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The role of TRPV4 in the regulation of retinal ganglion cells apoptosis in rat and mouse

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ABSTRACT

Retinal ganglion cell (RGC) damages are common in glaucoma, causing atrophy of the optic papilla, visual field damage, and visual loss. Transient receptor potential vanilloid 4 (TRPV4) is significantly expressed in the eyeball and is sensitive to mechanical and osmotic pressure. However, the specific role and mechanism of TRPV4 in glaucoma and RGC progression remain unclear. TRPV4 expression was detected in RGCs under different pressure culture conditions. We also explored the pressure effect on TRPV4 expression and the role and mechanism behind the functional regulation of RGCs. Immunofluorescence staining, western blotting, and TUNEL were utilized in this study. Our results established that TRPV4 was expressed in RGCs. TRPV4 expression was decreased at 40 mmHg and 60 mmHg, and the expression of BAX at 40 mmHg, 60 mmHg. Additionally, the expression of caspase 9 protein increased at 40 mmHg with the pressure increase compared with the conventional culture group. TUNEL staining revealed that the apoptosis rate of RGCs was elevated at 40 mmHg and 60 mmHg, compared with the traditional culture group. Therefore, the expression of BAX and caspase 9 increased, along with the apoptosis rate of RGCs compared with the control group. However, after TRPV4 antagonist treatment, the expression of BAX and caspase 9 decreased, and the apoptosis rate of RGCs decreased. Thus, TRPV4 may affect the mitochondrial apoptosis pathway, such as BAX and caspase 9, leading to the apoptosis of RGCs. The antagonists of TRPV4 could provide a new idea for clinically treating acute glaucoma.

1. Introduction

Globally, glaucoma affects 70 million people and is the second-largest eye disease among the three major blinding disorders [1,2]. Glaucoma causes irreversible visual impairment and is characterized by progressive optic nerve atrophy with varying degrees of visual field loss [3]. Research reports show glaucoma is expected to increase to 112 million by 2040 (4), constituting a heavy economic

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burden on society. Glaucoma drugs alone accounted for 38–52% of the total cost [5]. Various studies suggest that blindness due to glaucoma will continue to increase dramatically in the next few years, significantly burdening society.

Pupil block, iris hypertrophy, preciliary type and cause, anterior chamber stenosis, aqueous outflow disorder, and excessive excretion due to different causes are also the main factors in glaucoma incidence. The pathogenesis of glaucoma can be different based on its classification and cannot be summarized uniformly summarized. The common cause is increased aqueous humor, leading to elevated intraocular pressure and eye damage. Glaucoma can be treated in many ways, including drug, surgical, and laser treatments. The general principle is to reduce intraocular pressure and eye tissue damage and increase optic nerve protection. The axons of retinal ganglion cells (RGCs) form the optic nerve. The visual information received by the photoreceptor is transmitted to RGCs. During the clinical manifestations of glaucoma, the damage of RGCs severely and irreversibly affects the vision [6]. There is almost no way to repair the optic nerve and restore the visual field [7]. Therefore, preventing optic nerve injury, RGCs atrophy, and apoptosis are crucial to clinical prevention and treatment of glaucoma.

Transient receptor potential (TRP) channels are non-selective cation channel proteins widely distributed within the nervous system [8]. TRP channels are critical in human senses, including smell, hearing, touch, controlling temperature, and osmotic pressure, but the vision position is unclear [4,9]. Many studies have confirmed that TRP channels are essential in apoptosis. These channels are related to the pathological changes of ocular tissues in glaucoma and may become a therapeutic target [10–13]. Alkozi observed that GSK1016790 A, an agonist of transient receptor potential vanilloid 4 (TRPV4), could promote melatonin secretion, mediate intraocular pressure, and is related to GSK1016790 A concentration [14]. Western blot has confirmed that TRPA1, TRPC1, TRPC2, TRPC3, TRPC6, TRPM2, and TRPP2 are expressed within trabecular meshwork tissue and cells [15]. However, the function and mechanism of TRPV4 inside the retina remain unclear.

The present study enquired whether TRPV4 had protective effects against glaucoma. We also investigated the underlying mechanisms of TRPV4 on optic nerve damage. Therefore, the role of TRPV4 in the mitochondrial pathway of RGCs apoptosis during a high intraocular pressure environment could provide a basis for clinical prevention and treatment of optic atrophy due to glaucoma.

2. Materials and methods

2.1. Cell culture

We selected mouse RGC-5 (Shanghai FUHENG Biotechnology Co., Ltd., fh0369) and rat RGC (Shanghai YAJI Biotechnology Co., Ltd., YS2094C) as the experimental object. We used DMEM high glucose (Bi, bish0829) + 10% FBS (GIBCO, 10099141c) + 1% penicillin and streptomycin, 5% CO_2, and 37 °C for routine culture. Mouse RGCs and RGC-5 cells were made into single-cell suspensions and seeded in T25 as culture flasks at a density of 5×10^6/flask.

2.2. Establishment of cell sealing and pressurizing device

A disposable infusion set, butyl external turning rubber plug, blood pressure monitor, disposable medical three-way valve, scissors, and tweezers were included in the experimental materials. These were sterilized using a high-pressure steam device. The ultraviolet light of the ultra-clean work table was turned on for half an hour. The pressurization device was assembled with light in the ultra-clean work table, and the following operations were performed near the alcohol lamp. The infusion line was divided into two sections, the infusion set was cut from the upper end of the drip pot, and the upper end was connected with one end of the disposable medical T-type three-way valve. After removing the blood pressure band of the sphygmomanometer, the two segments were connected to the medical T-type three-way valve and the infusion device bottle plug, respectively. Then, the lid with filter paper was clamped. The 28 butyl external turning plug of the 100 ml saline bottle was punctured using a puncture device. The infusion hose with the cap was clamped with tweezers to assess the puncture port so that the lid of the disposable infusion line was stuck without leakage. The third end of the medical T-way valve was connected with an infusion hose using a cover. Finally, the pressurization device was assembled. The assembly is represented in Supplemental Fig. 1.

2.3. Cell treatment

RGC-5 cells were divided into four groups: standard culture, 0 mmHg, 40 mmHg, and 60 mmHg groups. Each pressure group was split into three groups: I. control, II. agonist, and III. antagonist groups. RGC-5 cells grew and fused more than 70%–80%. The old medium was sucked off, washed once using preheated PBS, and starved with serum-free DMEM medium for 4 h. After starving for 4 h, RGC-5 cells were incubated with an agonist or antagonist. The old medium was removed, washed with 1XPBS buffer once, and digested with 0.25% trypsin for about 3 min after 70–80% cell growth and fusion. The DMEM medium was added into the dish to terminate digestion and passaged based on 1:2–1:4.

In each pressure group, I. control, II. agonist, and III. antagonist groups were added having complete, 25 nM GSK1016790 A (sigma, g0798) and 10 μM HC067047 (MCE, hy-100208) DMEM complete culture medium. After 1 h of culture, the conventional culture group was placed in the incubator. The other pressure groups were kept in the closed pressure device. The conventional culture group was placed in the incubator for 24 h after setting the pressure at 0 mmHg (closed without pressure), 40 mmHg, and 60 mmHg.

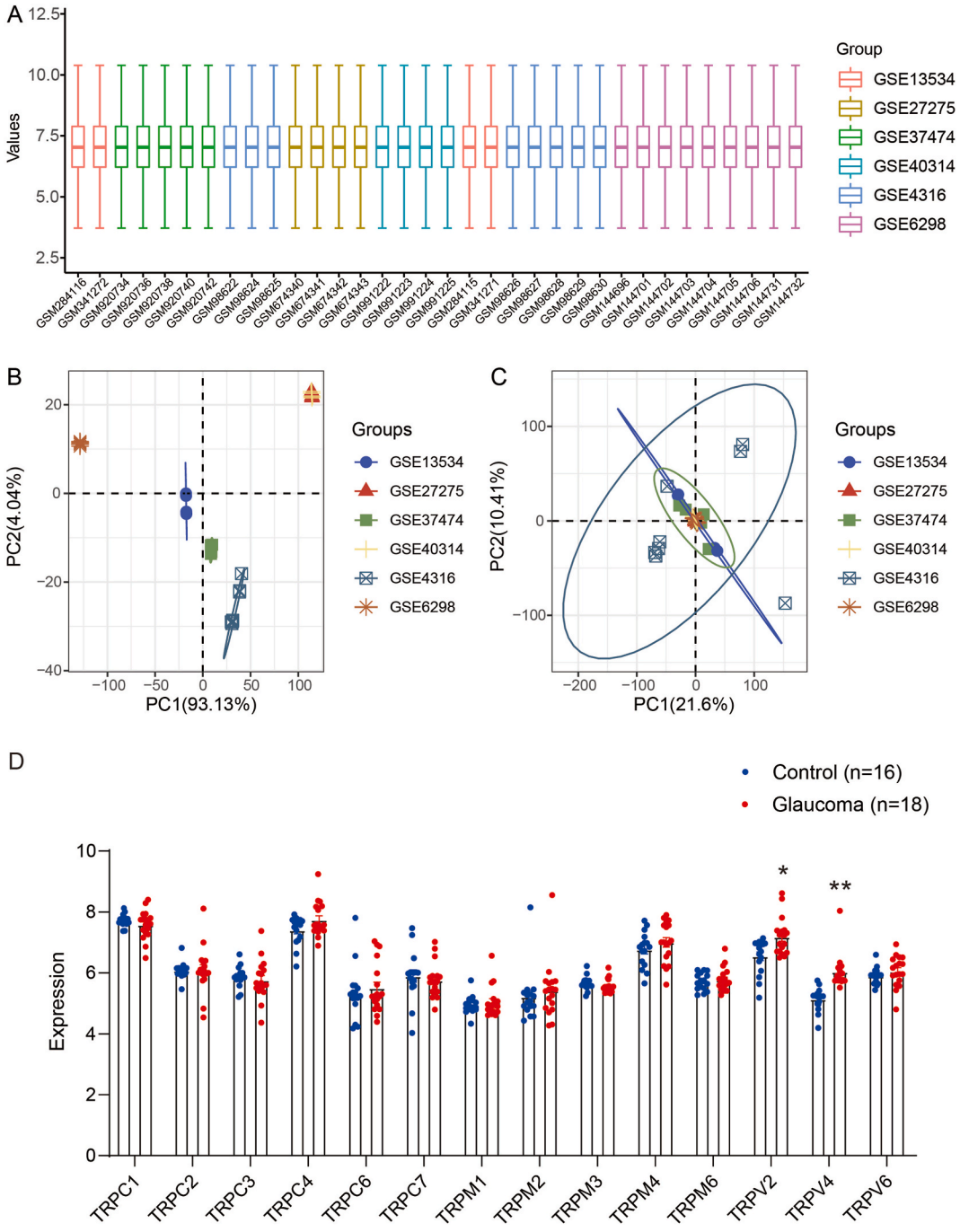

Fig. 1. The expression levels of TRPs in the normal and glaucomatous human cells (A) The boxplot of the normalized data. Different colors depict different datasets from (GSE13534), (GSE37474), (GSE4316), (GSE27275), (GSE40314), and (GSE6298). The rows represent samples, and the columns indicate the gene expression values across the samples. (B) Th PCA results before batch removal for the multiple datasets. The different colors demonstrate different datasets. The schematic diagram reveals that the six datasets have been separated without any intersection. (C) The PCA results after batch removal, as represented in the schematic diagram, indicate the intersection of six datasets for subsequent analysis. (D) The expression and distribution of TRP genes across different tissues. The abscissa describes different sample groups. The ordinate characterizes the expression distribution of the gene, and different colors represent different groups. The top-left depicts the significance p-value. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, asterisks (*) stand for significance levels. The statistical difference between the two groups was compared using the Wilcox test, and the significant difference between the three groups was tested using the Kruskal-Wallis test.

2.4. Immunofluorescence staining

A 14 mm diameter slide was placed into the 24-well plate and was covered evenly with 30 μl L-polylysine for 10 min. The slides were washed using 1XPBS for 5 min. RGC-5 cells were inoculated into the slides after UV irradiation for 1 h. The slides were divided according to the above groups. The slides were covered using 4% paraformaldehyde for 15–30 min when the cultured cell fusion rate was up to 80% and washed with PBS for 3 × 5 min. 0.3% Triton X-100 solution helped permeate the cellular membrane for 10 min. The slides were incubated using standard goat serum blocking solution for 30 min, washed with PBS for 3 × 1 min, and a primary antibody-containing TRPV4 (Abcam, ab39260) was added at 4 °C overnight. A fluorescent-labeled second antibody (a32731) was used for signal detection, which was observed using a fluorescence microscope (Olympus, bx53f2, laser Olympus, *o*-hglgps). The blue light of the nucleus was excited with ultraviolet light (excitation wavelength of 360–400 nm) and photographed.

2.5. Western blotting

The culture medium was removed from the well-growing cells and washed once with pre-cooled PBS. Then, radio-immunoprecipitation assay (RIPA) buffer supplemented with proteinase and phosphatase inhibitor cocktail (Thermo Fisher Scientific, 10,085,973) facilitated the lysis of cells. The supernatant was extracted and sub-packed after centrifugation. The BCA kit and microplate reader helped detect the protein concentration, and the sample was prepared by adding a loading buffer. The samples were separated on SDS-polyacrylamide gels and transferred to PVDF membranes, which were blocked using 5% skimmed milk powder for 1.5 h at room temperature and incubated with primary antibody at 4 °C overnight. The primary antibodies were: *anti*-TRPV4 (Abcam, ab39260), *anti*-BAX (cell signaling technology, 5023), *anti*-BCL-2 (Abcam, ab692), *anti*-caspase 9 (Abcam, ab32539), and *anti*-β-actin (Proteintech, 66,009-1-ig) antibodies. They were diluted in 5 ml of 5% BSA solution in 1:2000, 1:1000, 1:500, 1:5000, and 1:2000 orders. The membranes were incubated using an HRP-conjugated second antibody for 1 h at room temperature. The membranes were washed with 1xTBST solution three times for 10 min each time. ImageJ 1.48v software (http://imagej.nih.gov/ij) and gel image system 1D analysis software (version 4.2, www.bio-tanon.com.cn) helped analyze the image. The optical density values of Western blot bands were extracted using the software and compared with internal standards.

2.6. TUNEL staining

The cells (0.5×10^6 per well) were seeded in triplicate in six-well plates in DMEM using 0.1% FBS and left to adhere for 24 h at 37 °C. Next, cell death was determined using the one-step TUNEL cell apoptosis detection kit (Beyotime, C1082). The cells were detached from the plate, centrifuged, and fixed in 1% paraformaldehyde for 15 min on ice. Next, the fixed cells were washed with PBS, resuspended in 70% ice-cold ethanol, and stored for 12–18 h in a −20 °C freezer. Then, the samples were washed, resuspended in a DNA-labeling solution with reaction buffer, TdT (terminal deoxynucleotidyl transferase) enzyme, BrdUTP (deoxythymidine analog 5-bromo-2′-deoxyuridine 5′-triphosphate), and water and incubated for 60 min at 37 °C inside a water bath. Afterward, the samples were

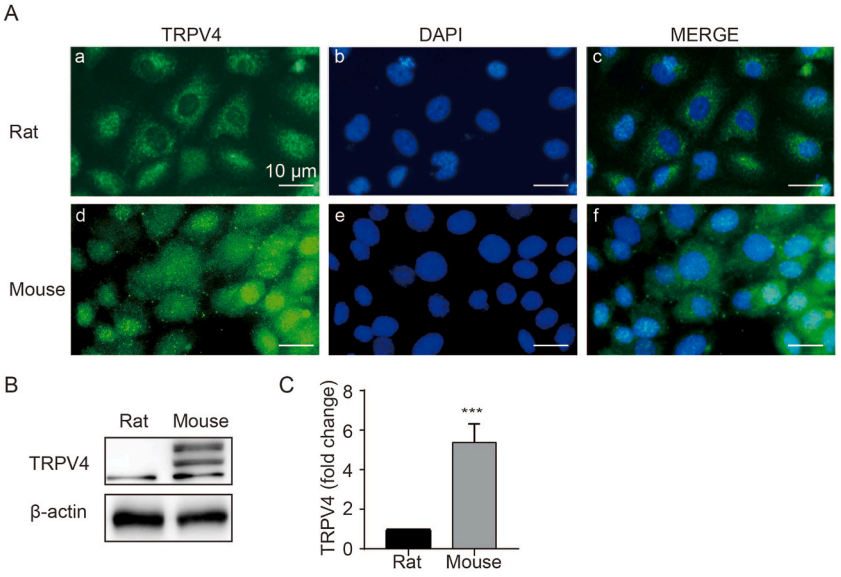

Fig. 2. TRPV4 channel protein expression in RGCs was verified using immunofluorescence and Western blot (A) DAPI staining depicted the cell nuclei in blue. Green indicates TRPV4 channel protein due to fluorescent second antibody staining; Eyepiece 10×, Objective 50×, scale bar: 10 μm. (B) TRPV4 expression in RGCs was verified using the Western blot. (C) Statistical analysis of Fig. 2B. All the data represent the mean ± SEM from a minimum of three independent experiments. ***P < 0.001 compared with 0 mmHg.

washed and set with BrdU-Alexa Fluor 488 antibody for 30 min at room temperature. Then, they were analyzed using an LSR II flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo software (FlowJo, Ashland, OR, USA).

2.7. Statistical analysis

All the data were analyzed by the SPSS 22.0 software using a *t*-test method for two independent samples. One-way ANOVA was used to evaluate between three groups. The values have been presented as mean ± standard error of the mean ($\overline{x} \pm$ SEM). The significance level was at $P < 0.05$. The experiments were performed at least thrice.

3. Results

3.1. TRPV4 was most intensively expressed in retinal ganglion cells

We analyzed the function of TRP channels in glaucoma in the human normal and glaucomatous cells using RNA sequencing (RNA-seq) datasets [(GSE13534), (GSE37474), (GSE4316), (GSE27275), (GSE40314), and (GSE6298)]. We performed the data analysis after ID conversion on the GEO data (array IDs corresponding to the genes), data standardization (unifying all the data under one order of magnitude), and removing batch effects (the effects due to different batch experiments) (Fig. 1A–C). As shown in Fig. 1D, TRPV2 and TRPV4 were significantly elevated in the glaucoma group. TRPV4 was used as the target gene because the down-regulation trend of TRPV4 (<0.01) was more evident than TRPV2 ($p < 0.05$) in the glaucoma group.

The transient receptor potential vanilloid 4 (TRPV4) channel could be opened using mechanical stimuli to mediate Ca^{2+} and Na^{+} influxes and could mediate glaucoma retinopathy. However, how TRPV4 activities affect primate RGC function remains unclear. First, the expression and localization of TRPV4 were detected in mouse RGC-5 and rat RGC. Immunofluorescence with TRPV4 antibody revealed that RGCs in good growth condition were fusiform adherent cells (Fig. 2A). TRPV4 was distributed within the cell membrane and mainly in the cytoplasm and most abundantly expressed at the nucleus edge. We also quantified the expression level of TRPV4 in RGC-5 and rat RGC. Western blot results revealed that TRPV4 was expressed in RGCs (Fig. 2B), and TRPV4 was more expressed in rat RGC than in mouse RGC-5 (Fig. 2C).

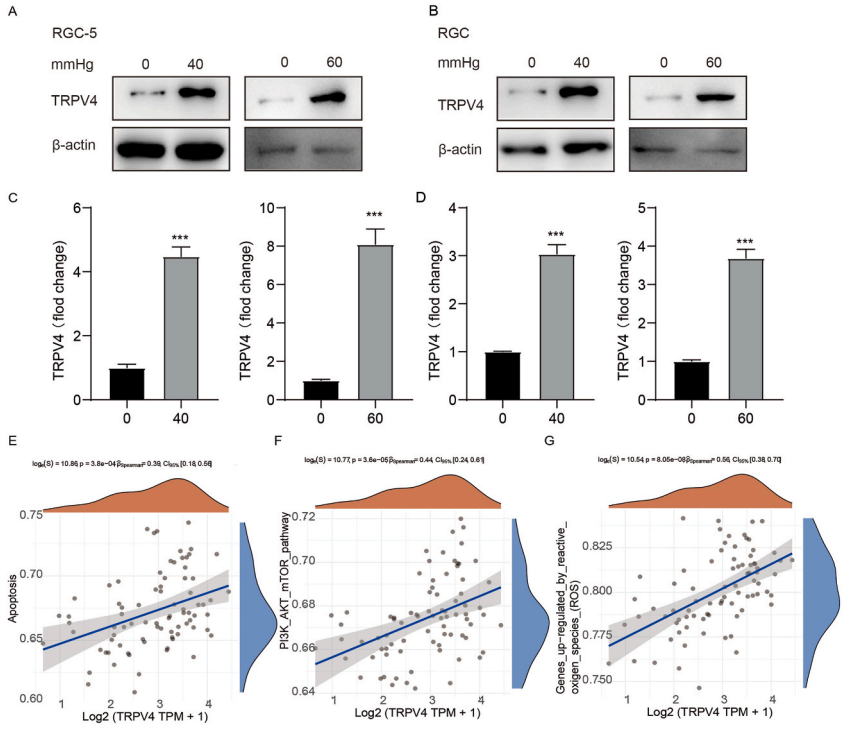

Fig. 3. TRPV4 expression levels in RGCs in the glaucoma model (A) TRPV4 expression in mouse RGC-5 was verified at 40 mmHg and 60 mmHg for 24 h. (B) TRPV4 expression in RGC was verified at 40 mmHg and 60 mmHg for 24 h. (C) TRPV4 level in A was quantified and compared using the 0 mmHg state. All the statistical data represent the mean ± SEM from a minimum of three independent experiments. **$P < 0.01$ and ***$P < 0.001$ compared using 0 mmHg. (D) TRPV4 level in B was quantified and compared using the 0 mmHg state. All the data indicate the mean ± SEM from at least three independent experiments. **$P < 0.01$ and ***$P < 0.001$ compared using 0 mmHg. (E) The correlation analysis of TRPV 4 and apoptotic proteins. (F) The correlation analysis of TRPV 4 and the PI3K-AKT-mTOR signaling pathway. (G) The correlation analysis between TRPV 4 and upregulated reactive oxygen species (ROS).

3.2. The expression and function of TRPV4 in retinal ganglion cells at elevated pressure

Furthermore, the glaucoma model with the mouse RGC-5 and rat RGC cell lines was stimulated to detect TRPV4 expression. Western blotting indicated that TRPV4 expression was reduced at 40 mmHg and 60 mmHg for 24 h than in the conventional culture group (Fig. 3A–D). TRPV4 expression was elevated in GRCs under 40 mmHg and 60 mmHg. Furthermore, the enrichment score of glaucomatous cells in each pathway was successively analyzed to obtain the correlation between the sample and pathway based on the GSEA algorithm (Fig. 3E–G). The relationship between TRPV4 and pathways was obtained by determining the correlation between TRPV4 expression and pathway score. TRPV4 was positively associated with apoptosis, PI3K-AKT-Mtor pathway, and gene upregulated by reactive oxygen species (ROS). Among them, the PI3K-AKT-mTOR pathway and gene upregulated by ROS-related pathways are also related to the function of apoptosis in cancers, rheumatoid arthritis, acute respiratory hypoxia, and reoxygenation [16–18].

3.3. RGC apoptosis increased with increasing pressure

Next, we determined whether the glaucoma model displayed altered apoptosis in RGCs. Western blotting indicated that BAX protein expression was enhanced at 40 mmHg ($P < 0.0001$) and 60 mmHg ($P = 0.002$, $P < 0.001$) for 24 h than in the 0 mmHg group. The BCL-2 level had decreased at 40 mmHg. Meanwhile, caspase 9 expression was significantly elevated at 40 mmHg and 60 mmHg. There was no significant difference in BCL-2 protein expression of RGC-5 in the pressure group of 60 mmHg than in the conventional culture group. These results are depicted in Fig. 4 (A-H). Furthermore, TUNEL analysis revealed that the apoptosis rate of RGC-5 was elevated at 40 mmHg and 60 mmHg for 24 h (Fig. 5A–C). Thus, the pressure of 40 mmHg and 60 mmHg can cause apoptosis of RGC-5. Rat RGC were operated in the same condition to confirm these phenomena (Sup. Fig. 1). The apoptosis rate of rat RGC was enhanced, and the expression of caspase 9 also elevated at 40 mmHg and 60 mmHg.

3.4. The effect of TRPV4 agonist on RGCs induced by pressure

RGCs cells were treated with 25 nM GSK1016790 A (TRPV4 agonists) 1 h before compression to investigate whether TRPV4 agonists affected their pressure-induced apoptosis. The levels of BAX, BCL-2, and caspase 9 were detected with a Western blot [19]. BAX protein expression was increased in the agonist group after 24 h of 40 mmHg and 60 mmHg. Moreover, BCL-2 protein expression was decreased in the 40 mmHg and 60 mmHg groups with GSK1016790 A after culturing for 24 h. Caspase 9 protein expression was higher in the agonist group than in the 40 mmHg group. Therefore, GSK1016790 A can promote RGCs apoptosis induced by 40 mmHg and 60 mmHg pressure (Fig. 6A–H).

Secondly, the apoptotic rate of RGC-5 treated with 25 nm GSK1016790 A for 1 h was detected before pressurization, which was increased at 40 mmHg and 60 mmHg (Fig. 7A–C). The same experiment was conducted in rat RGC (Sup. Fig. 2). GSK1016790 A could promote the apoptosis of RGCs cultured at 40 mmHg and 40 mmHg for 24 h. Therefore, TRPV4 agonists, GSK1016790 A, positively enhanced apoptosis in RGCs.

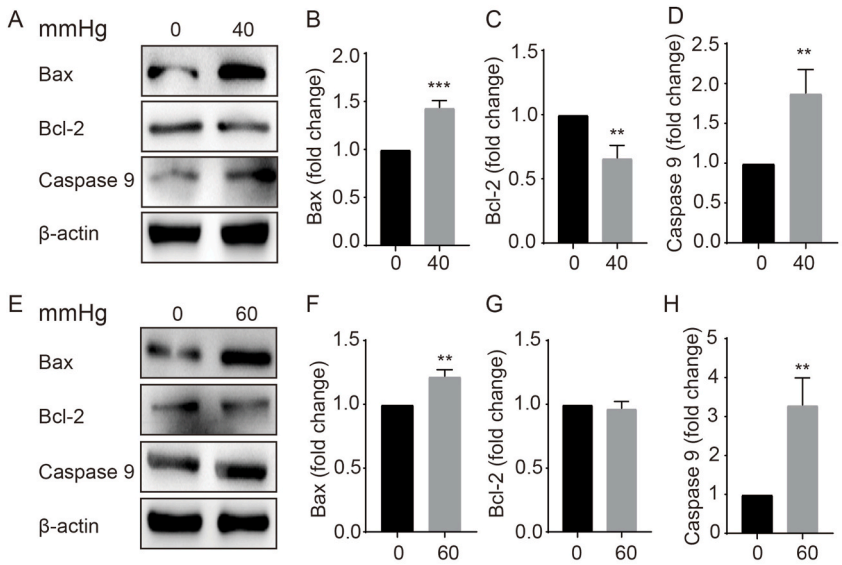

Fig. 4. Apoptosis of RGC-5 under pressure conditions (A–D) The expression of BAX, BCL-2, and caspase-9 in RGC-5 under 0 mmHg and 40 mmHg pressure after 24 h. (*E*–H) The expression of BAX, BCL-2, and caspase-9 in RGC-5 under 0 mmHg and 60 mmHg pressure after 24 h. All the statistical data are represented as mean ± SEM from at least three independent experiments. **P < 0.01 and ***P < 0.001 compared using 0 mmHg.

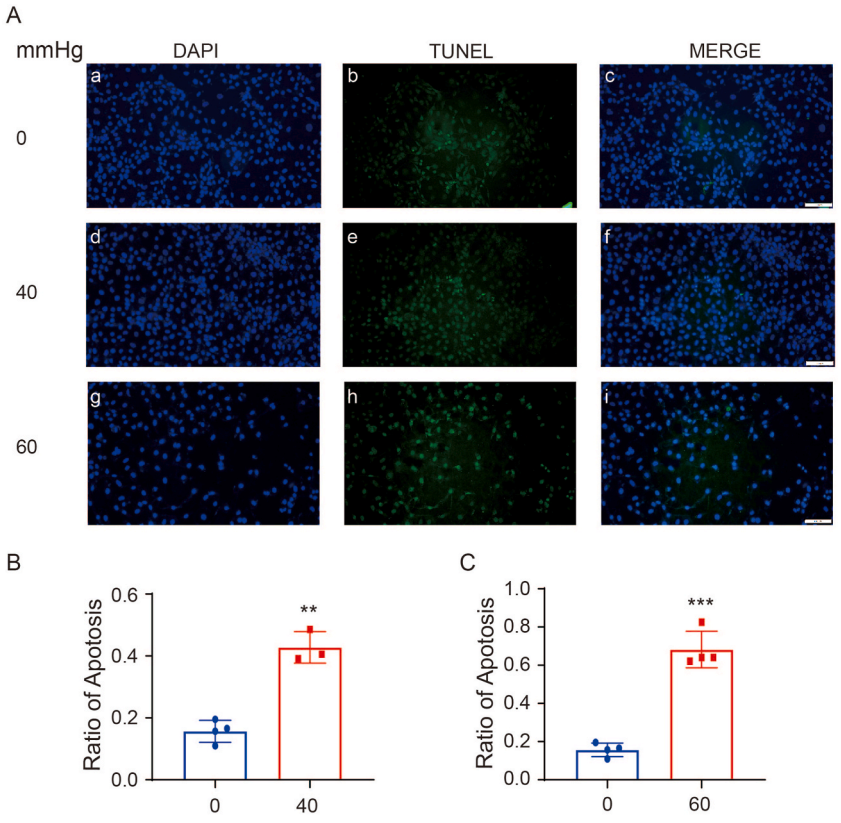

Fig. 5. The apoptosis of RGC-5 was detected using TUNEL staining (A) The blue color indicates the nuclear staining of all the cells in the conventional culture, 40 mmHg, and 60 mmHg groups. The green color consecutively depicts the nuclear staining of all the apoptotic cells in the 0 mmHg, 40 mmHg, and 60 mmHg groups. (B–C) The apoptosis rate of the 40 mmHg and 60 mmHg groups was compared and analyzed with the conventional culture group. All the statistical inputs are demonstrated as mean ± SEM from at least three independent experiments. **P < 0.01 and ***P < 0.001 compared using 0 mmHg.

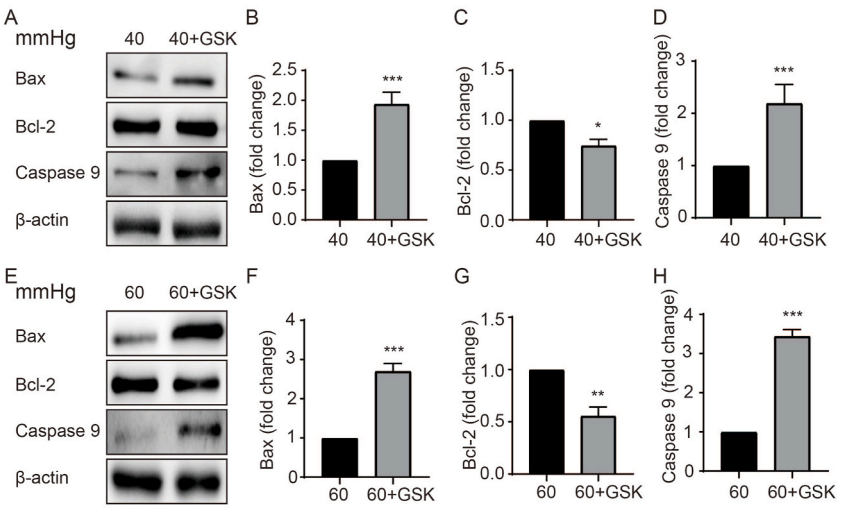

Fig. 6. GSK1016790 A effect on the expression of RGC-5 apoptotic protein (A–D) BAX, BCL-2, and caspase-9 protein expression at 40 mmHg with GSK1016790 A compared with the 40 mmHg group. All the data are represented as mean ± SEM from a minimum of three independent experiments. *P < 0.05 and ***P < 0.001 compared using 40 mmHg. (*E*–H) BAX, BCL-2, and caspase-9 protein expression at 40 mmHg with GSK1016790 A compared with the 60 mmHg group. All the statistical data demonstrate the mean ± SEM from at least three independent experiments. **P < 0.01 and ***P < 0.001 compared using 60 mmHg.

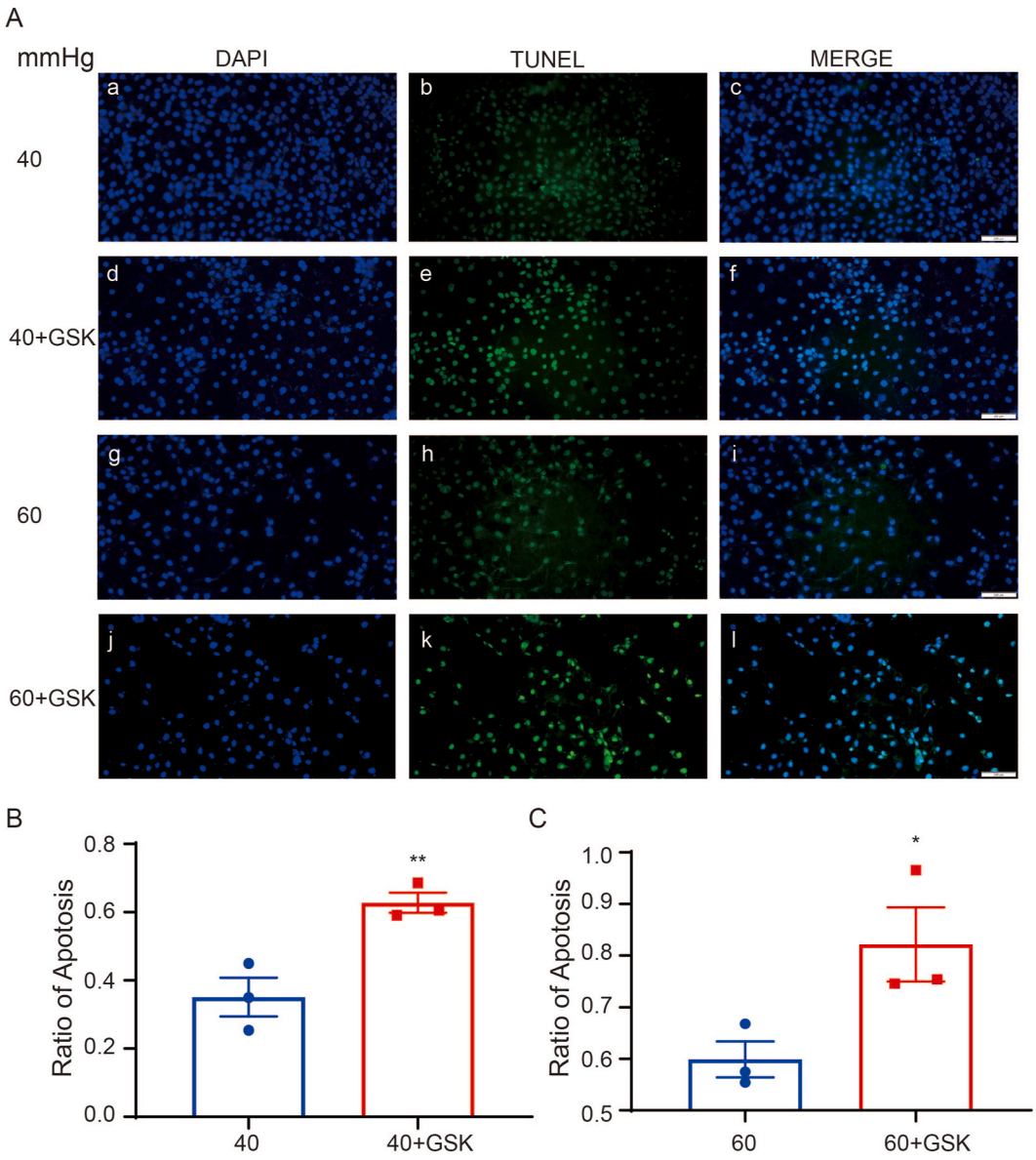

Fig. 7. GSK1016790 A effect on apoptosis of rate RGC-5 (A) Blue indicates the nuclear staining of all cells in each group; green characterizes the nuclear staining of all apoptotic RGC-5 cells. The third column demonstrates the merged map of apoptotic and all cells in each group. (B–C) Compared with the conventional culture group, the apoptosis rate of the 40 mmHg and 60 mmHg groups with GSK1016790 A stimulation. All the statistical inputs represent the mean ± SEM from at least three independent experiments. **P < 0.01 and ***P < 0.001 compared using non-GSK1016790 A stimulation.

3.5. The effect of TRPV4 antagonist on RGC-5 due to pressure

The above experiments revealed that TRPV4 expression was decreased in the glaucoma model. The glaucoma model cells (RGCs with pressure) were stimulated using TRPV4 agonist to enhance apoptosis. Then we explored the TRPV4 inhibitor on the apoptosis of glaucoma model cells. 10 μM HC067047 was incubated in RGCs with pressure [20]. BAX expression in the HC067047 group decreased more than in the control group after 24 h. The expression of BCL-2 protein was similar between the 40 mmHg and 60 mmHg groups after 24 h of culture. However, caspase 9 protein expression in the antagonist group decreased more than in the control group. Therefore, HC067047 could inhibit RGC-5 apoptosis caused by 40 mmHg and 60 mmHg (Fig. 8A–H).

Next, the apoptotic rate of RGC-5 was detected using the TUNEL technology. RGCs were treated using HC067047 at 40 mmHg and 60 mmHg. RGC-5 apoptotic rate was also decreased with HC067047 of glaucoma model cells (Fig. 9A–C). The apoptotic rate and caspase 9 level in rat RGC were also detected (Sup. Fig. 3). Thus, HC067047 can inhibit the apoptosis of RGCs in the glaucoma model. Furthermore, we normalized the data and analyzed the correlation between TRPV4 and BAX, BCL-2, and caspase 9, consistent with the

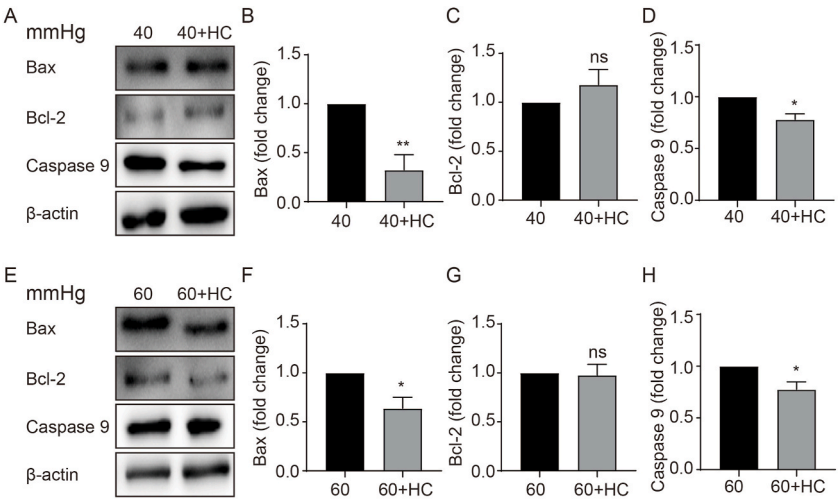

Fig. 8. HC067047 effect on the expression of RGC-5 apoptotic protein (A–D) BAX, BCL-2, and caspase-9 protein expression at 40 mmHg with HC067047 stimulation compared with the 40 mmHg group. All the data characterize the mean ± SEM from at least three independent experiments. *P < 0.05 and **P < 0.01 compared using 40 mmHg. (*E*–H) BAX, BCL-2, and caspase-9 protein expression at 40 mmHg with HC067047 stimulation compared with the 60 mmHg group. All the statistical data demonstrate the mean ± SEM from at least three independent experiments. *P < 0.05 compared using 60 mmHg.

previous data processing. BCL-2 showed a negative correlation, while BAX and caspase 9 were positively associated with TRPV4 (Fig. 10A–D). TRPV4 expression is decreased, its agonists promote apoptosis, and its inhibitors inhibit apoptosis by controlling BAX, BCL-2, and caspase 9 within the glaucoma model (Fig. 11).

4. Discussion

Glaucoma is the second leading reason of blindness after ametropia and cataracts, affecting vision worldwide [4,9] and demanding prevention and treatment. Progressive apoptosis of RGCs is a common pathological feature of various types of glaucoma [21]. The primary purpose of glaucoma treatment is to protect and prevent optic nerve damage. Thus, reducing intraocular pressure can alleviate the progressive death of RGCs in glaucoma, so studying the tolerance of RGCs to pressure is imperative. However, the damage of RGCs cannot be reversed. Therefore, understanding the specific mechanism of RGCs damage can provide theoretical support in preventing and treating glaucoma.

This study demonstrated that TRPV4 was expressed in mouse RGC-5 and rat RGC, and the TRPV4 expression level was elevated under the glaucoma model. TRP channel is vital in human physiological health. Inside the eye, TRPV4 is expressed in various eye tissues, including the corneal epithelium, ciliary body, and crystalline body [20,22,23]. Li et al. observed that TRPV4 also existed among RGCs of male Sprague Dawley rats. Immunohistochemical sections revealed that TRPV4 existed in the ganglion cell layer labeled with Brn3 and the whole nerve fiber layer [23]. TRPV4 channel may be associated with the physiological function of RGCs in controlling synaptic transmission [24]. Patel et al. showed that impaired TRPV4 channel activity contributed to trabecular meshwork dysfunction and elevated intraocular pressure within the primary open-angle glaucoma [25]. The high expression level of TRPV4 was correlated with elevated intraocular pressure [26], consistent with our findings.

TRPV4 is one of the mechanical gating channels that can conduct the physical stimuli some organisms receive, such as hydrostatic pressure sensitivity, osmotic pressure, membrane stretching, and temperature [24,27]. New research on TM cells also reported TRPV4 function in glaucomatous humans. It was observed that glaucomatous human TM cells had impaired activity of TRPV4 channels and disrupted TRPV4-eNOS signaling, contributing to TM dysfunction and increased IOP in glaucoma [25]. TRPV4 can regulate the calcium signal transduction across the cell membrane, suggesting that TRPV4 is pressure-sensitive in RGCs. Gao et al. confirmed that the functional TRPV4 channel was variably expressed in primate RGCs. TRPV4 reversed the bipolar cell current in the retina at about 18 mmHg, contributing to the pressure-related changes within glaucoma RGCs [28]. The colon of TRPV4$^{-/-}$ mice reacts to less than 90 mmHg, indicating an essential role of TRPV4 in the response of RGCs to pressure changes with pressure sensitivity [29]. Our study revealed that TRPV4 channel protein expression was reduced at 40 mmHg and 60 mmHg. These findings were consistent with previous studies that TRPV4 is sensitive to pressure. However, most evidence has not accurately simulated the IOP gradient similar to glaucoma. Our study mainly focuses on 40 mmHg and 60 mmHg, providing ideas for the expected fluctuation range of glaucoma IOP and TRPV4 regulation of RGCs to the pressure tolerance value.

High IOP is the leading risk factor for glaucoma, which can cause different degrees of damage to the optic nerve and retina [30,31]. In preventing and treating glaucoma, IOP is the only controllable and easy-to-measure risk factor [28]. Shang et al. utilized a self-made pressure device to pressurize RGC-5 to 100 mmHg and induce apoptosis. Their team also established that RGC-5 cell damage disease involves high hydrostatic pressure [32]. We set up a self-made pressure device to develop a glaucoma model and verify the effect of 40

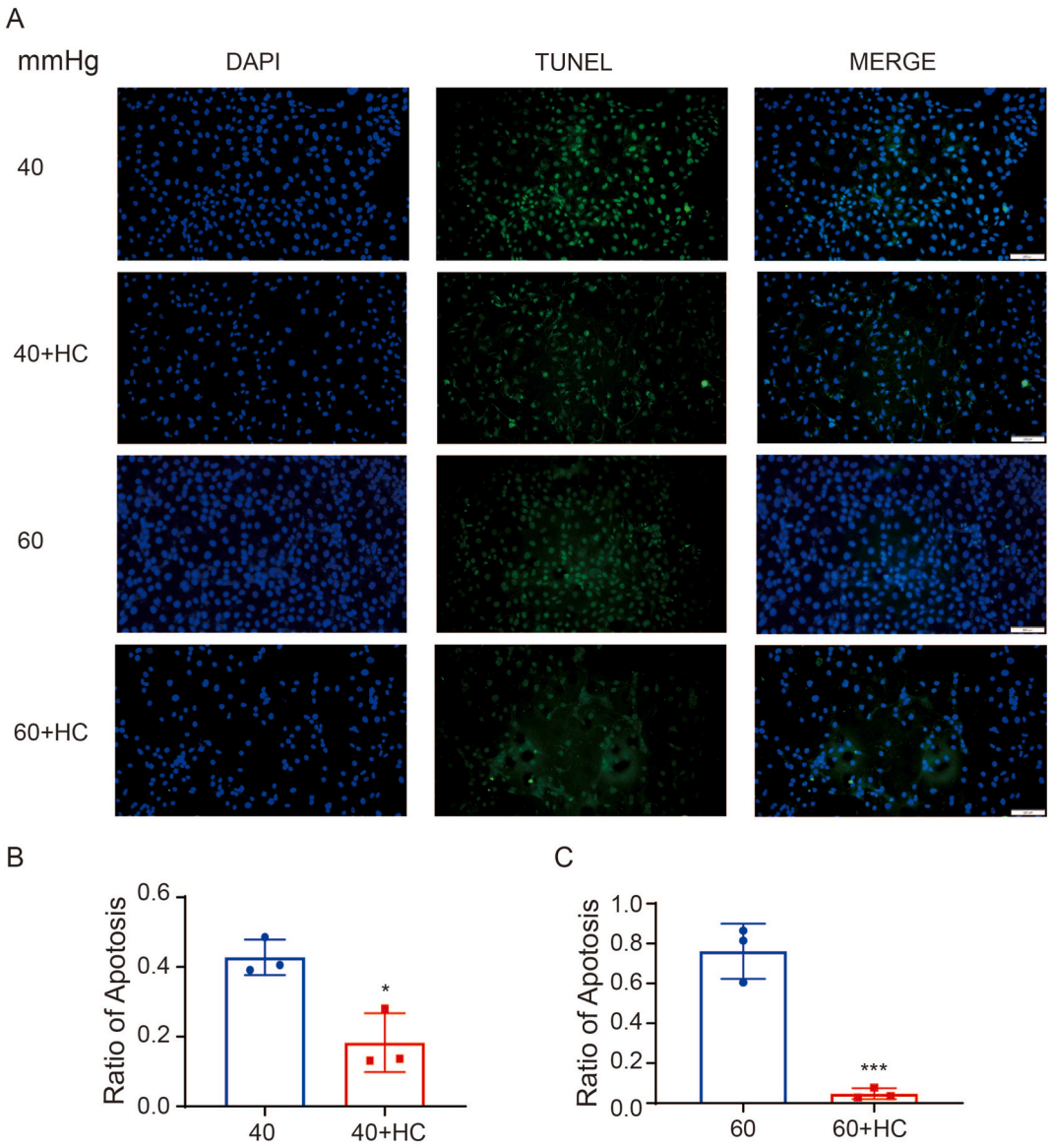

Fig. 9. HC067047 effect on apoptosis of rate RGC-5 (A) Blue represents the nuclear staining of all the cells in each group; green depicts the nuclear staining of all apoptotic RGC-5 cells. The third column indicates the merged map of apoptotic cells and all the cells in each group. (B–C) The apoptosis rate of the 40 mmHg and 60 mmHg groups with HC067047 stimulation compared with the conventional culture group. All the statistical data describe the mean ± SEM from at least three independent experiments. *P < 0.05 and ***P < 0.001 compared using non-HC067047 stimulation.

mmHg and 60 mmHg on RGCs apoptosis. Our study indicated that abnormal pressure rise could induce RGCs apoptosis. However, optic nerve axon deformation and optic ganglion cell aberration occur in normal-tension glaucoma [30]. Thus, high intraocular pressure is not the only glaucomatous optic nerve damage factor. This also explains the similarity in the expression of BCL-2 and caspase 9 in RGC-5 after 24 h of culture using 40 mmHg and 60 mmHg. Moreover, there is no difference in the expression of caspase 9 between RGCs and the standard culture group after 24 h under 60 mmHg pressure culture.

Studies have indicated that TRPV4 agonists can enhance the discharge rate of RGCs, leading to a large amount of Ca^{2+} influx in RGCs. Moreover, continuous TRPV4 activation will lead to the death of RGCs [33,34]. Additionally, TRPV4 antagonist enhances the survival rate of RGCs, and inhibition of TRPV4 can be a potential method for treating glaucoma [35,36]. TRPV4 channel agonist 4 has been reported α PDD (≤2 μM) and GSK (≤1 μM). The self-discharge rate for the action potential of the rhesus monkey, baboon, and RGCs was significantly elevated [37]. Selective TRPV4 agonists, including 4 α-PDD and GSK1016790 A, induced calcium influx within RGCs and enhanced their spontaneous discharge rate [23,38]. TRPV4 inhibition by HC067047 established that HC067047 could significantly inhibit the tube formation and migration of human retinal capillary endothelial cells [39]. Ryskamp stimulated retinal

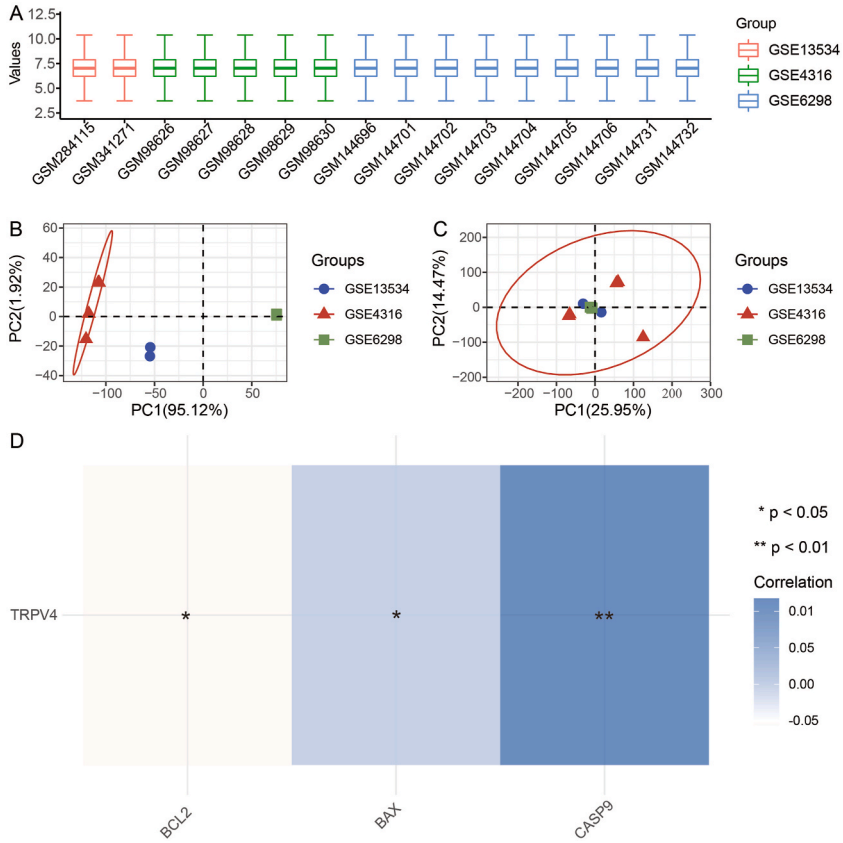

Fig. 10. The correlation between TRPV4 and apoptosis-related genes (A) The boxplot represents the normalized data. The different colors indicate various datasets from (GSE13534), (GSE4316), and (GSE6298). (B) The PCA results before batch removal for the multiple datasets. The different colors characterize the different datasets. The schematic diagram shows that the three datasets are separated without any intersection. (C) As shown in the schematic diagram, the PCA results after batch removal indicate the intersection of three datasets for subsequent analysis. (D) The analysis of the multi-gene correlation with TRPV4. A heatmap of the correlation between multiple genes and TRPV4, with abscissa and ordinate representing genes. The different colors depict the correlation coefficients (blue indicates positive correlation, whereas red characterizes negative correlation). The darker the color, the stronger the correlation. Asterisks (*) indicate significance levels, ** for $p < 0.01$, * for $p < 0.05$.

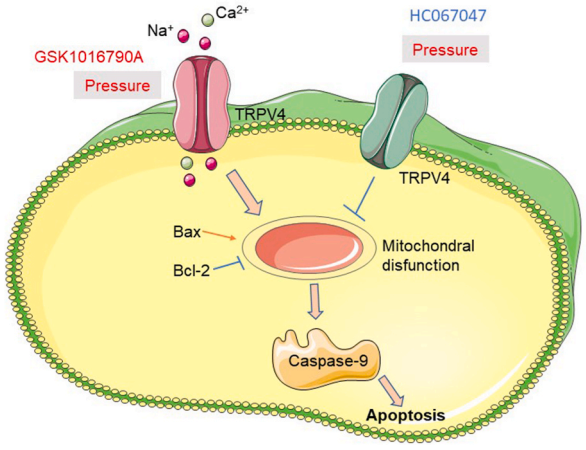

Fig. 11. Pattern diagram representing the regulation and mechanism of apoptosis within the glaucoma model.

cells from C57BL/6 J mice using 25 nM GSK1016790 A for calcium imaging, increasing Ca^{2+} in RGCs [40,41]. These studies are consistent with our results. However, they do not explain the role of the apoptosis and mitochondrial apoptosis pathways. Still, TRPV4 is essential in the physiological activities of RGC-5.

When TRPV4 expression increased, the apoptotic protein also increased. The presentation of BAX and caspase 9 in RGC-5 of the GSK1016790 A agonist group was higher than in the control group. The expression of BAX and caspase 9 in RGC-5 was reduced more by HC067047 (trv4 antagonist) than in the control group. Therefore, the TRPV4 channel can control RGC-5 apoptosis under increased intraocular pressure. TRPV4 channel protein can regulate the apoptosis of RGCs, agonists enhance, and antagonists inhibit RGC-5 apoptosis. Several common apoptotic proteins in the mitochondrial pathway are BAX, cytochrome C, anti-apoptotic protein BCL-2, caspase 9, etc. [42]. Our results indicate that activating TRPV4 can lead to intracellular Ca^{2+} influx in RGCs. Cytochrome C in the mitochondrial membrane of RGCs is promoted by upregulated BAX, leading to the apoptosis of RGCs, which the anti-apoptotic protein BCL-2 can reverse.

TRPV4 was expressed in mouse RGC-5 and mouse RGC; in glaucoma, TRPV4 expression was reduced with increased pressure. TRPV4 may impact the apoptosis of RGCs by affecting the expression of BAX, BCL-2, and caspase 9, essential in glaucoma. The presentation of TRPV4 in RGCs is sensitive to pressure with a particular regulatory effect on the apoptosis of RGCs. This provides ideas for preventing and treating optic nerve injury.

TRP are non-selective cationic receptor channels with wide distribution and expression in the eyeball. Their function is related to the normal physiological function of specific cellular and tissue structures and has a vital role in ophthalmic diseases. Elevated intraocular pressure is the most crucial risk factor for glaucoma. Thus, intraocular pressure lowering treatment can delay the occurrence and progression of glaucoma, the primary clinical treatment strategy for glaucoma. In our study, TRPV4 expression in retinal ganglion cell lines may be rapidly and effectively regulated under stress. The TRPV4 expression pattern is correlated with the viability and apoptotic index. Therefore, inhibiting the TRPV4 channel can relieve the damaging effects of stress on the cell viability and apoptosis of ganglion cells to a certain extent, with a protective effect on ganglion cells. Therefore, TRPV 4 blocking agent could be a new direction in treating glaucoma. However, it requires practice and demonstration in animal and clinical experiments. Many IOP-lowering drugs appear limited due to limitations outside of IOP control. Many preclinical studies on neuroprotective optic medicines have indicated that the nutritional nerves and reducing the apoptosis of retinal ganglion cells treat glaucoma with good prospects. However, only a few methods have been successfully translated into human clinical trials without investigating their safety and stability. Additionally, most patients lack a complete understanding of disease progression, leading to poor compliance, a common treatment problem. According to individualized demand alone or combined drugs, applying alternative eye drops and new administration methods, including ocular surface implants, intraocular administration, etc., may improve patient compliance and open up new ways for treating glaucoma.

Our study still has problems, such as setting the pressure value to 0 mmHg, 40 mmHg, and 60 mmHg can only mimic the intraocular pressure range of common clinical high hypertension glaucoma, not extending to 80 mmHg or 100 mmHg. Additionally, some differences exist in the results between RGC-5 and mouse RGCs.The sensitivity to pressure and drugs may differ due to the different expressions of TRPV 4 in these two cells. Therefore, the analysis may result from errors due to the differentiation of cell lines and the inevitable tools and equipment involved in the experimental process. We should conduct physical animal experiments to verify the damage to optic ganglion cells and their mechanism. Therefore, we must repeatedly repeat the experimental results to reduce the error. The causes of glaucoma-induced damage to optic ganglion cells are diverse, and a single down mechanism cannot explain their apoptosis. The increase in abnormal IOP could be one of the RGC risk factors. TRPV4 expression in RGCs is sensitive to pressure. It has a particular regulatory effect on the apoptosis of RGCs, providing ideas and an urgent direction for preventing and treating optic nerve damage.

Author contribution statement

Yi Wang, Wei Zhang: Conceived and designed the experiments.
Guozheng Xu, Changwei Shi: Performed the experiments.
Xiang Wang: Performed the experiments; Wrote the paper.
Jianfeng Qu: Analyzed and interpreted the data; Wrote the paper.
Hongmei Wang: Contributed reagents, materials, analysis tools or data.
Chunhua Liu: Analyzed and interpreted the data.

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Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e17583.

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