Calcification of Aortic Smooth Muscle Cells Isolated From Spontaneously Hypertensive Rats

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Abstract. Although hypertension and vascular calcification are well established as important risk factors for several cardiovascular diseases, the relationship between them is unknown. Here, we investigated whether hypertension is relevant to vascular calcification by examining aortic smooth muscle cells (SMCs) isolated from the descending thoracic aortas of Wistar Kyoto rats (WKY) as normotensive rats and spontaneously hypertensive rats (SHR), a typical rat model of hypertension. Cells were cultured in DMEM containing 10% FBS for 6 days after reaching confluence. Von Kossa staining revealed that the positively stained calcified area of aortic SMCs from SHR increased rapidly compared to that from WKY. The gene expressions of calcification-regulating proteins including msh homeobox homolog 2, Osterix (a master transcription factor for osteogenesis), and alkaline phosphatase (ALP) (a marker of vascular calcification) were significantly increased in aortic SMCs from SHR increased rapidly compared to that from WKY. The gene expressions of calcification-regulating proteins including msh homeobox homolog 2, Osterix (a master transcription factor for osteogenesis), and alkaline phosphatase (ALP) (a marker of vascular calcification) were significantly increased in aortic SMCs from SHR compared to SMCs from WKY. On the other hand, Runx2, another osteogenic transcription factor, did not upregulate. Furthermore, we confirmed that ALP activity was strongly increased in aortic SMCs from SHR compared to SMCs from WKY. These results suggest that aortic SMCs from SHR tend to become easily calcified via an Msx2-Osterix signaling pathway.

Keywords: calcification, aortic smooth muscle cell, spontaneously hypertensive rat, alkaline phosphatase, msh homeobox homolog 2

Introduction

Vascular calcification is a common finding in many cardiovascular diseases such as atherosclerosis, diabetes, chronic renal failure, aortic stenosis, and aortic aneurysm (1–3). Clinically, there are four different types of cardiovascular calcification, namely atherosclerotic calcification, medial artery calcification, cardiac valve calcification, and vascular calciphylaxis (4). Vascular calcification has been considered to be an end-stage process of “passive” mineral precipitation, which is associated with hyperphosphatemia (5). However, the mechanisms for vascular calcification in these clinical settings are still being elucidated (6).

Recent investigations have revealed that the mechanism of vascular calcification is similar to that of osteogenesis (7). Due to the presence of collagenous and noncollagenous proteins associated with bone mineralization in vascular smooth muscle cells (SMCs), vascular SMCs within developing atherosclerotic plaques play an important role in the deposition of extracellular matrix, resulting in vascular calcification (8). During vascular calcification, various vascular cells, including vascular SMCs, pericytes, fibroblasts, and macrophages, are transformed into osteoblast-like phenotypes, exhibit increased alkaline phosphatase (ALP) activity, and matrix vesicle formation, and show significant expressions of the genes for bone morphogenetic protein 2 (BMP2) and bone matrix proteins such as osteocalcin and osteopontin (9, 10). The gene for runt-related transcription factor 2 (Runx2), which is overexpressed in the presence of dexamethasone, a potent inducer of bone osteoblastic differentiation, is also expressed in bovine vascular SMCs (11).

Hypertension is known to be a risk factor for atherosclerosis (12, 13) and circulatory diseases such as cerebral apoplexy and heart attack. Severe atherosclerosis induces aortic calcification by increasing the arterial
rigidity (14, 15). Many researchers have examined the relationship between aortic calcification and hypertension. For example, calcification was found to be more common with hypertension in a retrospective study of 129 males with abdominal aortic aneurysms (16). On the other hand, a prospective open labeled study did not detect any correlations between aortic calcification and highly systolic or diastolic blood pressure (17, 18). However, there is controversy over whether hypertension predisposes the patient to aortic calcification (19).

In the present study, we isolated aortic SMCs from spontaneously hypertensive rats (SHR), a typical animal model for hypertension and compared their calcification areas and gene expressions of calcification-regulating proteins with those of SMCs from Wistar Kyoto rats (WKY) as normotensive rats by culturing the cells in DMEM containing 10% FBS for 6 days to clarify whether hypertension induces calcification in vascular tissues.

Materials and Methods

Materials

Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo). L-Glutamine was purchased from Nacalai Tesque, Inc. (Kyoto). Fetal bovine serum (FBS; Biofluids®) and Penicillin & Streptomycin (Gibco®) were obtained from Invitrogen Co. (Carlsbad, CA, USA). 2-Mercaptoethanol and sodium hydrogen carbonate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Reagents for reverse transcription reactions (Random Primer, 10 mM dNTP Mix, 5× First-strand Buffer, 0.1 M DTT, M-MLV RT, and DNase-, RNase-free distilled water) were purchased from Invitrogen Co. Ribonuclease inhibitor was obtained from TaKaRa Bio, Inc. (Shiga). All primers used for quantitative real-time PCR were obtained from Fasmac Co., Ltd. (Kanagawa). Power SYBR® Green PCR Master Mix was supplied by Applied Biosystems (Foster City, CA, USA). All chemicals used were of the highest purity commercially available. All solutions were made fresh at sufficiently high concentrations so that only very small aliquots had to be added to the culture medium.

Male WKY and SHR at 9 weeks of age were purchased from Charles River Japan, Inc. (Shizuoka). All experiments were performed according to the Animal Experiment Guidelines of the Laboratory of Animal Experiments for Hirosaki University School of Medicine. The animals were maintained for 1 week at a temperature of 22 ± 2°C and relative humidity of 57% on a 12-h light / 12-h dark (lights on 08:00 – 20:00) schedule and had free access to water and food.

Cell culture

At 10 weeks of age, the animals were anesthetized with ether. Aortic SMCs were obtained from the descending thoracic aorta by collagenase digestion as described by Chamley et al. (20). The culture medium used was DMEM (9.5 g/L) containing L-glutamine (0.584 g/L), 10% FBS, 10 U/L penicillin, and 10 µg/L streptomycin. Cells from passages 3 to 5 were used for all experiments. The cells were cultured at 37°C under 5% CO₂, and the medium was replaced with fresh medium every 3 days.

Total RNA preparation and cDNA synthesis

Total RNA was extracted using an RNeasy mini kit (Qiagen, Tokyo). After resuspension of the purified total RNA in RNase-free water, aliquots containing 1 µg of total RNA were used as a template for the reverse transcription reaction. To check the quality and concentration of the total RNA, 1 µl was directly analyzed using an RNA 6000 Nano Labchip Kit (Agilent Technology, Palo Alto, CA, USA).

Reverse transcription was carried out in a 14-µl reaction mixture containing 1 µg of total RNA and 10 nmol of dNTP Mix. After denaturation at 65°C for 5 min, the reaction mixture was cooled on ice for 2 min, and 200 U of M-MLV reverse transcriptase, 200 nmol of DTT, and 2 U of ribonuclease inhibitor were added to the reaction mixture. Extension was carried out at 37°C for 60 min and then 75°C for 15 min.

Von Kossa staining of aortic SMCs from SHR and WKY

After reaching confluence (day 0), cultured aortic SMCs from WKY and SHR were cultured in DMEM containing 10% FBS for 3 or 6 days. Calcium precipitation in the aortic SMCs was assessed by von Kossa staining. Briefly, the matrix was washed with phosphate-buffered saline (PBS) and incubated with 10% formalin in PBS for 30 min. After three washes with purified water, cultures were treated with 5% silver nitrate solution in the aortic SMCs was assessed by von Kossa staining. Briefly, the matrix was washed with phosphate-buffered saline (PBS) and incubated with 10% formalin in PBS for 30 min. After three washes with purified water, cultures were treated with 5% silver nitrate solution at room temperature for 30 min. The excess silver nitrate solution was completely washed away using purified water and the culture plates were exposed to sodium carbonate/formaldehyde solution for 5 min to allow color development. The von Kossa-stained areas were viewed by light microscopy and analyzed colorimetrically using the Image J software.

Quantitative real-time PCR

For real-time PCR reactions, the total reaction volume was adjusted to 26 µl and contained 3 µl of a 1:4 dilution of the first-strand reaction product, 1 µl of 10 µM specific forward and reverse primers, 8 µl of pure water and 13 µl of SYBR Green. Amplification and analysis...
of cDNA fragments were carried out using an ABI Prism 7700 (Applied Biosystems). The primers used for ALP, Msx2, Osterix, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were designed by Applied Biosystems (TaqMan® Probe & Primer Design) and are shown in Table 1. The cycling conditions were initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing/primer extension at 60°C for 1 min.

Amplification of the housekeeping gene G3PDH served as a normalization standard. The mRNA levels were measured as cycle threshold (Ct) levels and normalized by the individual G3PDH control Ct values. Relative gene expression was obtained by the ∆∆Ct method (∆Ct sample − ∆Ct G3PDH) for comparisons of the gene expression levels in each sample. The conversion between ∆∆Ct and the relative gene expression level was the fold induction (2−∆∆Ct) (21). Induction of gene expression was indicated as the fold increase compared to aortic SMCs from WKY at day 0.

Assay of ALP activity

After reaching confluence (day 0), aortic SMCs from WKY and SHR were cultured in DMEM containing 10% FBS for 6 days. ALP activity staining was performed with a TRACP & ALP Double-stain Kit (TaKaRa Bio). Positive ALP activity was characterized by the development of a violet color. The stained areas were analyzed colorimetrically using the Image J software.

Statistics

Multiple group comparisons were performed by one-way ANOVA, followed by Tukey’s procedure for comparisons of means. All data were expressed as the mean ± S.E.M., and differences with P<0.05 were considered to be significant.

Results

Calcium precipitation in cultured aortic SMCs

As shown in Fig. 1A, aortic SMCs from both SHR and WKY were weakly and similarly stained at day 0. After 6 days of culture, the number of nodules progressed markedly in aortic SMCs from SHR compared to SMCs from WKY, indicating an increase in calcium precipitation. The calcification areas of aortic SMCs from both WKY and SHR were increased at 3 days (Fig. 1B). After 6 days of culture, the calcification area was significantly larger in aortic SMCs from SHR compared to SMCs from WKY (Fig. 1B).

Analysis of the gene expressions of calcification-regulating proteins by quantitative real-time PCR

The gene expressions of calcification-regulating proteins just after aortic SMCs from WKY and SHR reached confluence were defined as the basal expression levels (day 0). The gene expressions of Msx2 and Osterix were significantly upregulated by 4.1- and 1.9-fold, respectively, in aortic SMCs from SHR at day 6 compared to the corresponding levels at day 0. On the other hand, the gene expression of Runx2 was decreased by 0.6-fold in aortic SMCs from SHR at day 6 compared to the corresponding levels at day 0. The gene expressions of Msx2 and Osterix were significantly upregulated by 4.1- and 1.9-fold, respectively, in aortic SMCs from SHR at day 6 compared to the corresponding levels at day 0. On the other hand, the gene expression of Runx2 was decreased by 0.6-fold in aortic SMCs from SHR at day 6 compared to the corresponding levels at day 0 (Fig. 2: A – C). In contrast, neither of these genes showed increased expression in aortic SMCs from WKY after 6 days of culture (Fig. 2: A – C). ALP gene expression in aortic SMCs from SHR at day 0 was 0.5-fold lower than the expression in SMCs from WKY, but increased by 2.1-fold after 6 days of culture (Fig. 3).

ALP activity

We further investigated whether the calcification of aortic SMCs from SHR was accelerated during 6 days of culture in DMEM containing 10% FBS by measuring the ALP activity, a typical marker of vascular calcifica-
Calcification of aortic SMCs from SHR was visualized by the development of a violet color. The ALP activity in aortic SMCs from SHR was strongly increased after 6 days of culture compared to the activity in SMCs from WKY (Fig. 4A). After 6 days of culture, the positively stained area for ALP activity in aortic SMCs from SHR was significantly increased compared to the corresponding area at day 0 (Fig. 4B).

**Discussion**

According to previous reports in the literature, vascular vessels in SHR exposed to some condition or treatment show a tendency to become easily calcified. One study reported that SHR fed a magnesium-free diet for 2 months (hypomagnesemic rats) showed higher calcification.
mean arterial pressure and widespread myocardial and renal tissue calcification (22). Another report showed that WKY, but not SHR, tolerated vitamin D intoxication (23). In the latter study, myocardial remodeling and aortic calcification occurred in SHR in response to vitamin D. These reports suggest that SHR have sensitivity towards various substances that result in calcification. However, the relationship between hypertension and vascular calcification remains to be clarified. In the present study, calcification of SMCs from the descending thoracic aortas of SHR was spontaneously accelerated by culture in DMEM containing 10% FBS for 6 days after the cells reached confluence. In our additional study, it was confirmed that the calcification was not induced in DMEM containing 1% FBS for 6 days after the cells reached confluence (unpublished observation). One possible explanation for this observation is that FBS contains some factor inducing calcification of aortic SMCs from SHR. Nonetheless, this is the first study to reveal that aortic SMCs from SHR without any calcification stimulus can easily become calcified compared to those from WKY. Calcification of the blood vessel in SHR may occur due to the tendency of SMCs to become easily calcified. However, it has not been confirmed whether this phenomenon is caused by hypertension. Some clinical reports have suggested the relationship between aortic calcification and hypertension. Allison et al. (24) demonstrated an association between hypertension and distal aortic calcification using multi-variable logistic regression. Kimura et al. (25) showed that there is an association between abdominal aortic calcification and high systolic blood pressure in patients on hemodialysis. Tsakiris et al. (26) reported that the prevalence of aortic calcification in essential hypertension is considerably higher compared to the general population. Essential hypertension seems to contribute to the concurrent appearance of aortic calcification. Comparative in vivo and in vitro investigations on the calcification of aortic SMCs between SHR rats and other hypertensive rats may provide further information.

After reaching confluence, vascular SMCs have been reported to escape from a dedifferentiated state (growth state) into a differentiated state or other phenotypes (27). We confirmed that gene expression of α-smooth muscle actin, a marker of vascular SMC differentiation, was increased during 6 days of culture after the cells reached confluence (unpublished observation). Calcification of aortic SMCs from SHR was accelerated after the cells reached confluence. These findings suggest that a phenotypic change or differentiation of aortic SMCs from SHR into osteoblast-like cells occurred and that this may be a trigger of calcification.

Along with mineralized nodule formation, aortic SMCs from SHR exhibited high expressions of osteogenic genes, that is, Msx2, Osterix, and ALP, compared to the expressions in SMCs from WKY. ALP activity was also strongly accelerated in aortic SMCs from SHR. Osterix was first identified as a transcript induced by BMP2 (a key regulator of osteoblast progenitors) and is an osteoblast-specific transcription factor required for ALP-dependent bone formation (28). BMP2 accelerates both Msx2 and Runx2, and Osterix is upregulated by Msx2 but not Runx2, in aortic SMCs (29). In this study,
Runx2 was not upregulated in aortic SMCs from SHR by 6 days of culture after the cells reached confluence. Therefore, the spontaneous calcification of aortic SMCs from SHR may be regulated via an Msx2-Osterix-ALP pathway.

In conclusion, aortic SMCs from SHR show a high potential to become calcified. During spontaneous calcification after the cells reached confluence, osteogenic genes such as Msx2, Osterix, and ALP were significantly upregulated. The calcification of aortic SMCs from SHR may be regulated through an Msx2-Osterix-ALP pathway. Further studies on the upstream of Msx2 signaling and comparative investigations of the calcification of aortic SMCs between SHR rats and other hypertensive rats may provide new insights into the contribution of hypertension to vascular calcification.

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