Role of matrix Glα protein in angiotensin II-induced exacerbation of vascular calcification

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Jia G, Stormont RM, Gangahar DM, Agrawal DK. Role of matrix Glα protein in angiotensin II-induced exacerbation of vascular calcification. Am J Physiol Heart Circ Physiol 303: H523–H532, 2012. First published July 13, 2012; doi:10.1152/ajpheart.00826.2011.—Vascular calcification predicts increased risk for cardiovascular events in atherosclerosis, diabetes, and end-stage kidney diseases. Matrix Glα protein (MGP), an inhibitor of calcification, limits calcium phosphate deposition in the vessel wall. There are many factors contributing to the progression of atherosclerosis, including hypertension, hyperlipidemia, the renin-angiotensin system, and inflammation. Angiotensin II (ANG II) plays a crucial role in the atherogenic process through not only its pressor responses but also its growth-promoting and inflammatory effects. In this study, we investigated the role of MGP in ANG II-induced exacerbation of vascular calcification in human vascular smooth muscle cells (VSMCs). The expression of MGP, calcification, and apoptosis in human VSMCs were examined by Western blot analysis, real-time PCR, in situ terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling, and enzyme-linked immunosorbent assay, respectively. Increase in VSMC calcification in human atherosclerotic plaques upregulates MGP expression and apoptosis in a negative feedback manner. ANG II inhibited MGP expression in VSMCs via and in vitro in a dose- and time-dependent manner through ANG II type 1 receptor and NF-κB signaling pathway. Meanwhile, MGP inhibited the calcification, caspase-3 activity, activation of runt-related transcription factor 2, and release of inflammatory cytokines by VSMCs induced by calcification medium (2.5 mM Pi) and ANG II in vitro. These observations provide evidence that ANG II exacerbates vascular calcification through activation of the transcription factors, runt-related transcription factor 2 and NF-κB, and regulation of MGP, inflammatory cytokines expression in human VSMCs.

VASCULAR CALCIFICATION is one of the major complications of cardiovascular disease and is associated with increased risk of morbidity and mortality (7). In the course of calcification formation, oxidized LDL can recruit T lymphocytes and macrophages to the lesion and induce the formation of atherosclerotic calcification by a process similar to endochondral ossification, osteoblast induction, and lamellar bone formation (5). Vascular smooth muscle cells (VSMCs) are the predominant cell type found in the arterial wall and are essential for the structural and functional integrity of the vessel. VSMCs can differentiate from a quiescent, contractile phenotype to a proliferative, synthetic phenotype following arterial injury and in atherosclerotic diseases (20). Indeed, VSMCs are capable of osteoblast transdifferentiation in calcifying arteries (5). Many factors, such as endotheлин-1, contribute to the development of vascular calcification (10). Recently, angiotensin II (ANG II) was found to play a key role in promoting differentiated VSMC phenotype, which can migrate to the subintimal space and contribute to the formation of calcification over the atherosclerotic plaque (17). The phenotypic switching and calcification of VSMCs have been found in the blood vessels and shown to positively correlate with the atherosclerotic plaque burden and increased risk of plaque instability (2).

Molecules regulating osteoblastic and chondrocytic differentiation, such as matrix Glα protein (MGP), fetuin-A, bone morphogenetic proteins, and runt-related transcription factor 2 (Runx2), have been identified in calcified lesions of blood vessels (23). MGP, one of the important inhibitors of calcification, is a 10-kDa protein containing 5-carboxyglutamic acid residues, can be expressed at high levels in smooth muscle cells, and serves as a calcification inhibitor in cartilage and vasculature (19). Previously, we found that ANG II plays many roles in the vascular pathology of cardiovascular diseases (3, 4). However, the cellular and molecular mechanisms of ANG II-induced atherosclerotic calcification and the effect on MGP in human VSMCs are not clear. In this study, we assessed the effect and the underlying mechanisms of ANG II on the expression of MGP, calcification, and apoptosis in human VSMCs.

MATERIALS AND METHODS

Human tissue specimens and culture of VSMCs. The experimental protocol for this study was approved by the Institutional Review Board of Creighton University. The tissue specimens were obtained from patients who underwent carotid endarterectomy (71.3 ± 6.5 yr, n = 303: H523–H532, 2012. First published July 13, 2012; doi:10.1152/ajpheart.00826.2011. 5%). Two or three-centimeter segments of the harvested human saphenous veins were cut open longitudinally with luminal surface facing upward. These tissues were cultured in smooth muscle cell (SMC) medium supplemented with 20% fetal bovine serum and incubated at 37°C in 5% CO2, and the medium was changed daily. VSMCs from the saphenous vein conduits were isolated by a method established in our laboratory (4). Briefly, after gentle removal of the endothelial cells and adventitia, the specimen was minced and digested with digestion media (containing elastase and collagenase). The isolated cells were cultured in 10% fetal bovine serum and incubated at 37°C in a humidified 5% CO2 atmosphere for 10–14 days and passaged. The subcultured VSMCs were used between passages 3 and 5. The confluent cells showed the characteristic hill-and-valley pattern associated with spindle-shaped VSMCs. The purity of isolated VSMCs was examined with positive immunostaining to smooth muscle α-actin and caldesmon. Before stimulation, cells were serum starved for 24 h in DMEM.

VSMC calcification. Because the transfected MGP plasmid has a maximum expression in VSMCs at 48–72 h, VSMCs were stimulated with free fetal bovine serum medium in the presence or absence of ANG II (10−7 mol/l) or calcification medium (SMC medium supple-
Fig. 1. The association between calcification, matrix Gla protein (MGP) expression, and apoptosis on vascular smooth muscle cells (VSMCs) from human carotid plaque and normal carotid artery. The calcification, MGP expression, and apoptosis were checked by von Kossa staining, immunohistochemistry, and in situ terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling, respectively. Histological serial adjacent sections were taken from human carotid plaque and normal carotid artery. Arrows indicate the calcification, MGP expression, and apoptosis positive cells, which were clearly visible in smooth muscle α-actin positive cells.
mented with 2.5 mM NaH₂PO₄) for 3 days. The von Kossa staining protocol was used to quantify calcification. Briefly, sections were incubated with 1% silver nitrate solution in a clear glass coplin jar placed under ultraviolet light for 20 min. The unreacted silver was removed with 5% sodium thiosulfate for 5 min. The sections were counterstained with nuclear fast red for 5 min, followed by dehydration through graded alcohol and cleared in xylene. Calcified SMCs were fixed with 70% ethanol, followed by staining with 2% Alizarin red-S (pH 4.2) for 5 min. The staining in sections was microscopically quantified with National Institutes of Health (NIH) Scion Image under the light microscope. The area of positive staining per millimeter squared was then calculated.

Quantification of calcium deposition. VSMCs were decalcified with 0.6 N HCl for 24 h. The calcium content in the supernatant was determined colorimetrically by the o-cresolphthalein complexone method, as previously described (6). The protein content was measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). The calcium content of the cell layer was normalized to protein concentration.

Immunostaining. Thin sections (6 µm) were treated with 3% peroxide in methanol for 15 min, followed by an overnight incubation with the primary antibody at 4°C. Rabbit primary monoclonal antibody directed against MGP (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) was used in these experiments. The immunopositivity in the plaque sections was microscopically quantified with NIH Scion Image analysis by randomly selecting four different areas of 1-mm length under the light microscope. The area of positive immunostaining per millimeter squared was then calculated. Meanwhile, VSMCs were plated onto four-well chamber slides at a density of 50,000 cells/well. The cells were fixed in 3.7% paraformaldehyde for 20 min at room temperature and incubated with anti-MGP or anti-NF-κB for 1 h at room temperature. After washing, cyanine-2 or cyanine-3 goat anti-rabbit IgG at a concentration of 1:200 was applied at room temperature for 1 h, followed by thorough washing and then staining with 4',6'-diamidino-2-phenylindole (300 nM). Staining was analyzed using fluorescence microscopy.

In situ terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling and annexin-V labeling and caspase-3 assay. DNA strand breaks were analyzed in 6-µm frozen sections of the saphenous vein by the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling reaction using a commercially available kit (Apopag Plus peroxidase in situ apoptosis detection kit S7101; Oncor, Gaithersburg, MD). The immunopositivity in the plaque sections was microscopically quantified with NIH Scion Image analysis by randomly selecting four different areas of 1-mm length under the light microscope. The area of positive staining per millimeter squared was then calculated.

Fig. 2. ANG II-induced inhibition of MGP expression in VSMCs via and in vitro. A: histological serial adjacent sections were taken from the ANG II (10⁻⁷ mol/l)-treated human saphenous vein with or without losartan (10⁻⁶ mol/l) for 24 h. Arrows indicate MGP expression positive cells, which were clearly visible in smooth muscle α-actin positive cells. B: VSMCs were stimulated by ANG II (10⁻⁷ mol/l) with or without losartan (10⁻⁶ mol/l) for 24 h, and expression of MGP was determined by immunostaining. Arrows indicate MGP expression (red) in cultured SMCs, and the nucleus was stained with blue color in 4',6'-diamidino-2-phenylindole.
MD). The reaction buffer was optimized for incorporation, which promotes antibody binding and minimizes any effects because of fluorescence quenching. The labeled DNA was detected using an anti-digoxigenin antibody fragment. Apoptosis was also quantified by measuring the activity of caspase-3 using the CPP32 colorimetric assay kit (MBL, Woburn, MA). Five milliliters of 1 mmol/l caspase-3 substrate and 50 μl of 2× reaction buffer/dithiothreitol mix were added, and the samples were incubated at 37°C for 1 h. Protease activity was quantified by spectrophotometer (Bio-Rad 3550; Bio-Rad, Hercules, CA) at 405 nm.

Quantitative real-time RT-PCR. The oligonucleotides were purchased from IDT (Integrated DNA Technologies), for human MGP (hMGP), NM_000900, forward, 5'-TGAAGAGCCTGATCCTTCTT-GCCA-3’; and reverse, 5'-TAGACGTTCGATCGATCTTCTT-3’. The real-time RT-PCR reaction was run in the CFX96 real-time system by using SYBR (Bio-Rad) as a dsDNA-specific binding dye. The reactions were cycled 40 times in the PCR after initial denaturation (95°C, 2 min) with the following parameters: 95°C, 15 s; and annealing and extension, 60°C, 1 min. The validation experiment proved the linear dependency of the threshold cycle (CT) value of both MGP and GAPDH concentration and consistency of ΔCT (MGP average CT-GAPDH average CT) in a given sample at different RNA concentration. MGP was indexed to the GAPDH using the following formula: 1/(2^ΔCT × 100%). The value of 2^ΔCT was calculated to demonstrate fold changes in MGP gene expression in stimulated cells compared with unstimulated cells.

Transfection of VSMCs. pN-FLAG-hMGP plasmid was kindly provided by Dr. Kristina I. Boström (David Geffen School of Medicine at University of California at Los Angeles). Transfection of VSMCs was performed using the nucleofection device to deliver electrical stimuli to the target cells and solutions (Amaxa Biosystems, Amaxa). The resulting transfection efficiency was more than 70% in VSMCs.

Western blot analysis. VSMCs were collected and lysed in lysis buffer, and the protein concentration of the lysate was determined by Bio-Rad protein assay. Proteins were resolved by SDS-PAGE and

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Fig. 3. ANG II-induced inhibition of MGP mRNA expression through ANG II type-1 receptor (AT1R) in human VSMCs. A: ANG II (10^-7 mol/l) inhibited MGP mRNA expression of VSMCs in a time-dependent manner. B: ANG II inhibited MGP mRNA expression of VSMCs at 24 h in a dose-dependent manner. C: IC50 of ANG II in the downregulation of MGP mRNA was 1.29 × 10^-7 mol/l. D: ANG II receptor antagonist losartan (10^-6 mol/l, AT1R receptor specific) and PD-123319 (10^-6 mol/l, ANG II type-2 receptor specific) were added to the cultured medium for 30 min and exposed to 10^-7 mol/l ANG II for an additional 24 h. E: calcification medium (2.5 mM Pi) induced MGP mRNA expression of VSMCs in vitro. Each bar represents the 3 independent experiments. *P < 0.01 compared with control group; #P < 0.01 compared with ANG II group.
transferred to Immobilon P membrane. The membranes were incubated overnight at 4°C with blocking buffer containing antibodies to FLAG, NF-κB-p65, and Runx2. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody and detected using an ECL kit (Pierce, Rockford, IL). As a loading control, nuclear protein Lamin B or cytosolic protein GAPDH was probed and visualized in all the blots.

**Measurements of IL-6, IL-8, and monocyte chemoattractant protein-1.** The concentration of IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1) in the culture supernatants was determined using an ELISA kit according to the manufacturer’s instructions (BD Biosciences, San Jose, CA). Briefly, the test samples were applied to a microtiter plate coated with an anti-human IL-6, IL-8, or MCP-1 monoclonal antibody, and the incubation was done at 37°C for 3 h.

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**Fig. 4. Involvement of the activation of transcription factor NF-κB in ANG II-inhibited MGP expression in human VSMCs.** A: ANG II (10^{-7} mol/l) induced NF-κB p65 nuclear translocation at 5, 15, and 30 min in VSMCs. Arrows indicate that NF-κB p65 were clearly visible in the part of VSMC nuclear. B: ANG II (10^{-7} mol/l) induced nuclear protein expression of NF-κB in VSMCs. C: VSMCs were stimulated by ANG II with or without NF-κB inhibitor II (20 nmol/l), and mRNA expression of MGP was determined by real-time PCR. Data are means ± SE of 3 independent experiments. #P < 0.01 compared with control group; *P < 0.01 compared with ANG II group.
Biotinylated anti-human IL-6, IL-8, and MCP-1 antibody solution was then added. After the incubation at room temperature for 30 min and washing 4 times, streptavidin-horseradish peroxidase was added, followed by the addition of a stabilized chromogen, tetramethylbenzidine. The color was read by spectrophotometer at 450 nm.

Statistical analysis. Data are presented as means ± SE. Statistical analysis was performed using Student’s t-test or ANOVA when appropriate to analyze statistically significant differences between groups. For this study, P < 0.05 was considered significant.

RESULTS

Association between calcification and MGP expression on VSMCs from human carotid plaque and normal carotid artery.

To explore the relationship between vascular calcification and MGP expression in atherosclerosis, we took advantage of the availability of human carotid endarterectomy specimens, which is a common model of atherosclerosis and lesion calcification. We first determined the difference in expression of calcification, MGP, and apoptosis on SMCs by comparing normal carotid artery and human carotid plaque. We found that the calcification, MGP, and apoptosis were all strongly expressed in the same area of human atherosclerotic plaques (Fig. 1). In contrast, the calcification and apoptosis were not observed in normal carotid artery except the evidence of mild MGP expression. There was statistically significant positive correlation between vascular calcification (5.3 ± 1.1 mm²) and MGP expression (11.6 ± 2.5 mm²) in plaque VSMCs (r² = 0.96) (Fig. 1).

ANG II inhibited MGP expression of VSMCs ex vivo and in vitro.

To investigate the effect of ANG II on vascular calcification and MGP expression, we isolated and cultured VSMCs from human saphenous vein. In both via (Fig. 2A) and in vitro (Fig. 2B) conditions, the MGP expression in the control VSMCs was observed. However, after stimulation with ANG II, the expression of MGP was very minimal in the organ culture of human saphenous vein and cultured VSMCs (Fig. 2, A and B). Furthermore, ANG II type-1 receptor (AT₁R) antagonist losartan relieved ANG II-inhibited MGP expression via and in vitro (Fig. 2).

ANG II inhibited MGP expression through AT₁Rs.

The expression of MGP in VSMCs was evaluated in a time- and dose-dependent manner. Similar to the reports by other researchers (8, 11), we could not find an effective MGP antibody...
to detect the protein in VSMCs by using Western blot analysis. Therefore, we used real-time PCR to investigate the MGP mRNA expression in VSMCs. After treatment with ANG II \((10^{-7} \text{ mol/l})\), the MGP mRNA expression was significantly inhibited at 3 h and remained at a low level for at least 24 h in the VSMCs (Fig. 3A). Also, ANG II decreased MGP mRNA expression in a dose-dependent manner with the maximal response of \(10^{-7}\) to \(10^{-6}\) mol/l at 24 h (Fig. 3B). The IC\(_{50}\) value of ANG II in the downregulation of MGP mRNA was \(1.29 \times 10^{-7}\) mol/l (Fig. 3C). To determine which AT receptor is involved in ANG II-inhibited MGP expression in VSMCs, the cells were stimulated with ANG II \((10^{-7}\text{ mol/l})\) after pretreatment for 30 min with AT\(_1\)R antagonist losartan \((10^{-6}\text{ mol/l})\) or the ANG II type-2 receptor antagonist PD-123319 \((10^{-6}\text{ mol/l})\). ANG II-induced effect on mRNA expression of MGP was inhibited by pretreatment with losartan but not by PD-123319 (Fig. 3D), suggesting the role of AT\(_1\)R in ANG II-inhibited MGP expression in VSMCs. Additionally, the MGP expression in VSMCs was induced by calcification medium \((2.5 \text{ mM P}_i)\) at 6 to 96 h in vitro (Fig. 3E), supporting the expression of MGP in response to calcification in a negative feedback manner.

**Involvement of NF-κB in ANG II-inhibited MGP expression.**

To determine the effect of ANG II on nuclear translocation of NF-κB, VSMCs were cultured in four-well chamber slides, serum starved for 24 h, and then treated with ANG II. Subcellular distribution of NF-κB was examined by immunofluorescence and Western blot analysis. Treatment with ANG II resulted in nuclear translocation of NF-κB and a steady increase in nuclear p65 protein of VSMCs at 5, 15, and 30 min (Fig. 4A and B). To confirm the role of NF-κB in ANG II-inhibited MGP mRNA expression, VSMCs were treated with NF-κB inhibitor II and subsequently exposed to ANG II for 24 h. ANG II significantly inhibited the MGP mRNA expression in VSMCs. However, the effect of ANG II on the MGP mRNA expression was significantly blocked by NF-κB inhibitor II (Fig. 4C), confirming the role of NF-κB in ANG II-induced inhibition of MGP expression.
MGP inhibited ANG II- and phosphate-induced calcification and caspase-3 activity in human VSMCs. We determined whether ANG II increases the capacity of calcification medium (2.5 mM P_i) to induce calcification and apoptosis of VSMCs. ANG II alone could neither induce the calcification nor increase the caspase-3 activity in VSMCs. However, ANG II enhanced the capacity of calcification medium-induced calcification and apoptosis of VSMCs. Because MGP serves as a calcification inhibitor in vessel wall, overexpression of MGP plasmid inhibited the calcification and caspase-3 activity of VSMCs induced by phosphate and ANG II (Fig. 5, A, C, and D). However, the overexpression of the cells with MGP did not completely inhibit the 2.5 mM P_i- and ANG II-induced calcification of VSMCs. This could be due to the inadequate protein expression using MGP plasmid. The MGP plasmid has a FLAG tag that becomes NH2-terminal after protein secretion and cleavage of the signal peptide. Because an effective antibody to detect the MGP expression was not available, we examined the protein expression of overexpression of MGP plasmid by using anti-FLAG antibody. We observed a distinctly greater degree of expression of MGP protein at 24 and 48 h in VSMCs (Fig. 5B). To determine the potential protective role of MGP on ANG II-induced exacerbation of vascular calcification, the VSMCs were stimulated by ANG II (10^-7 mol/l) with or without MGP. ANG II significantly induced bone morphogenetic protein (BMP)-2 expression. However, MGP did not inhibit ANG II-induced BMP-2 expression in VSMCs (Fig. 5E).

**DISCUSSION**

Human VSMCs spontaneously express the osteoblast transcription factor, Runx2, which is an osteochondrogenic marker and is found in human diabetic lesions, atherosclerotic intimal lesions, and aortic valve disease (9, 21). VSMCs also express a number of bone-associated, mineralization-regulating proteins such as alkaline phosphatase, MGP, osteopontin, and osteocalcin in vitro and in vivo (15). ANG II can induce cultured SMCs to undergo a phenotypic change characterized by the loss of SMC markers, smooth muscle 22α, and smooth muscle α-actin (16). In this study, we further examined the effect of ANG II-induced exacerbation of calcification, apoptosis, and MGP signaling pathway in human VSMCs.

MGP, a potent inhibitor of calcification, is constitutively expressed in the VSMCs of normal artery (13). Proteins involved in post-translational modification of MGP in VSMCs have carboxylated MGP, uncarboxylated MGP, carboxylated 1–53 MGP, carboxylated 54–84 MGP, nonphosphorylated 3–15 MGP, phosphorylated 3–15 MGP, uncarboxylated 35–54 MGP, and carboxylated 35–54 MGP (18). Carboxylation of Gla proteins is essential for their function. We confirmed the expression of MGP in VSMCs of human carotid artery and saphenous vein. Furthermore, VSMCs respond to and inhibit further calcification by increased MGP expression in the atherosclerotic lesions and in VSMCs stimulated with calcification medium. This is consistent with previous reports of increased MGP expression in calcified media and atherosclerotic lesions (1, 7). Thus VSMC calcification can increase MGP expression in VSMCs, and an increase in MGP expression may be an attempt by the cells to respond to and inhibit further calcification in a negative feedback manner in human atherosclerotic plaques.

In our study, ANG II inhibited MGP expression of VSMCs via AT1R. This is in contrast to another study where ANG II increased MGP mRNA levels by 20% in neonatal rat cardiac myocytes with significant increase in left ventricular MGP mRNA levels from 6 h to 2 wk when ANG II was administered by osmotic minipumps (10). The precise reason for such discrepancy is not clear. However, a variety of different factors can affect levels of MGP expression in vivo via in vitro and could also be cell specific (14, 22). It should be noted, in vivo an increase in MGP mRNA expression may be an attempt by the cells to respond to and inhibit further calcification with a negative feedback-like manner. In our findings, ANG II inhibited MGP expression of VSMCs from 3 to 24 h. However, ANG II alone could not induce the VSMC calcification for 3 days. Thus it is reasonable to speculate that MGP expression is not affected in a feedback phenomenon. We hypothesize that ANG II mediates its effect on the MGP expression via the activation of transcription factor, NF-κB. This could be supported by the findings that the treatment of the cells with ANG II resulted in nuclear translocation of NF-κB and a steady

![Angiotensin II](http://ajpheart.physiology.org/)
increase of nuclear p65 protein in ANG II-treated VSMCs. Other growth factors have also been found to regulate MGP expression. Insulin-like growth factor-1 causes a reduction in MGP mRNA expression, and basic fibroblast growth factor-2 increases MGP mRNA expression in cultured chondrocytes (12). These growth factors were, therefore, thought to act via MGP to alter chondrocyte differentiation.

Activation of ERK-1/2 increases expression of Runx2, which is a key regulator of osteoblastic differentiation; decreases the SMC lineage markers; and further generates the osteochondrogenic precursor state in VSMCs (20). In our study, we confirmed that ANG II induced the activation of transcription factor Runx2 in human VSMCs. However, although ANG II inhibited the expression of MGP and activated the transcription factor Runx2, ANG II alone could not induce the calcification and increase the apoptosis of VSMCs in vitro. This may be due to difference between the complex inflammatory environments which takes place in atherosclerosis versus the comparatively simple in vitro model. Thus this fact is not necessarily contradictory to our hypothesis. Furthermore, our data showed that ANG II enhanced the capacity of calcification medium-induced calcification and apoptosis of VSMCs. Thus the ability of ANG II to inhibit the expression of MGP can explain the increased calcification and apoptosis capacity in human VSMCs.

BMP-2 can provoke phosphate uptake and SMC phenotypic transition toward osteochondro-progenitors through ERK1/2-Runx2 signaling pathways. In our study, we found that ANG II can increase the BMP-2 expression and thus can exacerbate vascular calcification. The mechanism of the upregulation of BMP-2 induced by ANG II is related to NF-κB activation in human umbilical vein endothelial cells (24). Although MGP can antagonize the role of BMP2 and can inhibit VSMC calcification by inhibiting activity of Runx2 (20), in our study MGP did not inhibit ANG II-induced BMP-2 expression in VSMCs. The precise role and the underlying mechanism of MGP in the regulation of BMP-2 expression in VSMCs are still uncertain. The possibility that MGP may not be involved in the regulation of BMP-2 expression cannot be ruled out.

In our study we found three new functions for MGP: inhibition of calcium deposition, suppression of inflammation, and increase in cell survival. MGP inhibits both phosphate- and ANG II-induced mineral deposition via MGP3 direct inhibition of transcription factor Runx2. MGP is also protective against apoptosis due to its inhibitory effects on high extracellular mineral concentrations. MGP suppresses inflammation through its actions on ANG II, which is known to induce inflammatory cytokine secretion. It is possible that MGP affects the expression of inflammatory cytokines through inhibiting the activity of NF-κB, which was confirmed by our results that NF-κB inhibitor inhibited ANG II plus calcification culture medium-induced expression of inflammatory cytokines in VSMCs. However, NF-κB signaling pathway was not involved in the ANG II- and calcification culture medium-induced Runx2 expression. This could be due to the fact that the ERK-1/2 signaling pathway is involved in Runx2 activation (20). These data further support that NF-κB inhibitor may affect ANG II and phosphate medium-induced apoptosis of VSMCs but do not affect Runx2 expression. Obviously, further studies are warranted to elucidate and dissect cellular and molecular mechanisms underlying MGP-regulated calcification, apoptosis, and expression of inflammatory cytokines in human VSMCs.

In summary, ANG II exacerbates the vascular calcification through activation of the transcription factor, Runx2 and NF-κB, and regulation of MGP, inflammatory cytokines, and BMP-2 expression in human VSMCs (Fig. 7). These findings may explain, at least in part, vascular calcification in coronary and peripheral artery disease. Further understanding of the expression of MGP in human VSMCs has great clinical significance and could provide unique insights to the pathophysiological process of renin-angiotensin system and MGP in the pathogenesis of cardiovascular disease and to the development of therapeutic strategies that ultimately affect morbidity and mortality of human atherosclerosis.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
G.J., D.M.G., and D.K.A. conception and design of research; G.J. and R.M.S. performed experiments; G.J. and D.K.A. analyzed data; G.J. and D.K.A. interpreted results of experiments; G.J. prepared figures; G.J. drafted manuscript; G.J., R.M.S., D.M.G., and D.K.A. approved final version of manuscript; R.M.S., D.M.G., and D.K.A. edited and revised manuscript.

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