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Resveratrol attenuates high glucose-induced endothelial cell apoptosis via mediation of store-operated calcium entry

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Abstract The aim of this study was to evaluate the influence of resveratrol on HG-induced calcium entry in islet microvascular (MS-1) endothelial cells. MS-1 cells were pretreated with resveratrol or 2-APB (an inhibitor of store-operated calcium entry) and then incubated with high glucose. Cell viability was determined using the cell counting kit-8 method. Reactive oxygen species, endothelial apoptosis, and NO production were detected by DHE probe, TUNEL detection, and nitrate reductase assay kit. Protein levels of SOCE were detected by western blotting. Pretreatment with resveratrol significantly attenuated HG-induced endothelial apoptosis and improved cell viability. However, pretreatment with resveratrol and 2-APB abolished this effect, suggesting that the attenuation of HG-induced apoptosis by resveratrol may be associated with SOCE. Subsequent analyses indicated that HG induced the SOCE-related proteins, including TRPC1, Orai1, and Stim1. These results suggest that resveratrol pretreatment is associated with relieved HG-induced endothelial apoptosis at least partly via inhibition of SOCE-related proteins.

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Keywords Resveratrol · Store-operated calcium entry (SOCE) · Endothelial cells · Apoptosis · High glucose

Introduction

Atherosclerosis has been well established as a key pathophysiological feature of many cardiovascular diseases, including diabetes-associated vascular complications [1]. The integrity of endothelial cells (ECs) is the basis of overall vascular function, and apoptosis of ECs may be an important underlying mechanism of atherosclerosis [2, 3]. High glucose-induced apoptosis results in endothelial injury, which may eventually lead to microvascular dysfunction, causing cardiovascular and cerebrovascular complications. Indeed, EC apoptosis is not only considered as an initial trigger of the progression of atherosclerosis, but it is also indicated to be responsible for many acute vascular events related to atherosclerotic plaque instability, because loss of ECs can predispose individuals to arterial thrombosis, causing acute coronary occlusion and sudden death [4, 5]. Previous studies have suggested that in patients with diabetes mellitus (DM), a high glucose level is a toxic stimulus that may lead to EC senescence [6] and apoptosis [7, 8]. However, the exact mechanisms underlying HG-induced EC apoptosis remain to be determined.

Cytosolic calcium (Ca^{2+}) is an intracellular messenger that exerts many regulatory functions, including the determination of cell fate [9]. Endoplasmic reticulum (ER) calcium release can be stimulated through a variety of mechanisms. When thestromal interaction molecule (Stim1), as the ER calcium ion receptor, perceives calcium depletion, it initiates a change in the structure of the membrane and, with calcium ion release, activates the calcium channel protein (Orai1) to form a classical SOC

channel mediated by calcium influx [10]. Previous evidence indicated that high glucose stimulation of an intracellular calcium influx is an important mechanism of cell apoptosis [11]. Interestingly, the cellular flux can be regulated by the store-operated calcium channels, namely store-operated calcium entry (SOCE). Increasing evidence suggests that SOCE is extensively involved in the regulation of apoptosis of many non-excited cells, such as neuronal cells [12, 13] and hepatoma cells [14], via regulation of SOCE-related proteins, such as TRPC1, Orai1, and Stim1. However, the reported effects of changes in the expression of SOCE-related proteins on cellular apoptosis have been inconsistent in previous studies. For example, enhanced Orai1 and STIM1 expression as well as SOCE was observed in some cancer cells that are resistant to apoptosis [15, 16], whereas Stim1 and Orai1 were previously identified as pro-proliferative factors in vascular smooth muscle cells [17, 18]. Therefore, the potential roles of these proteins in HG-induced EC apoptosis and their exact roles during the pathogenetic process remain to be determined.

Resveratrol is an anti-inflammatory, antioxidant, and antiapoptotic polyphenol that exhibits many benefits for the cardiovascular system. Previous studies indicated that resveratrol may attenuate HG-related endothelial dysfunction via the regulation of the Akt/endothelial nitric oxide synthase (eNOS) pathway [19]. Moreover, administration of resveratrol may regulate the apoptosis of cancer cells via a SOCE-dependent mechanism [20]. Based on the above facts, we aimed to evaluate the potential effects of resveratrol pretreatment on HG-induced apoptosis in ECs. Moreover, the potential involvement of the SOCE process and changes in SOCE-related proteins were also evaluated.

Materials and methods

Cell culture

Cells of the MS-1 islet microvascular endothelial cell line, which were purchased from the Chinese Academy of Sciences Cell Bank, were cultured in basal medium consisting of Dulbecco's modified Eagle's medium (DMEM, Hyklong, USA) with 10% fetal bovine serum (FBS; Gibco, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin under standard conditions (5% CO_2 and 37 °C). Cells were synchronized in low-FBS media (0.5%) for 12 h prior to experiments and then exposed to culture medium (CON group) or 30 mmol/L glucose in FBS-containing media for 48 h (HG group). MS-1 cells were first preincubated with 50 µM resveratrol (Sigma, USA) or 100 µM 2-APB (SOC channel inhibitor) for 1 h and then treated with 30 mmol/L glucose (HG + Res) or HG + 2-APB. MS-1 cells were also treated with 50 µM resveratrol alone (Res) or with 2-APB (100 µM).

Cell viability measurement

MS-1 cells were incubated with culture medium or 30 mM glucose for 48 h. Cells were pretreated with either 50 µM resveratrol or 100 µM 2-APB for 1 h. Cell viability was measured with the cell counting kit-8 (CCK-8) (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. Briefly, 10 µL CCK8 solution was added to each well of a 96-well plate and incubated for 2 h at 37 °C. The optical density value was measured at an absorption wavelength of 490 nm. Cell viability was calculated and normalized to the control group.

TUNEL staining

MS-1 cells were incubated with culture medium or 30 mM glucose for 48 h. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining was used to detect DNA fragmentation in individual cells using a TUNEL fluorescence kit (fluorescein isothiocyanate, FITC) as described previously (Roche, Indianapolis, IN, USA) following the manufacturer's instructions. Briefly, MS-1 cells grown on coverslips were fixed with 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100. Then the cells were incubated with the TUNEL reaction mixture at 37 °C for 1 h. The stained cells were examined under a fluorescence microscope (Leica DMI3000 B, Germany).

Dihydroethidium (DHE) staining

Cells were seeded in 6-well plates after 48 h in culture, washed with phosphate-buffered saline (PBS), and mixed with 1 mL DHE probe solution (Sigma) at a concentration of 10 µM in each well. After incubation for 30 min at 37 °C, the cells were washed again with PBS and observed under a fluorescence microscope.

NO detection

NO production was detected by a nitrate reductase assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. Briefly, the solution (Griess Reagent I 50 µL + Griess Reagent II 50 µL) was added to each well of a 6-well plate and incubated for 5 min at 37 °C. The optical density value was measured at an absorption wavelength of 546 nm. NO production was calculated and normalized to the control group.

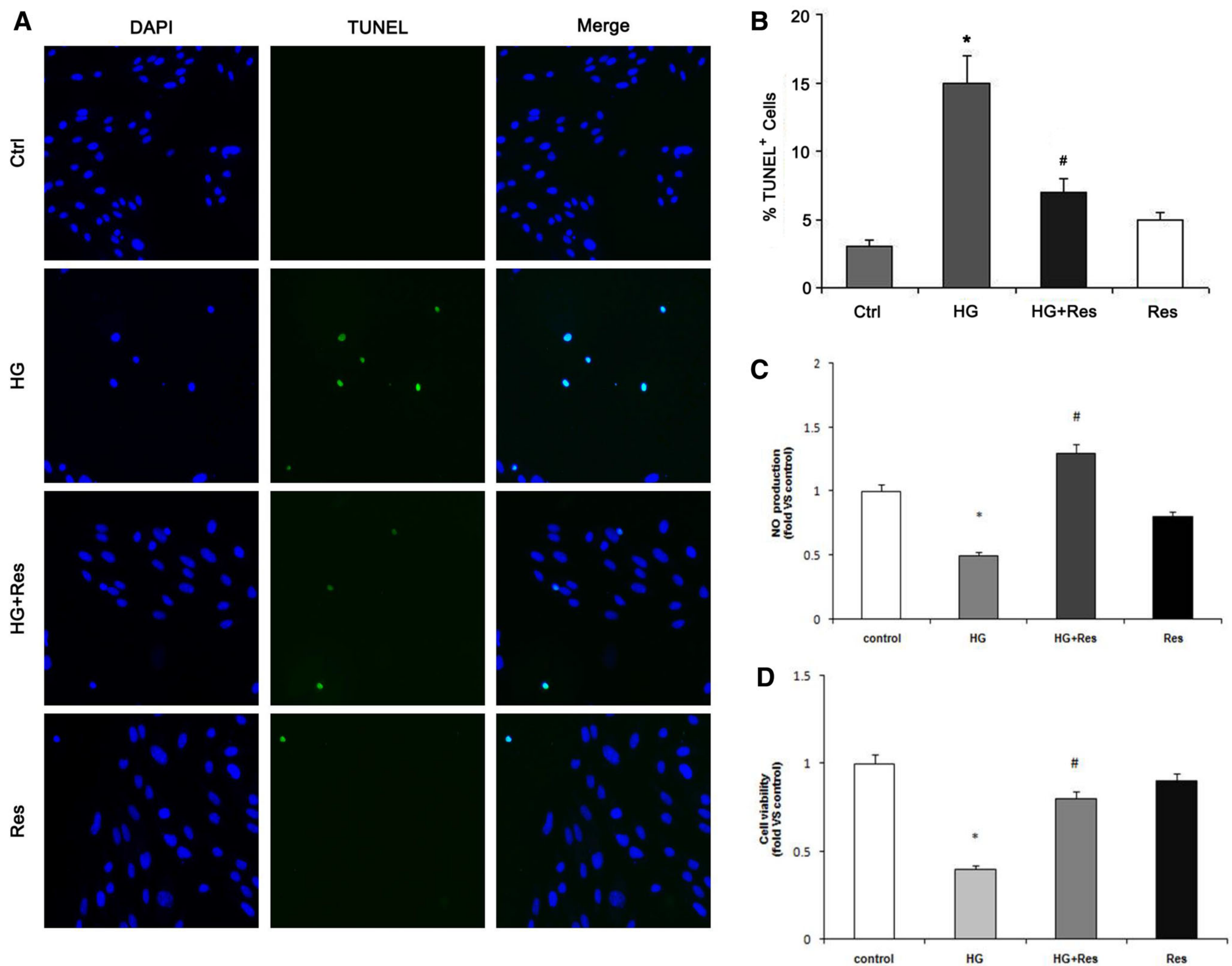


Fig. 1 Resveratrol suppressed HG-stimulated EC apoptosis and cell viability. **a** Representative images of TUNEL staining showing apoptotic cells (stained in green). Nuclei were stained blue with DAPI (magnification, ×100). **b** Quantification of the TUNEL staining. **c** Quantification of NO production. **d** Quantification of cell viability. Data are presented as mean ± SEM from five experiments. $*P < 0.05$ versus control group, $\#P < 0.05$ versus HG-treated group. (Color figure online)

Measurement of intracellular Ca^{2+} ($[Ca^{2+}]i$)

MS-1 cells were loaded with the calcium indicator Fura-2AM (5 μM) in HEPES-buffered saline. Changes in $[Ca^{2+}]i$ in individual cells were measured using an AquaCosmos system with band-pass filters for 340 and 380 nm. $[Ca^{2+}]i$ was calculated from the Fura-2 fluorescence ratio (F340/F380) using linear regression between adjacent points on a calibration curve generated by measuring F340/F380 in at least seven calibration solutions containing Ca^{2+} at concentrations ranging between 0 and 854 nM. The store-operated calcium channel (SOCC)-mediated influx of Ca^{2+} following stimulation with 1 μM thapsigargin (TG, a stimulator of SOCCs) during the change from Ca^{2+}-free conditions to 1.5 mM Ca^{2+} was measured as described previously [21].

Western blot analyses

Western blot analyses were conducted to measure the SOCE-related protein expression during HG-stimulated apoptosis of ECs. MS-1 cells were collected by centrifugation (700×g, for 10 min at 4 °C) and lysed. Cellular proteins were extracted from MS-1 cells. Total protein concentration was determined by Lowry's method using bovine serum albumin (BSA) as a standard. Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels. The protein was subsequently transferred onto a polyvinylidenedifluoride membrane by electroblotting for 3 h at 150 mA. Membranes were blocked for 1 h in Tris-buffered saline (TBS)/5% low-fat milk powder and incubated with the primary antibody overnight in TBS/5% BSA or 5% low-fat milk. The

secondary antibody, coupled with horseradish peroxidase (HRP), was applied for 1 h at room temperature. Chemiluminescence detection was performed using HRP Juice (PJK) (Thermo, USA) and a CCD camera (Bio-Rad, USA). Densitometric signals were quantified using Quantity One Bioanalysis software (Bio-Rad, Hercules, CA, USA). Anti-TRPC1 (1:1000 dilution), anti-Stim1 (1:1000 dilution), and anti-Orai1 (1:500 dilution) antibodies were purchased from Abcam, and anti-GAPDH (1:1000 dilution) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Statistical analyses

SPSS ver19.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analyses. All experiments were repeated at least thrice. Data are mean ± standard error of the mean (SEM). Groups of data were compared using analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Data that did not follow a normal distribution were analyzed with the Mann–Whitney test or Student's t test after log normalization. $P < 0.05$ was considered significant.

Results

Resveratrol inhibited HG-induced apoptosis and NO production in ECs

The results of TUNEL analyses are shown in Fig. 1. HG significantly induced apoptosis among MS-1 cells. However, pretreatment with resveratrol (50 µM, 1 h) in HG group significantly attenuated the apoptosis and of the ECs ($P < 0.05$), suggesting a protective effect of resveratrol against HG-induced EC apoptosis. In addition, resveratrol attenuated HG-mediated NO reduction and the reduction of cell viability. Compared with cells treated with 30 mM HG, the cells incubated with 50 µM resveratrol for 1 h exhibited a significant reversal of NO reduction and cell viability inhibition ($P < 0.05$; Fig. 1c, d).

Changes in reactive oxygen species (ROS) levels during HG-induced apoptosis of ECs

The measured cellular ROS levels are shown in Fig. 2. Culture in HG medium was associated with a significant increase in cellular ROS levels in MS-1 cells ($P < 0.05$). However, this increase in cellular ROS levels was inhibited to control levels in ECs by pretreatment with resveratrol (50 µM, 1 h). These results further confirm the potential antioxidant role of resveratrol in ECs.

Fig. 3 HG-stimulated apoptosis of ECs via exaggeration of SOCC-mediated $[Ca^{2+}]$i. **a** MS-1 ECs were preincubated with HG, 2-APB (a blocker of SOCC), or HG + 2-APB for different time periods. After the specified incubation periods, TG (1 µM)-induced store Ca^{2+} release and Ca^{2+} entry were measured. **b** and **c** 2-APB significantly suppressed HG-stimulated apoptosis of ECs (magnification, ×100). **d** Treatment with 2-APB significantly increased cell viability. Data are presented as mean ± SEM from five experiments. *$P < 0.05$ versus control group, #$P < 0.05$ versus HG-treated group ▶

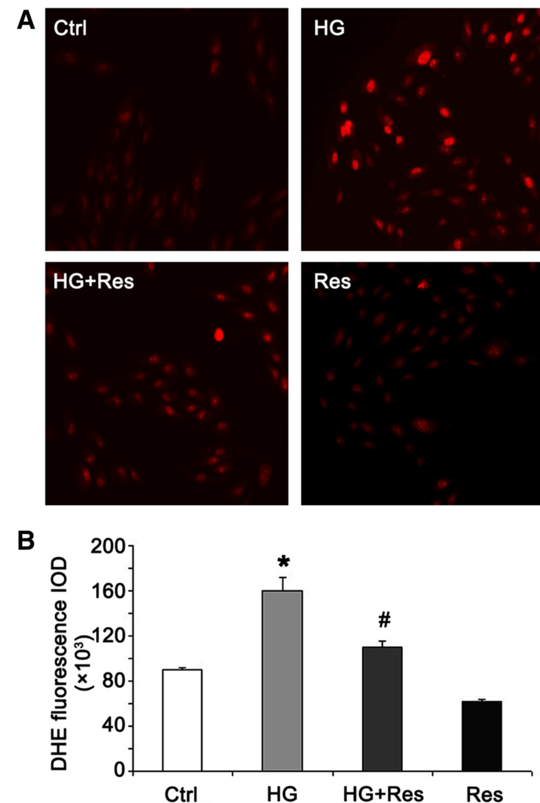
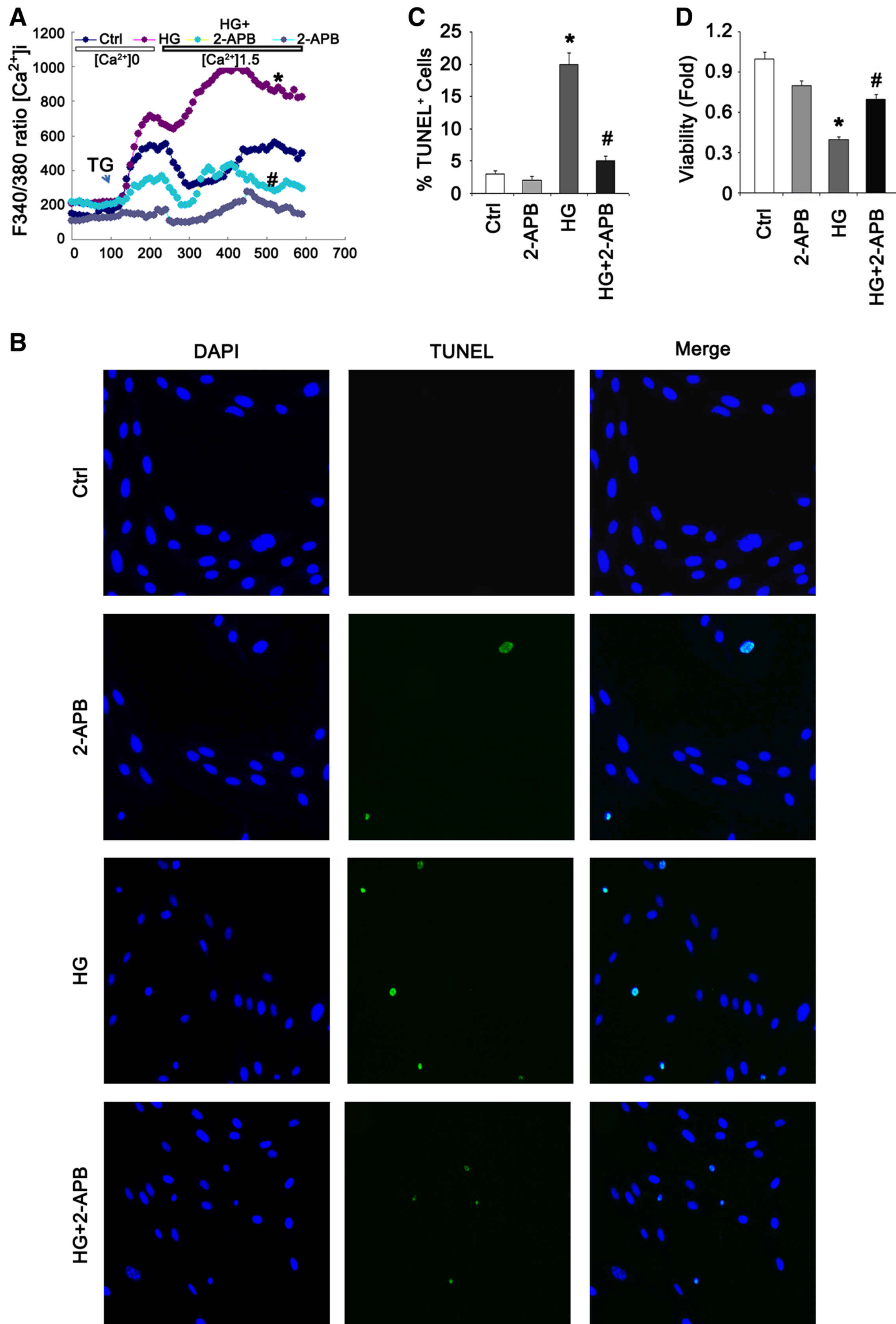


Fig. 2 Resveratrol significantly suppressed HG-stimulated ROS production. **a** Representative images of DHE detection of ROS (stained in red, magnification, ×100). **b** Quantification of ROS levels. Data are presented as mean ± SEM from five experiments. *$P < 0.05$ versus control group, #$P < 0.05$ versus HG-treated group. (Color figure online)

Changes in $[Ca^{2+}]$i during HG-induced apoptosis of ECs

Changes in the cellular $[Ca^{2+}]$i in ECs from each group are shown in Fig. 3. $[Ca^{2+}]$i was increased significantly in MS-1 cells after stimulation with TG, even with no Ca^{2+} present in the culture medium. After Ca^{2+} was added to the culture medium, HG exposure significantly increased $[Ca^{2+}]$i in MS-1 cells compared with levels in the control group ($P < 0.05$). However, 2-APB significantly reversed the HG-induced $[Ca^{2+}]$i increase. Moreover, 2-APB significantly increased cell viability and reversed HG-induced



A
F340/380 ratio [Ca2+]i
Ctrl
HG
HG+ 2-APB
2-APB
[Ca2+]0
[Ca2+]1.5
TG
*
#
C
% TUNEL+ Cells
Ctrl
2-APB
HG
HG+2-APB
D
Viability (Fold)
B
DAPI
TUNEL
Merge

MS-1 cell apoptosis, indicating that SOCE may be involved in the induction of EC apoptosis by HG.

Resveratrol inhibited HG-stimulated $[Ca^{2+}]i$ via suppression of SOCE-related proteins

The changes in $[Ca^{2+}]i$ following resveratrol pretreatment and HG exposure are shown in Fig. 4a. HG significantly increased $[Ca^{2+}]i$ in ECs. However, resveratrol significantly reversed the HG-stimulated $[Ca^{2+}]i$ in ECs, indicating that resveratrol might attenuate HG-induced EC apoptosis via abrogation of $[Ca^{2+}]i$ in ECs. Changes in the expression of SOCE-related proteins are shown in Fig. 4b–d. HG significantly induced EC expression of Orai1, TRPC1, and Stim1, and pretreatment with resveratrol significantly reversed the upregulation of Orai1 and TRPC1. However, Stim1 protein expression was not significantly affected by resveratrol pretreatment. Taken together, these results indicate that resveratrol might inhibit the HG-induced $[Ca^{2+}]i$ in ECs at least partly via regulation of the expression of SOCE-related proteins, especially TRPC1 and Orai1.

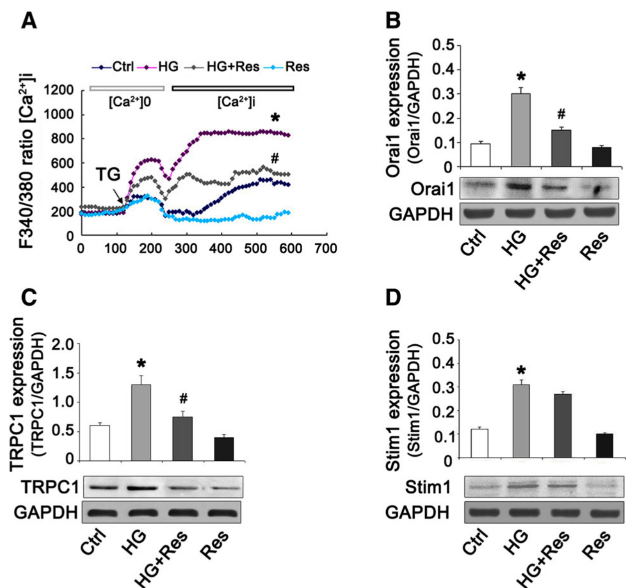


Fig. 4 Resveratrol inhibited HG-stimulated $[Ca^{2+}]i$ via suppressing the expression of TRPC1 and Orai1. **a** Arrow indicates time at which the cells were stimulated with TG. Data are presented as mean ± SEM from five experiments. **b** and **c** Resveratrol significantly reversed HG-induced expression of TRPC1 and Orai1. **d** Resveratrol had no effect on the expression of total Stim1. Representative plot is shown from five independent experiments. $*P < 0.05$ versus control group, $\#P < 0.05$ versus HG-treated group

Discussion

In the present study, we demonstrated that HG induced apoptosis among ECs via stimulation of a SOCE-related increase in $[Ca^{2+}]i$, as well as excessive ROS production and NO reduction. Interestingly, pretreatment with resveratrol attenuated the HG-induced EC apoptosis via restoration of cellular calcium homeostasis and expression of SOCE-related proteins, such as TRPC1 and Orai1 (supplemental figure). These results suggest that the SOCE-related increase in $[Ca^{2+}]i$ may be an important underlying mechanism of the pathogenesis of DM-induced EC apoptosis. Moreover, resveratrol may inhibit HG-induced EC apoptosis via regulation of SOCE-related protein expression, indicating the potential protective role of resveratrol against DM-related vascular complications.

Increased oxidative stress is a key contributor to the development and progression of EC apoptosis induced by HG. Accumulating evidence has demonstrated that HG-related ROS production may contribute to cellular DNA damage and apoptosis [22]. We demonstrated that resveratrol may inhibit the production of ROS induced by HG, which is consistent with the general antioxidative characteristics of resveratrol. These results suggest that the benefit of resveratrol on EC apoptosis may be related to its antioxidative effect.

Hyperglycemia due to DM has emerged as a major problem that threatens health and causes vascular dysfunction [22]. As indicated by previous studies, HG (30 mM glucose, for 24 h) has direct toxicity to human umbilical vein ECs [23]. In many chronic metabolic diseases, vascular endothelial integrity is affected by EC proliferation and apoptosis, which assures blood vessel function. The present study showed that HG significantly reduced cell viability and resveratrol abolished these effects. Therefore, restoration of injured ECs via regulation of EC proliferation and apoptosis may be of significance.

Intracellular Ca^{2+}, as a secondary messenger, regulates both cell survival and a massive increase in intracellular Ca^{2+}, which can cause cell apoptosis [24]. The present study suggests that HG induces apoptosis of ECs by causing intracellular Ca^{2+} overload via the regulation of a SOCE-related increase in intracellular Ca^{2+} and expression of SOCE-related proteins. This is consistent with the previous finding that SOCE-related calcium regulation may be an important mechanism for determination of the fate of non-excited cells, such as proliferation, apoptosis, and autophagy [25–27]. In view of the previous evidence that resveratrol may interact with SOCE-related proteins, we hypothesized that the regulation of SOCE and intracellular calcium levels may be involved in the benefits of resveratrol against HG-induced EC apoptosis. When resveratrol was added together with Ca^{2+}, we did not observe the inhibition of SOCE, suggesting that resveratrol did not function by inhibiting the Ca^{2+} channel pore. However, increasing the preincubation time with resveratrol before

Ca^{2+} addition did not inhibit SOCE unless resveratrol was added together with TG shortly before triggering the emptying of Ca^{2+} stores [28], which is consistent with our results. Subsequently, we found that resveratrol had no effects on the expression of total Stim1, but the HG-stimulated Orai1 and TRPC1 upregulation was significantly abrogated by resveratrol pretreatment. Previous evidence confirmed that SOCE is activated by depletion of Ca^{2+} stores in the endoplasmic reticulum (ER) and is mediated essentially by two classes of proteins, Stim and Orai [29]. Orai1 is a four-transmembrane-spanning domain protein that forms a pore through the coordination of four subunits that acts as a Ca^{2+} release-activated Ca^{2+} channel (CRAC). Orai1 silencing decreases the probability of SOC activation. Orai1 may be an essential subunit of the transient receptor protein channel (TRPC) that is required for TRPC proteins to sense calcium store depletion and, hence, to be store-operated [21]. In the present study, Orai1 and TRPC1 expression were significantly suppressed by resveratrol, which may partly explain the mechanism of Ca^{2+} influx.

Conclusion

Our experiments indicated that resveratrol pretreatment is associated with relieved HG-induced EC apoptosis at least partly via inhibition of SOCE-related proteins, suggesting the potential protective role of resveratrol against DM-related vascular complications. Further in vivo studies are needed to confirm our results.

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Authors' contributions Qiang Xu conceived of the study. Ting Lu carried out cell culture and Western blotting analysis, participated in TUNEL staining, and measurement of intracellular Ca^{2+}. Dayan Zhou performed the statistical analysis. Pan Gao drafted the manuscript. Liangyi Si participated in the design of the study. All authors contributed to and have approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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