The Roles of Aldosterone and Protein Kinase a Signaling in Vascular Smooth Muscle Cell Calcification

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Abstract

Vascular calcification is a disease prevalent in the elderly population. Intimal calcification, the dominant form, is calcification of the inner layer of the blood vessel wall and arises primarily after atherosclerotic plaque accumulation, thereby following the vascular distribution patterns of atherosclerosis. Medial calcification, calcium phosphate deposition in the middle layer of blood vessels, is much less common and is correlated with different disease states, such as type II diabetes and chronic kidney disease. It is widely recognized that vascular smooth muscle cells of the medial layer play a major role in the pathogenesis of vascular calcification.

This study focuses on how aldosterone and cAMP/PKA signaling affect the calcification of vascular smooth muscle cells. We investigated two independent pathways by which aldosterone may induce calcification. First, we discovered that aldosterone is a promoter of fibrosis, a potential mediator of vascular calcification, by inducing the expression of connective tissue growth factor. Second, we showed potential effects of aldosterone on phosphate/pyrophosphate imbalance, known to be an important determinant of calcification. In addition, aldosterone modulates expression of miRNAs predicted to regulate both pathways. Finally, PKA signaling has been previously shown to induce calcification of vascular smooth muscle cells by regulating phosphate/pyrophosphate metabolism. Here, we show that PKA signaling does, indeed, disrupt phosphate/pyrophosphate regulation; however, it appears to induce an anti-calcific response, directly contradicting the results of prior studies. In summary, aldosterone induces vascular medial calcification through fibrosis and phosphate/pyrophosphate imbalance, which is potentially regulated by miRNAs, and PKA signaling contributes to phosphate/pyrophosphate regulation in a protective manner against calcification.
List of Abbreviations

Aldo - aldosterone
ALPL – alkaline phosphatase
BMP-2 – bone morphogenetic protein 2
cAMP – cyclic adenosine monophosphate
CTGF – connective tissue growth factor
ENPP1 – ectonucleotide pyrophosphatase 1
FSK - forskolin
HAoSMCs – human aortic smooth muscle cells
MiRs - microRNAs
Pi – phosphate
PiT-1 – inorganic phosphate transporter 1
PPi - pyrophosphate
PKA – protein kinase A
RUNX2 – runt-related transcription factor 2
qRT-PCR – quantitative reverse transcriptase polymerase chain reaction
SM22α – smooth muscle 22 alpha
TGF-β – transforming growth factor
VSMC – vascular smooth muscle cell
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**Introduction**

Vascular calcification is characterized by inappropriate deposition of bone-like material in the walls of blood vessels, resulting in increased vessel-wall stiffness and blood flow dysregulation. Calcification of the coronary arteries has been shown to be present in > 97% of males and > 67% of females in the population above 70 yrs of age\(^1,2\) and is associated with cardiovascular morbidity and mortality.\(^3\) The most common form of vascular calcification, known as intimal calcification, is characterized by calcium deposition in the inner layer of the blood vessel walls secondary to atherosclerotic plaque formation.\(^4\) Vascular medial calcification, also known as Mönckeberg’s arteriosclerosis, is a less common form of vascular calcification in which calcium phosphate deposition is localized to the medial layer of the arterial wall. It is highly associated with chronic kidney disease and diabetes mellitus.\(^5,6\)

Although once thought to be a random process of calcium and phosphate deposition, vascular calcification is now known to be highly regulated. One mechanism by which this occurs is the acquisition of an osteoblast-like phenotype by vascular smooth muscle cells (VSMCs), which express markers of differentiated osteoblasts while concurrently downregulating factors associated with the smooth muscle contractile phenotype. Runt-related transcription factor 2 (RUNX2/Cbfa1) is a master regulator of osteoblast differentiation\(^7-9\) that has been implicated in VSMC calcification.\(^10-12\) Similarly, bone morphogenetic proteins (BMPs) play important roles in osteoinductive signaling,\(^13\) and BMP-2 has been most studied among the family of BMPs to be involved in vascular calcification.\(^14-16\)

It is well known that phosphate (pro-calcific) and pyrophosphate (anti-calcific) imbalances mediate vascular calcification. In patients with chronic kidney disease, which is associated with
medial calcification as stated above, serum phosphate levels are elevated due to the inability of the renal tubules to excrete phosphate. In vitro, elevated phosphate concentrations in culture media itself can induce calcification of VSMCs, upregulating osteoblastic markers such as RUNX2 and downregulating smooth muscle cell markers such as smooth muscle 22α (SM22α) and α-smooth muscle actin (α-SMA). In uremic rats, a high phosphate diet induced vascular medial calcification, and an ex vivo experiment confirmed decreased aortic compliance as a result of calcification in the setting of uremia. Novel magnesium and iron-based phosphate binders have been developed and shown to reduce medial calcification in chronic renal failure rat models. In addition, the inorganic phosphate transporter 1 (PiT-1), a sodium-dependent phosphate transporter that mediates phosphate uptake, appears to be a major factor in the calcification of vascular smooth muscle cells. Finally, phosphate can accumulate in the extracellular environment through matrix vesicle secretion, contributing to the existing calcium phosphate deposits.

Pyrophosphate (PPi) is a known inhibitor of calcification by directly inhibiting the growth of hydroxyapatite crystals. Ectonucleotide pyrophosphatase 1 (ENPP1) is a membrane-bound protein that cleaves nucleoside triphosphates into their corresponding monophosphates, releasing pyrophosphate. As its function is to produce pyrophosphate, ENPP1 is considered an inhibitor of calcification. Mutations of ENPP1 are known to cause diseases which involve soft tissue calcification, including generalized arterial calcification of infancy, pseudoxanthoma elasticum, and Hutchinson-Gilford Progeria syndrome. In addition, ENPP1 knockout mouse and rat models both have increased susceptibility to developing aortic calcification. Conversely, rat aortic smooth muscle cells grown in physiological concentrations of calcium and phosphate exhibited decreased calcium deposition when given pyrophosphate. Aortic calcium content was
significantly reduced in uremic rats with daily intraperitoneal injections of pyrophosphate.\textsuperscript{38} Figure 1 provides a summary of phosphate/pyrophosphate metabolic (dys)regulation in the setting of calcification. Of particular significance is alkaline phosphatase (ALPL), which cleaves pyrophosphate into phosphate, which subsequently induces calcification.

There is also growing evidence that the protein kinase A (PKA) pathway is implicated in VSMC calcification and that PKA regulates ENPP1 expression. Rat aortic smooth muscle cells treated with forskolin (FSK), an adenylate cyclase activator, markedly increased calcium deposition, which was associated with reduced extracellular pyrophosphate levels and ENPP1 expression.\textsuperscript{39} It has been further shown that in murine aortic cells, FSK-induced calcification was dependent on high extracellular phosphate concentrations. It is important to note that PKA regulation of ENPP1 has been studied exclusively in animal models and not in human VSMCs.

Aldosterone, a mineralocorticoid hormone, plays an important physiological role regulating fluid and electrolyte homeostasis as part of the renin-angiotensin-aldosterone system. Briefly, renin is an enzyme secreted by juxtaglomerular cells of renal afferent arterioles as a response to several stimuli, commonly from renal hypoperfusion secondary to arterial hypotension. Renin hydrolyzes angiotensinogen, constitutively released by the liver, into angiotensin I, which is then converted to angiotensin II by angiotensin-converting enzyme (ACE) in the endothelium and pulmonary capillaries. Angiotensin II causes arterial vasoconstriction and aldosterone secretion from the adrenal glands. Aldosterone then acts to reabsorb sodium in exchange for potassium in the distal tubules and collecting ducts of the kidneys, which also results in water reabsorption. Overall, this pathway counteracts the hypotensive state by direct vasoconstriction and fluid retention. However, there are extrarenal effects of aldosterone that have been well described.
Under certain pathologic conditions, such as cardiac and chronic kidney disease, aldosterone is markedly elevated and can lead to detrimental vascular effects including oxidative stress, inflammation, fibrosis, and impaired relaxation. It is well known that vascular smooth muscle cells express the mineralocorticoid receptor, and many recent studies have suggested a role for aldosterone in promoting vascular calcification. Rats given vitamin D₃ and oral nicotine to induce calcification showed reduced arterial calcium content when also given the aldosterone receptor antagonist, spironolactone. An in vitro study also showed that aldosterone increased calcium deposition and ALPL activity in bovine VSMCs. It is also known that aldosterone induces expression of BMP-2. In addition, aldosterone was found to stimulate PiT-1 expression in klotho-hypomorphic mice (characterized by growth deficits, rapid aging, hyperphosphatemia, and vascular and soft tissue calcification), suggesting a role for aldosterone in phosphate, and potentially pyrophosphate, regulation.

Another mechanism by which aldosterone can induce calcification is through fibrosis. Fibrocalcification has been previously described in pericardial tissue and valvular replacement grafts. Vascular fibrosis was noted to be greater in calcified tissue of the same rats as mentioned above given vitamin D₃ and oral nicotine. Connective tissue growth factor (CTGF/CCN2) is the second member of the CCN (CYR61/CTGF/NOV) family of matricellular proteins that are known to be regulators of cell adhesion and migration, angiogenesis, chondrogenesis, inflammation, and wound healing. It is well known that CTGF is a major determinant in the development of fibrosis of many tissue types through interaction with transforming growth factor beta (TGF-β), and inhibition of CTGF can prevent or potentially reverse fibrosis. Aldosterone is a known promoter of fibrosis, and its autonomous production in pulmonary artery endothelial cells has been shown to increase CTGF expression, inducing
pulmonary vascular fibrosis.\textsuperscript{55} CTGF has also been widely implicated in cardiovascular disease. For example, angiotensin II, a promoter of aldosterone secretion as described above, was shown to increase CTGF expression in cardiac fibroblasts in rats with heart failure.\textsuperscript{56} In addition, rats with experimentally induced myocardial infarction showed increased CTGF expression in the area of infarct.\textsuperscript{57} Interestingly, one study suggested that CTGF is a promoter of calcification in vascular smooth muscle cells isolated from mouse thoracic aortas.\textsuperscript{58} Exogenous CTGF given in vitro was shown to upregulate RUNX2 and ALPL, resulting in calcium deposition. Altogether, aldosterone may affect VSMC calcification through its actions on phosphate/pyrophosphate homeostasis and fibrosis.

The role of microRNAs (miRs) in VSMC calcification has become a major topic of investigation. MiRs are short, single-stranded sequences of \textasciitilde{22} nucleotides that bind to the 3’-untranslated region of mRNAs, resulting in mRNA degradation or inhibition of translation.\textsuperscript{59} One of the earliest studies by Goettsch et al. showed that miR-125b is reduced in calcified aortas of ApoE knockout mice, and inhibition of miR-125b in vitro induced calcification of human coronary artery smooth muscle cells (CASMCs).\textsuperscript{60} Qiao et al. showed that phosphate-induced calcification of human aortic smooth muscle cells had decreased expression of miR-205, and overexpression of miR-205 inhibited their transition into osteoblast-like cells.\textsuperscript{61} Similarly, in vitro miR-204 overexpression decreased RUNX2 protein levels and attenuated osteoblastic differentiation of mouse aortic smooth muscle cells, and in vivo overexpression alleviated vitamin D3-induced medial calcification.\textsuperscript{62} MiRs-29b, 133b, and 211 were also discovered to play roles in phosphate-induced vascular calcification in uremic rats by upregulating RUNX2 gene expression and suppressing inhibitors of osteoblast differentiation.\textsuperscript{63} MiR-30b and miR-30c levels were decreased in human CASMCs stimulated by BMP-2, and it was subsequently shown that miR-30b/c both
target RUNX2. There is also evidence of phosphate and pyrophosphate regulation by miRs-221 and 222 through changes of ENPP1 and PiT-1 expression. Finally, miR-34b/c were recently discovered to inhibit aldosterone-induced calcification of aortic smooth muscle cells isolated from uremic rats. Taken together, miRs play crucial and integrated roles in regulating VSMC calcification.

This study focuses on the mechanisms by which aldosterone and PKA induce calcification of human VSMCs. We believe aldosterone promotes calcification by affecting pro-fibrotic pathways and phosphate/pyrophosphate imbalance, and there may be a role for miRs in this process. In addition, we hypothesize that PKA activation disrupts phosphate/pyrophosphate homeostasis by shifting the equilibrium towards increased phosphate levels, thereby promoting vascular calcification.
Methods

Cell Culture

Human aortic smooth muscle cells (HAoSMC; Clonetics™, Lonza) were cultured at 37°C and 5% CO₂ in smooth muscle growth medium (SmGM™-2, Lonza), which includes 5% fetal bovine serum, 0.2% human basic fibroblast growth factor, 0.1% insulin, and 0.1% human epidermal growth factor. Cells were passaged at confluence (approximately every 3 days) with 0.5% trypsin/EDTA. Experiments were performed on cells from passages 4 to 8. Osteogenic medium was formulated by supplementing normal growth medium with 5 mM β-glycerophosphate.

Forskolin (FSK) and aldosterone (Aldo) treatment of HAoSMCs began one day after plating. Cells were harvested after 0, 4, 10, and 14 days of treatment with Aldo or FSK. Aldo treatments consisted of normal growth medium with or without 10⁻⁷ M Aldo⁶⁶ or osteogenic medium with or without 10⁻⁷ M Aldo. Similarly, FSK treatments consisted of normal growth medium with or without 10⁻⁶ M FSK³⁹ or osteogenic medium with or without FSK.

Alizarin Red Staining

Alizarin red staining was used to assess the extent of calcium deposition. Cells were treated in sterile chamber slides (Lab-Tek®) for 14 days with Aldo or FSK in normal medium or osteogenic medium. After treatment, cells were fixed with 4% formalin for 10 minutes. After washes with deionized water (diH₂O), cells were stained with 2% Alizarin Red S (ScienCell™) for 30 minutes. Cells were washed again with diH₂O and then imaged using a ChemiDocTouch™ machine (Bio-Rad). Densitometry was performed with ImageLab™ software (Bio-Rad).
RNA Isolation and Quantitation

The purification of total RNA, including miRNA, was performed by phenol-chloroform extraction. HAOsMCs were treated in 6-well culture plates with Aldo or FSK in normal medium or osteogenic medium. Cells were directly lysed with QIAzol lysis reagent (Qiagen). After addition of chloroform, the aqueous and organic layers were separated by centrifugation in Phase Lock Gel™ Heavy tubes (5 Prime). RNA from the aqueous layer was precipitated by addition of 100% ethanol. Subsequent RNA isolation was performed using the miRNeasy Mini Kit (Qiagen). RNA quantitation was performed using a NanoDrop® spectrophotometer (ND-1000). Absorbance at 260 nm wavelength was used to calculate RNA concentration. The 260/280 and 260/230 ratios were used to assess RNA purity. Minimum ratios (both 260/280 and 260/230) of 1.9 were used for downstream qRT-PCR experiments, and minimum ratios of 2.0 were required for miR Array experiments.

Reverse Transcription and Quantitative PCR of RNA and miRNA

Reverse transcription of up to 2.5 μg of RNA was performed with the SuperScript® VILO Master Mix (Invitrogen™). Reverse transcription of up to 10 ng of miRNA was performed with the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems®). The resulting cDNA was used for quantitative real-time polymerase chain reaction (qRT-PCR). TaqMan Universal Master Mix and human-based primers were purchased from Applied Biosystems. Genes for CTGF, ENPP1, SLC20A1, RUNX2, ALPL, and GAPDH were analyzed, with GAPDH serving as the internal control. PCR reactions were performed in triplicate, through a 7900 HT Fast RT-PCR system (Applied Biosystems). Quantitative analysis was performed with the comparative Ct method (ΔΔCt).67
Protein Extraction and Quantitation

HAoSMCs were seeded on 10 cm dishes and received the respective treatments. Treated cells were lifted and pelleted at 700 g for 10 min. Pelleted cells were frozen in -80°C until use. After freezing, cells were homogenized with inclusion of protease and phosphatase inhibitors (ThermoScientific). Protein quantitation was performed using the DC Protein Assay (Bio-Rad), which is similar to a Lowry Assay.68

Western Immunoblot

Protein samples were heated for at least 5 min at 95°C under reducing conditions in Laemmli’s Buffer (Boston BioProducts). Approximately 30 to 50 ug of protein per sample were separated by SDS-PAGE using Mini-PROTEAN® TGX™ Stain-Free™ Precast Gels (Bio-Rad). Proteins were then transferred to PVDF membranes using the Trans-Blot® Turbo™ Transfer System (Bio-Rad). The PVDF membranes were blocked for 1 hr at room temperature with 5% milk in TBS-Tween-20 (0.05%, Boston BioProducts). Membranes were then incubated with primary antibodies overnight at 4°C. Secondary antibody incubation was performed at room temperature for 1 hr. The primary antibodies used were: CTGF (Novus; 1:2500, anti-rabbit 2°Ab 1:5000), ENPP1/PC-1 (Santa Cruz; 1:500, anti-rabbit 2°Ab 1:2000), SLC20A1/PiT-1 (Abcam RabMAb; 1:5000, anti-rabbit 2°Ab 1:10,000), TNAP/ALPL (Abcam RabMAb; 1:10,000, anti-rabbit 2°Ab 1:10,000), RUNX2 (Abcam; 1:1000, anti-rabbit 2°Ab 1:5000), SM22α (Abcam; 1:5000, anti-rabbit 2°Ab 1:10,000), and β-Actin (Santa Cruz, HRP-conjugated, 1:20,000). Anti-rabbit HRP-conjugated secondary antibody was purchased from Sigma-Aldrich®. WesternBright™ chemiluminescent substrate (Advanta) was added to the membranes, and the signal was detected by a
ChemiDocTouch™ machine (Bio-Rad). Densitometry was performed with Image Lab™ software (Bio-Rad). Protein expression was normalized to β-Actin.

**Alkaline Phosphatase Activity**

HAoSMCs were treated with Aldo or FSK in normal medium or osteogenic medium for 0, 4, 10, and 14 days. Enzymatic activity of alkaline phosphatase was assessed using a colorimetric assay (Abcam) following the manufacturer’s instructions. In brief, treated cells were homogenized in assay buffer, and samples of the cell lysates were exposed to p-nitrophenylphosphate (pNPP) for 1 hr. Concentration of the reaction end-product, p-nitrophenol (pNP), was calculated with a standard curve after detecting the absorbance at 405 nm with a SpectraMax 190 spectrophotometer (Molecular Devices). All reactions were performed in duplicate. Alkaline phosphatase activity was calculated as Glycine Units, representing the amount of enzyme required to hydrolyze one micromole of pNPP per minute at pH 9.6 and 37°C. Alkaline phosphatase activity was normalized to total protein.

**MiRNA PCR Array**

HAoSMCs were seeded in 6-well culture plates and treated with normal medium or Aldo in normal medium for 4 days. Total RNA isolation was performed as described. Reverse transcription of all miRs was performed using the miScript II RT Kit (Qiagen). PCR reactions were performed using the miScript SYBR® Green PCR Kit (Qiagen). The miRNome miScript miRNA PCR Array v16 (Qiagen) was used to screen for the 1,066 most commonly expressed miRNAs in the human miRNome. Each array consisted of three 384-well plates, with each well containing oligo primers for different miRs, including positive and negative controls. The cDNA sample was individually
added to each well of the array panel for this purpose. Data analysis was performed using the online miScript miRNA PCR Array Data Analysis software provided by Qiagen, which uses the ΔΔCt method to assess relative changes of different miRs.

**Statistical Analysis**

All experiments were performed a minimum of 3 times in duplicate. The Shapiro-Wilk test was used to assess normality of sample sets. Data are presented as mean±SD. Two experimental groups were compared using paired two-sample t tests, and > 2 experimental groups were compared using 1-way ANOVA followed by a post hoc Tukey test. P < 0.05 was considered significant unless otherwise specified.
Results

Aldosterone Increases CTGF Expression in HAoSMCs

To investigate whether aldosterone promotes fibrosis in HAoSMCs, CTGF expression was assessed after treatment with Pi, Aldo, or Aldo + Pi for 0, 4, 10, and 14 days. A 1.8-fold increase in CTGF protein expression was observed in Aldo-treated cells at 14 days (p<0.1; Fig. 2A), and the increase in expression was observed as early as 4 days (1.26±0.17, p<0.05; Fig. 2B). The effect was accentuated (3.2-fold increase) with addition of Pi (p<0.1, Fig. 2A). However, the increase in CTGF protein expression was not reflected in changes in mRNA expression (Fig. 2C) over the time course of 0, 4, 10, and 14 days.

Induction of Calcification in Vascular Smooth Muscle Cells

To analyze calcium deposition by HAoSMCs in response to aldosterone, cells were treated with Pi, Aldo, Aldo + Pi, FSK, or FSK + Pi for 14 days and stained with Alizarin Red S. Nodules of calcium phosphate deposition were seen with greater density in cells treated with Pi alone compared to control (1.54±0.12, p<0.05; Fig 3). Similarly, staining of Aldo-treated cells showed increased levels of calcium deposition (1.67±0.17, p<0.05). The combination of Aldo and Pi did not show further changes in calcification compared to Aldo alone. These results show that Aldo and Pi individually stimulate VSMC calcification.

To observe the effect of PKA signaling on calcification, HAoSMCs were treated with FSK and FSK + Pi for 14 days. FSK-treated cells showed a marked decrease in calcium deposition compared with control (0.55±0.12, p<0.05; Fig. 3). This change was maintained even when Pi was
added in combination with FSK (0.57±0.12, p<0.05). This observation suggests that PKA activation is anti-calcific, contrary to expectation.

**ENPP1 Expression Increases in HAoSMC Calcification**

To assess whether pyrophosphate generation is reduced in calcifying human aortic smooth muscle cells, we analyzed ENPP1 expression over 14 days. Figure 4A shows an unexpected increase in ENPP1 expression with Aldo and Aldo + Pi treatments (1.3-fold and 1.7-fold, respectively; p<0.05) after 14 days. Interestingly, a transient decrease in ENPP1 expression was seen at 4 days with Aldo treatment (0.69±0.15, p<0.01; Fig. 4B). Cells treated with Pi alone also increased ENPP1 expression. After 14 days of treatment, ENPP1 protein levels were increased almost 1.7-fold (p<0.001; Fig. 4A), although the same changes in RNA expression were not observed (Fig. 4C) over a time course of 0, 4, 10, and 14 days. Taken together, Aldo and Pi both increase ENPP1 expression despite evidence of calcification.

We next examined whether activation of the PKA pathway affects ENPP1 expression in HAoSMC. ENPP1 protein expression was elevated 2.8-fold in cells treated with FSK alone for 14 days, which was further increased to 4.5-fold with the addition of Pi (p<0.001; Fig. 4A). Interestingly, at 4 days, a decrease in ENPP1 was observed with FSK treatment (0.50±0.11, p<0.05; Fig. 4B), as was the case with Aldo treatment above. ENPP1 mRNA expression was also elevated over this time course for FSK and FSK + Pi (Fig. 4C), consistent with the changes in ENPP1 protein levels.

**Alkaline Phosphatase Expression and Enzymatic Activity**
Alkaline phosphatase is known to hydrolyze pyrophosphate to produce inorganic phosphate, thereby promoting calcification. Hence, we measured both alkaline phosphatase expression and activity in HAoSMCs. Gene expression of ALPL, as determined by qRT-PCR, was elevated 1.5-fold in cells treated with Pi alone after 14 days (1.47±0.15, p<0.05; Fig. 5D). Aldo treatment did not elicit such change in RNA. In addition, we did not observe increases in ALPL protein expression (Fig. 5A, B) for Aldo, Pi, or Pi + Aldo treatments. In fact, a small decrease in protein expression was observed in cells treated with Pi or Aldo + Pi after 14 days, in contrast to what was observed in RNA expression (0.81±0.10 and 0.82±0.07, p<0.05). However, small increases in alkaline phosphatase activity were observed after 14 days of treatment with either Pi or Aldo (1.16±0.05 and 1.17±0.06, p<0.05; Fig. 5D). The combination of Pi + Aldo had produced an additive effect on enzymatic activity, producing a 1.4-fold change (1.41±0.10, p<0.01). Thus, although ALPL expression remained unchanged, both Aldo and Pi elevated activity of the enzyme.

Because we observed decreased calcification and increased ENPP1 expression in FSK-treated cells, the effect of FSK on ALPL expression and activity was also examined. ALPL mRNA expression decreased in a time-dependent manner for FSK-treated cells beginning at 4 days, resulting in 0.4-times lower expression at 14 days compared to control (p<0.01; Fig. 5C). The addition of Pi to FSK appeared to counteract the effects of FSK, with no overall change in RNA levels of ALPL with simultaneous treatment of FSK + Pi. In addition, FSK exerted no effect on protein expression of ALPL (Fig. 5B). Moreover, no change in ALPL enzymatic activity was observed in response to FSK or FSK + Pi (Fig. 5D).

Expression of PiT-1 in HAoSMCs
Because phosphate is a direct osteoinductive signal, the intracellular flux of phosphate would be important in the calcification process. To capture the relevance of phosphate uptake in aldosterone-induced HAoSMC calcification, we investigated the expression of PiT-1, a sodium-dependent phosphate transporter. By qRT-PCR and western blot, we did not observe any changes in RNA or protein expression in cells treated with Aldo or Pi at any given time point (Fig. 6A-C). The combination of Aldo + Pi also did not elicit any changes. Overall, PiT-1 expression was not changed in aldosterone or phosphate-induced VSMC calcification.

We next examined PiT-1 expression in cells treated with FSK to investigate whether the inhibition of calcification is associated with decreased phosphate uptake. FSK decreased protein expression of PiT-1 beginning at 4 days, with reduced expression of 0.5-times the control at the 14 day time point (p<0.05; Fig. 6A, B). Addition of Pi had no further effect on FSK-mediated PiT-1 downregulation. RNA expression of PiT-1 did not change with FSK or FSK+Pi treatments (Fig. 6C).

**Osteogenic Differentiation of HAoSMCs**

Because we observed decreased PiT-1 expression in FSK-treated cells, and Pi regulates smooth muscle phenotypic transition to osteoblast-like cells, we first investigated the effect of FSK and Pi on RUNX2, a master regulator of osteoblast differentiation, and SM22α, a marker of differentiated smooth muscle. RUNX2 expression did not change at the RNA or protein level at any time points in the experiments in response to FSK or Pi (Fig. 7A-C). However, FSK decreased SM22α protein expression by 2.0-fold after 14 days (p<0.05; Fig. 7D), which was accentuated to 2.8-fold with the inclusion of Pi (p<0.01).
Next, we again examined RUNX2 and SM22α in the context of aldosterone-induced vascular calcification. Cells treated with Aldo did not change RUNX2 protein levels at 4 and 10 days, but a small increase in expression of 1.2-fold was observed at 14 days (p<0.05; Fig. 7A, B). RUNX2 mRNA did not change at any time point (Fig. 7C). Protein expression of SM22α was measured after treatment with Aldo for 14 days (Fig. 7D). There was no change observed in SM22α expression in response to Aldo or Aldo + Pi. Overall, no major changes were observed in osteoblast or smooth muscle cell markers in aldosterone-induced HAoSMC calcification. Because there was only minor evidence of osteoblast-like differentiation within the 14 day timeframe of these experiments, we explored a different regulatory pathway, namely miRNAs, by which aldosterone promotes vascular calcification.

MiRNA Array

To see whether aldosterone-induced calcification of HAoSMCs involves miRs, a screen for the most commonly expressed miRs in the human miRNome was performed. Because Aldo decreased ENPP1 expression and increased CTGF expression at 4d, miR extraction of HAoSMCs either untreated or treated for 4 days with Aldo was performed. Reverse transcription of all miRs in the extract generated cDNA that was used for the PCR array. Each set of arrays consisted of three 384-well plates that assess expression of the 1,066 miRs most commonly expressed in the human miRNome. After analysis of relative miR expressions in the 4 day Aldo-treated sample compared to control, miRs with expression greater than two-fold difference were recorded.

Output of the miRNA array analysis is shown graphically in Figure 8. This experiment yielded 76 miRs with greater than two-fold increase and 86 miRs with greater than two-fold decrease in Aldo-treated HAoSMCs. Because we were interested in whether Aldo affects CTGF
and ENPP1 expression through miRs, miRs of greater than two-fold change recorded from the array analysis were cross-referenced to miRs predicted to target CTGF and ENPP1. MiR prediction databases utilized were miRanda,\textsuperscript{69} miRDB,\textsuperscript{70} and TargetScan.\textsuperscript{71} Table 1 shows miRs predicted to target ENPP1 with at least two-fold increased expression as well as miRs predicted to target CTGF with at least two-fold decrease in expression. These represent candidate miRs relevant to Aldo-induced changes in CTGF and ENPP1, potentially causing fibrocalcification of HAoSMTs.
Discussion

Aldosterone has been shown to induce vascular smooth muscle cell calcification through multiple pathways. CTGF plays a crucial role in the development of fibrosis and has also been shown to promote VSMC calcification. Aldosterone is known to increase CTGF expression in multiple cell types, and this signaling pathway may be important in aldosterone-induced calcification. Here, we confirmed that aldosterone increases calcium deposition of HAoSMCs. We additionally illustrate that aldosterone upregulates CTGF in HAoSMCs, which supports a hypothesis of fibrocalcification, by which aldosterone induces calcification by creating an initial, pro-fibrotic state.

Aldosterone also affects key regulators of phosphate/pyrophosphate homeostasis. Alkaline phosphatase is important in the formation of bone, and the increase in its activity supports observations of increased calcium deposition of vascular smooth muscle cells exposed to aldosterone. ENPP1 is recognized as an inhibitor of calcification by hydrolyzing ATP into pyrophosphate, and ENPP1 mutations are implicated in disease states characterized by ectopic calcification. This is the first study to show that aldosterone modulates ENPP1 expression. Interestingly, there appears to be a time-dependent discrepancy of pyrophosphate production of aldosterone-treated cells. ENPP1 expression was observed to decrease initially at 4 days, which is consistent with the concept that reduced pyrophosphate production would result in a disinhibition of calcification. However, later time points revealed a paradoxical increase in ENPP1 expression, which would theoretically increase pyrophosphate production and inhibit calcium deposition. It is possible to explain the rise in ENPP1 to be an intrinsic compensatory mechanism to prevent excessive calcification by producing pyrophosphate. At the same time, while there may be
increased pyrophosphate production, elevated activity of ALPL would convert the pyrophosphate into phosphate, resulting in increased osteoinductive signaling. Indeed, in the later stages when ENPP1 was observed to increase in aldosterone-stimulated cells, ALPL activity simultaneously increased, counteracting the inhibitory effects of ENPP1. Hence, it may not be sufficient to know the changes of ALPL and ENPP1 individually. Rather, the more relevant value may be the ALPL to ENPP1 ratio, perhaps a measurement of the phosphate/pyrophosphate ratio. It is the imbalance of phosphate and pyrophosphate, observed in diseases such as chronic kidney disease, that may be the more accurate predictor of vascular calcification.

An interesting finding in this work was that even as ALPL activity increased, ALPL protein expression remained unchanged. This suggests the presence of post-translational modification. Various glycosylation sites are present within the alkaline phosphatase molecule, which could aid in protein folding and subsequent stabilization.

A transition of vascular smooth cells towards an osteoblastic phenotype has been implicated in vascular calcification. An increase in RUNX2 protein expression was observed in aldosterone-stimulated vascular smooth muscle cells. Interestingly, there was no change seen in SM22α, a marker of well-differentiated smooth muscle, suggesting a maintenance of the smooth muscle phenotype. One explanation may be that aldosterone-induced transition of vascular smooth muscle cells to osteoblast-like cells is a very gradual process. The acquisition of a bone-forming phenotype may be seen earlier than the loss of a contractile phenotype.

Our findings from the miRNA array reveal candidate miRs that may be involved in aldosterone-induced VSMC calcification, particularly targeting ENPP1 and CTGF. Confirmation of changes in candidate miRs identified by the miR array must be performed by qRT-PCR. Pre-
miRs and anti-miRs of the confirmed miRs could be used to assess their relevance in aldosterone-induced calcification of HAoSMCs.

There is growing evidence that the PKA pathway is involved in vascular calcification. Most studies utilize forskolin, an activator of adenylate cyclase, to stimulate PKA signaling and have universally shown that FSK induces VSMC calcification. Most report a decrease in ENPP1 expression in response to FSK. Here, we provide counterevidence that PKA activation inhibits calcium deposition of human aortic smooth muscle cells. Moreover, it has a positive effect on ENPP1 expression, further supporting our findings of inhibition of calcification. Huang et al. also reported an upregulation of ENPP1 in response to FSK, but their experiments were performed in murine aortic smooth muscles that responded by calcifying. They also reported increased expression of alkaline phosphatase, which may shift the phosphate/pyrophosphate environment towards elevated phosphate levels, thereby inducing calcification (similar to aldosterone-induced calcification seen in this study). In contrast, we found no changes in ALPL protein expression or activity in FSK treated cells. Therefore, ENPP1 elevation likely produced an unopposed rise in extracellular pyrophosphate, which explains the marked reduction in calcium deposition after FSK stimulation. We also demonstrated a decrease in PiT-1 protein expression in response to FSK, though no change was seen in mRNA expression. As PiT-1 is responsible for the uptake of extracellular Pi, a driver of calcification, reduced levels of PiT-1 would prevent the osteoinductive signaling mechanism, which is consistent with our findings that FSK is anti-calcific. The lack of change in PiT-1 mRNA expression suggests post-transcriptional regulation of PiT-1, such as miRNA repression of translation.

Of note, this is not the first study showing inhibition of VSMC calcification through the PKA pathway. Adrenomedullin is a potent vasodilator and, like FSK, activates adenylate cyclase.
Cai et al showed that in rat aortic smooth muscle cells, adrenomedullin increased expression of osteopontin, an endogenous inhibitor of calcification, and decreased ALPL activity, ultimately attenuating vascular calcification. ENPP1 was not investigated in this study. It would be interested to see the effects of adrenomedullin on human aortic smooth muscle cells.

Finally, we showed that FSK does not cause a transition of HAoSMCs into osteoblast-like cells. There was no change in the RUNX2 transcription factor that is crucial for osteoblast differentiation. However, FSK reduced expression of SM22α, a smooth muscle cell marker, suggesting a decrease in the standard contractile phenotype of smooth muscle. Expression of smooth muscle specific genes, including SM22α, is highly regulated by serum response factor (SRF). One study has shown that PKA can phosphorylate SRF and inhibit its binding to the promoter regions of smooth muscle-specific genes. Thus, it is not unexpected that the decrease in SM22α expression resulted from FSK treatment.

Altogether, our findings indicate two pathways by which aldosterone induces vascular smooth muscle cell calcification, which may be regulated by miRNAs, and also illustrate that PKA signaling induced by forskolin inhibits calcification of human aortic smooth muscle cells.
Conclusions

Our results confirm that aldosterone induces the calcification of HAoSMCs. Fibrosis may be a determinant of this process as increased CTGF expression was observed. Elevation of alkaline phosphatase activity contributes to calcium deposition by converting pyrophosphate into inorganic phosphate, thereby decreasing inhibition and promoting osteoinductive signaling. Aldosterone-treated cells appeared to transition gradually toward an osteoblast-like phenotype over the period of observation (14 days). Finally, we discovered a novel connection between aldosterone and ENPP1 in the setting of VSMC calcification.

This study also contradicts most previous findings that PKA activation produces a pro-calcific state. Instead, all evidence in this study supports the argument that PKA induces a protective state against calcification. We discovered that forskolin decreases calcium deposition, and the mechanism most likely involves increased pyrophosphate production and decreased phosphate uptake. A major difference between this study and prior studies is the use of human aortic smooth muscle cells as opposed to rat aortic smooth muscle cells. It is possible that these two cell types respond differently to FSK and subsequent PKA activation. To our knowledge, no studies have compared cAMP and PKA signaling between the two cell types.
Limitations

Evidence of Aldosterone and Forskolin Activity

No confirmation of the immediate downstream activity of aldosterone or FSK was formally conducted. Aldosterone binds the mineralocorticoid receptor (MR). Spironolactone or eplerenone, both MR antagonists, could be administered to inhibit aldosterone and subsequently inhibit calcification. FSK activates adenylyl cyclase, converting ATP to cAMP. Determining intracellular levels of cAMP would be a direct measure of FSK activity. Furthermore, PKA activity could be assessed to confirm that the effects of FSK proceed through the PKA signaling pathway.

Assessment of Phosphate Uptake and Pyrophosphate Concentration

One of the motivations for studying ENPP1 is its effect on pyrophosphate homeostasis. Because ENPP1 is the primary producer of pyrophosphate, its expression is highly important in regulating pyrophosphate concentration. While this study investigates the expression of ENPP1, it does not directly address the primary concern of extracellular pyrophosphate levels. Changes in ENPP1 expression suggest corresponding changes in pyrophosphate concentration, but this assumption does not take into account other factors regulating pyrophosphate levels, such as alkaline phosphatase activity. Direct measurement of extracellular pyrophosphate levels would be necessary. In addition, it is important to note that while ENPP1 protein expression increased, we did not formally assess the activity of the enzyme. An ATP hydrolysis assay would directly address this issue.
Phosphate uptake was assumed to be directly correlated with PiT-1 expression. While PiT-1 is widely recognized as the primary phosphate transporter in VSMCs, other phosphate transporters may be relevant to vascular calcification. In fact, one study illustrated the redundancy of PiT-1 and PiT-2 in the setting of VSMC calcification.\textsuperscript{75} Hence, direct measurement of phosphate uptake, by radioactive labeling or other methods, would be necessary to confirm changes in phosphate transport.

**Aldosterone-Induced Fibrosis**

We found that aldosterone induces CTGF expression in HAoSMCs. While our results suggest cellular fibrotic activity, assessment of collagen deposition could be performed as more definitive evidence. Other indicators of fibrosis, such as transforming growth factor-β (TGF-β) or other extracellular matrix proteins, could also be explored.
Summary

This project investigates the roles of aldosterone and PKA signaling in the calcification of human aortic smooth muscle cells. Aldosterone is a known stimulator of fibrosis, and evidence supports its importance in vascular calcification. Here, we show that aldosterone induces pro-fibrotic and pro-calcific responses in vascular smooth muscle cells. We also established a novel connection between aldosterone and ENPP1, an endogenous producer of pyrophosphate. This suggests that aldosterone may alter phosphate/pyrophosphate homeostasis in the setting of vascular calcification. In addition, we determined potential candidate miRNAs that may be important in regulating aldosterone-induced vascular calcification. Activation of cAMP and the PKA pathway has recently been implicated in vascular smooth muscle cell calcification. This study directly contradicts previous findings and, instead, shows the anti-calcific consequences of PKA signaling. Our results suggest that activation of PKA shifts the phosphate/pyrophosphate balance towards pyrophosphate, providing a protective effect against calcification.
References

9. Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, Stamp GW, Beddington RS, Mundlos S, Olsen BR, Selby PB and Owen MJ. Cbfa1, a candidate gene for
cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell.* 1997;89:765-71.


**Figure 1. Regulation of Phosphate and Pyrophosphate.** The production of pyrophosphate (PPI) occurs both intracellularly and extracellularly. Within the cell, membrane bound ENPP1 hydrolyzes ATP to generate AMP and PPI, which is then transported into the extracellular space by progressive ankylosing protein homolog (ANKH). Extracellular ATP can also be cleaved by ENPP1 bound to the plasma membrane. Extracellular PPI can then inhibit the growth of hydroxyapatite crystals. Pyrophosphate can also be further cleaved into phosphate (Pi) by alkaline phosphatase (ALPL). Pi can then directly contribute to the growing hydroxyapatite crystal or be shuttled intracellularly by the sodium-dependent phosphate transporter (PiT-1). Intracellular Pi can modulate the smooth muscle phenotype, inducing differentiation into osteoblast-like cells. Pi can also be packaged into matrix vesicles that are released into the extracellular space and can contribute to calcium deposition.
Figure 2. Aldosterone Induces CTGF Expression. Cells treated with Pi, Aldo, or Aldo + Pi were analyzed for RNA and protein expression. A representative western blot (A) of CTGF expression in HAoSMCs treated for 14 days is shown. CTGF protein (B) and RNA (C) expression, quantified by western blot and qRT-PCR, is shown over a time course of 0, 4, 10, and 14 days. #p<0.1, *p<0.05 vs 0d (n=4).
Figure 3. Calcium Deposition of HAoSMCs. Cells in 6-well culture plates treated for 14 days with Aldo, Aldo + Pi, Pi, FSK, and FSK + Pi were stained with Alizarin Red. Relative calcium deposition compared to no treatment was assessed by densitometry. *p<0.05 vs no treatment (n=3).
Figure 4. Increase in ENPP1 Expression with Aldo and FSK. HAoSMC expression of ENPP1 was examined following the respective treatments. A) A representative western blot of ENPP1 expression at 14 days is shown. Protein expression was normalized to β-actin. *p<0.05, **p<0.01, ***p<0.001 vs untreated (n=6). B) Time course of ENPP1 protein levels was assessed
at 0, 4, 10, and 14 days for Aldo (left) and FSK (right) treatments. *p<0.05, ***p<0.001 vs 0d (n≥3). C) Relative gene expression, quantified by qRT-PCR and normalized to GAPDH, of ENPP1 across all time points in Aldo treatments (left) and FSK treatment (right) is shown. *p<0.05, **p<0.01 vs 0d (n≥6).
FIGURE 5

A

Protein Fold Change

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14 days

B

Protein Fold Change

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C

RNA Fold Change

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Figure 5. Alkaline Phosphatase Expression and Activity. HAoSsMCs treated for 0, 4, 10, and 14 days were assessed for ALPL expression and enzymatic activity. A) ALPL protein expression, determined by western blot after 14 days of treatment, is shown. Protein expression was normalized to β-actin. *p<0.05 (n=3). B) Time-dependent protein expression of ALPL is shown for Aldo (left) and FSK (right) treatments. *p<0.05 (n=3). C) Relative gene expression, quantified by qRT-PCR and normalized to GAPDH, of ALPL across all time points in Aldo treatments (left) and FSK treatment (right) are shown. *p<0.05, **p<0.01 vs 0d (n≥3). D) Alkaline phosphatase activity over 0, 4, 10, and 14 days was measured for Aldo- (left) and FSK- (right) treated cells and normalized to total protein. *p<0.05, **p<0.01 vs 0d (n≥4).
**Figure 6. FSK Decreases PiT-1 Expression.** HAoSMCs treated for 0, 4, 10, and 14 days were assessed for PiT-1 expression. A) PiT-1 protein expression, represented by western blot after 14 days of treatment, is shown. Protein expression was normalized to β-actin. *p<0.05 (n=4). B) Time-dependent protein expression of PiT-1 is shown for Aldo (left) and FSK (right) treatments. *p<0.05 (n=4), **p<0.01 (n≥3). C) Relative gene expression, quantified by qRT-PCR and
normalized to GAPDH, of ALPL across all time points in Aldo treatments (left) and FSK treatment (right) are shown.
FIGURE 7

A

RUNX2

β-Actin

Protein Fold Change

Pi

Aldo

FSK

14 days

B

Protein Fold Change

Pi

Aldo

FSK

0 4 10 14

C

RNA Fold Change

Pi

Aldo

FSK

0 4 10 14

0 4 10 14

0 4 10 14
Figure 7. RUNX2 and SM22α Regulation of Aldo and FSK. HAoS MCs treated for 0, 4, 10, and 14 days were assessed for expression of RUNX2 and SM22α. A) RUNX2 protein expression, represented by western blot after 14 days of treatment, is shown. Protein expression was normalized to β-actin. *p<0.05 (n=4). B) Time-dependent protein expression of RUNX2 is shown for Aldo (left) and FSK (right) treatments. C) Relative gene expression, quantified by qRT-PCR and normalized to GAPDH, of RUNX2 across all time points in Aldo treatments (left) and FSK treatment (right) are shown. D) Representative western blot is shown for SM22α after 14 days of treatment (left). E) A time course of SM22α protein expression is shown for Aldo (left) and FSK (right) treatments. *p<0.05, **p<0.01 (n≥3).
Figure 8. Aldosterone Induces Greater Than Two-Fold Changes in miRs. Untreated cells or cells treated with Aldo for 4 days were used to screen for miRs relevant to Aldo-induced calcification. Individual miRs are represented by circles. MiRs downregulated by Aldo have $\Delta$Ct values larger than those of control. Hence, $-\Delta$Ct values for those miRs in Aldo-treated cells are more negative than $-\Delta$Ct values in untreated cells. Accordingly, downregulated miRs appear above the 45° line, which indicates no change (blue), and upregulated miRs appear below. The plot also shows two parallel lines (black) above and below, both setting thresholds for minimums of two-fold change. Thus, miRs of less than two-fold change lie within the parallel lines, miRs with a minimum of two-fold decrease appear above the lines (red circles), and miRs with a minimum of two-fold increase fall below the threshold lines (green circles).
### TABLE 1

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Table 1. Candidate miRs in Aldo-Induced VSMC Calcification. MiRs of greater than 2-fold change obtained from the miRNA array analysis were matched with miRs predicted to target ENPP1 and CTGF.