

Impairment of autophagy mediates the uric-acid-induced phenotypic transformation of vascular smooth muscle cells

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Abstract

Hyperuricemia may be involved in the phenotypic transformation of vascular smooth muscle cells, thus promoting the occurrence of atherosclerosis, and autophagy may be one of the important links, but little is known about the specific molecular mechanism. We established a mouse model of hyperuricemia and studied the relationship between changes in autophagy levels and the phenotypic transformation of muscle cells. Our study found that high uric acid levels promote the phenotypic transformation of muscle cells by inhibiting autophagy, thus enhancing their proliferation and migration abilities. If autophagy is restored, phenotypic transformation may be reversed by reducing KLF4 levels. Thus, uric acid may induce the phenotypic transformation of muscle cells and promote the occurrence of atherosclerosis by disrupting normal autophagy.

Introduction

A large amount of epidemiological research has reported a close association between hyperuricemia and various chronic diseases, including coronary artery disease, kidney disease, hypertension, stroke, diabetes, metabolic syndrome, chronic obstructive pulmonary disease, and malignant tumors. Hyperuricemia has been increasingly recognized as a risk factor for atherosclerosis^[1, 2].

The specific mechanism by which hyperuricemia induces atherosclerotic lesions is not clear, although the existing literature indicates that the phenotypic transformation of vascular smooth muscle cells (VSMCs) is a critical step in the development of atherosclerosis^[3]. Uric acid (UA) is the final product of purine metabolism in humans and its synthesis is catalyzed by xanthine oxidase. Reactive oxygen species, including superoxide, are generated during purine metabolism, along with UA production. UA production may worsen endothelial function by reacting with superoxide and nitric oxide (NO), leading to reduced NO bioavailability and the increased production of peroxynitrite, a reactive oxidant^[4]. Endothelial dysfunction is an important risk factor for the development of atherosclerosis. UA induces VSMC proliferation and oxidative stress, leading to lipid deposition and the development of atherosclerotic lesions^[5]. Previous studies have shown that hyperuricemia induces endothelial dysfunction and accelerates atherosclerosis by interfering with the asymmetric dimethylarginine/dimethylarginine dimethylaminohydrolase 2 pathway^[6]. Some researchers have found that high levels of UA promote atherosclerosis through Nrf-2-mediated autophagy dysfunction and ferroptosis. Hyperuricemia significantly inhibits autophagy and protein levels of members of the NRF2/SLC7A11/GPX4 signaling pathway^[7]. Autophagy provides a protective effect under physiological conditions; however, it can be impaired under some pathological conditions. Our previous study showed that impaired autophagy, mediated by homocysteine under hyperhomocysteinemic conditions, contributes to the development of atherosclerosis^[8]. However, whether hyperuricemia impairs autophagy and leads to atherosclerosis has not yet been reported. Therefore, in this study, we aimed to investigate whether hyperuricemia affects VSMC autophagy and thus, influences endothelial cell phenotypic transformation, thereby providing new insights into the treatment of atherosclerosis.

Materials And Methods

Animals

Six-week-old male C57BL/6J mice (24 ± 0.5 g) were obtained from the Experimental Animal Center of Basic Medicine, Zhejiang Chinese Medical University, and randomized to the following four groups with eight mice in each group: control, rapamycin, rapamycin + UA, and rapamycin + UA + allopurinol groups. All rats were fed in the same environment, and all operations were performed under aseptic conditions. All experimental and animal operation procedures were approved by the Animal Ethics Committee of Shaoxing People's Hospital. The hyperuricemic model was induced using a combination of peritoneal injection of potassium oxonate at a dose of 250 mg/kg and intragastric administration of hypoxanthine at a dose of 300 mg/kg daily for 28 days. Rapamycin (2 mg/kg/d) was administered by gavage once daily for 28 days, and allopurinol (39 mg/kg/day) was dissolved in the drinking water for 21 days. After the experiment, the surviving mice were euthanized by an overdose of sodium pentobarbital, and the aortas were collected. The aortas were fixed in 4% paraformaldehyde for 12 h, embedded in paraffin, and serially sectioned (5 mm). Immunostaining and electron microscopy of the aortic roots were performed to detect aortic lesions.

Cell culture and treatment

Primary VSMCs (HA-VSMC line) and culture medium 231 were purchased from Cascade Biologics (Portland, OR, USA). Cells were cultured with different concentrations of UA for 48 h. Rapamycin (TargetMol, Boston, MA, USA) and MHY1485 were used to promote and inhibit autophagy, respectively. To knock out KLF4 in HA-VSMCs, the cells were transfected with an anti-*KLF4* siRNA using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA), according to the manufacturer's instructions.

Transmission electron microscopy

Aortic root specimens fixed with glutaraldehyde were examined by electron microscopy. The selected segments were dehydrated in graded ethanol and embedded in epoxy resin for transmission electron microscopic analysis (H-450; Hitachi, Tokyo, Japan).

Wound-healing assay

A wound-healing test was performed to evaluate cell migration. Cells were uniformly cultured in six-well plates until 80–90% confluent. Scratches of the same size were then formed perpendicular to the well plate with the tip of a sterile pipette. After washing thrice with phosphate-buffered saline (PBS), UA at the corresponding concentration was added to each well. Samples were photographed at 0 and 12 h using

an inverted microscope. The migration ability was measured as the area of migrating cells in the same visual field as the control. This experiment was performed thrice.

Transwell migration assay

VSMCs (2×10^4 cells per well) were inoculated into the upper chamber of a transwell system with a pore size of 8 μm . Meanwhile, 250 μL of culture medium containing different concentrations of UA was added to the upper chamber. VSMCs were incubated for 24 h at 37°C for migration detection. VSMCs in the upper chamber were washed and stained with crystal violet for 15 min. Migrated cells in the lower chamber were counted under a microscope in five randomly selected high-power fields.

Immunohistochemical staining

For immunohistochemical staining, aortic sections were quenched with 3% H_2O_2 , followed by treatment with citric acid buffer at 95°C for 15 min. After naturally cooling, the slides were washed with PBS containing Tween 20 (PBST). They were then incubated with the primary antibody, LC3b (ab192890, 1:200; Abcam, Cambridge, UK) at 4°C overnight, followed by the secondary antibody at 37°C for 45 min. The sections were stained with diaminobenzidine and then counterstained with hematoxylin. Stained sections were photographed at 200 \times magnification using a Leica DM3000 microscope (Leica, Wetzlar, Germany).

Cellular immunofluorescence

After the stained cells were cultured in a 32-well plate and the intervention factors were added, the cells were fixed with 4% paraformaldehyde for 20 min and incubated with 0.5% Triton X-100 in PBST for 20 min. After blocking with 10% homologous serum of the secondary antibody for 30 min, the cells were treated with primary antibodies against LC3 (Abcam, ab192890, 1:400) and α -SMA (Abcam, ab7817, 1:1,400) overnight at 4°C. The following day, the cells were incubated with DyLight 488 AffiniPure goat IgG(H + L) at room temperature in the dark for 2 h. The nuclei were counterstained with 0.5 $\mu\text{g}/\text{mL}$ 4',6-diamidino-2-phenylindole for 5 min. After each step, the cells were gently washed three times with PBS for 3 min each wash. Images were obtained using an Eclipse Ti-U fluorescence microscope ($\times 400$; Nikon, Tokyo, Japan).

Western blotting analysis

Protein samples were collected from the cells using radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China), and protein concentrations were assessed using a bicinchoninic acid assay to ensure that a consistent concentration was used for each protein sample. Electrophoresis was

performed using a sodium dodecyl sulfate polyacrylamide gel electrophoresis gel preparation kit (Beyotime) and the samples were then transferred to 0.45 µm and 0.22 µm polyvinylidene fluoride membranes (Millipore, Burlington, MA, USA). After 1 h of blocking at room temperature with Western Seal (Beyotime), the membranes were incubated with anti-LC3 (Abcam, ab192890, 1:1,000), anti-α-SMA (Abcam, ab7817, 1:1000), anti-P-mTOR (Abcam, ab109268, 1:1,000), anti-β-actin (Abcam, ab8226, 1:1,000), anti-KLF4 (Abcam, ab106629, 1:1,000), anti-OPN (Abcam, ab8448, 1:1,000), or anti-mTOR (Abcam, ab2732, 1:1,000) antibodies. Samples were gently shaken overnight at 4°C and incubated the following day with horseradish-peroxidase-labeled goat anti-mouse IgG (H + L; Abbkine, Inc., Redlands, CA, USA). The immune complex was irradiated with Beyo ECL Plus (Beyotime) using a dedicated tablet box (Beyotime). The gray levels of the target bands were identified using Quantity One 5.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each test was performed thrice.

Statistical analysis

Each test was performed thrice. All statistical analyses were performed using SPSS software (version 19.0; SPSS Inc., Chicago, IL, USA). The data obtained are shown as the mean ± standard deviation (SD). Intergroup differences were analyzed using a t-test, and data among multiple groups were compared using one-way analysis of variance, followed with Tukey's post hoc analysis. Two groups were compared using an unpaired Student's t-test. Statistical significance was set at $P < 0.05$.

Results

High uric levels promoted the phenotypic transformation of VSMCs *in vivo* and *in vitro*

First, we fed the mice with a high-purine diet for 2 months, and confirmed the successful establishment of a model of hyperuricemia by determining the serum UA concentrations of the mice (Fig. 1A). To determine whether hyperuricemia affected the phenotypic transformation of mouse arterial smooth muscle cells, we extracted mouse aortic tissue and determined the protein content using western blotting. As shown in Fig. 1B, the differentiation marker protein α-SMA was downregulated, and the dedifferentiation marker protein, OPN, was upregulated. In addition, using cultured cells, the *in vitro* immunofluorescence results were similar to the *in vivo* experimental results (Fig. 1C). Compared with the normal group, with the increase in UA concentration, the levels of α-SMA, a classical protein marker of VSMC phenotype transformation, decreased continuously, while OPN levels increased continuously. These results indicate that hyperuricemia promotes the phenotypic transformation of VMSCs *in vivo* and *in vitro*.

High UA concentrations promote the migration of VSMCs

The phenotypic transformation often leads to the migration of VSMCs. Therefore, we evaluated the effect of UA on the migration of VSMCs using scratch and transwell assays. The results showed that a high UA concentration promoted the migration of VSMCs and VSMC migration was positively correlated with UA concentration (Fig. 2A, 2B, and 2C).

A high UA concentration inhibited autophagy in VSMCs

We found that UA inhibited autophagy in VSMCs. With an increase in UA concentration, the fluorescence level of LC3B gradually decreased (Fig. 3A and 3D). Based on the *in vitro* experiments, we administered rapamycin, an inducer of mouse autophagy, and allopurinol, a UA-lowering drug. As shown in Fig. 3B and 3D, immunostaining of LC3B showed that the autophagy level was significantly higher in the rapamycin group than the control group. However, after UA intervention, the autophagy level decreased, but it increased again when the UA concentration was reduced by allopurinol, a UA-lowering drug. This indicates that high UA concentrations can regulate autophagy in VSMCs. Next, we examined autophagosome formation in aortic smooth muscle cells using transmission electron microscopy. We found that rapamycin significantly increased the number of autophagosomes, whereas UA inhibited autophagosome formation. The clearance of UA allowed the recovery of autophagy levels, which strongly indicated that UA regulates autophagosome formation (Fig. 3C).

A high UA concentration promotes the phenotypic transformation of VSMCs by regulating autophagy levels

Previous studies have shown a correlation between autophagy and phenotypic transformation. Therefore, we explored whether the UA-induced phenotypic transformation of VSMCs is associated with autophagy. After treating VSMCs with UA, mTOR was activated and α -SMA expression levels decreased. Rapamycin-induced autophagy enhancement may reduce the level of mTOR activation and enhance α -SMA expression. However, the use of autophagy inhibitors resulted in enhanced mTOR activation and decreased α -SMA expression levels, consistent with the results of UA treatment. These results indicate that high UA concentrations promote the phenotypic transformation of VSMCs by regulating autophagy (Fig. 4A and B).

Autophagy may inhibit the phenotypic transformation of VSMCs by reducing KLF4 levels

Previous studies have shown that the absence of KLF4 impairs autophagy, which adversely affects cell survival. KLF4 expression levels increased in mice fed a high-purine diet compared to the levels in normal mice (Fig. 5A), which was consistent with the findings of previous studies. To explore the role of KLF4 in high-UA-concentration-induced phenotypic transformation *in vitro*, we found that, in an environment with

a high UA concentration, KLF4 and OPN were highly expressed, and the induction of autophagy by rapamycin reduced the expression levels of KLF4 and OPN. However, transient silencing of KLF4 expression in UA-treated VSMCs using an anti-*KLF4* siRNA successfully downregulated OPN, a marker of contraction in VSMCs. The reversal effect of rapamycin on phenotypic transformation was extremely weak (Fig. 5B), suggesting that the reversal of phenotypic transformation by autophagy may be closely related to the reduction in KLF4 expression levels.

Discussion

In this study, we found that UA promoted the phenotypic transformation of VSMCs and induced their transformation from a “differentiation” type to a “dedifferentiation” type, and this transformation may be related to the effect of UA on autophagy. Further studies demonstrated that KLF4 mediated this effect.

There is a relationship between hyperuricemia and atherosclerosis. Hyperuricemia promotes an oxidative stress response and damages endothelial cells, which in turn, causes inflammatory reactions and induces atherosclerosis^[5]. The mechanism of endothelial dysfunction caused by a high UA concentration is that it induces the expression of pro-inflammatory factors (IL-1 β , IL-6, and TNF- α) by regulating pivotal inflammatory molecules, such as NF- κ B^[9, 10], leading to atherosclerosis. However, the specific molecular mechanism of hyperuricemia-induced atherosclerosis is far from clear, and new targets and signaling pathways need to be explored to optimize treatment.

We found that a high UA concentration promoted the phenotypic transformation of VSMCs and caused the transformation of highly differentiated contractile cells into synthetic cells in the normal vascular wall. Contractile VSMCs have weak proliferation and matrix synthesis abilities; are in the shape of spindle cells; are rich in regular myofilaments; and highly express contractile cell marker proteins, such as α -SMA^[11]. Synthetic VSMCs mainly play a role in maintaining the size of the vascular lumen through their synthetic function. Under the action of UA and other factors, VSMCs may dedifferentiate into synthetic cells, which are characterized by downregulated expression of differentiation marker genes; upregulated expression of the dedifferentiation marker protein, OPN; the disappearance of cell contraction function; and the formation and secretion of a large amount of extracellular matrix. Thus, the proliferation and migration abilities of VSMCs are enhanced, leading to the induction of atherosclerosis.

Autophagy is a biological process of cell self-degradation, in which garbage and damaged organelles are removed from cells and energy and nutrients are provided to maintain the normal physiological function of cells^[12]. Autophagy has an important effect on the proliferation and migration of VSMCs. By regulating autophagy, the proliferation and migration of VSMCs can be controlled, thereby affecting the growth and repair of blood vessels^[13]. Similarly, the level of autophagy in VSMCs plays an important role in the occurrence and development of atherosclerosis. The regulation of autophagy can reduce inflammatory responses and apoptosis and protect the survival and function of VSMCs. In conclusion,

autophagy plays an important role in muscle cells, affecting the growth and repair of blood vessels, and the occurrence and development of diseases^[14]. Previous studies have confirmed that autophagy is essential for the plasticity and phenotypic transformation of VSMCs. Brenner et al.^[15] found that the autophagy inhibitors, 3-methyladenine or spautin-1, stabilize the contraction phenotype of VSMCs and prevent their proliferation. Atorvastatin, an autophagically activated drug, protects VSMCs from TGF- β 1 and thus, reduces the formation of foam cells induced by calcification and lipid accumulation^[16]. However, whether autophagy plays a protective or a harmful role in atherosclerosis remains unclear. Our study showed that UA has an inhibitory effect on autophagy in vascular smooth muscle through the mTOR pathway. High UA concentrations decreased the levels of LC3b protein, which is a marker of mitochondrial autophagy, in VSMCs, indicating that a high UA concentration disrupted the normal autophagy level of cells. However, when the level of autophagy was restored by an autophagy promoter, the levels of the contractile cell marker protein, α -SMA, were also restored, indicating that a high UA concentration induced the phenotypic transformation of VSMCs by disrupting autophagy.

KLF4 is a transcription factor that participates in various biological processes, such as cell proliferation and differentiation, apoptosis, inflammation, and the cell cycle^[17]. Recent studies have shown that KLF4 is also involved in the regulation of autophagy and the progression of many cardiovascular diseases, including atherosclerosis. KLF4 also mediates the phenotypic transformation of VSMCs^[18]. Previous studies have shown that KLF4 regulates high-glucose-induced endothelial autophagy^[19], and KLF4 also acts as an upstream regulator of autophagy in diabetic nephropathy to alleviate endothelial cell damage^[20]. However, in this study, we found that mitochondrial autophagy reversed the phenotypic transformation of VSMCs by reducing KLF4 levels, suggesting an interaction between KLF4 and autophagy. Thus, KLF4 may be one of the key molecules mediating the UA-induced phenotypic transformation of VSMCs.

Although it is still uncertain whether UA is a direct risk factor for atherosclerosis, we have shown that UA has a significant effect on the function of VSMCs, which is related to the level of autophagy. Moreover, we explored the role of the key molecule, KLF4, in this process. There are still some limitations of this study, as the findings are based on mice and cultured cell lines, and they may not fully and accurately reflect the pathological changes in humans. Therefore, in our future research, we will carry out relevant clinical studies. In conclusion, we demonstrated that UA promoted the phenotypic transformation of VSMCs by affecting autophagy levels, which in turn, affected the expression levels of KLF4, suggesting that reducing UA levels *in vivo* may be an effective strategy to prevent the formation of atherosclerotic plaques.

Declarations

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none.

Ethics statement

The animal study was reviewed and approved by Experimental Animal Ethics Committee of Shaoxing People's Hospital. Written informed consent was obtained from the owners for the participation of their animals in this study. We confirm the study is reported in accordance with ARRIVE guidelines, and all methods were performed in accordance with the relevant guidelines and regulations.

Conflict of interest

All authors have no financial or personal relationships with other people or organizations to report that caused a conflict of interest in writing this paper.

Data availability statement:

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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Figures

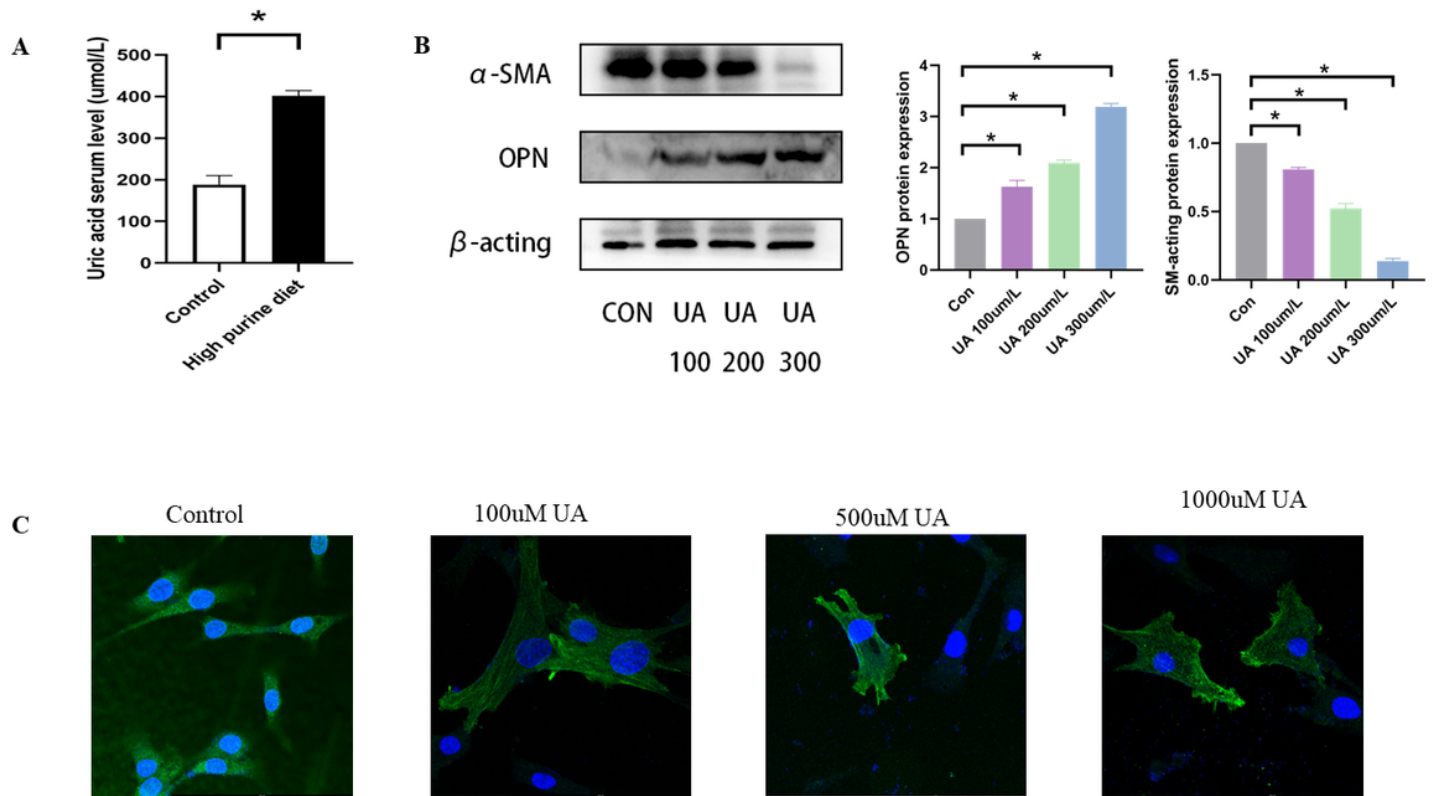


Figure 1

UA promotes the transition of vascular smooth muscle cells from a "differentiated" to an "dedifferentiation" state. (A) Serum uric acid levels in mice after a high-purine diet for 2 months. (B) Relative expression levels of the differentiation marker protein SM-actin and the undifferentiated marker protein OPN were determined by Western blot analysis. (C) Changes in the phenotype of VSMCs were observed by α-SMA fluorescence staining after treatment with uric acid in vitro culture: VSMCs became enlarged and deformed, lost their original spindle shape, and the distribution of α-SMA became uneven (*p<0.05).

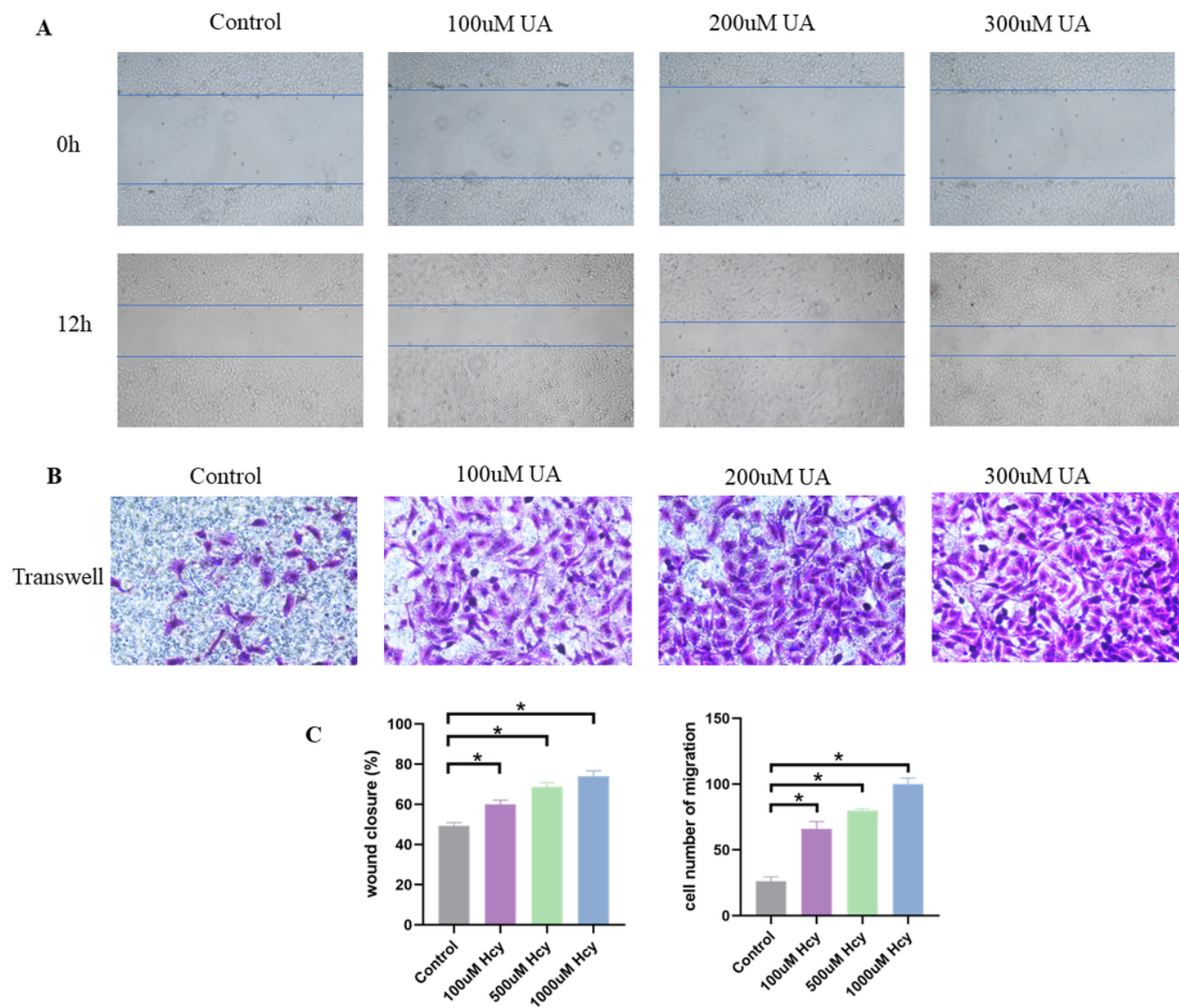


Figure 2

UA significantly promoted the migration ability of VSMC cells. (A) Scratch experiment was used to observe the migration ability of VSMC cells with different concentrations of UA. (B) Transwell experiment was used to observe the migration ability of VSMC cells under different UA (* $p < 0.05$).

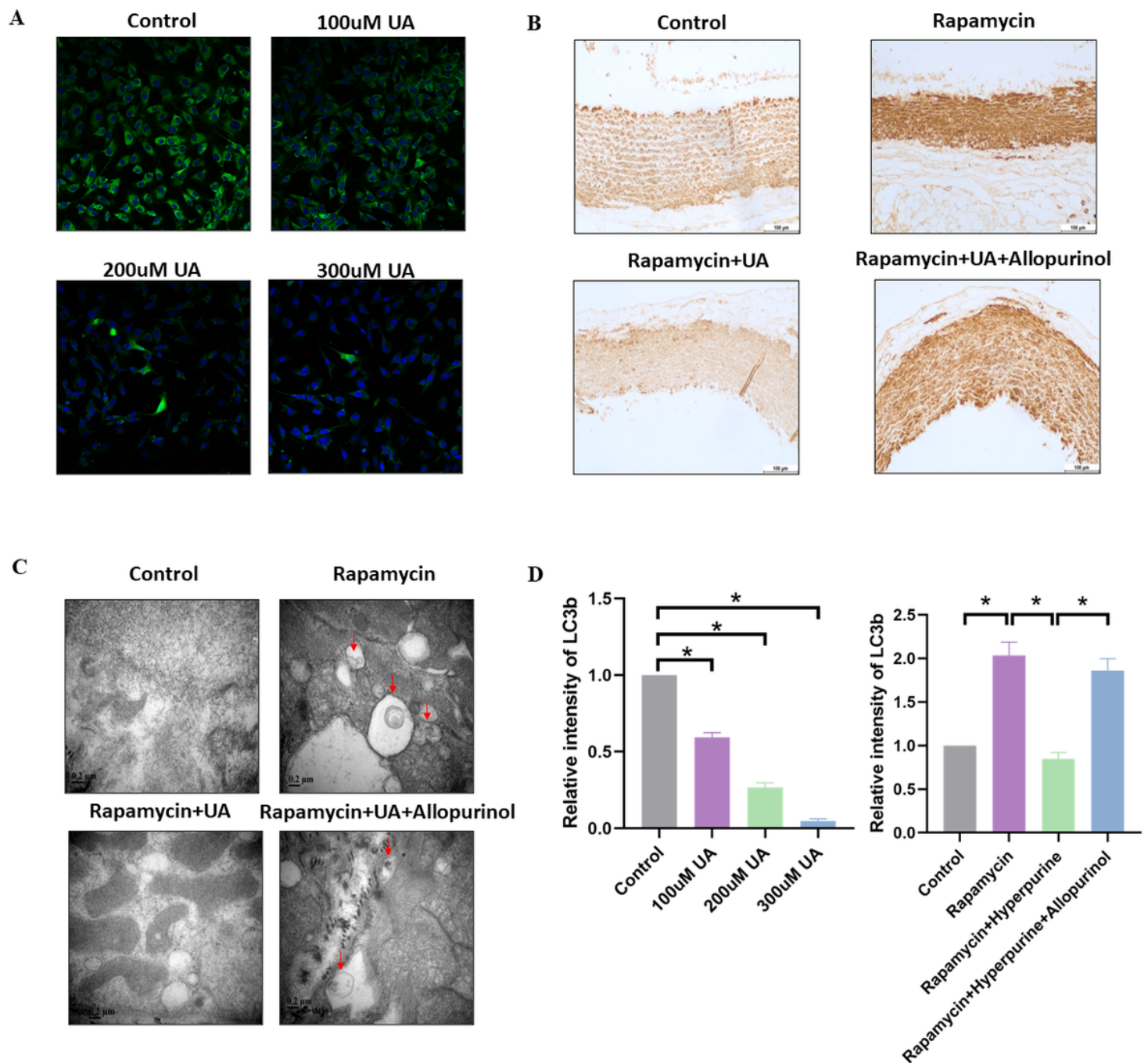


Figure 3

UA significantly inhibited the autophagy level of vascular smooth muscle cells. (A) The LC3 content of VSMC under the intervention of different concentrations of uric acid was detected by fluorescence staining. (B) Immunostaining was performed to detect the LC3 expression level in the aorta of mice in different groups. (C) The formation of autophagosomes in aortic smooth muscle cells was observed under the transmission electron microscope. (D) Relative quantification of fluorescence and immunostaining for LC3 (* $p < 0.05$).

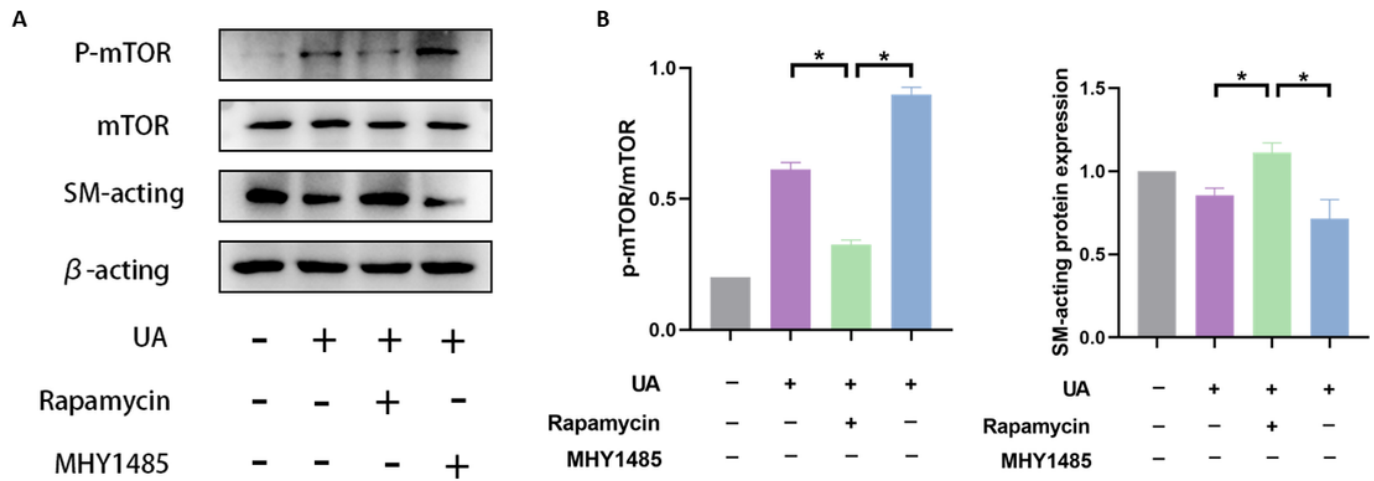


Figure 4

UA promotes phenotypic transformation of VSMC by inhibiting autophagy. (A) western blot was used to detect the relative expression levels of p-mTOR, mTOR and SM-actin in VSMC cells. (B) Relative quantitation of p-mTOR, mTOR, and SM-actin proteins (* $p < 0.05$).

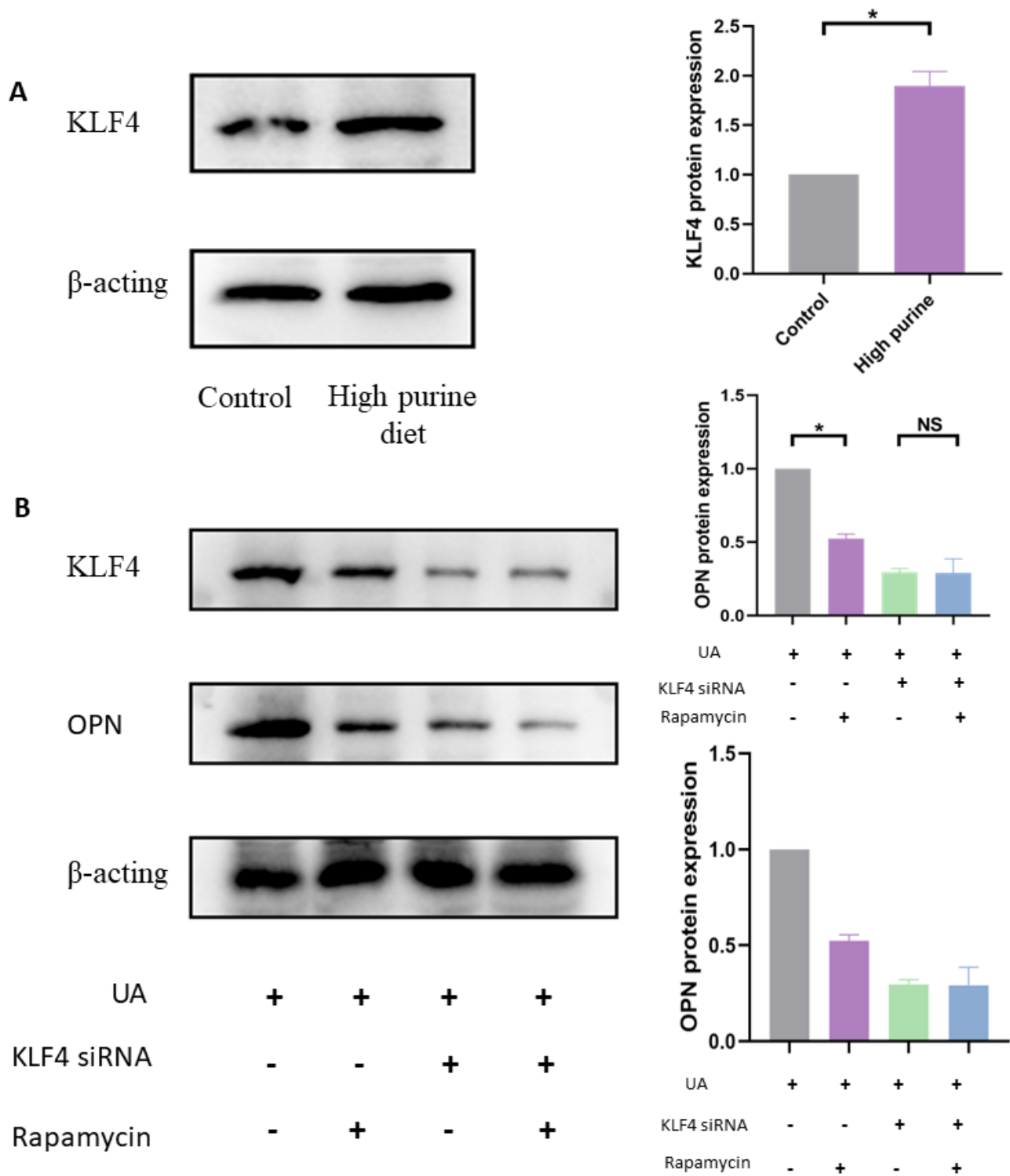


Figure 5

UA influences the phenotypic transformation of VSMC by regulating the KLF4 gene. (A) western blot was used to test the expression level of KLF4 in aorta of high purine diet fed mice and normal mice. (B) western blot examined the effect of autophagy on phenotypic transformation of VSMC cells in the presence or absence of the KLF4 gene (* $p < 0.05$).