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Klotho suppresses high phosphate-induced osteogenic responses in human aortic valve interstitial cells through inhibition of Sox9

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Abstract

Elevated level of blood phosphate (Pi) associated with chronic kidney disease (CKD) is a risk factor of aortic valve calcification. Aortic valve interstitial cells (AVICs) display osteogenic responses to high Pi although the underlying mechanism is incompletely understood. Sox9 is a pro-chondrogenic factor and may play a role in ectopic tissue calcification. Circulating and kidney levels of Klotho are reduced in patients with CKD. We hypothesized that Sox9 mediates high Pi-induced osteogenic responses in human AVICs and that Klotho inhibits the responses. Treatment of human AVICs with high Pi increased protein levels of Runt-related transcription factor 2 (Runx2) and alkaline phosphatase (ALP), and a prolonged exposure to high Pi caused calcium deposition. High Pi induced Sox9 upregulation through PKD and Akt activation. Knockdown of Sox9 essentially abolished the effect of high Pi on the osteogenic responses. Lower Klotho levels were observed in calcified aortic valve tissues. Interestingly, high Pi decreased Klotho levels in AVICs from normal valves, and treatment with recombinant Klotho markedly reduced the effect of high Pi on the levels of Sox9, Runx2, and ALP and suppressed calcium deposition. We conclude that high Pi induces human AVIC osteogenic responses through Sox9. Human AVICs express Klotho, and its levels in AVICs are modulated by high Pi and valvular calcification. Importantly, Klotho suppresses the pro-osteogenic effect of high Pi on human AVICs. These novel findings indicate that modulation of Klotho may have therapeutic potential for mitigation of valvular calcification associated with CKD.

Keywords

Phosphate; Aortic valve interstitial cells; Osteogenic responses; Sox9; Klotho

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Fei Li and Qingzhou Yao equal contribution

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Introduction

Calcific aortic valve disease (CAVD) is the leading cause for valve replacement in Europe and North America and increasingly affects the aging population. Approximately 2.8% of adults over 75 years old have aortic valve calcification [1, 2]. Currently, there is no effective pharmacological therapy for CAVD. Thus, it is important to understand the mechanism responsible for CAVD progression.

There is considerable evidence to suggest that chronic renal disease (CKD) is a risk factor for aortic valve calcification [3]. Aortic valve calcification in CKD patients is linked to the abnormal mineral metabolism characterized by the long-term elevation of serum phosphate (Pi) levels. The osteogenic responses of aortic valvular interstitial cells (AVICs) to high Pi are believed to contribute to the mechanism of aortic valve calcification [4, 5]. However, the mechanisms that mediate high Pi-induced osteogenic responses in human AVICs remain unclear.

In the past decade, increasing studies suggest that CAVD does not result from passive deposition of calcium–phosphate complexes on the injured valve surface but is an actively regulated pathological process mediated by the phenotype changes in AVICs, the most abundant cell type in valve tissue. Chronic stress is thought to cause prolonged activation of AVICs and may result in myofibroblastic as well as osteoblast-like phenotypic transition [6, 7]. Sox9, a master chondrogenesis factor, is upregulated in uremia [8] and participates in the stimulation of chondrogenic gene expression in vascular smooth muscle cells [9]. However, it has been reported that reduced Sox9 function promotes heart valve calcification [10]. In addition, Sox9 has also been found to inhibit bone morphogenetic protein 2 (BMP-2)-induced osteogenic responses in human mesenchymal stem cells [11]. It is unclear whether Sox9 is a pro-osteogenic factor in human aortic valves and whether it plays a role in high Pi-induced osteogenic responses in human AVICs.

Klotho was identified as an anti-aging protein [12], and its level is highest in the kidney [13]. The extracellular domain of this transmembrane protein can be cleaved off and released into the blood [14]. Klotho levels in the kidney and blood were lower in patients with CKD [15]. Klotho-deficient mice suffer from a wide variety of age-related disorders as well as tissue calcification, including aortic valve calcification [16, 17]. However, it remains unknown whether Klotho suppresses high Pi-induced osteogenic responses in human AVICs.

We hypothesized that high Pi induces the osteogenic responses in human AVICs through upregulation of Sox9 and that Klotho suppresses high Pi-induced AVIC osteogenic responses. The aim of the present study was to determine (1) how high Pi induces osteogenic responses in human AVICs (2) and whether Klotho had effect on high Pi-induced AVIC osteogenic responses and (3) the mechanism by which Klotho exerts its effect.

Materials and methods

Chemicals and reagents

Antibodies against Klotho and recombinant human Klotho protein (expressed by mouse myeloma cell line; endotoxin free) were purchased from Abcam (Cambridge, MA). Antibodies against Runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), Sox9, phosphorylated protein kinase D (PKD, also termed PKC μ), total PKD, phosphorylated protein kinase B (Akt), total Akt, and GAPDH were purchased from Cell Signaling, Inc. (Beverly, MA). The two antibodies against phosphorylated PKD recognize PKD ser916 and ser744/748, respectively. Specific PKD inhibitor CID755673 and specific Akt inhibitor MK2206 were purchased from Selleckchem (Houston, TX). Lentiviral short hairpin RNA (shRNA) constructs for Sox9 and Pit 2 were from the Functional Genomics Facility of University of Colorado. Lentiviral non-targeting shRNA expression construct was used as control. HiPerFect Transfection Reagent and other transfection-related reagents were purchased from Qiagen, Inc. (Valencia, CA). Medium 199 was purchased from Lonza (Walkersville, MD). Lipopolysaccharide (LPS), collagenase, and other reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Isolation and culture of AVICs

This study complied with the *Declaration of Helsinki* and was approved by the Institutional Review Board of University of Colorado. All patients provided written informed consent. Calcified aortic valve leaflets were obtained intraoperatively from patients undergoing aortic valve replacement at University of Colorado Hospital due to aortic stenosis. Normal aortic valve leaflets were collected from the explanted hearts of patients with cardiomyopathy and undergoing heart transplantation at the University of Colorado Hospital. Patients with a history of infective endocarditis, rheumatic heart disease, or a genetic syndrome were excluded. All valves were tricuspid. The valve leaflets from explanted hearts were thin and had no histological abnormality.

AVICs were isolated from normal and calcified valves by collagenase I digestion method as previously described [18, 19]. Briefly, valvular leaflets were digested in essential medium containing 1 mg/ml collagenase (type I) at 37 °C for 30 min. After removing endothelial cells by vortex, the leaflets were further digested with a fresh solution of 1 mg/ml collagenase for 4–6 h at 37 °C. After vortex and repeated aspirating to break up the tissue mass, the suspension was spun at 1000 rpm for 10 min to precipitate cells. Cells were resuspended and cultured in M199 growth medium, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum, in an incubator with 5% CO₂ at 37 °C. Cells of passages 3–6 were used for the experiments. When grown to 80–90% confluence, AVICs were stimulated with Pi of varying concentrations for different time periods. When needed, cells were pretreated with pharmacological reagents, including CID755673 (40 µmol/l), MK2206 (5 µmol/l), and recombinant Klotho 1 h prior to the addition of Pi. All experiments were repeated using cells isolated from four different donor valves.

Immunofluorescence staining

Detection of phospho-PKD, phospho-Akt, and Klotho proteins in valve tissues was performed using immunofluorescence staining. Frozen sections (5 μ m thick) were prepared and dried at room temperature for 30 min. After permeabilization with a methanol/acetone mixture, frozen sections were fixed in 4% paraformaldehyde and then were incubated with primary antibodies against phospho-PKD ser916 (1:50 dilution), phospho-Akt (1:50 dilution), or Klotho (1:50 dilution) overnight at 4 °C. After washing with PBS, sections were incubated with a Cy3-tagged secondary antibody (imaged on red channel). 4',6-Diamidino-2-phenylindole (DAPI) was used for nucleus counterstaining (imaged on blue channel). Alexa 488-tagged wheat germ agglutinin (WGA, green) was used to outline plasma membrane when needed.

AVICs were seeded in 8-well chamber slides and treated with or without high Pi (3 mmol/l) for indicated time period. If needed, recombinant Klotho (0.5 μ g/ml) was added 1 h prior to the addition of Pi. Immunofluorescence staining was performed as previously described to localize Sox9 in normal human AVICs [20].

Microscopy was performed with a Leica CTR5500 digital microscope (Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany).

Immunoblotting

Immunoblotting was applied to analyze Runx2, ALP, Sox9, Klotho, phosphorylated and total PKD, phosphorylated and total Akt, and GAPDH. Human aortic valve tissues and cells were homogenized or lysed in commercial sample buffer according to the manufacturer's instructions. The protein samples were resolved on 4 to 20% SDS-PAGE gels and then transferred onto nitrocellulose membranes using a wet transfer system. After blocking with 5% skim milk at room temperature for 2 h, membranes were incubated with primary antibodies at 4 °C overnight, followed by incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody diluted in 5% skim milk for 1 h. Protein bands were detected using the enhanced chemiluminescence system. Band density was analyzed using the National Institutes of Health ImageJ software (Wayne Rasband, National Institutes of Health, Bethesda, MD).

Alizarin red staining

For analysis of calcium deposition, AVICs were seeded on 24-well plates. When reaching 80% confluence, cells were treated with indicated interventions in a conditioning medium (growth medium supplemented with 10 mmol/l β -glycerophosphate, 10 nmol/l dexamethasone, 4 μ g/ml cholecalciferol, and 8 mmol/l CaCl_2) for 14 days. The medium was changed every 3 days. At the end of experiments, alizarin red staining and quantification of calcium deposition were performed as previously described [21].

Gene knockdown

Gene knockdown was performed as previously described [22]. AVICs were treated with recombinant lentivirus expressing control shRNA, Sox9 shRNA, or Pit 2 shRNA in TransDuxTM transduction reagent according to the manufacturer's instructions. Three days

later, cells were harvested for validation of knockdown or stimulated with high Pi for 72 h to determine the effect of knockdown on AVIC osteogenic responses.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences, version 17.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean $\pm$ standard error (SE). After confirming that all variables were normally distributed using the Kolmogorov–Smirnov test, Student's *t* test was applied for comparisons between two groups, and one-way ANOVA was used to analyze differences between multiple groups. $P < 0.05$ was accepted as statistically significant.

Results

High Pi induces osteogenic responses in human AVICs through the PKD and Akt pathways

To determine that the effect of high Pi has an effect on AVIC osteogenic responses, we simulated human AVICs of normal valves with high Pi (2 or 3 mmol/l) for 72 h and analyzed protein levels of Runx2 and ALP. As shown in Fig. 1a, high Pi increased cellular levels of Runx2 and ALP in a dose-dependent manner. To determine whether high Pi stimulation induces calcium deposition, we stimulated cells with high Pi for 14 days in a conditioning medium. Figure 1b shows that greater levels of calcium deposition were induced by high Pi. Thus, high Pi elevates the pro-osteogenic activity in human AVICs.

The PKD and Akt pathways have been found to play a critical role in vascular cell calcification [23, 24]. As shown in Supplemental Fig. 1, diseased aortic valves had higher levels of phosphorylated PKD and Akt. To address the role of PKD and Akt in mediating the osteogenic responses to high Pi, we stimulated cells with high Pi (3 mmol/l) for 0.5 to 8 h and observed that high Pi induces the phosphorylation of both Akt and PKD (Fig. 2a). However, no changes in the levels of phosphorylated PKD and Akt were observed in the same time course when high Pi is absent (Supplemental Fig. 2). Then, we applied specific pan inhibitors of PKD (CID755673) and Akt (MK2206) to verify the roles of these signaling pathways. As shown in Fig. 2b, inhibition of PKD markedly reduced the protein levels of Runx2 and ALP in cells treated with high Pi. To determine whether PKD plays a role in high Pi-induced calcium deposit formation, we stimulated cells with high Pi, in the presence or absence of CID755673 for 14 days in conditioning medium. Figure 2c shows that high Pi-induced calcium deposit formation was abolished by inhibition of PKD. Similarly, inhibition of Akt with MK2206 significantly reduced the expression of Runx2 and ALP and attenuated calcium deposition induced by high Pi (Fig. 2d, e). Taken together, these data reveal a critical role of PKD and Akt in mediating high Pi-induced osteogenic responses in human AVICs.

High Pi upregulates Sox9 through PKD and Akt

Sox9, a key transcriptional factor of chondrogenesis, has been implicated in valvular calcification [25]. A previous study shows that high Pi upregulates the expression of Sox9 and increases ALP activity in human aortic smooth muscle cells [26]. We found that the levels of Sox9 protein were increased in a dose-dependent manner in human AVICs exposed

to high Pi (Fig. 3a). Furthermore, high Pi induced Sox9 translocation from the cytoplasm to the nucleus (Fig. 3b). To determine whether PKD and Akt pathways are involved in high Pi-induced Sox9 upregulation, we pretreated human AVICs with PKD inhibitor or Akt inhibitor prior to high Pi stimulation. As shown in Fig. 3c, inhibition of PKD decreased Sox9 levels in human AVICs exposed to high Pi, and inhibition of Akt had a similar effect (Fig. 3d). Thus, high Pi induces Sox9 upregulation via the PKD and Akt pathways.

In exploration of the role of Pi transporters in high Pi-induced Sox9 upregulation, we found that high Pi increased the levels of Pit 2, but it had a minimal effect on Pit 1 levels (Supplemental Fig. 3A). Knockdown of Pit 2 with shRNA moderately attenuated the upregulation of Sox9 induced by high Pi (Supplemental Fig. 3B). It appears that Pi transporter Pit 2 is also involved in the upregulation of Sox9 expression by high Pi although its role is less important in comparison to that of PKD and Akt.

Sox9 mediates high Pi-induced osteogenic responses in human AVICs

To determine the role of Sox9 in AVIC osteogenic responses to high Pi, we treated cells for 72 h with lentivirus expressing Sox9 shRNA and then exposed them to high Pi. As shown in Fig. 4a, lentiviral Sox9 shRNA markedly reduced the levels of Sox9. Inhibition of Sox9 by shRNA suppressed high Pi-induced upregulation of Runx2 and ALP. Moreover, Sox9 knockdown attenuated AVIC calcium deposition induced by prolonged stimulation with high Pi (Fig. 4b). These data demonstrate that Sox9 plays a critical role in mediating AVIC osteogenic responses to high Pi.

We also stimulated human AVICs with LPS that has been shown to elevate AVIC pro-osteogenic activity in our previous studies [19, 27]. As shown in Supplemental Fig. 4, LPS failed to upregulate Sox9, whereas it upregulated the expression of BMP-2 and ALP in human AVICs. Conversely, it moderately reduced Sox9 levels. It seems that the role of Sox9 in AVIC osteogenic responses is stimulus-dependent. It is likely that the upregulation of Sox9 in human AVICs observed in the present study is specific for high Pi.

Calcified human aortic valves have reduced levels of Klotho

Patients with CKD have lower levels of Klotho in the kidney and blood [28]. However, it remains unclear whether human aortic valves express Klotho and whether high Pi and calcification have effects on valvular Klotho. In this study, we observed that Klotho protein was detectable in human aortic valve tissue, and calcified human aortic valves had lower levels of Klotho compared to normal aortic valves (Fig. 5a, b). In addition, we observed that high Pi decreased Klotho protein levels in AVICs from normal human aortic valves (Fig. 5c).

Klotho suppresses high Pi-induced osteogenic responses in human AVICs through suppressing Sox9 upregulation

To determine whether recombinant Klotho inhibits high Pi-induced osteogenic responses in human AVICs, we pretreated cells with different concentrations of recombinant Klotho (0.25 and 0.5 $\mu\text{g/ml}$) 1 h prior to high Pi treatment for 72 h. Klotho reduced the protein levels of Runx2 and ALP in cells exposed to high Pi (Fig. 6a). Additionally, calcium deposition in normal AVICs induced by a prolonged exposure to high Pi was attenuated by recombinant

Klotho (Fig. 6b). Furthermore, Klotho reduced the basal levels of Runx2 and ALP in AVICs isolated from calcified valves (Fig. 7). Thus, Klotho suppresses AVIC osteogenic responses to high Pi.

As Sox9 mediates high Pi-induced osteogenic responses and Klotho suppresses the response to high Pi, we conducted further experiment to examine whether and how Klotho inhibits Sox9 upregulation by high Pi. We treated normal AVICs with Klotho 1 h before the stimulation with high Pi. As shown in Fig. 8a, b, recombinant Klotho suppressed the upregulation of Sox9 by high Pi and prevented high Pi-induced Sox9 intranuclear translocation. Furthermore, Klotho inhibited the activation of PKD and Akt (Fig. 8c), two signaling molecules involved in the upregulation of Sox9 by high Pi. Together, these data reveal that Klotho suppresses the osteogenic responses in human AVICs through inhibition of PKD and Akt, which results in attenuated Sox9 upregulation.

Discussion

Numerous studies have confirmed that aortic valve calcification is an active process involving multiple mechanisms, including abnormal calcium or phosphate metabolism, valvular inflammation, and osteogenic reprogramming of AVICs. Elevated Pi levels in the circulation play an important role in aortic valve calcification associated with CKD. Currently, the precise mechanism underlying aortic valve calcification in CKD patients remains unclear. The present study demonstrates that high Pi elevates human AVIC pro-osteogenic activity, as evidenced by the upregulation of osteogenic biomarkers Runx2 and ALP, as well as calcium deposition, primarily through PKD-dependent and Akt-dependent Sox9 upregulation. In addition, our data show that human AVICs express Klotho, and Klotho levels in AVICs are negatively regulated by high Pi and calcification. Importantly, Klotho inhibits Sox9 upregulation to suppress the osteogenic responses of human AVICs to high Pi. These findings provide insights into the molecular mechanism underlying aortic valve calcification associated with CKD and indicate that Klotho may have therapeutic potential in attenuating aortic valve calcification.

The PKD and Akt pathways mediate high Pi-induced osteogenic responses

Several population-based studies demonstrate an increased prevalence of aortic valve calcification in patients with CKD [29, 30]. Studies on animal models of CKD also show that declined renal function and increased circulating levels of Pi are accompanied by the appearance of ectopic calcification in vascular and valvular tissues [31, 32]. Using a model of adenine-induced renal failure, Shuvy and colleagues demonstrated that rats fed with a high Pi diet develop aortic valve calcification [33]. Further, AVICs display greater mineral deposition in response to high Pi stimulation [5, 34]. However, the signaling mechanism remains incompletely understood. The results of the present study show that high Pi induces the phosphorylation of PKD and Akt, and inhibition of one of these two signaling pathways suppresses high Pi-induced upregulation of Runx2 and ALP and calcium deposit formation in human AVICs. Akt signaling has been implicated in AVIC osteoblast-like differentiation [35]. The results of the present study suggest that both Akt and PKD pathways mediate high Pi-induced osteogenic responses in human AVICs. It appears that these two signaling

pathways work in concert to mediate the upregulation of Runx2 and ALP, and blocking either of these two pathways would markedly attenuate the responses to high Pi.

The next question is how the Akt and PKD pathways upregulate the expression of Runx2 and ALP. The osteogenic transcription factor Runx2 is known to upregulate ALP level and activity [36]. The upregulation of Runx2 by high Pi may be responsible for the elevated ALP levels in human AVICs exposed to high Pi. A factor modulated by PKD and Akt may upregulate the expression of Runx2, and possibly ALP as well.

Sox9 plays a critical role in mediating high Pi-induced osteogenic responses

Sox9 is a master regulator of chondrogenesis and is required for chondrocyte proliferation, differentiation, and maturation [37]. It has been reported that Sox9 is overexpressed in stenotic human aortic valves [38], indicating a role of this factor in aortic valve calcification. However, Sox9 appears to have an anti-osteogenic effect in animal aortic valves. Mice with Sox9 deficiency have been found to develop heart valve calcification, and knockdown of Sox9 in murine heart valves in vitro results in increased Runx2 expression and calcium deposition [10]. In porcine AVICs, calcium nodule formation is associated with translocation of Sox9 from the nucleus to the cytoplasm [39]. Interestingly, the results of the present study show that high Pi upregulates Sox9 in human AVICs in a PKD-dependent and Akt-dependent manner and promotes Sox9 intranuclear translocation. Importantly, knockdown of Sox9 abolishes high Pi-induced expression of Runx2 and ALP, as well as the greater calcium deposition in human AVICs. Thus, Sox9 plays a pro-osteogenic role in human AVICs and mediates the osteogenic responses to high Pi. The discrepancy between our finding and the findings from the animal study [10] may be due to the difference between human and animal. In addition, heart valve calcification involves a complicated pathobiology. Other altered factors may be responsible for heart valve calcification observed in the Sox9-deficient mice.

LPS does not upregulate Sox9 levels in human AVICs although it induces the osteogenic responses. It appears that different stimuli utilize different mechanisms to mediate the osteogenic responses in human AVICs, and Sox9 upregulation appears to be specific to high Pi stimulation. It is likely that high Pi is responsible for the upregulation of Sox9 observed in this study. In this regard, high Pi has been reported to upregulate the expression of Sox9 in human endothelial cells and smooth muscle cells [26, 40]. The findings of the present study highlight a novel role of Sox9 in mediating the osteogenic responses of human AVICs to high Pi.

It should be noted that high Pi also elevates Pit 2 levels in human AVICs and that knockdown of Pit 2 suppresses the upregulation of Sox9 by high Pi. It appears that Pit 2 also plays a role in mediating high Pi-induced Sox9 upregulation. Future studies are needed to determine the relative role of Pit 2 in mediating the osteogenic responses to high Pi in human AVICs.

Klotho suppresses human AVIC osteogenic responses to high Pi

The anti-aging protein Klotho has attracted great attention because of its implication in diverse biological processes related to human longevity. Interestingly, aortic valve

calcification score of patients is negatively correlated with circulating Klotho levels [41]. The Klotho^{-/-} mouse model, which exhibits premature aging associated with hyperphosphatemia and low vitamin D, has been used to study age-related diseases, including CKD and vascular calcification. Klotho^{-/-} mice exhibit aortic valve calcification at the hinge region of the fibrosa side [16], a pathological change similar to human CAVD. Reduced expression of this protein has been observed in patients with CKD, and this may be one of the factors underlying the aortic valve calcification in CKD. However, the impact of high Pi on Klotho has not been examined, and the role of Klotho in AVIC osteogenic responses to high Pi is unknown.

We observed that Klotho protein is present in human aortic valves, and calcified aortic valve tissue and AVICs from such tissue have lower levels of Klotho. The reduction of Klotho in calcified aortic valves may be due to both disease and aging since patients with aortic valve calcification are usually older. Interestingly, high Pi decreases the levels of Klotho in AVICs isolated from normal human valves, and recombinant Klotho suppresses the upregulation of Runx2 and ALP and attenuates calcium deposition in normal human AVICs exposed to high Pi. More importantly, Klotho reduces the basal levels of Runx2 and ALP in AVICs isolated from calcified valves. Therefore, Klotho negatively modulates AVIC pro-osteogenic activity, and downregulation of Klotho in AVICs by high Pi may contribute to the elevated valvular pro-osteogenic activity associated with CKD and CAVD.

The anti-osteogenic effect of Klotho on human AVIC appears due to its suppression of Sox9 upregulation by high Pi since recombinant Klotho markedly reduces the upregulation of Sox9 that is critical in mediating high Pi-induced osteogenic responses in human AVICs. Our finding is consistent with a previous report showing that Klotho null mice have increased expression of Sox9 in aortic valve tissue [42]. Currently, the existence of a specific receptor for Klotho is a matter of debate. Nevertheless, our data show that Klotho inhibits PKD and Akt, two signaling molecules involved in the upregulation of Sox9 by high Pi. Together, the data of the present study reveal that Klotho suppresses the osteogenic responses to high Pi in human AVICs through attenuation of Sox9 upregulation by inhibition of PKD and Akt.

This study has several limitations. Although we observed that Sox9 plays a critical role in mediating high Pi-induced osteogenic responses in human AVICs and that Klotho attenuates Sox9 upregulation to suppress AVIC osteogenic responses to high Pi, it is important to confirm these in vitro findings in future studies using animal models. In addition, it remains unclear from this study what receptor mediates the effect of Klotho on human AVIC osteogenic responses to high Pi. Further studies are warranted to identify the receptor.

Conclusions

The present study demonstrates that high Pi induces osteogenic responses in human AVICs via PKD-dependent and Akt-dependent Sox9 upregulation. Klotho is present in human aortic valves and AVICs, and high Pi reduces Klotho levels in human AVICs. Furthermore, Klotho suppresses the pro-osteogenic effect of high Pi through inhibition of Sox9 upregulation.

These findings suggest that modulation of Klotho levels and/or function may have therapeutic potential for mitigation of aortic valve calcification associated with CKD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key messages

- CAVD associated with chronic kidney disease is a significant clinical problem.
- High phosphate upregulates Sox9 through AKT and PKD in human AVICs.
- Calcified human aortic valves have lower levels of Klotho.
- Klotho suppresses Sox9 upregulation and intranuclear translocation.
- Klotho inhibits high phosphate-induced osteogenic activity in human AVICs.

Fig. 1. High Pi induces osteogenic responses in human AVICs

a Human AVICs were treated with high Pi (2 or 3 mmol/l) for 72 h. Representative immunoblots and densitometric data show that high Pi upregulates the levels of Runx2 and ALP in human AVICs in a dose-dependent manner. **b** Human AVICs were treated with high Pi (3 mmol/l) for 14 days in the conditioning medium (growth medium supplemented with 10 mmol/l of β -glycerophosphate, 10 nmol/l of dexamethasone, and 8 mmol/l of CaCl_2). Representative images of alizarin red staining and spectrophotometric data show that cells exposed to high Pi form a greater amount of calcium deposits ($\times 10$ objective for original

magnification in the higher power images). Data are mean $\pm$ SE. $n = 4$ experiments using distinct cell isolates; * $P < 0.05$ vs. untreated control

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Fig. 2. PKD and Akt mediate the osteogenic responses induced by high Pi

a Human AVICs were treated with high Pi (3 mmol/l) for 0.5 to 8 h. Representative immunoblots and densitometric data show that high Pi induces rapid phosphorylation of Akt and delayed phosphorylation of PKD. **b** Human AVICs were treated with high Pi in the presence or absence of PKD inhibitor (CID755673, 40 μM) for 72 h. Representative immunoblots and densitometric data show that inhibition of PKD reduces the levels of Runx2 and ALP in human AVICs exposed to high Pi. **c** Human AVICs were treated with high Pi (3 mmol/l) in the presence or absence of PKD inhibitor (CID755673, 40 μmol/l) for

14 days in the conditioning medium. Representative images ($\times 10$ objective for original magnification in the higher power images) of alizarin red staining and spectrophotometric data show that inhibition of PKD attenuates calcium deposit formation induced by high Pi. **d** Inhibition of Akt by MK2206 (5 $\mu\text{mol/l}$) reduces the levels of Runx2 and ALP in cells exposed to high Pi. **e** Inhibition of Akt by MK2206 (5 $\mu\text{mol/l}$) attenuates calcium deposit formation ($\times 10$ objective for original magnification in the higher power images) in human AVICs induced by high Pi in the conditioning medium. Data are mean $\pm$ SE. $n = 4$ experiments using distinct cell isolates; * $P < 0.05$ vs. untreated control; # $P < 0.05$ vs. Pi alone or Pi + DMSO

Fig. 3. High Pi upregulates Sox9 through PKD and Akt

a Human AVICs were treated with high Pi (2 or 3 mmol/l) for 72 h. High Pi upregulates the levels of Sox9 protein in a dose-dependent manner. **b** Representative immunofluorescence images (original magnification, $\times 40$ objective) show that high Pi induces Sox9 (red) intranuclear translocation. Alexa 488-tagged wheat germ agglutinin (WGA, green) was used to outline plasma membrane. 4',6-Diamidino-2-phenylindole (DAPI, blue) was used for nuclear counterstaining. **c** Inhibition of PKD (CID755673, 40 μ mol/l) reduces the levels of Sox9 in cells exposed to high Pi. **d** Inhibition of Akt (MK2206, 5 μ mol/l) suppresses high

Pi-induced Sox9 expression. Data are mean $\pm$ SE. $n = 4$ experiments using distinct cell isolates; * $P < 0.05$ vs. untreated control; # $P < 0.05$ vs. Pi alone or Pi + DMSO

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Fig. 4. Sox9 mediates high Pi-induced osteogenic responses in human AVICs

a Human AVICs were pretreated with lentivirus expressing Sox9 shRNA (100 nmol/l) for 72 h and then treated with high Pi (3 mmol/l) for 72 h. Representative immunoblots show that knockdown of Sox9 with shRNA abolishes Sox9 upregulation and reduces the levels of Runx2 and ALP in cells exposed to high Pi. **b** Representative images ($\times 10$ objective for original magnification in the higher power images) of alizarin red staining show that knockdown of Sox9 attenuates high Pi-induced calcium deposit formation in human AVICs.

Data are mean $\pm$ SE. $n = 4$ experiments using distinct cell isolates; * $P < 0.05$ vs. control shRNA; # $P < 0.05$ vs. Pi alone or Pi + control shRNA

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Fig. 5. High Pi downregulates Klotho levels in human AVICs

a Calcified aortic valve tissues (from donors of 56 to 77 years old) have lower levels of Klotho compared to normal aortic valve tissues (from donors of 28 to 56 years old). Data are mean $\pm$ SE. $n = 4$ per group; * $P < 0.05$ vs. normal. **b** Representative images ($\times 40$ objective for original magnification) of immunofluorescence staining show that Klotho (*red*) is present in the interstitial cells and interstitial spaces of aortic valve tissue, and diseased aortic valves have lower levels of Klotho. 4',6-Diamidino-2-phenylindole (DAPI, *blue*) was used for nuclear counterstaining. Alexa 488-tagged wheat germ agglutinin (WGA, *green*) was used to

outline plasma membrane. **c** AVICs of normal valves were treated with high Pi (2 or 3 mmol/l) for 72 h. Representative immunoblots and densitometric data show that high Pi reduces Klotho protein levels in human AVICs. Data are mean $\pm$ SE. $n = 4$ experiments using distinct cell isolates; $*P < 0.05$ vs. untreated control

Fig. 6. Klotho suppresses high Pi-induced osteogenic responses in human AVICs

a AVICs of normal human valves were pretreated with different concentrations of recombinant Klotho (0.25 or 0.5 μg/ml) 1 h prior to high Pi treatment for 72 h. Representative immunoblots and densitometric data show that Klotho suppresses high Pi-induced Runx2 and ALP upregulation in human AVICs. **b** AVICs of normal human valves were treated with high Pi (3 mmol/l), in the presence or absence of Klotho (0.5 μg/ml) for 14 days in the conditioning medium. Representative images (×10 objective for original magnification in the higher power images) of alizarin red staining and spectrophotometric

data show that recombinant Klotho suppresses high Pi-induced calcium deposit formation. Data are mean $\pm$ SE. $n = 4$ experiments using distinct cell isolates; * $P < 0.05$ vs. untreated control; # $P < 0.05$ vs. Pi alone

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Fig. 7.

Klotho reduces Runx2 and ALP levels in AVICs of calcified valves. AVICs of calcified human aortic valves were treated with different concentrations of recombinant Klotho (0.25 or 0.5 $\mu\text{g/ml}$) for 72 h. Representative immunoblots and densitometric data show that recombinant Klotho reduces the levels of Runx2 and ALP in diseased AVICs in the absence of high Pi stimulation. Data are mean $\pm$ SE. $n = 4$ experiments using distinct cell isolates; * $P < 0.05$ vs. untreated control

Fig. 8. Klotho inhibits Akt and PKD phosphorylation and suppresses Sox9 upregulation induced by high Pi

a AVICs were pretreated with different concentrations of recombinant Klotho (0.25 or 0.5 μg/ml) 1 h prior to high Pi treatment for 72 h. Immunoblots and densitometric data show that recombinant Klotho suppresses high Pi-induced Sox9 upregulation. **b** AVICs were pretreated with recombinant Klotho (0.5 μg/ml) 1 h prior to high Pi (3 mmol/l) stimulation for 3 h. Representative immunofluorescence images (×40 objective for original magnification) show that Klotho inhibits Sox9 (red) intranuclear translocation induced by

high Pi. Alexa 488-tagged wheat germ agglutinin (WGA, *green*) was used to outline plasma membrane. 4',6-Diamidino-2-phenylindole (DAPI, *blue*) was used for nuclear counterstaining. **c** AVICs were pretreated with different concentrations of recombinant Klotho (0.25 or 0.5 $\mu\text{g/ml}$) 1 h prior to high Pi treatment for 2 h. Immunoblots and densitometric data show that recombinant Klotho inhibits high Pi-induced phosphorylation of Akt and PKD. Data are mean $\pm$ SE. $n = 4$ experiments using distinct cell isolates; * $P < 0.05$ vs. untreated control; # $P < 0.05$ vs. Pi alone