

Role of the PGE2 receptor in ischemia-reperfusion injury of the rat retina

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Purpose: To investigate the function and expression of the PGE2 receptors EP1–4 in rat retinal ischemia-reperfusion (I/R) injury and to determine the regulatory role of resveratrol (RES) in this process.

Methods: In vitro, we stimulated primary astrocytes extracted from the optic disc of rats with epidermal growth factor (EGF) and RES, and detected the location of EP1–4 expression with immunofluorescence. The expression of antigenic fibrillary acidic protein (GFAP), EGF receptor (EGFR), inducible NOS (iNOS), and EP1–4 in astrocytes was detected with western blotting. In vivo, we established an I/R injury model and RES treatment model with Sprague-Dawley rats. Changes in the thickness of the inner retina were observed with hematoxylin and eosin (H&E) staining. EP1–4 localization in the retina was observed with immunohistochemistry. The expression of COX-2, iNOS, and EP1–4 in the control and model groups was detected with western blotting.

Results: In this study, immunofluorescence and immunohistochemistry showed that EP1–4 are expressed in astrocytes and the rat retina. EGF stimulation increased the expression of EGFR, iNOS, EP1, EP2, and EP4 in astrocytes. The expression of EP1–4 was statistically significantly increased on the third day after model induction, and EP1–4 expression decreased to normal levels on day 7. EGF and RES mediated the decrease in the expression of EP2. RES treatment significantly reduced retinal damage and RGC loss, as demonstrated by the relatively intact tissue structure on day 7 observed with H&E staining. Moreover, inflammation was associated with this I/R injury model, as demonstrated by the early induction of proinflammatory mediators, and this inflammation was significantly attenuated after RES treatment.

Conclusions: These results indicate that the COX-2/PGE2/EPs pathway is involved in retinal damage and astrocyte inflammation. In addition, the results suggest that the neuroprotective effects of RES may be associated with decreased production of inflammatory mediators. These results suggest that the PGE2 receptor may be a key factor in the treatment of neurodegenerative diseases, and that RES may be used as a possible therapeutic strategy for glaucoma.

High intraocular pressure (IOP) has been reported to induce retinal ischemia injury, leading to the death and loss of retinal ganglion cells (RGCs); this process is known as one of the causes of blindness [1]. Research has indicated that the site of IOP-induced axonal damage in glaucoma is at the optic disc [2]. An increase in IOP can destroy the structure of the optic disc and produce obstacles for axoplasm transport in the lamina cribrosa area. These obstacles can result in many problems, such as inhibited transport of the ATP produced by mitochondria to the axonal membrane, reduced levels of RGC neurotrophic factor, and decreased production and translocation of axonal proteins, which impair the metabolism of RGCs and result in the loss of RGCs, as well as altered astrocyte activation [3]. Astrocytes are the major glial cell type in the optic disc and are responsible for providing homeostatic and metabolic support to the axons of RGCs [4]. Studying the

molecular mechanism of astrocytes in the optic disc will provide new ideas for the prevention of blindness [5].

After neural injury, quiescent astrocytes become reactive astrocytes [6]. Activated astrocytes increase the formation of intermediate filaments, promote extensive migration and proliferation [7], increase changes in cell morphology [8,9], synthesize extracellular matrices, and release inflammatory cytokines, such as COX-2, TNF- α , PGE2, and inducible NOS (iNOS) [10,11]. Astrocytes are crucial for providing metabolic and homeostatic functions to support RGC axons [12]. However, the specific molecular mechanism of the inflammatory reaction induced by the astrocytic response to increased IOP at the optic disc remains unclear.

Epidermal growth factor (EGF) has been implicated in the transformation of quiescent astrocytes into reactive astrocytes, and can be induced through changes in the actin cytoskeleton and the stimulation of chemotactic migration [7,10]. In vitro studies have shown that high IOP leads to the phosphorylation and nuclear transfer of EGR receptor

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(EGFR) in astrocytes at the optic disc, suggesting that EGFR is activated at high IOP and induces astrocyte cycle synthesis. The release of COX-2 and PGE2 [7,13] may be associated with high IOP optic nerve damage. EGFR is not detected in the astrocytes of normal adult brains. EGFR expression and glial cell aggregation are detected in glial cells with central nervous system (CNS) diseases. These results suggest that EGFR is involved in the pathological processes of nerve injury and degenerative diseases, resulting in the reactive proliferation of astrocytes at the site of nerve injury [7]. Pathological processes such as injury, degenerative diseases, and tumors cause the reactive proliferation of astrocytes in nerve injury sites [14]. COX-2 is a key enzyme involved in the synthesis of prostaglandins from arachidonic acid. In a cerebral ischemia experiment, the induction of COX-2 led to ischemic brain damage, and COX-2 was used as a proinflammatory factor that participated in the mechanism of secondary neuronal injury [15]. The same study showed that COX-2 expression was upregulated in Alzheimer disease, Parkinson disease, stroke, multiple sclerosis, amyotrophic lateral sclerosis, and other CNS diseases. The inhibition of COX-2 can reduce inflammatory-related neuronal death and cell damage [16]. PGE2, an important downstream product of COX-2 enzyme activity, is a key substance that mediates the inflammatory response [17]. PGE2 plays a different role in optic neuropathy by binding four different G-protein coupled receptors, EP1–4. Activation of the EP1 receptor mediates the development of cerebral ischemia and specifically inhibits the protective effect of the EP1 receptor on ischemic nerve damage [18]. The EP2/EP4 receptors are involved in intraocular inflammation and tumors, destroying the blood–aqueous barrier and local lymphocytic infiltration [19,20]. EP3/EP4 receptor signaling promotes wound healing and lymphangiogenesis in chronic inflammation and tumors [21,22]. NO is derived from L-arginine by the reaction catalyzed by members of the NO synthase (NOS) family, including neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). iNOS is involved in cell injury and host defense. Compared with nNOS and eNOS expression, iNOS expression is decreased in normal cells and tissues [23]. The relationship between COX-2/PGE2/EP1–4/iNOS and ocular hypertension in the inflammatory signaling pathway *in vivo* was investigated in this study.

Resveratrol (RES), a polyphenol found in red grapes and peanuts, has many potential benefits, including antioxidation, anti-inflammation, antiapoptosis, and antiaging properties [24]. In addition, RES may exert neuroprotective properties in neurodegenerative diseases, such as Alzheimer disease and cerebral ischemia-reperfusion (I/R) injury [25,26]. Moreover, no apparent adverse effects of RES have been reported in

either animal or human studies, making it a superb candidate for therapeutic use [26,27]. Several recent reports have shown that pretreatment or posttreatment with RES effectively protects injured astrocytes in experimental glaucoma models [28,29]. However, the precise mechanisms by which RES protects astrocytes are still largely unknown. Therefore, in this study, we not only investigated the function of PGE2 receptors in I/R injury in rats but also explored the function of RES in COX-2/PGE2/EP1–4/iNOS-mediated astrocyte protection.

METHODS

Tissue preparation: C57BL/6 male mice, 6–8 weeks of age, and Sprague-Dawley (SD) male rats, 2–3 months of age, were obtained from the Center of Laboratory Animal Science of Nanchang University. All procedures are approved by the Animal Care and Use Committees of Nanchang University Medical School and the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were housed under standard conditions and maintained in temperature-controlled rooms on a standard 12-h:12-h light-dark cycle.

Six pairs of adult donor eyes (age range, 30–50 years) were obtained from the Red Cross Society of China Jiangxi Branch. All experiments involving human samples were approved by the Ethics Committee of Affiliated Eye Hospital of Nanchang University. The study adhered to the ARVO statement on human subjects. All volunteers signed written informed consent documents in accordance with the principles of the Declaration of Helsinki. To obtain fresh eye tissues for protein, we restricted the globes in the present study to those that were received within 48 h postmortem. The previous medical and ocular histories of all donors were assessed to exclude donors with any eye disease. The retinas were processed as described below.

Primary cultures of optic nerve head (ONH) astrocytes: Primary optic disc astrocyte cultures were prepared from the anterior portions of the optic nerves of adult rats as described in our previous studies [7,30]. They were anesthetized by an intraperitoneal injection of 10% chloral hydrate (Macklin, Shanghai, China; 4 ml/kg body weight) before surgery. Briefly, after the rats were anesthetized, the optic disc was freed from the posterior pole of the eye and other neighboring tissues. The lamina cribrosa was dissected from the pre- and post-optic nerves under the microscope. The explants were placed in T-25 flasks and maintained DMEM/F-12 (Hyclone, Beijing, China) with 10% fetal bovine serum (FBS, Gibco, Life Technologies, Grand Island, NY) and 0.1%

Penicillin- Streptomycin-Amphotericin B Solution (10,000 U/ml penicillin, 10,000 µl/ml streptomycin, and 25 µl/ml amphotericin B). The explants were maintained in a humidified atmosphere of 95% air/5% CO₂ at 37 °C. Approximately 10 days later, cells migrated from the edges of the explants, and the amount of culture medium provided was increased. After another week, the explants were removed. The cultures were purified in primary neuroglial cell culture medium, which is a modified astrocyte-defined medium called selective medium (PriCells, Wuhan, China). Fourth-generation cells were used for the immunostaining of glial fibrillary acidic protein (GFAP) and vimentin to distinguish astrocytes from other cell types (Table 1). Additionally, the cells, which were >95% positive for GFAP, were trypsinized with 0.25% Trypsin-EDTA (BI), placed in six-well plates, and grown to confluence. Astrocytes were starved for 24 h in serum-free medium (0.1% FBS) before use in subsequent experiments.

Stimulus and inhibitor treatment of ONH astrocytes: For drug treatments, EGF (Sigma-Aldrich, St. Louis, MO) was diluted in acetic anhydride to make a stock solution, and then was further diluted in incubation media to obtain working solutions [30]. For EGF stimulation, astrocyte cultures were incubated with EGF (40 ng/ml) in serum-free medium at 37 °C

for 24 h, and the cell morphology and number were observed. The astrocytes were labeled with GFAP and vimentin fluorescent staining.

Experimental retina I/R model and drug treatment: SD rats aged 2–3 months were used. Under anesthesia by an intraperitoneal injection of 10% chloral hydrate (Macklin; 4 ml/kg body weight), transient, unilateral retinal ischemia was induced in rat eyes. The anterior chambers of both eyes were briefly cannulated, and the IOP in one eye was elevated above systolic blood pressure (approximately 110 mmHg) for 60 min, while the contralateral eye was cannulated and maintained at normal IOP. The rats were killed 0, 1, 3, or 7 days after retinal ischemia. The left eye in the control group was cannulated and maintained at normal IOP to serve as the baseline. RES (Sigma Chemical Co., Saint Louis, MO) was freshly prepared by dissolution and dilution in 25% ethanol. The experimental I/R rats treated with RES and the control condition rats treated with RES were injected with RES three times, 1 day before (250 mg/kg, intraperitoneal injection–i.p.), at the time of (250 mg/kg, i.p.), and 1 day after (250 mg/kg, i.p.) I/R retina injury, while the I/R and control groups that did not receive RES treatment received the same volume of 25% ethanol (i.p.).

TABLE 1. PRIMARY ANTIBODIES USED IN THE STUDY.

| Antibody | Source | Catalog No. | Type of Ab | Dilution | MW |
|------------|------------|-------------|------------|---------------------------------------|---------|
| EP1 | Cayman | 101,740 | Rabbit mAb | 1:100(IF) 1:100(IHC) 1:1000(WB) | 42 kDa |
| EP2 | Cayman | 101,750 | Rabbit mAb | 1:100(IF) 1:100(IHC) 1:1000(WB) | 52 kDa |
| EP3 | Cayman | 101,760 | Rabbit mAb | 1:100(IF) 1:100(IHC) 1:1000(WB) | 52 kDa |
| EP4 | Cayman | 101,775 | Rabbit mAb | 1:100(IF) 1:100(IHC) 1:1000(WB) | 65 kDa |
| GFAP | Sigma | G3893 | Mouse mAb | 1:100(IF) 1:1000(WB) | 50 kDa |
| Vimentin | CST | 5741S | Rabbit mAb | 1:100(IF) 1:1000(WB) | 57 kDa |
| EGFR | Santa Cruz | SC-31156 | Mouse mAb | 1:1000(WB) | 170 kDa |
| COX-2 | Cayman | 160,106 | Rabbit mAb | 1:1000(WB) | 75 kDa |
| iNOS | invitrogen | TB263105 | Rabbit mAb | 1:1000(WB) | 135 kDa |
| β-tuibulin | Santa Cruz | J10715 | Mouse mAb | 1:2500(WB) | 55 kDa |

CST, Cell Signaling Technology; IF, immunofluorescence; IHC, immunohistochemistry; WB, western blotting; MW, molecular weight

Immunocytochemistry and immunohistochemical analyses: Immunocytochemistry was performed using specific primary antibodies against GFAP, vimentin, or EP1–4 (Table 1). Cells grown on coverslips were fixed in 4% paraformaldehyde (PFA) at room temperature for 10 min and washed three times with PBS (IX, NaCl 0.137 M, KCl 0.0027 M, Na₂HPO₄ 0.01 M, KH₂PO₄ 0.0018 M, pH 7.4). The cells were then treated with 0.1% Triton X-100 (Solarbio, Beijing, China) in PBS for 10 min and washed three times again. Next, the cells were blocked in PBS containing 0.5% bovine serum albumin (BSA, Solarbio, Beijing, China) for 1 h at room temperature in a humidified chamber. For immunostaining, the coverslips were incubated with primary antibodies diluted in PBS containing 0.1% BSA overnight at 4 °C in a humidified chamber. The cells were then washed three times in PBS and incubated with Alexa Fluor®488 anti-mouse immunoglobulin G (IgG; H + L), Alexa Fluor®488 anti-rabbit IgG (H + L), or Alexa Fluor®594 anti-rabbit IgG (H + L; working dilution, 1:200; donkey polyclonal; Life Technologies, Eugene, OR) for 1 h at room temperature. After additional washes in PBS, the coverslips were mounted with 4',6-diamidino-2-phenylindole (DAPI) and stored at 4 °C in the dark or viewed under a confocal microscope (ZEISS LSM800, Zeiss, Jena, Germany).

For immunohistochemistry of rat retinal tissue, the tissue first underwent perfusion fixation through the left cardiac ventricle with ice-cold 4% PFA in 0.1 M PBS (pH 7.4). Then, the eyes were enucleated and fixed in 4% PFA for 1 day, dehydrated, embedded in paraffin, sectioned, deparaffinized, and antigen repaired. The tissue was then blocked in PBS containing donkey serum and 0.1% Triton X-100 for 1 h at room temperature in a humidified chamber. The sections were stained with primary antibodies against EP1–4 overnight at 4 °C in a humidified chamber. After washing several times, the sections were incubated with peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L; working dilution, 1:200) for 60 min at room temperature, washed three times in PBS, and drained. The antigen–antibody complex was visualized with 3,3'-diaminobenzidine tetrahydrochloride, and the sections were counterstained with hematoxylin and examined using a microscope.

Western blot analyses: Retinal tissue and astrocyte cells were lysed in radio immunoprecipitation assay (RIPA) buffer supplemented with phosphatase inhibitors. The lysates were processed with ultrasound and centrifuged. Protein concentration was determined using the BCA assay (Vazyme Biotech Co., Ltd., Nanjing, China). Protein supernatant containing loading buffer was stored at –20 °C before use. For western blot analysis, aliquots containing an equal amount of protein

were analyzed with Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels and were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Darmstadt, Germany). The membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% nonfat milk. The membranes were washed three times in TBST and incubated with the appropriate primary antibodies at 4 °C overnight. Afterward, the membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h at room temperature and then washed three times in TBST again. Finally, the target proteins on the membranes were detected with an EasySee Western Blot Kit (TRANS, Beijing, China), and digital images were collected, which were subsequently analyzed with [Image J](#).

Data analysis: All data are expressed as the mean ± standard deviation (SD) from at least three independent experiments. [Image J](#) and [Graph Pad Prism 5.0](#) software were used for statistical analysis. All data were analyzed with a paired t test or with one-way ANOVA (ANOVA) and the post hoc Tukey test to determine the significance of the response. A p value of less than 0.05 was considered statistically significant.

RESULTS

Expression of EP1–4 in the retina and astrocytes: We analyzed the protein levels of EP1–4 in mouse, rat, and human retinas (Figure 1A). We confirmed that EP1–4 are expressed in the retinas of all three species, and are expressed at different levels in the different species. The protein levels of EP1 and EP2 were the highest in rat retinas, while EP3 and EP4 were the highest in human retinas. The protein levels of EP1–4 were the lowest in mouse retinas. Therefore, we used rats as experimental models. This is valuable for the study of human retinal EP receptors. Using specific antibodies to detect the PGE2 receptor protein, immunohistochemistry clearly demonstrated the presence of the EP receptor in the cells located in several layers of the retina. EP1 and EP3 receptors were expressed in the nerve fiber layer (NFL), ganglion cell layer (GCL), and inner plexiform layer (IPL; Figure 1B,D), while EP1 and EP3 were localized in the cytoplasm of the RGCs (shown with an arrow). EP2 and EP4 receptors were expressed in the NFL, GCL, IPL, and inner nuclear layer (INL; Figure 1C, E), while EP2 and EP4 were located in the nuclei of RGCs (shown with an arrow).

To elucidate the localization of the EP1–4 receptors in astrocytes, we cultured astrocytes isolated from the optic discs of adult rats. We performed immunofluorescence staining on cultured astrocytes after a selective culturing process. More than 95% of the cells were GFAP-positive astrocytes (Figure 1F). Additionally, these cultured astrocytes

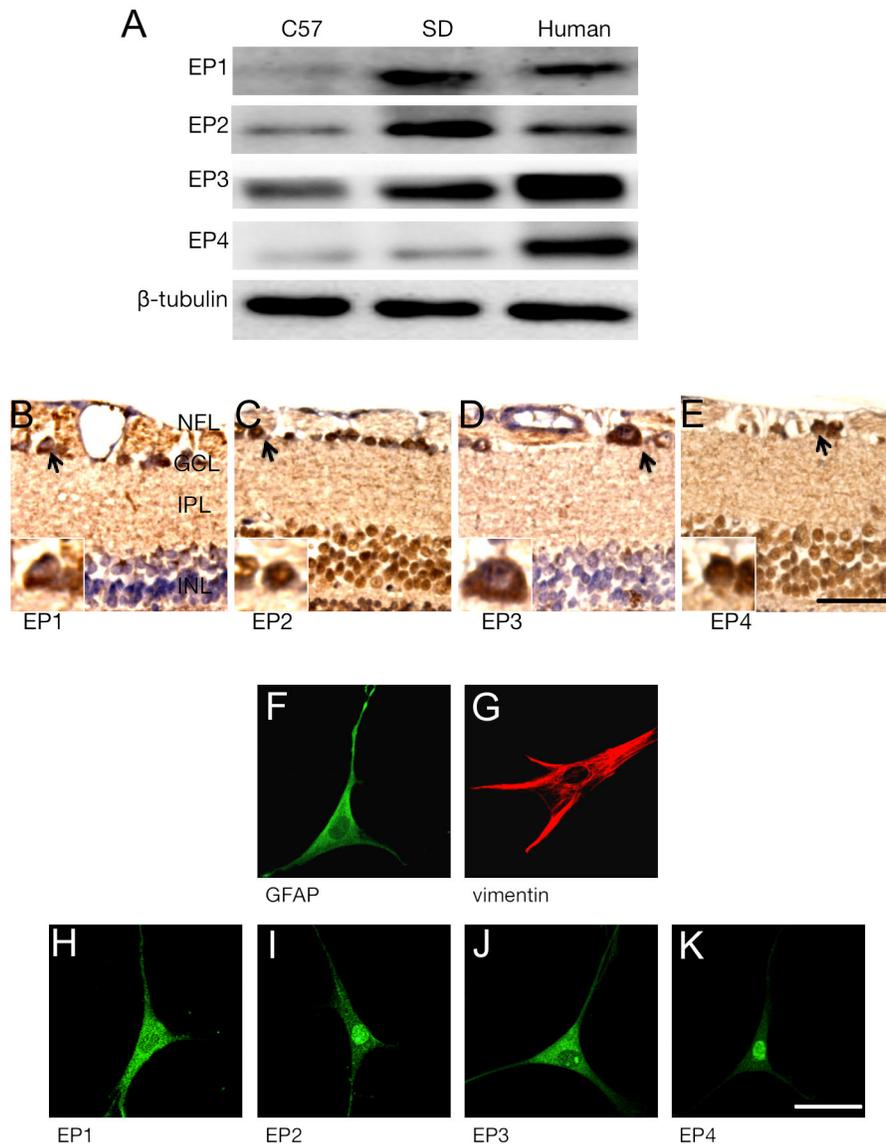


Figure 1. Expression of EP1–4 in retina and astrocytes. **A**: Expression of EP1–4 in adult mouse, rat, and human retinas. **B–E**: The localization of the EP1–4 receptors in the rat retina. **B**: Expression of EP1 in the rat retina. **C**: Expression of EP2 in the rat retina. **D**: Expression of EP3 in the rat retina. **E**: Expression of EP4 in the rat retina. Expression of EP1 and EP3 receptors in RGC cytoplasm (shown with an arrow). Expression of EP2 and EP4 receptors in the nucleus of an RGC (shown with an arrow). **F**, **G**: Microphotographs of astrocytes immunostained with glial fibrillary acidic protein (GFAP) and vimentin antibodies. **H–K**: Microphotographs of astrocytes immunostained with EP1–4 antibodies. (40X). The scale bar represents 50 μm . RGC: Retinal ganglion cell.

consistently expressed protein markers of astrocytes such as vimentin (Figure 1G). Immunofluorescence staining showed that EP1 and EP3 are expressed in the cytoplasm of astrocytes (Figure 1H, J), while EP2 and EP4 are expressed in the nucleus and cell membrane of astrocytes (Figure 1I, K).

PGE2 receptors are involved in the regulation of the EGF-dependent activation of astrocytes: Compared to the untreated controls, the astrocytes treated with EGF became

hypertrophied with enlarged cell bodies and thickened processes (Figure 2A), similar to the observations reported in previous studies [7]. The morphological changes in the astrocytes and the elevated GFAP immunoreactivity elucidated the presence of reactive astrocytes in response to EGF [7]. These data suggest that quiescent astrocytes become reactive astrocytes when primary rat optic nerve astrocytes are treated with EGF. Immunoblotting confirmed that GFAP expression

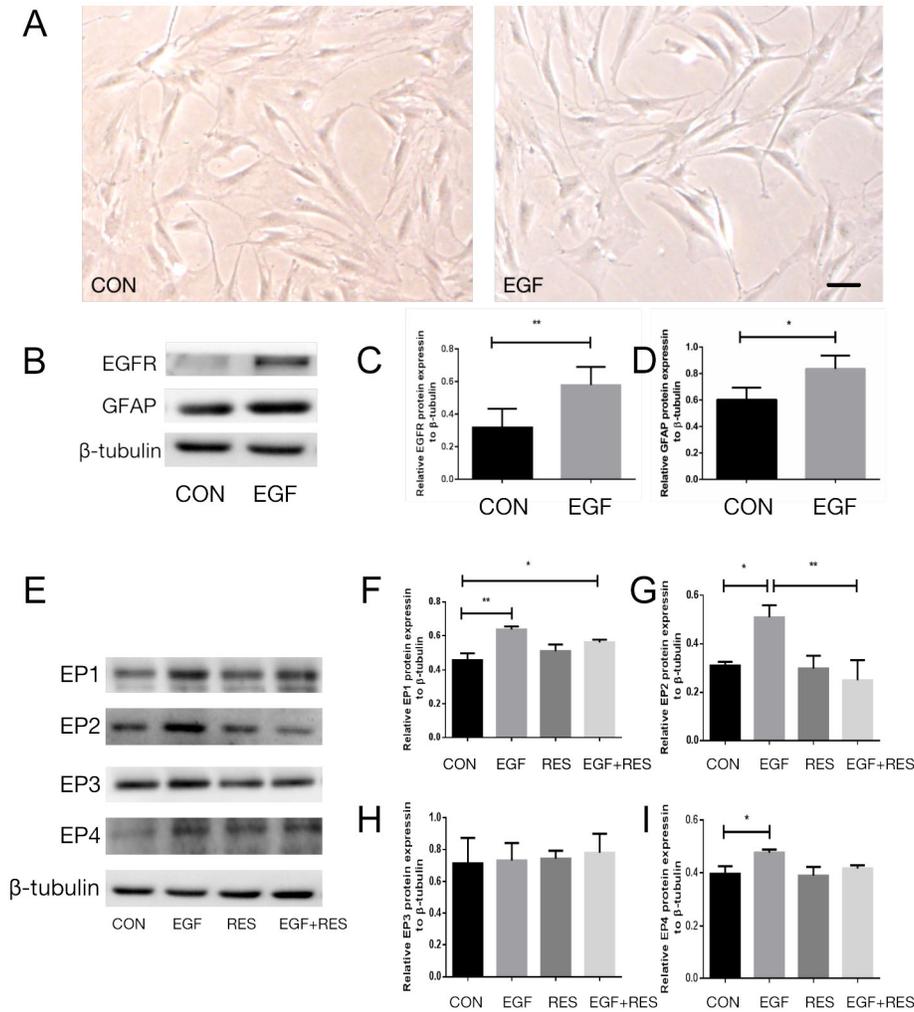


Figure 2. The PGE2 receptors are involved in the regulation of the EGF-dependent activation of astrocytes. **A:** The morphological changes in astrocytes in response to epidermal growth factor (EGF). Astrocytes were treated without EGF or with 40 ng/ml EGF for 24 h (10X). The scale bar represents 50 μ m. **B-D:** EGFR and glial fibrillary acidic protein (GFAP) were increased after treatment with EGF. Cells were immunoblotted with the EGFR and GFAP antibodies for immunoblotting. **E-I:** The expression level of EP1–4 was evaluated with western blotting. β -tubulin was used as a loading control in all samples. Data were shown as mean \pm standard error of the mean (SEM; * p <0.05, ** p <0.01, n =6).

was increased after exposure to EGF, which indicate the presence of activated astrocytes [31] (p <0.05; Figure 2B,D). EGF can induce increased GFAP expression and morphological changes in astrocytes. Quiescent astrocytes can be induced to become reactive astrocytes through the upstream signaling of the EGFR pathway (p <0.01; Figure 2B,C) [32]. To investigate whether the PGE2 receptor is involved in the regulation of the EGF-dependent activation of astrocytes, EP1–4 expression was detected in cultured ONH astrocytes after treatment with 40 ng/ml EGF for 24 h with immunoblotting. The results showed that EP1, EP2, and EP4 were statistically significantly upregulated in the EGF-treated group compared to the control

group (p <0.01, p <0.05 and p <0.05, respectively; Figure 2E–I). However, there was no statistically significant change in the expression of EP3. These results demonstrate that the PGE2 receptor was involved in the regulation of the EGF-dependent activation of optic disc astrocytes.

RES has previously exhibited neuroprotective properties against the pathophysiological alteration of cerebral ischemia [33–35]. To elucidate the role of RES, astrocytes were treated with EGF (40 ng/ml) and RES (100 μ M), and the expression of EP1–4 in astrocytes was detected with western blotting. The results showed that the level of EP2 in the reactive

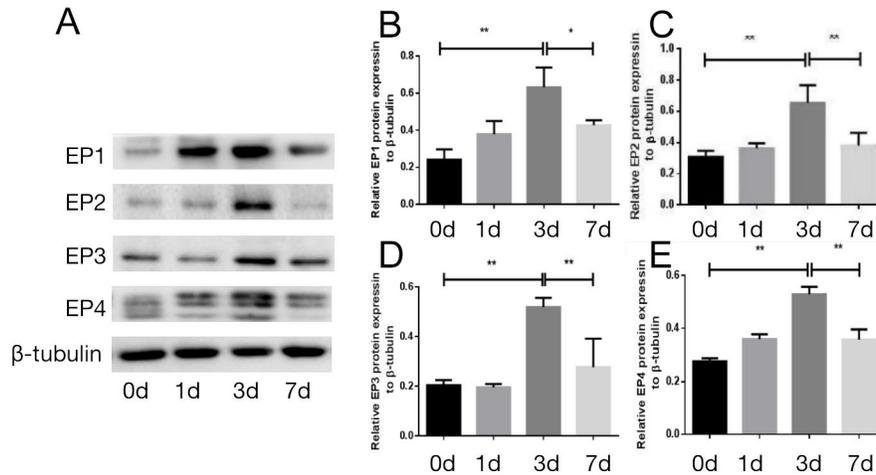


Figure 3. The PGE2 receptor is involved in I/R injury. A-E: Ischemia-reperfusion (I/R) injury markedly upregulated the expression of EP1-4 at day 3, and β -tubulin was used as a loading control in all samples. Data were shown as mean \pm standard error of the mean (SEM; * $p < 0.05$, ** $p < 0.01$, $n = 6$).

astrocytes of the optic disc was statistically significantly reduced by treatment with EGF and RES (100 μ M) for 24 h ($p < 0.01$; Figure 2G). These results demonstrated that RES can suppress the EGF-mediated inflammatory reaction of optic disc astrocytes.

*PGE2 receptor is involved in I/R injury*²⁸²: To fully understand the role of PGE2 receptors, we also performed in vivo experiments. In the in vivo experiment, western blotting showed a remarkable elevation in EP1-4 expression ($p < 0.05$, $p < 0.01$, respectively; Figure 3A-E) at day 3 in the I/R injury group compared with the control group. These results may have demonstrated that EP1-4 participate in I/R injury to the rat retina, which is consistent with the in vitro results.

RES delays RGC loss and attenuates I/R injury-induced COX-2 and iNOS expression: Western blotting have shown remarkable elevation in the expression of COX-2 at day 1 ($p < 0.05$ and $p < 0.01$, respectively; Figure 4A) and remarkable elevation in the expression of iNOS at day 3 ($p < 0.05$ and $p < 0.01$, respectively; Figure 4C) after I/R injury. To realize the effects of RES on the release of proinflammatory factors in the retina after I/R injury, we conducted a more in-depth study of COX-2 and iNOS. Western blotting showed that RES statistically significantly reduced the expression of COX-2 and iNOS after I/R injury ($p < 0.05$ and $p < 0.01$, respectively; Figure 4B,D). Importantly, RES had no effect on the expression of COX-2 and iNOS in normal rat retinas (Figure 4B,D). To further evaluate the effect of RES on I/R injury protection, we investigated the general histopathological changes in the retina at 7 days postinjury with H&E staining. The retinal structure of the normal control group was intact and orderly

(Figure 4E), and RES had no effect on the retinal structure of the control group was intact and orderly (Figure 4E), and RES had no effect on the retinal structure of the control group and exhibited no toxic effects on the retinal structure ($p < 0.05$; Figure 4E,F). As shown in Figure 4, remarkable tissue loss was observed in the retina after I/R injury, and the structural disorder and the number of RGCs decreased. However, I/R-induced retinal damage was statistically significantly attenuated, and retinal structural integrity was statistically significantly preserved in the RES-treated I/R group. The most important observation was that RGCs were preserved. Taken together, these results demonstrate that I/R injury leads to inflammation in the retina, which can be prevented with RES treatment.

CONCLUSION: There are many factors that can cause RGC damage, including I/R injury and inflammation, and these factors can lead to irreversible blindness or glaucoma [36]. The involvement of inflammatory mediators in the pathological changes in the glaucoma optic nerve has become an increasingly popular topic in molecular research. PGE2 is an important mediator of inflammation [37], and the most widely produced prostaglandin in the body [38]. PGE2 is generally considered to be a proinflammatory molecule associated with redness, swelling, and pain [38]. Accumulating data suggest that PGE2 not only participates in initiation but may also actively contribute to the resolution of inflammation [37]. Thus, the present research on PGE2 is meaningful. These different physiological effects of PGE2 are mediated by the four PGE2 receptor subtypes, EP1-4 [39]. In this study, we used EGF-activated ONH astrocytes and a rat retinal I/R

injury model to investigate PGE2 receptors. Encouragingly, we found that PGE2 receptors are involved in reactive astrocytes and in retinal I/R injury. In addition, the results showed that RES could decrease the production of proinflammatory mediators in reactive astrocytes and during retinal I/R injury. Collectively, these data provide compelling evidence that PGE2 receptors, especially the EP2 receptor, are involved in the pathological changes in the optic nerve in glaucoma,

and that RES may be a promising therapeutic intervention for glaucoma.

COX-2 is the rate-limiting enzyme in the biosynthesis of prostaglandins from arachidonic acid and known to be constitutively present in the normal retina [40]. However, there is controversy regarding the expression of COX-2. COX-2 is an early gene product of related damage that can be rapidly

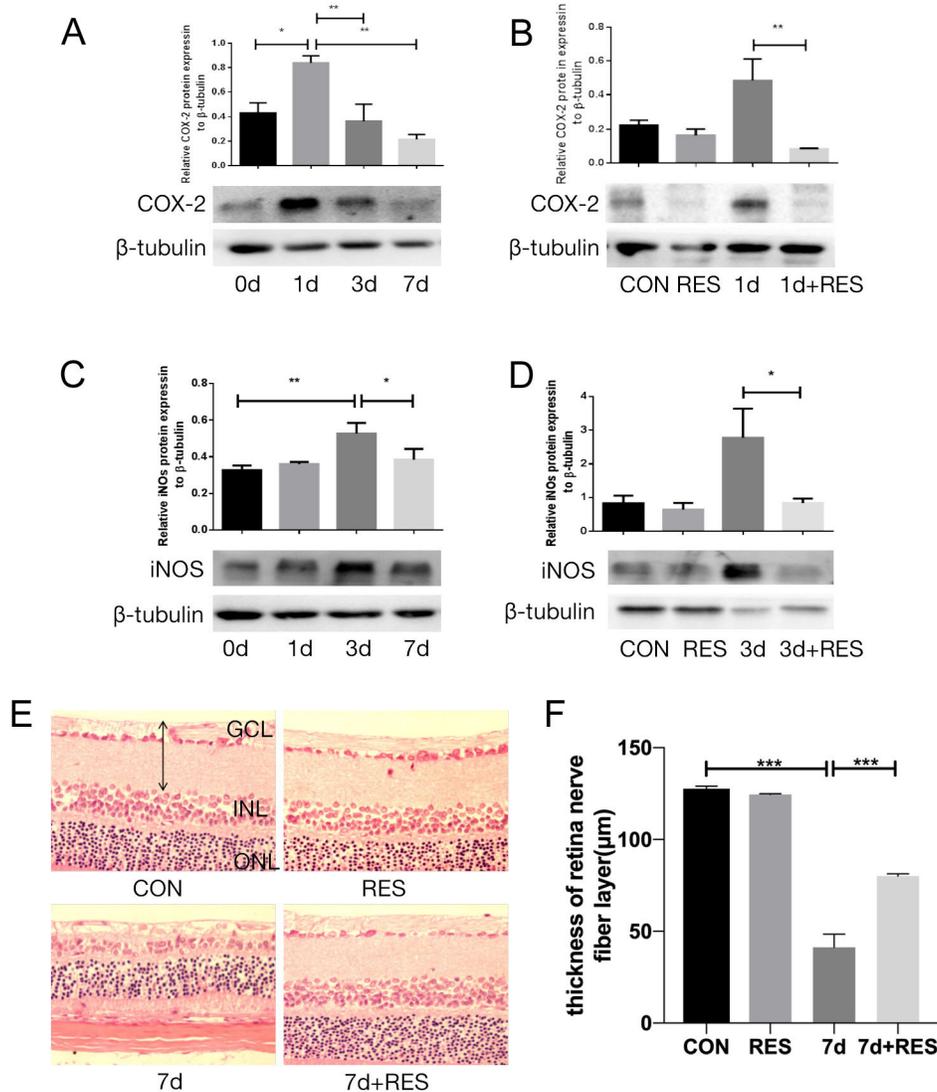


Figure 4. RES delays RGC loss and attenuates gliosis-related inflammation from I/R injury. **A-D**: The effect of resveratrol (RES) on the production of pro-inflammatory mediator. Ischemia-reperfusion (I/R) injury markedly upregulated the expression of COX-2 at day 1 (**A**) and iNOS at day 3 (**C**), but RES statistically significantly inhibited the production of these two proinflammatory mediators (**B, D**). β-tubulin was used as a loading control in all samples. Data were shown as mean ± standard error of the mean (SEM; *p < 0.05, **p < 0.01, n = 6). **E, F**: RES treatment inhibited retinal tissue loss after I/R injury. **E**: Hematoxylin and eosin (H&E) staining of retinal cross-sections showed the degeneration of retinal ganglion cells (RGCs) and the decreased thickness of retinal tissue at 7 days after I/R injury, and this effect was markedly inhibited with RES treatment. Representative images from the control group, control group plus RES, 7-day I/R group, and 7-day I/R group plus RES (40X). **F**: Effects of RES on retinal tissue after I/R injury. Retinal tissue thickness was assessed by H&E staining and Image J analysis. Data are shown as mean ± SEM (**p < 0.01, ***p < 0.001, n = 4).

induced to participate in the regulation of the inflammatory response and is expressed in retinal degeneration-related lesions [16]. In this study, the *in vitro* cell culture experiments did not show significant changes in COX-2, whereas COX-2 reactivity was significantly increased in the early stage (1 day post-I/R injury) of inflammatory injury stimulation, with statistical significance in *in vivo* studies. The differences between the *in vitro* and *in vivo* experiments prompt the question of whether COX-2 is involved in the inflammatory immune response through optic disc astrocytes. Studies have shown that perhaps the neurodegenerative response of COX-2 in the early stages of acute ischemic injury occurs through the horizontal cells, amacrine cells, RGCs, and Müller cells of the retina [40]. Thus, we will continue to further explore changes in the expression of COX-2 after EGF stimulation in different cell types.

PGE2 is a major product of COX-2 and acts on four G-protein coupled receptors, EP1–4 [41]. The stimulation of prostanoid EP1 receptors elevates the level of intracellular inositol phosphate and Ca²⁺. The stimulation of prostanoid EP2 or EP4 receptors activates adenylyl cyclase, thus increasing levels of intracellular cAMP [42], whereas the stimulation of prostanoid EP3 receptors inhibits adenylyl cyclase [41]. Several studies demonstrated the presence of prostanoid EP2 and EP4 receptors in neuronal and vascular cells in the retina [43]. However, these receptors have not been fully investigated. Our studies demonstrated the presence of prostanoid EP receptors in the astrocytes. The EP3 receptor has three transcriptional splice variants [44,45]. There was no marked change in the expression of EP3 in the *in vitro* experiment, which may be related to the complex function of EP3. However, it is still necessary to repeat the experiment. The changes in EP1, EP2, and EP4 were pronounced in the experiment, similar to many previous studies that reported increased expression in activated astrocytes exposed to external damage factors that mediate inflammatory responses. We also detected EP receptors in the rat retina. In the rat retinal I/R injury model, the expression of EP1–4 and iNOS was statistically significantly increased on the third day, which was consistent with the COX-2/PGE2/EPs signaling pathway. The initial (day 1) response of the proinflammatory factor COX-2 mediated the increased expression of the downstream PGE2 receptors and regulated the release of the cellular inflammatory factor iNOS. Because I/R injury and inflammation can contribute to irreversible blindness or glaucoma [36], the PGE2 receptor may be a target for neuroprotective intervention in these diseases.

RES possesses antioxidant, anti-inflammatory, anti-apoptotic, antiaging, and neuroprotective properties [24,26].

Notably, the neuroprotective effects of RES show the ability of RES to cross the blood–brain barrier, indicating that RES can likely easily cross the blood–retinal barrier. Additionally, the superb safety profile of RES supports its potential as a safe therapeutic intervention in numerous diseases [28]. It has been reported that pretreatment or posttreatment with RES, primarily by oral administration, provides neuroprotection in glaucoma, but the underlying mechanisms are largely unknown [28]. Many studies have shown that the oral bioavailability of RES was less than 1%, which may have caused the discrepancies between the *in vitro* and *in vivo* RES studies (pretreatment and posttreatment). We further investigated the mechanisms underlying the protective effects of RES on glaucoma. The expression of the EP2 receptor in astrocytes is increased under EGF stimulation, and RES can inhibit the overexpression of EP2. Because stimulation of the EP2 receptors elevates intracellular cAMP levels [42], RES may play an important role in the cAMP signaling pathway. In a future study, we will use EP2 knockout mice to study the EP2 function in the retina. Our previous studies reported that COX-2 is a proinflammatory mediator that could lead to the secondary injury of RGCs [7,13]. iNOS is another primary inducible inflammatory enzyme that plays a degenerative role in glaucoma [46]. The present results demonstrated the remarkably increased expression of COX-2 at 1 day and iNOS at 3 days post-I/R injury, both of which were attenuated with RES treatment. The histopathological observations in the present study indicated that I/R-induced retinal tissue degeneration was statistically significantly attenuated, and that structural integrity was markedly maintained with RES administration. The inflammatory process is inhibitory; therefore, reducing the pathological damage of diseases and protecting the function of the optic nerve and neurons. However, is the retinal protection provided by RES specific to a PGE2 receptor? Can we increase the expression of EP receptors after RES injection on day 3 of the model? These questions will be addressed in future studies under the influence of specific diseases.

Overall, the present study showed that the COX-2/PGE2/EPs pathway is involved in irreversible damage to the optic nerve in EGF-activated ONH astrocytes and rat retina I/R-induced models, and that RES can inhibit this effect. In addition, the present results suggest that the neuroprotective effects of RES may be associated with decreased production of inflammatory mediators. Taken together, these results further demonstrate the potential therapeutic effect of RES on glaucoma-induced I/R injury. Therefore, we hypothesized that the regulation of PGE2 receptor function may be a key factor in the treatment of neurodegenerative diseases. To better control the progression of glaucoma in clinical practice,

we used RES to inhibit the inflammatory response of glial cell activation to protect retinal function, which is a new direction in the study of drug treatment for glaucoma-related diseases. We still need to work together to uncover treatments for glaucoma and glaucoma-related diseases.

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