• Basic Research •

Pigment epithelium-derived factor protects retinal ganglion cells from hypoxia-induced apoptosis by preventing mitochondrial dysfunction

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Abstract

• AIM: To investigate the potential of pigment epithelium-derived factor (PEDF) to protect the immortalized rat retinal ganglion cells-5 (RGC-5) exposed to $CoCl_2$-induced chemical hypoxia.

• METHODS: After being differentiated with staurosporine (SS), RGC-5 cells were cultured in four conditions: control group cells cultured in Dulbecco 's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 μmol/mL streptomycin and penicillin (named as normal conditions); hypoxia group cells cultured in DMEM containing 300 μmol/mL $CoCl_2$; cells in the group protected by PEDF were first pretreated with 100 ng/mL PEDF for 2h and then cultured in the same condition as hypoxia group cells; and PEDF group cells that were cultured in the presence of 100 ng/mL PEDF under normal conditions. The cell viability was assessed by MTT assay, the percentage of apoptotic cells was quantified using Annexin V-FITC apoptosis kit, and intra-cellar reactive oxygen species (ROS) was measured by dichloro-dihydro-fluorescein diacetate (DCFH-DA) probe. The mitochondria-mediated apoptosis was also examined to further study the underlying mechanism of the protective effect of PEDF. The opening of mitochondrial permeability transition pores (mPTPs) and membrane potential (Δψm) were tested as cellular adenosine triphosphate (ATP) level and glutathione (GSH). Also, the expression and distribution of Cyt C and apoptosis inducing factor (AIF) were observed.

• RESULTS: SS induced differentiation of RGC-5 cells resulting in elongation of their neurites and establishing contacts between outgrowths. Exposure to 300 μmol/mL $CoCl_2$ triggered death of 30% of the total cells in cultures within 24h. At the same time, pretreatment with 100 ng/mL PEDF significantly suppressed the cell death induced by hypoxia (P<0.05). The apoptosis induced by treatment of $CoCl_2$ was that induced cell death accompanied with increasing intracellar ROS and decreasing GSH and ATP level. PEDF pretreatment suppressed these effects (P<0.05). Additionally, PEDF treatment inhibited the opening of mPTPs and suppressed decreasing of Δψm in RGC-5 cells, resulting in blocking of the mitochondrial apoptotic pathway.

• CONCLUSION: Pretreatment of RGC-5 cells with 100 ng/mL PEDF significantly decreases the extent of apoptosis. PEDF inhibits the opening of mPTPs and suppresses decreasing of Δψm. Moreover, PEDF also reduces ROS production and inhibits cellular ATP level's reduction. Cyt C and AIF activation in PEDF-pretreated cultures are also reduced. These results demonstrate the potential for PEDF to protect RGCs against hypoxic damage *in vitro* by preventing mitochondrial dysfunction.

• KEYWORDS: pigment epithelium-derived factor; $CoCl_2$; retinal ganglion cells-5; reactive oxygen species; reduced glutathione hormone; mitochondrial permeability transition pores; membrane potential; adenosine triphosphate; Cyt C; apoptosis-inducing factor

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INTRODUCTION

Death of retinal ganglion cell (RGC) is a common feature of many eye diseases, such as glaucoma, retinal ischemia, and optic neuropathy[1]. The RGCs death may involve various mechanisms, for example, reactive oxygen species (ROS), nitric oxide, and excitatory amino acids[2-5]. It has been shown that RGCs die through apoptosis in models

of acute optic nerve lesion (axotomy and crush), experimental glaucoma and human glaucoma[6-10].

In humans, visual information entering the eye needs to be processed in the retina before transmitted into visual center in brain *via* the optic nerve axons[11-12]. The RGCs transfer visual information from the retina to the lateral geniculate nucleus (LGN), and partially to another cortical region, including superior colliculus (SC), coordinating eye movement. Like other neurons in the central nervous system (CNS), RGCs fail to regenerate: once they die, they are lost forever. Thus, glaucoma, as the view of a neurodegenerative disease, ultimately induces irreversible change in neuronal function. Several risk factors have been proposed to contribute to glaucoma progression, including elevated intraocular pressure, age genetic background, thinner corneal thickness and vascular dysregulation[13-19].

RGCs death plays a key role in some ocular pathologies, such as retinal vessel occlusion, diabetic retinopathy and glaucoma. The main cause of irreversible neuronal injury and visual loss is ischemia which is a main trigger of RGCs death and it is accompanied with many progressive changes, such as increase of glutamate excite toxicity and free radical production, decrease of adenosine triphosphate (ATP) stores, ions imbalance, and apoptosis, which eventually lead to RGCs death[20-21]. Importantly, reperfusion following ischemia may potentiate oxidative stress that plays additional role in the RGCs degeneration[22].

Neurotrophins are diffusible trophic molecules that exert a potent survival effect on adult CNS neurons undergoing degeneration induced by variety of intrinsic and extrinsic stimuli. Pigment epithelium-derived factor (PEDF) is a 50 kDa glycoprotein that belongs to the superfamily of serine protease inhibitors, secreted by retinal pigment epithelial (RPE) cells, which was first identified in the conditioned medium of cultured fetal human RPE cells[23]. There are several reviews about PEDF functions in various aspects, such as potent neuroprotective and neurotrophic activities, while its anti-angiogenic effect was confirmed to participate in many aspects of ocular diseases pathogenesis[24]. Several experimental results indicate that after treatment of the ischemic retina with PEDF, the retinal neurons can survive from the damage caused by ischemia. Notably, PEDF shows dramatic effects on the neovascular response that is the feature of retinal ischemia[25-28]. In addition, PEDF acts as a survival factor for a wide range of cultured cells including cerebellar granule cells, hippocampal neurons, and spinal cord motor neurons, protecting these cells against glutamate toxicity and plays the role as an antagonist of apoptosis[29-33]. Nevertheless, only few works about the function of PEDF on protecting the RGCs under hypoxia have been reported. The exact mechanism of its protective action is still need to be revealed.

In the present study, we investigate the neuroprotective potential of PEDF on $CoCl_2$-mimetic hypoxia-induced apoptosis in rat retinal ganglion cells-5 (RGC-5) and explore its possible mechanisms.

MATERIALS AND METHODS

Cell Culture The transformed retinal ganglion cell line, RGC-5, was received from Zhong Shan Medical Center. The cell line RGC-5 originally derives from postnatal day one rat retinal cells transformed with Ψ2 E1A virus[34]. It was grown in high-glucose Dulbecco's modified Eagle's medium (DMEM; Hyclone, USA) containing 10% fetal bovine serum (FBS; Hyclone, USA). RGC-5 cells were cultured in a CO_2 incubator (ESPEC, Japan) at 37℃ with 95% air and 5% CO_2. The number of cells would be doubled after 18-20h. We passaged cells at a ratio of 1:4 in every 2d. The passages 10-20 of RGC-5 cells at 80%-90% confluence were used in all experiments. RGC-5 was induced to differentiate into highly branched, no-mitotic cells through the protocol developed by Frassetto *et al*[35]. Briefly, RGC-5 was firstly cultured as previously described for 24h, then they were seeded into normal growth medium with only 1% FBS (to slow the proliferation rate of the cells) containing staurosporine (SS) 316 nmol/L. After the treatment in this normal growth medium for 24h, the cells were then processed for analysis.

$CoCl_2$-mimetic Hypoxia Injury $CoCl_2$ was used to cultured cells to cause hypoxia. $CoCl_2$ (Sigma, USA) was dissolved in distilled H_2O, and a 0.2-μm filter was used to make the solution sterile. Based on the preliminary study, we pretreated cells with final concentration of 100 ng/mL PEDF in subsequent experiments (PeproTech, USA) for 2h, and then treated them with 300 μmol/L $CoCl_2$ for 24h.

Measurement of Cell Viability Cells were cultured in 96-well plates with density of 5×10^3 cells/well and 1% FBS for 24h under various conditions: control group (C), hypoxia damage group (H), PEDF pretreatment group (P+H), and PEDF group (P). Then MTT (5 mg/mL) was added and incubated 4h, after that we used DMSO to dissolve the generated formazan crystals. The absorbance of the crystalline at 490 nm was analyzed with a Tecan Genios (Tecan, Germany). The cell viability of H group, P+H and P groups were represented as the percentage of viable cells relative to cell viability of the control group.

DAPI Staining DAPI was used to stain RGC-5 cells to visualize fragmented nuclei (Beyotime, China). After being pretreated for 24h, cells were fixed with methanol for 10min following stained with DAPI for 5min at room temperature. Finally, a fluorescence microscope (SIV ZN, Japan) was used to detect RGC-5 cells.

Annexin V-FITC/PI Assay Annexin V-FITC/PI detection kit (Jiankangyuan, China) was used to detect apoptosis following the manufacturer's instructions. Briefly, the harvested cells were resuspended in binding buffer with the density of 1×10^6 cells/mL. Every 1×10^5 cells were mixed with 5 μL Annexin V-FITC and 10 μL PI. The samples were kept in the dark at

room temperature for 15min. Flow cytometry (FACS-400, USA) was used to detect fluorescence. The percentage of apoptosis was expressed with the number of Annexin V(+)/PI(-) cells and the number of Annexin V(+)/PI(+) cells.

Assay for Intracellular Reactive Oxygen Species Intracellular redox state levels were detected using the fluorescent dye dichloro-dihydro-fluorescein diacetate (DCFH-DA; GENMED, USA). Briefly, we washed cells with phosphate buffered saline (PBS) once and incubated in the PBS containing DCFH-DA for 30min at 37℃. Intracellular fluorescence was measured by flow cytometry (FACS-400, USA).

Assessment of Total Intracellular Glutathione The assay is based on that glutathione (GSH) with dinitrothio cyanobenzene (DNTB; NJJC, China) can form a kind of yellow compound. Briefly, after maintaining cell cultures in the presence of different substances for 24h, approximately 1×10^6 cells were resolved. The cracked cells were added buffer solution and centrifuged at 4000 rpm for 10min. Supernatant samples were transferred to a microplate and added with DNTB, waiting for 5min detected 405 nm absorbance using a Tecan Genios (Tecan, German).

Analysis of Mitochondrial Membrane Potential Lipophilic and cationic probe, JC-1 (Beyotime, China) was used for the detection of the mitochondrial membrane potential ($\Delta\psi m$) of RGC-5 cells with the fluorescent. Briefly, cells were seeded in 6-well plates, while incubating with JC-1 staining solution (5 μg/mL) for 20min at 37℃ after appropriate treatments. After washing cells twice with JC-1 staining buffer, fluorescence intensity was detected by a flow cytometry (FACS-400, USA). Mitochondrial JC-1 monomers and aggregates respectively showed as green and red. The fluorescence ratio of red to green indirectly reflects the $\Delta\psi m$ of RGC-5 cells in different conditions.

Detection of Opened Mitochondrial Permeability Transition Pores Opened mitochondrial permeability transition pores (mPTPs) of RGC-5 cells was detected by calcein-cobalt, which was included in a mPTP assay kit (GenMed Scientifics Inc., USA). RGC-5 cells were seeded in 6-well plates (2.5×10^5 cells/well), culturing 24h. After that, cells were treated with various conditions for 24h. Cells were incubated with reagent B and C as ratio 1:50 for 20min at 37℃, following by washing with reagent A as described in the manufacturer's instruction. After that, cells were washed twice again with reagent A. Finally, the flow cytometry was used to detect fluorescence intensity (FACS-400, USA).

Detection of Cellular Adenosine Triphosphate Levels We used firefly luciferase based ATP assay kit to measure cellular ATP levels (Beyotime, China). Briefly, we pretreated RGC-5 cells with or without PEDF, followed by 24h treatment of $CoCl_2$. Cells were harvested and centrifuged at 12 000× g for 5min. Every 100 μL ATP detection working dilution was added in 100 μL supernatant, then mixed the components in 24-well plates. Luminance (RLU) represented the protein concentration, which was measured by a Tecan Genios (Tecan, German). Each treatment group was determined by generated standard curves. Total ATP levels were exhibited as μmol/g protein.

Immunofluorescence Analysis In order to identify RGC-5, cells were cultured in a 6-well plate on coverslips, and then treated with appropriate medium. After cells were 80%-90% confluent fixed with 4% paraformaldehyde, ruptured membrane with Triton X-100, 5% normal goat serum was used for blocking at 37℃, then after 30min incubated with Thy-1 (Abcam, 1:500), Brn-3C (Sigma, 1:200), GFAP(Abcam, 1:3000), GABA-B (Millipore, 1:3000) and NMDA (Sigma, 1:200) respectively overnight at 4℃ and subsequently incubated with a combination of secondary antibodies goat anti-chicken IgY (Abcam, 1:500) and FITC-anti-rabbit IgG (Kehao, China 1:100) then stained with DAPI 5min. Finally, we examined the coverslips with a fluorescence microscope (SIV ZN, Japan). Assay of expression of Cyt C and apoptosis inducing factor (AIF) were carried on with same method as described above.

Statistical Analysis All experiments were carried out three times independently. The results were expressed in terms of mean±standard deviation (SD) values, and tested for statistical differences by analysis of variance, using the one-way ANOVA method. When the calculated P value was <0.05, we take the differences are significant.

RESULTS

Pigment Epithelium-derived Factor Decreases Hypoxia-induced Apoptosis of Retinal Ganglion Cells The purity of RGC-5 cultures was confirmed by detecting expression of Thy-1, Brn-3c, GABA-B receptor and NMDA in the cytoplasm and absence of glial marker GFAP expression (Figure 1).

To prove the protective functions of PEDF on RGC-5 cells under the condition of hypoxia, morphological changes of various treated RGC-5 cells were analyzed. In control group, RGC-5 cells cultured in normal condition without any treatment exhibited morphological characters of mature ganglion cells with long extensions. In the hypoxia group, with prolonged exposure time, increased cells were observed rounded up (Figure 2). MTT assays were used to quantitate cell viability. The viability of RGC-5 cells decreased to 69%±1.5% after hypoxic exposure 24h, while pretreated RGC-5 cells with PEDF exhibited higher level of cell viability to 86%±3% ($P<0.05$ *vs* H group). Notably, the viability of RGC-5 cells in C group and P+H group exhibited no significant differences (Figure 3). We used DAPI-staining to determine nuclear changes under various conditions of RGC-5 cells. RGC-5 cells exposed to hypoxic condition expressed clear changes in chromatin morphology, as condensation and fragmentation, exhibiting bright blue labeling, and the changes were weakened by PEDF (Figure 4).

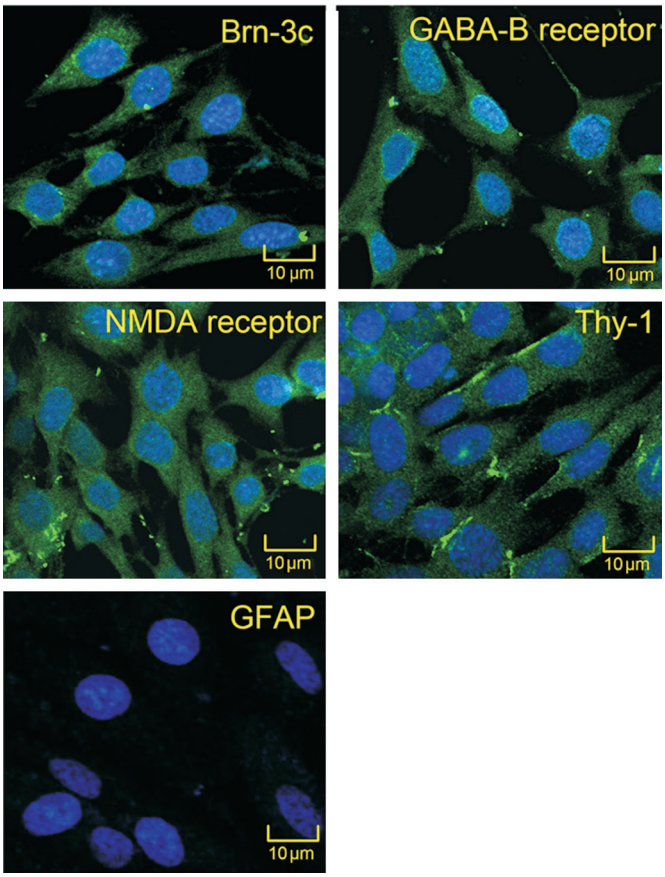

Figure 1 Cell identity.

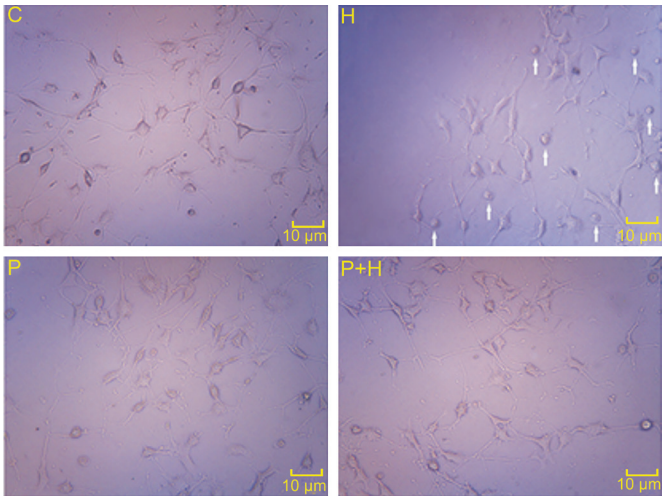

Figure 2 Phase-contrast micrographs of RGC-5 cells RGC-5 cells were differentiated by staurosporine following exposed to hypoxia for 24h in different groups. C: Control group; H: Hypoxia group; P+H: PEDF pretreated group; P: PEDF group.

Annexin V-FITC/PI assay was also used for further analysis of the anti-apoptotic effects of PEDF (Figure 5). Our data support the previous finding showed that $CoCl_2$ induce apoptosis of RGC-5 cells. The percentage of apoptotic cells after 24h was significantly increased after $CoCl_2$ treatment (34.82%±2.17% *vs* 4.39%±1.23%, $P<0.05$). However, pretreatment of these cells with PEDF decreased this number significantly (12.46%±0.31%, $P<0.05$). It is thus clear that PEDF suppressed apoptosis of RGC-5 cells, which induced by hypoxia.

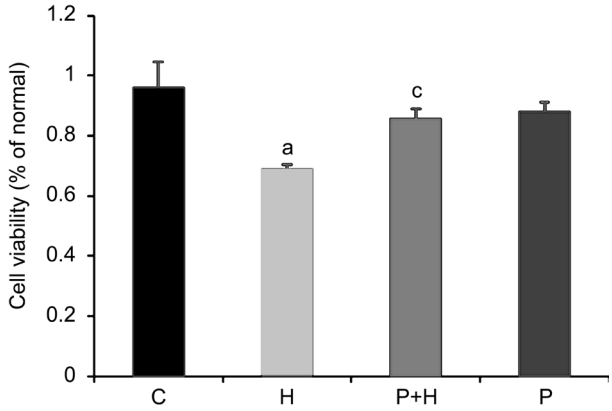

Figure 3 MTT assay Cell viability of RGC-5 cells exposed to hypoxia for 24h in different group, measured by MTT assay ($^aP<0.05$ as compared to the C group; $^cP<0.05$ as compared to the H group).

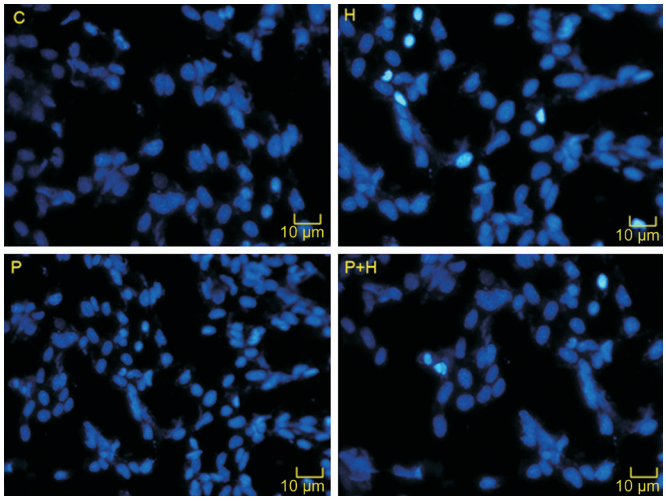

Figure 4 DAPI staining Photomicrographs of RGC-5 cells nuclear stained with DAPI (in different conditions).

Hypoxia Induced the Elevation of Intracellular Reactive Oxygen Species Increased intracellular ROS in cells and tissues is a cause of damage in many pathophysiological conditions. Plenty of work have documented that hypoxia could induce the elevation of intracellular ROS[36]. In our study, we examined the role of ROS in the death of RGC-5 cells. Consistent with earlier studies, an increasing level of ROS was observed in RGC-5 cells exposed to hypoxic stress, 477.27±53.87 ($P<0.05$). However, PEDF suppressed hypoxia-induced ROS increase in RGC-5 cells (190.18±13.31, $P<0.05$) (Figure 6).

We carried out experiments to measure the level of cellular total anti-oxidant GSH to further evaluate the effect of hypoxia on RGC-5 cells. As shown in Figure 7, hypoxia decreased the GSH from 45.97±17.26 to 10.63±5.33 ($P<0.05$). When the RGC-5 cells were pretreated with PEDF before the application of hypoxia, no significant decrease of GSH was observed. There were no significant differences between PEDF group and control group, indicating that the cellular redox status is deteriorated when cells are exposed to the hypoxia, and PEDF protects RGC-5 cells from it.

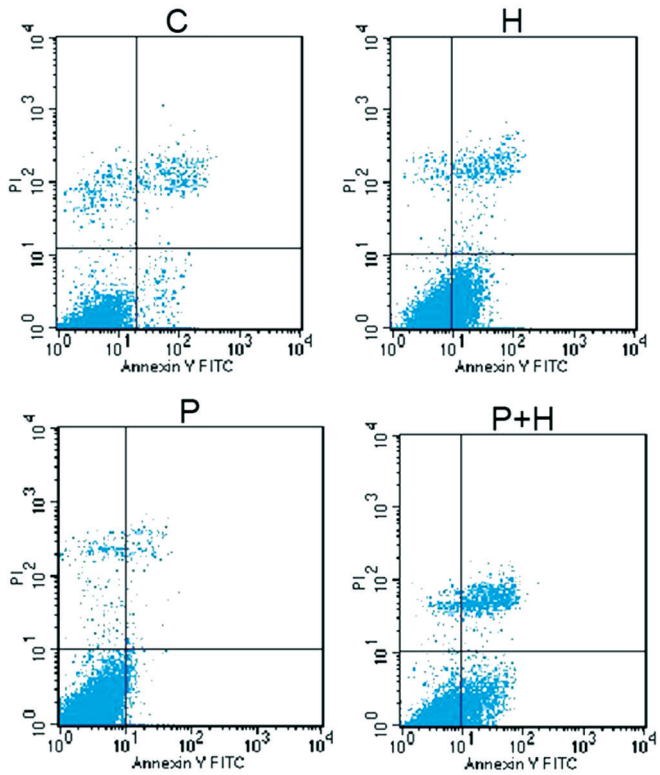

Figure 5 Annexin V-FITC/PI staining Apoptotic cells were counted as percentage *via* staining by Annexin V-FITC/PI and analyzed with flow cytometry. The area of Annexin V(+)/PI(−) and Annexin V(+)/PI(+) were included in apoptotic cells.

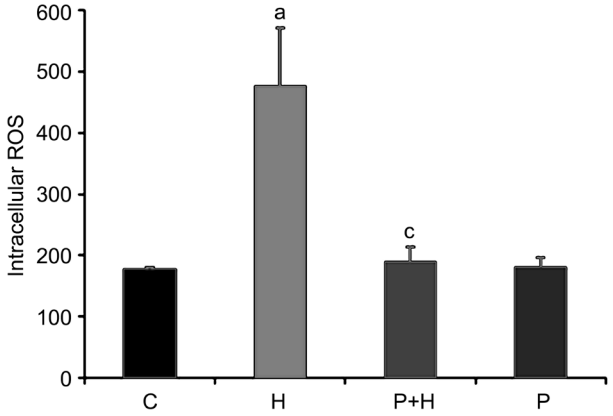

Figure 6 Intracellular ROS The effect of PEDF on the ROS production in RGC-5 cells exposed to hypoxia. ROS was measured using DCFH-DA cultures exposed to normoxia or hypoxia for 24h under the condition with or without PEDF. Results are expressed as the means±SD. ^{a}P<0.05 compared to the C group; ^{c}P<0.05 compared to the H group.

Pigment Epithelium-derived Factor Inhibits the Opening of Mitochondrial Permeability Transition Pores Elevated Ca^{2+} level triggers the opening of mPTPs during hypoxia, then initiated mPTP furthermore induces to mitochondrial dysfunction[37]. Therefore, distribution of green fluorescence, which emitted from calcein, was monitored by the calcein-cobalt method as a result of intact mPTPs to examine the influence of PEDF on mPTPs. After 24h induction of hypoxia, significantly higher level of the opening of mPTPs in PEDF-

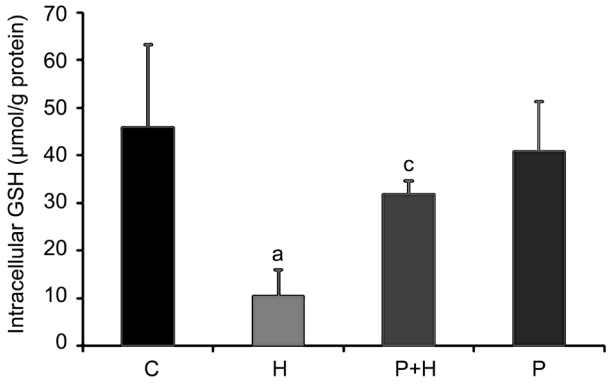

Figure 7 Intracellular GSH The effect of PEDF on the intracellular GSH in RGC-5 cells exposed to hypoxia. Cells culture exposed to normoxia or hypoxia for 24h in the presence or absence of PEDF. Results are expressed as the means±SD. ^{a}P<0.05 compared to the C group; ^{c}P <0.05 compared to the H group.

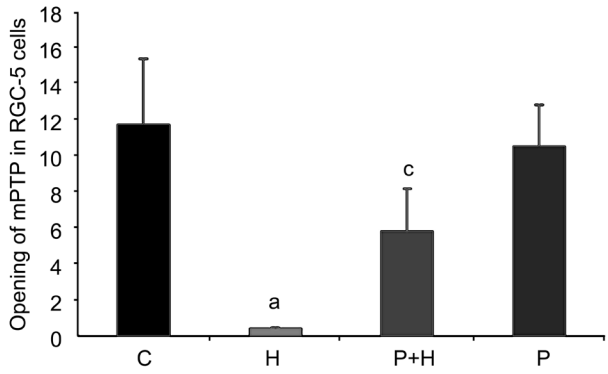

Figure 8 Opening of mPTPs The normalized relative fluorescence units (NRFU) of calcein exhibit the opening of mPTPs. RGC-5 cells were pretreated with or without PEDF and exposed to normoxia or hypoxia for 24h. Results exhibit as the means±SD. ^{a}P<0.05 compared to the C group, ^{c}P<0.05 compared to the H group.

treated cultures was observed in fluorescence measurement (Figure 8), with the ratio of M2/M1 changing from (0.47±0.17, P<0.05) in H group to (5.87±2.34) in H+P group. Our results thus clearly demonstrated the ability of PEDF on reducing the extent of opened mPTPs in hypoxic condition.

Pigment Epithelium-derived Factor Prevents Mitochondrial Dysfunction Δψm and level of cellular ATP indicate mitochondrial activity. Therefore, we further investigated the effects of PEDF on mitochondrial function based on the above parameters. After 24h hypoxic exposure, RGC-5 cells exhibited lower Δψm values (P<0.05). However, the Δψm of PEDF-treated cells and normal cells showed no marked difference (Figure 9). Except the above index, cellular ATP level also exhibits mitochondrial function. In this study, after 24h treatment of hypoxia, cellular ATP level drastically decreased to 0.51±0.03 μmol/g protein as Figure 10 shows. In contrast, pretreatment of cultured RGC-5 cells with PEDF still contains relatively high cellular ATP content of 0.81±0.01 μmol/g protein (P<0.05). There were no marked differences between P group and C group (Figure 10). Thus these findings

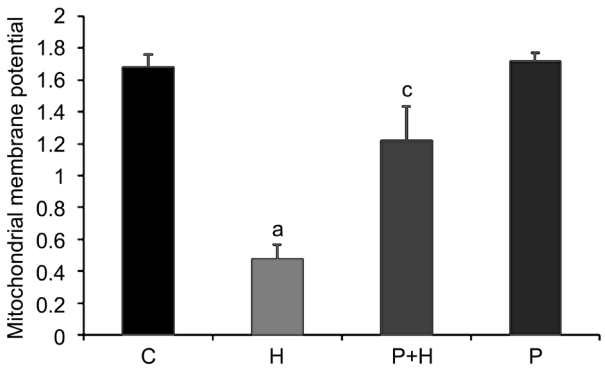

Figure 9 Mitochondria Δψm Mitochondrial Δψm of RGC-5 cells were determined using JC-1. RGC-5 cells were exposed to normoxia or hypoxia for 24h in the condition with or without PEDF. Results show as the mean±SD. $^{a}P<0.05$ compared to the C group; $^{c}P<0.05$ compared to the H group.

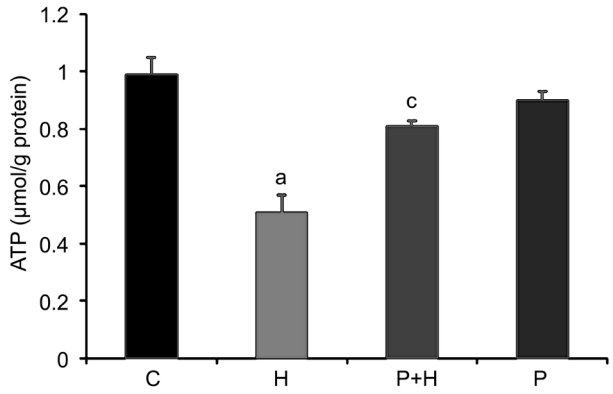

Figure 10 Cellular ATP Cellular ATP concentration of RGC-5 cells were detected after exposed to normoxia or hypoxia 24h cultured with or without PEDF. Results are expressed as the mean±SD. $^{a}P<0.05$ compared to the C group; $^{c}P<0.05$ compared to the H group.

directly demonstrated that PEDF has the effect of protecting mitochondrial function during hypoxia.

Pigment Epithelium-derived Factor Affects Cyt C Involved Mitochondrial Apoptotic Pathway Cyt C entering to the cytoplasm mediates mitochondrial activation of apoptosis. Importantly, we also observed a statistically significant difference between the expression of Cyt C in the hypoxia group and the control group. During the process of apoptosis, Cyt C dispersed while its concentration in cytosol increased. The increase of red fluorescence in PEDF pretreated group is lower, which indicates PEDF inhibits Cyt C releasing to cytoplasm from mitochondria (Figure 11).

Pigment Epithelium-derived Factor Affects Apoptosis Inducing Factor Apoptotic Pathway We also found difference between the expression of AIF in H group compared with C group, and results showed that AIF dispersed in nucleus while its concentration increased in cytosol. Weaker red fluorescent labeling in cytoplasm was observed in PEDF group, which suggests that PEDF inhibits the release of AIF (Figure 12).

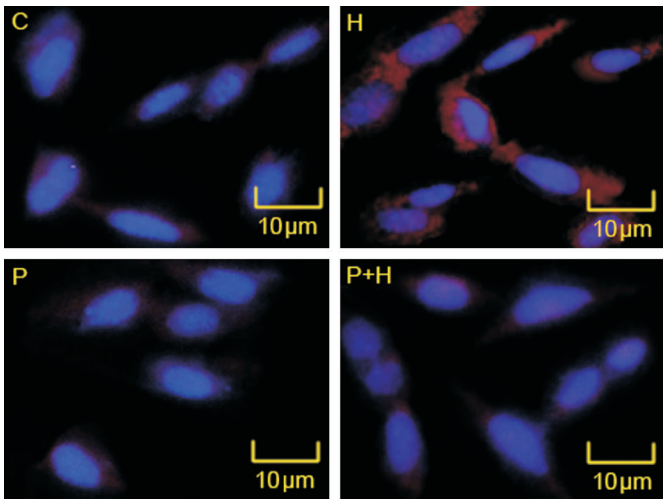

Figure 11 Expression and distribution of Cyt C Effects of PEDF on the expression and distribution of Cyt C in RGC-5 cells exposed to hypoxia for 24h. Cyt C immunoreactivity showed red in RGC-5 with nuclear counter stained with DAPI (blue).

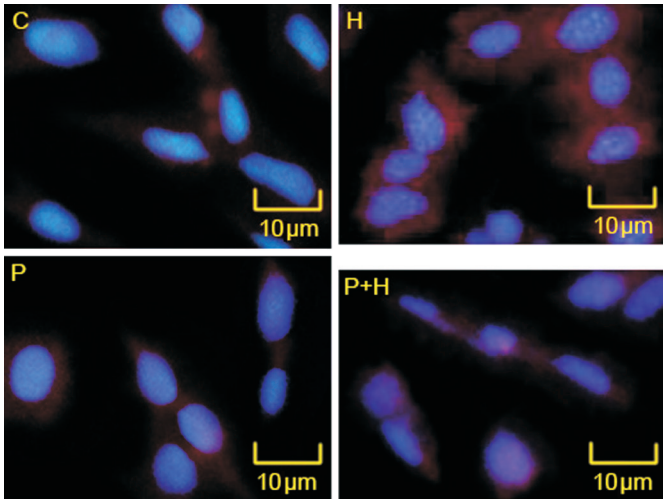

Figure 12 Expression and distribution of AIF Effects of PEDF on the expression and distribution of AIF in RGC-5 exposed to hypoxia for 24h. The bright red fluorescence indicates IF immunoreactivity (red) in RGC-5 cells.

DISCUSSION

We used the RGC-5 cell line to elucidate that PEDF can suppress hypoxia-induced apoptosis in RGCs with the focus on mitochondrial function. Many endogenous trophic factors have protective functions in models of neurodegenerative diseases. Among them, PEDF is a cell survival factor secreted by the RPE and widely expresses in most regions of nervous system[38-39]. The cytoprotective effects of PEDF have previously been observed in cerebellar granule cells and retinal pericytes[40]. Reduced extracellular concentration of PEDF leads to neuronal damage due to various cell-damaging conditions including hypoxia[41]. In our hypoxia model, PEDF preserved cells from damage. We used 100 ng/mL PEDF on RGC-5 and found the total number of apoptotic cells decreased from about 30% by 15%, meaning that PEDF obviously provides neuroprotective effect. Furthermore, we used reliable methods to identify apoptosis and viability of cells, including the Annexin V-FITC/PI assays to detect apoptosis. Annexin

V-FITC/PI assay showed that percentage of apoptotic cells was 34.82%±2.17% *vs* 12.46%±0.31% (P<0.05) in C group and P group respectively. The MTT assays also provided strong evidence for cell viability to support our hypothesis that PEDF plays an important role in neuroprotective effects on RGCs.

$CoCl_2$ is a common agent to induce hypoxia is shown by the previous studies[42-43], and in our work using RGC-5, we prove that $CoCl_2$ contributes to hypoxia-induced damage. Gene and protein expression are influenced by $CoCl_2$ simulated hypoxia, which is similar to ischemia[44]. $CoCl_2$ induced hypoxia breaks the balance of hypoxia-inducible factor-1 (HIF-1), between the degradation and the consequent accumulation, thereby expression of heat shock protein-27 increases[45]. Moreover, in $CoCl_2$-treated RGC-5 accumulation of HIF-1 protein also induces generation of β-amyloid peptide. In addition, $CoCl_2$ could active caspase-3/8, produce ROS and fission anti-apoptotic protein Mcl-1 to induce apoptosis as shown various *in vitro* studies[46-47]. And both *in vivo* and *in vitro* studies show that RGCs are sensitive to oxidative stress especially in pathological situations. In the experimental studies, $CoCl_2$ is used in the hypoxia model and there are a lot of works providing evidences that $CoCl_2$ induces apoptosis while damaging retinal photoreceptor[48-49].

ROS plays an important role in retinal cells injury through changing cellular redox potentials while reducing ATP levels, which indirectly induce apoptosis with depleted GSH in diabetic retinopathy and glaucoma[50-52]. The imbalance between oxidative product and anti-oxidants results in dysfunction and destruction of cells, and further leads to tissue injury. Plenty of studies demonstrate that PEDF has a beneficial aspect in protecting retinal endothelial cell from the increased ROS induced by high glucose levels. In our study, we first describe the similar protective function is also effective on RGC-5. $CoCl_2$ significantly stimulates the ROS activation (477.27±53.87), while pretreatment with PEDF could prevent the ROS increasing (190.18±13.31, P<0.05). Here we evaluate the possible connection between PEDF and mitochondria function. It is well known that mitochondria is a key component for cells including RGCs. Mitochondrial dysfunction plays an important role in hypoxia-induced apoptosis in RGCs. Mitochondrial permeability transition is a critical factor in mediating mitochondrial dysfunction. Morphological disintegration and functional damage of mitochondria occur when the cell death is initiated[53]. From our current research, we provide further evidence that the anti-apoptotic function of PEDF prevents mitochondrial dysfunction by suppressing the mitochondrial permeability transition and obstructing the Cyt C apoptotic pathway. During hypoxia, oxygen rapidly depletes then ATP levels decrease, thus induce ionic gradients in cells are imbalance which lead to subsequent neuronal depolarization. Mitochondrial permeability transition pores are non-specific pores that connect the internal and outer membranes of mitochondria. The molecule diameter up to about 1500 Da is allowed to cross the mitochondrial membrane. Under the hypoxia situation, enormous amounts of Ca^{2+} flow into cells, while adenine nucleotide depletes and cellular ROS levels elevate. These trigger the opening of mPTPs, furthermore, mitochondrial permeability transition occurs. Our observation that PEDF could suppress the opening of mPTPs (5.87±2.34 *vs* 0.47±0.17, P<0.05) proves the protective role of PEDF in the regulation of mPTPs.

Occurrence of the mitochondrial permeability transition in a cell induces a series of danger which directly threaten its survival, including bioenergetic, biosynthetic, and redox[9,54]. The mitochondrial inner membrane is permeable to protons when the mitochondrial permeability transition happens, uncoupling of the electron respiratory chain occurs which results in the destruction of membrane potential, furthermore induces stop generation of mitochondrial ATP[55]. The hypoxia-induced injury of mitochondrial Δψm and declined ATP level is relieved on RGC-5, which are pretreated by PEDF. Our study supports neuroprotection effect of PEDF in the view of preventing mitochondrial dysfunction. The results exhibit in this study also is consistent with previous studies of cerebella granular cells exposed to glutamate. PEDF were demonstrated has the capability in strengthening the mitochondrial activity of cells[56].

Another threatening consequence of altered mitochondrial permeability is the apoptotic proteins release from the mitochondria into the cytosol[53-55]. Because of Cyt C is a critical component of the electron transfer chain, mitochondrial function is damaged when the mitochondrial permeability transition is triggered and Cyt C releases from the mitochondria into the cytosol. In this research, comparing to the cells cultured in hypoxia medium, there is relatively lower cytosolic Cyt C level in PEDF-pretreated cultures, thus proves that there is correlation between PEDF and the protection of mitochondrial function. Furthermore, once Cyt C enters the cytosol, apoptotic peptidase activating factor 1 (Apaf-1) and dATP are activated to assemble an apoptosome. Moreover, apoptosome collects and activates procaspase-9, which further leads to activation of other downstream caspase to launch apoptosis[55].

AIF causes nuclear chromatin condensation and further affects mitochondria, leading to a drop in the Δψm and triggering the release of Cyt C as shown by immunofluorescence staining[57-59]. Moreover, the translocation of AIF occurs in the process of cell death *in vivo*, in models of retinal degeneration. AIF releasing from mitochondria appears to universally associate with cell death. Here we showed elevation of AIF in the RGC-5 in the hypoxic condition and its decrease following protection with PEDF.

Although the underlying mechanisms are not very clear and require further study, the data from our study clearly

demonstrate that PEDF plays an important role in preventing signaling pathways of apoptosis.

In conclusion, our study provides evidence that PEDF can protect RGC-5 from hypoxia-induced mitochondrial dysfunction while inhibiting apoptosis. Its effects include inhibiting the opening of mPTPs and suppressing the decrease of Δψm. Moreover, PEDF also reduces ROS production and inhibited cellular ATP level's reduction which are important index of mitochondrial dysfunction. And the research elucidates PEDF has an effect on inhibiting release of apoptogenic protein. At the same time, PEDF, as a neurotrophins to be new treatment, still requires further *in vivo* studies to ensure the efficiency of therapy in protecting functions on RGCs. PEDF as a highly attractive treatment application in the ophthalmic clinic, the long-term effects in retinal diseases have to be investigated.

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