



The Key Role of Liraglutide in Preventing Autophagy of Vascular Smooth Muscle Cells in High Glucose Conditions

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Background: The glucagon-like peptide-1 (GLP-1) receptor agonist liraglutide (LIRA) is a potential hypoglycemic drug with anti-atherosclerosis (AS) effects. Autophagy in the vascular smooth muscle cells (VSMCs) facilitates AS. However, the role of autophagy in the anti-AS mechanism of LIRA remains unclear.

Aims: To examine the role and mechanisms of autophagy in LIRA's improvement of the biological characteristics of VSMCs in high glucose conditions.

Study Design: Experimental animal study.

Methods: VSMCs isolated from the thoracic aorta of male SD rats were subjected to a high glucose (HG) condition (25 mM) in Dulbecco's Modified Eagle's Medium with or without LIRA, the GLP-1 receptor antagonist exendin9-39 (Exe9-39), a phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002), and autophagy inhibitors (3-methyladenine [3-MA] and bafilomycin A1 [Baf A1]). Acridine

orange staining, western blotting, transmission electron microscopy, and mCherry-GFP-LC3 transfection were performed to evaluate the autophagy flux. Additionally, VSMC migration, calcification, proliferation, and apoptosis in HG conditions were observed.

Results: Addition of LIRA alone or in combination with autophagy inhibitors significantly downregulated Beclin, increased the LC3-II/LC3-I ratio, and upregulated p62 in VSMCs in HG conditions. Furthermore, autophagolysosome formation was markedly curbed after treatment with LIRA and/or autophagy inhibitors. Inhibition of autophagy by LIRA and/or the autophagy inhibitors attenuated VSMC phenotype conversion, proliferation, migration, and calcification and promoted VSMC apoptosis in HG conditions. This protective role of LIRA was augmented by LY294002, but inhibited by Exe9-39.

Conclusion: LIRA plays a significant role in the improvement of the biological features of VSMCs in HG conditions.

INTRODUCTION

Atherosclerosis (AS) is a vascular complication of diabetes mellitus.¹ The vascular smooth muscle cells (VSMCs) may proliferate and migrate abnormally in hyperglycemic conditions.²⁻⁴ High glucose (HG) levels reportedly facilitate the phenotypic change of VSMCs from contractile to synthetic.^{2,5} These synthetic VSMCs secrete various cytokines, including core-binding factor subunit alpha-1, osteoprotegerin, alkaline phosphatase (ALP), and cellular matrix metalloproteinases, which promote vascular calcification.⁶ HG levels suppress the apoptosis of VSMCs and aggravate AS.⁷ As a hypoglycemic drug, glucagon-like peptide 1 (GLP-1) analogs regulate the secretion of insulin and suppress the release of glucagon. Reportedly, therapy with liraglutide (LIRA) can lower the mortality of patients with cardiovascular disease facing an increased risk of

type-II diabetes.⁸ LIRA binds to β -cell-specific receptors via the activation of the adenosine enzyme, resulting in the enhancement of glucose-dependent insulin secretion, inhibition of glucagon secretion, reduction of appetite, and delay in gastric emptying.⁹ Considering the positive impact of its long-term use in inhibiting the symptoms of obesity, hyperlipidemia, and hypertension, GLP-1 can be used as an anti-AS drug.^{8,10} Additionally, GLP-1 possesses a direct/indirect protective role in VSMCs,² macrophages,¹¹ and other cardiovascular-associated complications. The efficiency of HG levels on VSMCs is highly associated with phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway. The GLP-1 analog LIRA reverses the effect of HG levels on the proliferation, migration, and apoptosis of VSMCs by inhibiting this pathway, thereby protecting VSMCs from AS.^{2,11} Moreover, LIRA weakens the β -glycerophosphate-induced calcification of VSMCs via



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the PI3K/AKT pathway,¹² which is strongly associated with its mechanism of action in HG conditions.

Autophagy, strongly conserved in evolution, occurs extensively in eukaryotic cells. It is a process in which the damaged/denatured/senescent organelles and proteins are transported into the lysosome to be digested and degraded. Autophagy is crucial for both homeostasis and cell survival¹³ and can be found in the pathophysiological process of various illnesses, particularly in the development of AS. Autophagy occurs in various cells such as ECs, macrophages, and VSMCs, and is implicated in the development of AS.^{14,15} Excessive or impaired autophagy in VSMCs may reportedly facilitate AS by affecting migration, calcification, proliferation, and apoptosis.^{16,17}

In VSMCs, autophagy is frequently observed in the emergence and occurrence of cardiovascular-associated diseases, and LIRA exhibits a protective role. LIRA can reportedly trigger the protection of cardiomyocytes and pancreatic islet cells under HG conditions by impacting autophagy.^{18,19} However, its ability to influence autophagy in VSMCs under HG conditions and inhibit AS development remains unknown. Thus, herein, we aimed to assess the impact of LIRA on the calcification, proliferation, migration and apoptosis of VSMCs in HG conditions via autophagy.

MATERIALS AND METHODS

Animals

All experiments were performed in accordance with the regulations of HMU's Institutional Animal Care & Use Committee. Additional procedures were carried out in accordance with the guidelines for animal research based on *Animal Research Reporting of In-Vivo Experiments*.²⁰

Reagents

The LIRA used in our study was provided by JSENB (Wan Chai, Hong Kong, China). The early-phase autophagy inhibitor (3-methyladenine [3-MA]), PI3K inhibitor (LY294002), and primary antibodies against LC3-II, LC3-I, Beclin-1, and β -actin were obtained from Sigma-Aldrich (St Louis, MO, USA). The p62 and proliferating cell nuclear antigen (PCNA) primary antibodies were obtained from Wanleibio (Shenyang, China). Smooth muscle myosin heavy chain (SM-MHC) primary antibodies and SM22 were provided by Abclonal (Wuhan, China), and the Calponin primary antibodies were provided by Proteintech (Wuhan, China). The late-phase autophagy inhibitor Bafilomycin A1 (Baf A1) was obtained from MCE (Monmouth Junction, NJ, USA). Exe9-39 was obtained from AnaSpec (San Jose, California, USA). Trypsin, fetal bovine serum (FBS), and Dulbecco's Modified Eagle Medium (DMEM) were obtained from Gibco (MA, USA).

Cell culture

VSMCs from SD rats were meticulously isolated from the thoracic aorta via modified standard procedures.²¹ The thoracic aorta of the rat was removed, and the endothelial cells were acquired from the vascular lumen by inserting a disinfected toothpick twice.

Thereafter, a pair of tweezers was used to carefully remove the vascular adventitia. Subsequently, the aortic tissue was cut into small pieces (1 mm²), placed in the DMEM media with 15% FBS, and incubated (5% CO₂, 37 °C).²² Thereafter, via trypsinization, the cells were transferred to new flasks, and passages 4-8 were adopted for in vitro assays. Mature VSMCs were identified based on the expression of alpha-smooth muscle actin (α -SMA).

Glucose and LIRA treatment

Prior to administering the treatments, the cells were serum-starved for 24 hours before being cultivated for two weeks. The media was replaced once every two days. Rat VSMCs were subcultured to until 80% confluence was attained. In the LY294002-treated group, the drugs were added 1 h before exposure to HG conditions. In the other VSMCs groups, the drugs were added 30 m before exposure to HG conditions.

Acridine orange staining

The cells were smeared on coverslips and processed. Subsequently, they were incubated with PBS and 5 μ g/ml of acridine orange (AO) (Sigma-Aldrich, USA) for 15 min. After being washed with PBS four times, an inverted fluorescence microscope was used to observe the VSMCs.

Transfection with mCherry-GFP-LC3-expressing adenovirus

The VSMCs were transfected with mCherry-GFP-LC3-expressing adenovirus plasmid within 12 h. Thereafter, the VSMCs were treated with different agents at specified concentrations. The autophagic flux of VSMCs transfected with Ad-mCherry-GFP-LC3 was observed under scanning confocal microscopy.

Transmission electron microscopy analysis

Samples from four groups (cells treated with glucose, LIRA, or glucose + LIRA and control) were fixed in 0.1 mol/l of sodium cacodylate. The cells were immersed in propyleneid/LX-112 solution (1:1; Ladd Research Industries,) after being dehydration with ethanol. Ultrathin sections were stained using lead citrate and uranyl acetate and observed via transmission electron microscopy (TEM) (Jeol-100 CX II;).

Proliferation assay

The cells were incubated with serum-free DMEM in 96-well culture plates for 24 h. Thereafter, proliferation assay was performed using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, USA) following the manufacturer's guidelines. The colorimetric reagent (WST8) was added to each well, and the absorbance value (450 nm) was measured.

Transwell migration and invasion assay

Invasion ability was determined using 24-well Transwell plates (Millipore, Massachusetts, USA) using a protocol with a transwell insert pore diameter of 0.3 μ m.²³ The VSMCs were treated (24 h, 37 °C) as above. The cells were washed with PBS three times and re-suspended (37 °C) in serum-free DMEM. In 0.2 ml DMEM, cells

(1×10^5) and 0.8 ml of 20% FBS-containing DMEM were placed in the upper and lower compartments of the Transwell, respectively. The cells were incubated for 12 h at 37 °C. Cells migrating to the bottom of the upper compartment were immobilized using 4% formaldehyde (for 20 min) and immersed in a hematoxylin staining solution for 15 min. The slides were observed under a light microscope.

In vitro scratch wound assay

A standard scratch wound experiment was performed to determine the migration capacity of VSMCs.²⁴ Confluent VSMCs were incubated with DMEM for 24 h. Thereafter, the cells were scratched with a 200 μ l pipette tip and incubated under different conditions for another 24 h. The wounds were examined at the beginning and 24th hour. Migration of the VSMCs was detected by measuring its width using ImageJ.²⁵

Apoptosis determination

The annexin V-FITC kit (BD Biosciences, USA) was used to identify cells undergoing apoptosis. The cells were cultured in the serum-free DMEM media for 24 h. Thereafter, they were treated under various conditions for 48 h. The cells were washed with PBS, centrifuged, and re-suspended in the binding buffer (500 μ l). Subsequently, they were stained with 5 μ l annexin V-FITC and propidium iodide (PI) solution for 15 min without direct light. A flow cytometer (FACSARIA; BD Biosciences) was used to detect the proportion of cells demonstrating annexin V-FITC- and PI-positive signals.

ALP activity

Following 13 days of incubation, the cells were re-suspended in 100 μ l of PBS and subjected to three freeze-thaw cycles. The suspension was centrifuged (12,000 rpm) to acquire the supernatant, which was conserved at -20 °C for analyses. ALP activity was evaluated using an ALP activity kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's guidelines.

Western blotting

After being washed with PBS, the cells were lysed by being placed on ice for 1 h in a 200 μ l phenylmethylsulfonyl fluoride-containing (100 mM) radio-immunoprecipitation assay buffer. Thereafter, the cell suspension was centrifuged (12,000 rpm, 4 °C) for 5 min, and the supernatant was obtained. Subsequently, the total protein concentration was estimated using the bicinchoninic acid kit (Thermo Fisher Scientific). Thereafter, each sample of 10- μ g protein was treated with 12% SDS-PAGE before being transferred to a nitrocellulose membrane. The samples were cultured with the primary (Beclin and LC3-II/I for 1:10,000 dilution; p62, PCNA, SM22, and SM-MHC for 1:1,000 dilution; Calponin for 1:500 dilution) and secondary (1:5,000 or 50,000 dilutions, Burlingame, CA, the States) antibodies. Subsequently, an enhanced chemiluminescence advance western blotting (WB) kit (GE Healthcare, UK) was used for the detection of proteins. Quantity One (version 4.62; Bio-Rad Laboratories, USA) was used to determine and quantify band intensities (Loading control: β -actin).

Statistical analysis

Each in vitro test was carried out in at least three independent experiments. All numerical results are presented as means and standard deviations (SDs). All data analyses were performed using SPSS (version 26; IBM Corp, Armonk, NY, USA). Statistical differences between conditions were analyzed using one-way ANOVA and Tukey's post-hoc test. A *p*-values of < 0.05 was considered statistically significant.

RESULTS

LIRA suppresses VSMC autophagy in HG conditions

To determine whether VSMC autophagy in HG conditions is alleviated by LIRA, WB was used to detect autophagy-associated markers such as Beclin, LC3, and p62. Compared with the controls, the HG group exhibited a higher LC3-II/LC3-I ratio ($p = 0.0231$) and Beclin ($p < 0.001$) expression, while p62 ($p < 0.001$) was downregulated (Figure 1a-c). However, treatment with LIRA alone or in combination with autophagy inhibitors (3-MA or Baf A1) significantly decreased the LC3-II/LC3-I ratio ($p = 0.0217$) and Beclin ($p < 0.001$) expression, while p62 was upregulated ($p < 0.001$) (Figure 1a-d). To further determine whether LIRA affects autophagic flux in HG conditions, AO staining, Ad-mCherry-GFP-LC3 transfection, and TEM were performed. AO staining revealed HG-induced autophagolysosomal formation within the cells, as demonstrated by the enhanced orange-red fluorescence (Figure 2a). Treatment with LIRA alone or in combination with the autophagy inhibitor Baf A1 inhibited the AO-positive cells ($p = 0.0375$; Figure 2a). Compared to that in the control group, the yellow dots (indicating autophagosomes) and red dots (indicating autophagolysosomes) in the HG group increased. Treatment with LIRA reduced the number of yellow and red dots ($p < 0.001$; Figure 2b). TEM revealed a remarkable decrease in autophagolysosomes after LIRA treatment (Figure 2c). These findings suggest that VSMC autophagy flux in HG conditions is blocked by LIRA.

LIRA regulates the GLP-1 receptor pathway

To better understand the mechanisms underlying the role of LIRA in autophagy, the VSMCs were pretreated with LY294002, followed by LIRA and HG. WB revealed that PI3K inhibition downregulated Beclin-1 ($p = 0.0283$), reduced the LC3-II/LC3-I ratio ($p < 0.001$), and upregulated p62 ($p < 0.001$) in HG condition (Figure 1a-d). LY294002 facilitated the inhibitory function of LIRA on autophagy (Figure 1). Furthermore, LIRA suppressed the VSMC autophagy in HG conditions by activating the GLP-1 receptor pathway. To determine whether the GLP-1 receptor pathway contributed to LIRA's impact on autophagy, the VSMCs were pretreated with a GLP-1 receptor antagonist (Exe9-39). Subsequently, they were treated with LIRA and HG. Pretreatment with Exe9-39 upregulated Beclin-1 ($p = 0.0057$), increased the LC3-II/LC3-I ratio ($p = 0.0073$), and downregulated p62 in the HG + Exe9-39 + LIRA group but not in the HG and LIRA group ($p = 0.0052$, Figure 1a-d). This indicated that Exe9-39 can lower the inhibitory effect of LIRA on autophagy in HG conditions. Thus, LIRA influences VSMC autophagy in HG conditions by activating the GLP-1 receptor pathway.

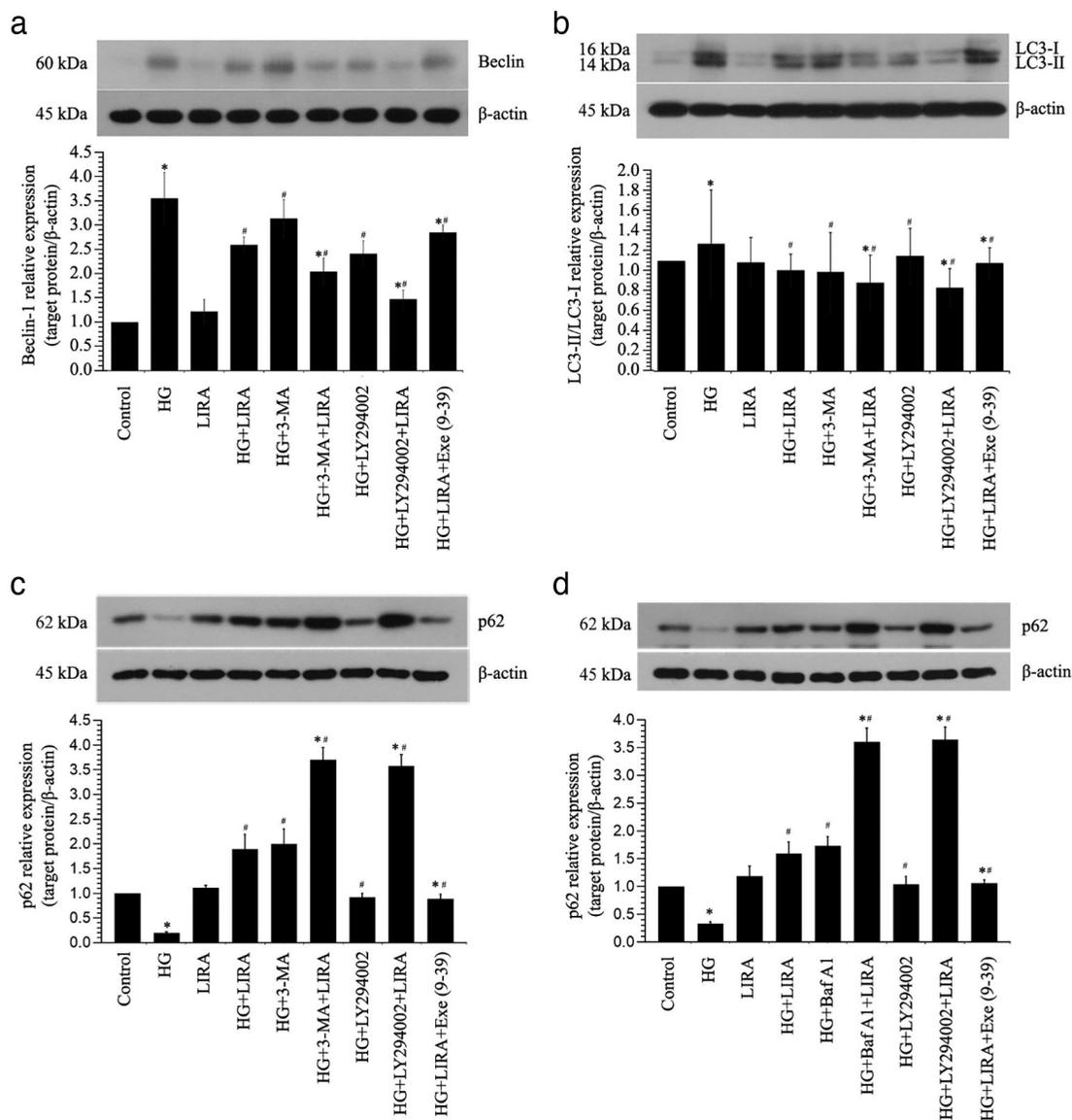


FIG. 1. LIRA affects the autophagy-associated markers in VSMCs under HG conditions. VSMCs subjected to HG conditions were pretreated with LIRA alone or in combination with autophagy inhibitors (3-MA or Baf A1), LY294002, or Exe 9-39. The relative protein levels of the autophagy-related markers (a) Beclin-1, (b) LC3-II/LC3-I ratio, and (c, d) p62 were detected.

* $p < 0.05$ vs. control; # $p < 0.05$ vs. HG; ** $p < 0.05$ vs. HG + LIRA.

Data from three independent experiments are presented as mean \pm standard deviation.

HG, high glucose; VSMCs, vascular smooth muscle cells; LIRA, liraglutide; 3-MA, 3-methyladenine; Baf A1, Bafilomycin A1; PI3K, phosphoinositide 3-kinase; GLP-1, glucagon-like peptide-1; Exe 9-39, exendin9-39.

LIRA mediates calcification, migration, proliferation, and apoptosis of VSMCs via autophagy under HG condition

The effects of LIRA on autophagy-mediated VSMC behaviors, including proliferation, migration, calcification, and apoptosis, were further investigated. The phenotypic transformation from a contractile to synthetic state plays a significant role in VSMC proliferation.²⁶ Therefore, we aimed to detect the changes in phenotype markers. The expression of PCNA was higher ($p < 0.001$) and that of SM-MHC ($p < 0.001$), SM22 ($p < 0.001$),

and Calponin ($p < 0.001$) was lower in the HG group than in the control (Figure 3a-d). However, LIRA alone or in combination with the autophagy inhibitors (3-MA or Baf A1) reversed the HG-induced changes in the phenotype markers. The CCK-8 analysis revealed that treatment with LIRA suppressed the VSMC proliferation in HG conditions by blocking autophagy ($p < 0.001$; Figure 3e). Additionally, treatment with LIRA significantly decreased the number of invading cells ($p < 0.001$, Figure 4a; $p = 0.0013$, Figure 5a), wound area colonization ($p < 0.001$; Figure 4b-c, Figure 5b-c), ALP activity ($p < 0.001$; Figure 4d,

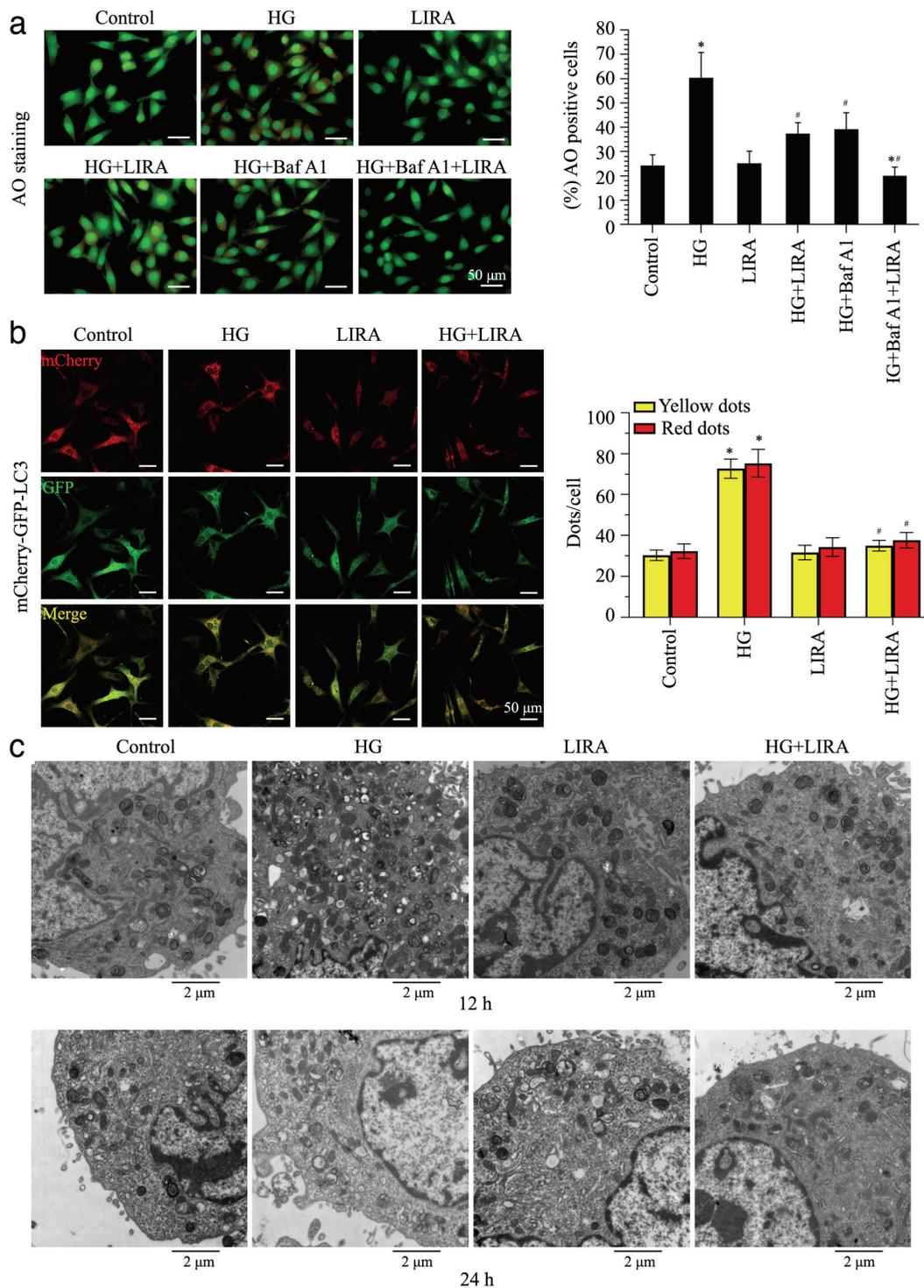


FIG. 2. LIRA inhibits autophagolysosome formation in VSMCs under HG conditions. VSMCs subjected to HG conditions were pretreated with LIRA alone or in combination with a late-stage autophagy inhibitor (Baf A1). (a) AO staining of acidic vesicles (orange-red fluorescence) in VSMCs was detected (magnification, x 400). (b) Representative confocal images of GFP-LC3 and mCherry-LC3 fluorescent puncta in VSMCs (magnification, x 400). GFP/mCherry double-positive (autophagosome) and mCherry single-positive (autophagolysosome) dots in the VSMCs were quantified. (c) Autophagolysosomes were observed in the VSMCs using a TEM (magnification, x 20,000).

* $p < 0.05$ vs. control; # $p < 0.05$ vs. HG.

Data from three independent experiments are presented as mean \pm standard deviation.

HG, high glucose; VSMCs, vascular smooth muscle cells; LIRA, liraglutide; Baf A1, Bafilomycin A1; AO, acridine orange; TEM, transmission electron microscope.

Figure 5d), and apoptosis rate ($p = 0.0023$, Figure 4e; $p < 0.001$, Figure 5e) in VSMCs in HG conditions. Pretreatment of the VSMCs with autophagy inhibitors (3-MA or Baf A1) may have potentiated these effects (Figure 4 and 5). Thus, LIRA can inhibit the migration, calcification and proliferation of VSMCs through autophagy and induce VSMC apoptosis in HG conditions. However, the specific underlying mechanism remains unclear and requires further investigation.

DISCUSSION

We found that the GLP-1 receptor agonist LIRA suppressed the proliferation, calcification, and migration of VSMCs and induced VSMCs apoptosis in HG conditions by blocking autophagy, potentially preventing the development of AS. The study data revealed a novel perspective of the anti-AS mechanisms of LIRA.

During the pathophysiological process of AS, autophagy in VSMCs is of great importance. Zhao et al.²⁷ demonstrated that

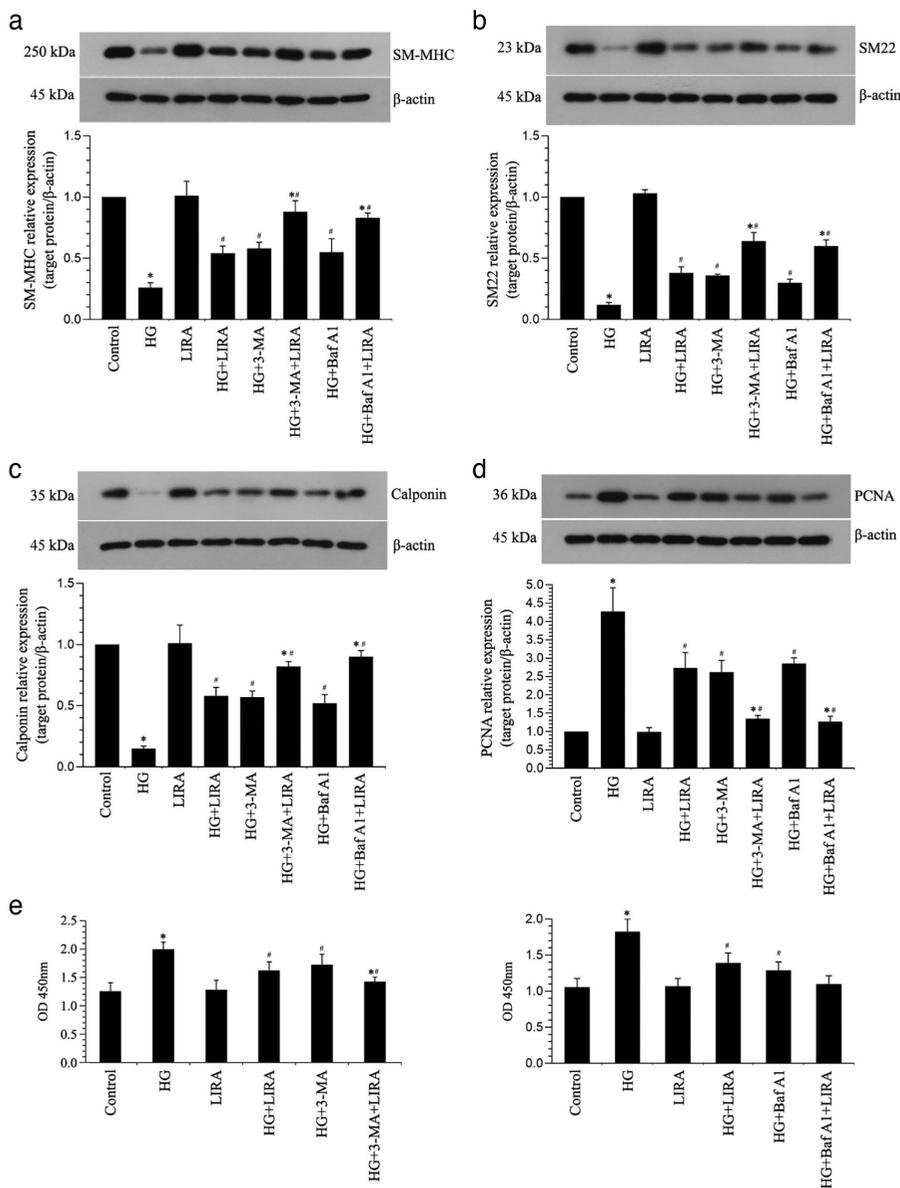


FIG. 3. LIRA suppresses phenotype conversion in and proliferation of VSMCs under HG conditions. VSMCs subjected to HG conditions were pretreated with LIRA alone or in combination with autophagy inhibitors (3-MA or Baf A1). The relative protein levels of the contractile phenotype markers (a) SM-MHC, (b) SM22, (c) Calponin, and the synthetic phenotype marker (d) PCNA were quantified. (e) VSMC proliferation under HG conditions was evaluated using a CCK-8 assay with or without 3-MA and with or without Baf A1.

* $p < 0.05$ vs. control; # $p < 0.05$ vs. HG; ** $p < 0.05$ vs. HG + LIRA.

Data from three independent experiments are presented as mean \pm standard deviation.

HG, high glucose; VSMCs, vascular smooth muscle cells; LIRA, liraglutide; 3-MA, 3-methyladenine; Baf A1, Bafilomycin A1; SM-MHC, smooth muscle myosin heavy chain; PCNA, proliferating cell nuclear antigen; CCK-8, Cell Counting Kit-8.

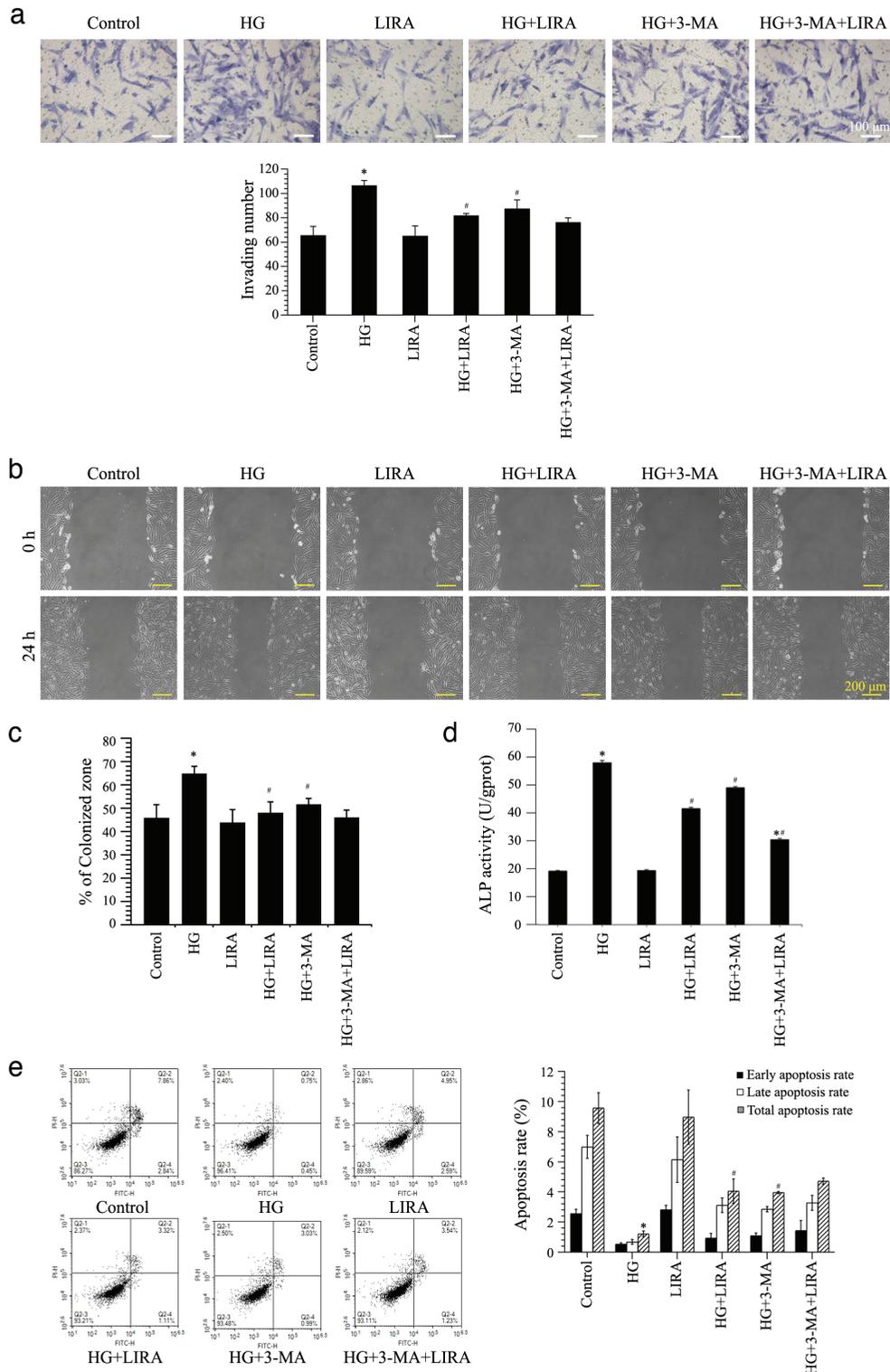


FIG. 4 LIRA contributes to 3-MA-mediated migration suppression, calcification reduction, and VSMC apoptosis under HG conditions. VSMCs under HG conditions were pretreated with LIRA alone or in combination with 3-MA. (a) The invading ability of the VSMCs was evaluated using Transwell assay (magnification, x 200). (b, c) Migrating cells were detected using the scratch wound assay (magnification, x 100). (d) ALP activity was measured using an ALP activity kit. (e) Apoptosis of VSMCs was determined using a flow cytometer.

* $p < 0.05$ vs. control; # $p < 0.05$ vs. HG; ** $p < 0.05$ vs. HG + LIRA.

Data from three independent experiments are presented as mean \pm standard deviation.

3-MA, 3-methyladenine; HG, high glucose; VSMCs, vascular smooth muscle cells; LIRA, liraglutide; ALP, alkaline phosphatase.

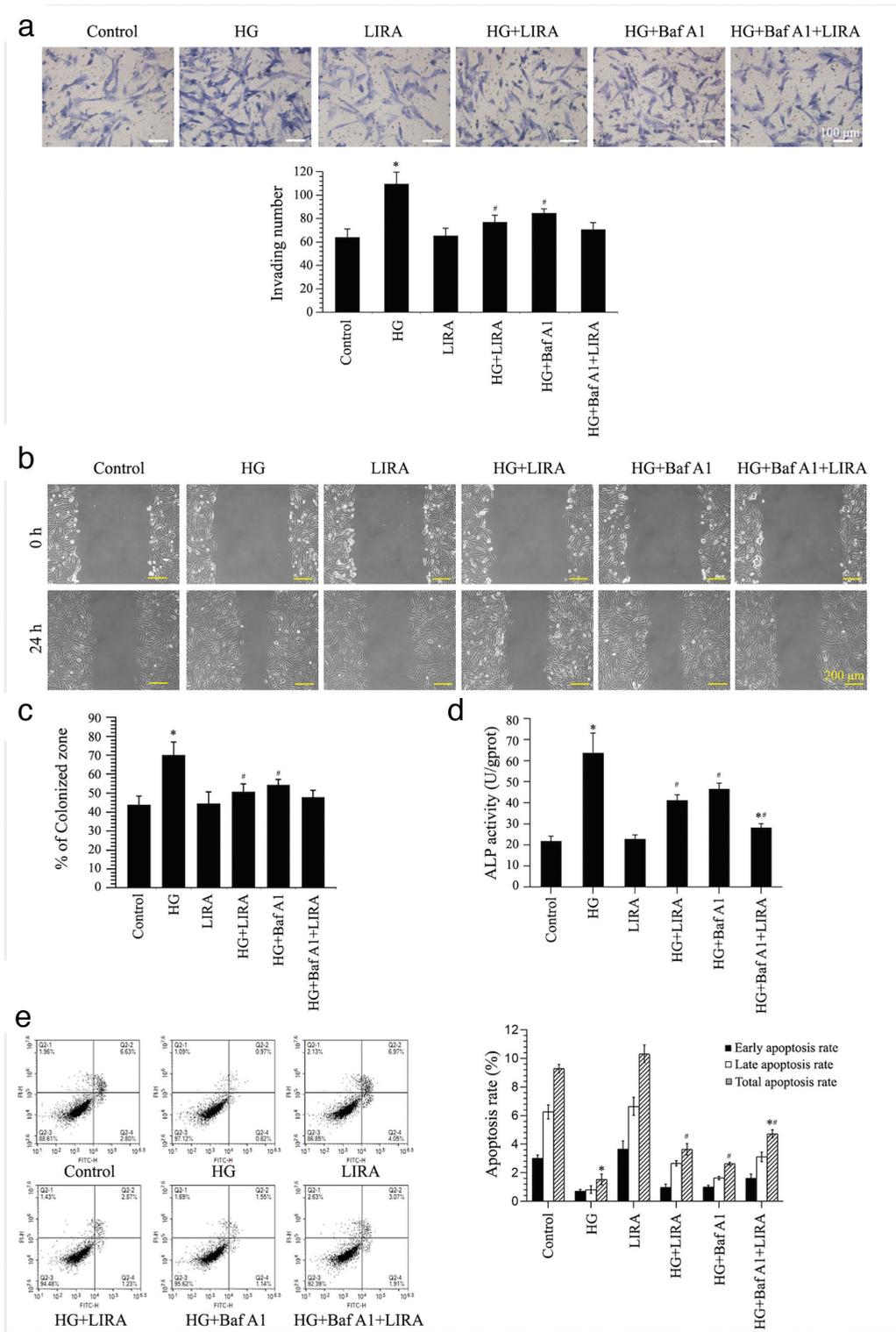


FIG. 5. Migration inhibition, calcification reduction, and apoptosis mediated by Baf A1 are potentiated by LIRA in VSMCs under HG conditions. VSMCs under HG conditions were pretreated with LIRA alone or in combination with Baf A1. (a) The invading ability of VSMCs was evaluated using Transwell assay (magnification, x 200). (b, c) Migrating cells were detected using the scratch wound assay (magnification, x 100). (d) ALP activity was measured using an ALP activity kit. (e) Apoptosis of VSMCs was determined using a flow cytometer.

* $p < 0.05$ vs. control; # $p < 0.05$ vs. HG; ** $p < 0.05$ vs. HG + LIRA.

Data from three independent experiments are presented as mean \pm standard deviation.

Baf A1, Bafilomycin A1; HG, high glucose; VSMCs, vascular smooth muscle cells; LIRA, liraglutide; ALP, alkaline phosphatase.

LIRA inhibits autophagy in human renal tubular ECs in HG conditions. Additionally, LIRA attenuates the autophagy of the pulmonary artery's smooth muscle cells by inhibiting the autophagy-related gene (Atg)-5/Atg-7/Beclin-1/LC3 pathway.²⁸ The extent of autophagy are determined by the changes in the LC3-II/LC3-I ratio and Beclin expression and the accumulation of p62.²⁹ We found that LIRA effectively lowered the LC3-II/LC3-I ratio, downregulated the Beclin expression, and upregulated p62, demonstrating the inhibitory impact of LIRA on VSMC autophagy in HG conditions.

Autophagy of VSMCs and the PI3K/AKT pathway are reportedly related.^{30,31} LIRA partly exerts beneficial effects on VSMC behaviors by suppressing the extracellular signal-regulated kinase (ERK) 1/2 and PI3K/AKT pathways.² We demonstrated that LY294002 enhanced the inhibitory impact of LIRA on VSMC autophagy in HG conditions, indicating that LIRA-mediated autophagy suppression is associated with the PI3K/AKT pathway. Furthermore, the GLP-1 receptor agonist LIRA ameliorates vascular remodeling-related diseases.³² Our study results indicate that the GLP-1 receptor antagonist Exe9-39 abolished the inhibitory effect of LIRA on VSMC autophagy in HG conditions, as demonstrated by the increase in Beclin expression, LC3-II/LC3-I ratio, and p62 levels.

Alteration in the VSMC phenotype leads to poor contractility, vascular calcification, and VSMC apoptosis, which aids in the progression of AS.³³ Activation of autophagy in VSMCs in vascular diseases is crucial for its survival and plasticity.³⁴ García-Miguel et al.³⁵ demonstrated that tumor necrosis factor- α promotes the migration and proliferation of VSMCs by activating autophagy. Additionally, PDGF-induced autophagy contributes to the phenotypic transition and apoptosis inhibition of VSMCs.^{36,37} Furthermore, autophagy promotes VSMC calcification by enhancing ALP activity.³⁸ We discovered that the activation of autophagy in HG conditions resulted in phenotypic modulation and the cellular stress response of VSMCs. Administration of LIRA alone or in combination with autophagy-specific inhibitors (3-MA, Baf A1) reversed the phenotypic modulation, inhibited proliferation, migration, and calcification, and induced the apoptosis of VSMCs. These findings indicate that the inhibition of autophagy by LIRA curbs the phenotypic transition of VSMCs and alleviates their injury under HG conditions. Thus, LIRA may be effective against AS and for vascular remodeling.

Some studies have findings contradictory to those of the present study. Chen et al.³⁹ demonstrated that HG conditions inhibit VSMC autophagy via the AMPK and mTOR signaling pathways. In another study, rapamycin, an mTOR pathway inhibitor and autophagy inducer, was found to inhibit the phenotypic transition and excessive proliferation of VSMCs, preventing post-angioplasty restenosis.⁴⁰ Despite the differences in results between these studies and our study, the outcomes are related to the degree of autophagy. Specifically, under physiological conditions, autophagy can suppress VSMC proliferation, which suppresses the development of AS. However,

excessive or insufficient autophagy of VSMCs can promote AS development.^{16,41} To determine whether autophagy plays a role in the anti-AS influence of LIRA, the VSMCs were pretreated 3-MA and Baf A1, and subsequently exposed to LIRA. Our study results indicate that LIRA suppresses the migration, calcification, and proliferation of VSMCs and induces the VSMC apoptosis by suppressing autophagy under HG conditions. Furthermore, the use of autophagy inhibitors potentiated these effects. LIRA reportedly dampens insulin cell apoptosis in HG conditions by promoting autophagy.¹⁸ Additionally, LIRA can exert an anti-apoptotic effect via autophagy activation in cardiomyocytes under HG condition.¹⁹ These discrepancies are most likely associated with the different cell types utilized in various experimental settings. These results also denote the possible different mechanisms of LIRA in different tissues, cell types, and diseases.

We have demonstrated the potential protective impact of the GLP-1 receptor agonist LIRA on vasculature in this study. LIRA prevents VSMC phenotype conversion, proliferation, calcification, and migration and induces VSMC apoptosis via inhibition of autophagy and the PI3K pathway. Although further studies are required, our data indicate that autophagy is the key target for LIRA to ameliorate the biological characteristics of VSMCs under HG conditions.

Ethics Committee Approval: Harbin Medical University, number: 20190228, date: 2019.02.28.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authorship Contributions: Concept- L.S., M.H., G.Q., Y.X., Z.C., L.G., H.K.; Design- L.S., M.H., G.Q., Y.X., Z.C., L.G., H.K.; Data Collection or Processing- L.S., M.H., G.Q., Y.X., Z.C., L.G., H.K.; Analysis or Interpretation- L.S., M.H., G.Q., Y.X., Z.C., L.G., H.K.; Literature Search- L.S., M.H., G.Q., Y.X., Z.C., L.G., H.K.; Writing- L.S., M.H., G.Q., Y.X., Z.C., L.G., H.K.

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