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RESEARCH ARTICLE

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Eriodictyol inhibits high glucose-induced oxidative stress and inflammation in retinal ganglial cells

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Abstract

Diabetic retinopathy (DR) is one of the most common microvascular complications of diabetes mellitus and is considered as a leading cause of blindness. Oxidative stress and inflammation are significant drivers for the development of DR. Eriodictyol, a flavonoid compound, was proved to possess anti-inflammatory, antioxidative, and antidiabetic activities. However, the role of eriodictyol in DR has not been unveiled. In the current study, we explored the protective effects of eriodictyol on high glucose (HG)-induced rat retinal ganglial cells (RGCs). The results suggested that eriodictyol improved cell viability of HG-induced rat RGC-5 cells in a dose-dependent manner. Eriodictyol reduced the reactive oxygen species production and increased the activities of superoxide dismutase, glutathione peroxidase and catalase in rat RGC-5 cells in response to HG stimulation. The production of proinflammatory cytokines including tumor necrosis factor alpha and interleukin-8 was diminished after eriodictyol treatment. Eriodictyol also suppressed cell apoptosis induced HG in rat RGC-5 cells. Furthermore, eriodictyol enhanced the nuclear translocation of nuclear factor erythroid-2 (E2)-related factor 2 (Nrf2) and elevated the expression of antioxidant enzyme heme-oxygenase-1 (HO-1). These findings suggested that eriodictyol protects the RGC-5 cells from HG-induced oxidative stress, inflammation, and cell apoptosis through regulating the activation of Nrf2/HO-1 pathway.

KEYWORDS

diabetic retinopathy (DR), eriodictyol, inflammation, nuclear factor erythroid-2 (E2)-related factor 2/heme-oxygenase-1 (Nrf2/HO-1) pathway, oxidative stress

1 | INTRODUCTION

Diabetic retinopathy (DR) is one of the major and most common microvascular complications of diabetes mellitus.[1,2] DR may lead to neuronal dysfunction, retinal vascular leakage, and cell apoptosis, resulting in damage in the retina.[1,3] Thus, DR is considered as a leading cause of blindness. The incidence of DR is highly associated with the illness time of patients with diabetes mellitus.[4,5] Therefore, there is a need to take some strategy to prevent the occurrence of DR. Over the past decade, increasing research have demonstrated that oxidative stress and inflammation are significant drivers of diabetic complications, including DR.[6,7] Thus, the focus now has been towards inhibiting the oxidative stress and inflammation and the related pathways to prevent DR.[3]

Nuclear factor erythroid-2 (E2)-related factor 2 (Nrf2) is a transcription factor that has been confirmed to act as an

important regulator of oxidative stress.[8] Normally, Nrf2 is held in the cytosol by its negative regulator, Kelch-like ECH-associated protein 1 (Keap1), and kept in an inactive complex. However, in response to oxidative stress, Nrf2 is released from Keap1 and translocates into the nucleus where it binds to antioxidant response elements in the promoter region of various antioxidant genes, such as heme-oxygenase-1 (HO-1), NAD(P)H dehydrogenase quinone-1 (NQO-1), and detoxifying enzymes.[8] In addition, Nrf2 also plays a role in the regulation of inflammation through interaction with nuclear factor kappa B, which is linked to inflammatory response and cytokine production.[9] Plenty of studies have proven that Nrf2 might be a therapeutic target for inhibiting oxidative stress and inflammation.

Eriodictyol, a flavonoid compound that exists in many fruits and vegetables, was proved to possess anti-inflammatory and antioxidative activity.[10,11] In addition, it has been reported that eriodictyol attenuates early retinal and plasma abnormalities in streptozotocin-induced diabetic rats.[12] Besides, several previous studies demonstrated that eriodictyol exerts its anti-inflammatory and antioxidative activity via regulating the Nrf2 pathway.[10,13] However, the role of eriodictyol in DR remains unclear. Thus, we examined the effects of eriodictyol on oxidative stress, inflammation, and apoptosis induced by high glucose in retinal ganglial cells (RGCs).

2 | MATERIALS AND METHODS

2.1 | Cell culture and treatment

The rat RGC-5 cells were provided by the Department of Ophthalmology, Second Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China). Cells were cultured in Dulbecco modified Eagle medium containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO), penicillin (100 U/mL; Sigma), and streptomycin (100 μg/mL; Sigma). The cells were grown in a saturated humidified incubator with 5% CO_2 at 37°C. For the glucose stimulation studies, the cells were incubated with 5.5 mM normal glucose (NG) or 33.3 mM high glucose (HG). For the eriodictyol treatment, cells were pretreated with different concentrations of eriodictyol (≥98.0%; Sigma; 5, 10, and 20 μM), followed by further exposure to NG or HG for 24 hours.

2.2 | Cell transfection

RGC-5 cells were transiently transfected with small interfering RNA (siRNA) targeting Nrf2 (si-Nrf2; Invitrogen, Thermo Fisher Scientific). After 24 hours, the transfected cells were subjected to HG with or without the presence of eriodictyol.

2.3 | Cell viability assay

The cell viability of RGC-5 cells was determined using the 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assay. RGC-5 cells (2×10^4 cells/well) were seeded on to 96-well plates and cultured for 24 hours. After prescribed treatments, MTT solution was added to each well and the samples were incubated for 4 hours. The formazan was dissolved by adding dimethyl sulfoxide (DMSO), and then the optical density was taken at 490 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek Instruments, Winooski, VT).

2.4 | Cell apoptosis assay

Cell apoptosis was determined using a Cell Death Detection ELISA Kit (Roche Applied Science, Indianapolis, IN). The cytosolic DNA-histone complex generated by the RGC-5 cells during apoptotic DNA fragmentation was assessed by incubating with antihistone antibody and anti-DNA antibody according to the manufacturer's protocol.

2.5 | Measurement of reactive oxygen species

Measurement of reactive oxygen species (ROS) was performed by monitoring the oxidation of 2′, 7′-dihydrodichlorofluorescein diacetate (H_2DCF-DA; Sigma) to dichlorofluorescein (DCF), which has the fluorescence activity. The RGC-5 cells were harvested and then treated with H_2DCF-DA (10 μM), and then the DCF fluorescence was analyzed by flow cytometry (Becton-Dickinson, San Jose, CA).

2.6 | Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨm) was measured using the lipophilic fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1; Cayman Chemicals, MI). After treatment, cells were washed with phosphate buffered saline (PBS), and ΔΨm was examined using a JC-1 Assay Kit according to manufacturer's instructions. JC-1 fluorescence was measured using a Becton-Dickinson FACScalibur analytical flow cytometer (BD Biosciences, San Jose, CA). The ratio of red (530 nm) to green (590 nm) fluorescence of JC-1 was calculated.

2.7 | Detection of superoxide dismutase, glutathione peroxidase, and catalase

The activities of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in the medium were assessed with the use of specific kits that are purchased from Beyotime Biotechnology (Shanghai, China) according to the manufacturer's protocol.

2.8 | Detection of tumor necrosis factor alpha and interleukin-8

Cell culture supernatants of RGC-5 cells with different treatments were centrifuged and collected. The levels of tumor necrosis factor alpha (TNF-α) and interleukin (IL)-8 in samples were detected using ELISA commercial kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. All assays were performed in triplicate and the mean values were then calculated.

2.9 | Western blot analysis

RGC-5 cells were washed with PBS and the cell lysates were prepared using ice-cold lysis buffer (150 mM NaCl, 0.5% deoxycholic acid, 50 mM Tris-HCl, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate [SDS], and 1% Nonider-P40) containing the protease inhibitor phenylmethanesulfonyl fluoride. The preparation of nuclear and cytoplasmic proteins was conducted using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Biotechnology). Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. After blocking with 5% skim milk in Tris-buffered saline with Tween 20 (TBS-T) buffer, the membrane was washed with TBS-T buffer for three times. The membrane was incubated with the primary antibodies bcl-2, bax, cleaved caspase-3, Nrf2, HO-1, and β-actin (1:500; Abcam, Cambridge, MA) at 4°C for 12 hours. After washing for three times, the membrane was additionally incubated with the corresponding horseradish peroxidase-bound secondary antibody (1:3000; Abcam) for 1 hour at room temperature. Chemoluminescence reagents (Thermo Fisher Scientific, Waltham, MA) were added for the visualization of the respective protein bands.

2.10 | Statistical analysis

All data were expressed as means ± standard deviation. One-way analysis of variance was performed to compare the statistical differences among multiple groups using GraphPad Prism 5.0 (San Diego, CA). *P* value less than 0.05 indicated a statistical significance.

3 | RESULTS

3.1 | Effect of eriodictyol on cell viability in HG-stimulated RGC-5 cells

First, we examined the effect of eriodictyol on cell viability in RGC-5 cells cultured with NG for 24 hours. The results of MTT assay showed that eriodictyol at the concentration of 5, 10, and 20 μM did not affect the viability of RGC-5 cells (Figure 1A). In addition, we observed that cell viability of RGC-5 cells treated with HG was markedly decreased when compared with the cells treated with NG. However, eriodictyol treatment improved cell viability in HG-stimulated RGC-5 cells, in a dose-dependent manner (Figure 1B).

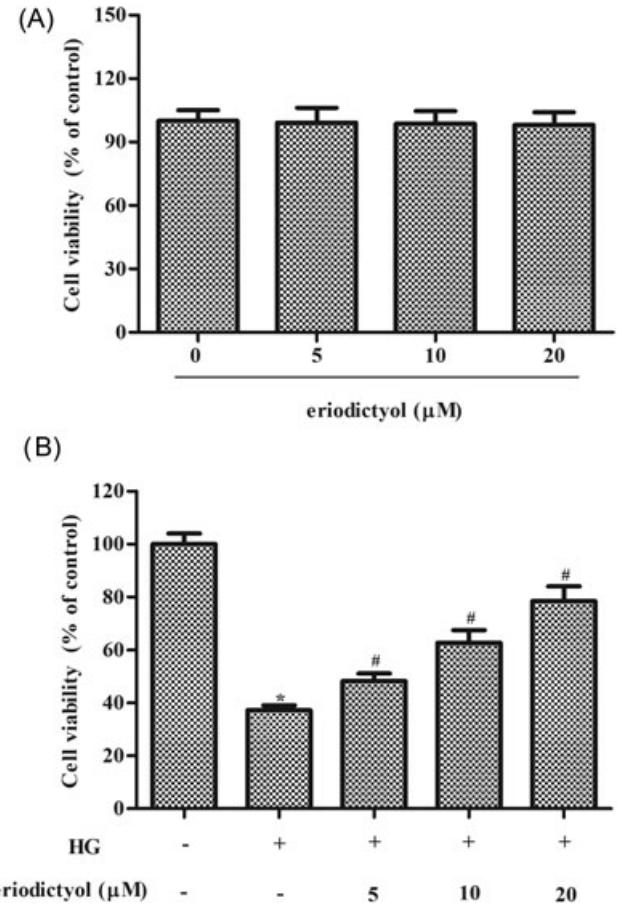

FIGURE 1 Eriodictyol improves cell viability in HG-stimulated RGC-5 cells. A, RGC-5 cells were stimulated with different concentrations of eriodictyol (0, 5, 10, and 20 μM) under NG condition for 24 hours. And then the cell viability was measured using MTT assay. B, RGC-5 cells were stimulated with NG or HG for 24 hours in the presence or absence of eriodictyol (5, 10, and 20 μM). Cell viability was measured using MTT assay. $^{*}P < 0.05$ versus control cells; $^{\#}P < 0.05$ versus HG-stimulated cells. HG, high glucose; MTT, 3-(4,5)-dimethylthiahiazo (-z-y1)-3, 5-di-phenytetrazoliumromide; NG, normal glucose; RGC, retinal ganglial cells

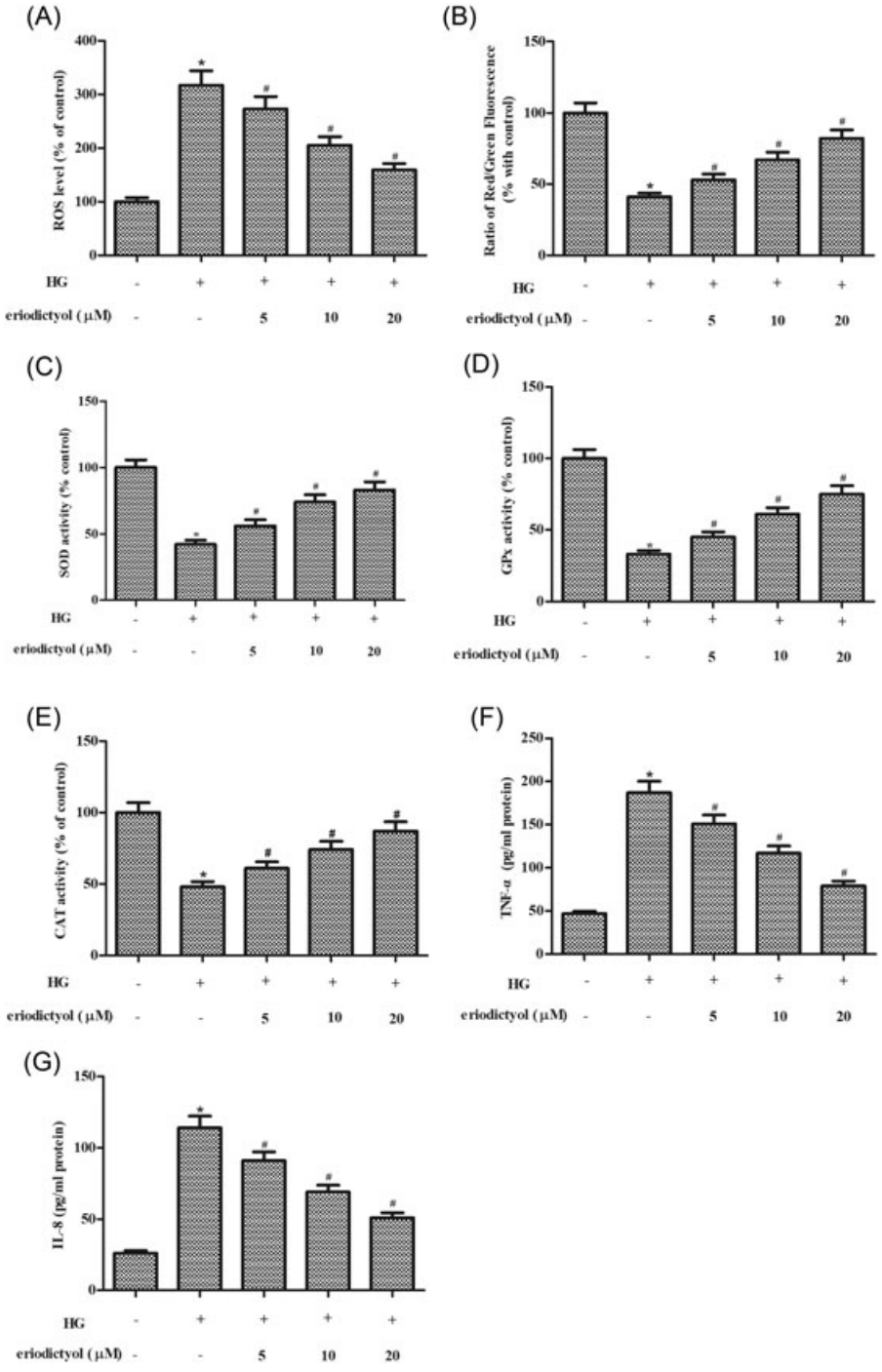

FIGURE 2 Eriodictyol inhibits oxidative stress and inflammatory response in HG-stimulated RGC-5 cells. RGC-5 cells were cultured with NG or HG plus different concentrations of eriodictyol (5, 10, and 20 μM) for 24 hours. Then, to evaluate the effect on HG-induced oxidative stress in RGC-5 cells, the production of ROS (A), the change of mitochondrial membrane potential (ΔΨm), and the activities of SOD (C), GPx (D) and CAT (E) were detected. The production of proinflammatory cytokines including TNF-α (F) and IL-8 (G) was determined using ELISA kits. $^{*}P < 0.05$ versus control cells; $^{\#}P < 0.05$ versus HG-stimulated cells. CAT, catalase; ELISA, enzyme-linked immunosorbent assay; GPx, glutathione peroxidase; HG, high glucose; IL-8, interleukin-8; MTT, 3-(4,5)-dimethylthiahiazo (-z-y1)-3, 5-di-phenytetrazoliumromide; NG, normal glucose; RGC, retinal ganglial cells; SOD, superoxide dismutase; TNF-α, tumor necrosis factor alpha

3.2 | Effects of eriodictyol on oxidative stress and inflammatory response in RGC-5 cells exposed to HG stimulation

To evaluate the effect on HG-induced oxidative stress in RGC-5 cells, the production of oxidative stress markers including ROS, SOD, GPx, and CAT were detected. As shown in Figure 2A, HG induced the production of ROS in RGC-5 cells, whereas the induction was attenuated by eriodictyol treatment (5, 10, and 20 μM). Eriodictyol also maintained the ΔΨm in HG-treated RGC-5 cells (Figure 2B). In addition, the activities of SOD, GPx, and CAT were decreased after HG stimulation. Eriodictyol treatment (5, 10, and 20 μM) increased the SOD, GPx, and CAT activities in response to HG stimulation (Figures 2C-E).

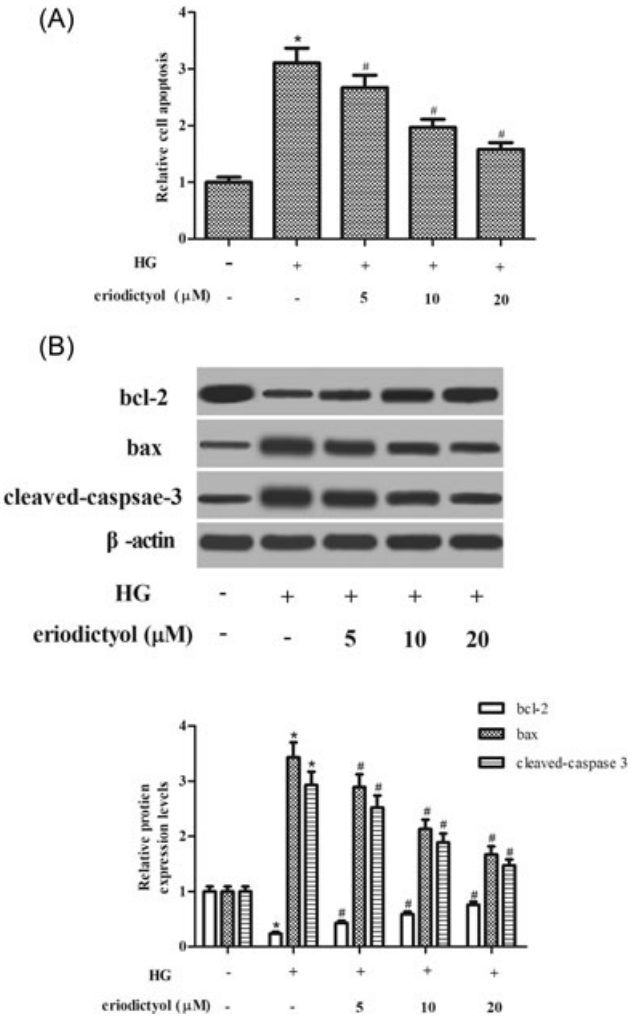

FIGURE 3 Eriodictyol represses cell apoptosis in HG-stimulated RGC-5 cells. RGC-5 cells were cultured with NG or HG plus different concentrations of eriodictyol (5, 10, and 20 μM) for 24 hours. A, Cell apoptosis was assessed using ELISA to detect cytosolic DNA-histone complex generated during apoptotic DNA fragmentation. B, Furthermore, the expressions of cell apoptosis related proteins including bax, bcl-2, and cleaved caspase-3 were measured by Western blot analysis. $^{*}P < 0.05$ versus control cells; $^{\#}P < 0.05$ versus HG-stimulated cells. ELISA, enzyme-linked immunosorbent assay; HG, high glucose; NG, normal glucose; RGC, retinal ganglial cells

To investigate the effect of eriodictyol on HG-induced inflammatory response, the production of proinflammatory cytokines including TNF-α and IL-8 was determined. The results of ELISA showed that HG caused significant increase in TNF-α and IL-8 production, however, the eriodictyol treatment mitigated the induction effect of HG (Figures 2F and 2G). The results indicated that eriodictyol suppressed the HG-induced inflammatory response in RGC-5 cells.

3.3 | Effect of eriodictyol on cell apoptosis in RGC-5 cells exposed to HG stimulation

Cell apoptosis was assessed by detecting cytosolic DNA-histone complex generated during apoptotic DNA fragmentation. As indicated in Figure 3A, cell apoptosis was markedly increased in the cells stimulated with HG, while the cell apoptosis was inhibited by eriodictyol treatment in a dose-dependent manner. Furthermore, the expressions of cell apoptosis-related proteins were measured by Western blot analysis. As shown in Figure 3B, the expressions of bax and cleaved caspase-3 were increased, while the bcl-2 expression was decreased in HG-induced RGC-5 cells compared with that in control cells. However, the changes in the expressions of bax, bcl-2, and cleaved caspase-3 were reversed by eriodictyol treatment.

3.4 | Effect of eriodictyol on the activation of Nrf2/HO-1 pathway in HG-stimulated RGC-5 cells

To assess the effect of eriodictyol on the activation of Nrf2/HO-1 pathway, the expression of HO-1 and the Nrf2 levels in cytoplasm and nucleus were measured by Western blot analysis. As shown in Figure 4A, the Nrf2 level in the nucleus was increased after HG stimulation. Besides, the expression of HO-1 was induced in HG-induced RGC-5 cells. The results indicated that HG-induced HO-1 expression and nuclear translocation of Nrf2. We also observed that eriodictyol treatment enhanced the increase in HO-1 expression and Nrf2 nuclear translocation, indicating that eriodictyol enhanced the activation of Nrf2/HO-1 pathway in HG-induced RGC-5 cells. To further investigate the role of the Nrf2/HO-1 signaling pathway in the protective effect of eriodictyol, RGC-5 cells were transfected with si-Nrf2. The expression levels of Nrf2 and HO-1 were significantly decreased after transfection with si-Nrf2 in RGC-5 cells (Figure 4D). In addition, knockdown of Nrf2 significantly attenuated the effects of eriodictyol on cell viability (Figure 4G), ROS level (Figure 4H), TNF-α production (Figure 4I) and cell apoptosis (Figure 4J) in RGC-5 cells.

4 | DISCUSSION

DR is the primary cause of visual impairment among the working-age population. Increasing evidence indicates that oxidative stress is the major mechanism associated with the pathogenesis of DR.[14,15] Under normal physiological conditions, ROS are continuously produced to support

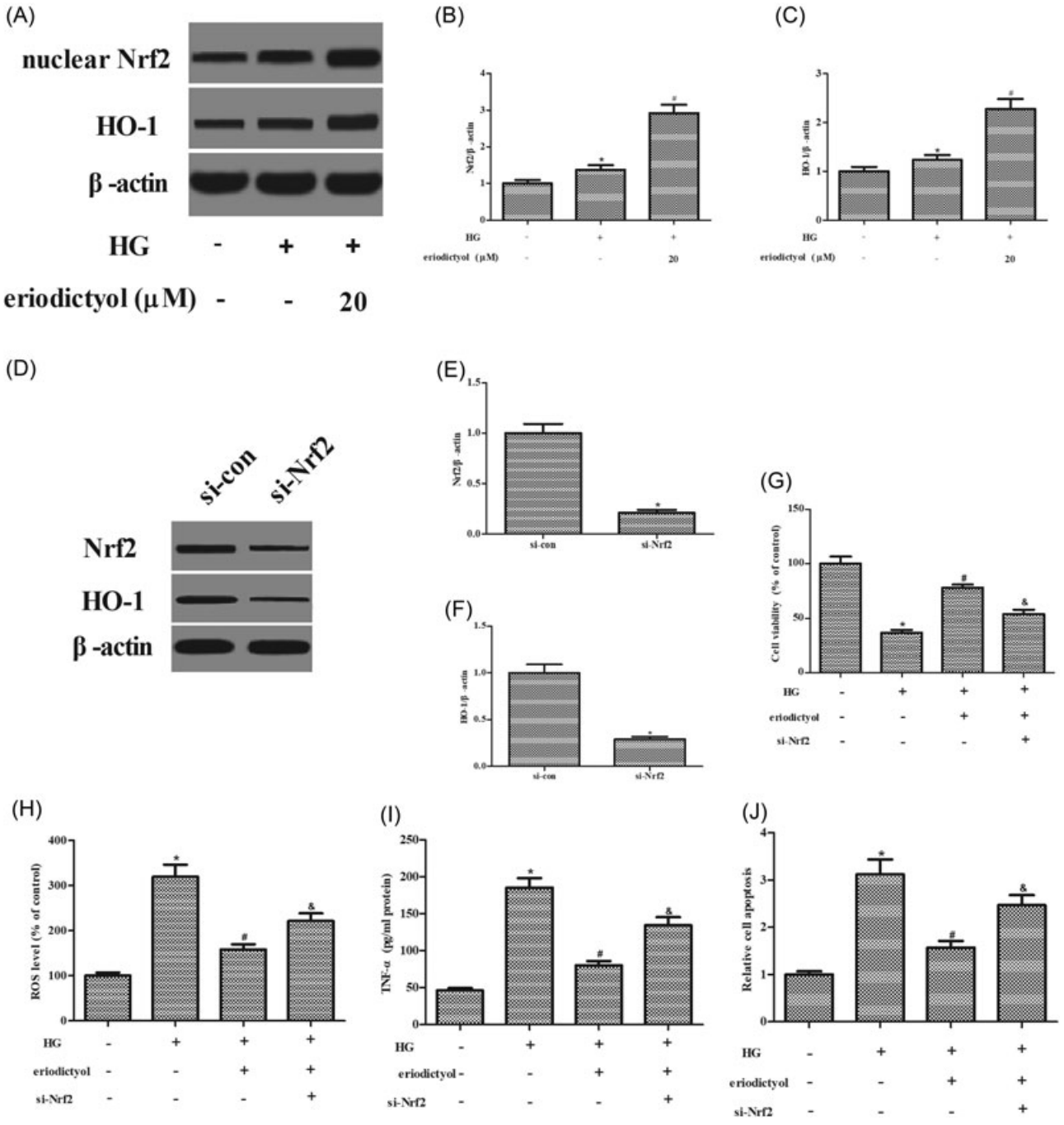

FIGURE 4 Eriodictyol induces the activation of Nrf2/HO-1 pathway in HG-stimulated RGC-5 cells. A, After eriodictyol (0, 5, 10, and 20 μM) and HG treatments, Western blot was performed to determine the expression of HO-1 and the Nrf2 levels in cytoplasm and nucleus. B and C, Quantification analysis was performed using Gel-Pro Analyzer version 4.0 software. D, RGC-5 cells were transiently transfected with si-Nrf2 for 24 hours. The protein expression levels of Nrf2 and HO-1 were detected using Western blot. E and F, Quantification analysis was performed using Gel-Pro Analyzer version 4.0 software. The transfected cells were subjected to HG with or without the presence of eriodictyol. G, Cell viability was measured using MTT assay. H and I, The production of ROS and TNF-α was determined using ELISA kits. J, Cell apoptosis was assessed. $^{*}P < 0.05$ versus control cells; $^{\#}P < 0.05$ versus HG-stimulated cells; $^{\&}P < 0.05$ versus HG+eriodictyol group. ELISA, enzyme-linked immunosorbent assay; HG, high glucose; HO-1, heme-oxygenase-1; MTT, 3-(4,5)-dimethylthiahiazo (-z-y1)-3, 5-di-phenytetrazoliumromide; Nrf2, nuclear factor erythroid-2 (E2)-related factor 2; RGC, retinal ganglial cells; ROS, reactive oxygen species; TNF-α, tumor necrosis factor alpha

normal cellular functions. However, ROS is excessively generated under HG condition.[14,16] It is well known that oxidative stress is a consequence of excess ROS generation, indicating a causal link between elevated glucose and increased oxidative stress.[16] ROS is capable to induce mitochondria dysfunctions, and further reduces the antioxidant defense capability, leading to an enhanced oxidative stress. The elevated oxidative stress leads to the damage of DNA, lipids, proteins, carbohydrates, and disruption in cellular homeostasis, and ultimately results in cell apoptosis.[14] Besides, ROS also activates some other metabolic pathways that contribute to the development of DR.[15] In addition, ROS is considered as a strong stimulus for the release of some proinflammatory cytokines, which play a major role in the activation of subsequent inflammatory responses.[17] Taken together, oxidative stress and inflammation are considered as critical contributors to the development of DR. Increasing evidence points to specific mechanism-based strategies for preventing DR by targeting both oxidative stress and inflammatory.

Eriodictyol, a flavonoid presented in citrus fruits, has been found to have anti-oxidative and anti-inflammatory effects.[10,18] Li et al[10] reported that eriodictyol elevated the activities of SOD, CAT, and GPx, as well as inhibited the production of malondialdehyde, thiobarbituric acid reactive substances (TBARS), TNF-α and IL-1β in kidney tissues.[10] Ferreira et al[18] demonstrated that eriodictyol combined with other citrus flavanones hesperidin and eriocitrin protects against inflammation and oxidative stress caused by high-fat diet in C57BL/6J mice, as evidenced by the increased total antioxidant capacity, and decreased IL-6, TBARS, macrophage chemoattractant protein-1, and C-reactive protein (hs-CRP) in serum. In addition, eriodictyol increased insulin-induced glucose uptake in human hepatocellular liver carcinoma cells (HepG2) and differentiated 3T3-L1 adipocytes under HG condition.[19] Moreover, eriodictyol attenuates the degree of retinal inflammation and plasma lipid peroxidation, and preserves the blood-retinal barrier (BRB) integrity in streptozotocin-induced diabetic rats.[12] These findings suggest that eriodictyol possesses antidiabetic property, which may be partially mediated by its antioxidative and anti-inflammatory effects. Therefore, we hypothesized that eriodictyol could be effective against DR. In the current study, we found that eriodictyol attenuated the reduced cell viability and increased cell apoptosis in HG-stimulated RGC-5 cells. Eriodictyol also inhibited the production of ROS and improved the activities of SOD, GPx, and CAT, as well as suppressed the production of TNF-α and IL-8.

Nrf2 is a redox sensitive transcription factor that plays an essential role in regulating the physiological response to oxidative stress via regulation of multiple genes encoding antioxidant proteins and phase II detoxifying enzymes.[8] During the development of DR, the role of Nrf2 antioxidant response pathway is to act as a cytoprotective mechanism in the retina to overcome oxidative stress.[20-22] In the human Müller glial cell line MIO-M1, Nrf2 inhibition by small interference RNA transfection significantly downregulates the antioxidant gene expression and exacerbates *tert*-butyl hydroperoxide and hydrogen peroxide-induced oxidative stress.[21] On the contrary, Nrf2 activation strongly induces the expressions of Nrf2 target gene and inhibits the oxidant-induced ROS production.[21] Compared with wild-type mice, superoxide levels are significantly increased in Nrf2 knockout streptozotocin-induced diabetic mice. Diabetic Nrf2 knockout mice also exhibit a decrease in retinal glutathione and an increase in TNF-α level when compared with wild-type mice. Furthermore, the early onset of BRB dysfunction and exacerbation of neuronal dysfunction are observed in diabetic Nrf2 knockout mice.[21] In view of its important roles, Nrf2 is used as a new therapeutic target for the DR treatment. Our work proved that eriodictyol enhanced the nuclear translocation of Nrf2 and elevated the expression of antioxidant enzyme HO-1, indicating that eriodictyol induced the activation of the Nrf2/HO-1 pathway in HG-induced RGC-5 cells.

In summary, the results suggested that the natural flavonoid compound, eriodictyol, suppressed the HG-induced oxidative stress, inflammation, and apoptosis in RGC-5 cells. Eriodictyol enhanced the activation of the Nrf2/HO-1 pathway in HG-induced RGC-5 cells. These findings suggested that eriodictyol protected the RGC-5 cells from HG-induced injury through regulating the Nrf2/HO-1 pathway, indicating that eriodictyol might be a new therapeutic strategy for the management of DR.

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CONFLICTS OF INTEREST

There is no conflicts of interest to be declared by the authors.

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