Ligustrazine protects against chronic hypertensive glaucoma in rats by inhibiting autophagy via the PI3K-Akt/mTOR pathway

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Purpose: Glaucoma is a leading cause of global irreversible blindness, and characterized by the progressive loss of retinal ganglion cells (RGCs). Ligustrazine (TMP) is a natural product that has shown beneficial effects on various diseases. This study aimed to determine whether ligustrazine produces a therapeutic effect on glaucoma and to investigate its underlying mechanisms.

Methods: A rat chronic hypertensive glaucoma model was induced by episcleral vein cauterization (EVC). Adult Sprague-Dawley (SD) rats were intraperitoneally administered TMP at a dose of 80 mg/kg once a day, from two days before EVC to one month after EVC. To elucidate the role of the mammalian target of rapamycin (mTOR) and phosphoinositide 3-kinase (PI3K), TMP-treated experimental rats were co-treated with the mTOR inhibitor rapamycin (5 mg/kg) or the PI3K inhibitor Ly294002 (10 mg/kg). The intraocular pressure (IOP) of the experimental and control rats was measured every six days. Retinal cells were examined by hematoxylin-eosin and terminal deoxynucleotidyltransferase-mediated biotinylated UTP nick end labeling (TUNEL) staining, as well as transmission electron microscopy. Immunohistochemistry and western blot analysis were performed to measure proteins involved in apoptosis and autophagy.

Results: Ligustrazine protected retinal cells from death in experimental glaucoma rats, which was not due to the lowering of IOP, but could be attributable to direct suppression of retinal cell apoptosis. In glaucoma rats, autophagy was markedly activated in retina cells, as evidenced by increased numbers of autophagosomes and the expression of autophagy-related proteins (ATG5 and LC3-II/I). Notably, such alterations in glaucoma rats were almost completely reversed by ligustrazine. The suppressive effects of ligustrazine on apoptosis and autophagy of retina cells were markedly attenuated by the mTOR inhibitor rapamycin or the PI3K inhibitor Ly294002. Additionally, ligustrazine significantly increased the protein levels of phosphorylated PI3K (p-PI3K), protein kinase B (p-Akt), and mTOR (p-mTOR) in glaucoma rats, whereas such increases were attenuated by rapamycin or Ly294002.

Conclusions: These results demonstrate that ligustrazine is protective in experimental glaucoma by inhibiting autophagy via the activation of the PI3K-Akt/mTOR pathway, providing compelling evidence that ligustrazine is potentially therapeutic for patients with glaucoma.
against H₂O₂-induced injuries in rat retinal cell cultures [13] and mouse primary RGCs [14], suggesting the therapeutic potential of ligustrazine in treating glaucoma. However, the in vivo effect of ligustrazine on glaucoma and its underlying mechanisms remain largely unknown. Rat chronic hypertensive glaucoma models induced by episcleral vein cauterization (EVC) have been used extensively to investigate the pathogenesis of glaucoma and to explore new treatments for this disease [15]. In the present study, we evaluated the effect of ligustrazine on glaucoma in an EVC-induced rat chronic hypertensive glaucoma model. Our data suggested that ligustrazine is neuroprotective in experimental glaucoma by inhibiting autophagy in RGCs via the PI3K-Akt/mTOR pathway.

METHODS

Chemicals and reagents: Ligustrazine (TMP; sigma, 183,938-25G) was purchased from Sigma-Aldrich (St. Louis, MO). Ly294002 (MCE, #HY-10108, 2-morpholin-4-yl-8-phenylchromen-4-one) and rapamycin (MCE, #HY-10219) were obtained from MedChem Express (MCE, Monmouth Junction, NJ). All other reagents were obtained from commercial vendors and were of the highest purity available. Animals: The experimental protocol was approved by the Animal Ethics Committee at the Affiliated Traditional Chinese Medicine Hospital of Guangzhou Medical University. All experimental procedures were performed in compliance with the National Institute of Health's guide for the care and use of laboratory animals. Specific-pathogen-free adult Sprague-Dawley (SD) rats (weight: 200–300 g, both male and female) were purchased from Southern Medical University (Guangdong, China; Batch No. 44002100023747), and housed in an environmentally controlled animal breeding facility (temperature: 22 ± 2 °C, humidity: 60 ± 5%, 12h dark/light cycle). All rats were given water and food ad libitum.

Episcleral vein cauterization: The glaucoma model with high IOP was induced by EVC on the rats’ right eyes, as previously described [16]. In brief, rats were anesthetized with xylazine hydrochloride (5 mg/kg) and ketamine hydrochloride (10 mg/kg), and three drops of a topical anesthetic (alcaline) were instilled into the right eyes. A radial surgical incision was cut along the limbus. The fascia, muscles, and upper bulbar conjunctiva were separated to expose the underlying episcleral vein. A heated cautery pen was used to cautereize the three episcleral veins. The unoperated eye served as a control. After the operation, the conjunctival sac was flushed with normal saline and treated with antibiotic ointment.

Animal treatments: A total of 25 rats were randomly assigned to five groups (n = 5/per group). 1) Control group: The right eyes in the control group were sham-operated by similarly isolating the veins without cauterization. Once a day, an equal amount of normal saline was injected intraperitoneally from two days before the sham operation to one month after the sham operation. 2) Operated group: Once a day, an equal amount of normal saline was injected intraperitoneally from two days before EVC to one month after EVC. 3) TMP group: Once a day, TMP at a dose of 80 mg/kg was injected intraperitoneally from two days before EVC to one month after EVC. 4) TMP + Ly294002 group: Once a day, TMP at a dose of 80 mg/kg was injected intraperitoneally from two days before EVC to one month after EVC. Ly294002 at a dose of 10 mg/kg was injected intraperitoneally at the same time, from two days before EVC (once a day) to one month after EVC (twice a week). 5) TMP + rapamycin group: TMP at a dose of 80 mg/kg was injected intraperitoneally from two days before EVC to one month after EVC. Rapamycin at a dose of 5 mg/kg was injected intraperitoneally at the same time, from two days before EVC (once a day) to one month after EVC (once every other day). In previous studies, 80 mg/kg ligustrazine was used to improve atherosclerosis or suppress pulmonary artery smooth muscle cell proliferation in rats [17,18]. Thus, we used 80 mg/kg ligustrazine in this study. Additionally, the dosages of rapamycin and Ly294002 were also chosen in accordance with previous literature [19,20]. No animals were excluded during the study.

Measurement of intraocular pressure: The IOP of the experimental and control eyes was measured using a Tono-Pen AVIA Handheld Tonometer once every six days. The rats were anesthetized with xylazine hydrochloride (5 mg/kg) and ketamine hydrochloride (10 mg/kg), and three drops of alcaline were instilled into the experimental and control eyes, respectively. IOP was measured five times per eye at the same time by the same person. No animals were excluded from the study.

Tissue preparation: All rats were deeply anesthetized using a single intraperitoneal injection of xylazine hydrochloride (10 mg/kg) and ketamine hydrochloride (75 mg/kg), and euthanized one month after EVC. The eyes were enucleated carefully, leaving 2 mm of the optic nerve attached to the globe. After the eyes were washed with saline, the extra-orbital muscles and fat were removed. The resulting eyes were fixed in a freshly prepared fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) for 2 h at room temperature. The cornea was punctured to expedite the penetration of fixative. Following additional 24 h fixation, the lens were removed, and the remaining eyes were dehydrated using graded ethanol (70% ethanol for 1 h, 80% ethanol for 1 h, 90% ethanol for 1
All data are presented as mean± SEM. 22

**Transmission electron microscopy:** Retinal sections (100 μm) were cut with a vibratome, fixed with 4% glutaraldehyde in a 0.1 mmol/l cacodylate buffer (pH 7.4) for 1 h, and then with 1% osmium tetroxide in a 0.1 mmol/l cacodylate buffer for 2 h. Sections were rinsed with distilled water and treated with 1% aqueous uranyl acetate overnight. Next, they were dehydrated in an ascending series of ethanol to 100%, followed by dry acetone, and then embedded in Durcupan ACM. Ultrathin sections (0.1 μm) were cut and mounted on Formvar-coated slot grids and stained with 3% lead citrate. Sections were examined using a Zeiss transmission electron microscope (Zeiss Inc., Thornwood, NY).

**Western blot:** Retinas were homogenized in RIPA lysis buffer (Cwbio Bio Inc., Beijing, China) to extract the total protein according to the manufacturer’s protocol. Equally loaded proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. Next, the membranes were blocked with a solution of nonfat powdered milk for 1.5 h at room temperature and incubated overnight with the appropriate primary antibodies (p-Akt [Abcam, ab38449], AKT [Abcam, ab182729], p-PI3K [Abcam, ab127617], PI3K [Abcam, ab189403], p-mTOR [Abcam, ab131538], mTOR [Abcam, ab2732], LC3 [Abcam, ab48394]), followed by incubation with the corresponding secondary antibodies at room temperature for 1 h. Blot imaging was conducted using ECL western blotting detection reagents. Bands of western blot were quantified using the open-source ImageJ software, version 1.53 (NIH, Bethesda, MD). Each band intensity was normalized relative to its respective loading control bands. Values were expressed as the ratio of the average of the control group.

**Immunohistochemistry:** ATG5 protein expression was assessed with immunohistochemistry (IHC). The paraffin retinal sections were deparaffinized, rehydrated, and stained with ATG5 antibody (Abcam, ab108327). All sections were performed and stained simultaneously. Images of immunohistochemical sections were magnified to x40 and examined using the open-source ImageJ software version 1.53 (NIH, Bethesda, MD). Five randomly selected areas were captured for analysis. The pixel count for each area was used, and a score was assigned to each area based on a pixel-by-pixel analysis. The positive area ratio was scored as 0 (0%), 1 (1%–25%), 2 (26%–50%), 3 (51%–75%), or 4 (76%–100%) [21].

**Statistical analysis:** All data are presented as mean± SEM (n=5). The data were statistically analyzed using GraphPad Prism software, version 7.00. The differences between groups were analyzed using a one-way analysis of variance (ANOVA) followed by two-by-two comparisons using Tukey’s test. p<0.05 is considered significant.

**RESULTS**

**Ligustrazine has no effect on intraocular pressure in chronic hypertensive glaucoma rats:** As shown in Figure 1, the average IOP was 7.94 ± 1.39 mm Hg in rats before surgery. It should be noted that the IOP in this study is lower than the previously reported IOP of awake rats (ranging from 18 to 52 mm Hg) [22]. Although the exact causes for such a difference are unknown, the IOP values should be comparable because all the experiments were performed with the same instrument and under the same conditions. The IOP in the control rats with sham surgery did not show elevation throughout the study. In contrast, EVC gradually increased the IOP, which reached a plateau around six days after surgery. Consequently, the IOP of the operated rats was about 2.6 times higher than that of the control rats throughout the experiment. Nonetheless, the IOP in operated rats was not significantly altered by TMP, TMP + rapamycin, or TMP + LY294002. These findings suggest that TMP has no effect on the IOP of EVC-induced glaucoma rats.

**Ligustrazine rescues apoptotic retinal cells in chronic hypertensive glaucoma rats:** Figure 2A shows representative histological appearance of retinal cross-sections observed by H&E staining. Control rats displayed a well-organized outer nuclear layer (ONL), outer plexiform layer (OPL), inner
nuclear layer (INL), inner plexiform layer (IPL), and ganglion cell layer (GCL). RGCs in the control rats were arranged in a single layer with regular shapes. In contrast, EVC-operated rats showed loose retinal layers, and their RGCs were disorganized with irregular shapes.

Ligustrazine significantly rescued the retinal injury induced by EVC in operated rats, as evidenced by the organized cell layers. Although TMP + rapamycin and TMP + Ly294002 also attenuated retinal injury in operated rats, they were less effective compared to TMP alone.

Apoptosis is thought to play an important role in the degeneration of the retina. To further elucidate the effect of TMP, TUNEL staining was performed on retina sections, and the percentages of TUNEL-positive cells were quantified (Figure 2B,C). Compared to control rats, numerous TUNEL-positive cells were observed in the INL and GCL.
of operated rats. Ligustrazine significantly reduced TUNEL-positive cells in operated rats, and the retinal staining in ligustrazine rats was almost as light as that of control rats. Compared with operated rats treated with ligustrazine alone, more TUNEL-positive cells were observed in operated rats treated with TMP + rapamycin and TMP + Ly294002. Quantitative analysis of TUNEL confirmed that ligustrazine completely rescued apoptotic cells in operated rats, whereas rapamycin and Ly294002 significantly attenuated the effect of ligustrazine. Taken together, these findings suggest that the anti-apoptotic effect of ligustrazine on retina cells in EVC-induced glaucoma rats is suppressed by mTOR or PI3K inhibitors.

Ligustrazine inhibits autophagy in the retinal cells of chronic hypertensive glaucoma rats: Autophagy and apoptosis are known to be interconnected through multiple crosstalk mechanisms. To determine whether autophagic activity was altered by ligustrazine, we examined autophagosomes (APs) in retinal sections using transmission electron microscopy (EM). As shown in Figure 3A, double- and multiple-membrane APs were occasionally observed in the cytoplasm of RGCs of sham-operated (Control) rats or EVC-operated rats treated with vehicle (Operated), TMP (TMP), TMP+Rapamycin (TMP+Rapamycin), or TMP+Ly294002 (TMP+Ly294002). Three eyes were examined in each experimental period.

Figure 3. Transmission electron micrographs of RGCs in the GCL and inner plexiform layer (IPL). Representative ultrastructural images show the presence of double- or multiple-membrane autophagic vesicles (box) containing cell organelles in the cytoplasm of ganglion cell layer (GCL) retinal ganglion cells (RGCs) and dendrites of IPL RGCs of sham-operated (Control) rats or EVC-operated rats treated with vehicle (Operated), TMP (TMP), TMP+Rapamycin (TMP+Rapamycin), or TMP+Ly294002 (TMP+Ly294002). Three eyes were examined in each experimental period.

Ligustrazine activates the PI3K-Akt/mTOR signaling pathway in retinal cells of chronic hypertensive glaucoma rats: To further evaluate the autophagic mechanisms, we evaluated the expression of several autophagy-related proteins in rat retinas. ATP5 is indispensable for the formation of the autophagic vesicle, and its dysfunction results in autophagy inhibition [23]. IHC staining showed that the ATP5 protein level was significantly increased in the retinas of operated rats compared to control rats (Figure 4A,B). Ligustrazine decreased the protein expression of ATP5 back to the control level in operated rats, but the effect was significantly attenuated by rapamycin and Ly294002. Western blot analysis revealed that operated rats had lower protein levels of p-mTOR, p-Akt and p-PI3K, but higher protein levels of LC3B-I and -II than the control rats did (Figure 4C,D). Such alterations of autophagy-related proteins in operated rats were almost completely reversed by ligustrazine. Notably, both rapamycin and Ly294002 suppressed the effect of ligustrazine on the protein expression of autophagy-related proteins in operated rats. Taken together, the anti-autophagic effect of ligustrazine on the retina in EVC-induced glaucoma rats is due to the activation of the PI3K-Akt/mTOR signaling pathway.
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DISCUSSION

Glaucoma is characterized by the chronic and progressive loss of RGCs. The mechanisms of RGC death in glaucoma are attributed to both apoptosis and autophagy, which dynamically occur in the pathogenesis of glaucoma. It should be noted that the role of autophagy in glaucoma may be dependent on the animal models. Autophagy was reported to promote the survival of RGCs in glaucoma mice induced by optic nerve transection but to induce the apoptosis of RGCs in glaucoma rats induced by EVC [24,25]. Autophagy is activated early after IOP elevation and precedes the significant loss of RGCs, suggesting that autophagy may promote the initiation of apoptosis in the RGCs of chronic hypertensive glaucoma rats [5,26]. Therefore, inhibiting autophagy holds promise to prevent retinal cell degeneration in chronic hypertensive glaucoma.

The present study showed that chronic IOP elevation resulted in markedly increased apoptotic and autophagic activities in rat retinas, as evidenced by increased TUNEL-positive cells, an increased number of APs, and the expression of autophagy-related proteins. Ligustrazine significantly rescued retina injury in chronic hypertensive glaucoma rats by inhibiting both apoptosis and autophagy of retinal cells. Our data provide compelling evidence that ligustrazine is potentially therapeutic for patients with glaucoma.
Several studies have demonstrated the regulatory effect of ligustrazine on autophagy in various experimental disease models. Ligustrazine protects against the apoptosis of bone marrow-derived mesenchymal stem cells (BMSCs) by promoting autophagy [27]. Ligustrazine ameliorates lipopolysaccharide (LPS)-induced brain inflammation by activating autophagy [12]. Additionally, ligustrazine protects against bupivacaine-induced neurotoxicity in human neuroblastoma SH-SY5Y cells by the activation of autophagy [28]. In contrast, the present study showed that ligustrazine significantly inhibited retinal cell autophagy in chronic hypertensive glaucoma rats. This is unsurprising because of the complicated role of autophagy in the pathogenesis of different diseases. Autophagy can be activated by various factors, such as starvation, oxidative stress, ischemia, and misfolded proteins, as well as by the levels of ATP and growth factors. Additionally, it is agreed that autophagy promotes or inhibits cell death, depending on the internal and external environment and cell type [29]. Accordingly, RGC death is differentially regulated by autophagy under different circumstances. For example, inhibition of autophagy with 3-methyladenine significantly increased the apoptosis of RGCs in diabetic retinas but decreased the apoptosis of RGCs in glaucomatous retinas [7]. To the best of our knowledge, the present study is the first to demonstrate the anti-autophagic activity of ligustrazine in glaucoma.

Autophagy is modulated by multiple signaling pathways, including the PI3K-Akt/mTOR pathway [30]. In the present study, ligustrazine increased phosphorylation of PI3K, Akt and mTOR in the retinas of chronic hypertensive glaucoma rats, and this increase was attenuated by the mTOR inhibitor rapamycin and the PI3K inhibitor Ly294002. It should be noted that neither rapamycin nor Ly294002 completely reversed the inhibitory effect of ligustrazine on autophagy in glaucoma rats. Thus, ligustrazine inhibited retinal cell autophagy in chronic hypertensive glaucoma rats at least partially through activation of the PI3K-Akt/mTOR signaling pathway. It should also be noted that only one dose of ligustrazine, rapamycin, or Ly294002 was used in the present study, which may underestimate the importance of PI3K-Akt/mTOR pathway underlying the therapeutic effect of ligustrazine. In contrast, ligustrazine was reported to inhibit the PI3K-Akt/mTOR signaling pathway in the lung tissue of paraquat-treated mice [31] and in the brains of rats with lipopolysaccharide-induced brain inflammation [12]. It should be noted that the PI3K-Akt/mTOR pathway could be activated or inhibited by various factors. Its activators include epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), insulin and calmodulin (CaM), whereas phosphatase and tensin homolog (PTEN), glycogen synthase kinase 3 beta (GSK3β) and homeobox protein 9 (HB9) are inhibitors of this pathway. Our results suggest that ligustrazine may have different effects on the PI3K-Akt/mTOR pathway. Future studies are required to elucidate the exact underlying mechanism of ligustrazine-mediated activation of the PI3K/Akt/mTOR pathway in glaucoma.

In conclusion, we report that ligustrazine could protect retinal cells from death in a rat chronic hypertensive glaucoma model. Ligustrazine-mediated protection appears to be attributable to the inhibition of autophagy via the activation of the PI3K-Akt/mTOR pathway. Our study provides compelling evidence for the neuroprotective effect of ligustrazine in experimental glaucoma and supports the notion that ligustrazine is potentially therapeutic for patients with glaucoma.

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