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ORIGINAL ARTICLE

Warfarin calcifies human aortic valve interstitial cells at high-phosphate conditions via pregnane X receptor

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Abstract

Warfarin, a vitamin K antagonist, is the most common anticoagulant used to prevent thromboembolisms associated with atrial fibrillation or following valvular surgery. Although several studies have revealed that long-term warfarin use accelerates aortic valve calcification and the development of aortic stenosis (AS), the detailed mechanism for this phenomenon remains unclear. Therefore, our aim was twofold: to establish the conditions for warfarin-induced calcification of human aortic valve interstitial cells (HAVICs) using high-inorganic phosphate (Pi) conditions and to investigate the underlying mechanism. We prepared and cultured HAVICs from aortic valves affected by calcific aortic valve stenosis (AS group) and aortic valves affected by aortic regurgitation but without any signs of calcification (non-AS group). Under Pi concentrations of 3.2 mM, warfarin significantly increased the calcification and alkaline phosphatase (ALP) activity of AS but not non-AS group HAVICs. Furthermore, gene expression of bone morphogenetic protein 2 (BMP2), a calcigenic marker, was significantly increased following 7 days of warfarin treatment. Warfarin-induced calcification of AS group HAVICs at 3.2 mM Pi was significantly inhibited by dorsomorphin, a Smad inhibitor, and the pregnane X receptor (PXR) inhibitors, ketoconazole and coumestrol, but was unaffected by SN-50, an NF-κB inhibitor. Warfarin was also able to increase BMP2 gene expression at a physiological Pi concentration (1.0 mM). Furthermore, excess BMP2 (30 ng/mL) facilitated warfarin-induced ALP upregulation and HAVIC calcification, an effect which was significantly reduced in the presence of coumestrol. Together, our results suggest that warfarin accelerates calcification of HAVICs from AS patients via the PXR–BMP2–ALP pathway.

Keywords Aortic valve stenosis · Pregnane X receptor · Calcification · Warfarin · Bone morphogenetic protein 2

Introduction

Aortic valve stenosis (AS) is the most common heart valve disorder in developed countries [1, 2]. It often occurs in older adults, with an estimated incidence of 4.6% in patients over 75 years old [3, 4]. Calcified aortic valve disease (CAS) is the most prevalent form of AS worldwide. It is characterized by substantial thickening of aortic valve leaflets through fibrosis and extensive focal ectopic calcification [5]. The aortic valve calcification is irreversible and the most viable available treatments are either surgical aortic valve replacement, which is extremely invasive for patients [6], or transcatheter aortic valve replacement (TAVR) for frail older adult patients who have a higher risk for complications during and after open surgery [7]. Indeed, approximately 20% of CAS patients are deemed unsuitable for valve replacement because of its invasiveness [8]. As there is a clear need to develop a medical treatment for this condition, it is necessary to elucidate the detailed mechanism of aortic valve calcification. Although much research has been focused on the mechanism of aortic valve calcification in AS [9, 10], no effective non-invasive treatment has been developed to date.

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Anticoagulation treatment is necessary for the prevention and treatment of thromboembolic and embolic complications following surgical valve replacement or atrial fibrillation [11]. Warfarin, a vitamin K antagonist, is the most commonly prescribed postoperative anticoagulation drug. It decreases blood clotting by lowering the supply of active vitamin K to below the concentration necessary for the biosynthesis of the coagulation factors II, VII, IX, and X. This is achieved via the inhibition of vitamin K epoxide reductase, an enzyme essential for the reactivation of oxidized (inactive) vitamin K in the liver [12, 13]. However, several studies have established that warfarin use is associated with the acceleration of valvular and vascular calcification, including the aorta and coronary arteries [14–16]. Warfarin promotes aortic valve calcification by inhibiting the vitamin K-dependent matrix Gla protein (MGP) [17]. MGP is a vitamin K-dependent 10-kDa secreted protein synthesized by vascular smooth muscle cells that functions via five calcium ion-binding gamma-carboxyglutamic acid (Gla) residues. A recent study has elucidated that MGP inhibits vascular and valvular calcification by sequestering signal bone morphogenetic protein 2 (BMP2) and reducing BMP signaling [18]. This supports our previous work that also concluded that BMP2 plays a critical role in aortic valve calcification [19].

The pregnane X receptor (PXR) is a ligand-activated nuclear receptor that is a master regulator of xenobiotic metabolism, and is also involved in several human diseases such as inflammation, cancer, and diabetes [20, 21]. PXR is also activated by vitamin K: vitamin K2, a warfarin antagonist, has been shown to regulate collagen assembly through the action of PXR, which results in bone formation [22]. In addition, it has been recently reported that warfarin also interacts with PXR to upregulate the expression of several cytochrome P450 enzymes [23].

In a previous study, we revealed that high concentrations of inorganic phosphate (Pi) induced the calcification of human aortic valve interstitial cells (HAVICs) via the activation of alkaline phosphatase (ALP), but independently of calcification factors such as BMP2 and Runx2 [24, 25]. In this study, we sought to elucidate whether warfarin induces calcification of HAVICs at high-Pi concentrations. Our results indicated that at a Pi concentration of 3.2 mM, warfarin accelerated the calcification of HAVICs obtained from patients with AS (AS group) by elevating BMP2 gene expression and alkaline phosphatase (ALP) activity. We further investigated the molecular mechanism of warfarin-induced calcification of HAVICs from the AS group and found critical roles for PXR and BMP2 in aortic valve calcification.

Materials and methods

Materials

Warfarin, ketoconazole, coumestrol, tumor necrosis factor-α (TNF-α), sodium phosphonoformic acid (PFA), and dimethyl sulfoxide (DMSO) were obtained from Wako Pure Chemicals (Osaka, Japan). Dorsomorphin, SB239063, U-0126, SR-12813, and collagenase type V were purchased from Sigma-Aldrich (St. Louis, MO, USA). SN-50 and BMS345541 were obtained from Cayman Chemical (Ann Arbor, MI, USA). Alpha modified Eagle's medium (α-MEM) was obtained from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum (FBS, Biofluids™), penicillin and streptomycin (Gibco™; Gaithersburg, MD, USA) were obtained from Invitrogen (Carlsbad, CA, USA). All primers used for the quantitative real-time polymerase chain reactions (qPCRs) were obtained from Fasmac (Kanagawa, Japan). Power SYBR® Green PCR Master Mix was supplied by TOYOBO (Osaka, Japan). All chemicals used were of the highest purity that was commercially available. All solutions were freshly made at sufficiently high concentrations so that only very small volumes were required to be added to the culture medium. The final concentration of DMSO added to HAVICs never exceeded 0.1% (v/v). This DMSO concentration was sufficiently low to elicit no effects on the cells or assays.

Isolation and culture of HAVICs

All patients gave written informed consent, and the study was approved by the Institutional Review Board of the Hirosaki University Hospital. Additional informed consent was obtained from all participants for whom identifying information is included in this article. Human aortic valves were obtained from patients with calcific aortic valve stenosis (AS group, $n = 6$, age: 64.3 ± 5.8 years, male:female = 3:3) and from patients with aortic aneurysm or aortic regurgitation without any signs of calcification (non-AS group, $n = 4$, age: 66 ± 10 years, male:female = 3:1), who underwent surgical aortic valve replacement at Hirosaki University Hospital (Aomori, Japan). There were no statistically significant differences in clinical factors associated with AS between these two groups (data not shown).

Human aortic valve specimens were gently cut into 2 ± 1-mm pieces and washed by α-MEM containing 10% FBS. HAVICs were isolated by collagenase digestion. Briefly, collagenase type V (1 mg/ml) and minced valve

specimens are incubated for 2 h at 37 °C in the presence of 95% O_2 and 5% CO_2 (34). HAVICs isolated were cultured in α-MEM containing 10% FBS, 1 U/ml sodium penicillin G, and 0.5 U/ml streptomycin. Cells from passage four were used in all experiments. After HAVICs reached 80–90% confluency, they were further cultured in the presence or absence of warfarin for 7 days. The medium was replenished every 3 days [26].

Induction of HAVIC calcification

To examine warfarin-induced HAVIC calcification, HAVICs were seeded into a 12- or 96-well plate and grown for 3 days in α-MEM containing 10% FBS, 1 U/ml sodium penicillin G, and 0.5 U/ml streptomycin. After HAVICs reached 80–90% confluency, they were further cultured with or without warfarin (1 μM) in medium including inorganic phosphoric acid (Pi 1.0–3.8 mM) for 7 days or excess BMP2 (30 ng/ml) for 21 days.

Evaluation of HAVIC calcification

The degree of calcification of cultured HAVICs was measured by Alizarin Red S staining. Briefly, the matrix was washed phosphate-buffered saline (PBS) and incubated with 10% buffered formalin for 15 min. After three washes with purified water, cultures were treated with Alizarin Red S solution (200 mg/ml) for 5 min at a room temperature. The excess Alizarin red S solution was completely washed away using purified water to color development. Stained cells were examined under a digital camera (Nikon, Tokyo, Japan). For quantification of staining, 100 mM aqueous cetyl-pyridinium chloride solution was added to each well (12-well plate: 200 μl, 96-well plate: 50 μl) and incubated to solubilize and release Alizarin Red S dye from the extracellular matrix, then quantified by spectrophotometry at 550 nm by microplate reader, Bio-Rad Model 680 (Hercules, CA, USA) [27].

To measure ALP activity as a marker of HAVIC calcification at 0, 7 and 21 days, we used the LabAssay ALP Kit (Wako Pure Chemicals, Osaka, Japan) according to the manufacturer's instructions. Briefly, cells were washed twice with PBS and then treated with a cell lysis buffer containing 100 μl of 0.05% (v/v) Triton X-100 in PBS and centrifuged. The supernatants were assayed using *p*-nitrophenylphosphate as a substrate. One unit was defined as the activity producing 1 nmol of *p*-nitrophenol for 30-min incubation at 37 °C.

Measurement of gene expression

Total RNA was isolated from HAVICs using the QuickGene RNA cultured cell kit S (Fujifilm, Tokyo, Japan). An aliquot of total RNA (10 ng) was reverse transcribed to cDNA using ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). Each real-time polymerase chain reaction (RT-PCR) contained 3 μl of a 1:4 dilution of the first-strand reaction product, 0.6 μl each of 10 μM specific forward and reverse primers, 0.4 μl 50× ROX reference dye, 5.4 μl of pure water, and 10 μl of THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan), giving a final reaction volume of 20 μl. The cDNA was amplified using an ABI PRISM 7000 (Life Technologies, Carlsbad, CA) under the following reaction conditions: 40 cycles of PCR (95 °C for 15 s, and 60 °C for 1 min) following an initial denaturation (95 °C for 1 min). National Center for Biotechnology Information (NCBI) Primer BLAST (Bethesda, MD, USA) was used to design the primers for ALP, BMP2, pregnane X receptor (PXR), CYP3A4, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and their sequences are shown in Table 1. The expression level of the G3PDH housekeeping gene was used as an internal standard for normalization. The relative abundance of target mRNA was calculated using the 2[-delta delta C(T)] method [28]. Real-time PCR data were represented as cycle threshold (C_t) levels and normalized to control G3PDH C_t values.

The effects of various drugs on warfarin-induced calcification of HAVICs

Primary cultured HAVICs were seeded into a 96-well plate and cultured for 3 days until they reached 80–90% confluency. Batches of cells were then individually pretreated with various pathway inhibitors: Smad1/5/8 phosphorylation [dorsomorphin (3 μM)], NF-κB p65 subunit translocation into nucleus [SN-50 (10 μM)], MAP kinase signaling [U-0126 (10 μM) and SB239063 (3 μM)], PXR activity [ketoconazole (30 μM) and coumestrol (10 μM)], and inorganic phosphate transporter-1 [sodium phosphonoformate (0.1 mM)[for 1 h. Cells were subsequently treated

Table 1 Primers used for quantitative real-time PCR

Gene symbol	GenBank Accession no.	Sequences (5′–3′)
ALP	NM_000478	Forward: agaaccccaaaggcttcttc
		Reverse: cttggcttttccttcatggt
BMP2	NM_001200	Forward: cggactgcggtctcctaa
		Reverse: ggaagcagcaacgctagaag
PXR	NM_001065	Forward: gcttcagaaaaccacctcagaca
		Reverse: caataatgccggtactggttcttc
CYP3A4	NM_001202855	Forward: aagggatggcaccgtaagtg
		Reverse: ctggtgttctcaggcacaga
G3PDH	NM_002046	Forward: tgcaccaccaactgcttagc
		Reverse: ggcatggactgtggtcatgag

with warfarin (1 μM) up to 21 days in physiological Pi (1.0 mM), high-Pi (3.2 mM), or excess BMP2 (30 ng/mL) condition. We assessed HAVIC ectopic calcification by measuring Alizarin Red S staining and ALP activity. The expression of calcification genes was examined by real-time PCR.

Statistical analysis

All statistical analyses were carried out using KyPlot 5.0 software (Kyenslab, Tokyo, Japan). Group comparisons were performed by one-way analysis of variance (ANOVA) with the Student–Newman–Keuls post hoc correction procedure. Comparisons between two independent data sets were assessed using the Student's *t* test. Values are presented as mean ± SEM of results from all replicates; probability values of $P < 0.05$ were considered to be statistically significant.

Results

Establishment of optimal warfarin-induced HAVIC calcification conditions

To establish an effective assessment method of warfarin-induced aortic valve calcification, we initially investigated an appropriate concentration of Pi for use in our experiments. For HAVICs, we confirmed that the mean threshold concentration of Pi for calcification was 3.2 mM (Fig. 1a). Calcification was induced at a minimal level at this Pi concentration in HAVICs from both the AS and non-AS groups. Therefore, we used 3.2 mM Pi in our subsequent experiments. To establish that this high-Pi concentration does not affect HAVIC calcification via BMP2–ALP signaling, we investigated HAVIC calcification induced by TNF-α, a potent stimulator of calcification that acts via the BMP2–ALP pathway [24]. Figure 1b shows that, although TNF-α (30 ng/mL) alone induced AS group HAVIC calcification, it strongly accelerated HAVIC calcification in the presence of 3.2 mM Pi. This effect was completely suppressed by treatment with sodium phosphonoformate (PFA), an inorganic phosphate transporter-1 (PiT-1) inhibitor.

To assess the effect of warfarin on HAVIC calcification, HAVICs obtained from AS or non-AS patient groups were cultured in high-Pi medium in the presence or absence of warfarin (1 μM) for 7 days. Warfarin alone did not accelerate HAVIC calcification in HAVICs from either group; however, in high-Pi (3.2 mM) medium, the calcification of AS group HAVICs was markedly accelerated by warfarin, but non-AS HAVICs were unaffected (Fig. 1c). These data suggested that HAVICs from patients with AS were highly sensitive to warfarin-induced calcification in high-Pi conditions.

Warfarin accelerates the calcification of HAVICs

We then investigated the effect of a range of warfarin concentrations on the calcification of HAVICs from AS patients in high-Pi (3.2 mM) medium. After reaching 90% confluency, HAVICs were cultured in the presence (0.1–1 μM) or absence of warfarin for 7 days. We observed that warfarin induced the calcification of HAVICs in a dose-dependent manner (Fig. 2a). Spectrophotometric quantification of calcification following Alizarin Red S staining also showed that calcification was accelerated by warfarin (Fig. 2a). In addition, PFA significantly inhibited warfarin-induced calcification of HAVICs in high-Pi medium (Fig. 2b).

To investigate the molecular mechanism of warfarin-induced calcification of AS group HAVICs, we investigated if BMP2 gene expression and ALP activity are affected by warfarin treatment of AS group HAVICs in high-Pi medium. When AS group HAVICs were cultured with warfarin (1 μM) in high-Pi medium for 7 days, we found a significant increase in BMP2 gene expression and ALP activity (Fig. 2c, d). In non-AS group HAVICs, BMP2 expression and ALP activation did not alter (Fig. 2e, f). We then separately investigated the inhibitory effects of dorsomorphin (3 μM), a Smad1/5/8 phosphorylation inhibitor, and SN-50 (10 μM), an NF-κB inhibitor, to examine if warfarin-induced calcification of AS group HAVICs is induced via the NF-κB–BMP2–ALP pathway, analogous to TNF-α-induced HAVIC calcification [24]. We observed that dorsomorphin, but not SN-50, significantly inhibited warfarin-induced AS group HAVIC calcification in high-Pi medium (Fig. 2g). Although the MAP kinase pathway is also possibly involved in aortic valve calcification, two inhibitors of this pathway, U-0126 (10 μM, an ERK inhibitor) and SB239063 [3 μM, a p38 mitogen-activated kinase (MAP) kinase inhibitor], did not inhibit warfarin-induced calcification of AS group HAVICs in high-Pi medium (data not shown). These data suggest that warfarin accelerates AS group HAVIC calcification via the BMP2–ALP pathway.

Effect of PXR inhibitors on warfarin-induced AS group HAVIC calcification in high-Pi medium

To confirm whether warfarin-induced calcification stimulates PXR, we investigated the effects of two PXR inhibitors,

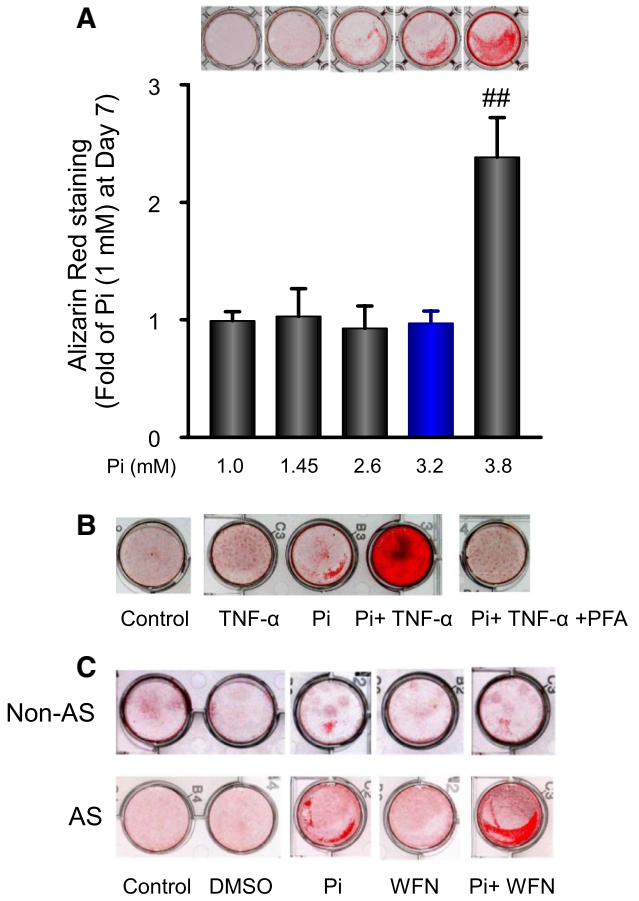


Fig. 1 Establishment of warfarin-induced calcification conditions of HAVICs using a range of phosphate concentrations. HAVICs were cultured in α-MEM containing 10% fetal bovine serum (FBS). After reaching 90% confluency (Day 0), HAVICs were further cultured in medium containing a range of inorganic phosphate (Pi) concentrations (1.0–3.8 mM) for 7 days. **a** Representative images of Alizarin Red S staining (upper images) of HAVICs from both the AS and non-AS groups and quantification of Alizarin Red S staining on day 7 following cetyl-pyridinium chloride extraction. The amount of released dye was quantified by spectrophotometry at 550 nm. All ratios were calculated versus the Pi (1.0 mM) group on day 7. Bars represent the mean ± SEM ($n = 4$). A significant difference ($P < 0.01$) when compared with control cells (Pi = 1.0 mM) or Pi = 3.2 mM on day 7 is denoted by 'Hash'. The mean threshold concentration of Pi for calcification was 3.2 mM (Blue bar). **b** Representative images of Alizarin Red S staining of HUVECs following tumor necrosis factor-α (TNF-α, 30 ng/ml)-induced calcification in high-phosphate (Pi) medium (3.2 mM). Sodium phosphonoformate (PFA, 0.1 mM) was used as a selective inhibitor for PiT-1, a phosphate transporter. **c** Typical images of Alizarin Red S staining of HAVICs prepared from aortic valves affected by calcific aortic valve stenosis (AS) and HAVICs prepared from aortal valves affected by aortic regurgitation but without any signs of calcification (non-AS) in the presence or absence of 1 μM warfarin in high-Pi medium (color figure online)

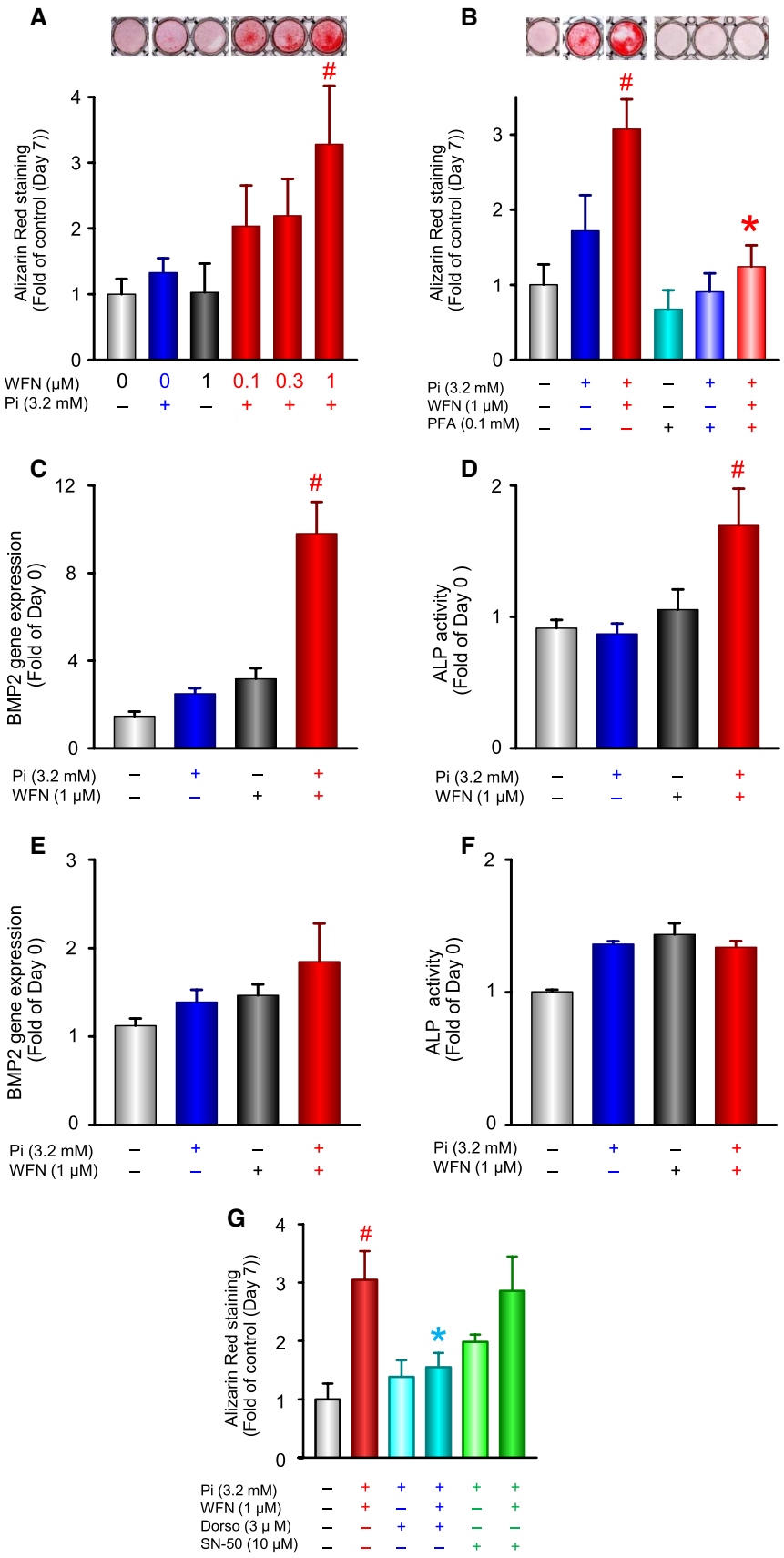
ketoconazole [29] and coumestrol [30], on the calcification of AS group HAVICs in high-Pi medium. As shown in Fig. 3a, both ketoconazole and coumestrol strongly suppressed warfarin-induced AS group HAVIC calcification in high-Pi medium. Spectrophotometric quantification of calcification using Alizarin Red S dye supported these data (Fig. 3b, c). We also observed a significant decrease in warfarin-induced BMP2 gene expression (Fig. 3d) and ALP enzyme activity (Fig. 3e) in high-Pi medium in the presence of either ketoconazole or coumestrol. However, warfarin had no effect on PXR gene expression (Supplemental Fig. 1a). Although there was no significant difference of the PXR gene expression between AS and non-AS group HAVICs, in non-AS group HAVICs, warfarin tended to strongly suppress PXR gene expression (Supplemental Fig. 1b).

It is well known that CYP3A4 is a prototypical target gene of PXR. In warfarin-induced AS group HAVIC calcification, CYP3A4 gene expression was strongly decreased in high-Pi medium (Supplemental Fig. 1c). Further, in normal phosphate condition, warfarin strongly decreased CYP3A4 gene expression in both AS and non-AS group HAVICs on Day 3 (Supplemental Fig. 1d).

We further investigated whether a selective PXR agonist, SR-12813, induces AS group HAVIC calcification. SR-12813 (3 μM) markedly induced AS group HAVIC calcification like warfarin (Supplemental Fig. 2a). This calcification by SR-12813 occurred together with BMP2 gene expression (Supplemental Fig. 2b). However, in this condition, SR-12813 largely decreased CYP3A4 gene expression (Supplemental Fig. 2c). These data suggest that warfarin-induced calcification of HAVICs from the AS patients occurs via the PXR–BMP2–ALP pathway.

Warfarin induces BMP2 gene expression in AS group HAVICs

Next, we sought to determine whether warfarin treatment alone was able to induce BMP2 gene expression in HAVICs. We cultured AS group HAVICs for 3 days in the presence of warfarin (1 μM) at a physiological Pi concentration (1.0 mM) and observed a significant increase in BMP2 gene expression. Furthermore, this effect was significantly inhibited in the presence of coumestrol (Fig. 4a). While, in non-AS group HAVICs, warfarin did not affect the BMP2 gene expression (Fig. 4b). In addition, we demonstrated that culturing AS group HAVICs for 21 days in the presence of warfarin and excess BMP2 (30 ng/mL) at physiological Pi


A
Alizarin Red staining (Fold of control (Day 7))
WFN (μM) 0 0 1 0.1 0.3 1
Pi (3.2 mM) – + – + + +
B
Alizarin Red staining (Fold of control (Day 7))
Pi (3.2 mM) – + + – + +
WFN (1 μM) – – + – – +
PFA (0.1 mM) – – – + + +
C
BMP2 gene expression (Fold of Day 0)
Pi (3.2 mM) – + – +
WFN (1 μM) – – + +
D
ALP activity (Fold of Day 0)
Pi (3.2 mM) – + – +
WFN (1 μM) – – + +
E
BMP2 gene expression (Fold of Day 0)
Pi (3.2 mM) – + – +
WFN (1 μM) – – + +
F
ALP activity (Fold of Day 0)
Pi (3.2 mM) – + – +
WFN (1 μM) – – + +
G
Alizarin Red staining (Fold of control (Day 7))
Pi (3.2 mM) – + + + + +
WFN (1 μM) – + – + – +
Dorso (3 μ M) – – + + – –
SN-50 (10 μM) – – – – + +

◂**Fig. 2** Warfarin induces calcification of AS group HAVICs in high-Pi medium. AS group HAVICs were cultured in α-MEM containing 10% fetal bovine serum (FBS). After reaching 80–90% confluency (Day 0), the HAVICs were further cultured for 7 days (Day 7). **a** Representative images of Alizarin Red S staining (upper images) of AS group HUVECs and quantification of Alizarin Red S staining on day 7 following cetyl-pyridinium chloride extraction. The amount of released dye was quantified by spectrophotometry at 550 nm. All ratios were normalized to the untreated control values on day 7. White bar: untreated cells; blue bar: cells treated with high (3.2 mM) Pi (+) but without (−) warfarin (WFN); black bar: cells treated with 1 μM WFN (+) and physiological (−) concentrations of Pi (1.2 mM); red bars: cells treated with WFN (0.1–1 μM) and high (3.2 mM) Pi (+). Bars represent the mean ± SEM (n = 5). A significant difference ($P < 0.05$) when compared with untreated AS group control cells, AS group cells treated with high Pi (3.2 mM) only, and AS group cells treated with warfarin (1 μM) only on day 7 is denoted by 'Hash'. **b** Representative images of Alizarin Red S staining (upper images) of AS group HUVECs and quantification of Alizarin Red S staining on day 7 following cetyl-pyridinium chloride extraction. All ratios were normalized to control values on day 7. White bar: untreated cells; blue bar: cells treated with high (3.2 mM) Pi (+) alone; red bar: cells treated with 1 μM WFN (+) and high (3.2 mM) Pi (+); cyan blue bar: cells treated with 0.1 mM sodium phosphonoformate [PFA, (+)] alone; light blue bar: cells treated with high (3.2 mM) Pi (+) and 0.1 mM PFA (+); light red bar: cells treated with high (3.2 mM) Pi (+), 1 μM WFN (+), and 0.1 mM PFA (+). Bars represent the mean ± SEM (n = 5). A significant difference ($P < 0.05$) when compared against untreated control AS group cells and AS group cells treated with high Pi (3.2 mM) only on day 7 is denoted by 'Hash' and a significant difference ($P < 0.05$) when compared against AS group cells treated with both Pi (3.2 mM) and warfarin (1 μM) is denoted by 'Asterisk'. **c**, **d** BMP2 gene expression (**c**) and ALP activity (**d**) in AS group HAVICs were measured on day 7. All ratios were calculated versus the control group on day 0. Relative gene expression levels were determined by normalizing measured values to those obtained for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH). White bar: untreated cells; blue bar: cells treated with high (3.2 mM) Pi (+); black bar: cells treated with 1 μM WFN (+); red bars: cells treated with 1 μM WFN (+) and high (3.2 mM) Pi (+). Bars represent the mean ± SEM (n = 4). A significant difference ($P < 0.05$) when compared with untreated control AS group cells, AS group cells treated with high Pi (3.2 mM) only, and AS group cells treated with warfarin (1 μM) only on day 7 is denoted by 'Hash'. **e**, **f** BMP2 gene expression (**e**) and ALP activity (**f**) in non-AS group HAVICs were measured on day 7. All ratios were calculated versus the control group on day 0. Relative gene expression levels were determined by normalizing measured values to those obtained for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH). White bar: untreated cells; blue bar: cells treated with high (3.2 mM) Pi (+); black bar: cells treated with 1 μM WFN (+); red bars: cells treated with 1 μM WFN (+) and high (3.2 mM) Pi (+). Bars represent the mean ± SEM (n = 3). G: Quantification of Alizarin Red S staining on day 7 following cetyl-pyridinium chloride extraction. The amount of released dye was quantified by spectrophotometry at 550 nm. All ratios were normalized to the control value on day 7. White bar: untreated cells; red bar: cells treated with 1 μM WFN (+) and high (3.2 mM) Pi (+); light cyan blue bar: cells treated with high (3.2 mM) Pi (+) and 3 μM dorsomorphin, an inhibitor of Smad1/5/8 phosphorylation [Dorso, (+)]; cyan blue bar: cells treated with high (3.2 mM) Pi (+), 1 μM WFN (+), and 3 μM Dorso (+); light green bar: cells treated with high (3.2 mM) Pi (+) and 10 μM SN-50 (+), an NF-κB inhibitor; green bar: cells treated with high (3.2 mM) Pi (+), 1 μM WFN (+), and 10 μM SN-50 (+). Bars represent the mean ± SEM (n = 4). A significant difference ($P < 0.05$) when compared with untreated AS group control cells on day 7 is denoted by 'Hash' and a significant difference ($P < 0.05$) when compared with untreated control AS group cells, AS group cells treated with high Pi (3.2 mM) only, and AS group cells treated with warfarin (1 μM) only on day 7 is denoted by 'Asterisk' (color figure online)

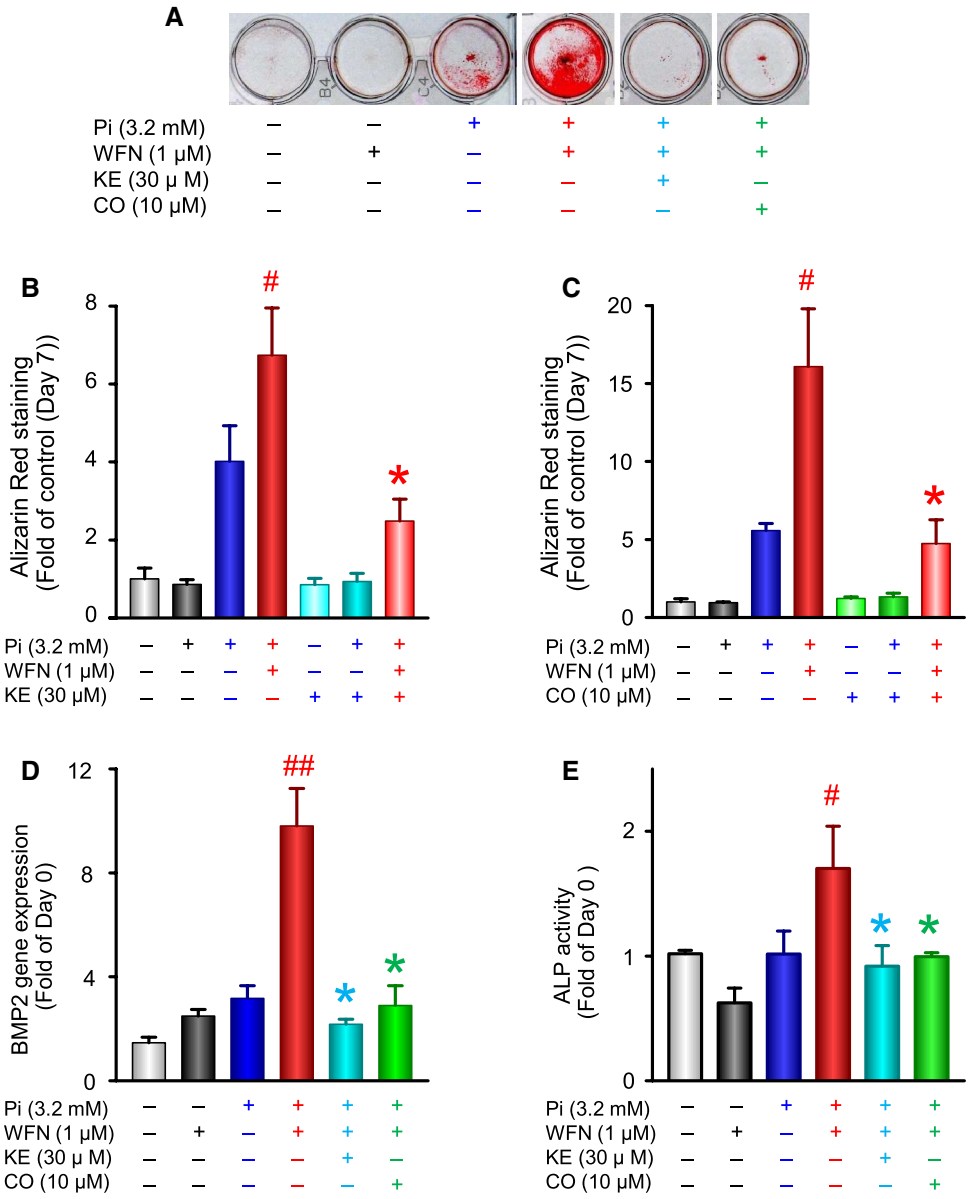
concentrations (1.0 mM) could induce significant calcification (Fig. 4c) and ALP activation (Fig. 4d). Both these effects were inhibited in the presence of coumestrol.

Together, our results suggest that warfarin can stimulate BMP2 gene expression via PXR and induces AS group HAVIC calcification at high-Pi concentrations (3.2 mM) as well as excess BMP2 (30 ng/mL) at physiological Pi concentrations (1.0 mM).

Discussion

With life expectancies increasing, aortic stenosis (AS) remains the most prevalent heart valve disease in developed countries [1, 2]. The number of older adult patients requiring treatment for AS is proving a challenge as the most viable treatment is surgical aortic valve replacement, which is a highly invasive procedure with a substantial risk of complications [8]. In recent years, transcatheter aortic valve replacement has emerged as a promising option to treat frailer patients as it is significantly less invasive. However, non-structural valve dysfunction of prosthetic aortic valve caused by calcification remains an issue. Warfarin, which inhibits vitamin K-dependent clotting factors, is the most commonly prescribed anticoagulation drug to treat and prevent blood clots associated with atrial fibrillation and deep vein thrombosis. It is also used for thrombosis prevention following surgical aortic valve replacement or repair [9]. However, several studies have shown that long-term intake of warfarin induces the acceleration of calcification in arteries and valves [14, 16, 31]. The molecular mechanism underlying this phenomenon remains unclear.

Warfarin increases systemic calcification, including that of coronary arteries and valves, by inhibiting the vitamin K-dependent matrix gamma-carboxyglutamate Gla protein (MGP) [17, 32]. However, warfarin has no effect on MGP gene expression (data not shown). Warfarin has been shown to inhibit Vitamin K2 activity, which exerts its effects by binding to and activating the pregnane X receptor (PXR) [23]. Especially, (R)-(+)-Warfarin directly interacts with PXR to upregulate the expression of several cytochrome P450 enzymes [23]. Therefore, in this study, we examined if PXR is involved in warfarin-induced calcification of AS group aortic valves. We initially found that warfarin significantly upregulated BMP2 gene expression at high-phosphate concentrations, while non-AS group HAVICs have low susceptibility on high-Pi-induced calcification and did not promote BMP2 expression. We demonstrated that after the BMP2 activation induces ALP activation through the distal-less homeobox 5 gene expression, resulting in AS but not non-AS group HAVIC calcification in normal phosphate medium [19]. To investigate a possible link between the upregulated BMP2 expression and PXR activity, we



used the PXR inhibitors, ketoconazole and coumestrol, to assess the effect of PXR inhibition on warfarin-induced calcification of AS group HAVICs. We observed that both ketoconazole and coumestrol significantly inhibited calcification under the same conditions. Furthermore, BMP2 gene expression and ALP activity were also inhibited. To examine if additional pathways were involved, we assessed the effect of a range of other pathway inhibitors targeting PiT-1, Smad4, MAPK, and NF-κB on warfarin-induced calcification of AS group HAVICs under high-phosphate conditions. Although SB239063 (a MAPK inhibitor) and SN-50 (an NF-κB inhibitor) had no effect, PFA (a PiT-1 inhibitor) and dorsomorphin (a Smad1/5/8 phosphorylation inhibitor) did inhibit warfarin-induced calcification and ALP activity in high-phosphate conditions. Together, these data indicate that warfarin-induced calcification in high-phosphate conditions occurs via the PXR-BMP2–ALP pathway. Figure 5 shows our proposed mechanism of warfarin-induced calcification of AS group HAVICs, namely warfarin accelerates BMP2 gene expression via the translocation of PXR into the nucleus, which stimulates the expression of calcification genes. This results in ALP activation, which, via Smad1/5/8 phosphorylation, drives the calcification process.

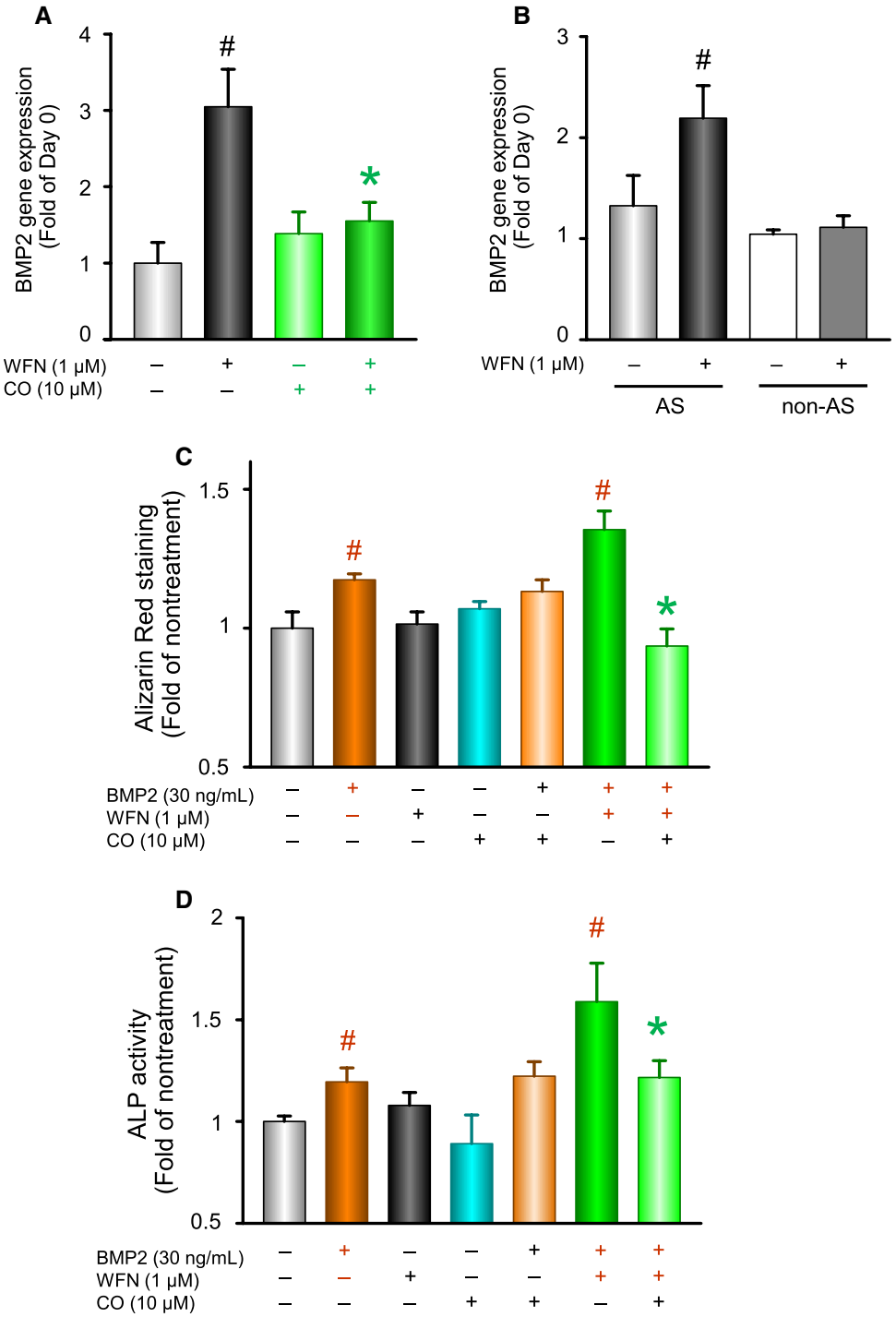
It is well known that SR-12813 is a selective PXR agonist. We further investigated whether SR12813 induces AS group HAVIC calcification. In high Pi, we demonstrated that SR-12813 also induced HAVIC calcification together

◂**Fig. 3** Warfarin-induced calcification of AS group HAVICs in high-Pi medium is inhibited by inhibitors of the pregnane X receptor (PXR). AS group HAVICs were cultured in α-MEM containing 10% fetal bovine serum (FBS). After reaching 90% confluency (Day 0), HAVICs were further cultured for 7 days (Day 7). **a**–**c** Representative images of Alizarin Red S staining (**a**) and quantification of Alizarin Red S staining on day 7 following cetyl-pyridinium chloride extraction (**b**, **c**). The amount of released dye was quantified by spectrophotometry at 550 nm. All ratios were normalized to the untreated control value on day 7. White bar: untreated cells; black bar: cells treated with 1 μM warfarin [WFN, (+)] only; blue bar: cells treated with high (3.2 mM) Pi (+) only; red bar: cells treated with high (3.2 mM) Pi (+) and 1 μM WFN (+); light cyan blue bar: cells treated with 30 μM ketoconazole, a pregnane X receptor (PXR) inhibitor [KE, (+)]; cyan blue bar: cells treated with high (3.2 mM) Pi (+) and 30 μM KE (+); light green bar: cells treated with 10 μM coumestrol [CO, (+)], another PXR inhibitor; green bar: cells treated with high (3.2 mM) Pi (+) and 10 μM CO (+); light red bar: cells treated with high (3.2 mM) Pi (+), 1 μM WFN (+), and 30 μM KE (+) (**b**) or 10 μM CO (+) (**c**). Bars represent the mean ± SEM ($n = 4$). A significant difference ($P < 0.05$) when compared with untreated control AS group cells, AS group cells treated with high Pi (3.2 mM) only, and AS group cells treated with warfarin (1 μM) only on day 7 is denoted by 'Hash' and a significant difference ($P < 0.05$) when compared against AS group cells treated with both Pi (3.2 mM) and warfarin (1 μM) on day 7 is denoted by 'Asterisk'. **d**, **e** BMP2 gene expression (**d**) and ALP activity (**e**) in AS group HAVICs were measured on day 7. All ratios were calculated versus the control group on day 0. Relative gene expression levels were determined by normalizing measured values to those obtained for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH). White bar: untreated cells; black bar: cells treated with 1 μM warfarin [WFN, (+)] only; blue bar: cells treated with high (3.2 mM) Pi (+); red bar: cells treated with high (3.2 mM) Pi (+) and 1 μM WFN (+); cyan blue bar: cells treated with high (3.2 mM) Pi (+) only, 1 μM warfarin (+), and 30 μM KE (+); green bar: cells treated with high (3.2 mM) Pi (+), 1 μM WFN (+), and 10 μM CO (+). Bars represent the mean ± SEM ($n = 4$). A significant difference ($P < 0.05$ and 0.01) when compared against untreated control AS group cells AS group cells treated with Pi (3.2 mM) only, and AS group cells treated with warfarin (1 μM) only on day 7 is denoted by 'Hash' and 'Double Hash', respectively, and a significant difference ($P < 0.05$) when compared with AS group cells treated with both Pi (3.2 mM) and warfarin (1 μM) on day 7 is denoted by 'Asterisk' (color figure online)

with the acceleration of BMP2 gene expression. However, both warfarin and SR-12813 tend to strongly decrease the gene expression of CYP3A4, a prototypical target gene of PXR, in HAVICs (Supplemental Figs. 1c, 1d, and 2c). Interestingly, although there was no significant difference of the basal PXR gene expression between AS and non-AS group HAVICs, in non-AS group HAVICs, warfarin tended to strongly suppress PXR gene expression (Supplemental Fig. 1b). These results suggest a possibility that the difference in PXR gene expression between AS and non-AS group HAVICs is closely related to the susceptibility to warfarin-induced HAVIC calcification at high-Pi concentration. In the next study, we must investigate the role of PXR on AS group HAVIC calcification using various PXR activators and inhibitors, pharmacologically and molecular biologically. Further, there is a report that in cardiac tissue CYP3A4 gene is rarely expressed [33]. To solve the role of CYP3A4 gene expressed by PXR in AS group HAVICs, we should investigate the CYP3A4's oxidation reactivity of various substrates and CYP3A4 protein expression using AS and non-AS group HAVICs, and hepatic cells as a positive control. These future investigations maybe present a new knowledge on HAVIC calcification through PXR activation.

Warfarin decreases blood coagulation by blocking the activity of the liver enzyme vitamin K oxide reductase, which is required for the synthesis of the functional clotting factors II, VII, IX, and X [10, 11]. Controversially, the warfarin antagonist, vitamin K2 (VK2), has also been proposed to play a role in tissue calcification: a study showed that VK2 was able to regulate collagen assembly through the action of the pregnane X receptor (PXR), leading to bone formation [22]. However, other research studies have indicated that VK2 supplementation inhibited the acceleration of vascular calcification [34, 35]. Therefore, further work is required to understand the relationship between VK2 and warfarin-induced calcification.

To date, no effective drugs have been developed to inhibit ectopic aortic valve calcification [36]. Son and colleagues reported that the administration of statins protected human aortic smooth muscle cells and HAVICs from phosphate-induced calcification by inhibiting apoptosis via restoration of the Gas6–Axl pathway [37]. The JUPITER trial (Justification for the use of statins in primary prevention trial) was developed to evaluate the effects of rosuvastatin in the primary prevention of cardiovascular disease [38]. However, a specific critique of the JUPITER trial concluded that its results did not support the use of statins for the primary prevention of cardiovascular diseases and raised troubling questions concerning the involvement of commercial sponsors [39]. Therefore, VK2 could be a viable candidate for development as a new therapy to inhibit AS ectopic calcification. We believe that our assay to assess warfarin-induced


A
BMP2 gene expression (Fold of Day 0)
WFN (1 μM)
CO (10 μM)
B
BMP2 gene expression (Fold of Day 0)
WFN (1 μM)
AS
non-AS
C
Alizarin Red staining (Fold of nontreatment)
BMP2 (30 ng/mL)
WFN (1 μM)
CO (10 μM)
D
ALP activity (Fold of nontreatment)
BMP2 (30 ng/mL)
WFN (1 μM)
CO (10 μM)

◂**Fig. 4** Warfarin induces BMP2 gene expression in AS group HAVICs. **a** Real-time PCR analysis of BMP2 mRNA expression in AS group HAVICs following culturing for 3 days (Day 3) in the presence or absence of coumestrol (CO) at physiological Pi concentrations (1.0 mM). All ratios were calculated versus the control group on day 0. Relative gene expression levels were determined by normalizing measured values to those obtained for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH). White bar: untreated cells, black bar: cells treated with 1 μM warfarin [WFN, (+)] only, light green bar: cells treated with 10 μM coumestrol [CO (+)] only, and green bar: cells treated with 1 μM warfarin (+) and 10 μM CO (+). Bars represent the mean ± SEM ($n = 4$). A significant difference ($P < 0.05$) when compared against untreated AS control cells is indicated with 'Ash' and a significant difference ($P < 0.05$) when compared against AS cells treated with warfarin (1 μM) is indicated by 'Asterisk'. **b** BMP2 mRNA expression was compared between AS and non-AS group HAVICs cultured for 3 days at physiological Pi concentrations (1.0 mM) in the absence or presence of warfarin. All ratios were calculated versus the control group on day 0. Relative gene expression levels were determined by normalizing measured values to those obtained for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (G3PDH). White bar: untreated cells, black bar: cells treated with 1 μM warfarin [WFN, (+)] only. Bars represent the mean ± SEM ($n = 3$). A significant difference ($P < 0.05$) when compared against untreated AS control cells is indicated with 'Hash'. **c**, **d** Quantification of Alizarin Red S staining of AS group HAVICs (**b**) and ALP activity (**c**) on day 21 in the presence or absence of BMP2, warfarin (WFN), or coumestrol (CO) at physiological Pi concentrations (1.0 mM). The amount of released dye was quantified by spectrophotometry at 550 nm following cetylpyridinium chloride extraction. All ratios were calculated versus the control group on day 0. White bars: untreated cells; brown bars: cells treated with 30 ng/mL BMP2 (+); black bars: cells treated with 1 μM WFN (+) only; cyan bars: cells treated with 10 μM CO (+) only; light brown bars: cells treated with 30 ng/mL BMP2 (+) and 10 μM coumestrol (+); green bars: cells treated with 30 ng/mL BMP2 (+) and 10 μM WFN (+); light green bars: cells treated with 30 ng/mL BMP2 (+), 1 μM warfarin (+) and 10 μM coumestrol (+). Bars represent the mean ± SEM ($n = 4$). A significant difference ($P < 0.05$) when compared with untreated control AS cells is indicated with 'Ash' and a significant difference ($P < 0.05$) when compared against AS cells treated with BMP2 and warfarin is indicated by 'Asterisk' (color figure online)

calcification of AS group HAVICs would provide a good model to investigate the interaction between warfarin and VK2.

To conclude, we have demonstrated that warfarin increases PXR activity to accelerate aortic valve calcification via the elevation of BMP2 expression and ALP activity. Our data contribute towards the understanding of the aortic valve calcification mechanism and could aid the future development of new therapies for the treatment and prevention of aortic valve stenosis.

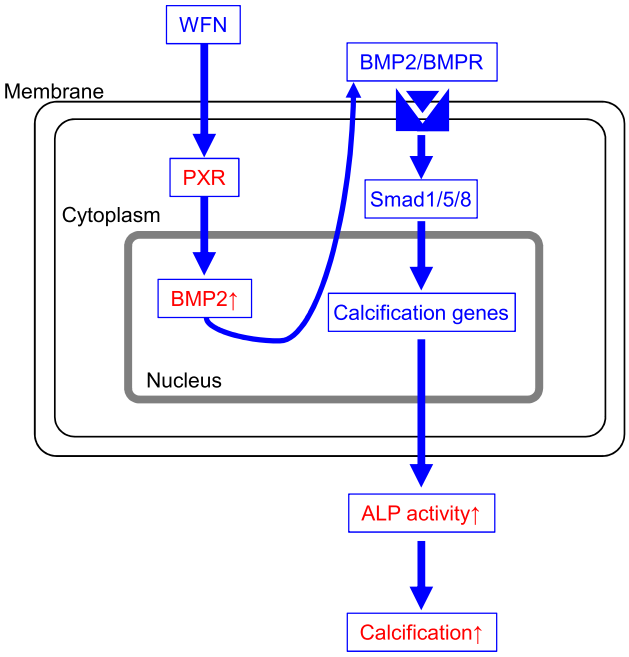


Fig. 5 Proposed mechanism of warfarin-induced calcification of AS group HAVICs. The filled arrows indicate the pathways supported by the data obtained in this study. Warfarin accelerates BMP2 gene expression via the translocation of PXR into the nucleus, which stimulates the expression of calcification genes. This results in ALP activation, which, via Smad1/5/8 phosphorylation, drives the calcification process (color figure online)

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

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