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Paeoniflorin Protects Retinal Pigment Epithelial Cells from High Glucose-Induced Oxidative Damage by Activating Nrf2-Mediated HO-1 Signaling

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Abstract

Oxidative stress due to hyperglycemia damages the functions of retinal pigment epithelial (RPE) cells and is a major risk factor for diabetic retinopathy (DR). Paeoniflorin is a monoterpenoid glycoside found in the roots of *Paeonia lactiflora* Pall and has been reported to have a variety of health benefits. However, the mechanisms underlying its therapeutic effects on high glucose (HG)-induced oxidative damage in RPE cells are not fully understood. In this study, we investigated the protective effect of paeoniflorin against HG-induced oxidative damage in cultured human RPE ARPE-19 cells, an *in vitro* model of hyperglycemia. Pretreatment with paeoniflorin markedly reduced HG-induced cytotoxicity and DNA damage. Paeoniflorin inhibited HG-induced apoptosis by suppressing activation of the caspase cascade, and this suppression was associated with the blockade of cytochrome c release to cytoplasm by maintaining mitochondrial membrane stability. In addition, paeoniflorin suppressed the HG-induced production of reactive oxygen species (ROS), increased the phosphorylation of nuclear factor erythroid 2-related factor 2 (Nrf2), a key redox regulator, and the expression of its downstream factor heme oxygenase-1 (HO-1). On the other hand, zinc protoporphyrin (ZnPP), an inhibitor of HO-1, abolished the protective effect of paeoniflorin against ROS production in HG-treated cells. Furthermore, ZnPP reversed the protective effects of paeoniflorin against HG-induced cellular damage and induced mitochondrial damage, DNA injury, and apoptosis in paeoniflorin-treated cells. These results suggest that paeoniflorin protects RPE cells from HG-mediated oxidative stress-induced cytotoxicity by activating Nrf2/HO-1 signaling and highlight the potential therapeutic use of paeoniflorin to improve the symptoms of DR.

Key Words: Paeoniflorin, Retinal pigment epithelial cells, High glucose, Oxidative stress, Nrf2/HO-1

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INTRODUCTION

Paeoniae Radix, the root of *Paeonia lactiflora* Pall., is a traditional herbal medicine in East Asian countries, including Korea, and is used to treat a variety of conditions, including dementia, inflammation, pain disorders, traumatic injuries, and immune disorders (Mu *et al*., 2024; Zhou *et al*., 2024). This herbal medicine contains glycosides, phenolic acids, tannins, flavones, steroids, and essential oils, and glycosides are considered to be the main active ingredients (Li *et al*., 2021; Ma *et al*., 2024). Glucosides extracted from Paeoniae Radix have been shown to have a wide range of health benefits, including antioxidant, antifibrotic, anti-inflammatory, and immunomodulatory properties (Lu *et al*., 2024; Wang *et al*., 2024; Xu *et al*., 2024). For example, in the context of ocular diseases, these glucosides have been reported to improve eye dryness and fatigue and to prevent ocular diseases, such as dry eye due to Sjögren's syndrome in humans and non-obese diabetic mouse models (Li *et al*., 2013; Jiang *et al*., 2023; Cui *et al*., 2024).

Paeoniflorin, a water-soluble monoterpene glucoside, is also found in the aquatic fern Salvinia molesta but is the major glucoside in Paeoniae Radix (Choudhary *et al*., 2008; Li *et al*., 2021; Ma *et al*., 2024). Recently, Sun *et al*. (2024) reported that paeoniflorin effectively alleviates diabetic retinopathy (DR) by suppressing the expression of vascular endothelial growth factor A and attenuating high glucose (HG)-induced cytotoxicity and inflammatory responses in human retinal pigment epithelial (RPE) cells, which physiologically and structurally support photoreceptors. This result supports previous studies, which showed that paeoniflorin has an anti-inflammatory effect on 4-hydroxynonenal, a major by-product of oxidative stress caused by lipid peroxidation in RPE cells (Yang *et al*., 2019). Paeoniflorin has also been reported to reduce all-trans-retinal (atRAL)-induced mitochondrial dysfunction and endoplasmic reticulum stress in RPE cells, and thus, to attenuate atRAL-induced cell damage achieved through the inhibition of NADPH oxidase 1-derived reactive oxygen species (ROS) generation and the activation of AMP-activated kinase signaling (Zhu *et al*., 2018). Furthermore, paeoniflorin abrogated the expression of hyperosmolar-induced inflammatory factors in human corneal epithelial cells and significantly reduced dry eye symptoms by reducing tear production, ocular surface inflammation, and corneal epithelial detachment in a mouse model (Zhao *et al*., 2019). In addition, paeoniflorin attenuated extracellular matrix remodeling by reducing the oxidative stress induced by transforming growth factor-β 2, a multifunctional profibrotic cytokine, in trabecular meshwork cells, which play a key role in determining intraocular pressure values (Hu *et al*., 2024a). Meanwhile, Zeng *et al*. (2022) reported that paeoniflorin might reduce diabetic cataract formation by inhibiting oxidative damage and epithelial-mesenchymal transition by activating Sirtuin 1 in HG-exposed lens epithelial cells. In addition, Zhu *et al*. (2017) suggested that paeoniflorin might have a preventive effect on DR by upregulating cytokine signaling 3 expression and downregulating matrix metalloproteinase-9 activity in HG-treated retinal microglia and streptozotocin-induced diabetic mice. These results suggest that paeoniflorin protects major eye cells, including human RPE cells, from various harmful factors that might impede the initiation and progression of DR.

Under diabetic conditions, increased blood glucose levels activate several biochemical pathways that enhance retinal cell glucose uptake and metabolism, and thus, induce oxidative stress through ROS generation (Goldney *et al*., 2023; Zhang *et al*., 2024). Although paeoniflorin has been reported to block hydrogen peroxide (H_2O_2)-induced oxidative stress in human RPE cells (Wankun *et al*., 2011), no study has been conducted to determine whether it can protect against HG-induced oxidative stress. Recently, there has been increasing recognition of the importance of nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that neutralizes ROS and restores redox balance, as a major target of paeoniflorin. For example, paeoniflorin attenuated acute lung injury caused by particulate matter by inhibiting oxidative stress and inflammation-mediated pyroptosis by activating the Nrf2 signaling pathway (Zhou *et al*., 2024). The importance of this Nrf2 intervention has been demonstrated in various disease models, including oxidative stress-induced acute kidney injury, cardiac hypertrophy, ischemic cardiovascular disease, neurological disease, photodamage, vitiligo, brain injury, and lung injury models (Lu *et al*., 2020; Wang *et al*., 2020, 2021a; Jiang *et al*., 2021; Ren *et al*., 2023; Xing *et al*., 2023). Interestingly, paeoniflorin inhibited the HG-induced apoptosis of Schwann cells, which directly intervene in the induction of diabetic peripheral neuropathy by blocking the production of ROS through Nrf2 activation (Yang *et al*., 2016). Nevertheless, the involvement of Nrf2 in the protective effect of paeoniflorin against HG-mediated oxidative stress-induced cell damage in ocular cells, including RPE cells, has not been adequately addressed. Therefore, we aimed to establish the role played by Nrf2 in the protective effect of paeoniflorin against HG-induced oxidative damage in human RPE ARPE-19 cells.

MATERIALS AND METHODS

Cell culture and treatment

ARPE-19 cells (American Type Culture Collection, Manassas, VA, USA) were cultured as described previously (Park *et al*., 2024). All the materials used for cell culture were purchased from WelGENE (Gyeongsan, Korea). A stock solution (100 mM) of paeoniflorin (Sigma-Aldrich Co., St. Louis, MO, USA) was prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich Co.) and diluted to the required concentrations with culture medium before treating cells. The highest concentration of DMSO in the medium treated with stock solution of paeoniflorin was less than 0.05%, which did not cause cytotoxicity. Cells were cultured in media containing different concentrations of paeoniflorin and D-(+)-glucose (Sigma-Aldrich Co.) for 48 h or pretreated with 100 μM paeoniflorin alone or together with 10 μM zinc protoporphyrin (ZnPP, Sigma-Aldrich Co.) for 2 h and then treated with 30 mM D-(+)-glucose for 48 h.

Cytotoxicity assay

An MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Thermo Fisher Scientific, Waltham, MA, USA) assay was used to evaluate the effect of paeoniflorin on HG-induced cytotoxicity, as previously described (Kang *et al*., 2024a), and an LDH Activity Assay Kit (Sigma-Aldrich Co.) was used to determine lactate dehydrogenase (LDH) release.

Apoptosis analysis by flow cytometry

Apoptotic cells were quantified using an Annexin V/Propidium Iodide (PI) Apoptosis Detection Kit (BD Bioscience,

Franklin Lakes, NJ, USA). Briefly, cells exposed to HG in the presence or absence of paeoniflorin were collected, washed with phosphate-buffered saline (PBS), suspended in binding buffer, and treated with Annexin V-FITC and PI buffer for 20 min (Jeon *et al*., 2024a). Cell suspensions were subjected to flow cytometry (BD Accuri™ C6 Plus Flow Cytometer, BD Biosciences).

Analysis of apoptosis based on nuclear morphological changes

4′,6′-diamidino-2-phenylindole (DAPI) staining was used to observe nuclear morphological changes. Cells cultured under different conditions were fixed with paraformaldehyde solution (Sigma-Aldrich Co.) and then stained with 1 μg/mL DAPI solution (Thermo Fisher Scientific) at room temperature (RT). DAPI-stained nuclei morphologies were observed under a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Apoptosis analysis by DNA fragmentation detection

To detect DNA fragmentation, cells were lysed in lysis buffer (5 mM ethylene-diamine-tetraacetic acid, 10 mM Tris-HCl [pH 7.4], 0.5% Triton X-100, 0.1 mg/mL proteinase K, and 150 mM NaCl) for 30 min at RT. DNA in supernatants was extracted using a phenol-chloroform-isoamyl alcohol mixture (Sigma-Aldrich Co.), precipitated with ethanol, subjected to electrophoresis on agarose gel at 70 V, stained with ethidium bromide (EtBr, Sigma-Aldrich Co.), and observed under ultraviolet (UV) light using a microplate reader (FilterMax F3/F5 Multi-Mode Microplate Reader, Molecular Devices, Sunnyvale, CA, USA).

Protein isolation and immunoblotting

Whole cell lysates of cells cultured under various conditions were prepared, as previously described (Kang *et al*., 2024b). Mitochondrial and cytoplasmic fractions were isolated using a Mitochondria/Cytosol Fractionation Kit (Sigma-Aldrich Co.). Equal amounts of protein extracted from cells in each treatment group were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to membranes (Bio-Rad Lab., Hercules, CA, USA), which were hybridized then with primary antibodies against the target proteins and incubated with secondary antibodies conjugated to horseradish peroxidase. Proteins were detected using an Enhanced Chemiluminescence (ECL) Detection Kit Sigma-Aldrich Co.). The primary (Table 1) and secondary antibodies used for immunoblotting were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Cell Signaling Technology (Danvers, MA, USA), Thermo Fisher Scientific and Abcam (Cambridge, UK). Cytochrome c oxidase subunit IV (COX IV) and β-actin were used as loading controls for mitochondrial and cytosolic fractions, respectively.

Caspase-3 activity assay

Caspase-3 activity was measured using a Caspase-3 Assay Kit (Abcam), utilizing the hydrolysis of fluorescent substrate peptides by activated caspases. Briefly, after resuspending cells in the cell lysis buffer provided, supernatants were reacted with substrates, and the concentrations of p-nitroaniline released from substrates were determined using a microplate reader (Park *et al*., 2024).

DNA damage analysis using the comet assay

A Comet Assay Kit (Trevigen, Gaithersburg, MD, USA) was used to determine whether DNA strand breaks were induced in cell nuclei. Briefly, cells exposed to HG in the presence or absence of paeoniflorin were collected and subjected to the comet assay. Randomly selected images were acquired using a fluorescence microscope.

Mitochondrial membrane potential (MMP) assay

5,5,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimi-dazoylcarbocyanine iodide (JC-1) fluorescent dye was used to analyze MMPs, which are indicators of mitochondrial membrane stability. Collected cells were stained with 10 μM JC-1 (Thermo Fisher Scientific) for 30 min at RT. JC-1 aggregates and monomer frequencies were promptly monitored by flow cytometry, as previously described (Ni *et al*., 2024).

ROS generation assay

Intracellular ROS levels were analyzed by 2’,7’-dichlorofluorescein diacetate (DCF-DA) staining to assess the antioxidant activity of paeoniflorin. In brief, harvested cells were incubated with 10 μM DCF-DA solution (Thermo Fisher Scientific), and ROS levels were measured by flow cytometry (Jeon *et al*., 2024b). Cells stained with DCF-DA were also observed under a fluorescence microscope to assess ROS levels.

Statistical analyses

The results are presented as mean ± standard deviation (SD) of at least three independent experiments. All statistical analyses were performed using one-way ANOVA and Tukey’s *post hoc* test in GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA), with a p value of <0.05 indicating sta-

Table 1. List of antibodies used in this study

Antibody	Species raised	Dilution	Product Code	Source
Caspase-3	Rabbit polyclonal	1:1000	#9662	Cell Signaling Technology Inc.
PARP	Mouse monoclonal	1:1000	sc-8007	Santa Cruz Biotechnology Inc.
γH2AX	Mouse monoclonal	1:500	MA1-2022	Thermo Fisher Scientific Inc.
Cytochrome *c*	Mouse monoclonal	1:1000	sc-13560	Santa Cruz Biotechnology Inc.
Nrf2	Mouse monoclonal	1:1000	sc-518036	Santa Cruz Biotechnology Inc.
p-Nrf2	Rabbit polyclonal	1:500	PA5-67520	Thermo Fisher Scientific Inc.
Keap1	Mouse monoclonal	1:1000	ab119403	Abcam
HO-1	Mouse monoclonal	1:1000	sc-136960	Santa Cruz Biotechnology Inc.
COX IV	Rabbit polyclonal	1:1000	#4844	Cell Signaling Technology Inc.
β-actin	Mouse monoclonal	1:1000	sc-47778	Santa Cruz Biotechnology Inc.

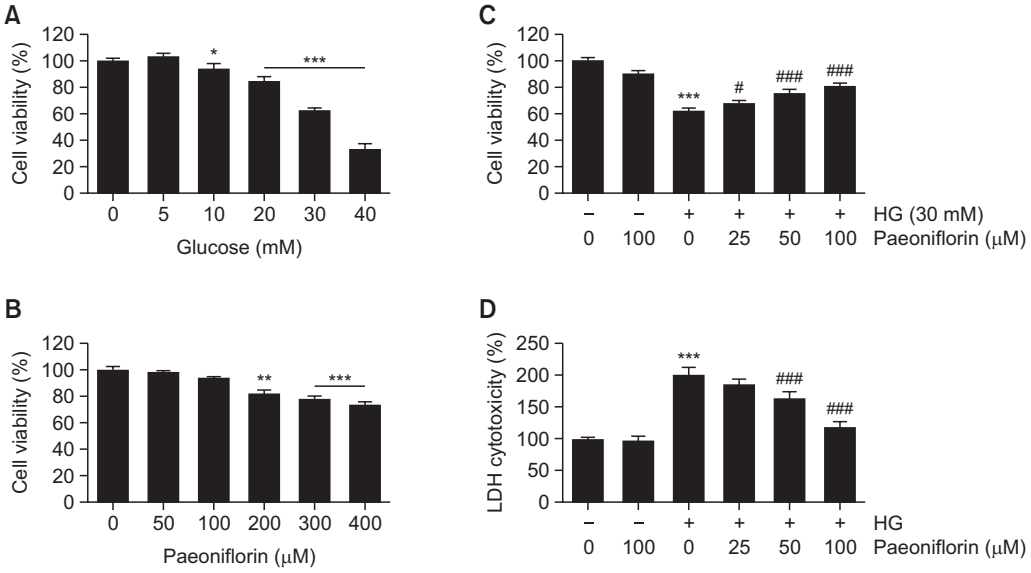

Fig. 1. Suppression of HG-induced cytotoxicity by paeoniflorin in ARPE-19 cells. Cells were treated with different concentrations of glucose (0, 5, 10, 20, 30 and 40 mM, A) or paeoniflorin (0, 50, 100, 200, 300 and 400 μM, B) for 48 h, or with the indicated concentrations of paeoniflorin for 2 h, and then treated with HG (30 mM D-(+)-glucose) for 48 h (C). Cell viability was determined using an MTT assay. (D) Relative levels of LDH released into cell supernatant were determined using an LDH activity assay kit. Values are the means ± SD for at least three independent experiments, and analysis of variance followed by Tukey's *post hoc* test showed significant differences (**p*<0.05, ***p*<0.01 and ****p*<0.001 *vs.* control cells; $^{\#}p$<0.05 and $^{\#\#\#}p$<0.001 *vs.* HG-treated cells).

tistical significance.

RESULTS

Inhibition of HG-induced cytotoxicity by paeoniflorin

MTT assay results showed that the viability of ARPE-19 cells cultured in a medium containing glucose or paeoniflorin gradually decreased concentration-dependently (Fig. 1A, 1B). Based on these results, the hyperglycemic concentration was set at a glucose concentration of 30 mM, which resulted in a viability of ~60%. The pretreatment concentration of paeoniflorin used to investigate its protective effect against HG conditions was ≤ 100 μM, at which it did not affect cell viability. Pretreatment with paeoniflorin at concentrations below 100 μM attenuated the HG-mediated reduction in cell viability in a concentration-dependent manner (Fig. 1C). Paeoniflorin also significantly blocked LDH leakage, a marker of cell damage, in HG-treated cells (Fig. 1D). These results demonstrate that paeoniflorin effectively blocks HG-induced cytotoxicity in ARPE-19 cells.

Reduction of HG-induced apoptosis by paeoniflorin

We investigated whether paeoniflorin suppresses cytotoxicity under HG conditions by inhibiting HG-induced apoptosis. Flow cytometric analysis by annexin V/PI staining showed that the proportion of annexin-positive cells, an indicator of apoptosis, significantly increased in HG-treated cells (Fig. 2A) and that pretreatment with paeoniflorin significantly reduced this proportion in a concentration-dependent manner. Similarly, DAPI staining results revealed that compared with normal cells, cells cultured under HG conditions showed increased nuclear morphological changes characteristic of apoptosis, such as nuclear fragmentation, chromatin condensation, and increased apoptotic bodies, and that these apoptotic features were significantly reduced in paeoniflorin pretreated cells (Fig. 2B, 2C). We also investigated whether DNA fragmentation occurred in cells treated with HG and pretreated or not with paeoniflorin. Furthermore, oligonucleosomal-sized DNA fragments, a characteristic of apoptosis, were significantly increased in HG-treated cells but not in paeoniflorin-pretreated cells (Fig. 2D). In addition, the expression of the pro-form of caspase-3, a key effector of caspase-dependent apoptosis, was reduced by paeoniflorin, whereas the enzymatic activity and degradation of poly(ADP-ribose) polymerase (PARP), a key caspase-3 substrate, were increased in HG-treated cells (Fig. 2E, 2F). However, caspase-3 activation and PARP cleavage were substantially attenuated in the presence of paeoniflorin. Thus, the blockade of HG-induced cytotoxicity by paeoniflorin in ARPE-19 cells was found to be closely correlated with the inhibition of apoptosis.

Alleviation of DNA damage caused by HG by paeoniflorin

We also evaluated whether the reduction of HG-induced cytotoxicity and apoptosis by paeoniflorin was related to the inhibition of DNA damage. The expression of the phosphorylated form of H2AX (γH2AX), which is upregulated by increases in double-strand DNA breaks, was increased after HG treatment (Fig. 2E), and the formation of comet tails, an indicator of DNA double-helix breakage, was markedly increased in HG-treated cells (Fig. 2G). However, these expressional increases in DNA damage markers were largely abolished by paeoniflorin. These results suggest that the protective effect of paeoniflorin on ARPE-19 cells exposed to HG was due to the suppression of apoptosis and DNA damage.

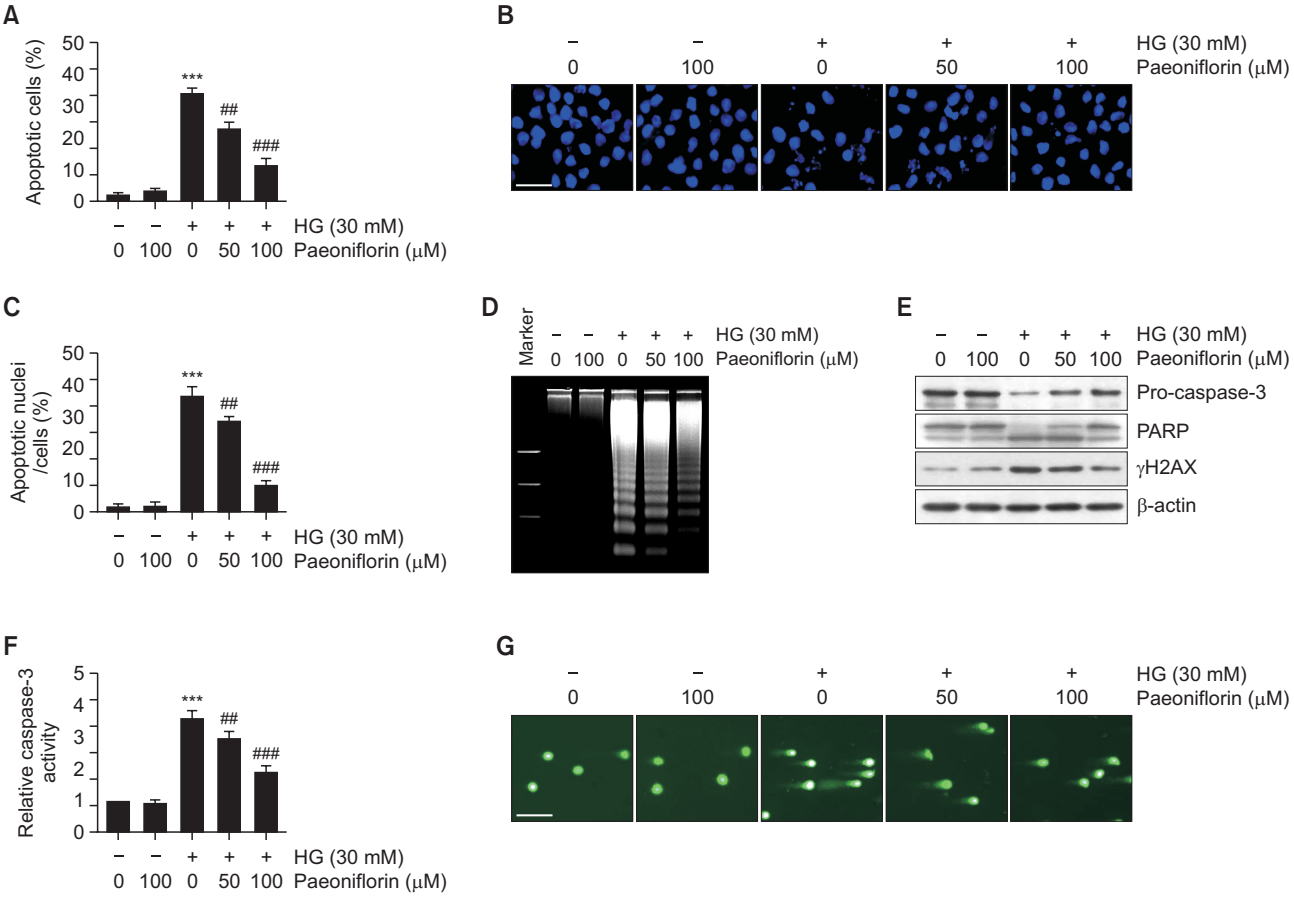

Fig. 2. Amelioration of HG-induced apoptosis and DNA damage by paeoniflorin in ARPE-19 cells. Cells were pretreated with or without paeoniflorin (0, 50 and 100 μM) for 2 h and then stimulated with HG (30 mM D-(+)-glucose) for 48 h. (A) Apoptosis was analyzed by annexin V/PI staining. Each number represents the total frequency of cells in the early stage (annexin V positive) of apoptosis and cells in the late stage (annexin V and PI double positive) of apoptosis. (B, C). Morphological changes in nuclei were observed using a fluorescence microscope after DAPI staining (1 μg/mL, scale bar 75 μm). Representative images (B) and the proportion of apoptotic nuclei (C). (D) DNA was isolated from cells and separated by agarose gel electrophoresis to visualize DNA fragmentation. (E) Changes in caspase-3, PARP, and γH2AX expressions were determined using total protein levels. β-actin served as a loading control. (F) Caspase-3 activity was assessed using a commercially available kit. (G) Extent of DNA damage was determined using a Comet assay (scale bar 50 μm). (A, C) Values are the means ± SD for at least three independent experiments, and analysis of variance followed by Tukey's *post hoc* test showed significant differences (***p<0.001 *vs.* control cells; $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001 *vs.* HG-treated cells).

Attenuation of HG-induced mitochondrial impairment by paeoniflorin

Changes in MMP, an indicator of mitochondrial stability, were investigated to determine whether paeoniflorin-mediated protection against HG-induced cytotoxicity was due to the maintenance of mitochondrial homeostasis. Flow cytometric analysis by JC-1 staining revealed an increase in the proportion of JC-1 monomers in HG-treated cells (Fig. 3A), indicating loss of MMP due to mitochondrial depolarization, and this effect was impeded by paeoniflorin pretreatment. In addition, the expression of cytochrome c, which is present in the space between the outer and inner mitochondrial membranes in normal cells, was upregulated in the cytosolic fraction but downregulated in the mitochondrial fraction of HG-treated cells, and these effects were effectively blocked by paeoniflorin (Fig. 3B). These results show that paeoniflorin enhanced the mitochondrial stability of ARPE-19 cells under HG conditions.

Inhibition of ROS production and activation of Nrf2 signaling in HG-treated cells by paeoniflorin

The effect of paeoniflorin on HG-induced ROS production was investigated to determine whether the antioxidant activity of paeoniflorin was responsible for its protective effect on HG-induced cytotoxicity. Fluorescence microscopy of DCF-DA stained cells showed that paeoniflorin pretreatment marked reduced HG-induced increases in intracellular ROS levels (Fig. 4A), and flow cytometry results concurred (Fig. 4B). In addition, changes in the expressions of Nrf2 and heme oxygenase 1 (HO-1, a representative downstream antioxidant enzyme of Nrf2) were used to determine whether Nrf2/HO-1 signaling was involved in the ROS-scavenging effect of paeoniflorin. Immunoblotting results demonstrated that the expression of phosphorylated Nrf2 (p-Nrf2; the activated form of Nrf2) was slightly increased in cells treated with paeoniflorin or HG alone but significantly increased in cells treated with paeoniflorin plus HG. However, the total protein level of Nrf2 remained un-

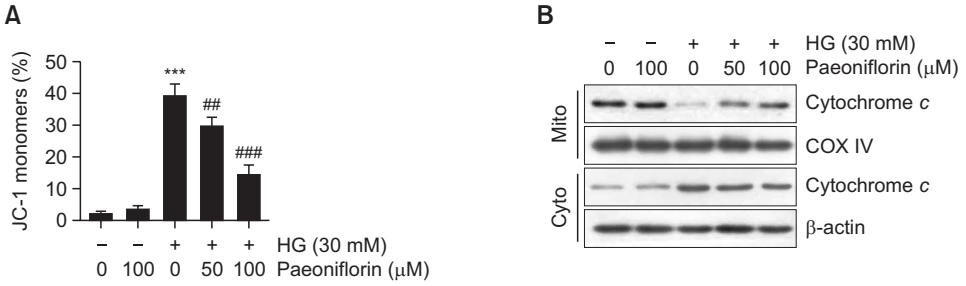

Fig. 3. Attenuation of HG-induced mitochondrial dysfunction and cytosolic release of cytochrome c by paeoniflorin in ARPE-19 cells. (A) Cells were treated with HG (30 mM D-(+)-glucose) in the presence or absence of paeoniflorin (0, 50 and 100 μM) for 48 h, JC-1 stained, and subjected to followed by flow cytometry. Changes in JC-1 monomer ratio (indicating MMP loss) are shown. JC-1, a cationic carbocyanine dye, shows voltage-dependent accumulation in mitochondria and begins to form J aggregates in mitochondria. Since it remains as a monomer upon depolarization of the mitochondrial membrane, the high frequency of monomers in paeoniflorin-treated cells indicates the loss of MMP. Values are the means ± SD for at least three independent experiments, and analysis of variance followed by Tukey's *post hoc* test showed significant differences (***p<0.001 *vs.* control cells; $^{\#\#}p$<0.01 and $^{\#\#\#}p$<0.001 *vs.* HG-treated cells). (B) Changes in cytochrome c expression were analyzed using mitochondrial (Mito) and cytosolic fractions (Cyto). COX IV and β-actin were used as loading controls for the mitochondrial and cytosolic fractions, respectively.

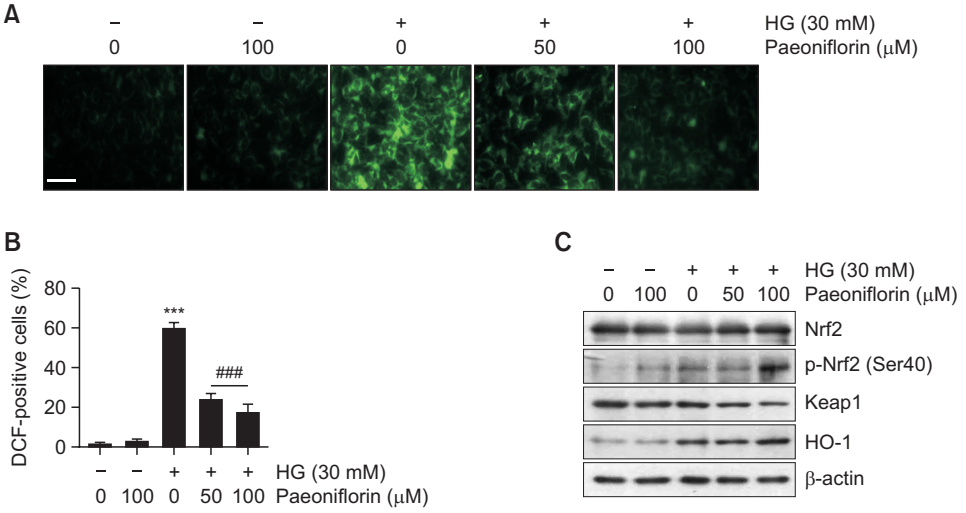

Fig. 4. Attenuation of ROS production and induction of Nrf2-mediated HO-1 by paeoniflorin in HG-treated ARPE-19 cells. Cells were pretreated with or without paeoniflorin (0, 50 and 100 μM) for 2 h and then stimulated with HG (30 mM D-(+)-glucose) for 1 h (A, B) or 48 h (C). (A) After DCF-DA staining, fluorescence intensities, representing ROS production, were measured under a fluorescence microscope (scale bar 50 μm). (B) Changes in intracellular ROS levels were investigated by flow cytometry after DCF-DA staining. Values are the means ± SD for at least three independent experiments, and analysis of variance followed by Tukey's *post hoc* test showed significant differences (***p<0.001 *vs.* control cells; $^{\#\#\#}p$<0.001 *vs.* HG-treated cells). (C) Changes in the expressions of Nrf2, HO-1, and Keap1 were investigated using total proteins isolated from cells.

changed, whereas that of Kelch-like ECH-associated protein 1 (Keap1), a negative regulator of Nrf2, decreased (Fig. 4C). Therefore, these results suggest that the antioxidant activity of paeoniflorin against HG in ARPE-19 cells is related to the activation of Nrf2-mediated HO-1.

Role of HO-1 in the antioxidant activity and preservation of mitochondrial function of paeoniflorin under HG conditions

Because the expression and activity of HO-1 were increased after co-treating cells with HG and paeoniflorin, we also investigated whether HO-1 activation was involved in the antioxidant activity of paeoniflorin. Inhibition of HG-induced ROS accumulation by paeoniflorin was significantly reduced in the presence of ZnPP, a pharmacological inhibitor of HO-1 (Fig. 5A). Additionally, the paeoniflorin-induced maintenance of mitochondrial membrane stability and inhibition of cytochrome c efflux from mitochondria to cytosol in HG-treated ARPE-19 cells were lost when HO-1 was inhibited (Fig. 5B, 5C), demonstrating that the antioxidative effect of paeoniflorin in ARPE-19 cells under HG conditions was mediated by the activation of HO-1, and thus, by the inhibition of mitochondrial damage.

Attenuation of the protective effect of paeoniflorin against HG-induced cytotoxicity by inhibition of HO-1 activity

Since the antioxidant and mitochondrial protective effects

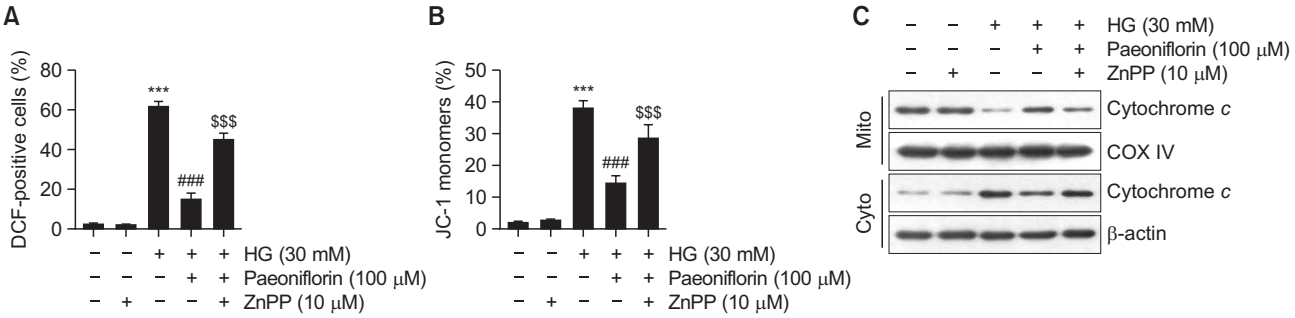

Fig. 5. Loss of the ROS scavenging and mitochondrial protective effects of paeoniflorin after inhibiting HO-1 activity in HG-treated ARPE-19 cells. Cells were pretreated with 100 μM paeoniflorin and 10 μM ZnPP for 2 h and then stimulated with HG (30 mM D-(+)-glucose) for 1 h (A, B) or 48 h (C-E). (A) Changes in intracellular ROS levels were measured by DCF-DA staining. (B) Flow cytometric analysis was performed on JC-1-stained. (A, B) Values are the means ± SD for at least three independent experiments, and analysis of variance followed by Tukey's *post hoc* test showed significant differences (***p<0.001 *vs.* control cells; ###p<0.001 *vs.* HG-treated cells; $$$$p$<0.001 *vs.* HG and paeoniflorin-treated cells). (C) Changes in cytochrome c expression were analyzed in mitochondrial and cytosolic fractions isolated from cells.

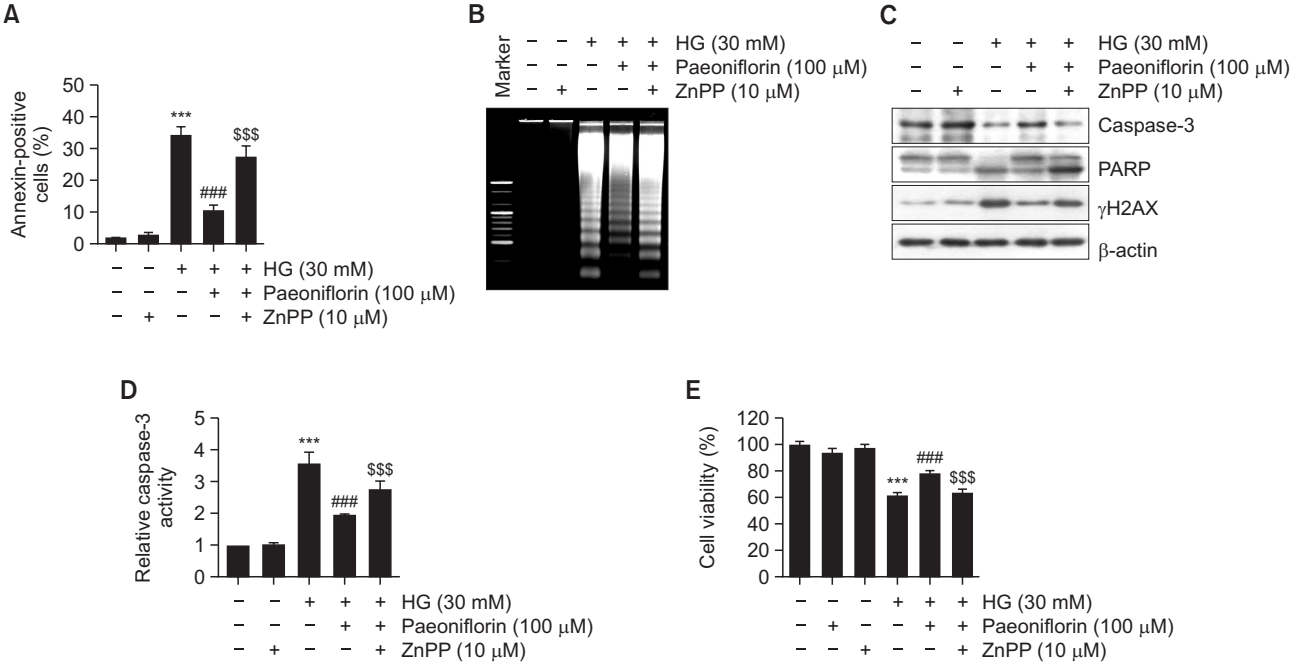

Fig. 6. Abrogation of the protective effect of paeoniflorin against HG-induced apoptosis and cytotoxicity by HO-1 inhibition in ARPE-19 cells. Cells were pretreated with 100 μM paeoniflorin and 10 μM ZnPP for 2 h and then stimulated with HG (30 mM D-(+)-glucose) for 48 h. (A) The average degree of apoptosis (annexin V-positive cells) determined by Annexin V/PI staining is shown. (B) DNA fragmentation was visualized by agarose gel electrophoresis. (C) Caspase-3, PARP, and γH2AX expressions were determined using total proteins. (D) Caspase-3 activity was assessed using a commercial assay kit. (E) Cell viabilities were determined using an MTT assay. (A, D, E) Values are the means ± SD for at least three independent experiments, and analysis of variance followed by Tukey's *post hoc* test showed significant differences (***p<0.001 *vs.* control cells; ###p<0.001 *vs.* HG-treated cells; $$$$p$<0.001 *vs.* HG and paeoniflorin-treated cells).

of paeoniflorin were diminished by HO-1 inactivation in ARPE-19 cells treated with HG, we evaluated the effect of HO-1 inactivation on other anticytotoxic activities of paeoniflorin. The neutralizing effect of paeoniflorin on HG-induced apoptosis and DNA damage was abolished in the presence of ZnPP, as determined by flow cytometry, immunoblotting, caspase-3 activity, and agarose gel electrophoresis (Fig. 6A-6D). In addition, the inhibitory effect of paeoniflorin on the HG-mediated reduction in cell viability was abolished after inhibiting HO-1 activity (Fig. 6E). These results show that the antioxidant potential of paeoniflorin in ARPE-19 cells under HG conditions is dependent on Nrf2/HO-1 axis activation.

DISCUSSION

Hyperglycemia-induced DR is the most common cause of blindness, and HG is primarily responsible for activating the

oxidative stress that affects all cellular components of the retina (Gao *et al*., 2023; Zhang *et al*., 2024). Although hormone therapy and vascular endothelial growth factor inhibitors are the main treatments used to treat DR, their efficacies are limited, and many side effects can occur (Muns *et al*., 2023; Cheng and Liu, 2024). Therefore, reducing oxidative stress caused by HG may be an alternative strategy for treating DR, and increasing interest has been shown in the application of natural products with high antioxidant activity (Liang *et al*., 2024; Akpoveso *et al*., 2023). Although paeoniflorin, a strong antioxidant, has been reported to be efficacious in various DR-associated ocular disease models, research on its ability to protect ocular cells from oxidative stress-induced damage by HG is limited. In this study, we investigated whether paeoniflorin protects against HG-induced oxidative damage in human RPE ARPE-19 cells, a model of hyperglycemia. MTT assay results showed that paeoniflorin significantly blocked HG-induced inhibition of cell viability under non-cytotoxic conditions. Since the MTT assay reflects total mitochondrial activity in terms of viable cell numbers (Plumb, 2004), the blocking of HG-induced cytotoxicity by paeoniflorin might be related to the inhibition of apoptosis due to the maintenance of mitochondrial homeostasis.

Most cellular damage induced by oxidative stress involves DNA damage, and unrepaired DNA damage contributes to the induction of apoptosis (Wang *et al*., 2021b; Li *et al*., 2024). Consistent with our results, paeoniflorin has been previously reported to block DNA damage caused by oxidative stimuli, such as UV radiation and endotoxins, in keratinocytes, thymocytes, and macrophages (Lee *et al*., 2006; Li *et al*., 2007; Kim and Ha, 2009). In addition, our results showed that paeoniflorin blocked HG-induced ARPE-19 cell apoptosis by neutralizing MMP loss, caspase-3 activation, PARP fragmentation, and cytochrome c release into cytosol, thus highlighting the importance of mitochondrial stability in the attenuation of HG-induced cytotoxicity. Paeoniflorin also markedly eliminated ROS production in the HG environment, as was reported for RPE cells treated with atRAL and H_2O_2 (Wankun *et al*., 2011; Zhu *et al*., 2018). These results demonstrate that the potent antioxidant activity of this glycoside is also involved in the inhibition of RPE cell apoptosis. Moreover, the suppression of HG-induced ROS production by paeoniflorin was blocked by ZnPP, which is consistent with previous findings (Jiang *et al*., 2021; Wang *et al*., 2021a; Ren *et al*., 2023; Xing *et al*., 2023), indicated that the inhibition of ROS accumulation by paeoniflorin occurred through Nrf2-mediated HO-1 activation.

Under physiological conditions, Nrf2 exists in the cytoplasm in a Keap1-bound form and is degraded via the ubiquitin-proteasome pathway (Saha *et al*., 2020; Ngo *et al*., 2023). However, when cells are exposed to Nrf2 inducers or oxidative stressors, phosphorylation of Nrf2 liberates it from Keap1, and the phosphorylated product translocates to the nucleus to transcriptionally activate antioxidant genes, including HO-1 (Liu *et al*., 2021; Ngo *et al*., 2023). HO-1 is an Nrf2-dependent downstream gene and an enzyme that catabolizes heme into free iron, carbon monoxide, and biliverdin, which is then metabolized to bilirubin, a potent antioxidant, by bilirubin reductase (Chiang *et al*., 2021; Consoli *et al*., 2021). Our results show that the expression and activity of HO-1 were significantly enhanced in cells treated with HG in the presence of paeoniflorin compared to cells treated with paeoniflorin or HG alone. Furthermore, this was associated with increased Nrf2 phosphorylation and the downregulation of Keap1 protein, indicating that paeoniflorin enhanced Nrf2/HO-1 axis activation under HG-induced oxidative conditions.

In RPE cells, as in other cells, Nrf2 activation increases antioxidant defense and mitigates the production of ROS (Bellezza, 2018; Zhang *et al*., 2023). Aging and oxidative stress increase ROS production in retinal pigment epithelium. This increase in ROS is directly related to the weakening of Nrf2 activation, the apoptosis of retinal cells, and the onset and progression of ocular diseases, such as age-related macular degeneration (AMD) (Hyttinen *et al*., 2019; Hu *et al*., 2024b). In addition to redox balance, Nrf2 has been shown to regulate several genes involved in diverse physiological processes required for cell survival and proliferation, including mitochondrial biogenesis and homeostasis (van der Horst *et al*., 2022; Bhat *et al*., 2024; Luchkova *et al*., 2024). For example, mice lacking the Nrf2 gene exhibited an AMD-like pathology, and autophagy in RPE cells was found to be involved in oxidative damage and inflammation responses following mitochondrial dysfunction (Zhao *et al*., 2011; Cano *et al*., 2021). Yang *et al*. (2023) also reported that acteoside, a phenylpropanoid glycoside with antioxidant activity, reduced HG-induced ROS production, inhibited mitochondria-mediated apoptosis, and increased the expression of Nrf2 target genes via Nrf2 activation in RPE cells. They also observed that Nrf2 knockdown prevented this phenomenon. In addition, when RPE cells were exposed to oxidative inducers, such as H_2O_2, the expressions of Nrf2 and HO-1 were significantly reduced, which reduced mitochondrial quality and induced apoptosis. However, Nrf2 activators blocked these changes, while HO-1 inhibitors abrogated the blocking effect (You *et al*., 2021; Chen *et al*., 2022; Hsu *et al*., 2022; Park *et al*., 2022, 2024). Moreover, gomisin A, a major bioactive substance of *Schisandra chinensis* fruit, prevented the inhibition of HG-induced osteoblast differentiation by scavenging ROS and maintaining mitochondrial biogenesis through the Nrf2-mediated upregulation of HO-1, and these effects were blocked by ZnPP pretreatment (Takanche *et al*., 2020). Similarly, myricitrin, an antioxidant flavonoid, attenuated HG-induced cardiac endothelial cell death by inhibiting ROS production and preserving mitochondrial function while increasing Nrf2 expression and HO-1 transcriptional activity, and ZnPP pretreatment also abolished this protective effect of myricitrin (Zhang *et al*., 2016). Consistent with the results of these studies, we found that the HO-1 inhibitor ZnPP abrogated the paeoniflorin-mediated inhibition of HG-induced ROS production, suppression of mitochondrial dysfunction, apoptosis, and cytotoxicity, thus highlighting the importance of the Nrf-2/HO-1 axis in protective effects mediated by paeoniflorin against HG-mediated oxidative stress in ARPE-19 cells.

The present study demonstrates that the protective effect of paeoniflorin on human RPE ARPE-19 cells under HG conditions mimicking hyperglycemia is mediated by maintaining mitochondrial homeostasis by inhibiting ROS production. The study shows that the ROS-scavenging activity of paeoniflorin may contribute to ARPE-19 cell survival under HG-induced oxidative conditions by suppressing DNA damage and apoptosis *via* Nrf-2-mediated HO-1 activation, thereby alleviating the initiation and progression of DR (Fig. 7). However, various intracellular signaling pathways other than the Nrf-2/HO-1 signaling axis might also be involved in the antioxidant activity of paeoniflorin. Therefore, we suggest further studies, including animal experiments, be conducted to identify the upstream

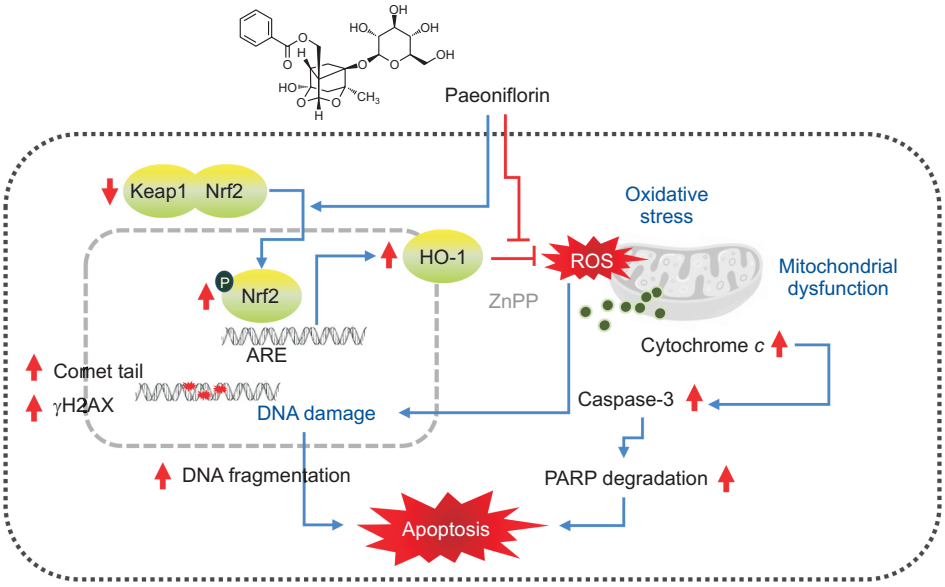

Fig. 7. Schematic diagram showing the protective effect of paeoniflorin against HG-mediated oxidative stress-induced cytotoxicity in RPE ARPE-19 cells. ARE, antioxidant response element; HO-1, heme oxygenase-1; Keap1, Kelch-like ECH associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; ZnPP, zinc protoporphyrin; γH2AX, phosphorylated form of H2AX.

kinases involved in paeoniflorin-induced Nrf-2 activation and to elucidate how Nrf-2/HO-1 signaling is related to various signaling pathways involved in redox regulation and mitochondrial homeostasis.

In conclusion, the results of the present study provided compelling evidence that paeoniflorin significantly suppresses cytotoxicity, DNA damage, and apoptosis in HG-treated ARPE-19 cells and that its apoptosis-blocking effect is related to the blockade of caspase-3 activation and PARP degradation. Paeoniflorin also maintained the integrity of mitochondrial membranes in HG-treated cells, as evidenced by MMP improvement and the inhibition of cytochrome *c* efflux into cytosol, and thus, reduced ROS production. Moreover, Keap1 expression was further downregulated in cells co-treated with paeoniflorin and HG, compared to that in cells treated with paeoniflorin or HG alone, whereas Nrf2 phosphorylation and HO-1 expression were enhanced, indicating that Nrf2/HO-1 signaling is activated by paeoniflorin under oxidative conditions. However, when the activity of HO-1 was artificially reduced, the ROS scavenging, anti-apoptotic, and cytotoxicity inhibitory effects of paeoniflorin against HG were abolished, which emphasized the importance of Nrf-2/HO-1 signaling as a target of the antioxidant activity of paeoniflorin. Therefore, our data suggest that paeoniflorin, an Nrf-2 activator, has potential use as a therapeutic agent for the prevention and treatment of hyperglycemia-induced DR by protecting RPE cells from oxidative injury.

CONFLICT OF INTEREST

The authors have no conflicts of interest relevant to this study to disclose.

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AUTHOR CONTRIBUTIONS

Cheol Park: Writing of manuscript, Design of the work, and Analysis of data. Hee-Jae Cha: Conceptualization, Writing & revision of manuscript, and Acquisition of data. Su Hyun Hong: Visualization and Data curation. Jeong Sook Noh: Analysis of data and Conceptualization. Jeong Sook Noh: Investigation and Analysis of data. Gi Young Kim: Visualization and Methodology. Sang Hoon Hong: Data curation and Resources, and Analysis of data. Jung-Hyun Shim: Review & editing of manuscript. Jin Won Hyun: Project administration, Supervision, and Review & editing of manuscript. Yung Hyun Choi: Resources, and Review & editing of manuscript.

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