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Apigenin prevents TNF-α induced apoptosis of primary rat retinal ganglion cells

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Abstract

TNF-α has recently been identified to be a mediator of retinal ganglion cell (RGC) death, while glial cells are relatively protected against this death stimulus. Exposure of RGCs to TNF-α is thought to contribute to RGC apoptosis. Apigenin is a flavone with powerful anti-inflammatory properties that exists naturally in various plants and Chinese medicine. In our study, MTT assays showed that apigenin significantly inhibited the decrease of RGC viability induced by TNF-α in a dose-dependent manner. Pretreatment with apigenin prevented TNF-α-induced apoptosis in a dose-dependent manner as shown by flow cytometry. The production of ATP and the total oxygen uptake were also promoted after apigenin administration. TNF-α stimulation led to a significant reduction of bcl-2 and enhancement of bax, which was reversed by apigenin treatment. Apigenin treatment also alleviated the increased caspase-3 activity induced by TNF-α. Moreover, luciferase reporter assay indicated that apigenin dose-dependently decreased NF-κB activation induced by TNF-α, but had no significant effect on activation of AP-1. Collectively, these data demonstrated that apigenin alleviated TNF-α-induced apoptosis through inhibition of caspase-dependent apoptotic pathway and activation of nuclear factor-kappaB. Therefore, apigenin may be developed as an anti-apoptotic drug to treat retinopathy.

Key words: TNF-α, apigenin, retinal ganglion cells, apoptosis, NF-κB.

Introduction

Glaucoma is a complex disease characterized by the degeneration of the optic nerve and the apoptosis of retinal ganglion cells (RGCs) (1, 2). It is believed that the death of RGCs is associated with high intraocular pressure (IOP), therefore conventional glaucoma therapy has focused on lowering IOP (3). However, IOP reduction alone is not sufficient to prevent the glaucomatous damage even if IOP can be substantially lowered (4). Thus, it is urgent to develop new and effective approach to prevent RGC death.

In the central nervous system, excitotoxic and ischemic injuries can increase the production of the cytokines such as tumor necrosis factor-α (TNF-α). These receptors mediate cellular proliferation, differentiation, apoptosis and survival (5, 6). Among them, TNF-α has been shown to play a pivotal role through regulation the pro- and anti-apoptotic signaling pathways and cell proliferation and inflammation (7, 8). Increasing studies have shown that TNF-α and its receptor-1 are increased in RGCs of glaucoma patients (9). *In vitro*, ischemia and elevated hydrostatic pressure lead to up-regulated TNF-α production and then induce apoptosis in RGCs (10). *In vivo*, TNF-α intravitreal injection induces axonal degeneration and delayed loss of RGC cell bodies. Apigenin is a natural plant flavonoid that is contained in vegetables, fruits, herbs and wines that are brewed from natural ingredients. It has been recognized as a bioactive flavonoids which possess anti-cancer, antioxidant, and anti-inflammatory properties (11, 12). Studies both *in vitro* and *in vivo* have shown that apigenin could scavenging free radicals and alleviate kainic acid-induced excitotoxicity by quenching reactive oxygen species in hippocampal neurons (13, 14). In the present study, we investigated the neuroprotective effects of apigenin against TNF-α-induced apoptosis in primary cultured RGCs.

Materials and methods

Reagents

Apigenin (HLPC content 98%) was purchased from Shanghai Winherb Medical S & T Development Co. Ltd (Shanghai, China). Dulbecco's modified Eagle media (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). ATP assay kit was obtained from Beyotime (Jiangsu, China). The Annexin-V-FITC Apoptosis Detection Kit was purchased from BD Biosciences, USA. The Lipofectamine 2000 reagent was obtained from Invitrogen Life Technologies (Grand Island, NY, USA). The luciferase reporter assay system and avian myeloblastosis virus reverse transcription system were obtained from Promega (Madison, WI, USA).

Cell Culture

RGCs were prepared according to the method described previously (15), with minor modifications. All of the procedures described in this study were performed in accordance with the Guide for the Care and Use of Laboratory Animals. Briefly, neonatal Sprague Dawley rats were sacrificed by decapitation, and their eyes were rapidly removed and immersed in a calcium- and magnesium-free (CMF) salt solution (0.1 M Dulbecco's phosphate-buffered saline (PBS); calcium- and magnesium-free; Gibco, Grand Island, NY).

Approximately 30 eyes were harvested for each

experiment. The retinas were rapidly isolated and incubated at 37 °C for 25 min in CMF containing 0.1% trypsin. Then, cells were exposed for 5 min with antimacrophage antibody (mouse). The cell suspensions were then incubated for 30 min on a Petri dish coated with a goat antimouse immunoglobulin G (H + L chain) antibody. Suspension cells were harvested and incubated for 1 h in a dish coated with an anti-Thy-1.1 antibody. The cells that adhered to the dish were then trypsinized (0.1% trypsin for 10 min), then adhered cells were diluted to 1 x 10^5 cells/ml and placed on dishes or glass coverslips that had previously been coated with 50 mg/ml poly-L-ornithine. Cells were incubated in B27-supplemented neurobasal culture medium without serum. The cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C, and the medium was changed every 3 days. Cells were used after 5 days.

MTT Assay

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl) -2,5- diphenyltetrazolium bromide (MTT) assay. Briefly, RGCs were seeded in 96-well dishes at 1 × 104 to 2 × 104 cells per well, and pretreated with or without Apigenin for 2h. Each well was then supplemented with 10 μL MTT (Sigma) and incubated for 4 h at 37 °C. The medium was then removed, and 150 μL dimethyl sulfoxide (Sigma) were added to solubilize the MTT formazan. The optical density was read at 490 nm.

Flow Cytometry

To estimate the number of apoptotic cells, RGCs were fluorescently labeled by addition of 20 μL of binding buffer, 5 μL of Annexin V-FITC and 5 μL of propidium iodide. After the incubation at room temperature in dark for 15 min, cells were applied to flow cytometry analysis. A minimum of 10,000 cells in the gated region was analyzed by BD FACS Calibur Flow Cytometer. Results were interpreted by the percentage of total cells appearing in each quadrant.

ATP assay

The level of intracellular ATP was determined using the ATP Bioluminescence Assay Kit. Cultured cells were lysed with a lysis buffer, followed by centrifugation at 12,000 × g for 1 min at 4°C. Finally, the level of ATP was determined by mixing 50 μl of the supernatant with 50 μl of luciferase reagent, which catalyzed the light production from ATP and substrate. The emitted light was linearly related to the ATP concentration and measured using a microplate luminometer.

Oxygen consumption

To evaluate the ability of cellular oxygen consumption (VO2) during drug treatment, the Micro Respirometry System (Strathkelvin, Mitocell S200) was used to measure oxygen content of culture media. Before the assay, primary RGCs just isolated from rats were pretreated in low oxygen conditions for 2 h in suspension culture with drugs.

Real-time RT-PCR assay

RGCs were cultured and treated as described above. The total RNA was extracted using Trizol reagent. First-strand cDNA synthesis was performed using 1ug of total RNA and an avian myeloblastosis virus reverse transcription system. The primers were designed using primer express software (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR was performed using the SYBR Green PCR Master Mix and ABI Prism 7300 Sequence Detection System (Applied Biosystems). All data were analysed using the expression of the gene encoding β-actin as a reference. The sequences of the primers used are available upon request. The gene primers were as follows: BCL-2 forward primer, 5'- AGCATGCGACCTCTGTTTGA-3' and reverse primer, 5'- TCACTTGTGGCCCAGGTATG-3'. Caspase-3 forward primer, 5'- ATGCAGTGTTGGTGTTGGTG-3' and reverse primer, 5'- AGTCCATCGACTTGCTTCCA-3'. Bax forward primer, 5'- AGTCCTCACTGCCTCACTCA-3' and reverse primer, 5'- CGTTCCCCATTCATCCCAGG-3'. The relative expression levels were calculated according to the formula $2^{-\Delta Ct}$, where ΔCt was the difference in threshold cycle (Ct) values between the target gene and the endogenous control.

Caspase Activity

RGCs treated or non-treated with apigenin and TNF-α were subjected to caspase activity assay as described before (16). In brief, protein extracts were harvested in isolation buffer (10 mM of Tris-HCl buffer, pH 7.6, 5 mM of MgCl2, 1.5 mM of potassium acetate, 2 mM of dithiothreitol) supplemented with protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany). General activities of caspase-3 were determined by enzymatic cleavage of chromophore p-nitroanilide (pNA) from the substrates N-acetyl-Asp-Glu-Val-Asp-pNA (DEVD-pNA). The proteolytic reaction was carried out in isolation buffer containing 50 μg of cytosolic protein and 50 μM specific caspase substrate. The reaction mixtures were incubated at 37 °C for 1 h, and measured by monitoring A405 using a 96-well plate reader.

Transient transfection and luciferase reporter assay

The luciferase reporter construct 3 × NF-κB-LUC (NF-κB responsive elements) or AP-1 was transiently transfected into RGCs grown in 24-well plates respectively using the lipofectamine-2000 reagent according to the manufacturer's instructions. The luciferase reporters construct driven by three copies of the NF-κB response elements from B. M. Forman (Department of Gene Regulation and Drug Discovery, Beckman Research Institute of City of Hope National MedicalCenter, Duarte, CA, USA). A plasmid expressing the gene encoding β-galactosidase driven by the cytomegalovirus (CMV) promoter (Clontech Laboratories, Palo Alto, CA, USA) was simultaneously cotransfected as an internal control. The medium was replaced 4 h after transfection. After 24 hours transfection, RGCs were treated with the indicated concentrations of apigenin and TNF-α for additional 24 h and harvested for luciferase reporter assays as described previously (17).

Statistical analysis

Differences between groups were analyzed using the two-sided t test and ANOVA with $P < 0.05$ considered statistically significant.

Results

Apigenin protects primary rat retinal ganglion cells (RGCs) from TNF-α-mediated apoptosis

To investigate the effect of apigenin on the apoptosis of RGCs, cell viability was determined using MTT assays. Apigenin significantly inhibited the decrease of RGC viability induced by TNF-α in a dose-dependent manner (Fig 1A). Quantitative evaluation of apoptosis through annexin V-FITC/PI staining was analyzed by Flow Cytometry. As shown in Fig 1B, the rate of apoptotic cells rose to 36.23% with the treatment of TNF-α (100ng/ml) for 24 h. Furthermore, pretreatment with apigenin prevented TNF-α-induced death in a dose-dependent manner. As apigenin accelerated the amount of RGCs, the production of ATP (Fig. 1C) and the total oxygen uptake (Fig. 1D) were also promoted significantly. Taken together, it suggested that apigenin had a strong anti-apoptotic effect in primary rat retinal ganglion cells.

Apigenin inactivates caspase-dependent apoptotic signaling pathway in RGCs

Dysfunction induced by the decreased population of RGCs is regarded as an important factor in the pathogenesis of various eye diseases. Neural apoptosis is generally believed to be mediated by two distinct steps, caspase-dependent and caspase-independent pathways. TNF-α caused a change of the expression of Bcl-2, Bax and caspase-3 activation, as markers of cytokine-induced apoptosis. Moreover, the damage was reversed by apigenin (Fig. 2A and B). Caspase-3 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways. As shown in Fig 2C and 2D, we examined the expression and activation of caspase-3. TNF-α promoted 2-fold activation of caspase-3, but not effect caspase-3 mRNA level. Apigenin treatment alleviated the increased caspase-3 activity induced by TNF-α.

Apigenin Suppressed TNF-α -Induced Activation of NF-κB in RGCs

Nuclear factor kappa B (NF-κB), as a transcription factor, is thought to play an important role in onset of RGCs apoptosis (18, 19). To determine the effects of apigenin on the inhibition of NF-κB during apoptosis using the luciferase reporter assay, the plasmid (pGL3-3 × NF-κB-Luc) was transiently transfected into RGCs. The results indicated that apigenin dose-dependently decreased NF-κB activation induced by TNF-α (Fig.3A). The nuclear transcription factor AP-1, composed of dimers of Fos and Jun proteins, has been linked to a startling breadth of cellular events including cell trans-

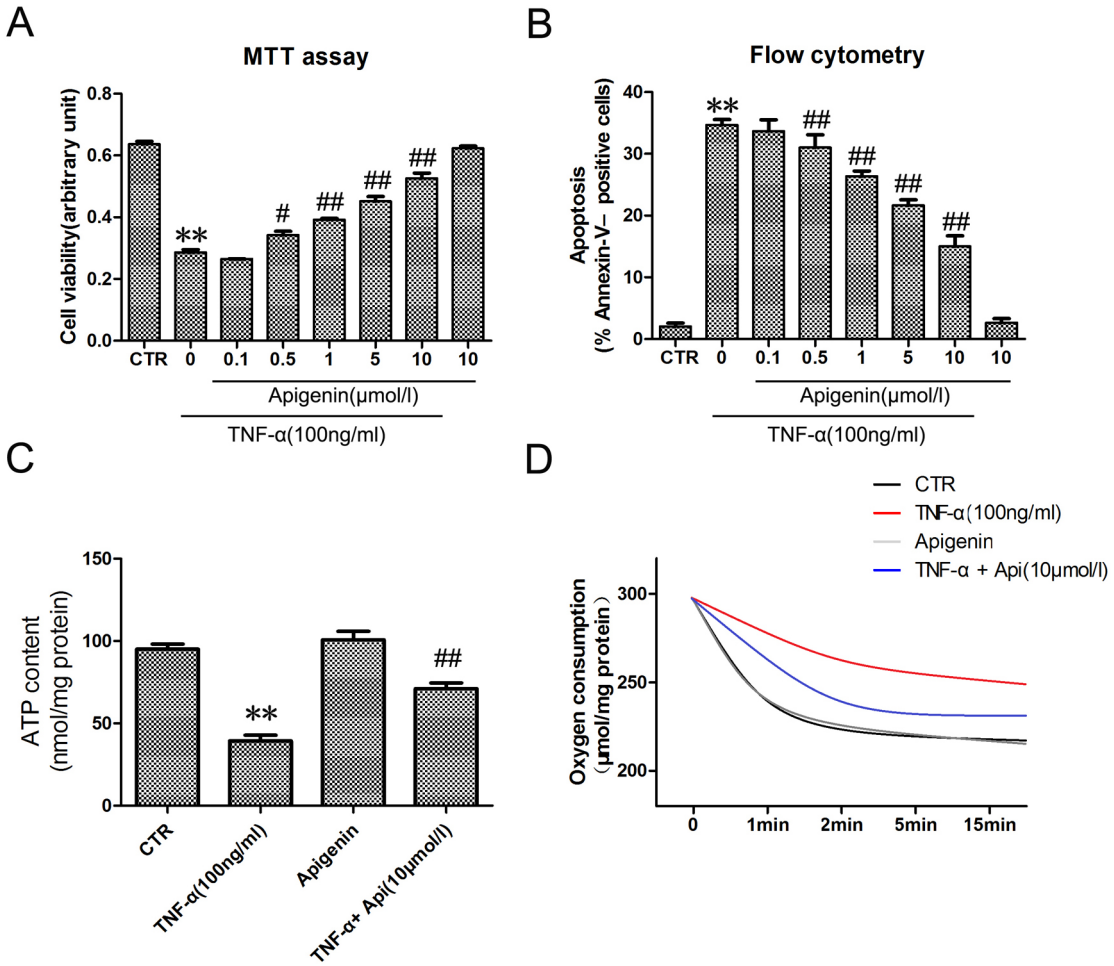

Figure 1. Apigenin inhibited TNF-α-induced apoptosis in primary rat retinal ganglion cells. (A) Cells were treated with the indicated concentrations of apigenin and TNF-α for 24 h, analyzed by MTT assay. (B) Cells were stained with Annexin V-FITC and PI, analyzed by flow cytometry. Data are expressed as % of Annexin V-FITC-positive and PI-negative cells (early stage of apoptosis). (C) The intracellular ATP concentrations were evaluated after exposure to apigenin(10μmol/l) and TNF-α(100ng/ml) for 24 h. Before the oxygen consumption test (E), cells were pre-treated in low oxygen conditions for 2 h in suspension culture with apigenin. Values are the means ± SD (n = 3) of three independent experiments.*$P < 0.05$, ** $P < 0.01$ *vs*. control (DMSO), $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* group treated with TNF-α (100ng/ml).

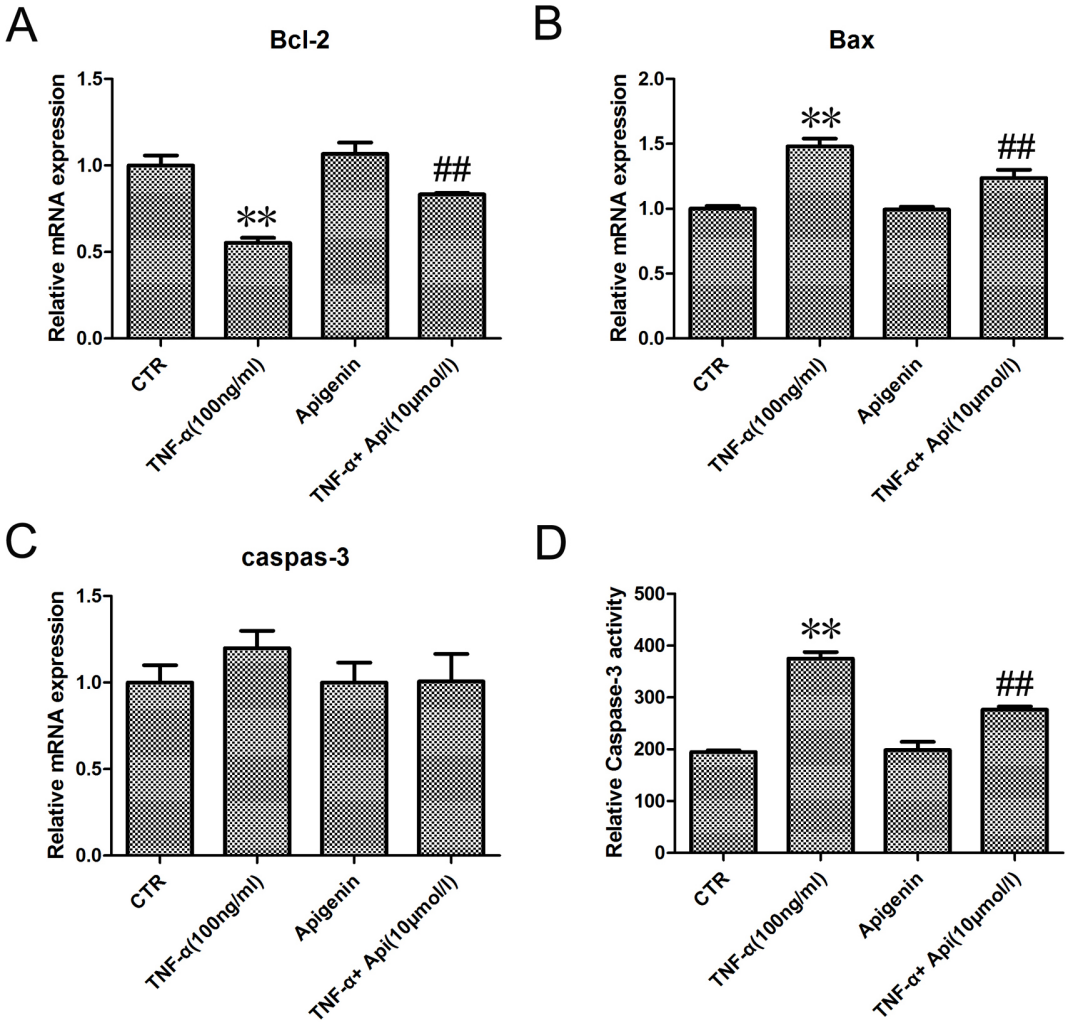

Figure 2. The effect of apigenin on caspase-dependent pathway in RGCs. Cells were treated with apigenin(10μmol/l) and TNF-α(100ng/ml) for 16 h. (A-C) The transcripts of apoptotic genes were analyzed by real-time RT-PCR assays. (D) caspase-3 activation assay .Values are the means ± SD (n = 3) of three independent experiments.*$P < 0.05$, ** $P < 0.01$ *vs*. control (DMSO), $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs* group treated with TNF-α (100ng/ml).

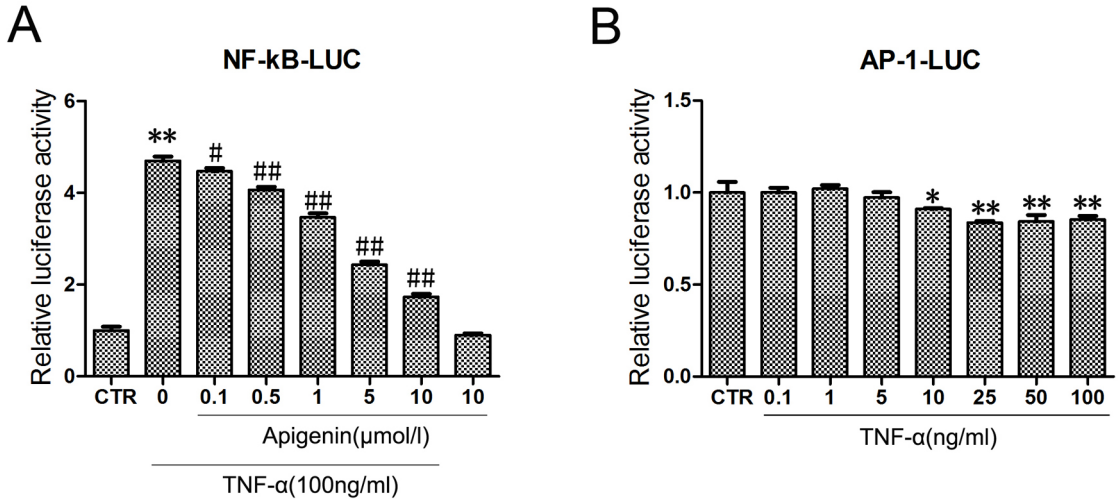

Figure 3. The effect of apigenin on TNF-α -induced activation of NF-κB in RGCs. Cells were treated with the indicated concentrations of apigenin and TNF-α for 16 h. (A) Apigenin dose-dependently reduced NF-κB activation in luciferase reporter assay. (B) After cells were treated with the indicated concentrations of TNF-α for 24 h, AP-1 activation was determined by luciferase reporter assay. Values are the means ± SD (n = 3) of three individual experiments. * $p < 0.05$, ** $p < 0.01$ *vs*. ctr (DMSO); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs*. group treated with TNF-α (100ng/ml).

formation, proliferation, differentiation and apoptosis (17). However, TNF-α had no significant effect on activation of AP-1 (Fig.3B). These data suggested that NF-κB might be a mediator for TNF-α -initiated toxicity in RGCs and activated NF-κB was significantly reversed by apigenin.

Discussion

TNF-α is a pro-inflammatory cytokine which plays an important role in the pathogenesis of glaucoma (20). In the present study, we confirmed that TNF-α induces apoptosis of primary rat RGCs, and we further showed that apigenin could prevent the cytotoxic effects of

TNF-α on RGCs.

In the eye, TNF-α has been detected in induced uveitis, hereditary retinal degeneration, glaucoma, proliferative vitreoretinopathy, and diabetic retinopathy (21, 22). Several studies have demonstrated that apigenin treatment produced some neuroprotective effects with *in vivo* and *in vitro* experimental models. Apigenin has been used for its anxiolytic, sedative and antidepressant effects. Apigenin isolated from *Carduus crispus* showed protection against kainate-induced excitotoxicity by quenching reactive oxygen species as well as inhibiting glutathione peroxidase depletion in hippocampal neurons (13). Apigenin isolated from *Elsholtzia rugulosa* exhibited protective effects against Aβ -induced toxicity by regulating redox imbalance and strengthening barrier function in rat cerebral microvascular endothelial cells (23). In our study, apigenin had a strong anti-apoptotic effect in primary rat retinal ganglion cells induced by TNF-α.

Dysfunction induced by the decreased population of RGCs is regarded as an important factor in the pathogenesis of various eye diseases. Neural apoptosis is generally believed to be mediated by two distinct steps, caspase-dependant and caspase-independent pathways (24, 25). In our study, TNF-α stimulation led to a significant reduction of bcl-2 and enhancement of bax. However, apigenin treatment reversed such effects. Caspase-3 is activated in the apoptotic cell both by extrinsic (death ligand) and intrinsic (mitochondrial) pathways (26). We examined the expression and activation of caspase-3 and found that TNF-α promoted the activation of caspase-3, but not effect caspase-3 mRNA level.

NF-κB is a family of ubiquitously expressed transcription factors that control the expression of hundreds of genes involved in inflammation, immune cell activation and cell survival (27). NF-κB and activator protein 1 (AP-1) transcription factors can regulate many important biological and pathological processes. NF-κB plays a dual role in apoptosis depending on different external stimuli (28). Reactive oxygen species including hydrogen peroxide and superoxide will cause the neuronal ischemia/reperfusion injury through NF-κB pathway (29). Recent study also showed that it played an important role in onset of RGCs apoptosis (30). Activation of the AP-1 transcription factor was activated by inflammatory cytokines of the TNF family. So we try to detect AP-1 activation in RGCs (fig.3). In our results, only NF-κB could be activated by TNF-α. Therefore, we suggested apigenin inhibited NF-κB activation specifically.

In summary, our study demonstrated that apigenin alleviated TNF-α-induced apoptosis through inhibition of caspase-dependent apoptotic pathway and activation of nuclear factor-κB. Therefore, apigenin may be developed as an anti-apoptotic drug to treat retinopathy.

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