Original Article Crocin protects retinal pigment epithelial cells from oxidative stress through suppression of the MAPK signaling pathway

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Abstract: Oxidative stress damage to retinal pigment epithelial (RPE) cells is thought to play a critical role in the pathogenesis of age-related macular degeneration (AMD). Crocin, a pharmacologically active component of *Crocus sativus* L., was reported to have anti-antioxidant and anti-apoptotic properties. However, whether crocin protects against oxidative stress-induced retinal damage in RPE cells is still unknown. Therefore, in this study, we investigated the protective effect of crocin against oxidative stress-induced cell death in ARPE-19 cells and its underlying mechanism. Our results demonstrated that crocin significantly attenuated H_2O_2 -induced ARPE-19 cell injury by suppressing LDH release. Crocin also decreased H_2O_2 -induced oxidative stress though scavenging ROS accumulation and MDA peroxidation. Furthermore, crocin reversed H_2O_2 -induced Bcl-2 down-regulation and Bax overexpression, inhibited caspase-3 activation, p38 MAPK and JNK phosphorylation in ARPE-19 cells. In conclusion, these data suggest that crocin protects against H_2O_2 -induced oxidative stress through suppression of the MAPK signaling pathway in ARPE-19 cells. Thus, crocin may be useful for the prevention or treatment of AMD.

Keywords: Crocin, retinal pigment epithelial (RPE) cells, oxidative stress, apoptosis

Introduction

Age-related macular degeneration (AMD) is a leading cause of visual impairment among the elderly in developed countries [1]. It is characterized by morphological a functional abnormalities in the macular retinal pigment epithelial (RPE) cells. Oxidative stress damage to RPE cells is thought to play a critical role in the pathogenesis of AMD. It was reported that exposure of oxidative stress to RPE cells may promote apoptosis and lead to the development of AMD [2, 3], and AREDS (age-related eye disease study) confirmed that the specific antioxidants and mineral components could preserve vision and reduce the disease progression in patients with AMD [4]. Therefore, antioxidants may reduce the damage caused by oxidative stress in AMD.

Crocus sativus L. (or saffron), which belongs to the Iridaceae family, is often used as a culinary spice or an anodyne or tranquilizer in traditional

Chinese medicine. Crocin is a pharmacologically active component of Crocus sativus L. It has been reported that crocin may have antiarthritic, anti-inflammatory, anti-antioxidant and anti-apoptotic properties [5-8]. For example, one study demonstrated that crocin effectively inhibited sesamol-induced oxidative stress and apoptosis in human platelets [9]. In addition, crocin has been reported to be useful in the treatment of retinal damage. Chen et al. confirmed that crocin prevents retinal ischaemia/reperfusion injury-induced apoptosis in retinal ganglion cells through the PI3K/Akt signaling pathway [10]. Crocin also protects retinal ganglion cells against oxidative stress-induced damage through the mitochondrial pathway and activation of NF-kB signaling pathway [11]. However, whether crocin protects against oxidative stress-induced retinal damage in RPE cells is still unknown. Therefore, in this study, we investigated the protective effect of crocin against oxidative stress-induced cell death in ARPE-19 cells and its underlying mechanism.

Materials and methods

Cell culture and treatment

The human retinal pigment epithelial cell line (ARPE-19) was purchased from American type Tissue Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA) 1 mM sodium pyruvate, 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified chamber at 37°C and 5% CO₂. After reaching 80% confluence, ARPE-19 cells were plated in a 96-well culture plate (4×10^4 cells/well) for 24 h, then incubated with different concentrations of crocin (10, 50 and 100 µM).

Exposure of cells to H_2O_2

After treatment with crocin, the culture media was removed and, after washing with PBS, the cells were exposed to 500 μ M H₂O₂ for 1 h in phenol red free DMEM medium.

Cell viability assay

Cell viability was measured with a Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. In brief, ARPE-19 cells were pre-treated with various concentrations of crocin (10, 50 and 100 µM) for 24 h prior to exposure with 500 μ M H₂O₂ for 24 h. CCK-8 solution (10 μ l) was added to each well and the plates were incubated for 2 h. The absorbance of each well was measured at 450 nm using a microplate reader (Omega Bio-Tek, Inc., Norcross, GA, USA) with the culture medium as a blank. Cell viability was calculated using the following formula: cell viability (%) = [absorbance (with crocin)-absorbance (blank)]/[absorbance (without crocin)-absorbance (blank)] × 100.

LDH assay

The LDH Release Kit is a colorimetric assay for the quantification of cell death based on the measurement of lactate dehydrogenase activity released from the cytosol of dead cells into the medium. In brief, the medium from each well was removed, fractionated by centrifugation (240 g for 10 min), and 25 μ L of the supernatant solution were placed in a 96-well plate. Phosphate buffered saline (PBS, 75 μ L) was added to each well, followed by freshly prepared LDH reaction mixture (100 μ L). After 30 min of incubation at room temperature in the dark, the absorbance of each solution at 490 nm was measured using a plate reader.

Measurement of accumulation of intracellular reactive oxygen species, SOD and malondialdehyde

The generation of intracellular reactive oxygen species (ROS) was measured by fluorescence of 2',7'-dichlorofluorescein (DCF) [12]. The fluorescence intensity of the supernatant was measured with a plate reader (Wallac; PerkinElmer) at 485 nm excitation and 535 nm emission. Subsequently, the protein content of each well was determined using a commercial kit (Beyotime Institute of Biotechnology), and the ROS level of each well was calculated as the fluorescence intensity of each well/the protein content of each well.

Intracellular SOD activity was assayed with a commercially available assay kit (Jiancheng Biochemical, Inc., Nanjing, China). The optical density at 550 nm was measured by a microplate reader. And malondialdehyde (MDA) level was determined by a method based on the reaction with thiobarbituric acid [13].

Western blot

Total protein was extracted using radio-immunoprecipitation assay (RIPA) lysis buffer containing a mixture of protease inhibitor, and protein concentrations were determined using the Pierce BCA Protein Assay Kit (Pierce, Rockford, USA). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto PVDF membranes (Millipore Corp., Billerica, MA, USA). The membranes were blocked in 5% non-fat milk in TBST buffer (5 mM Tris-HCl, pH 7.4, 136 mM NaCl, 0.1% Tween 20) for 1 h at room temperature before hybridization with primary antibodies against p38, p-p38, JNK, p-JNK and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Primary antibody was removed by washing the membranes three times in TBST, and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (Abcam, Cambridge, UK). Bound proteins were visualized by enhanced chemiluminescence (ECL) detection system (Amersham, Little Chalfont, UK). The grey intensity analysis was performed using Image-Pro



Figure 1. Crocin prevents H₂O₂-induced decrease in ARPE-19 cell viability. A. ARPE-19 cells were pretreated with increasing concentrations of crocin (10-100 μ M) 24 h, cell viability was measured with the CCK-8 assay. B. After culturing with H₂O₂ for 24 h, with or without probucol pretreatment, cell viability was assessed. C. Cellular cytotoxicity was assessed by LDH releasing. Values are mean ± S.E.M. of three independent experiments. *P<0.05 vs. control; #P<0.05 vs. H₂O₂.

Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis

Data are presented as means ± S.E.M. Statistical significance was evaluated with one-way



Figure 2. Crocin protects against H_2O_2 -induced apoptosis in ARPE-19 cells. ARPE-19 cells were pre-treated with increasing concentrations of crocin (10-100 μ M) prior to exposure with 500 μ M H_2O_2 for 24 h. A. Western blot analysis using cleaved caspase-3, Bax and Bcl-2 antibodies was performed. GAPDH was served as the loading control. B. Quantitative analysis was performed by measuring the intensity relative to the control. Values are mean ± S.E.M. of three independent experiments. *P<0.05 vs. control; #P<0.05 vs. H_2O_2 .

ANOVA followed by LSD post hoc analysis. In all comparisons, the level of significance was set at P<0.05.

Results

Crocin prevents H₂O₂-induced decrease in ARPE-19 cell viability

To determine the potential cytotoxicity of crocin, ARPE-19 cells were incubated with various concentrations (10, 50 and 100 μ M) of crocin for 24 h prior to performing a CCK-8 assay. The results showed that crocin did not exhibit any significant cytotoxic effects at concentrations of 10-100 μ M (**Figure 1A**). Moreover, RPE cells exposed to H₂O₂ for 24 h exhibited significantly decreased the viability and increased the release level of LDH compared with the control. Whereas, crocin prevents H₂O₂-induced decrease cell viability and increase LDH release in ARPE-19 cells, exhibiting a dose-dependent manner (**Figure 1B** and **1C**).



Figure 3. Crocin inhibits H_2O_2 -induced the levels of ROS, SOD and MDA in ARPE-19 cells. ARPE-19 cells were pre-treated with increasing concentrations of crocin (10-100 μ M) prior to exposure with 500 μ M H_2O_2 for 24 h. A. ROS generation tested with DCF-DA staining expressed as ratio relative to controls. B. Intracellular SOD activity was assayed with a commercially available assay kit. C. MDA level was determined by a method based on the reaction with thiobarbituric acid. Values are mean \pm S.E.M. of three independent experiments. *P<0.05 vs. control; #P<0.05 vs. H_2O_2 .

Crocin protects against H_2O_2 -induced apoptosis in ARPE-19 cells

The next experiment was performed to investigate the effect of crocin on cell apoptosis in H_2O_2 -induced ARPE-19 cells. As expected, Western blot analysis showed that H_2O_2 significantly increased the expression of Bax and caspase-3 proteins, and decreased Bcl-2 expression. However, in the crocin (10, 50 and 100 μ M)-pretreated group, the expression of Bax and caspase-3 was down-regulated, and the expression of Bcl-2 was up-regulated in a dosedependent manner (**Figure 2**).

Crocin inhibits H_2O_2 -induced the levels of ROS, SOD and MDA in ARPE-19 cells

Then, we measured intracellular ROS levels by DCF fluorescence. As indicated in Figure 3A, as compared with the control group, H₂O₂ significantly increased the level of ROS in ARPE-19 cells. While, H₂O₂ exposure for 24 h in the presence of crocin significantly inhibited the intracellular ROS level in ARPE-19 cells. Furthermore, we observed that H₂O₂ notably decreased the SOD activity, whereas, crocin prevented the H₂O₂-induced decrease in SOD activity in ARPE-19 cells (Figure 3B). Conversely, after treatment with H₂O₂, the MDA activity was increased compared to control levels. However, with H₂O₂ exposure, pretreatment with crocin significantly inhibited the level MDA in ARPE-19 cells (Figure 3C).

Crocin attenuates p38 MAPK and JNK phosphorylation in H₂O₂-induced ARPE-19 cells

Furthermore, we investigated the effect of crocin on the activation of the p38 MAPK and ERK signaling pathways in ARPE-19 cells. As expected, H_2O_2 significantly increased the phosphorylation of p38 MAPK and JNK. The phosphorylation of p38 MAPK and JNK was potently inhibited by treatment with crocin, while the levels of p38 MAPK and JNK were not obviously affected (**Figure 4**).

Discussion

In this study, our results demonstrated that crocin significantly attenuated H_2O_2 -induced ARPE-19 cell injury by suppressing LDH release. Crocin also decreased H_2O_2 -induced oxidative stress though scavenging ROS accumulation and MDA peroxidation. Furthermore, crocin reversed H_2O_2 -induced Bcl-2 down-regulation and Bax overexpression, inhibited caspase-3 activation, p38 MAPK and JNK phosphorylation in ARPE-19 cells.



Figure 4. Crocin attenuates p38 MAPK and JNK phosphorylation in H_2O_2 -induced ARPE-19 cells. ARPE-19 cells were pre-treated with increasing concentrations of crocin (10-100 μ M) prior to exposure with 500 μ M H_2O_2 for 24 h. A. Western blot analysis using p38, p-p38, JNK and p-JNK antibodies was performed. B. Quantitative analysis of p-p38/p38. C. Quantitative analysis of p-JNK/JNK. Values are mean \pm S.E.M. of three independent experiments. *P<0.05 vs. control; #P<0.05 vs. H_2O_2 .

Hydrogen peroxide is one of the reactive oxygen species affecting RPE cells. The oxidant H_2O_2 was used in this study for oxidative stress in RPE cells. In the present study, we showed that crocin protects RPE cells by increasing the viability in ARPE-19 cells exposed to H_2O_2 .

There is increasing evidence that oxidant stress-induced death of RPE cells may contribute to the onset of AMD [3, 14, 15]. Previous studies demonstrated that H_2O_2 , which is generated physiologically during outer segment phagocytosis, causes mitochondrial dysfunction and promotes apoptosis correlating with

increased Bax expression and decreased Bcl-2 expression in human RPE cells [16, 17]. Caspases are key mediators of cell death and caspase-3 is an executioner for the death program in a variety of cells in response to oxidant. and oxidative stress induces caspase-3 activation in RPE cells [18, 19]. In line with these results, in this study, we observed that H_2O_2 significantly increased the expression of Bax and caspase-3 proteins, and decreased Bcl-2 expression. However, crocin inhibited the expression of Bax and caspase-3 proteins, and increased Bcl-2 expression in ARPE-19 cells, suggesting that crocin can protect ARPE-19 cells from the cellular apoptosis induced by oxidative stress.

Excessive ROS is one major cause of AMD. Lipid peroxidation-derived adducts also caused the apoptosis and physiological dysfunction in RPE cells. MDA, the main lipid peroxidation product, induces apoptosis in human lens epithelial and RPE cells [20]. Cellular anti-oxidant enzymes, including SOD, provide a substantial defense network against the accumulation of reactive oxygen intermediates and oxidative stress. Previous study showed that crocin treatment significantly increased the level of activity of glutathione, enhanced the activity of total superoxide dismutase (T-SOD), and decreased the activity level of ROS and MDA after IR injury [21]. Consistent with the prior study, in this study, we observed that crocin showed significant protection against H₂O₂-induced increases in ROS accumulation and lipid peroxidation, and H₂O₂-induced decreases in SOD activity. These results suggest again that crocin is a potent antioxidant in RPE cells.

It is known that oxidative stress can activate all three major MAPK signal cascades [22-24]. Activation of ERK1/2 usually leads cells to growth and survival, while activation of p38 MAPK and JNK also plays a critical role in cell growth, senescence, apoptosis and survival [25, 26]. It was reported that inhibition or knockdown of p38 MAPK by chemical inhibitors or siRNA induces cell death under oxidative stress, while activation of p38 MAPK enhances cell survival in ARPE-19 cells [27]. Ho *et al.* reported that activation of two stress kinases (JNK and p38) occurs during H_2O_2 stimulation, and H_2O_2 -mediated ARPE-19 death was significantly reduced by their specific inhibition [28]. In line with the previous studies, herein, we observed that H_2O_2 significantly increased the phosphorylation of p38 MAPK and JNK. However, when the cells were pre-treated with crocin, the phosphorylation of p38 MAPK and JNK could be suppressed. These results suggest that crocin protects against H_2O_2 -induced oxidative stress through suppression of the MAPK signaling pathway in ARPE-19 cells.

In conclusion, these data suggest that crocin protects against H_2O_2 -induced oxidative stress through suppression of the MAPK signaling pathway in ARPE-19 cells. Thus, crocin may be useful for the prevention or treatment of AMD.

Disclosure of conflict of interest

None.

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