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Effect of BDNF-TrKB pathway on apoptosis of retinal ganglion cells in glaucomatous animal model

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Abstract. – OBJECTIVE: The aim of this paper is to investigate the effect of BDNF-TrKB pathway on AOH by accessing its regulatory role in retinal ganglion cell apoptosis.

MATERIALS AND METHODS: Acute ocular hypertension (AOH) model in rats was established by anterior chamber perfusion to increase intraocular tension. Rats were randomly divided into AOH group, control group and k252a group, with ten rats in each group. Rats were sacrificed 72 h after animal procedures and eyeballs were harvested. HE staining was used to observe retinal structural changes at different time points. Immunohistochemical staining was used to observe the BDNF-positive cells in retinal tissues. TUNEL staining was conducted to measure apoptosis of retinal ganglion cells. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Western blot were performed to detect mRNA and protein levels of BDNF, TrKB, PI3K and ERK1 in retinal tissues, respectively.

RESULTS: HE and TUNEL staining showed significant pathological changes and abundant apoptotic cells in rat retina of AOH group and k252a group compared with those of the control group ($p<0.05$). The number of survived retinal ganglion cells in the AOH group was lower than that of the control group ($p<0.05$). K252a group had the lowest number of survived retinal ganglion cells. Immunohistochemical results showed that BDNF was rarely expressed in rat retinal tissues of the control group, which was remarkably pronounced in the AOH group and k252a group. The number of BDNF-positive cells in the k252a group was higher than that in the AOH group ($p<0.05$). RT-PCR and Western blot indicated that mRNA and protein levels of relative genes in BDNF-TrKB and PI3K/ERK1 pathways were upregulated in AOH group ($p<0.05$), but were significantly downregulated in k252a group ($p<0.05$).

CONCLUSIONS: BNDF-TrKB pathway exerts a protective effect on retina against acute ocular hypertension by reducing retinal cell apoptosis.

Key Words:
BDNF, Glaucoma, Ganglion cells, Apoptosis.

Introduction

Glaucoma is an ophthalmic emergency characterized by elevated intraocular pressure. It is a common blinding eye disease following myopia and cataract. About 65% of glaucoma patients may eventually lead to irreversible unilateral or bilateral blindness if timely treatment is lacked[1,2]. Optic nerve damage is the most serious pathological change in glaucoma, mainly manifesting as apoptosis of retinal ganglion cells (RGCs), which will eventually cause the irreversible damage of the optic nerve[3,4]. The pathogenesis of glaucoma has not been comprehensively elucidated. Lucas et al[5] found that excessive glutamate accumulation results in selective damage to retinal inner cells. Russo et al[6] found that massive accumulation of glutamate in retina induced by neuronal glutamate transporters would lead to retinal ischemia[7]. Bagnis et al[8] suggested that intraocular pressure elevation may increase the concentration of glutamic acid in the vitreous body of patients with primary open angle glaucoma, thereafter leading to the apoptosis of ganglion cells.

BDNF is mainly synthesized in the brain. Perez et al[9] found that BDNF is expressed in the rat retinal ganglion cell layer, proximal end of the fibroblast layer and the proximal end of the core layer. The expression of high-affinity tyrosine kinase receptor B (TrkB) can be observed in the inner retina, retinal nerve fiber layer and optic nerve in embryonic rats. RGCs exert a protective response when the optic nerve is damaged, accompanied by the upregulated expressions of BDNF and its receptor[10]. Iwabe et al[11]

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detected that blockage of BDNF transport promotes the survival rate of ganglion cells in high intraocular pressure model in dogs. Hirsch et al[12] observed that 2 days after complete transection of rat optic nerves, the amount of RGCs increases by 2 times, and the amount of TrkB-expressing RGCs also increases by 50%. Pease et al[13] showed that the interruption of BDNF retrograde transport and accumulation of TrkB receptor of optic nerve papilla are the major pathogenic factors of RCGs death in acute and chronic high intraocular pressure rat models. Mo et al[14] indicated that overexpressed BDNF by lentivirus injection in the ganglion cell layer increases the survival rate of RGCs and reduces the apoptotic rate. Therefore, we believed that BDNF plays an important role against glaucoma-induced apoptosis.

In this study, AOH rat model was established by anterior chamber perfusion to elevate intraocular pressure. After k252a intervention, we detected pathological lesions and cell apoptosis in retinal tissues. Subsequently, the regulatory effect of BDNF-TrKB pathway on AOH was explored, to provide the basis for the treatment of AOH.

Materials and Methods

Animals and Groups

Thirty healthy male Sprague-Dawley (SD) rats weighing 180-250 g without eye diseases were housed in SPF (specific pathogen-free) environment. Rats had free access to water and food. The relative humidity of the feeding room was 55%, and the temperature was 22°C. Rats were kept under a standard 12/12 light-dark circle; they were randomly divided into control group, AOH group and k252a group, with 10 rats in each group. This study was approved by the Animal Ethics Committee of Qingdao University Animal Center.

Preparation of Animal Model

After fasting 12 h, rats were anesthetized by intraperitoneal injection. Compound Tropicamide Eye Drops were applied on their right eyes to dilate the pupils. The surface of the right eyes was anesthetized with benoxinate. The anterior chamber of the rat was injected with normal saline. The puncture needle was fixed by the glue cloth and the infusion bottle was raised to 150 cm above. Disconnection of the fundus blood vessels, retinal ischemia, red reflector of the fundus and pale conjunctiva were observed under an ophthalmoscope. The recovery of blood supply in the retina can be observed after pulling out the puncture needle. The right eyes were coated with tetracycline ointment to prevent infection.

After the procedures of eyeball pressure, pupil dilation was performed with atropine. 10 pmol/μL k252a solution or isodose saline was slowly injected into the vitreous cavity under the microscope using a 10 μL syringe needle. The needle was pulled out 30 s after injection to prevent drug spillover.

Sample Collection

Rats were sacrificed by exsanguination from abdominal aorta (weighed 200-280 g at the time of sacrifice) under 10% chloral hydrate (0.4 g/kg, i.p.) anesthesia. About 10 ml of blood was extracted by exsanguination. No heart beating indicated the animal death. No rat exhibited signs of peritonitis following the administration of 10% chloral hydrate. The eyeball was quickly extirpated by cutting off fascia of the outer canthus in the right eye. 0.5 mm optic nerve was retained and the eyeball was removed completely. The eyeballs were rinsed with saline and then soaked in the eyeball fixative.

Paraffin Embedded Retinal Tissues and Hematoxylin and Eosin (HE) Staining

Eyeball samples were washed by flowing water, dehydrated in ascending series of ethanol, cleared in xylene and embedded in paraffin. Paraffin-embedded samples were cut for several sections. The neutral gum was dripped into the center of the specimen, and then the slides were sealed. Three randomly selected fields in each sample were observed for retinal structural changes using a microscope.

Detection of Apoptosis by TUNEL

The tissue sections were dewaxed, washed, hydrated and fixed. Detection of apoptosis was performed according to TUNEL kit instructions (Beyotime, Shanghai, China). Three randomly selected fields in each group were observed and captured. The number of the apoptotic cells and the total cells in three non-overlapping fields of each sample were counted under high magnification. AI = (number of apoptotic cells /number of total cells) ×100%.

Detection of BDNF Expression in Rat Retina by Immunohistochemistry

The slice was dewaxed, hydrated, and incubated with 3% H_2O_2 at room temperature. Antigenic heat repair was carried out, followed by BDNF

antibody incubation overnight at 4°C. Tissue samples were incubated with IgG for 2 h at 37°C. Diaminobenzidine (DAB) was used for the color reaction, and then the neutral resin was sealed and air dried. Three randomly selected fields in each sample were selected for the analysis of BDNF-positive rate by Image-pro-plus immunohistochemical image software.

Count of Retinal Ganglion Cells

Cy3 was stimulated by green fluorescence with a fluorescence microscope, and the number of positive-expressed RCGs in a certain area (0.235 mm^2) was counted in 20-times field. The count area of each retina included about 20-30 regions. The average value (A) of the positive cells in all the counting regions was calculated. The number of ganglion cells in the whole retina was calculated according to the formula: A/0.212 mm^2 ×retinal area (mm^2).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The total RNA was extracted by TRIzol method (Invitrogen, Carlsbad, CA, USA). The content of the RNA sample was determined by the acid protease apparatus, and then diluted to 0.5 μg/μL with diethyl pyrocarbonate (DEPC) water. Complementary Deoxyribose Nucleic Acid (cDNA) was synthesized according to TaKaRa RNA PCR Kit (AMV) Ver.3.0 kit (Otsu, Shiga, Japan). Primer sequences were shown in Table I. Reverse transcription products were used as templates to prepare PCR reaction system for PCR reaction. Data were recorded and analyzed after PCR reaction termination.

Western Blot

The homogenate was allocated and the supernatant was used to quantify the protein. Electrophoretic and transmembrane were carried out. 5% skim milk was used for blocking non-specific sites for 90 min at room temperature. Membranes were incubated at 4°C overnight with primary antibodies. The corresponding secondary antibody labeled by peroxidase was added for incubation at room temperature for 2 h. Images were obtained by continuous exposures through the UVP chemiluminescence imaging system. The final results were semi-quantitatively analyzed based on the target protein relative to β-actin optical density.

Statistical Analysis

Statistical analysis was carried out by Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA). The data were expressed by $\bar{x} \pm s$. The *t*-test was used in comparison between two different groups. Single factor analysis of variance was used in comparison among groups followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ indicated statistically significant.

Results

Pathological Observation of Retina

Rats in the control group did not show significant pathological changes in the retina. In the AOH group, edema of the inner retina and the inner plexiform layer were significant. The ganglion cell layers decreased, and the retinal structure was slightly disorganized. In k252a group, the retina got thinned, the ganglion cells markedly decreased, and the retinal structure was seriously disorganized (Figure 1A).

Detection of Apoptotic Retinal Cells

In the control group, apoptotic retinal cells were rare. AOH group showed much more apoptotic retinal cells than that of the control group. The number of apoptotic cells increased significantly in the k252a group, which was higher than that in the AOH group, and the difference was statistically significant ($p<0.05$, Figure 1B).

The Survival of Retinal Ganglion Cells

In the AOH group, the number of survived retinal ganglion cells was significantly lower than that in the control group, which was further reduced by k252a intervention ($p<0.05$, Figure 2A).

Expression of BDNF in Retina

BDNF-positive staining can be observed in each group. The positive expression of BDNF in

Table I. Primer sequence.

Gene name	Primer sequence
BDNF	5'-AGCTGTGCGGACCCATGG-3'
	5'-GAACCGCCAGCCAATTCTC-3'
TrKB	5'-CCAAGAGGCTAAATCCAGTCC-3'
	5'-CCAGGTTACCAACATCCCAATA-3
PI3K	5'-CATCACTTCCTCCTGCTCTAT-3'
	5'-CAGTTGTTGGCAATCTTCTTC-3'
ERK1	5'-CTAAACCACATCGGGAACCT-3'
	5'-TACTTCCGGGCTTTGATGGA-3'
GAPDH	5'-CCCCAATGTATCCGTTGTG-3'
	5'-CTCAGTGTAGCCCAGGArGC-3'

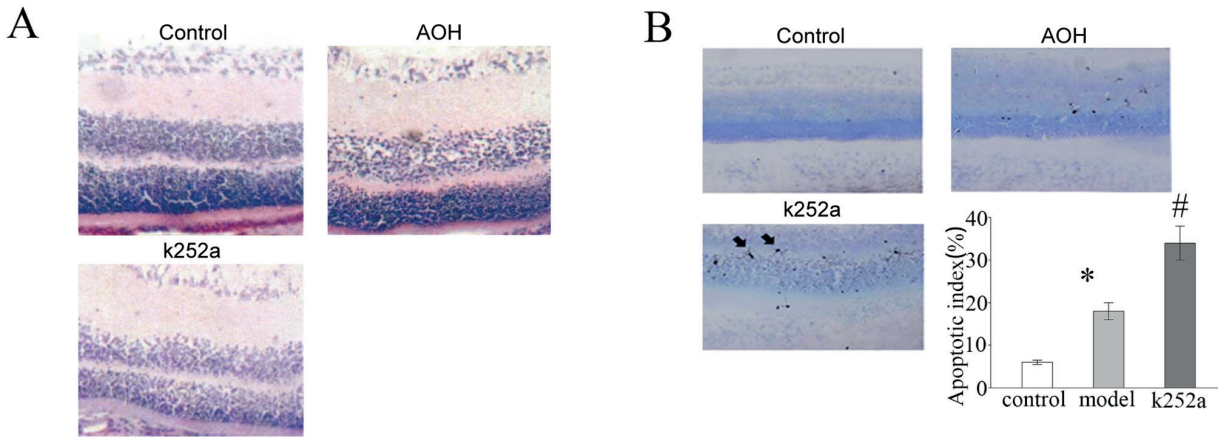

Figure 1. HE staining and TUNEL staining in rat retina (magnification 200×). ***A,*** HE staining in rat retina of control group, AOH group and k252a group. ***B,*** TUNEL staining in rat retina of control group, AOH group and k252a group. Comparison of apoptotic rate in three groups. *: Compared with the control group, the difference was statistically significant (p<0.05); #: Compared with the AOH group, the difference was statistically significant (p<0.05).

the retina of the control group was rare. The number of BDNF-positive cells in AOH group was remarkably higher than that of the control group (p<0.05), which was the highest in the k252a group (p<0.05, Figure 2B).

Transcription Levels of BDNF-TrKB and PI3K/ERK1 in Retina

Compared with the control group, the transcriptional levels of BDNF-TrKB and PI3K/ERK1 in the AOH group remarkably increased (p<0.05). However, transcriptional levels in the k252a group were significantly lower than those in the AOH group (p<0.05, Figure 3A-3D).

Protein Expressions of BDNF-TrKB and PI3K/ERK1 in Retina

Compared with the control group, the expression levels of BDNF-TrKB and PI3K/ERK1 markedly increased (p<0.05). In the k252a group, expression levels of all these proteins decreased compared with those of the AOH group (p<0.05,

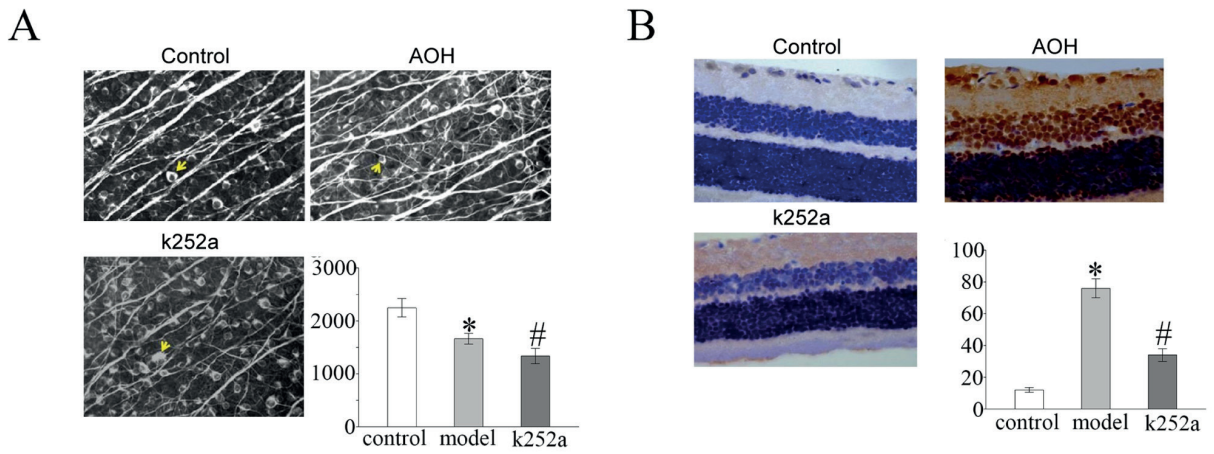

Figure 2. Immunohistochemical staining of RGCs and BDNF (magnification 200×). ***A,*** RGCs in the control group, AOH group and k252a group. Comparison of survived RGCs in three groups. ***B,*** Immunohistochemical staining of BDNF in the control group, AOH group and k252a group. Comparison of number of positive cells in three groups. *: Compared with the control group, the difference was statistically significant (p<0.05); #: Compared with the AOH group, the difference was statistically significant (p<0.05).

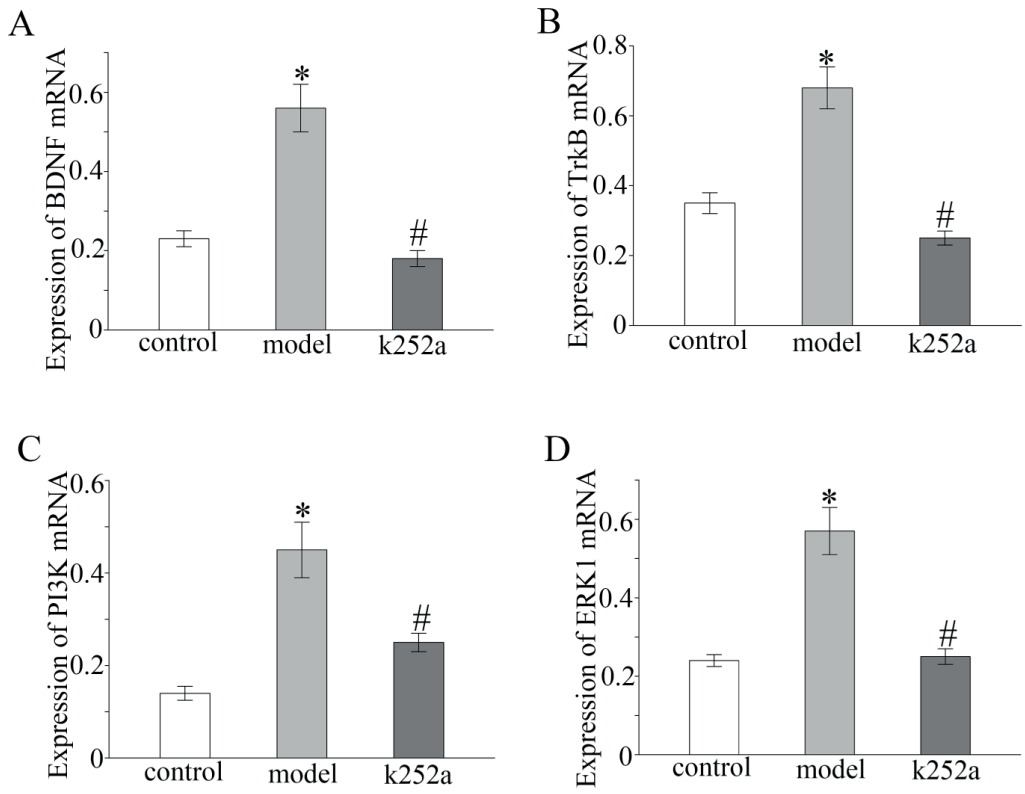

Figure 3. Transcriptional levels of BDNF-TrKB and PI3K/ERK1. ***A,*** The mRNA level of BDNF in three groups. ***B,*** The mRNA level of TrKB in three groups. ***C,*** The mRNA level of PI3K in three groups. ***D,*** The mRNA level of ERK1 in three groups. *: Compared with the control group, the difference was statistically significant ($p<0.05$); #: Compared with the AOH group, the difference was statistically significant ($p<0.05$).

Figure4A-D). Protein changes of BDNF-TrKB and PI3K/ERK1 in the retina were identical to transcription level changes.

Discussion

Glaucoma is an ophthalmic emergency characterized by elevated intraocular pressure. Visual dysfunction has resulted from impaired optic nerves and pathways. Lack of timely treatment would lead to irreversible vision loss[15,16]. The etiology of glaucoma is not yet very clear. It is currently believed that the occurrence of glaucoma is closely related to anatomy, genetic susceptibility and mental factors[17,18]. Retinal optic nerve damage, manifesting as characteristic optic atrophy, is the major pathological performance of glaucoma[19]. Sands and Barish[20] have shown that glaucomatous optic neuropathy is associated with denaturation of trans synaptic neurons in corpora geniculatum externum and primary visual cortex. Histopathological studies showed that the early-stage pathological lesions of glaucoma first occur in the lamina layer. The main manifestations include the loss of the axons, blood vessels and collagen cells, accompanied by the accumulation of the temporal and lower pole nerve fibers in the optic disc[21]. With the further development of the disease course, the optic disk is eventually depressed and could be reversed.

Intraocular pressure (IOP) is the pressure of eyeball contents on the wall of the eyeball. The change of eyeball content inevitably causes IOP. Dynamically balanced aqueous humor circulation is the most important factor that maintains IOP stability[22]. Histopathologically increased IOP leads to the interruption of retinal or optic nerve flow and insufficiency of blood supply. The main pathological changes are the apoptosis of retinal ganglion cells, eventually leading to the irreversible damage to the optic nerves[23]. Therefore, protection of ganglion cells, inhibition of further retinal damage and IOP-induced apoptosis have

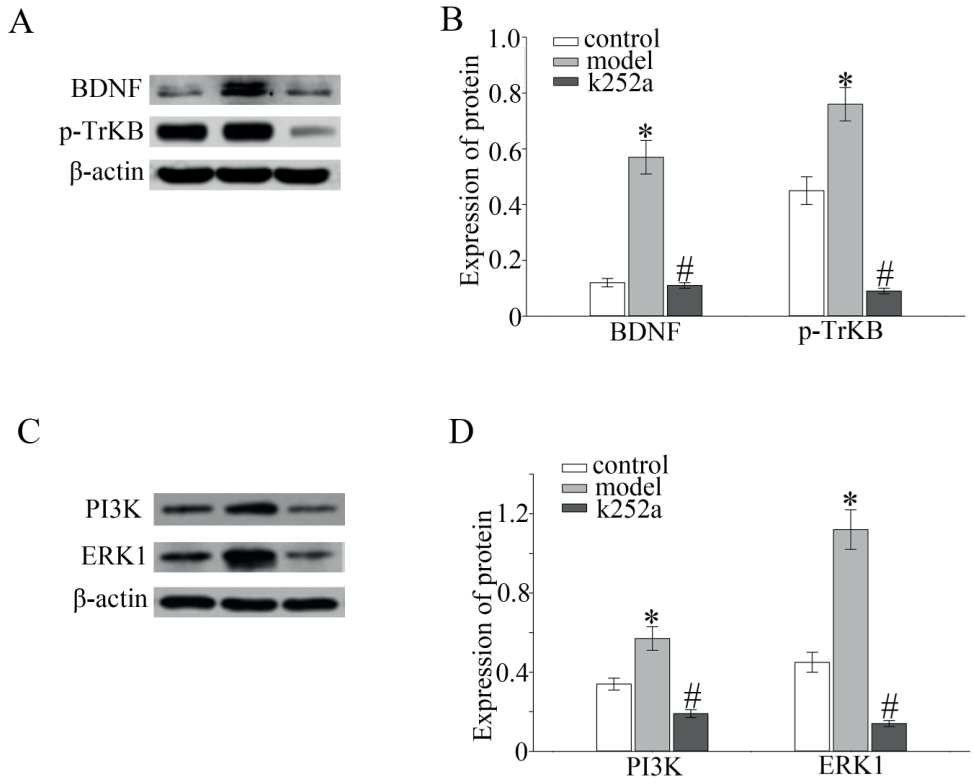

Figure 4. Protein expressions of BDNF-TrKB and PI3K/ERK1. ***A,*** Protein expression of BDNF in three groups. B, Protein expression of TrKB in three groups. ***C,*** Protein expression of PI3K in three groups. ***D,*** Protein expression of ERK1 in three groups. *: Compared with the control group, the difference was statistically significant ($p<0.05$); #: Compared with the AOH group, the difference was statistically significant ($p<0.05$).

become the latest research focuses. It is generally believed that effective inhibition of retinal ganglion cell apoptosis is the key to vision retention. The inhibition of the apoptotic ganglion cells is of great value in the treatment of glaucoma[24].

BDNF is a member of the neurotrophin family. It has been widely studied because of its significant inhibitory effect on neuronal apoptosis. BDNF plays an important role in maintaining neuronal growth, differentiation, repair and regeneration in nerve injury. Expressions of BDNF and its receptors may be the basis of its local effects. BDNF is capable of inhibiting secondary neuronal apoptosis after spinal cord injury[25]. It protects central and peripheral nerve damages, and promotes the growth of neuron axons[22,23,26]. Wordinger et al[24] firstly found that BDNF and its receptor are expressed in trabecular cells and trabecular tissues. Also, BDNF participates in autocrine and paracrine, and can be expressed in the photoreceptor layer and inner layer of the retina[27]. BDNF and its main receptor TrKB are widely expressed in the eye. BDNF is found to inhibit RGCs apoptosis after high intraocular pressure injury[28-30]. It is known that BDNF regulates TrKB mainly through the MAPK/PI3K/ERK pathway. The upstream activator Ras activates the MAPK kinase, thus further stimulating the MAPK/ERK pathway. Subsequently, activated ERK can phosphorylate the cAMP response-element protein Ser133 and the Ser308 site of protein kinase B. Finally, a series of gene expressions and anti-apoptotic pathways are activated[31,32].

In this study, the pathological lesions and apoptosis in retinal tissues were pronounced in the AOH rat model, which were worse after blocking the BDNF-TrKB pathway by k252a treatment. Immunohistochemical staining showed that BDNF reactivity increased in the AOH group, which was remarkably downregulated in the k252a group. Besides, the expression levels of BDNF, TrKB, PI3K and ERK1 in AOH group were upregulated. However, these expressions were markedly downregulated in the k252a group. It can be seen that RGCs immediately exert protective response after the optic nerve injury by upregulating expressions of BDNF and its receptors. Pease et al[13]

found the interruption of BDNF retrograde transport and the accumulation of TrkB receptor of optic nerve papilla in rats with acute and chronic high intraocular pressure and glaucomatous monkeys, which are the major pathogenesis of RCGs death. However, the specific dose, administration approaches and clinical evaluation of BDNF treatment are still needed to be further explored[33-35].

Conclusions

We showed that BNDF-TrKB pathway exerts a protective effect on the retina against acute ocular hypertension by reducing retinal cell apoptosis.

Conflict of interest

The authors declare no conflicts of interest.

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