Protective effect of leukemia inhibitory factor on the retinal injury induced by acute ocular hypertension in rats

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Abstract. Glaucoma is one of the leading causes of irreversible blindness worldwide. As such, neuroprotective therapy is essential for the treatment of this disease. Leukemia inhibitory factor (LIF) is a member of the IL-6 cytokine family and the LIF signaling pathway is considered to be one of the major endogenous factors mediating neuroprotection in the retina. Therefore, the present study aimed to investigate the possible effects of LIF in acute ocular hypertension (AOH). The intraocular pressure in rat eyes was raised to 110 mmHg for 1 h by infusing the anterior chamber with normal saline to establish the AOH model. In the treatment group, LIF was then injected into the vitreous cavity after AOH was ceased. The retinal tissues were obtained after the termination of AOH, and H&E staining was conducted to assess the morphological damage. The number of retinal ganglion cells (RGCs) was counted using the Fluoro-Gold retrograde staining method. TUNEL staining was used to determine the extent of apoptosis among the retinal cells. In addition, the protein expression levels of cleaved caspase-3, poly (ADP-ribose) polymerase (PARP), STAT3 and components of the AKT/mTOR/70-kDa ribosomal protein S6 kinase (p70S6K) signaling pathway were examined by western blotting. The results showed that AOH induced tissue swelling and structural damage in the retina, which were reversed by LIF injection. In the LIF treatment group, RGC loss was significantly inhibited and the quantity of TUNEL-stained cells was also significantly reduced, whereas the expression of cleaved caspase-3 and PARP was decreased. Furthermore, increased phosphorylation of STAT3, AKT, mTOR and p70S6K was observed after LIF treatment. By contrast, pretreatment with the STAT3 inhibitor C188-9 or the PI3K/AKT/mTOR inhibitor LY3023414 reversed the LIF-induced inhibition of RGC loss. These results suggested that exogenous LIF treatment inhibited the retinal damage induced by AOH, which was associated with the activation of STAT3 and mTOR/p70S6K signaling. Therefore, LIF may serve a role in neuroprotection for glaucoma treatment.

Introduction

Glaucoma is one of the leading causes of irreversible blindness worldwide, the prevalence of which is projected to reach 111.8 million by 2040, with ~10% of patients succumbing to blindness (1). Glaucoma is a chronic neurodegenerative disease that is characterized by the progressive loss of retinal ganglion cells (RGCs), including the neurons and their axons, resulting in structural and functional defects in the visual field (2). Intraocular pressure (IOP) reduction is considered to be the most promising intervention strategy to protect the optic nerve from glaucomatous damage (3,4). However, the deterioration of glaucomatous neuropathy cannot be prevented in some patients, for whom lowering the IOP is either insufficient or difficult to achieve (5). Furthermore, accumulating evidence suggests that optic nerve damage can continue despite effective IOP reduction (3-6). Therefore, the possible use of neuroprotective strategies to prevent visual loss in glaucoma is garnering the attention of this research field (7,8).

A number of causes have been reported to be responsible for RGC damage and death in glaucoma, including IOP elevation, ischemia/reperfusion (I/R) damage of the retina, oxidative stress, glutamate neurotoxicity, neurotrophic growth factor deprivation and immune disturbance (9). Acute ocular hypertension (AOH) mimics the pathophysiological process of acute glaucomatous damage (10), as well as I/R injury to the retina (11,12). Therefore, animal models of AOH are frequently used for glaucoma research. Aberrant IOP increase induces stress and strain to the eye, resulting in the compression, deformation and remodeling of the lamina cribrosa to...
induce mechanical axonal damage and disruption in axonal transport (13,14). Therefore, retrograde delivery of essential neurotrophic factors, such as brain-derived neurotrophic factor and its receptor, interleukin-6 and neural growth factor, to RGCs from the central nervous system is interrupted (15). I/R damage is also reported to be involved in retinal damage induced by AOH (16). During ischemia, glutamate is released in the retina and induces the death of neurons expressing ionotropic glutamate (N-methyl-D-aspartate) receptors (17). RGC apoptosis has been reported to be caused by the activation of caspase signaling induced by the abnormally high concentration of glutamate (18).

Several cytokines have been previously found to be involved in the pathophysiology of glaucoma, such as tumor necrosis factor-α, interleukin-1β, interleukin-6 and interleukin-18 (19). In particular, leukemia inhibitory factor (LIF) is a member of the IL-6 cytokine family and has been reported to be present in the retina (20,21). The LIF signaling pathway is considered to be one of the major endogenous factors mediating neuroprotection in the retina (20,21). Mechanistically, LIF activates the Janus kinase (JAK)/STAT3, PI3K/AKT and ERK1/2 signaling pathways to facilitate the neuroprotection of retina from injury (22-27). Photoreceptor injury or degeneration activates a subset of Müller glial cells into expressing LIF, which initiates a neuroprotective signaling cascade between photoreceptors and Müller cells (20,28). These signaling events include activation of the JAK/STAT3 pathway (29-31), which result in the upregulation of the expression of several genes, including STAT3 (18). In addition, LIF was shown to protect the degenerative retina from apoptosis (32). Activation of the mTOR/70-kDa ribosomal protein S6 kinase (p70S6K) signaling pathway has also been reported to be activated by exogenous LIF (33), which may be responsible for neuroprotection against ischemic brain injury (34).

Our previous study on a rat AOH model has revealed that LIF and LIF receptor protein expression is upregulated associated with activation of the STAT3 and AKT signaling pathways (35). Therefore, in the present study the potential effects of exogenous LIF treatment on retinal damage induced by AOH in rats were investigated.

Materials and methods

Animals. The protocol of the present study was approved by the Experimental Animal Ethics Committee of Xiamen University, School of Medicine (Xiamen, China; approval no. 20150306155209) and followed the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic Vision Research (https://www.arvo.org/About/policies/arvo-statement-for-the-use-of-animals-in-ophthalmic-and-vision-research/). Adult male Sprague-Dawley rats (aged 8-12 weeks old; weight 250±30 g; n=140) were obtained from the Shanghai SLAC Laboratory Animal Co., Ltd. The rats were maintained on a 12-h light-dark cycle (~20°C; humidity ~50%) and were dark-adapted for ≥2 h before any experiments. All animals had access to food (standard lab chow) and water ad libitum. All efforts were made to minimize the number of animals used and their suffering. Prior to AOH induction and drug injection procedures, deep anesthesia was induced by an intraperitoneal injection of pentobarbital sodium (30 mg/kg; Sinopharm Chemical Reagent Co., Ltd.).

Induction of AOH. The experimental procedure has previously been described (35). Briefly, following the topical administration of 0.5% proparacaine (Alcon), the eye pupil was diluted with 0.5% tropicamide (Alcon). Under a Spot OPMI 11 operation microscope (Carl Zeiss AG), the anterior chamber of the eye was cannulated by 7-scalp infusion acupuncture needle connected to a 500-ml container of sterile saline. Only the right eyes were chosen for the experiment; the left eyes remained untouched and served as control. The IOP was then elevated to ~110 mmHg by raising the height of the saline container to 150 cm above the eye for 60 min. The infusion needle was then removed before further experiments on the rats were performed.

LIF injection. After the needle was removed and AOH was ceased, intravitreal injection of 1 µg/µl LIF was conducted immediately. Topical anesthesia by 0.5% proparacaine eyedrops (Alcon Inc.) was performed as needed. A total volume of 5 µl LIF was injected into the vitreous through the par plana using a 33G microsyringe (Hamilton Co.). For the untreated AOH group, a dose of 5 µl PBS was injected instead of LIF solution.

In another set of experiments, to test the involvement of STAT3 and the PI3K/AKT/mTOR signaling pathway in the effect induced by LIF injection, the pretreatment of intravitreal injection through the par plana of either the STAT3 inhibitor C188-9 (10 mM; Selleck Chemicals) or the PI3K/AKT/mTOR inhibitor LY3023414 (50 nM; Selleck Chemicals), was conducted 3 h prior to LIF injection (~2 h before AOH induction). A dose of 5 µl PBS was used as a control. C188-9, LY3023414 and PBS injections were all performed under topical anesthesia by 0.5% proparacaine and general anesthesia by pentobarbital sodium (30 mg/kg).

Histological assessment of the ocular tissue. One day or 3 days after AOH induction, rats were sacrificed by anesthetic overdose with an intraperitoneal injection of pentobarbital sodium (150 mg/kg), and the eyeball was immediately emulsified and frozen (~20°C) in the optimal cutting temperature compound (Sakura Finetek Japan Co., Ltd.). The eyeball was sectioned along the meridian to a thickness of 10 mm to assess the histological changes in the anterior part of the eyes. Tissue preparations were stained with hematoxylin (3 min) and eosin (1 min) at room temperature and viewed under a light optical microscope (Nikon Co., cTokyo, Japan). The retinal thickness was measured using Image-Pro Plus 6.0 (Media Cybernetics) and the data were proceeded for statistical analysis.

Fluoro-Gold (FG) retrograde labeling and cell counting of the RGCs. RGCs were retrogradely labeled with Fluoro-Gold™ (Fluorochrome, LLC) 7 days before the induction of AOH. The procedures were described in our previous reports (9,31). Briefly, the rats were deeply anesthetized with 30 mg/kg pentobarbital sodium (i.p.) and placed in a prone position on the stereotaxic apparatus (RWD Life Science Co. Ltd.). RGC labeling of both eyes was conducted by injecting 4% FG into
the superior colliculus at 6.0 mm caudal to the bregma and 1.0 mm lateral to the midline on both sides (3 µl each), to a depth of 5.0 mm from the surface of the skull. A total of 3 days after LIF or PBS injection, the rats were sacrificed by overdose of pentobarbital sodium before the eyes were enucleated and fixed in 4% paraformaldehyde solution for 40 min at room temperature. The retinas were dissected free and flatly mounted onto a glass slide. The FDA-labeled RGCs were then identified under a fluorescence microscope (Leica DM2500; Leica Microsystems GmbH) with a wide band ultraviolet filter (0.1% fluoroglow solution in distilled water with a pH of 4.5, Ex 414 nm, Em 541 nm). RGCs were manually counted by another investigator to blinded to the experiment protocols. For each quadrant of the retina, three images were captured at 1, 2 and 3 mm radially from the optic disc in the identical retinal preparation (magnification, x10); the total number of RGCs was counted in all four quadrants using the image analysis program Image J (version 1.52; National Institutes of Health).

**Western blotting.** The rat retinas were dissected free after the global enucleation and homogenized with lysis buffer (Solarbio Science & Technology, China), and the protein concentration was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Inc.). SDS-PAGE (12%) of the protein (20 µg per lane) was performed for 1-2 h and then transferred onto a PVDF membrane (MilliporeSigma). After blocking with 2% bovine serum albumin (Ameresco, Inc.), TUNEL staining (37˚C; 1 h) of the retinal sample was performed using a TUNEL assay kit (Promega Corporation), whereas the cell nuclei were stained with 50 mM DAPI (37˚C; 15 min, Vector Laboratories, Inc.). As a positive control, sections were incubated (room temperature; 10 min) with 0.5 µg/ml DNase I (Promega Corporation) to confirm the TUNEL assay kit. TUNEL-positive cells were observed and counted under fluorescence microscopy. The TUNEL-positive cells in each section were counted and quantified as per mm² of the retina using the image analysis program Image J. A total of six images on x20 magnification were used from two sections per animal.

**Statistical analysis.** All data are presented as the mean ± standard deviation. One-way ANOVA followed by Tukey's multiple comparisons were performed using SPSS (version 17.0; SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**LIF alleviates retinal damage by AOH.** H&E staining was used to assess the effect of LIF on retinal histopathology induced by AOH. As shown in Fig. 1A (n=3), on day 1 after AOH, the thickness of the inner nuclear layer (INL) was decreased, whereas that of the inner plexiform layer (IPL) was markedly increased. The nuclei in the RGC layer also appeared larger compared with those in the normal control retina. On day 3 after AOH, a marked decrease in the IPL was observed. After LIF treatment, the change in the thickness of the IPL was reversed compared with AOH group without LIF treatment. The change in the total retinal thickness (TRT) is presented in Fig. 1B and C (n=3/group). At 1 day after AOH, the TRT increase in both AOH and LIF treatment groups was statistically significant compared with the control (both P<0.05). At 3 days after AOH, significant decrease in the TRT was noticed in AOH rats compared with the control (P<0.001), while the TRT in AOH rats treated with LIF was significantly reversed compared with the AOH group (P<0.001).

**LIF improves RGC survival after AOH.** An FG tracer was used to label RGCs to assess the effect of LIF on RGC survival after AOH (Fig. 2A). Quantitative analyses indicated that the FG-labeled RGC density in the AOH model retina (1,572.6±21.3/mm²) was significantly less compared with that in the normal control retina (2,131.2±85.6/mm²; P<0.001) (Fig. 2B). In retinae that received intravitreal LIF treatment, the RGC density was significantly higher compared with those in the AOH group without LIF treatment. The nuclear layers in the AOH model retina were markedly increased. The nuclei in the RGC layer also appeared larger compared with those in the normal control retina.
of TUNEL-positive cells was increased significantly in the AOH group (127.6±30.0/mm², n=3) compared with that in the normal control group (1.8±3.3/mm², n=3) (P<0.001). Accordingly, 1 day after AOH, the protein expression levels of cleaved-caspase-3 and PARP, indicators of apoptotic activity in the tissue, were significantly increased (Fig. 3C-E). After treatment with intravitreal LIF injection, the number of apoptotic cells (26.9±13.3/mm², n=3) was reduced significantly compared with that in the AOH group (P<0.001; Fig. 3B). In addition, the expression of cleaved-caspase-3 (P<0.001) and PARP (P<0.01) were significantly reduced compared with that in the AOH group (Fig. 3C-E).

**LIF upregulates the JAK/STAT signaling pathway after AOH.** As reported previously, the expression of STAT3, AKT/mTOR/p70S6K signaling pathways components peaked at around day 3 post AOH (35). The level of STAT3 phosphorylation in the rat retina was tested 3 days after AOH. A significant increase in the phosphorylation of STAT3 was observed only in the LIF treatment group compared with the AOH group (P<0.001, n=3/group; Fig. 4).

**Figure 1. Protective effect of LIF on the rat retina 1 and 3 days after AOH.** (A) Morphological changes in the rat retina. On day 1 after AOH, the thickness of the INL was decreased, whereas that of the IPL was increased. On day 3 after AOH, the IPL decreased markedly. After LIF treatment, the change in the thickness of the IPL was reversed compared with AOH group without LIF treatment. Scale bar, 100 µm. Changes in retinal thickness (B) 1 day and (C) 3 days after AOH. n=3/group. *P<0.05 and **P<0.001. AOH, acute ocular hypertension; LIF, leukemia inhibitory factor; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PR (IS/OS), photoreceptor (inner segment/outer segment).
Inhibition of JAK/STAT3 or PI3K/AKT/mTOR pathways reverses LIF-induced RGC protection after AOH. Compared with that in the AOH retina treated with LIF and PBS intravitreal injection, the RGC density was significantly lower in AOH rats receiving intravitreal injection of the JAK/STAT3 inhibitor C188-9 or with the PI3K/AKT/mTOR inhibitor LY3023414 (both P<0.01; Fig. 6, n=4/group).

Figure 2. FG retrograde labeling to determine the effects of LIF on RGC survival after AOH. (A) FG retrograde labeling of RGCs; 1, 2 and 3 mm indicate the radial distances to the optic disc in the identical retinal preparation for each treatment condition. Scale bar, 200 µm. (B) The results of RGC counting under the fluorescence microscope (Leica DM2500). For each quadrant of the retina, three images were captured at 1, 2 and 3 mm radially from the optic disc in the identical retinal preparation (magnification, x10). RGCs were manually counted by an investigator blinded to the experiment protocols. *P<0.001, n=4/group. (C) Partial enlargement (magnification, x10) of an FG-labeled image. The red arrows refer to examples of RGCs. Scale bar, 40 µm. AOH, acute ocular hypertension; FG, Fluoro-Gold; LIF, leukemia inhibitory factor; RGC, retinal ganglion cell.
Discussion

In the present study, the effects of exogenous LIF on the survival of RGCs was investigated in AOH model rats. When injected into the vitreous, LIF significantly inhibited the retinal atrophy and RGC loss induced by AOH. Furthermore, apoptosis was reduced after LIF injection. Activation of the AKT/mTOR/p70S6K and JAK/STAT signaling pathways may be associated with these neuroprotective effects of LIF.

Several mechanisms are involved in the pathological changes in the retina after AOH. In the early stages, direct stress on the inner retina leads to the death of RGCs and...
axonal damage (13,14). IOP elevation may also directly obstruct retinal blood vessels and decrease retinal blood flow (36). I/R damage of the retina also serves a key role in neuronal cell death in the latter pathological stages (37,38). In the present study, tissue edema and disorder in cell arrangement were observed 1 day after AOH, followed by atrophy of the retina and cell loss 2 days later. It has been observed that the degree of neuronal loss is associated with the duration of AOH (I/R) imposed on the eye (11,12). Retinal thinning and RGC loss are more evident with longer reperfusion times (11,12). Treatment with intravitreal LIF injection prevented this RGC loss and atrophy of the retina after AOH. However, the underlying mechanism of this type cell death after I/R induced by AOH, as well as
the protective effects of LIF, remain largely unknown and warrant further study.

Apoptosis inhibition is reported to be associated with the neuroprotective effect of LIF in the retina. In a previous model of light-mediated retinal injury, apoptosis of photoreceptor cells triggers the expression of LIF from Müller cells (20). LIF accumulation then promotes the expression of fibroblast growth factor-2 (18) and activates the STAT3 signaling pathway (39) to protect photoreceptors from apoptosis. In cultured human neural progenitor cells, exogenous LIF has also been reported to inhibit caspase-mediated apoptosis (40). In the present study on a rat AOH model, TUNEL-stained cells were noticed in the RGC, INL and ONL layers 24 h after AOH. After treatment with exogenous LIF, the apoptotic cells were significantly reduced compared with those in the untreated AOH group, suggesting that inhibition of apoptosis is associated with the protective effects of LIF on the RGC loss induced by AOH. The protein expression of cleaved caspase-3 and PARP was also reduced in the LIF treatment group, which is in accordance with the aforementioned observation, since both cleaved caspase-3 and PARP are indicators of apoptosis activation (41,42). Activation of caspase family members, especially caspase-3, by the accumulation of neurotoxic glutamate, is considered to be one of the major mechanisms responsible for RGC death observed in glaucoma (43). Nevertheless, other mechanisms aside from apoptosis may be responsible for the neuroprotective effects of LIF against RGC damage and death. Indeed, it has been previously shown that LIF can block amyloid β-mediated induction of autophagy-related activity in HT-22 mouse hippocampal cells. In addition, suppression of the autophagy marker, light chain 3II, by LIF has also been observed in a Drosophila model of Alzheimer's disease (44). However, whether autophagy-related mechanisms are involved in the neuroprotective effects of LIF against AOH-induced retinal damage requires further investigation.

Our previous study showed that AOH activated the expression of intrinsic LIF and LIF receptors in the retina, which is accompanied by the upregulation of STAT3 activity and AKT protein expression (35). It was therefore hypothesized that this LIF activation can exert a protective effect against retinal damage induced by AOH through the JAK/STAT and AKT signaling pathways. In the present study, exogenous LIF treatment in AOH prevented RGC damage/death whilst also upregulating the STAT3 pathway. In contrast to what occurred in AOH without treatment, when treated with exogenous LIF, the ratio of phosphorylated/non-phosphorylated JAK/STAT and AKT signaling pathway components increased markedly, indicating the enhancement of these pathways' activation. It has also been reported that LIF is involved in protecting photoreceptors against light-induced injury, predominantly through activation of the JAK/STAT3 pathway (20,45). In addition, increased, although not statistically significant, phosphorylation of AKT in the retina after LIF injection was also observed in the present study, which is accompanied by the increased phosphorylation of mTOR and p70S6K. It has been shown that the mTOR/p70S6K signaling pathway has a crucial effect in modulating axonal protein synthesis and neurite growth during the development or recovery of the nervous system in response to injury (46). RGC counting using FG labeling revealed the inhibition of LIF-induced neuroprotection against RGC loss by potent STAT3 or PI3K/AKT/mTOR pathway inhibitors. Results of the present study therefore suggested that the neuroprotective effects of LIF against AOH-induced retinal damage may be associated with the activation of both the STAT3 and the mTOR/p70S6K signaling pathways.

The present study has several limitations. First of all, the effect of LIF on RGC survival after AOH needs to be monitored for a longer time, and a combination of any functional assessment would be valuable. Additionally, the mechanism underlying the neuroprotection of LIF injection needs further investigation and the involvement of the signaling pathways need to be clarified. Nevertheless, the present study demonstrated the neuroprotective effects of exogenous LIF treatment against retinal damage observed in AOH, which suggests that LIF may serve a role in the neuroprotective treatment for glaucoma. Further studies are needed to confirm this, as well as the neuroprotective effects of LIF treatment in patients with glaucoma.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
JL, CH and RW designed the study. JL and RW were responsible for the data collection. JL, RG, CH and YW conducted the experiments. JL, RG and RW were responsible for data analysis. JL and CH confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
The present study was approved by the Ethics Review Committee of Xiamen University (Xiamen, China; approval no. 20150306155209).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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