Original Research

AGEs promote calcification of HASMCs by mediating Pi3k/AKT-GSK3β signaling

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1. Abstract

This study aimed to investigate the effects of advanced glycation end products (AGEs) on the calcification of human arterial smooth muscle cells (HASMCs) and to explore whether AGEs can promote the calcification of HASMCs by activating the phosphoinositide 3-kinase (PI3K)/AKT-glycogen synthase kinase 3 beta (GSK3-β) axis. Cultured HASMCs were divided into five groups: blank control group, dimethyl sulfoxide (vehicle) group, AGEs group, LY294002 (AKT inhibitor) group, and TWS119 (GSK3-β inhibitor) group. Cells were pretreated with either vehicle, LY294002, or TWS119 for 2 hours followed by incubation with AGEs (25 μg/mL) for 5 days, and the expression levels of proteins in each group were analyzed by western blotting. AGE treatment promoted HASMC calcification, which coincided with increased expression of p-AKT and p-GSK3-β (serine 9). Also, AGEs upregulated the expression of osteoprotegerin and bone morphogenetic protein, and these effects were suppressed by LY294002 but enhanced by TWS119. In conclusion, AGEs promote calcification of HASMCs, and this effect

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is ameliorated by inhibition of AKT activity but potentiated by inhibition of GSK3-β activity. Hence, AGEs trigger HASMC calcification by regulating PI3K/AKT-GSK3-β signaling.

2. Introduction

Vascular calcification is a progressive disease [1–3] that is accompanied by phenotypic changes in the vascular smooth muscle cells (VSMCs) that manifest mainly as calcification in the intimal or medial layers of the involved arteries [4]. A growing body of evidence suggests that advanced glycation end products (AGEs) and their receptors (receptors for AGE, RAGE) play an important role in the initiation and progression of vascular calcification [5, 6]. Mechanistically, AGEs bind to RAGE present on the membrane of VSMCs [7], which in turn promotes activation of the Wnt/β-catenin signaling pathway to trigger vascular calcification [8, 9]. However, whether other signaling pathways are involved in AGE-induced vascular calcification remains largely unknown.

Protein kinase-B (AKT) is a lipid kinase that plays an important part in the inflammatory and allergic processes [10]. The phosphatidylinositol 3-kinase (PI3K)/AKT signal pathway and its downstream effector glycogen synthase kinase 3 beta (GSK3-β), the serine/threonine kinase, have been shown to be critical mediators of multiple cellular events such as cell proliferation, differentiation, and apoptosis [11, 12]. In the field of vascular calcification, one mechanism by which AKT activation and GSK3-β inhibition promote vascular calcification is the potentiation of Runx2 activity [13, 14], a transcriptional factor for osteogenesis [15].

While the AGE/RAGE axis and Wnt/β-catenin signaling have been shown to collaboratively contribute to VSMC calcification [16], little is known about any link between AGEs and the PI3K/AKT-GSK3-β pathway. In the present study, we aimed to investigate the effects of AGEs on VSMC calcification and potential involvement of PI3K/AKT-GSK3-β signaling in this process.

3. Materials and methods

3.1 Culture of human aortic SMCs (HASMCs)

HASMCs were obtained from ScienCell American and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 U/mL) in a 37 °C incubator with humidified air containing 5% CO_2. The HASMCs were previously characterized [16]. The culture medium was replenished twice per week. First, HASMCs were divided into the following three groups: HASMCs were treated with vehicle (control group), AGEs (Biovision, Japan), β-phosphoglycerine (10 mM) (Beyotime Biotechnology, Shanghai, China), or AGEs + β-phosphoglycerine for 5 days. Also, the following five groups were designed: (1) the blank control group, (2) the dimethyl sulfoxide (DMSO, vehicle) group, (3) the AGEs group, in which cells were treated with 25 μg/mL AGEs in culture for 5 days, (4) the LY294002 group, in which cells were pretreated with LY294002 (Selleck, USA), an AKT inhibitor, at 20 μM for 2 hours followed by AGEs treatment, and (5) the TWS119 group, in which cells were pretreated with TWS119 (Selleck, USA), an inhibitor of GSK3-β, at 10 μM for 2 hours followed by AGEs treatment. HASMCs of passages 3–6 were used for this study.

3.2 Von Kossa staining

Briefly, HASMCs were seeded on glass slides and cultured in an incubator (37 °C, 5% CO_2). When cells reached approximately 40–50% confluency, they were fixed with 4% paraformaldehyde at 4 °C on a shaker for 15 min, followed by three repeated washes with distilled H_2O. Next, the cells were incubated with 0.5% silver nitrate (Beyotime Biotechnology, Shanghai, China) at room temperature under sunlight for 20 min, washed twice with distilled H_2O and visualized using a phase microscope. Calcification was quantified using the software Motic Images Advanced.

3.3 Western blot analysis

Briefly, whole cell lysates were extracted from cultured HASMCs with radioimmunoprecipitation assay (RIPA) buffer (Beyotime Biotechnology, Shanghai, China), and the total protein concentrations determined with the Bradford method (Beyotime Biotechnology, Shanghai, China). An equal amount of protein lysate per sample was loaded onto 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gel (Sigma, USA) and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). The PVDF membrane was blocked with 5% non-fat dried skim milk powder for 1 hour at room temperature. The membrane was then incubated with the primary antibody of interest, including osteoprotegerin (OPG) and bone morphogenetic protein 2 (BMP-2) (Abcam, Cambridge, UK. Dilution 1 : 500) at 4 °C overnight, followed by another incubation with the appropriate secondary antibody (dilution 1 : 2000) for 1 hour at room temperature. The specific protein bands were visualized with an ECL Plus kit (Beyotime Biotechnology, Shanghai, China) and quantified with the Quantity One software (BioRad, USA).

3.4 AKT knockdown by siRNA

The initial experiments confirmed the transfection efficiency of AKT-siRNA (Ruibo Biotec, Guangzhou, China). Briefly, HASMCs were transfected with AKT siRNA using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. After transfection, cells were cultured at 37 ° in a 5% CO_2 atmosphere

for another 6 hours, and then the medium was replaced with complete medium. The efficiency of AKT knockdown was evaluated by Western blotting.

3.5 GSK3-β knockdown by lentiviral-mediated siRNA expression

Lentiviral vectors expressing green fluorescent protein (GFP) (LV-GFP) or siRNA against GSK3-β (LV-GSK3-β-RNAi) were provided by Shanghai Genechem Co. Ltd (China). HASMCs were infected with LV-GFP as a control or LV-GSK3-β-RNAi at a multiplicity of infection (MOI) of 100. Green fluorescence was observed at 72 hours post-infection, and screening of positive cells was performed with 4 μg/mL puromycin (Sigma, USA) for 1 week after obtaining 70–80% cell fusion. The expression level was evaluated by Western blotting.

3.6 Statistical analysis

All data were analyzed using SPSS 19.0 statistical software. The measurement data were expressed by means $\pm$ standard deviations (SDs), and single-factor analysis of variance (ANOVA) was used for data comparison among multiple groups, followed by q test for two-group comparison. Differences were considered statistically significant at a level of $P < 0.05$.

4. Results

4.1 Effect of AGEs on HASMC calcification

We next examined the effects of AGEs on HASMC calcification via Alizarin Red staining (Fig. 1A) and Von Kossa staining (Fig. 1B). HASMCs were treated with vehicle (control group), AGEs, β-phosphoglycerine (10 mM), or AGEs + β-phosphoglycerine for 5 days. As expected, no calcified plaques were observed in the control group. However, calcified plaques were observed in both the AGEs and the β-phosphoglycerine groups, while the highest number of calcified plaques was observed in the AGEs + β-phosphoglycerine group (Fig. 1). Collectively, these findings indicated that either AGEs or β-phosphoglycerine promote the calcification of HASMCs and that they have synergistic effects on the calcification of HASMCs. Hence, AGEs and PI3K cooperatively trigger HASMC calcification.

4.2 Effects of AGEs on AKT and GSK3-β expression

We next investigated the effects of AGEs on the expression of AKT and GSK3-β by western blotting. As shown in Fig. 2A, AGEs upregulated the expression of p-AKT and p-GSK3-β (serine 9) in a dose-dependent manner with the concentration of 25 μg/mL having the greatest effect, while AGEs showed no significant effects on the total levels of AKT and GSK3-β. Notably, GSK3-β phosphorylation at serine 9 suppresses the ability of GSK3-β to phosphorylate substrates [17]. Thus, we chose the concentration of 25 μg/mL AGEs to test the temporal effects of AGEs treatment. As shown in Fig. 2B, after 5 min of treatment with 25 μg/mL AGEs, the greatest effects on the expression levels of p-AKT and p-GSK3-β were observed. Our findings suggest that AGEs activate the AKT signaling pathway and inhibit the downstream GSK3-β signaling.

4.3 Effects of AKT on HASMC calcification

To investigate the involvement of the AKT signaling pathway in HASMC calcification induced by AGEs, HASMCs were divided into four groups: a normal control group, DMSO (vehicle) group, AGEs group, and AGEs + LY294002 group (in which the cells were pretreated with LY294002, a specific inhibitor of AKT, followed by AGEs treatment for 5 days. Consistent with the above observations, the expression levels of p-AKT and p-GSK3-β were significantly increased by AGEs compared with the control and DMSO treatments, and these increases were attenuated by LY294002 (Fig. 3A). The results confirmed that the inhibitor significantly reduced the activation of AKT. The expression of p-GSK3-β in the LY294002 group was significantly reduced compared with that in the AGEs group (Fig. 3A), further indicating that AGEs activate AKT signaling and inhibit the downstream GSK3-β signaling.

To further investigate the involvement of the AKT signaling pathway in HASMC calcification, the cells were pretreated with LY294002 for 2 hours and then incubated with 25 μg/mL AGEs for 5 days. As shown in Fig. 3B, compared with the control and DMSO groups, the AGEs group showed significantly increased expression of OPG and BMP-2, both of which are osteogenic factors. This upregulation was suppressed by LY294002 pretreatment.

AKT-siRNA treatment was used to deplete the expression of AKT. As shown in Fig. 3C, AKT expression was significantly decreased in the AKT-siRNA group compared with the expression levels in the control and NC groups. Compared with the corresponding levels in the AGEs group, the expression levels of OPG, BMP-2, and β-catenin were up-regulated in the AKT-siRNA group (Fig. 3D). Hence, AGEs promote the expression of osteogenic factors in cultured HASMCs, and this effect is alleviated by inhibition of the AKT signaling pathway.

4.4 Effects of GSK3-β on HASMC calcification

Because AGEs activated the AKT signaling pathway but inhibited the activity of GSK3-β, we next investigated the role of GSK3-β in AGE-mediated HASMC calcification. HASMCs were divided into the following four groups: control, DMSO, AGEs, and AGEs + TWS119 groups. In the AGEs + TWS119 group, cells were pretreated with TWS119, which is a specific inhibitor of GSK3-β, followed by AGEs treatment for 25 min. Compared with control and DMSO groups, the AGEs group showed significantly upregulated expression of p-GSK3-β, which was further potentiated by TWS119 pretreatment

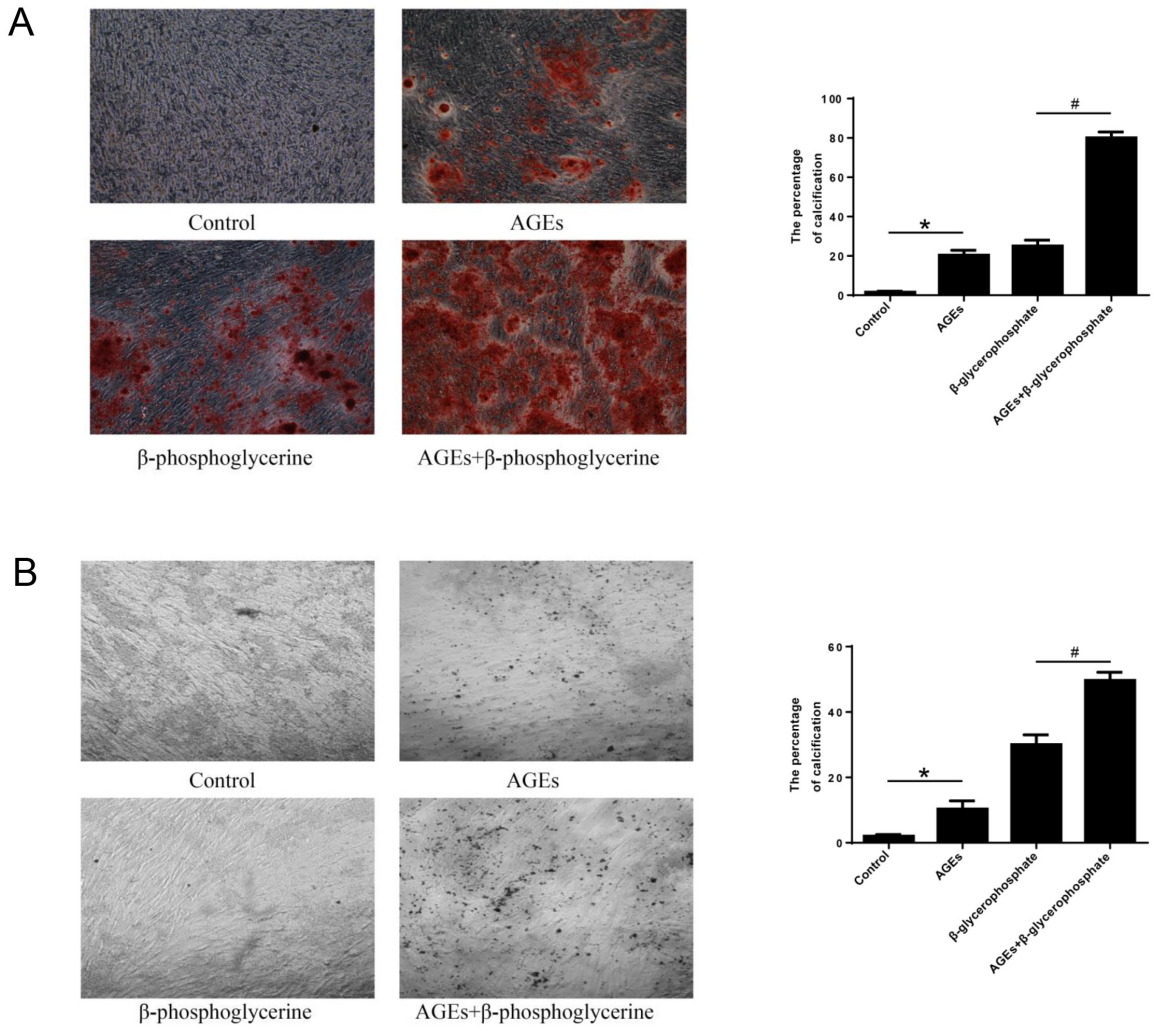

Fig. 1. Effect of AGEs on calcification of HASMCs. Cultured HASMCs were treated with vehicle (control), AGEs, β-phosphoglycerin, or AGEs + β-phosphoglycerin. Calcification of HASMCs was observed by Alizarin Red staining (A) and Von Kossa staining (B) after 14 days of treatment. The ratio of calcified cells was calculated using Motic Images Advanced 3.2. *$P < 0.05$, versus control group; #$P < 0.05$, versus β-phosphoglycerin group.

(Fig. 4A). We further pretreated the cells with LY294002 or TWS119 for 2 hours followed by treatment with 25 μg/mL AGEs for 5 days. The expression levels of OPG and BMP-2 were significantly reduced in the AGEs + LY294002 group ($P < 0.05$) compared with the AGEs group (Fig. 4B), but significantly up-regulated in the AGEs + TWS119 group ($P < 0.05$).

To further investigate the effects of GSK3-β on HASMC calcification, GSK3-β was knocked down by adenovirus-mediated siRNA expression, and Ad-GFP was used as a control. The adenoviral infection efficiency after 72 hours of infection in either group was over 90% (Fig. 5A). As expected, GSK3-β expression was significantly decreased in the Ad-GSK3-β-RNAi group compared with normal control (Ad-GFP) group (Fig. 5B). Cells of the blank control group, Ad-GFP group, and Ad-GSK3-β-RNAi group were co-cultured with 25 μg/mL AGEs for 5 days. The expression levels of OPG, BMP-2, and β-catenin in the GSK3-β knockdown group were significantly up-regulated compared with those in the control and Ad-GFP groups (Fig. 5C). These results indicated that AGEs promoted HASMC calcification, and this process was enhanced by inhibiting GSK3-β. Thus, GSK3-β may play a key role in AGE-induced HASMC calcification.

To further validate the role of the PI3K/AKT-GSK3-β signaling pathway in AGE-triggered HASMC calcification, the cells were divided into the following four groups: control, AGEs, AGEs + LY29002, and AGEs + TWS199. Except for cells in the control group, those in the other three groups were treated with 25 μg/mL AGEs for 14 days. Von Kossa staining was used to detect calcification in each group. As shown in Fig. 6, the amount of calcified plaques was significantly lower in the AGEs + LY294002 group but higher in the AGEs + TWS119 group compared with the AGEs group (Fig. 6).

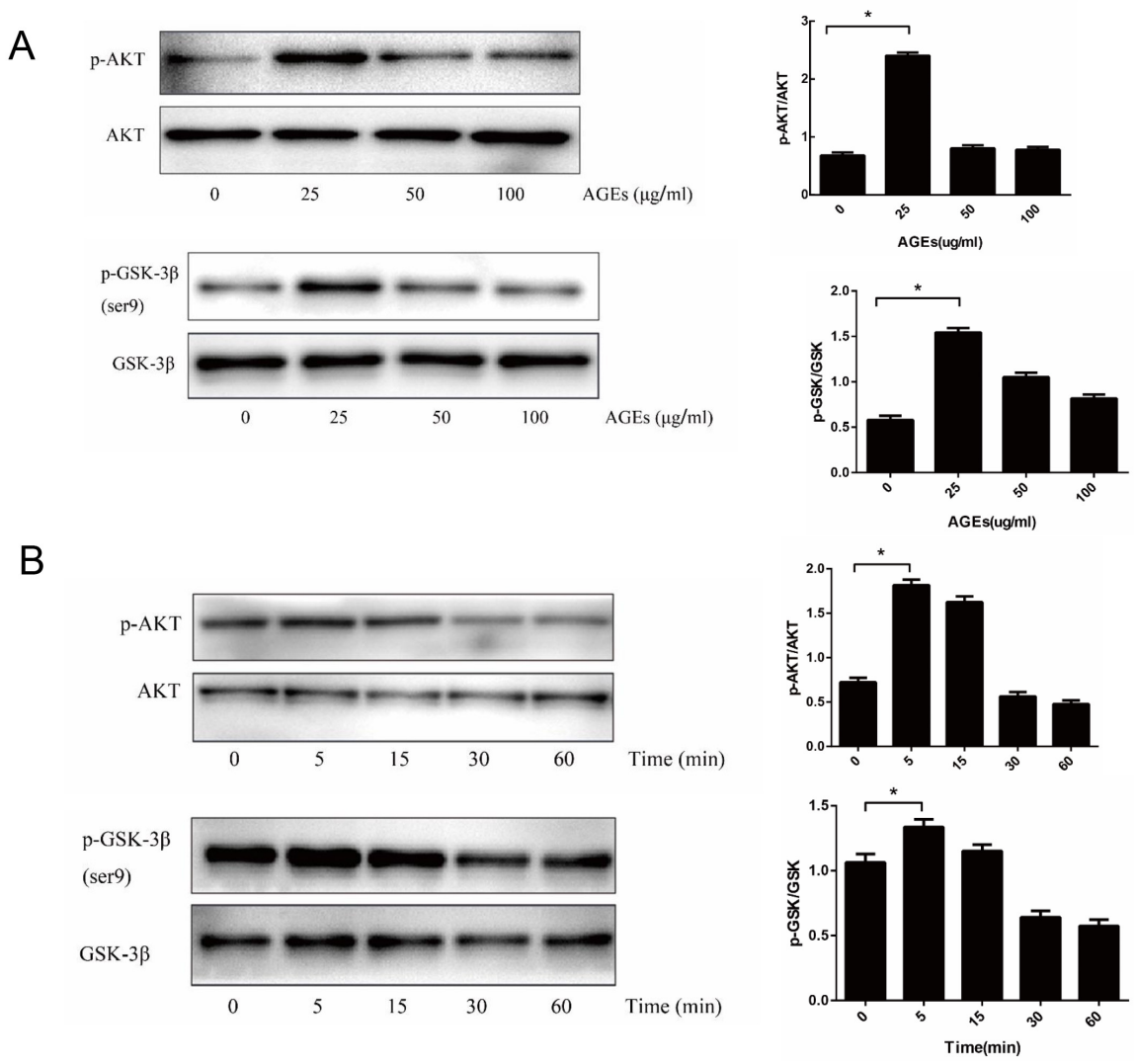

Fig. 2. Effects of AGEs on AKT and GSK3-β expression. (A) Effects of different concentrations of AGEs on the expression levels of p-AKT and p-GSK3-β. (B) Effects of AGEs on the expression levels of p-AKT and p-GSK3-β at different time points as indicated.

5. Discussion

The major findings from this study included the following: (1) AGEs promoted HASMC calcification, which coincided with increased AKT activity and decreased GSK3-β activity; (2) inhibition of AKT activity attenuated AGE-induced HASMC calcification; (3) suppression of GSK3-β activity potentiated AGE-induced HASMC calcification; and (4) AGEs increased Wnt/β-catenin activity. Thus, we conclude that AGEs promote HASMC calcification, at least in part, by mediating PI3K/AKT-GSK3-β signaling.

Vascular calcification is a pathological change involved in a variety of cardiovascular diseases. It increases arterial stiffness, which causes systolic hypertension, and is associated with increased morbidity, mortality, stroke, and amputation rates [18]. Initially, vascular calcification was considered to be a passive pathological process, but recent studies have shown that it is an active but controllable process regulated mainly by the phenotypic transformation of VSMCs [19–21]. The initiation and progression of calcification are governed by multiple factors, including an abnormal inflammatory response and lipid metabolism. During the calcification process, the SMCs, macrophages, and fibroblasts in vascular media undergo a bone-like phenotypic transformation to form matrix vesicles, which increases the expression of calcification-related genes such as alkaline phosphatase, leading to calcium deposits in blood vessels and vascular calcification [22, 23]. Indeed, in the present study, we observed increased expression of OPG and BMP-2 in calcified HASMCs, further supporting the above findings.

AGEs are stable and irreversible end products, which are derived from non-enzymatic reaction of reducing sugars with amino acid components. The levels of AGEs are significantly elevated in patients with some diseases such as diabetes mellitus [22]. Previous studies showed

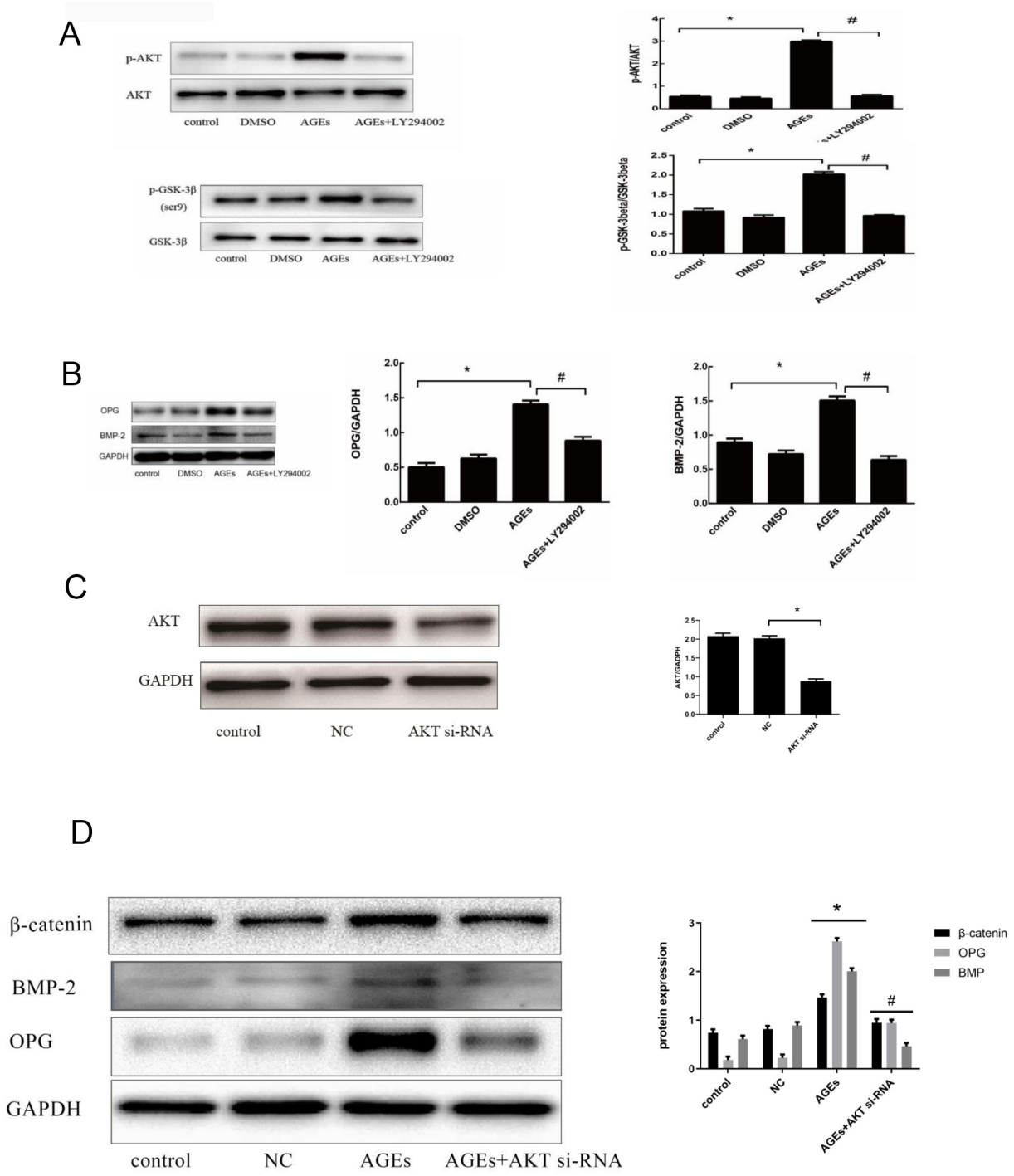

Fig. 3. Effect of AKT on HASMC calcification. (A) LY294002 inhibited the AGE-induced upregulation of p-AKT (top panel) and p-GSK3-β (bottom panel) in cultured HASMCs. Quantitated data are shown in the graphs in the left panels. (B) LY294002 attenuated the AGE-induced upregulation of osteogenic factors OPG and BMP-2 in cultured HASMCs. Quantitated data are shown in the graphs in the left panels. Western blotting was performed using whole cell lysates purified from cultured HASMCs that were divided into control, DMSO (vehicle), AGEs, and AGEs + LY294002 groups. In the AGEs + LY294002 group, cells were pretreated with LY294002 for 2 hours followed by AGEs stimulation for 30 min. *$P < 0.05$, versus control group; #$P < 0.05$, versus AGEs group. (C) AKT- siRNA depleted the expression of AKT in cultured HASMCs. (D) AKT-siRNA attenuated the AGE-induced upregulation of osteogenic factors OPG and BMP-2 in cultured HASMCs. *$P < 0.05$, versus control group; #$P < 0.05$, versus AGEs group.

that AGEs regulate the biological behavior of VSMCs in a concentration-dependent manner. Li *et al.* [24] found that AGEs promote rabbit VSMC proliferation at a low concentration (1~10 μg/mL), while a high concentration of AGEs over 40 mg/L significantly impairs cell proliferation and migration, which is accompanied by increased apop-

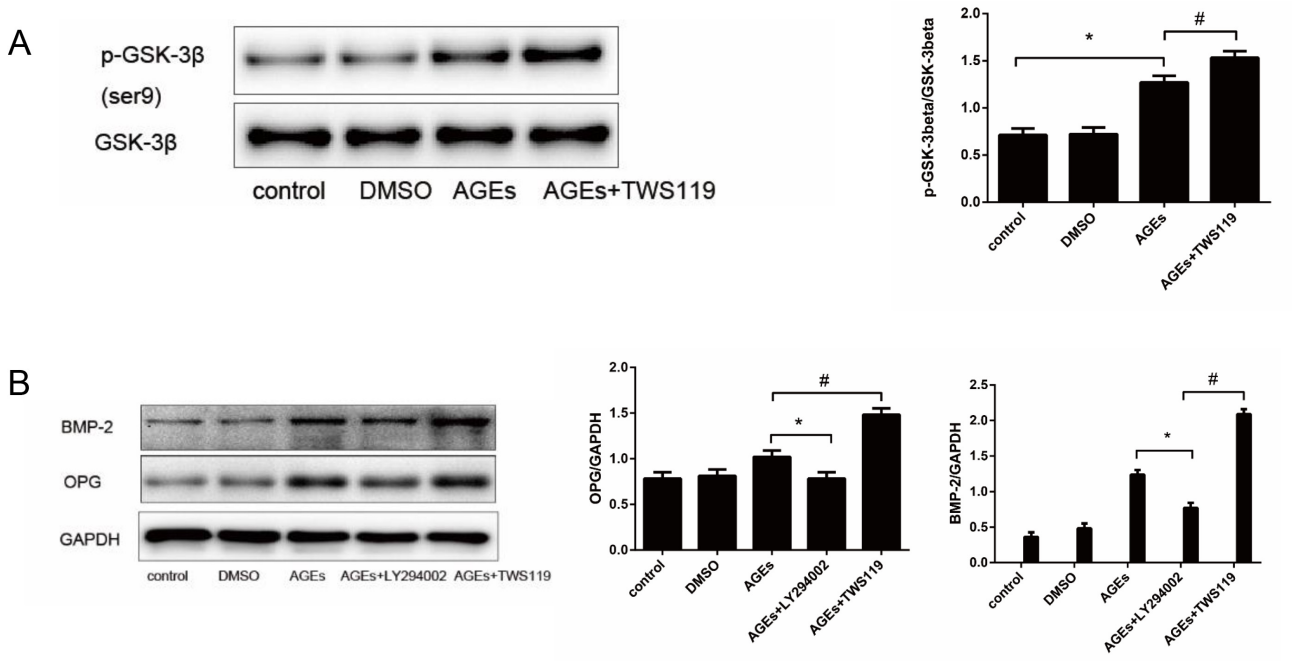

Fig. 4. Effect of GSK3-β on HASMC calcification. (A) TWS199 increased the expression of p-GSK3-β. (B) TWS199 promoted the AGE-induced expression of osteogenic factors, BMP-2 and OPG, in HASMCs. Western blotting was performed using whole cell lysates purified from cultured HASMCs that were divided into control, DMSO (vehicle), AGEs, AGEs + LY294002, and AGEs + TWS199 groups. In the AGEs + LY294002 and AGEs + TWS199 groups, cells were pretreated with LY294002 or TWS199 for 12 hours followed by AGE stimulation for 30 min. $*P < 0.05$, versus control group; $\#P < 0.05$, versus AGEs group.

tosis and calcification. Similarly, a high concentration of AGEs in the serum of diabetic patients induces apoptosis and calcification of VSMCs [25]. Through the induction of medial arterial calcification and the formation of calcified plaques, AGEs significantly contribute to the pathogenesis of diabetes-linked atherosclerosis [26, 27]. Our previous study also showed that AGEs induce the expression of their receptor, RAGE, and in combination with RAGE, AGEs promote calcification of HASMCs by activating the Wt/β-catenin signaling pathway [28]. Consistent with these previous findings, in the present study, AGE treatment significantly increased the number of calcified plaques in HASMCs in a dose-dependent manner and acted cooperatively with β-phosphoglycerine to promote HASMC calcification.

It has been well established that AGEs and RAGE play a key role in arterial calcification. However, after the binding of AGEs to RAGE, it remains unclear how exactly the signals are transmitted from the cell membrane to the nucleus to activate downstream signal transductions, thereby leading to changes in cellular activities. Increasing evidence suggests that the PI3K/AKT signaling pathway is involved in artery calcification. Okazaki *et al*. [29] studied human vascular smooth muscle dells (HVSMC) calcification induced by inflammatory mediators and found that the PI3K/AKT axis promotes HVSMC calcification by regulating the expression of alkaline phosphatase (ALP). Also, a recent study suggested that the PI3K/AKT signaling pathway is implicated in the osteoblast differentiation [30]. In line with previous reports, in the present study, AGE treatment increased p-AKT levels but did not alter the level of total AKT. Moreover, the increased AKT activity was functionally involved in AGE-mediated HASMC calcification, as evidenced by the observation that suppression of AKT activity by LY294002 attenuated the calcified plaque formation caused by AGEs. We also observed that LY294002 pretreatment significantly reduced the expression levels of OPG and BMP-2, both of which may synergize to promote calcification [31]. Moreover, AKT is required for BMP-2-promoted osteogenesis and vascular calcification [32]. Thus, our findings further confirmed the functional link between AKT and BMP signaling in the pathogenesis of vascular calcification.

On the other hand, previous studies implicated GSK3-β in vascular calcification. For instance, suppression of GSK3-β was shown to be involved in lithium chloride-promoted calcium deposition of VSMCs and in delayed fracture healing observed in connexin 43-null mice [33]. In agreement with these findings, we also found in the present study that AGEs potentiated HASMC calcification, which coincided with decreased GSK3-β activity.

Our previous observations regarding the role of GSK3-β in AGE-mediated HASMC calcification were further supported by the application of TWS119, a GSK3-β inhibitor, and by Ad-GSK3-β-RNAi-mediated GSK3-β knockdown, which showed that the suppression of

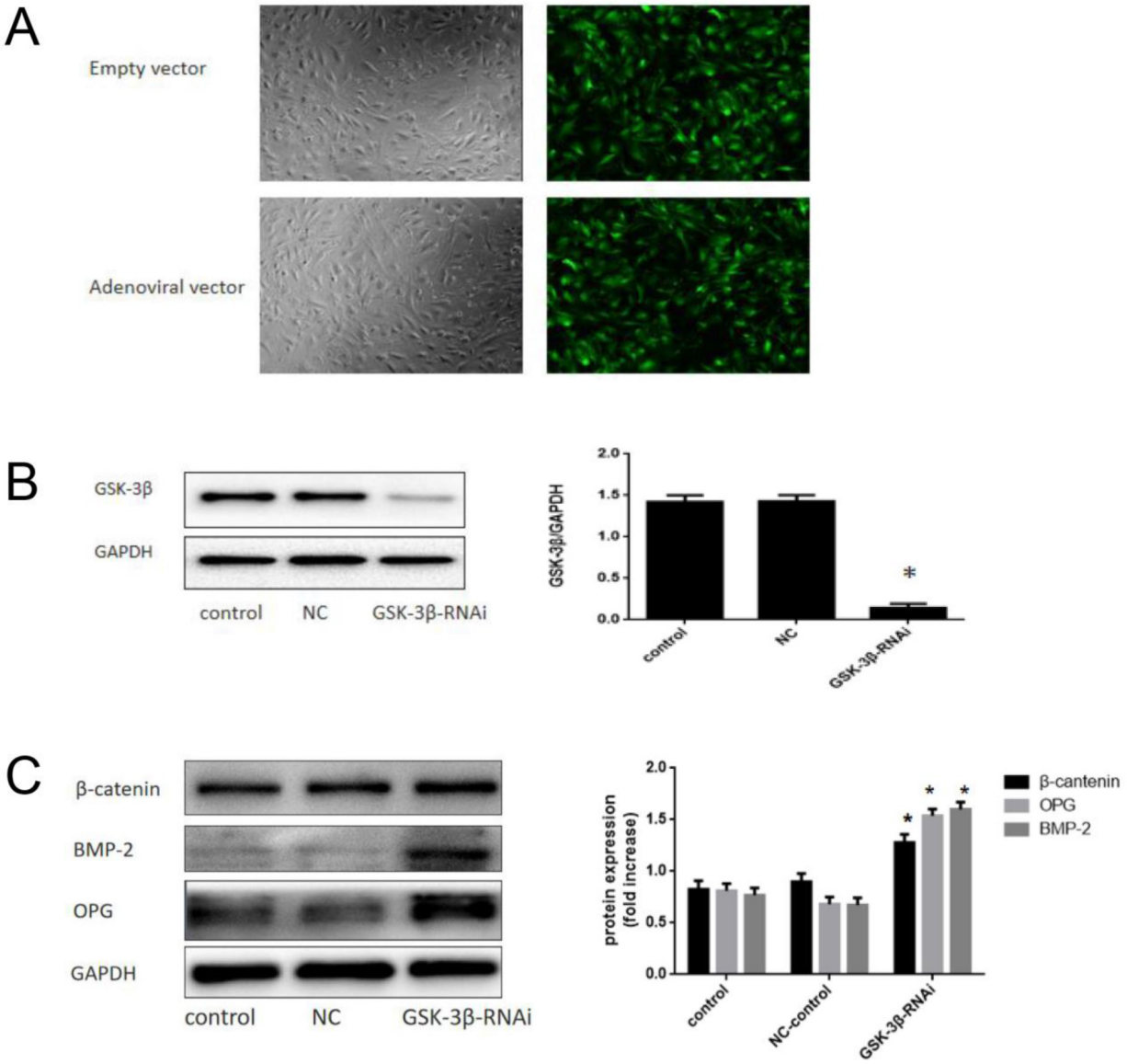

Fig. 5. GSK3-β knockdown promotes HASMC calcification. (A) Images showing HASMCs infected with Ad-GFP and Ad-GSK3-β-RNAi. Left, brightfield; right, fluorescence imaging of GFP expression. Magnification: 20×. (B) Western blot showing efficient knockdown of GSK3-β by Ad-GSK3-β-RNAi. NC, Ad-GFP group. *$P < 0.05$, versus NC group. (C) Western blot showing that GSK3-β knockdown promoted the expression of osteogenic factors, β-catenin, BMP-2 and OPG, in HASMCs. *$P < 0.05$, versus NC group.

GSK3-β activity significantly increased the calcification of HASMCs. Taken together, these findings support the notion that GSK3-β is also involved in the HASMC calcification induced by AGEs.

In conclusion, we demonstrated in the present study that AGEs promote HASMC calcification by activating PI3K/AKT signaling and suppressing GSK3-β activity. We also showed that the activated Wnt/β-catenin signaling contributes to AGE-induced HASMC calcification. Our findings suggest that regulation of the above-mentioned pathways may provide a potential novel strategy for the prevention and treatment of the vascular calcification that occurs in a number of cardiovascular diseases.

6. Author contributions

All authors contributed substantially to the preparation of this review. QCH designed and completed the experiments, as well as drafted and wrote the manuscript. YL, JWW and WJM edited and provided critical review of the manuscript. GY, YPW, KQX, LZ, and XFX helped with the design of the study. All authors discussed and confirmed the final manuscript.

7. Ethics approval and consent to participate

Not applicable.

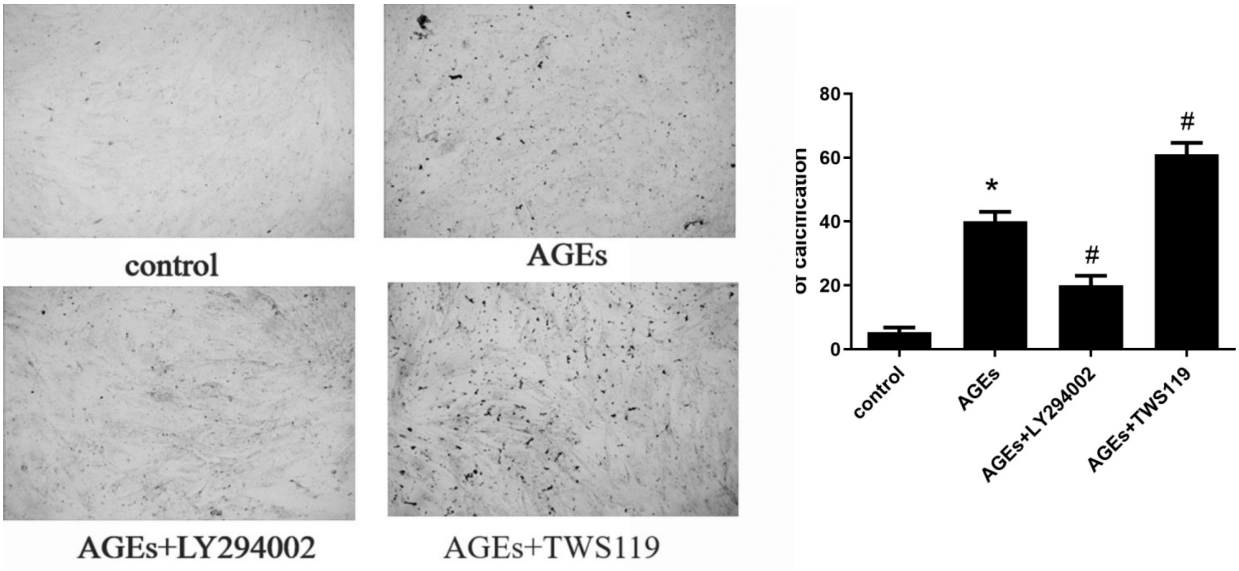

Fig. 6. Effects of suppression of AKT and GSK3-β on HASMC calcification. Cultured HASMCs were divided into control, AGEs, AGEs + LY294002, and AGEs + TWS199 groups. In the AGEs + LY294002 and AGEs + TWS199 groups, cells were pretreated with LY294002 or TWS199 for 2 hours followed by AGEs stimulation for 14 days. Calcification was detected by Von Kossa staining. $*P < 0.05$, versus control group; $\#P < 0.05$, versus β-phosphoglycerin group.

8. Acknowledgment

None.

9. Funding

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10. Conflict of interest

The authors declare that they have no conflict of interest.

11. Data availability statement

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Abbreviations: AGEs, advanced glycation end products; AKT, protein kinase B; BMP-2, bone morphogenetic protein 2; BSA, bovine serum albumin; DAPI, 4', 6-diamidino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GFP, green fluorescent protein; GSK3-β, glycogen synthase kinase 3 beta; HASMCs, human aortic vascular smooth muscle cells; OPG, osteoprotegerin; PI3K, phosphatidylinositol 3-kinase; PVDF, polyvinylidene difluoride; RAGE, receptors for AGE; VSMCs, vascular smooth muscle cells.

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