Down-regulated miR-145 rescues insulin resistance in endothelial cells

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Research article

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Abstract

Background: MiR-145 is involved in insulin resistance (IR) in liver cells, but its effects in human umbilical vein endothelial cells (HUVECs) induced by IR remains unclear. This study took this as the starting point, aiming to find a potential target for the treatment of related disease. Methods: HUVECs were respectively treated with glucose of 15, 30, 45 mmol/L, or insulin of 1, 2, 3, 4, 5 μmol/L on the basis of high-glucose (33.3 mmol/L). MiR-145 mimics and miR-145 inhibitor were severally transfected into HUVECs with or without IR (4 μmol/L insulin + high-glucose). Quantitative real-time polymerase chain reaction (qRT-PCR) assay determined the miR-145 expression in HUVECs after treatment and transfection. The glucose consumption and glycogen contents of cells were appraised by glucose oxidase-peroxidase and anthranone-sulfuric acid methods, respectively. The apoptotic rates were ascertained using the flow cytometry. The expressions of apoptosis-related indicators Bcl-2 and Bax were analyzed by western blot (WB) and qRT-PCR assays. Results: The expression of miR-145 was increased in IR models and incremental glucose concentrations. The glucose consumption and glycogen content were down-regulated in IR-induced HUVECs, which were enhanced by over-expressed miR-145 but reversed by down-regulation. Moreover, over-expression of miR-145 aggravated the apoptosis of IR-induced HUVECs, while the inhibition of miR-145 had a completely opposite effect. Accordingly, up-regulated miR-145 obviously reduced Bcl-2 level and enhanced Bax expression in IR models, which was contrary to the down-regulated miR-145. Conclusion: Down-regulated miR-145 rescued IR in endothelial cells, which might be a conceivable treatment for IR of endothelial cells.

Background

With the rapid development of economy and the improvement of living standards, the incidence of diabetes mellitus is also obviously increasing, and gradually showing a younger trend, among which type 2 diabetes mellitus is the most common [1]. Vascular disease is one of the main complications of diabetes, in which vascular endothelial cell injury is the primary link [2, 3]. Endothelial cells play a crucial role in maintaining the stability of vascular structure and function. On the one hand, vascular diastolic dysfunction may lead to hypertension; in addition, it can also cause changes in the structure of blood vessels that can lead to diseases such as hardening of the arteries [4]. Several studies have shown that insulin resistance (IR) is often associated with endothelial dysfunction, which is a pivotal link in the development of type 2 diabetes [5-7]. Normally, insulin binds to its receptors on endothelial cells and provokes the production of nitric oxide by endothelial cells through specific cell signaling pathways, thus maintaining the biological characteristics of vascular dilation and increased blood flow [8]. However, stimulation factors such as high-glucose can induce abnormal insulin signals and IR, which may give rise to the destruction of endothelial cell integrity and lead to endothelial cell dysfunction [9, 10]. Thus, correcting IR has a positive significance in the treatment of endothelial cell injury.

MicroRNAs (miRNAs), a class of highly conserved non-coding RNAs composed of about 22 actinic acid, which specifically binds to the 3'-untranslated region (3’UTR) of target gene sequence to inhibit its post-transcriptional translation or damage target gene [11]. MiRNAs were previously discovered in the

pathological process of tumor [12], but existing studies have shown that they are involved in a variety of cellular physiological and pathological processes [13, 14]. Recent studies have found that various miRNAs take part in the regulation of endothelial cell function, such as miR-200b [15], miR-142 [16], miR-126 [17], and so on. Moreover, there are increasing indications demonstrate that miRNAs also act as pivotal regulators in the generation, secretion, and action of insulin [18]. An animal study reported that miR-145 was involved in IR of liver cells, but the role and mechanism of miR-145 in IR of endothelial cells remains indistinct, so this paper mainly explored the effects and action principle of miR-145 in IR of endothelial cells.

Here, we induced IR in human umbilical vein endothelial cells (HUVECs) by high-glucose, and up-regulated and down-regulated the expression level of miR-145 in cells to probe into the influence of miR-145 on IR of endothelial cells, including analysis of glucose consumption, glycogen content and apoptosis. The purpose of this study was to investigate the effects of miR-145 on IR endothelial cells, so as to provide a potential mean for the treatment of type 2 diabetes mellitus and thus improve the therapeutic effect of patients.

Methods

Cell culture

HUVECs were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA). HUVECs were grown in a humidified environment at 37°C supplemented with 5% CO_2, and prepared for our subsequent experiments. Cells at passages 3-9 were applied in our study.

Morphological observation

33.3 mmol/L of glucose was determined as high-concentration based on previous literatures [19, 20]. To determine the effect of high-glucose on cells, cultured HUVECs were obtained and placed in serum-free medium containing 33.3 mmol/L of high-glucose. For comparison, HUVECs only incubated in serum-free medium were served as controls. After 24 h, the morphology of HUVECs incubated with or without high-glucose was observed under the inverted microscope (Eclipse TS-100, Nikon, Tokyo, Japan).

Cell treatment

To investigate the role of glucose in miR-145 expression, HUVECs were respectively treated with different concentrations of glucose (15, 30, 45 mmol/L). Quantitative real-time polymerase chain reaction (qRT-PCR) assay was used to determine the expression of miR-145 in glucose-treated HUVECs after 6 h. Meanwhile, the gene expression in HUVECs treated with 30 mmol/L of glucose was further examined at different action times (0, 0.5, 1, 2, and 4 h).

For the establishment of IR cell models, HUVECs were treated with insulin of 1, 2, 3, 4, 5 μmol/L on the basis of high-glucose (33.3 mmol/L). HUVECs co-treated with insulin (4 μmol/L) and high-glucose (33.3 mmol/L) were considered as IR models, cells only treated with high-glucose were used as a single comparison, and untreated cells were taken as a blank control. After 6 h of intervention with insulin of different concentrations, glucose consumptions in HUVECs were detected by glucose oxidase-peroxidase method. In controls and IR-treated cells, intracellular glycogen contents of cells were respectively assessed after intervention for 24 and 48 h through anthrone-sulfuric acid method; the morphology of cells after 24 h of treatment were observed with the Eclipse TS-100 inverted microscope (Nikon, Tokyo, Japan). Moreover, the expression of miR-145 in controls, IR-treated and glucose-treated cells were measured via qRT-PCR assay.

Cell transfection

For further exploring the effects of miR-145 on IR-induced HUVECs, miR-145 mimics (#MCH01320, Applied Biological Materials, Vancouver, Canada) and miR-145 inhibitor (#MIH01320, Applied Biological Materials, Vancouver, Canada) were severally transfected into HUVECs (3×10^5) with or without IR. Lipofectamine 2000 (Invitrogen, California, USA) was used for cell transfection according to the manufacturer's protocol. For comparison, un-transfected HUVECs were used as controls, cells transfected with scrambled sequence were served as a negative comparison (NC), and cells only treated with IR were considered as models. Transfected cells were prepared for following experiments, including qRT-PCR, glucose consumption, glycogen content, flow cytometry and western blot (WB) analysis.

qRT-PCR assay

The total RNA of cells was isolated by the Trizol reagents (Invitrogen, Carlsbad, California, USA). The concentration and integrity of isolated RNA was quantified via a NanoDrop Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) and 1% agarose modified gel electrophoresis, respectively. The cDNA was synthesized from the 1 μg of isolated RNA with the PrimeScript RT Master Mix Perfect Real Time (TaKaRa, Shiga, Japan) based on the manufacturer’s instructions. The Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) was utilized to ascertain miR-145 expression following the sets: pre-degeneration for 5 min at 95°C, and 35 cycles of 15 s at 95°C and 30 s at 60°C. U6 or GAPDH acted as the internal control, and $2^{-\Delta\Delta Ct}$ method [21] was employed to evaluate miR-145 expression. Sequence: The sequences of primers were shown in Table 1 and synthesized by Gene Pharma (Shanghai, China).

Glucose consumption detection

The glucose oxidase-peroxidase reagent (Robio, Shanghai, China) was used to appraise the glucose consumption of insulin-treated HUVECs and transfected cells. The absorbance at wavelength of 500 nm was detected by Elx-800 microplate reader (Bio-Tek, Vermont, USA), and the glucose in culture was calculated according to the manufacturer's descriptions.

Intracellular glycogen content

The glycogen contents of HUVECs after transfection and insulin treatment were measured by anthranone-sulfuric acid method (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Following the manufacturer's instructions, lye was added into treated HUVECs for boiling water bath for 20 min, and then the developer was instilled into cells for boil water bath for another 5 min. Finally, the absorbance was measured by a landscape photometer (NP80, Implen Gmb H, Germany) at the wavelength of 620 nm, and the glycogen contents of cells were analyzed.

Cell apoptosis

The apoptotic rates of HUVECs after transfection were ascertained by the flow cytometry analysis. For the detection, transfected cells were stabilized in 70% ice-cold ethanol for 1 h, and then stained with 10 μL Annexin V and 5 μL propidium iodide (PI). The apoptotic rates of cells were evaluated using the FACSCalibur flow cytometer (BD Biosciences, USA) following the manufacturer's instructions.

WB analysis

The expressions of apoptosis-related proteins (Bcl-2 and Bax) in transfected HUVECs were analyzed by WB assays. For the assays, the total protein from cells was isolated using RIPA buffer (Solarbio, Beijing, China). The concentration of total protein was determined through the Bicinchoninic Protein Assay kit (BCA, Pierce, Rockford, IL, USA). Next, the total protein (50 μg) were separated onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime, Shanghai, China) and then shifted onto polyvinylidene fluoride (PVDF) membranes. Then, the membranes were blocked with 5% non-fat dried milk for 2 h. The primary antibodies at dilution rate of 1:1000 were added overnight at 4°C, including Bcl-2 (ab59348, Abcam, USA), Bax (ab32503, Abcam, USA), and GAPDH (ab8245, Abcam, USA) acted as the internal reference. The corresponding secondary antibodies goat anti-rabbit IgG H&L (HRP; 1:7000, ab97051, Abcam, USA) and goat anti-mouse IgG H&L (HRP; 1:1000, ab150113, Abcam) were added for 1 h at room temperature. The blots signals were developed by an enhanced chemiluminescence-detecting kit (Thermo Fisher, MA, USA), and the results were normalized to GAPDH.

Statistical analysis

Statistical Package of the Social Sciences 20.0 software (SPSS, Inc., Chicago, USA) was used for data analysis. The measurement data were presented as mean ± standard deviation (SD). The comparison between groups was performed by Student's *t*-test or one-way analysis of variance (ANOVA). All experiments were carried out in triplicate. $P<0.05$ was considered as statistically significant.

Results

High-glucose promoted miR-145 expression in HUVECs

After 24 h of treatment with high-glucose, the contour of HUVECs was un-sharp and the cells were arranged in disorder. In contrast, the shape of control cells was long fusiform, adherent and tightly arranged, with large and round nuclei (Figure 1A). The results of qRT-PCR assays revealed that the expression of miR-145 in HUVECs were increased gradually under the action of glucose at different concentrations (15, 30, 45 mmol/L) (P<0.001, Figure 1B). In addition, with the increase of time, the expression of miR-145 in glucose-treated (30 mmol/L) cells was first up-regulated and then slightly decreased, but always higher than the initial state (P<0.001, Figure 1C).

The expression of miR-145 in IR models was up-regulated

In the analysis of glucose consumption, compared with the HUVECs without insulin, the glucose consumption ability of cells was decreased gradually with the augment of insulin concentration, with the weakest ability at concentration of 4 and 5 μmol/L (P<0.05, Figure 2A). Accordingly, the glycogen contents of IR models at 24 and 48 h were observably down-regulated in comparison with controls (P<0.05, Figure 2B). The body of the control cells was full and arranged neatly like flagstone, with a large number and no obvious morphological damage. However, the number of IR models was less, the edges were blurred, and the cells were apoptotic (Figure 1C). Similar to the effect of high-glucose, miR-145 expression level in IR model cells was accelerative significantly (P<0.001, Figure 1D).

Down-regulated miR-145 rescued the IR in HUVECs

In cell transfection experiments, miR-145 mimics enhanced the glucose consumption ability of IR cell models, while miR-145 inhibitor weakened this ability of models (P<0.05, Figure 3A). Correspondingly, miR-145 mimics obviously suppressed glycogen synthesis of IR models, whereas miR-145 inhibitor had the exact opposite effect on glycogen content (P<0.05, Figure 3B). As for the flow cytometry analysis, the apoptosis rate of IR models was raised evidently, which could be enhanced by over-expressed miR-145 and reversed by miR-145 inhibitor (P<0.05, Figure 3C-D). Interestingly, down-regulated miR-145 had no significant effects on non-IR cells, whereas over-expressed miR-145 reduced glucose consumption and glycogen synthesis, and accelerated cell apoptosis (P<0.05, Figure 3A-D). qRT-PCR assays indicated that miR-145 mimics intensified the auxo-action of IR on miR-145 expression, while miR-145 inhibitor restrained this effect (P<0.05, Figure 3E). Furthermore, WB and qRT-PCR analysis confirmed that Bcl-2 expression level was notably reduced in the IR models, while Bax expression level was up-regulated. The up-regulated miR-145 positively enhanced the expression of apoptosis-related proteins in IR models, and had the same effect in non-IR cells; on the contrary, the down-regulation of miR-145 rescued the expressions of apoptosis-related proteins in IR models, but had little effect on non-IR cells (P<0.05, Figure 4).

Discussion

IR refers to the impairment of insulin in promoting glucose uptake and utilization, resulting in the metabolic effect of body on insulin is lower compared with expected normal level, thus making it difficult to control glucose level. IR is not only an important factor in the development of type 2 diabetes mellitus,

but also a key cause of endothelial cell injury [22]. IR interacts with endothelial cells injury, high-concentrations of insulin can disrupt cell signaling pathways and make cells insensitive to insulin, leading to endothelial dysfunction [23]. In previous studies, high-glucose was commonly used to induce IR in cells [24]. In the present study, we found that endothelial cell proliferation was sparse observably under the action of high-glucose compared with untreated cells, which was similar to the findings of anterior research [25]. Moreover, adding 4-5 μmol/L of insulin on the basis of high-glucose can observably suppress glucose consumption and glycogen synthesis, and the cell morphology was apoptotic, which was similar to the results of Udumula MP et al. [26], meaning the successfully establishment of IR cell models. As for the gene expression in endothelial cells, this study determined that the expression level of miR-145 was increased in IR models, as same as the trend of that in cells treated with incremental glucose concentration, without time dependence. The outcomes of our experiments suggested that miR-145 may be involved in the regulation of IR in endothelial cells.

In previous reports, miR-145 has been determined to be associated with the occurrence and development of various diseases including gastric cancer [27], colorectal cancer [28], prostate cancer [29], and so on. Based on the analysis of Wen F et al. [30], the over-expression of miR-145 suppressed the glucose uptake of liver cells and stimulated insulin resistance in cells. Here, we found that glucose consumption and glycogen content in IR models could be enhanced by over-expressed miR-145, which could be improved with the gene down-regulation. These findings indicated that the up-regulation of miR-145 aggravated IR of endothelial cells, while miR-145 inhibition had an alleviate effect on IR. Moreover, it was worth pointing out that the up-regulation of miR-145 can directly regulate glucose consumption and glycogen synthesis in endothelial cells, while the down-regulation had no obvious effects. Similar to this study, in liver cancer cells, Li Q et al. [31] reported that over-expressed miR-145 inhibited insulin-induced glucose consumption by targeting pyruvate kinase M2 (PKM2), which further testified that the raise of miR-145 can induce IR in endothelial cells via regulating glucose metabolism.

In addition, this study also found that IR had an evident apoptosis-promoting effect on endothelial cells, which was enhanced by the over-expression of miR-145 and reversed through the down-regulation of gene. In cancer cells, Chen Z et al. showed that miR-145 over-expression decreased the proliferation of prostate cancer cells and promoted cell apoptosis [32]. Anton L et al. [33] also found that transfection of miR-145 could restrain cervical cell multiplication through increasing apoptosis and blocking cell cycle. In brief, these studies indicated that the over-expression of miR-145 expedited apoptosis in cells of various diseases, which may be a potential target for the treatment of some diseases. To be more precise, we further determined that, even under the action of miR-145 mimics, IR could further induce the increase of gene expression in endothelial cells, suggesting that the up-regulation of miR-145 and the occurrence of IR were mutually reinforcing.

For in-depth exploration, this paper also measured the expressions of proteins and mRNAs related to cell apoptosis, including Bcl-2 and Bax. Currently, Bcl-2/Bax is the main target molecule for the study of apoptosis molecular mechanism. Bcl-2 is the main regulator of cell apoptosis, which has the functions of promoting cell transformation from G1 phase to S phase, repairing chromosome damage, and prolonging

cell survival by antagonizing the pro-apoptotic molecule Bax at the early stage of cell differentiation [34]. Contrary to Bcl-2, Bax is an essential substance for cell apoptosis and as a pro-apoptotic factor, which may be achieved by inducing the release of Caspase [35]. Shortly, Bcl-2/Bax ratio determines the trend of apoptosis, that is, the up-regulation of Bcl-2 inhibits apoptosis, while up-regulated Bax promotes cell death. In this study, both WB and qRT-PCR analysis confirmed that up-regulated miR-145 obviously reduced Bcl-2 level and enhanced Bax expression in IR models, while the inhibition of miR-145 had a completely opposite effect. These outcomes further demonstrated that elevated miR-145 reinforced IR-induced endothelial cell apoptosis, while down-regulation of miR-145r relieved this situation.

In conclusion, miR-145 was observably up-regulated in the endothelial cells induced by insulin resistance. Contrary to the effect of up-regulated miR-145, inhibition of miR-145 rescued glucose consumption and glycogen content in IR-induced endothelial cells, which might be a conceivable therapeutic direction for IR of endothelial cells, benefiting patients with type 2 diabetes mellitus.

List Of Abbreviations

IR= insulin resistance

HUVECs= human umbilical vein endothelial cells

WB= western blot

miRNAs= MicroRNAs

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

No human and animals are involved in this research.

Consent for publication

Not applicable.

Availiability of Data and Materials

The datasets used and/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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Not applicable.

Authors' contributions

Substantial contributions to conception and design: JW, ZD

Data acquisition, data analysis and interpretation: ZD, LL

Drafting the article or critically revising it for important intellectual content: JW

Final approval of the version to be published: All authors

Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of the work are appropriately investigated and resolved: JW

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from the nucleus in healthy interphasic cells. *Cell cycle (Georgetown, Tex)* 2017, **16**(21):2108-2118.

Table

Table 1 Primer base sequence

	Forward (5’-3’)	Reverse (5’-3’)
5	GGTCCAGTTTTCCCAGG	CAGTGCGTGTCGTGGAGT
	GAGACAGCCAGGAGAAATCA	CCTGTGGATGACTGAGTACC
	GCTGGACATTGGACTTCCTC	CTCAGCCCATCTTCTTCCAG
	AGAAGGCTGG GGCTCATTTG	AGGGGCCATC CACAGTCT TC
	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

Figures

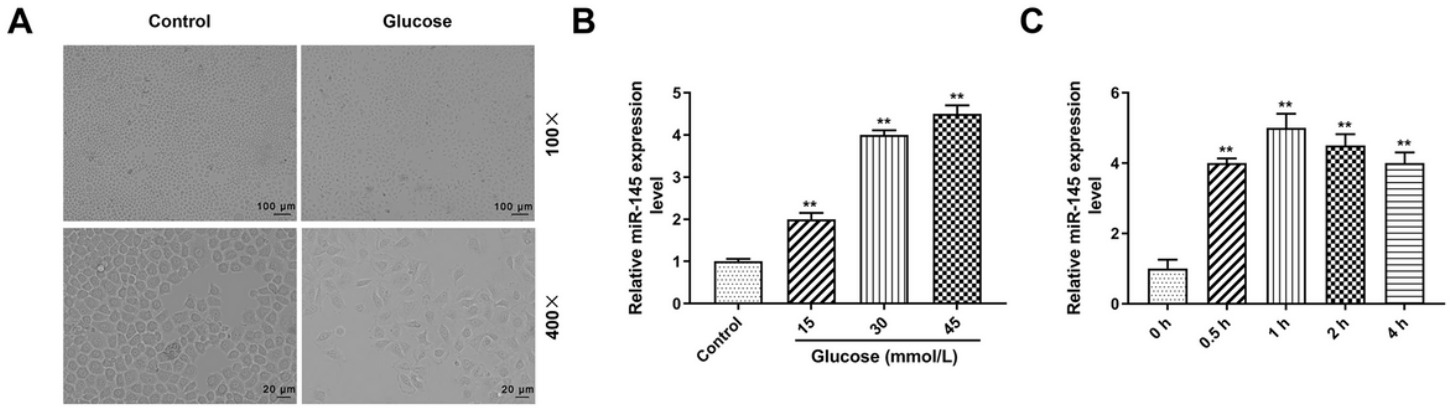

Figure 1

High-glucose promoted miR-145 expression in human umbilical vein endothelial cells (HUVECs). (A) Microscopic pictures of glucose-treated HUVECs (33.3 mmol/L) and untreated controls. (B) Quantitative real-time polymerase chain reaction (qRT-PCR) assay assessed the expression of miR-145 in HUVECs treated with glucose at different concentrations (0 (Control), 15, 30, 45 mmol/L). (C) The miR-145 expression in HUVECs treated with 30 mmol/L of glucose was further examined at different action times (0, 0.5, 1, 2, and 4 h) via qRT-PCR assay. **P<0.001, vs. Control, or 0 h. n=3.

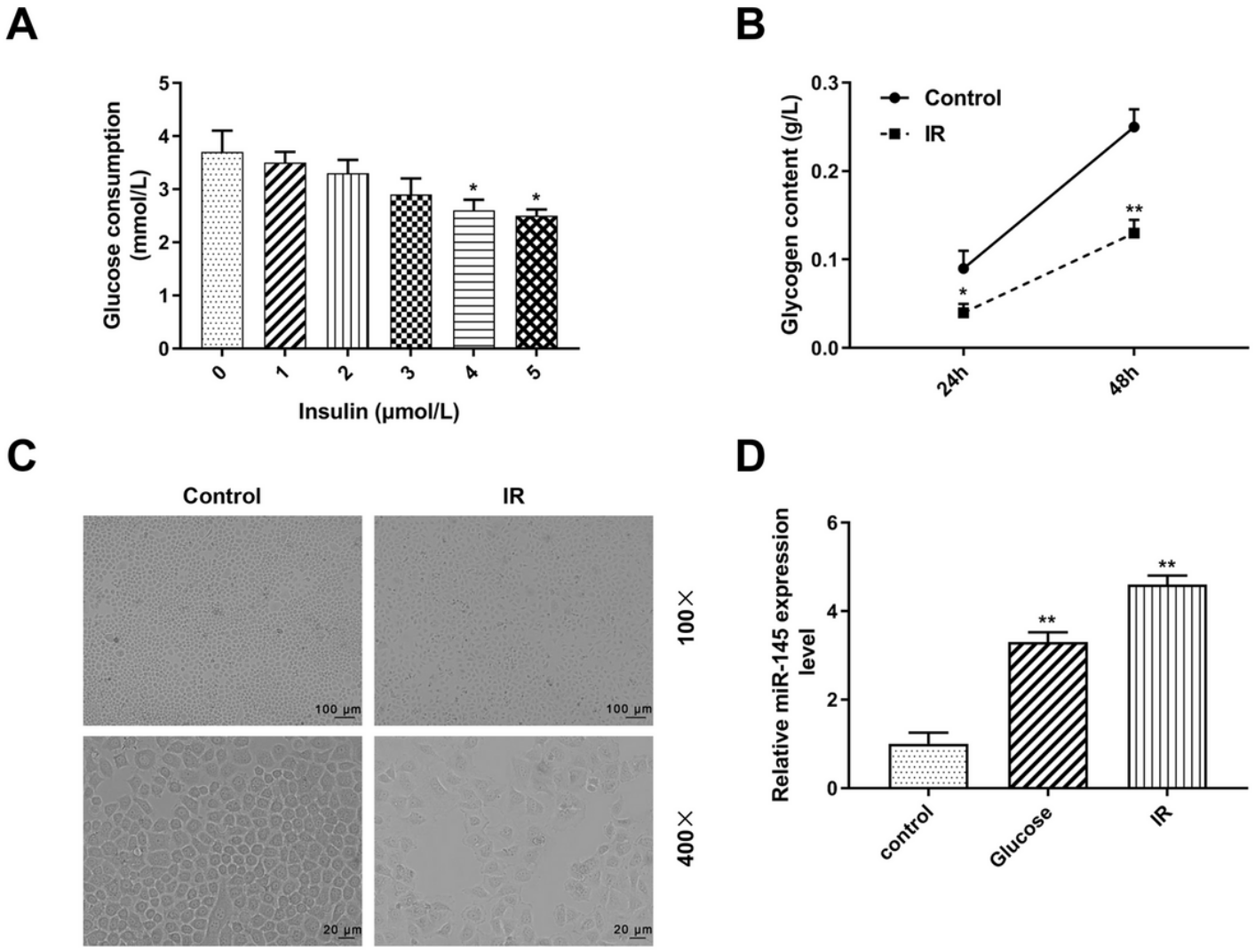

Figure 2

The expression of miR-145 in insulin resistance (IR) models was up-regulated. (A) The glucose consumptions in human umbilical vein endothelial cells (HUVECs) were detected after intervention with insulin of different concentrations (0, 1, 2, 3, 4, 5 μmol/L) by glucose oxidase-peroxidase method. (B) In controls and IR-treated cells, intracellular glycogen contents of cells were respectively assessed after intervention for 24 and 48 h through anthrone-sulfuric acid method. (C) The morphologies of controls and IR-treated cells were observed under the microscope. (D) The expression of miR-145 in controls, IR-treated and glucose-treated cells were measured via quantitative real-time polymerase chain reaction (qRT-PCR) assay. **P<0.001, vs. 0 μmol/L of insulin, or Control. n=3.

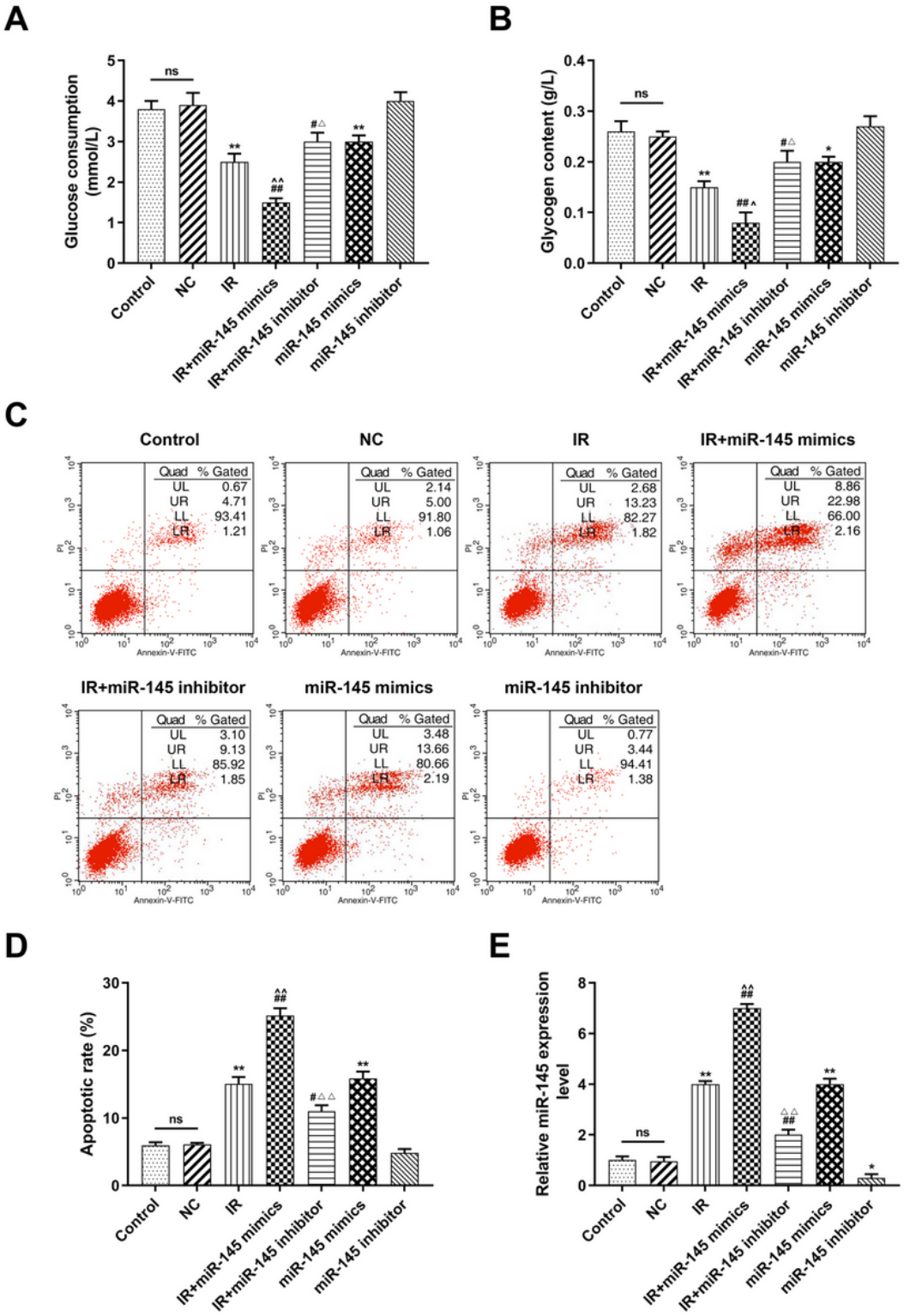

Figure 3

Down-regulated miR-145 rescued the insulin resistance (IR) in human umbilical vein endothelial cells (HUVECs). In this figure, miR-145 mimics and miR-145 inhibitor were severally transfected into HUVECs with or without IR. For comparison, un-transfected HUVECs were used as controls, cells transfected with scrambled sequence were served as a negative comparison (NC), and cells only treated with IR were considered as models. (A) The glucose consumptions in transfected cells were detected by glucose

oxidase-peroxidase method. (B) Intracellular glycogen contents of transfected cells were assessed through anthrone-sulfuric acid method. (C, D) The apoptotic rates of transfected cells were analyzed by flow cytometry. (E) The expression of miR-145 in transfected cells was measured by quantitative real-time polymerase chain reaction (qRT-PCR) assay. *P<0.05, **P<0.001, vs. control or NC; #P<0.05, ##P<0.001, vs. IR; ^P<0.05, ^^P<0.001, vs. miR-145 mimics; △P<0.05, △△P<0.001, vs. miR-145 inhibitor. n=3.

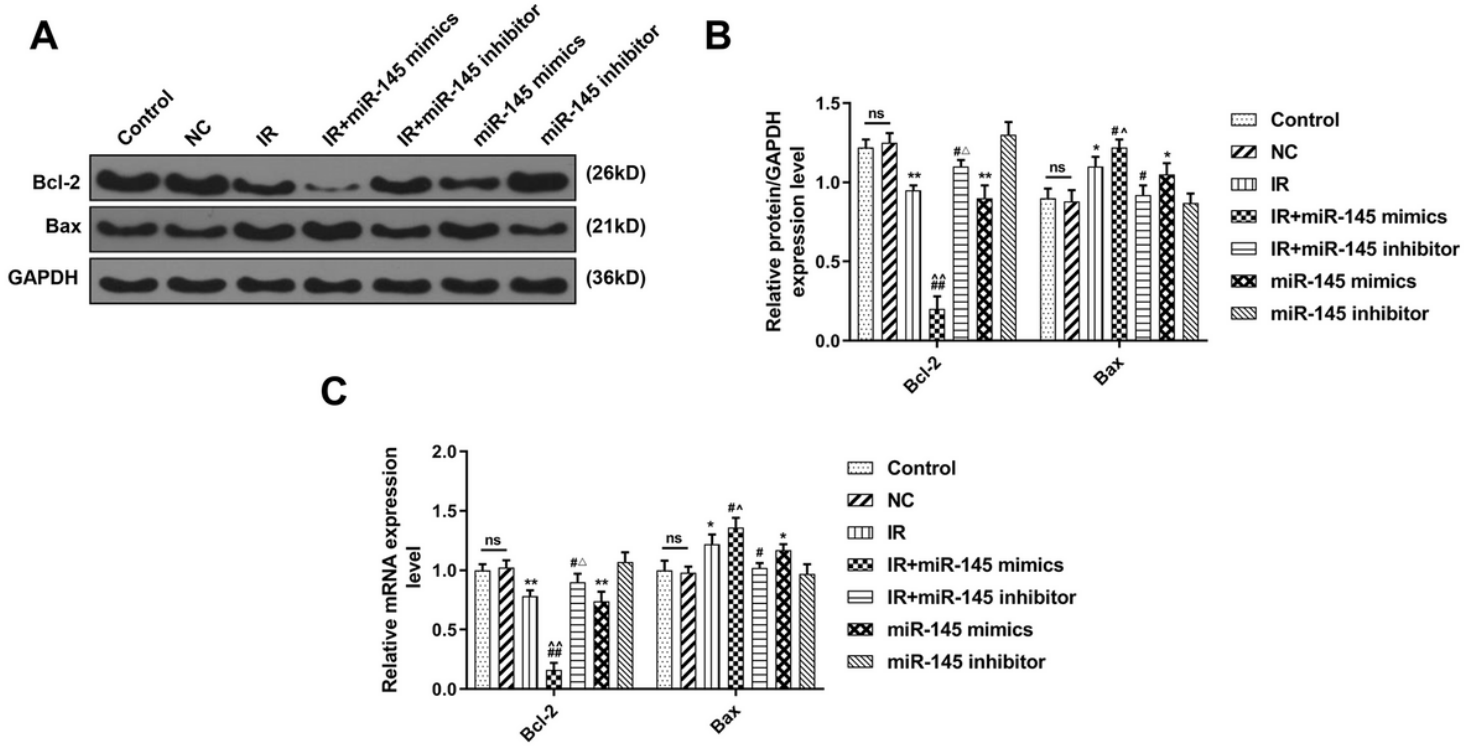

Figure 4

Down-regulated miR-145 suppressed the apoptosis of insulin resistance (IR) cell models. In this figure, miR-145 mimics and miR-145 inhibitor were severally transfected into HUVECs with or without IR. For comparison, un-transfected HUVECs were used as controls, cells transfected with scrambled sequence were served as a negative comparison (NC), and cells only treated with IR were considered as models. (A, B) Western blot (WB) and (C) quantitative real-time polymerase chain reaction (qRT-PCR) analysis determine the apoptosis-related proteins and mRNAs (Bcl-2 and Bax) in transfected cells. *P<0.05, **P<0.001, vs. control or NC; #P<0.05, ##P<0.001, vs. IR; ^P<0.05, ^^P<0.001, vs. miR-145 mimics; △P<0.05, vs. miR-145 inhibitor. n=3.