival, we counted the number of VSMCs per cross-sectional area of aorta to determine whether there was a reduction in VSMCs within the medial layer. VSMC cell densities in PiT-1<sup>fl/fl</sup> and PiT-1<sup>∆sm</sup> were 3565±378 and 4158±332 cells/mm² (mean±SEM), respectively, showing no statistical difference between genotypes (P=0.27).

**SMC-Specific Deletion of PiT-1 Did Not Alter Phosphate-Induced Calcification of Aortic Rings and Cultured VSMCs**

To determine whether compensatory circulating factors might explain the lack of effect of PiT-1 deletion on AMC in uremic mice, in vitro calcification of aortic rings and isolated VSMCs was assessed. Aortas were harvested from PiT-1<sup>fl/fl</sup> and PiT-1<sup>∆sm</sup> mice to generate aortic rings, and calcification was induced by incubation in high-phosphate media for 9 days. As shown in Figure 3A, there was no significant difference in calcification between the 2 groups. Similar to the results in aortic rings, we found no significant difference in the extent of calcification between PiT-1<sup>∆sm</sup> and PiT-1<sup>fl/fl</sup> in cultured VSMCs after treatment with high-phosphate media for 6 to 10 days (Figure 3B).

**Deletion of PiT-1 in Mouse VSMCs Had No Effect on Phosphate Uptake**

Sodium-dependent phosphate uptake kinetics were determined in cultured VSMCs from PiT-1<sup>fl/fl</sup> and PiT-1<sup>∆sm</sup> mice and revealed no significant differences between the 2 genotypes when nonlinear curve fitting assuming Michaelis–Menten kinetics was performed (P=0.6; Figure 3C). The V<sub>max</sub> was measured as 0.315±0.042 and 0.285±0.038 pmol/μg protein per minute with a K<sub>m</sub> of 0.223±0.061 and 0.205±0.057 mmol/L for the PiT-1<sup>fl/fl</sup> and PiT-1<sup>∆sm</sup> VSMCs, respectively. Sodium-dependent uptake was unchanged in the presence of 0 to 1 mmol/L phosphonoformic acid, an inhibitor of type II NaPi transport (Figure 3D). As the K<sub>i</sub> of phosphonoformic acid for PiT-1 and PiT-2 is in the range of 2.5 to 5 mmol/L, lack of inhibition by phosphonoformic acid at submillimolar concentrations indicated that type II transporters were not contributing to phosphate uptake in the cultured VSMCs. This finding is consistent with the reverse transcription PCR data that showed no expression of type II transporters in mouse VSMCs (Figure 1 in the online-only Data Supplement).

**VSMC-Specific Knockdown of PiT-1 Was Associated With PiT-2 Upregulation**

Because sodium-dependent phosphate uptake was unchanged in PiT-1 knockout cells compared with controls, we suspected...
that PiT-2 might be compensating for the loss of PiT-1 in VSMCs. Real-time quantitative PCR showed that aortic media PiT-2 mRNA was 2-fold higher in PiT-1∆sm mice compared with PiT-1fl/fl mice. All other tissues examined showed no difference in PiT-2 expression between the genotypes

Table. Serum Chemistries

<table>
<thead>
<tr>
<th>Treatment Group (n=16)</th>
<th>Pre-CrD Parameters, mg/dL</th>
<th>CrD Parameters, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>Ca</td>
</tr>
<tr>
<td>PiT-1Δsm</td>
<td>8.5±1.6</td>
<td>9.4±0.2</td>
</tr>
<tr>
<td>PiT-1fl/fl</td>
<td>8.1±1.3</td>
<td>9.3±0.2</td>
</tr>
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</table>

BUN was measured in all mice. Pre-CrD Ca and P were measured from 6 mice per group. CrD Ca and P were measured in 3 mice per group. Data are mean±SE. Parameters were not significantly different between groups. BUN indicates blood urea nitrogen; Ca, calcium; n, number of mice in each treatment group; and P, phosphate.

Figure 2. Aortic calcification in uremic, high-phosphate–fed mice. A, Aortic calcium content was not different (P=0.35) between the high-phosphate–fed chronic kidney disease mice in the PiT-1∆sm group vs the PiT-1fl/fl group. Data are mean±SE and n=16 per group. B, Von Kossa staining (brown) with hematoxylin counterstain (blue) showing medial calcification in aortas from (i) PiT-1Δsm mouse and (ii) PiT-1fl/fl mouse. Scale bars, 50 μm. C, Aortic sections from PiT-1Δsm (i, iii, and v) and PiT-1fl/fl (ii, iv, and vi) mice showing similar hematoxylin and eosin staining (i and ii), eosin fluorescence (iii and iv) with arrowheads showing elastin strand breaks, and SM22α staining with methyl green nuclear counterstain (v and vi), with arrows pointing to smooth muscle cell nuclei within the calcified area that have lost or have much reduced SM22α staining compared with adjacent noncalcified areas. Scale bars, 25 μm. PiT-1 indicates type III sodium-dependent phosphate cotransporter 1.

Figure 3. Aortic ring calcification and phosphate uptake in PiT-1Δsm (∆SM) and PiT-1fl/fl (∆fl) vascular smooth muscle cells (VSMCs). A, Calcification of mouse aortic rings in response to normal (1 mmol/L; open bars) or elevated (2.6 mmol/L; black bars) phosphate, n=5 per group. Data are given as means±SD. B, Fold difference in calcification of PiT-1Δsm VSMCs relative to PiT-1fl/fl VSMCs after treatment with 2.6 mmol/L phosphate. Data are the average of 4 separate experiments using cell passages 6–8 and were treated for 9–12 days in duration. Expressed as means±SEM. C, Radiolabeled phosphate transport assays in VSMCs from PiT-1Δsm (circles) and PiT-1fl/fl (squares) mice. Data are expressed as means±SD. D, Lack of inhibition of phosphate uptake by the type II NaPi inhibitor phosphonoformic acid (PFA). Circles are PiT-1Δsm, squares are PiT-1fl/fl. Closed symbols are in the presence of sodium, open symbols are in the absence of sodium. Data are expressed as means±SD. PiT-1 indicates type III sodium-dependent phosphate cotransporter 1.

Overexpression of PiT-2 in PiT-1 Knockdown VSMCs Restored Phosphate Uptake and Phosphate-Induced Calcification

We previously reported that knockdown of PiT-1 expression in human VSMCs using shRNA decreased phosphate uptake, phosphate-induced calcification, and osteogenic differentiation without compensation by PiT-2.3 Thus, to determine whether PiT-2 could functionally compensate for the loss of PiT-1 in VSMCs, we overexpressed PiT-2 by retroviral transduction into PiT-1 knockdown human VSMCs that were previously shown to express ≈80% less PiT-1 than controls.21 Overexpression of PiT-2 was confirmed by quantitative PCR (Figure 5A, graph) and Western blotting (Figure 5A,
PiT-2 expression was increased 2-fold in PiT-1 knockdown VSMCs compared with the scrambled control (Figure 2). Sodium-dependent phosphate uptake was significantly decreased by ~80% compared with the control cells (Figure 3A). PiT-2 mRNA expression was reduced by ~55% in PiT-1–deficient mouse VSMCs compared with the scrambled control (Figure 6C).

Knockdown of PiT-2 in PiT-1–Deficient Mouse VSMCs Decreased Phosphate Transport and Phosphate-Induced Calcification

To determine whether PiT-2 was functionally compensating for PiT-1 in the PiT-1Δsm VSMCs, we transduced these cells with scrambled control or PiT-2–specific shRNA to create PiT-1/PiT-2 double knockdown VSMCs. In these cells, PiT-2 mRNA expression was reduced by ~80% compared with the control cells (Figure 6A). Subsequently, sodium-dependent phosphate uptake was significantly decreased by ~35% (Figure 6B) and phosphate-induced calcification was significantly decreased by ~20% in PiT-1 knockdown VSMCs compared with the scrambled control (Figure 6C).

Discussion

Overall, our studies show that deletion of PiT-1 selectively from VSMCs does not alter the extent of AMC in CKD mice fed a high-phosphate diet. Similarly, sodium-dependent phosphate uptake in VSMCs, as well as calcification of explanted aortic rings, and VSMC primary cultures treated with high-phosphate media were equivalent in PiT-1Δsm and PiT-1fl/fl mice. Interestingly, PiT-2 expression was increased 2-fold in PiT-1Δsm VSMCs compared with PiT-1fl/fl mice, suggesting that this type III NaPi family member might be compensating for the loss of PiT-1. A compensatory role was supported by in vitro studies because overexpression of PiT-2 restored calcification in cultured PiT-1–deficient human VSMCs; in addition, knockdown of PiT-2 in PiT-1Δsm VSMCs diminished calcification. These are the first studies to demonstrate a redundant role for PiT-1 and PiT-2 in VC.

Previous work in our group demonstrated a role for PiT-1 in phosphate-induced human VSMC calcification and osteochondrogenic transformation in vitro.21 Knockdown of PiT-1 by shRNA suppressed phosphate-induced calcification and osteochondrogenic phenotype change but did not block SMC apoptosis induced by high phosphate under serum-free conditions. Others have seen similar results in osteoblast cultures where knockdown of PiT-1 reduced matrix mineralization and reduced expression of osteoblast markers such as osteopontin.35,36 More recently, Sugita et al37 found that cellular phosphate transport and ATP synthesis mediated by PiT-1 were critical for chondrogenesis in mice. Together, these studies support the hypothesis that PiT-1 is a key player in elevated phosphate-induced calcification. Thus, it was surprising that no differences in SMC phenotype modulation or AMC were observed between PiT-1Δsm and PiT-1fl/fl mice after CKD and high-phosphate feeding. Furthermore, no difference was observed in calcification of cultured aortic rings from PiT-1Δsm and PiT-1fl/fl mice, suggesting that compensation by circulating factors was unlikely. Finally, isolated VSMC from PiT-1Δsm and PiT-1fl/fl mice showed no difference in sodium-dependent phosphate uptake kinetics in vitro. These findings led us to consider a potential cell autonomous compensatory mechanism in SMCs deficient in PiT-1.

We discovered that PiT-2 mRNA expression was upregulated in aortas in response to developmental loss of PiT-1 specifically in mouse SMCs. This finding was distinct from our previous findings in human VSMC, where levels of PiT-2 were low and unchanged after shRNA knockdown of PiT-1, pointing to a potential developmental compensatory
mechanism (discussed in more detail below). The increase in PiT-2 expression in PiT-1∆sm aortas was selective because no change in other known sodium-dependent phosphate cotransporters was detected. The increase in PiT-2 compensated for PiT-1 in phosphate transport and VC in vitro. Overexpression of PiT-2 in PiT-1–deficient human SMC restored phosphate uptake and calcification after elevated phosphate treatment. Furthermore, siRNA knockdown of PiT-2 in PiT-1∆sm VSMCs diminished both phosphate uptake and calcification. Together, these data support the hypothesis that PiT-2 could compensate for PiT-1 in both sodium-dependent phosphate uptake and phosphate-induced calcification.

Although we were able to knockdown PiT-2 mRNA levels by $\approx80\%$ in PiT-1∆sm VSMC, we observed only a 35% decrease in sodium-dependent phosphate uptake and 20% decrease in phosphate-induced calcification. There are several possible explanations for this observation. First, a low density of PiT-2 transporter may be sufficient, within the duress of a high-phosphate milieu, to initiate the signaling cascade that results in VSMC calcification. Second, because decline in phosphate uptake and phosphate-induced calcification were comparable, our data might suggest that the phosphate uptake function of PiT-2 is most critical for VSMC calcification. Finally, a combination of these mechanisms may be acting to explain the results.

A review of the literature shows that regulation of PiT-1 and PiT-2 expression is specific to the cell type, physiological state,
and treatment agent. Byskov et al. (in MC3T3-E1 and NIH3T3 cell lines) and our laboratory (unpublished data in mouse VSMCs) have shown that the expression of PiT-1 and PiT-2 is variable based on cell density in vitro, whereby increased cell density results in lower PiT-1 and PiT-2 mRNA expression. Bone morphogenetic protein-2 has been shown to increase PiT-1, but not PiT-2, mRNA expression in MC3T3-E1 osteoblast cell and human VSMCs in vitro. Likewise, platelet-derived growth factor-BB increased PiT-1 mRNA and protein in rat VSMCs with no change in PiT-2. Our data showing PiT-2 upregulation in VSMCs in response to PiT-1 deletion are in agreement with other reports. Beck et al. demonstrated that in mice with a global deletion of PiT-1, PiT-2 was upregulated in the embryonic liver, and Byskov et al. showed PiT-2 mRNA upregulation in MC3T3-E1 after PiT-1 shRNA knockdown. However, we are the first to demonstrate that PiT-2 can functionally compensate for PiT-1 in VSMC calcification.

As mentioned above, PiT-2 mRNA expression was upregulated in VSMCs in response to developmental loss of PiT-1 specifically in mouse SMCs, and this finding was in contrast to results obtained when shRNA was used to more acutely knockdown PiT-1 in cultured human SMCs. Although the underlying mechanisms of developmental compensation are often enigmatic, the increased level of PiT-2 expression observed in the PiT-1 knockout mice, together with published literature, suggests ≥2 potential compensatory mechanisms. In the first hypothetical mechanism, a decrease in PiT-1 triggers a regulatory switch, which in turn results in increased transcriptional activation of PiT-2, either through increased expression or post-translational stabilization of a transcriptional activator, or increased accessibility of the PiT-2 promoter. Molecular cloning and sequence analysis have revealed several potential cis-acting elements in the PiT-2 promoter, including a CACCC (Kruppel-like factor) binding site, thyroid hormone response elements, serum response elements, retinoic acid response elements, and vitamin D3-response elements. Examination of the endogenous activity of these candidate upstream regulators and their interaction with the PiT-2 promoter in vivo may reveal important information about the mechanism of compensation. Importantly, a KLF family member was recently shown to regulate expression of PiT-1 during erythroid maturation. Alternately, the compensatory mechanism may be independent of transcriptional regulation. A decrease in phosphate levels is known to result in enhanced mRNA stability of PiT-2. It is possible that the decrease in phosphate intake caused by the absence of PiT-1 during development results in a prolonged half-life of PiT-2 and, in turn, the higher levels of PiT-2 mRNA that we observed by quantitative PCR (Figure 4A). Future analysis of PiT-1/PiT-2 double knockout models along with evaluation of candidate PiT-2 transcriptional regulators and examination of PiT-2 mRNA stability in vivo will be required to test these 2 hypotheses. Finally, potential species differences in PiT compensation cannot be ruled out.

The idea that both PiT-1 and PiT-2 may be important regulators of VC is further strengthened by recent findings in rare human genetic syndromes. Wider et al. found that mutations in PiT-2 associated with impaired phosphate transport caused familial idiopathic basal ganglia calcification, a condition characterized by mineralization of capillaries, as well as small arteries and veins of the basal ganglion region. Although the exact mechanism for this effect is unknown, the authors speculated that the loss of PiT-2 function and regional phosphate homeostasis might lead to increased PiT-1 function in VSMC, thereby promoting osteochondrogenic differentiation and calcification. Furthermore, the overexpression of PiT-1 has been reported in fibroblasts derived from patients with Werner syndrome, an autosomal recessive disorder caused by mutations in RecQ DNA helicase that is characterized by premature aging and soft tissue calcification.

In summary, although we have shown that the extent of AMC in high-phosphate-fed uremic mice was not different either between the PiT-1<sup>Δ<sub>atahydrome</sub></sup> and PiT-1<sup>flox/flox</sup> or in vivo using VSMC primary culture from these mice, we discovered increased PiT-2 expression in the PiT-1<sup>Δ<sub>atahydrome</sub></sup> VSMCs. Using in vitro methods, we demonstrated that PiT-2 overexpression could compensate for phosphate uptake and phosphate-induced calcification in PiT-1-deficient VSMCs, whereas knockdown of PiT-2 reduced phosphate uptake and calcification. Taken together our data suggest redundant roles for PiT-1 and PiT-2 in phosphate-driven AMC.

Sources of Funding
This study was funded by National Institutes of Health grants to Dr Giachelli (R01 HL62329 and R01 HL081785), W.L. Lau and M.H. Crouthamel received funding from T32 HL007828 (NHBLII), and W.L. Lau was also funded by T32 DK007467 (NIDDK).

Disclosures
None.

References
2632 Arterioscler Thromb Vasc Biol November 2013


**Significance**

Vascular calcification (VC) is a major risk factor for cardiovascular morbidity and mortality, and elevated phosphate has been identified as a key inducer of vascular calcification via procalcific effects on vascular smooth muscle cells. We identified a novel function for the sodium-dependent phosphate transporter, PIT-2, as a mediator of elevated-phosphate–induced vascular calcification in vitro and in vivo. Moreover, we provide mechanistic insight into compensatory mechanisms that operate in smooth muscle cells to protect against phosphate transporter deficiency. Importantly, as our studies were in progress, PiT-2 was identified as the causative gene for idiopathic basal ganglion calcification in people, and thus our studies noting compensatory mechanisms for phosphate transporters may help to explain how mutation of PIT-2 might lead to compensatory changes that actually facilitate vascular calcification. Clinically, our data provide a cautionary note on compensatory pathways that should be considered when attempting to translate inhibition of phosphate transport to clinical therapies.
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Arterioscler Thromb Vasc Biol. 2013;33:2625-2632; originally published online August 22, 2013;
doi: 10.1161/ATVBAHA.113.302249
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Sodium-dependent phosphate cotransporters and phosphate-induced calcification of vascular smooth muscle cells: Redundant roles for PiT-1 and PiT-2

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SUPPLEMENTAL MATERIAL

Supplemental Tables
Table I: PCR primers and probes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Taqman probe</th>
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<tbody>
<tr>
<td>SLC17A1</td>
<td>Applied Biosystems TaqMan® Gene Expression Assay: Mm00436577_m1</td>
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<td></td>
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<tr>
<td>SLC17A4</td>
<td>Applied Biosystems TaqMan® Gene Expression Assay: Mm00621610_m1</td>
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<tr>
<td>SLC17A7</td>
<td>Applied Biosystems TaqMan® Gene Expression Assay: Mm00812886_m1</td>
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<tr>
<td>SLC34A1</td>
<td>GAGCCCTTCACAAGACTCATCAT</td>
<td>CGGCAATGCTGGTGATCA</td>
<td>n.a.</td>
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<td>SLC34A2</td>
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<tr>
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<td>PiT-1</td>
<td>TTCCTTGTCCGTGCCTCATT</td>
<td>AATGGTAAGCTCGTAAGCCATT</td>
<td>CCGTAAGGCAGATCC</td>
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<td>PiT-2</td>
<td>GACCGTGGAAACGCTAAATGG</td>
<td>CTCAGGAAGGACGCAGACATCAA</td>
<td>CATGGTTGGTTCAGCTG</td>
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<tr>
<td>GAPDH</td>
<td>ACCACAGTCATGGCCATC</td>
<td>TCCACCACCTGGTGGTGA</td>
<td>n.a.</td>
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Table II. Echocardiography data.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Left ventricular wall thickness (mm)</th>
<th>Left ventricular end-diastolic dimension (mm)</th>
<th>Ejection fraction (%)</th>
<th>Fractional shortening (%)</th>
<th>Heart rate (beats per minute)</th>
<th>Body weight (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PiT-1&lt;sup&gt;lox/lox&lt;/sup&gt;</td>
<td>0.83 ± 0.04</td>
<td>3.53 ± 0.19</td>
<td>75.3 ± 2.6</td>
<td>43.2 ± 2.2</td>
<td>558 ± 26</td>
<td>28.8 ± 2.5</td>
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<tr>
<td>PiT-1&lt;sup&gt;Δsm&lt;/sup&gt;</td>
<td>0.9 ± 0.03</td>
<td>3.36 ± 0.31</td>
<td>74.9 ± 3.6</td>
<td>42.8 ± 3.3</td>
<td>572 ± 29</td>
<td>29.6 ± 0.5</td>
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</table>

Table III. Osteochondrogenic Gene Expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold difference</th>
<th>CKD-fl/fl /nonCKD-fl/fl</th>
<th>p=</th>
<th>Fold Difference</th>
<th>CKD-Δsm /CKD- fl/fl</th>
<th>p=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteopontin</td>
<td>42</td>
<td>0</td>
<td></td>
<td>1.2</td>
<td></td>
<td>n/s</td>
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<td>Runx2</td>
<td>4.3</td>
<td>0.000207934</td>
<td>0.6</td>
<td>0.6</td>
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<td>n/s</td>
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<tr>
<td>Osteoprotegerin</td>
<td>2.6</td>
<td>.00815541</td>
<td>0.9</td>
<td>n/s</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RNA sequencing as described in the Methods section was used to determine fold differences in mRNA levels between groups (all fed high phosphate). CKD=chronic kidney disease; fl/fl= PiT-1<sup>lox/lox</sup>; Δsm= PiT-1<sup>Δsm</sup>; n/s=not significant.
Figure I. Cotransporter gene expression in VSMCs. A) Reverse transcription PCR determination of SLC17 (type I), SLC34 (type II), and SLC20 (type III) sodium-dependent phosphate cotransporter mRNA expression in cultured mouse VSMCs and positive control tissues listed. B) Reverse transcription PCR of the house keeping gene Gapdh. Only type III transporters (PiT-1 and PiT-2) were detected in VSMCs.
Figure II: PCR amplification of genomic DNA using PiT-1 primers spanning exon 2 through exon 5.

Addition of LoxP sites into the PiT-1 gene results in a 150 base pair increase in amplicon size in the PiT-1\textsuperscript{fl/fl} VSMCs compared to wild-type PiT-1. Cre mediated deletion of exon 3 and 4 in the PiT-1\textsuperscript{∆sm} VSMCs results in a 450 bp reduction in amplicon size, compared to PiT-1\textsuperscript{flox/flox} VSMCs.
Figure III. Selective deletion of PiT-1 from VSMCs in PiT-1<sup>∆sm</sup> mice. Real time quantitative PCR determination of PiT-1 mRNA expression in various tissues. mRNA levels were derived from standard curves and normalized to 18s, and data expressed as ratio of mean mRNA levels in PiT-1<sup>∆sm</sup> tissue relative to mean levels in PiT-1<sup>flx/flx</sup> tissue, error bars represent standard deviation. PiT-1 was undetectable in isolated aortic media, and was significantly decreased in whole aorta. There was a non-significant trend for decreased expression in the heart and small intestine. No significant difference in expression levels was noted in the other tissues surveyed.
Materials and Methods:

VSMC-specific Pit-1 deletion
Generation of mice carrying the floxed Pit-1 conditional allele (Pit-1\(^{\text{lox/lox}}\)) was previously described\(^1\). The conditional allele has \(\text{loxP}\) sites flanking exons 3 and 4; removal of these exons in the presence of Cre recombinase results in an early stop codon after position 120, removing 83% of the full-length protein that includes 4 of the 6 residues important for phosphate transport\(^1\). Sm22-Cre\(^{-/0}\) mice that express Cre from a smooth muscle specific Sm22\(\alpha\) promoter\(^2\) were kindly provided by Dr. David Dichek (University of Washington). Pit-1\(^{\text{lox/lox}}\) and Sm22-Cre\(^{-/0}\) lines were bred onto DBA/2J background and C57/Bl6 backgrounds until congenic. Genotyping of mice using DNA from tail biopsies was done using the HotSHOT method as previously described\(^1\).

Reverse-transcription PCR for VSMC NaPi transporters
VSMCs were isolated from the aortic media of healthy wild-type mice as previously described\(^3\). RNA from VSMCs at passage 10 was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA), and first-strand cDNA was made using the Omniscript Reverse Transcriptase kit from Qiagen. To identify NaPi transporters expressed by mouse VSMCs, intron-spanning mouse SLC20 and SLC34 primers were designed using PrimerExpress software V2.0 (Applied Biosystems, Foster City, CA) (supplemental Table I). SLC17 pre-mixed primer/probe sets were ordered from Applied Biosystems (for SLC17A1, A4 and A7). Positive control mouse cDNA was as follows: kidney for SLC17A1, SLC34A1, SLC34A2 and SLC34A3; small intestine for SLC17A4; brain for SLC17A7; and cementoblast OCCM for Pit-1 and Pit-2.

Taqman real-time quantitative PCR (qPCR)
To confirm selective knockdown of Pit-1 from VSMCs, analysis of Pit-1 and Pit-2 expression in various tissues was performed (normalized to 18s rRNA). Intron-spanning mouse SLC20 primers/probes were designed using Primer-Express software V2.0 (Applied Biosystems, Foster City, CA). The TaqMan probes use a FAM (fluorochrome reporter) tag at the 5'-end and a MGB quencher at the 3'-end. Aortic media (pooled from 2–4 mice), aorta, heart, lung, liver, kidney, bladder, skeletal muscle, small intestine, bone marrow, ovaries and testes from healthy knockout and control mice were harvested. Two animals were analyzed per group, and if a difference in gene expression was found, this was confirmed by analyzing an additional two mice per group. Tissues were pulverized in liquid nitrogen, and RNA was extracted using chloroform and Trizol (Invitrogen, Carlsbad, California) then further purified using the RNeasy Mini Kit (Qiagen). First-strand cDNA was made from 1 \(\mu\)g total RNA using the Omniscript Reverse Transcriptase kit from Qiagen. Amplification and detection of cDNA of interest were carried out in 96-well optical plates on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with TaqMan Universal PCR 2x master mix in a final volume of 25 \(\mu\)L per reaction. Each reaction was performed in triplicate at 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Products were analyzed with the manufacturer’s software, SDS 1.1 (Applied Biosystems). Standard curves were generated from cDNA of mouse cultured cementoblast OCCM. Gene mRNA expression was normalized to the housekeeping gene 18S, then normalized to the Pit-1\(^{\text{lox/lox}}\) control using the formula \(2^{-\Delta\Delta Ct}}\) where \(\Delta\Delta Ct=(Ct_{\text{transporter gene}} - Ct_{18S})-(Ct_{\text{Pit-1\(^{\text{lox/lox}}\) transporter gene}} - Ct_{18S})\).

Western blotting.
Aortas were pulverized in liquid nitrogen and collected in lysis buffer containing 50mM Tris-HCl [pH 7.4], 150mM NaCl, 1% Triton-X100, 0.5% Sodium Deoxycholate, 0.1% SDS, 1mM EDTA, 1mM EGTA, and protease inhibitors. Cultured mouse and human VSMCs were lysed in the same buffer. 30μg protein lysate was run on a 10% SDS PAGE gel and transferred to PVDF membrane. For mouse lysates, membranes were blocked and incubated in either primary chicken anti-rat PiT-1 antibody or rabbit anti-PiT-2 antibody (provided by Dr. M. Levi) at a 1:1000 dilution overnight and incubated in the secondary (Donkey Anti-Chicken-horse radish peroxidase (HRP) or Goat Anti-Rabbit-HRP from Jackson ImmunoResearch) at a 1:2000-1:5000 dilution for 1 hr at room temperature. For human lysates, primary rabbit anti-human PiT-2 antibody obtained by immunizing rabbits to the human PiT-2 peptide GAKANDDDSTIPLTGAAGETLGTSEGTSAGSHPRAAYGRAL including amino acids 275 to 315 followed by peptide affinity purification of serum using Sulfo-Link kit from Pierce Biotechnologies, Rockford IL) was used at a 1:200 dilution overnight. SuperSignal WestDura ECL reagents (Thermo Scientific) were used to detect HRP. Amido black staining was used to confirm equal loading. Densitometry using ImageJ was used to quantify relative protein levels.

Induction of CKD and treatment protocol
The two in vivo experimental groups (on calcification prone DBA/2J background) were PiT-1 Δsm mice with CKD (n = 16) and PiT-1 flox/flox littermates with CKD (controls, n = 16). Female mice aged 8-12 weeks underwent the 2-step surgical procedure for partial renal ablation as previously described 4-6. Briefly, during surgery 1, the right kidney was exposed, decapsulated, and electrocauterized. After a 2-week recovery period, left total nephrectomy was performed (surgery 2). Surgeries were performed under isoflurane anesthesia (Isoflurane, Baxter). One week after surgery 2, CKD mice were placed on a normal phosphate (0.5%) or high-phosphate diet (1.5%) that was starch-based and also contained 0.6% calcium, 0.07% magnesium and 1540 IU/kg vitamin D3 (Dyets, Bethlehem, PA). Mice had access to food and water ad libitum and were maintained on a 12-hour day/night cycle in a specific pathogen-free environment, according to the Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Washington, Seattle WA.

After 4 weeks on diet, mice were anesthetized with 40-90 mg/kg pentobarbital, followed by exsanguination via cardiac puncture. Aortas were harvested and dissected into several parts for tissue analysis: 1) aortic arch snap-frozen in liquid nitrogen for calcium quantitation; 2) thoracic aorta fixed in methyl Carnoy’s solution followed by paraffin embedding for histology; 3) abdominal aorta snap-frozen in liquid nitrogen for RNA analysis.

Serum chemistries
Fasting baseline (pre-CKD) serum was collected from the saphenous vein one week before surgery 1. Non-fasting blood for blood urea nitrogen (BUN) was drawn one week after surgery 2. Blood collection via cardiac puncture was done at time of termination following a minimum 2-hour fasting period. Serum was collected using serum separator tubes (BD 365956) with centrifugation at 1000g for 10 minutes. BUN was analyzed using the QuantiChrom™ Urea Assay Kit (BioAssay Systems, Hayward, CA). Terminal serum phosphorus, calcium and alkaline phosphatase were analyzed with a standard bioanalyzer at Phoenix Central Laboratory (Everett, WA). Complete blood count (CBC) from non-CKD mice was processed on a Hemavet 950 (Drew Scientific, Oxford, CT).
**Echocardiography**

Echocardiographic experiments were performed to measure left ventricular (LV) wall thickness, LV end-diastolic dimension (LVEDD), and ejection fraction (EF) using a VisualSonics VEVO 770 system equipped with a 707B scan head, as previously described\(^7,8\). Mice were lightly anesthetized with 1\% isoflurane. Data were measured in M mode from the short axis. LVEF (in \%) was calculated as follows: 

\[
\text{LVEF} = \left(\frac{\text{LV Vol; d} - \text{LV Vol; s}}{\text{LV Vol; d}}\right) \times 100,
\]

where (LV Vol; d) and (LV Vol; s) are LV volume at diastole and systole, respectively. LV Vol (\(\mu\text{L}\)) was calculated as: 

\[
\text{LV Vol} = \left(\frac{7.0}{2.4 + \text{LVID}}\right) \times (\text{LVID})^3 \times 1000.
\]

LVID is LV internal diameter and it was measured at both diastole and systole. Left ventricle fractional shortening (LVFS, in \%) was calculated as follows: 

\[
\text{LVFS} = \left(\frac{\text{LVID; d} - \text{LVID; s}}{\text{LVID; d}}\right) \times 100.
\]

**Quantitative Biochemical Analysis of Aortic Calcium**

Aortic arch segments were snap-frozen in liquid nitrogen, lyophilized, and decalcified with 0.6 \(\text{N HCl}\) at 37°C for 24 hours. The calcium content of the supernatant was determined colorimetrically with the \(o\)-cresolphthalein complexone kit from Teco Diagnostics (Anaheim, CA) as previously described\(^9\). Aortic calcium content was normalized to the dry weight of the tissue and expressed as \(\mu\text{g Ca/mg dry weight}\).

**Histochemical/immunohistochemical analysis**

Staining protocols performed on thoracic aorta sections included 1) hematoxylin/eosin, 2) von Kossa staining with methyl green counterstain, and 3) SM22\(\alpha\) (ab10135, Abcam; 1 \(\mu\text{g/mL}\)). VSMC cell density in the aorta was evaluated from H&E stained sections, using the “photomerge” and “count” tools in Adobe Photoshop CS3 version 10.0.1. Cell count was done on sections taken from 6 mice per group, and average cell number per unit area was calculated (expressed as \#cells per \(\text{mm}^2\)).

**Transcriptional Profiling**

RNA was prepared from aortas of PiT-1\(^{fl/fl}\) and PiT-1\(^{Δsm}\) and submitted to a sequencing facility (the High-Throughput Genomics Unit, Department of Genome Sciences, University of Washington; [http://www.htseq.org/index.html](http://www.htseq.org/index.html)), where a sequencing platform-specific chemistry was utilized to produce cDNA and sequencing was carried out using platform-specific protocols, producing reads of 36 bp in length. These data were analyzed using tools of the open-source Tuxedo protocol as previously described\(^10\). Briefly, reads were trimmed with fastq-mcf, and aligned to the mm9 genome using TopHat. Transcript quantitation was performed on each sample by Cufflinks. All pairwise comparisons were done with Cuffdiff.

**Phosphate-induced calcification of explanted mouse aortic rings**

Mouse aortas were harvested from 8-10 week old DBA/2J mice and perivascular fat was removed. Aortas were cut into 2–3 mm length aortic rings that were cultured in individual wells of a 24-well plate in DMEM containing 100 U/mL penicillin, 100 \(\mu\text{g/mL}\) streptomycin, 0.25 \(\mu\text{g/mL}\) fungizone, 5\% FBS and 1\(\text{mmol/L}\)phosphate. Calcification was induced by culture in high phosphate 2.6\(\text{mM}\) media for 9 days. At the termination of the experiment, aortic rings were snap-frozen in liquid nitrogen, lyophilized, and decalcified with 0.6 \(\text{N HCl}\) at 37°C for 24 hours. The calcium content of the supernatant was determined colorimetrically with the \(o\)-cresolphthalein complexone kit from Teco Diagnostics (Anaheim, CA).
Aortic calcium content was normalized to the dry weight of the tissue and expressed as µg Ca/mg dry weight.

**Mouse and human aortic VSMC**

Mouse vascular SMCs were prepared from aortas of 5-week-old C57/Bl6 PiT-1^flex/flex^ or PiT-1^∆sm^ transgenic mice as described previously. Briefly, the media was carefully stripped from the thoracic and upper part of abdominal aorta under a dissection microscope and cut into 5-mm pieces. Residual endothelial and adventitial cells were removed by 20 minute digestion of the media pieces in culture medium containing 12.5% FBS and 1 mg/mL collagenase (Worthington). The media was then digested in culture medium containing 12.5% FBS, 1 mg/mL collagenase, and 0.5 mg/mL elastase (Sigma-Aldrich) at 37°C for 1 hour. The resulting cell suspension was pelleted by centrifugation at 800g for 5 minutes. The cells were resuspended in DMEM culture medium containing 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B (1% pen/strep/amph), and 20% FBS. Subcultured SMCs were maintained in DMEM culture medium containing 1% pen/strep/amph and 10% FBS after passage 2. These cells were not used in any assay past passage 9. Confirmation of Cre/loxP genetic recombination was done through genotyping of isolated VSMCs (primers spanned exon 2 through intron 4: Ex2F sequence CTCATCCTGGGCTTCATCAT and int4R3 sequence TTCCTCCTGAATGCCCTCT). Human aortic SMC were isolated as previously described.

**Phosphate uptake assays**

Phosphate uptake assays were performed as previously described with cultured VSMCs. Briefly, VSMCs were incubated in Earle’s buffered salt solution (EBSS) containing various concentrations of phosphate (including H_3^33PO_4 obtained from PerkinElmer Life Science, Inc., Boston, MA). Sodium-dependent phosphate uptake was determined by subtracting uptake in the presence of EBSS containing choline chloride from uptake in EBSS containing sodium chloride. Uptake values were normalized to cellular protein content. Non-linear curve fitting was performed using the Michaelis-Menten equation using GraphPad Prism 5 software.

**Phosphate-induced calcification of cultured VSMCs**

VSMC calcification was induced by treatment with calcification media supplemented with NaH2PO4/Na2HPO4 to a final concentration of 2.6 mmol/L phosphate, with 10% FBS (human VSMCs) or 3% FBS (mouse VSMCs). Calcium content of the cultures was determined using the o-cresolphthalein complexone method as previously described and normalized to protein content.

**Knockdown of PiT-2 by shRNA in PiT-1^∆sm^ VSMC**

Mouse PiT-2 specific or control shRNA retroviral constructs (Origene Technologies, Inc., Rockville, MD) were transfected into Phoenix-Eco packaging cells to generate retrovirus. The conditioned media containing the retrovirus was harvested and filtered with a 0.45µm filter. VSMCs from PiT-1^∆sm^ mice were transduced using polybrene by centrifugation at 800g with retrovirus for 1 h every 24 h for three consecutive days. The infected cells were selected with 3 µg/mL puromycin for 4 days. Knockdown of PiT-2 was verified by qPCR.

**Retroviral transduction and overexpression of PiT-2 in PiT-1-knockdown human aortic VSMCs**
Isolation-immortalization of human aortic VSMCs\textsuperscript{12} and generation of VSMCs stably expressing PiT-1 siRNA using the pSUPER RNA interference system (Oligoengine, Seattle, WA)\textsuperscript{13} have previously been described. Cells were routinely cultured in growth media (DMEM containing 15% FBS, 1.4 mmol/L phosphate, 100 U/mL of penicillin and 100 mg/mL of streptomycin). A full-length human PiT-2 cDNA cloned from human newborn aortic SMCs by reverse transcription PCR and was inserted into the retroviral vector pBMN-IRES-PURI, and stable expression of human PiT-2 was established in PiT-1 siRNA cells. Overexpression of PiT-2 was verified by reverse transcription PCR.

**Statistical analysis**

SPSS software v16.0 (SPSS, Chicago, IL) was used to compare group means (for data with >2 groups) using one-way ANOVA with Tukey post-hoc analysis. Means between 2 groups was compared using the paired student t-test. Significance for all tests was set at $p<0.05$. For RNA sequence analysis, all pairwise comparisons were done with Cuffdiff.

