Crocin protects retinal ganglion cells against H_2O_2-induced damage through the mitochondrial pathway and activation of NF-κB

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Abstract. Glaucoma is a degenerative nerve disorder that results in irreversible blindness. It has been reported that the apoptosis of retinal ganglion cells (RGCs) is a hallmark of glaucoma. Oxidative stress is one of the major factors that cause apoptosis of RGCs. Crocin has many beneficial effects, including anti-oxidant and anti-apoptotic actions. However, the mechanism by which crocin protects against oxidative stress-induced damage to RGCs remains unclear. The present study aimed to investigate the mechanism by which crocin protects RGC-5 cells against H_2O_2-induced damage. H_2O_2 was used to establish a model of oxidative stress injury in RGC-5 cells to mimic the development of glaucoma *in vitro*. Different concentrations (0.1 and 1 μM) of crocin were added to test whether crocin was capable of protecting RGCs from H_2O_2-induced damage. WST-1, lactic dehydrogenase (LDH) release and Annexin V/FITC assays were then performed. Levels of reactive oxygen species (ROS) were detected using a ROS assay kit, mitochondrial membrane potential ($\Delta\Psi$m) was analyzed by JC-1 staining, caspase-3 activity was examined using a Caspase-3 assay kit, and the protein levels of Bax, Bcl-1 and cytochrome *c* were measured using western blot analysis. In addition, the protein level of phosphorylated nuclear factor-κB (p-NF-κB) p65 was also evaluated using western blot analysis. The results showed that crocin protected RGC-5 cells from apoptosis, decreased LDH release and enhanced cell viability. Additional experiments demonstrated that crocin decreased ROS levels, increased $\Delta\Psi$m, downregulated the protein expression of Bax and cytochrome *c*, promoted Bcl-2 protein expression and activated NF-κB. Taken together, the findings of this study indicate that crocin prevented H_2O_2-induced damage to RGCs through the mitochondrial pathway and activation of NF-κB.

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Abbreviations: RGCs, retinal ganglion cells; $\Delta\Psi$m, mitochondrial membrane potential; LDH, lactic dehydrogenase; IR, ischemia/reperfusion; ROS, reactive oxygen species; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; OD, optical density

Key words: crocin, retinal ganglion cell, hydrogen peroxide, mitochondrial pathway

Introduction

Glaucoma is a degenerative nerve disorder, which is characterized by optic atrophy and visual field defects, and results in irreversible blindness. It has been reported that almost 64.3 million patients suffered from glaucoma worldwide in 2013, and this number is likely to increase to 76 million in 2020 and 111.8 million in 2040 (1). The hallmark of glaucoma is the apoptosis of retinal ganglion cells (RGCs), which are the only efferent neurons that convey visual signals from the retina to the brain. Several risk factors, including elevated intraocular pressure (2), oxidative stress (3), elevated glutamate (4) and aging (5), have been considered to accelerate RGC apoptosis in glaucoma, among which, oxidative stress is considered as the final common pathway in glaucoma (6). Several studies have reported that oxidative stress can lead to apoptosis of RGCs through activation of the mitochondrial pathway (7), and that apoptosis is the main cause of RGC loss (8). Thus, identifying a way of inhibiting oxidative stress-induced apoptosis in RGCs may provide an effective therapy for glaucoma.

Oxidative stress is able to destroy mitochondrial membrane potential ($\Delta\Psi$m), induce mitochondrial DNA damage and the release of apoptosis-related factors, and thus, trigger apoptosis (9). Mitochondria play an important role in the functioning and survival of RGCs (10,11), and mitochondrial dysfunction has been observed in glaucoma patients (12). Mitochondrial dysfunction is regarded as an early event in the mitochondrial apoptotic pathway. In the mitochondrial apoptotic pathway, mitochondrial dysfunction and the activation of pro-apoptotic

Bcl-2 family members has been demonstrated to induce the release of cytochrome *c*, which forms the apoptosome complexes, and contributes to the activation of caspase-9 and the cleavage of caspase-3 (8,13,14). The mitochondrial-dependent apoptosis of RGCs has been previously investigated (15). As an important consequence of mitochondrial dysfunction caused by oxidative stress, excessive reactive oxygen species (ROS) are capable of mediating mitochondrial permeability transition and the release of pro-apoptotic proteins, and thus, stimulate the mitochondrial apoptotic pathway (9).

Saffron is a traditional medicine that is frequently used in clinical therapy. The clinical therapeutic effects of saffron have been demonstrated in cancer (16), hypertension (17), insomnia and anxiety (18), cerebral ischemia (19) and depression (20). The effect of saffron on retinal diseases has also been demonstrated by improving focal macular electrorectinogram parameters (21), inhibiting cell death induced by intense light (22), and treating macula lutea and ischemic retinopathy caused by old age (23). Crocetin and crocin are the two major active ingredients of saffron. Crocetin can is capable of preventing the retinal damage induced by oxidative and endoplasmic reticulum stresses through inhibition of the activity of caspase-3 and -9 (24), and of protecting the retina from ischemic damage via the inhibition of oxidative stress (25). Crocin has also been shown to exert a protective effect on retinal ischemia/reperfusion (IR) injury-induced apoptosis of RGCs (26). However, the mechanism by which crocin protects against oxidative stress-induced damage toof RGCs remains unclear.

In the present study, we investigated the protective effects of crocin on RGCs under oxidative stress. Hydrogen peroxide (H_2O_2) was used to establish a model of oxidative stress injury in RGCs to mimic RGC injury in glaucoma *in vitro*. The anti-apoptotic effect of crocin was determined, and the mitochondrial-mediated apoptosis pathway was examined to determine the anti-apoptotic mechanism of crocin. In addition, the activity of phosphorylated nuclear factor-κB (p-NF-κB) p65 was also measured using western blot analysis.

Materials and methods

Cell culture. RGC-5 cells, obtained from the American Type Culture Collection (Cat. no. PT6600; ATCC, Manassas, VA, USA), were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (both from Gibco, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (both from Sigma-Aldrich, St. Louis, MO, USA). Cells were grown in a humidified incubator with 5% CO_2 at 37˚C and passaged every three days. Second generation RGCs were used in our experiments.

Establishment of a model of oxidative stress injury in RGCs and crocin treatment. The cells were equally divided into five groups, which were treated with different concentrations of H_2O_2 (0, 200, 400, 800 and 1,000 μM) for 16 h. Cell viability and lactic dehydrogenase (LDH) release were tested to investigate the cell injury induced by H_2O_2 in RGCs, and an appropriate concentration was chosen to establish the model of oxidative stress injury in RGCs.

RGC-5 cells were pre-treated with 0.1 and 1 μM of crocin (Sigma-Aldrich) for 24 h, and no drugs were added to the control group. The cells were then subjected to oxidative insult with H_2O_2 (800 μM) for 16 h and collected for subsequent experiments.

WST-1 cell proliferation assay. Cell viability was determined using a WST-1 assay (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, the cells were cultured as described above. WST-1 reagent (10 μl) was then added to each well, and incubated for 4 h at 37˚C. The optical density (OD) was read at 440 nm using a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany).

LDH release assay. LDH release was determined using a LDH cytotoxicity detection kit (Takara Bio, Tokyo, Japan). Briefly, the cells were cultured in 96-well plates and 100 μl cell suspensions of RGCs were collected to assess the LDH activity. Fresh reaction mixture (100 μl) was then added to each well and incubated at room temperature for 30 min. The absorbance was determined at 490 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Annexin V/FITC assay. Apoptotic cells were quantified using a FITC Annexin V apoptosis detection kit (BD Biosciences, Piscataway, NJ, USA). Approximately $1x10^5$ cells were collected and resuspended with 100 μl of binding buffer. Then, 5 μl of FITC Annexin V and 5 μl of propidium iodide (PI) were added to stain cells for 15 min at room temperature in the dark. Subsequently, 400 μl of 1X binding buffer was added prior to analysis by flow cytometry (BD Biosciences).

ROS assay. The level of intracellular ROS was evaluated using a ROS assay kit (Beyotime Biotech, Jiangsu, China). Briefly, the cells were harvested and washed by 1X buffer, and then stained with 20 μM of dichloro-dihydro-fluorescein diacetate (DCFH-DA; 1:1,000) for 20 min at 37˚C. The signal was read at excitation/emission (Ex/Em) wavelengths of 488/525 nm filter after the cells were further washed three times using PBS. Cells treated with ROSup (provided with the ROS assay kit) only were used as negative controls.

Measurement of $\Delta\Psi m$. JC-1 fluorescent dye 9 (Beyotime Biotech) was used to measure $\Delta\Psi m$. The cells were collected and incubated with JC-1 staining solution at 37˚C for 15 min in a 5% CO_2 incubator, and then resuspended with 500 μl of preheated incubation buffer. The green fluorescence (JC-1-monomer) was viewed at Ex/Em 490/530 nm, and the red fluorescence (JC-1-aggregate) was viewed at Ex/Em wavelengths of 525/590 nm.

Caspase-3 activity assay. The enzymatic activity of caspase-3 was detected by a caspase-3 assay kit (Abcam, Cambridge, MA, USA). Briefly, the cells were suspended in lysis buffer and incubated on ice for 10 min. Reaction buffer and DEVD-AFC substrate were then added prior to being read at Ex/Em wavelengths of 400/505 nm.

Western blot analysis. The total protein was extracted from RGC-5 cells using RIPA (Beyotime Biotech) and its concentration was determined using a bicinchoninic acid (BCA) assay. The proteins were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membranes (Amersham;

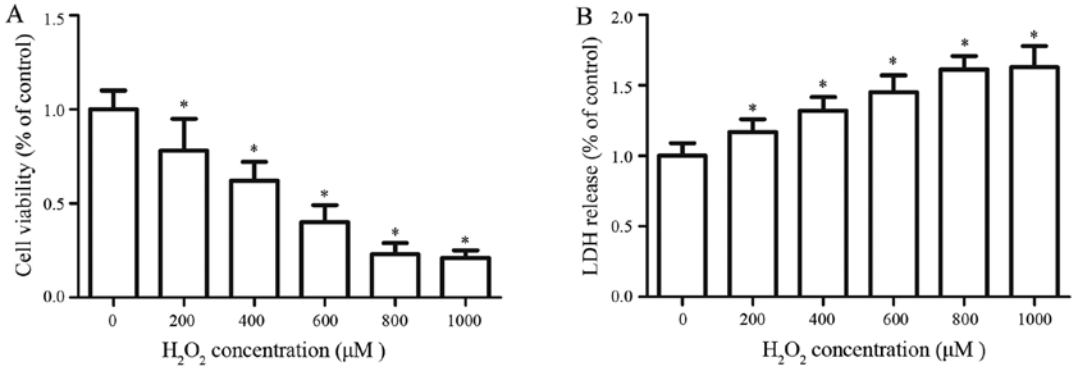

Figure 1. Effects of H_2O_2 on cell viability and lactic dehydrogenase (LDH) release in (RGC)-5 cells. RGC-5 cells were pre-treated with 0.1 and 1 μM of crocin for 24 h prior to being exposed to 800 μM of H_2O_2. (A) Cell viability was determined by a WST-1 assay. (B) LDH release was analyzed by an LDH cytotoxicity assay kit. The columns indicate the means ± SEM. *P<0.05 vs. the control group.

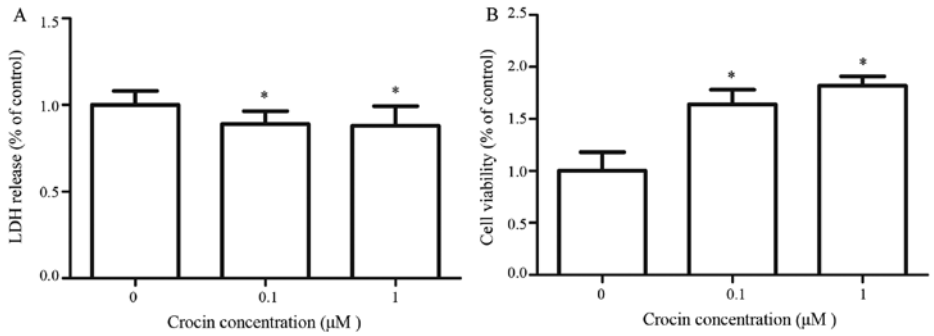

Figure 2. Effects of crocin on the release of lactic dehydrogenase (LDH) and cell viability in RGC-5 cells. RGC-5 cells were pre-treated with 0.1 and 1 μM of crocin for 24 h prior to being exposed to 800 μM of H_2O_2. (A) LDH release was analyzed by an LDH cytotoxicity assay kit. (B) Cell viability was determined by a WST-1 assay. The column indicates the means ± SEM. *P<0.05 vs. the control group.

GE Healthcare Europe GmbH, Freiburg, Germany). The membranes were blocked with 5% (v/v) dried milk and probed with anti-Bax, anti-Bcl-2, anti-cytochrome *c* (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti p-NF-κB p65 (Cell Signaling Technology, Inc., Danvers, MA, USA) at 4˚C overnight. Subsequently, HRP-conjugated goat anti-mouse IgG (Bioworld Technology, Inc., St. Louis Park, MN, USA) was added and incubated with the membranes for 1 h at room temperature. β-actin (Cell Signaling Technology, Inc.,) was used as the reference protein.

Statistical analysis. Data are presented as the means ± SEM. Statistical comparisons were performed using the Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of different concentrations of H_2O_2 on cell viability and LDH release in RGC-5 cells. To establish a model of oxidative stress injury in RGCs, different concentrations of H_2O_2 were used to evaluate the cytotoxicity to select an appropriate concentration. The results showed that H_2O_2 is capable of decreasing RGC-5 cell viability in a dose-dependent manner; however, there was no significant difference between concentrations of 800 and 1,000 μM (Fig. 1A). As the concentration of H_2O_2 increased, there was a corresponding gradual increase in the release of LDH (Fig. 1B). The LDH release assay and the WST-1 assay showed a very low gradient from 800 to 1,000 μM. Thus, an H_2O_2 concentration of 800 μM was used to establish the a model of oxidative stress injury in RGCs.

Effects of crocin on cell viability and LDH release in oxidative stress-injured RGC-5 cells. LDH is a stable cytoplasmic enzyme that is present in all cells. When the plasma membrane is damaged, LDH is rapidly released into the culture supernatant, thus, LDH release in the culture supernatant is a measure of cytotoxicity. To evaluate the anti-cytotoxic effect of crocin on RGC-5 cells, an LDH assay was performed using an LDH cytotoxicity assay kit. LDH release in RGC-5 cells was significantly decreased in the presence of crocin. There was no significant difference in the LDH release between crocin concentrations of 0.1 and 1 μM (P>0.05) (Fig. 2A). To understand the cytoprotective effects of crocin in H_2O_2-insulted RGC-5 cells, a WST-1 assay was performed to determine cell viability. Crocin significantly enhanced RGC-5 cell viability in H_2O_2-insulted cells (P<0.05), and this effect was not concentration-dependent (P>0.05) (Fig. 2B). These results indicated that crocin could enhanced the cell viability of RGC-5 cells that have been injured by H_2O_2.

Effect of crocin on the apoptosis of oxidative stress-injured RGC-5 cells. H_2O_2-induced apoptosis of RGC-5 cells was analyzed using flow cytometry through Annexin V-FITC/PI

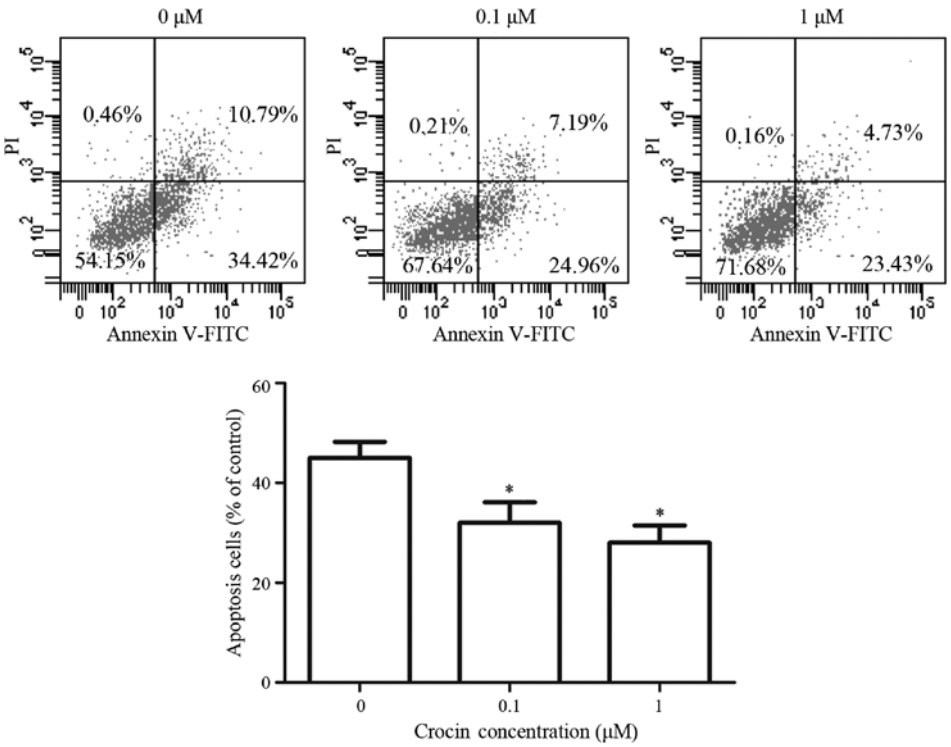

Figure 3. Crocin inhibits H_2O_2-induced apoptosis of RGC-5 cells. RGC-5 cells were pre-treated with 0.1 and 1 μM of crocin for 24 h prior to being exposed to 800 μM H_2O_2. Apoptotic cells were detected by a FITC Annexin V apoptosis detection kit. The columns indicate the means ± SEM. *P<0. 05 vs. the control group.

staining. The percentages of apoptotic cells decreased from 45.39% without crocin, to 32.15% in the presence of 0.1 μM and 28.16% in the presence of 1 μM crocin (Fig. 3). Crocin significantly inhibited H_2O_2-induced apoptosis in RCG-5 cells and there was no significant difference in the percentages of apoptotic cells between crocin concentrations of 0.1 and 1 μM.

Effect of crocin on the production of ROS in oxidative stress-injured RGC-5 cells. Intracellular ROS is an oxidative stress indicator in cells that plays an important role in apoptosis induction under physiological and pathological conditions (27). ROS are released from the mitochondria, and excessive ROS are able to disrupt the ΔΨm in return (27). In the present study, excessive ROS was generated in H_2O_2-injured cells (Fig. 4). With the addition of crocin, the intracellular ROS content was markedly reduced compared with the control group, and the difference between crocin concentrations of 0.1 and 1 μM was not significant. These results indicated that crocin exerted an antioxidant effect on the oxidative stress-injured RGC-5 cells.

Effect of crocin on ΔΨm in oxidative stress-injured RGC-5 cells. Mitochondria are closely associated with cell apoptosis, and a decrease in ΔΨm is considered as one of the earliest hallmark events in the cascade reaction process of apoptosis (28). To examien the effect of crocin on H_2O_2-induced ΔΨm disruption, the lipophilic cation JC-1 was used to evaluate ΔΨm. Crocin significantly increased ΔΨm in oxidative stress-injured RGC-5 cells (P<0.05). A significant difference between crocin concentrations of 0.1 and 1 μM was identified, which suggested that the mitochondria-dependent pathway may be involved in the protective effect of crocin on H_2O_2-injured cells (Fig. 5).

Effects of crocin on the activity of caspase-3 and the expression of Bcl-2, Bax and cytochrome c in oxidative stress-injured RGC-5 cells. Caspase-3 is the final effector in the mitochondria-mediated apoptotic pathway (9). Cytochrome *c*, the anti-apoptotic factor Bcl-2 and pro-apoptotic factor Bax are the key regulating factors in the mitochondrial pathway (14). To investigate the effect of crocin on the apoptosis of RGC-5 cells, we detected the expression of Bcl-2, Bax and cytochrome *c* using western blot analysis. As shown in Fig. 6A, the expression level of Bcl-2 was markedly higher in the crocin groups than the control group (P<0.05), and the difference between 0.1 and 1 μM crocin was not significant (Fig. 6B). There was also a significant difference in the expression of Bax between the crocin groups and the control group (P<0.05) (Fig. 6C), while no significant difference was observed between 0.1 and 1 μM crocin. Cytochrome *c* release in oxidative-stress-injured RGC-5 cells was significantly suppressed at crocin concentrations of 0.1 and 1 μM (Fig. 6D), and the inhibitory effect of 1 μM of crocin was significantly stronger than that of 0.1 μM of crocin.

Caspases are aspartic acid proteases containing cysteine, which selectively cleave the target protein of aspartate residue, and thus, induce cell apoptosis. In caspase-dependent signaling, caspase-3 is one of the most important effector caspases, and its activation is the final step of apoptosis (29). To investigate the effect of crocin on H_2O_2-induced activation of caspase-3, we used a caspase-3 assay kit to detect caspase-3 activity. The results showed that crocin treatment significantly inhibited the activation of caspase-3 activity, while there was no significant difference between 0.1 and 1 μM crocin (Fig. 6E).

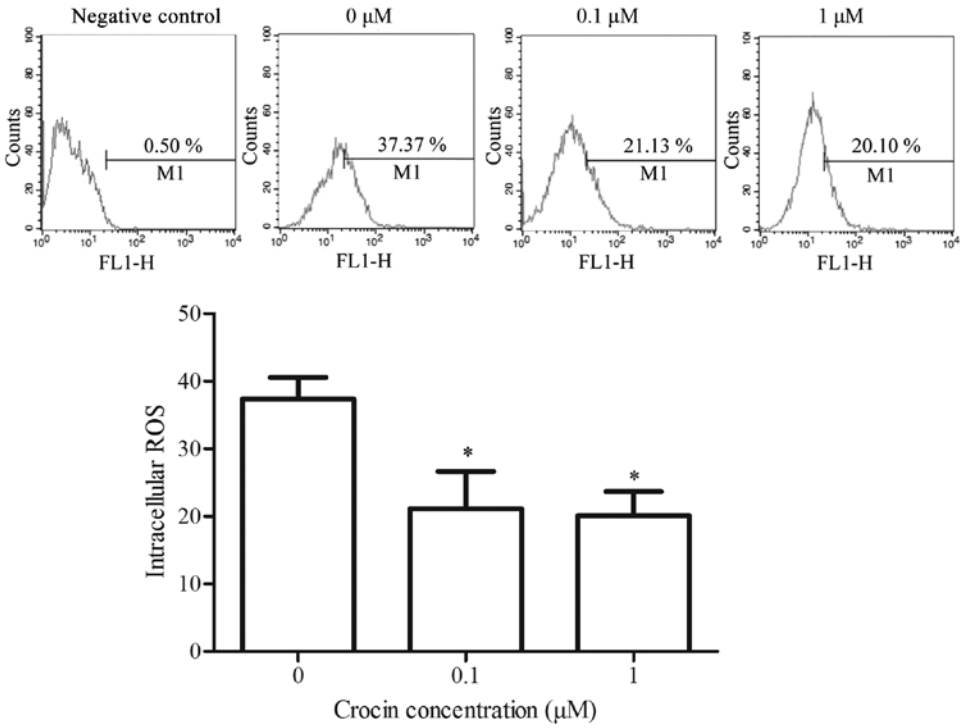

Figure 4. Crocin reduces intracellular reactive oxygen species (ROS) production. RGC-5 cells were pre-treated with 0.1 and 1 μM crocin for 24 h prior to being exposed to 800 μM H_2O_2. The level of intracellular ROS was evaluated using flow cytometry. The columns indicate the means ± SEM. *P<0.05 vs. the control group.

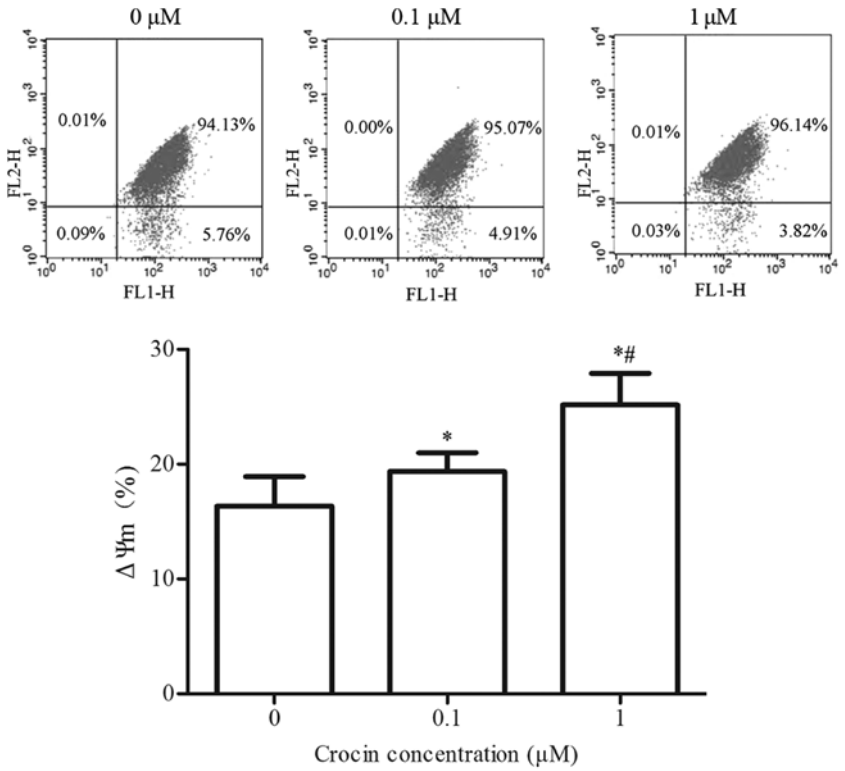

Figure 5. Crocin increases mitochondrial membrane potential (ΔΨm) in oxidative-stress-injured retinal ganglion cells (RGCs). RGC-5 cells were pre-treated with 0.1 and 1 μM crocin for 24 h before being exposed to 800 μM H_2O_2. A mitochondrial membrane potential assay kit was used to measure ΔΨm. The columns indicate means ± SEM. *P<0.05 vs. control group; #P<0.05 vs. 0.1 μM crocin.

Effect of crocin on p-NF-κB 65 in oxidative stress-injured RGC-5 cells. NF-κB is a family of nuclear transcription factors that include the subunits Rel, p65, RelB, p50 and p52, which influence cell apoptosis by regulating the expression of

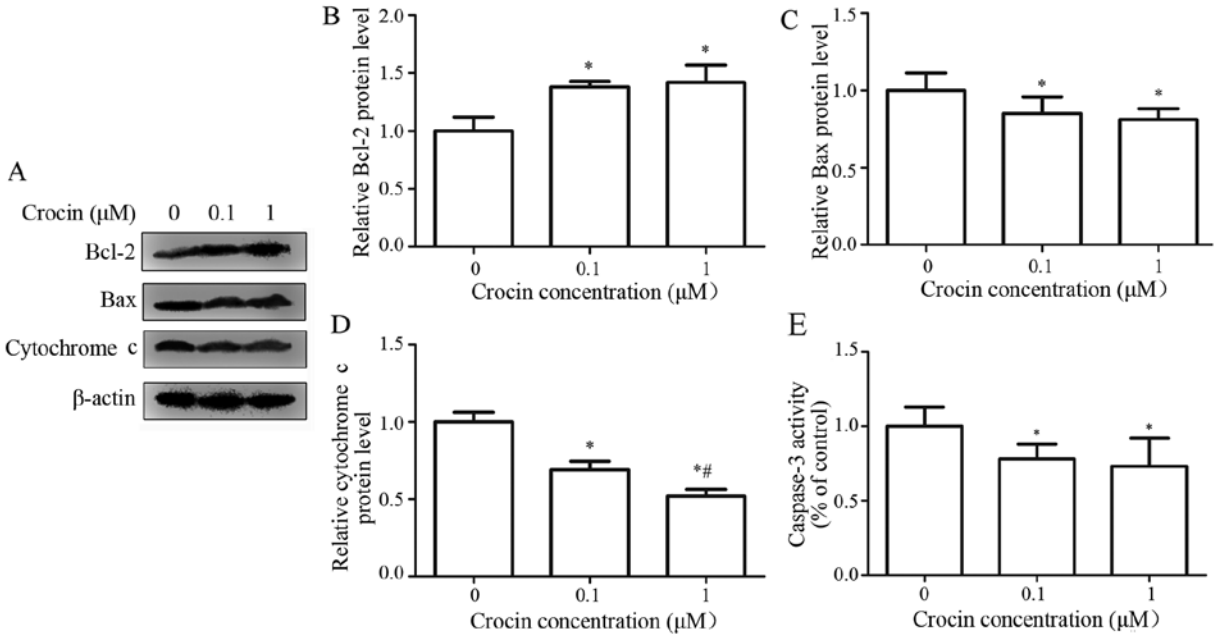

Figure 6. Effects of crocin on the activity of caspase-3 and the expression of Bcl-2, Bax and cytochrome *c* in oxidative stress-injured RGC-5 cells. RGC-5 cells were pre-treated with 0.1 and 1 μM crocin for 24 h prior to being exposed to 800 μM H_2O_2. (A) Representative western blot image shows the expression of Bcl-2, Bax and cytochrome *c* in all the groups, and the columns show the quantification of (B) Bcl-2, (C) Bax and (D) cytochrome *c*. (E) The enzymatic activity of caspase-3 was detected by a caspase-3 assay kit. The columns indicate the means ± SEM. *P<0.05 vs. control group; #P<0.05 vs. 0.1 μM crocin.

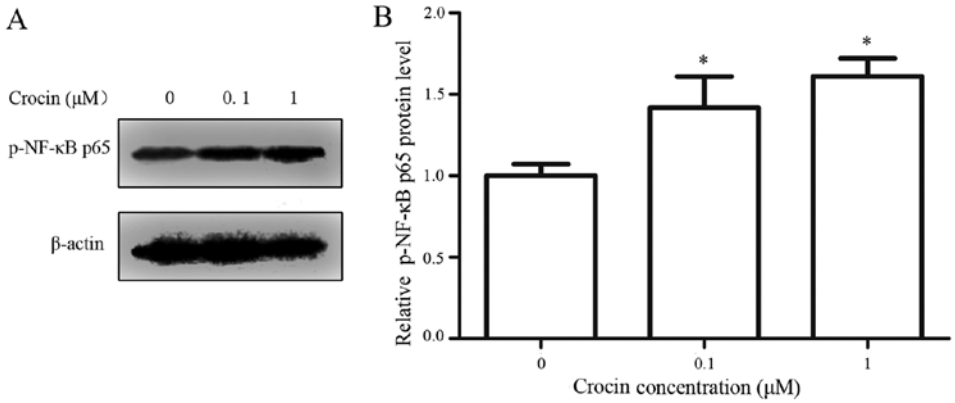

Figure 7. Crocin increases the level of p-nuclear factor-κB (NF-κB) p65 protein in oxidative stress-injured retinal ganglion cells (RGCs). RGC-5 cells were pre-treated with 0.1 and 1 μM crocin for 24 h prior to being exposed to 800 μM H_2O_2. Western blot analysis was used to measure the level of p-NF-κB p65 protein. The columns indicate the means ± SEM. *P<0. 05 vs. control group.

cell survival genes (30). The phosphorylation of the transactivation p65 subunit is essential for efficient transcriptional activation by NF-κB (31). Thus, the level of p-NF-κB p65 was measured using western blot analysis in the present experiment. The results showed that the relative p-NF-κB p65 level was markedly increased in the presence of crocin (Fig. 7), which indicates that crocin upregulated the activity of NF-κB in oxidative stress-injured RGC-5 cells.

Discussion

In glaucoma, elevated intraocular pressure is the most significant risk factor for accelerated RGC death. It is widely accepted that oxidative damage in response to pressure elevation is an important underlying mechanism of elevated intraocular pressure-induced cell damage and neuronal death (32,33). Thus, H_2O_2 was used to establish a model of oxidative stress injury in RGCs to mimic RGC injury in glaucoma *in vitro*. The LDH and WST-1 assay results showed that H_2O_2 decreased cell viability and increased LDH release. Efficiency was highest at a concentration of 800 μM, therefore a concentration of 800 μM H_2O_2 was used to establish the model of oxidative stress injured in RGCs for subsequent experiments.

Crocin is one of the active ingredients of saffron, which is frequently used as a traditional medicine for its antitoxic properties (34). The anti-apoptotic and antioxidant effects of crocin have been stated in several studies (35,36). Qi *et al* (26) have reported that crocin injections prevented apoptosis of RGCs subsequent to retinal IR injury. In the present study, we

detected changes in the cell viability and apoptosis of H_2O_2-insulted RGC-5 cells by WST-1 and Annexin V/PI staining *in vitro,* respectively. Our results were consistent with those of Mehri et al (37), who reported that crocin enhanced cell viability and reduced apoptosis. In addition, we also detected LDH release in H_2O_2-insulted RGCs using an LDH cytotoxicity assay kit. LDH release was significantly decreased by crocin concentrations of 0.1 and 1 μM. Taken together, these results suggest that crocin prevented H_2O_2-induced damage to RGCs.

One of the important mechanisms by which crocin exerts its biological effects is its ability to modulate the redox status of organisms. Evidence has suggested that overproduction of ROS plays an important role in the protective effects of crocin in serum-deprived and hypoxic PC12 cells (38). Mousavi et al (39) have confirmed that crocin decreased the production of ROS induced by glucose in PC12 cells. To determine the effects of crocin on the production of ROS in H_2O_2-injured RGC-5 cells, we determined the production of ROS by performing a cellular ROS assay. The results showed that H_2O_2-induced production of ROS was significantly suppressed by crocin, suggesting that crocin is capable of reducing the ROS level and suppressing H_2O_2-induced oxidative stress in RGC-5 cells.

There are two main pathways of oxidative stress-induced apoptosis: mitochondrial- and death receptor-mediated pathways (40). In the mitochondrial pathway, Bcl-2 and Bax are the key regulators. Bcl-2 inhibits apoptosis by suppressing cytochrome *c* release and caspase activation, while Bax promotes apoptosis by inducing the release of cytochrome *c*, which then triggers the downstream apoptosis event (29). On the other hand, the release of apoptosis-related factor cytochrome *c* may also be inhibited by the rise of the $\Delta\Psi$m, which decreased intimal permeability (41). Our results show that crocin effectively prevented H_2O_2-induced apoptosis by increasing $\Delta\Psi$m, downregulating Bax and cytochrome *c* and caspase-3, and upregulating Bcl-2. This finding indicates that crocin stabilized the mitochondria and inhibited apoptosis mediated by the mitochondrial pathway, thereby protecting RGCs from apoptosis.

NF-κB activity helps cells to avoid the sustained phase of JNK activation which has been demonstrated to activate the mitochondrial apoptotic pathway (42), and thus, promotes cell survival (43,44). NF-κB plays an important role in the apoptosis of RGCs mediated by H_2O_2 (45,46). The present study revealed that the level of p-NF-κB p65 was significantly higher in the crocin groups than in the control group. This result suggests that crocin initiated the activation of NF-κB in the presence of H_2O_2, thereby reducing H_2O_2-induced apoptosis.

Taken together, our results demonstrate that crocin is capable of protecting H_2O_2-injured RGC-5 cells from apoptosis through the mitochondrial pathway, and by upregulating the activity of NF-κB.

Acknowledgements

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