RPS4Y1 Promotes High Glucose-Induced Endothelial Cell Apoptosis and Inflammation by Activation of the p38 MAPK Signaling

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Aim: Endothelial dysfunction is a key pathological basis for diabetes mellitus complications, including diabetic nephropathy, diabetic retinopathy, and diabetic cardiomyopathy. This study aimed to reveal the functional role of ribosomal protein S4 Y-linked 1 (RPS4Y1) in endothelial dysfunction.

Methods: Human umbilical vein endothelial cells (HUVECs) were subjected to high glucose. The expression of RPS4Y1 in cells was overexpressed or silenced by plasmid or siRNA transfection. MTT assay, flow cytometry, JC-1 probe, scratch test, tube formation, and ELISA were conducted to assess the effects of RPS4Y1 on cell. Western blot was performed to assay the downstream signaling of RPS4Y1. The inhibitors of p38, ERK, and Jnk were used to treat cells to validate the involvement of them in RPS4Y1-mediated endothelial dysfunction.

Results: RPS4Y1 was upregulated in HUVECs in response to high glucose in both dose- and time-dependent manners. Overexpression of RPS4Y1 induced viability loss, apoptosis, and inflammation, but inhibited cell migration and tube formation. Silence of RPS4Y1 impacted these aspects in a contrary trend. The phosphorylation of p38 rather than ERK and Jnk was activated by RPS4Y1. In addition, the dysfunction of HUVECs mediated by RPS4Y1 was attenuated by SB203580 (a specific inhibitor of p38 signaling).

Conclusion: The highly expressed RPS4Y1 in endothelial cells may contribute to high glucose-induced dysfunction through regulating p38 MAPK signaling. RPS4Y1 might be a potential therapeutic target for treating diabetes mellitus complications.

Keywords: RPS4Y1, HUVEC, diabetes mellitus, high glucose, p38 MAPK signaling

Introduction

Diabetes mellitus is a kind of metabolic disorder characterized by chronic hyperglycemia that is mainly caused by the defects of insulin secretion and/or use. As estimated by the International Diabetes Federation (IDF), there are approximately 300 million patients with diabetes mellitus around the world, and 80% of them are in low-income countries.1 Several genetic and lifestyle factors, like age, pregnancy, smoking, and obesity are thought to be involved in the pathogenesis of diabetes mellitus.2 Along with the increase in aging and obesity of population, the incidence of diabetes mellitus is increased greatly in the recent decades.2,3

Endothelial cells are a layer of highly differentiated flat cells located in the inner wall of blood vessels and lymphatics. Vascular endothelium consisting of endothelial cells is the main barrier to maintaining vascular permeability and is the central target of metabolites. The deleterious impacts of blood high glucose are found in...
various kinds of cells, including endothelial cells. In response to high glucose, the synthesis of diacylglycerol is promoted, protein kinase C is activated, and the level of advanced glycation end products is increased, which further induces the reactive oxygen species (ROS) and leads to endothelial cell dysfunction. The dysfunction of endothelial cells is a key pathological basis for diabetes mellitus complications, including diabetic nephropathy, diabetic retinopathy, and diabetic cardiomyopathy. Therefore, maintaining the normal endothelial function has been considered as an effective therapeutic intervention in patients with diabetes mellitus.

Ribosomal protein S4 Y-linked 1 (RPS4Y1) codifies for ribosomal protein S4 that locates at chromosome p11.31. RPS4Y1 is ubiquitously expressed and it plays a significant role in correct development. It has been reported that RPS4Y1 together with RPS4Y2 have essential roles in Turner syndrome. RPS4Y1 was highly expressed in the placental samples of preeclampsia patient, and RPS4Y1 contributed to the inhibition of trophoblast cell migration and invasion. RPS4Y1 was also found to be expressed in endothelial cells, but its function in endothelial cells under the event of diabetes mellitus is still unknown.

In the present study, we aimed to reveal the functional roles of RPS4Y1 in endothelial cells under high glucose conditions, which was widely conducted to mimic the diabetes mellitus microenvironment. The preliminary underlying mechanism of RPS4Y1 was also investigated. The findings of this work suggest evidence of gene target therapy in treating diabetes mellitus complications.

Materials and Methods
HUVECs Culture
Human umbilical vein endothelial cells (HUVECs) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in endothelial cell growth medium (Gibco, Rockville, MD, USA) containing 5 mM glucose. For complete culture medium, 10% fetal bovine serum (Gibco) was supplemented, and cells were maintained in a humidified incubator with 5% CO2 at 37°C. For high glucose culture, additional glucose (Gibco) with different concentrations (5, 15, 25, and 30 mM) was added in the culture medium. The cells were treated with high glucose for 0, 4, 8, or 24 h. To eliminate the adverse effects of high glucose on cells, we added different concentrations of mannitol (Sigma-Aldrich, St. Louis, MO, USA) to balance the osmotic pressure in each group.

To inhibit the p38, ERK, and Jnk signaling, 30 μM SB203580 (MedChemExpress, Monmouth Junction, NJ, USA), 30 μM PD98059 (MedChemExpress), or 10 μM SP600125 (MedChemExpress) were used to treat cells for 24 h, respectively.

Cell Transfection
RPS4Y1 overexpression plasmid and RPS4Y1 siRNA (5’-GGAAAGGGCATCGACTTACT-3’) were purchased from Ribobio (Guangzhou, China). The empty vector and scrambled siRNA were used as blank controls. For transfection, HUVEC cells with high confluence (60–70%) were collected into the culture medium and allowed to be cultured in the humidified incubator at 37°C overnight. Subsequently, the corresponding plasmid or siRNA was transfected into the cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. After 48 h, cells were collected for subsequent experiments.

MTT
Cell viability was measured using an MTT assay. In brief, the treated HUVECs were added into 96-well plates with a concentration of 5 × 10⁴ cells per well. Following 12 h of incubation, 20 μL MTT solution (Solarbio, Beijing, China) was added for an additional 4 h of incubation at 37°C. After the removal of the supernatant, 100 μL DMSO (Sigma-Aldrich) was used to dissolve the purple formazan. The absorbance value of each well at 490 nm was detected under a microplate reader (Bio-Rad, Hercules, CA, USA).

Flow Cytometry
Cell apoptosis was detected by Annexin V-FITC Apoptosis Detection kit (BD Biosciences, CA, USA) according to the manufacturer’s instructions. Briefly, the treated HUVECs were harvested, followed by incubation with 10 μL Annexin V-FITC for 10 min and PI for 5 min in the dark. Ultimately, the apoptosis of cell (5 × 10⁴ per sample) was calculated by using the flow cytometer (BD Biosciences).

Measurement of Mitochondrial Membrane Potential (∆ψm)
The ∆ψm was detected by the JC-1 probe (Beyotime, Shanghai, China) based on the manufacturer’s instruction. In brief, 5 × 10⁵ HUVECs following treatment were
harvested and incubated with JC-1 solution for 20 min at 37°C. The HUVECs were rinsed with JC-1 buffer, and the fluorescence intensity was measured under a fluorescence microscope (Olympus, Tokyo, Japan).

Scratch Test
Following transfection and high glucose treatment, 5 × 10^5 HUVECs were collected and seeded in the 6-well culture plates, which were pre-marked on the back of the plates. A 20 μL pipette was used to create a scratch in the cultures. The non-adherent cells were removed by three washes of PBS, and the cultures were maintained at 37°C for 24 or 48 h. The scratch was photographed, and the wounded distance was calculated.

Tube Formation Assay
Matrigel (BD Biosciences) was pre-cold at 4°C overnight and then mixed with a serum-free culture medium (1:3). The mixture was added to 24-well plates (300 μL per well), and the HUVECs following transfection and high glucose treatment were added. After 24 h of incubation at 37°C, the cultures were photographed. Tube/cord lengths of capillary-like structures were calculated by using the ImageJ software (National Institutes of Health, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)
Following transfection and high glucose treatment, the supernatant of HUVECs was collected and used for ELISA assay. The levels of IL-1β, IL-6, TNF-α, and IL-8 were measured by the commercial analyzing kits (Abcam, Cambridge, MA), according to the manufacturer’s instruction.

Real-Time Quantitative RT-PCR
Total RNA was isolated from HUVECs using an RNA simple total RNA kit (Tiangen, Beijing, China). Reverse transcription was performed by the Fast Quant RT Kit (Tiangen) according to the manufacturer’s instructions. Quantitative real-time PCRs were performed using the Super Real PreMix Plus SYBR Green (Tiangen) on a QuantStudio 6 Flex system. The relative quantification of RPS4Y1 gene was calculated using 2-ΔΔCt method. β-actin served as the internal control. The primer sequences used in the RT-PCR procedure are listed as follows. RPS4Y1, 5’-CCCTGCTGGATTCTGATGGTG-3’ (forward), 5’-GCTGCTACTGCAATTT AGCCAC-3’ (reverse); β-actin, 5’-GATTCCTATGTGGGCGACGA-3’ (forward), 5’-AGGTCTCAACATGATCTGGG-3’ (reverse).

Western Blot
HUVECs were lysed with RIPA buffer (Beyotime, Shanghai, China), and total protein was obtained. The protein concentration was measured by using the BCA protein assay kit (Tiangen). After processing with loading buffer, proteins (50 μg) were subjected to SDS/PAGE (10–12% gels), and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). The blot was blocked with 5% nonfat milk for 2 h at room temperature and incubated with primary antibodies overnight at 4°C. The primary antibodies used were listed as following: RPS4Y1 (1:1000; RayBiotech, Norcross, GA, USA), NOX-4 (1:1000; Abcam), IL-1β (1:1000; Abcam), IL-6 (1:1000; Abcam), TNF-α (1:1000; Abcam), p38 (phospho Y182, 1:1000; Abcam), ERK1/2 (1:1000; Abcam), ERK1/2 (phospho T202/T185, 1:1000; Abcam), Jnk1/2 (1:1000; Abcam), Jnk1/2 (1:1000; Abcam), IL-8 (1:1000; Biorbyt, San Francisco, CA, USA), and p38 (1:500; Biorbyt). After washing, blots were incubated with appropriate HRP-conjugated secondary antibody (1:1000; Abcam) for 2 h. The protein bands were visualized and revealed by chemiluminescence using an ECL detection kit (Millipore).

Statistical Analysis
All the assays were performed at least three times independently. Data are presented as mean ± standard deviation. Statistical significance was assessed by Student’s t-test or one-way ANOVA. p < 0.05 was considered to be a significant difference.

Results
RPS4Y1 Was Highly Expressed in HUVECs in Response to High Glucose
First, the expression changes of RPS4Y1 in HUVECs following high glucose treatment were measured. In order to eliminate the adverse effects of osmotic pressure caused by high glucose on cells, the same dose of mannitol was used. Glucose-sensitive protein NOX-4 was tested as a positive control. As seen in Figures 1A and B, the mRNA (p<0.05) and protein levels of RPS4Y1 were remarkably increased by 24 h of glucose treatment in a dose-dependent manner. In
addition, the mRNA (p<0.05) and protein levels of RPS4Y1 was elevated by 35 mM glucose in a time-dependent manner (Figures 1C and D). HUVECs were treated by 35 mM glucose for 24 h in the following studies.

RPS4Y1 Induced the Apoptosis of HUVECs

RPS4Y1 overexpression plasmid and the specific siRNA against RPS4Y1 were, respectively, transfected into HUVECs to see the functional impacts of RPS4Y1. As seen in Figures 2A and B, mRNA (p<0.05) and protein levels of RPS4Y1 were elevated by transfection with the overexpression plasmid, while it was silenced by the siRNA transfection. Overexpression of RPS4Y1 significantly inhibited cell viability, while RPS4Y1 silence promoted cell viability (p<0.05, Figure 2C). RPS4Y1 overexpression significantly induced cell apoptosis (p<0.05), but RPS4Y1 silence failed to inhibit apoptosis (p>0.05, Figure 2D), possibly due to the apoptosis being already very low. JC-1 staining results showed that RPS4Y1 overexpression induced mitochondrial depolarization, while RPS4Y1 siRNA inhibited mitochondrial depolarization (p<0.05, Figure 2E).

RPS4Y1 Enhanced the Dysfunction and Inflammation in HUVECs

The function and inflammation of HUVECs following transfection were then tested. Figures 3A and B showed that the migrating and tube formation capacities of HUVECs were inhibited by RPS4Y1 overexpression, while promoted by RPS4Y1 silence (p<0.05). In addition, RPS4Y1 overexpression significantly increased the levels of pro-inflammatory cytokines, including IL-1β (Figure 3C), IL-6 (Figure 3D), TNF-α (Figure 3E), and IL-8 (Figure 3F). The protein expression of IL-1β, IL-6, TNF-α, and IL-8 was increased by RPS4Y1 overexpression (Figure 3G). Silence of RPS4Y1 impacted those proteins in a contrary trend.

RPS4Y1 Enhanced the Apoptosis of HUVECs Induced by High Glucose

Data in Figure 4A displayed that transfection of HUVECs with RPS4Y1 overexpression plasmid significantly accelerated the viability loss induced by high glucose, while RPS4Y1 siRNA attenuated the viability loss (p<0.05). The apoptosis induced by high glucose was further accelerated by RPS4Y1 overexpression plasmid but attenuated by RPS4Y1 siRNA (p<0.05, Figure 4B). In line with this, JC-1 staining results indicated that the mitochondrial...
Depolarization induced by high glucose was enhanced by RPS4Y1 overexpression plasmid but attenuated by RPS4Y1 siRNA (p<0.05, Figure 4C).

**RPS4Y1 Enhanced the Dysfunction and Inflammation in HUVECs Induced by High Glucose**

The migrating capacity of HUVECs was analyzed by scratch test. As seen in Figure 5A, high glucose significantly inhibited the wound closure (p<0.05). Transfection of cells with RPS4Y1 overexpression plasmid further inhibited the wound closure, while RPS4Y1 siRNA significantly promoted the wound closure (p<0.05). In addition, the tube formation capacity of HUVECs was inhibited by high glucose and further aggravated by RPS4Y1 overexpression plasmid (p<0.05, Figure 5B). RPS4Y1 siRNA impacted the tube formation in a contrary trend (p<0.05).

The concentration of pro-inflammatory cytokines in the supernatant of HUVECs following transfection and high glucose treatment was analyzed. The IL-1β (Figure 5C), IL-6 (Figure 5D), TNF-α (Figure 5E), and IL-8 (Figure 5F) levels were significantly elevated by high glucose (all p<0.05). Transfection of cells with RPS4Y1 overexpression plasmid further increased the levels of these cytokines, while RPS4Y1 siRNA decreased the levels (p<0.05). Protein expression of IL-1β, IL-6, TNF-α, and IL-8 induced by high glucose was also enhanced by RPS4Y1 overexpression plasmid, while suppressed by RPS4Y1 siRNA (Figure 5G).

**RPS4Y1 Activated p38 MAPK Signaling to Mediate HUVECs Apoptosis**

MAPK is a widely studied signaling that plays a vital role in regulating endothelial cell survival, apoptosis, inflammation, and dysfunction.18,19 By performing Western blot analysis, the phosphorylation of p38 was found to be
promoted by RPS4Y1 overexpression plasmid, while suppressed by RPS4Y1 siRNA (Figure 6A). Both RPS4Y1 overexpression plasmid and RPS4Y1 siRNA showed no obvious effects on the phosphorylation of ERK and Jnk.

For further investigation, the specific inhibitors of p38 (SB203580), ERK (PD98059) and Jnk (SP600125) were used. Figure 6B shows that the phosphorylation of p38, ERK and Jnk was respectively inhibited by SB203580, PD98059 and SP600125. SB203580 significantly attenuated RPS4Y1-induced apoptosis (p<0.05, Figure 6C), while PD98059 and SP600125 showed no significant impacts on apoptosis (p>0.05).

**RPS4Y1 Activated p38 MAPK Signaling to Mediate HUVECs Dysfunction and Inflammation**

It was shown that SB203580 significantly attenuated the impacts of RPS4Y1 overexpression on HUVECs migration (p<0.05, Figure 7A) and tube formation (p<0.05, Figure 7B). Both PD98059 and SP600125 had no significantly impacts on HUVECs migration and tube formation (p>0.05).

Treating HUVECs with SB203580 also significantly attenuated RPS4Y1 induced the increase of IL-1β (Figure 7C), IL-6 (Figure 7D), TNF-α (Figure 7E), and IL-8 (Figure 7F) levels (all p<0.05). PD98059 and SP600125 could not change the concentration of these cytokines in the supernatant (p>0.05).

**Discussion**

High blood glucose is one of the main causal factors of endothelial dysfunction in diabetes mellitus, thereby setting the stage for long-term complications. In the present study, high glucose was used to treat HUVECs for 24 h to mimic an in vitro model of endothelial dysfunction. It was found that high glucose induced a significant cell death and inflammation, and suppressed the migrating and tube formation capacities of HUVECs, which was in line with
Further studies demonstrated that RPS4Y1 was up-regulated in response to high glucose in HUVECs. The elevated RPS4Y1 expression in HUVECs contributed to viability loss, mitochondrial-dependent apoptosis, and inflammation induced by high glucose. RPS4Y1 overexpression, on the other hand, impaired the normal function of HUVECs, including the migrating and tube-formation capacities. On the contrary, silence of RPS4Y1 impacted these aspects in a contrary trend. p38 signaling pathway, rather than ERK and Jnk signaling, was activated by RPS4Y1. We further revealed that RPS4Y1 accelerated high glucose induced endothelial dysfunction possibly via regulating p38 signaling.

Ribosome protein genes are well-known structural components of the ribosome, which is the essential machinery of protein translation. In mammalian cells, the regulated production of ribosome-related genes is important in all organisms to maintain a functional proteome. In addition, ribosome-related genes are involved in extra-ribosomal functions, like DNA repair, apoptosis and cellular homeostasis. In response to high glucose, the ribosome-related genes, as a kind of stress-responsive genes, are up- or down-regulated. The ribosome-related genes then contribute to the regulation of various signaling pathways through direct binding with the main components or the upstream factors of signaling pathways. For example, the silence of ribosomal protein S3 inhibited NF-κB signaling through binding to the p65 subunit of the NF-κB complex via its KH domain. Ribosomal protein L11 activated p53 signaling by inhibiting oncoprotein MDM2. Through mediation of the diverse signaling pathways, the dysregulated ribosome-related genes confer cells to sense or adapt to changes in environmental conditions, including high glucose. In the current study, RPS4Y1 as a ribosome-related gene was found to be highly expressed in HUVECs under high glucose. In addition, highly expressed RPS4Y1 further activated p38 MAPK signaling to induce dysfunction of HUVECs. It seems that the upregulated RPS4Y1 may be involved in the mechanism of endothelial dysfunction, which we have confirmed in the following in vitro experiments.

Figure 4 RPS4Y1 enhanced the apoptosis of HUVECs induced by high glucose. HUVECs were transfected with RPS4Y1 overexpression plasmid, RPS4Y1 siRNA or the negative controls (empty vector and si-NC). Following transfection, cells were treated by 35 mM glucose for 24 h (H-Glucose group). (A) The viability, (B) apoptosis, and (C) Δψm were detected by MTT, flow cytometry, and JC-1 probe, respectively. *p<0.05 vs the indicated group.
density lipoproteins, and high glucose.\textsuperscript{29,30} In this study, the viability loss of HUVECs induced by high glucose could be accelerated by RPS4Y1 overexpression, while attenuated by RPS4Y1 silence. RPS4Y1 inhibited cell viability possibly by inducing mitochondrial dependent apoptosis, as evidenced by the increase in mitochondrial depolarization. RPS4Y1 contributed to cell death, on the other hand, might be through mediating pro-inflammatory cytokines, including IL-1β, IL-6, TNF-α, and IL-8. In addition, the normal function of endothelial cells, including migration and tube formation, was impaired by RPS4Y1 overexpression, while improved by RPS4Y1 silence. All these results suggested the significant role of RPS4Y1 in endothelial dysfunction. This study is similar to a previous study in which the regulatory function of lncRNA MALAT1 on endothelial cell proliferation, migration, and tube formation was revealed.\textsuperscript{31} However, we for the first time demonstrated RPS4Y1 as one of the mediators in endothelial dysfunction.

MAPK is one of the major signaling pathways that is responsible for transmitting stimulation signals from the cell surface to the nucleus and mediating the subsequent cell response. MAPK comprises three family members, p38, ERK, and Jnk, and all of them are implicated in a wide range of cellular progresses, like proliferation, death, inflammation, differentiation, and stress response.\textsuperscript{32,33} Of note, MAPK signaling is essential for glucose homeostasis.\textsuperscript{34} Patients with diabetes mellitus...
show significant activated MAPK signaling, suggesting MAPK signaling as one key node linking endothelial dysfunction and diabetes mellitus.\textsuperscript{35–37} In addition, p38 is a critical contributor to the regulation of endothelial cell inflammation and apoptosis in response to high glucose.\textsuperscript{35,38,39} Inhibition of p38 signaling activation has been considered to be effective in attenuating endothelial cell dysfunction induced by high glucose.\textsuperscript{40,41} In this study, p38 signaling, rather than ERK and Jnk signaling, was found to be activated by RPS4Y1 in HUVECs. Further in vitro studies for the first time demonstrated that RPS4Y1 contributed to endothelial cell dysfunction, possibly through activation of p38 signaling. The potential mechanism by which RPS4Y1 modulates p38 activity needs to be studied in the future.

**Conclusion**

This work showed the upregulated RPS4Y1 expression in HUVECs in response to high glucose. The elevated RPS4Y1 level accelerated high glucose-induced dysfunction of endothelial cells. Additionally, RPS4Y1...
contributed to endothelial dysfunction, possibly via p38 MAPK signaling. These findings suggested RPS4Y1 as a potential therapeutic target for treating diabetes mellitus complications.

Data Sharing Statement
The datasets used and analyzed during the current study are available from the corresponding authors on reasonable requests.
Ethics Approval and Informed Consent
Animal and human experiments were not included in this study.

Author Contributions
All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding
This work was supported by National Natural Science Foundation, (Project Approval Number: 81973628); National Natural Science Foundation, (Project Approval Number: 81673793); Ningbo Natural Science Foundation, (Project Approval Number: 81673793); Ningbo Natural Science Foundation, (Project Approval Number: 81973628); Ningbo Natural Science Foundation, (Project Approval Number: 81673793); Ningbo Natural Science Foundation, (Project Approval Number: 81673793). This work was supported by National Natural Science Foundation which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure
The authors declare that they have no conflicts of interest.

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