Quercetin Regulates Inflammatory Response of Retinal Ganglion Cells by Affecting the Tumor Necrosis Factor Receptor Associated Factor 6/Transforming Growth Factor-β-Activated Kinase-1 Signaling Pathway

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To investigate the protective effect of quercetin on the lipopolysaccharide-induced inflammatory response of retinal ganglion cell line 5 cells and to explore its possible molecular mechanism. Retinal ganglion cell line 5 cells were cultured in vitro and assigned into blank control group, lipopolysaccharide (1.0 μg/ml) induction group, lipopolysaccharide+low dose (0.25 μg/ml) quercetin group, lipopolysaccharide+medium dose (0.5 μg/ml) quercetin group and lipopolysaccharide+high dose (1.0 μg/ml) quercetin group. The relative survival rate of retinal ganglion cell line 5 cells was determined by cell counting kit-8 assay and the levels of inflammatory factors interleukin-6 and tumor necrosis factor-alpha were detected using enzyme-linked immunosorbent assay kits. In addition, the levels of tumor necrosis factor receptor-associated factor 6, phosphorylation-transforming growth factor-beta-activated kinase 1 and transforming growth factor-beta-activated kinase 1 were measured by western blotting. The results of cell counting kit-8 assay revealed that quercetin could dramatically elevate the relative survival rate of retinal ganglion cell line 5 cells. The levels of inflammatory factors interleukin-6 and tumor necrosis factor-alpha in different quercetin groups were significantly lower than those in lipopolysaccharide induction group and the levels of interleukin-6 and tumor necrosis factor-alpha in cell supernatant declined with the increase of quercetin concentration in a concentration-dependent manner. In addition, the expression levels of tumor necrosis factor receptor-associated factor 6 and phosphorylation-transforming growth factor-beta-activated kinase 1 in different quercetin groups were remarkably lower than those in lipopolysaccharide induction group and they also declined with the increase of quercetin concentration in a concentration-dependent manner. Quercetin displays a protective effect against the lipopolysaccharide-induced inflammatory response of retinal ganglion cell line 5 cells and its mechanism is related to suppression of the activation of the tumor necrosis factor receptor-associated factor 6/transforming growth factor-beta-activated kinase 1 signaling pathway.

Key words: Quercetin, lipopolysaccharide, glaucoma, inflammatory response, retinal ganglion cell
Acid (NMDA)-induced damage in BV-2 cells, whose mechanism may be related to its inhibition on the Tumor Necrosis Factor Receptor-Associated Factor 6 (TRAF6)/Transforming Growth Factor-Beta-Activated Kinase 1 (TAK1) signaling pathway. In this study, therefore, the inflammatory cell model was established using RGCs cultured \textit{in vitro} to study the protective effect of quercetin against the Lipopolysaccharide (LPS) induced inflammatory response of RGC-5 cells and to preliminarily explore its possible mechanism.

RGC-5 cells were purchased from Wuhan Biofavor Biotech Co., Ltd., Quercetin was purchased from Shaanxi Zhongxin Biotechnology Co., Ltd., Dulbecco's Modified Eagle Medium (DMEM) was purchased from Shanghai Guge Biological Co., Ltd., trypsin was purchased from Sigma (USA), Cell Counting Kit-8 (CCK-8) kits were purchased from MSK, Bicinechonic Acid (BCA) protein concentration assay kits (batch No. P0012S-07) were purchased from Shanghai Guge Biological Co., Ltd., and Interleukin-6 (IL-6) and Tumor Necrosis Factor-Alpha (TNF-α) assay kits were purchased from Shanghai Huamei Biotechnology Co., Ltd., Electrogeneated Chemiluminescence (ECL) solution was bought from Sigma, Beta (β)-actin, TRAF6, TAK1 and Phosphorylation-Transforming Growth Factor-β-Activated Kinase 1 (p-TAK1) antibodies were all bought from Cell Signaling Technology (USA), goat anti-rabbit Immunoglobulin G (IgG) secondary antibodies were bought from LI-COR Biosciences (USA), a single-person clean bench and a CB15C02 cell incubator were bought from Beijing Liuyi Instrument Factory, a Victor3 1420 Multilable Counter microplate reader was bought from BD (USA), an HD-3000 gel imager was bought from Shanghai Shangtian Precision Instrument Co., Ltd., and a 7230 G ultraviolet visible spectrophotometer was bought from Shanghai Jinghua Technology Instrument Co., Ltd. RGC-5 cells were cultured in complete DMEM in a 5 % CO₂ constant humidity incubator at 37°. When about 90 % of the cells adhered to the wall, the old medium was discarded and the cells were washed with sterile PBS and digested with trypsin. After the cells became round, the digestion was terminated with medium, followed by centrifugation and resuspension. Finally, the cells were sub-cultured at 1:3 ratio. RGC-5 cells in the logarithmic growth were harvested, digested, centrifuged and resuspended and the cell density was adjusted. Then the cells were inoculated into a 96-well plate (1×10⁴ cells/well) and cultured for 24 h. After the medium was discarded, quercetin at different concentrations (0.25, 0.5 and 1.0 mg/ml) and LPS (1.0 μg/ml) were added for intervention for 24 h. Then the medium in the plate was aspirated and serum-free medium containing 10 μl of CCK-8 reagent was added into each well for culture for 1 h. Finally, the optical density of each well was measured at a wavelength of 450 nm using a microplate reader. RGC-5 cells in the logarithmic growth were harvested, washed, digested and resuspended. They were inoculated into a 6-well plate (5×10⁵ cells/well), with 6 replicates in each group, cultured in the 5 % CO₂ incubator at 37° overnight and then subjected to intervention with quercetin at different concentrations (0.25, 0.5 and 1.0 mg/ml) and LPS (1.0 μg/ml) for 24 h. The cell supernatant was harvested to detect the levels of inflammatory factors IL-6 and TNF-α using ELISA kits in strict accordance with the instructions. The cells were lysed and the protein concentration was measured. The lysate was added with loading buffer and heated at 100° for 10 min. Then 40 μg of proteins were loaded in each well and separated by 12 % Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (voltage: 70 V for spacer gel and 120 V for separation gel). After separation, the protein was transferred onto the nitrocel lulose membrane at 275 mA for 60 min and the membrane was blocked with 5 % skim milk powder at room temperature for 1 h. The primary antibodies were used for incubation overnight and the secondary antibodies were used for incubation at room temperature for 1 h. Finally, the membrane was washed for 3 times followed by color development and imaging. Statistical Package for the Social Sciences (SPSS) 26.0 software was used for statistical analysis. Normally distributed measurement data were expressed as mean±standard deviation and compared between two groups using Least Significant Difference (LSD)-t test in analysis of variance, p<0.05 was considered statistically significant. The cell survival rate in LPS group was significantly lower than that in control group (p<0.05), indicating that LPS can inhibit the proliferation of RGC-5 cells. After intervention with low, medium and high dose quercetin, the cell proliferation was obviously enhanced and the relative survival rate also rose with the increase in the drug concentration, showing concentration dependence (fig. 1). The levels of IL-6 and TNF-α in cell supernatant in LPS group were significantly higher than those in control group (p<0.05), indicating that LPS can induce the release of IL-6 and TNF-α in RGC-5 cells. After intervention with low, medium and high dose quercetin, the levels of inflammatory factors in RGC-5 cells significantly
declined and they were also decreased with the increase in the drug concentration, showing concentration dependence (fig. 2). The levels of TRAF6 and p-TAK1 in LPS group were higher than those in control group (p<0.05). After intervention with low, medium and high dose quercetin, the levels of TRAF6 and p-TAK1 in RGC-5 cells declined with statistically significant differences and they were also decreased with the increase in the drug concentration, showing concentration dependence (fig. 3). The retina is an important photoreception organ able to perceive visual signals followed by further processing. In the case of damage, however, visual disorders or blindness will be caused. RGCs are cells responsible for retinal signal output, which process and transmit the signals through the retinal neural circuit to the visual center in the form of action potentials, further producing normal vision. Under a variety of stimuli, retinal oxidative stress will be caused, the Nuclear Factor Kappa B (NF-κB) signaling pathway will be activated, the transcription of downstream target genes will be initiated and the interaction between inflammatory factors (IL-6 and TNF-α) and adhesion molecules will be induced, further leading to pathological processes such as retinal capillary occlusion and non-perfusion, endothelial injury and neovascularization. Therefore, the eye can be well protected from damage by inhibiting the inflammatory response of RGCs. Traditional Chinese medicine has definite efficacy in the treatment of glaucoma. Quercetin is a natural flavonoid active substance and flavonoids can ameliorate the inflammatory factor-induced inflammatory injury of neurons, so quercetin has a good pharmacological effect in the prevention and treatment of inflammatory diseases. In the present study, the effects of quercetin at different concentrations on RGC-5 cells were observed by CCK-8 assay. It was found that 0.1-2.0 mg/ml quercetin had no significant effects on the proliferative activity and morphology of RGC-5 cells. Therefore, RGC-5 cells were treated with 0.25, 0.5 and 1.0 mg/ml quercetin in this study. The inflammatory cell model was established by LPS in vitro and the protective effect of quercetin against inflammatory injury in RGC-5 cells was observed. The results revealed that quercetin could greatly increase the relative survival rate of RGC-5 cells and the inflammatory factors IL-6 and TNF-α could be...
obviously reduced by different doses of quercetin in a concentration dependent manner. TRAF6, an adapter protein, can transmit signals mediated by a variety of receptors on the membrane, including those mediated by the toll-interleukin-1 receptor family\[14\]. LPS can cause the ubiquitination of TRAF6 and then TRAF6 together with TAK1 forms complexes to further activate IkappaB kinase kinases (IKKs) and promote the transfer of NF-κB to the nucleus, ultimately resulting in inflammatory response, including the increase in the levels of IL-6 and TNF-α. It can be seen that inhibiting the TRAF6/TAK1 signaling pathway can reduce the production of inflammatory factors and alleviate the inflammatory injury of cells. Besides, the expression of TRAF6 shows an increasing trend in injury of RGCs, which may be related to the pathogenesis of glaucoma\[15\]. In the present study, it was found that quercetin could significantly inhibit the expressions of TRAF6 and p-TAK1 and weaken the activation of the TRAF6/TAK1 signaling pathway, further reducing the inflammatory response. In conclusion, quercetin can exert an anti-inflammatory effect through inhibiting the activation of the TRAF6/TAK1 signaling pathway and reducing the inflammatory factors IL-6 and TNF-α.

Conflict of interests:

The authors declared no conflicts of interest.

REFERENCES