Therapeutic Effects of Puerarin Against Anterior Ischemic Optic Neuropathy Through Antiapoptotic and Anti-Inflammatory Actions

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PURPOSE. This study investigated the therapeutic effects of puerarin (PR) on a rat model of anterior ischemic optic neuropathy (rAION).

METHODS. The neuroprotective effects of PR on rAION were evaluated using flash visual-evoked potentials (FVEP), retrograde labeling of retinal ganglion cells (RGCs), TUNEL assay of the retina, optical coherence tomography (OCT) images of optic nerve width, and ED1 staining of the optic nerve (ON). The inflammatory response of ON and Akt signaling pathways were analyzed through Western blot. M2 polarization was determined by immunostaining and immunoblotting in ONs.

RESULTS. In FVEP analysis, the amplitude of P1-N2 and the RGC density in the PR-treated group were 2.3- and 1.6-fold higher than those in the PBS-treated group, respectively (P < 0.05). The number of apoptotic RGC in the PR-treated group was 2.8-fold lower than that in the PBS-treated group. OCT images demonstrated that PR treatment–reduced ON edema in the acute phase compared to PBS treatment (P < 0.05). Macrophage infiltration was reduced by 5.2-fold by PR treatment compared with the PBS treatment (P < 0.05). PR treatment inhibited the levels of iNOS, IL-1β, and TNF-α, induced the levels of IL-10, Arg1, and Fizz1 in the rAION model. The levels of p-Akt1 and C/EBPβ in the PR-treated group increased by 3.4-fold and 5.89-fold compared with those in the PBS-treated group (P < 0.05). Inhibition of Akt activation reduced the number of M2 macrophage in the PR-treated group (P < 0.05).

CONCLUSIONS. PR treatment provided the neuroprotective effects in the rAION model, which may lead to new clinical applications.

Keywords: rat model of anterior ischemic optic neuropathy (rAION), inflammation, M2 macrophage polarization, Akt1, puerarin (PR), optic nerve, retinal ganglion cells

Nonarteritic anterior ischemic optic neuropathy (NAION) is the most common form of acute optic neuropathy in aged people, with an estimated annual incidence of 2.3 to 10.2 per 100,000 people in the United States and Taiwan, at least 6000 new cases a year.1–3 NAION is characterized clinically by acute, painless, visual loss with swelling of the optic disc leading to optic disc atrophy.4 The most common pathogenesis of NAION is caused by transient nonperfusion or hypoperfusion of the optic nerve head (ONH).5 Optic Nerve (ON) ischemia induces a chain reaction of inflammation and edema, eventually resulting in retinal ganglion cell (RGC) death and vision loss.6,7 Effective treatments for NAION are yet to be established.

In a rat model of anterior ischemic optic neuropathy (rAION), the breakdown of the blood–ON barrier (BOB) occurs within few hours after the induction of an infarct, followed by the infiltration of extrinsic macrophages and the activation of resident microglia at the core of the ischemic ON.8–12 ON inflammation is responsible for certain ON damage that occurs in NAION.7,13 However, activated macrophages can improve neuronal survival and play a key role in phagocytosis and the removal of myelin debris.14 Macrophages can be polarized into M1 and M2 phenotypes and can be classified by their surface markers.15 M1 macrophages induce inflammation, inhibit cell proliferation, and cause tissue damage, whereas M2 macrophages can reduce inflammation and promote cell proliferation and tissue repair.15 In addition, the activated M2 phenotype of microglia and macrophages has been reported to exert neuroprotective effects on some experimental models.12,16–19 Therefore, the activation of M2-phenotype macrophages and the inhibition of proinflammatory cytokines may be vital for ON protection in the rAION model.12,17

Puerarin (PR), a known isoflavone,20 is a primary bioactive ingredient derived from the root of Pueraria lobata, widely known as Gegen in traditional Chinese medicine. PR has been demonstrated to exert a broad spectrum of pharmacological effects, such as vasodilation,21 cardioprotection,22 neuroprotection,23 antioxidant,24 anticancer,25 anti-inflammation,26 and inhibition of diabetic ocular complications.27 Recently, the neuroprotective effects of PR have been reported in some experimental models, such as those of spinal cord injury28,29 and cerebral ischemia injury.30,31 The antiapoptotic capacity of PR was reported in RGC damage through the inhibition of the...
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JNK/p38 MAPK pathway. Moreover, PR treatment could inhibit the apoptosis of retinal pigment epithelial cells in diabetic rats by reducing peroxynitrite levels and iNOS expression. PR treatment exerted anti-inflammatory effects on the rat model of cerebral injury through the inhibition of NF-κB signaling pathway. In addition, PR can modulate the PI3k/Akt signaling pathway to exert anti-inflammatory and antiapoptotic effects on cerebral and kidney injuries.  

The PI3K/AKT signaling pathway not only regulates macrophage survival, migration, and proliferation but also orchestrates the response to different metabolic and inflammatory signals in macrophages. Activation of the PI3K/Akt pathway is critical for controlling proinflammatory and anti-inflammatory responses in Toll-like receptor (TLR)-stimulated macrophages. A study reported that the negative immune regulator TIPE2 promotes M2 macrophage polarization through the PI3K/Akt signaling pathway. Akt1 and Akt2 kinase isoenzymes play distinct roles in the regulation of macrophage polarization. Thus, we believe that Akt1 activation plays a key role in M2 microglia and macrophage polarization in the rAION model. We hypothesize that the administration of PR may induce M2 polarization through Akt1 activation to produce neuroprotective effects on the rAION model.  

Thus, the purpose of this study is to evaluate whether the treatment with PR provides neuroprotective effects in the rAION model and whether Akt1 is involved in the macrophage and microglia polarization.

**MATERIALS AND METHODS**

**Study Design**

In this study, we investigated the neuroprotective effects of PR and the effects of PR on microglia/macrophage polarization in the rAION model (Fig. 1). In the therapeutic evaluation, after the successful induction of anterior ischemic optic neuropathy (AION) in rats, 24 rats were randomized into the following 2 groups: treatment with phosphate-buffered saline and treatment with intraperitoneal injection of PR alone immediately after the rAION procedure for a total of 3 consecutive days. Treatment on the first three days is a critical window to control vascular permeability in rAION. The other 12 rats received sham laser treatment without a photosensitizing agent to serve as normal controls. The rats in each group were evaluated by flash visual-evoked potentials (FVEPs), retrograde labeling of RGC, OCT imaging of the ONH, TUNEL assay of the RGC layer, and ED1 and CD206 (the mannose receptor is a known M2 macrophage marker for determining macrophage polarization) staining of ON sections. The protein levels of the proinflammatory cytokines TNFα, IL-1β, and iNOS; the anti-inflammation cytokine IL-10; M2 microglia and macrophage polarization markers (Arg1 and Fizz1); and the markers of Akt signaling pathway (Akt, p-Akt1, p-Akt2, and C/EBPβ) were measured in ON samples through Western blot at day 3 post-rAION.

To confirm PR-induced Akt1-dependent M2 polarization, another 12 rats were randomized into the following 2 treatment groups: treatment with intraperitoneal injection of PR or treatment with vitreous cavity injection of the Akt inhibitor (LY294002) plus intraperitoneal injection of PR immediately after the rAION procedure for a total of 3 consecutive days. The CD206 staining of ON sections was performed in these two groups to evaluate the level of M2 polarization.

**Experimental Animals**

The male Wistar rats, weighing 150 to 180 g (7–8 weeks old), were maintained in filter-top holding cages. Rats were obtained from the breeding colony of BioLASCO Co., Taiwan. Animal care and experimental procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all animal experiments were approved by the Institutional Animal Care and Use Committee at the Laboratory Animal Center, Tzu Chi University. An intramuscular injection consisting of a cocktail of ketamine (100 mg/kg; Merial, France) and xylazine (10 mg/kg; Health-Tech Pharmaceutical Co., Taipei, Taiwan) was administered to induce general anesthesia. Alcaine was applied for local anesthesia, and Mydrin-P was applied for pupil dilation in all experiments.

**Administration and Formulation of PR**

Commercial PR with a purity of 80% (P5555, Sigma-Aldrich Corp., St. Louis, MO, USA) was prepared in injection water. The PR drug used in previous studies was referenced in this study. In the PR-treated groups, PR was administered at a concentration of 50 mg/kg. The dose of PR is based on previous study in the experimental spinal cord injury. All treatments and the vehicle were applied through intraperitoneal injection after rAION induction for 3 days thereafter.

**Intravitreal Injections of the Pi3k/Akt Pathway Inhibitor LY294002**

After rAION induction, 300 mM in 2 μL of LY294002, a Pi3k/Akt pathway inhibitor, was injected into the vitreous cavity of rat eyes; then, rats were administered a daily intraperitoneal injection of 50 mg/kg of PR immediately for 3 days thereafter. Four weeks after surgery, rats were killed through CO2 insufflation.

**rAION Induction**

The rAION induction method used in this study was the same as that used in our previous study. Briefly, Rose Bengal (RB; Sigma-Aldrich Corp.) was administered intravenously through the tail vein by using a 28-gauge needle (2.5 mM RB in PBS/1 ml/kg animal weight) after general anesthesia. After the administration of RB and pupil dilatation, we illuminated the ON head region with an argon laser. The sham laser treatment group did not receive an RB injection. The right optic discs of rats were directly treated with an argon green 532 nm/500 lm/80 mW spot laser (MC-500 Multicolor laser, Nidek Co., Ltd., Tokyo, Japan) with 12 pulses of 1-second duration each. Laser settings were the same as those used in our previous studies.

**FVEP Recordings**

FVEPs were recorded 4 weeks after rAION induction. A visual electrodiagnostic system (Espion, Diagnosys LLC, Gaithersburg, MA, USA) was used to measure FVEPs. The first positive-negative wave was defined as the P1 wave, and the first negative-going wavelet was defined as the N1. The amplitudes of the P1-N2 wave were compared among the groups (*n* = 6 rats in each group) to estimate visual function.

**Retrograde Labeling of RGCs by Fluorogold**

The retrograde labeling of RGCs was performed 1 week before rats were euthanized. The detailed methods and protocol of...
FluoroGold labeling have been described in our previous studies. In brief, the retinas were evaluated at distances of 1 mm from the center of ONH for RGC calculation to obtain the central RGC densities. We calculated at least eight randomly selected areas in the central regions (approximately 40% of the central area) of each retina, and data are averaged and presented as the mean density of RGCs per retina. RGC densities were calculated by ImageMaster 2D Platinum software.

ON and Retinal Sample Preparation

A segment of the ON about 5 to 7 mm in length between the optic chiasm and the eyeball was harvested upon killing at 2 weeks. The nerve was immediately frozen at −80°C for later studies. Rats that showed axotomy or massive hemorrhage upon examination of the optic nerve histologic sections were excluded from the study.

Rats were euthanized, and their eyes were enucleated and fixed in 4% paraformaldehyde. The eyeballs and ONs were separated and transferred to 30% sucrose; the samples were stored at 4°C until they settled at the bottom of the tubes. Retina and ON cross sections of 20 μm were obtained using a cryostat.

TUNEL Assay

The retina cross-section boundaries were traced using an immerse marker to create a hydrophobic barrier to retain solutions on tissues. Tissues were washed with 100 μL of 1X PBS for 5 minutes, followed by proteinase K (20 μg/mL), to increase permeability (proteinase K in 0.3% Triton X-100, 1X PBS [pH 7.2]) at room temperature. Tissues were then equilibrated with 100 μL of equilibration buffer at room temperature for 10 minutes. Then, 50 μL of the TdT reaction mix was applied to the tissue area, and slides were incubated at 37°C in a humid chamber for 60 minutes. Slides were washed and counterstained with 4',6-diamidino-2-phenylindole (DAPI, 0.3% Triton X-100, 1x PBS [pH 7.2], 1:300). Slides were mounted with 7:3 glycerol: PBS and stored at 4°C or were subjected to image acquisition with appropriate filter sets in a confocal microscope (Carl Zeiss Meditech Inc., Thornwood, NY, USA). The TUNEL-positive cells in the RGC layer of each sample were counted in 10 high-powered fields (HPF, ×400). Three sections per eye were averaged, and there were six rats in the group. TUNEL-positive cells were calculated by imaging software (ImageMaster 2D Platinum; GE Healthcare, Chicago, IL, USA) in this study.

IHC on ON Tissues

The ED1 antibody reacted against extrinsic macrophages and intrinsic microglia. Monoclonal antibodies of ED1 (1:100, Abcam, Cambridge, MA, USA) were used. CD206 is known M2 macrophage markers. Polyclonal antibody of CD206 (1:100, Abcam) were used to determine macrophage polarization. Samples were incubated in a primary antibody overnight at 4°C. A secondary antibody conjugated with fluorescein isothiocyanate (FITC and Rhodamine, 1:100, Jackson Immuno Research Laboratories, West Grove, PA, USA) was incubated at room temperature for 1 hour. Counterstaining was performed using DAPI (1:1000, Sigma-Aldrich Corp.). Images were taken within 2 mm posterior to the ONH. For comparison, ED1-positive cells were calculated in 6 high-power fields (HPFs; ×400 magnification) at the ON lesion site.

OCT Imaging of ONH

A Phoenix Micron IV retinal microscope with image-guided OCT was used for imaging. This system uses spectral domain OCT, which provides a longitudinal resolution of 1.8 μm and a...
transverse resolution of 3 μm with a 3.2-mm field of view and 1.2-mm imaging depth at the retina. After general anesthesia, rats were placed on the imaging platform and their heads were positioned at an angle allowing the penetration of light vertical to the cornea from the temporal side. The optic nerve width (ONW) was linearly scanned through the center of the optic disc and the distance of Bruch’s membrane opening was measured. OCT laser was calibrated before the measurements were done. To avoid false readings, the whole ONH was scanned and then the Bruch membrane opening along with the hyaloid artery as a landmark was used to double check the OCT scanned location. At least three clear captures were obtained for each eye. Quantitative measurements of the Bruch membrane opening were conducted using built-in Insight software (Phoenix Research Labs, Pleasanton, CA, USA). Edema was measured by comparing the Bruch’s membrane opening distance between the sham-operated rats and the rAION-induced rats to evaluate the change of optic disc width. This procedure was performed at days 1, 3, 7, 14, and 28 post-rAION.

**Western Blotting**

Rats were euthanized, and their eyes were enucleated. The ON samples were homogenized and stored at −80°C for further analysis. A protein assay was performed using a BCA protein assay kit. For immunoblotting, 30 μg of protein was separated on a 10% bis-acrylamide gel. Proteins were transferred onto polyvinylidene difluoride membranes. After the transfer, membranes were blocked with 5% nonfat dry milk for 1 hour, followed by an overnight incubation with iNOS, IL-1β, TNF-α, IL-10, Arg1, FIZZ 1, total Akt, p-Akt2 (Ser 474), p-Akt1 (Ser 473), C/EBPβ, and GAPDH (Sigma-Aldrich Corp.) primary antibody at 4°C. Membranes were washed, followed by incubation with a secondary antibody conjugated to HRP against the appropriate host species for 1 hour at room temperature. Membranes were then developed using an enhanced chemiluminescent substrate, and images were taken in a western blot analyzer. The relative density was calculated using ImageJ software (https://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). In brief, the mean gray value was measured to quantify the protein band in the quantification software. For band detection, the rectangle tool was used to select a region of interest. For band quantification, the area under the curve of the detected band was used to represent the band density. The final relative density is the ratio of protein of interest to internal loading control.

**Statistical Analysis**

All statistical analyses were performed using a GraphPad Prism. Data are presented as the mean ± standard deviation. The Kruskal/Wallis-test was applied for comparison among groups. A protein assay was performed using a BCA protein assay kit. For immunoblotting, 30 μg of protein was separated on a 10% bis-acrylamide gel. Proteins were transferred onto polyvinylidene difluoride membranes. After the transfer, membranes were blocked with 5% nonfat dry milk for 1 hour, followed by an overnight incubation with iNOS, IL-1β, TNF-α, IL-10, Arg1, FIZZ 1, total Akt, p-Akt2 (Ser 474), p-Akt1 (Ser 473), C/EBPβ, and GAPDH (Sigma-Aldrich Corp.) primary antibody at 4°C. Membranes were washed, followed by incubation with a secondary antibody conjugated to HRP against the appropriate host species for 1 hour at room temperature. Membranes were then developed using an enhanced chemiluminescent substrate, and images were taken in a western blot analyzer. The relative density was calculated using ImageJ software (https://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). In brief, the mean gray value was measured to quantify the protein band in the quantification software. For band detection, the rectangle tool was used to select a region of interest. For band quantification, the area under the curve of the detected band was used to represent the band density. The final relative density is the ratio of protein of interest to internal loading control.

**RESULTS**

**Treatment With PR Preserved Visual Function**

In evaluation of visual function, FVEPs showed that the P1-N2 amplitudes in the sham, PBS-treated, and PR-treated groups were 61.2 ± 7.3 μV, 18.6 ± 3.2 μV, and 42.8 ± 7.56 μV, respectively (Fig. 2). The P1-N2 amplitude was 2.5-fold higher in the PR-treated group than in the PBS-treated group (P < 0.05).

**Treatment With PR Preserved RGC Survival**

The RGC densities of the sham, PBS-treated, and PR-treated groups in the central retinas were 1676.3 ± 206.9, 786.4 ± 235.5, and 1281.5 ± 157.8/mm², respectively (Fig. 3A). The RGC density in the PR-treated group was 1.6-fold higher than that in the PBS-treated group (P < 0.05; Fig. 3B).

**Treatment With PR Inhibited RGC Apoptosis**

On retina sections, the number of TUNEL-positive cells in the sham, PBS-treated, and PR-treated groups was 0.75 ± 0.24, 18.21 ± 2.22, and 6.53 ± 2.82, respectively (Fig. 4A). The number of TUNEL-positive cells in the PR-treated group was 2.8-fold lower than that in the PBS-treated group (P < 0.05; Fig. 4B).

**Treatment With PR-Reduced ONH Edema**

One day after rAION induction, ONH width was 253.67 ± 7.7 μm in the sham group, 410 ± 19.1 μm in the PBS-treated group, and 316.7 ± 20.2 μm in the PR-treated group. A significant difference was observed in ONH width between the PBS-treated and PR-treated groups (P = 0.047). Three days after rAION induction, ONH width was 264.0 ± 14.0 μm in the sham group, 385.0 ± 15.5 μm in the PBS-treated group, and 267.7 ± 15.0 μm in the PR-treated group. The ONH width was significantly lower in the PR-treated group than in the PBS-treated group (P = 0.0068). No statistical differences were observed between the PR-treated and PBS-treated groups at days 7, 14, and 28 post-rAION. PR treatment reduced ONH edema in the acute phase of rAION (Fig. 5).

**PR Treatment Prevented Macrophage Infiltration in the ON Tissue**

Blood-borne macrophage infiltration into the ON tissue is considered a primary response to tissue inflammation in rAION. The ED1 antibody was used for staining the newly synthesized (extrinsic) macrophage, and cells on the ON were counterstained with DAPI (Fig. 6A). IHC staining demonstrated that the PR treatment reduced the number of ED1-positive cells in ON sections. The number of ED1-positive cells per HPF was 1.22 ± 0.2 in the sham group, 56.2 ± 10.5 in the PBS-treated group, and 10.9 ± 7.3 in the PR-treated group (Fig. 6B). PR treatment reduced 5.2-fold of ED1-positive cells compared with PBS treatment in the rAION model (P < 0.05). These results indicate that PR treatment could reduce blood-borne macrophage infiltration in the ON tissue after ON infarct.

**PR Treatment Reduced Proinflammatory Cytokine Production and Enhanced Anti-Inflammatory Cytokine Production in rAION**

The levels of proinflammatory cytokines and anti-inflammatory cytokines in the ON tissue showed that the protein levels of iNOS, TNF-α and IL-1β were increased by 3.49-fold (P = 0.0038), 5.23-fold (P < 0.001), and 10.97-fold (P < 0.001), respectively, in the PBS-treated group compared with the sham group (Fig. 7). The IL-10 expression decreased by 2.81-fold (P = 0.048) after rAION induction compared with the sham group (Fig. 7B). PR treatment reduced the expression of the proinflammatory cytokines iNOS, IL-1β, and TNF-α by 4.53-fold (P = 0.002), 4.53-fold (P < 0.001), and 9.79-fold (P < 0.001), respectively, compared with PBS treatment (Fig. 7B). The IL-10 expression level increased by...
FIGURE 2. Evaluation of the recovery of injured optic nerves by FVEPs in the rAION model. (A) Representative FVEP tracings at 4 weeks after rAION induction in the sham group, the PBS-treated group, and the PR-treated group. (B) Bar charts demonstrating the P1-N2 amplitude. The values of amplitude are expressed as the mean ± SD in each group (n = 6 in each group). The P1-N2 amplitude was 2.3-fold higher in the PR-treated group than in the PBS-treated group (P < 0.05). The asterisk indicates P < 0.05 using the Kruskal–Wallis test.

FIGURE 3. Survival of RGCs in rAION-induced rats with PBS treatment or PR treatment at 28 days post rAION induction. (A) Representative of flat-mounted central retinas and the morphometry of RGCs in each group by Fluoro-Gold retrograde labeling at 4 weeks after rAION induction. (B) The RGC density of the central retina in each group. The data are expressed as the mean ± SD for each group (n = 6). The RGC density in the PR-treated group was 1.6-fold higher than that in the PBS-treated group (P < 0.05). The asterisk indicates P < 0.05 using the Kruskal–Wallis test.
1.67-fold \((P = 0.032)\) in the PR-treated group than in the PBS-treated group (Fig. 7B). IHC staining of ON sections also showed that IL-1\(\beta\) expression was higher in the PBS-treated group than those in the sham group and the PR-treated group (Fig. 7C). Besides, IL-10 expression in ON was less in the PBS-treated group than in the sham group and in the PR-treated group (Fig. 7C).

PR Treatment Enhanced M2 Microglia and Macrophage Polarization

The specific protein levels of Arg1, Fizz1, and CD206 (markers of M2 microglia and macrophage) of ON demonstrated that the level of Arg1 in the PR-treated group increased by 3.4-fold compared with in the PBS-treated group \((P = 0.01)\). The level

**Figure 4.** Analysis of RGC apoptosis in the RGC layer with TUNEL assay at 4 weeks after rAION induction. (A) Representative images of apoptotic cells in the RGC layers among each group, double stained. The apoptotic cells (TUNEL-positive cells) in green were stained with TUNEL staining, and the nuclei of RGCs in blue were labeled with DAPI staining. (B) Quantification of TUNEL-positive cells per high-power field. The amounts are expressed as the mean ± SD for each group \((n = 6)\). The number of TUNEL-positive cells in the PR-treated group was 2.8-fold lower than that in the PBS-treated group \((P < 0.05)\). The asterisk indicates \(P < 0.05\) using the Kruskal–Wallis test.

**Figure 5.** Cross-sectional OCT images of a retina crossing the optic disc. (A) Linear scan across the optic nerve head. (B, C) Representative ONW profiles of the sham, rAION, rAION + PR groups at days 1 and 3. (D) ONH width profile in the course of time. The ONH width was significantly lower in the PR-treated group than in the PBS-treated group at day 1 and day 3 post-rAION. Data are expressed as mean ± SD; *\(P \leq 0.05\), **\(P \leq 0.01\); \(n = 6\).
FIGURE 6. ED1 immunostaining of the ON section for evaluating inflammatory infiltration of macrophages. (A) Representative images of ED1 immunostaining in ON sections in each group. (B) Quantification of ED1-positive cells per high-power field. The data are expressed as the mean ± SD in each group (n = 6). PR treatment reduced 5.2-fold of ED1-positive cells compared with PBS treatment in the rAION model (P < 0.05). The asterisk indicates P < 0.05 using the Kruskal–Wallis test.

FIGURE 7. The levels of pro-inflammation cytokines iNOS, IL-1β, TNF-α and anti-inflammation cytokine IL-10 in ON tissue. (A) Analysis of expression levels of iNOS, IL-1β, TNF-α and IL-10 expression by using Western blotting. (B) Quantification of the protein bands of iNOS, IL-1β, TNF-α, and IL-10. (C) Representative IHC staining of ON section for IL-1β and IL-10 expression. Data are expressed as mean ± SD. Treatment with PR reduced the levels of the proinflammatory cytokines iNOS, IL-1β, and TNF-α by 4.53-fold, 4.53-fold, and 9.79-fold compared with PBS treatment. The IL-10 expression level increased by 1.67-fold in the PR-treated group than in the PBS-treated group (Fig. 7B). *P < 0.05, **P < 0.01, ***P ≤ 0.001.
of Fizz1 increased by 7.99-fold in the PR-treated group compared with in the PBS-treated group ($P = 0.0032$; Fig. 8A, 8B). IHC staining of ONs demonstrated that the number of CD206-positive cells increased in the PR-treated group compared with in the PBS-treated group (Fig. 8C). These results indicate that treatment with PR induced M2 microglia and macrophage polarization in the rAION model.

**PR Treatment Promoted Akt1 Activation to Induce M2 Polarization**

To verify whether PR treatment induces M2 polarization through Akt1 activation, the protein levels of Akt, p-Akt1, p-Akt2, and C/EBP$\beta$ were determined in the sham, PBS-treated, and PR-treated groups through Western blot. The expression levels of p-Akt1, p-Akt2, and C/EBP$\beta$ were determined at 3 days postinfarct (Figs. 9A, 9B). The levels of p-Akt1 and C/EBP$\beta$ increased by 3.4-fold ($P = 0.042$) and 5.89-fold ($P = 0.038$), respectively, in the PR-treated group compared with in the PBS-treated group ($P = 0.72$). This result demonstrates that PR treatment could induce Akt1 activation to enhance C/EBP$\beta$ expression in the rAION model.

To confirm the role of the Akt1-dependent signaling pathway in macrophage and microglia polarization, we administered intravitreal injections of an Akt inhibitor (LY294002) in the PR-treated group to detect CD206- (M2 microglia and macrophage markers) positive cells in ON sections. The results demonstrated that the Akt inhibitor eliminated the effect of PR on M2 polarization (Fig. 9C). Enhanced M2 microglia and macrophage polarization suggest that the neuroprotective effects of PR are Akt1 signaling dependent in the rAION model.

**DISCUSSION**

In this study, we observed that the administration of PR (50 mg/kg/day) for 3 days exerted neuroprotective effects on the rAION model, which was evidenced by both the FVEPs and morphometry of RGCs. The TUNEL assay of retina sections also proved that apoptotic RGCs decreased after PR treatment in the rAION model. OCT findings demonstrated that PR treatment reduced ONH edema in the acute phase of rAION. We also found that macrophage infiltration into the ON tissue was inhibited by PR treatment. Moreover, PR treatment reduced the proinflammatory cytokine expression and induced anti-inflammatory cytokine expression in the rAION model. Remarkably, PR treatment induced M2 microglia and macrophage polarization in the ON after rAION induction. We observed that PR treatment maintained Akt1 activation and induced C/EBP$\beta$ expression in the rAION model. Inhibition of Akt1 activation in PR-treated rats resulted in the suppression of M2 polarization after ON infarct.

This study is the first to report on the neuroprotective effects of PR on the rAION model. Notably, PR treatment showed protective effects preventing RGC apoptosis after ON infarct. A study demonstrated that PR attenuated N-methyl-D-aspartic acid-induced apoptosis and RGC damage through the JNK/p38 MAPK pathway. Other neuroprotective effects of PR are mediated through the preservation of PI3k/Akt signaling. In addition, some studies have proven that the activation of the PI3K–Akt signaling pathway exerts protective effects on injured RGCs. The PI3K–Akt signaling pathway is activated in response to environmental stimuli, such as cytokines/chemokines and hormones, to regulate cell survival, proliferation, and differentiation. In this study, we observed that treatment with PR maintained Akt1 activation after ON infarct. However, no difference was
observed in Akt2 activation between PBS and PR treatment. A study demonstrated that rapamycin-induced activation of autophagy and the PI3K/Akt1/mTOR/CREB pathway(s) are crucial in the neuroprotection of amyloid-beta-insulted hippocampal neurons in a rat model of Alzheimer disease. Thus, we suggest that Akt1 activation contributes to RGC protection more than Akt2 activation does in the rAION model.

AKT signaling is considered to be an activation-dampening signal that controls NO and inflammatory cytokine production after TLR signaling and promotes anti-inflammatory cytokines such as IL-10. However, PI3K activates downstream kinase AKT that may exist as three different isoforms, namely AKT1, AKT2, and AKT3. A study also demonstrated that AKT1 and AKT2 isoforms play opposing roles in macrophage polarization. AKT1 KO macrophages demonstrate enhanced iNOS and IL-12 production and bacterial clearance. These effects were mediated by the induction of the pro-M1 factor miR-155 that suppresses target CCAAT/enhancer-binding protein beta C/EBPbeta, a transcription factor that is a master regulator of M2 differentiation. By contrast, AKT2 deficiency has the opposite effect, resulting in macrophages that express C/EBPbeta and signature M2 markers such as Arg-1, YM1, and the regulatory cytokine IL-10. In this study, we demonstrated that PR induced Akt1 activation, not Akt2, and expression of the M2 polarization marker on day 3 postinfarct. We believe that Akt1 activation plays a key role in M2 polarization in the rAION model.

Macrophages can polarize and are classified according to their surface markers, cytokine and chemokine profiles, and functions. They have anti-inflammatory effects and can reduce immune reactions by releasing cytokines as well as increasing inflammation and stimulating the immune system. M1 macrophages secrete proinflammatory cytokines (e.g., TNF-z, IL-1β, and IL-6) and free radicals (iNOS) and have proinflammatory, bactericidal, and phagocytic effects. M2 macrophages produce anti-inflammatory cytokines (e.g., IL-10) and growth factors, such as platelet-derived growth factor, transforming growth factor beta 1, and vascular endothelial growth factor, and exert anti-inflammatory, proangiogenesis, and wound-healing effects.

Our previous studies have demonstrated that microglia and macrophage phenotype switching from M1 to M2 reduced proinflammatory cytokine expression after ON infarct, such as for TNF-z and IL-1β to prevent the subsequent cytokine-induced ON injuries. We believe that treatment with PR can induce M2 microglia and macrophage polarization to reduce ON inflammation after ON infarct.

In summary, we observed that treatment with PR effectively reduced ONH edema, macrophage infiltration, ON inflammation, and RGC apoptosis in the rAION model. PR treatment can maintain Akt1 activation, and activation of Akt1 is required to trigger M2 polarization after ON infarct. These novel findings provide insight into the role of PR in neuroprotection and the role of Akt1 in microglia and macrophage polarization. Through different mechanisms, this alternative therapy may serve as a potential strategy in the treatment of ischemic ON injury.

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References


