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Original Paper

Inhibition of Phosphate-Induced Vascular Smooth Muscle Cell Osteo-/Chondrogenic Signaling and Calcification by Bafilomycin A1 and Methylamine

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Key Words
Calcification • Senescence • Vesicular pH • H^+ pump • Phosphate • TGFß1 • NFAT5 • Alkaline phosphatase • Osteoinductive signaling

Abstract
Background/Aims: Excessive phosphate concentrations trigger vascular calcification, an active process promoted by osteoinduction of vascular smooth muscle cells (VSMCs) with increased expression and activity of transcription factor RUNX2 (Core-binding factor α1, CBFA1), alkaline phosphatase (ALPL), TGFß1, transcription factor NFAT5, and NFAT5-sensitive transcription factor SOX9. The osteoinductive signaling and vascular calcification of hyperphosphatemic klotho-hypomorphic mice could be reversed by treatment with NH_4Cl, effects involving decrease of TGFß1 and inhibition of NFAT5-dependent osteoinductive signaling. Known effects of NH_4Cl include alkalinization of acidic cellular compartments. The present study explored whether osteo-/chondrogenic signaling could be influenced by alkalinization of acidic cellular compartments following inhibition of the vacuolar H^+ ATPase with bafilomycin A1 or following dissipation of the pH gradient across the membranes of acidic cellular compartments with methylamine. ***Methods:*** Primary human aortic smooth muscle cells (HAoSMCs) were treated with high phosphate to trigger osteo-/chondrogenic signaling and calcification in the absence or presence of bafilomycin A1 or methylamine. Calcium content was determined using a QuantiChrom Calcium assay, ALP activity by a colorimetric assay and transcript levels by quantitative RT-PCR. ***Results:*** High phosphate increased significantly the calcium deposition, *CBFA1* and *ALPL* mRNA expression as well as alkaline phosphatase activity in HAoSMCs, all effects ameliorated by both, bafilomycin A1 and methylamine. High phosphate further significantly up-regulated the mRNA levels of *TGFB1*, *NFAT5* and *SOX9*, effects significantly blunted by additional treatment with bafilomycin A1 or methylamine. Treatment of HAoSMCs with human TGFß1 protein or high phosphate up-regulated *NFAT5*, *SOX9*, *CBFA1* and *ALPL* mRNA expression to

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similarly high levels which could not be further increased by combined treatment with high phosphate and TGFß1. Bafilomycin A1 failed to reverse the osteo-/chondrogenic signaling triggered by high phosphate together with TGFß1. ***Conclusions:*** Inhibition of the vacuolar H^+ ATPase or dissipation of the pH gradient across the membranes of acidic cellular compartments both disrupt osteo-/chondrogenic signaling and calcium deposition in VSMCs, observations supporting the hypothesis that vascular calcification requires acidic cellular compartments.

Introduction

Vascular calcification, a hallmark of aging [1-4], is promoted by hyperphosphatemia [5], which accelerates aging and increases cardiovascular risk [6]. Excessive plasma phosphate concentrations with subsequent mineral bone disorder (MBD) and vascular calcification are particularly decisive for the mortality of patients with chronic kidney disease (CKD)[4, 7].

Vascular calcification is, however, not simply due to precipitation of $CaHPO_4$ salts, but results from a complex active process [8] involving reprogramming of vascular smooth muscle cells (VSMCs) into osteo-/chondrogenic phenotypes [9]. The osteo-/chondrogenic reprogramming of VSMCs is triggered by increased extracellular phosphate concentrations [5]. Signaling involved in osteo-/chondrogenic differentiation of VSMCs include up-regulation of CBFA1/RUNX2 (Core-binding factor α1), an osteogenic transcription factor [10] orchestrating the transformation of VSMCs into osteoblast-like cells [11-13]. CBFA1-dependent genes include the alkaline phosphatase [12, 14-16], which degrades the calcification inhibitor pyrophosphate [10, 17]. CBFA1 expression is up-regulated by the TGFß1-sensitive [18] transcription factor NFAT5 (nuclear factor of activated T-cells 5) [19], an effect involving the transcription factor SOX9 [19]. SOX9 is up-regulated in uremia [20] and participates in the stimulation of chondrogenic gene expression in VSMCs [21]. TGFß1 thus stimulates osteogenic signaling [22-25]. Vascular osteoinduction is closely associated with VSMC senescence [26], which parallels vascular aging and injury [27].

A recent study disclosed an inhibitory effect of NH_4Cl on osteogenic signaling and vascular calcification [28]. NH_4^+ may dissociate thus yielding NH_3, which easily crosses membranes, thus entering cells and cellular compartments. In acidic compartments, NH_3 binds H^+ and is thus trapped as NH_4^+ [29]. As a result, NH_4Cl alkalinizes acidic cellular compartments, which is known to prevent maturation of several proteins including TGFß1 [30]. It was thus hypothesized that NH_4^+ is effective by alkalinizing acidic cellular compartments. However, at least in theory, NH_4Cl may inhibit calcification by triggering acidosis [31-33]. Alternative methods leading to alkalinisation of acidic intracellular compartments include inhibition of the vacuolar H^+ ATPase with bafilomycin A1 [34] and the dissipation of the pH gradient across the vacuolar membrane with methylamine [35].

The present study explored the effect of bafilomycin A1 and of methylamine on osteo-/chondrogenic signaling and calcification induced by high phosphate in primary human aortic smooth muscle cells.

Materials and Methods

Cell culture of HAoSMCs

Primary human aortic smooth muscle cells (Invitrogen, Life Technologies) were routinely cultured in Waymouth's MB 752/1 medium and Ham's F-12 nutrient mixture (1:1, Gibco, Life Technologies) supplemented with 10% FBS (Gibco, Life Technologies) and 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, Life Technologies). HAoSMCs were grown to confluency and used in all experiments from passages 4 to 10. Where indicated, HAoSMCs were treated with 2 mM β-glycerophosphate (Sigma-Aldrich), with 3 mM sodium phosphate buffer, pH 7.4 (Sigma-Aldrich), with 500 nM bafilomycin A1 (Sigma-Aldrich, solved in

DMSO), with 1 mM methylamine (Sigma-Aldrich) and/or with 10 ng/ml human TGFβ1 (R&D Systems, solved in 4 mM HCl solution containing 1mg/ml BSA). Equal amounts of vehicle were used as control.

Quantitative RT-PCR

HAoSMCs were washed with PBS and total RNA was isolated using Trifast Reagent (Peqlab) according to the manufacturer's instructions. Reverse transcription of 2 µg RNA was performed using oligo(dT)$_{12-18}$ primers (Invitrogen) and SuperScriptIII Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was performed with the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories) and iQ™ Sybr Green Supermix (Bio-Rad Laboratories) according to the manufacturer's instructions. The following human primers were used (5'→3' orientation):

TN alkaline phosphatase fw: GGGACTGGTACTCAGACAACG;
TN alkaline phosphatase rev: GTAGGCGATGTCCTTACAGCC;
CBFA1 fw: GCCTTCCACTCTCAGTAAGAAGA;
CBFA1 rev: GCCTGGGGTCTGAAAAAGGG;
GAPDH fw: GAGTCAACGGATTTGGTCGT;
GAPDH rev: GACAAGCTTCCCGTTCTCAG;
NFAT5 fw: GGGTCAAACGACGAGATTGTG;
NFAT5 rev: GTCCGTGGTAAGCTGAGAAAG;
SOX9 fw: AGCGAACGCACATCAAGAC;
SOX9 rev: CTGTAGGCGATCTGTTGGGG;
TGFB1 fw: CAATTCCTGGCGATACCTCAG;
TGFB1 rev: GCACAACTCCGGTGACATCAA.

The specificity of the PCR products was confirmed by analysis of the melting curves. All PCRs were performed in duplicate and relative mRNA fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method using GAPDH as internal reference.

Calcium content

HAoSMCs were treated for 14 days with 3 mM sodium phosphate buffer as calcification media. Fresh media with agents were added every 2-3 days. After the incubation period, HAoSMCs were decalcified for 24 hours at 4°C in 0.6 M HCl. The calcium content was determined colorimetrically using a QuantiChrom Calcium assay kit (BioAssay Systems) according to the manufacturer's protocol. After washing with PBS, HAoSMCs were lysed with 0.1 M NaOH/0.1% SDS. Calcium content was normalized to total protein concentration as assessed by the Bradford assay (Bio-Rad Laboratories).

Alkaline phosphatase (ALP) activity assay

For determination of cellular ALP activity, HAoSMCs were treated for 7 days. Fresh media with agents were added every 2-3 days. After the incubation period, HAoSMCs were washed with PBS and assayed for ALP activity using the ALP colorimetric assay kit (Abcam) according to the manufacturer's protocol. ALP activity was normalized to total protein concentration as assessed by the Bradford assay (Bio-Rad Laboratories).

Statistics

Data are provided as arithmetic means ± SEM, *n* represents the number of experiments. Normality was tested with Shapiro-Wilk test. Statistical testing was performed by one-way Anova followed by Tukey-test for homoscedastic data or Games-Howell test for heteroscedastic data. Non-normal data was tested by the Steel-Dwass method. Results with $p<0.05$ were considered statistically significant.

Results

In order to explore whether vacuolar pH impacts on vascular calcification, experiments have been performed in primary human aortic smooth muscle cells (HAoSMCs) treated with high phosphate with or without inhibition of the vacuolar H^+ pump with bafilomycin

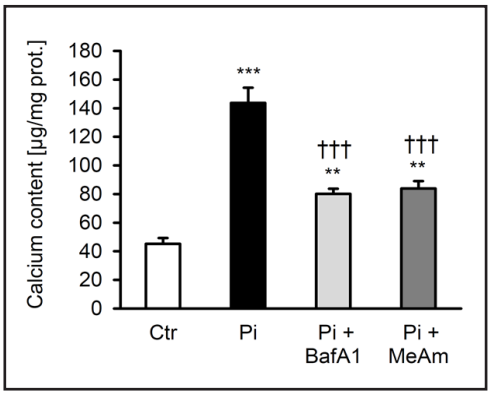

Fig. 1. Bafilomycin A1 and methylamine interfere with phosphate-induced calcification in primary human aortic smooth muscle cells. Arithmetic means ± SEM (n=8; µg/mg protein) of calcium content in HAoSMCs after 14 days of treatment with control (white bar, Ctr), with 3 mM sodium phosphate alone (black bar, Pi), with 3 mM sodium phosphate and 500 nM bafilomycin A1 (light grey bar, Pi+BafA1) or with 3 mM sodium phosphate and 1 mM methylamine (dark grey bar, Pi+MeAm). **(p<0.01), ***(p<0.001) indicates statistically significant difference from control treated HAoSMCs; †††(p<0.001) indicates statistically significant difference from HAoSMCs treated with 3 mM sodium phosphate alone.

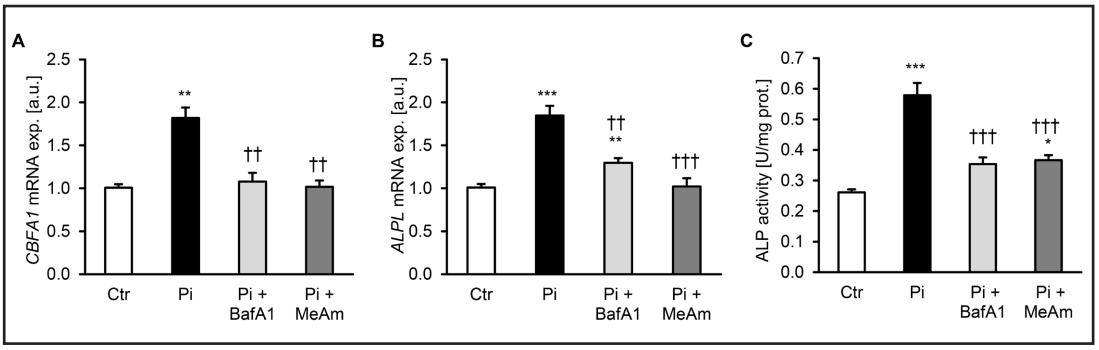

Fig. 2. Bafilomycin A1 and methylamine inhibit phosphate-induced osteoinductive signaling in primary human aortic smooth muscle cells. Arithmetic means ± SEM (n=10; arbitrary units, a.u.) of *CBFA1* (A) and *ALPL* (B) relative mRNA expression in HAoSMCs after 24 hours of treatment with control (white bars, Ctr), with 2 mM β-glycerophosphate alone (black bars, Pi), with 2 mM β-glycerophosphate and 500 nM bafilomycin A1 (light grey bars, Pi+BafA1) or with 2 mM β-glycerophosphate and 1 mM methylamine (dark grey bars, Pi+MeAm). Arithmetic means ± SEM (n=4; U/mg protein) of alkaline phosphatase activity (C) in HAoSMCs after 7 days of treatment with control (white bars, Ctr), with 2 mM β-glycerophosphate alone (black bars, Pi), with 2 mM β-glycerophosphate and 500 nM bafilomycin A1 (light grey bars, Pi+BafA1) or with 2 mM β-glycerophosphate and 1 mM methylamine (dark grey bars, Pi+MeAm). * (p<0.05), **(p<0.01), ***(p<0.001) indicates statistically significant difference from control treated HAoSMCs; ††(p<0.01), †††(p<0.001) indicates statistically significant difference from HAoSMCs treated with 2 mM β-glycerophosphate alone.

A1 and with or without dissipation of the pH gradient across the vacuolar membrane with methylamine. In a first step, the calcium deposition in HAoSMCs was determined. As illustrated in Fig. 1, the calcium content of HAoSMCs increased significantly following 14 days of treatment with calcification media containing 3 mM sodium phosphate. The effect on calcium deposition was significantly blunted in the presence of either bafilomycin A1 (500 nM) or methylamine (1 mM). Thus, alkalinisation of acidic intracellular compartments inhibits the phosphate-induced calcification of HAoSMCs.

A further series of experiments explored whether the effects of bafilomycin A1 and methylamine on calcium content were paralleled by corresponding effects on osteo-/chondrogenic signaling. To this end, the transcript levels of the osteogenic transcription factor *CBFA1* (Core-binding factor α1, RUNX2) and of alkaline phosphatase (*ALPL*) were determined. As shown in Fig. 2, the *CBFA1* and *ALPL* mRNA expression were significantly up-regulated following 24 hours treatment with 2 mM β-glycerophosphate as phosphate donor in HAoSMCs,

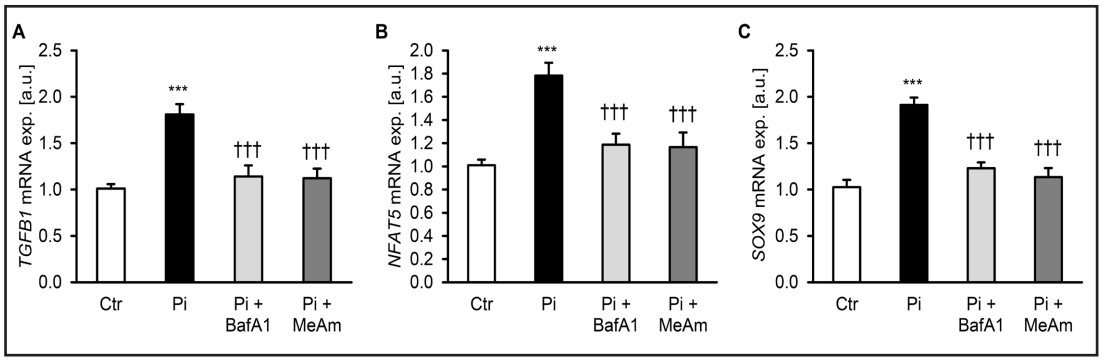

Fig. 3. Bafilomycin A1 and methylamine interfere with phosphate-induced TGFß1 transcription and signaling in primary human aortic smooth muscle cells. Arithmetic means ± SEM (n=10; arbitrary units, a.u.) of *TGFB1* (A), *NFAT5* (B) and *SOX9* (C) relative mRNA expression in HAoSMCs after 24 hours of treatment with control (white bars, Ctr), with 2 mM β-glycerophosphate alone (black bars, Pi), with 2 mM β-glycerophosphate and 500 nM bafilomycin A1 (light grey bars, Pi+BafA1) or with 2 mM β-glycerophosphate and 1 mM methylamine (dark grey bars, Pi+MeAm). ***(p<0.001) indicates statistically significant difference from control treated HAoSMCs; †††(p<0.001) indicates statistically significant difference from HAoSMCs treated with 2 mM β-glycerophosphate alone.

an effect significantly blunted in the presence of either bafilomycin A1 or methylamine (Fig. 2A, B). Bafilomycin A1 and methylamine similarly interfered with the induction of alkaline phosphatase activity in HAoSMCs (Fig. 2C). Treatment for 7 days with 2 mM β-glycerophosphate increased significantly the alkaline phosphatase activity in HAoSMCs, an effect again significantly blunted following treatment with either bafilomycin A1 or methylamine. Taken together, bafilomycin A1 and methylamine inhibited osteo-/chondrogenic transformation of HAoSMCs and calcium depositions under high phosphate conditions.

A further series of experiments explored whether bafilomycin A1 and methylamine similarly impact on TGFß1 expression and signaling. Therefore, quantitative RT-PCR was employed to elucidate the effects on the transcript levels of *TGFB1*, TGFß1-sensitive transcription factor *NFAT5* and NFAT5-downstream transcription factor *SOX9*. As illustrated in Fig. 3, the β-glycerophosphate-induced *TGFB1*, *NFAT5* and *SOX9* mRNA expression was significantly blunted and virtually abrogated by additional treatment with either bafilomycin A1 or methylamine in HAoSMCs.

Additional experiments addressed the role of TGFß1 on osteo-/chondrogenic signaling. As shown in Fig. 4, treatment for 24 hours of HAoSMCs with human TGFß1 protein mimicked the effects of β-glycerophosphate and significantly increased *NFAT5*, *SOX9, CBFA1* and *ALPL* mRNA expression. No further significant increase of *NFAT5*, *SOX9, CBFA1* and *ALPL* mRNA expression could be observed upon combined treatment of HAoSMCs with TGFß1 together with β-glycerophosphate (Fig. 4).

A final series of experiments explored whether the influence of high phosphate and of alkalinisation of acidic intracellular compartments on osteo-/chondrogenic signaling was secondary to their effects on TGFß1 by additional treatment of HAoSMCs with human TGFß1 protein. As a result, the transcript levels of *NFAT5*, *SOX9, CBFA1* and *ALPL* were again significantly up-regulated following treatment with human TGFß1 protein in HAoSMCs (Fig. 5). Bafilomycin A1 again abrogated the β-glycerophosphate-induced *NFAT5*, *SOX9*, *CBFA1* and *ALPL* mRNA expression. The effects of bafilomycin A1 on β-glycerophosphate-induced osteo-/chondrogenic signaling were completely reversed by the addition of human TGFß1 protein (Fig. 5). Thus, alkalinization of acidic cellular compartments inhibits the osteo-/chondrogenic dedifferentiation of HAoSMCs at least partially by downregulation of *TGFB1* and TGFβ1-dependent signaling.

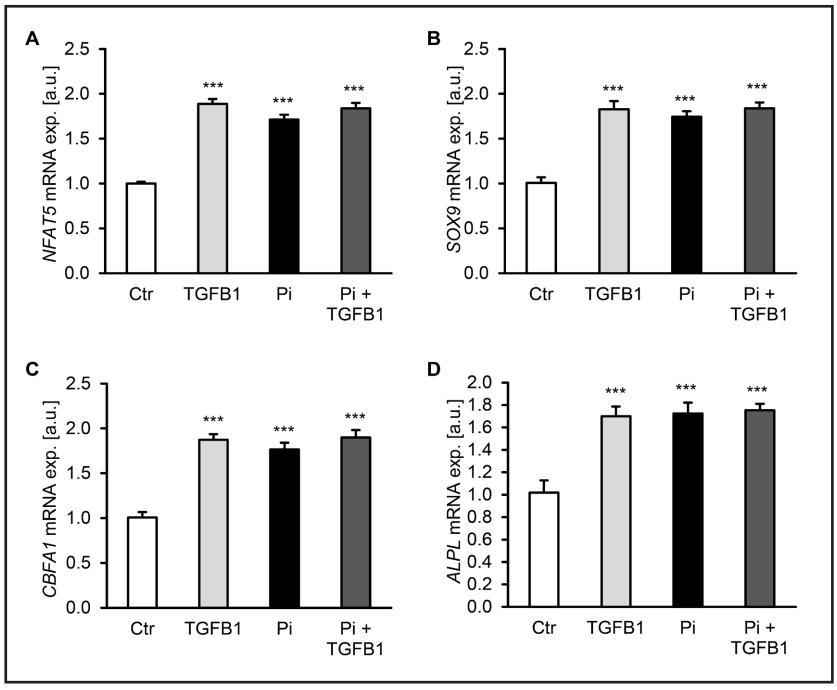

Fig. 4. TGFß1 mimics the effect of phosphate on osteo-/chondrogenic signaling in primary human aortic smooth muscle cells. Arithmetic means ± SEM (n=4; arbitrary units, a.u.) of *NFAT5* (A), *SOX9* (B), *CBFA1* (C) and *ALPL* (D) relative mRNA expression in HAoSMCs after 24 hours of treatment with control (white bars, Ctr), with 10 ng/ml human TGFß1 alone (light grey bars, TGFß1), with 2 mM β-glycerophosphate alone (black bars, Pi) or with 2 mM β-glycerophosphate and 10 ng/ml human TGFß1 (dark grey bars, Pi+TGFß1). ***(p<0.001) indicates statistically significant difference from control treated HAoSMCs.

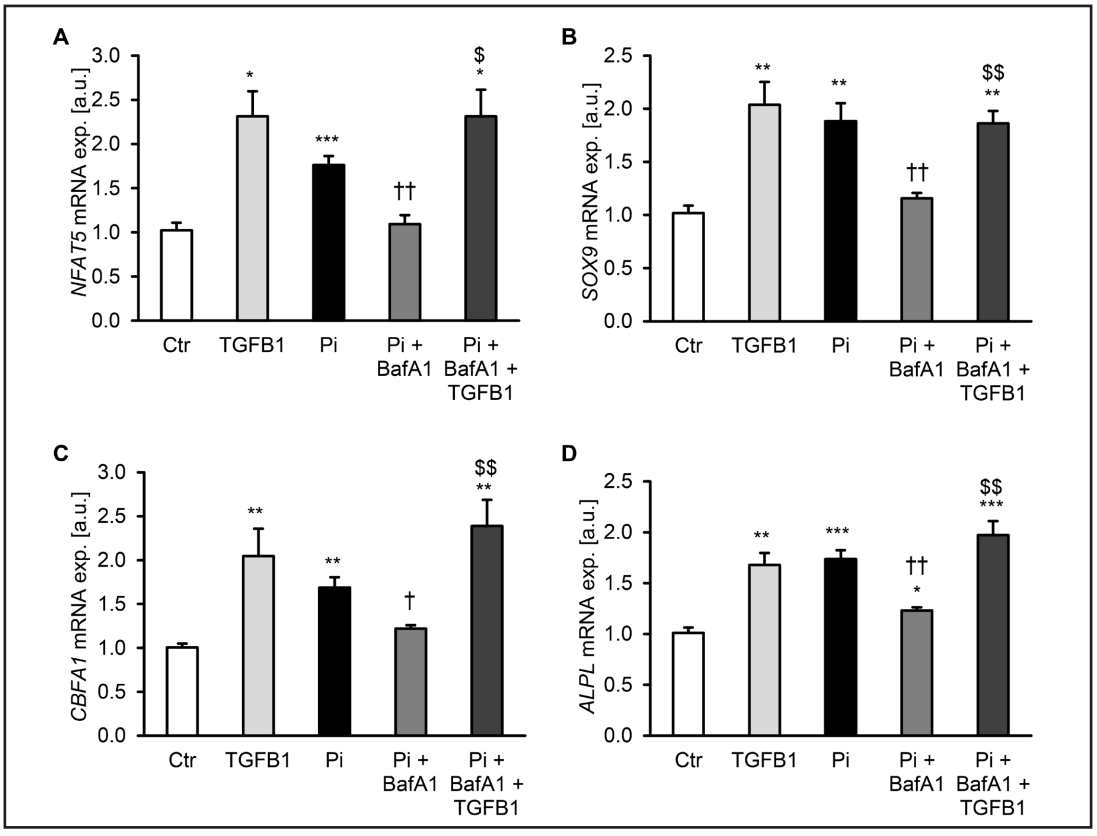

Fig. 5. TGFß1 reverses the effect of bafilomycin A1 on phosphate-induced osteo-/chondrogenic signaling in primary human aortic smooth muscle cells. Arithmetic means ± SEM (n=8; arbitrary units, a.u.) of *NFAT5*

(A), *SOX9* (B), *CBFA1* (C) and *ALPL* (D) relative mRNA expression in HAoSMCs after 24 hours of treatment with control (white bars, Ctr), with 10 ng/ml human TGFß1 alone (light grey bars, TGFß1), with 2 mM β-glycerophosphate alone (black bars, Pi), with 2 mM β-glycerophosphate and 500 nM bafilomycin A1 (middle grey bars, Pi+BafA1) or with 2 mM β-glycerophosphate, 500 nM bafilomycin A1 and 10 ng/ml human TGFß1 (dark grey bars, Pi+BafA1+TGFß1). *($p<0.05$), **($p<0.01$), ***($p<0.001$) indicates statistically significant difference from control treated HAoSMCs; †($p<0.05$), ††($p<0.01$) indicates statistically significant difference from HAoSMCs treated with 2 mM β-glycerophosphate alone. $($p<0.05$), $$($p<0.01$) indicates statistically significant difference between HAoSMCs treated with Pi+BafA1 and Pi+BafA1+TGFß1.

Discussion

The present study reveals that both, bafilomycin A1 and methylamine counteract osteoinductive signaling in primary human aortic smooth muscle cells (HAoSMCs) under high phosphate conditions. Treatment of HAoSMCs with high phosphate triggered osteo-/chondrogenic reprogramming as reflected by up-regulation of osteogenic markers *CBFA1* and *ALPL*, of *TGFB1* and TGFß1-sensitive transcription factors *NFAT5* and *SOX9* expression, as well as alkaline phosphatase activity and calcium deposition. All those effects in HAoSMCs were ameliorated by both, bafilomycin A1 and methylamine. Methylamine and bafilomycin A1 are both known to interfere with lysosomal acidification [36-38]. Bafilomycin A1 may further decrease the cytoplasmic pH, while methylamine rather increases cytoplasmic pH [37, 38]. Thus alkalinization of acidic intracellular compartments rather than alterations of cytosolic pH may account for the inhibitory effects of bafilomycin A1 and methylamine on osteoinductive signaling.

Treatment of HAoSMCs with TGFß1 fully mimics the effect of high phosphate on the osteo-/chondrogenic signaling. The effects of phosphate and TGFß1 were not additive. The effect of phosphate with exogenously added TGFß1 on osteo-/condrogenic transformation was insensitive to bafilomycin A1. Alkalinization of acidic cellular compartments thus interferes with osteo-/chondrogenic signaling by affecting a mechanism upstream of TGFß1. Alkalinization of acidic cellular compartments may also prevent intracellular calcium overload and apoptosis of vascular smooth muscle cells (VSMCs) [39]. The present observations do not allow safe conclusions on the mechanisms involved in bafilomycin A1 and methylamine sensitivity of TGFß1 expression and function.

Alkalinization of acidic cellular compartments has previously been shown to impede the maturation of TGFß1, an effect presumably due to inhibition of pH-sensitive luminal enzymes [30]. Impaired acidification of endosomes could further disrupt receptor/ligand dissociation and interfere with TGFß1 receptor externalization [40, 41]. How an alkalinization of the acidic cellular compartments affects the *TGFB1* transcript levels, remains, however, elusive. TGFß1 is known to be up-regulated by excessive extracellular phosphate concentrations [42] and to stimulate cellular senescence thus contributing to aging and vascular osteoinduction [42]. Conversely, interference with TGFß1 signaling counteracts vascular calcification [43].

In view of previous [28] and present observations, alkalinization of acidic cellular compartments may be a novel therapeutic option in the prevention of vascular calcification. The treatment may be particularly important in patients with chronic kidney disease (CKD), which suffer from hyperphosphatemia and mineral bone disorder (MBD) [15, 44]. In CKD patients stimulation of osteo-/chondrogenic reprogramming [45] leads to vascular calcification, which in turn triggers cardiovascular events [46], the leading cause of death in those patients [47]. Osteo-/chondrogenic reprogramming and vascular calcification may affect further clinical conditions. NFAT5 is up-regulated by hyperglycaemia [48] and NFAT5-dependent osteo-/chondrogenic transformation of VSMCs may thus lead to vascular calcification in diabetes [49]. NFAT5 is further up-regulated and thus osteo-/chondrogenic reprogramming presumably fostered by dehydration [50], inflammation [50], hypoxia [51] and ischemia [51].

Conclusions

The present observations reveal that bafilomycin A1 and methylamine disrupt osteo-/chondrogenic transformation and calcification of VSMCs and thus lend further support for the hypothesis that the pH of acidic cellular compartments plays a decisive role in osteoinductive signaling. The present observations further show that bafilomycin A1 does not reverse the activation of the osteoinductive signaling by TGFß1.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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