Azelnidipine Inhibits Msx2-Dependent Osteogenic Differentiation and Matrix Mineralization of Vascular Smooth Muscle Cells

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Summary

Vascular calcification is a critical role in vascular calcification. Calcium channel blockers (CCBs) have been shown to improve outcomes in atherosclerotic vascular disease, it remains unknown whether CCBs have an effect on the process of vascular calcification. Here we investigated whether CCBs inhibit osteogenic differentiation and matrix mineralization of vascular smooth muscle cells induced by Msx2, a key factor of vascular calcification. Human aortic smooth muscle cells (HASMCs) were transduced with adenovirus expressing MSX2 and were treated with 3 distinct CCBs. Azelnidipine, a dihydropyridine subclass of CCBs, significantly decreased alkaline phosphatase (ALP) activity of Msx2-overexpressed HASMCs, whereas verapamil and diltiazem had no effect. Furthermore, azelnidipine, but not verapamil and diltiazem, significantly decreased matrix mineralization of Msx2-overexpressing HASMCs. Azelnidipine significantly attenuated the induction of ALP gene expression by Msx2, a key transcription factor in osteogenesis, while it did not reduce enzymatic activity of ALP. Furthermore, azelnidipine inhibited the ability of Msx2 to activate the ALP gene, but had no effect on Notch-induced Msx2 expression. Given that L-type calcium channels are equally blocked by these CCBs, our results suggest that azelnidipine inhibits the Msx2-dependent process of vascular calcification by mechanisms other than inhibition of calcium channel activity. (Int Heart J 2012; 53: 331-335)

Key words: Calcification, Calcium channel blocker, Transcription factor, Atherosclerosis

Vascular calcification is commonly seen with aging, end-stage renal disease, diabetes, and atherosclerosis, and is closely associated with an increased risk of cardiovascular morbidity and mortality.1,2 Although vascular calcification has been considered to be a passive and unregulated process, accumulating evidence shows that it is an active and tightly regulated phenomenon that resembles bone development. In which vascular smooth muscle cells (VSMCs) undergo a phenotypic switch to osteoblastic phenotypes in response to a variety of osteogenic regulatory factors.3-5 Among these osteogenic regulatory factors, Msx2 is known as a key regulator of vascular calcification.6,7 Originally identified as a homeodomain transcription factor responsible for osteogenesis,8 Msx2 is now known to modulate the formation of medial artery calcification by producing the adventitial Wnt signal.9 Furthermore, we have recently reported that an Msx2-dependent Notch signaling pathway is also involved in the development of atherosclerotic/fibrocalcific calcification.10,11 Notch signaling, either independently or cooperatively with osteoinductive bone morphogenetic protein 2 (BMP2), activates the Msx2 gene in VSMCs, and induces osteogenic differentiation and mineralization. Furthermore, histochemical analysis of human specimens has suggested that Notch signaling is involved in the early phase of atherosclerotic/fibrocalcific calcification formation. Thus, we proposed that the Notch/Msx2-axis plays a critical role in vascular calcification.

Calcium channel blockers (CCBs) have been used worldwide mainly to treat patients with hypertension, and numerous clinical trials, such as PREVENT and ALLHAT, have provided evidence that CCBs improve the outcomes of these patients.12,13 One apparent mechanism of these beneficial effects is the blood pressure-lowering effect by CCBs through the blocking of L-type calcium channels in vascular smooth muscle cells (VSMCs). On the other hand, other mechanisms beyond a blood pressure-lowering effect, such as an anti-inflammatory or antioxidant effect, have also been reported, and this is known as the pleiotropic effect of CCBs.14 Notably, azelnidipine, a dihydropyridine (DHP)-based L-type CCB, has been reported to possess unique antiatherogenic properties; azelnidipine has a high affinity for tissues because of its high lipid solubility and exerts potent antioxidant effects. In fact, azelnidipine is reported to cause longer relaxation of smooth muscle cells and more enhanced NO production in endothelial cells than other CCBs.15,16 Furthermore, we have previously reported that azelnidipine down-regulates gene expression of molecular components of the renin-angiotensin-aldosterone system,17 suggesting a novel mechanism for the vasoprotective and renoprotective effect of azelnidipine. Despite the potential beneficial effect of azelnidipine on vascular cells, it has not yet been determined whether azelnidipine inhibits the osteogenic...
differentiation of VSMCs and further development of vascular calcification.

Here, we tested the hypothesis that azelnidipine inhibits the Msx2-dependent process of vascular calcification. We show that azelnidipine, but not other CCBs, inhibits osteogenic differentiation and mineralization of VSMCs overexpressing Msx2 or NICD, through an inhibition of the transactivating function of Msx2.

**METHODS**

**Drugs:** Verapamil, diltiazem, and SigmaFAST™ BCIP/NBT, a substrate for ALP, were purchased from Sigma-Aldrich. Azelnidipine was kindly donated by Daiichi-Sankyo Co., Ltd. Calf intestine ALP was purchased from CALZYM Laboratory.

**Cell culture:** Primary human aortic smooth muscle cells (HASMCs) were purchased from Kurabo. These cells were maintained as described previously, and cells from passage 7 to 15 were used.

**Measurement of alkaline phosphate (ALP) activity:** ALP activity of various cells was measured using LabAssay ALP (Wako Pure Chemical Industries), according to the manufacturer’s protocol. ALP activity was normalized to total protein determined with a Bio-Rad protein assay solution (Bio-Rad Laboratories).

**Von Kossa staining:** The cells were fixed with 4% formaldehyde. After fixation, they were exposed to 5% aqueous AgNO3. To induce mineralization in the HASMC culture (Figures 1C and 3C), culture media were supplemented with inorganic phosphate (Pi) so that the media Pi concentration was 1.8 mmol/L.

**Plasmid and generation of adenoviruses:** Msx2 expression plasmid was kindly provided by Dr. Nishimura of Osaka University School of Dentistry. Adenovirus encoding Msx2 was produced using the Gateway system (Invitrogen). Its protein expression was confirmed by Western blotting (data not shown). A control adenovirus (Ad-LacZ) and an adenovirus expressing the intracellular domain (ICD) of Notch1 (termed Ad-N1-ICD) were created as described elsewhere.

**RNA isolation, RT-PCR, and real-time PCR:** Total RNA was isolated from various cells using IsoGen reagent (Nippon Gene) and reverse-transcribed using an RT-PCR kit (Takara Biotech) according to the manufacturer’s protocol. Real-time PCR was performed with an Mx3000 instrument (Stratagene). The reaction was carried out using SYBR green master mix (Toyobo) according to the manufacturer’s protocol. The relative quantities of transcripts were determined using a standard curve and normalized against GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA. The gene-specific primers were as follows: human MSX2, forward 5'-AAGAAAACAGGGCTTGGTGCCCTC-3', reverse 5'-GGCCAGTTCCGTGCAGAAACAG

TA-3', mouse Msx2, forward 5'-TGGAAAAACAGGGCTTGGTGCCCTC-3', reverse 5'-GTCTATGGAAGGGTGAGGT-3'; GAPDH, forward 5'-ACCCAGTCCATGCACCATCAC

-3', reverse 5'-TCCACACCTGGTTGCTGTA-3'; human ALP, forward 5'-CCGGCTCAGAAAT

TCCTGATCT-3', reverse 5'-CCAAAACAGGAGAGTGCGTCTC-3'.

**Statistical analysis:** Data are expressed as the mean ± SEM. Multiple comparisons were carried out by 1-way ANOVA with post hoc correction. The Student t test was used for pairwise comparison between groups. For all analyses, values of P < 0.05 were considered significant.

**RESULTS**

Azelnidipine, but not verapamil and diltiazem, attenuated ALP activity in HASMCs: Msx2 is emerging as a critical transcription factor that regulates osteogenic differentiation of VSMCs and vascular calcification. Here we employed 3 different sub-classes of CCBs, azelnidipine, verapamil, and diltiazem, to determine whether these CCBs have an effect on Msx2-induced osteogenic conversion of HASMCs in vitro. To this end, we examined the effects of CCBs on alkaline phosphatase (ALP) activity, a marker of osteogenic differentiation of HASMCs as well as osteogenesis. First, we used BCIP/NBT, a substrate for ALP, to visualize ALP activity in HASMCs. As shown in Figure 1A, in the absence of CCBs, adenovirus-mediated overexpression of Msx2 strongly induced ALP activity in HASMCs. Interestingly, azelnidipine clearly abrogated Msx2-induced ALP activity of HASMCs, whereas neither verapamil (phenyl-
alkylamines) nor diltiazem (benzothiazepines) had a measurable effect (Figure 1A). In order to determine more precisely the effect of these CCBs on Mx2-overexpressing HASMCs, we next measured the ALP activity of these cells. Consistent with the results in Figure 1A, azelnidipine, but not verapamil or diltiazem, markedly inhibited Mx2-induced ALP activity of HASMCs (Figure 1B).

We next examined whether azelnidipine inhibits matrix mineralization, as well as ALP activity, of Mx2-overexpressing HASMCs. Similar to the results in Figures 1A and B, azelnidipine, but not verapamil and diltiazem, inhibited Mx2-induced matrix mineralization of HASMCs (Figure 1C). These results suggest that azelnidipine inhibits the Mx2-dependent process of osteogenic differentiation of HASMCs independent of Ca channel blockade because neither verapamil nor diltiazem had effects on this process.

Azelnidipine did not decrease enzymatic activity of ALP: Azelnidipine is known to have a higher affinity for tissues because of its high lipid solubility, and therefore azelnidipine is maintained in membrane longer than other CCBs and exerts longer inhibition of L-type Ca channel-dependent voltage. Given that ALP is an ectoenzyme that removes extracellular pyrophosphate, a strong inhibitor of vascular calcification, we hypothesize that azelnidipine in membranes of HASMCs directly interacts with extracellular ALP, thus attenuating its activity and protecting against vascular calcification. Therefore, we next examined the effect of azelnidipine on ALP activity prepared from calf intestine. However, azelnidipine had no effect, if any, on the enzymatic activity of prepared ALP either as visualized using BCIP/NBT (Figure 2A) or as determined by measurement of ALP activity (Figure 2B).

Azelnidipine selectively inhibits Mx2-induced ALP expression at transcription level: We have shown previously that azelnidipine down-regulates the gene expression of molecular components of the renin-angiotensin-aldosterone system. Similarly, Isaka, et al reported that azelnidipine exerts its potent inhibitory effects on the expression of the genes relevant to steroid synthetases such as CYP11-beta-1 and CYP11-beta-2...
and antiproliferation effects on VSMCs, have been described.

Discussion

In this study, we demonstrate that azelnidipine inhibits osteogenic differentiation of VSMC by suppressing the transactivating function of Mxs2, a key regulator of osteogenesis.

CCBs have been shown in numerous clinical trials such as PREVENT, ALLHAT, and VALUE to reduce cardiovascular events.\(^{12,13,14}\) The beneficial effect of CCBs is generally attributed to the lowering of blood pressure. However, many pleiotropic actions of CCBs beyond lowering of blood pressure, such as anti-inflammatory effects, antioxidant effects, and antiproliferation effects on VSMCs, have been described.\(^{15}\) Because these pleiotropic actions are largely due to a blockade of Ca\(^{2+}\) influx into VSMCs, and elevated extracellular Ca\(^{2+}\) alone is sufficient to accelerate matrix mineralization of VSMC in vitro,\(^{20}\) we first expected that L-type CCBs in general equally affect osteogenic conversion and matrix mineralization of VSMCs by inhibiting Ca\(^{2+}\) influx into VSMCs. Surprisingly, however, we found that azelnidipine, but not verapamil and diltiazem, inhibited Mxs2-dependent osteogenic differentiation and matrix mineralization of VSMCs (Figure 1). These findings led us to speculate that azelnidipine exerts its protective effect against Mxs2-induced vascular calcification independent of L-type calcium channel antagonism and resultant inhibition of Ca\(^{2+}\) influx into VSMCs.

Because azelnidipine is known to have a potent antioxidant effect, we examined whether it contributes to the inhibition of Mxs2-induced osteogenic differentiation and mineralization. Our results showed that the potent antioxidant apocynin had no effects on the Mxs2-mediated ALP induction (data not shown), suggesting that the ability of Mxs2 to induce ALP gene expression is not impaired by antioxidants.

Next, we tested the effects of azelnidipine on enzymatic activity of ALP, given that azelnidipine is lipophilic and likely to be highly maintained in VSMC membranes and thus has a potential to directly interact with ectoenzymes including ALP. Contrary to our assumption, azelnidipine did not inhibit the enzymatic activity of ALP (Figure 2). Instead, RT-PCR analysis showed that an increase in ALP mRNA levels by Mxs2 is abrogated by azelnidipine, suggesting that azelnidipine inhibits Mxs2-driven transcription of the osteogenic genes in VSMCs. More specifically, we can envisage that such inhibitory effects on osteogenic gene expression are independent of calcium channel antagonism because neither verapamil nor diltiazem abrogated transcription of the ALP gene (Figure 2). The effects of CCBs on gene expression independent of calcium channel blockade have been described. Recently, calcium-independent pathways were reported to participate in the effects of CCBs on the expression of several genes such as c-myb, c-fos, c-jun proto-oncogenes, and transcription factors in VSMCs.\(^{21,22}\) Obviously, further studies should be carried out to understand how azelnidipine inhibits Mxs2-dependent osteogenic differentiation and matrix mineralization of VSMCs.

Vascular calcification is histoanatomically classified into 4 types: atherosclerotic/fibrocalcific, cardiac valve, medial artery calcification, and vascular calciphylaxis.\(^{23}\) As for medial artery calcification, Towler, et al have shown that Mxs2 plays a central role in its formation.\(^{24}\) We have recently shown that Notch1 and Mxs2 are strongly expressed in human atherosclerotic lesions containing calcification, and that the Notch-Mxs2 axis induces osteogenic differentiation of VSMCs, either independently or cooperatively with BMP2. It is interesting to note that azelnidipine did not inhibit phosphate/Runx2-induced osteogenic differentiation and matrix mineralization of VSMCs (data not shown), which further confirms our hypothesis that inhibition of Mxs2-dependent transcription by azelnidipine is gene-specific.

The present study has several limitations. First, we used 1,000 nmol/L azelnidipine, which was much higher than the plasma concentration produced by the clinical dose (approximately less than 100 nmol/L). There is a general consensus that 1,000 nmol/L CCB is considerably high but this concentration is relevant to a clinical setting because the tissue concentration of azelnidipine is higher than that in the plasma due to its high lipid solubility.\(^{25}\) Second, we have not yet determined whether this effect is unique to azelnidipine, or if other third-generation DHP-based CCBs have similar effects. In particular, amlodipine is known to exert pleiotropic effects such as inhibition of platelet aggregation and stimulation synthesis,
which are distinct from calcium channel antagonism,\textsuperscript{14} and therefore its effect on the vascular calcification process should also be examined in future studies.

**Conclusions:** We showed that azelnidipine inhibited the Msx2-dependent process of osteogenic differentiation and matrix mineralization by specifically blocking the ability of Msx2 to induce the ALP gene expression. Studies to test whether azelnidipine might be beneficial for protection against vascular calcification as well as atherosclerosis should be warranted.

**References**