Zoledronate inhibits phosphate and bone morphogenetic protein 2-induced extracellular calcification of vascular smooth muscle cells *in vitro*

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Abstract. The aim of this study was to explore the effects of the bisphosphonate zoledronate on calcification induced by inorganic phosphate (Pi) and/or bone morphogenetic protein 2 (BMP-2) and the underlying mechanisms. Primary vascular smooth muscle cells (VSMCs) from rats were treated with 3 mM Pi or 3 mM Pi/BMP-2, with and without addition of zoledronate; 1.4 mM Pi served as a control. Calcium deposits, expression of core binding factor α-1 (Cbfa-1), osteopontin (OPN), parathyroid pituitary-specific transcription factor (Pit)-1 and Pit-2, and Pi uptake of VSMCs was determined. The calcification of VSMCs induced by elevated Pi or Pi/BMP-2 was significantly inhibited by zoledronate. The expression of Cbfa-1, OPN and Pit-1 was increased significantly after treatment with an elevated level of Pi or Pi/BMP-2, and this expression was significantly suppressed by addition of zoledronate. Pi uptake of VSMCs increased following treatment with elevated Pi and significantly decreased by addition of zoledronate. These results indicated that zoledronate effectively inhibited calcification induced by Pi/BMP-2, and this may have been achieved by means of the downregulation of expression of calcification-related proteins and uptake of Pi.

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Abbreviations: ANOVA, analysis of variance; BPs, bisphosphonates; BMP-2, bone morphogenetic protein 2; CVD, cardiovascular disease; CKD, chronic kidney disease; Cbfa-1, core binding factor α-1; DMEM, Dulbecco's minimum essential medium; EBSS, Earle's buffered salt solution; OPN, osteopontin; Pit, parathyroid pituitary-specific transcription factor; Pi, phosphate; SMCs, smooth muscle cells; VSMCs, vascular smooth muscle cells

Key words: vascular calcification, zoledronate, bone morphogenetic protein 2, osteopontin, core binding factor α-1, phosphate uptake

Introduction

Vascular calcification is highly prevalent and is a major contributor to cardiovascular disease (CVD) in patients with chronic kidney disease (CKD). Susceptibility to vascular calcification is in part genetically determined and actively regulated by diverse inducers and inhibitors. One of these inducers, hyperphosphatemia, promotes vascular calcification, and the control of arterial calcification is now recognized as a means to prevent CVD events in patients with CKD (1,2).

Vascular calcification is an active, cell-mediated process that results from an imbalance between the promoters and inhibitors of mineralization (3,4). Several molecules that normally regulate osteoblast differentiation and bone formation have been found in calcifying vessels, such as osteonectin, osteocalcin, matrix Gla protein and bone morphogenetic protein 2 (BMP-2) (5-7). Elevated phosphate (Pi) levels also induce smooth muscle cell (SMC) calcification and osteogenic phenotypic modulation (8,9).

Bisphosphonates (BPs) are widely used in the treatment of diseases associated with excessive osteoclast-mediated bone resorption, such as osteoporosis (10,11). The classical pharmacological effects of BPs appear to result from two key properties: their affinity for bone mineral and their inhibitory effects on osteoclasts. Mineral binding affinities differ among the clinically used BPs, and this may influence their differential distribution within bone, their biological potency and their duration of action (12,13). It is reported that ibandronate prevents experimentally induced arterial calcification in uremic rats (14). These findings extend the link between bone remodeling and vascular calcification of CKD, opening perspectives toward novel therapeutic strategies. However, whether zoledronate, a new third generation bisphosphonate, may serve as an inhibitor of calcification and by what mechanism it may function is not known. Thus, we designed and completed the present *in vitro* study.

Materials and methods

Cell culture and identification. Rat vascular smooth muscle cells (VSMCs) were grown in Dulbecco's minimum essential

medium (DMEM; Gibco, Carlsbad, CA, USA). The type and purity of VSMCs were further confirmed using an α-smooth muscle actin antibody (Sigma-Aldrich, St. Louis, MO, USA), which indicated >95% positive staining for these cells. VSMCs (4th to 8th passages) were made quiescent by serum starvation in 0.4% FBS for 24 h for use in all of the experiments in this study.

Cellular calcification assay. Calcification of VSMCs was induced by 3 mM Pi (but not by DMEM alone), 1.4 Pi or BMP-2 in our pilot study. Therefore, VSMC calcification was induced by incubation with calcifying medium (growth medium supplemented with NaH_2PO_4/Na_2HPO_4 to 3 mM Pi), and 1.4 mM Pi served as the control. Human recombinant BMP-2 (R&D Systems, Minneapolis, MN, USA) and/or zoledronate (Novartis Pharmacy AG, Basel, Switzerland) were added every 2 days during the treatment period. Calcium deposited in the extracellular matrix was extracted with 0.6 N HCl for 24 h. The calcium content of the HCl supernatants was determined using the o-cresolphthalein complex one method (Calcium Assay kit; Bioassays, Hayward, CA, USA) and normalized relative to the protein concentration of the same culture well.

Western blot analysis. Protein expression of core binding factor α-1 (Cbfa-1) and osteopontin (OPN) in VSMCs was determined by western blotting. The specific signal was detected using an enhanced chemiluminescence system (Cell Signaling Technology, Beverly, MA, USA).

Real-time PCR. Levels of rat Pit-1 and Pit-2 mRNAs were determined by quantitative real-time PCR performed using a SYBR GreenER two-step qRT-PCR kit (Invitrogen, Carlsbad, CA, USA) and an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The comparative CT method was used for quantification, as recommended by the manufacturer, using GAPDH as the endogenous reference. The primers used for PCR amplification were: i) Rat Pit-1 forward primer: 5'-CCGTCAGCAACCAGATCAACTC-3' and reverse primer: 5'-CCCATGCAGTCTCCCACCTTG-3', generating an amplified fragment of 121 bp (NM_031148); ii) Rat Pit-2 forward primer: 5'-CTATTCCAAGAAGAGGCTCCG-3' and reverse primer: 5'-TCAGGATCGGTCAGCTCAG-3', generating an amplified fragment of 126 bp (NM_017223); iii) Rat GAPDH forward primer: 5'-ATGACTCTACCCACG GCAAG-3' and reverse primer: 5'-TACTCAGCACCAGC ATCACC-3', generating an amplified fragment of 136 bp (NM_017008).

Pi uptake assay. VSMCs were seeded into 24-well plates at 10^5 cells/well. Transport was initiated by addition of 0.3 ml of the above-mentioned medium containing the labeled substrate $H_3{}^{32}PO_4$ to confluent VSMCs. The uptake was stopped by washing the cell monolayers three times with 1 ml of ice-cold Earle's buffered salt solution (EBSS). The cells were solubilized with 0.5 ml of 0.1 N NaOH/0.1% SDS, and the radioactivity of 100-μl aliquots was counted by standard liquid scintillation techniques (Packard 2500 TR/AB; Packard Instruments, Meriden, CT, USA). Sodium-dependent Pi uptake was determined by subtracting the uptake in the presence of EBSS containing chorine from the uptake in the presence of EBSS containing sodium. Uptake values were normalized based on the protein content of the cell culture.

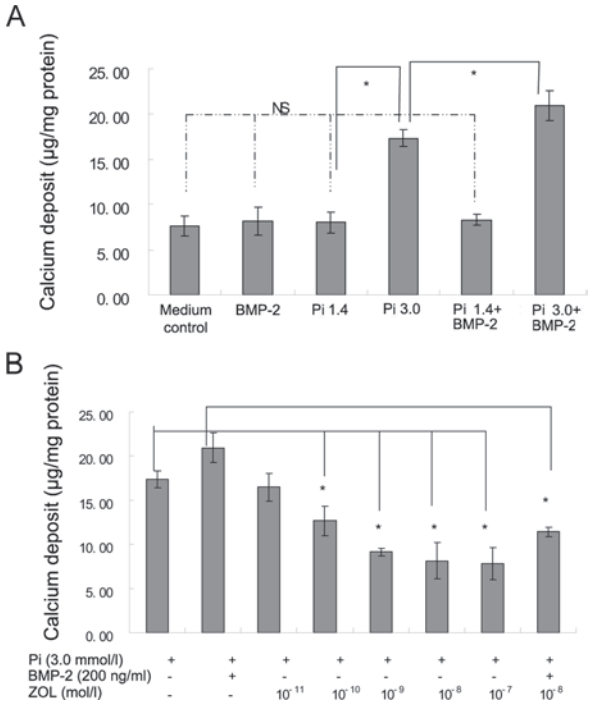

Figure 1. (A) Calcium deposition of VSMCs induced by elevated Pi and Pi/BMP-2. (B) Calcium deposition of VSMCs induced by elevated Pi and Pi/BMP-2 was inhibited by zoledronate (ZOL) in a dose-dependent manner.

Statistical analysis. Data analyses were conducted using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Differences among groups were determined by analysis of variance (ANOVA), and the Tukey's test method was used for post-hoc testing. $p<0.05$ denoted statistical significance.

Results

Zoledronate inhibits Pi- and Pi/BMP-2-induced VSMC calcification. Elevated Pi (3 mM Pi) significantly induced calcification of VSMCs in comparison to 1.4 mM Pi ($p<0.05$). The calcium deposition became more severe after treatment with both BMP-2 and 3 mM Pi compared to 3 mM Pi alone ($p<0.05$; Fig. 1A). Zoledronate significantly inhibited the calcium deposition of VSMCs treated with 3 mM Pi in a dose-dependent manner (Fig. 1B). Zoledronate (10^{-8} mM) also significantly decreased the calcium deposition of VSMCs induced by the addition of both 3 mM Pi and 200 ng/ml BMP-2 (Fig. 1B).

Zoledronate inhibits the expression of Cbfa-1 and OPN upregulated by BMP-2 and elevated Pi. Results demonstrated that 3 mM Pi significantly upregulated the expression of Cbfa-1 and OPN compared to that of the control (1.4 Pi; Fig. 2A and B). Moreover, the expression of Cbfa-1 and OPN was further increased after treatment with both BMP-2 and 3 mM Pi compared to 3 mM Pi alone (Fig. 2C and D). Zoledronate significantly suppressed the expression of Cbfa-1 and OPN upregulated by either 3 mM Pi (Fig. 2A and B) or both 3 mM Pi and BMP-2 (Fig. 2C and D).

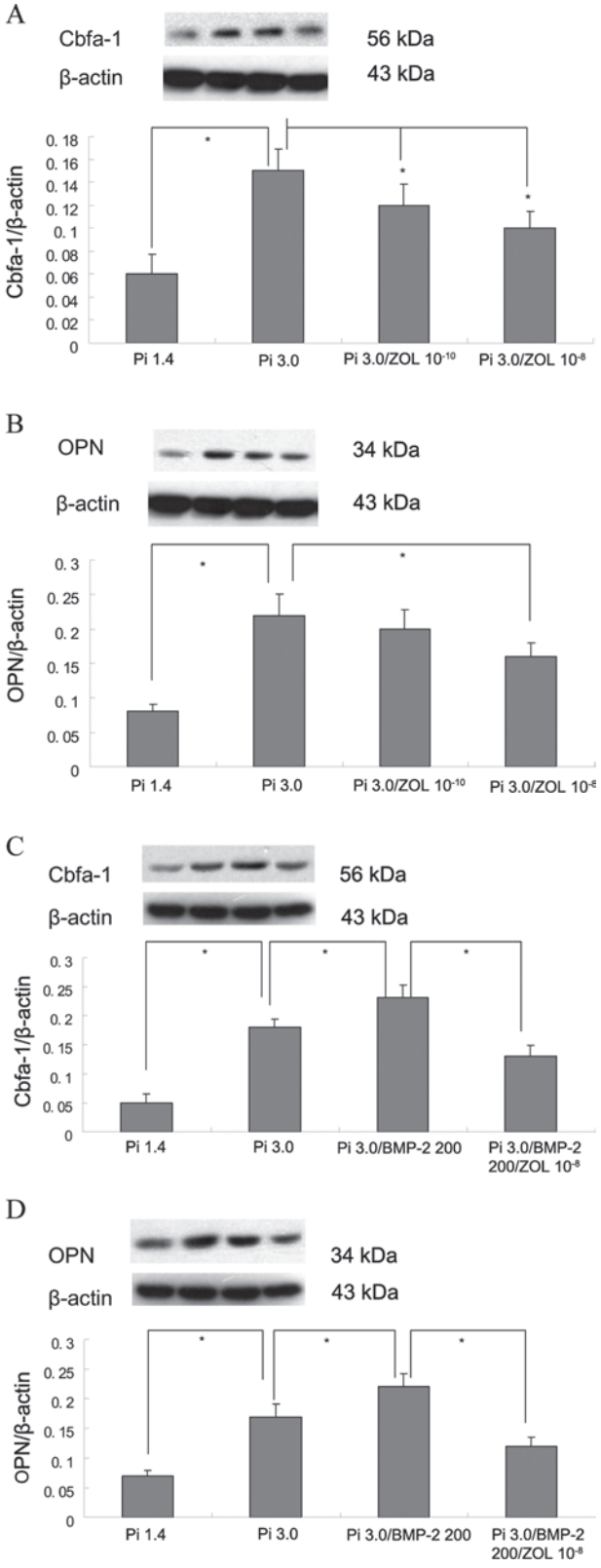

Figure 2. (A) Expression of Cbfa-1 in VSMCs treated with elevated Pi was inhibited by zoledronate (ZOL). (B) Expression of OPN in VSMCs treated with elevated Pi was inhibited by zoledronate. (C) Expression of Cbfa-1 in VSMCs treated with Pi/BMP-2 was inhibited by zoledronate. (D) Expression of OPN in VSMCs treated with Pi/BMP-2 was inhibited by zoledronate.

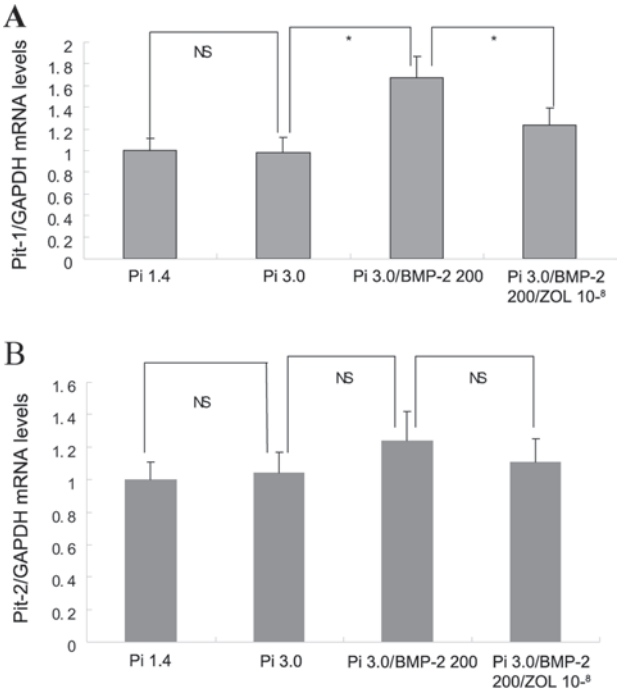

Figure 3. (A) Overexpression of Pit-1 mRNA induced by Pi/BMP-2 was suppressed by zoledronate (ZOL). (B) However, expression of Pit-2 mRNA was not altered by the addition of Pi, Pi/BMP-2 or zoledronate.

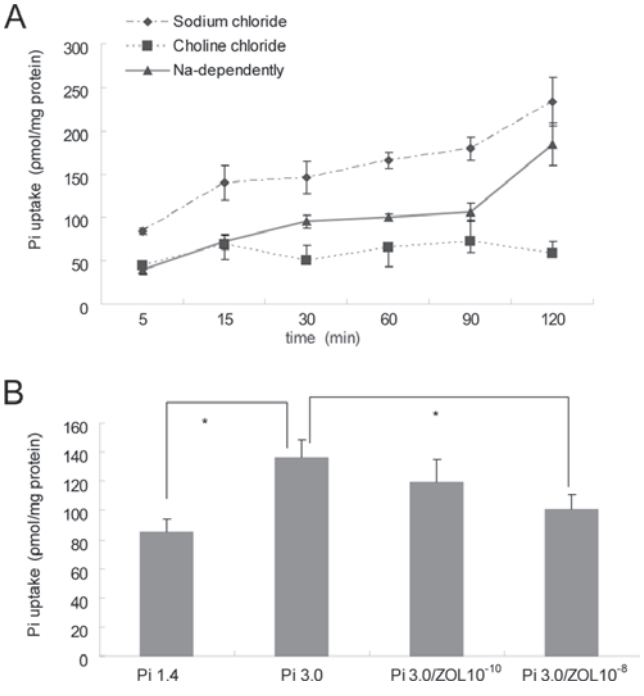

Figure 4. (A) Pi uptake of VSMCs was increased in the presence of elevated Pi in a time-dependent manner. (B) This Pi uptake was inhibited by zoledronate (ZOL).

Zoledronate suppresses the expression of Pit-1 mRNA, but not the expression of Pit-2. The expression of Pit-1 mRNA in VSMCs increased significantly after treatment with both BMP-2 and 3 mM Pi compared to 3 mM Pi alone ($p<0.05$), and this overexpression of the Pit-1 mRNA was inhibited by the addition of zoledronate ($p<0.05$; Fig. 3A). However, the mRNA expression of Pit-2 was not significantly different among the elevated Pi, Pi/BMP-2 and zoledronate groups (Fig. 3B).

Zoledronate inhibits Pi uptake of VSMCs. The uptake of Pi by rat VSMCs increased gradually after treatment with elevated Pi in a time-dependent manner (5-120 min; Fig. 4A). The Pi uptake by VSMCs was significantly increased in the 3 mM Pi group compared to that of the 1.4 mM Pi group, and this was significantly inhibited by the addition of zoledronate ($p<0.05$; Fig. 4B).

Discussion

In the present study, we demonstrated that an elevated level of Pi induced calcification of rat VSMCs; furthermore, we confirmed that this calcification was enhanced by the addition of BMP-2. This is the first report showing that BMP-2 is involved in the process of calcification induced by elevated Pi levels. BMPs are part of the TGF-β superfamily, and BMP-2 is associated with calcific arteriopathy (8). Expression of BMP-2 is also found in calcified human atherosclerotic lesions (7,8). In addition, treatment of calcifying vascular or SMCs *in vitro* with BMP-2 results in enhanced calcification (15,16). Thus, BMP-2 may play an important role in the regulation of bone formation as well as vascular calcification under conditions of high Pi.

Our results demonstrated that expression of Cbfa-1 and OPN in VSMCs was upregulated after stimulation with elevated Pi and BMP-2, and this was consistent with the observed calcification, as the expression of Cbfa-1 and OPN in SMCs usually serve as markers of osteochondrogenic phenotype transition (16,17). Thus, elevated Pi and BMP-2 may induce SMCs to transition to an osteoblast-like phenotype, and this may contribute to cell calcification. On the other hand, our data showed that addition of BMP-2 upregulated Pit-1 expression under conditions of elevated Pi, indicating that BMP-2 may promote vascular calcification via increased Pi uptake. As such, zoledronate likely inhibited calcification by means of either inhibition of Pit-1 expression and/or decreased Pi uptake. However, neither zoledronate nor inorganic Pi influenced expression of the Pit-2 mRNA in our experiments. VSMCs appear to respond to elevated Pi levels by undergoing an osteochondrogenic phenotype change and by mineralizing their extracellular matrix through a mechanism requiring sodium-dependent Pi cotransporters (18,19).

It is interesting that zoledronate was found to effectively inhibit the calcification of VSMCs induced by elevated Pi and BPM-2. Zoledronate also inhibited expression of Cbfa-1 and OPN induced by elevated Pi and BMP-2, and this was consistent with its suppression of calcification. Cbfa-1 and OPN have previously been described as markers of VSMC transition to osteoblast-like cells. BPs have been widely used in the treatment of excessive bone resorption, hypercalcemia and osteoporosis (12,13). Etidronate has been reported to decrease the intima-media thickening of carotid arteries (20). Therefore, the above-mentioned data indicate that zoledronate suppressed calcification induced by elevated Pi and BMP-2, and the mechanism was likely due to inhibition of Cbfa-1 and OPN expression in VSMCs. At the same time, zoledronate also inhibited cell calcification, and this was probably via the suppression of Pit-1 upregulation and subsequent decreased Pi transport into cells. However, further studies are required to confirm these findings.

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