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Original Research

miR-4463 Regulates Hypoxia-Induced Autophagy and Apoptosis by Targeting ULK1 in Endothelial Cells

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Abstract

Background: Our previous study revealed aberrant miR-4463 expression in the vascular tissues of patients with arteriosclerosis obliterans of the lower extremities (ASO), but the role of miR-4463 was largely ambiguous. In the current study, we aimed to explore the function of miR-4463 in hypoxia-induced endothelial cells and determine its molecular mechanisms. **Methods**: CCK-8 assay and flow cytometry were performed to evaluate cell viability and apoptosis. Adenovirus carrying mRFP-GFP-LC3 was employed to monitor cellular autophagy, and mitochondrial membrane potential was determined by JC-1 staining. Moreover, dual-luciferase reporter gene assay, qPCR, western blot and siRNA analysis were carried out to explore the potential molecular mechanisms. **Results**: Hypoxia significantly elevated the miR-4463 expression in primary human umbilical vein endothelial cells (HUVEC). Overexpression of miR-4463 inhibited hypoxia-induced autophagy by suppressing the formation of autophagosomes and autolysosomes, resulting in reduced cell viability and increased apoptosis, and these effects were reversed by miR-4463 inhibitor. Furthermore, activation of autophagy induced by miR-4463 inhibitor attenuated HUVECs apoptosis in hypoxic conditions. Mechanically, the results of the dual-luciferase reporter gene assay discovered that miR-4463 directly targeted Unc-51 like kinase 1 (ULK1). The silence of ULK1 blocked miR-4463 inhibitor-activated autophagy and further facilitated apoptosis under hypoxic conditions. **Conclusions**: Our findings indicate that miR-4463 is an essential regulator of hypoxia-induced autophagy and apoptosis in endothelial cells via directly targeting ULK1. Inhibition of miR-4463 might be a potential strategy to protect endothelial cells and maintain vascular function in patients with lower limb ischemia and its complications.

Keywords: miR-4463; autophagosomes; autolysosomes; apoptosis

1. Introduction

Ischemic vascular diseases cause substantial injuries to the vascular endothelial cells, eventually bringing damage to surrounding tissues [1]. Macroautophagy, also known as autophagy, is an adaptive response to stressful conditions, including starvation, hypoxia, endoplasmic reticulum (ER) stress and infection, in which both intracellular organelles and cytoplasmic proteins are degraded to produce energy and substrates for organelles' re-synthesis to maintain cell functions during those stressful situations [2–6]. During the progression of ischemia-induced injury, promoting autophagy plays a protective role in ischemia- or ischemia/reperfusion (I/R)-induced tissue damage through inhibition of apoptosis and oxidative stress [3,7–9]. It is widely considered that autophagy is essential for cell survival, differentiation, and homeostasis under ischemia or hypoxia stresses [10–13]. However, the underlying regulatory mechanisms are still not fully understood.

microRNA (miRNAs), which are endogenous, noncoding, and single-stranded RNA molecules, are involved in regulating various pathophysiologic processes, including apoptosis, cell proliferation, differentiation, and autophagy [14]. Several miRNAs are reported as essential regulators of autophagy and apoptosis in ischemic diseases [3,15–17]. Liu *et al.* [18] reported that 14 miRNAs were differentially expressed in a mouse aortic endothelial cell model of chronic intermittent hypoxia and 6 miRNAs of them are apoptosis- or autophagy-related target genes. Mo *et al.* [19] reported that inhibition of miR-195 promoted cell proliferation and activation of autophagy in endothelial progenitor cells under hypoxic conditions. Gao *et al.* [20] reported that miR-19a protected cardiomyocytes against hypoxia-induced lethality at least in part via autophagy activation. Besides, Li Z revealed that miR-26b was an inhibitory regulator of brain microvascular endothelial cell autophagy and survival by targeting ULK2 under oxygen-glucose deprivation/reoxygenation conditions [21]. These results revealed that miRNAs play extensive and critical regulatory roles in autophagy when cells encounter ischemia and hypoxia.

Our previous study has demonstrated that miR-4463 expression was altered in the vascular tissues of patients with arteriosclerosis obliterans (ASO) of the lower extremities as well as in endothelial cells under hypoxic conditions [22]. However, the functions of miR-4463 in hypoxic conditions and whether miR-4463 participates in hypoxia-induced autophagy are still unknown. In this study, we

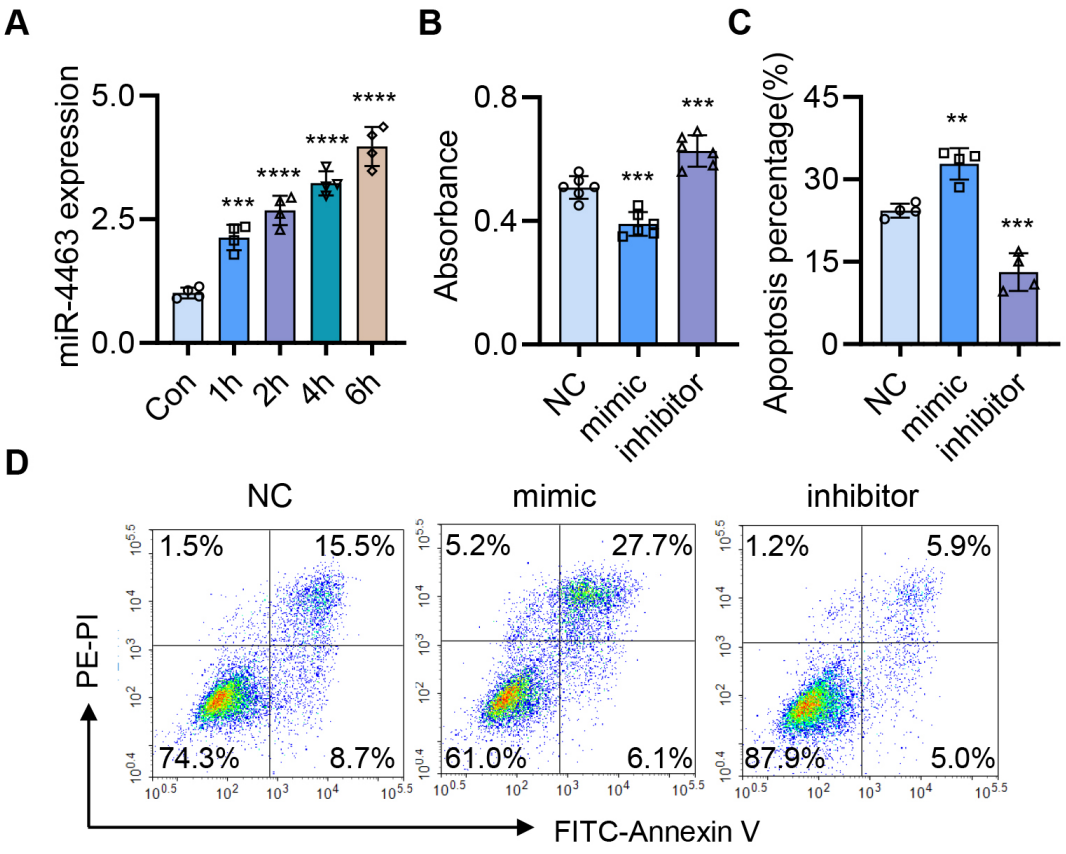

Fig. 1. The expression and effect of miR-4463 on HUVECs apoptosis under hypoxic conditions. (A) Hypoxia elevated miR-4463 expression in HUVECs. ***$p < 0.001$, ****$p < 0.0001$, compared with control. (B) The effect of miR-4463 on HUVECs cell viability was detected by CCK-8 assay. (C,D) The histogram (C) and flow cytometry chart (D) of miR-4463 on HUVECs apoptosis were determined by flow cytometry under hypoxic conditions.

aimed to investigate the role of miR-4463 in regulating autophagy and apoptosis under hypoxic conditions and elaborate on the relative mechanisms.

2. Materials and Methods

2.1 Cell Culture

Primary human umbilical vein endothelial cells (HUVECs) were purchased from ATCC and grown in DMEM low glucose medium (Gibco, Grand Island, USA) with 10% FBS (Gibco, Grand Island, USA), 100 U/mL penicillin, 100 U/mL streptomycin at 37 (C with 5%CO_2/95% air (control group). HUVECs were cultured in serum-free DMEM without glucose medium (Gibco, Grand Island, USA) at 37 (C in a tri-gas incubator with 1%O_2/5%CO_2/94% N_2 (Hypoxia group) to imitate ischemia-induced vascular starvation. HUVECs were cultured in a hypoxia environment for 1 h, 2 h, 4 h and 6 h. Autophagy inhibitor 3-methyladenine (3-MA) was purchased from Hanbio (Hanbio, Shanghai, China). HUVECs were exposed to 3-MA (5 mmol/L) for 2 h prior to treatment with FBS and glucose deprivation and hypoxia for a further 6 h.

2.2 Transfection

miR-4463 mimic, miR-4463 inhibitor, and negative control (NC) were purchased from Guangzhou RiboBio Co., LTD. ULK1 siRNA and vehicle sequence were purchased from Santa Cruz Biotechnology. miR-4463 mimic (50 nmol/L), miR-4463 inhibitor (100 nmol/L), NC (100 nmol/L), ULK1 siRNA (100 nmol/L), and vehicle (100 nmol/L) were transfected in HUVECs with Ribo FECT^{TM} CP Transfection Kit (RiboBio, Guangzhou, China).

2.3 Adenovirus Transfection

Adenovirus mRFP-GFP-LC3 (Ad-mRFP-GFP-LC3) was purchased from Hanbio (Hanbio, Shanghai, China). HUVECs were cultured in a dish with a diameter of 35 mm until the cell density reached 40%–60%. The medium was replaced with 2 mL fresh medium and 5 μg/mL polybrene. 10 μL Ad-mRFP-GFP-LC3 (10^{10} PFU) were added to each dish and cultured in normal conditions. 24 h later, cells were transfected with NC/miR-4463 mimic/inhibitor. Transfection efficiency of Ad-mRFP-GFP-LC3 was confirmed by fluorescence microscope after transfection for 48 h. Then cells were treated with hypoxia and observed by

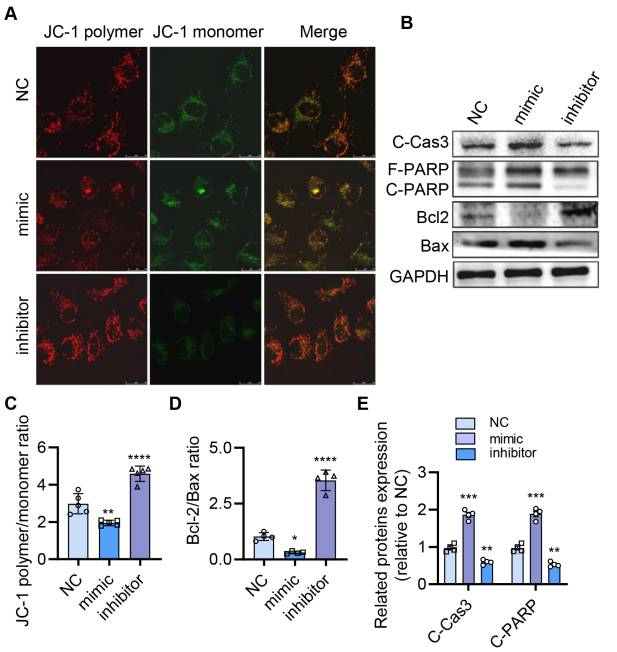

Fig. 2. miR-4463 regulates MMP and Bcl-2/Bax ratio in HUVECs under hypoxic conditions. (A,C) MMP levels were detected by JC-1 staining. (B,D,E) Levels of C-Caspase 3, F-PARP, C-PARP, Bcl-2 and Bax were determined by western blot (B) and relative quantitative analysis (D,E). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with NC. C-Cas 3, cleaved caspase 3; F-PARP, full-length PARP; C-PARP, cleaved PARP.

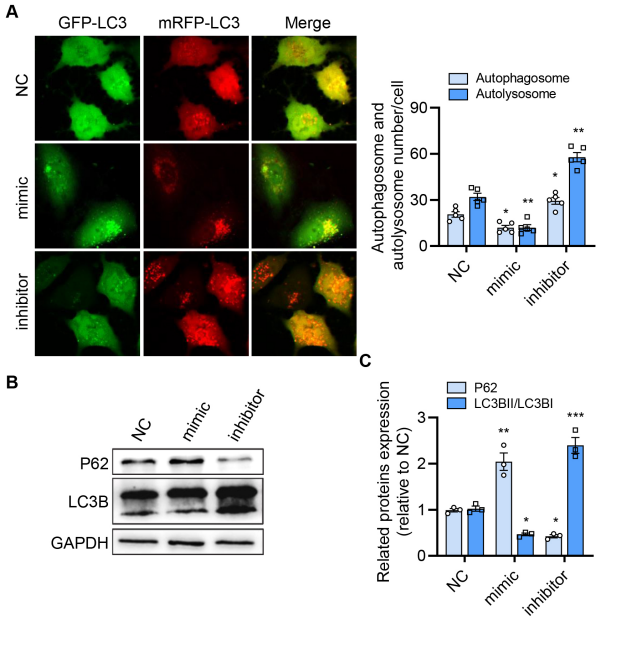

Fig. 3. The effect of miR-4463 on autophagy HUVECs under hypoxic conditions. (A) The autophagosomes and autolysosomes were observed in HUVECs after co-transfection with Ad-mRFP-GFP-LC3 and NC/miR-4463 mimic/inhibitor under hypoxic conditions by laser confocal microscopy. (B,C) The expression of P62 and LC3B was determined by western blot (B) and relative quantitative analysis (C). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with NC.

laser scanning confocal microscope. The mRFP and GFP were used to mark and trace LC3. The yellow spots suggest autophagosomes while red spots indicate autolysosomes.

2.4 RNA Extraction and Real-Time qPCR

Total RNA was isolated according to instructions using miRNA Mini Kit (QIAGEN, Hilden, Germany). Then 500 ng total RNA was reverse-transcribed to cDNA by miScript II RT Kit (QIAGEN, Hilden, Germany). miRNA expression analysis was carried out using the miScript SYBR Green PCR kit in the ABI StepOne Plus PCR system (Applied Biosystems). snRU6 (U6) was used as a reference gene for miR-4463. For ULK1 detection, 500 ng total RNA was reverse-transcribed to cDNA by ReverTra Ace qPCR RT Kit (TOYOBO, Shanghai, China), and the qPCR was carried out with QuantiNova SYBR Green PCR Kit (QIAGEN, Hilden, Germany). GAPDH was used as an internal reference gene. The sequence of ULK1 and GAPDH mRNA primers is as follows: ULK1F: CCT GCT GAG CCG AGA ATG, ULK1R: CTG CTT CAC AGT GGA CGA CA. GAPDHF: ATGCTGGCGCTGAGTACGTC, GAPDHR: GGTCATGAGTCCTTCCACGATA. Each experiment was performed in triplicate, and the $2^{-\Delta\Delta Ct}$ method was applied to analyze data.

2.5 Cell Viability Assay

HUVECs were plated in 96-well plates by 3×10^3 cells per well. Cells were transfected and treated as described previously. After treatment with hypoxia, 10 μL cell counting kit-8 (CCK-8) solution (Beyotime, Shanghai, China) and 100 μL medium were mixed and added to 96-well plates. After incubating the cells at 37 °C for 1 h, the absorbance was assessed at 450 nm.

2.6 Cell Apoptosis

HUVECs apoptosis was determined by flow cytometry using an Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, San Jose, USA) according to the manufacturers' instructions.

2.7 Mitochondrial Membrane Potential

The mitochondrial membrane potential was performed by a Mitochondrial Membrane Potential Assay Kit with JC-1 (Beyotime, Shanghai, China). Briefly, HUVECs were planted in a plate and treated with transfection and hypoxia. Then HUVECs were incubated with JC-1 working solution for 20 min at 37 °C and observed by confocal microscopy. The polymer of JC-1 in the mitochondrial matrix was shown as red fluorescence, while the monomer of JC-1 in the cytoplasm represents unhealthy cells (green fluores-

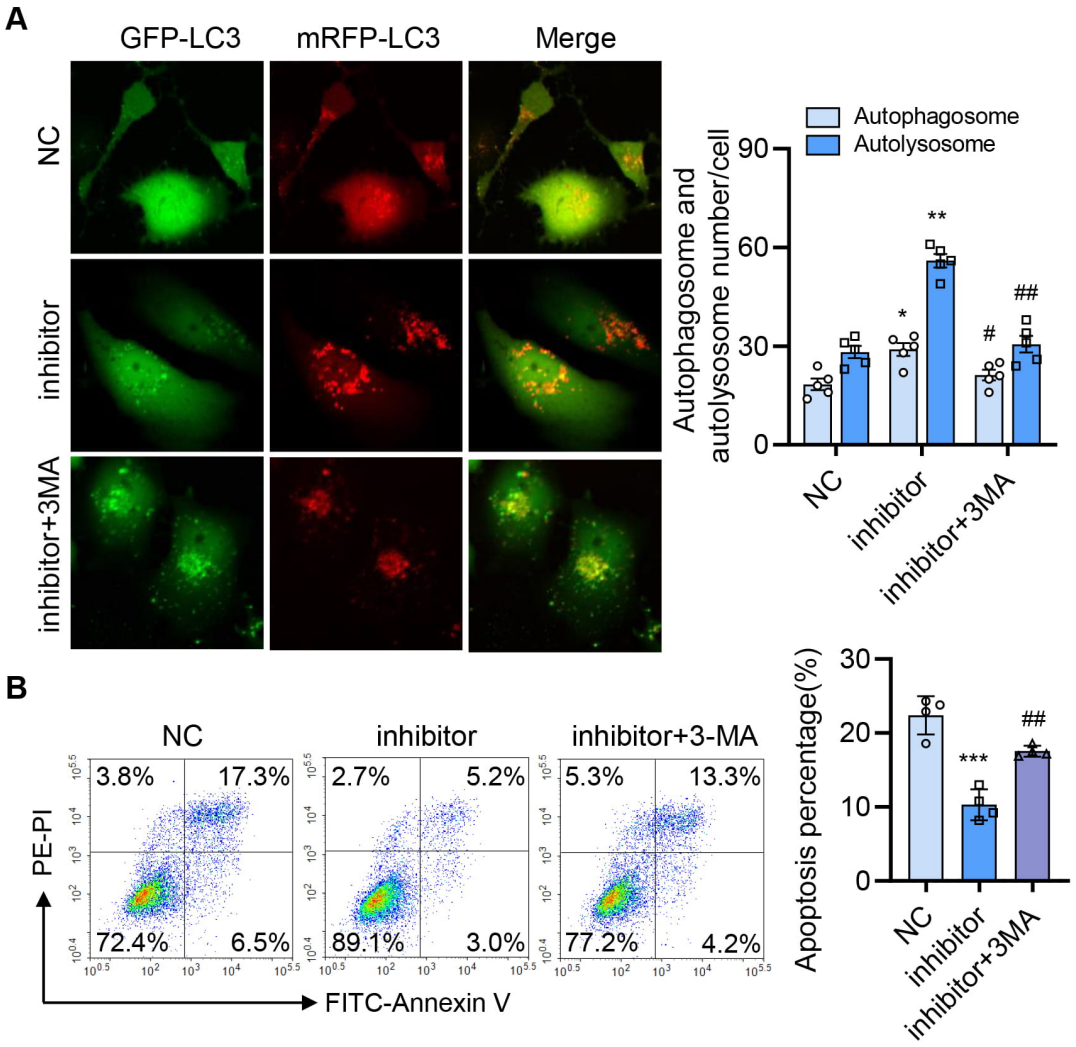

Fig. 4. Inhibition of autophagy deteriorates hypoxia-induced apoptosis in HUVECs. HUVECs were transfected with Ad-mRFP-GFP-LC3 and then treated with NC, miR-4463 inhibitor, miR-4463 inhibitor combined with 3-MA. Later, HUVECs were exposed to hypoxia for 6 h. (A) The autophagosomes and autolysosomes were observed and counted. (B) The flow cytometry chart and histogram were determined. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with NC. #$p < 0.05$, ##$p < 0.01$, compared with inhibitor.

cence). The fluorescence signal intensity was calculated by Image J software (National Institutes of Health, USA).

2.8 Western Blot

HUVECs were lysed by RIPA buffer, and 30 μg of total protein was loaded and separated on 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocked with BSA, the PVDF membrane was incubated in primary antibody overnight at 4 °C (Rabbit anti-LC3B: 1:800, RRID: AB2137707; Rabbit anti-P62: 1:1000; Rabbit anti-cleaved-caspase 3: 1:1000, RRID: AB2070042; Rabbit anti-PARP: 1:1000, RRID: AB659884; Rabbit anti-ULK1: 1:1000, Rabbit anti-p-ULK1 ser-555: 1:1000, RRID: AB10707365; Cell Signaling Technology, Danvers, USA; Mouse anti-GAPDH:1:3000, Beyotime, Shanghai, China). GAPDH was used as a loading control. Signals were detected after incubation with horseradish peroxidase-conjugated secondary antibodies (1:5000, Bioworld, Shanghai, China) for 1 h at room temperature. The images were scanned by Fusion solo 4 (Vilber, Paris, France), and analyzed using Quantity One software 4.2.3 (Bio-Rad, USA).

2.9 Luciferase Reporter Assay

The wild type (WT) and mutant (MUT) 3'UTR of ULK1 mRNA were cloned and ligated to the vector to generate the recombinational vector. For the luciferase report assay, HUVECs were cultured in 96-well plates and transfected with the recombinational vector and miR-4463 mimic, recombinational vector, and control by Lipofectamine 3000. The cells were analyzed with a dual-luciferase reporter assay system (Promega, Wisconsin, USA) accord-

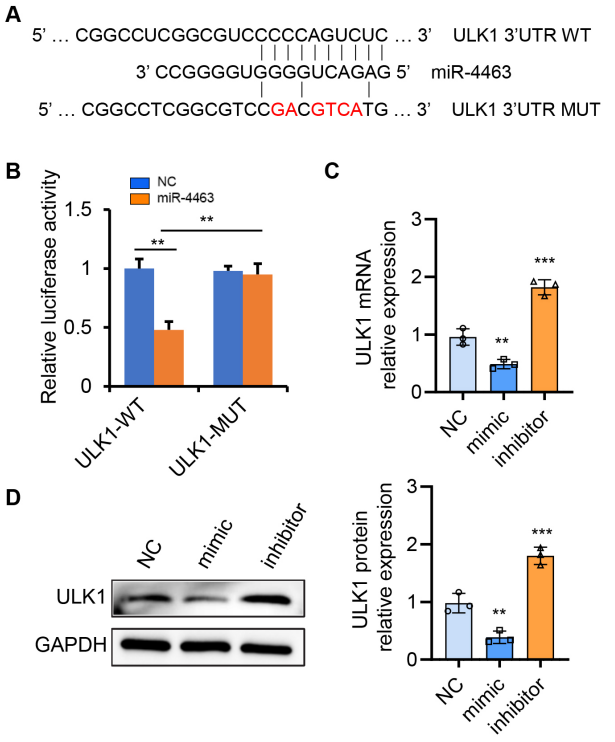

Fig. 5. miR-4463 directly targets ULK1. (A) The predicted binding sites of miR-4463 within ULK1 3'UTR and its mutated version by mutagenesis are as shown. (B) Down-regulation of luciferase activity by ULK1 3'UTR was dependent on miR-4463. $^{**}p < 0.01$, compared with each other. (C,D) miR-4463 negatively regulates ULK1 at mRNA (C) and protein (D) levels. $^{**}p < 0.01$, $^{***}p < 0.001$, compared with NC.

ing to the manufacturer's instructions with a Lumat LB 9507 luminometer (Berthold, Nashua, USA).

2.10 Statistical Analysis

Experimental data were expressed as mean ± standard error of the mean (SEM) and analyzed with SPSS 19.0 software (International Business Machines Corporation, USA). Student *t*-Test was used to analyze the data between the two groups. One-way ANOVA was performed to investigate the difference between multiple comparisons, and the Student-Newman-Keuls method was used to analyze each other. $p < 0.05$ was considered a significant statistical difference. All results were reproduced in at least three independent experiments.

3. Results

3.1 miR-4463 Accelerates HUVEC Apoptosis under Hypoxic Conditions

To confirm the effects of hypoxia on miR-4463 expression, we detected miR-4463 levels in HUVECs treated with hypoxia. We found that hypoxia elevated miR-4463 levels in a time-dependent manner (Fig. 1A). To investigate the functional role of miR-4463 in HUVECs under hypoxic conditions, we detected the cell viability in HUVECs after transfection with miR-4463 mimic, inhibitor, and NC following treatment with hypoxia for 6 h. The results showed that miR-4463 mimic reduced cell viability and accelerated HUVEC apoptosis while miR-4463 inhibitor treatment exerted opposite effects on HUVECs under hypoxic conditions (Fig. 1B–D). We further detected the mitochondrial membrane potential (MMP, $\Delta\Psi m$) since the decrease in MMP is a marker of early apoptosis in living cells. We observed that miR-4463 mimic suppressed the MMP and inhibited the Bcl-2/Bax ratio compared with NC group (Fig. 2A–E). Moreover, miR-4463 mimic aggravated the cleavage of caspase 3 and PARP in HUVECs under hypoxic conditions. To the opposite, miR-4463 inhibitor showed opposite effects on HUVECs (Fig. 2A–E). Taken together, these results suggest that miR-4463 regulates apoptosis via influencing MMP and Bcl-2/Bax ratio.

3.2 miR-4463 Regulates Autophagy under Hypoxic Conditions

To explore whether miR-4463 regulates HUVECs autophagy under hypoxic conditions, we further determined the autophagosomes and autolysosomes distribution in HUVECs co-transfected with Ad-mRFP-GFP-LC3 and NC, miR-4463 mimic or miR-4463 inhibitor. We observed that up-regulation of miR-4463 decreased autophagosomes and autolysosomes compared with NC group in HUVECs under hypoxic conditions. Down-regulation of miR-4463 showed opposite effects (Fig. 3A). Consistent with the effects of miR-4463 on autophagosomes and autolysosomes, miR-4463 mimic suppressed the transformation of LC3I to LC3II and sustained P62 level. In contrast, down-regulation of miR-4463 promoted the LCB3II expression and P62 degradation (Fig. 3B,C). In conclusion, these results indicate that miR-4463 regulates autophagosomes and autolysosomes to influence autophagy progression.

3.3 Down-Regulation of miR-4463 Attenuates Apoptosis by Promoting Autophagy

The above results exhibited that miR-4463 inhibitor remarkably decreased apoptosis and promoted autophagy in HUVECs under hypoxic conditions. To clarify the probable correlation of autophagy with miR-4463-mediated apoptosis, we used 3-MA to inhibit autophagy in HUVECs with miR-4463 knockdown. We observed that 3-MA reversed the promotive effect of miR-4463 inhibitor on the formation of autophagosomes and autolysosomes (Fig. 4A). Furthermore, 3-MA abolished the protection of miR-4463 inhibitor from hypoxia-induced apoptosis (Fig. 4B). These results suggest that down-regulation of miR-4463 inhibits apoptosis induced by hypoxia through enhancing autophagy.

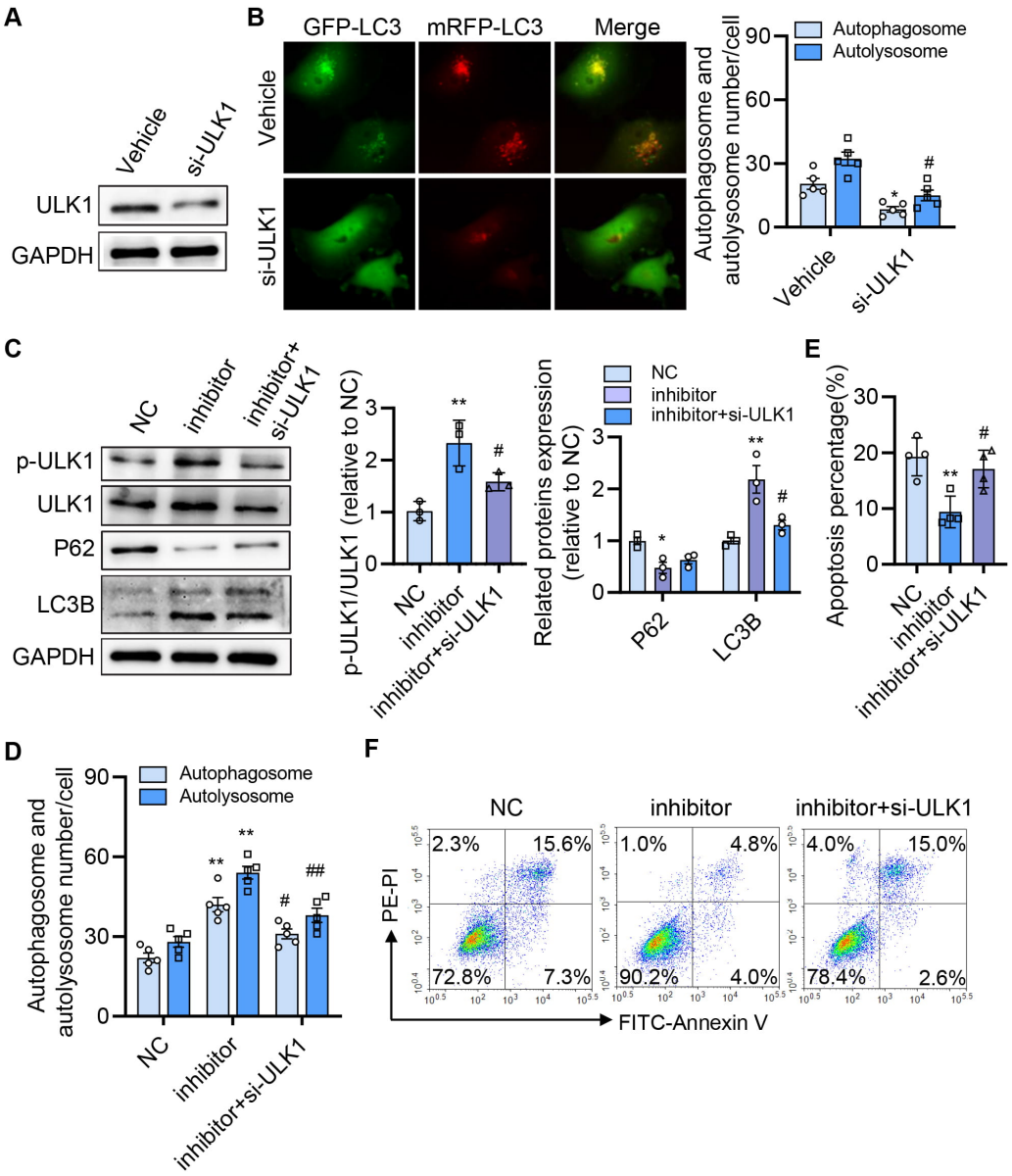

Fig. 6. Silence of ULK1 blocks miR-4463-regulated autophagy in HUVECs under hypoxic conditions. (A) ULK1 siRNA (si-ULK1) decreased the protein level of ULK1. (B) The autophagosomes and autolysosomes were observed and counted in HUVECs after transfection with Vehicle and si-ULK1 and further treated with hypoxia for 6 h. (C) The levels of p-ULK1, ULK1, P62 and LC3B were determined by western blot. (D–F) HUVECs were transfected with NC, miR-4463 inhibitor and miR-4463 inhibitor with si-ULK1, and further treated with hypoxia. The number of autophagosomes and autolysosomes was calculated (D) and the apoptosis rate (E,F) was determined. *$p < 0.05$, **$p < 0.01$, compared with NC. #$p < 0.05$, ##$p < 0.01$, compared with inhibitor.

3.4 miR-4463 Directly Targets ULK1

To explore the probable molecular mechanism of the effect of miR-4463 on autophagy and apoptosis, we predicted the potential target genes of miR-4463 using TargetScan and miRDB. ULK1 attracted our attention among these predicted target genes. As shown in Fig. 5A, the wild type (WT) and mutated (MUT) sequences of ULK1 3'-UTR were cloned and ligated to the luciferase reporter plasmid. The dual-luciferase report gene assay results showed that miR-4463 reduced the luciferase reporter activity significantly compared to NC, and mutations in ULK1 3'-UTR binding sites reversed the effects (Fig. 5B), indicating that ULK1 is a target gene of miR-4463. Besides, miR-4463 mimic decreased the ULK1 expression at both mRNA and protein levels while miR-4463 inhibitor showed an opposite effect under hypoxic conditions (Fig. 5C,D). These data suggest that miR-4463 directly targeted the ULK1 3'UTR region to negatively regulate its expression at translational and post-translational levels.

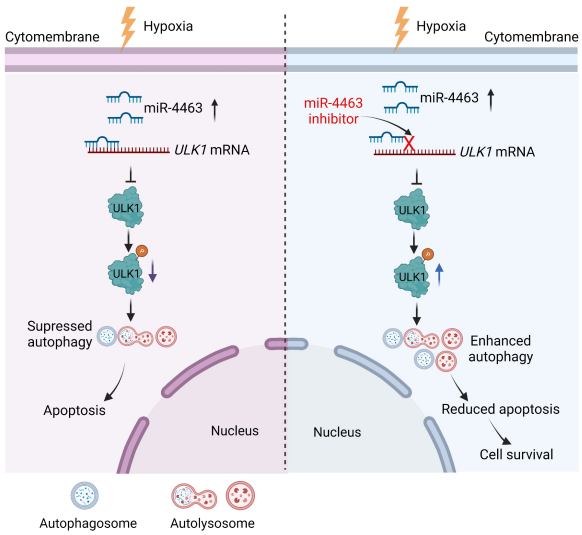

Fig. 7. The schematic diagram of the role of miR-4463 in autophagy and apoptosis under hypoxic conditions. Hypoxia promotes miR-4463 expression in HUVECs. Down-regulation of miR-4463 enhances autophagy activation to improve cell survival by targeting ULK1 to accelerate the formation of autophagosomes and autolysosomes.

3.5 miR-4463 Mediates Autophagy and Apoptosis Through ULK1

To elaborate on whether miR-4463 mediates autophagy and apoptosis via ULK1 in hypoxic conditions, we introduced ULK1 siRNA to knockdown ULK1. As shown in Fig. 6A, ULK1 siRNA (si-ULK1) significantly knocked down the ULK1 protein level compared with transfection vehicle. Knockdown of ULK1 significantly suppressed the number of autophagosomes and autolysosomes in HUVECs under hypoxic conditions (Fig. 6B). Moreover, we observed that ULK1 siRNA reversed the effects of miR-4463 inhibitor on autophagosome and autolysosome formation, total and phosphorylated ULK1 and P62 levels, P62 level, and LC3BI-to-LC3BII transition under hypoxic conditions (Fig. 6C,D). Knockdown of ULK1 reversed the effects of miR-4463 inhibitor-regulated cell apoptosis in HUVECs (Fig. 6E,F), which indicates that ULK1 is responsible for miR-4463 inhibitor-regulated autophagy and apoptosis.

4. Discussion

Studies have revealed that miRNAs are involved in autophagy and apoptosis. However, the mechanisms are not yet fully understood. Here, we discovered that miR-4463 was highly expressed in HUVECs under hypoxic conditions. Down-regulation of miR-4463 decreased hypoxia-induced apoptosis through maintaining MMP in HUVECs. Moreover, down-regulation of miR-4463 promoted the formation of autophagosomes and autolysosomes by targeting ULK1 to strengthen autophagy and promote survival.

Researches have reported that aberrant miR-4463 expression is observed in patients with polycystic ovary syndrome (PCOS) [23] and patients with ASO [22]. Our previous study found that H_2O_2 induced the expression of miR-4463 in HUVECs [24]. Similar to this result, we observed that miR-4463 expression was increased in a time-dependent manner under hypoxic conditions. Studies have reported that autophagy exerts a protective role under hypoxic stress, and miRNAs play a significant role in this process. Xu *et al.* [25] reported that miR-210 promoted autophagy to preserve cell survival in endometriotic cells suffering from hypoxia. miR-17-5p induced protective autophagy to develop anti-apoptosis role in VSMCs [26]. Consistent with previous studies, we discovered that miR-4463 was a momentous regulator of apoptosis and autophagy under hypoxic conditions. Inhibition of miR-4463 played a protective role in sustaining cell survival through strengthening autophagy. The transformation from LC3I to LC3II implicates that LC3 has been recruited to autophagosomal membranes [27]. P62 is required for the formation and the degradation of polyubiquitin-containing bodies by autophagy [28,29]. We found that miR-4463 regulated the transformation from LC3I to LC3II and P62 expression. These findings improve our understanding of the regulation of miR-4463 on autophagy and apoptosis.

ULK1, as a mammalian homolog of yeast Atg1, acts as an initiator to transduce signals to downstream mediators and thereby regulate autophagy [30]. Recently, studies revealed that ULK1 expression was correlated with hypoxia treatment. ULK1 level was transcriptionally induced by chronic or short-term hypoxia, but decreased under prolonged hypoxic conditions [30,31]. And Allavena G *et al.* [30] reported that under conditions of amino acid and serum deprivation, the ULK1 protein was rapidly depleted. These results suggest that a variety of factors coordinately controls the cellular level of ULK1. Previous studies reported several miRNAs, including miR-372, miR-93, miR-26a, miR-17, modulated cell survival and apoptosis by targeting ULK1 [32–35]. In this study, we verified that miR-4463 directly targeted ULK1 at the transcriptional and post-transcriptional levels. In the process of autophagy, phosphorylation of ULK1 at one set of highly conserved sites (ULK1 Ser317, Ser467, Ser555, Ser637) has been implicated in modulating the formation of the autophagosomes. Here we observed that miR-4463 inhibitor promoted ULK1 phosphorylation at Ser555 in hypoxic conditions and increased autophagosomes and autolysosomes along with LC3BI-to-LC3BII transition. The blockage of these effects by ULK1 siRNA suggests that miR-4463 regulates autophagic activity at least partially through the interaction of miR-4463 with ULK1 mRNA and mediating ULK1 phosphorylation at Ser555.

5. Conclusions

In conclusion, our results demonstrate that hypoxia promotes miR-4463 expression in HUVECs. Down-regulation of miR-4463 enhances autophagy activation to promote cell survival by targeting ULK1 to accelerate the formation of autophagosomes and autolysosomes (Fig. 7). These findings suggest that miR-4463 might be a critical regulatory factor in promoting endothelial cell survival when exposed to hypoxia. Further studies are warranted to fully understand the relationship between the pathway regulated by miR-4463 under hypoxic conditions on a more fundamental level, which also must be involved in animal models and clinical samples in the future. Nonetheless, our data indicate that inhibiting miR-4463 might be a potential target for the survival of endothelial cells and the maintenance of vascular function in patients with lower limb ischemia and its complications.

Abbreviations

ASO, arteriosclerosis obliterans; HUVECs, human umbilical vein endothelial cells; ULK1, Unc-51 like kinase 1; ER, endoplasmic reticulum; I/R, ischemia/reperfusion; CCK-8, cell counting kit-8; FITC, fluorescein isothiocyanate; MMP, mitochondrial membrane potential.

Author Contributions

XZ conceived the study and revised the manuscript. XH performed the experiments and wrote the paper. FW and YZ carried out western blot experiments. JW and YL revised the paper. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest. JW is serving as the guest editor of this journal. We declare that JW had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to VDG.

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