Liraglutide mitigates changes in migration, proliferation, calcification and apoptosis of vascular smooth muscle cells under high glucose conditions via autophagy

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Abstract

Background Liraglutide, a glucagon-like peptide-1 (GLP-1) analogue, is a new hypoglycemic drug with anti-atherosclerosis (AS) effects. Vascular smooth muscle cells (VSMCs) play vital roles in the occurrence and development of diabetic AS. The autophagy of VSMCs is closely related to AS. To understand the mechanisms of the anti-AS effects of liraglutide, autophagy and related biological characteristics were studied in VSMCs under high glucose conditions.

Methods VSMCs were isolated from the thoracic aorta of Sprague-Dawley rats and cultured in normal or high glucose media. Following pretreatment with liraglutide, VSMCs were treated with inhibitors of autophagy (3-methyladenine [3-MA]), phosphoinositide 3-kinase signaling (PI3K; LY294002), or the GLP-1 receptor (GLP-1R; exendin9-39 [Exe9–39]). Expression of autophagy-related proteins LC3-II/LC3-I and Beclin-1 were detected by western blot, autophagosomes were detected by transmission electron microscopy (TEM), and autophagic vesicles were detected by acridine orange (AO) staining. Cell proliferation, migration, and apoptosis were also measured. Cell calcification was analysed using an alkaline phosphatase (ALP) viability assay.

Results Our results suggested that the migration, proliferation, calcification and autophagy of VSMCs under high glucose conditions were increased while apoptosis was decreased. Pretreatment with liraglutide mitigated those effects. Furthermore, an inhibitor of autophagy and a PI3K antagonist further promoted the effect of liraglutide, while the GLP-1R antagonist impaired the effect of liraglutide on VSMCs.

Conclusion Our results suggest a mechanism where liraglutide mediates the migration, proliferation, apoptosis and calcification of VSMCs under high glucose conditions by affecting autophagy. The GLP-1R and PI3K signaling pathways are also involved during this process. We propose that autophagy in VSMCs may be an important mechanism of action of liraglutide in diabetic AS.

Background

Atherosclerosis (AS) is one of the vascular complications of diabetes[1]. It has been shown that vascular smooth muscle cells (VSMCs) play vital roles in AS through proliferation, migration, calcification, and apoptosis[2–4]. Hyperglycemia can cause abnormal proliferation and migration of VSMCs[4–6]. In addition, high glucose can promote the conversion of VSMCs from a contractile phenotype to a synthetic phenotype[4, 7]. Moreover, these synthetic VSMCs can secrete a variety of cytokines such as cellular matrix metalloproteinases (MMPs) and biomarkers such as osteoprotegerin (OPG), alkaline phosphatase (ALP) and core-binding factor subunit alpha-1 (Cbfα-1), which then promote the development of AS and vascular calcification[8]. A previous report showed that high glucose inhibits the apoptosis of VSMCs, and apoptosis as well as insufficient clearance of apoptotic bodies can further aggravate the development of AS[9]. Glucagon-like peptide-1 (GLP-1), a hypoglycemic drug, can regulate insulin secretion and inhibit the release of glucagon. It has been shown that treatment with liraglutide can

decrease the mortality of cardiovascular (CV) patients with a high risk of type 2 diabetes [10]. Long-term GLP-1 treatment can improve risk factors of AS including obesity, hypertension and hyperlipidemia, and therefore function as anti-AS drugs[10, 11]. In addition, GLP-1 also has either direct or indirect protective effects on cardiomyocytes[12–14], endothelial cells[15, 16], macrophages[17], VSMCs[4] and many other CV-related cells. Previous studies have shown that the function of high glucose on VSMCs is related to the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway, and that the GLP-1 analogue liraglutide reduces the effects of high glucose on proliferation, migration and apoptosis of VSMCs through the inhibition of the PI3K/AKT signaling pathway, ultimately protecting VSMCs against AS[4, 18]. Furthermore, liraglutide can also reduce β-glycerophosphate-induced calcification of VSMCs through the PI3K/AKT signaling pathway[19]. Therefore, the PI3K/AKT signaling pathway is closely related to the mechanism of action of liraglutide on VSMCs under high glucose conditions.

Autophagy is a process of cellular degradation that is widely present in eukaryotic cells and is highly conserved in evolution. Autophagy involves the digestion and degradation of damaged, denatured or senescent proteins or organelles in the lysosome. It is of great significance for homeostasis and cell survival[20]. Autophagy is also involved in pathophysiological processes of many diseases, and in particular in the development of AS. Autophagy that occurs in a variety of cells, including endothelial cells, macrophages and VSMCs, is involved in AS[21, 22]. Previous studies have shown that impaired or excessive autophagy in VSMCs can promote AS by affecting their proliferation, migration, calcification and apoptosis[23, 24].

Autophagy in VSMCs is highly involved in the occurrence and development of CV-related diseases, and liraglutide has a protective effect against CV diseases. Liraglutide can protect pancreatic islet cells and cardiomyocytes under high glucose conditions by affecting autophagy[25, 26]. However, whether liraglutide can affect autophagy in VSMCs under high glucose conditions to achieve its anti-AS effect remains unclear. Therefore, this study aims to explore whether liraglutide affects the migration, proliferation, calcification and apoptosis of VSMCs through autophagy and to explore the specific mechanisms whereby liraglutide achieves anti-AS effects.

Methods

Animals

Male Sprague Dawley rats (5–8 weeks) were provided by the Laboratory Animal Center of Harbin Medical University (China). All procedures were conducted in accordance with the rules of the Institutional Animal Care and Use Committee of the University. Additional procedures were also performed following Animal Research Reporting of *In Vivo* Experiments (ARRIVE) guidelines on animal research[27].

Reagents

The liraglutide used in this study was purchased from JSENB Company (Wan Chai, Hong Kong, China). The PI3K inhibitor (LY294002), the autophagy inhibitor (3-methyladenine [3-MA]), as well as primary antibodies raised against LC3-I, LC3-II, Beclin-1 and β-actin were purchased from Sigma-Aldrich (St Louis,

MO, USA). Exendin9–39 (Exe9–39) was purchased from AnaSpec (San Jose, CA, USA). Trypsin, fetal bovine serum (FBS), and Dulbecco's Modified Eagle's medium (DMEM) were all purchased from Gibco (MA, USA).

Cell incubation

VSMCs from Sprague Dawley rats were carefully isolated from the thoracic aorta using standard procedures with some modifications[28]. Briefly, the thoracic aorta was removed from the rat, and a sterile toothpick was inserted into the vascular lumen twice to remove endothelial cells. The vascular adventitia was then carefully stripped using tweezers. Next, the aortic tissue was cut into several small pieces (1 mm^2) and plated in DMEM media with 15% FBS and cultured at 37 °C with 5% CO_2. The cells were subcultured into new flasks by trypsinization, and passages 4–8 were used for *in vitro* experiments. The biomarker alpha-smooth muscle actin (α-SMA) was used to identify mature VSMCs.

Glucose and liraglutide treatment

Rat VSMCs were subcultured and grown until the cells reached 80% confluence. Cells were then serum-starved for 24 hr, then cultured for two weeks prior to the start of various treatments. During this time, media was changed once every two days. The VSMC treatment groups included: control (5 mM glucose); high glucose (HG; 25 mM glucose); LIRA (100 nM liraglutide); HG + LIRA (25 mM glucose and 100 nM liraglutide); HG + LY294002 (25 mM glucose and 50 μM LY294002[4]); HG + LIRA + LY294002 (25 mM glucose, 100 nM liraglutide, and 50 μM LY294002); HG + 3-MA (25 mM glucose and 5 mM 3-MA[29]); HG + LIRA + 3-MA (25 mM glucose, 100 nM liraglutide and 5 mM 3-MA) and HG + LIRA + Exe9–39 (25 mM glucose, 100 nM liraglutide, and 200 nM Exe9–39[30, 31]). Treatment with HG + LY294002 and HG + LIRA + LY294002, were added 1 hr prior to high glucose exposure. All other groups of VSMCs were pretreated with the drugs detailed above for 30 min before high glucose exposure.

Proliferation assay

Cells were seeded into 96-well culture plates with serum-free DMEM for 24 hr. The Cell Counting Kit-8 (Dojindo Molecular Technologies, USA) was used to determine proliferation following the manufacturer's instructions. Briefly, the colorimetric reagent (WST8) was added to the individual wells. Following a 1 hr incubation at 37ºC, the absorbance value at a wavelength of 450 nm was assessed.

Transwell migration assay

Cell migration was measured using 24-well Transwell plates (Millipore, MA, USA) following a previously published protocol[32]. Briefly, VSMCs were treated as previously described for 24 hr at 37 °C. The cells were then washed with phosphate-buffered saline (PBS) three times and subsequently resuspended in serum-free DMEM at 37 °C. Cells (1×10^5) in 0.2 mL DMEM were seeded into the upper chamber of a Transwell. Meanwhile, 0.8 mL DMEM with 20% FBS was added to the lower chamber of the Transwell. These cells were incubated at 37 °C for 12 hr. Formaldehyde (4% for 20 min) was used to fix the cells that had migrated to the underside of the top chamber, and cells were then immersed into a hematoxylin staining solution for 15 min. Slides were finally observed under a light microscope.

In vitro *scratch wound assay*

The migration ability of VSMCs was determined using standard scratch wound experiments[33]. Briefly, confluent VSMCs were incubated with DMEM for 24 hr. A scratch was made using a 200 μL pipette tip, and cells were incubated for an additional 24 hr under the various treatment conditions. The wounds were analyzed at two time points (0 and 24 hr), and the capacity of VSMCs to migrate was determined by measuring the width of the wounds using ImageJ[34].

Assessment of cell apoptosis

The annexin V-FITC kit (BD Biosciences, USA) was used to evaluate apoptosis. Briefly, cells were incubated in DMEM without serum for 24 hr, then treated under the various conditions for 48 hr. After washing with PBS, cells were centrifuged, re-suspended in 500 μL binding buffer and stained with 5 μL of an annexin V-FITC and propidium iodide solution for 15 min, in the absence of direct light. The percentage of cells with both annexin V-FITC- and propidium iodide-positive signal was determined by flow cytometry (FACSAria, BD Biosciences).

Cell staining with acridine orange

Cells plated on coverslips were treated for different conditions. Cells were then incubated with 5 μg/ml acridine orange (AO; Sigma-Aldrich, USA) in PBS for 15 min. After washing with PBS four times, VSMCs were observed under an inverted fluorescence microscope.

Alkaline phosphatase activity

After 13 days of culture, cells were collected and resuspend into 100 μL PBS, followed by three freeze-thaw cycles. The suspension was then centrifuged (12000 rpm) and the supernatant was carefully collected and stored at -20 °C until further use. An alkaline phosphatase (ALP) activity kit (Nanjing Jiancheng Bioengineering Institute, China) was used to measure ALP activity following the provided instructions.

Transmission electron microscopy analysis

All samples from four different groups treated by control, glucose, liraglutide or combination of glucose and liraglutide were fixed in 0.1 mol/L sodium cacodylate with 1% OsO_4. After dehydration using ethanol, cells were impregnated with a solution of propylenoid/LX-112 (Ladd Research Industries, 1:1). Uranyl acetate and lead citrate were used to stain ultrathin sections, which were examined using the Jeol-100 CX II transmission electron microscope (TEM).

Western blotting

Cells were washed in PBS and lysed for 1 hr on ice in 200 μL radioimmunoprecipitation assay (RIPA) buffer supplemented with phenylmethylsulfonyl fluoride (PMSF; 100 mM). After lysis, the cell suspension was centrifuged at 12,000 rpm for 5 min at 4 °C, and the supernatant was collected. A bicinchoninic acid (BCA) kit (Thermo Fisher Scientific) was used to evaluate the concentration of total protein. Subsequently, 10 μg protein of each sample was subjected to 12% SDS-PAGE electrophoresis, followed by transfer to a nitrocellulose membrane. After incubation with primary antibodies (1:10,000) and secondary antibodies

(1:50,000) (Burlingame, CA, USA), proteins were detected using an enhanced chemiluminescence (ECL) Advance western blotting kit (GE Healthcare, UK). Band intensity was determined and quantified using Quantity One 4.62 software (Bio-Rad Laboratories, USA). β-actin served as a loading control.

Statistical analysis

SPSS package (SPSS Inc., USA) was used for all statistical analyses in this study. Results were expressed as mean ± standard deviation (SD) unless otherwise mentioned. Data were compared using one-way analysis of variance (ANOVA). A p value < 0.05 was considered statistically significant.

Results

Liraglutide inhibits high glucose-induced autophagy in VSMCs

To understand the effects of liraglutide on the autophagy, VSMCs were treated with liraglutide for 12 hr or 24 hr. Autophagy was detected at 12 hr by western blot and fluorescent staining with AO and autophagosomes of VSMCs were observed at 12 hr and 24 hr by TEM. Compared with the control group, the HG group had an increased ratio of LC3-II/LC3-I and Beclin-1 protein ($p < 0.01$, Fig. 1), and the number of orange fluorescently labeled autophagic vesicles was also increased (Fig. 2). The number of VSMCs autophagosomes in the HG group also significantly increased compared with the control group at both the 12 hr and 24 hr timepoint (Fig. 3). The autophagy inhibitor (3-MA) and PI3K antagonist (LY294002) promoted the effect of liraglutide on the high glucose-induced autophagy of VSMCs ($p < 0.05$, Fig. 1), whereas the GLP-1R antagonist (Exe9–39) reduced this effect ($p < 0.05$, Fig. 1). Compared with the control group, pretreatment with liraglutide had no significant effect on the above mentioned indicators of autophagy ($p > 0.05$).

Liraglutide affects high glucose-induced autophagy through PI3K signaling pathway

To study the underlying mechanisms of liraglutide on autophagy, VSMCs were pretreated with the PI3K antagonist LY294002 under high glucose conditions, then treated with liraglutide and markers of autophagy were detected. The results showed that inhibition of PI3K signaling decreased high glucose-induced autophagy (as indicated by levels of Beclin-1, LC3-II, and the LC3-II/LC3-I ratio) in VSMCs ($p < 0.05$). In addition, LY294002 further promoted the inhibitory effect of liraglutide on autophagy ($p < 0.05$, Fig. 1). Our previous study revealed that liraglutide can inhibit the upregulation of AKT protein induced by high glucose in VSMCs[4]. Taken together, these data suggest that liraglutide affects high glucose-induced autophagy of VSMCs through the PI3K signaling pathway.

Liraglutide affects autophagy of VSMCs by activating the GLP-1R pathway

To determine whether the GLP-1R pathway is involved in the effect of liraglutide on autophagy, VSMCs were pretreated with the GLP-1R antagonist Exe9-39 under high glucose conditions, and then treated with liraglutide. Levels of autophagy were then measured. The results showed increased levels of proteins including Beclin-1 and LC3-II, as well as an increase in the LC3-II/LC3-I ratio ($p < 0.05$), suggesting that Exe9-39 can alleviate the inhibitory effect of liraglutide on high glucose-induced autophagy. Therefore, liraglutide influences autophagy in VSMCs by activating the GLP-1R pathway (Fig. 1).

Liraglutide mediates proliferation, migration, calcification and apoptosis of VSMCs through autophagy under high glucose conditions

Our previous study indicated that high glucose promots proliferation, migration, and calcification of VSMCs while inhibiting apoptosis. It was subsequently investigated whether liraglutide could affect these bioactivities and if autophagy was involved. As shown in Fig. 4, liraglutide can attenuate the upregulated proliferation (Fig. 4A), migration (Fig. 4B, C), and calcification (Fig. 4E) as well as the decreased apoptosis (Fig. 4D) in VSMCs induced under high glucose conditions ($p < 0.01$). Pretreatment of VSMCs with an inhibitor of autophagy (3-MA) can reduce these effects ($p < 0.05$; Fig. 4). Furthermore, 3-MA can promote the effect of liraglutide on the proliferation, migration, apoptosis and calcification of VSMCs induced by high glucose ($p < 0.05$ vs HG + LIRA; Fig. 4).

Discussion

This study shows that liraglutide can affect the proliferation, migration, calcification and apoptosis of VSMCs through its effect on autophagy, thereby achieving anti-AS effects. These data also reveal a new perspective regarding the anti-AS biological mechanisms of liraglutide.

AS is a serious cardiovascular condition associated with complications of diabetes. Vascular remodeling is vital to the process of AS, which affects pathologic processes in VSMCs, including proliferation, migration, calcification and apoptosis[8, 35–37]. High glucose conditions promote proliferation, migration and calcification, but inhibit apoptosis[4, 8, 38–41].

Autophagy in VSMCs also plays an important pathophysiological role in AS[21–23]. Xue *et al*. showed that the autophagy of gastric smooth muscle cells was significantly increased after 24 hr of culture with high-glucose[42]. Chen *et al*. showed that high glucose inhibits the autophagy of VSMCs in the superior mesenteric artery[43]. This study indicated that high glucose can induce autophagy in VSMCs and that liraglutide can inhibit autophagy induced by high glucose. This is similar to the results from Zhao *et al*., which showed that high glucose can induce autophagy in human renal tubular endothelial cells (HK-2 cells), while liraglutide can inhibit this autophagy in a dose-dependent manner[44]. However, another study showed that high glucose can inhibit cardiomyocyte autophagy and that liraglutide can increase the level of cardiomyocyte autophagy[26, 45]. Therefore, autophagy in different cell types might participate in the progression of disease through diverse mechanisms, and high glucose and liraglutide might affect autophagy in different cell types through a variety of mechanisms.

However, the specific mechanism of autophagy in VSMCs is still unclear. The PI3K/AKT signaling pathway can regulate cell proliferation and migration, and inhibit cell apoptosis[46, 47]. Previous studies have found that high glucose can induce dynamic changes in VSMCs through the PI3K/Akt pathway[4, 21–24]. Moreover, our previous data also showed that liraglutide exerts beneficial effects on VSMCs partly through the inhibition of extracellular signal-regulated kinase (ERK)1/2 and PI3K/AKT pathways[4]. Therefore, PI3K/AKT signaling possibly plays a large role in the tissue damage induced by AS, which may be an important pathway for GLP-1 to exert cardiovascular protection. A variety of signaling pathways

including mammalian target of rapamycin (mTOR), 5'-AMP-activated protein kinase (AMPK), tumor protein 53 (p53) as well as PI3K/AKT are involved in autophagy[48–50]. Vasopressin inhibits autophagy and promotes cell proliferation of VSMCs cultured with high glucose through the PI3K/AKT/mTOR signaling pathway[51]. In this study, to further investigate the specific mechanisms of the effect of liraglutide on autophagy in VSMCs under high glucose conditions, the PI3K inhibitor LY294002 was used. The results showed that high glucose affects autophagy in VSMCs through the PI3K/AKT signaling pathway. This is consistent with previous studies that have indicated that the effect of high glucose on the biological characteristics of VSMCs is dependent on the PI3K/AKT pathway[4, 41, 52, 53].

Moreover, several cells types, including cardiomyocytes, macrophages, and VSMCs all express the GLP-1R, which mediates the anti-inflammatory and anti-proliferative effects of GLP-1[16, 54, 55]. To investigate if the effects of liraglutide in high glucose-treated cells occur via GLP-1R, VSMCs were pretreated with Exe9–39. Indeed, Exe9–39 treatment abolished the beneficial effects of liraglutide treatment.

How liraglutide protects cardiovascular in VSMCs remains unclear. Previous studies indicated that autophagy is closely related to AS and biological changes of VSMCs. Li *et al*. found that the secreted protein sonic hedgehog (SHH) can induce autophagy, which can promote the proliferation of VSMCs[56]. Marina *et al*. found that tumor necrosis factor alpha (TNF-α) promotes the proliferation and migration of VSMCs through autophagy[57]. Salabei *et al*. found that platelet-derived growth factor (PDGF) can further promote the phenotypic transition and calcification of VSMCs by promoting autophagy[58]. Aleksandar *et al*. have shown that early autophagy promotes an increase of ALP, Runt-related transcription factor 2 (Runx2), and bone morphogenic protein 2 (BMP2) in cells, thereby promoting the occurrence of cell calcification[59]. Park *et al*. found that reactive oxygen species (ROS) regulate the autophagy of VSMCs induced by PDGF and exert an anti-apoptotic effect[60]. The present study showed that autophagy induced by high glucose can promote proliferation, migration and calcification but inhibit apoptosis, which is consistent with the literature cited above.

However, there are certain studies that are inconsistent with the findings of the present study. One study showed that high glucose induces the activation of mTOR in VSMCs, thereby inhibiting autophagy[51]. Chronic hyperglycemia inhibits AMPK activation and autophagy, thereby promoting VSMC proliferation and migration[61, 62]. Research by Chen *et al*. showed that high glucose can upregulate endothelin type B (ETB) receptors and inhibit VSMC autophagy through AMPK and mTOR signaling pathways[43]. These studies have shown that high glucose promotes VSMC proliferation and migration by inhibiting autophagy. In addition, rapamycin (an inhibitor of the mTOR pathway and inducer of autophagy) can prevent phenotypic transition and excessive proliferation of VSMCs, and therefore can prevent restenosis after angioplasty. These data indicate that autophagy in VSMCs might exert an anti-AS effect by inhibiting phenotypic transition and excessive proliferation[63]. Although the results of these studies are different from the current study, all of the outcomes are considered to be related to the degree of autophagy. Specifically, under physiological conditions, autophagy of VSMCs can inhibit cell proliferation, thereby further inhibiting the development of AS. However, excessive autophagy or

insufficient VSMC autophagy can promote the development of AS[23, 64]. To study whether autophagy was involved in the anti-AS effect of liraglutide, the inhibitor of autophagy 3-MA was used to pretreat VSMCs under high glucose conditions and cells were then exposed to liraglutide (HG + LIRA). The results indicated that liraglutide can affect the proliferation, migration, calcification and apoptosis of VSMCs by inhibiting autophagy under high glucose conditions. Li *et al.* showed that liraglutide can inhibit insulin cell apoptosis induced by high glucose by promoting autophagy[25]. Yu *et al.* showed that liraglutide and exenatide can achieve anti-apoptotic effects by promoting autophagy in cardiomyocytes under high glucose[26]. Those differences are most likely related to the different cell types used in different experimental setttings. These results also suggest that the mechanisms of liraglutide in different tissues and cell types, and in diverse diseases, might be not the same.

Conclusions

The evidence presented here suggests that high glucose conditions promote autophagy of VSMCs and that pretreatment with liraglutide reduces this high glucose-induced autophagy. PI3K inhibitors can inhibit the autophagy of VSMCs by high glucose, and liraglutide can reduce these effects suggesting that liraglutide affects the high glucose-induced autophagy of VSMCs through the PI3K pathway. In addition, GLP-1R antagonists can block autophagy in VSMCs exposed to high glucose and liraglutide. Therefore, liraglutide mediates autophagy through the GLP-1R. Treatment with 3-MA, an inhibitor of autophagy, can reduce the proliferation, migration, calcification and apoptotic effects on VSMCs caused by high glucose, and further alleviates these effects of liraglutide on VSMCs under high glucose. All these data indicate that liraglutide mediates proliferation, migration, calcification, and apoptosis of VSMCs caused by high glucose conditions through autophagy, which reveals new mechisms by which liraglutide excerts its anti-AS effect through autophagy and new perspectives for clinical anti-AS therapeutics.

List Of Abbreviations

3-MA: 3-methyladenine; AKT: protein kinase B; ALP: alkaline phosphatase; AMPK: 5’-AMP-activated protein kinase; AO: :acridine orange; ARRIVE:Animal Research Reporting of In Vivo Experiments; AS: :anti-atherosclerosis; BCA: bicinchoninic acid; BMP2: morphogenic protein 2; Cbfα-1: core-binding factor subunit alpha-1; CV: :cardiovascular; DMEM: Dulbecco’s Modified Eagle’s medium; ERK: extracellular signal-regulated kinase; ETB: endothelin type B; Exe9-39: GLP-1R antagonist; FBS: fetal bovine serum; GLP-1: glucagon-like peptide-1; HG: :high glucose; LIRA: liraglutide; MMPs: metalloproteinases; mTOR: mammalian target of rapamycin; OPG: osteoprotegerin; P53: :tumor protein 53; PDGF: platelet-derived growth factor; PI3K: phosphatidylinositol 3-kinase; PMSF: phenylmethylsulfonyl fluoride; RIPA: radioimmunoprecipitation assay; ROS: reactive oxygen species; SHH: protein sonic hedgehog; TEM: transmission electron microscopy; TNF-α: tumor necrosis factor alpha; VSMCs: vascular smooth muscle cells; WST8: colorimetric reagent; α-SMA: alpha-smooth muscle actin

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

LLS participated in research concept and design, carried out the molecular biology studies, and wrote the manuscript. ZYJ participated in study design, performed statistical analyses, and drafted the manuscript. SSY made a significant contribution in the manuscript correction and modification, and participated in providing the supplementary part of the manuscript. XDL performed data analyses and interpretation, and critically revised the manuscript. XC participated in in vitro experiments, performed data collection and analyses. MMC participated in experiments, collection and assembly of data, and wrote the manuscript. XYL wrote the manuscript. HYKcritically revised the manuscript. XYJ conceived the study, participated in its design and coordination, helped to draft the manuscript, and provided final approval of the article. All authors read and approved the final manuscript.

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Figures

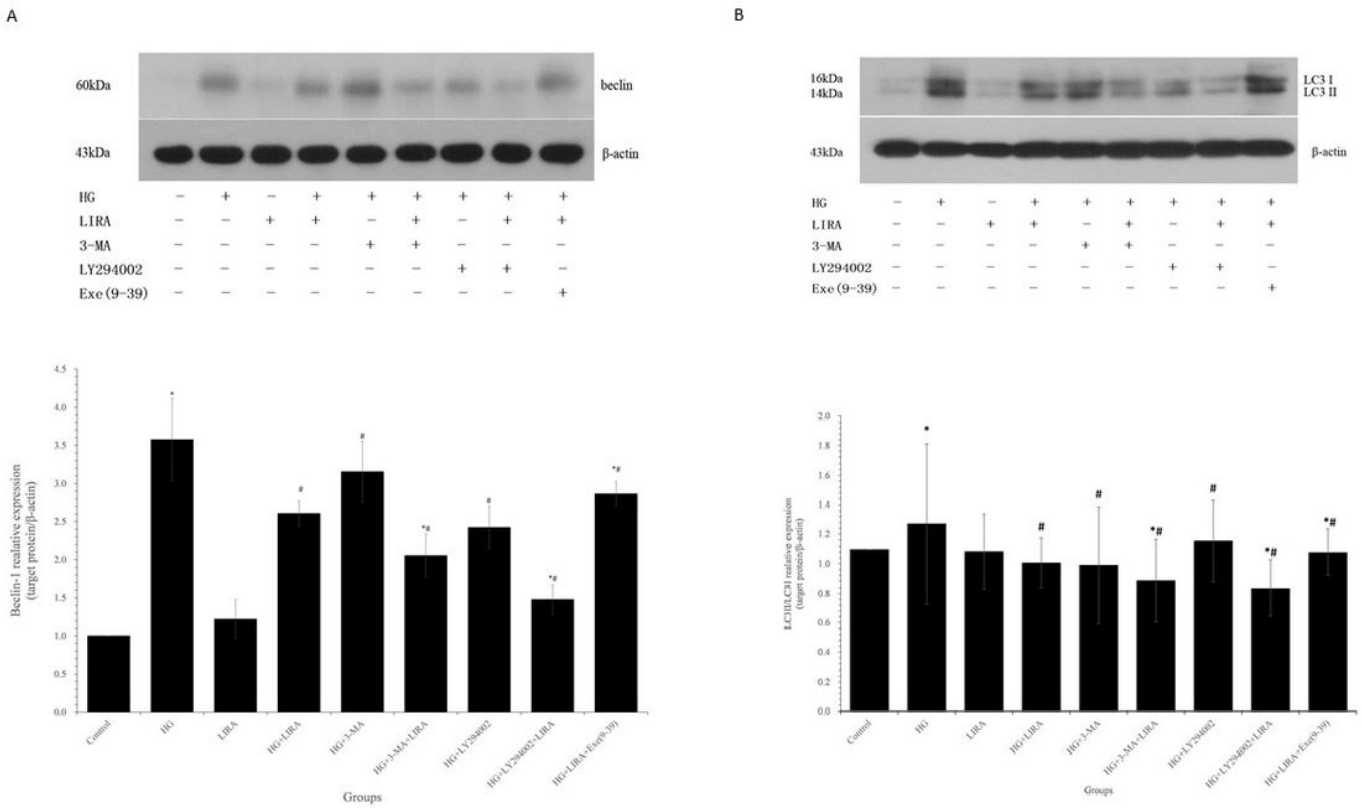

Figure 1

Liraglutide (LIRA) inhibited high glucose (HG)-induced autophagy in cultured vascular smooth muscle cells (VSMCs). VSMCs were pretreated with LIRA alone or in combination with LY294002 or Exe9-39, and then culutured in the presence of HG for 12 hr. (A) Relative expression of autophagy protein marker Beclin-1,. *p < 0.01 vs. control; #p < 0.05 vs. HG; *#p < 0.05 vs. HG + LIRA; (B) LC3-II /LC3-I ratio in VSMCs; *p < 0.01 vs. control; #p < 0.05 vs. HG; *#p < 0.05 vs. HG + LIRA. The results from three independent experiments are presented as mean ± standard deviation. HG, high glucose; LIRA, liraglutide; LY294002, PI3K inhibitor; 3-MA , 3-methyladenine; Exe9–39, Exendin9–39.

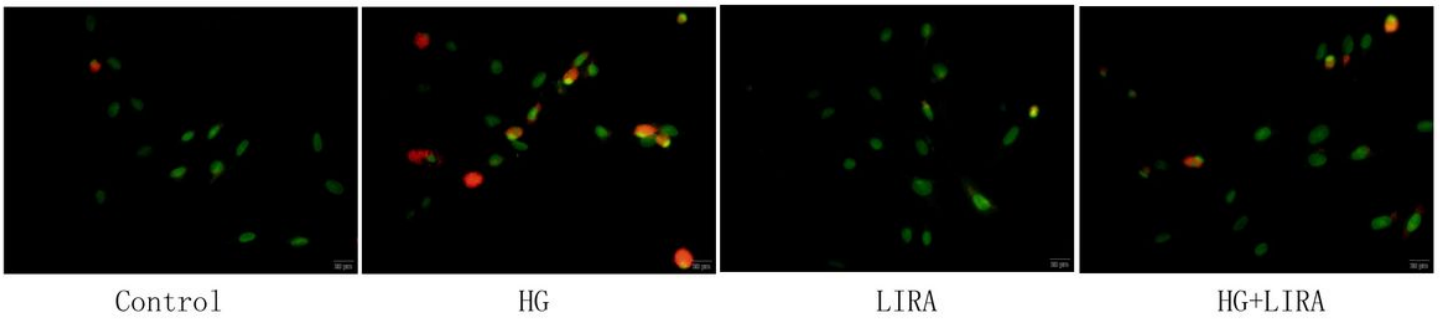

Figure 2

Liraglutide (LIRA) inhibited high glucose (HG)-induced autophagy in cultured vascular smooth muscle cells. VSMCs were pretreated with LIRA and then culutured in the presence of HG for 12 hr. Autophagosomes were detected using acridine orange fluorescent staining. Representative images of acridine orange staining in VSMCs (magnification, ×400).

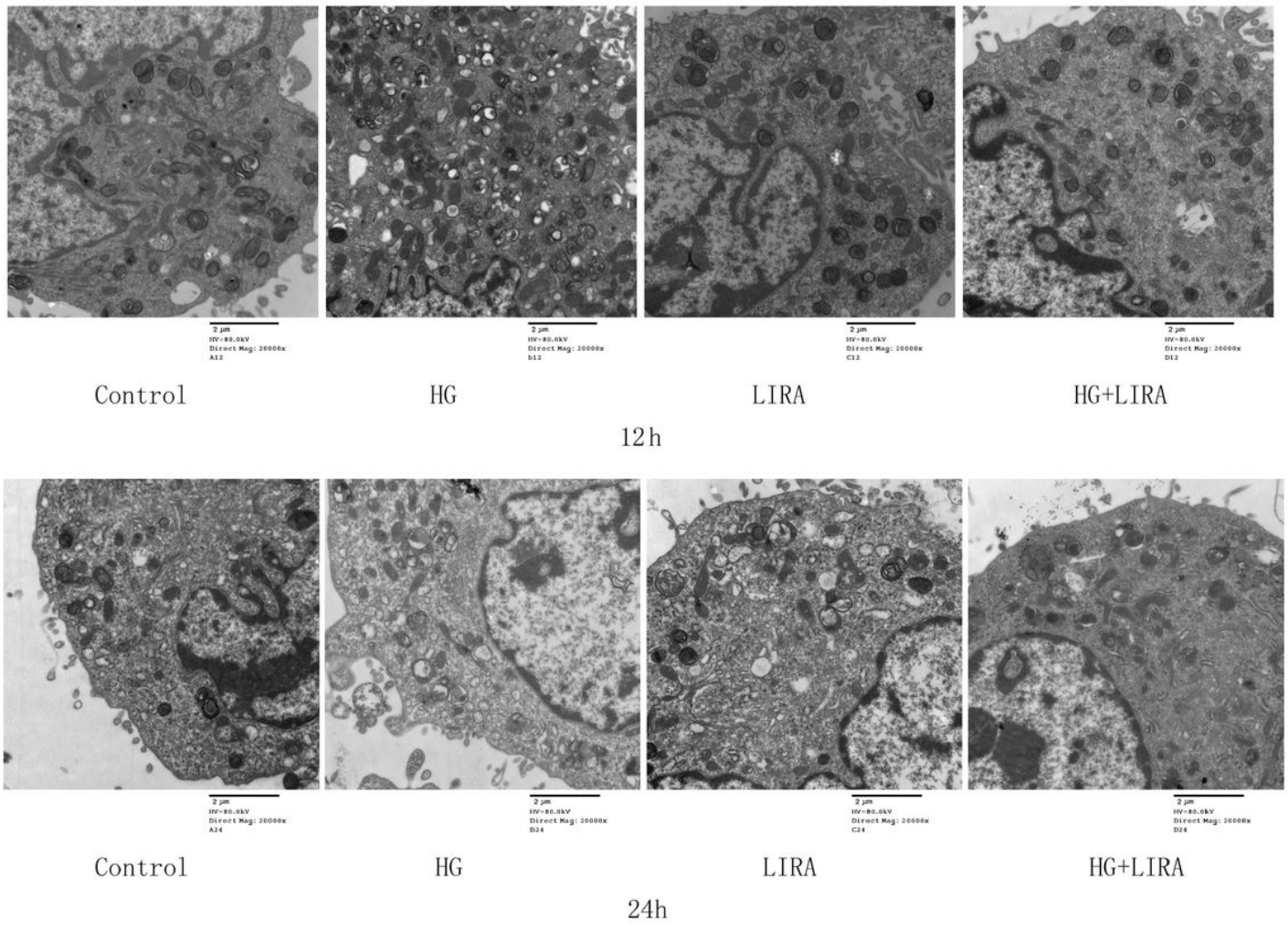

Figure 3

Liraglutide (LIRA) inhibited high glucose (HG)-induced autophagy in cultured vascular smooth muscle cells (VSMC). VSMCs were pretreated with LIRA and then culutured in the presence of HG for 12 hr or 24 hr. Electron microscopy was used to observe autophagy in VSMCs. (magnification, ×20,000).

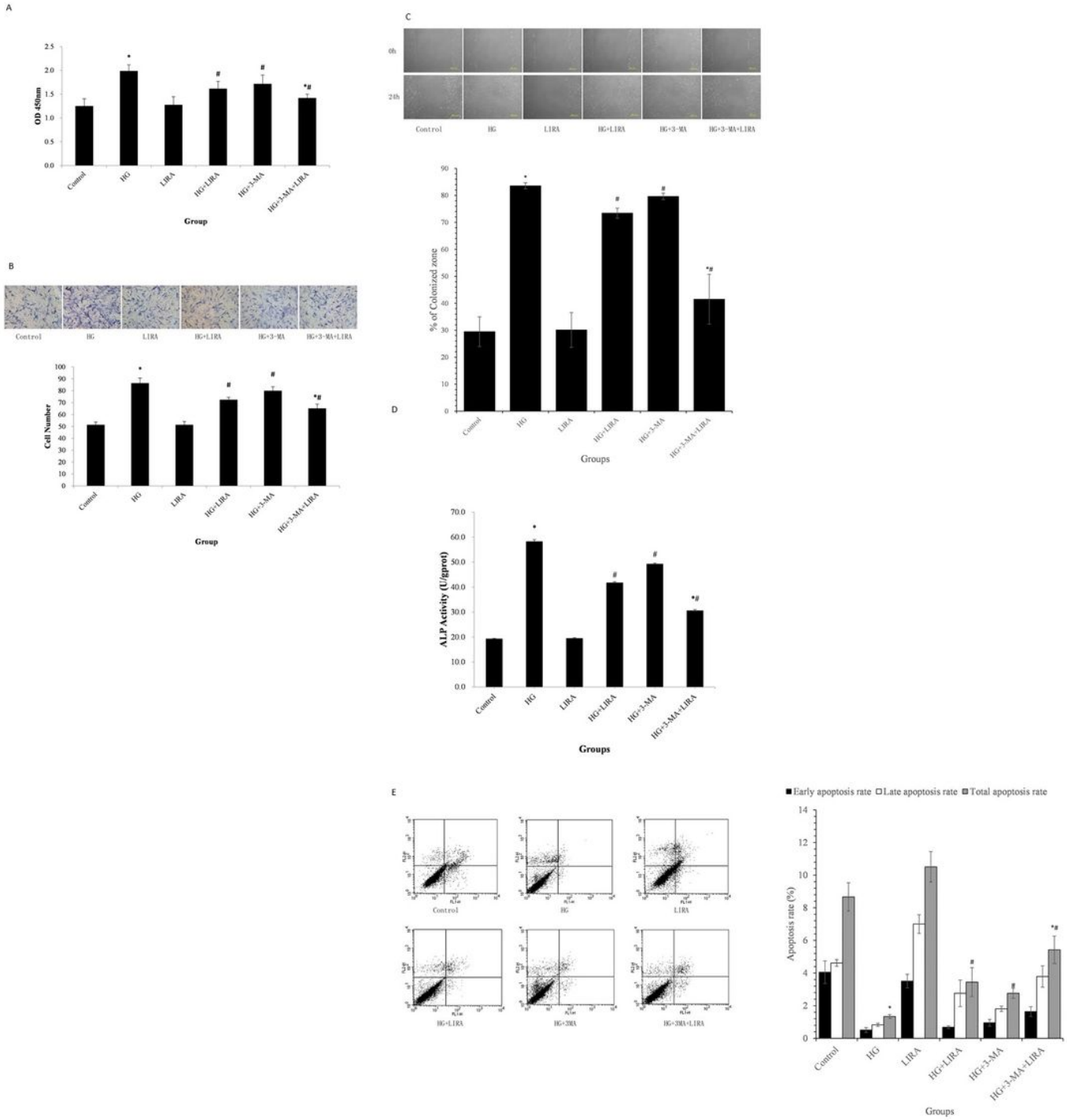

Figure 4

Liraglutide (LIRA) mediates proliferation, migration, calcification and apoptosis of vascular smooth muscle cells (VSMC) by high glucose (HG) through autophagy. VSMCs were pretreated with LIRA alone or

combination with 3-MA, and then culutured in the presence of HG for 24 hr (proliferation and Transwell migration assay) or 48 hr (scratch wound assay and apoptosis). A. The proliferation analysis by CCK-8, *p < 0.01 vs. control; #p < 0.05 vs. HG; *#p < 0.05 vs. HG + LIRA. B. The migration of VSMCs in the Transwell migration assay (200× magnification). *p < 0.01 vs. control; #p < 0.05 vs. HG; *#p < 0.05 vs. HG + LIRA. C. Migrating cells in the scratch wound assay; *p < 0.01 vs. control; #p < 0.05 vs. HG; *#p < 0.01 vs. HG + LIRA. D.Viability analysis by alkaline phosphatase (ALP) kit analysis *p < 0.01 vs. control; #p < 0.05 vs. HG; *#p < 0.01 vs. HG + LIRA. E. Apoptosis rates of cells; *p < 0.01 vs. control; #p < 0.05 vs. HG; *#p < 0.05 vs. HG + LIRA. Data from three independent experiments are expressed as mean ± standard deviation.