Schizandrin A Protects Human Retinal Pigment Epithelial Cell Line ARPE-19 against HG-Induced Cell Injury by Regulation of miR-145

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Diabetic retinopathy (DR) is a serious complication of diabetes, which is the main cause of blindness among adults. Traditional Chinese medicines (TCMs) have been proven to delay the development of DR. Nonetheless, the effect of Schizandrin A (SchA) on DR remains uninvestigated. The present study aimed to probe the protective effect of SchA on high-glucose (HG)-induced injury in ARPE-19 cells. We observed that SchA accelerated cell proliferation, prohibited apoptosis, and restrained pro-inflammatory cytokines (monocyte chemoattractant protein-1 [MCP-1], interleukin-6 [IL-6], and tumor necrosis factor alpha [TNF-α]) and reactive oxygen species (ROS) level in HG-stimulated cells. Additionally, miR-145 expression was upregulated in HG and SchA co-treated cells, and miR-145 inhibition reversed the protective effect of SchA on HG-managed ARPE-19 cells. Interestingly, downregulated myeloid differentiation factor 88 (MyD88) was found in HG and SchA co-treated cells, and upregulation of MyD88 was observed in miR-145 inhibitor-transfected cells. Additionally, SchA hindered nuclear factor κB (NF-κB) and p38 mitogen-activated protein kinase (p38MAPK) signaling pathways in HG-treated ARPE-19 cells. The findings validated that SchA could protect ARPE-19 cells from HG-induced cell injury by regulation of miR-145.

INTRODUCTION

Diabetic retinopathy (DR) is one of the most common microvascular complications of diabetes, which is a reflection of metabolic disorders of diabetes and the damage of the endocrine system and blood system on the retina.1,2 The morbidity of DR is increased with the course of diabetes, which is approximately 44.4% in 5 years; however, after 7 years, the incidence is increased to 56%.3 The occurrence of DR is linked to various risk factors, such as hyperglycemia, abnormal metabolism of polyethanol, cell apoptosis, and free radical action.4,5 Recently, hyperglycemia has been considered to be a major cause of DR damage.6 The management for DR includes laser surgery, vitrectomy, and injection of corticosteroids or anti-vascular endothelial growth factor (VEGF) agents into the eye.7 However, the effectiveness of the treatment of DR is still dissatisfied. With the wide application of traditional Chinese medicines (TCMs) in eye diseases, scholars have shown great interest in the treatment of DR by TCMs.

Increased evidence has demonstrated that TCMs and their active ingredient, such as Salvia miltiorrhiza Bunge, Radix pueraria, and Astragalus membranaceus (Fisch.) Bunge, could effectively delay the occurrence and development of DR, as well as improve the quality of life of patients.8-10 Schizandrin A (SchA) is a dominating lignin, separates from the dry fruits of Schisandra chinensis (Turcz.) Baill.11 The extensive pharmacological properties of SchA, such as anti-tussive, anti-cancer, enzyme reduction, improved immunity, and anti-aging effects, have been reported in recent studies.12,13 A study from Song et al.14 revealed that SchA could inhibit microglia-mediated neuron inflammation by regulation of tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6)/nuclear factor κB (NF-κB) and Janus kinase 2 (JAK2)/signal transducers and activators of transcription (STAT3) signaling pathways. However, the impact of SchA on DR is still ambiguous.

Proteins of p53 and CyclinD1 are closely linked to control of the cell cycle.15 Bax, Bcl-2, and caspase-3 are key regulators of apoptosis-associated pathways, which affect cell apoptosis progression.16 These factors are widely researched in the initiation and development of DR.17-19 Reactive oxygen species (ROS) include O2−, OH, and H2O2, which have been confirmed to directly oxidize and damage DNA, protein, and lipids directly. ROS can also be used as a functional molecular signal to activate a variety of stress-sensitive signaling pathways, which are closely linked to insulin resistance and impaired function of beta cells.20,21 Monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), and TNF-alpha (TNF-α) are important pro-inflammatory factors, which are closely associated with the development of inflammation-induced diseases.22,23 Myeloid differentiation factor 88 (MyD88), NF-κB, and p38 mitogen-activated protein kinase (p38MAPK) are crucial signaling pathways.
pathways, which are also researched in diverse diseases, including DR. These pathways participated in the regulation of the pathogenesis of DR. Therefore, exploring these factors contribute to a better understanding of the occurrence and development mechanism of DR.

Hence, we used the adult retinal pigment epithelial cell line ARPE-19 as the experimental cell line in the research, and we explored the effect of SchA on cell behaviors of cell proliferation and apoptosis, as well as the relevant factors of p53/CyclinD1, Bax/Bcl-2/caspase-3, MCP-1/IL-6/TNF-α, and ROS level in ARPE-19 cells under high-glucose (HG) conditions. The underlying mechanism was uncovered through regulation of MyD88, NF-κB, and p38MAPK signaling pathways. The findings in the current study might provide a potential therapeutic method for future DR treatment.

RESULTS

SchA Alleviated HG-Induced ARPE-19 Cell Injury

In the study, five concentrations of SchA (10, 20, 30, 40, and 50 μM) were used to stimulate ARPE-19 cells, and cell viability was determined by using a Cell Counting Kit-8 (CCK-8) assay. In Figure 1A, results show that the viability of ARPE-19 cells was decreased by SchA at the concentrations of 40 and 50 μM (p < 0.05). There was no effect of SchA at 10, 20, and 30 μM on the viability of ARPE-19 cells. Subsequently, ARPE-19 cells were pretreated with SchA at the concentrations of 10, 20, and 30 μM, and then received HG treatment. The results showed that HG treatment significantly decreased cell viability compared with the control group (p < 0.01). However, SchA significantly increased the viability of ARPE-19 cells at 20 μM (p < 0.05) and 30 μM (p < 0.01) in HG-treated cells (Figure 1B). The subsequent experiments were selected 30 μM SchA for the treatment of ARPE-19 cells.
After treatment with HG, upregulated p53 and downregulated CyclinD1 were found in these treated ARPE-19 cells, as shown in Figures 1C and 1D (p < 0.01 or p < 0.001). Additionally, cell apoptosis results revealed that HG treatment significantly increased the percentage of apoptotic cells (p < 0.001) and upregulated Bax and cleaved caspase-3 protein levels and downregulated Bcl-2 protein level (all p < 0.001, Figures 1E and 1F). Furthermore, the mRNA levels and concentrations of MCP-1, IL-6, and TNF-α as well as the ROS level were all increased by HG treatment (p < 0.001, Figures 1G–1I). In contrast, all of the above results were reversed by SchA in HG-treated ARPE-19 cells (p < 0.05, p < 0.01 or p < 0.001, Figures 1C–1I). These data suggested that HG-induced ARPE-19 cell injury was alleviated by SchA, indicating the protective effect of SchA on HG-injured ARPE-19 cells.

SchA Promoted miR-145 Expression in HG-Treated Cells

ARPE-19 cells were pretreated with 10, 20, and 30 μM SchA and then received HG treatment, and the relative expression level of miR-145 was determined. Non-treated cells acted as a control group. The results showed that HG treatment significantly downregulated miR-145 expression compared with the non-treated group (p < 0.001, Figure 2). However, the expression level of miR-145 was promoted by SchA at 10 (p < 0.01), 20 (p < 0.001), and 30 μM (p < 0.001; Figure 2). The results hinted that SchA upregulated miR-145 expression in a dose-dependent manner in HG-treated cells.

SchA Alleviated HG-Induced ARPE-19 Cell Injury by Mediation of miR-145 Expression

The above study showed the promoting effect of SchA on miR-145 expression, and therefore we supposed that miR-145 might participate in regulating the protective effect of SchA on HG-injured ARPE-19 cells. To clarify the postulate, the expression vector of miR-145 inhibitor was transfected into ARPE-19 cells, and the effects of miR-145 inhibitor on these cellular biological processes were subsequently assessed. In Figure 3A, miR-145 expression was decreased in miR-145 inhibitor-transfected cells compared with that in negative control (NC)-transfected cells (p < 0.01). Additionally, we observed that miR-145 inhibition significantly declined cell viability (p < 0.001), increased p53 protein level (p < 0.001), and decreased CyclinD1 protein level (p < 0.01) in HG- and SchA-treated cells (Figures 3B–3D). Cell apoptosis was significantly induced by miR-145 inhibition (p < 0.001, Figure 3E); additionally, the protein levels of Bax and cleaved-caspase-3 were increased and the protein level of Bcl-2 was decreased by miR-145 inhibition in HG- and SchA-treated cells (Figure 3F). Results in Figures 3G–3I revealed that miR-145 inhibition markedly increased the mRNA levels and concentrations of MCP-1, IL-6, and TNF-α and the ROS level in HG- and SchA-treated cells (all p < 0.001). These data indicated that miR-145 inhibition overturned the effect of SchA on HG-injured ARPE-19 cells.

MyD88 Was a Direct Target of miR-145

We next investigated the regulatory effect of SchA and miR-145 on MyD88 expression level. In Figure 4A, the protein and mRNA levels of MyD88 were significantly upregulated in HG-treated cells (p < 0.001), but downregulated in HG and SchA co-treated cells (p < 0.001). Additionally, in miR-145 inhibitor-transfected cells, the protein and mRNA levels of MyD88 were also increased (p < 0.001, Figure 4B). To further explore the relationship between miR-145 and MyD88, a luciferase reporter assay was performed. The results in Figure 4C show that co-transfection of miR-145 mimic and wild-type (WT)-MyD88 significantly reduced the luciferase activity compared with the NC group (p < 0.05). There was no significant alteration in co-transfection of miR-145 mimic and mutant (Mut)-MyD88 cells. These results hinted that MyD88 might be a direct target of miR-145 in ARPE-19 cells.

SchA Inhibited the Activation of NF-κB and p38MAPK Signaling Pathways in HG-Treated ARPE-19 Cells

To uncover the potential mechanism of SchA in protecting ARPE-19 cells against HG-induced injury, the signaling pathways of NF-κB and p38MAPK were examined. Figures 5A and 5B show that HG treatment upregulated the protein levels of phosphorylated (p-)IkBα and p-p65 in ARPE-19 cells (p < 0.001). However, SchA notably inhibited p-IkBα and p-p65 protein levels in HG-treated ARPE-19 cells. Meanwhile, we observed that the protein level of p-p38MAPK was increased by HG treatment (p < 0.05, Figures 5C and 5D). However, the protein level of p-p38MAPK was decreased in HG and SchA co-treated ARPE-19 cells (p < 0.05, Figures 5C and 5D). These data indicated that SchA blocked NF-κB and p38MAPK signaling pathways in HG-treated ARPE-19 cells. These signaling pathways might play an important role in the regulation of SchA protecting ARPE-19 cells against HG-induced injury.

DISCUSSION

In this study, we explored the protective effect of SchA on HG-injured ARPE-19 cells. Results showed that SchA significantly alleviated...
HG-induced injury in ARPE-19 cells. The expression level of miR-145 was upregulated by SchA, and miR-145 suppression reversed the protective effect of SchA on HG-injured ARPE-19 cells. MyD88 was observed to be downregulated in ARPE-19 cells after treatment with HG and SchA, meanwhile upregulated in miR-145 inhibitor-transfected cells. Additionally, MyD88 was predicted as a direct target of miR-145. Finally, we found that SchA blocked the NF-κB and p38MAPK pathways in HG-treated ARPE-19 cells.

Figure 3. miR-145 Inhibition Reversed the Protective Effect of SchA on HG-Injured ARPE-19 Cells

(A) Expression vector of miR-145 inhibitor and its NC were transfected into ARPE-19 cells, and the relative expression of miR-145 in these transfected cells was determined by quantitative real-time PCR assay. (B–I) The transfected cells were pretreated with SchA (30 μM) and then received HG treatment for 48 h: (B) cell viability and (C) p53 and CyclinD1 protein levels were assessed by CCK-8 and western blot assays, (D) quantitative analysis of p53 and CyclinD1 proteins, while (E) apoptosis, (F) Bax, Bcl-2, and pro/cleaved-caspase-3 protein levels, (G) relative mRNA expressions, (H) concentrations of MCP-1, IL-6, and TNF-α, and (I) ROS level were assessed by flow cytometry, western blot, quantitative real-time PCR, ELISA and DCFH-DA staining. Data are shown as mean ± SD and were determined by three independent repeated experiments. *p < 0.05, **p < 0.01, ***p < 0.001 versus control group; *p < 0.05, **p < 0.01, ***p < 0.001 versus HG group; #p < 0.05, ##p < 0.01, ###p < 0.001 versus the HG+SchA+NC group.
Our research explored the functions of SchA in miR-145 expression and the relationship between SchA and miR-145 is still unclear. In vivo, adjusting other miRNAs. Moreover, to our best knowledge, little evidence has been uncovered on the anti-inflammatory effect of SchA. In our study, we explored the regulatory effect of SchA on HG-induced cell injury through studying cell growth, pro-inflammatory factor expression, and ROS level in ARPE-19 cells. We found that SchA significantly increased cell proliferation, reduced apoptosis, and inhibited MCP-1, IL-6, and TNF-α expression and secretion, and it reduced ROS generation in HG-treated ARPE-19 cells, indicating the protective effect of SchA on HG-injured ARPE-19 cells.

Emerging studies have demonstrated that microRNAs (miRNAs) are important regulators in the pathogenesis of diabetes and the related microvascular complications. In DR, Kovacs et al. first explored the effect of miRNAs on DR and identified the different expression patterns of these miRNAs in the retinas of diabetic rats. Additionally, a study reported that miR-200b could mediate VEGF-mediated alterations in DR. miR-145 is one of the important miRNAs, and the neuro-restorative effects of miR-145 on diabetes stroke rats have been reported in the research from Cui et al. Furthermore, a study from Chen et al. demonstrated that miR-145 could alleviate HG-induced proliferation and migration in vascular smooth muscle cells (VSMCs) by targeting Rho-associated kinase 1 (ROCK1). In the literature, SchA has been reported to alleviate lipopolysaccharide (LPS)-triggered HαCaT cell injury via modulating miR-127, to inhibit TPC-1 cell proliferation, migration, and invasion via declining miR-429, as well as to enhance chemosensitivity of colon carcinoma cells to 5-fluorouracil via upregulating miR-195. Therefore, SchA does not specifically regulate miR-145, which can exert the functions via adjusting other miRNAs. Moreover, to our best knowledge, the relationship between SchA and miR-145 is still unclear. In our research, we explore the functions of SchA in miR-145 expression, and we further probe whether miR-145 joins in mediating the effect of SchA in HG-affected ARPE-19 cell behaviors. In our study, the experiments revealed that miR-145 expression was increased by SchA in HG-treated ARPE-19 cells. Interesting results indicated that miR-145 suppression reversed the protective effect of SchA on ARPE-19 cell injury induced by HG. These data indicated that miR-145 might be a vital regulator in SchA-protecting ARPE-19 cells.

MyD88 is a key junction molecule in the Toll-like receptor (TLR) signaling pathway, which plays an important role in the transmission of upstream information and the development of diseases. A recent study demonstrated that MyD88 was associated with inducing various inflammatory responses in animal models of DR. In the present study, our interest was mainly focused on exploring the role of MyD88 in HG- and SchA-treated ARPE-19 cells, and in the relationship between MyD88 and miR-145. The results revealed that MyD88 expression was downregulated in HG- and SchA-treated ARPE-19 cells, and MyD88 was indicated as a direct target of miR-145 in ARPE-19 cells. These data indicated that MyD88 might be an important mediator in regulating the effect of SchA on HG-injured ARPE-19 cells. Further studies are still needed to clarify this postulate.
ARPE-19 cells. The data hinted that the NF-κB and p38MAPK pathways might be involved in SchA alleviating HG-induced ARPE-19 cell injury.

Conclusion
Taken together, the study demonstrated that SchA protected ARPE-19 cells against HG-induced injury by regulation of miR-145 and inactivation of the NF-κB and p38MAPK signaling pathways. The findings not only clarified the protective effect of SchA on HG-injured ARPE-19 cells, but also provided a novel insight for the treatment of DR. Further studies are necessary for exploring the clinical efficacy of SchA on DR.

MATERIALS AND METHODS

Cell Culture
ARPE-19 cells obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) were cultured in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), and 10% fetal bovine serum (FBS, Gibco), 100 μg/mL streptomycin, and 100 U/mL penicillin were added to the medium. Cells were incubated at 37°C with 95% air and 5% CO2.

Cell Treatment
ARPE-19 cells were cultured in six-well culture dishes with normal glucose condition (5.5 mM), and the culture medium was then added to the cells for 24 h before switching to HG treatment (30 mM). The cells were then incubated in 37°C in a humidified chamber with 5% CO2 for 48 h. SchA was obtained from the National Institutes for Food and Drug Control (Beijing, China). It was diluted in methyl alcohol and the concentrations were adjusted from 0 to 50 μM. The same concentrations of methyl alcohol were utilized to dispose of ARPE-19 cells as the blank control groups. Subsequently, ARPE-19 cells were then pretreated with SchA for 24 h before HG treatment.

CCK-8 Assay
Cell viability was examined by using a CCK-8 (Dojindo Molecular Technologies, Gaithersburg, MD) assay. After treatment with SchA alone or co-treatment with HG, the CCK-8 solution (10 μL) was added to the each culture well, and the culture plate was placed in an incubator with 95% air and 5% CO2, and incubated for 1 h at 37°C. The absorbance was read at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

ELISA
After treatment with SchA and HG, the treated cell culture supernatant was collected from the culture plates. The concentrations of pro-inflammatory cytokines of MCP-1, IL-6, and TNF-α were examined by using the corresponding ELISA kit (R&D Systems, Abingdon, UK) according to the protocols supplied by the manufacturer.

ROS Assay
ARPE-19 cells were seeded onto a six-well plate and treated with SchA and HG. After washing twice with PBS, these treated cells were incubated with 10 μM 2',7'-dichlorofluorescein diacetate (DCFH-DA, Jiansheng, Nanjing, China) for 20 min, 37°C, in the dark condition. Then, cells were resuspended in 500 μL of PBS and the fluorescent
intensities were measured using a flow cytometer (488 nm excitation, 521 nm emission).

**Cell Transfection**

Expression vectors of miR-145 inhibitor, miR-145 mimic and the NC were synthesized by GenePharma (Shanghai, China). All of these cell transfections were conducted by using Lipofectamine 3000 reagent (Life Technologies, MD, USA) following the manufacturer’s protocol.

**Luciferase Reporter Assay**

The 3’ UTR sequence of MyD88 was amplified by PCR and was inserted into the pmir-Report vector (Promega, Madison, WI, USA). The vectors were co-transfected with the miR-145 mimic and the NC. After transfection for 48 h by using the DharmaFECT Duo transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA), the luciferase assays were performed by using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s instructions.

**Quantitative Real-Time PCR**

Total RNA was isolated from treated or transfected cells by using TRizol reagent (Invitrogen, Carlsbad, CA, USA) and treated with DNase I (Promega). A first-strand cDNA synthesis kit (Takara, Otsu, Shiga, Japan) was performed to synthesize the cDNA in this experiment. A quantitative real-time PCR assay was performed to determine the relative expression of miR-145 as well as MCP-1, IL-6, TNF-α, and MyD88 by using a TaqMan miRNA assay supplemented with TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) and a SYBR Green PCR kit (Takara) according to the instructions of the manufacturers. U6 and β-actin served as the corresponding controls, and the relative quantification was calculated by using the 2^(-ΔΔCt) method. Primer sequences were as follows: miR-145, forward, 5'-ACA CTC CAG CTG GGG TCC TGC AGT TT T CCC AGG A-3' and reverse, 5'-TGG TGT CGT GGA GTG C-3'; U6, forward, 5'-CTC GCT TCG GCA GCA CA-3' and reverse, 5'-AAC GCT TCA CGA ATT TGC GT-3'; MCP-1, forward, 5'-GCT CAT AGG CAT AGG CAC CTT-3' and reverse, 5'-GGG ATC CTC CGG CCA CTT-3'; TNF-α, forward, 5'-GCT CAT AGG CAT AGG CAC CTT-3' and reverse, 5'-GGG ATC CTC CGG CCA CTT-3'; IL-6, forward, 5'-TGG AGA TGT CTG AGG CTC ATT-3' and reverse, 5'-CGC TTG TGG AGA AGG AGT TC-3'; MyD88, forward, 5'-ATA GCC ACC AGC ATG CAC-3' and reverse, 5'-AGG GTC CTT ACC AGG TA-3'; β-actin, forward, 5'-AGG GAC GAA TGG ACC CAA TCA TT-3'.

**Western Blot Assay**

The protein used for the western blot assay was extracted by using radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China), and the concentrations of these protein samples were quantified by using a bicinchoninic acid (BCA) protein assay kit (Fierce, Appleton, WI, USA). The equivalent proteins were separated on gels by SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking with 5% BSA, these membranes were incubated with primary antibodies of p53 (ab131442), CyclinD1 (ab166633), Bax (ab182733), Bcl-2 (ab32124), pro-caspase-3 (ab32150), cleaved-caspase-3 (ab2302), MyD88 (ab133739), total (t)-IκBζ (ab178846), p-IκBζ (ab334362), t-p65 (ab32536), p-p65 (ab86299), t-p38MAPK (ab170099), p-p38MAPK (ab47363), and β-actin (ab6276) (from Abcam, Cambridge, UK) at 4°C overnight. Subsequently, membranes were washed with TBS with Tween 20 (TBST), and the secondary antibody of horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG; ab205718, 1:2,000, Abcam) was added to incubate for 1 h at room temperature. After this, 200 μL of Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was supplemented to cover the surface of the membrane, and the signals were captured. The intensity of the bands was quantified using Image Lab software (Bio-Rad).

**Statistical Analysis**

The data were obtained from three independent experiments and are presented as the mean ± SD. Statistical analyses were performed using SPSS 19.0 statistical software (SPSS, Chicago, IL, USA). The p values were calculated by using a one-way ANOVA. A p value of <0.05 was considered to indicate a statistically significant result.

**AUTHOR CONTRIBUTIONS**

C.Q. and G.W. conceived and designed the experiments, P.Y. and S.L. performed the experiments and analyzed the data, J.W. and Y.D. contributed reagents/materials/analysis tools, and S.L. and Y.D. wrote and revised the manuscript. All authors read and approved the final version of the manuscript.

**CONFLICTS OF INTEREST**

The authors declare no competing interests.

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