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Liraglutide alleviates H_2O_2-induced retinal ganglion cells injury by inhibiting autophagy through mitochondrial pathways

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ABSTRACT

Retinal ganglion cells (RGCs), which exist in the inner retina, are the retinal neurons which can be damaged in the early stage of diabetic retinopathy (DR). Liraglutide, a glucagon-like peptide-1 (GLP-1) analog, exerts biological functions by binding the receptor (GLP-1R), the expression of which in RGC-5 cells was first shown by our team in 2012. It was reported that liraglutide prevented retinal neurodegeneration in diabetic subjects. However, the involvement of mechanisms such as autophagy and mitochondrial balance in liraglutide-induced retinal protection is unknown. Here, we aimed to investigate the protective effects of liraglutide and explore the potential mechanisms of liraglutide-induced retinal RGC protection. RGC-5 cells were treated with H_2O_2 and/or liraglutide. Cell viability was detected with the CCK-8 kit. The axon marker GAP43, autophagy and mitophagy indicators LC3A/B, Beclin-1, p62, Parkin, BCL2/Adenovirus E1B 19 kDa protein-interacting protein 3-like (BNIP3L) and the key regulator of mitochondrial biogenesis PGC-1α were examined via western blot analysis. Autophagy was also evaluated using the ImageXpress Micro XLS system and transmission electron microscopy (TEM). Reactive oxygen species (ROS), mitochondrial membrane potential and fluorescent staining for mitochondria were also measured using the ImageXpress Micro XLS system. Our results showed that pretreatment with liraglutide significantly prevented H_2O_2-induced cell viability decline, mitochondrial morphological deterioration and induction of autophagy, which appeared as increased expression of LC3 II/I and Beclin-1, along with p62 degradation. Moreover, liraglutide suppressed the H_2O_2-induced decline in GAP43 expression, thus protecting cells. However, rapamycin induced autophagy and blocked the protective process. Liraglutide also provided mitochondrial protection and appeared to alleviate H_2O_2-induced ROS overproduction and a decline in mitochondrial membrane potential, partially by promoting mitochondrial generation and attenuating mitophagy. In conclusion, liraglutide attenuates H_2O_2 induced RGC-5 cell injury by inhibiting autophagy through maintaining a balance between mitochondrial biogenesis and mitophagy.

1. Introduction

The prevalence of diabetes has attracted widespread attention in all populations and has become a predominant health threat worldwide. Diabetic retinopathy (DR) is one of the most severe chronic complications of diabetes, which predominantly accounts for preventable blindness in working-aged adults [1]. An accumulating body of evidence indicates that retinal neurodegeneration and microangiopathy jointly participate in the pathogenesis of DR, and neuropathy precedes microangiopathy [2]. Retinal ganglion cells (RGCs) are the solely afferent neurons by which visual information is delivered to the visual center. It is currently recognized that despite systemic and ocular therapies being used, a considerable proportion of patients will still progress to vision-threatening DR. New approaches targeting the early stage of DR have emerged. Glucagon-like peptide-1 (GLP-1), a gut incretin hormone secreted by intestinal L cells, has been considered to be an important intelligent hypoglycemic agent, with respect to its multiple biological effects, including effective glycemic control, lipid metabolism, weight loss, blood pressure reduction, organ protection and significant neurotrophic action, through binding with the receptor, GLP-1R [3]. Importantly, our team firstly proved that GLP-1R exists in RGC-5 cells and highlighted its protective mechanisms in 2012 [4,5]. Furthermore, emerging evidence has demonstrated that administration of liraglutide, a GLP-1 analog, prevented retinal neurodegeneration

Abbreviations: AMPK, AMP-activated kinase; BNIP3L, BCL2/Adenovirus E1B 19 kDa protein-interacting protein 3-like; CaMK, calcium/calmodulin-dependent kinase; CaMKKβ, Ca^{2+} calmodulin dependent protein kinase kinase beta; DR, diabetic retinopathy; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; JC-1, 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanineiodide; GCN5L1, GCN5-like protein 1, MTG, Mito-tracker Green; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator-1 alpha; RGCs, retinal ganglion cells; ROS, reactive oxygen species; TEM, transmission electron microscopy

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including neural apoptosis, glial activation, and electroretinographical abnormalities [6]. However, the mechanisms have not been fully elucidated.

Autophagy is a cellular process for removal of impaired proteins and organelles. Several studies have focused on autophagy in RGCs. Russo et al. [7] revealed a disorder of autophagy at the baseline in a retina that had suffered ocular ischemia/reperfusion injury. Park [8] and Piras et al. [9] suggested that originally, the activation of autophagy in the dendrites of RGCs represented a pro-survival mechanism, but then occurred in cytoplasm and exacerbated cell death. Accurate regulation of autophagy in dendrites and cytoplasm is pivotal for maintaining axonal integrity and cell survival. Physiologists have also confirmed that the delicate balance between mitochondrial biogenesis and mitophagy was the key issue in cellular homeostasis [10]. As enriched mitochondria develop inside axons, mitochondrial balance, combined with regulation of autophagy, lays the foundation for RGC protection and may play a potential role in preventive and therapeutic approaches to DR. Several studies have shown that GLP-1 dynamically regulates autophagy in multiple organs [11–13]. Moreover, GLP-1 analogs have been suggested to maintain mitochondrial integrity, mitigate autophagy and ameliorate ischemia-reperfusion injury in murine steatotic liver [14]. Therefore, in this study, we speculate that liraglutide may participate in the protection of RGC-5 cells by regulating autophagy depending on mitochondrial pathways.

2. Material and methods

2.1. Cell culture and treatment

RGC-5 cells were obtained from the laboratory of Fudan University and cultured with DMEM (Hyclone, SH30022.01) containing 10% fetal bovine serum (BI, 04-001-1ACS) in a 5% CO_2 humidified atmosphere at 37 °C. During the logarithmic growth phase, cells were pretreated with liraglutide (10 nM) (Novo Nordisk) for 2 h, with or without exposing to H_2O_2 (20 μM) for 24 h. Additionally, cells were also incubated with liraglutide for 2 h, with or without rapamycin (RA, 10 nM) for 1 h and then exposed to H_2O_2 for 24 h.

2.2. Measurement of cell viability

Cell viability was detected with the CCK-8 detection kit (Dojingdo Laboratories, CK04). RGC-5 cells were seeded in a 96-well microplate (Costar, 3599) at a density of 9000 cells per well and treated with different concentrations of H_2O_2 (10, 15, 20, 25 and 30 μM) for 24 h, or pretreated with different concentrations of liraglutide (1, 10, 50, 100, 500 and 1000 nM) for 2 h, with or without 20 μM H_2O_2 for 24 h. Subsequently, the wells were incubated with CCK-8 reagent (10 μL per well) and the plate was placed at 37 °C for 1 h. A monochromator microplate reader was used to count viable cells via absorbance measurements at a wavelength of 450 nm. The optical density value equaled OD value.

2.3. Western blot analysis

Equal amount of protein extracted from each group were isolated with 8% and 12% SDS–PAGE and transferred to polyvinylidene difluoride membrane. After blocking for 1 h with 5% milk, membranes were incubated with primary antibodies LC3A/B (1:1000 dilution, CST, 4108), p62 (1:2000 dilution, Abcam, 56416), Beclin-1 (1:1000 dilution, CST, 3738), GAP43 (1:2000 dilution, Abcam, 16053), Parkin (1:1000 dilution, Abcam, 15954), BNIP3L (1:1000 dilution, CST, 12396), PGC-1α (1:1000 dilution, Merck Millipore, ST1202), β-actin (1:2000 dilution, ZSJQ-BIO, TA-09), GAPDH (1:2000 dilution, CST, 2118) overnight at 4 °C, followed by horseradish peroxidase-conjugated secondary antibodies (1:2000, ZSGB-BIO, ZB2301, ZB2305). Then the membranes were detected via scotography with X-ray film.

2.4. Transmission electron microscopy

After fixing in 4% paraformaldehyde and 2.5% glutaraldehyde in a 0.1 M phosphate buffer for 2 h, RGC-5 cells were fixed with 1% OsO4 and washed, followed by dehydrating with graded alcohol and embedding in epoxy resin. Ultrathin sections were placed on copper grids, stained with uranyl acetate and lead citrate and examined by 80 kV with a Zeiss model electron microscope.

2.5. Immunofluorescence assay

RGC-5 cells were seeded in a 24-well plate (Costar, 3524) and treated. Subsequently, cells were fixed with ice-cold (−20 °C) methyl alcohol for 30 min at room temperature and penetrated with 0.5% Triton-X solution for 20 min. After blocking cells with 5% goat serum for 30 min at room temperature, cells were treated with the primary antibody against LC3A/B (1:200 dilution, CST, 4108) overnight at 4 °C. Next, the plate was washed with 0.5% Triton-X three times and probed with FITC-conjugated anti-rabbit secondary antibody (1:200, ZSGB-BIO, ZF0311) in a humidified container for 1 h at room temperature. Finally, the cells were rinsed with 0.5% Triton-X three times and nuclei were stained with DAPI (Beyotime, C1005). The images were recorded using ImageXpress Micro XLS system and Multi Wavelength Cell Scoring module was used to analyze the data. The cells were selected based on both DAPI and FITC staining by setting the maximal and minimal diameter, and the minimal fluorescence intensity relative to the background from both channels. The LC3A/B was traced automatically by setting the minimal and maximal width and minimal fluorescence intensity above background. The integrated intensity/cell which represented the fluorescence intensity of each cell was used to measure LC3A/B expression. The fluorescence intensity of each cell was calculated as the total fluorescence intensity divided by the number of cells.

2.6. Determination of reactive oxygen species (ROS)

2′,7′-Dichlorofluorescin diacetate (DCFH-DA, Beyotime, S0033) (10 mM) were added to cells for 25 min at 37 °C in the dark, then the cells were rinsed with serum-free DMEM three times. ImageXpress Micro XLS system was used to observe fluorescence and Multi Wavelength Cell Scoring module was used to analyze the data. The integrated intensity/cell which represented the fluorescence intensity of each cell was used to reflect ROS production in different groups.

2.7. Measurement of mitochondrial membrane potential

5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanineiodide (JC-1, Beyotime, C2006) was used to detect mitochondrial membrane potential. RGC-5 cells were treated and stained with JC-1 solution (10 mg/ml) for 25 min at 37 °C and washed twice with buffer (pre-cooled at 4 °C), followed by detection with the ImageXpress Micro XLS system and analysis with the Multi Wavelength Cell Scoring module. Mitochondrial membrane potential is shown as the ratio of red to green fluorescence intensity.

2.8. Fluorescent staining for mitochondria loaded with Mito-Tracker Green

Cells were treated with Mito-Tracker Green FM (MTG, Beyotime, C1048) for 30 min at 37 °C in the dark. Working fluid was removed, followed by rinsing twice with pre-warm DMEM. Nuclei were determined by using Hoechst 33342 dye (Beyotime, C1022). The pictures were recorded using ImageXpress Micro XLS system and the data was analyzed using the Multi Wavelength Cell Scoring module. The integrated intensity/cell which represented the fluorescence of each cell was used to measure MTG expression in different groups.