RESEARCH

Molecular mechanisms and effects of urocortin II on rat adventitial fibroblast calcification induced by calcified medium

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Abstract

The present study aimed to assess the role of urocortin II (UII) in the process of vascular calcification *in vitro* by using a calcification model, to detect the changes in the mRNA and protein levels of associated markers in rat adventitial fibroblasts (AFs) during their phenotypic transformation to osteoblast cellsto clarify the main signal transduction pathway of UII responsible for regulating vascular calcification and AF phenotypic transformation of osteoblast cells, and to prove that UII was an endogenous factor promoting vascular calcification, so as to provide an effective experimental basis for the clinical regulation of related diseases caused by vascular calcification. Finally, we successfully constructed the calcified cell model, found that UII was an endogenous substance regulating vascular calcification, regulated the vascular calcification by promoting apoptosis and inhibiting autophagy through up- and downregulated BAX and BCL-2/BECLIN 1 (BECN1) level, and the Wnt/β-catenin signaling pathway was involved.

Introduction

Vascular calcification is a common condition noted in aging, atherosclerosis, hypertension, diabetes, dyslipidemia, valvular heart disease, and chronic kidney disease (1, 2). It is caused by heterotopic deposition of hydroxyapatite on vascular walls (3). Vascular calcification increases the risk of heart disease, atherosclerotic plaque rupture, and stroke. It is an important risk factor for the increased incidence of cardiovascular disease-associated morbidity and mortality (4, 5). Therefore, vascular calcification is attracting increased attention worldwide. At present, various research studies have contributed to intima calcification, middle membrane calcification, and heart valve calcification. Previous clinical studies demonstrated that adventitia calcification is also possible. However, research studies on adventitia calcification are limited. Therefore, additional studies are required to elucidate the pathogenesis of vascular calcification.

Originally, vascular calcification was considered to be the result of passive degeneration of blood vessels, marking the aging of blood vessels. However, a growing number of studies have shown that vascular calcification is an active process regulated by a variety of molecular signaling pathways (4, 5, 6, 7), which are highly similar to those involved in bone formation (7, 8). Boström *et al.* demonstrated that during calcification, the morphology of rat vascular mesenchymal cells (VSMCs) was altered from

the original contractile phenotype to the osteoblast-like phenotype. The expression levels of the original contractile markers, such as α-SMA, were decreased, while the expression levels of Runx2, BMP-2, and other osteoblast-like markers were increased, which enabled the deposition of bone matrix in the blood vessels (9, 10). Apoptosis and autophagy are two pathways that induce cell death, which are closely associated with vascular calcification. Previous studies have shown that VSMCs release apoptotic bodies during the induction of apoptosis to promote cellular calcification. Autophagy has also been shown to regulate cellular calcification through multiple mechanisms of action (11).

Urocortin II (UII) is a neurocyclic peptide composed of 11 amino acid residues, which is currently considered to be a vasoactive peptide (12). Immunohistochemical and quantitative PCR analyses were used to measure the expression levels of UII in human atherosclerotic lesions. The data indicated that the expression levels of UII were increased (13). Rakowski *et al.* demonstrated that the use of the UII antagonist SB-611812 against UII could reduce the thickness of new intima and increase the lumen flow in a rat carotid artery angioplasty restenosis model (14). These studies suggested that UII played an important role in vascular dysfunction and remodeling. However, the mechanism of UII in the calcification of adventitia fibroblasts remains unclear.

The purpose of the present study was to investigate whether UII promoted adventitia fibroblast calcification by inducing apoptosis and inhibiting autophagy and to explore the possible mechanism of action. We hypothesized that UII promoted AF calcification by inducing apoptosis and inhibiting autophagy. To verify this hypothesis, we constructed an *in vitro* calcification model using AFs and investigated the effects of UII on the calcification of AFs and its possible mechanism of action.

Materials and methods

Cell culture

AFs were isolated from the thoracic aortas of 4- to 6-week-old male Wistar-Kyoto rats weighing 120–180 g and cultured according to the tissue adherence method (15). Animal experiments for AFs acquirement were carried out at the Molecular Biology Center of the First Central Clinical College of Tianjin Medical University, China. Briefly, for the rats, i.v. anesthesia was performed with 3% sterile saline solution of sodium pentobarbital according to 30 mg/kg body weight and were finally sacrificed by medullotomy after the experiments. These operations were strictly complied with the Helsinki Declaration and approved by the Ethics Committee of the Tianjin Medical University (TMU-2020-LLSC-0106). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Cat#11965092, Gibco) supplemented with 10% fetal bovine serum (Gibco) and maintained in a humidified atmosphere of 5% CO_2 at 37°C. AFs were cultured at 80–90% confluence and passaged with 0.125% trypsin-EDTA. Cells from passages three to six were used.

The following reagents were used: fetal bovine serum (FBS), high glucose DMEM, penicillin, streptomycin (Cat#85886, Sigma–Aldrich), alizarin red solution, β-glycerol phosphate, ascorbic acid, dexamethasone (Sigma), antibodies against GAPDH (Cat#AP0063, Bioworld, USA), antibodies against Bcl-2, Bax, Beclin1 and LC3 (Cell signaling), alkaline phosphatase assay kit, and calcium (Ca) test kit (Nanjing Jiancheng Biological Engineering Institute). The primers for the RT-PCR assay were synthesized by BGI Genomics.

Grouping

The cells were seeded into six-well plates at a density of 1×10^5 cells/L and maintained in DMEM containing 10% FBS. Following 24 h of incubation, the cells were divided into the following seven groups: (i) normal control group, which contained cells maintained in DMEM without other treatments, (ii) calcification group, including cells that were treated with 10 mmol/L β-glycerophosphate+0.05 mmol/L ascorbic acid+100 mmol/L dexamethasone, (iii) calcification+UII group, which included cells treated with UII at different concentrations (10^{-7}, 10^{-8}, and 10^{-9} mol/L) in the presence of the calcified medium, and (iv) inhibitor group, which contained calcified medium and UII (10^{-9} mol/L), and cells were treated with or without DKK-1 (50 ng/mL). The medium was replaced with fresh medium every 3 days. Following 15 days, the medium was removed and the cells were used for different experiments.

Measurement of alkaline phosphatase activity

The cells were seeded in six-well plates. The medium was removed and the cells were washed thrice with PBS. The alkaline phosphatase (ALP) activity was measured according to the manufacturer's instructions with a kit (A0592, Nanjing Jiancheng Biological Engineering Institute). In brief, 300 μL Triton X-100 (0.1%) was added to each well, followed by incubation at 4°C for 40 min. The lysed cells were transferred to a centrifuge tube. The

cells were centrifuged and the supernatant was used for detection. The incubation was performed at 37°C. The ALP activity was measured by using an atomic absorption spectrophotometer at 520 nm. The hydrolysis of para-nitrophenyl phosphate, which is used as a chromogenic substrate, within 1 min may produce 1 μmol of p-nitrophenol, which requires 1 unit of ALP activity.

Measurement of calcium content

The cells were seeded into six-well plates. The medium was removed and the cells were washed thrice with PBS. The calcium content was measured according to the manufacturer's instructions with a kit (C004-2-1 Nanjing Jiancheng Biological Engineering Institute). For decalcification, 300 μL of 0.6 M HCl was added to each well, followed by incubation at room temperature for 24 h. The supernatant was used for detection. The calcium content was measured using an atomic absorption spectrophotometer at 610 nm.

Alizarin red staining

The cells were seeded into six-well plates. The medium was removed, and the cells were washed in PBS twice and fixed in 4% paraformaldehyde (1 mL/well) for 30 min. Following removal of formalin, the cells were washed in PBS twice, followed by incubation with 0.1% alizarin red (1 mL/well) for 15 min. Following washing with PBS, the cells were observed under a light microscope in order to detect the calcified nodules. Three samples were selected from each group, and one field was randomly selected from each sample at ×100. The number of calcified nodules was compared between the groups. The calcified nodules were orange in color.

RT-PCR

The cells were harvested and total RNA was extracted with TRIzol® reagent (Invitrogen). The mRNA expression levels were determined by an SYBR green-based real-time PCR assay using a standard protocol as follows: predenaturation at 95°C for 5 min and a total of 35 cycles of denaturation at 95°C for 45 s, annealing at 58°C for 45 s, and extension at 72°C for 50 s, followed by a final extension at 72°C for 10 min. The experiments were performed thrice and GAPDH was used as an internal control. The relative mRNA expression levels of each gene were determined. The primers used for PCR are shown in Table 1.

Western blotting

The cells were harvested with lysis buffer. The lysates were collected, and the protein concentration was determined using the BCA Protein Assay Kit (A045-4, Nanjing Jiancheng Biological Engineering Institute). The protein samples were separated on 12% SDS-PAGE gels, transferred to polyvinylidene fluoride membranes (Millipore), and blocked with 5% non-fat milk/TBST for 2 h at room temperature. The membranes were subsequently incubated overnight at 48°C with primary antibodies. The membranes were washed with TBST three times and incubated with secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) (Millipore). The density of the bands was quantified with the Quantity One image analysis software (Bio-Rad). The optical density (OD) of each band was determined and subsequently normalized to that of GAPDH.

Statistical analysis

All data are expressed as mean ± s.e.m. The mean differences between the groups were analyzed by one-way ANOVA or *t*-test using SPSS 22.0 statistical software. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

AFs can induce calcification *in vitro*

It has been previously reported that the *in vitro* culture of VSMC in the presence of calcified medium can induce

Table 1 Primer sequence list.

Gene	Primers (5'–3')	
	Forward	Reverse
ALP	TGCAGTACGAGCTGAACAGG	GTCAATTCTGCCTCCTTCCCA
Opn	5'-TCAGCTGGATGACCAGAGTG	TTGGGGTCTACAACCAGCAT
Runx2	TGG CTT TGG TTT CAG GTT AGG	TGG AGA TGT TGC TCT GTT CG
BMP-2	TGA GGA TTA GCA GGT CTT TGC-3'	TCT CGT TTG TGG AGT GGA TG
Gapdh	GGC TGC CCA GAA CAT CAT	CGG ACA CAT TGG GGG TAG

their calcification. To assess whether high calcium and phosphorus can induce calcification of rat AFs, the cells were cultured in a calcified medium, and the ALP activity and intracellular calcium contents were detected at different time points. By extending the incubation time, the ALP activity was gradually increased and reached its peak on the ninth day of culture. The results were significantly different compared with the control group ($P < 0.05$). The intracellular calcium content was significantly increased following 9 days of induction compared with that of the control group ($P < 0.05$), and the increase noted in ALP activity levels and in the calcium content was time-dependent (Fig. 1A). The highest calcium content was detected on the 15th day of induction. Therefore, the induction time selected for subsequent experiments was 15 days. Alizarin red staining was performed on the cells following 15 days of induction of calcification. The results indicated that the cells in the control group were long and spindle-shaped, while the cells in the calcification group were diamond-shaped (data not shown). Following alizarin red staining, a large number of orange calcium nodules were observed in the calcified group (Fig. 1B), whereas these nodules were not present in the control group. The results demonstrated that AFs could induce calcification *in vitro* following incubation with the calcified medium.
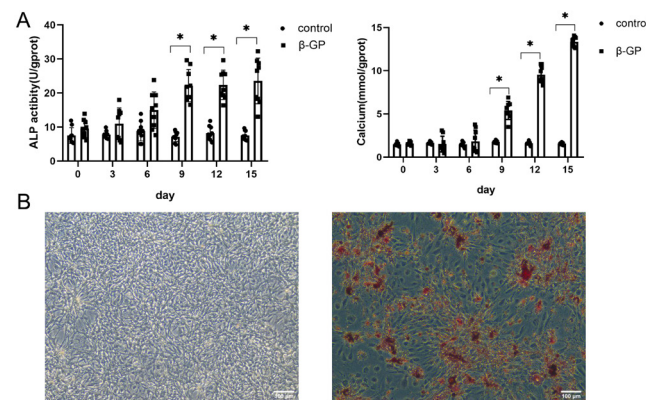
UII increases the calcification of AFs

In order to explore whether UII exerts an effect on the calcification of AFs, different concentrations of UII were added in the calcified medium to induce calcification. ALP

Figure 1
Rat extravascular outer membrane cells induce calcification *in vitro*. (A) By using the calcification-induced medium, the ALP activity and the intracellular calcium content were increased following the time of culture. (B) In culture, intracellular calcium nodules were clearly observed for 15 days with cissarin red staining (100×). In each group, the cells were seeded into six parallel wells for every single index detection.
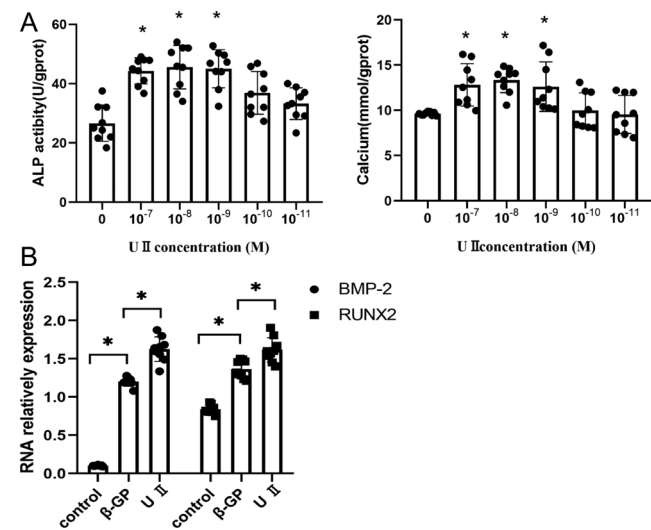
activity and intracellular calcium content were detected following 15 days of incubation. The data indicated that 10^{-9}, 10^{-8}, and 10^{-7} M UII significantly increased ALP activity ($P < 0.05$) (Fig. 2A). The intracellular calcium content was significantly higher than that of the calcified group ($P < 0.05$) following 15 days of cell incubation with 10^{-9}, 10^{-8}, and 10^{-7} M UII in the medium (Fig. 1A). Increased vascular calcification is associated with increased expression levels of osteoblast-specific markers, such as BMP-2 and Runx2 (16). RT-PCR results indicated that the mRNA expression levels of the osteoblast-specific markers *BMP-2* and *Runx2* were significantly increased in the calcified group compared with those of the control group ($P < 0.05$). Compared with the calcified group, the mRNA expression levels of *BMP-2* and *Runx2* were further increased in the presence of 10^{-9} M UII, and the differences were statistically significant ($P < 0.05$) (Fig. 2B).

UII promotes calcification by inducing apoptosis and inhibiting autophagy

In order to further explore the mechanism of UII in promoting cell calcification, the changes in the expression levels of apoptotic and autophagic proteins were assessed by Western blot analysis. Compared with the control

Figure 2
UII aggravates the calcification of vascular outer membrane cells in rats. (A) The incubation of the cells with different concentrations of UII demonstrated that high concentrations of UII could increase ALP activity and intracellular calcium content. (B) The expression levels of specific osteogenesis-related genes were detected using the RT-PCR assay, and the data indicated that the levels between all four osteogenesis-related genes were upregulated at 15 days following induction of calcification. UII caused an upregulation in the expression levels of osteogenesis-related genes. In each group, the cells were seeded into six parallel wells for every single index detection.

group, the expression levels of the pro-apoptotic protein Bax were upregulated, while those of the antiapoptotic protein BCL-2 were downregulated in the calcified group. The increase in the concentration of UII caused an upregulation in the expression levels of the BAX protein and downregulation in the expression levels of the BCL-2 protein in a concentration-dependent manner (Fig. 3A). Compared with the control group, the expression levels of the autophagy-related protein BECLIN1 (BECN1) and the ratio of LC3-II/LC3-I were increased in the calcification group, indicating that intracellular autophagy levels were enhanced following incubation with the calcified medium. However, the expression levels of the autophagy-related protein BECLIN1 and the ratio of LC3-II to LC3-1 were decreased following the addition of UII to the calcium content in a concentration-dependent manner (Fig. 3B). In conclusion, UII inhibited autophagy during calcification.

## UII promotes calcification by activating the Wnt/β-catenin pathway

Activation of the Wnt/β-catenin pathway is one of the key factors involved in cell calcification. In order to investigate whether UII promotes the calcification of AFs via the Wnt/β-catenin pathway, the cells were treated with DKK-1, which is a Wnt/β-catenin pathway inhibitor. The results of the Western blot analysis indicated that the expression levels of the BAX protein were upregulated, whereas the expression levels of the BCL-2 protein were downregulated following incubation of the cells with calcified medium for 15 days. The expression levels of BAX were further upregulated following the addition of UII in the calcium content, whereas the expression levels of BCL-2 were downregulated. The data indicated that DKK-1 reversed the pro-apoptotic effect of UII (Fig. 4A). Figure 3B indicated

that UII inhibited the expression levels of the autophagy-related protein BECLIN 1, whereas it could decrease the LC3-II/LC3-1 ratio. DKK-1 intervention caused the upregulation of the expression levels of the autophagy-related protein BECLIN 1 and an increase in the LC3-II/LC3-I ratio, which partially restored the autophagy levels of the cells, suggesting that UII may promote calcification in AF cells through the Wnt/β-catenin pathway.

To further confirm that the Wnt/β-catenin inhibitor DKK-1 can inhibit UII-induced cell calcification, the cells were simultaneously treated with UII+DKK-1, and the formation of intracellular calcium nodules was detected following 15 days of culture by alizarin red staining (Fig. 4C). The data indicated that following DKK-1 intervention, the number of calcium nodules induced by UII was significantly reduced.

Discussion

Vascular calcification refers to the abnormal deposition of calcium and phosphorus crystals on the vascular wall, which is divided into the three following types: intima calcification, middle membrane calcification, and outer membrane calcification (17). Vascular calcification is one of the complications of cardiovascular disease and has increased in incidence and severity in the past years. It is considered to be an important factor leading to the high mortality of cardiovascular disease. Currently, no effective treatment is available for vascular calcification. Therefore, the exploration of the pathogenesis of vascular calcification and the identification of effective diagnostic and prevention targets at the molecular level are urgently required.
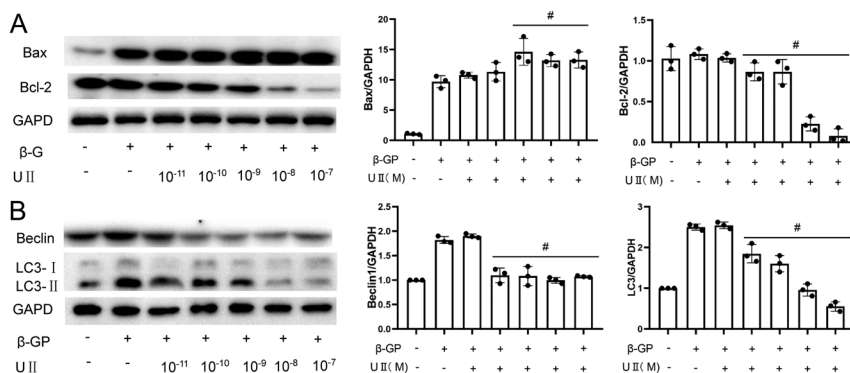
Figure 3

UII promotes cell calcification by inducing apoptosis and inhibiting autophagy. WB analysis indicated that following 15 days of calcification, the expression levels of the apoptotic protein BAX were increased, whereas those of the antiapoptotic protein BCL-2 were decreased. The expression levels of the autophagy-related proteins BECLIN 1 and LC3 were upregulated. UII caused further upregulation of the expression levels of the apoptotic protein BAX and downregulation of the expression levels of the apoptotic protein BCL-2. The expression levels of the autophagy-related proteins Beclin1 and LC3 were downregulated. The data indicated that UII exacerbated cell calcification by promoting apoptosis and inhibiting autophagy. In each group, the cells were seeded into six parallel wells for every single index detection.
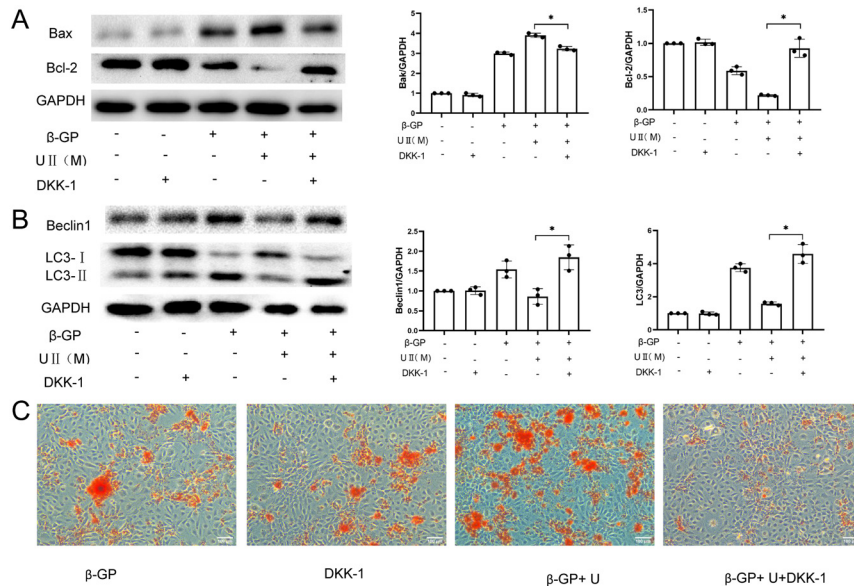
Figure 4

DKK-1, the inhibitor of the Wnt/β-catenin pathway, inhibits the effect of UII. (A and B) WB analysis indicated that treatment of cells with the Wnt/β-catenin inhibitor DKK-1 reversed the effects of UII, causing downregulation of the apoptotic protein BAX, upregulation of the antiapoptotic protein BCL-2, and upregulation of the autophagy-related proteins BECLIN 1 and LC3. (C) Sissarin red staining demonstrated that significant calcium nodules were observed in the intracellular fraction following 15 days of induction with calcified media. The simultaneous addition of the UII culture indicated significantly denser intracellular calcium nodules. The increase in the concentration levels of DKK-1 resulted in significantly smaller orange calcium nodules being present in the cells. In each group, the cells were seeded into six parallel wells for every single index detection.

A growing number of studies have shown that vascular calcification is an active process regulated by a variety of molecular signaling pathways (4, 5, 6, 7), which are highly similar to those involved in bone formation (7, 8). Previous studies have shown that TGF-β1 can induce calcification of rat extravascular membrane cells in the calcification medium (17). However, the present study demonstrated that significant calcification of AF cells could be induced *in vitro* by 10 mmol/L β-glycerophosphate+0.05 mmol/L ascorbic acid+100 mmol/L dexamethasone without the addition of exogenous cytokines. This was determined by alizarin red S staining and measurement of ALP activity levels and intracellular calcium content. In addition, the experimental results indicated that UII could further increase ALP activity and intracellular calcium content. These results were partially consistent with the classical calcification theory, while they further had proven the calcification process without exogenous cytokines, especially for the rare outer membrane calcification.

Factors such as oxidative stress, inflammation, hyperglycemia, hyperblood phosphorus, apoptosis, and even mechanical stress damage of blood flow can induce the differentiation of calcified cells to osteoblast phenotype through different cell conduction pathways. Cells with osteogenic phenotypes include the increased expression of osteogenesis-related proteins and osteogenesis-related transcription factors, such as bone-forming proteins, *Msx2* gene, transcription factors *Cbfal1* and *Osterix*, and alkaline phosphatase. These factors regulate the deposition or dissolution of calcium–phosphorus inorganic complexes in the vessel matrix and thus calcification formation or calcification dissolution. Certain studies have implied that in the process of vascular calcification, VSMCs transform into osteoblast-like cells and express specific osteogenic markers, resulting in the deposition of bone matrix, which is an important feature and mechanism of vascular calcification (18). The calcification conditions of AF cells used in the present study are consistent with those reported in previous studies for VSMCs, suggesting that the mechanism of AF cell calcification may be partially consistent with that of VSMC calcification. It has been reported that during the calcification process of VSMCs, the cells express osteogenic transcription factors, such as *Runx2*, which promote the expression levels of downstream bone-related proteins, such as bone morphogenetic protein BMP-2, and facilitate the active differentiation of cells into osteoblast-like cells (19). This mediated the deposition of the bone matrix in the blood vessels (19). In the present study, a similar mechanism was observed. The mRNA expression levels of *BMP-2* and *Runx2* were significantly increased in AFs following the incubation of the cells in the calcified medium for 15 days. UII could further promote the mRNA expression levels of *BMP-2* and *Runx2*. In the present study, calcium salt deposition and phenotypic transformation of osteoblasts suggest that AFs could be calcified by calcium-rich medium, which extended the classification of vascular calcification.

The mechanism of vascular calcification is complex, involving various signaling pathways, such as autophagy and apoptosis, activation of the Wnt/β-catenin signaling pathway, and induction of ER stress. As an adaptive response to cellular stress, autophagy is critical in maintaining vascular structure and function. Previous studies have shown increased levels of autophagy during

vascular calcification (20, 21). Autophagy can inhibit vascular calcification through a variety of mechanisms. In *in vitro* experiments, phosphorus increased the levels of autophagy in VSMCs and the number of autophagosomes, thereby inhibiting the apoptotic and calcification processes (22). Additional studies have shown that autophagy can inhibit the oxidative stress of VSMCs and the inflammatory response of vascular endothelial cells, which regulates lipid metabolism and reduces vascular calcification (19, 23). LC3 and BECLIN 1 are two typical markers of autophagy. The results of the Western blot analysis indicated that the LC3-II/LC3-I ratio and the expression levels of BECLIN 1 protein were increased in AF cells incubated with calcified medium for 15 days, while UII treatment decreased LC3-II/LC3-I ratio and BECLIN 1 expression in a concentration-dependent manner.

Apoptosis involves the induction of vascular calcification, and its inhibition can in turn inhibit the calcification process (20, 21). Shi *et al.* demonstrated that fibroblast growth factor 21 could regulate the CHOP and caspase-12 signaling pathways by endoplasmic reticulum stress so as to reduce vascular smooth muscle cell apoptosis and inhibit vascular calcification (22). In addition, the increased concentration of Pi or Ca^{2+} in the medium induced the formation and release of matrix vesicles, such as apoptotic bodies, from the plasma membrane of VSMCs, leading to the calcification of the extracellular matrix, which may be the nucleation site for vascular calcification (24). The findings demonstrated that the intracellular apoptotic levels were increased during the calcification induced by the calcified medium in AFs, which was consistent with previous studies. Concomitantly, intracellular autophagy levels were also increased, which may be the result of the cells regulating themselves against calcification. However, UII increased the apoptotic levels and inhibited autophagy, which aggravated the calcification of AF cells .

Previous studies have shown that the Wnt/β-catenin pathway is an important signaling pathway involved in the process of osteogenesis and vascular calcification. Activation of the Wnt/β-catenin pathway can promote the expression levels of calcification factors and ultimately initiate and promote the occurrence and development of vascular calcification by upregulating ALP activity. In contrast to this mechanism of action, the inhibition of the Wnt/β-catenin pathway has been reported to protect against vascular calcification (25, 26, 27). To determine whether the Wnt/β-catenin pathway plays a role in AF calcification, the cells were treated with DKK-1, which is a Wnt/β-catenin pathway inhibitor. The results indicated that inhibition of the Wnt/β-catenin pathway inhibited

apoptosis and promoted autophagy of AFs, which is consistent with the report of Liu *et al.* (25). Concomitantly, alizarin red staining was used to detect the formation of intracellular calcium nodules following induction for 15 days. The data indicated that following DKK-1 intervention, the number of calcium nodules induced by UII was significantly reduced. These results further demonstrated that UII promoted AF calcification partially by activating the WNT/β-catenin pathway.

In conclusion, the present study demonstrated that the calcium content could induce the calcification of rat AF cells, whereas UII could aggravate the degree of calcification by promoting apoptosis and inhibiting calcification. Moreover, the inhibition of the WNT/β-catenin pathway could alleviate the calcification induced by UII. Eventually, the changes of vascular elasticity and stretching force caused by cell calcification will seriously affect the physiological functions and even cause irreversible functional damage. Therefore, it is vital to improve tissue elasticity and tensile force through clinical intervention while explaining the calcification mechanism.

Conclusions

The culture of AF cells for 15 days in the calcified medium could successfully construct the calcified cell model. UII can increase calcium deposition and ALP activity in AF calcified cells in a concentration-dependent manner, suggesting that it is an endogenous substance regulating vascular calcification. Following UII intervention, the protein levels of BAX were significantly upregulated, while the protein levels of BCL-2 and BECLIN 1 were downregulated. The LC3-II/LC3-I ratio was upregulated, suggesting that UII regulated vascular calcification by promoting apoptosis and inhibiting autophagy. The Wnt/β-catenin signaling pathway blocker DKK-1 inhibited apoptosis and autophagy induced by UII and reduced the number of calcium nodules, suggesting that the ability of UII to promote cell calcification may be related to the activation of the Wnt/β-catenin signaling pathway.

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