

The X box binding protein 1/C/EBP homologous protein pathway induces apoptosis of endothelial cells under hyperglycemia

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Abstract. Venous endothelial cell apoptosis can be induced by endoplasmic reticulum (ER) stress, thus serving an important role in the formation of deep venous thrombosis. X-box binding protein 1 (XBP1) is a protein associated with ER. The present study aimed to explore the function of XBP1/C/EBP homologous protein (CHOP) pathway in the process of endothelial cell apoptosis under hyperglycemia. Small interfering (si)RNAs targeting XBP1 and CHOP were designed to downregulate the expression of XBP1 and CHOP in human umbilical vein endothelial cell, respectively. Flow cytometry was used to determine cell apoptosis. The expression of XBP1, glucose-regulated protein 78 (GRP78), CHOP, Puma, cleaved caspase-3 and Cytochrome c was evaluated by western blotting. There were seven groups of cells that were used in the present study: i) Control (5.5 mM D-glucose); ii) hypertonic (hypertonic control, 27.8 mM mannitol and 5.5 mM D-glucose); iii) 16.7 mM D-glucose; iv) 33.3 mM D-glucose; v) 33.3 mM + NC (33.3 mM D-glucose incubated with NC); vi) 33.3 mM + si-XBP1 (33.3 mM D-glucose incubated with siRNA against XBP1); and vii) 33.3 mM + si-CHOP (33.3 mM D-glucose incubated with siRNA against CHOP). Compared with the control, the apoptosis rate of human umbilical vein endothelial cells (HUVECs) increased greatly with the increase in the concentration of D-glucose. Compared with the 33.3 mM D-glucose group, the HUVECs incubated with 33.3 mM D-glucose and si-XBP1 or 33.3 mM D-glucose and si-CHOP demonstrated a significantly lower apoptosis rate. Compared with the control, XBP1, GRP78, CHOP, Puma, cleaved caspase-3 and cytochrome c were significantly upregulated in the hypertonic, 16.7 mM D-glucose, 33.3 mM D-glucose

and 33.3 mM + negative control (NC) groups. Compared with the 33.3 mM D-glucose group, the expression levels of XBP1, GRP78, CHOP, Puma, cleaved caspase-3 and cytochrome c in the 33.3 mM + si-XBP1 or 33.3 mM + si-CHOP groups significantly decreased. High dosage of glucose induced endothelial cell apoptosis by promoting the expression of apoptotic proteins by activating endoplasmic reticulum stress. XBP1/CHOP may be a potential target for the treatment of deep vein thrombosis as one of the key pathways regulating ERS by regulating apoptosis of endothelial cells.

Introduction

Deep venous thrombosis (DVT) is a major threat to psychological health (1). It has high morbidity and its early diagnosis is difficult, as evidence by ~100,000 patients being diagnosed with DVT between 2007-2016 in China alone (2). The pathogenesis of DVT is complicated, in which endothelial cells, leukocytes, platelets, coagulation factors and the fibrinolytic system are involved (3). The structural disorder and dysfunction of venous endothelial cells are the initiating factors of DVT, which impact the development and process of DVT by regulating the systole and diastole of vessels (4). Structural disorder and dysfunction of venous endothelial cells modulates the adherence, activation, recruitment and interaction between platelets and leukocytes and disrupts the balance of coagulation/anticoagulation and fibrinolysis/antifibrinolysis (4). The endothelial cell injury caused by endoplasmic reticulum stress (ERS) serves an important role in the formation of DVT (5). Endothelial cells protect the vessels as the first barrier by regulating blood flow, participating in material exchange, preventing lipid leakage and inhibiting platelet aggregation and thrombogenesis (6). In addition, endothelial cells, especially in new vessels, exert a secretory function as the highly-developed endoplasmic reticulum is visible under an electron microscope, which makes endothelial cells highly allergic to the factors that induce ERS, including Ca²⁺ metabolism imbalance and oxidative stress stimulation (7,8). ERS is reported to be involved in multiple types of vascular diseases, such as atherosclerosis (9) and Kawasaki disease (10) by inducing the apoptosis of endothelial cells (11). Transcriptional factor X-box binding protein 1 (XBP1) is an important mediator

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in the process of ERS signal transduction in mammalian cells (12). With the development of ERS, inositol-requiring kinase1 (IRE1), an important transmembrane protein molecule in the endoplasmic reticulum cavity, disconnects with glucose-regulated protein 78 to be oligomerized and auto-phosphorylated, hence inducing specific splicing on XBPI mRNA to combine with ERS reaction components in the nucleus, such as the unfolded protein reaction target molecule glucose-regulated protein 78 (GRP78) (13,14). As a result, the relative expression level of certain ERS-related genes, including activating transcription factor 6 (ATF6) and eukaryotic translation initiation factor 2a (eIF2a) (15), is elevated at the transcriptional level (16).

XBPI is a novel protein that is closely related to protein folding and endoplasmic reticulum construction; it is an important transcriptional factor in the leucine zipper protein family (17). XBPI functions as a significant signal regulator for the ERS reaction by binding with the X box cis-acting element located in the promoter region of the major histocompatibility complex gene (18). In the process of ERS, the unspliced X-box binding protein-1 (XBPI-u) composed of 261 amino acids is transformed to spliced X-box binding protein-1 (XBPI-s) composed of 376 amino acids via transcriptional activation in the presence of IRE1. XBPI-s-regulated ERS normally promotes the survival of cells at the early stage of diseases (19,20). However, the persistent activation of ERS will finally result in tissue over-apoptosis with the continuous activation of ERS (21). In addition, C/EBP homologous protein (CHOP) is one of the important factors involved in the ERS-mediated apoptotic pathway (22). Following ERS, the expression level of CHOP is elevated, which further induces apoptosis (23).

The present study explored the impact of XBPI/CHOP signaling pathway on the apoptosis of endothelial cells under the stimulation of hyperglycemia to provide the fundamental basis for the treatment of DVT. Small interfering (si) RNA technology was used to downregulate XBPI in HUVECs, followed by stimulation of hyperglycemia and measurement of the change of apoptotic rate and the expression level of downstream proteins of XBPI/CHOP pathway. In addition, CHOP was also knocked down using siRNA, followed by stimulation of hyperglycemia and the change in apoptosis rate was measured. The results of the present study may elucidate a potential biomarker for the clinical diagnosis and treatment of DVT.

Materials and methods

Cell culture and treatments. HUVECs (cat. no. iCell-h110) were purchased from iCell Bioscience Inc. and cultured in DMEM (cat. no. KGM12800S-500; Nanjing KeyGen Biotech Co., Ltd.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin/streptomycin at 37°C with 5% CO₂. D-glucose (cat. no. G116307) was purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. and had a purity >99.5%.

Cell transfection. HUVECs culture medium was changed to DMEM without serum when the density of cells reached 70%. A total of ~125 µl of Opti-MEM (Takara Bio Inc.)

was added into 2 Eppendorf tubes with the cells at a density of 1x10⁶ cells/tube. One tube was filled with 5 µl of Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) and one was filled with 12.5 µl of siRNA (Takara Bio Inc.). After incubation for 15 min and mixing the 2 tubes, the mixture was added into the 6-well-plate and placed into a cell incubator after the cell density reached 70%. After 4 h of transfection at 37°C, 1 ml of complete DMEM containing 20% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) was added into each well. The sequences of the siRNAs targeting XBPI and CHOP are shown in Table I. The negative control (NC) for siXBPI and for siCHOP was non-targeting (Takara Bio, Inc.). The sequences of the NC are presented in Table I. The aforementioned agents were added to the six-well-plate after transfection for 48 h. The cells were collected for subsequent experimentation after another 48 h of incubation at 37°C. Untransfected HUVECs were taken as the control group.

Groups of cells. The use of D-glucose to injure endothelial cells has been previously reported (24), and 5.5 mM is the concentration of D-glucose. The effect of different concentrations of D-glucose on endothelial cell proliferation was assessed by conducting a CCK8 assay (25). It was determined that 33.3 mM D-glucose had the most significant inhibitory effect on cell proliferation (data not shown). There were 7 groups of cells in the present study: i) Control (5.5 mM D-glucose); ii) hypertonic (hypertonic control, 27.8 mM mannitol and 5.5 mM D-glucose); iii) 16.7 mM D-glucose; iv) 33.3 mM D-glucose; v) 33.3 mM + NC (33.3 mM D-glucose incubated with NC); vi) 33.3 mM + si-XBPI (33.3 mM D-glucose incubated with siRNA against XBPI); and vii) 33.3 mM + si-CHOP (33.3 mM D-glucose incubated with siRNA against CHOP). The 33.3 mM D-glucose group was representative of the hyperglycemic condition.

Western blotting. RIPA lysis buffer (cat. no. P0013D; Beyotime Institute of Biotechnology) was used to isolate the proteins from HUVECs at a density of 1x10⁶ cells/group. Protein (~35 µg/lane) was separated on a 12% SDS-polyacrylamide gel. The gel was transferred to a polyvinylidene difluoride membrane (MilliporeSigma). The membrane was blocked with 5% skimmed milk in Tris buffered saline/0.1% Tween-20 (pH 7.4) for 1 h at room temperature and incubated overnight at 4°C with the following primary rabbit anti-human antibodies: XBPI (1:1,000; cat. no. AF5110; Affinity Biosciences, Ltd.), CHOP (1:1,000; cat. no. AF5110, Affinity Biosciences, Ltd.), GRP78 (1:1,000; cat. no. AF5366; Affinity Biosciences, Ltd.), Puma, (1:1,000; cat. no. AF5173; Affinity Biosciences, Ltd.), caspase-3 (1:1,000; cat. no. Ab2302; Abcam), cytochrome C (1:1,000; cat. no. AF0146; Affinity Biosciences, Ltd.) and GAPDH (1:2,000; cat. no. TA-08; OriGene Technologies, Inc.). A horseradish peroxidase (HRP)-conjugated antibody against rabbit IgG (1:5,000; cat. no. ZB-2305; OriGene Technologies, Inc.) was used as a secondary antibody that was incubated at room temperature for 1.5 h. Blots were incubated with the ECL reagents (Beyotime Institute of Biotechnology) and exposed to Tanon 5200-multi (Tanon Science and Technology Co., Ltd.) to detect protein expression. Image J software V1.8.0 (National Institutes of Health)

Table I. Sequences of siRNAs targeting XBP1 and CHOP.

siRNAs	siRNA sequences (5'-3')
CHOP (human) siRNA-1	S: AGAAAGAACAGGAGAAUGATT A: UCAUUCUCCUGUUCUUCUTT
CHOP (human) siRNA-2	S: GGAGGAAGACCAAGGGAGATT A: UCUCUUUGGUCUCCUCCTT
CHOP (human) siRNA-3	S: AGGAGAAAGAACAGGAGAATT A: UUCUCCUGUUCUUCUCCUTT
XBP1 (human) siRNA-1	S: CGAAAGAAGGCUCGAAUGATT A: UCAUUCGAGCCUUCUUCGTT
XBP1 (human) siRNA-2	S: AGUGGUAGAUUUAGAAGAATT A: UUCUUCUAAAUCUACCACUTT
XBP1 (human) siRNA-3	S: GGUAUUGACUCUUCAGAUUTT A: AAUCUGAAGAGUCAAUACCTT
NC	S: UUCUCCGAACGUGUCACGUTT A: ACGUGACACGUUCGGAGAATT

Si, small interfering; CHOP, C/EBP homologous protein; XBP1, X box binding protein 1; NC, negative control; S, sense; A, antisense.

was used to quantify the relative expression level of target proteins. GAPDH was used as a loading control. A total of 3 independent experiments were performed.

Flow cytometry for cell apoptosis. HUVECs were collected in 1.5 ml tubes. Each tube was added with 10 μ l fluorescently labeled Annexin V-FITC reagent [cat. no. AP101-100-kit; Multi Sciences (Lianke) Biotech Co., Ltd.] and 5 μ l of propidium iodide (PI) reagent [AP101-100-kit; Multi Sciences (Lianke) Biotech Co., Ltd.]. Each tube was incubated for 10 min at room temperature. Cells (~200 μ l) were added into flow tubes containing 2 ml of PBS and tested by flow cytometry (FACS Aria III; BD Biosciences). The data were analyzed using FlowJo V10.8 software (BD Biosciences). A total of 3 independent experiments were performed and both early apoptosis and late apoptosis were detected.

Statistical analysis. All tests were performed using GraphPad Prism 5 software (GraphPad Inc.) and data were presented as the mean \pm SD. Statistically significant differences for continuous variables were determined by one-way ANOVA with the post hoc Tukey's test. $P < 0.05$ was considered to indicate a statistically significant difference. Three statistical replicates were performed for each experiment.

Results

CHOP and XBP1 knockdown in HUVECs. Western blotting was performed to detect the interference efficiency of CHOP, XBP1 and interference vectors in endothelial cells. Compared with the control (untransfected HUVECs), CHOP was significantly downregulated in the siRNA CHOP-2 and siRNA CHOP-3 groups, especially in the siRNA CHOP-3 group ($^*P < 0.05$; Fig. 1A). The expression of XBP1 decreased greatly in the siRNA XBP1-3 groups compared with that in the control ($^*P < 0.05$; Fig. 1B). Hence, siRNA CHOP-3 and

siRNA XBP1-3 were selected for use in subsequent experiments.

Knockdown of CHOP or XBP1 inhibits the apoptosis of HUVECs. Apoptosis was detected by performing flow cytometry. Compared with the control, the apoptosis rate of HUVECs increased greatly with the increase in the concentration of D-glucose ($^*P < 0.05$; Fig. 2). Compared with the 33.3 mM D-glucose group, the HUVECs incubated with 33.3 mM D-glucose and si-XBP1 or 33.3 mM D-glucose and si-CHOP showed significantly lower apoptosis rates ($^*P < 0.05$; Fig. 2). In addition, compared to the 33.3 mM+si-XBP1 group, a slightly lower apoptotic rate was observed in the 33.3 mM+si-CHOP group (Fig. 2). The results revealed that XBP1 and CHOP knockdown suppressed high glucose-induced endothelial cell apoptosis.

Knockdown of CHOP or XBP1 suppresses the expression of GRP78, Puma, cleaved caspase-3 and cytochrome c. Western blotting was performed to detect the expression levels of XBP1, GRP78, CHOP, Puma, cleaved caspase-3 and cytochrome C. Compared with the control, XBP1, GRP78, CHOP, Puma, cleaved caspase-3 and cytochrome c were significantly upregulated in the hypertonic, 16.7 mM D-glucose, 33.3 mM D-glucose, and 33.3 mM + NC groups ($^*P < 0.05$; Fig. 3). Compared with the 33.3 mM D-glucose group, the expression levels of XBP1, GRP78, CHOP, Puma, cleaved caspase-3, and cytochrome c in the 33.3 mM + si-XBP1 or 33.3 mM + si-CHOP groups significantly decreased ($^*P < 0.05$; Fig. 3). In addition, compared with the 33.3 mM + NC group, lowest expression level of Puma was observed in the 33.3 mM+si-XBP1 group, while the lowest expression level of GRP78 and cytochrome c was observed in the 33.3 mM + si-CHOP group ($^*P < 0.05$; Fig. 3). The results indicated that XBP1 and CHOP knockdown inhibited ER stress and apoptotic gene expression in high glucose treated endothelial cells.

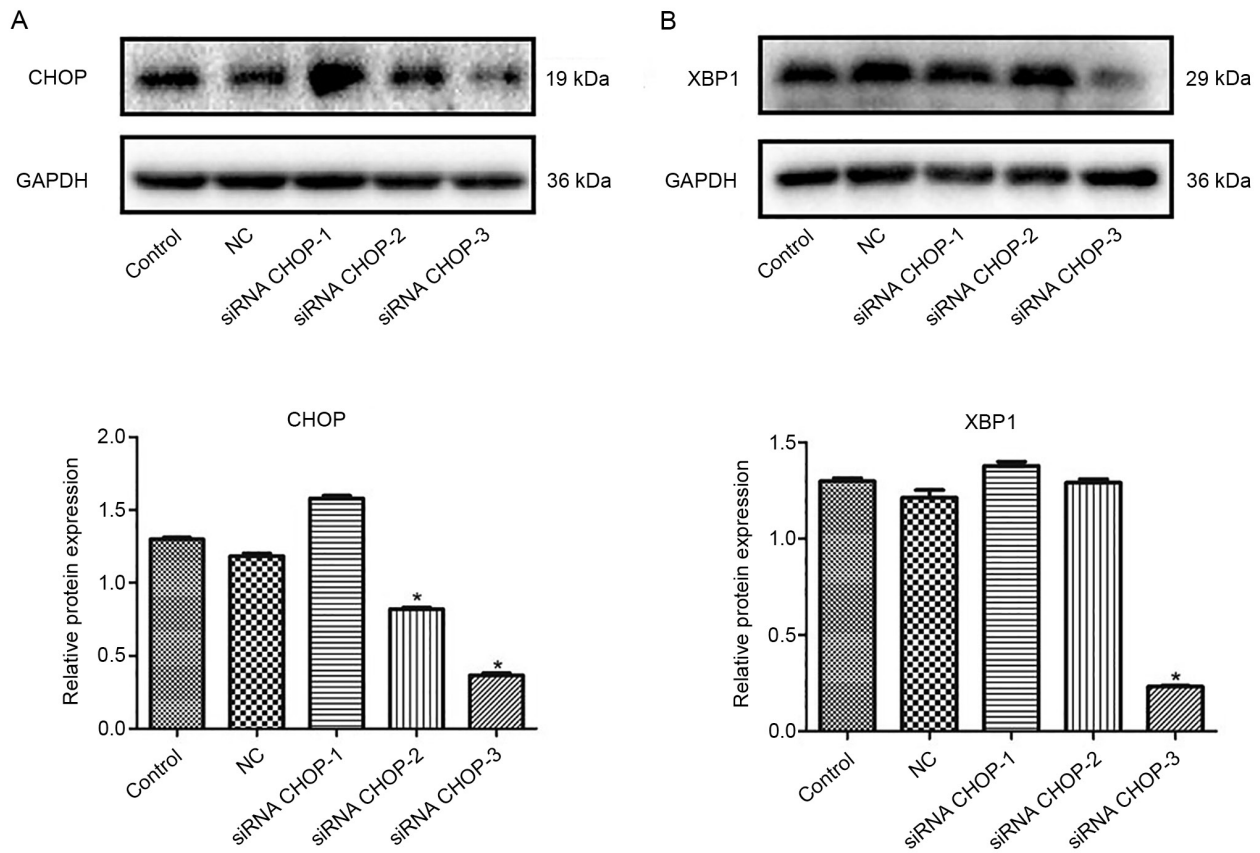


Figure 1. Identification of transfection efficacy. The protein expression levels of (A) CHOP and (B) XBPI-3 were detected by western blotting. * $P < 0.05$, vs. Control. Si, small interfering; CHOP, C/EBP homologous protein; XBPI, X box binding protein 1; control, untransfected HUVECs.

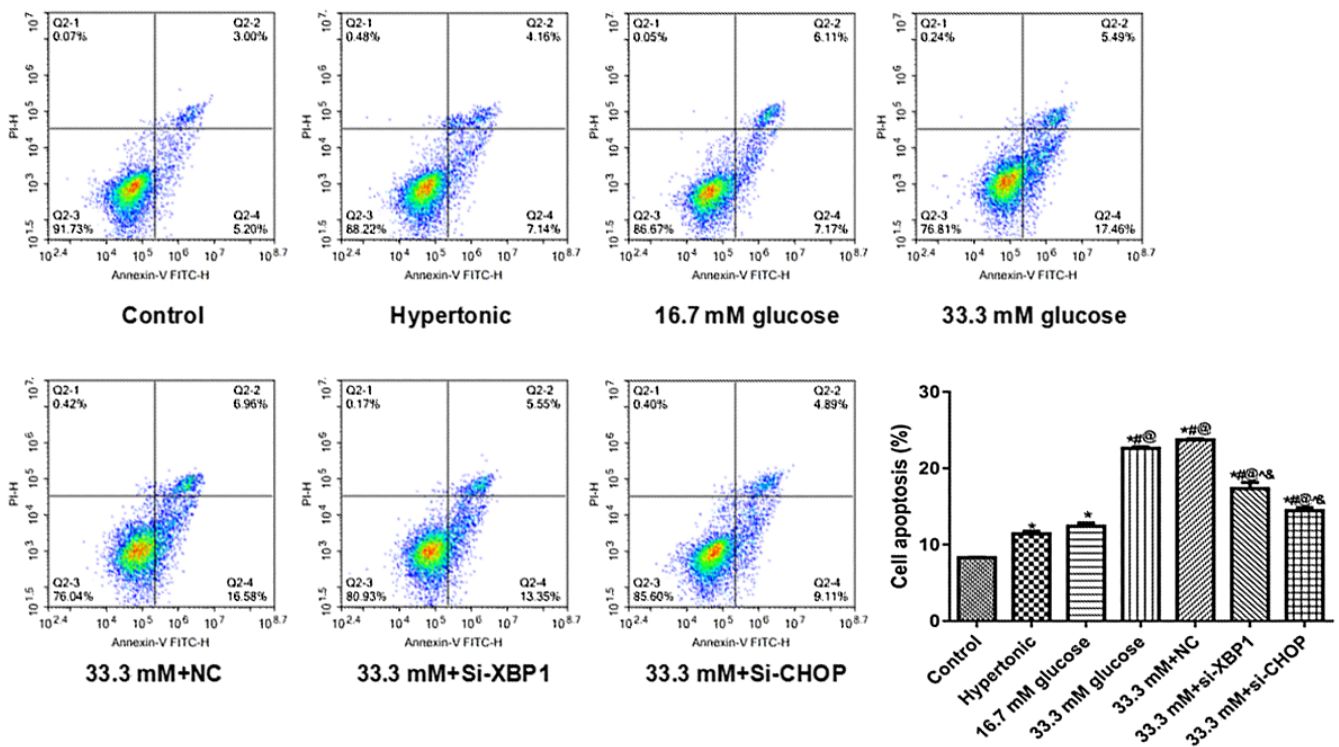


Figure 2. Effects of knocking down XBPI or CHOP on apoptotic rate. Flow cytometry was performed to detect the effects of Si-XBPI and Si-CHOP on the apoptosis of high glucose induced endothelial cells. The gating strategies for the flow cytometry assay were as follows: i) Early stage of HUVECs apoptosis (Annexin V⁺PI⁻); ii) advanced stage of HUVECs apoptosis (Annexin V⁺PI⁺); iii) normal HUVECs (Annexin V⁻PI⁻); and iv) necrotic HUVECs (Annexin V⁻PI⁺). * $P < 0.05$ vs. Control, [#] $P < 0.05$ vs. Hypertonic, [@] $P < 0.05$ vs. 16.7 mM D-glucose, [^] $P < 0.05$ vs. 33.3 mM D-glucose, [&] $P < 0.05$ vs. 33.3 mM+NC. Si, small interfering; NC, negative control; CHOP, C/EBP homologous protein; XBPI, X box binding protein 1; PI, propidium iodide; control, untransfected and untreated HUVECs.

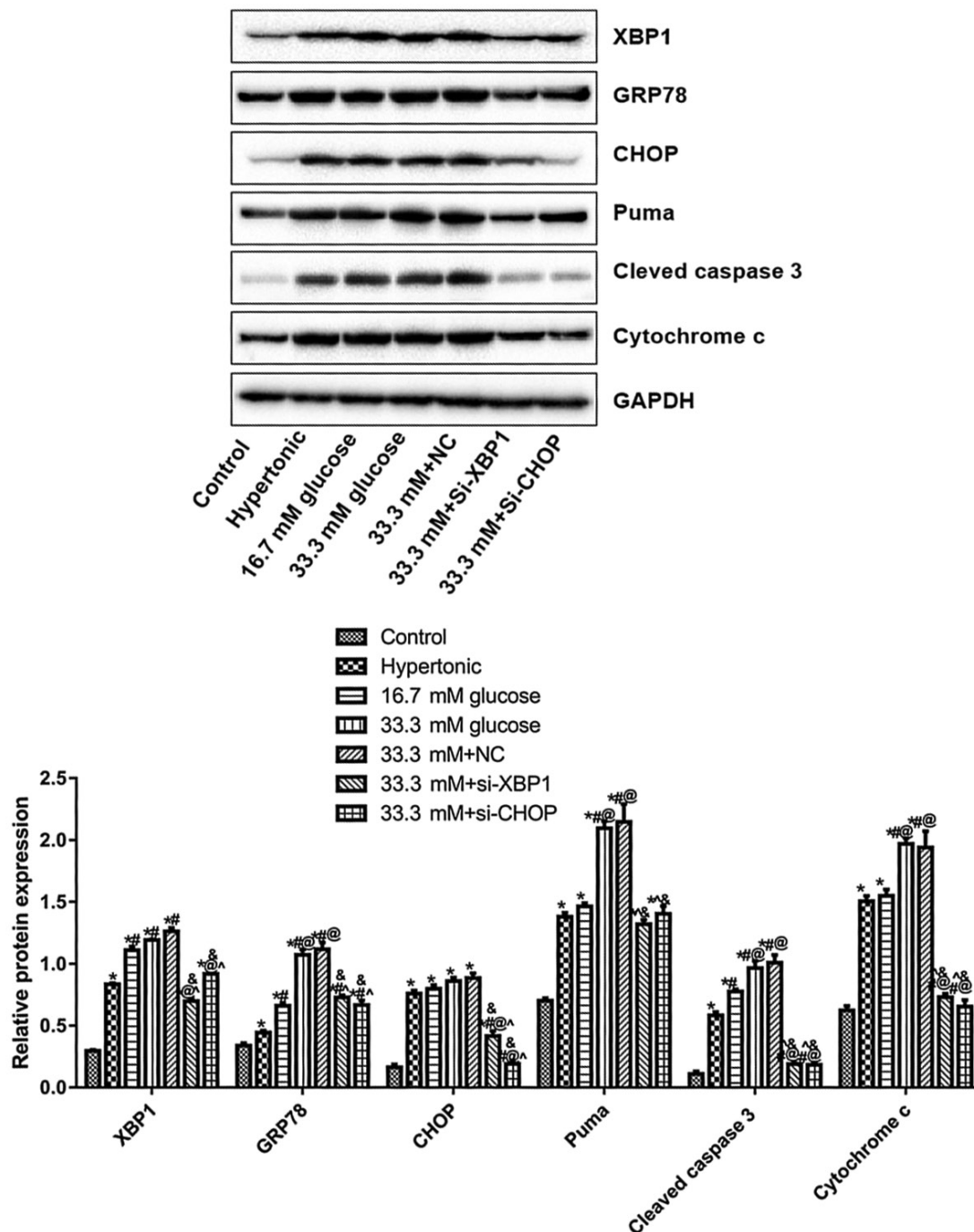


Figure 3. Effects of knocking down XBP1 or CHOP on the expression level of GRP78, puma, cleaved caspase-3 and Cytochrome c. The expression levels of XBP1, GRP78, CHOP, puma, cleaved caspase-3 and Cytochrome c were evaluated by western blotting. * $P<0.05$ vs. Control, # $P<0.05$ vs. Hypertonic, @ $P<0.05$ vs. 16.7 mM D-glucose, ^ $P<0.05$ vs. 33.3 mM D-glucose, & $P<0.05$ vs. 33.3 mM+NC. Three independent experiments were performed. Si, small interfering; NC, negative control; CHOP, C/EBP homologous protein; XBP1, X box binding protein 1; control, untransfected and untreated HUVECs.

Discussion

Normal venous endothelium has antithrombosis effects and venous wall injury is one of the important factors that can result in thrombogenesis (26). Local continuous platelet aggregation adheres to the endothelium when collagen is exposed due to endothelial cell injury, meanwhile, the coagulation system is initiated. The permeability of the endothelium is promoted by the dysfunction of the endothelium, which results in the adhesion of leucocytes to release certain inflammatory factors, such

as IL-6, TNF- α and IL-1 β . Fibrin deposition is inhibited by the released inflammatory factors to suppress the fibrinolytic system, which contributes to the formation of the prethrombotic state (27). High glucose concentration-induced ERS results in the dysfunction of endothelial cells, the inhibition of cell proliferation and cell death, thereby contributing to injury to vessels and various vascular diseases, including diabetic vascular disease (28,29). Autophagy, apoptosis, inflammation and senescence of endothelial cells can be induced by high dosage of glucose (30). XBP1 is a central regulator in the

process of ERS signal transfer in mammals (19). When ERS arises in the cells, IRE1, which is located in the endoplasmic reticulum lumen, is separated from GRP78 to be activated by oligomerization and autophosphorylation, resulting in the specific splicing of XBP1 mRNA. The expression of ERS-related genes is upregulated when XBP1-s binds with the ERS reaction components in the nucleus (16,31,32). The survival rate of cells is promoted by activating IRE1 artificially under ERS, which indicates that XBP1 serves an important role in cell survival and apoptosis (33). XBP1 is reported to induce endothelial cell injury, cell apoptosis and coagulation leading to thrombogenesis (34). CHOP is an important signal factor mediating ERS and cell apoptosis; it induces cell apoptosis through excessive ER stress (35). ERS was initiated in primary neonatal mouse cardiomyocytes by stimulation with high concentration of glucose and the expression of XBP1 and CHOP was observed to be upregulated (36). By inhibition of the activation of XBP1 or downregulation of the expression of XBP1, XBP1 splicing was suppressed and CHOP was upregulated, which indicated that the transcription and expression of CHOP could be regulated by XBP1 to induce the apoptosis of mouse cardiomyocytes (36). Consistent with the above reports, in the present study, elevated expression level of both XBP1 and CHOP could be induced by high glucose concentration. ERS in endothelial cells, denoted by upregulated XBP1 and CHOP, was stimulated by treatment with 16.7 mM glucose, which induced a 1 and 2-fold increase in the expression of XBP1 and CHOP, respectively. In the present study, a 0.92 and 2.20-fold increase in the expression level of XBP1 and CHOP, respectively were observed in 33.3 mM glucose treated endothelial cells. In the present study, the findings related to apoptosis demonstrated that it was induced by the upregulation of XBP1 and CHOP and high concentrations of glucose in a dose-dependent manner, which indicated that the XBP1/CHOP signaling pathway exerted important roles in the processing of endothelial cell apoptosis. On the contrary, apoptosis was suppressed by downregulating the expression of XBP1 or CHOP, which further verified the involvement of XBP1 and CHOP in the process of high glucose induced apoptosis.

GRP78 is a type of molecular chaperone in the endoplasmic reticulum, and high expression of GRP78 can be regarded as the symbol of ERS (37). The increased distribution of GRP78 on the cell membrane exerts a regulatory function on cell apoptosis and cell proliferation (38). GRP78 and CHOP were upregulated in Sertoli cells by a high dosage of glucose, which indicated that the activation of the CHOP signaling pathway under ERS is the mechanism underlying the pro-apoptotic effects of high glucose concentration (39). In the present study, it was found that the expression of GRP78 was upregulated by a high dosage of glucose, which was reversed by downregulating the expression level of XBP1 or CHOP.

Puma is an important pro-apoptotic gene and serves an important role in the initiation of cell apoptosis and the induction of numerous other diseases, such as hepatocyte injury and bone marrow hyperplasia (40). Puma is reported to be involved in p53-dependent or independent cell apoptosis and tumor processing (40-42). Puma is significantly upregulated when apoptosis occurs (43). Caspase is the operator of apoptosis, which is responsible for the transfer, transduction and integration of apoptotic signals (44). Tiong *et al* (45) reported that

Puma was upregulated under hyperglycemia in Schwann cells and Cazanave *et al* (46) claimed that the mRNA and protein level of CHOP in adipocytes was positively related to that of Puma. In the present study, the expression of Puma could be elevated under high glucose concentrations. Approximately 1.1 and 1.9-fold increases in Puma expression were observed in 16.7 mM glucose-treated and 33.3 mM glucose-treated endothelial cells, respectively. In contrast, high protein expression of Puma induced by high concentration of glucose was suppressed by downregulating XBP1 or CHOP in the present study.

Caspase-3 is one of the most important apoptotic operators in the caspase family and exists in the cytoplasm as an inactivated proenzyme post synthesis (47). Caspase 3 is activated by stimulating apoptotic signals that degrades multiple types of protein substrates, including pro-caspase-3, pro-caspase-6, pro-caspase-9 and DNA-dependent protein kinase (DNA-PK), in the processing of apoptosis (48). The complex composed of caspase-9 and Cytochrome c is one of the inducers of the activation of caspase-3, which is also reported to be involved in the mitochondrial apoptosis pathway (49). Mitochondrial permeability transition pores are opened under the stimulation of active oxygen and ATP, which contribute to the imbalance of the H⁺ concentration and differing pressure between the inside and outside of the mitochondria (50). Cytochrome c, which is located within the mitochondria is released into the cytoplasm as a result of differing pressure (31). By binding with caspase-9, cell apoptosis can be induced by Cytochrome c (31). Jiang *et al* (51) reported that the expression level of cleaved-caspase-3 and Cytochrome c in rat cartilage endplate cells was significantly elevated under hyperglycemia. Similarly, in the present study, the expression of Puma, cleaved caspase-3 and Cytochrome c was upregulated by the high dosage of glucose (33.3 mM), which was reversed by knocking down XBP1 and CHOP.

To sum up, in the present study the expression level of proapoptotic proteins, such as GRP78, Puma, caspase-3, and cytochrome c, were elevated by hyperglycemia in a dose-dependent manner, which further contributed to the apoptosis of HUVECs. Following inhibition of the XBP1/CHOP signal pathway, the expression level of pro-apoptotic proteins was suppressed, which further inhibited the apoptosis of endothelial cells. However, the current study has limitations. The present study only investigated the effect of the XBP1/CHOP pathway on ER stress in high glucose induced cells, meaning that the effect of ER stress inhibition on the prevention and treatment of DVT was not investigated *in vivo*. Future studies should therefore focus on *in vivo* assessment. Taken together, XBP1/CHOP may be a potential target for the treatment of DVT as one of the key pathways regulating ERS processing through mediating cell apoptosis.

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Availability of data and materials

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

Authors' contributions

MT conceived and designed the current study. JL acquired, analyzed and interpreted the data. YH performed statistical analysis. YZ made substantial contributions to conception and design, drafted the manuscript, and revised it for important intellectual content. All authors have read and approved the final manuscript. MT and YZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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