• Basic Research •

GPR120/FFAR4 protects retinal vascular endothelial cells against high glucose injury *via* suppressing ROS-ERS mediated apoptosis

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Abstract

● **AIM:** To evaluate the role of reactive oxygen species-endoplasmic reticulum stress (ROS-ERS) in the cellular protection of G protein-coupled receptor 120 (GPR120/FFAR4) against high glucose (HG) induced human retinal vascular endothelial cell (HRVEC) injury and its underlying mechanisms.

● **METHODS:** HRVECs were divided into the control group, GW9508 (an agonist of GPR120) group, HG group, and HG+GW9508 group. The cell proliferation and apoptosis were assessed by cell counting kit-8 and annexin V-FITC/PI apoptosis detection kit, respectively. Western blotting analysis was performed to assess the protein expressions of Bax, Bcl-2, activating transcription factor 6 (ATF6), PKR-like endoplasmic reticulum kinase (PERK), and inositol-requiring enzyme 1 (IRE1). The ROS assay kit was used for the detection of ROS production. Then the cells were transfected with siRNA of GPR120 and the ROS level and protein levels of ATF6, PERK, and IER1 were compared.

● **RESULTS:** GW9508 promoted the proliferation of HRVECs, which was significantly reduced by the stimulation of HG. GW9508 remarkably reduced the apoptosis rate of HRVECs under HG and the expression of proapoptotic protein Bax, while increased the expression of antiapoptotic protein Bcl-2. Under HG condition, a significant increase of ROS production was noticed in HRVECs, and GW9508 treatment greatly decreased it. The over-expressions of ERS-related proteins ATF6, PERK, and IER1 under HG were down-regulated by GW9508 treatment. After successfully transfected with siGPR120, the effects of GW9508 on the production of ROS as well as the expressions of ATF6, PERK, and IER1 were reversed.

● **CONCLUSION:** GPR120 protects HRVECs against HG induced apoptosis, and suppressing ROS-ERS pathway is one of the mechanisms involved. Activation of GPR120 may be considered as a potential therapeutic target for diabetic retinopathy.

● **KEYWORDS:** GPR120/FFAR4; high glucose; apoptosis; oxidative stress; retinal vascular endothelial cells
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INTRODUCTION

Diabetes mellitus (DM) is a global public health disease and will affect 642 million adults by 2040. Diabetic retinopathy (DR) affects 1 in 3 people with DM and remains a leading cause of blindness among the working-age population of industrialized countries[1]. Diabetic macular edema (DME) and proliferative diabetic retinopathy (PDR) in late stage are the major vision-threatening endpoints of patients with DM. Most current therapies focus on PDR and DME, including laser photocoagulation, intravitreal pharmacologic agents, and vitrectomy, however, the treatment of DR remains challenging[2]. Prolonged exposure to the hyperglycemia leads to the activation of a variety of interconnecting signaling pathways that contribute to DR pathology, such as activation of the protein kinase C (PKC) and plasma kallikrein-kinin (PKK) pathway, as well as accumulation of advanced glycation end products (AGEs)[3]. However, the pathological mechanism of DR is still not fully understood. Improved appreciation of the hyperglycemia-associated pathophysiology in the neurovascular unit may provide opportunities to prevent disease at an earlier stage of DR. It is expected that in the coming years, novel treatments based on the understanding of

the pathogenesis of DR will be developed and address the need for vascular and neuroprotection for the benefit of early DR[4].

Reactive oxygen species (ROS) is a free radical that regulate many signaling molecules as intracellular and intercellular second messengers. ROS is maintained in equilibrium in normal biological conditions, however, its excessive generation under stimulation can lead to biological process called oxidative stress[5]. While the retina is susceptible to ROS because of high-energy demands and exposure to light, excessive accumulation of ROS will induce mitochondrial damage, cellular apoptosis, inflammation, and structural and functional alterations in retina. Extensive research considers hyperglycemia-induced oxidative stress as the main pathogenesis of DR, therefore, oxidative stress reduction and restoration of retinal antioxidant system should be one of the key modalities to fight against this disorder[6]. Endoplasmic reticulum (ER) is the site for regulating protein synthesis, folding and aggregation, and Ca^{2+} storage, and regulating cell stress response to govern cellular functions. Endoplasmic reticulum stress (ERS) occurs when misfolded and unfolded proteins accumulate and Ca^{2+} balance is disturbed. Chronic or irreversible ERS triggers unfolded protein response (UPR)-initiated cellular dysfunction and cell death, serving as major contributors to many diseases[7-8]. Several studies emphasized the association of ERS with the initiation and progression of DM and associated microvascular complications, including DR, suggesting ERS as a putative therapeutic target for the treatment of DR[9-10].

G protein-coupled receptors (GPCRs) activated by free fatty acids (FFAs) have emerged as new and exciting drug targets due to their potential for pharmacological transformation[11]. Among GPCRs, GPR120, later named free fatty acid receptor 4 (FFAR4), has been widely studied since its discovery[12-13]. GPR120 shows a complex pharmacological activity and different functions, including neuroprotective, antiproliferative, wound-healing, anti-inflammatory, and antidiabetic properties[14-15], with beneficial effects in metabolic disorders. Furthermore, GPR120 agonists are identified as novel antidiabetic drugs in type 2 DM[16-17]. In experimental model of type 2 diabetes, unsaturated fatty acids activated GPR120 to exert protective effects on the retina from DR progression[18]. However, the molecular mechanisms by which GPR120 protects DR is still unclear. In this study, the role and regulatory mechanism of GPR120 in high glucose (HG) treated retinal vascular endothelial cells *in vitro* was investigated, which may provide more theoretical insight for the treatment of DR.

MATERIALS AND METHODS

Cell Grouping Human retinal vascular endothelial cell (HRVECs; Shanghai Zhong Qiao Xin Zhou Biotec, China) was regularly cultured in a M199 medium (Procell, China) containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin solution (Gibco, USA) at 37℃ and 5% CO_2. The cells in a logarithmic phase were seeded onto the 96-well plates at a density of 5×10^3 cells/well overnight. Then, the cells were divided into four groups according to different treatment as following: control (cultured in M199 medium), GW9508 [50 μmol/L GW9508 (Cayman Chemical, USA), an agonist of GPR120[19-20], was added to the medium], HG (25 mmol/L D-glucose was added to the medium), HG+GW9508 (50 μmol/L GW9508 and 25 mmol/L D-glucose were added to the medium). All cells were treated for 24h.

Cell Proliferation Cell counting kit-8 (CCK-8) proliferation and cytotoxicity assay kit (MCE, USA) was used to determine the proliferation of HRVECs. Briefly, the cells in each group were treated for 24h in the 96-well plates. Then, 10 μL of CCK-8 solution was added to each well. After incubated at 37℃ for 4h, the absorbance (A) of each well was detected at 450 nm with a microplate reader (Flexstation®, Molecular Devices, USA). The cellular proliferation rate =[(As-Ab)/(Ac-Ab)]×100%. As refers to the absorbance of experimental well containing cells, medium, CCK-8 solution and reagents. Ab refers to the absorbance of blank well containing medium and CCK-8 solution. Ac refers to the absorbance of control well containing cells, medium and CCK-8 solution.

Cell Apoptosis Annexin V-FITC/PI apoptosis detection kit (Elabscience, China) was utilized to was evaluated the cellular apoptosis rate of HRVECs. The instructions from the manufacturer were strictly followed. Briefly, the cells in different treatment groups were digested with 0.25% trypsin without EDTA. Then, the cells were collected, centrifuged at 1500 rpm for 5min, and washed twice with phosphate buffer saline (PBS). After that, the cells were resuspended in 500 μL binding buffer. Then, 5 μL Annexin V-FITC and 5 μL PI were added to the buffer and the cells were incubated for 15min at room temperature avoiding light. The cellular apoptosis was analyzed by a flow cytometer (CytoFLEX, Beckman Coulter, USA) within 1h.

Western Blotting Western blotting analysis was carried out to assess the protein expressions of Bax, Bcl-2, activating transcription factor 6 (ATF6), PKR-like endoplasmic reticulum kinase (PERK), and inositol-requiring enzyme 1 (IRE1). The cells were fully lysed on ice with the RIPA lysate containing PMSF (Beyotime, China), homogenized and centrifuged at 12 000 rpm at 4℃ for 5min. BCA protein assay kit (Beyotime, China) was used to determine the protein concentration of the sample. The protein was then placed in a boiling water bath for denaturation, applied to SDS-PAGE gel for electrophoresis, transferred to PVDF membrane, and soaked in Tris buffered saline with Tween-20 (TBST) containing 5% skimmed milk

powder for 2h on a shaker. PVDF membrane was incubated with primary antibody overnight at 4℃, washed well with TBST, and then incubated with secondary antibody for 2h at 37℃ on a shaker. The protein bands were then visualized *via* ECL chemiluminescence (Thermo Scientific, USA) and quantified using Image J software (NIH, USA). The primary antibodies were as following: Bax (1:2000, Proteintech, China), Bcl-2 (1:1000, Proteintech, China), ATF6 (1:1000, Proteintech, China), PERK (1:1000, Abcam, USA), p-PERK (1:1000, CST, USA), IRE1 (1:1000, Affinity, USA), p-IRE1 (1:1000, Affinity, USA), GPR120 (1:1000, Affinity, USA), and β-actin (1:1000, Boster, China).

ROS Production ROS assay kit (Beyotime, China) was used for the detection of ROS production in HRVECs by using fluorescent probe DCFH-DA. The cells in the logarithmic growth phase with good growth condition were taken and 5×10^5 cells/well were seeded onto a 6-well plate and cultured overnight at 37℃. Cells were treated for 24h according to the grouping scheme. After that, the culture fluid was removed and 1 mL DCFH-DA diluted with serum-free medium at 1:1000 was added to each well. The plate was placed in an incubator for 20min at 37℃. The cells were washed three times with serum-free medium to fully remove DCFH-DA that did not enter the cells. After resuspend with 500 μL PBS, the cells were collected and detected by flow cytometery.

siGPR120 Transfection The cells in the logarithmic growth phase were seeded onto 6-well plates at 2×10^5 cells/well and incubated at 37℃ and 5% CO_2 overnight. Serum-free M199 medium was replaced two hours before transfection. HRVECs were transfected with 20 μmol/L siRNA of GPR120 (Tsingke Biotec, China) by using Lipofectamine™ 2000 Reagent (Invitrogen, USA) according to the manufacturer's instructions. The cells transfected with nonspecific sequence was set as the negative control (NS) group. After transfection for 24h, the culture medium was replaced and different reagents were added according to the grouping method. Then, the cells were cultured for another 24h before analyzing the protein expression of GPR120.

Statistical Analysis Statistical analysis was performed with SPSS 19.0 and Graphpad Prism 6.0. All quantitative data from at least three repeated experiments were expressed as the mean±standard deviation. Differences between groups were compared using one-way ANOVA, followed by LSD post hoc test when they conformed to normal distribution and homogeneity of variance. The significance level was determined at 0.05.

RESULTS

GPR120 Activation Inhibits High Glucose Induced Injury of HRVECs To mimic hyperglycemia environment in DR, glucose at the concentration of 25 mmol/L was used to treat HRVECs. According to CCK-8 assay results, the proliferation rate of HRVECs was reduced after HG stimulation (Figure 1A). In addition, HG treatment increased the apoptosis rate of HRVECs (Figure 1B) and the protein expression of Bax and decreased the protein expression of Bcl-2 (Figure 1C) in HRVECs. Next, to figure out the role of GPR120 in DR cell model, the agonist GW9508 was used to treat HRVECs after HG stimulation. CCK-8 assay revealed that GW9508 offset HG-induced decrease in the proliferation rate of RGCs (Figure 1A). Flow cytometry demonstrated that GW9508 attenuated the promotive effects of HG on apoptosis of HRVECs (Figure 1B). The elevation in Bcl-2 protein level and decline in Bax protein level caused by HG in HRVECs were counteracted by GW9508 (Figure 1C). All these results revealed that 25 mmol/L of glucose has an effect on inducing cell injury, and activation of GPR120 by GW9508 inhibits cell injury of HG-treated HRVECs.

GPR120 Activation Inhibits ROS and ERS in High Glucose-Treated HRVECs DCFH-DA staining assay suggested that ROS production of HRVECs was significantly promoted by HG treatment. The enhancement of ROS in HG-treated HRVECs was neutralized by GW9508 (Figure 2). As Figure 3 showed, increased protein expression of ATF6, p-PERK and p-IRE1 was observed in HG group compared with control group, suggesting that ERS was promoted in HRVECs under HG stimulation. However, GW9508 treatment in HG model attenuated the increase of these proteins which indicated that activation of GPR120 inhibited HG-induced ERS in HRVECs (Figure 3). To sum up, activation of GPR120 is able to inhibit ROS and ERS in HRVECs under HG condition.

Effects of GW9508 on ROS and ERS in High Glucose-Treated HRVECs Mediated by GPR120 Activation of GPR120 by its agonist GW9508 was proved to have an inhibitory effect on ROS production and ERS in HG-treated HRVECs. However, whether the chemical GW9508 works or whether it works by activating the receptor GPR120 is unclear. Here, knockdown of GPR120 by siRNA was performed. As Figure 4 showed, the expression of GPR120 in HRVECs was successfully silenced by transfection with siGPR120. Then, the production of ROS and expressions of ERS-related proteins were detected. As Figure 5 showed, ROS fluorescein intensity was remarkably reduced by GW9508 in HG-treated HRVECs in both HG+GW9508+NS and HG+GW9508+siGPR120 groups. In addition, siGPR120 weaken the effects of GW9508 on ROS production under HG when compared with that of NS treatment. Similarly, the protein expressions of ATF6, p-PERK and p-IRE1were decreased by GW9508 in HG-treated HRVECs, and siGPR120 attenuated the effects of GW9508 on the expression of these proteins under HG conditions

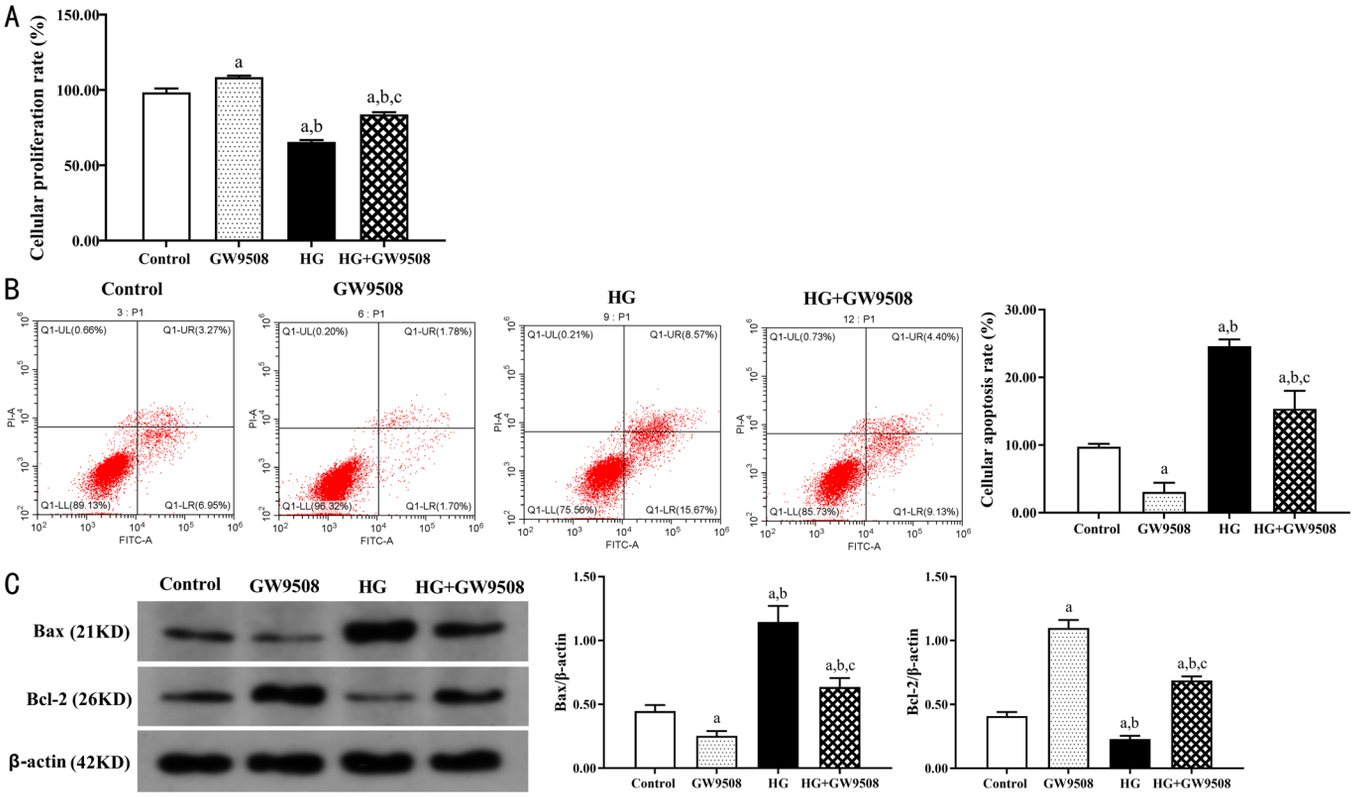

Figure 1 Activation of GPR120 by GW9508 inhibits injury of HG-treated HRVECs A: Proliferation of HRVECs in four groups (control, GW9508, HG, and HG+GW9508) was assessed by CCK-8 assay; B: Apoptosis of h HRVECs in the four groups was tested by flow cytometry; C: Expression of apoptosis-related proteins Bax and Bcl-2 in HRVECs in four groups was evaluated by Western blotting. *n*=3, [a]*P*<0.05 *vs* control group; [b]*P*<0.05 *vs* GW9508 group; [c]*P*<0.05 *vs* HG group. GPR120: G protein-coupled receptor 120; HG: High glucose; HRVECs: Human retinal vascular endothelial cells.

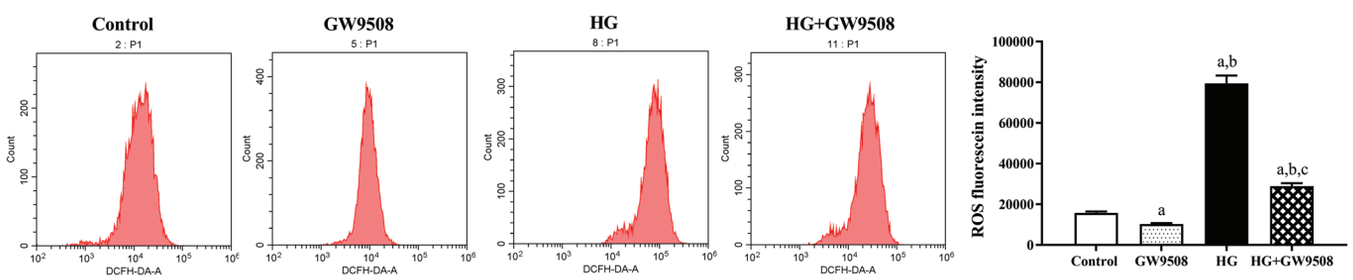

Figure 2 Activation of GPR120 by GW9508 restrains HG-induced reactive oxygen species overproduction in HRVECs DCFH-DA staining assay was performed and reactive oxygen species levels in HRVECs in four groups (control, GW9508, HG, and HG+GW9508) were measured. *n*=3, [a]*P*<0.05 *vs* control group; [b]*P*<0.05 *vs* GW9508 group; [c]*P*<0.05 *vs* HG group. GPR120: G protein-coupled receptor 120; HG: High glucose; HRVECs: Human retinal vascular endothelial cells.

(Figure 6). On the whole, the inhibitory effects of GW9508 on ROS and ERS in HG-treated HRVECs were abolished by knockdown of GPR120, suggesting that the effects of GW9508 are directly mediated by GPR120.

DISCUSSION

As one of the most common and challenging ocular complications of diabetes, DR poses a serious threat to vision and has gradually become a leading cause of blindness worldwide[21]. Among various retinal cells, retinal vascular endothelial cells constitute an important component of the highly selective inner blood retinal barrier (BRB). Breakdown of the BRB and the resultant vascular permeability strongly correlates with vision loss in DR[22]. In view of the importance of retinal vascular endothelial cells and to mimic the insult of them occurring in diabetes, HRVECs were treated with HG to investigate the viability and apoptosis as well as the protective effects of GPR120 on these cells. Consistent with previous reports[23-24], we demonstrated that HG induced decreased proliferation rate of HRVECs and increased cellular apoptosis rate. Several genes involved in the regulation of apoptosis, Bax and Bcl-2 are mostly investigated. Activated by apoptosis-inducing factors, Bax leads to oligomerization and insertion into mitochondrial membrane, resulting in changes in mitochondrial membrane permeability and release of cytochrome c (CytC), which forms a poly complex with apoptotic protease activator (Apaf-1). This complex can recruit

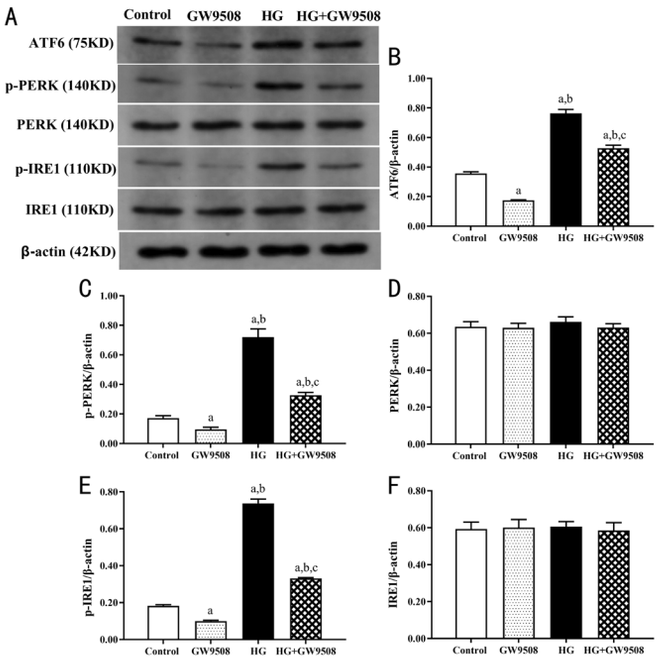

Figure 3 Activation of GPR120 by GW9508 restrains HG-induced endoplasmic reticulum stress in HRVECs A: Expression of endoplasmic reticulum stress-related proteins (ATF6, PERK, and IER1) in HRVECs of four groups (control, GW9508, HG, and HG+GW9508) was detected by Western blotting; B-F: Statistical comparison of protein expressions of ATF6, p-PERK, PERK, p-IRE1, and IRE1 among the four groups. *n*=3, [a]*P*<0.05 *vs* control group; [b]*P*<0.05 *vs* GW9508 group; [c]*P*<0.05 *vs* HG group. GPR120: G protein-coupled receptor 120; HG: High glucose; HRVECs: Human retinal vascular endothelial cells; ATF6: Activating transcription factor 6; PERK: PKR-like endoplasmic reticulum kinase; IRE1: Inositol-requiring enzyme 1.

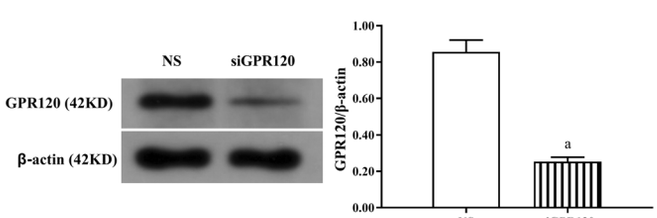

Figure 4 Expression of GPR120 in HRVECs was successfully silenced by transfection with siGPR120 Expression of GPR120 protein in HRVECs of two groups (NS and siGPR120) was detected by Western blotting. *n*=3, [a]*P*<0.05 *vs* NS group. GPR120: G protein-coupled receptor 120; NS: Negative control; HRVECs: Human retinal vascular endothelial cells.

the precursor of caspase-9 in the cytoplasm, which can be cleaved and activated to cleaved caspase-9, and then activate downstream caspase-3 and caspase-7, causing cell apoptosis. Bcl-2 is expressed in mitochondrial outer membrane, ER and nuclear membrane, and inhibits cell apoptosis by inhibiting the release of CytC from mitochondria or binding with Apaf-1 to inhibit caspase activity[25-26]. In this study, we found that the expression of Bax was up-regulated and Bcl-2 expression was down-regulated under HG conditions, confirming the

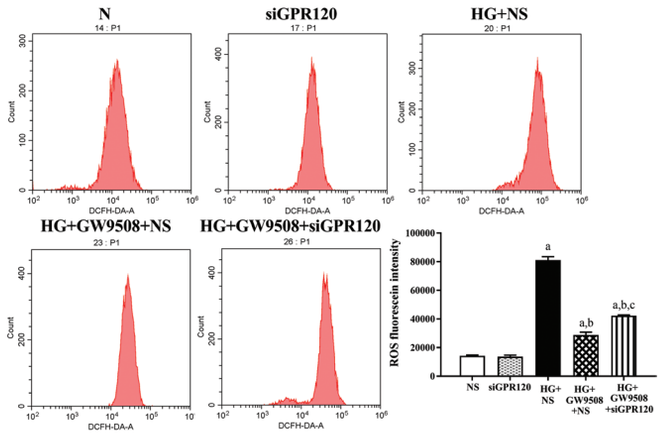

Figure 5 Knockdown of GPR120 attenuates the effect of GW9508 on HG-induced ROS overproduction in HRVECs DCFH-DA staining assay was performed and ROS levels in HRVECs in five groups (NS, siGPR120, HG+NS, HG+GW9508+NS, and HG+GW9508+siGPR120) were measured. *n*=3, [a]*P*<0.05 *vs* NS group; [b]*P*<0.05 *vs* HG+NS group; [c]*P*<0.05 *vs* HG+GW9508+NS group. GPR120: G protein-coupled receptor 120; ROS: Reactive oxygen species; HG: High glucose; NS: Negative control; HRVECs: Human retinal vascular endothelial cells.

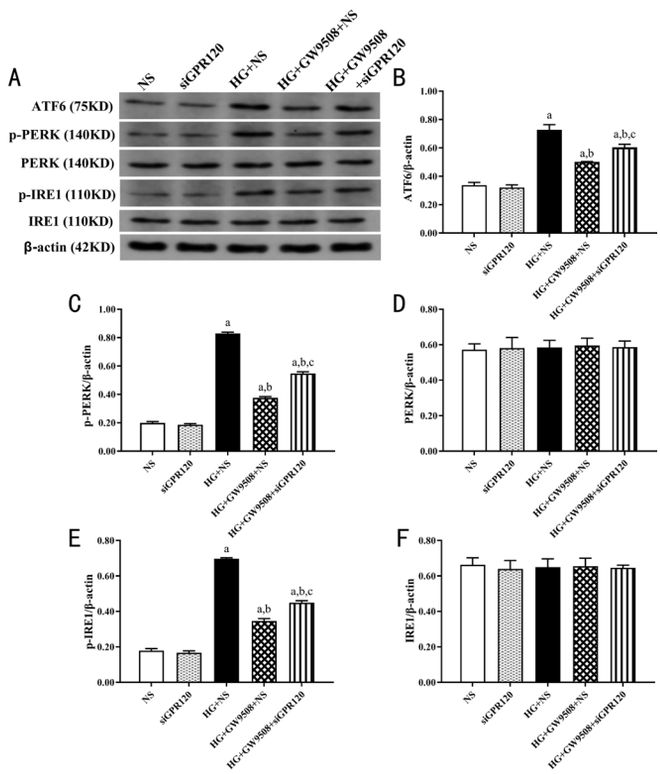

Figure 6 Knockdown of GPR120 attenuates the effect of GW9508 HG-induced ERS in HRVECs A: Expression of ERS-related proteins (ATF6, PERK, and IRE1) in HRVECs of five groups (NS, siGPR120, HG+NS, HG+GW9508+NS, and HG+GW9508+siGPR120) was detected by Western blotting; B-F: Statistical comparison of protein expressions of ATF6, p-PERK, PERK, p-IRE1, and IRE1 among the five groups. *n*=3, [a]*P*<0.05 *vs* NS group; [b]*P*<0.05 *vs* HG+NS group; [c]*P*<0.05 *vs* HG+GW9508+NS group. GPR120: G protein-coupled receptor 120; ERS: Endoplasmic reticulum stress; HG: High glucose; NS: Negative control; HRVECs: Human retinal vascular endothelial cells; ATF6: Activating transcription factor 6; PERK: PKR-like endoplasmic reticulum kinase; IRE1: Inositol-requiring enzyme 1.

importance of enhanced activity of pro-apoptotic Bax gene and reduced activity of anti-apoptotic Bcl-2 gene in augmenting HG induced apoptosis of HRVECs[27].

To date, several FFARs that are activated by the FFAs of various chain lengths, including FFAR4 (GPR120) have been identified and found to have key physiological functions in the regulation of metabolism and immune responses in health and disease[15]. Recent evidences suggest that GPR120 has a protective potential in diabetes and its complications[16,28]. Furthermore, GPR120 can be detected in the retina of diabetic mice and modulating its gene and protein expression can protect the retina from progression of DR[18]. However, there are few studies on the role of GPR120 in the pathology of DR. In the present study, we discuss whether GPR120 can protect HRVECs against HG injury *in vitro*. To analyze the effect of GPR120, GW9508 (3-4-3-phenyloxyphenylmethyl aminophenyl propanoic acid), a selective and potent small-molecule agonist of GPR120[29] was chosen to treat HRVECs. We confirmed that GW9508 increased the cellular proliferation rate and expression of Bcl-2, while reduced cellular apoptosis rate and Bax expression of HRVECs under HG environment. These results suggested that GW9508 protect HRVECs with HG-induced injury.

ROS is the most prominent molecule involved in cell signaling, and excessive ROS results in ERS and activate UPR, a set of tightly controlled regulatory programs, to protect the cells and restore the normal function of ER. It is generally believed that early mild UPR is beneficial to the recovery of cell homeostasis, however, if ERS is persistent and intense and the adaptive UPR fails to eliminate unfolded or misfolded proteins, apoptosis will occur to remove the stressed cells[30-31]. Since ROS and subsequent ERS stimulated by HG play critical roles in apoptosis of retinal vascular endothelial cells[30-31] and the pathogenesis of DR[6,10], we further evaluated the role of ROS and ERS pathway in the enhancement of HRVECs survival by GW9508. Our results showed that HG induced significant cellular apoptosis in HRVECs, which was accompanied by increased intracellular levels of ROS and ERS, shown as elevated expression levels of three important transmembrane proteins on the ER, namely ATF-6, PERK, and IRE1. Under normal conditions, ATF-6, PERK and IRE1 bind to glucose regulated protein 78 (GRP78) as inactive complexes. In ERS mode, these three transducers are separated from GRP78 and activated respectively to start the UPR[32]. Under HG conditions, treatment with GW9508 decreased the level of ROS and the expressions of ATF-6, PERK, and IRE1.

Our finds revealed that GW9508, the agonist of GPR120, possess protective effects against HG induced apoptosis of HRVECs and inhibitory effects on ROS-ERS pathway. However, whether GPR120 directly participate in these processes could not be affirmed, since GW9508 is also the agonist of GPR40[33]. Therefore, we finally observed the effects of GW9508 on ROS and ERS after silencing GPR120 by using siRNA transduction. Knockdown of GPR120 remarkably abolished the inhibitory effects of GW9508 on ROS production and the expressions of ATF-6, PERK, and IRE1, suggesting that GPR120 directly mediates the effects of GW9508 on HRVECs. In conclusion, activation of GPR120 is involved in inhibiting HG-induced injury of retinal endothelial cells primarily via the ROS-ERS signaling pathway. GW9508 might be a potential therapeutic strategy for DR. However, the molecular mechanisms whereby GPR120 regulates HG induced oxidative stress and ERS need to be studied. In addition, experiments on other retinal cells, the animal models of DR and even tissue specimen from patients with DR should be carried out to confirm the role of GPR120.

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

Conflicts of Interest: Wang LZ, None; **Yan JJ,** None.

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