miR-450a-5p eliminats MGO-induced insulin resistance via targeteting CREB

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Research article

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Abstract

Background miR-450a-5p was involved in fat formation, but its role in insulin resistance remains unclear. This study further investigated the effects of miR-450a-5p in endothelial cells, with the aim of finding a potential target for diabetes mellitus. Methods Human umbilical vein endothelial cells (HUVECs) were severally treated with low-glucose, high-glucose, methylglyoxal (MGO), and insulin only or plus MGO. miR-450a-5p was up-regulated or down-regulated in treated HUVECs. miR-450a-5p expression in cells was measured by quantitative real-time polymerase chain reaction (qRT-PCR) assays. The cell activity was determined through MTT experiments. Transwell assay and oil red O staining were used for the detection of cell invasion and fat formation. The expressions of eNOS/AKT pathway-related proteins in cells were assessed by Western blot (WB) analysis. Furthermore, the target gene of miR-450a-5p was analyzed by double-luciferase reporter analysis, and its influence in eNOS/AKT pathway was estimated. Results miR-450a-5p decreased obviously in endothelial cells with high-glucose and MGO. Through in vitro cell experiments, we knew that MGO could not only intensify the activity of endothelial cells, but also accelerate cell invasion and fat accumulation, which could be reversed by up-regulated miR-450a-5p. Moreover, MGO inhibited eNOS/AKT pathway activation and NO release mediated by insulin, which were eliminated by up-regulated miR-450a-5p. Furthermore, CREB was the target gene of miR-450a-5p that had an activation effect on the eNOS/AKT pathway. Conclusions Up-regulated miR-450a-5p eliminated MGO-induced insulin resistance via targeting CREB, which might be a potential target to improve insulin resistance and benefit patients with related diseases.

Background

Diabetes mellitus (DM), a common metabolic disorder, is featured by a chronically raised level of blood glucose caused by insulin dysfunction [1]. Methylglyoxal (MGO) plays a pivotal role in the occurrence and development of DM, which is a highly active dicarbonyl compound and a ubiquitous product of cell metabolism [2]. Concretely, MGO is an inherent by-product of glycolysis with cell permeability, the aggregation of which is harmful because it is the most active compound to induce glycosylation products in cells [3]. As a result, MGO will induce insulin resistance that further aggravates pathoglycemia and dyslipidemia, thus promoting the progress of the disease [4]. In addition, MGO can easily react with proteins, fats and nucleic acids to form advanced glycation end products, leading to the occurrence of cataract, retinopathy, kidney disease, vascular disease and other diabetic complications [5–8]. In recent years, although several MGO scavengers have been developed, there is no clear clinical plan for the treatment of insulin resistance induced by MGO [9]. Therefore, finding a new therapeutic target to relieve MGO-induced insulin resistance may have a positive significance in the cure of DM.

MicroRNAs (miRs) are a class of single-stranded non-coding RNAs with length of approximately 18–23 nucleotides, which exert post-transcriptional modification via binding to the 3'untranslated region of target gene, so as to affect the stability and translation of mRNA. It is estimated that miRNAs account for about 1% of the genome of nuclear organisms, and the regulatory range of their target genes covered 30% of all genes [10]. A recent study has found that miRNAs played an important role in the pathogenesis

of DM by regulating the synthesis, secretion and signal transduction of insulin [11]. For example, Li X [12] indicated that miR-375 regulated the expressions of insulin secretion-related genes to control the progression of DM. In addition, some scholars have found that miRNAs also exerted a vital function in insulin signaling pathway and insulin resistance, such as miR-143, miR-145, miR-29, etc. In related fields, miR-450a-5p can secrete various factors to regulate fat formation through paracrine signals [13]. Correlatively, previous animal studies have shown that the formation of fat may be associated with the onset of DM [14]. Nevertheless, the role and mechanism of miR-450a-5p in insulin resistance of endothelial cells remains unclear.

Herein, we explored the relationship between miR-450a-5p and MGO, and further analyzed the effects and mechanisms of miR-450a-5p on MGO-induced human umbilical vein endothelial cells (HUVECs), including cell activity, invasion, fat formation and insulin resistance. The purpose of this study was to further analyze the action mechanism of miR-450a-5p in the insulin resistance induced by MGO, so as to provide a potential target for related therapies, thus delaying the progression of DM and improving the prognosis of patients.

Methods

Cell culture and treatment

HUVECs were provided by the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultivated in EGM-2 Bullet Kit from Lonza (Walkersville, MD, USA). For treatment, cells were starved in medium supplemented with 0.25% albumin bovine serum (BSA) for 16 h. Then, cells were treated with low-glucose (5 mmol/L; Sigma, St Louis, USA), high-glucose (25 mmol/L; Sigma, St Louis, USA), or low-glucose combined with xylose (4.5 mmol/L; Sigma, St Louis, USA) for 24, 48, 72 h. In addition, different concentrations of MGO (0, 250, 500, 1000 μ mol/L; Sigma, St Louis, USA) were added into HUVECs for 16 h, respectively. For a stronger contrast, cells were also pre-disposed with aminoguanidine bicarbonate salt (AG; 4 mmol/L; Sigma, St Louis, USA), and then treated with 500 μ mol/L MGO for 16 h. Furthermore, insulin (100 nmol/L; Eli Lilly Florence, Italy) only or combined with MGO (500 μ mol/L) were supplemented into HUVECs for 10 min. For comparison, untreated cells (0 μ mol/L) were served as controls. Experimentally, quantitative real-time polymerase chain reaction (qRT-PCR) assays were performed in treated HUVECs to determine the expression levels of miR-450a-5p.

Cell transfection

In order to probe into the effects of miR-450a-5p in HUVECs, cell transfection was conducted in HUVECs. In detail, mimic control, miR-450a-5p mimic (#miR10001545-1-5, RiboBio, Guangzhou, China), inhibitor control, miR-450a-5p inhibitor (#miR20001545-1-5, RiboBio, Guangzhou, China), mimic plus negative control, and mimic combined with CREB (NM_009952.2, Han Bio, Shanghai, China) were severally transfected into MGO-treated cells. Moreover, miR-450a-5p mimic was also transfected

into HUVECs after co-treatment with MGO and insulin. Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, California, USA) was used for cell transfection in accordance with manufacturer's instructions.

qRT-PCR assay

The expression levels of relevant mRNAs in HUVECs were measured by qRT-PCR assays. Concretely, total RNA was separated from HUVECs using TRIzol reagent (Invitrogen, Carlsbad, California, USA). NanoDrop–2000c spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) was adopted to assess the quality of RNA, while the integrity was determined by 1% agarose modified gel electrophoresis. Then, 1 µg of separated RNA was reverse transcribed into cDNA with the PrimeScript RT Master Mix Perfect Real Time (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. qRT-PCR assay was conducted with the ABI Prism 7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA) following the reaction conditions: total for 30 cycles of hot start for 1 min at 94°C, denaturation for 1 min at 94°C, annealing for 45 s at 50°C, and elongation for 2 min at 72°C, then final elongation for 8 min at 72°C. The sequences of primers were shown in Table 1 and synthesized by Gene Pharma (Shanghai, China). Using GAPDH or U6 as internal reference, and the data were evaluated with the comparative $2^{-\Delta\Delta Ct}$ method [15].

Cell viability

MTT assay was used to measure the viability of transfected HUVECs. Briefly, HUVECs were seeded in 96-well plates (1×10^4 cells/well). After co-treatment with MGO treatment and transfection, 20 µL MTT reagent (Sigma, USA) was added at 24, 48, and 72 h to ascertain the viability of cells, and the optical densities (OD) value was read at 570 nm with the ELX–800 Biotek plate reader (Winooski, USA).

Transwell assay

A 24-well Transwell chamber (Corning, MA, USA) was performed to detect the invasion ability of HUVECs after MGO treatment and transfection. In brief, the upper chambers were pre-covered with matrigel (BD Bioscience, CA, USA), while the lower chamber was supplemented with media containing 10 % fetal bovine serum (FBS; Gibco, USA). Resuspended HUVECs (5×10^4 cells) were shifted into the upper chambers for 8 h. Then, migrated cells were fixed with methanol and stained with the 0.1% crystal violet solution for 15 min. Nikon Eclipse TS–100 inverted microscope (Tokyo, Japan) was used for microscopic observation.

Oil red O staining

Fat accumulation in HUVECs was assessed by oil red O staining. Cells were washed by phosphate buffer saline (PBS) and fixed with 10% formaldehyde at room temperature for 10 min. After MGO treatment and transfection, HUVECs were incubated with 0.5% Oil red O solution (Gibco, USA) for 15 min, and then washed with water for 5 min. Stained HUVECs were photographed and viewed under the DP73 Olympus microscope (Tokyo, Japan).

Griess reaction

NO in HUVECs culture medium was measured by Griess reaction. After treatment and transfection, cell culture medium was gathered, and the supernatant fraction after centrifugation (1000 *g*, 15 min) was taken as a sample solution for the measurement of NO using Nitrate/nitric Assay Kit Colorimetric (Sigma, USA). This assay formed a chromophoric azo-derivative that can be detected at 540~570 nm with the microplate reader (Spectra MAX190; MD, USA).

Double-luciferase reporter analysis

Targetscan 7.2 (<http://www.targetscan.org/>.) revealed that CREB was the potential target of miR-450a-5p. For determination, double-luciferase report system detection kit (Promega, Madison, Wisconsin, USA) was used for double-luciferase reporter analysis. Tersely, HUVECs were seeded in 24-well plates (110⁵ cell/well) for 24 h. Then, miR-450a-5p mimic or blank control was transfected into HUVECs combined with wild type CREB (CREB-WT) or its mutant type (CREB-MUT) using Lipofectamine™ 2000. After 48 h, the cells were lysed and the luciferase activity was evaluated with the GloMax® Discover Multimode Microplate Reader (GM3000, Promega, USA).

Western blot (WB) analysis

The expressions of related proteins in HUVECs were measured by Wb assays. Total proteins of cells were separated by the RIPA buffer (Solarbio, Beijing, China), and Bicinchoninic Protein Assay kit (BCA, Pierce, Rockford, IL, USA) was used for quantitation. 30 µg of total protein was isolated with the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime, Shanghai, China), and then shifted onto the polyvinylidene fluoride (PVDF) membranes. Then, 5% non-fat dried milk was used to seal the membranes for 2 h. Primary antibodies were subsequently added overnight at 4°C, including p-eNOS (1:2000, ab184154, Abcam, USA), p-eNOS (1:1000, ab138430, Abcam, USA), eNOS (1:1000, ab76198, Abcam, USA) p-Akt (1:1000, #9271, Cell Signalling Technology, USA), Akt (1:1000, #9272, Cell Signalling Technology, USA), and GAPDH (1:1000, ab8245, Abcam, USA) acted as the internal control. Subsequently, the homologous 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 another 1 h at room

temperature. The bands were developed by an enhanced chemiluminescence-detecting kit (Thermo Fisher, MA, USA).

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 $\pm$ standard deviation (SD). The comparison between groups was performed by Student's *t*-test or one-way analysis of variance (ANOVA). All experiments were repeated in triplicate. $P < 0.05$ was considered as statistically significant.

Results

Over-expressed miR-450a-5p inhibited viability, migration, and lipid formation of MGO-induced HUVECs

Based on qRT-PCR analysis, the expression level of miR-450a-5p in HUVECs was observably decreasing after 72 h of high-glucose treatment ($P < 0.001$), while low-glucose had no obvious effect on miR-450a-5p expression (Figure 1A). Moreover, the addition of MGO clearly inhibited the expression of miR-450a-5p in HUVECs, which could be reversed by AG ($P < 0.001$; Figure 1B, C). In order to further investigate the effects of miR-450a-5p and MGO on the cells, transfection was performed in the MGO-induced cells. The results revealed that miR-450a-5p was obviously up-regulated by mimic and down-regulated via inhibitor ($P < 0.001$; Figure 2A), which means the transfection was successful. Interestingly, MTT results showed that over-expressed miR-450a-5p prominently restrained the viability of MGO-induced HUVECs after transfection for 48 and 72 h ($P < 0.001$; Figure 2B), while down-regulated miR-450a-5p had no significant effect on cell viability. In Transwell assay, microscopic and quantitative analysis indicated that over-expressed miR-450a-5p observably suppressed the migration of MGO-induced HUVECs ($P < 0.001$; Figure 2C, D), whereas down-regulated miR-450a-5p had no significant effect on cell migration. In the oil red O staining, lipid formation showed an orange appearance. Experimental images viewed that over-expressed miR-450a-5p significantly reduced the lipid accumulation of MGO-induced HUVECs (Figure 2D), while the lipid accumulation of cells was still high after down-regulating miR-450a-5p.

Over-expressed miR-450a-5p reduced the insulin resistance of MGO-induced HUVECs by targeting CREB

WB analysis suggested that insulin promoted the phosphorylation of eNOS and AKT, which could be reversed by MGO, and the addition of miR-450a-5p relieved the effects of MGO on eNOS/AKT pathway ($P<0.001$; Figure 3A-C). Analogously, Griess reaction displayed that MGO inhibited the insulin-induced NO release, which could be relieved by up-regulated miR-450a-5p ($P<0.001$; Figure 3D). For in-depth exploration, Targetscan7.2 forecasted the position 108–114 of CREB1 3'UTR was the binding site of miR-450a-5p (Figure 4A). Double-luciferase reporter analysis indicated that the luciferase activity of HUVECs was observably depressed after co-transfection with miR-450a-5p and CREB-WT, which verified that CREB was the target gene of miR-450a-5p ($P<0.001$; Figure 4B). Moreover, MGO promoted the expression of CREB in HUVECs, whereas miR-450a-5p had the completely opposite effect ($P<0.001$; Figure 4C). As for the impacts of CREB in eNOS/AKT pathway, WB experiment revealed that the up-regulation of CREB reduced the phosphorylation of eNOS/AKT pathway in HUVECs induced by insulin ($P<0.001$; Figure 5).

Discussion

MGO can be obtained from food or produced in the body. *In vivo*, the main pathway of MGO production is glycolysis, and its content is abnormally increased due to the DM or hyperglycemia [16]. As MGO is hyper-responsiveness with short half-life, as well as the human body contains a variety of enzymes degrading MGO, so MGO can be self-cleared by the body under normal physiological conditions [17]. In abnormal conditions, the obstacle of MGO clearing pathway will lead to the gradual accumulation of MGO content, which can not only directly cause damage to various cells in the body, but also induce the mass-generation of advanced glycation end products [3]. In previous studies, miRNAs have been proved to be closely related to the content of MGO, such as miR-30b [18], miR-214 [19], miR-9a-3p [20], etc. In this study, the expression of miR-450a-5p showed a down-regulated trend in HUVECs with high-glucose status, which was also decreased with the increase of MGO concentration. Therefore, it can be speculated that miR-450a-5p might participate in the glycolysis process and play a role in the pathogenesis of DM.

From *in vitro* cell experiments, this paper further discovered that MGO could not only enhance the activity of HUVECs, but also promote cell invasion and fat formation, which was consistent with the previous report [21]. Interestingly, the over-expression of miR-450a-5p could reverse the actions of MGO in HUVECs, reduce cell activity, and inhibit cell invasion and fat accumulation, suggesting that miR-450a-5p could improve MGO-induced cell damage. In other fields, it has been verified that the over-expression of miR-450 observably reduced the proliferation and invasion of lung cancer cells, as well as controlled the growth of tumors [22]. Moreover, on the one hand, up-regulated miR-450a-5p exerted an inhibitory effect in the invasion of ovarian cancer cells; on the other hand, miR-450a-5p also regulated cell apoptosis by blocking cell cycle [23]. These findings further implied that up-regulated miR-450a-5p could alleviate cell deterioration, which might be beneficial to the related diseases.

As for the therapy of DM, it is well known that insulin is the most commonly used drug in the treatment of the disease. Unsatisfactorily, the continuous accumulation of MGO can cause the occurrence and

development of insulin resistance in the body [24]. In related pathways, insulin was confirmed to have the function of protecting endothelial cells through modulating the activation of eNOS/AKT pathway [25]. Concretely, eNOS is an endothelial nitric oxide synthase, which can directly affect the ability of endothelial cells to synthesize NO [26]. Correlatively, the phosphorylation of AKT activates eNOS, thereby promoting the production of NO and maintaining the normal function of endothelial cells [27]. In short, insulin has the effect of activating eNOS/AKT pathway and promoting the release of NO, thus preventing endothelial cells from damage, which is the same as the results of this study. On the contrary, we also found that the addition of MGO inhibited insulin-mediated eNOS/AKT pathway activation, whereas up-regulated miR-450a-5p eliminated the resistance of MGO to insulin. It could be seen that miR-450a-5p had a reverse effect on insulin resistance mediated by MGO.

For in-depth exploration, we discovered that CREB had a binding site with miR-450a-5p through prediction websites and double-luciferase reporter analysis, which demonstrated that CREB was the target gene of miR-450a-5p. Furthermore, miR-450a-5p reversed the CREB-promoting effect of MGO. In the past, the initial research on the function of CREB mainly focused on the nervous system, and it is known that CREB had a wide range of biological functions including learning and memory regulation [28]. In recent years, the research field of CREB has gradually expanded; some scholars pointed out that activated CREB can promote the proliferation and migration of endothelial cells [29]. In addition, activated CREB promoted insulin resistance and broke glucose balance in the body [30]. In this study, we learned that CREB obviously restrained the phosphorylation of eNOS and AKT, prompting that CREB had an activation effect on the eNOS/AKT pathway. In related studies, Niwano K et al. [31] also reported that CREB competitively bound to the cAMP/ATF reactive element, so as to regulate eNOS gene expression in endothelial cells. These finding prompted that miR-450a-5p might relieve MGO-induced insulin resistance via targeting CREB.

Conclusions

In conclusion, miR-450a-5p decreased obviously in endothelial cells with high-glucose and MGO of certain concentrations (500, 1000 $\mu\text{mol/L}$). Through *in vitro* cell experiments, we knew that MGO could not only intensify the activity of endothelial cells, but also accelerate cell invasion and fat accumulation, which could be reversed by up-regulated miR-450a-5p instead of down-regulation. Moreover, MGO inhibited eNOS/AKT pathway activation and NO release mediated by insulin. Nevertheless, up-regulated miR-450a-5p eliminated the resistance of MGO to insulin via targeting CREB, which might be a potential therapeutic target to improve MGO-induced insulin resistance and benefit patients with related diseases.

List Of Abbreviations

HUVECs = Human umbilical vein endothelial cells

MGO = methylglyoxal

qRT-PCR = quantitative real-time polymerase chain reaction

WB = Western blot

DM = Diabetes mellitus

miRs = 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 or animals are involved in this research.

Consent for publication

Not applicable.

Availability of data and material

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

Competing interests

The authors declare no conflicts of interest.

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Authors' contributions

Substantial contributions to conception and design: CW, LM

Data acquisition, data analysis and interpretation: CW, LM

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

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: All authors

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Table 1

Table 1 Primer base sequence

Gene	Forward (5'-3')	Reverse (5'-3')
miR-450a-5p	CGATCGGTTTTGCGATGTGTTCC	ATCCAGTGCAGGGTCCGAGG
CREB	TGCCACATTAGCCCAGGTA	GCTGTATTGCTCCTCCCT
GAPDH	AGAAGGCTGG GGCTCATTTG	AGGGGCCATC CACAGTCT TC
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

Figures

Figure 1

Methylglyoxal (MGO) down-regulated the expression of miR-450a-5p in human umbilical vein endothelial cells (HUVECs). (A) Quantitative real-time polymerase chain reaction (qRT-PCR) assay measured the expression of miR-450a-5p in HUVECs after treatment with low-glucose (5 mM), high-glucose (25 mM), or low-glucose combined with xylose (4.5 mM) for 24, 48, 72 h. (B) qRT-PCR assay determined the expression of miR-450a-5p in HUVECs after treatment with different concentrations of MGO (0, 250, 500, 1000 μM). (C) qRT-PCR assay determined the expression of miR-450a-5p in HUVECs after treatment with control, MGO only or combined with aminoguanidine bicarbonate salt (AG). **P<0.001, vs. low-glucose, 0 Mm, or Control; ##P<0.001, vs. MGO. n=3.

Figure 2

Over-expressed miR-450a-5p inhibited viability, migration, and lipid formation of methylglyoxal (MGO)-induced human umbilical vein endothelial cells (HUVECs). In this figure, mimic control, miR-450a-5p mimic, inhibitor control, and miR-450a-5p inhibitor were severally transfected into MGO-treated HUVECs. (A) Quantitative real-time polymerase chain reaction (qRT-PCR) assay measured the expression of miR-450a-5p in MGO-treated HUVECs after transfection. (B) MTT assay detected the optical densities (OD) values of MGO-induced HUVECs after transfection for 24, 48 and 72h. (C) Quantitative analysis of Transwell assay in MGO-treated HUVECs after transfection. (D) Microscopic images of Transwell and oil red O staining experiments in MGO-induced HUVECs after transfection. ^{AA}P<0.001, vs. Control; ^{**}P<0.001, vs. MGO + mimic control; ^{##}P<0.001, vs. MGO + inhibitor control. n=3.

Figure 3

Over-expressed miR-450a-5p inhibited the insulin resistance of methylglyoxal (MGO)-induced human umbilical vein endothelial cells (HUVECs). In this figure, HUVECs were severely disposed with control, insulin (Ins), MGO, insulin combined with MGO, miR-450a-5p mimic plus MGO, or mimic plus MGO and insulin. (A-C) Western blot (WB) bands and quantitative analysis determined the expressions of eNOS/AKT pathway-related proteins in HUVECs. (D) Griess reaction assessed the release of NO in HUVECs. ^P<0.001, vs. Control; *P<0.001, vs. Ins; ##P<0.001, vs. Ins + MGO. n=3.

A

	Predicted consequential pairing of target region (top) and miRNA (bottom)	Site type
Position 108-114 of CREB1 3' UTR	5' ...UUCUUUUUUUUCUAUGCGCAAAAC...	7mer-A1
hsa-miR-450a-5p	3' UAUAAUCCUUGUGUAGCGUUUU	

B

C

Figure 4

CREB was the target gene of miR-450a-5p. (A) Targetscan7.2 forecasted the position 108-114 of CREB1 3'UTR was the binding site of miR-450a-5p. (B) Double-luciferase reporter analysis measured the luciferase activity of human umbilical vein endothelial cells (HUVECs) after transfection with miR-450a-5p mimic plus wild type CREB (CREB-WT) or its mutant type (CREB-MUT). (C) Quantitative real-time polymerase chain reaction (qRT-PCR) assay measured the expression of CREB in methylglyoxal (MGO)-induced HUVECs after transfection with mimic control, miR-450a-5p mimic, mimic plus negative control (NC) or CREB. ^^P<0.001, vs. Blank, or Control; **P<0.001, vs. MGO + mimic control; ##P<0.001, vs. MGO + mimic control + NC. n=3.

Figure 5

Over-expressed miR-450a-5p reduced the insulin resistance of methylglyoxal (MGO)-induced human umbilical vein endothelial cells (HUVECs) by targeting CREB. Western blot (WB) bands and quantitative analysis determined the expressions of eNOS/AKT pathway-related proteins in MGO-induced HUVECs after severely dispose with control, insulin (Ins), MGO, MGO + Ins, MGO + miR-450a-5p mimic + negative control (NC), MGO + Ins + mimic + NC, MGO + mimic + CREB, or MGO + Ins + mimic + CREB. [^]P<0.001, vs. Blank, or Control; ^{**}P<0.001, vs. MGO + mimic control; ^{##}P<0.001, vs. MGO + mimic control + NC. n=3.