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Research Article

Krüppel-like factor 4 promotes autophagy in macrophages under high glucose concentration by inhibiting the AKT/mTOR signaling pathway

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Abstract

Background: Diabetic atherosclerosis (AS) is the main cause of disability and death in diabetes. In the progression of AS, autophagic activity plays an important role. Krüppel-like factor 4 (KLF4) is a member of the zinc finger protein transcription factor family and is believed to play a protective role in the pathogenesis of atherosclerosis. This study aimed to explore the role of KLF4 in diabetic atherosclerosis and the autophagic mechanism.

Methods: A diabetic mouse model was established and the expression level of KLF4 protein in the aorta of the mice was detected after a high-fat diet. The effects of KLF4 on cholesterol content, apoptosis, autophagy-related proteins, and the AKT/mTOR signaling pathway of THP-1 macrophages were also evaluated.

Results: The expression level of KLF4 protein in the aorta of diabetic mice was decreased. Meanwhile, overexpression of KLF4 in THP-1 macrophages significantly decreased cholesterol accumulation, increased beclin-1 expression, decreased P62 expression, enhanced LC3 fluorescence intensity decreased cell apoptosis and p-mTOR and p-AKT expression were decreased under the condition of high glucose. After the reduction of KLF4 expression, the result is reversed.

Conclusion: KLF4 induces autophagy by inhibiting the AKT/mTOR pathway and alleviates cholesterol deposition in THP-1 macrophages under high glucose concentration.

Abbreviations

DM: Diabetic Mellitus; AS: Atherosclerosis; ox-LDL: oxidized Low-Density Lipoprotein; KLF4: Krüppel-Like Factor 4; siRNA: small Interfering RNA; THP-1: Human Monocytes Leukemia Cells; mTOR: mammalian Target of Rapamycin; PMA: Phorbol-12-Myristate-13-Acetate; LV: Lentiviral; NC: Negative Control.

Background

Macrovascular complications of Diabetic Mellitus (DM), characterized by Atherosclerosis (AS), such as coronary atherosclerotic heart disease, stroke, and peripheral artery disease, are the main causes of disability and death in diabetes [1]. In the early stage of AS, an inflammatory response is triggered by endothelial injury, and monocytes are recruited to sub-endothelium [2] and differentiate into macrophages under the action of various cytokines. Macrophages engulf lipids (especially oxidized low-density lipoprotein, ox-LDL) to form foam cells [3], which is an important step in the formation and development of AS plaques. Along with plaque progresses, macrophage aggregation increases, leading to macrophage apoptosis, inflammation, and plaque necrosis, eventually leading to cardiovascular and cerebrovascular events [4,5]. So, early intervention in AS formation targeting macrophage-

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derived foam cells has become the main direction for the prevention of AS.

The zinc finger protein transcription factor (Krüppel-like factors, KLFs) family is a class of binding proteins that regulate gene transcription [6]. Many studies have shown that family member KLF4 is closely related to endothelial cells, macrophages, other inflammatory cells, and pro-inflammatory factors [3], which may be particularly relevant to AS. Therefore, it is believed that KLF4 plays an important role in the AS pathogenesis, but its mechanism in diabetic AS is not completely clear.

In the progression of AS, autophagic activity plays an important role in combating oxidative stress and cellular self-protection [7]. Impairment of autophagy function increases lipid deposition in mouse arteries, leading to AS [8]. Studies have reported that autophagy is involved in lipid balance metabolism and cholesterol efflux [9]. In recent years, studies have found that inhibiting the mammalian Target of Rapamycin (mTOR) becomes a novel approach for the stability of atherosclerotic plaques [10]. Selective inhibition of the PI3K/Akt/mTOR pathway can effectively induce autophagy, which ultimately reduces the accumulation of macrophages in plaques, promotes plaque stability, and reduces inflammation factors secretion [11]. Studies have shown that in mouse embryonic fibroblasts, KLF4 plays a significant role in the regulation of autophagy and inhibits mTOR activity [12]. At the same time, KLF4 promotes IL-4-induced macrophage polarization to M2 and elevates the expression of the key protein MCP-1-inducible protein (MCPIP), while experiments showed that knockout of MCPIP inhibits macrophage autophagy [13]. Therefore, we speculate that KLF4 induces autophagy in macrophages by inhibiting mTOR, thereby promoting the stability of atherosclerotic plaques and delaying the occurrence and development of diabetic atherosclerosis.

Materials and methods

Animal model

Wild eight-week-old female Balb/c mice (SiPeiFu Beijing Biotechnology Co., Ltd.) were housed in a temperature-controlled animal facility under a 12 h light/dark cycle with food and water ad libitum. They were randomly divided into the diabetic group (DM group, n = 5) and normal control group (NC group, n = 5), maintained on a high-fat and sugar diet composed of (by mg) 10% sugar, 10% lard, 5% yolk, 1% cholesterol, and 0.2% bile salt and standard chow diet, described in the previous study [14]. Eight weeks later, the DM group was intraperitoneally injected with 80 mg/kg 2% STZ once a day for three consequent days [14]. Mice with blood glucose ≥ 16.7 mmol/L were considered as DM mice. The NC group was simultaneously injected with sodium citrate buffer. Four weeks later, mice were sacrificed by cervical dislocation. The aortic arches were dissected from the iliac bifurcation to remove external fatty deposits and collected for the following *in vitro* experiment. All animal procedures were approved by Animal Ethical and Welfare of Tianjin Medical University Chu Hsien-I Memorial Hospital (Approval No. DXBYY-IACUC-2022056).

Cell culture

Human monocytic THP-1 cells were obtained from Tianjin Cosmo Biotechnology Co., Ltd. Cells were grown at a density of 5 × 10^5 cells/mL in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin solution (Biological Industries, Israel) in an incubator at 37 °C, 5% CO_2. In all experiments, THP-1 cells were differentiated using 25 nM phorbol-12-myristate-13-acetate (PMA) (Beyotime, Shanghai, China) over 48 h, followed by a recovery period of 24h in culture in the absence of PMA [15].

Cell transfection

Three synthetic small interfering RNAs (siRNA) targeting KLF4 and siRNA negative control (si-NC) were generated by GenePharma, China. SiRNA sequences are listed below (Table 1). They were transfected into cells, at a final concentration of 50 nM, through the Lipofectamine 2000 System (Invitrogen, USA) [16], according to the manufacturer's instructions.

On the other hand, the KLF4 sequence was subcloned into the pcDNA3.1 vector (Y3616, GenePharma, China) for the plasmid construction to over-express KLF4, and the empty vector was the control. Vectors were transfected into cells using Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions.

Cell counting Kit-8 (CCK-8) assay

Cell viability was analyzed by the Cell Counting Kit-8 (CCK-8) (Solarbio, BeiJing, China) according to the manufacturer's instructions. THP-1 cells were cultured in 96-well plates (2×10^3 cells with 100 µL culture medium per well) and incubated with different concentrations of glucose (5.6, 12.7, 25.5, 33 mmol/L) and different hours (0, 12, 24, 36 h). Then, 10 µL of CCK8 solution was added to each well of the 96-well plate and incubated in the dark for 4 h [17]. Subsequently, absorbance was evaluated at 450 nm with a microplate reader (Tecan, Switzerland). All experiments were repeated three times.

Oil red O staining

Oil-red O Staining was used to evaluate the red lipid droplets produced in THP-1 cells [18]. After glucose treatment for 24 h, each group was rinsed in PBS, and fixed in 4% paraformaldehyde for 30 min. The Oil red O solution (IO1720, Solarbio, BeiJing, China) was diluted in deionized water in a 3:2 ratio and added to cells for 10 min at room temperature. Destained and rinsed with 60% isopropyl alcohol to remove

Table 1: Primer sequences for cell transfection.

Gene	Sequence
KLF4-Homo-1	5'- GGAGAAGACACUGCGUCAATT -3' 5'-UUGACGCAGUGUCUUCUCCTT-3'
KLF4-Homo-2	5'-GGUCAUCAGCGUCAGCAAATT-3' 5'-UUUGCUGACGCUGAUGACCTT-3'
KLF4-Homo-3	5'-CAAAGAGUUCCCAUCUCAATT-3' 5'-UUGAGAUGGGAACUCUUUGTT-3'
KLF4-siRNA NC	5'-UUCUCCGAACGUGUCACGUTT-3' 5'-ACGUGACACGUUCGGAGAATT-3'

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excess dye. Counterstain was performed with hematoxylin (H9627, Solarbio, BeiJing, China) for 5 min and rinsed twice with PBS. Slides were covered with glycerol gelatin and observed under an inverted microscope (Olympus, Japan). Image J pro plus software (version 6.0) (National Institutes of Health, USA) was used to calculate the area of oil red O staining.

Cholesterol content determination

Total cholesterol levels in cells were measured using an enzymatic colorimetric assay [19]. The cells were collected from six-well plates and then crushed using ultrasonic waves. Subsequently, the total cholesterol levels of the cells were determined using a total cholesterol assay kit (Elabscience Biotechnology, China) according to the manufacturer's instructions. Absorbance values (OD values) were observed by a microplate reader with a wavelength of 510 nm.

Terminal deoxynucleotidyl transferase (TdT) - mediated dUTP nick end labeling (TUNEL) assay

The apoptosis cells were determined using the TUNEL Apoptosis kit (Elabscience Biotechnology, China) [20], according to the manufacturer's instructions. Briefly, immerse the naturally dried cell smear into the fixing solution at 4 °C for 20min. The fixed sample was immersed in PBS for 3 times, 5 minutes each time. For the prepared Triton X-100 permeable solution, 0.2% Triton X-100 was added with PBS and mixed for immediate use. The specimen was immersed in permeable solution and treated at room temperature for 5 minutes, then immersed in PBS 3 times and rinsed for 5 minutes each time. Configuration of the label working liquid. The dosage of each sample in the experimental group is: 35 μL balance liquid (1x) was added with 10 μL fluorescent labeling solution and 5 μL TDT enzyme. Each sample was added with 100 μL balancing solution (1x) and equilibrated at room temperature for 20min. Absorbent paper absorbs the balance liquid, each sample drops 50 μL of labeled working liquid, covers the slide, and puts it in a wet box at 37 °C for 60 minutes without light reaction. The sample was immersed in PBS 3 times and rinsed for 5min each time. DAPI was added to absorbent paper after drying, incubated at room temperature for 5 minutes away from light, and the nucleus was re-dyed. The sample was immersed in PBS and rinsed 4 times, 5 minutes each time. The excess liquid is sucked up with absorbent paper and sealed with a fluorescent quenching agent. Observe with a fluorescence microscope (OLYMPUS FV1000, Japan) at an excitation wavelength of 450 nm and an emission wavelength of 515 nm - 565 nm. 200 - 400 cells were counted for the calculation of the percentage of the cells with dark nuclei.

Immunofluorescence staining

Immunofluorescence staining was performed to detect the expression of LC3 in THP-1 cells [21]. Cells were fixed with 4% polymerized formaldehyde for 20 min on coverslips and washed 3 times with PBS. Subsequently, Triton X-100 (0.5% mass fraction) (Solarbio, Beijing, China) was added for 10 minutes and blocked with bovine serum (2% mass fraction) (Gibco, USA) for 1 hour at room temperature. Then, an anti-LC3 antibody (A5179; BIMAKE, USA; 1:1,000) was added and incubated overnight at 4 °C. The next day, the coverslips were washed 3 times with PBS, and then FITC secondary antibody (Cell Signaling Technology, USA; 1: 500) was added and incubated at room temperature for 2 hours. Next, the cells were observed under the fluorescence microscope. Image J Pro Plus software was used to calculate the average fluorescence intensity for semiquantitative analysis.

Western blot analysis

Buffer A cell lysates (Cell Signaling Technology, USA) containing a protease and phosphatase inhibitor cocktail (Roche Diagnostics, Switzerland) were added to cells collected from all groups of THP-1 cells or tissues obtained from the aorta of mice, and homogenized for 30 min at 4 °C. Total protein extracts were quantified using the Bicinchoninic acid protein assay kit (Thermo Fisher Scientific) and were separated by 10% SDS-PAGE, then transferred to a nitrocellulose membrane. Subsequently, the membranes were blocked with 5% fat-free dry milk at room temperature for 1 h. Immunoblotting analysis was performed as previously described [22]. The blots were incubated with human anti-AKT (A5031; BIMAKE; 1:1,000), anti-p-AKT (Ser 473) (A5030, BIMAKE; 1:1,000), anti-mTOR (AF6308; Affinity; 1:1,000), anti- p-mTOR (Ser2448) (AF3308; Affinity; 1:1,000), anti- LC3 (A5179; BIMAKE; 1:1,000), anti-Beclin1 (AF5128; Affinity; 1:000), anti-p62/SQSTM1 (ab56416; Abcam; 1:1000), anti-KLF4 (A5391; BIMAKE; 1:1,000), anti-β-actin (ab8226; Abcam; 1:2,000) overnight at 4 °C. The membranes were again washed with PBS and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibodies (P0448; Agilent Technologies Inc., Santa Clara, USA; 1:2,000) at room temperature for 1 h. Proteins were finally examined by an enhanced chemiluminescence system (Bio-Rad Laboratories, USA). Band intensities were quantified by densitometry using Image J Software.

Quantitative real-time polymerase chain reaction (qRT-PCR) assay

Total RNA from cells was extracted using the RNeasy mini kit (Qiagen GmbH, Germany) according to the manufacturer's protocol. The RNA concentration was analyzed by a Pan-wavelength microplate reader (BioTek Instruments, USA). Then, cDNA was synthesized by reverse transcriptase (Takara, Beijing, China) and amplified using SYBR Premix reagent (Takara, Japan) for qRT-qPCR for 40 cycles on an ABI 7500 system. The RNA expression level of β-actin was used to normalize the RNA expression level of KLF4 with the Ct value. The $2^{-\Delta\Delta Ct}$ method was used to calculate the fold change differences in gene expression [16]. The primers were constructed by Sangon Biotech Co. Ltd. (ShangHai, China). KLF4: Forward: 5′-ACTCGCCTTGCTGATTGTCT-3′, Reverse: 5′-GGCCGAGATCCTTCTTCTTT-3′. β-actin: Forward: 5′-GGCACTC TTCCAGCCTTCCT-3′and Reverse: 5′-GCACTGTGTTGGCGTACAGG-3′. Each sample was determined in triplicate.

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Statistical analysis

Statistical analyses were performed using Statistical Product and Service Solutions 22.0 software (SPSS, USA). Data are represented as mean ± SD. Student's t-test or one-way analysis of variance (ANOVA) was used for comparisons between groups. The difference was considered statistically significant when *p<0.05.

Results

The expression of the KLF4 protein is decreased in the aorta of DM mice

Wild Balb/c mice were fed a high-fat diet and diabetes was induced using STZ as previously described [14]. After 4 weeks, the blood from the inner canthus was collected and the mice were sacrificed. During the experiment, the blood glucose of the experimental group was always at a high level and remained stable, and the plasma TG and TC levels of the DM group were significantly higher than those of the NC group (p < 0.05) (Table 2). KLF4 expression in the aorta of DM mice was significantly lower than that of non-DM mice (Figure 1), indicating that high glucose negatively regulated the expression of KLF4.

Glucose-stimulated cell proliferation in a concentration- and time-dependent manner

To choose the appropriate concentration of glucose and the appropriate timing of treatment in the following experiment, we performed the CCK-8 assay to detect the proliferation activity of THP-1 cells and found that glucose-stimulated cell proliferation in a concentration and time-dependent manner. We found that by increasing the glucose concentration, the OD value gradually increased compared to the glucose concentration at 5.6mmol/L (***p < 0.01). Furthermore, 25.5 mmol/L had a higher OD value than 12.7 mmol/L (*p < 0.05), but the highest glucose concentration (33 mmol/L) had no statistical differences compared to 25.5 mmol/L (Figure 2A).

Subsequently, the OD value gradually increased with prolongation of the intervention time and was statistically significant compared to cells at 0 h (Figure 2B) (*p < 0.05, *** p < 0.01). Cells incubated with a glucose concentration at 24h and 36h presented higher OD values than at 12h (*p < 0.05, ***p < 0.01, respectively), while OD values between 24 h and 36 h did not have statistical significance. Therefore, the results enabled us to proceed with the *in vitro* experiments under 25.5 mmol/L high glucose concentration for 24 h.

KLF4 inhibits lipid deposition in macrophages when exposed to high glucose concentration

To evaluate KLF4's function on macrophages, we performed oil red staining to observe the lipid droplets change in THP-1 cells after KLF4 lentivirus or siRNA transfection. The lentivirus and siRNA validations in THP-1 cells were performed by qRT-PCR, respectively. After transfection of three synthetic siRNAs (1,2,3) of KLF4, the expression level of KLF4 in the siRNA 1 group decreased significantly, as shown in Figure 3A. Therefore, siRNA 1, was selected as KLF4 siRNA for the following experiments.

Table 2: Comparison of blood glucose and blood lipid between two groups of mice ($x \pm s$).

Group	Number	Blood glucose (mmol/L)	TG (mmol/L)	TC (mmol/L)
DM	5	17.17 ± 1.47*	2.25 ± 0.07*	7.13 ± 0.83*
NC	5	5.40 ± 0.35	1.26 ± 0.11	2.25 ± 0.10

*: Compared with the control group, p < 0.01. TG: Triglyceride; TC: Total Cholesterol.

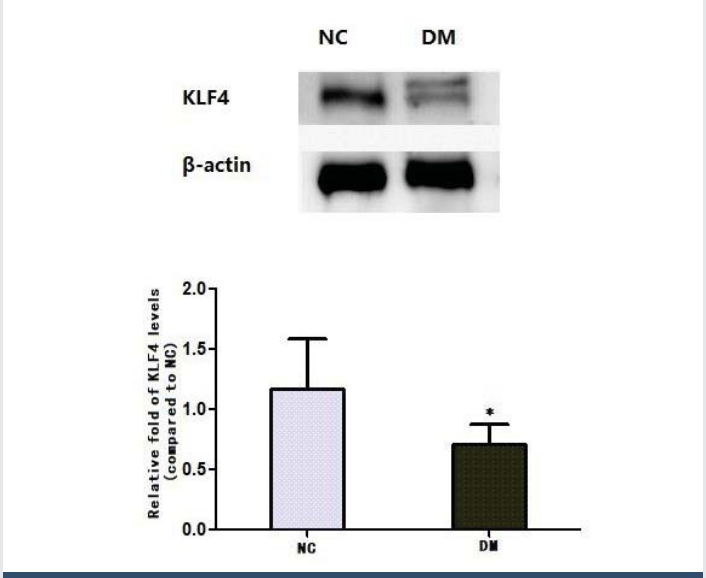

Figure 1: KLF4 expression in the aorta of DM mice was reduced compared to non-diabetic mice. KLF4 expression was evaluated by Western blotting in the aorta of DM mice and non-diabetic mice (*p < 0.05 *vs.* NC). NC: Negative Control; DM: Diabetic Mellitus.

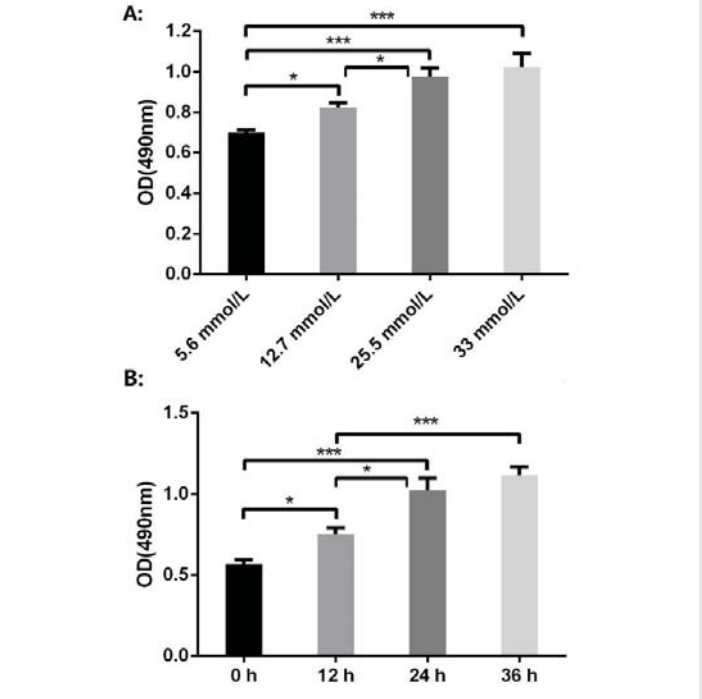

Figure 2: Glucose stimulated THP-1 cell proliferation in a concentration- and time-dependent manner. The CCK-8 assay was used to assess the influence of high glucose on the viability of THP-1. Results are shown as means ± SD. Figure 2A showed that the OD value gradually increased compared to the glucose concentration at 5.6mmol/L, while 25.5 mmol/L had higher OD values than 12.7 mmol/L. The OD value gradually increased with time prolongation and 24 h exposure to 25.5 mmol/L glucose was statistically significant compared to cells at 12 h shown in Fig. 2B (*p < 0.05, ***p < 0.01).

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KLF4 expression level was significantly increased after KLF4 lentivirus transfection (**$p < 0.01$) (Figure 3B).

After KLF4 removal, oil red staining showed that THP-1 cells produced foam cells rich in lipids in the cytoplasm (Figure 4). However, after overexpressing KLF4, the lipid droplets in THP-1 cells under a glucose concentration of 25.5 mmol/L were significantly reduced (Figure 4).

KLF4 decreases the intracellular total cholesterol content in THP-1 cells

To evaluate the effect that KLF4 has on cholesterol formation in THP-1 cells under 25.5mmol/L glucose concentration, the cholesterol kit was used to determine the total cholesterol level in cells after KLF4 knockdown or overexpression. After KLF4 siRNA transfection, we observed an increase in cholesterol content in human THP-1 cells, while after KLF4 lentivirus transfection, the cholesterol content decreased in THP-1 cells (Figure 5).

KLF4 overexpression increased macrophage apoptosis under high glucose concentrations

To assess how KLF4 affects macrophage apoptosis after high glucose (25.5mmol/L) intervention for 24h, TUNEL staining was performed. The number of apoptotic cells (red) in the Si-KLF4 group increased compared to the control group, while the number of apoptotic cells did not change much in the LV-KLF4 group (Figure 6A). At 36h, the number of apoptotic cells increased even more in the Si-KLF4 group (red), indicating that as time passed, the apoptotic cells increased (Figure 6B). These findings suggest that KLF4 may reduce apoptosis of THP-1 macrophages at high glucose concentrations.

THP-1 cells overexpressing KLF4 upregulate autophagy to inhibit the progression of diabetic atherosclerosis

To assess the effect of KLF4 on autophagy during diabetic atherosclerosis, changes in LC3, a specific marker of autophagy, were determined. *In vitro* immunofluorescence indicated that the intensity of LC3 fluorescence was stronger after KLF4 overexpression, while it decreased after KLF4 knockdown, indicating that the level of autophagy was significantly decreased after KLF4 knockdown (Figure 7A). Then the

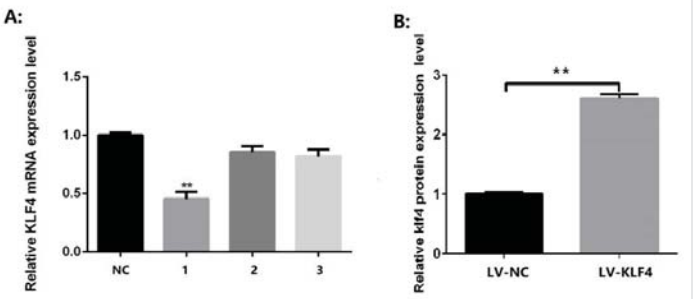

Figure 3: Transfection of KLF4 lentivirus and siRNA. Figure 3A: After transfection of three synthetic siRNAs (1,2,3) of KLF4, the expression level of KLF4 in the siRNA1 group was significantly reduced as proved by qRT-PCR (**$p < 0.01$). Figure 3B: qRT-PCR showed that KLF4 expression level was significantly increased after KLF4 lentivirus transfection (**$p < 0.01$). LV: Lentiviral; NC: Negative Control.

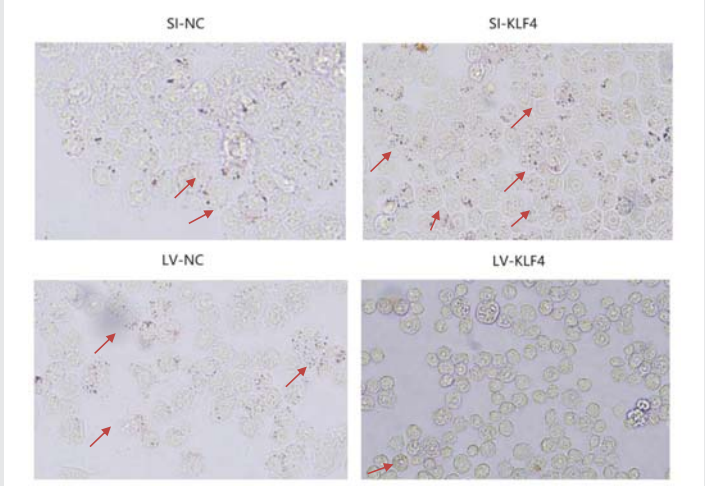

Figure 4: KLF4 inhibits lipid deposit in THP-1 cells under 25.5 mmol/L glucose concentration. After transfection with KLF4 siRNA, THP-1 cells promoted the formation of many red lipid droplets in the cytoplasm, while overexpression of KLF4 decreased lipid deposition. ×200. LV: Lentiviral; SI: siRNA; NC: Negative Control.

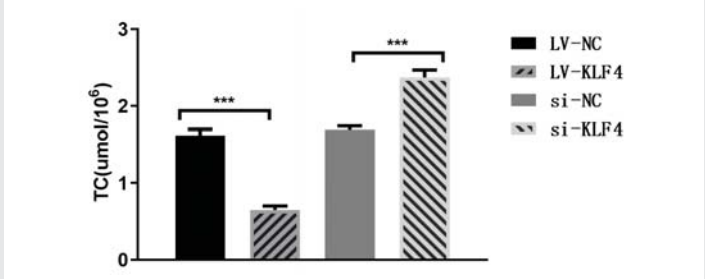

Figure 5: KLF4 inhibits the intracellular total cholesterol level in THP-1 cells. KLF4 knockdown or overexpression increased or decreased total TC levels, respectively. The cholesterol kit measured the TC levels in cells after specified transfections (***$p < 0.01$); LV: Lentiviral; NC: Negative Control; TC: Total Cholesterol.

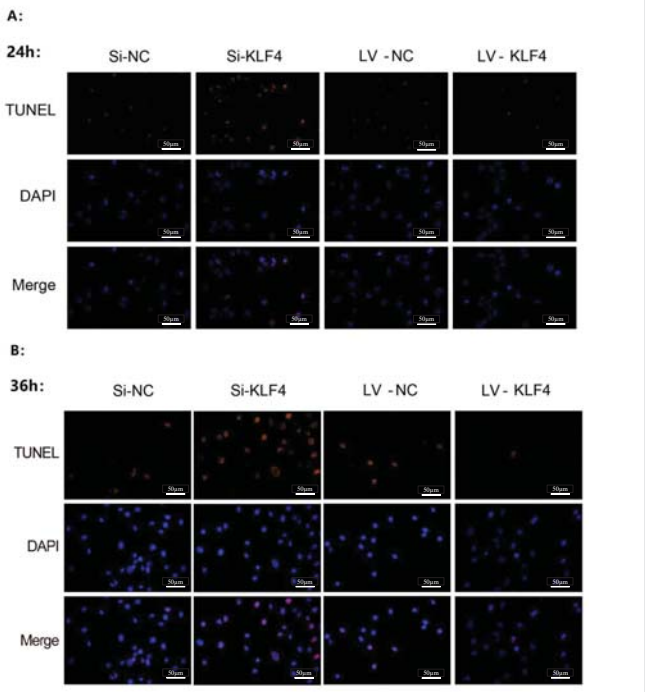

Figure 6: KLF4 reduced THP-1 cell apoptosis under high glucose concentrations. The results of TUNEL staining showed apoptotic cells after transfection with KLF4 siRNA or lentivirus under 25.5mmol/L high glucose concentration at 24h (Figure 6A) or 36h (Figure 6B), respectively. LV: Lentiviral; Si: siRNA; NC: Negative Control. Red staining stands for the apoptotic cells.

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fluorescence was quantified to the mean gray value and the results were consistent with the LC3 fluorescence intensity graphics (Figure 7B). These data illustrated the important role of KLF4 in the regulation of autophagy to exert its protective effect against diabetic atherosclerosis. Then, western blotting was used to detect expression levels of proteins related to autophagy. KLF4 knockdown decreased beclin1 (Figure 8A) and LC3 (Figure 8C) protein expression levels while increasing p62 (Figure 8B) expression level. On the opposite, KLF4 overexpression increased beclin1 (Figure 8A) and LC3 (Figure 8C) protein expression levels and reduced p62 (Figure 8B) expression levels.

KLF4 activates autophagy in THP-1 cells under high-glucose concentration through negative feedback regulation of mTOR signaling pathway

To study the mechanism of KLF4-induced autophagy activation in THP-1 cells, we used western blotting to detect expression levels of proteins related to the mTOR signaling pathway (Figure 9). Total AKT and mTOR expression levels were similar, but after KLF4 overexpression, the expression levels of p-AKT and p-mTOR decreased (*$p < 0.05$). In contrast, after KLF4 elimination, the expression level of p-AKT and p-mTOR increased (*$p < 0.05$) (Figure 9). So, it was inferred that KLF4 activates autophagy in THP-1 cells under high-glucose concentration through negative feedback regulation of the mTOR signaling pathway.

Discussion

As we know, diabetic patients are prone to developing atherosclerosis due to high glycemic toxicity [23]. Meanwhile, high glucose can enhance the toxic effects of ox-LDL [24,25], which eventually leads to the promotion and initiation of atherosclerosis formation.

KLF4 has been reported to be involved in the development of atherosclerosis [26,27]. As a transcription factor containing the zinc finger domain, KLF4 has been shown to regulate various cellular activities, including cell metabolism, cell cycle,

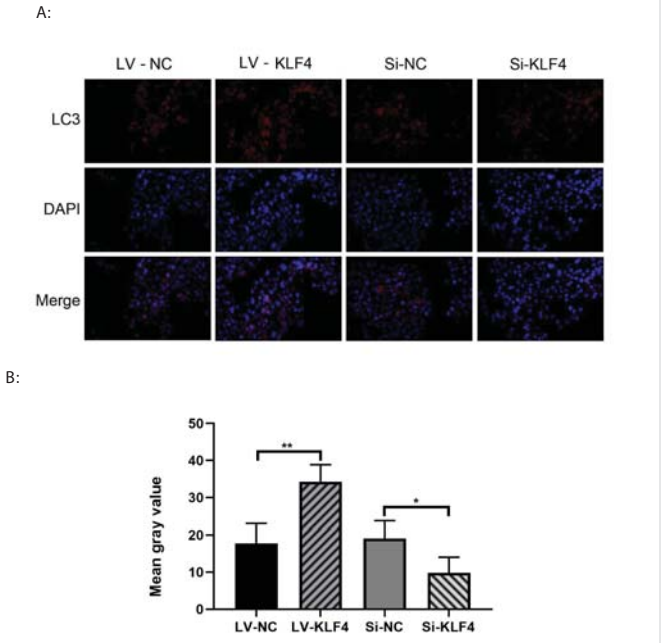

Figure 7: KLF4 overexpression upregulates autophagy in THP-1 cells. An immunofluorescence assay was used to evaluate the expression of LC3 in THP-1 cells under 25.5 mmol/L glucose concentration. KLF4 overexpression enabled greater LC3 fluorescence intensity, while KLF4 knockdown decreased LC3 fluorescence intensity (**$p < 0.01$, *$p < 0.05$). LV: Lentiviral; NC: Negative Control.

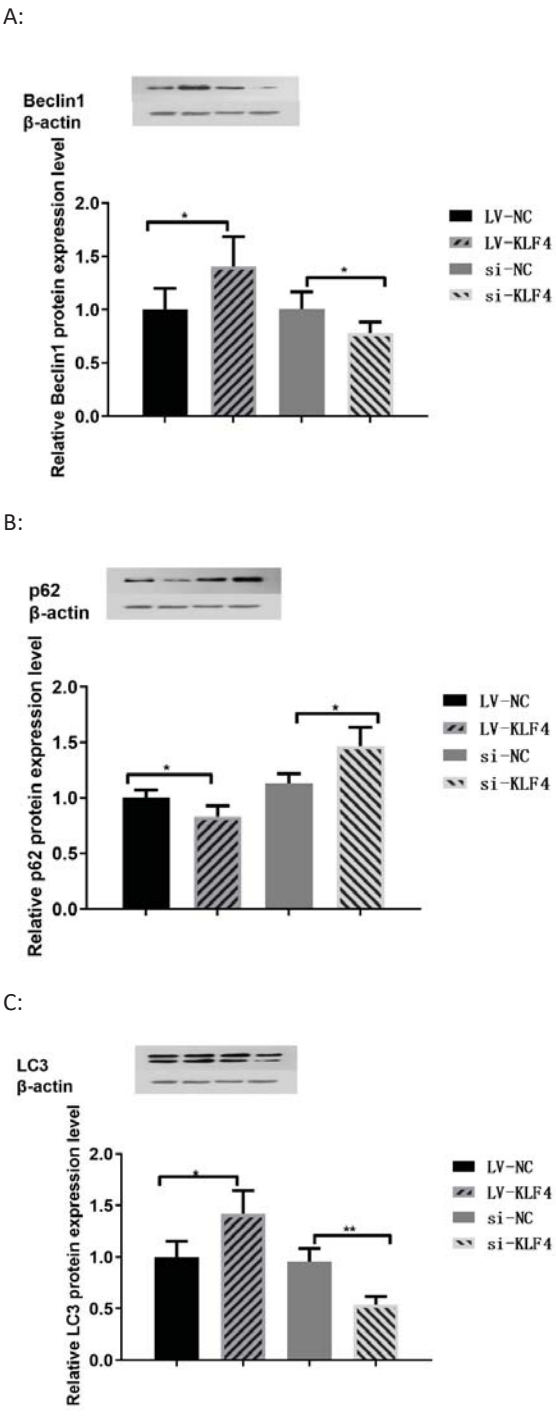

Figure 8: KLF4 activates autophagy in THP-1 cells. KLF4 knockdown decreased beclin1 (Figure 8A) and LC3 (Figure 8C) protein expression levels while increasing p62 (Figure 8B) expression level. KLF4 overexpression showed the opposite results. Bar graphs showing the quantitative evaluation of the level of beclin-1, LC3, and p62, respectively, in each group (*$p < 0.05$, **$p < 0.01$). LV: Lentiviral; NC: Negative Control.

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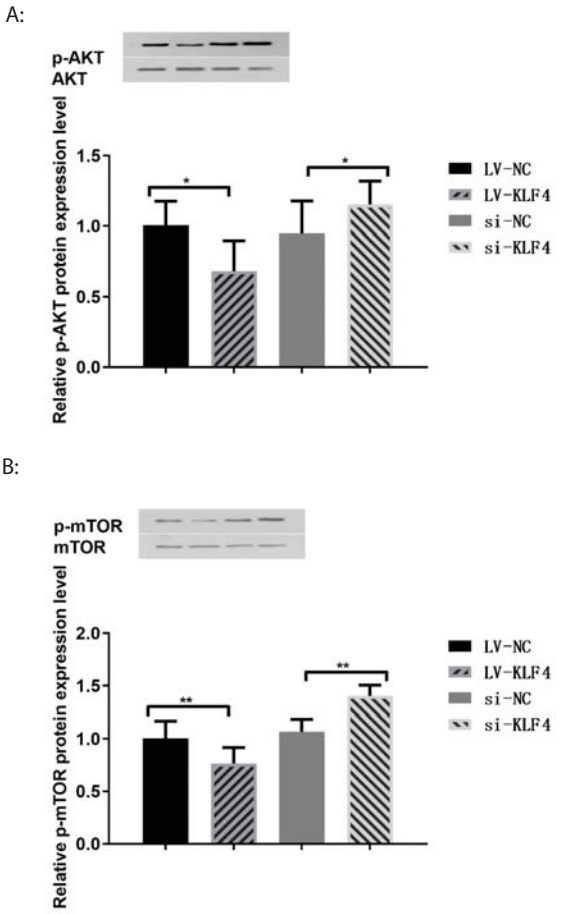

Figure 9: KLF4 activates autophagy in THP-1 cells through the AKT/mTOR signaling pathway. KLF4 knockdown induced p-AKT and p-mTOR expression while KLF4 overexpression decreased p-AKT (Figure 9A) and p-mTOR (Figure 9B) expression, respectively. Bar graphs showing the quantitative evaluation of the level of p-AKT/AKT, p mTOR /mTOR in each group (*$p < 0.05$). p: phosphorylated; LV: Lentiviral; NC: Negative Control; A: LV-NC; B: LV-KLF4; C: si-NC; D: si-KLF4.

apoptosis, and autophagy [28]. Furthermore, it has been found to be a key regulator of macrophage polarization, in which KLF4 expression increased strongly in M2 macrophages, while it decreased significantly in M1 macrophages [29]. Furthermore, in human umbilical vein endothelial cells KLF4 negatively regulates the expression of vascular cell adhesion molecule -1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1), which are important in the development of atherosclerosis [30].

In this study, we found that the expression of KLF4 decreased in the aorta of diabetic mice. Since we have previously found that the aortic cholesterol content and plaque increased in diabetic mice [14], we deduce that KLF4 is involved in the development of diabetic atherosclerosis. After that, we induced human monocyte THP-1 into a macrophage. The results showed that KLF4 plays a key role in the regulation of cholesterol uptake in THP-1 macrophages under high glucose concentration. Meanwhile we found that KLF4 plays a key role in regulating cholesterol uptake in THP-1 cells under high glucose concentration. When the cholesterol load exceeds the threshold at which cells can respond, excess cholesterol cannot be released from the cell, inducing the release of inflammatory factors, oxidative stress, and apoptosis, leading to cellular dysfunction [31]. Furthermore, the addition of high glucose slightly weakened the ability to respond to the cholesterol load, resulting in increased cholesterol accumulation. We also found that the apoptosis in the KLF4 overexpression group decreased. The enhanced apoptosis of THP-1 cells leads to the exacerbation of diabetic AS. On the contrary, the KLF4 siRNA group increased THP-1 cell apoptosis when exposed to high glucose. Previous studies have found that cholesterol can induce the death of macrophage-derived foam cells by activating the mitochondrial apoptosis pathway and the endoplasmic reticulum apoptosis pathway [32]. So, we try to find more contact between apoptosis and the development of diabetic AS.

In exploring the mechanism of diabetic atherosclerosis, it was found that autophagic flow has an influence that cannot be ignored [33], which is beneficial to maintaining cell function. As known, harmful substances may be engulfed and lysed during autophagy, a self-clearing cellular activity [34]. Autophagy markers p62 and LC3-II are present in cells isolated from plaques, representing dysfunctioning or decreased autophagy [35]. Abnormal autophagy of macrophages stimulates plaque instability by inducing necrosis and apoptosis [36]. The autophagolysosomal system of macrophages in human and mouse atherosclerotic plaques is decreased [37]. Therefore, some studies believe that the origin of autophagy dysfunction in atherosclerosis is not the destruction of the formation of autophagosomes, but the destruction of lysosomal mediated degradation of its contents. In addition, severe oxidative stress and cholesterol crystals in advanced atherosclerotic plaques can cause lysosomal membrane damage and reactivated autophagy plays this beneficial role by weakening apoptosis and pro-inflammatory signals of plaque macrophages [37]. The above illustrates the importance of normal autophagy in reducing atherosclerotic plaque burden and preventing cardiovascular disease. Inhibition of mammalian targets of rapamycin (mTOR) has become a new approach to stabilizing atherosclerotic plaques [38]. Selective inhibition of the PI3K/Akt/mTOR pathway can effectively induce autophagy, ultimately reduce the accumulation of macrophages in plaques, promote plaque stability, and reduce the secretion of inflammatory factors [11].

Many studies have demonstrated that KLF4 is a well-recognized regulator of autophagy [33]. Diabetic hyperglycemia suppresses KLF4 expression [39], but there is no evidence that KLF4 participates in autophagy in diabetic atherosclerosis. Furthermore, KLF4 also exerts an effect on smooth muscle cells (SMC) [40] and cardiomyocytes [41] to improve autophagy. Salmom, et al. found that KLF4 can bind to the beclin-1 promoter region with KLF2 or zinc finger protein 148, respectively, and improve the formation of aortic aneurysms by increasing the level of autophagy in human and mouse SMC [41]. The latest study found that the regulatory autophagy function of KLF4 is achieved in part by Sirt6 expression [33]. At the same time, KLF4 promotes IL-4-induced macrophage polarization to M2 and promotes the expression of the key protein MCPIP, while its knockout inhibits macrophage autophagy [13]. Therefore, we deduce that KLF4 regulates the

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progression of atherosclerosis by modulating the autophagy function of diabetic macrophages.

Regarding the specific mechanism in the relationship between KLF4 and autophagy, many studies have tried to find out. Hsieh, et al. demonstrated that KLF4 overexpression in endothelial cells restricted autophagy and delayed vascular aging by binding directly to the KLF4 binding site (CA/GCCC box) in several core ATGs, showing the direct regulation of KLF4 in autophagy [42]. Riz, et al. demonstrated that in myeloma, KLF4 can directly bind to the promoter region of the sequestosome-1 gene encoding p62, thus mediating autophagy in association with the autophagy gene (ATG) [43]. We found that KLF4 promotes autophagy in THP-1 cells exposed to high glucose concentration, through the AKT / mTOR pathway. As we know, AKT can activate its substrate, mTOR, either by directly phosphorylating mTOR or by inactivating tuberous sclerosis complex 2 (TSC2), thus enhancing the activation of mTOR [44]. In this study, we found that KLF4 promoted autophagy in THP-1 macrophages exposed to high glucose concentration through the AKT/mTOR pathway and alleviated cellular cholesterol deposition. Therefore, KLF4 was inferred to regulate the progression of atherosclerosis by regulating the autophagy function of diabetic macrophages. However, despite numerous studies, the specific mechanism by which KLF4 regulates autophagy under high glucose needs more research.

The strengths of the study

The strength of this study is that it has provided evidence that KLF4 could induce autophagy in macrophages by inhibiting mTOR, thereby promoting the stability of atherosclerotic plaques and delaying the occurrence and development of diabetic atherosclerosis, providing a new idea for the clinical development and treatment of new drugs for diabetic atherosclerosis.

Research limitations

We have described the KLF4 overexpression or siRNA-based KLF4 inhibition and its effect on autophagy, cholesterol deposition, and apoptosis index in macrophages in vitro experiments only. In the future, we will provide evidence to investigate the expression of autophagy markers (LC3 and beclin1) and the AKT and mTOR in the plaque macrophages and advance more significant scientific knowledge in the research area focusing on understanding the role of KLFs in the pathogenesis of the cardiovascular disease and metabolic disorders.

Conclusion

This study found that KLF4 plays a role in the regulation of autophagy in macrophages under high glucose concentration and preliminarily explored its mechanism of action, elucidating that KLF4 inhibits cellular lipid deposition and cholesterol content by activating macrophage autophagy under high glucose concentration, thus alleviating the development of diabetic atherosclerosis. The AKT/mTOR pathway may be a key pathway involved in the regulation of atherosclerosis, indicating the importance of KLF4 in the development of diabetic atherosclerosis. The study revealed one of the mechanisms of diabetic atherosclerosis from the perspective of autophagy, providing new ideas for the prevention and treatment of diabetic macrovascular disease.

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Authors' contributions

RZ designed the study and revised the manuscript. SC and TW collected the data and analyzed them. TW produced the initial draft of the manuscript. PY revised the manuscript. All authors have read and approved the final submitted manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Consent for publication

All authors agree to publish this article in Archive of Gerontology and Geriatrics and they have no competing interests.

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