Biochemical and Biophysical Research Communications 391 (2010) 1087–1092

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Tumor necrosis factor-α increases alkaline phosphatase expression in vascular smooth muscle cells via MSX2 induction

Hye-Lim Lee, Kyung Mi Woo, Hyun-Mo Ryoo, Jeong-Hwa Baek *

Department of Cell and Developmental Biology, School of Dentistry and Dental Research Institute, Seoul National University, Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 1 December 2009
Available online 14 December 2009

Keywords:
Vascular smooth muscle cells
Tumor necrosis factor-α
MSX2
NF-κB
Alkaline phosphatase

ABSTRACT

Vascular calcification is implicated in many diseases including atherosclerosis and diabetes. Tumor necrosis factor-α (TNF-α) has been shown to promote vascular calcification both *in vitro* and *in vivo*. However, the molecular mechanism of TNF-α-mediated vascular calcification has not yet been fully defined. Therefore, in this study, we aimed to investigate whether MSX2 acts as a crucial regulator in TNF-α-induced vascular calcification and to define the regulatory mechanism of MSX2 induction in human vascular smooth muscle cells (VSMCs). TNF-α increased the expression of osteogenic marker genes including RUNX2, osterix, alkaline phosphatase (ALP), and bone sialoprotein, and it also promoted matrix mineralization in VSMCs. In addition, TNF-α enhanced MSX2 expression in a dose- and time-dependent manner. MSX2 over-expression alone induced ALP expression, whereas knockdown of MSX2 with small interfering RNA completely blocked TNF-α-induced ALP expression. New protein synthesis was dispensable for MSX2 induction by TNF-α, and the inhibition of NF-κB by BAY-11-7082 or by dominant negative IκBα abolished the TNF-α-directed induction of MSX2 expression. However, inhibition of NADPH oxidase did not affect MSX2 expression. In conclusion, our study suggests that TNF-α directly induces MSX2 expression through the NF-κB pathway, which in turn induces expression of ALP, a key molecule in mineralization, in VSMCs.

Introduction

Vascular calcification is commonly observed in many diseases such as diabetes, end-stage renal disease (ESRD), and advanced atherosclerosis [1]. Among several types of vascular calcification, medial calcification is a characteristic feature of diabetes and ESRD and is considered a significant predictor of morbidity and mortality in cardiovascular disease [1–3]. Vascular calcification has been shown to be regulated by a variety of factors including inflammatory cytokines, bioactive peptides, and oxidized lipids [4,5]. Tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine, contributes to the calcification of vascular smooth muscle cells [6]. It has been demonstrated that TNF-α promotes *in vitro* vascular calcification and induces atherosclerotic lesions in coronary arteries *in vivo* [7,8]. However, the molecular mechanism of TNF-α-directed vascular calcification remains unclear.

Vascular calcification is similar to the process of bone mineralization. Recent studies showed that factors regulating bone mineralization, such as bone morphogenetic protein-2 and transforming growth factor-β, are expressed and play important roles in calcified vascular regions [9,10]. Vascular smooth muscle cells (VSMCs), which comprise the tunica media, play an important role in vascular calcification and express noncollagenous bone-associated matrix proteins such as osteopontin and osteocalcin [11,12].

MSX2 encodes a homeobox transcription factor that is involved in the regulation of osteoblast proliferation and differentiation [13]. MSX2 acts as a lineage switching regulator, since it drives osteogenic differentiation but suppresses adipogenic differentiation in multipotent mesenchymal progenitor cells [14]. Interestingly, a recent study showed that Msx2 promotes aortic valve and medial calcification *in vivo* by creating an osteogenic environment through the induction of the Wnt signaling pathway. [15]. Msx2-expressing cells up-regulate the expression of multiple canonical Wnt ligands, down-regulate the antagonist Dikkopf homologue-1 and enhance aortic canonical Wnt signaling. A subsequent study performed by the same group showed that TNF-α regulates the aortic Msx2-Wnt calcification cascade that contributes to aortic mineralization in diabetic mice [16]. However, the molecular mechanism of TNF-α action has not yet been clearly defined.

Therefore, in this study, we aimed to investigate whether MSX2 acts as a crucial regulator in TNF-α-induced vascular calcification and to define the regulatory mechanism in terms of signal transduction. In the present study, we showed that TNF-α directly pro-

* Corresponding author. Address: Department of Cell and Developmental Biology, School of Dentistry, Seoul National University, 28 Yeongun-dong, Jongno-gu, Seoul 110-749, Republic of Korea. Fax: +82 2 741 3193.
E-mail address: baekjh@snu.ac.kr (J.-H. Baek).

0006-291X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2009.12.027

motes MSX2 expression through the NF-κB pathway, which in turn induces the expression of alkaline phosphatase (ALP), a key molecule in mineralization, in VSMCs.

Materials and methods

Materials. Recombinant human TNF-α was purchased from R&D Systems (Minneapolis, MN, USA). The easy-BLUE™ and *Star*Taq™ reagents were from iNtRON Biotechnology (Sungnam, Korea), the AccuPower RT-PreMix was from Bioneer (Daejeon, Korea), and the SYBR *Premix Ex Taq*™ was purchased from TaKaRa (Otsu, Japan). PCR primers were synthesized by TaKaRa Korea (Seoul, Korea). Anti-NF-κB RelA and anti-lamin B antibodies as well as goat anti-rabbit horseradish peroxidase (HRP)-conjugated IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The NE-PER Nuclear and Cytoplasmic Extraction Reagent was from PIERCE Biotechnology (Rockford, IL, USA). The Alkaline Phosphatase staining kit, CALCIUM assay kit, cycloheximide, diphenyleneiodonium (DPI), and anti-Msx2 antibody were purchased from Sigma (St. Louis, MO, USA) and the BAY-11-7082 reagent was from Calbiochem (Gibbstown, NJ, USA). The Cell Counting Kit-8 (CCK-8) used to assay cell proliferation and cytotoxicity was from Dojindo Molecular Technologies (Gaithersburg, MA, USA). The dominant negative (*dn*) IκBα construct was a kind gift from Prof. H. Kim at Wonkwang University in Korea.

Cell culture and induction of osteogenic differentiation. Primary human VSMCs (ATCC CRL-1999) were maintained in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate. To induce osteogenic differentiation, sub-confluent vascular smooth muscle cells were cultured with differentiation medium consisting of growth medium supplemented with 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid.

ALP staining. VSMCs were seeded at a density of 5×10^5 cells per well in a four-well plate and cultured in differentiation medium for seven days. At the end of the culture period, the cells were fixed and subjected to ALP staining using an Alkaline Phosphatase staining kit.

RNA extraction, reverse transcription PCR (RT-PCR), and real-time PCR. Semi-quantitative RT-PCR or real-time PCR was performed to evaluate mRNA expression. Total RNA was isolated using easy-BLUE™ RNA Extraction Reagents. Complementary DNA was synthesized from 1 μg of total RNA using the AccuPower RT-PreMix and was subsequently used for PCR amplification. Semi-quantitative RT-PCR was performed using *Star*Taq™ in the range of linear amplification and the PCR products were electrophoresed on a 1.2% agarose gel and visualized under UV light by ethidium bromide staining. Real-time PCR was performed using the SYBR *Premix Ex Taq*™ and an AB 7500 Fast Real-Time system (Applied Biosystems; Foster City, CA, USA). Each sample was analyzed in triplicate, and target genes were normalized to the reference housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Fold differences were then calculated for each treatment group using normalized C_T values for the control.

Human genes and their primer sequences for semi-quantitative RT-PCR were as follows: MSX2-forward (f) 5′-CCTTTACCACATCCCAGCTC-3′, MSX 2-reverse (r) 5′-GTGGCATAGAGTCCCACAGG-3′; ALP-f 5′-CGTGGAACATTGATCTG-3′, ALP-r 5′-CCAAACAGGAGAGTCGCTTC-3′; OSTERIX (OSX)-f 5′-GCAGCTAGAAGGGAGTGGTG-3′, OSX-r 5′-GCAGGCAGGTGAACTTCTTC-3′; RUNX2-f 5′-GGTACCAGATGGGACTGTGG-3′, RUNX2-r 5′-GAGGCGGTCAGAGAACAAAC-3′; bone sialoprotein (BSP)-f 5′-CAGAGGCTCACTCCCTTGAG-3′, BSP-r 5′-CTTCTTGGGAAGCTGGATTG-3′; smooth muscle α-actin (ACTA2)-f 5′-GGCACCACTGAACCCTAAGG-3′, ACTA2-r 5′-TCTCCAGAGTCCAGCACAAT-3′; GAPDH-f 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′, and GAPDH-r 5′-CATGTGGGCCATGAGGTCCACCAC-3′.

Primer sequences used in real-time PCR were as follows: MSX2-f 5′-GCCATTTTCAGCTTTTCCAG-3′, MSX2-r 5′-CCCTGAGGAAACAAGACC-3′; ALP-f 5′-ACCATTCCCACGTCTTCACATTTG-3′, ALP-r 5′-AGACATTCTCTCGTTCACCGCC-3′; GAPDH-f 5′- CCATCTTCCAGGAGCGAGATC-3′, and GAPDH-r 5′- GCCTTCTCCATGGTGGTGAA-3′.

Nuclear extract preparation and Western blot analysis. After appropriate treatment, VSMCs were washed with phosphate-buffered saline (PBS) and scraped into lysis buffer consisting of 10 mM Tris–Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 0.2 mM sodium orthovanadate, 1 mM PMSF, 1 μg/ml aprotinin, 1 μM leupeptin, and 1 μM pepstatin, and they were sonicated briefly. Nuclear extracts were then prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions. Protein concentrations were determined using a modified Bradford method. Each sample containing equal amounts of protein was subjected to SDS–PAGE. The proteins separated in the gel were subsequently electro-transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with the relevant primary antibody, followed by incubation with the HRP-conjugated secondary antibody. Immune complexes were visualized using Supex reagent and luminescence was detected with a LAS1000 (Fuji PhotoFilm; Tokyo, Japan).

Quantification of calcium deposition. The calcium content deposited in a calcified matrix was determined by using a CALCIUM assay kit. Briefly, VSMCs were plated at a density of 5×10^5 cells per well in a four-well plate and cultured for 21 days in differentiation medium. At the end of the culture period, the cells were washed with calcium- and magnesium-free PBS and the calcified matrix was decalcified with 0.6 M HCl for 24 h. A calcium reagent working solution was then added to each sample according to manufacturer's instructions and the absorbance was measured at 575 nm using a microplate reader.

MSX2 knockdown using small interfering RNA (siRNA). MSX2 *si*RNA and control *si*RNA (ON-TARGET*plus* Non-targeting *si*RNA #2 D-001810-02-05) were purchased from Dharmacon (Chicago, IL, USA). Transfection into VSMCs was performed according to the manufacturer's instructions.

Results and discussion

TNF-α enhances the osteogenic differentiation of VSMCs

Because TNF-α has been shown to promote the calcification of vascular cells *in vitro* [7,17], we first examined whether TNF-α enhanced osteogenic marker gene expression in human VSMCs. For this purpose, VSMCs were cultured in osteogenic differentiation medium in the presence or absence of TNF-α (10 ng/ml) for 14 days. Semi-quantitative RT-PCR results showed that TNF-α increased the mRNA expression of osteogenic marker genes such as RUNX2, OSX, ALP, and BSP and decreased that of ACTA2, a phenotypic marker of VSMCs (Fig. 1A). Cytochemical staining of ALP also showed that TNF-α up-regulated ALP activity in VSMCs (Fig. 1B). These results suggest that TNF-α promotes the phenotypic conversion of VSMCs to osteoblasts in an osteogenic environment. To further examine the osteoblastic characteristics of these cells, calcium deposition in the extracellular matrix was measured after 21 days of culture in differentiation medium. Consistent with the RT-PCR and ALP staining data, TNF-α significantly increased calcium deposition in the extracellular matrix (Fig. 1C). These data suggest that TNF-α promotes the calcification of VSMCs by enhancing the expression of osteoblastic genes.

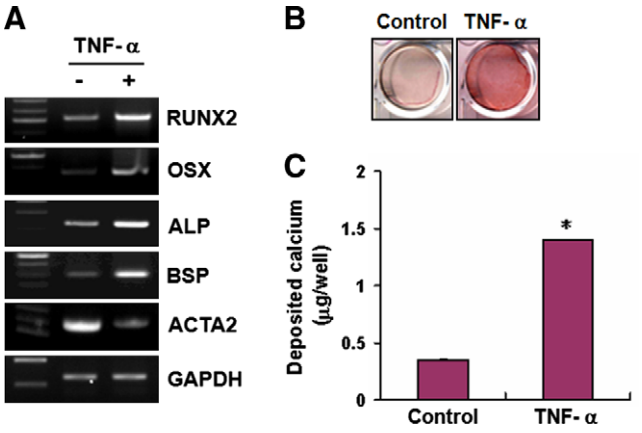

Fig. 1. TNF-α enhances the differentiation of vascular smooth muscle cells under osteogenic stimulation. Vascular smooth muscle cells (VSMCs) were cultured in osteogenic differentiation medium for 14 days (A), 7 days (B), or 21 days (C) in the presence or absence of TNF-α (10 ng/ml). Semi-quantitative RT-PCR (A), ALP staining (B) and measurements of calcium deposition (C) were performed after the culture period. OSX, osterix; ALP, alkaline phosphatase; BSP, bone sialoprotein; ACTA2, smooth muscle α-actin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; * $p < 0.01$ (Student's *t*-test).

TNF-α increases MSX2 expression in VSMCs

Because MSX2 has been shown to play an important role in vascular calcification [15,16], we aimed to investigate whether the TNF-α-induced osteogenic differentiation of VSMCs involves enhanced MSX2 expression. VSMCs were treated with increasing concentrations of TNF-α for 24 h, after which MSX2 mRNA expression levels were analyzed by RT-PCR. TNF-α increased MSX2 mRNA expression in a dose-dependent manner (Fig. 2A). Increases in MSX2 mRNA expression occurred concomitantly with an increase in ALP expression after TNF-α treatment at both 12 and 24 h (Fig. 2B). Nuclear expression of MSX2 protein was also up-regulated by TNF-α treatment, a finding consistent with the RT-PCR results (Fig. 2C). These results show that TNF-α enhances the expression of MSX2 as well as that of osteogenic marker genes in VSMCs.

TNF-α-induced ALP expression requires MSX2 expression

ALP is an osteoblast differentiation marker and is required for bone matrix mineralization [18]. It has been also implicated in bovine vascular smooth muscle cell calcification and in aortic valvular calcification [11,19]. Because the above results suggest that there is a possible correlation between TNF-α-induced MSX2 and ALP mRNA expression, we next examined whether there is some hierarchical relationship between MSX2 and ALP in TNF-α-induced responses. We treated VSMCs with TNF-α in the presence of cycloheximide, a protein synthesis inhibitor, and then examined MSX2 and ALP mRNA expression. Cycloheximide did not affect TNF-α-induced MSX2 expression but it almost completely abrogated ALP expression (Fig. 2D). This result implies that new protein synthesis is required for the TNF-α-induced expression of ALP but not MSX2, and that ALP may be downstream of MSX2 in this pathway.

To determine whether MSX2 is required for ALP expression in VSMCs, we first observed the effect of MSX2 over-expression. When MSX2 was over-expressed, ALP was highly expressed even in the absence of TNF-α (Fig. 3A). In cells over-expressing MSX2, TNF-α treatment exerted little inducing effect on ALP expression, suggesting that over-expressed MSX2 alone is sufficient to induce ALP expression in VSMCs (Fig. 3A). This result indicated that TNF-α-induced increases in MSX2 expression may be responsible for the up-regulation of ALP mRNA expression by TNF-α.

To further define the correlation between MSX2 and ALP expression induced by TNF-α, we examined the expression levels of ALP after MSX2 knockdown by *si*RNA. VSMCs were transfected with *siMSX2* or non-targeting control *si*RNA and then treated with TNF-α. To confirm the efficiency of *siMSX2*, we examined the levels of MSX2 mRNA and protein expression. Western blot and real-time PCR results showed that *siMSX2* clearly suppressed both basal and TNF-α-induced MSX2 expression (Fig. 3B and C *left panel*). Furthermore, TNF-α-induced ALP expression was also completely blocked by MSX2 knockdown (Fig. 3C *right panel*). These results showed that TNF-α-induced ALP expression is dependent on MSX2 expression, suggesting that MSX2 is the newly synthesized protein required for TNF-α-induced ALP expression, as shown in Fig. 2D. It is still unclear whether MSX2 directly up-regulates ALP expression or not. It has been reported that the mouse ALP promoter contains

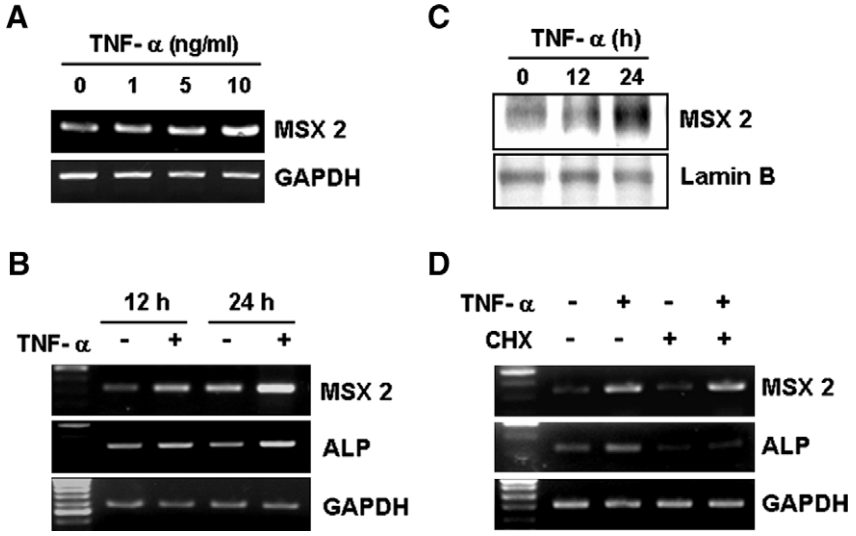

Fig. 2. TNF-α up-regulates MSX2 expression in VSMCs. (A) The cells were cultured in differentiation medium for 24 h in the presence of TNF-α at the concentrations indicated. (B, C) VSMCs were cultured in differentiation medium for the times indicated in the presence of TNF-α (10 ng/ml). At the end of culture period, semi-quantitative RT-PCR (A, B) or immunoblot analysis (C) was performed. Nuclear lamin B was used as a loading control. (D) VSMCs were incubated for 24 h in the presence of the indicated reagents. CHX, 10 μg/ml cycloheximide; TNF-α, 10 ng/ml. Semi-quantitative RT-PCR showed that CHX treatment almost completely abrogates the expression of ALP mRNA, indicating that TNF-α-induced ALP expression requires new protein synthesis.

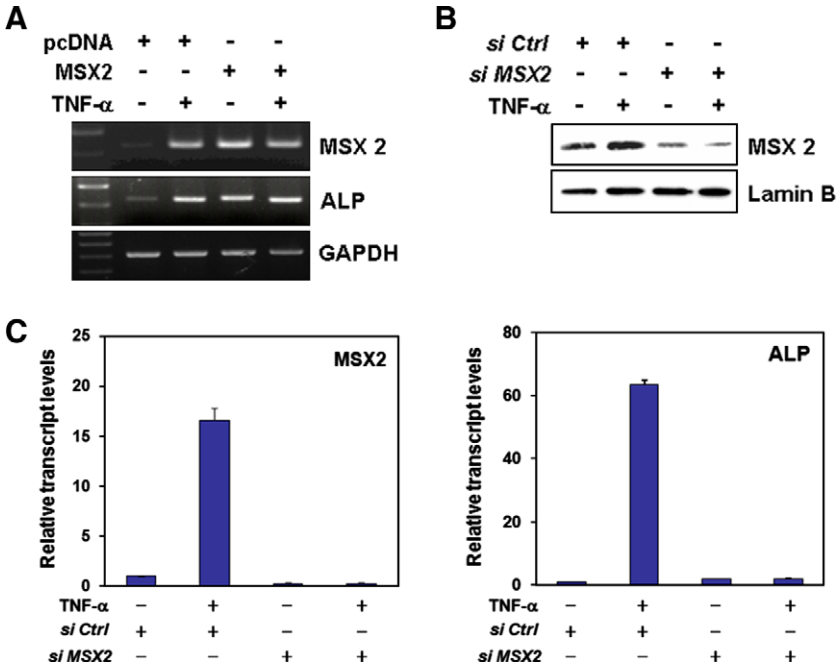

Fig. 3. MSX2 expression is required for TNF-α-induced ALP expression. (A) MSX2 over-expression is sufficient to induce ALP expression. After transfection of cells with an empty (pcDNA) or MSX2 expression vector, the cells were treated with TNF-α for 24 h. Total RNA was extracted and semi-quantitative RT-PCR was performed. (B, C) TNF-α-induction of ALP expression was completely blocked by MSX2 knockdown. For the cellular knockdown of MSX2, VSMCs were transfected with MSX2 *si*RNA (*siMSX2*) or control *si*RNA (*siCtrl*). The cells were then further incubated with or without TNF-α for 24 h, and immunoblot analysis (B) or real-time PCR (C) was performed. MSX2 knockdown was verified with both mRNA and protein levels.

an Msx2-responsive element and that Msx2 suppresses ALP expression in murine osteoblastic cell lines [20]. However, other studies have shown that *in vivo* over-expression of Msx2 promotes both bone formation and vascular calcification via canonical Wnt signaling [16,21], and that canonical Wnt signaling increases ALP activity [22]. Because our results present that MSX2 over-expression increases ALP expression in VSMCs, it is likely that MSX2 stimulates ALP expression indirectly via WNT signaling.

TNF-α-induced MSX2 expression occurs via the NF-κB pathway

To clarify the link between the activation of the TNF receptor and MSX2 expression, we investigated TNF-α-activated downstream signaling pathways. TNF-α mainly activates mitogen-activated protein kinases and the NF-κB pathway. Because our previous study revealed that c-Jun-N-terminal kinase activation was not involved in TNF-α-induced Msx2 expression in bone cells (unpublished data), we focused on the NF-κB pathway in this study. BAY-11-7082, a potent NF-κB inhibitor, was used to functionally block the NF-κB pathway. At a concentration of 10 μM, BAY-11-7082 did not affect the proliferating activity of VSMCs (Supplementary Fig. 1). NF-κB inhibition by BAY-11-7082 dramatically suppressed the TNF-α-induced expression of both MSX2 and ALP (Fig. 4A). Immunoblot analysis also confirmed that NF-κB inhibition completely suppressed TNF-α-induced MSX2 expression (Fig. 4B). In cells over-expressing MSX2, ALP expression was maintained despite the inhibition of NF-κB (Fig. 4A), suggesting that NF-κB activation is required for TNF-α-induced MSX2 expression in VSMCs but not directly associated with ALP transcription. To further provide evidence of NF-κB involvement in TNF-α-induced MSX2 expression, *dn*IκBα was over-expressed in VSMCs. Over-expression of *dn*IκBα completely inhibited TNF-α-induced NF-κB RelA nuclear translocation (Fig. 4D). RT-PCR and immunoblot results showed that TNF-α-induced MSX2 expression was abolished when NF-κB was sequestered in the cytoplasm by *dn*IκBα (Fig. 4C). ALP expression was also suppressed by *dn*IκBα. Taken together, these results demonstrate the involvement of the NF-κB pathway in TNF-α-induced MSX2 expression.

Because TNF-α is also known as a trigger signal for NADPH oxidase-dependent production of reactive oxygen species (ROS) and some studies have shown that NF-κB activation is downstream of NADPH oxidase [23–25], we investigated whether NADPH oxidase and/or ROS are involved in TNF-α-induced MSX2 expression in VSMCs. First of all, we observed whether TNF-α enhances ROS production in VSMCs. Over the course of 12 h of incubation, we could not observe any increase in ROS production with TNF-α treatment (Supplementary Fig. 2A). To check whether NADPH oxidases are working in these cells, we incubated VMSCs in the presence of DPI (10 μg/ml), an NADPH oxidase inhibitor. DPI significantly reduced basal ROS production and further suppressed ROS levels in the presence of TNF-α (Supplementary Fig. 2A). Real-time PCR and immunoblot analysis showed that DPI did not affect TNF-α-induced MSX2 expression (Supplementary Fig. 2B and C). These data indicate that TNF-α does not facilitate NADPH oxidase-dependent ROS generation under these experimental conditions and that TNF-α activation of the NF-κB pathway is independent of the NADPH oxidase-ROS system in VSMCs.

In this study, we demonstrated that TNF-α directly up-regulates MSX2 via the activation of NF-κB in VSMCs. To our knowledge, this is the first study to report that TNF-α induces MSX2 expression via the NF-κB pathway in VSMCs. These results are consistent with previous reports showing that TNF-α induces Msx2-Wnt signaling in aortic adventitial myofibroblasts in diabetic $Ldlr^{-/-}$ mice [16]. Towler and colleagues have provided working model for vascular calcification in diabetes that explains the interactions between oxidative stress and vascular Bmp-Wnt signaling, in that they suggested the role of TNF-α-generated ROS in endothelial cells as a paracrine signal to induce Msx2 expression in aortic myofibroblasts [26]. However, our data indicate that TNF-α can directly increase MSX2 expression in VSMCs and that this induction is

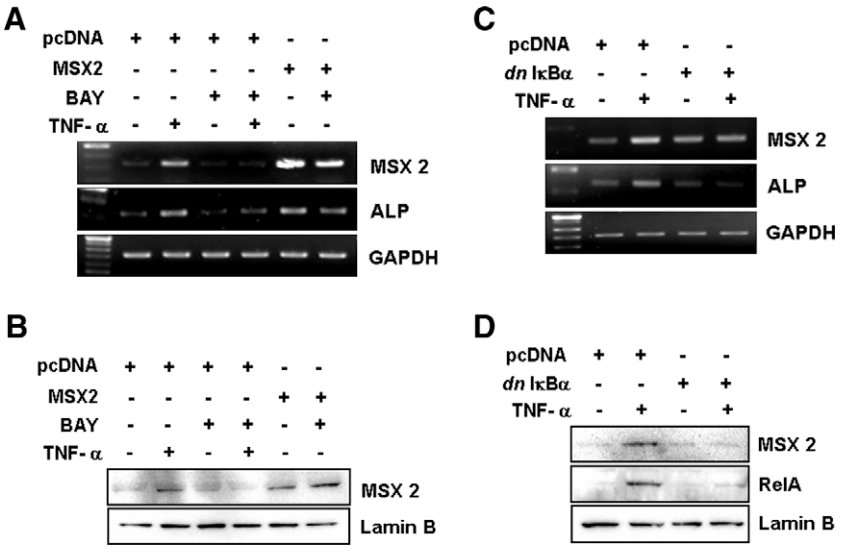

Fig. 4. TNF-α stimulates MSX2 expression via the NF-κB pathway. (A, B) NF-κB inhibitor treatment blocked TNF-α-induced MSX2 expression. VSMCs were incubated for 24 h in the presence of the indicated reagents. When indicated, cells were transfected with an MSX2 expression vector or pcDNA and incubated for 6 h before the reagents were added to the culture medium. After the end of the incubation period, semi-quantitative RT-PCR (A) or immunoblot analysis (B) was performed. BAY; 10 μM BAY-11-7082. (C, D) Over-expression of a dominant negative (*dn*) IκBα mutant suppressed TNF-α-mediated MSX2 expression. VSMCs were transfected with *dn*IκBα or pcDNA. After 6 h, the cells were incubated with TNF-α for 24 h and semi-quantitative RT-PCR (C) or immunoblot analysis (D) was performed. The effect of *dn*IκBα over-expression was confirmed by blocking NF-κB RelA nuclear translocation.

independent of ROS production. Because vascular calcification is also observed in advanced atherosclerotic lesions and TNF-α is also mainly produced and secreted from macrophages and T lymphocytes, which are the predominant cell types found in advanced atherosclerotic lesions [27], we suggest that TNF-α produced by inflammatory cells infiltrated in advanced atherosclerotic lesions directly induces the osteogenic conversion of VSMCs via MSX2 expression, thus contributing at least in part to vascular calcification.

In summary, our finding that TNF-α directly induces MSX2 expression, which is a prerequisite to ALP expression indispensable for bio-mineralization, suggests that MSX2 may be a crucial regulator in TNF-α-directed vascular calcification. In addition, our study showed that NF-κB pathway but not NADPH oxidase-ROS system is responsible for TNF-α-directed MSX2 expression in human VSMCs. But further study is necessary to verify whether MSX2 stimulates ALP expression directly or indirectly via canonical WNT signaling.

Acknowledgments

This work was supported by the Basic Research Program of the Korea Science & Engineering Foundation (R01-2005-000-106650) and by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (KRF-2006-000-E00093).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.12.027.

References

[1] R. Vattikuti, D.A. Towler, Osteogenic regulation of vascular calcification: an early perspective, Am. J. Physiol. Endocrinol. Metab. 286 (2004) E686–E696.
[2] S. Lehto, L. Niskanen, M. Suhonen, T. Ronnemaa, M. Laakso, Medial artery calcification. A neglected harbinger of cardiovascular complications in non-insulin-dependent diabetes mellitus, Arterioscler. Thromb. Vasc. Biol. 16 (1996) 978–983.
[3] G.M. London, A.P. Guerin, S.J. Marchais, F. Metivier, B. Pannier, H. Adda, Arterial media calcification in end-stage renal disease: impact on all-cause and cardiovascular mortality, Nephrol. Dial. Transplant. 18 (2003) 1731–1740.
[4] F. Parhami, A.D. Morrow, J. Balucan, N. Leitinger, A.D. Watson, Y. Tintut, J.A. Berliner, L.L. Demer, Lipid oxidation products have opposite effects on calcifying vascular cell and bone cell differentiation. A possible explanation for the paradox of arterial calcification in osteoporotic patients, Arterioscler. Thromb. Vasc. Biol. 17 (1997) 680–687.
[5] F. Parhami, Y. Tintut, J.K. Patel, N. Mody, A. Hemmat, L.L. Demer, Regulation of vascular calcification in atherosclerosis, Z. Kardiol. 90 (Suppl. 3) (2001) 27–30.
[6] A. Shioi, Mechanism of vascular calcification, Clin. Calcium 12 (2002) 1059–1066.
[7] Y. Tintut, J. Patel, F. Parhami, L.L. Demer, Tumor necrosis factor-alpha promotes in vitro calcification of vascular cells via the cAMP pathway, Circulation 102 (2000) 2636–2642.
[8] Y. Fukumoto, H. Shimokawa, A. Ito, T. Kadokami, Y. Yonemitsu, M. Aikawa, M.K. Owada, K. Egashira, K. Sueishi, R. Nagai, Y. Yazaki, A. Takeshita, Inflammatory cytokines cause coronary arteriosclerosis-like changes and alterations in the smooth-muscle phenotypes in pigs, J. Cardiovasc. Pharmacol. 29 (1997) 222–231.
[9] K. Bostrom, K.E. Watson, S. Horn, C. Wortham, I.M. Herman, L.L. Demer, Bone morphogenetic protein expression in human atherosclerotic lesions, J. Clin. Invest. 91 (1993) 1800–1809.
[10] K.E. Watson, K. Bostrom, R. Ravindranath, T. Lam, B. Norton, L.L. Demer, TGF-beta 1 and 25-hydroxycholesterol stimulate osteoblast-like vascular cells to calcify, J. Clin. Invest. 93 (1994) 2106–2113.
[11] A. Shioi, Y. Nishizawa, S. Jono, H. Koyama, M. Hosoi, H. Morii, Beta-glycerophosphate accelerates calcification in cultured bovine vascular smooth muscle cells, Arterioscler. Thromb. Vasc. Biol. 15 (1995) 2003–2009.
[12] K. Mori, A. Shioi, S. Jono, Y. Nishizawa, H. Morii, Dexamethasone enhances in vitro vascular calcification by promoting osteoblastic differentiation of vascular smooth muscle cells, Arterioscler. Thromb. Vasc. Biol. 19 (1999) 2112–2118.
[13] D. Davidson, The function and evolution of Msx genes: pointers and paradoxes, Trends Genet. 11 (1995) 405–411.
[14] S.L. Cheng, J.S. Shao, N. Charlton-Kachigian, A.P. Loewy, D.A. Towler, MSX2 promotes osteogenesis and suppresses adipogenic differentiation of multipotent mesenchymal progenitors, J. Biol. Chem. 278 (2003) 45969–45977.
[15] J.S. Shao, S.L. Cheng, J.M. Pingsterhaus, N. Charlton-Kachigian, A.P. Loewy, D.A. Towler, Msx2 promotes cardiovascular calcification by activating paracrine Wnt signals, J. Clin. Invest. 115 (2005) 1210–1220.
[16] Z. Al-Aly, J.S. Shao, C.F. Lai, E. Huang, J. Cai, A. Behrmann, S.L. Cheng, D.A. Towler, Aortic Msx2-Wnt calcification cascade is regulated by TNF-alpha-dependent signals in diabetic Ldlr$^{-/-}$ mice, Arterioscler. Thromb. Vasc. Biol. 27 (2007) 2589–2596.
[17] A. Shioi, M. Katagi, Y. Okuno, K. Mori, S. Jono, H. Koyama, Y. Nishizawa, Induction of bone-type alkaline phosphatase in human vascular smooth

muscle cells: roles of tumor necrosis factor-alpha and oncostatin M derived from macrophages, Circ. Res. 91 (2002) 9–16.
[18] M. Murshed, D. Harmey, J.L. Millan, M.D. McKee, G. Karsenty, Unique coexpression in osteoblasts of broadly expressed genes accounts for the spatial restriction of ECM mineralization to bone, Genes Dev. 19 (2005) 1093–1104.
[19] R.J. Levy, F.J. Schoen, W.B. Flowers, S.T. Staelin, Initiation of mineralization in bioprosthetic heart valves: studies of alkaline phosphatase activity and its inhibition by $AlCl_3$ or $FeCl_3$ preincubations, J. Biomed. Mater. Res. 25 (1991) 905–935.
[20] Y.J. Kim, M.H. Lee, J.M. Wozney, J.Y. Cho, H.M. Ryoo, Bone morphogenetic protein-2-induced alkaline phosphatase expression is stimulated by Dlx5 and repressed by Msx2, J. Biol. Chem. 279 (2004) 50773–50780.
[21] S.L. Cheng, J.S. Shao, J. Cai, O.L. Sierra, D.A. Towler, Msx2 exerts bone anabolism via canonical Wnt signaling, J. Biol. Chem. 283 (2008) 20505–20522.
[22] G. Rawadi, B. Vayssiere, F. Dunn, R. Baron, S. Roman-Roman, BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop, J. Bone Miner. Res. 18 (2003) 1842–1853.
[23] Y.Y. Lo, T.F. Cruz, Involvement of reactive oxygen species in cytokine and growth factor induction of c-fos expression in chondrocytes, J. Biol. Chem. 270 (1995) 11727–11730.
[24] L.S. Yoshida, S. Tsunawaki, Expression of NADPH oxidases and enhanced H_2O_2-generating activity in human coronary artery endothelial cells upon induction with tumor necrosis factor-alpha, Int. Immunopharmacol. 8 (2008) 1377–1385.
[25] Q. Li, N.Y. Spencer, F.D. Oakley, G.R. Buettner, J.F. Engelhardt, Endosomal Nox2 facilitates redox-dependent induction of NF-kappaB by TNF-alpha, Antioxid. Redox Signal. 11 (2009) 1249–1263.
[26] J.S. Shao, Z.A. Aly, C.F. Lai, S.L. Cheng, J. Cai, E. Huang, A. Behrmann, D.A. Towler, Vascular Bmp Msx2 Wnt signaling and oxidative stress in arterial calcification, Ann. N. Y. Acad. Sci. 1117 (2007) 40–50.
[27] G.K. Hansson, L. Jonasson, B. Lojsthed, S. Stemme, O. Kocher, G. Gabbiani, Localization of T lymphocytes and macrophages in fibrous and complicated human atherosclerotic plaques, Atherosclerosis 72 (1988) 135–141.