Intermedin1-47 inhibits high phosphate-induced vascular smooth muscle cell calcification by regulating Wnt/β-catenin signaling

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Abstract. Vascular calcification is a major risk factor for cardiovascular disease and accounts for a large proportion of deaths from cardiovascular disease in patients with chronic kidney disease. The high incidence, rapid progression and irreversibility of vascular smooth muscle cell (VSMC) calcification in patients has attracted attention. In the present study, the effect of intermedin1-47 (IMD1-47), an important isoform of intermedin, was investigated on the calcification of rat cardiovascular VSMCs induced by high phosphate (HP). To stimulate osteoblast-like differentiation and calcification in rat VSMCs, 10 mM β-sodium glycerophosphate was used. The VSMCs were then treated with three doses of IMD1-47 and the effects of IMD1-47 on VSMC calcification, on the expression of osteogenic markers [osteoprotegerin, Runt-related transcription factor 2 (Runx2) and osteopontin (OPN)] and on alkaline phosphatase (ALP) activity were assessed. HP treatment significantly enhanced the cellular calcium content of VSMCs, the expression of osteogenic markers, and ALP activity, while IMD1-47 significantly reversed these effects in a dose-dependent manner. The protein expression levels of Wnt1, Wnt3a and active β-catenin were determined and it was found that IMD1-47 significantly inhibited their expression. Following β-catenin silencing, the protein expression levels Runx2 and OPN were increased compared with the IMD1-47 treatment alone, indicating a role for the Wnt/β-catenin pathway in the effects of IMD1-47 on osteogenic markers. The present study suggested that IMD1-47 inhibited HP-induced VSMC calcification by regulating the Wnt/β-catenin signaling pathway.

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Introduction

Vascular calcification refers to the abnormal ectopic calcification resulting from the deposition of calcium or phosphates in soft tissues (1). Vascular calcification has been identified as a major risk factor for cardiovascular disease (2). Cardiovascular disease is responsible for >50% of the total number of mortalities of patients with chronic kidney disease (CKD) (3). Therefore, the high incidence, rapid progression and irreversibility of vascular smooth muscle cell (VSMC) calcification has attracted attention.

The mechanism of vascular calcification is similar to that of bone and cartilage ossification (4). Possible mechanisms leading to vascular calcification in patients with CKD may involve VSMC injury, VSMC phenotypic transition from the original myoblast to an osteoblast/chondrocyte-like phenotype and the inhibition of vascular calcification accompanied by an increase of pro-calcification factors (5). The central event of vascular calcification is the phenotypic transition of VSMCs, from the original myoblast type to the osteogenesis/chondrocyte-like phenotype, followed by ectopic osteogenesis (6). Phosphate has an important role in this process by increasing the expression of Runt-related transcription factor 2 (Runx2) and other proteins, such as alkaline phosphatase (ALP) and bone morphogenic proteins (7-9).

Intermedin (IMD), also known as adrenomedullon 2, belongs to the calcitonin gene-related peptide family. It is a cardiovascular polypeptide and brain-gut peptide composed of 47 amino acids (10,11). IMD mRNA is expressed in various tissues, including the kidney, pituitary gland, hypothalamus, and stomach (12). In total, three isoforms have been identified (IMD1-47, IMD8-47 and IMD1-53) that are cleaved from the prepropeptide and have different biological activities (10,13). For example, IMD1-53 attenuates VSMC calcification (14,15), however, the mechanism has not been elucidated. In addition, the administration of IMD1-47 leads to vasodilation and marked hypotension through calcitonin-related receptor complexes, and increases coronary blood flow and cardiac function by releasing NO (16). However, the effects of IMD1-47 on the calcification of rat cardiovascular VSMCs, to the best of our knowledge, has not been studied previously. Therefore, in the present study, the effect of IMD1-47, an important isoform of IMD, was investigated on the calcification of rat

cardiovascular VSMCs induced by exposure to high phosphate (HP). Furthermore, the mechanism of action was examined by investigating the involvement of the Wnt/β-catenin pathway.

Materials and methods

VSMC culture and HP treatment. Cardiovascular VSMCs were isolated from the thoracic aorta of Sprague Dawley rats (7-8 weeks old, 220-240 g) using a previously described method (17,18). A total of 6 male rats were used. All of the rats were housed in the animal center of Shanghai Fourth Rehabilitation Hospital at 23-25˚C, relative humidity (50-70%) conditions and 12 h light/12 h dark cycle, and allowed to free access to food and water. The thoracic aorta was dissected into roughly 5 mm^2 sections, and digested with collagenase (3 mg/ml) and elastase (1 mg/ml) for 4 h at 37˚C in DMEM (Gibco; Thermo Fisher Scientific, Inc.). Staining of α-smooth muscle actin was used to confirm the purity of the VSMCs (data not shown), as described by Wang *et al* (19). The VSMCs were then cultured in DMEM containing 10% FBS (Beyotime Institute of Biotechnology) and 1% penicillin/streptomycin. All cells were cultured for 5-7 passages before use in experiments. When cells reached a confluency of 80-90%, the cells were treated with fresh DMEM medium with or without 10 mmol/l β-sodium glycerophosphate (Cayman Chemical Company) for 8 days. Alizarin red staining was used to confirm the efficiency of HP to induce VSMC calcification (data not shown). The optical density value of the alizarin red exacted from cells was measured with a spectrophotometery at 570 nm to determine the calcification. The detailed method was described by Zhu *et al* (20).

Experimental design. To investigate the effects of IMD1-47 on HP-induced calcification in VSMCs, VSMCs were randomly assigned into six groups: Control, HP alone (hereafter HP group); HP with IMD1-47 treatment (0.1, 0.5 or 1 nM IMD1-47; hereafter HP+IMD1-47 groups); and IMD1-47 treatment alone (hereafter IMD1-47 group). Similar concentrations of IMD was used as reported in a previous study (14). IMD1-47 was purchased from Phoenix Europe GmbH. Cells in the control group were incubated with complete DMEM/10% FBS media, while cells in the HP group were incubated with DMEM supplemented with 10 mmol/l β-sodium glycerophosphate and 10% FBS for 8 days. The three HP+IMD1-47 groups were incubated with 0.1 (low dose), 0.5 (medium dose) or 1 (high dose) nM IMD1-47 in HP medium for 8 days. The IMD1-47 group was incubated with 0.5 nM IMD1-47 in DMEM with 10% FBS.

To confirm the efficiency of the β-catenin small interfering (si)RNA, the cells were divided into three groups: Control, scrambled siRNA and β-catenin siRNA. The expression of active β-catenin was determined using western blotting. The β-catenin siRNA sequence was 5'-CAGGGGGUUGUGGUU AAGCUCUU-3' and the scramble siRNA sequence was 5'-TTC TCCGAACGTGTCACGT-3'. Then, to investigate the effect of β-catenin silencing on VSMC calcification, the cells were divided into five groups: Control, HP, IMD1-47, scrambled siRNA and β-catenin siRNA. For siRNA transfection, 60 pmol siRNA was transfected into VSMCs with Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). 6 h later, the culture medium was replaced with fresh DMEM with 10% FBS. After 48 h, the VSMCs in the control group received no treatment, and the VSMCs in the other groups received HP treatment for 8 days; the VSMCs in the IMD1-47, scrambled siRNA and β-catenin siRNA groups were also treated with 0.5 nM IMD1-47 for 8 days.

Determination of cellular calcium content using fluorescence. VSMCs ($2.5x10^4$ cells/cm^2) were cultured on coverslips and received HP or IMD1-47 treatments for 8 days. At the time of examination, after 8 days of treatment, the VSMCs were placed in PBS with 10 μmol/l Fluo-3 AM (Sigma-Aldrich; Merck KGaA) in a dark room at 37˚C for 40 min. The stained cells were then visualized using laser scanning confocal microscopy (magnification, x200) and the cellular calcium content was quantitatively analyzed using Leica LAS AF Lite software v2.6.3 (Leica Microsystems GmbH). The change in the relative fluorescence intensity of Fluo-3 indicated the change in cellular calcium content.

Detection of cellular calcium content using colorimetry. To detect cellular calcium content using a colorimetric method, VSMCs (in 6-well plates) were washed twice with PBS and then incubated with 0.6 M HCl at 37˚C for 24 h for decalcification. The supernatant was discarded and the cells were washed with PBS three times. The cells were then incubated with 0.1 M NaOH and 0.1% SDS for 30 min to lyse the cells. A Calcium Quantitative Detection kit (Roche Diagnostics) was used to determine the cellular calcium content according to the manufacturer's protocol and the bicinchoninic acid method was used to determine the cellular protein concentration for normalization. The final calcium content was calculated as μg/mg protein.

Measurement of IMD levels in the cell culture medium. To examine the relationships between HP and IMD, the levels of IMD in the cell culture medium was measured after cells were treated with HP using the radioimmunoassay method. Briefly, after cells were treated with normal culture media or the cell culture media supplemented with 10 mmol/l β-sodium glycerophosphate for 8 days, the cell culture media was collected and centrifuged at 1,600 x g for 15 min at 4˚C. The supernatant was loaded onto a Sep-Pak C18 cartridge (Waters Corporation) equilibrated with 0.5 mmol/l acetic acid. After elution with 50% CH_3CN containing 0.1% trifluoroacetic acid, the sample was lyophilized and the residue was dissolved in radioimmunoassay buffer, and analyzed using an IMD radioimmunoassay kit (Phoenix Pharmaceuticals, Inc.), according to the manufacturer protocol.

Reverse transcription-quantitative (RT-q) PCR assay for osteoprotegerin (OPG), Runx2, osteopontin (OPN) and β-catenin. The mRNA levels of OPG, Runx2, OPN and β-catenin were determined by RT-qPCR. After cells were treated with HP or IMD1-47 as aforementioned, total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Inc.). In total, 2 μg of total RNA was reverse transcribed using the Verso™ cDNA kit (ABgene; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. qPCR was performed using SYBR Premix Ex Taq (Takara

Table I. Sequences of primers used for reverse transcription-quantitative PCR.

Gene	Sense primer (5'-3')	Antisense primer (5'-3')
OPG	ATCATTGAATGGACAACCCAGG	TGCGTGGCTTCTCTGTTTCC
Runx2	CCGTCCATCCACTCTACCAC	ATGAAATGCTTGGGAACTGC
OPN	GTTGGTGGAGGATGTCTG	TACTTGGAAGGGTCTGTG
β-catenin	GCCAAGTGGGTGGTATAGAG	CTGGGTATCCTGATGTGC
GAPDH	AACGGATTTGGTCGTATTG	GGAAGATGGTGATGGGATT

Runx2, Runt-related transcription factor 2; OPG, osteoprotegerin; OPN, osteopontin.

Biotechnology Co., Ltd.) in a Thermal Cycler Dice (Takara Biotechnology Co., Ltd.), following the manufacturer's instructions. The qPCR reactions were conducted using the following reaction conditions: 95˚C for 15 sec, followed by 35 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The primer sequences are listed in Table I. Each experiment was repeated three times. Relative mRNA expression levels of OPG, Runx2, OPN and β-catenin were normalized to GAPDH as an internal control and calculated using the $2^{-\Delta\Delta Cq}$ method (21).

Determination of ALP activity. To determine the activity of ALP, after VSMCs were treated with HP or IMD1-47 for 8 days (in 6-well plates), they were rinsed with PBS three times, and then 500 μl of 0.1% Triton X-100 was added to each well and incubated at 4˚C overnight. The cells were then disrupted by repeated shaking and centrifuged at 18,000 x g for 3 min at 4˚C to collect the supernatant. Finally, the ALP activity in the supernatant was measured following a chemiluminescence method, which was described previously (22).

Western blotting. First of all, the proteins were extracted from VSMCs using lysis buffer (Beyotime Institue of Biotechnology) containing 50 mM HEPES, 150 mM NaCl, 10% (vol/vol) glycerol, 1 mM EDTA (pH 8.0), 1% (vol/vol) NP-40, 1 mM DTT, 1 mM PMSF, and protease and phosphatase inhibitors (Beyotime Institue of Biotechnology, pH 7.4). The protein concentration was determined using a BCA kit (Beyotime Institue of Biotechnology). To measure protein expression by western blotting, 25 μg of total cellular protein was separated using 10% polyacrylamide gels. The proteins were then transferred to polyvinylidene fluoride membranes and blocked in TBS/0.1% Tween-20 (TBST) containing 5% milk at room temperature for 1 h. Membranes were then rinsed with TBST and shaken for 5-10 min. The membranes were incubated with the following primary antibodies overnight at 4˚C: OPG (0.2 μg/ml; cat. no. O1139; Sigma-Aldrich; Merck KGaA), Runx2 (5 μg/ml; cat. no. SAB1412247; Sigma-Aldrich; Merck KGaA), OPN (1:1,000; cat. no. SAB1306579; Sigma-Aldrich; Merck KGaA), β-actin (1 μg/ml; cat. no. A1978; Sigma-Aldrich; Merck KGaA), Wnt1 (1:1,000; cat. no. SAB2102711; Sigma-Aldrich; Merck KGaA), Wnt3a (1 μg/ml; cat. no. SAB1400757; Sigma-Aldrich; Merck KGaA) and active-β-catenin (1:1,000; cat. no. 05-665; Sigma-Aldrich; Merck KGaA). The following day, the membranes were rinsed with TBST three times and incubated with goat anti-rat IgG antibody, HRP conjugate (cat. no. AP136P, 1:5,000, Sigma-Aldrich; Merck KGaA) for 2 h at 25˚C. The membranes were rinsed three times in TBST. Protein bands were visualized using an Electro-Chemi-Luminescence Substrate kit (Rahn AG) and quantified using the Quantity One software 4.6.2 (Bio-Rad Laboratories, Inc.).

Statistical analysis. SPSS 13.0 statistical software for Windows (SPSS, Inc.) was used to analyze all the data using one-way ANOVA followed by the Student-Newman-Keuls post hoc test. Data are expressed as the mean ± SEM. $P<0.05$ was considered to indicate a statistically significant difference. Experiments were repeated three times.

Results

Effects of HP and IMD1-47 on VSMC calcification. The cellular calcium content in VSMCs was determined using fluorescence (Fig. 1A) and colorimetric (Fig. 1B) methods. HP treatment significantly increased the cellular calcium content of VSMCs compared with untreated control group ($P<0.05$). Treatment with the low dose of IMD1-47 did not significantly change the cellular calcium content, however, exposure to the medium and high IMD1-47 doses significantly decreased the cellular calcium content of VSMCs compared with the HP alone group ($P<0.05$). Treatment with the medium IMD1-47 dose alone did not significantly change the cellular calcium content of the VSMCs, compared with the untreated control group. Of note, treatment of VSMCs with HP alone did not affect the endogenous levels of IMD in the cell culture medium compared with the control group ($P>0.05$).

Effects of HP and IMD1-47 on osteogenic proteins and mRNAs in VSMCs. The expression levels of osteogenic proteins OPG, Runx2 and OPN were determined using western blotting. Representative western blotting results are shown in Fig. 2A and quantification of the fold increases in OPG, Runx2 and OPN protein expression levels compared with the control group are shown in Fig. 2B-D. The relative mRNA expression levels of OPG, Runx2 and OPN are shown in Fig. 2E. HP significantly enhanced the protein and mRNA expression levels of OPG, Runx2 and OPN compared with the control ($P<0.05$). Although the low IMD1-47 dose had no significant effect on the expression of either of these factors, the medium and high doses of IMD1-47 significantly decreased the protein and mRNA expression levels of OPG, Runx2 and OPN compared with the HP alone group ($P<0.05$). Treatment with

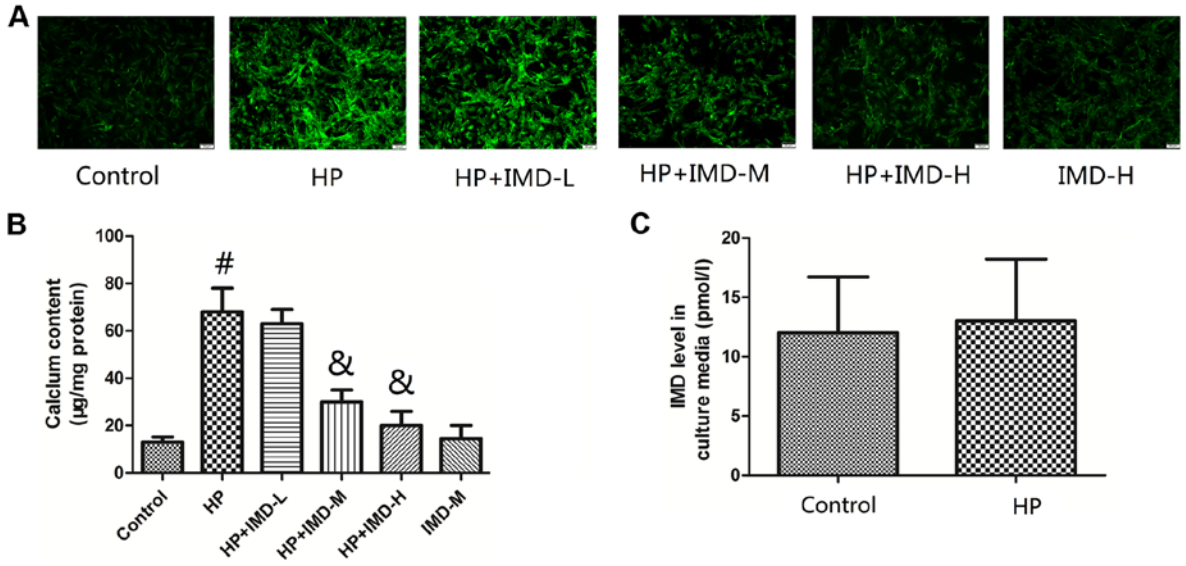

Figure 1. Effects of HP and IMD exposure on VSMC calcification. The cellular calcium content in VSMCs was detected using (A) fluorescence (green signal represents the cellular calcium aggregates) and (B) colorimetric methods. Scale bar, 50 μm. (C) The levels of IMD in the cell culture media was determined using a radioimmunoassay method after VSMCs were treated with HP. Data are expressed as the mean ± SEM. n=8. $^{\#}$P<0.05 vs. control; $^{\&}$P<0.05 vs. HP. HP, high phosphate; IMD, intermedin1-47; VSMCs, vascular smooth muscle cells; L, low dose; M, medium dose; H, high dose.

the medium IMD1-47 dose alone (without HP) did not significantly change the protein or mRNA expression levels of OPG, Runx2 or OPN compared with the control (P>0.05).

Effects of HP and IMD1-47 on ALP activity in VSMCs. The effects of HP and IMD1-47 on ALP activity are shown in Fig. 3. HP treatment significantly increased ALP activity compared with the control (P<0.05). The low dose of IMD1-47 had no significant effect on ALP activity, however, the medium and high IMD1-47 doses significantly decreased ALP activity compared with the HP alone group (P<0.05). Treatment with the medium IMD1-47 dose (without HP) did not significantly change the ALP activity compared with the control (P>0.05).

Effects of HP and IMD1-47 on the Wnt/β-catenin pathway. Wnt1, Wnt3a and active β-catenin protein expression levels were determined by western blotting (Fig. 4), with the aim to investigate the mechanism driving the effects of HP and IMD1-47. HP treatment significantly increased the protein levels of Wnt1 and Wnt3a (P<0.05; Fig. 4B and C). The low dosage of IMD1-47 significantly decreased Wnt1 and Wnt3a expression compared with the HP alone group. Furthermore, HP treatment significantly enhanced the protein expression levels of active β-catenin compared with the control (P<0.05; Fig. 4D). All IMD1-47 doses tested significantly decreased the protein expression levels of active β-catenin compared with the HP alone group. Treatment with the medium IMD1-47 dose alone (without HP) did not significantly change the protein levels of Wnt1, Wnt3a or active β-catenin compared with the control group (P>0.05).

β-catenin silencing abolishes the effects of IMD1-47 on Runx2 and OPN in VSMCs. The efficiency of the β-catenin siRNA was confirmed by western blotting. Transfection of the cells with scrambled siRNA did not significantly alter the protein expression levels of β-catenin; however, transfection with the β-catenin-specific siRNA significantly reduced the active β-catenin protein expression levels (Fig. 5A). Next, the protein expression levels of the osteogenic proteins Runx2 and OPN were determined in VSMCs in the control, vehicle, IMD1-47, scrambled siRNA and β-catenin siRNA treatment groups (Fig. 5B and C). Except for the control group, VSMCs in all groups received HP treatment, and VSMCs in the IMD1-47, scrambled siRNA and β-catenin siRNA groups also received the medium IMD1-47 dose. The results demonstrated that the scrambled siRNA did not significantly change the levels of Runx2 and OPN compared with IMD1-47 treatment, however, β-catenin siRNA significantly increased the levels of Runx2 and OPN expression compared with the IMD1-47 treatment group (P<0.05; Fig. 5B and C).

β-catenin silencing abolishes the effect of IMD1-47 on ALP activity in VSMCs. ALP activity was measured in VSMCs in the control, vehicle, IMD1-47, scrambled siRNA and β-catenin siRNA treatment groups. Transfection with scrambled siRNA had no significant effect on ALP activity, however, β-catenin siRNA significantly increased the ALP activity compared with the IMD1-47 treatment group (P<0.05; Fig. 6).

Discussion

VSMCs are the major cellular components of the vascular membrane. As the osteogenic-like phenotypic transition of VSMCs is the structural basis of vascular calcification and a central event in cardiovascular calcification, VSMCs have been intensely studied in recent years (18,23). In the present study, 10 mM β-sodium glycerophosphate was used to stimulate osteoblast-like differentiation and calcification in rat VSMCs. These VSMCs were then treated with three different doses of IMD1-47 and the effects of HP and IMD1-47 on VSMC calcification, the expression of osteogenic markers (OPG, Runx2 and OPN) and ALP activity were investigated. HP

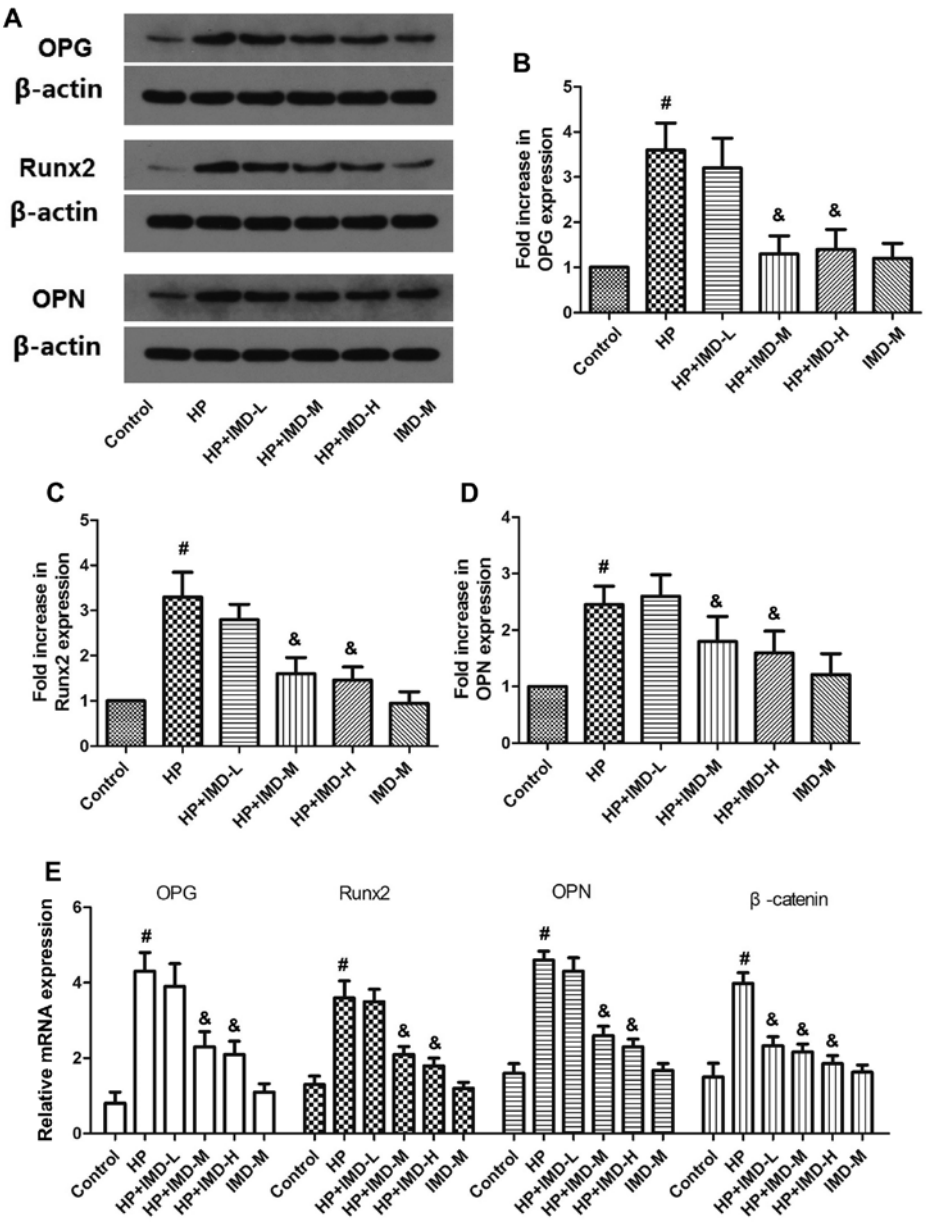

Figure 2. Effects of HP and IMD on osteogenic protein and mRNA expression in vascular smooth muscle cells. (A) Representative images from western blot analysis for the protein expression levels of osteogenic proteins. (B) Quantification of western blot results for OPG, (C) Runx2 and (D) OPN. (E) Relative mRNA expression levels of OPG, Runx2 and OPN were detected by reverse transcription-quantitative PCR. Data are expressed as the mean ± SEM. n=8. #P<0.05 vs. control; &P<0.05 vs. HP. HP, high phosphate; IMD, intermedin1-47; L, low dose; M, medium c dose; H, high dose; Runx2, Runt-related transcription factor 2; OPG, osteoprotegerin; OPN, osteopontin.

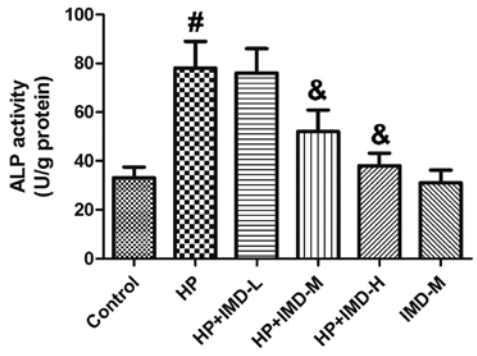

Figure 3. Effects of HP and IMD on ALP activity in vascular smooth muscle cells. Data are expressed as the mean ± SEM. n=8. #P<0.05 vs. control; &P<0.05 vs. HP. HP, high phosphate; IMD, intermedin1-47; ALP, alkaline phosphatase; L, low dose; M, medium dose; H, high dose.

treatment significantly enhanced the cellular calcium content of VSMCs, the expression of osteogenic markers and ALP activity, while IMD1-47 significantly reversed these effects in a dose-dependent manner. Treatment with IMD1-47 alone did not affect the cellular calcium content of VSMCs, the expression of osteogenic markers or ALP activity compared with the control untreated cells. To characterize the underlying mechanism, the protein expression levels of Wnt1, Wnt3a and active β-catenin were measured, and the results demonstrated that HP significantly enhanced the expression of Wnt1, Wnt3a and active β-catenin. IMD1-47 significantly reversed the effect of HP on the expression of these factors. Notably, treatment with IMD1-47 alone did not affect the expression of Wnt1, Wnt3a or active β-catenin. When VSMCs were transfected with β-catenin siRNA, the protein levels of Runx2 and OPN were

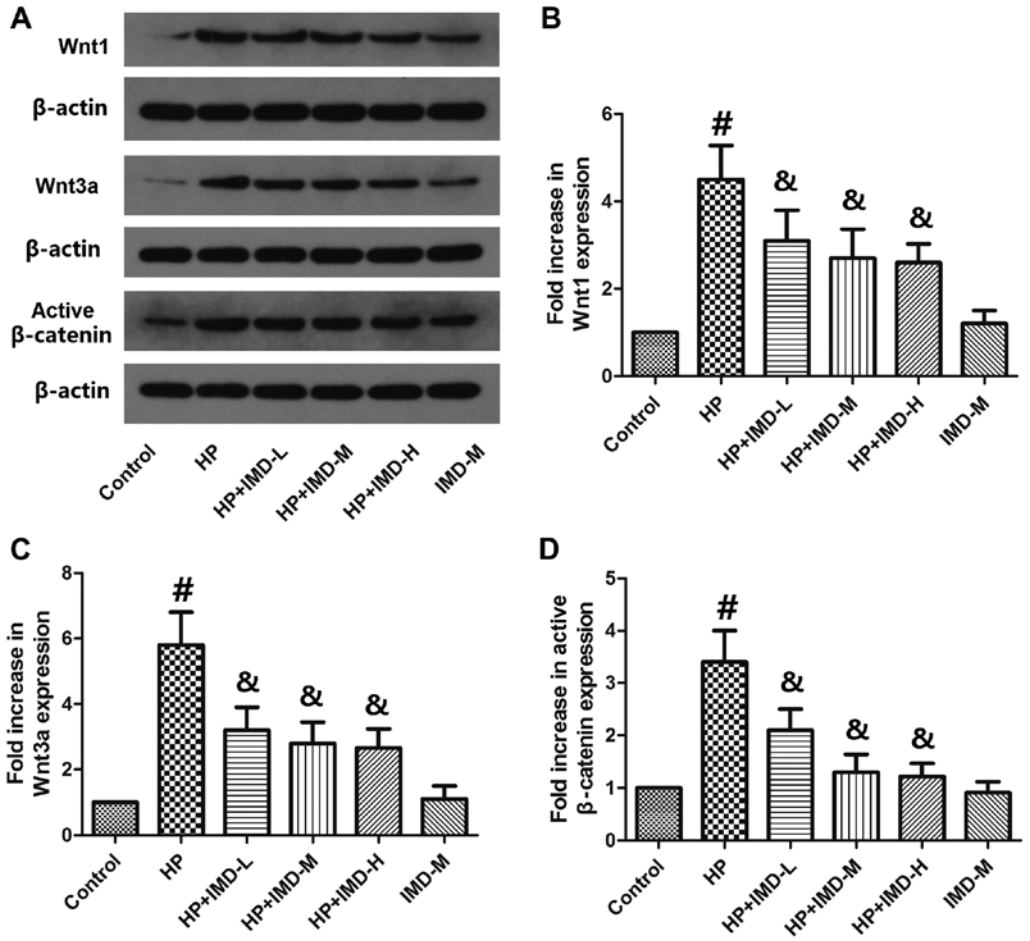

Figure 4. Effects of HP and IMD on the Wnt/β-catenin pathway in vascular smooth muscle cells. (A) Representative images from western blot analysis for the protein expression levels of Wnt1, Wnt3a and active β-catenin. (B) Quantification of western blot results for Wnt1, (C) Wnt3a and (D) active β-catenin. Data are expressed as the mean ± SEM. n=8. $^{\#}$P<0.05 vs. control; $^{\&}$P<0.05 vs. HP. HP, high phosphate; IMD, intermedin1-47; L, low dose; M, medium dose; H, high dose.

significantly increased compared with the IMD1-47 group, indicating a role for the Wnt/β-catenin pathway in the effects of IMD1-47 on the expression of osteogenic markers.

The process of vascular calcification is similar to that of bone and cartilage formation (24). Mineralized vesicles, containing hydroxyapatite and bone matrix proteins, including OPG and OPN, are associated with the mineralization of bone and cartilage (25). The phenotype of VSMCs in the blood vessel changes significantly during the vascular calcification process (23). When calcification occurs, VSMCs migrate and actively proliferate, producing a large amount of extracellular matrix (23). Furthermore, some atherosclerosis-related factors confer osteoblast-like characteristics on VSMCs (26,27). Runx2, a key transcription factor in vascular calcification, is also expressed in mineralized VSMCs (28). These previous studies have shown that the osteoblast-like phenotypic transition of VSMCs is closely related to vascular calcification. The results of the present study demonstrated that HP treatment significantly increased the cellular calcium content of VSMCs, indicating that HP effectively induced VSMC calcification. Both medium and high IMD1-47 doses significantly decreased the cellular calcium content of VSMCs, suggesting an effect of IMD1-47 on VSMC calcification. It is also possible that HP may have affected the endogenous levels of IMD1-47 in VSMCs, thereby inducing VSMC calcification. To explore this hypothesis, the IMD levels in the cell culture media were measured after treatment of the VSMCs with HP. The results showed that the levels of IMD were not affected by HP treatment, indicating that HP treatment alone did not regulate the endogenous expression of IMD.

Changes in the expression levels of the osteogenic markers OPG, Runx2 and OPN were assessed in VSMCs. HP treatment significantly enhanced the protein and RNA expression levels of OPG, Runx2 and OPN compared with untreated control cells. Both medium and high IMD1-47 doses inhibited the HP-induced increases in the expression of OPG, Runx2 and OPN. Runx2 is a specific factor in the phenotypic transition of VSMC to an osteoblast-like cell phenotype and is a member of the core binding factor family (29). It was reported that a deficiency in Runx2 significantly decreased the activation of NF-κB and the formation of osteoclast-like cells (30). Runx2 is also expressed in calcified VSMCs, not only in osteoblasts (31). Runx2 is important for the regulation of the expression of ALP and is a molecular marker of osteogenic transition (32). The regulation of Runx2 by IMD1-47 indicated that IMD1-47 may directly regulate the core binding factor family and interfere with the osteoblast-like phenotypic transition of VSMCs.

ALP is an early marker of osteoblast formation and is secreted into the extracellular matrix during

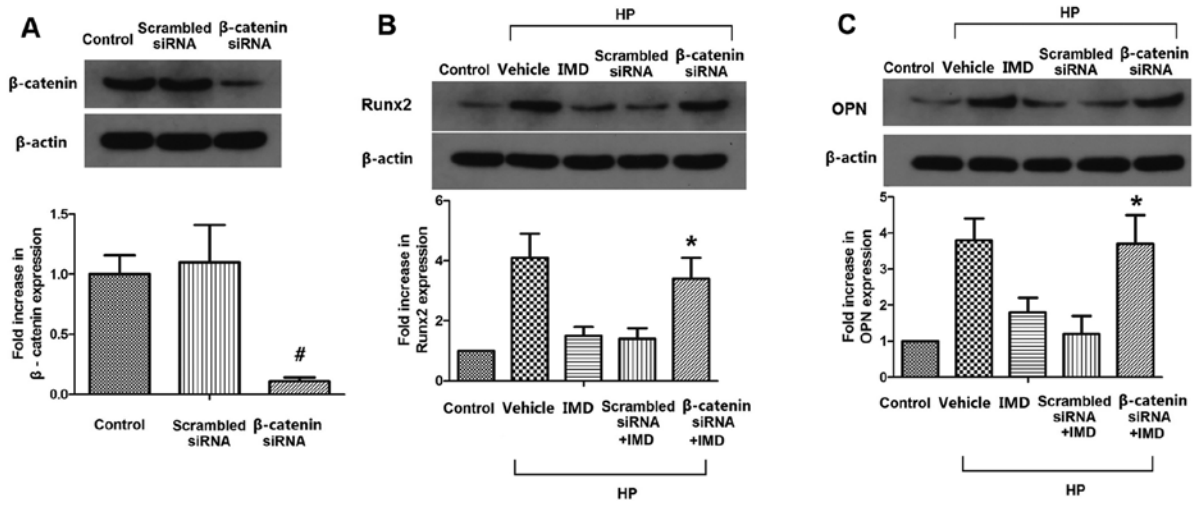

Figure 5. Effects of β-catenin siRNA and IMD on the expression of osteogenic markers in VSMCs. (A) Active β-catenin protein levels in VSMCs treated with β-catenin siRNA or scrambled siRNA. (B) The protein expression levels of the osteogenic proteins Runx2 and (C) OPN were determined in VSMCs treated with β-catenin siRNA. Data are expressed as the mean ± SEM. n=8. #P<0.05 vs. scrambled siRNA; *P<0.05 vs. scrambled siRNA+IMD. siRNA, small interfering RNA; IMD, intermedin1-47; VSMCs, vascular smooth muscle cells; Runx2, Runt-related transcription factor 2; OPN, osteopontin; HP, high phosphate.

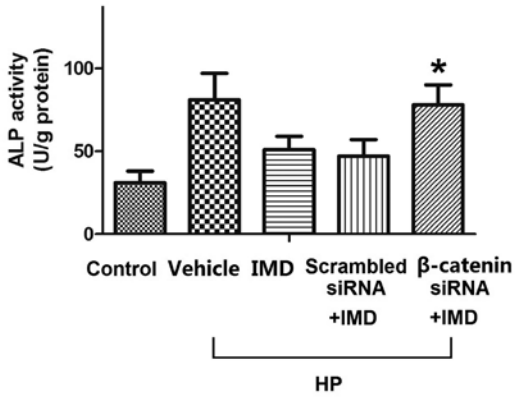

Figure 6. Effects of β-catenin siRNA and IMD on ALP activity in vascular smooth muscle cells. Data are expressed as the mean ± SEM. n=8. *P<0.05 vs. scrambled siRNA+IMD. siRNA, small interfering RNA; IMD, intermedin1-47; ALP, alkaline phosphatase; HP, high phosphate.

osteogenic/chondrogenic bone mineralization (33). High concentrations of ALP promote the accumulation of phosphate, making it insoluble, which results in phosphate crystallization and mineralization (34). ALP is expressed at low levels in normal VSMCs, however, it is highly expressed in calcified blood vessels. Levamisole, a specific ALP inhibitor, has been reported to inhibit VSMC calcification in a dose-dependent manner (35). In the present study, ALP activity in VSMCs was significantly enhanced by treatment with HP for 8 days. With increasing IMD1-47 concentrations, ALP activity was found to decrease compared with the HP group. However, treatment with IMD1-47 alone did not affect the ALP activity, indicating that IMD1-47 effectively inhibited HP-induced osteoblast formation and mineralization.

The Wnt/β-catenin signaling pathway in VSMCs has an important role in arterial calcification (36). In β-catenin knock-out mice, the bone formation process is significantly inhibited, and bone marrow mesenchymal progenitor cells are prevented from differentiating into osteoblasts (37). Furthermore, activation of the Wnt/β-catenin pathway was reported to promote the expression of bone formation-related factors, such as Runx2, OPG, osteoblast-specific gene, bone morphogenic protein, cyclin Dl and matrix metalloproteinase 7 (38). Phenotypically transformed VSMCs acquire the characteristics of osteoblast-like cells, such as ALP expression on the plasma membrane, and secretion of type I collagen and OPN, which are regulated by the Wnt/β-catenin pathway (39). It has also been reported that Wnt/β-catenin is activated at the site of cardiovascular calcification (40-42). The treatment of diabetic mice with the Wnt signaling pathway inhibitor DKK1 inhibited aortic mineralization and early bone formation-related factor activation (43). In addition, the degree of aortic calcification and sclerosis can be reduced by inhibiting the Wnt/β-catenin signaling pathway. For instance, when the Wnt/β-catenin pathway was inhibited, the expression of bone formation-associated factors collagen type 1 α 1 chain, Runx2 and NADPH oxidase 1 were decreased (42). In arterial calcification caused by both diabetes and chronic renal failure, the expression of Wnt3a and Wnt7a in the arterial wall was significantly increased (43). Similar with previous studies (40-43), the present study found that HP significantly increased the protein expression levels of Wnt1, Wnt3a and active β-catenin, while IMD1-47 significantly decreased their levels compared with the HP group. When cells were treated with IMD1-47 and β-catenin siRNA, osteogenic protein levels in VSMCs were significantly increased compared with the group treated with IMD1-47 alone. Similarly, β-catenin siRNA significantly increased the activity of ALP compared with the IMD1-47 treatment group alone. These results indicated that HP treatment may induce VSMC calcification through the activation of Wnt1/Wnt3a and β-catenin. By contrast, IMD1-47 may inhibit VSMC calcification by suppressing the activation of the Wnt/β-catenin pathway.

In conclusion, the present study indicated that IMD1-47 inhibited VSMC calcification induced by high levels of phosphate by suppressing the Wnt/β-catenin signaling pathway. The present study adds a new dimension to the

current understanding of the biological effects of IMD1-47. The present study revealed the protective effect and mechanism of IMD1-47 on VSMC calcification, and provided an experimental basis for its potential use in the clinic.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ designed the study, conducted some of the experiments and prepared the manuscript. NT performed the experiments. JZ collected and analyzed the data, and interpreted the results. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Animal Welfare and Ethics Committee of Shanghai XuHui Central Hospital (approval no. XHCH-2018-032).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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