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High glucose induces the apoptosis of HUVECs in mitochondria dependent manner by enhancing VDAC1 expression

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Mellithemia are diabetic patients' emblematic syndrome, which would induce vasculopathy resulting from apoptosis of vascular endothelial cells. The pathological mechanism of high glucose-induced apoptosis of vascular endothelial cells is investigated in the present study utilizing HUVEC cells. As high glucose-induced apoptosis is caused by elevated mitochondrial permeability-mediated release of mitochondrial cytochrome c, voltage-dependent anion channel (VDAC1), the controller of mitochondrial permeability, and its regulator Bax were investigated. Our results suggest that upregulation of VDAC1 is the central event in high glucose-induced cell apoptosis, since silencing VDAC1 reduced high glucose-induced upregulation of mitochondrial/cellular Bax; thus silencing VDAC1 recovered the high glucose-reduced binding of Bax to VDAC1, which finally reduced the high mitochondrial permeability. Besides, high glucose increased VDAC1 expression by elevating the expression of SREBP1 and SREBP2, the tanscriptional factor of VDAC1. Those findings indicate that SREBP1 or SREBP2/VDAC1 could be novel targets for the prevention of diabetic vasculopathy.

1. Introduction

Diabetes mellitus is a chronic systemic and metabolic disease caused by genetic and environmental factors conjointly (Mehnoosh et al. 2012). According to the WHO, diabetes' global incidence will increase from 3% to 5% from 1995 to 2025, and the age brackets of the patients will be from more than 65 years old to 45-54 years old (Van et al. 2010). By 2030, the number of Chinese diabetics are likely to double and achieve 42.3 millions (Chen et al. 2010). Diabetes has been listed as the third chronic and non-communicable diseases of the world, after cardiovascular disease and cancer (Shi and Hu, 2014). Diabetic complications can involve all tissues and organs of the body. Diabetes can even cause disability and death of patients when severe symptoms occur (van der Feltz-Cornelis et al. 2010). Diabetic cardiovascular complications are the most common complications of diabetes (Turan 2010). The dysfunction of endothelial cells plays an important role in the occurrence and development of diabetic cardiovascular complications (Leal et al. 2010) .

The dysfunction of mitochondria is crucial during endothelial cells apoptosis. The Voltage Dependent Anion Channel (VDAC) located in the Outer Mitochondrial Membrane (OMM), plays an important role in the regulation of mitochondrial membrane permeability and is essential for cytochrome c release during apoptosis of cells (Shoshanbarmatz et al. 2010). The VDAC protein consists of three subtypes including VDAC1, VDAC2 and VDAC3, while vascular endothelial cells express the VDAC1 isoform overwhelmingly (Shoshan-Barmatz and Ben-Hail 2012). VDAC1 acts as the pathway for the movement of many small molecule substances out of the mitochondria (Shoshan-Barmatz and Golan 2012). Physiologically, VDAC1 mediated ATP and other small molecules transporting from mitochondria to cytoplasm (Rosano 2011). Pathologically, VDAC1 open and lead to Cytochrome C (Cytc) release into the cytoplasm (Shoshan-Barmatz et al. 2010). The binding of Bax and VDAC1 can open VDAC1, thus increasing mitochondrial membrane permeability, which leads to cell apoptosis induced by Cytc (Tomasello et al. 2013) .

The present study investigated the pro-apoptotic effect of high glucose on human umbilical vein endothelial cells (HUVECs) and the possible mechanisms implicated. The results indicated that high glucose could induce HUVEC apoptosis through up-regulation of VDAC1, which led to the enhancement of interaction between Bax and VDAC1 and subsequent mitochondrial cytochrome c release. The findings of this study will provide new approaches and potential targets for the prevention of diabetic cardiovascular complications.

2. Investigations and results

2.1. High glucose induced apoptosis in HUVECs

As shown by Hoechst staining and MTT assay in HUVECs, high concentrations of glucose (16.5 and 33 mM) induced a dramatic increase in cell apoptosis (Fig. 1A) and decreased cell viability (Fig. 1B). Immunoblot analysis indicated that the exposure of HUVECs to high glucose resulted in a significant activation of caspase 3, while the level of Bax protein remained unchanged (Fig. 1C). JC-1 staining revealed that high glucose caused a noticeable decrease in mitochondrial membrane potential in HUVECs (Fig. 1D). These findings suggested that high glucose can induce apoptosis in HUVECs, which might be related to impairment of mitochondrial function. Immunoblot analysis indicated that exposure to high glucose for 96 h resulted in a notable reduction of mitochondrial cytochrome c (Fig. 1E), while cytochrome c in whole cell lysates remained unchanged (Fig. 1F). Those findings suggested that high glucose increases mitochondrial membrane permeability and induces cytochrome c release to the cytosol. It is widely recognized that VDAC1 controls mitochondrial membrane permeability in HUVECs, which is regulated by Bax. Immunoblot demonstrated that the exposure to high glucose increased the mito-

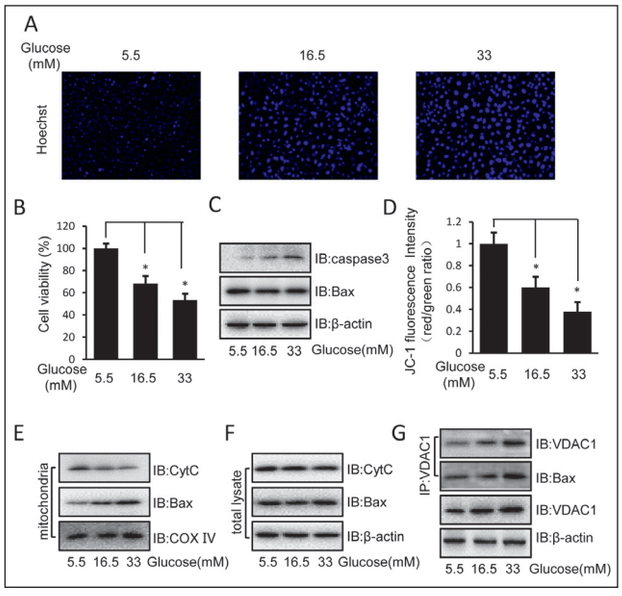

Fig. 1: Cells were cultured in medium with 5.5, 16.5 and 33 mM glucose for 96 h. (A) Cells were stained with the fluorescent nuclear dye Hoechst 33258. (B) MTT assay (C) Cell extracts were immunoblotted by antibodies against Bax, caspase 3 or β-actin. (D) Cells stained with JC-1 and then analyzed by flow cytometry for fluorescence emission at 530 nm (JC-1 FL1) and 575 nm (JC-1 FL2). The ratio of FL2/FL1 represented the mitochondrial membrane potential.(E) Mitochondria extracts were immunoblotted by antibodies against Bax, Cytc or COX4. (F) Cell extracts were immunoblotted by antibodies of Bax, Cytc or β-actin. (G) Cell extracts were immunoprecipitated by VDAC1 antibody; the blots were immunoblotted by Bax and VDAC1 antibody. β-Actin in extracts was immunoblotted as input. The data are presented as the mean ± S.E., *p<0.05.

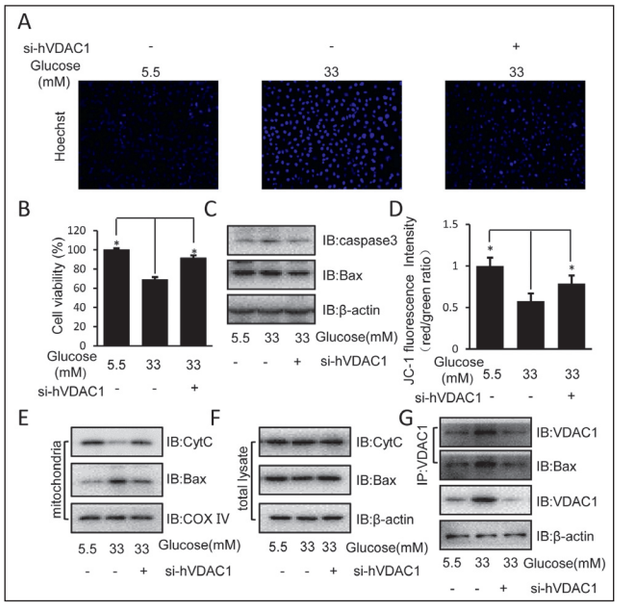

Fig. 2: HUVECs were transfected by si-RNA. 24 h later, cells were treated with 5.5 and 33 mM glucose for another 96 h. (A) Cells stained with the fluorescent nuclear dye Hoechst 33258. (B) MTT assay. (C) Proteins from total cell extracts were immunoblotted by antibodies of caspase 3, Bax or β-actin. (D) Cells stained with JC-1 and then analyzed by flow cytometry for fluorescence emission at 530 nm (JC-1 FL1) and 575 nm (JC-1 FL2). The ratio of FL2/FL1 represented the mitochondrial membrane potential.(E) Mitochondria extracts were immunoblotted by antibodies against Bax, Cytc or COX4. (F) Cell extracts were immunoblotted by antibodies of Bax, Cytc or β-actin (G) Cell extracts were immunoprecipitated by VDAC1 antibody; the blots were immunoblotted by Bax and VDAC1 antibody. β-Actin in extracts was immunoblotted as input. The data are presented as the mean±S.E., *p<0.05.

chondrial level of Bax (Fig. 1E), but did not affect Bax expression in total cell lysates (Fig. 1F). This suggested that high glucose enriched Bax on mitochondria. Immunoprecipitation analysis indicated an enhanced interaction between VDAC1 and Bax (Fig. 1G), which is consistent to the increased mitochondrial abundance of Bax.

2.2. Knockdown of VDAC1 reversed the apoptosis induced by high glucose

It is well defined that VDAC1 plays an important role in regulating mitochondrial membrane permeability and subsequent mitochondrial function. Base on the increment in the protein level of VDAC1 in high glucose-treated HUVECs (Fig. 2A), it is necessary to clarify whether the downregulation of VDAC1 is capable of reversing the apoptosis caused by high glucose. The reduction of VDAC1 expression was achieved by transfecting siRNA (Fig. 2G). Knockdown of VDAC1 effectively reversed high glucose-induced apoptosis (Fi.g 2A), decrease in cell viability (Fig. 2B), and reduction of mitochondrial membrane potential (Fig. 2D). The activation of caspase 3 and upregulation of mitochondrial Bax induced by high glucose in HUVECs were also revised by knockdown of VDAC1 (Fig. 2C, E). Besides, knockdown of VDAC1 suppressed the interaction between VDAC1 and Bax (Fig. 2G) and release of mitochondrial cytochrome c into the cytosol (Fig. 2F).

2.3. High glucose enhances VDAC1 expression at the transcriptional levels in HUVECs

To investigate the mechanisms by which high glucose stimulates VDAC1 expression in HUVECs, we first checked the transcription level of VDAC1 by RT-qPCR, and found that both 16.5 and 33 mM glucose significantly increased VDAC1 mRNA levels (Fig. 3A). The proteasome inhibitor MG132, blocking VDAC1 protein degradation, was utilized to test the effect of high glucose on the protein stability of VDAC1. As shown in Fig. 3B, MG132 did not change the high glucose-induced increment in VDAC1 protein levels, suggesting that high glucose regulates VDAC1 expression at the transcriptional levels instead of promoting protein degradation.

2.4. High glucose upregulates VDAC1 expression through stimulating the transcriptioanl activity of SREBP1 or SREBP2

It has been reported that SREBP1, SREBP2 and NRF2 are transcription factors of VDAC1. Fig. 4A-C shows that high glucose increased mRNA levels of SREBP1, SREBP2 and NRF2. Then we examined the protein levels of full-length and cleaved SREBP1 and SREBP2 (Fig. 4D) as well as the phosphorylation levelof NRF2 (Fig. 4E) in HUVECs. The results indicated that high glucose increased the expression and induced the activation of SREBP1 and SREBP2 but had no effect on NRF2. Consistently, the Akt, the activator of SREBP1 and SREBP2, was also activated by high glucose via phosphorylation (Fig. 4E). Luciferase assay indicated that SREBP1 and SREBP2 induced the transcription of

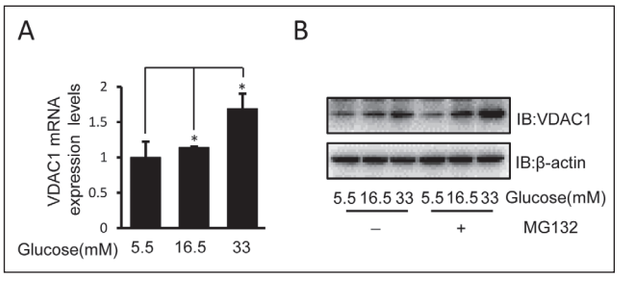

Fig. 3: (A) HUVECs were culture in DMEM containing 5.5 ,16.5 and 33 mM glucose for 96 h. Total RNA was isolated from cells to detect mRNA level of VDAC1 by RT-qPCR and normalized to GAPDH mRNA level. (B) HUVECs were cultured in DMEM containing 5.5, 16.5 and 33 mM glucose for 88 h.Then cells were treated by MG132 (50 μM) for another 8 h. Cell extracts were immunoblotted against antibodies of VDAC1 or β-actin.

VDAC1, which was further promoted by high glucose (Fig. 4G). These findings indicated that high glucose induced up-regulation of VDAC1 protein was derived by its stimulation on the expression and activation of SREBP1 or SREBP2.

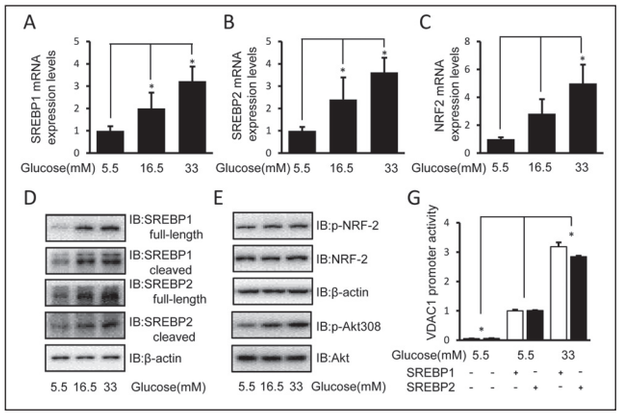

Fig. 4: HUVECs were culture in DMEM containing 5.5 ,16.5 and 33 mM glucose for 96 h. (A)Total RNA was isolated from cells to detect mRNA level of SREBP1 by RT-qPCR and normalized to GAPDH mRNA level. (B)Total RNA was isolated from cells to detect mRNA level of SREBP2 by RT-qPCR and normalized to GAPDH mRNA level. (C)Total RNA was isolated from cells to detect mRNA level of NRF2 by RT-qPCR and normalized to GAPDH mRNA level. (D) Cell extracts were immunoblotted against of SREBP1 full-length, SREBP1 cleaved, SREBP2 full-length, SREBP2 cleaved antibodies or β-actin. (E) Cell extracts were immunoblotted against of P-NRF-2, NRF-2, P-Akt, Akt antibodies or β-actin. (F) The expression vectors of SREBP1 (100 ng) or SREBP2 were co-transfected with pGL3- VDAC1 construct (200 ng) containing VDAC1 promotorand pRL-TK-Renilla (10 ng) into cells. 48 h later, cells were harvested for luciferase activity assay. The data are expressed as the mean ± S.E., *p<0.05.

3. Discussion

Diabetic vascular disease, the common complication of diabetes, is the main cause of diabetic death and disability (Cohen and Tong 2010). Apoptosis of endothelial cells is proved to be the initiating factor and pathological basis of diabetic angiopathy (Sena et al. 2013). In the present study, we found the apoptosis-inducing effect of high glucose in HUVECs and the possible mechanisms implicated, aiming to explore appropriate targets for the prevention of diabetic vascular complications.

In this study, we demonstrated that high glucose induced apoptosis in HUVECs and it might be attributed by the increase of mitochondrial membrane permeability (Yu et al. 2011). In support of these findings, high glucose caused a marked decrease in mitochondrial membrane potential and mitochondrial cytochrome c content (Fig. 1D and Fig. 1E). The level of VDAC1 plays a crucial role in regulating mitochondrial membrane permeability. We found that the exposure to high glucose resulted in significant enhancement of VDAC1 expression. At the same time, the content of mitochondrial Bax protein was notably increased by high glucose treatment (Dang et al. 2010). The increased mitochondrial Bax protein level was able to interact with VDAC1 and subsequently increased mitochondrial membrane permeability (Šileikytė et al. 2011). Immunoprecipitation analysis provided further evidence that high glucose could enhance the interaction between VDAC1 and Bax protein, and thus the increased mitochondrial membrane permeability could be explained.

To verify whether upregulation of VDAC1 was a central event in high glucose-induced cell apoptosis, we utilized siRNA against VDAC1 to reduce VDAC1 expression in HUVECs. Knockdown of VDAC1 effectively prevented high glucose-induced cell apoptosis and reduction in mitochondrial membrane potential (Fig. 2). The restoration of mitochondrial cytochrome c provided further evidence for the recovery of mitochondrial membrane permeability, which might be related closely to the reduction in mitochondrial level of Bax and the interaction between Bax and VDAC1 (Fig. 2). As discussed above, knockdown of VDAC1 resulted in a decreased interaction between VDAC1 and Bax (Chacko et al. 2010). In consistence with our findings, previous studies have demonstrated that knockdown of VDAC1 could resulted in decreased expression of apoptotic proteins (Sharaf et al. 2012) .

To elucidate the mechanisms by which high glucose regulates VDAC1 expression, we examined the level of VDAC1 mRNA and found that high glucose increased mRNA level of VDAC1 by stimulating its transcription (Fig. 3A). In addition, MG132, a proteasome inhibitor that suppressed VDAC1 protein degradation, did not affect high glucose-induced increase in VDAC1 protein, providing further evidence that high glucose regulated VDAC1 expression at transcription level (Fig. 3A and 3B). It has been reported that SREBP1 (You et al. 2017), SREBP2 (Lynch et al. 2016) are important transcription factors of VDAC1. According to our research, high glucose enhanced the expression of SREBP1 and SREBP2 and activated them via Akt pathway, and thus upregulated VDAC1 expression.

In conclusion, this study indicated that exposure to high glucose stimulates VDAC1 transcription in HUVECs. These changes enhanced interaction between Bax and VDAC1, subsequently increases mitochondrial membrane permeability and ultimately promotes cell apoptosis. It is implied that VDAC1 might be a potential target for suppression of vascular endothelial cell apoptosis and prevention of diabetic vascular complications.

4. Experimental

4.1. Cell culture

HUVEC cell line, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), were maintained in DMEM containing 5.5 mM glucose, 10 % fetal bovine serum (Gibco, MD, USA), 100 U/mL penicillin, 100 µg/mL streptomycin (Gibco, MD, USA) at humidified atmosphere of 95 % air and 5 % CO_2 at 37 ºC.

4.2. Cell viability assay

Cell viability was evaluated by MTT assay. HUVEC cells were seeded into 96-well plates at a density 2×10^4 cells/well and cultured for 12 h. Then the cells were incubated in fresh medium containing 16.5 or 33 mM glucose for 96 h. MTT (Beyotime Biotech, Shanghai, China) solution was added with final concentration of 5 mg/mL for one-hour incubation. The formazan precipitate was dissolved in 200 µL dimethylsulfoxide (DMSO) and the absorbance at 570 nm was determined with a microplate reader (SpectraFluor, Tecan, Männedorf, Switzerland). Cell survival was expressed as the percentage of formazan absorbance of control wells.

4.3. Cell apoptosis analysis

HUVEC apoptosis was determined by Hoechst 33258 staining with the Boytime Staining Assay kit (Beyotime Biotech, Shanghai, China). After rinsing three times with PBS, cells were incubated with 10 µg/L Hoechst 33258 for 10 min at room temperature and then washed with PBS. The coverslips were mounted with glycerol and the cells were observed with a fluorescence microscope with an excitation wavelength at 346 nm and an emission wavelength at 460 nm.

4.4. Isolation of mitochondria

HUVEC cells were resuspended in mitochondria isolation reagents (Beyotime Biotech, Shanghai, China) and placed on ice for 15 min. After homogenization, the solution was centrifuged at 600 x g, 4 ºC for 10 min. The supernatants were collected and centrifuged again at 11000 x g, 4 ºC for 10 min. The pellet was mitochondria collected for the subsequent experiments.

4.5. Assessment of mitochondrial membrane potential with JC-1 staining

After digested with trypsin and washed twice with PBS, HUVECs were suspended in 1 ml JC-1 staining solution (1×, Beyotime Biotech, Shanghai, China) and incubated at 37 °C for 20 min. Thereafter, the cells were collected again through centrifugation and suspended in 500 µl staining buffer. The results were detected and analyzed with flow cytometry.

4.6. Immunoprecipitation and immunoblotting

HUVEC cells were lysed with RIPA buffer. Lysates were mixed in 3×loading buffer. For immunoprecipitation, cell extracts were incubated with primary antibody at 4 °C for 2 h and subsequently with protein A-sepharose beads (P001-3, 7-Sea Biotech, Shanghai, China) for another 1 h at 4 °C. Then protein A-sepharose beads were washed twice with lysis buffer and boiled at 100 °C for 10 min mixed with 2×loading buffer. Immunoblotted with indicated primary antibodies and horseradish

peroxidase-conjugated secondary antibodies (1:5000 dilution; Anti-Rabbit IgG, #7074; Anti-mouse IgG, #7076; Cell Signaling Technology (Beverly, MA) and visualized using Tanon-5200 Chemiluminescence Imager (Tanon, Shanghai, China) with ECL western blotting substrate (Millipore Co. Bedford, USA). Primary antibodies purchased from Cell Signaling Technology (Beverly, MA) were as follows: ACTIN (#3700, 1:1000 dilution), Bax (#5023, 1:1000 dilution), cytochrome c (#4272,1:1000 dilution), Cleaved Caspase3 (#9661:1:1000 dilution), Phospho-Akt (#13038,1:1000 dilution), Akt (#9272:1:1000 dilution). Antibodies against SREBP1 full-length (#199318, 1:1000 dilution) , SREBP2 full-length (#30682, 1:1000 dilution) ,COX4 (#202554, 1:1000 dilution) , Phospho-NRF2 (#76026:1:1000 dilution) and NRF2 (#62352,1:1000 dilution) were purchased from abcam (Cambridge, MA). Antibodies against VDAC1 (sc-390996, 1:1000 dilution) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against SREBP1 cleaved (NB100-2215, 1:500 dilution) and SREBP2 cleaved (AF7119, 1:500 dilution) were purchased from Novus Biologicals (Littleton, USA).

4.7. Quantitative real-time PCR (RT-qPCR) analysis

Total RNAs from HUVEC cells was extracted using Trizol (Takara, Shiga, Japan). Real-time PCR amplifications were carried out using StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) and Thunderbird SYBR Master Mix (TOYOBO, Osaka, Japan). Primer sequences were as follows: VDAC1, 5′- GGCCTGACGTTTACAGAGAAA-3′ (forward), 5′- TGCAAGCTGATCTTCCACAGT-3′ (reverse);SREBP1,5′- CGGAACCATCTTGGCAACAGT-3′(forward),5′- CGCTTCTCAATGGCGTTGT-3′ (reverse); SREBP2, 5′- AACGGTCATTCACCCAGGTC-3′ (forward), 5′- GGCTGAAGAATAGGAGTTGCC-3′ (reverse); NRF2, 5′-TTCCCGGTCACATCGAGAG-3′ (forward),5′- TCCTGTTGCATACCGTCTAAATC-3′(reverse);GAPDH,5′- GGAGCGAGATCCCTCCAAAAT-3′ (forward), 5′-GGCTGTTGTCATACTTCTCATGG-3′ (reverse). The relative expression levels of target genes were calculated using $2^{-\Delta\Delta Ct}$ method.

4.8. Vectors

pGL3-VDAC1 containing human VDAC1 promoter were kindly provided by Dongping Wei (The First Hospital of Nanjing, Nanjing, Jiangsu, China). pCMV SREBP1 (Addgene plasmid #32017) and pCMV SREBP2 (Addgene plasmid #32018) were gift from David Sabatini.

4.9. Luciferase assay

HUVEC cells were seeded onto 24-well plates, transfected with luciferase reporter plasmids using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, cells were incubated for 4 h in 250 µL of serum-free DMEM containing pGL3-VDAC1, pRL-TK-Renilla (10 ng), and expression vectors, such as pCMV SREBP1 or pCMV SREBP2 (100ng) as well as Lipofectamine 2000 (3 µL). Then HUVECs were grown in DMEM with 10 % FBS for 24 h and subsequently incubated for 48 h in the complete medium described above different concentrations of glucose. Luciferase activity was measured using the Dual-luciferase reporter assay System (Promega, Madison, WI) and normalized to Renilla luciferase values. Measurements for three biological replicates were taken in triplicate and averaged.

4.10. Knock-down of VDAC1

Knockdown of endogenous VDAC1 in HUVEC cells was performed by infection with siRNA targeting VDAC1 which were obtained from Genepharma (Suzhou, China). The following sequences (names underlined) were used (nucleotide positions are provided for sense (S) and antisense (AS) sequences): si-hVDAC1- S: 238 5′ACACUAGGCACCGAGAUUA3′-256 and AS: 5′UAAUCUCGGUGCCUAGUGU3′. Cells were transfected with 5–100 nmol/l siRNA-hVDAC1 using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc).

4.11. Statistical analysis

Data were presented as mean±SEM and analyzed by one-way ANOVA followed by Bonferroni–DunnN tests. Values of $P < 0.05$ were considered statistically significant.

Conflicts of interest: None declared.

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