

FTY720 Protects Retinal Ganglion Cells in Experimental Glaucoma

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PURPOSE. To investigate the neuroprotective effects of sphingosine-1-phosphate (S1P) analogue fingolimod (FTY720) in experimental glaucoma in rats.

METHODS. A unilateral chronic ocular hypertensive model was established by injections of microbeads into the anterior eye chamber of adult Sprague-Dawley rats. Fingolimod was administered to one group of rats intraperitoneally every week for 3 months. The scotopic threshold response (STR) was recorded to assess the function of the inner retina. Changes in cell density in the ganglion cell layer (GCL) were evaluated by hematoxylin and eosin staining on retinal sections and axonal count of the optic nerve was performed using Bielschowsky's silver staining. Effects of drug treatment on activation of Akt and Erk1/2 were evaluated using Western blotting by assessing phosphorylation levels of these proteins. The expression of S1P receptors in the optic nerve head region was also evaluated using Western blotting and immunohistochemistry.

RESULTS. Administration of FTY720 reduced the loss of STR amplitude in glaucomatous eyes ($P < 0.05$). Counting and plotting the cell numbers/axonal density showed significant neural preservation in the GCL and the optic nerve ($P < 0.05$). An increased phosphorylation level of Akt and Erk1/2 following FTY720 administration was observed. Both S1P1 and S1P5 receptors were found to be expressed in the retina and the expression of S1P1R was upregulated in experimentally-induced glaucoma.

CONCLUSIONS. This study demonstrates, for the first time, that FTY720 could act as a neuroprotective agent to protect retinal ganglion cells in experimental glaucoma. Administration of this drug significantly reduces the structural and functional loss of the inner retina elicited indicating that it may potentially be used to attenuate neuronal loss and optic nerve damage in glaucomatous patients.

Keywords: glaucoma, neuroprotection, S1P

Retinal ganglion cell (RGC) loss and optic nerve cupping are the hallmark features of glaucomatous optic neuropathy. Increased IOP is considered as the most important risk factor of glaucoma, but many glaucoma patients continue to develop progressive visual field loss regardless of the normalization of IOP. Although there is still insufficient evidence to support the notion of including neuroprotective agents in the management of glaucomatous patients,¹ there is a need to pursue neuroprotection as an additional therapy for glaucoma, as control of IOP by itself, may not be sufficient to hinder the progression of RGC degeneration.²⁻⁴

Sphingosine-1-phosphate (S1P), a signaling lipid, plays crucial roles in a wide variety of cellular functions, including cell growth and survival, angiogenesis, proliferation, neuritogenesis, cell motility and migration, and lymphocyte trafficking.⁵ Sphingosine-1-phosphate is the ligand for a family of five G-protein-coupled receptors, named S1P1R to S1P5R, respectively. S1P1R is mainly expressed on neuronal cell bodies and shows a widespread expression in the central nervous system including the retina. Fingolimod, or FTY720, belongs to the S1PR modulator group of molecules and behaves as a full agonist on S1P1R, S1P3R, S1P4R, and S1P5R at low nanomolar

concentrations in the form of its active metabolite FTY720-phosphate (FTY720-P).^{6,7} Fingolimod is increasingly being used in relapsing multiple sclerosis patients as an immunosuppressive compound based on its effects on lymphocyte migration via the S1P1 signalling.⁸ Recently, FTY720 has also been shown to exhibit potential neuroprotective activity in vivo⁹ as well as in cultured cortical neurons against excitotoxic damage¹⁰ and the neuroprotective effect is thought to be independent of its immunosuppressive effect.⁹ Although studies using FTY720 in the models of cerebral ischemia elicited different results,¹¹⁻¹⁴ a most up-to-date meta-analysis suggested that FTY720 can protect cortical neurons against ischemia and can decrease infarct volume in rodents.¹⁵ In addition, FTY720 also plays a neuroprotective role in the retina,^{5,16} and administration of this drug was found to be able to provide protective effects for photoreceptors against light-induced retinal degeneration.⁹ In this study we examined whether FTY720 depicts any protective effects in an experimental model of glaucoma to prevent RGC degeneration and functional loss induced by exposure to chronic ocular hypertension.

Intraocular injection of microbeads in rodents has been described by us and others, and is widely used as a chronic

experimental model of glaucoma with sustained and mild elevation of IOP and subsequent retinal degeneration and RGC apoptosis.¹⁷⁻²¹ In this study, we used this well-established model as a platform to evaluate the neuroprotective effects of FTY720 in vivo against glaucomatous stress.

METHODS

Animals

Male Sprague-Dawley rats with a body weight of 300 to 350 g (10–12 weeks; Animal Research Centre, Perth, Australia) were used. All animals were maintained in an air-conditioned room with controlled temperature ($21 \pm 2^\circ\text{C}$) and fixed daily 12-hour light/dark cycles. All procedures involving animals were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were anaesthetized with an intraperitoneal (i.p.) injection of ketamine (75 mg/kg) and medetomidine (0.5 mg/kg) for all the procedures including the intraocular injection and electrophysiological recordings.

Microbead Injection and FTY720 Administration

For microbead injection, animals were placed on a heating pad during the procedure and both pupils were dilated with topical tropicamide 1% and anesthetized with proparacaine 0.5% drops. Polystyrene microspheres of 10 μm in size were injected with a concentration of 1.0×10^6 beads/mL and volume of 10 μL (FluoSpheres; Invitrogen, Carlsbad, CA, USA) followed by an air bubble²¹ using a Hamilton syringe connected to disposable 33-G needles (TSK Laboratory, Tochigi, Japan). All procedures were performed using an operating microscope (OPMI-11; Carl Zeiss, Oberkochen, Germany) with care taken to avoid needle contact with the iris or lens. A randomly selected eye was injected, leaving the fellow eye as the control, which was injected with the same volume of supernatant. Microbead injection was performed weekly between week 0 and week 4 and then fortnightly from week 6. The IOP was followed up in rats using an iCare TonoLab (Tonovet; Icare, Helsinki, Finland). All IOP measurements were post anesthesia. Dose-dependent effects of FTY720 have been recognized and dose of systemic administration of FTY720 has been optimized. A dose of 5 mg/kg was shown to be effective in protecting the outer retina of rodents,⁹ and doses between 3 and 10 mg/kg have been widely used in other studies.^{7,22} The pharmacological effects of FTY720 appeared to be dose dependent at lower dosage and then reach a plateau with the increase of dose. As FTY720 has a half-life of 6 to 9 days,²³ to minimize the side effects of the drug it was administered weekly (i.p. injection) from week 1 using a dose of 5 mg/kg initially dissolved in dimethyl sulfoxide and then diluted in saline (1:1), and the control group was injected using the vehicle.

Scotopic Threshold Response Recording

Scotopic threshold response (STR) recording was carried out using an electroretinogram (ERG) machine (OcuScience, Rolla, MO, USA)²⁴ and a similar ERG recording protocol as we have described.²⁵ Briefly, after overnight dark-adaptation, animals were anaesthetized and placed on a warm pad. A gold wire ring electrode (Roland Consult, Brandenburg, Germany) was placed on the center of the cornea to serve as the positive lead and the reference electrode was provided by a stainless steel needle, which was inserted into the skin over the forehead. Another needle electrode was inserted into the tail two-thirds from its

base as the ground. Dim stimulation ($-3.4 \log \text{cd}\cdot\text{s}/\text{m}^2$) was delivered 30 times at a frequency of 0.5 Hz.

Histology

Animals were killed after 3 months with an overdose of anesthetics and then perfused transcardially with 4% paraformaldehyde. For the morphology study, eyes were fixed in 4% paraformaldehyde overnight, processed in an automatic tissue processor (Leica, Wetzlar, Germany), and embedded in paraffin. Care was taken to ensure that the orientations of the eyes were identical by using tissue marking dye and 7- μm thick sagittal sections of the eye were made using a rotary microtome (Carl Zeiss). Cell density in the retinal GCL was determined for each eye by counting the number of cells in the GCL over a distance of 500 μm (from 100–600 μm to the edge of the optic disc) for both superior and inferior retina on three consecutive sections. Bielschowsky's silver staining was used for axonal analysis in the optic nerve as we have described²⁵ and axonal density was determined for each nerve by counting the number of axons in 12 standardized microscopic fields of 100 μm^2 . For immunohistochemistry study, eye balls were subjected to 4% paraformaldehyde fixation followed by overnight incubation in 30% sucrose, cryosections (15 μm) were then permeabilized with cold ethanol and incubated with primary antibodies (1:100), diluted in 4% normal horse serum overnight at 4°C . Sections were then subjected to incubation with Alexa-Fluor 488 goat anti-rabbit immunoglobulin G (IgG; 1:400 in Tris phosphate buffered saline) for 1 hour in the dark and mounted on glass slides.²⁶ The pictures were captured using a microscope (Carl Zeiss).

Western Blotting

The optic nerve head region of the retina (mainly contains RGC axons) was precisely excised from the retina under the surgical microscope. The tissue was mixed in lysis buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; [Sigma-Aldrich, St. Louis, MO, USA], pH 7.4, 1% Triton X-100, 2 mM EDTA) containing protease inhibitors (10 $\mu\text{g}/\text{mL}$ aprotinin, 10 μM leupeptin, 1 mM phenylmethyl sulfonyl fluoride) and phosphatase inhibitors (1 mM NaVO_3 , 100 mM NaF, 1 mM Na_2MoO_4) and sonicated. Insoluble materials were removed by centrifugation at 15,000g for 10 minutes at 4°C . Protein concentrations were determined using the BCA protein assay kit (Pierce, Rockford, IL, USA). Samples were then run on a NuPAGE 10% Bis-Tris gel in MOPS SDS running buffer (Invitrogen) and electroblotted to an iBlot gel transfer polyvinylidene fluoride membrane (Invitrogen). Next, membranes were blocked in Tris buffered saline (TBS) (20 mM Tris-HCl, pH 7.4, 0.15M NaCl) containing 5% skimmed milk powder for 1 hour. After blocking, membranes were probed overnight at 4°C with primary antibodies. Membranes were then washed with TBS buffer and incubated with horseradish peroxidase (HRP)-labeled secondary antibody for 1 hour at room temperature. After extensive washing, antibody detection was accomplished with Supersignal West Pico Chemiluminescent substrate (Pierce). Signals were detected using an automated luminescent image analyzer (ImageQuant LAS 4000; GE Healthcare, Little Chalfont, UK). The densitometric analysis of the band intensities was performed using the ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).²⁷

Statistical Analysis

The STR responses, cell density in the GCL, axonal density in the optic nerve, as well as protein expression from the

glaucomatous eyes were compared with those from the control eyes. A comparison between the rat groups with and without FTY720 treatment was also carried out using the Student's *t*-test. The data were plotted using GraphPad Prism software (version 6.0; GraphPad, La Jolla, CA, USA), and a *P* value of less than 0.05 was considered statistically significant.

RESULTS

FTY720 Restores the Loss of STR Response Induced by Ocular Hypertension

All eyes injected with microbeads experienced a sustained increase of IOP for 3 months, while the control eyes depicted no elevation of IOP (Fig. 1). There was also no significant difference in the IOP measurement between the FTY720 treatment group and the control group. The positive STR (pSTR) is very useful for RGC assessment in rodents and is considered to be RGC-driven²⁸ in contrast to the negative STR (nSTR), which is the predominant STR in humans but may be more amacrine cell-based. Figure 2 shows representative pSTR traces recorded from the rat eyes with and without FTY720 treatment. There was a significant reduction in pSTR amplitude in the glaucomatous eyes (Fig. 2A, left panel). This functional deterioration was, however, relatively preserved following FTY720 administration (Fig. 2A, right panel). The pSTR amplitude in the glaucomatous eyes with FTY720 treatment was approximately 3-fold higher compared with that from the group without FTY720 treatment ($48.04 \pm 8.12 \mu\text{V}$ vs. $16.95 \pm 12.03 \mu\text{V}$, $P < 0.01$).

FTY720 Prevents Cell Loss in the GCL and Axonal Loss in the Optic Nerve

Ganglion cell layer cell counts were performed on paraffin embedded cross-sectional slides stained with hematoxylin and eosin. As previously reported, no attempt was made to distinguish RGCs from displaced amacrine cells or other neuron-like cells, but morphologically distinguishable glial cells and vascular endothelial cells were excluded.²⁹ Representative cross-sectional pictures of the rat retina are displayed in Figure 3A. Intraocular pressure-induced cell loss in GCL decreased from 66.8% to 21.9% after systemic administration of FTY720. As there are not only RGCs but also amacrine cells in the GCL, we also carried out axonal analysis using Bielschowsky's silver staining to confirm the protective effects of FTY720 on RGCs. Similar to the observations in functional changes (pSTR) and cellular degeneration in the GCL, axonal loss in experimental glaucoma was also significantly attenuated by FTY720 treatment. Axonal loss in experimental glaucoma was reduced from 38.8% to 27.1% after FTY720 treatment. ($P < 0.01$, Fig. 4).

FTY720 Upregulates the Akt and Erk 1/2 Phosphorylation

The phosphatidylinositol-3 kinase (PI3K)/Akt and the mitogen-activated protein kinase (MAPK, also known as extracellular signal-regulated kinases 1/2 or Erk1/2) signaling are the prominent survival pathways for retinal ganglion cells,²⁸ and FTY720 administration has been shown to be associated with the activation of these two pathways in cultured neuronal cells¹⁰ as well as in the rat model of stroke *in vivo*.¹³ In this study, Western blotting of the optic nerve head tissue from glaucomatous eyes depicted a reduction of Akt phosphorylation, and Akt activity was found to be significantly upregulated in the rat group with FTY720 treatment ($P < 0.05$, Figs. 5A, 5B). The expression of Erk 1/2 demonstrated a similar trend,

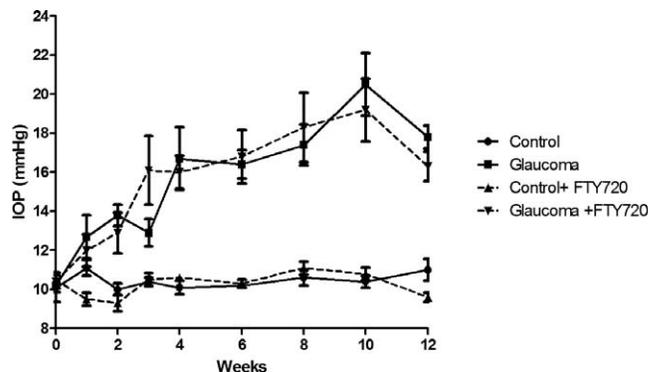


FIGURE 1. Sustained IOP elevation in rats with microbead injection over 3 months ($n = 12$ per group).

the phosphorylation level of Erk 1/2 also significantly reduced in experimental glaucoma but noticeably increased in the rats that received FTY720 injections ($P < 0.05$, Figs. 5A, 5C).

FTY720 Modulates the Expression of S1P1 Receptor

The expression of FTY720 receptors, S1P1R and S1P5R, in the optic nerve head region was also investigated using Western blotting. A significant upregulation of S1P1R expression was found in glaucomatous eyes (Figs. 6A, 6B), however, there was no change observed in the expression of S1P5R. Interestingly, the increase of S1P1R expression under glaucomatous condition was reversed in group with the FTY720 treatment (Figs. 6A, 6B). Immunohistochemistry further confirmed the manner of S1P1R expression in the inner retina (Fig. 6C), which was augmented in the glaucoma exposed eyes but not in the ones receiving treatment with FTY720.

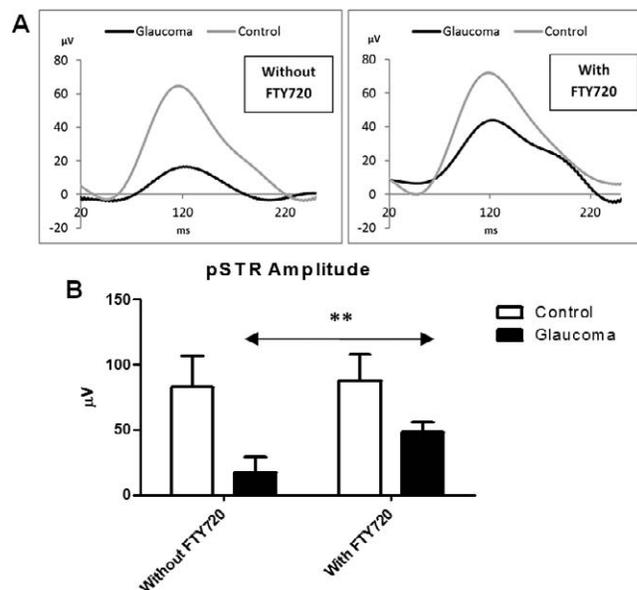


FIGURE 2. (A) Positive scotopic threshold responses of control and glaucomatous eyes without/with Fingolimod treatment. (B) Quantification of the pSTR amplitude shows that FTY720 protects the RGC function in experimental glaucoma. $**P < 0.01$, *t*-test, $n = 8$ per group.

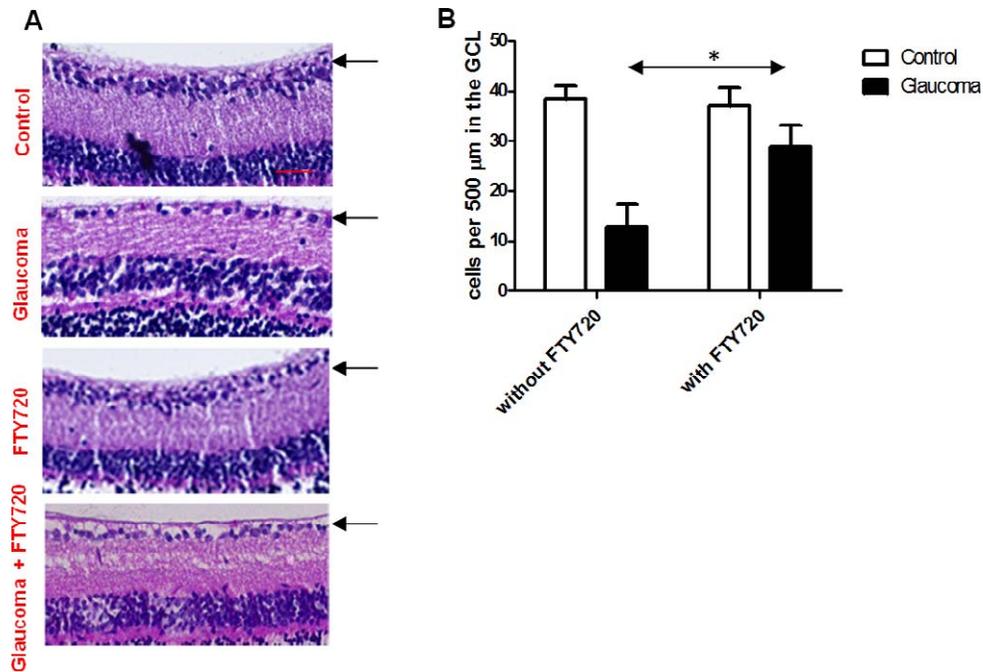


FIGURE 3. (A) Representative cross-sectional pictures of the rat retina. The GCL is indicated by arrows. Scale bar: 10 μm; (B) cell count shows neuroprotective effect of FTY720 for the cells in the GCL. **P* < 0.05, *t*-test, *n* = 4 per group.

DISCUSSION

Fingolimod was developed as a medication originally for relapsing multiple sclerosis. It acts as a functional antagonist of the S1PR on lymphocytes, and as a result, lymphocytes no longer respond to S1P gradients and tend to be confined to lymph nodes.⁸ The neuroprotective potential of FTY720 has recently been noticed and the expression of S1PRs has been proven widely distributed in the central nervous system,

including the retina.⁵ Although some reports suggested that FTY720 might not be neuroprotective,^{14,22,30} other studies have actually provided strong evidence showing that this drug can impart neuroprotection to the brain against ischemic insults,^{10,13,31-33} which has further been confirmed by a meta-analysis.¹⁵ The protective role of FTY720 also appears to be dose-dependent.^{9,31} On the other hand, FTY720 may be associated with reversible macular edema, in 0.3% patients treated with 0.5 mg FTY720.²⁶ However, a recent report

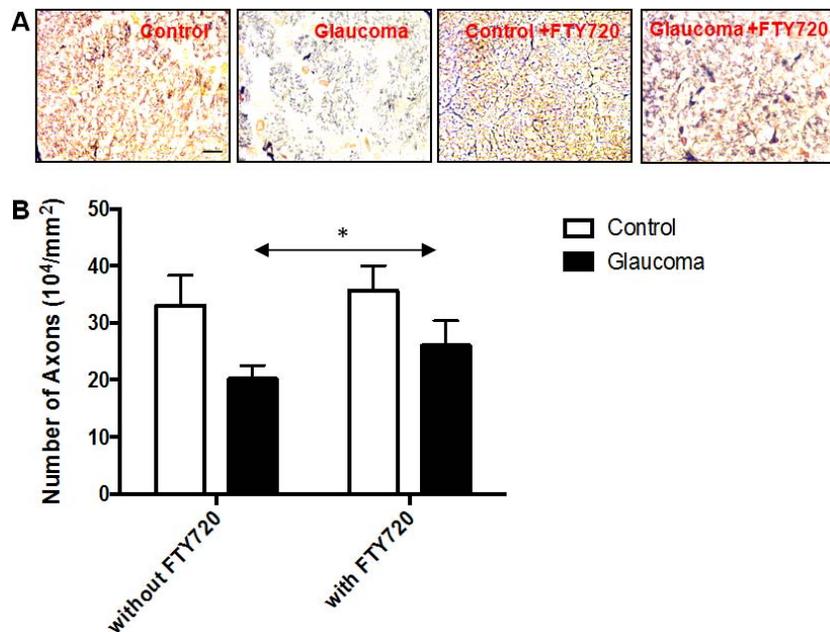


FIGURE 4. (A) Bielschowsky's silver staining of the optic nerves. Scale bar: 20 μm; (B) axonal density analysis. Axonal loss in the optic nerve secondary to experimental glaucoma (38.8%) was reduced to 27.1% by FTY720 treatment. **P* < 0.05, *t*-test, *n* = 4 per group.

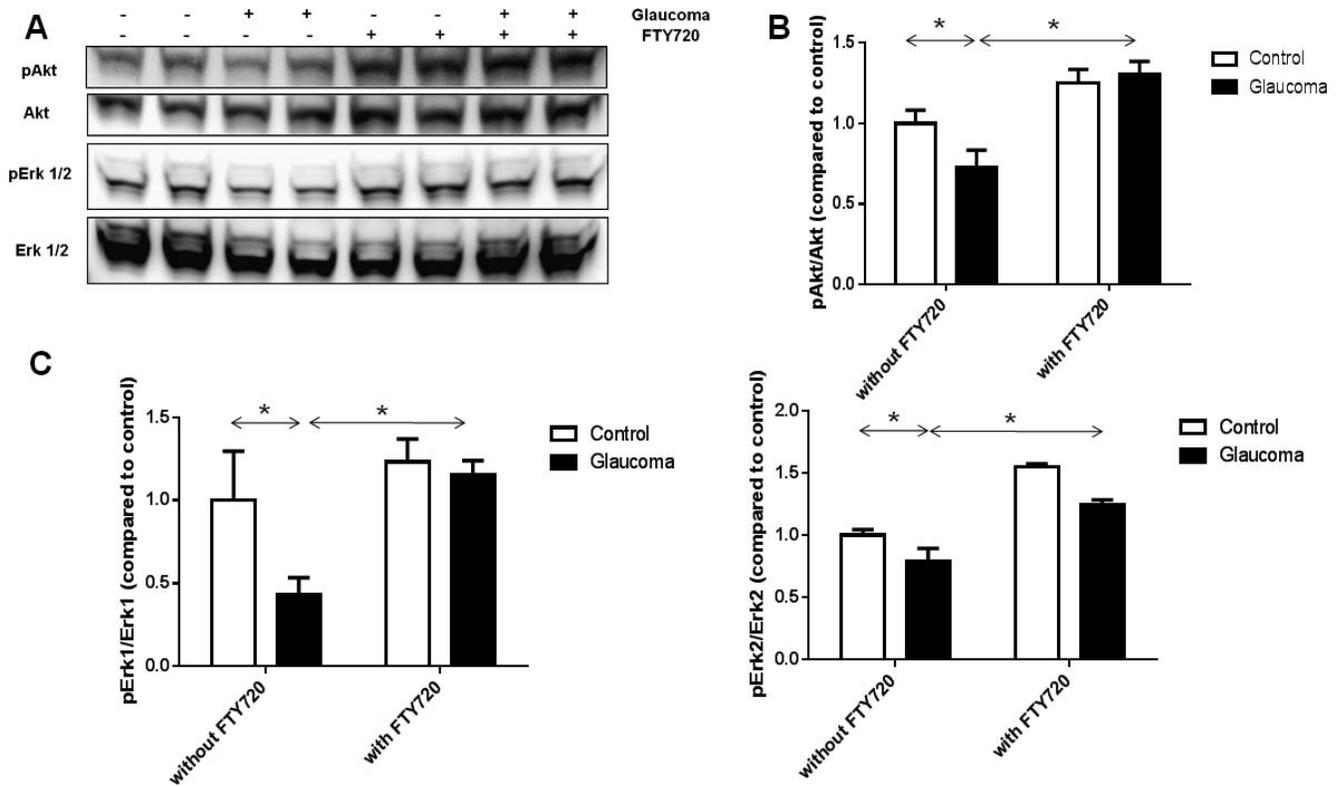


FIGURE 5. (A) Phosphorylation of Akt and Erk 1/2 were up-regulated following FTY720 treatment. (B) Densitometric analysis showed a significant reduction of Akt phosphorylation in glaucoma and an upregulation of pAkt/Akt ratio after FTY720 injections. (C) Erk1/2 activity was also upregulated by FTY720 treatment. * $P < 0.05$; $n = 4$.

demonstrated that FTY720 actually acts as a neuroprotective molecule in the photoreceptors in rat retina⁹ indicating its potential protective effects in macular degeneration and retinitis pigmentosa. In this study, we evaluated the neuroprotective effect of FTY720 using a well-established rat model of experimental glaucoma. Our results suggest that FTY720 can also protect retinal cells in the inner retina in vivo. This finding was confirmed by functional evaluation using STRs as well as by histologic analysis on retinal and optic nerve cross-sections.

The mechanisms of how FTY720 plays its neuroprotective role in the central nervous system remain unknown. The Akt and Erk 1/2 signaling along with the phospholipase $C\gamma$ (PLC γ) pathway are considered to be important downstream survival pathways of the brain-derived neurotrophic factor (BDNF)/TrkB signaling, which plays a prominent role in RGC survival.²⁸ The downstream signaling of S1P has been shown to overlap with the BDNF/TrkB signaling, including but not limited to the PI3K/Akt, MAPK/Erk, and PLC pathways.^{34,35} Activation of the Akt and Erk 1/2 signaling by FTY720 treatment has been proven in neuronal culture¹⁰ as well as by using the rodent model of stroke.¹³ In the current study, we confirmed that the phosphorylation level of Akt and Erk 1/2 were upregulated in the optic nerve head region following FTY720 administration. However, more investigations are required to completely unravel the interactions between the BDNF/TrkB and the S1P signaling in neuronal survival mechanisms. Systemic effects of FTY720 on immunomodulation may also play a role in neuroprotection. Additionally, FTY720 may also elicit its protective effects by decreasing ceramide levels caused by inhibiting ceramide synthases^{9,36} through the de novo pathway.

Most of the clinical effects of FTY720 are thought to be mediated via the S1P1 receptor.^{7,37} Fingolimod initially acts as an agonist of the S1P1R on the targeted cells and then becomes a highly potent functional antagonist, resulting in internalization of the S1P1 receptors. It was suggested that loss of S1P1 through functional antagonism by FTY720-P is the primary mechanism of the pharmacological effects of FTY720. In the current study, we found a downregulation in S1P1R expression following FTY720 treatment (Fig. 6), which may be due to the long-term effects of receptor internalization and subsequent decreased protein turn-over. This suggested that the neuroprotective effects of FTY720 in experimental glaucoma were also generated by functional antagonism of the drug. Interestingly, S1P1R activated by FTY720-P may retain signaling activity even after receptor internalization.³⁷ The expression of S1P1R in this microbead-induced glaucoma model was significantly upregulated and this finding was in accordance with previous observations in optic neuritis models.²² However, we did not observe any responsive changes in the expression of S1P5R, indicating that the neuroprotective effects of FTY720 in the retina are mainly mediated by the S1P1 receptor, similar to the pharmaceutical effects of this drug on lymphocytes and endothelial cells.³⁷

The pharmacological effects of FTY720 in the retina appear to be dramatically influenced by the timing and dosage of drug administration.⁹ Fingolimod given prior to the onset of neuronal damage results in better neuroprotective outcomes. In this study, FTY720 treatment was initiated on day one when the IOP was still normal. Glaucoma patients are usually diagnosed only when optic nerve damage has already occurred and visual field defects are present, thus future studies should commence FTY720 therapy after neuronal damage has been

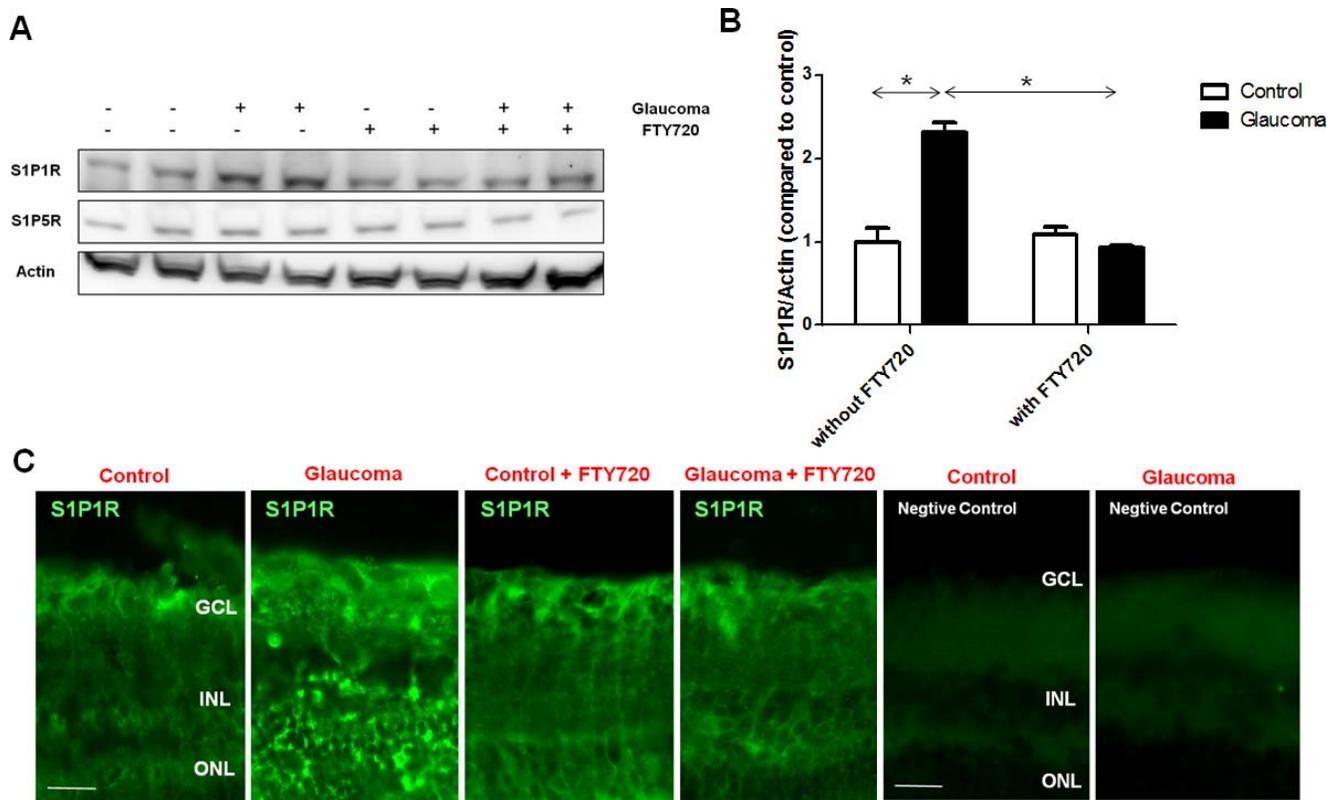


FIGURE 6. (A) The expression of S1P1R and S1P5R in the optic nerve head region by western blots. (B) Densitometric analysis demonstrated a significant up-regulation of S1P1R expression in experimental glaucoma. The expression of S1P1R was reduced following FTY720 treatment. * $P < 0.05$; (C) immunostaining confirmed the upregulation of S1P1R expression in the inner retina under glaucomatous condition. Scale bars: 10 μm ; INL, inner nuclear layer; ONL, outer nuclear layer.

induced, which would be more applicable to the clinical scenarios.

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