**Original Article**

**Exendin-4 protects retinal pigment epithelial cells against oxidative stress in vitro via PI3K/Akt-STAT3 activation**

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**Abstract:** The glucagon-like peptide 1 (GLP-1) analogue Exendin-4 (Ex-4) could perform anti-oxidative effects against oxidative damage both in vitro and in vivo. However, little was known about the direct protection of Exendin-4 on RPE cells against oxidative stress and its underlying mechanism. The present study examined the protective effects of Exendin-4 on RPE cells against H₂O₂ treatment in vitro and retina I/R injury in vivo. The results indicated that Ex-4 exerted protective effects under H₂O₂ condition on RPE cells and attenuated the oxidative stress-induced damage, as evidenced by the results of cellular viability, cytotoxicity, apoptosis and western blotting. However, the protective effects of Ex-4 were mainly abrogated by p-Akt inhibitor or p-STAT3 inhibitor. In conclusion, the Ex-4 was able to protect RPE cells from H₂O₂ induced oxidative damage in vitro and attenuate retinal injury from I/R in vivo, which was closely related to PI3k/Akt and p-STAT3 signaling.

**Keywords:** Retinal pigment epithelial cells, oxidative stress, retinal I/R injury, PI3K/Akt-STAT3

**Introduction**

The retinal pigment epithelium (RPE) is a monolayer of pigmented cells lying in the interface between the neural retina and the choriocapillaris [1, 2]. It performs various functions in the visual system, such as forming the part of the outer blood-retinal barrier (BRB), absorbing the light energy, transporting some metabolic end products and interacting closely with photoreceptors in the visual cycle [2]. What’s more, the retinal pigment epithelium could also contribute to the maintenance of the choriocapillaris and photoreceptors structural integrity via secreting some growth factors. The pathomorphological changes of PRE cells were therefore closed to many ophthalmology diseases [3, 4]. For example, the immigration and proliferation of RPE cells refer to be the important events in proliferative vitreoretinopathy (PVR) [5]. What’s more, the dysfunction of PRE cells could also lead to the Age-Related Macular Degeneration (ARMD) and Retinitis Pigmentosa [6, 7].

Evidences indicated that RPE cells were very sensitive to oxidative stress [8, 9]. The overproduction of reactive oxygen species (ROS) under oxidative stress condition would cause the cellular apoptosis or death of RPE cells, as well as some complicated inflammatory response [9-11]. The resulted cellular dysfunctions of RPE cells were closed relatively with the pathomorphological changes of the visual system [12]. Therefore, the oxidative stress damage of RPE cells has becoming a focus concerned in the research field. Previously studies suggested that, the administration of some anti-oxidant could be used to protect the RPE cells against oxidative stress damage, yet the underlying mechanism were confusion and need to be further explored [10, 13-15].

The Exendin-4 (Ex-4) is a kind of glucagon-like peptide 1 (GLP-1) analogue, which was proved to be a glucagon-like protein-1 (GLP-1) receptor agonist [16, 17]. Previously studies suggested that the Exendin-4 could perform anti-oxidative effects in oxidative stress animal models, such as myocardial or cerebral ischemia reperfusion injury, hind limb ischemia etc. Some papers also proved the potential anti-oxidative effect of Exendin-4 on RPE cells, yet the direct evi-
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Evidence of Exendin-4 protective effect against oxidative stress was lacking [18-21].

Besides, PI3K/Akt signaling pathway is essential for the cellular survival and the activation of PI3K/Akt had been proved to be able to suppress the cell apoptosis [22, 23]. The activation of the PI3K/Akt signaling was evolved in some cellular models after treated with Exendin-4, which may potential resulted in a series of bio-modulation, like oxidative stress protective effect [23-25]. Thus we speculated that the PI3K/Akt signaling might also play important roles in the protective effect of Exendin-4 against ROS injury.

Based on these, the present study aims to investigate the protective effects of Exenin-4 on RPE under oxidative stress (OS) condition and the potential roles of PI3K/Akt signaling pathway in it. The protective effects of Exendin-4 on retinal I/R injury were also investigated in vivo in the present study.

Materials and methods

Cell culture

The retinal pigment epithelial cells ARPE-19 cells were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and cultured in flasks in DMEM/F12 medium containing 10% FBS, 50 units/mL penicillin, and 50 μg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO2. The medium was replaced very two days.

Effect of Exendin-4 on H2O2-induced damage

RPE cells were seeded (5000 cells/cm²) and cultured for 24 hours. Exendin-4 (10 nM, Sigma) along, or with combination of 10 μM PI3K inhibitor (LY294002, Cayman Chemical) and 20 μM p-STAT3 inhibitor (Stattic, Sigma), was administrated to the culture medium. After 1 h, a final concentration of 250 μM hydrogen peroxide (H2O2) was applied to induce oxidative stress. The cells were cultured for 24 h for LDH assay, MTT assay, TUNEL staining and Western Blotting. All groups were tested in triplicate.

Lactate dehydrogenase (LDH) release

The oxidative stress induced cellular damage was evaluated by Lactate dehydrogenase (LDH) release assay according to the manufacturer’s protocol. Shortly, the treated cells’ supernatant was centrifuged at 12, 000 rpm for 6 min. 120 μL of the resulted supernatant was incubated with 60 μL LDH working solution for 30 minutes in dark place. The absorbance at 490 nm was analyzed using a micro-plate reader (Bio-RAD 680, USA).

Assessment of cellular apoptosis

The TUNEL staining for cellular apoptosis was performed to evaluate the apoptosis extent according to the manufacturer’s instructions of TUNEL detection kit (Nanjing Jiancheng Bio-engineering Institute, Nanjing, China). Shortly, the 0.1% Triton X-100 solution permeabilized samples were treated with the TUNEL working solution for 60 minutes under dark condition. The nuclear was then stained with DAPI for 10 min. The samples were observed with an inverted microscope (OLYMPUS IX70-SBF, Olympus). For each group, photographs of 10 random fields were taken under a fluorescence microscope (Nikon, Tokyo, Japan). The apoptosis level was calculated as (Annexin V+/Total cells) × 100%.

MTT assessment for cellular viability

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide reduction (MTT) assay was applied for cell viability detection. The samples were incubated with 5 mg/mL of MTT working solution for 4 h. After that, formazan crystals was solved by DMSO and The absorbance at 490 nm was analyzed using a micro-plate reader (Bio-RAD 680, USA).

Western blotting

The cell total protein lysis was obtained and the concentration was measured by protein assay kit (Byeotime, China). 10 μg of protein were transferred electrophoretically onto a polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). After blocking with 2% nonfat milk for 1 h, the samples were incubated overnight with primary antibodies. The following primary antibodies were used for the Western Blotting: anti-p-Akt (1:1000, CST, USA), anti-Akt (1:1000, CST, USA), anti-p-STAT3 (1:1000, CST, USA), anti-STAT3 (1:1000, CST, USA), anti-Bcl-2 (1:1000, CST, USA), anti-Bax (1:1000, CST, USA), anti-Caspase-3 (Cp3, 1:1000, CST, USA). GAPDH (1:8000, Abmart, Shanghai, China) expression was used as an internal control. The optical density of each band was determined with analysis software (Quantity One, Bio-Rad) and normalized.
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All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Chinese PLA general hospital. Every effort was made to minimize the animal suffering. The SD rats were anesthetized with sodium pentobarbital, 30 mg/kg IV. Ligation of external carotid arteries for 60 min was used to induce ischemia/reperfusion (I/R) injury in the rat retina. After ligation, observation of the episcleral veins was applied for confirmation of reperfusion. After the surgery, the

Figure 1. Protective effects of Exendin-4 on Retinal Pigment Epithelial Cells against Oxidative Stress in vitro: Representative staining images of TUNEL staining (A) and quantitative analysis of apoptotic cells (D); LDH release (B) and cell viability (C) of different groups; (E-J) Western blotting and quantitative analysis of p-Akt/Akt, p-STAT3/STAT3, p-Akt/Akt, Caspase-3 (Csp 3)/GAPDH, Bcl-2/GAPDH, Bax/GAPDH. *p < 0.05.

Induction of retinal ischemia/reperfusion (I/R) injury in rat

All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Chinese PLA general hospital. Every effort was made to minimize the animal suffering. The SD rats were anesthetized with sodium pentobarbital, 30 mg/kg IV. Ligation of external carotid arteries for 60 min was used to induce ischemia/reperfusion (I/R) injury in the rat retina. After ligation, observation of the episcleral veins was applied for confirmation of reperfusion. After the surgery, the
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0.9% saline solution dissolved Exendin-4 (10 μg/kg) was injected into the intravitreal of rats for every 12 hours, with or without the combination of PI3K inhibitor (10 μmol/kg) or STAT3 inhibitor.

Figure 2. PI3k inhibitor abrogated the protective effects of Exendin-4. Representative staining images of TUNEL staining (A) and quantitative analysis of apoptotic cells (D) among the H₂O₂ treated control group, Exendin-4 treated group (Exendin-4) and Exendin-4 & PI3k inhibitor treated group (PI3k inhibitor); LDH release (B) and cell viability (C) of different groups; (E-J) Western blotting and quantitative analysis of p-Akt/Akt, p-STAT3/STAT3, p-Akt/Akt, Caspase-3 (Csp 3)/GAPDH, Bcl-2/GAPDH, Bax/GAPDH. *p < 0.05.
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inhibitor (20 µmol/kg). The I/R injury rat received saline vehicle injections were set as negative control and the non-treated animals were set as positive control. After 1 week, the rats were dilled with overdose of sodium pentobarbital and the eyeballs were collected for histological analysis [16]. Only the right eye was used in the present study.

Histological analysis

3-5 µm thick sections of retina samples along the vertical meridian were stained with H&E. The thickness of total retina Outer Nuclear Layer (ONL), Inner nuclear layer (INL) and Inner Plexiform Layer (IPL) were calculated according to the H&E staining image of every group. 5 samples from each group and 3 random fields of each sample were selected for the measurement.

Statistical analysis

All data are presented as mean ± SEM. Significance was calculated with one-way ANOVA followed by the post hoc test for multiple comparisons. A difference at P < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS Statistics (version 16.0, IBM Co., Chicago, USA).

Results

Protective effects of Exendin-4 on RPE cells under oxidative stress condition

The H$_2$O$_2$ was used to induce in vitro oxidative stress in the present study. As shown in Figure 1, H$_2$O$_2$ treatment resulted in cellular apoptosis and impaired cellular function, as indicated by remarkably increased cell apoptosis and LDH release. H$_2$O$_2$ treatment also induced a significant loss of RPE cell viability. However, the increased cellular apoptosis and impaired viability could be attenuated via administration of Exendin-4. To explore the possible mechanism involved in the protective effect of Exendin-4, we measured some anti-apoptotic and apoptotic proteins. Western blotting results indicated that oxidative stress induced by H$_2$O$_2$ significantly decreased the p-Akt/Akt, p-STAT3/STAT3, Bcl-2 levels and increased the pro-apoptotic proteins Cp3 and Bax, but the oxidative stress damage was all lessened by Exendin-4. These data indicated that Exendin-4 exerted certain protection on RPE under oxidative stress condition.

PI3k inhibitor abrogated the protective effects of Exendin-4

In order to explore the protective effects of Exendin-4 on RPE and the potential role of PI3K and STAT3 signaling, the PI3k inhibitor was added together with the Exendin-4 under oxidative stress condition. As shown in Figure 2, compared with Exendin-4 administration alone, cells treated with PI3k inhibitor and Exendin-4 displayed remarkably higher level of the LDH release, apoptotic cell and lower level of cellular viability. The western blotting results indicated that PI3k inhibitor could abrogate the Exendin-4 induced up-regulation of p-Akt/Akt, p-STAT3/STAT3 and Bcl-2 levels. Meanwhile, down-regulation of pro-apoptotic proteins Cp3 and Bax that resulted from Exendin-4 were markedly recovered after adding of PI3k inhibitor as well. The results suggested that the protective effects of Exendin-4 on H$_2$O$_2$ treated RPE were neutralized when the PI3k inhibitor was administrated to the cells.

p-STAT3 inhibitor abrogated the protective effects of Exendin-4

To further explore the potential role of STAT3 signaling in the protective effects of Exendin-4, as well as its relation with PI3K/Akt signaling, the p-STAT3 inhibitor was also supplemented during the Exendin-4 administration under oxidative stress condition. As shown in Figure 3, both the LDH release and apoptotic cells were augmented by p-STAT3 inhibitor. The cell viability was also remarkably decreased after p-STAT3 inhibitor adding. Western blotting results indicated that supplemented p-STAT3 inhibitor had little effect on exendin-4-resulted p-Akt/Akt level. However, p-STAT3 inhibitor could still abrogate the Exendin-4 induced up-regulation of p-STAT3/STAT3, Bcl-2 levels and down-regulation of pro-apoptotic relative proteins Cp3 and Bax contents. The results indicated that p-STAT3 inhibitor could neutralize the rescue effects of Exendin-4 on H$_2$O$_2$ treated RPE, while it has no relative effects with p-Akt/Akt level.

In vivo protection of Exendin-4

The I/R injury of retina was applied for investigation of in vivo protective effect of Exendin-4. As show in Figure 4A, 4B, the thickness of total retina Outer Nuclear Layer (ONL), Inner nuclear layer (INL) and Inner Plexiform Layer (IPL) were significantly increased as compared with the I/R injured group. However, adding of either
Figure 3. p-STAT3 inhibitor abrogated the protective effects of Exendin-4. Representative staining images of TUNEL staining (A) and quantitative analysis of apoptotic cells (C) among the H₂O₂ treated control group, Exendin-4 treated group (Exendin-4) and Exendin-4 & p-STAT3 inhibitor treated group (p-STAT3 inhibitor); LDH release (B) and cell viability (D) of different groups; (E-J) Western blotting and quantitative analysis of p-Akt/Akt, p-STAT3/STAT3, p-Akt/Akt, Caspase-3 (Csp 3)/GAPDH, Bcl-2/GAPDH, Bax/GAPDH. *p < 0.05.

PI3k inhibitor or p-STAT3 inhibitor could neutralize the protective effects of Exendin-4 and resulted in decreased ONL, INL and IPL thickness.
Discussion

It has been demonstrated that glucagon-like peptide 1 (GLP-1) analogue, Exendin-4, could perform anti-oxidative effects against oxidative damage both in vitro and in vivo. However, little is known about the direct evidence of Exendin-4 protective effects against oxidative stress on
RPE cells as well as its underlying mechanism. The present study examined the protective effects of Exendin-4 on RPE cells against H$_2$O$_2$ treatment in vitro and on retina I/R injury in vivo. Western blotting results also indicated that the protective effect of Exendin-4 against oxidative stress damage was closed to PI3k/Akt and p-STAT3 signaling.

Previously studies proved that Glucagon-like peptide-1 (GLP-1) could bind to GLP-1R to activate many intracellular signaling, and mainly of these cellular signaling were restricted to pancreatic beta-cells [16, 17, 26]. Recently evidences showed that many peripheral tissues, such as central and peripheral nervous systems, heart, kidney etc. could also express GLP-1R. Especially, Puddu etc. found that the presence of functional GLP-1R was also expressed in RPE cells and it could be directly activated by GLP-1. The GLP-1 and its longer-acting analogue Exendin-4 were proved to have protective effects against a variety of oxidative injury in vivo and in vitro [19, 27].

Consistently, our results showed that Ex-4 exerts protective effects against H$_2$O$_2$ treatment on RPE cells and attenuated the oxidative stress induced damage, such as decreased cellular viability, and increased cytotoxicity and apoptotic cells percentage. The underlying mechanisms of Exendin-4 protective effect was proved to involve the activation of PI3K-Akt signaling and apoptotic related mitochondrial pathway. These suggested that Ex-4/GLP-1R might potentially contribute to rescue RPE cells from oxidative stress damage via PI3K-Akt signaling. Our results confirmed that the administration of Ex-4 to RPE cells would significantly attenuate the decreased the p-Akt/Akt, p-STAT3/STAT3 and Bcl-2 levels, as well as increased Cp3 and Bax caused by H$_2$O$_2$ treatment. However, the protective effects of Ex-4 were mainly abrogated by adding p-Akt inhibitor or p-STAT3 inhibitor. The increased p-Akt/Akt by Ex-4 in H$_2$O$_2$ treated RPE cells was not affected by p-STAT3 inhibitor, which suggested that p-STAT3 functioned as a downstream target of PI3k/Akt signaling during the Ex-4 protection process [20, 28-30].

In conclusion, we have shown that the Ex-4 was able to protect RPE cells from H$_2$O$_2$ induced oxidative damage in vitro and retina from I/R injury. The activation of PI3k/Akt and the downstream effector Stat3 might involve in the process. Thus stimulation of GLP-1R might provides a useful anti-oxidation solution for the treatment RPE related retinal diseases.

Disclosure of conflict of interest

None.

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