Cellular and Molecular Biology

Journal homepage: www.cellmolbiol.org

Influences of Rapamycin on Retinal Ganglion Cells in Rats with Acute High Intraocular Pressure Through Regulating COX-2

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ARTICLE INFO

Original paper

Article history:
Received: September 11, 2021
Accepted: December 19, 2021
Published: February 28, 2022

Keywords:
acute high intraocular pressure, rapamycin, COX-2, apoptosis, inflammation

ABSTRACT

The study aimed to explore the influences of rapamycin on the retinal ganglion cells in rats with acute high intraocular pressure through regulating cyclooxygenase-2 (COX-2). 36 Sprague-Dawley rats were randomly assigned to the normal group (n=12), model group (n=12) and rapamycin group (n=12). The rats in the normal group were normally fed, those in the model group were prepared the model of acute high intraocular pressure and injected with normal saline, and those in the rapamycin group were given rapamycin. At 7 d after the operation, sampling was performed. The expressions of COX-2 and Caspase-3 were detected via immunohistochemistry, and their protein expressions were determined using Western blotting (WB). Quantitative polymerase chain reaction (qPCR) was conducted to measure the messenger ribonucleic acid (mRNA) expression levels, and cell apoptosis was evaluated using terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay. The content of interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α) was determined via enzyme-linked immunosorbent assay (ELISA). Compared with those in the normal group, the positive expression levels rose substantially in the other two groups, and those in the rapamycin group were notably lower (p<0.05). The relative protein expression levels in the model group and rapamycin group were higher, and the rapamycin group exhibited remarkable decreases (p<0.05). In comparison with the normal group, the other two groups had considerably raised relative mRNA expression levels and those in the rapamycin group were lower (p<0.05). The cells in the model and rapamycin groups had a higher apoptosis rate, and the apoptosis rate of cells in the rapamycin group was lower (p<0.05). Compared with that in the normal group, the content of IL-6 and TNF-α was elevated in the other two groups and their content in the rapamycin group was lower. Rapamycin inhibits COX-2 to repress inflammation and apoptosis, thereby exerting a protective effect on the retinal ganglion cells in rats with acute high intraocular pressure.

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Introduction

Acute high intraocular pressure is one of the important pathological processes in glaucoma, and such a pathological high intraocular pressure can cause vision loss and even blindness in patients, which has been considered as the second leading cause of blinding eye diseases (1, 2). Therefore, efficacious treatment of acute high intraocular high pressure in the onset of glaucoma is of great significance for treating glaucoma, alleviating the pathological injuries of retinal ganglion cells, and protecting them.

In the onset of glaucoma, acute high intraocular pressure can induce multiple pathological reactions such as inflammation and cell apoptosis, resulting in grave pathological injuries to retinal ganglion cells. Studies have demonstrated that (3-6) cyclooxygenase-2 (COX-2) plays a pivotal role in inflammation, cell apoptosis and other pathological injuries and can promote vasodilation at injurious sites and inflammatory responses and aggravate cell apoptosis and optic nerve damage. The aberrantly highly expressed COX-2 in acute high intraocular pressure regulates inflammation and cell apoptosis to hinder the repair of retinal ganglion injury.

Rapamycin, as a clinically commonly applied

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immunosuppressant, has favorable anti-inflammation and anti-apoptosis effects (7, 8). The present study, therefore, seeks to observe its influences on the retinal ganglion cells in rats with acute high intraocular pressure through the regulation of COX-2.

Materials and methods

Laboratory animals and grouping

A total of 36 female Sprague-Dawley rats weighing (200±10) g were randomly divided into the normal group (n=12), model group (n=12) and rapamycin group (n=12). All the rats were raised in the Laboratory Animal Center, and given with purified water and adequate feed daily in a 12/12 h light-dark cycle, as required by the Laboratory Animal Ethics Committee.

Main reagents

Rapamycin(CST, USA), anti-COX-2 antibody and anti-Caspase-3 antibody (Abcam, USA), immunohistochemistry kit (Fuzhou Maxim Biotech. Co., Ltd.), enzyme-linked immunosorbent assay (ELISA) kit (Wuhan Boster Biological Technology, CO., Ltd.), terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) kit (Fuzhou Maxim Biotech. Co., Ltd.), AceQ quantitative polymerase chain reaction (qPCR) SYBR Green Master Mix kit and HiScript II Q RT SuperMix for qPCR [+genomic deoxyribonucleic acid (+gDNA) wiper] kit (Vazyme Biotech, Nanjing), optical microscope (Leica DMI 4000B/DFC425C, Germany), and fluorescence qPCR instrument (ABI 7500, USA).

Modeling and treatment in each group

The rats were first intraperitoneally injected with 10% chloral hydrate at a dose of 5 mL/kg. Following successful anesthesia, they were placed in the prone position, and the anterior chamber was punctured along the corneal limbus using a side port paracentesis knife under a surgical microscope, and accessed by an injector to draw about 0.1 mL of aqueous humor along with the side port paracentesis knife. Subsequently, about 0.1 mL of the viscoelastic agent was infused, and the model of acute high intraocular pressure was obtained.

The rats in the normal group were normally fed, and after the acute high intraocular pressure model was established in the rats as above, the rats in the model group were intraperitoneally injected with normal saline, while those in the rapamycin group with rapamycin at 2 mg/kg. After intervention for 7 consecutive days, the materials were sampled.

Sampling

First, 6 anesthetized rats in each group were fixed by perfusing paraformaldehyde until the limbs of rats became stiff. Then, the eyeball tissues were removed, soaked in paraformaldehyde and fixed for an additional 48 h. Besides, the eyeball tissues of the remaining 6 rats anesthetized in each group were directly sampled, and preserved in EP tubes in an ultra-low temperature refrigerator for later use.

Immunohistochemistry

The paraffin-embedded tissues were first sectioned to be 5 μmthick, extended in warm water at 42°C, mounted, and baked to prepare paraffin-embedded tissue sections. Then, the paraffin-embedded tissue sections were soaked successively in xylene solution and gradient ethanol for routine deparaffinization and hydration, respectively. Subsequently, the resulting sections were immersed in citrate buffer and subjected to complete antigen retrieval through heating for 3 min and braising for 5 min for 3 times in a microwave oven. Afterward, the rinsed tissue sections were added dropwise with endogenous peroxidase blocker, reacted for 10 min, rinsed and sealed using goat serum in drops for 20 min. After the goat serum blocking solution was discarded, the tissue sections were incubated with the anti-COX-2 and anti-Caspase-3 primary antibodies (1:200) in a refrigerator at 4°C overnight. On the next day, the rinsed sections were added dropwise with the secondary antibody solution, reacted for 10 min, fully rinsed, reacted with streptomycin avidin-peroxidase solution for 10 min, and added with DAB in drops for color development. Finally, the cell nuclei were counter-stained with hematoxylin, and the sections were sealed and observed.

Western blotting (WB)

The cryopreserved eyeball tissues were first added with lysis buffer, bathed on ice for 1 h and centrifuged at 14,000g in a centrifuge for 10 min. Then, proteins were quantified by the BCA method, and their concentration in the tissues was calculated based on

the absorbance and standard curves of proteins measured using a microplate reader. Subsequently, the proteins in the tissue specimens were denaturalized and isolated via SDS-PAGE which was terminated when the marker protein stayed at the bottom of the glass plate in a straight line. Later, the isolated proteins were transferred onto a PVDF membrane and reacted with the sealing solution for 1.5 h, incubated successively with the anti-COX-2 primary antibody (1:1,000), anti-Caspase-3 primary antibody (1:1,000) and secondary antibodies (1:1,000), and rinsed. Finally, the proteins were reacted with a chemiluminescent reagent for 1 min in the dark for complete image development.

qPCR assay

The preserved eyeball tissues were added to the ribonucleic acid (RNA) extraction reagent to extract the total RNAs therein. Then, the extracted total RNAs were reversely transcribed into complementary DNAs (cDNAs) using the reverse transcription kit. Subsequently, qPCR was completed according to the following steps: reaction at 53°C for 5 min, pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 s and annealing at 62°C for 30 s, for 35 cycles using the designed reaction system (20 μL). Finally, the differences in the expressions of target genes were computed based on the calculated ΔCt value. The primer sequences are detailed in Table 1.

Table 1. List of primer sequences

Name	Primer sequence
COX-2	Forward: 5' TAAGCGTGGCAGTGTCTTAG 3' Reverse: 5' GTGCACCCATGCTCCGAGGT 3'
Caspase-3	Forward: 5' TGCCTTAATGGCAGTGTCTTAG 3' Reverse: 5' TTCTAAGGCCTTATGCCGAATA 3'
GAPDH	Forward: 5' ACGGCAAGTTCAACGGCACAG 3' Reverse: 5' GAAGACGCCAGTAGACTCCACGAC 3'

TUNEL assay

The tissues embedded in paraffin in advance were first made into 5 μm-thick sections, placed in warm water at 42°C for extending, mounted, baked and prepared into paraffin-embedded tissue sections. Then, these sections were routinely de-paraffinized and hydrated through immersing successively in xylene solution and gradient ethanol. Subsequently, the resulting sections were added dropwise with TdT reaction solution, reacted in the dark for 1 h, and incubated with deionized water in drops for 15 min to terminate the reaction. Afterward, the sections were added dropwise with hydrogen peroxide to block the activity of endogenous peroxidases, reacted with working solution added in drops for 1 h, rinsed, and added with DAB solution in drops for color development. Finally, the rinsed sections were sealed and observed.

Detection of interleukin (IL)-6 and tumor necrosis factor-alpha (TNF-α) using ELISA

The fresh eyeball tissues were taken out and fully ground to pieces in a grinder. Then, according to the instructions of the ELISA kit, the sample and standard were separately loaded into a plate, and the plate was added with biotinylated antibody and enzyme-conjugated substance working solution, and washed. Finally, the products were detected at 450 nm in a microplate reader.

Statistical methods

SPSS 20.0 software was employed for statistical analysis. *t*-test, corrected *t*-test and nonparametric test were performed for data conforming to normal distribution and homogeneity of variance, those conforming to normal distribution and heterogeneity of variance, and those dissatisfying normal distribution and homogeneity of variance, respectively. Rank sum test was used for ranked data, and chi-square was adopted to test enumeration data.

Results and discussion

COX-2 and Caspase-3 expressions detected via immunohistochemistry

As shown in Figure 1, positive cells are tan, and there were fewer cells positive for COX-2 and Caspase-3 in the normal group, but more in the other two groups. The statistical results revealed that the average optical density of the cells positive for COX-2 and Caspase-3 rose notably in the model group and rapamycin group compared with that in the normal group, showing a statistically significant difference ($p<0.05$), while the average optical density of the positive cells in rapamycin group was considerably lower than that in the model group, with a statistically significant difference ($p<0.05$) (Figure 2).

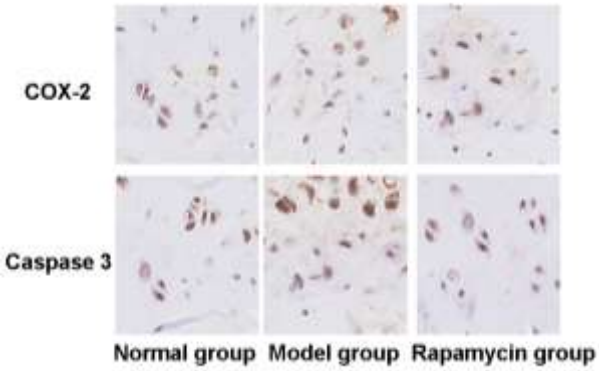

Figure 1. Immunohistochemistry results (×200).

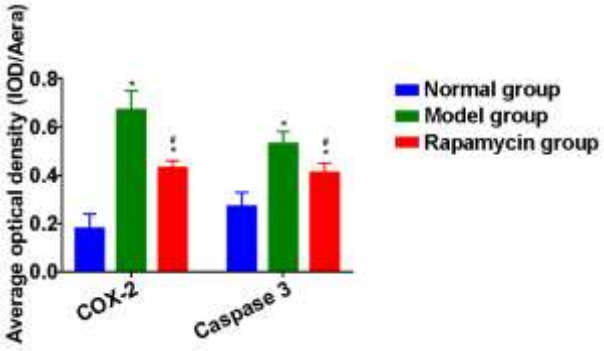

Figure 2. The average optical density of positive cells in each group. Note: *p<0.05 vs. normal group, and #p<0.05 vs. model group.

WB results

As shown in Figure 3A, the normal group had fewer expressed COX-2 and Caspase-3 proteins, while there were more expressed COX-2 and Caspase-3 proteins in the other two groups. According to the statistics (Figure 3B), the relative protein expression levels of COX-2 and Caspase-3 in the model group and rapamycin group were substantially higher than those in the normal group, displaying statistically significant differences (p<0.05). Moreover, their relative protein expression levels in the rapamycin group were notably lower than those in the model group, and the differences were statistically significant (p<0.05).

qPCR assay results

In comparison with those in the normal group, the relative messenger RNA (mRNA) expression levels of COX-2 and Caspase-3 rose markedly in the other two groups, with statistically significant differences (p<0.05), whereas their relative mRNA expression levels in the rapamycin group were prominently lower than those in the model group, showing statistically significant differences (p<0.05) (Figure 4).

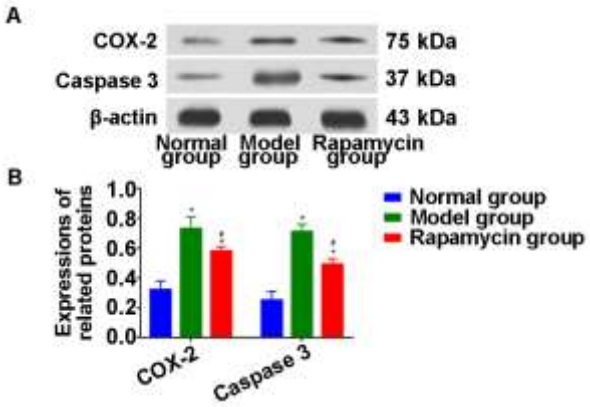

Figure 3. Expressions of related proteins detected via WB. A: Protein expressions of COX-2 and Caspase-3 detected via WB. B: Relative protein expression levels of COX-2 and Caspase-3 measured using WB. Note: *p<0.05 vs. normal group, and #p<0.05 vs. model group.

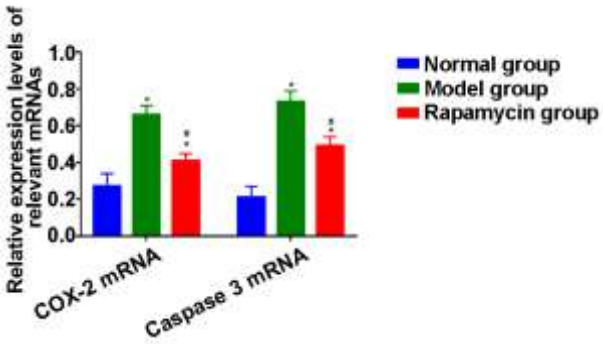

Figure 4. Relative expression levels of relevant mRNAs in each group. Note: $^{*}p$<0.05 vs. normal group, and $^{\#}p$<0.05 vs. model group.

Cell apoptosis rate measured via TUNEL assay

As shown in Figure 5A, TUNEL-positive apoptotic cells are tan, and the normal group had fewer apoptotic cells than the other two groups. Compared with that in normal, the cell apoptosis rate was markedly raised in both the model group and rapamycin group, with a statistically significant difference (p<0.05), whereas rapamycin exhibited a lower cell apoptosis rate than the model group, and the difference was statistically significant (p<0.05) (Figure 5B).

Content of IL-6 and TNF-α determined using ELISA

The content of IL-6 and TNF-α rose notably in the model and rapamycin groups compared with that in

the normal group, displaying a statistically significant difference (p<0.05), while their content in the rapamycin group was substantially lower than that in the model group, with a statistically significant difference (p<0.05) (Figure 6).

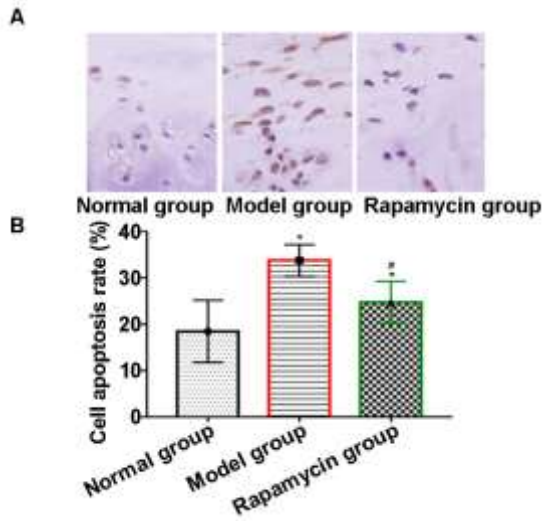

Figure 5. Cell apoptosis rate in each group. A: Apoptotic cells detected via TUNEL assay. B: Comparison of cell apoptosis rate among all groups. Note: *p<0.05 vs. normal group, and #p<0.05 vs. model group.

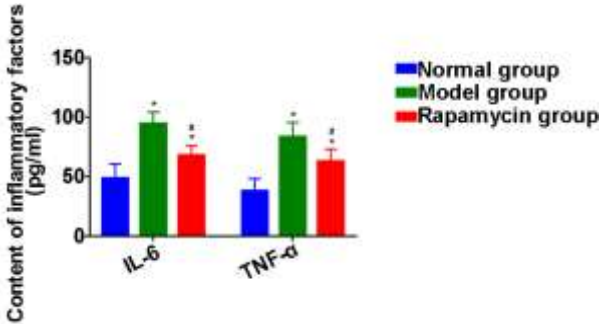

Figure 6. Content of inflammatory factors in each group. Note: *p<0.05 vs. normal group, and #p<0.05 vs. model group.

As a clinically researched blinding disease, glaucoma tends to cause vision loss and even blindness in patients, and studying the physiological and pathological reactions in glaucoma has important implications for the clinical treatment of glaucoma. Acute high intraocular pressure, one crucial pathological process in glaucoma, often results in retinal ganglion cell apoptosis and even necrosis, exacerbating optic nerve damage (9-11). Studies have unraveled that (12,13) inflammation and cell apoptosis are vital pathological reactions in acute high intraocular high pressure, and in acute high intraocular pressure injury, high intraocular pressure-induced vascular injury and other damage factors cause the increase in the release of local inflammatory factors and other cytokines in the optic nerve. Of them, inflammatory factors IL-6 and TNF-α can further activate several downstream signaling pathways to exert a crucial regulatory effect on cell apoptosis, abnormally raising the expression of apoptosis effector molecule Caspase-3 to mediate cell apoptosis. As a result, the optic nerve damage is worsened, which is not conducive to the repair of the optic nerve with glaucoma-induced injury. Additionally, the important cyclooxygenase COX-2 can regulate inflammation and cell apoptosis in organisms. According to the studies (14-16), COX-2 is closely associated with oxygen-free radicals and modulates their generation and release to participate in injury. Owing to the action of injurious factors, COX-2 can catalyze oxygen free radicals to be involved in the retinal ganglion cell membrane, thereby exerting pivotal regulatory effects on the physiological functions and post-injury repair of retinal ganglion cells. COX-2 also bears a close relationship with prostaglandin, and regulates its production and release to regulate inflammation, further modulating such inflammatory factors as IL-6 and TNF-α and apoptosis effector molecule Caspase-3 to exert an important regulatory effect on the apoptosis of retinal ganglion cells (17-20). According to the results of the present study, COX-2 was highly expressed in the eyeball tissues of rats with acute high intraocular pressure, and inflammatory factors IL-6 and TNF-α and apoptosis effector molecule Caspase-3 were also aberrantly highly expressed, with a substantially elevated cell apoptosis rate, indicating that COX-2 plays a critical role in the eyeball tissues of rats with acute high intraocular pressure and that the abnormally high expressions of COX-2, IL-6, TNF-α and Caspase-3 cause the apoptosis of massive cells.

As an immunosuppressive agent commonly used in the clinic, rapamycin possesses potent anti-apoptosis and anti-inflammation effects. The present study found that after rapamycin intervention, the rats with acute high intraocular pressure had notably decreased expression levels of COX-2, IL-6, TNF-α and Caspase-3 in eyeball tissues, with a remarkably lowered cell apoptosis rate, implying that rapamycin

has favorable inhibitory effects on the apoptosis and inflammation probably by inhibiting COX-2 expression. Therefore, it can be concluded that rapamycin represses COX-2 to inhibit inflammation and apoptosis, thereby protecting the retinal ganglion cells in rats with acute high intraocular pressure.

Acknowledgments

Not applicable.

Funding

Natural Science Foundation of Tianjin(Grant number: 16JCQNJC12700); National Natural Science Foundation of China(Grant number: 81500745).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contribution

YG wrote the manuscript. YG and XF were responsible for the establishment of an animal model. BW performed Immunohistochemistry. HL performed Western blotting and qPCR. XS performed a TUNEL assay. YK performed ELISA. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the ethics committee of Tianjin Eye Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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