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ORIGINAL ARTICLE

Fasudil attenuates glial cell-mediated neuroinflammation via ERK1/2 and AKT signaling pathways after optic nerve crush

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Abstract

To investigate the functional role of fasudil in optic nerve crush (ONC), and further explore its possible molecular mechanism. After ONC injury, the rats were injected intraperitoneally either with fasudil or normal saline once a day until euthanized. RGCs survival was assessed by retrograde labeling with FluoroGold. Retinal glial cells activation and population changes (GFAP, iba-1) were measured by immunofluorescence. The expressions of cleaved caspase 3 and 9, p-ERK1/2 and p-AKT were detected by western blot. The levels of the pro-inflammatory cytokines were determined using real-time polymerase chain reaction. Fasudil treatment inhibited RGCs apoptosis and reduced RGCs loss demonstrated by the decreased apoptosis-associated proteins expression and the increased fluorogold labeling of RGCs after ONC, respectively. In addition, the ONC + fasudil group compared had a significantly lower expression of GFAP and iba1 compared with the ONC group. The levels of pro-inflammatory cytokines were significantly reduced in the ONC + fasudil group than in the ONC group. Furthermore, the phosphorylation levels of ERK1/2 and AKT (p-ERK1/2 and p-AKT) were obviously elevated by the fasudil treatment. Our study demonstrated that fasudil attenuated glial cell-mediated neuroinflammation by up-regulating the ERK1/2 and AKT signaling pathways in rats ONC models. We conclude that fasudil may be a novel treatment for traumatic optic neuropathy.

Keywords Fasudil · Neuroinflammation · Retinal ganglion cells · p-ERK1/2 · p-AKT · Optic nerve crush

Introduction

Traumatic optic neuropathy (TON), characterized by significant visual impairment and even blindness, is an irreversible injury of the optic nerve caused bytrauma [1–7]. The main mechanism of TON is retinal ganglion cells (RGCs) apoptosis [7] and axon degeneration, which may be mediated by glial cells dysfunction and optic nerve inflammation. Optic nerve crush (ONC) have been reported and share similar features, indicating that ONC is an excellent working animal model of TON. However, the underlying pathophysiologic and molecular mechanisms remain far from clear.

Inflammation is the basic response to optic nerve injury [8]. Growing evidence shows that ONC triggers inflammatory response produced by activated retinal glial cells, further aggravates the loss of RGCs [9–12]. Indeed, neuroinflammation involves the activation of glial cells and the release of proinflammatory cytokines [13]. Retinal glial cells, including microglia, astrocytes, Müller cells and so

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on, play crucial roles in maintaining retinal homeostasis and protecting retinal neurons [14]. Under pathological conditions, glial cells become activated and produce pro-inflammatory cytokines [15–17] such as IL-1β, IL-6, COX-2, and TNF-α [18]. These pro-inflammatory cytokines can convey neurotoxicity to RGCs [16–19]. In view of this, agents aimed at modulating the neuroinflammatory responses have attracted the attention of researchers. Although many agents have been tested in animal models where they have been effective in attenuating glial cell-mediated neuroinflammation [8, 20, 21]. To date, however, there is no effective clinical treatment for TON. Thus, searching for novel agents that can attenuate glial cell-mediated neuroinflammation may be particularly beneficial [7, 22].

Recently, some studies provided evidence that the ERK1/2 and Akt pathways mediated neuroprotection after injury [23–26]. Pernet et al. have shown that the activation of ERK1/2 by ciliary neurotrophic factor attenuated glial cells-mediated neuroinflammation and increased RGCs survival after optic nerve injured [27]. Besides, the ERK1/2 pathways was known to participate in LPA1-driven microglial activation [28]. Furthermore, compound C (CC) inhibited neuroinflammation and protected nerve through the activation of the ERK1/2 and AKT pathways [29, 30]. Moreover, G-CSF may play a neuroprotective role by activating AKT phosphorylation after ONC [31, 32]. In addition, Brimonidine fueled optic nerve regeneration after injury by activating ERK1/2 [23]. Therefore, the activation of ERK1/2 and AKT signaling pathways may inhibit the activation of glial cells and attenuate glial cells-mediated neuroinflammation.

Fasudil, a selective Rho-Associated Protein Kinase (ROCK) inhibitor [4, 5, 7, 33–35], is primarily used to treat subarachnoid hemorrhage (SAH) [34, 36], pulmonary hypertension [37, 38], cardiovascular diseases [39] and cerebral vasospasms after subarachnoid hemorrhage [40]. Most studies to date showed beneficial treatment effects of fasudil in treating central nervous system disease in animal models, such as stroke [41], cerebral infarct [42], cerebral vasospasm [33, 42, 43], Parkinson [11, 44], and traumatic spinal cord injury [33, 36]. Mounting amount of evidence have shown that the neuroprotective and cardioprotective effects of fasudil was via the AKT and ERK1/2 pathways [45–50]. Furthermore, fasudil attenuates the inflammatory response by shifting of microglial M1 polarization toward the M2 phenotype [51, 52]. Also, fasudil has been found to attenuate glial cells-mediated neuroinflammation in a New Zealand rabbit optic nerve injury model [7]. However, the mechanism by which fasudil attenuates glial cells-mediated neuroinflammation in the rats ONC model remain unclear.

The purpose of this research, therefore, is to verify the hypothesis that fasudil attenuates glial cells-mediated neuroinflammation via activating the ERK1/2 and AKT signaling pathways in the rats ONC model.

Materials and methods

Animals

Adult male Sprague Dawley rats (10 weeks, weight 250 ± 30 g) were cared for by the Medical Laboratory Animal Center of Guangxi Medical University (Nanning, China). All experiments were approved by the Ethics Committee of the People's Hospital of Guangxi Zhuang Autonomous Region and performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

ONC model

The ONC model was constructed on 10-week-old rats as described previously with slight modification [50, 53–56]. 1% Pentobarbital Sodium (50 mg/kg) was injected intraperitoneally to anesthesia rats. After anesthesia, the eye was routinely disinfected. The left optic nerve was surgically exposed and squeezed 2 mm behind the eyeball for 10 s. After the operation, funduscopic examination revealed that the retinal blood supply remained intact, and then ofloxacin ointment was applied topically. The right optic nerve was exposed but without crushed. The study of Berkelaar et al. suggests that axotomy causes a loss of RGCs in a delayed pattern, and the number of RGCs was significantly reduced at days 14 [57]. Besides, there are studies also have shown that ONC caused a significant decrease of RGCs and a increase of retinal glial cells, which peaks on day 14 [58–61]. Therefore, the 14-day time point was chosen for the subsequent experiments. All rats were euthanized by CO2 pneumoperitoneum 14 days after ONC.

Fasudil administration

The rats were randomly assigned to four groups: control group (sham surgery control), fasudil group, ONC group, and ONC + fasudil group). Animals were given an intraperitoneal injection of normal saline or fasudil (10 mg/kg/day, 30 rats) (Sigma Chemical Co., St. Louis, MO) once daily for 14 consecutive days following model building. The choice of concentrations of fasudil for this study were based on previous research [33, 62, 63]. None of the rats were lost or died during the specified survival time.

Retrograde labeling of RGCs with FluoroGold

The retrograde labeling of FluoroGold (FG) has been used for morphometry analysis of RGCs after injury as described

in previous studies [64, 65]. To distinguish RGCs from glial cells, RGCs were retrogradely labeled with FG (4%, Colorado). Briefly, 7 days before the establishment of rats ONC model [59, 66–68], midline incisions of approximately 1.2 cm in length were made on the rat head, exposing the sagittal and lambda suture. Holes (1 mm diameter) were then drilled into the skull 2.5 mm rostral to lambda and 1.2 mm lateral to the sagittal sutures based on rat brain stereotaxic coordinates. 4 µl FG was injected 3 mm deep into the superior colliculi using a 10 µl microsyringe. Following euthanasia of the rats, eye balls were enucleated immediately and fixed with 4% paraformaldehyde (PFA). Retinas dissected and flat mounted. Labeled RGCs were counted in three areas (62,500 μm^2 each) per retinal quadrant ($n > 6$ in each group) and the average was calculated under fluorescence microscope (Leica Microsystems; Mannheim, Germany). RGC survival percentage was defined as RGCs density ratios between left eyes with an right eyes multiplied by 100 [69, 70].

Western blot analysis

Western blot were performed as described previously [50, 55, 71]. Retinal tissues of 5 rats each group were collected and stored at − 80 °C. Retinal proteins were extracted using RIPA buffer (Beyotime) and quantified using the BCA assay (Thermo). After SDS-PAGE, PVDF membranes were blocked with 5% non fat milk, followed by overnight incubation with primary antibodies for mouse monoclonal anti-caspase-3, mouse monoclonal anti-caspase-9, rabbit polyclonal anti-cleaved-caspase-3, rabbit polyclonal anti-cleaved-caspase-9, mouse monoclonal anti-p44/42 (ERK1/2), rabbit monoclonal anti-Phospho-p44/42 MAPK (p-ERK1/2), mouse monoclonal anti-AKT, mouse monoclonal anti-Phospho-AKT, or rabbit polyclonal anti-β-actin at 4℃. All labeled antibodies were purchased from Cell Signaling Technology, except antibody against β-actin (Abcam). After three 5-min PBST washes, the membranes were incubated with secondary antibodies (SouthernBiotech) for additional 1 h at room temperature. Finally, the bands were visualized by ECL Plus(Amersham), and quantified by ImageJ software (NIH, Bethesda, MD).

Immunofluorescence and cell counting of microglial and macroglia

Eyes from euthanized rats were removed, fixed overnight in 4% PFA, cryoprotected with 30% sucrose afterwards. Tissues were embedded in optimum cutting temperature compound (OCT, Leica Biosystems, Shanghai, China) overnight and 7 µm thick frozen sections were prepared for immunofluorescence staining. After being washed with PBS, nonspecific sites were blocked by incubation with 5% bovine serum albumin (BSA) for 2 h Subsequently, the sections were incubated overnight with either rabbit polyclonal anti-iba1 (a microglial cells marker; #019-19741, Wako) or rabbit monoclonal anti-GFAP (an astrocyte marker; #MAB3402, Millipore) primary antibody in a humidified box, followed by Alexa Fluor 488 or 555 (Invitrogen) secondary antibodies 2 h at room temperature and finally DAPI (Sigma-Aldrich). Finally, imaging of the stained sections were photographed using confocal microscopy (Germany). The number of microglia or macroglia was calculated and then average was worked out. Finally, the numbers of iba1-positive cells and GFAP-positive cells were quantified using the cellcounter tool in ImageJ.

Quantitative real-time PCR (qPCR)

Sample collection, RNA isolations and qPCR were carried out as previously described [53, 72]. Total RNA of retinal cells was extracted by Trizol (Invitrogen) and reversed-transcribed into cDNA by using a cDNA first-strand synthesis kit (Fermentas). The Primers were designed to amplify within a 200-bp length according to the NCBI Primer-Blast (Table 1). PCR steps was as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fluorescence signals were detected during the extended phase. Each sample was detected three times with the 6-point standard curve. qPCR was performed with SYBR Green (Biotool, Houston). The results were expressed as percentages. The expression of target genes calculated as normalized ratio and normalized to β-actin using the $2^{-\Delta\Delta Cq}$ method.

Table 1. The primer sequences of TNF-α, IL-6, IL-1β, COX-2 and ß-actin

Gene	Forward primer sequences	Reverse primer sequences	Length (bp)
TNF-α	5-CCACGCTCTTCTGTCTACTG-3	5-GCTACGGGCTTGTCACTC-3	145
IL-6	5-AGCCACTGCCTTCCCTAC-3	5-TTGCCATTGCACAACTCTT-3	156
IL-1β	5-TGTGATGTTCCCATTAGAC-3	5-AATACCACTTGTTGGCTTA-3	131
COX-2	5-AGAGTCAGTTAGTGGGTAGT-3	5-CTTGTAGTAGGCTTAAACATAG-3	170
β-actin	5-GTCAGGTCATCACTATCGGCAAT-3	5-AGAGGTCTTTACGGATGTCAACGT-3	147

Statistical analysis

All of the values were presented as mean ± standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA) followed by Turkey's posthoc multiple comparison tests. All statistical analysis were done using GraphPad Prism 6.0 (GraphPad Software). A p-value < 0.05 was considered statistically significant. Each experiment involved at least three repetitions.

Results

Fasudil reduced the loss of RGCs after ONC

To investigate the protective effects of fasudil on optic nerve injury induced by ONC, FG retrograde labeling was performed in all groups, and the surviving RGCs were visible as a round shape (Fig. 1a–d). FG-positive RGCs were remained viable 14 days post-ONC, but in significantly decreased number(1043 ± 52 cells/mm^2, Fig. 1b, e, $^{\#}p < 0.001$). Notably, fasudil dramatically inhibited the loss of RGCs and improved survival after ONC (1890 ± 83 cells/mm^2, $^{**}p < 0.001$). After 14 days of treatment, no notable differences in the number of RGCs were found between the control (2642 ± 182 cells/mm^2) and control + fasudil (2530 ± 231 cells/mm^2) groups, indicating that fasudil had no cytotoxicity to RGCs. These results suggested that fasudil played a neuroprotective role in the ONC animal model.

Fasudil attenuated apoptosis of RGCs after ONC

ONC is an acute axonal damage model that leads to cell apoptotic in RGCs. Caspase-3 and caspase-9 are the principal effector caspases in the execution of apoptotic cell death [25, 73, 74]. To further explore the impact of fasudil on RGCs apoptosis, we used western blot to assess the expression of apoptosis-related proteins as mentioned above (Fig. 2a). The relative amount of cleavage of caspases 3 or 9, normalized to respective total protein as a loading control, was calculated as a ratio. The results showed that there was no significant difference in the protein expression level of total caspases 9 and 3 between all groups. Of note, the ONC group showed statistically increased levels of cleavage of caspases 3 and 9 ($^{\#}p < 0.001$). However, after treatment with fasudil, these expression levels were significantly reduced ($^{**}p < 0.001$). In general, these results indicate that fasudil may upregulate the phosphorylation levels of caspase 3 and 9, which is further favorable to the anti-apoptotic effect on RGCs (Fig. 2b, c).

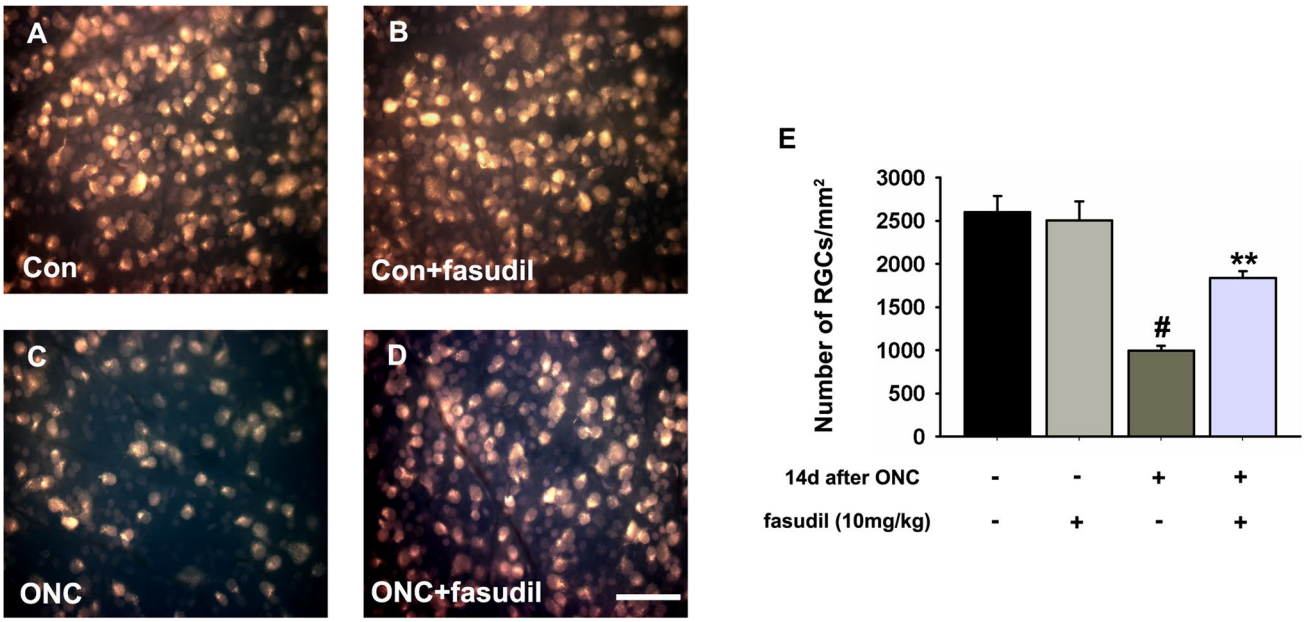

Fig. 1 The protective effects of fasudil in RGCs after ONC. The photos of retina in each group were captured using a fluorescence microscope. Scale bar = 100 μm. **a** Con group; **b** fasudil group; **c** ONC group; **d** ONC + fasudil group. **e** The number of FG-positive RGCs in each group was counted (mean ± SEM, n = 6). ($^{\#}p < 0.001$ vs. the control group; $^{**}p < 0.001$ vs. the ONC group). *Con* control, *ONC* optic nerve crush, *RGCs* retinal ganglion cells

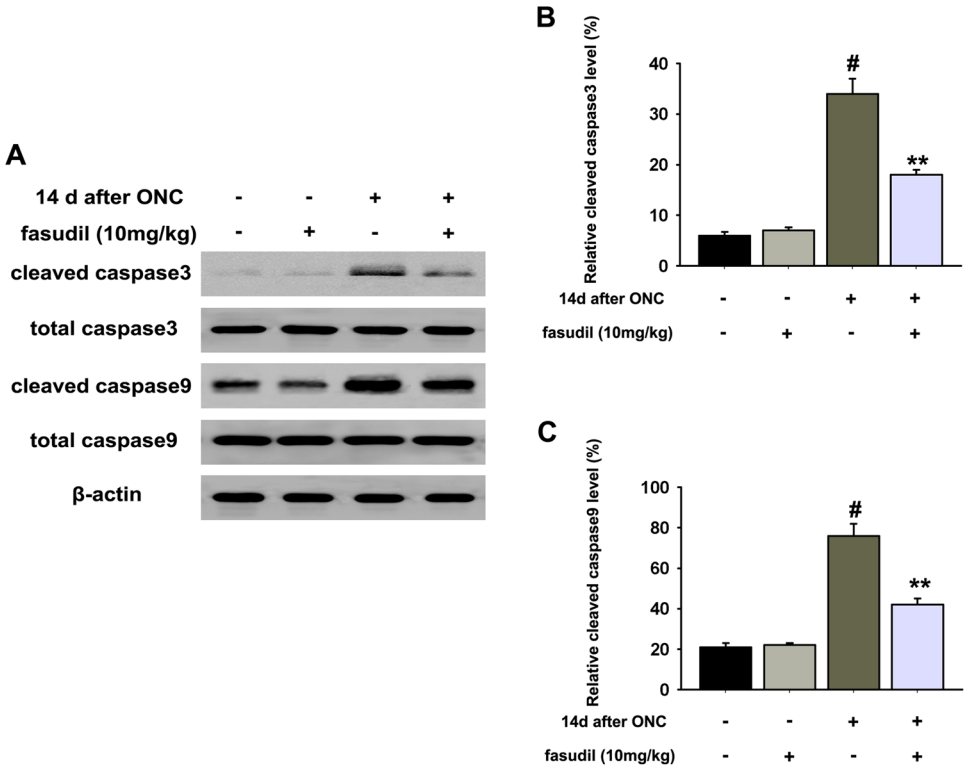

Fig. 2 Fasudil inhibited ONC-induced caspase-3/9 activation in the retina after ONC. **a** Western blot analysis assessed the protein level of total caspases 9/3 and cleavage of caspases 9/3. β-actin was used as a loading control. Gel bands were subjected to densitometric analysis, and the relative cleaved-caspase-3/9 level was calculated as cleaved-caspase-3/9 level divided by totalcaspase3/9 level (mean ± SEM, n = 10). ($^{\#}p < 0.001$ vs. the control group, $^{**}p < 0.001$ vs. the ONC group). *ONC* optic nerve crush

Fasudil restrained the activation of retinal glial cells after ONC

The retinal glial cells undergo significantly morphological and behavioral changes and switch from a quiescent state to a highly active state after ONC injury [22, 75]. Then, to determine whether the effect of fasudil on retinal glial cells was correlated with its neuroprotective function, the expressions of the GFAP and iba-1 were detected and the number of microglia or macroglia cells was counted by immunofluorescence (Fig. 3). As showed in Fig. 3a, the expression of GFAP and iba-1 was dramatically elevated in the ONC group compared with the control group ($^{\#}p < 0.001$), and was markedly reduced after fasudil treatment ($^{**}p < 0.001$). In addition, ONC resulted in the increases in the number of microglia and macroglia cells ($^{\#}p < 0.001$). However, these expansion in cell numbers were inhibited by fasudil treatment ($^{**}p < 0.001$). Overall, our results suggested that fasudil inhibited the activation of retinal glial cells after ONC.

Fasudil inhibited optic nerve inflammation after ONC

Then, to further investigate the anti-inflammatory effects of fasudil, we determined the mRNA level of IL-6, IL-1β, COX-2, and TNF-α in the retina by qPCR. As expected, the mRNA expression levels of these proinflammatory cytokines were significantly increased after ONC ($^{\#}p < 0.001$), while were decreased after treatment with fasudil ($^{**}p < 0.001$) (Fig. 4a–d). Our results indicated that fasudil inhibited the mRNA expression of proinflammatory cytokines after ONC. Namely, fasudil played a key anti-inflammatory role during optic nerve provoked by ONC.

Fasudil up-regulated ERK1/2 and AKT pathway after ONC

Previous studies demonstrated that fasudil administration leads to ERK1/2 and AKT phosphorylation [27, 76]. To explore whether fasudil protect RGCs from apoptosis via ERK1/2 and AKT pathways, the total and phosphorylated levels of ERK1/2 and AKT were detected by western blot (Fig. 5a). The relative amount of phosphoprotein levels, normalized to total level of the corresponding protein as a loading control respectively was calculated as a ratio. As expected, there was no significant difference between the total levels of ERK1/2 or AKT for each group. Moreover, the phosphorylation levels of ERK1/2 and AKT increased slightly after ONC, but the change was not statistically ($^{\#}p > 0.05$). And we considered this change was triggered by ONC stress. Following treatment with fasudil, the levels of the above phosphorylated protein significantly increased (Fig. 5, $^{**}p < 0.001$). The above results further implicate that fasudil can activate the ERK1/2 and AKT pathway, and the activation of these signalings can inhibited the activation of retinal glial cells and the apoptosis of RGCs after ONC.

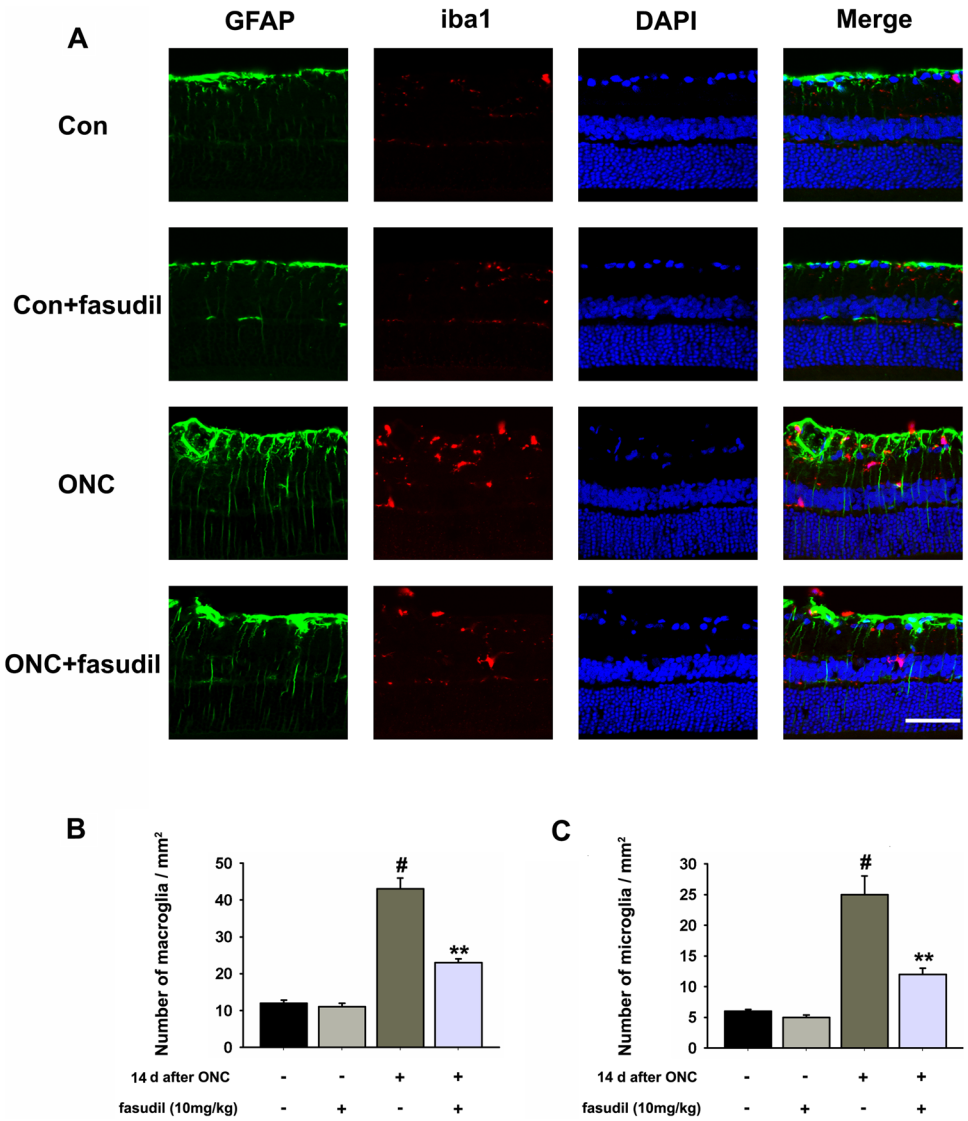

Fig. 3 Fasudil exerted its rewarding effect through inhibition of ONC-induced glial cells activation. **a** Retinal sections were stained with the GFAP (green), Iba1 (red) and DAPI (blue), and viewed on a confocal microscope. Scale bar = 100 μm. The stained cells were dropped onto slides and imaged by confocal microscopy. Densitometric analysis was used to quantify the number of macroglia (**b**) and macroglia (**c**). Values are expressed as mean ± s.e.m. *$p < 0.05$; Data were means ± SEM of multiple experiments (n = 8; #$p < 0.001$ vs. the control group; **$p < 0.001$ vs. the ONC group). *GFAP* glial fibrillary acidic protein, *Iba1* ionized calcium binding adapter molecule 1, *ONC* optic nerve crush. (Color figure online)

Discussion

TON is a serious vision threatening condition. However, there is no proven-effective therapy and interventions that can reverse the optic nerve degeneration and recover the vision. In this present study, our results demonstrated that fasudil directly attenuated RGCs apoptosis and increased the survival rate of RGCs via inhibiting caspase3 and caspase9 cleavage in retina after ONC. In addition, fasudil administration specifically attenuates glial cells-mediated neuroinflammation, played a neuroprotective and anti-inflammatory role by up-regulating the phosphorylation of ERK1/2 and AKT in rats ONC model. In general, these findings provided convincing evidence that fasudil exerts neuroprotective effects through attenuating glial cells-mediated neuroinflammation.

Fasudil, a widely used compound for many preclinical studies, may offer a novel therapeutic option for TON due to its potential neuroprotective and anti-inflammatory properties [4]. Yamamoto et al. revealed that fasudil had a concentration-dependent neuroprotective effect against ROCK activation in retina after ONC by inhibiting RGC apoptosis [67]. Moreover, fasudil could ameliorates damage of the optic nerve through Rho/ROCK signaling pathway, suggesting that fasudil is efficacious for the treatment of ONC injury [7, 35, 36]. Our results displayed that the fasudil treated group had significantly more viable RGCs after ONC as compared to the untreated group, suggesting that fasudil could resist the apoptosis of RGCs after ONC.

The effector caspases become activated through cleavage by initiator caspases, and lead to RGCs apoptosis after ONC [5, 25]. Fasudil suppressed inflammation-induced caspase activation that ultimately inhibited apoptosis [77]. Increasing evidence suggested that the activations of caspase-3 and caspase-9 pathways were up-regulated after RGCs injury [5,

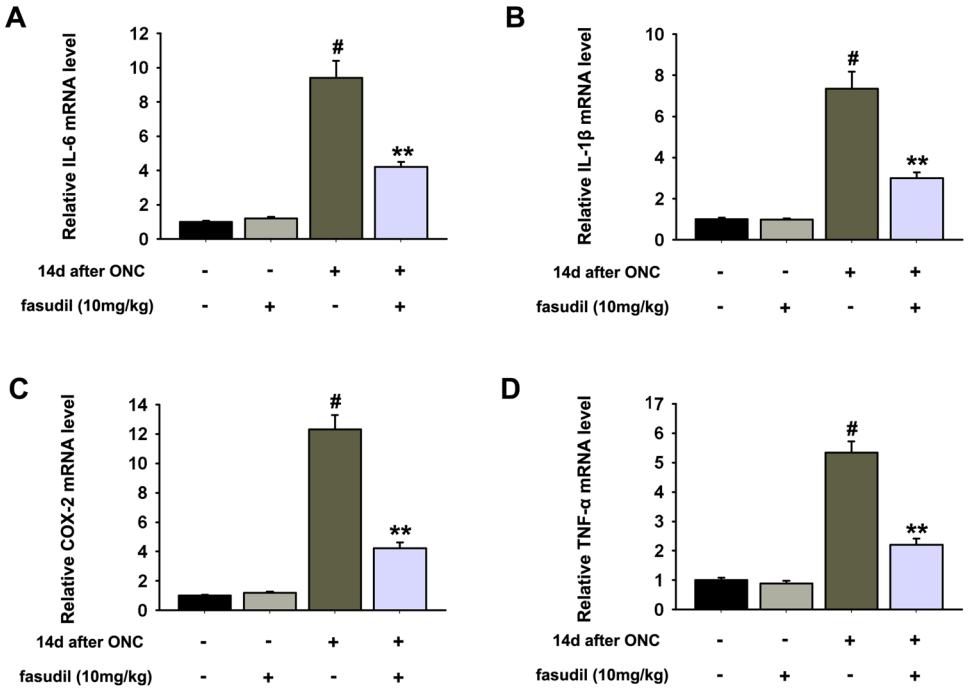

Fig. 4 Fasudil played an anti-inflammatory role in ONC, as reflected by transcriptional inhibition of proinflammatory cytokines. The mRNA levels of proinflammatory cytokines genes were assayed via qPCR (mean ± SEM, n = 6). **a** IL-6, **b** IL-1β, **c** COX-2, and **d** TNF-α ($^{\#}p < 0.001$ vs. the control group; $^{**}p < 0.001$ vs. the ONC group). IL-6, interleukin 6; IL-1β, interleukin 1β; COX-2, cyclooxygenase-2; TNF-α, tumor necrosis factor-α; ONC, optic nerve crush

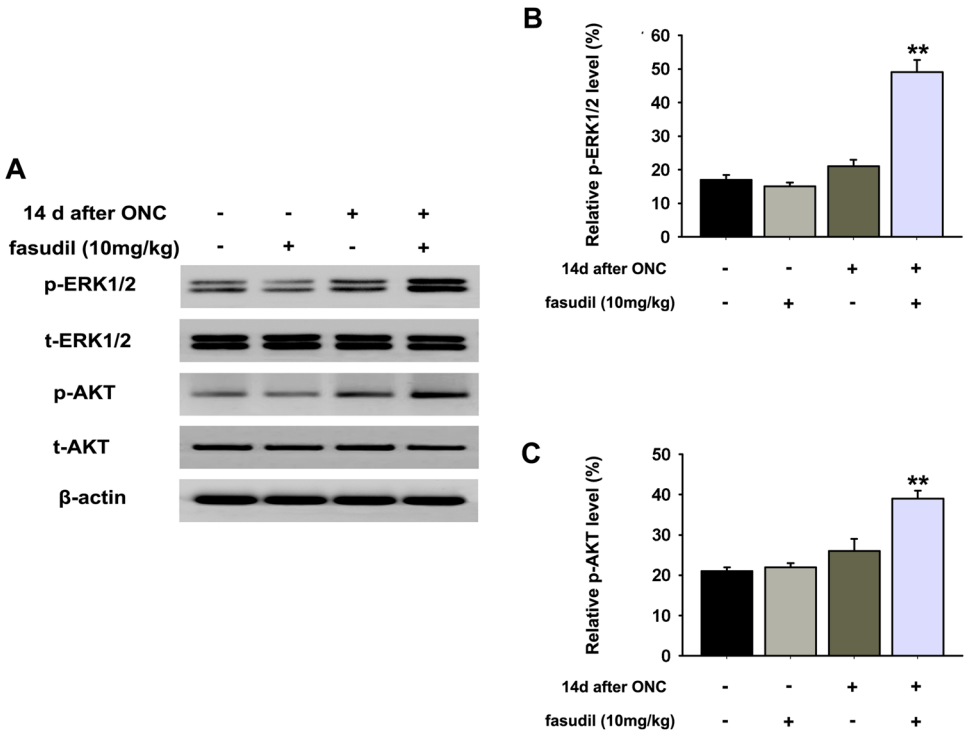

Fig. 5 Fasudil induce activation of ERK1/2 and AKT pathways after ONC. **a** Western blot analysis assessed the phosphorylated and total levels of ERK1/2 and AKT. β-actin served as an internal loading control. Densitometric analysis of bands was performed, and relative p-ERK1/2 (**b**) or p-AKT (**c**) level was calculated as phosphorylated proteins level divided by the corresponding values of total proteins level, respectively (mean ± SEM, n = 10). ($^{**}p < 0.001$ vs. the ONC group). *ONC* optic nerve crush, *ERK1/2* extracellular regulated protein kinases ½, *AKT* protein kinase B, *p* phosphorylated, *t* total

78–81], while the inhibition of cleaved caspase of 3 and 9 significantly enhances RGCs survival rate [5, 82, 83]. Furthermore, fasudil was able to inhibit caspase-3 activation and protect optic nerve [7]. Our findings are in line with prior studies that fasudil treatment significantly inhibited RGCs apoptosis related proteins expression after ONC.

Retinal glial cells were verified as the critical cells in the neuroinflammatory processes of TON, which were cause optic nerve inflammation and contributed to RGCs apoptosis when they were activated [53, 84, 85]. Therefore, inhibiting the activation of retinal glial cells that further reduce optic nerve inflammation may provide a suitable and effective

treatment for ONC. Activated retinal glial cells may upregulate the expression of GFAP and iba-1 and promotes the release of proinflammatory cytokines, thus resulting in neuroinflammation. Prior research have documented that fasudil can inhibit the conversion of retinal glial cells from M2 to M1 [51, 52, 86, 87]). In the hippocampus of the cigarette smoke-exposed mice model, chronic fasudil administration suppressed the overproduction of cytokines (IL-1β, IL-6 and TNF-α) and lessened the inflammatory impairments [88]. Our study detected that fasudil significantly inhibited the activation of retinal glial cells, which were manifested by decreased the expression of GFAP and iba-1. Moreover, fasudil inhibited the mRNA expression of proinflammatory factors after ONC. Thus, we speculate that the anti-apoptosis mechanism of fasudil might be associated with the inhibition of glial cell-mediated neuroinflammation.

Recently, ERK1/2 and AKT pathways have been demonstrated to protect against cell apoptosis, and some agents exert neuroprotective effects by activating ERK1/2 signaling pathways in the retina [89, 90]. Moreover, overexpression of MEK1 mutants resulted in a conspicuous increase in p-ERK1/2 levels further attenuated neuroinflammation and improve RGCs survival after optic nerve injury [91]. The activation of AKT and ERK1/2 can lead to phosphorylation of pro-apoptotic BAD (thus inactivation) and activation of anti-apoptotic Bcl-2 protein, which may work together to improve RGCs survival [44, 92]. Notably, fasudil attenuate neuroinflammation and prevent the nerve from damage via ERK1/2 and AKT pathways. All the results above were consistent with our founding.

In summary, we reveal that the number of RGCs were significantly decreased, retinal glial cells were activated, which was accompanied by slight elevation (no statistical significance) in the phosphorylation of AKT and ERK1/2 in the retina after ONC. However, fasudil treatment significantly attenuated glial cell-mediated neuroinflammation and further enhanced RGCs survival by a concomitant increase in p-ERK1/2 expression along with p-AKT expression in the rats ONC model. Hence, these findings may promote the useful application of fasudil in the treatment of TON.

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Author contributions QL, LJ and FX conceived and designed the experiments. ZM, BH, NL and MZ performed the establishment of model and the intraperitoneal injection. The retrograde labeling of RGCs with FluoroGold was performed by WH, HH and JL. WH, LJ and WY performed the western blot analysis and immunofluorescence staining experiments. FT, CS and JL performed the qPCR. WH, QL, LJ, FT analyzed and interpreted the data. WH, QL, LJ, and LC edited the paper. HZ, SZ, ML, LC and FX read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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