



## Molecular and Cellular Pharmacology

## Crocetin prevents retinal degeneration induced by oxidative and endoplasmic reticulum stresses via inhibition of caspase activity

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## ABSTRACT

Crocetin is a carotenoid that is the aglicone of crocin, which are found in saffron stigmas (*Crocus sativus* L.) and gardenia fruit (*Gardenia jasminoides* Ellis). In this study, we investigated the effects of crocetin on retinal damage. To examine whether crocetin affects stress pathways, we investigated intracellular oxidation induced by reactive oxygen species, expression of endoplasmic reticulum (ER) stress-related proteins, disruption of the mitochondrial membrane potential ( $\Delta\Psi_m$ ), and caspases activation. *In vitro*, we employed cultured retinal ganglion cells (RGC-5, a mouse ganglion cell-line transformed using E1A virus). Cell damage was induced by tunicamycin or hydrogen peroxide ( $H_2O_2$ ) exposure. Crocetin at a concentration of 3  $\mu M$  showed the inhibitory effect of 50–60% against tunicamycin- and  $H_2O_2$ -induced cell death and inhibited increase in caspase-3 and -9 activity. Moreover, crocetin inhibited the enzymatic activity of caspase-9 in a cell-free system. *In vivo*, retinal damage in mice was induced by exposure to white light at 8000 lx for 3 h after dark adaptation. Photoreceptor damage was evaluated by measuring the outer nuclear layer thickness at 5 days after light exposure and recording the electroretinogram (ERG). Retinal cell damage was also detected with Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining at 48 h after light exposure. Crocetin at 100 mg/kg, p.o. significantly inhibited photoreceptor degeneration and retinal dysfunction and halved the expression of TUNEL-positive cells. These results indicate that crocetin has protective effects against retinal damage *in vitro* and *in vivo*, suggesting that the mechanism may inhibit increase in caspase-3 and -9 activities after retinal damage.

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## 1. Introduction

Oxidative stress is caused by reactive oxygen species, such as hydrogen peroxide ( $H_2O_2$ ), superoxide ( $O_2^{\cdot-}$ ), and hydroxyl radical ( $HO\cdot$ ). In particular, the retina consumes significant amounts of oxygen in the body and easily produces reactive oxygen species.

Endoplasmic reticulum (ER) stress is caused by several biochemical and physiological stimuli that result in the accumulation of unfolded proteins in the ER lumen (Kaufman, 1999). In response to ER stress, cells have developed a self-protective signal transduction pathway termed the unfolded protein response, which includes increased expression of the molecular chaperone [immunoglobulin heavy-chain protein (BiP)], translational attenuation, and ER-associated degradation (Cudna and Dickson, 2003). However, if the damage is too severe to repair, the unfolded protein response ultimately activates an apoptotic pathway (Oyadomari et al., 2002) involving a C/EBP homologous protein (CHOP) (Wang et al., 1996). Recent indications

suggest that ER stress also activates the mitochondrial apoptotic pathway (Deniaud et al., 2008).

The mitochondrial apoptotic pathway integrates a broad spectrum of extracellular and intracellular stresses. The intracellular stimuli include oxidative stress, DNA damage, and protein misfolding (Crow et al., 2004). These stresses induce the activation of Bax/Bak involving members of the BCL-2 family protein, the opening of permeability transition pores, disruption of the mitochondrial membrane potential ( $\Delta\Psi_m$ ), release of cytochrome *c*, activation of caspases, and cell death. Jimbo et al. reported that ER stress triggers caspase-8 activation, resulting in caspase-9 activation via Bid processing (Jimbo et al., 2003). Reactive oxygen species production and treatment with  $H_2O_2$  cause membrane depolarization and cytochrome *c* release (Sato et al., 1997). These results indicate that the mitochondrial apoptotic pathway may form a pivotal part of the induction of apoptosis by ER stress and oxidative stress. In addition, light exposure leads to these stresses in photoreceptor cells (Cruickshanks et al., 1993; Yang et al., 2008).

Light exposure leads to photoreceptor degeneration (Noell et al., 1966; Shahinfar et al., 1991), and several epidemiologic studies have suggested that long-term history of exposure to light may have some

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impacted on the incidence of age-related macular degeneration (Taylor et al., 1992). Photoreceptor cell death has been reported to be induced by calcium levels, reactive oxygen species, mitochondria, and so on. Intracellular calcium levels increase and reactive oxygen species generation has been shown to cause ER stress (Yang et al., 2008). Thus, oxidative stress and ER stress are likely to be involved in the pathogenesis of light-induced retinal damage.

Crocin, the glycoside of crocetin, is found in saffron stigmas (*Crocus sativus* L.) and gardenia fruit (*Gardenia jasminoides* Ellis) (Ichi et al., 1995; Li et al., 1999). In these plants, crocetin is postulated to be synthesized from zeaxanthin by enzymatic cleavage and then glycosylated to crocins (Bouvier et al., 2003; Camara and Bouvier, 2004). Saffron has been largely used in traditional medicine (Abdullaev, 1993), and antiapoptotic characterization in the retina has been reported (Maccarone et al., 2008). Therefore, in this study, we examined the protective effects of crocetin on retinal damage *in vitro* and *in vivo*. We investigated the effects on tunicamycin- and H<sub>2</sub>O<sub>2</sub>-induced neurotoxicity in RGC-5 and light-exposure-induced retinal damage in mice.

## 2. Materials and methods

### 2.1. Materials

The RGC-5 line was kindly gifted from Dr. Neeraj Agarwal (UNT Health Science Center, Fort Worth, TX, USA). RGC-5 cells have been shown to be retinal ganglion cells based on the positive expression of Thy-1, Brn-3C, neuritin, NMDA receptor, GABA-B2 receptor, and synaptophysin and the negative expression of glial fibrillary acidic protein (GFAP), HPC-1, and 8A1 (Krishnamoorthy et al., 2001). Drugs and sources were as follows: trolox (Sigma-Aldrich, St. Louis, MO, USA), H<sub>2</sub>O<sub>2</sub> (Wako, Osaka, Japan), tunicamycin (Wako), Hoechst 33342 (Molecular Probes, Eugene, OR, USA), propidium iodide (PI; Molecular Probes), and crocetin (Riken Vitamin Co., Ltd., Tokyo, Japan).

### 2.2. Retinal ganglion cell (RGC-5) culture

RGC-5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin (Meiji Seika Kaisha Ltd., Tokyo, Japan), and 100 µg/ml streptomycin (Meiji Seika) under a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37 °C. The RGC-5 cells were passaged by trypsinization every 3 to 4 days, as described in our previous report (Shimazawa et al., 2005).

### 2.3. Tunicamycin or H<sub>2</sub>O<sub>2</sub>-induced cell death assay

RGC-5 cells were seeded at 1 × 10<sup>3</sup> cells per well in 96-well plates and then incubated for 24 h. The entire medium was then replaced with fresh medium containing 0.5% or 1% FBS, and crocetin and trolox were pretreated for 1 h; then tunicamycin was added (at a final concentration of 2 µg/ml) or H<sub>2</sub>O<sub>2</sub> (0.3 mM). Crocetin and trolox were dissolved in dimethyl sulfoxide (DMSO) and diluted with PBS containing 1% DMSO (final concentration, 0.1%). Trolox at a concentration of 100 µM was used as a typical antioxidant (Nakajima et al., 2008). Nuclear staining assays were carried out after an additional 27 h of incubation. At the end of the culture period, Hoechst 33342 ( $\lambda_{\text{ex}} = 360 \text{ nm}$ ,  $\lambda_{\text{em}} > 490 \text{ nm}$ ) and PI ( $\lambda_{\text{ex}} = 535 \text{ nm}$ ,  $\lambda_{\text{em}} > 617 \text{ nm}$ ) were added to the culture medium for 15 min at final concentrations of 8.1 µM and 1.5 µM, respectively. Hoechst 33342 freely enters living cells and then stains the nuclei of viable cells, as well as those that have suffered apoptosis or necrosis. Propidium iodide is a membrane-impermeable dye that is generally excluded from viable cells. Images were collected using an Olympus IX70 inverted epifluorescence microscope (Olympus, Tokyo, Japan). The

total number of cells was counted in a blind manner (by M.Y.) and calculated the percent of PI positive cells.

### 2.4. Radical scavenging-capacity assay

RGC-5 cells were seeded at 2 × 10<sup>3</sup> cells per well in 96-well plates, and then incubated. After incubating for 1 day, cells were washed with 1% FBS DMEM. After a 1-h pretreatment with crocetin or trolox, 10 µM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H<sub>2</sub>DCFDA; Molecular Probes) (the radical probe) was added to load the cell culture. After 20 min, the cell culture medium was replaced with fresh 1% FBS DMEM in the presence or absence of crocetin or trolox to remove any extracellular CM-H<sub>2</sub>DCFDA (inactive for reactive oxygen species), the radical probe was converted to 2',7'-dichlorodihydrofluorescein diacetate (DCFH) (active for reactive oxygen species) by the action of intracellular esterase. To generate the radical species, we added 1 mM of H<sub>2</sub>O<sub>2</sub> as the radical probe loading medium. Fluorescence was measured, after H<sub>2</sub>O<sub>2</sub> had been present for various time periods, using a SkanIt RE for Varioskan Flash 2.4 (a microplate reader) (Thermo Fisher Scientific, Waltham, MA, USA) at excitation/emission wavelengths of 485/535 nm. To try to detect the ·OH, we used 2-[6-(4'-amino) phenoxy-3 H-xanthen-3-on-9-yl] benzoic acid (APF; Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan). The cells were loaded with APF by incubation for 20 min at 37 °C in Hanks/Hepes buffer solution. H<sub>2</sub>O<sub>2</sub> was added to the Hanks/Hepes buffer solution of APF, containing APF (10 µM), and then iron (II) perchlorate hexahydrate (Wako) was added. H<sub>2</sub>O<sub>2</sub> reacts with reduced iron (FeII) in the Fenton reaction to generate ·OH. Fluorescence was measured at excitation/emission wavelengths of 490/515 nm.

Radical integrals were calculated by integrating the area under the CM-H<sub>2</sub>DCFDA fluorescence intensity curve after a 20 min treatment with reactive oxygen species-generating compounds.

### 2.5. Mitochondrial membrane potential ( $\Delta\Psi_m$ )

To measure  $\Delta\Psi_m$ , the fluorescent dye JC-1 (Cell Technology, Mountain View, CA, USA) was used. JC-1 exists as a monomer at low  $\Delta\Psi_m$  and emits green fluorescence but forms aggregates and emits red fluorescence at higher  $\Delta\Psi_m$  levels. After cells were treated with H<sub>2</sub>O<sub>2</sub> in the presence or absence of 3 µM crocetin or 100 µM trolox for 24 h, cells were collected by trypsinization and incubated for 15 min at 37 °C with JC-1. Thereafter, cells were washed with loading buffer and measured using SkanIt RE for Varioskan Flash 2.4 (Thermo) with respective excitation/emission wavelengths of 485/535 nm for the monomer and 550/600 nm for the aggregates.

### 2.6. Western blot analysis

RGC-5 cells were washed with PBS, harvested, and lysed in RIPA buffer (Sigma) supplemented with protease inhibitor cocktail (Sigma) and phosphate inhibitor cocktails 1 and 2 (Sigma). Lysates were centrifuged at 12,000 × g for 15 min at 4 °C. Supernatants were collected and boiled for 5 min in SDS sample buffer (Wako). Equal amounts of protein were subjected to 10% SDS-PAGE gradient gels and then transferred to poly (vinylidene difluoride) membranes. After being blocked with Block Ace (Snow Brand Milk Products Co. Ltd., Tokyo, Japan) for 30 min, the membranes were incubated with the primary antibody [mouse anti-BIP/GRP78 antibody (BD Transduction Laboratories, Lexington, KY, USA), mouse anti-GADD 153 (B-3) (Santa Cruz Biotechnology, CA, USA), Affinity-purified goat anti-human/mouse Bid (R&D Systems, Minneapolis, MN, USA), or mouse anti-β-actin antibody (Sigma)]. Subsequently, the membrane was incubated with the secondary antibody [HRP-conjugated goat anti-mouse IgG (Pierce Biotechnology, Rockford, IL, USA) or rabbit anti-goat IgG, (H + L), peroxidase conjugated (Thermo)]. The immunoreactive

bands were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology) and the LAS-4000 luminescent image analyzer (Fuji Film Co., Ltd., Tokyo, Japan).

### 2.7. Caspase-9 assay

To measure caspase-9 activity, Caspase-Glo 9 Assay (Promega Corp., Madison, WI, USA) was used. RGC-5 cells were seeded at  $1 \times 10^4$  cells per well in 96-well plates and then incubated for 24 h at 37 °C. After cells were treated with H<sub>2</sub>O<sub>2</sub> or tunicamycin in the presence or absence of 3 μM of crocetin or 100 μM trolox for 15 h, Caspase-Glo 9 Reagent was added with a 1:1 ratio of the reagent volume to the sample volume. Thereafter, cells were incubated for 2 h at 37 °C, and the absorbance at a particular wavelength was measured using SkanIt RE for Varioskan Flash 2.4.

### 2.8. Caspase-3 assay

To measure caspase-3 activity, CaspACE™ Assay System, Fluorometric (Promega) was used. RGC-5 cells were seeded at  $2 \times 10^6$  cells per 10-cm dishes and then incubated for 24 h at 37 °C. After cells were treated with H<sub>2</sub>O<sub>2</sub> or tunicamycin in the presence or absence of 3 μM of crocetin or 100 μM of trolox for 24 h, cells were collected by trypsinization and suspended in cell lysis buffer. Cell lysates were mixed in CaspACE™ Assay substrates and incubated for 1 h at 37 °C. The fluorescence was measured using SkanIt RE for Varioskan Flash 2.4 with excitation/emission wavelengths of 360/460 nm.

### 2.9. In vitro protease assay

RGC-5 cells were washed with PBS, collected by trypsinization, and centrifuged at  $1000 \times g$  for 5 min at 4 °C. The cell pellets were washed with PBS, centrifuged at  $1500 \times g$  for 5 min at 4 °C, and suspended in cell lysis buffer. After 20 min, cell lysates were incubated with 3 μM of crocetin for 1 h at 4 °C. Caspase-9 was activated by adding cytochrome c from bovine heart (Sigma) and 2'-deoxyadenosine 5'-triphosphate sodium salt solution (dATP; Sigma) for 30 min at 37 °C (Tsuruma et al., 2006). Thereafter, Caspase-Glo 9 Reagent was added with a 1:1 ratio of the reagent volume to the sample volume and incubated for 2 h at 37 °C. The absorbance at a particular wavelength was measured using SkanIt RE for Varioskan Flash 2.4.

### 2.10. Animals

Male adult ddY mice and SD rats (Japan SLC, Hamamatsu, Japan) were kept under 12 h light/12 h dark conditions and had ad libitum access to food and water. All experimental procedures were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University.

### 2.11. Crocetin analysis

The concentrations of plasma and aqueous humor crocetin were determined with HPLC analysis. Crocetin was dissolved in 0.5% carboxymethylcellulose (CMC) immediately before use, and was orally administered at doses of 10–100 mg/kg. At 1 h after the administration, each mouse was anesthetized with pentobarbital, and blood was collected from the caudal vena cava with a heparinized syringe. At 1.5 h after the administration, each rat was anesthetized with diethylether, and blood was collected from tail vein, and aqueous humor was collected with a heparinized syringe. Plasma was prepared by centrifugation of blood at  $3000 \times g$  for 30 min at 4 °C and stored at –80 °C. The plasma or aqueous humor (100 μl) was mixed with 2.0 ml of methanol and centrifuged (3000 rpm, 10 min). The supernatant (1.7 ml) was evaporated under nitrogen gas. The residue was dissolved in 2.0 ml of alkaline buffer (50 mM Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>–50 mM

Na<sub>2</sub>CO<sub>3</sub> buffer of pH 10) and loaded onto a solid-phase extraction cartridge (Oasis HLB Extraction Cartridge, Nihon Waters, Japan) pre-conditioned with methanol (2.0 ml) and alkaline buffer (2.0 ml). The cartridge was washed with water (2.0 ml) and hexane (2.0 ml). The analyte was eluted with methanol (2.0 ml), and the eluate was concentrated to dryness under nitrogen gas. The residue was re-constituted in 200 μl of methanol and filtered with 0.45 μm of Millipore filter for HPLC analysis.

Crocetin was quantified by the reversed-phase HPLC method using Atlantis T3 column (2.1 mm × 150 mm, Nihon Waters). The injection volume was 20 μl, and the mobile phase consisted of water with 0.1% trifluoroacetic acid (A) and mixture of methanol: water (9:1, v/v) with 0.1% trifluoroacetic acid (B). The gradient started with 50% B to 100% for 35 min. The flow rate was 0.2 ml/min, and the chromatogram was monitored at 430 nm. The crocetin concentration was calculated from the peak area with the calibration curve of the authentic crocetin standard.

### 2.12. Exposure to light

After dark adaptation for 24 h, the pupils were dilated with 1% cyclopentolate hydrochloride eye drops (Santen, Osaka, Japan) 30 min before exposure to light. The mice were exposed to 8000 lx of white fluorescent light (Toshiba, Tokyo, Japan) for 3 h in cages with a reflective interior. After being exposed to light, the mice were kept under normal light/dark conditions.

Crocetin was dissolved in 0.5% CMC immediately before use, and was orally administered at a dose of 20 mg/kg or 100 mg/kg at 1 h before exposure to light, and then once daily for 5 days.

### 2.13. Electroretinogram

Electroretinograms (ERGs) were recorded at 5 days after the exposure to light (Mayo, Aichi, Japan). The mice were maintained in a completely dark room for 24 h. They were anesthetized with an intraperitoneal injection of ketamine (120 mg/kg) and xylazine (6 mg/kg). The pupils were dilated with 1% tropicamide and 2.5% phenylephrine (Santen). Recordings were made from the left eyes, with a golden-ring electrode (Mayo) placed on the cornea and a reference electrode (Nihon Kohden, Tokyo, Japan) through the tongue. A neural electrode (Nihon Kohden) was inserted subcutaneously near the tail. All procedures were performed in dim red light, and the mice were kept warm during the entire procedure. The amplitude of the a-wave was measured from the baseline to the maximum a-wave peak, and the b-wave was measured from the maximum a-wave peak to the maximum b-wave peak.

### 2.14. Histological analysis

In mice under anesthesia produced by an intraperitoneal injection of sodium pentobarbital (80 mg/kg, Nembutal®, Dainihon Sumitomo, Co., Ltd., Tokyo, Japan), each eye was enucleated and kept immersed for at least 24 h at 4 °C in a fixative solution containing 4% paraformaldehyde. Six paraffin-embedded sections (thickness, 4 μm) cut through the optic disc of each eye were prepared in the standard manner, and stained with hematoxylin and eosin. The damage induced by light exposure was then evaluated, with six sections from each eye used for the morphometric analysis. Light-microscope images were photographed, and the thickness of the outer nuclear layer from the optic disc was measured at 240-μm intervals on the photographs in a blind manner. Data from three sections (selected randomly from the six sections) were averaged for each eye.

### 2.15. TUNEL staining

TUNEL staining was performed according to the manufacturer's protocols (In Situ Cell Death Detection Kit; Roche Biochemicals,

Mannheim, Germany) to detect retinal cell death induced by exposure to light. The mice were anesthetized with pentobarbital sodium at 80 mg/kg, i.p., at 48 h after exposure to light for 3 h. The eyes were enucleated, fixed overnight in 4% paraformaldehyde, and immersed for 2 days in 25% sucrose with PBS. The eyes were then embedded in a supporting medium for frozen-tissue specimens (OCT compound, Tissue-Tek; Miles Laboratories, Naperville, IL, USA). The retinal sections were cut in 10- $\mu$ m thicknesses on a cryostat at  $-25^{\circ}\text{C}$ , and stored at  $-80^{\circ}\text{C}$  until staining. After being washed twice with PBS, the sections were incubated with terminal deoxyribonucleotidyl transferase (TdT) enzyme at  $37^{\circ}\text{C}$  for 1 h. The sections were washed 3 times in PBS for 1 min at room temperature. Sections were subsequently incubated with an anti-fluorescein antibody-peroxidase (POD) conjugate at room temperature in a humidified chamber for 30 min, and then developed using diaminobenzidine (DAB) tetrahydrochloride peroxidase substrate. Light-microscope images were photographed, and the labeled cells were counted in the outer nuclear layer at a distance between 375 and 625  $\mu$ m from the optic disc in two areas of the retina. The numbers of TUNEL-positive cells were averaged for these two areas, and the value was plotted as the number of TUNEL-positive cells.

### 2.16. Statistical analysis

Data are presented as the means  $\pm$  S.E.M. Statistical comparisons were made using Student's *t*-test and one-way ANOVA followed by Dunnett's test [using STAT VIEW version 5.0 (SAS Institute, Cary, NC, USA)].  $P < 0.05$  was considered to indicate statistical significance.

## 3. Results

### 3.1. Effects of crocetin and trolox on cell damage induced by tunicamycin or $\text{H}_2\text{O}_2$ in RGC-5 culture

Representative photographs of Hoechst 33342 and PI staining are shown in Fig. 1A and C. Hoechst 33342 stains all cells (live and dead cells), whereas PI stains only dead cells. Pretreatment with crocetin at concentrations of 0.1 to 3  $\mu$ M protected against tunicamycin-induced cell death in a concentration-dependent manner; the effect was significant for the 3- $\mu$ M concentration. In contrast, trolox at a concentration of 100  $\mu$ M did not affect cell death (Fig. 1B). Crocetin also protected against  $\text{H}_2\text{O}_2$ -induced cell death; the effect was significant at the 1- and 3- $\mu$ M concentrations. Trolox at a concentration of 100  $\mu$ M inhibited cell death (Fig. 1D).

### 3.2. Effects of crocetin on the intracellular oxidation of DCFH and APF induced by $\text{H}_2\text{O}_2$ or $\text{HO}\cdot$

To investigate the effect of crocetin on  $\text{H}_2\text{O}_2$  or  $\text{HO}\cdot$  production, we employed radical scavenging-capacity assays using reactive oxygen species-sensitive probes, CM- $\text{H}_2\text{DCFDA}$ , and APF. The kinetics of DCFH and APF oxidation by reactive oxygen species (monitored as fluorescence generation) are shown in Supplemental Fig. 1A and B.  $\text{H}_2\text{O}_2$  was added at a concentration of 1 mM, and trolox at concentrations of 1 to 100  $\mu$ M significantly scavenged these  $\text{H}_2\text{O}_2$  radicals, but crocetin did not have an effect (Supplemental Fig. 1C). The  $\text{HO}\cdot$  radicals generated by treatment with  $\text{H}_2\text{O}_2$  at a concentration of 1 mM plus Fe perchlorate (II) at a concentration of 100  $\mu$ M were scavenged by trolox at concentrations of 1 to 100  $\mu$ M, although crocetin did not affect the  $\text{HO}\cdot$  radicals (Supplemental Fig. 1D).

### 3.3. Effects of crocetin on $\text{H}_2\text{O}_2$ -induced $\Delta\Psi_m$ disruption

An increase in mitochondrial permeability leads to a disruption of  $\Delta\Psi_m$ . To examine whether crocetin might prevent the disruption of  $\Delta\Psi_m$  induced by  $\text{H}_2\text{O}_2$ , we used lipophilic cation JC-1 to evaluate  $\Delta\Psi_m$ .

Compared with the control,  $\text{H}_2\text{O}_2$  significantly decreased  $\Delta\Psi_m$ , and trolox partially prevented this effect of  $\text{H}_2\text{O}_2$ , although crocetin did not prevent mitochondrial damage (Supplemental Fig. 2).

### 3.4. Effects of crocetin on the expression of ER stress-related proteins after treatment with tunicamycin

To investigate whether crocetin might affect the ER stress pathway, we detected BiP and CHOP expression, which responds to ER stress, and the truncated Bid (tBid), which is activated by ER stress and leads to mitochondrial damage, using Western blot (Fig. 2A and B). Tunicamycin significantly up-regulated BiP and CHOP expression, but crocetin at a concentration of 3  $\mu$ M did not inhibit the expression of BiP and CHOP (Fig. 2C). Similarly, tunicamycin increased tBid, but crocetin at a concentration of 3  $\mu$ M did not affect the increase of tBid (Fig. 2D).

### 3.5. Effects of crocetin on tunicamycin- and $\text{H}_2\text{O}_2$ -induced caspase-3 and -9 activation

To investigate the effect of crocetin on tunicamycin- and  $\text{H}_2\text{O}_2$ -induced activation of caspase-3 and -9, we used CaspACE™ Assay System, Fluorometric and Caspase-Glo 9 Assay. Tunicamycin significantly increased caspase-3 and -9 activation, and crocetin at a concentration of 3  $\mu$ M significantly inhibited the activation of these caspases (Fig. 3A and B), but trolox at a concentration of 100  $\mu$ M did not (data not shown).  $\text{H}_2\text{O}_2$  also significantly increased caspase-3 and -9 activation, and crocetin at a concentration of 3  $\mu$ M and trolox at a concentration of 100  $\mu$ M significantly inhibited the activation of caspase-3 and -9 (Fig. 3C and D).

Furthermore, we examined the inhibitory effects of crocetin on caspase-9 activation in a cell-free system. The addition of cytochrome c and deoxyadenosine triphosphate (dATP) in the cell lysate significantly increased caspase-9 activation, and crocetin at a concentration of 3  $\mu$ M inhibited activation (Fig. 4). Therefore, crocetin might affect caspase-9 activity or activation directly.

### 3.6. Plasma and aqueous humor concentrations of crocetin after oral administration in mice and rats

Plasma concentrations of crocetin in mice were determined by HPLC analysis at 1 h after oral administration of 10 and 100 mg/kg and the results were  $4.5 \pm 1.0 \mu\text{M}$  (mean  $\pm$  SEM,  $n = 4$ ) and  $109.6 \pm 13.7 \mu\text{M}$  ( $n = 5$ ), respectively. On the other hand, at 1.5 h after oral administration of 50 mg/kg, the plasma and aqueous humor concentrations of crocetin in rats were  $62.5 \pm 13.7 \mu\text{M}$  and  $2.0 \pm 0.5 \mu\text{M}$  ( $n = 5$ ), respectively (Table 1).

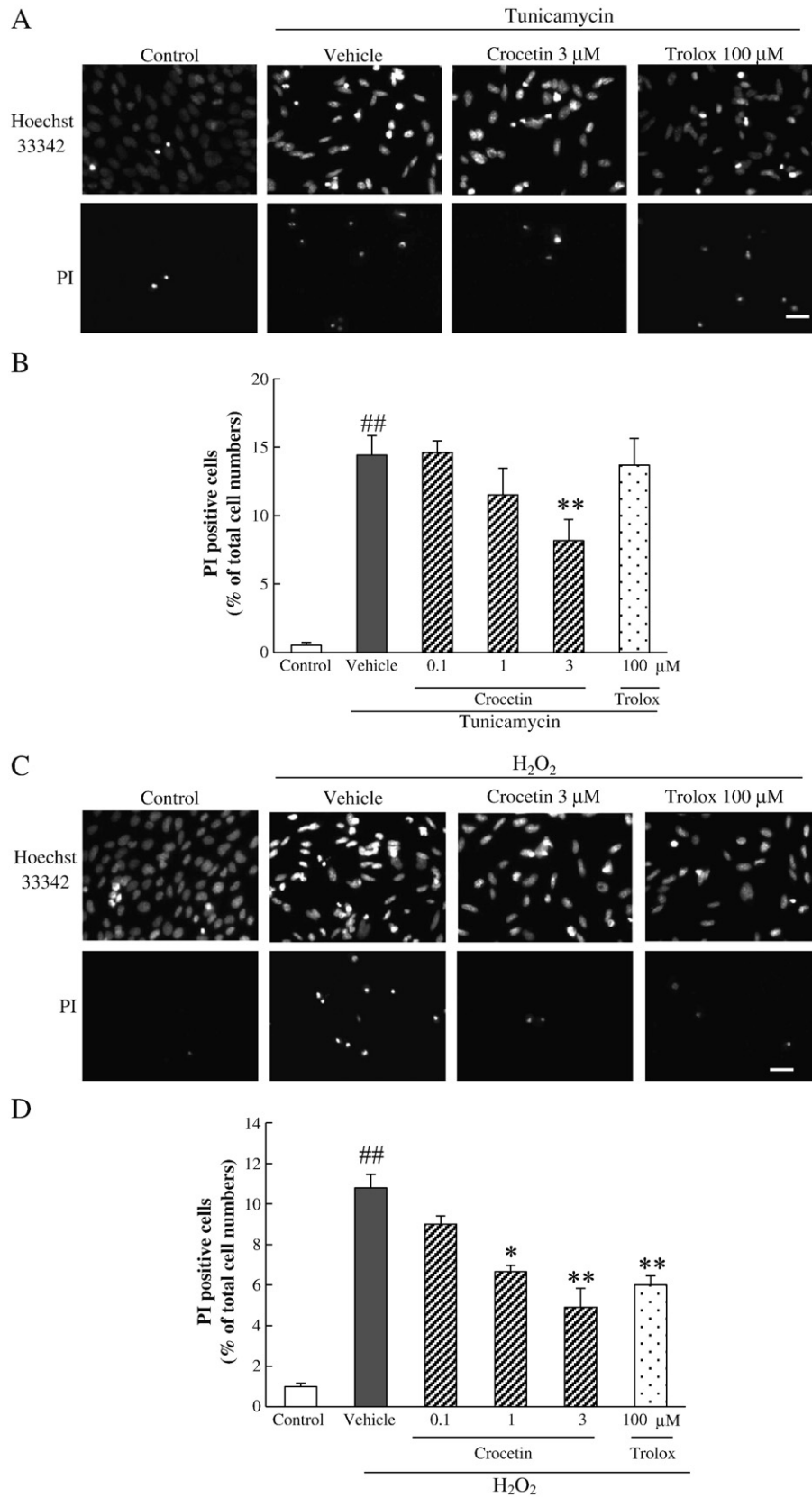
### 3.7. Effects of crocetin on light-induced retinal functional damage in mice

The functional consequences of crocetin were evaluated by recording the electroretinographic (ERG) response. The a-wave shows the function of the photoreceptors, and the b-wave reflects bipolar cells and Müller cell function. Therefore, the decrease of a- and b-wave amplitudes shows retinal dysfunction. Both a- and b-wave amplitudes were significantly reduced at 5 days after 8000 lx light exposure for 3 h, and in the crocetin-treated (100 mg/kg, p.o.) group, a decrease in the a- and b-wave amplitudes was significantly prevented compared with the vehicle group (Fig. 5A to C).

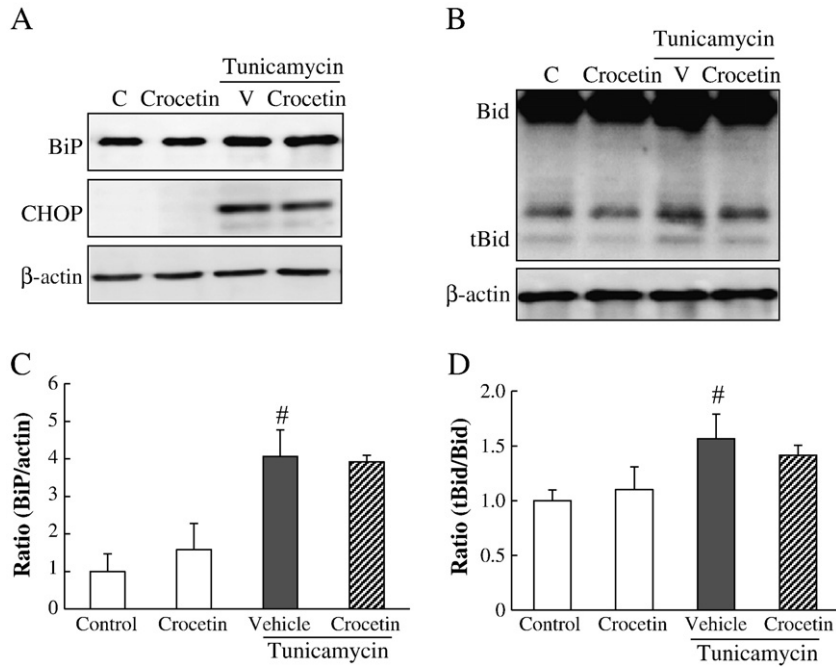
### 3.8. Effects of crocetin on light-induced photoreceptor histology and cell death

In the histological evaluation, representative retinal images from the optic nerve were taken at 5 days after light exposure (Fig. 6A). The





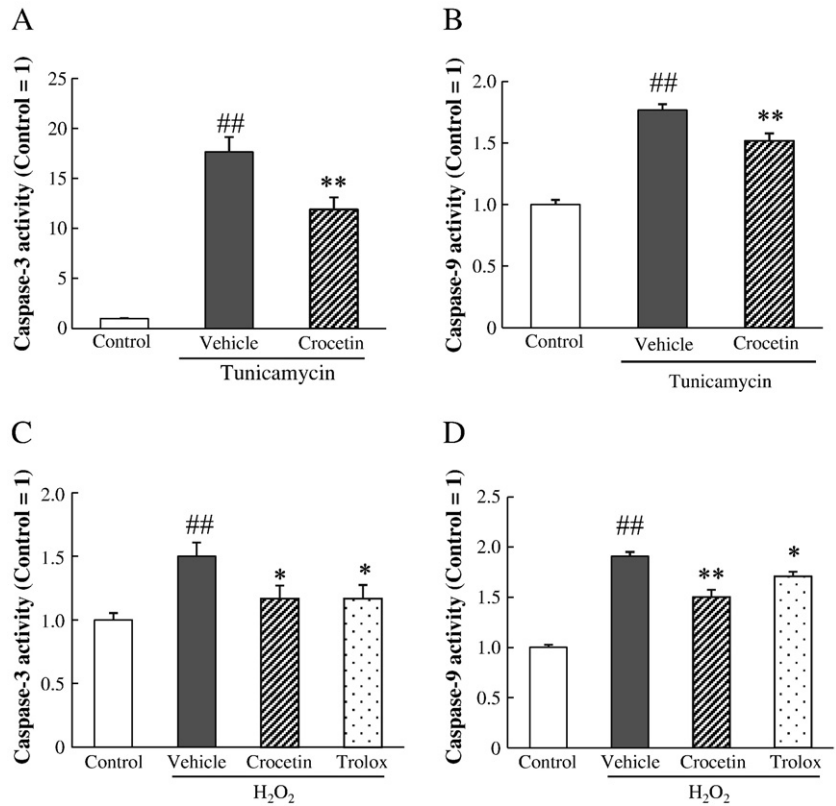
**Fig. 1.** Effects of crocetin and trolox on cell damage induced by tunicamycin or  $\text{H}_2\text{O}_2$  in RGC-5 culture. Representative fluorescence microscopy of Hoechst 33342 and PI staining (added at 27 h after tunicamycin (A) or  $\text{H}_2\text{O}_2$  (B) treatment). The number of PI positive cells increased at 27 h after tunicamycin (A) or  $\text{H}_2\text{O}_2$  (B) treatment. Trolox was used as a typical antioxidant. Data are shown as mean  $\pm$  S.E.M. ( $n=6$  or 8). <sup>\*</sup> $P<0.05$ , <sup>\*\*</sup> $P<0.01$  vs. tunicamycin or  $\text{H}_2\text{O}_2$ , <sup>##</sup> $P<0.01$  vs. control.



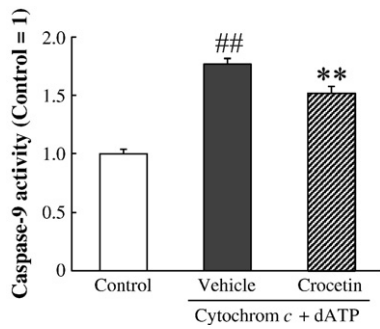
**Fig. 2.** Effects of crocetin on BiP, CHOP, and tBid expression using Western blotting in RGC-5. (A, B) Pretreatment with vehicle (lanes 1 and 3) or with crocetin (3 μM) at either (lanes 2 and 4) was followed by 24 h of additional incubation with 2 μg/ml tunicamycin (lanes 3 and 4). Representative band images showed (A) BiP and CHOP expression and (B) Bid and tBid expression. (C) BiP and CHOP expressions were quantified by densitometry and corrected by reference to β-actin. (D) Bid expression was quantified by density for tBid/Bid. Data are shown as mean ± S.E.M. (n = 4 or 6). <sup>#</sup>P < 0.05 vs. Control. C: Control, V: Vehicle.

thickness of the outer nuclear layer was remarkably thinned in the vehicle group (Fig. 6A-b) vs. the normal group (Fig. 6A-a). The crocetin-treated (100 mg/kg, p.o.) group suppressed the damage

(Fig. 6A-d). The thickness of the outer nuclear layer was measured in 240-μm steps; the data were averaged and reported in Fig. 6B. Crocetin at a concentration of 100 mg/kg, p.o., significantly protected



**Fig. 3.** Effects of crocetin against tunicamycin or H<sub>2</sub>O<sub>2</sub>-induced caspase-3 and -9 activity. (A, C) Caspase-9 activity was evaluated by using Caspase-Glo® 9 Assays. It was measured after 15 h of pretreatment of (A) tunicamycin (2 μg/ml) or (C) H<sub>2</sub>O<sub>2</sub> (0.3 mM). (B, D) Caspase-3 activity was evaluated by using CasPACE™ Assay System, Fluorometric. It was measured after 24 h of pretreatment (B) tunicamycin or (D) H<sub>2</sub>O<sub>2</sub>. Concentrations of 3 μM and 100 μM of crocetin and trolox, respectively, were used. Data are shown as mean ± S.E.M. (n = 5 or 6). <sup>\*</sup>P < 0.05, <sup>\*\*</sup>P < 0.01 vs. tunicamycin or H<sub>2</sub>O<sub>2</sub>, <sup>##</sup>P < 0.01 vs. control.



**Fig. 4.** Effects of crocetin on caspase-9 activity in a cell-free system. Caspase-9 activation was induced by the addition of cytochrome *c* and dATP. The effects of crocetin (3  $\mu$ M) on caspase-9 activation in the cell lysates of RGC-5 were evaluated with Caspase-Glo 9 Assays. The cell lysates were incubated for 2 h at 37 °C, and the luminescence was measured. Data are shown as mean  $\pm$  S.E.M. ( $n=6$ ). \*\* $P<0.01$  vs. cytochrome *c* and dATP, ## $P<0.01$  vs. control.

**Table 1**

Plasma and aqueous humor concentrations of crocetin after oral administration in mice and rats.

	Doses (mg/kg)	Time (h)	<i>n</i>	Concentrations ( $\mu$ M)	
				Plasma	Aqueous humor
Mouse	10	1	4	4.5 $\pm$ 1.0	–
	100	1	5	109.6 $\pm$ 13.7	–
Rat	50	1.5	5	62.5 $\pm$ 13.7	2.0 $\pm$ 0.5

Blood of mice was collected at 1 h after the administration of crocetin (10 or 100 mg/kg, p.o.). Blood and aqueous humor of rats were collected at 1.5 h after the administration of crocetin (50 mg/kg, p.o.). The plasma and aqueous humor concentrations of crocetin were determined by HPLC analysis. Data are shown as means  $\pm$  S.E.M.

the retinal superior area from 240  $\mu$ m to 1440  $\mu$ m, and the inferior area from 240  $\mu$ m to 1680  $\mu$ m.

To show light-induced apoptotic cell death and the effect of crocetin on cell death, we investigated the expression of TUNEL-positive cells at 48 h after exposure to light (Fig. 6C). TUNEL-positive cells were observed only in the outer nuclear layer, and not in any other retinal area (Fig. 6C-b). On the other hand, TUNEL-positive cells in the normal group were not found in any retinal area (Fig. 6C-a).

Quantitative analysis showed that exposure to light in the mouse retina significantly increased the number of TUNEL-positive cells in the superior and inferior outer nuclear layer at 48 h (vs. control) (Fig. 6D). Crocetin at a concentration of 100 mg/kg, p.o., significantly reduced the number of TUNEL-positive cells.

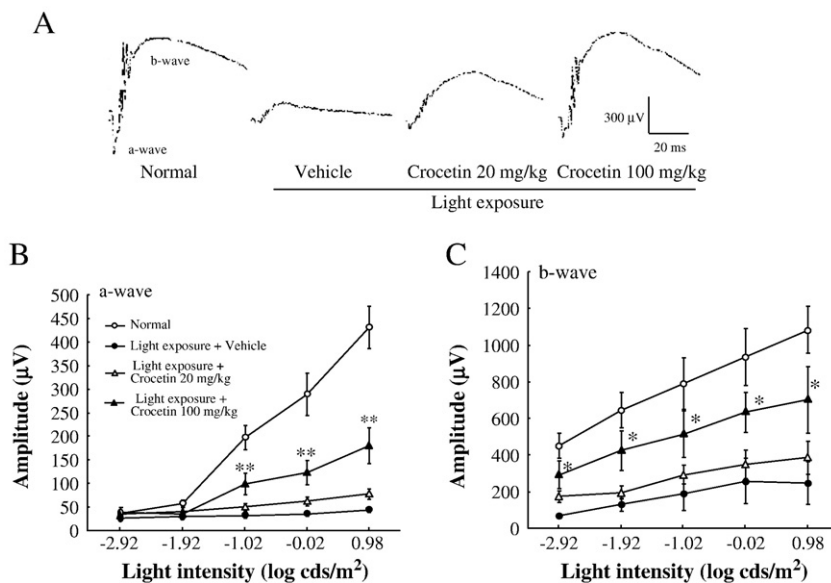
#### 4. Discussion

In the present study, we found that in RGC-5 *in vitro*, crocetin inhibited cell death induced tunicamycin- and H<sub>2</sub>O<sub>2</sub>-induced cell death by the inhibition of caspases activities, and that in mice *in vivo*, crocetin significantly protected against light-induced retinal damage.

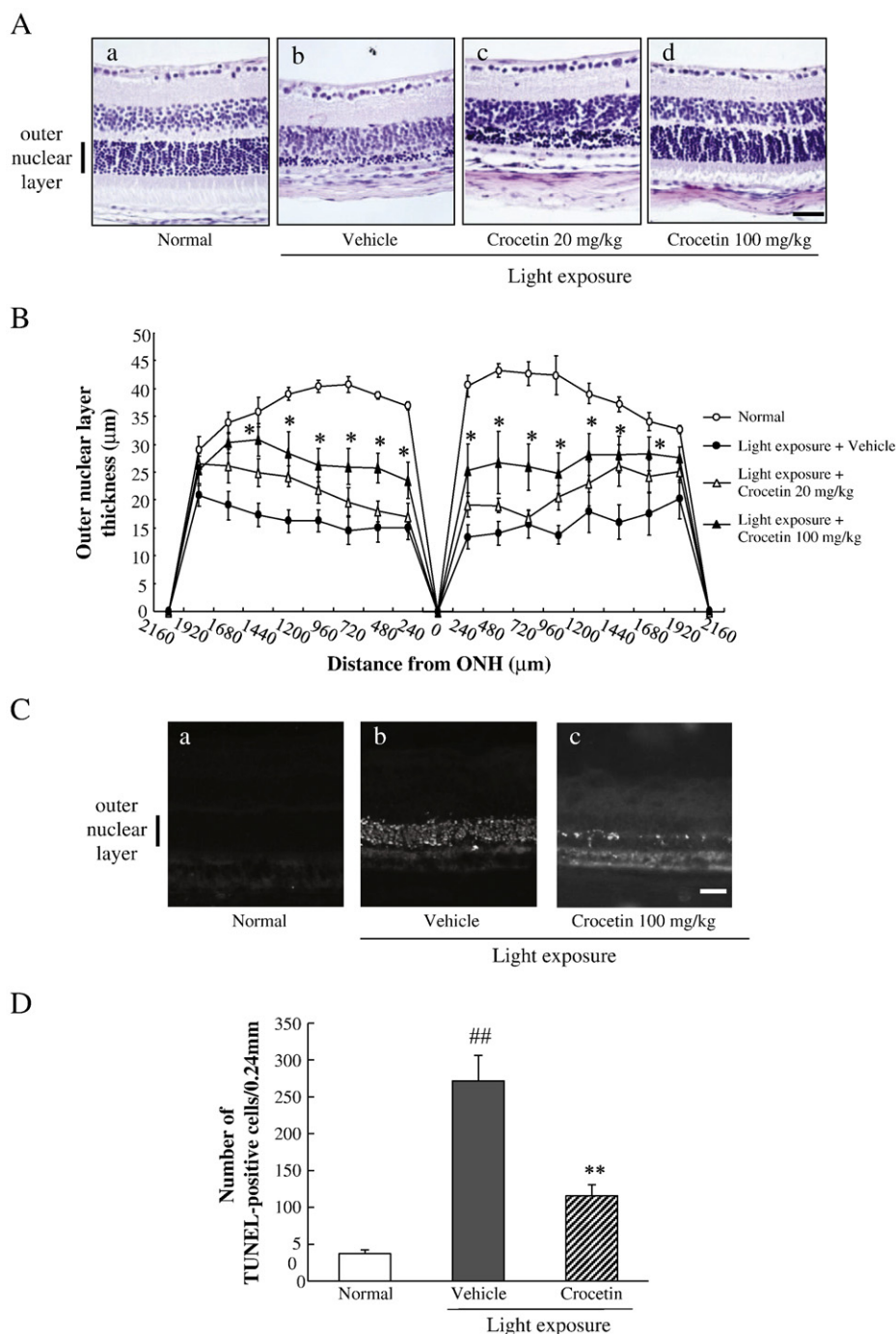
*In vitro*, crocetin at a concentration of 3  $\mu$ M inhibited retinal cell death induced by tunicamycin and H<sub>2</sub>O<sub>2</sub>, whereas trolox at a concentration of 100  $\mu$ M protected against H<sub>2</sub>O<sub>2</sub>-induced cell death but did not have any effect on tunicamycin-induced cell death (Fig. 1). Since crocetin has been reported to have antioxidant activity (Ochiai et al., 2004; Kanakis et al., 2007), we investigated the effects of crocetin on H<sub>2</sub>O<sub>2</sub> and HO $\cdot$  production. However, crocetin at a concentration of 3  $\mu$ M had no effect (Supplemental Fig. 1). A previous study indicated the antioxidant activity of crocetin was tested by the DPPH $\cdot$  antioxidant activity assay, and the IC<sub>50</sub> value was 17.8  $\pm$  1  $\mu$ g/ml (Kanakis et al., 2007). In our study, the concentration of crocetin that inhibited cell death was approximately 1  $\mu$ g/ml (the molecular weight of crocetin is 328.4). This result suggests that the protective effects of crocetin against tunicamycin- and H<sub>2</sub>O<sub>2</sub>-induced cell death were independent of its antioxidant activity in our experiments.

Since crocetin protected against an ER stressor, tunicamycin, we thought that they might alter the expression of ER stress-related proteins (BiP, CHOP, and tBid). However, crocetin did not alter the expression levels of BiP, CHOP, or tBid observed after tunicamycin application (Fig. 2). These data suggest that the ER stress-defensive effects of crocetin may be independent of alterations in the levels of protein expression induced by ER stress.

Next, we focused on the common apoptosis pathway activated by ER stress and oxidative stress. Arduino et al. reported that tunicamycin induces changes in the mitochondrial function, impairing mitochondrial membrane potential and overexpressing caspase-9 in NT2 neuron-like cells (Arduino et al., 2009). Moreover, H<sub>2</sub>O<sub>2</sub> clearly increased the levels of Bax by 1.5-fold and caspase-3 by 1.8-fold in



**Fig. 5.** Effects of crocetin on light-induced retinal functional damage after light exposure. The effects of crocetin were evaluated by recording the electroretinogram (ERG) at 5 days after exposure to light in mice. (A) Representative ERG recording in non-treated, light exposure (8000 lx) plus vehicle-treated and light exposure plus crocetin (20 or 100 mg/kg, p.o.). (B, C) Intensity response functions for dark-adapted (B) a-wave and (C) b-wave amplitudes. The crocetin-treated (100 mg/kg, p.o.) group showed significantly preserved a- and b-wave amplitudes compared with the vehicle group. Data are shown as mean  $\pm$  S.E.M. ( $n=6$  to 9). \* $P<0.05$ , \*\* $P<0.01$  vs. light exposure plus the vehicle-treated group (vehicle).



**Fig. 6.** Effects of crocetin on light-induced retinal morphological change. (A) Representative photographs (hematoxylin and eosin staining of retinal sections) showing (a) non-treated, (b) light exposure (8000 lx) plus vehicle-treated, (c) light exposure plus crocetin at 20 mg/kg, p.o. and (d) light exposure plus crocetin at 100 mg/kg, p.o. (B) Thickness of the outer nuclear layer was measured at 5 days after light exposure. The outer nuclear layer was measured at 240- $\mu$ m intervals from the optic disc. (C) Representative photographs (TUNEL staining of retinal sections) showing (a) non-treated, (b) light exposure (8000 lx) plus vehicle-treated and (c) light exposure plus crocetin at a concentration of 100 mg/kg, p.o. (D) Quantitative analysis of the number of TUNEL-positive cells in the outer nuclear layer at 48 h after light exposure. Superior area was left side of optic nerve and inferior area was another side. Data are shown as means  $\pm$  S.E.M. ( $n = 6$  to 9). \* $P < 0.05$  vs. light exposure plus the vehicle-treated group (vehicle). \*\* $P < 0.01$  vs. non-treated group (normal).

the RGC-5 cell culture (Koriyama et al., 2009). These reports indicated that increased mitochondrial permeability may play an important role in cell death induced by ER stress and oxidative stress. Thus, we evaluated the pharmacological action of crocetin against the mitochondrial apoptosis pathway. However, crocetin did not prevent mitochondrial damage (Supplemental Fig. 2). This result suggests that crocetin may not prevent the inhibition of  $\Delta\Psi_m$  depolarization. Therefore, we focused on caspase-3 and -9, which are downstream from the  $\Delta\Psi_m$  loss. Crocetin at a concentration of 3  $\mu$ M inhibited this

activation after treatment with tunicamycin and  $H_2O_2$  (Fig. 3). Moreover, crocetin inhibited caspase-9 activity in the cell lysate under conditions where cytochrome c and dATP existed (Fig. 4). This is the first report as far as we know that crocetin has the ability to inhibit the enzymatic activity of caspase-9. Further studies will be needed to determine how crocetin inhibits caspase-9 activity. In general, carotenoids are accumulated in cell membranes; however, Ochiai et al. have reported that most of the crocin localizes in cell membranes, but it is incorporated in the cells in a time-dependent manner (Ochiai



et al., 2004). Thus, crocetin may interact with caspase-9 directly and affect caspase-9 activity in cells.

Excessive light exposure induces many reactive oxygen species, including free radicals, and their production can be overcome by a retinal defensive mechanism. It has been reported that superoxide dismutase (SOD) is induced in the rat retina after light exposure (Yamamoto et al., 1999), and mutant SOD1 mice, which have a gain of catalytic functions such as increased peroxidase activity, are highly susceptible to environmental light-induced retinal degeneration (Mit-tag et al., 1999). Recently, after light exposure, the ER stress sensors, including glucose-regulated protein-78 (GRP78/BiP), caspase-12, phospho-eukaryotic initiation factor 2 alpha (eIF2 $\alpha$ ), and phospho-pancreatic ER kinase (PERK), were significantly up-regulated in a time-dependent manner (Yang et al., 2008). Therefore, it is very conceivable that oxidative stress and ER stress cause retinal damage in a coordinated manner under the light exposure condition. Light exposure is also related to caspase activation. Kanan et al. have reported that caspase-9 activity increased by greater than 150% in response to light in 661 W cells (Kanan et al., 2007). Furthermore, procaspase-3 protein is temporally up-regulated in the retina after *in vivo* exposure to blue light, and the up-regulation is coupled with activation of caspase-3 and concomitant induction of apoptosis in the photoreceptor cells (Wu et al., 2002). Thus, *in vivo*, we evaluated the effects of crocetin on exposure to white light at 8000 lx for 3 h after dark adaptation.

Light exposure for 3 h induced retinal dysfunction. In electroretinographic (ERG) response, both a- and b-wave amplitudes were significantly decreased by light exposure. The a-wave shows the function of the photoreceptors, and the b-wave reflects bipolar cells and Müller cell function. Therefore, the decrease of a- and b-wave amplitudes shows retinal dysfunction. Orally administered crocetin at a concentration of 100 mg/kg prevented decreases both the a-wave and b-wave amplitudes of flash ERG. These findings indicate that crocetin can attenuate photoreceptor and inner retinal dysfunction induced by light exposure. Moreover, crocetin at a concentration of 100 mg/kg, *p.o.*, inhibited the decrease in the outer nuclear layer thickness and expression of TUNEL-positive cells (Figs. 5 and 6). We also measured plasma and aqueous humor concentrations of crocetin after oral administration in mice and rats at doses of 10–100 mg/kg, and the aqueous humor concentration was approximately 2  $\mu$ M, which was sufficient to reduce cell death *in vitro*. Maccarone et al. have reported that saffron protected photoreceptors from retinal stress, maintaining both morphology and function after exposure to damaging light in the mammalian retina (Maccarone et al., 2008). In early age-related macular degeneration, short-term oral Saffron supplementation improved retinal flicker sensitivity in novel and unexpected ways, possibly beyond their antioxidant properties (Falsini et al., *in press*). Moreover, orally administered crocins are hydrolyzed to crocetin before and during intestinal absorption, and absorbed crocetin is partly metabolized to mono- and diglucuronide conjugates (Asai et al., 2005). In this study, we support these reports, and we can clarify the protective effects of saffron are caused by crocetin, and crocetin is absorbed in the intestine, reaches the retina, and may show neuroprotective effects and has retinal protective effects against light-induced damage by inhibition of caspases activity.

In the present study, we have shown that crocetin inhibits the retinal damage induced by tunicamycin and H<sub>2</sub>O<sub>2</sub> *in vitro*, and *in vivo*, that crocetin protected against light-induced photoreceptor degeneration. Moreover, the mechanism may inhibit increases in caspase-3 and -9 activity. Taken together, these results suggest that crocetin may become a possible prophylactic agent for degenerative diseases of the retina such as age-related macular degeneration.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ejphar.2010.09.081.

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