Presence and regulation of cannabinoid receptors in human retinal pigment epithelial cells

Yan Wei, Xu Wang, Ling Wang

1Department of Ophthalmology, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, People's Republic of China; 2Drug Discovery and Design Centre, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, People's Republic of China

Purpose: Cannabinoid receptors have been detected in neuron cells and proposed as potential therapeutic agents in neurodegenerative disorders because of their involvement in controlling neural cell survival and death. However, their presence and role in human retinal pigment epithelial (RPE) cells, which play a key role in initiating and developing age related macular degeneration (ARMD), have never been investigated. Here we analyzed the expression of and changes in cannabinoid receptors (CB1 and CB2) and one enzyme responsible for endocannabinoid hydrolysis, fatty acid amide hydrolase (FAAH), in RPE cell oxidative damage process, a cellular model of ARMD.

Methods: Primary human RPE cells and cells from the ARPE-19 cell line were cultured and exposed to H2O2 for 24 h to induce oxidative damage. Real time RT–PCR, immunofluorescent staining, and western blot methods were performed to study the expression of and changes in CB1 and CB2 receptors, and FAAH. Cell viability and reactive oxygen species (ROS) production were measured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and a dichlorofluorescein (DCF) assay, respectively. PI3K/Akt and ERK1/2 protein expression and activation of signaling molecules were assessed by western blot analysis.

Results: By using real time RT–PCR, immunofluorescent staining and western blot methods, we showed that human RPE cells express CB1, CB2, and FAAH. Meanwhile, oxidative stress can upregulate CB1 and CB2 receptor expression, and downregulate FAAH expression. The CB1/CB2 receptor agonist, CP55,940, and the CB2 receptor agonist, JWH015 significantly protected RPE cells from oxidative damage. In addition, CP55,940 significantly reduced the levels of intracellular ROS, strengthened oxidative stress-induced activation of PI3K/Akt and reduced activation of the ERK1/2 signal pathway.

Conclusions: The results demonstrate the expression and regulation of CB1 and CB2 receptors and FAAH in human RPE cells. The modulation of cannabinoid receptor tone warrants consideration for future therapeutic strategies of ARMD.

The endocannabinoid system (ECS), a new intercellular communication network, has been shown to play a crucial role in neurodegenerative disorders' pathogenesis [1,2]. The ECS consists of the following: the cannabinoid receptors, which include Cannabinoid receptor 1 (CB1)/CB2 receptors and non-CB1/CB2 receptors; the endogenous ligands, primarily anandamide (AEA) and 2-arachidonylglycerol (2-AG); the enzymes responsible for the endogenous ligands' degradation, notably fatty acid amide hydrolase (FAAH), and biosynthesis; and the specific uptake mechanisms [3].

Both cannabinoid receptors subtypes, CB1 and CB2, are abundantly expressed in neurons and can protect neurons from oxidative damage [4]. Cannabinoid receptor induction of neuroprotection has been observed in animal models of some neurodegenerative disorders, including Alzheimer disease [5], Huntington's disease [6], multiple sclerosis [7], and Parkinson disease [8]. However, the presence and regulation of cannabinoid receptors in retinal neurodegenerative disorders have been studied far less than those in brain and other organ systems.

Age-related macular degeneration (ARMD) is a late-onset neurodegenerative retinal disease, which predominates in the elderly as a cause of irreversible and profound vision loss [9]. ARMD shares many common clinical and pathogenetic properties with other neurodegenerative disorders. Currently, there is no ideal therapy to prevent or cure ARMD. Intake of antioxidants is the only established treatment for those with the atrophic or “dry” form of ARMD [10]. Although treatment options for those with the neovascular or “wet” form have increased recently, even these treatments are largely ineffective for reversing existing visual impairment [11]. Thus, the development of strategies for preventing ARMD assumes great importance; and new treatment approaches should focus on the initial insults that lead to the disease's progression. ARMD initial pathogenesis includes degeneration, dysfunction, or loss of RPE cells caused by oxidative injury [12].

Previous studies have demonstrated the presence of endocannabinoids (AEA and 2-AG) in retinas by gas
chromatography and mass spectrometry [13-15]. CB1 receptor, a cannabinoid receptor enriched in neuronal tissue, was localized in the inner and outer plexiform layers of a human retina [16]. Unlike CB1 receptor, CB2 receptor and FAAH were undetectable in the human retina, but were found in rat retina, mostly in the ganglion cells and in the soma of dopamine amacrine cells and large cells [17,18]. The function of ECS in the eye, in addition to regulating photoreception and neurotransmission in the retina, and the effects on intraocular pressure and ocular blood vessels [19-24], also encompasses neuroprotective effects against retinal neurotoxicity [25,26]. This background suggests a functional ECS in the retina. Eyes of patients with ARMD have been show increase levels of endocannabinoids (AEA) in their retina [27]. Therefore, we hypothesized that ECS, acting as a novel signaling system in the retina, may play a substantive role in the ARMD pathophysiological process. Because most effects of endocannabinoids are mediated by binding to cannabinoid receptors and terminated by degradation enzyme FAAH [28]. Thus, the present study aims to examine the expression of CB1 and CB2 receptors, and FAAH in human RPE cells, and their changes in oxidative stress conditions, using a cellular model of ARMD [29]. Our findings may set the basis for the potential pharmacological modulation of cannabinoid receptor tone as a novel therapeutic target in ARMD.

METHODS

Primary human RPE cells and ARPE-19 cell line culture:
Human RPE cells were obtained from eye bank donor eyes, which were cut across the posterior pole, and the vitreous and neural retina were removed. Eyes were obtained from three male human donors between 30 and 50 years of age. None of the donors had a known history of eye disease. The eyes at time of receipt were all integral. RPE cells were isolated within 4 to 16 h after death. The remaining eyecup was washed with phosphate buffered saline (PBS, 8.00 g NaCl, 0.20 g KCl, 0.24 g KH₂PO₄ and 1.44 g Na₂HPO₄ in 1 l distilled water, pH 7.4) and incubated with 0.025% trypsin-ethylene diamine tetraacetic acid (EDTA; Invitrogen-Gibco, Carlsbad, CA) in a humidified chamber at 37 °C. The cells then were scraped gently and seeded in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen-Gibco) containing 15% fetal bovine serum (FBS; Invitrogen-Gibco).

A human retinal pigment epithelial cell line (ARPE-19) was purchased from American Type Culture Collection (ATCC; Manassas, VA). The cells were maintained in DMEM/F12 medium (Invitrogen-Gibco) supplemented with 10% FBS (Invitrogen-Gibco) and 1% penicillin and streptomycin, and were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. The medium was changed every two days. The ARPE-19 cells were used within 10 generations. Since cells of lower generations were quite sensitive to oxidative stress.

Cell treatment: The ARPE-19 cells were seeded in flat-bottomed microculture 96 well plate (15,000 cells/well) and allowed to adhere for 24 h. Dose–response assays were performed on the ARPE-19 cells to determine the 50% inhibiting concentration (IC₅₀) of hydrogen peroxide (H₂O₂). The cells were treated with 0 to 500 μM H₂O₂ in serum-free and phenol-free DMEM-F12 medium for 24 h. The 30% H₂O₂ stock solution was used within its three-month expiration limit. Working solutions of H₂O₂ were made fresh and added to the medium.

CP55,940 (Sigma, St. Louis, MO) is a nonselective CB1 and CB2 cannabinoid receptor agonist. ACEA (Tocris Bioscience, Ellisville, MO) is a selective CB1 agonist. JWH015 (Cayman, Ann Arbor, MI) is a selective CB2 receptor agonist. ARPE-19 cells were preincubated with various concentrations of CP55,940, ACEA, and JWH015 for 15 min before exposure to 200 μM H₂O₂ for 24 h in serum-free, phenol-red-free DMEM and F12 media at 37 °C.

For each concentration of H₂O₂ and the compounds, five wells were analyzed. Each experiment was performed at least three times.

RNA extraction: Total RNA was isolated from primary human RPE cells, ARPE-19 cells and, H₂O₂ (200 μM)-treated ARPE-19 cells using the RNeasy Total RNA System (RNeasy Mini Kit, Qiagen, Valencia, CA) following the manufacturer’s recommendation, and then treated with RNase-free DNase I to remove any contaminating genomic DNA. The isolated RNA had optic density (OD) 260/280 ratios greater than or equal to 2.0 [30]. To synthesize a cDNA template for PCR, we reverse transcribed 1 μg of total RNA with oligo-(dT) primer and reverse transcriptase (ReverTra Ace, Toyobo Co., Ltd., Osaka, Japan). The quality of first-strand cDNA was confirmed by PCR with β-actin primers.

Real-time reverse transcription-polymerase chain reaction (Real-time RT–PCR): Real-time RT–PCR was performed for quantitative analysis according to the standard protocol using the SYBR Green PCR Master Mix (Toyobo Co., Ltd., Osaka, Japan). PCR conditions for CB1 and CB2 were as follows: after initial denaturation at 95 °C for 5 min, 40 cycles were performed at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, followed by a 10 min extension at 72 °C. For FAAH, the conditions were as follows: after initial denaturation at 95 °C for 5 min, 40 cycles were performed at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min, followed by a 10 min extension at 72 °C.

Primers used were shown in Table 1. Quantification analysis of CB1, CB2, and FAAH mRNA was normalized with β-actin as reference. The specificity of PCR amplification products was checked by performing a dissociation melting curve analysis. Relative multiples of changes in mRNA expression were determined with the relative comparative threshold (CT) method.
Immunofluorescent staining: CB1 and CB2 expression in the ARPE-19 cells was determined by immunofluorescent staining. In brief, ARPE-19 cells were grown to confluence in chamber slides (Nalgene-Nunc, Lab-Tek, Naperville, IL). The growth medium was aspirated and the cells were washed three times with PBS, and then fixed with 4.0% paraformaldehyde for 20 min at 4 °C. After the cells had been washed with PBS, they were permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature. Subsequently, CB1 and CB2 expression in the ARPE-19 cells was determined by immunofluorescent staining using a 1:100 dilution of anti-CB1 (rabbit polyclonal, Abcam, Cambridge, UK) or anti-CB2 (rabbit polyclonal, Abcam), respectively, for 6 h at 4 °C. After the cells had been rinsed with PBS, they were probed with 1:250 goat anti-rabbit FITC (Pierce, Rockford, IL) for 1 h at room temperature. Their nucleus was counterstained with 4’,6-diamidino-2-phenylindole (Molecular Probes, Invitrogen-Gibco, Carlsbad, CA). Images were obtained with a fluorescent microscope (Olympus IX 81) at 20X objective with 1.5X optical zoom (Olympus IX 81, Olympus Optical, Tokyo, Japan).

MTT assay for cell viability: An 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay is a qualitative index of cell viability. Mitochondrial and cytosolic dehydrogenases of living cells reduce the yellow tetrazolium salt (MTT) to produce a purple formazan dye that can be detected spectrophotometrically [31]. After ARPE-19 cells were preincubated with various concentrations of CP55,940, ACEA, and JWH015 for 15 min followed by exposure to 200 μM H2O2 for 24 h. Then, MTT (Sigma, St. Louis, MO) was added to a final concentration of 0.5 mg/ml and incubated for 4 h at 37 °C. The culture medium was then removed and the remaining blue precipitate was solubilized in dimethyl sulfoxide, followed by reading absorbance at 570 nm in a plate reader using 630 nm as a reference (Spectra Max 340; Molecular Devices, Sunnyvale, CA). This reading was divided by the adjusted absorbance reading of untreated cells in control wells to obtain the percentage of cellular survival.

Reactive oxygen species determination: The intracellular reactive oxygen species (ROS) level is an important biomarker for oxidative stress. An increased ROS level generally indicates increased oxidative stress. Relative ROS level was determined by the formation of a fluorescent Dichlorofluorescein (DCF) compound on oxidation of the nonfluorescent, reduced DCF-DA [32]. Cells were incubated with 10 μM DCF-DA at 37 °C for 30 min, and then washed twice with PBS. Relative fluorescence was measured using a fluorescence plate reader at 485 nm excitation and 535 nm emission wavelength (Wallace, Perkin-Elmer, Warrington, UK).

| Gene   | Primer (5′-3′)                                      |
|--------|----------------------------------------------------|
| CB1    | F: TTCCCTCTTGTGAAGGCAGCTGC<br>R: TCTTGACGTTCCTGGATGC |
| CB2    | F: TTTTCTTCTTCTGCCATGGCTG<br>R: TTTTTGGCTGACCCCAG   |
| FAAH   | F: GCCTGGAAGTGAACTAAAGGACC<br>R: CCATACGCTTGCCACTCCGCG |
| β-actin| F: GATGAGATTGGCATGCGCTT<br>R: GAGAAGTGGGGTGGCCTT       |

Table 1. Primer sequences used for real time RT–PCR.
Western blot analysis: We plated the ARPE-19 cells into six-well plates (150,000 cells/ml). To evaluate the expression of CB1 and CB2 receptors and FAAH, we treated the cells with 200 μM H2O2 in serum-free and phenol-free DMEM and F12 medium for 24 h. As for the expression of PI3K/Akt and ERK1/2 protein, the cells were pretreated with or without 1 μM CP55,940 for 15 min and then exposed to 200 μM H2O2 for 24 h. After the treatment, the cells were rinsed twice with ice-cold PBS, then scraped into cell lysis buffer and centrifuged at 13400x g for 10 min at 4°C. Protein levels were determined using the bicinchoninic acid (BCA) protein assay (Pierce). Next, 15 μg of total protein were solubilized in 2% sodium dodecyl sulfate (SDS) sample buffer, separated on 10% SDS-polyacrylamide gel by electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes by electroblotting. The blots were washed in Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat dairy milk, and incubated in antibodies to 1:1000 dilution of rabbit polyclonal CB1 and CB2 receptors antibodies (Abcam), 1:1,000 dilution of mouse polyclonal FAAH antibody (Abcam), 1:3,000 dilution of rabbit polyclonal PI3K/Akt and ERK1/2 antibodies (Cell Signaling Technology, Beverly, MA), and 1:10,000 dilution of mouse monoclonal GAPDH antibody (Cell Signaling Technology) at 4 °C overnight. Blots were washed three times, incubated with 1:3,000 horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Pierce) or 1:20,000 HRP-conjugated goat anti-mouse IgG (Pierce) and developed using chemiluminescence (SuperSignal West Pico Luminescent; Pierce) according to the manufacturer’s instructions.

Statistical analysis: Data were presented as the mean ±standard error of the mean (SEM) of the results of two or three separate experiments, as specified in the figure legends. Data were analyzed using ANOVA or a Student’s t-test with the SPSS software. A p-value <0.05 was considered significant.

RESULTS

Effect of H2O2 on ARPE-19 cell viability: The MTT assay for cell viability was used to quantify the ARPE-19 cell cytotoxic response to H2O2. H2O2 produced a progressive, cytotoxic effect in the ARPE-19 cells, beginning at a dose of 100 μM, with 5.7% cytotoxicity and reaching a maximum toxicity at 77.5% damaged cells measured at 500 μM after 24 h of incubation (p<0.05; Figure 1). The results indicate that treating ARPE-19 cells with H2O2 for 24 h caused a dose-dependent decrease in their viability with the half maximal inhibitory concentration (IC50) of 200 μM.

Expression of and changes in cannabinoid receptors and FAAH in RPE cells: As Figure 2A shows, real time RT–PCR revealed primary human RPE cells expressed CB1, CB2, and FAAH mRNA. ARPE-19 cells were treated with 200 μM H2O2 for 24 h, and the changes in CB1, CB2, and FAAH mRNA expression were determined by quantitative RT–PCR. The results showed that H2O2-treated ARPE-19 cells had a 7.02 fold increased CB1 mRNA expression, a 5.68 fold increased CB2 mRNA expression, and a 35.7 fold decreased FAAH mRNA expression, compared to untreated cells (Figure 2B). Consistent with the results of RT–PCR, CB1 and CB2 protein expression increased and FAAH protein expression decreased in 200 μM H2O2 -treated ARPE-19 cells (Figure 2C). Similar results were obtained with immunofluorescence assays (Figure 2D). CP55,940 attenuates H2O2-induced cytotoxicity and ROS generation in ARPE-19 cells: As Figure 3A shows, 200 μM H2O2 (24 h) induced a significant (42%) decrease in ARPE-19 cell viability.
cell viability. Pretreating ARPE-19 cells with CP55,940 for 15 min significantly protected them against H₂O₂-induced toxicity at concentrations of 0.1, 0.3, and 1 μM, with, respectively, 88%, 98%, and almost 100% of the control. Treatment with H₂O₂ at 200 μM for 24 h induced a significant increase in intracellular ROS, approximately 1.7 times compared to the control. Pretreatment with 1 μM CP55,940 for 15 min significantly inhibited ROS generation (23% reduction; Figure 3B). ARPE-19 cells treated with 0, 0.01, 0.1, and 1 μM CP55,940 alone showed no significant effect on viability compared to the untreated control cells.

CP55,940 enhanced H₂O₂-induced activation of PI3K/Akt and reduced activation of ERK1/2: To address the potential role of PI3K/Akt in mediating CP55,940 protection of ARPE-19 cells from oxidative injury, we assessed phosphorylation of PI3K/Akt by western blot analysis. The results showed that PI3K/Akt is activated by H₂O₂. Pretreating ARPE-19 cells with 1 μM CP55,940 followed by 200 μM H₂O₂ enhanced PI3K/Akt activity compared to cells treated with 200 μM H₂O₂ alone (Figure 4A). LY 294002, a specific inhibitor of PI3K/Akt, was used to block PI3K/Akt activation. ARPE-19 cells were pretreated with 20 μM LY294002 for 15 min in the presence or absence of CP55,940, followed by an H₂O₂ challenge for 24 h. As Figure 4B shows, inhibition of PI3K/Akt abrogated CP55,940 protection of ARPE-19 cells from oxidative injury.

U0126, a specific inhibitor for the ERK1/2 pathway, was used to evaluate whether ERK1/2 is involved in H₂O₂-induced ARPE-19 cell oxidative damage. ARPE-19 cells were pretreated with the selective inhibitor for ERK1/2 before exposure to H₂O₂, and cell viability levels were determined. Viability levels increased significantly in the presence of 20 μM U0126 (Figure 4C). Because of that, their phosphorylated ERK1/2 (p-ERK1/2) level was examined by western blot analysis, which showed that ERK1/2 was activated by H₂O₂ pretreatment ARPE-19 cells with CP55,940 reduced p-ERK1/2 activity, compared to cells treated with H₂O₂ alone (Figure 4A).

CB2 receptor agonist attenuates H₂O₂-induced cytotoxicity in ARPE-19 cells: As Figure 5 shows, pretreating ARPE-19 cells with JWH015, a CB2 receptor agonist, for 15 min resulted in a significant protection against H₂O₂-induced toxicity at concentrations of 0.1, 1, 5, and 10 μM, with, respectively, 62%, 68%, 86%, and 95% of the control. Pretreatment with CB1 receptor agonist (AECA) for 15 min showed no cytoprotective effect (Figure 5).

**DISCUSSION**

To the best of our knowledge, this is the first demonstration of the presence of CB1, CB2, and FAAH in human RPE cells, which play a key role in ARMD initiation and development. Plenty of evidence in the previous literature suggests that oxidative stress is a contributing factor to RPE dysfunction in ARMD [33]. The results led us to further analyze the cannabinoid receptors and FAAH expression changes in RPE cells induced by oxidative stress. Most studies have used human RPE cells as the in vitro study model of ARMD, either primary or cell line. One of the drawbacks of using primary RPE cells is that it provides a limited number of cells and may lack a consistent cellular background. The ARPE-19 cell line overcomes this problem while maintaining the morphological and functional characteristics of primary cells. In addition, ARPE-19 cells respond to oxidative stress (H₂O₂, media starvation, etc.) in a fashion similar to that of primary cultured
human RPE cells. Therefore, we have used the ARPE-19 cell line for most of our studies. \( \text{H}_2\text{O}_2 \) added to the culture medium was used as a chemical oxidant. This agent is convenient and biologically relevant, especially for the RPE cells. \( \text{H}_2\text{O}_2 \) has been found in ocular tissues in vivo [34] and can be produced by the RPE cells as a reactive oxygen intermediate during photoreceptor outer segment phagocytosis [35]. For these reasons, we selected \( \text{H}_2\text{O}_2 \) for our studies and performed a series of dose response assays to determine the working concentration that led to a consistent and reliable cytotoxicity.

By using real-time RT–PCR and the western blot method, we showed that oxidative stress can upregulate \( \text{CB}1 \) and \( \text{CB}2 \) receptor expression and downregulate \( \text{FAAH} \) expression in a cell model of ARMD. These results agree with a previous comparative analytical study, which showed that endocannabinoids (AEA) increased in the retina from human ARMD eyes [27]. As a lipid soluble substance, AEA cannot be stored in vesicles; and therefore it is synthesized on demand and travels, in a retrograde direction, across the postsynaptic membrane to the presynaptic membrane, where it activates presynaptic \( \text{CB}1 \) and \( \text{CB}2 \) receptors [36]. After cellular uptake, AEA is degraded via the enzyme FAAH [14]. Variation of ECS also can be observed in other neurodegenerative disorders, such as Parkinson disease, Alzheimer disease, and multiple sclerosis [2,37,38]. The variation of ECS may be an endogenous response to maintain endocannabinoid homeostasis and regulate the pathologic function of neuron cells [39,40]. In line with the endocannabinoid homeostasis theory, there are now several

![Diagram](image1.png)

Figure 4. CP55,940 modulates phosphorylation of PI3K/Akt and ERK1/2. A: Representative western blot analysis shows that 1 \( \mu \text{M} \) CP55,940 enhanced \( \text{H}_2\text{O}_2 \)-induced activation of p-PI3K/Akt and reduced activation of p-ERK1/2 in ARPE-19 cells. B: Protective effects of PI3K/Akt inhibitor (LY294002) on \( \text{H}_2\text{O}_2 \)-induced decrease in cell viability measured by the MTT assay. ARPE-19 cells were pretreated with or without 20 \( \mu \text{M} \) LY294002 for 15 min in the presence or absence of 1 \( \mu \text{M} \) CP55,940 before exposure to 200 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) for 24 h. Asterisk (*) represents the correlation significant at the p<0.05 level and suggest a significant increase in cell viability as compared to \( \text{H}_2\text{O}_2 \)-treated group. Hash mark (#) represents the correlation significant at the p<0.05 level and suggest a significant decrease in cell viability as compared to \( \text{H}_2\text{O}_2 \)-treated group. C: Protective effects of selective ERK1/2 inhibitor (U0126) on \( \text{H}_2\text{O}_2 \)-induced decrease in cell viability measured by the MTT assay. ARPE-19 cells were pretreated with 20 \( \mu \text{M} \) U0126 for 15 min before exposure to 200 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) for 24 h. Asterisk (*) represents the correlation significant at the p<0.05 level and suggest a significant decrease in cell viability as compared to control group. Asterisks and hash mark (#) represents the correlation significant at the p<0.05 level and suggest a significant increase in cell viability as compared to \( \text{H}_2\text{O}_2 \)-treated group. Data are expressed as mean±SEM of results in three separate experiments, each experiment performed in duplicate.

![Diagram](image2.png)

Figure 5. JWH015, but not ACEA, attenuates \( \text{H}_2\text{O}_2 \)-induced cytotoxicity. A: Inhibition of \( \text{H}_2\text{O}_2 \)-induced decrease in ARPE-19 cell viability by JWH015. ARPE-19 cells were pretreated with 0–10 \( \mu \text{M} \) JWH015 for 15 min before exposure to 200 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) for 24 h, and their viability was measured by the MTT assay. Asterisk (*) represents the correlation significant at the p<0.05 level and suggest a significant increase in cell viability as compared to \( \text{H}_2\text{O}_2 \)-treated group. B: ACEA showed no significant effect on \( \text{H}_2\text{O}_2 \)-induced decrease in cell viability. ARPE-19 cells were pretreated with 0–10 \( \mu \text{M} \) ACEA for 15 min before exposure to 200 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) for 24 h, and their viability was measured by MTT assay.
examples of the successful use of ECS-directed drugs used to alleviate the clinical symptoms of neurodegenerative diseases in animal models [2,41,42]. These results suggest that cannabinoid receptors may be potential targets for therapeutic interventions for ARMD.

We also introduced CP55,940, which is a nonselective CB1/CB2 receptor agonist in oxidative stress-induced RPE cellular damage. We found that CP55,940 protected ARPE-19 cells from oxidative stress-induced cell damage and intracellular ROS generation in a dose-dependent way with excellent efficacy. We further explored whether CP55,940 induced cytoprotective signaling pathways while rescuing RPE cells from oxidative damage. The PI3K/Akt- and ERK1/2-mediated survival signal pathways had been suggested to protect RPE cells from oxidative stress [43,44]. We therefore addressed whether CP55,940 could modulate PI3K/Akt and ERK1/2 pathways, and found that CP55,940 could enhance H2O2-induced activation of PI3K/Akt and reduce the activation of the ERK1/2 pathway. We further investigated whether the selective activation of CB1 or CB2 receptor could protect RPE cells from oxidative stress induced damage. We used a cell viability assay to determine viability in cells treated with H2O2. We demonstrated that CB2 receptor agonist, but not CB1 receptor agonist, significantly protected human RPE cells from oxidative stress. Several studies have shown CB1 receptor activation to mediate neuroprotection, but this is not a universal finding [45,46]. This neuroprotective effect could be related to different cell types and pathological conditions.

In conclusion, we demonstrated the expression of CB1 and CB2 receptors and FAAH in human RPE cells, and their changes in oxidative stress conditions. The RPE cells perform vital functions for safeguarding photoreceptor cells against oxidative stress and are involved in the pathogenesis of ARMD [47]. These findings open up the attractive possibility that a correlation exists between endocannabinoid homeostasis and the onset of ARMD. However, as we observe inhibition, rather than the more usual stimulation, of ERK signaling, the involvement of receptors other than CB1 as well as CB2 in the effects of CP55,940 or JWH015 cannot be excluded. More studies using siRNA or specific CB1 and CB2 receptor antagonists should be performed to examine whether manipulating the levels of cannabinoid receptors could be a novel pharmacological approach to treat ARMD in the future.

ACKNOWLEDGMENTS
We greatly appreciate the support received from Shanghai Leading Academic Discipline Project S30205 and Shanghai Science and Technology Committee 08JC1415600.

REFERENCES
1. Martinez-Orgado J, Fernandez-Lopez D, Lizasoain I, Romero J. The seek of neuroprotection: introducing cannabinoids. Recent Pat CNS Drug Discov 2007; 2:131-9. [PMID: 18221224]
2. van der Stelt M, Mazzola C, Esposito G, Matias I, Petrosino S, De Filippis D, Micale V, Steardo L, Drago F, Iuvone T, Di Marzo V. Endocannabinoids and beta-amyloid-induced neurotoxicity in vivo: effect of pharmacological elevation of endocannabinoid levels. Cell Mol Life Sci 2006; 63:1410-24. [PMID: 16732431]
3. Gomez-Ruiz M, Hernandez M, de Miguel R, Ramos JA. An overview on the biochemistry of the cannabinoid system. Mol Neurobiol 2007; 36:3-14. [PMID: 17952645]
4. Galve-Roperh I, Aguado T, Palazuelos J, Guzman M. Mechanisms of control of neuron survival by the endocannabinoid system. Curr Pharm Des 2008; 14:2279-88. [PMID: 18781978]
5. Grundy RI. The therapeutic potential of the cannabinoids in neuroprotection. Expert Opin Investig Drugs 2002; 11:1365-74. [PMID: 12387700]
6. Pazos MR, Sagredo O, Fernandez-Ruiz J. The endocannabinoid system in Huntington's disease. Curr Pharm Des 2008; 14:2317-25. [PMID: 18781982]
7. Pryce G, Ahmed Z, Hankey DJ, Jackson SJ, Croxford JL, Pocock JM, Lendert C, Petzold A, Thompson AJ, Giovannini G, Cuzner ML, Baker D. Cannabinoids inhibit neurodegeneration in models of multiple sclerosis. Brain 2003; 126:2191-202. [PMID: 12876144]
8. de Lago E, Fernandez-Ruiz J. Cannabinoids and neuroprotection in motor-related disorders. CNS Neurol Disord Drug Targets 2007; 6:377-87. [PMID: 18220777]
9. Javitt JC, Zhou Z, Maguire MG, Fine SL, Willke RJ. Incidence of exudative age-related macular degeneration among elderly Americans. Ophthalmology 2003; 110:1534-9. [PMID: 12917168]
10. Age-Related Eye Disease Study Research Group. SanGiovanni JP, Chew EY, Clemons TE, Ferris FL 3rd, Gensler G, Lindblad AS, Milton RC, Seddon JM, Sperduto RD. The relationship of dietary carotenoid and vitamin A, E, and C intake with age-related macular degeneration in a case-control study: AREDS Report No. 22. Arch Ophthalmol 2007; 125:1225-32. [PMID: 17846363]
11. Incorvia C, Campa C, Parmeggiani F, Menzione M, D'Angelo S, Della Corte M, Rinaldi M, Romano M, Dell'omo R, Costagliola C. 12-month retrospective study and review of photodynamic therapy with verteporfin for subfoveal choroidal neovascularization in age-related macular degeneration. Retina 2008; 28:289-97. [PMID: 18301052]
12. Drobek-Słowik M, Karczewicz D, Safranow K. The potential role of oxidative stress in the pathogenesis of the age-related macular degeneration (AMD). Postepy Hig Med Dosw (Online) 2007; 61:28-37. [PMID: 17245315]
13. Straiker A, Stella N, Piomelli D, Mackie K, Karten HJ, Maguire G. Cannabinoid CB1 receptors and ligands in vertebrate retina: localization and function of an endogenous signaling system. Proc Natl Acad Sci USA 1999; 96:14565-70. [PMID: 10588745]
14. Bisogno T, Delton-Vandenbroucke I, Milone A, Lagarde M, Di Marzo V. Biosynthesis and inactivation of N-arachidonoylthetanolamine (anandamide) and N-docosahexaenoylthetanolamine in bovine retina. Arch Biochem Biophys 1999; 370:300-7. [PMID: 10577359]
15. Chen J, Matias I, Dinh T, Lu T, Venezia S, Nieves A, Woodward DF, Di Marzo V. Finding of endocannabinoids in human eye
tissues: implications for glaucoma. Biochem Biophys Res Commun 2005; 330:1062-7. [PMID: 15823551]

16. Straiker AJ, Maguire G, Mackie K, Lindsey J. Localization of cannabinoid CB1 receptors in the human anterior eye and retina. Invest Ophthalmol Vis Sci 1999; 40:2442-8. [PMID: 10476817]

17. Yazulla S, Studholme KM, McIntosh HH, Deutsch DG. Immunocytochemical localization of cannabinoid CB1 receptor and fatty acid amidase hydrolyase in rat retina. J Comp Neurol 1999; 415:80-90. [PMID: 10540359]

18. Lu Q, Straiker A, Lu Q, Maguire G. Expression of CB2 cannabinoid receptor mRNA in adult rat retina. Vis Neurosci 2000; 17:391-5. [PMID: 10750830]

19. Schlicker E, Timm J, Goethert M. Cannabinoid receptor-mediated inhibition of dopamine release in the retina. Naunyn Schmiedebergs Arch Pharmacol 1996; 354:791-5. [PMID: 8971741]

20. Yazulla S, Studholme KM, McIntosh HH, Fan SF. Cannabinoid receptors on goldfish retinal bipolar cells: electron-microscope immunocytochemistry and whole-cell recordings. Vis Neurosci 2000; 17:391-401. [PMID: 10910107]

21. Fan SF, Yazulla S. Biphasic modulation of voltage-dependent currents of retinal cones by cannabinoid CB1 receptor agonist WIN 55212–2. Vis Neurosci 2003; 20:177-88. [PMID: 12916739]

22. Straiker A, Sullivan JM. Cannabinoid receptor activation differentially modulates ion channels in photoreceptors of the tiger salamander. J Neurophysiol 2003; 89:2647-54. [PMID: 12740409]

23. Fan SF, Yazulla S. Reciprocal inhibition of voltage-gated potassium currents (I K(V)) by activation of cannabinoid CB1 and dopamine D1 receptors in ON bipolar cells of goldfish retina. Vis Neurosci 2005; 22:55-63. [PMID: 15842741]

24. Struik ML, Yazulla S, Kamermans M. Cannabinoid agonist WIN 55212–2 speeds up the cone response to light offset in goldfish retina. Vis Neurosci 2006; 23:285-93. [PMID: 16638179]

25. El-Remessy AB, Khalil IE, Matraagoon S, Abou-Mohamed G, Tsai NJ, Roon P, Caldwell RB, Caldwell RW, Green K, Liou GI. Neuroprotective effect of (-)Delta9-tetrahydrocannabinol and cannabidiol in N-methyl-D-aspartate-induced retinal neurotoxicity: involvement of peroxynitrite. Am J Pathol 2003; 163:1997-2008. [PMID: 14578199]

26. Yazulla S. Endocannabinoids in the retina: from marijuana to neuroprotection. Prog Retin Eye Res 2008; 27:501-26. [PMID: 18725316]

27. Matias I, Wang JW, Moriello AS, Nieves A, Woodward DF, Di Marzo V. Changes in endocannabinoid and palmitoylethanolamide levels in eye tissues of patients with diabetic retinopathy and age-related macular degeneration. Prostaglandins Leukot Essent Fatty Acids 2006; 75:413-8. [PMID: 17011761]

28. Piomelli D, Giffrida A, Calignano A, Rodriguez de Fonseca F. The endocannabinoid system as a target for therapeutic drugs. Trends Pharmacol Sci 2000; 21:218-24. [PMID: 10838609]

29. Liang FQ, Godley BF. Oxidative stress-induced mitochondrial DNA damage in human retinal pigment epithelial cells: a possible mechanism for RPE aging and age-related macular degeneration. Exp Eye Res 2003; 76:397-403. [PMID: 12634104]

30. Chomczynski P, Sacchi N. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. Nat Protoc 2006; 1:581-5. [PMID: 17406285]

31. Bernas T, Dobrucki J. Mitochondrial and nonmitochondrial reduction of MTT: interaction of MTT with TMRE, JC-1, and NAO mitochondrial fluorescent probes. Cytometry 2002; 47:236-42. [PMID: 11933013]

32. Wang H, Joseph JA. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic Biol Med 1999; 27:612-6. [PMID: 10490282]

33. Hollyfield JG, Bonilha VL, Rayborn ME, Yang X, Shadrach KG, Lu L, Ufret RL, Salomon RG, Perez VL. Oxidative damage-induced inflammation initiates age-related macular degeneration. Nat Med 2008; 14:194-8. [PMID: 18223565]

34. Halliwell B, Clement MV, Long LH. Hydrogen peroxide in the human body. FEBS Lett 2000; 486:10-3. [PMID: 11108833]

35. Jin GF, Hurst JS, Godley BF. Rod outer segments mediate mitochondrial DNA damage and apoptosis in human retinal pigment epithelium. Curr Eye Res 2001; 23:11-9. [PMID: 11821981]

36. Wilson RI, Nicoll RA. Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. Nature 2001; 410:588-92. [PMID: 11279497]

37. Baker D, Pryce G, Croxford JL, Brown P, Pertwee RG, Makriyannis A, Kanhokar A, Layward L, Fezza F, Bisogno T, Di Marzo V. Endocannabinoids control spasticity in a multiple sclerosis model. FASEB J 2001; 15:300-2. [PMID: 11156943]

38. Di Marzo V, Hill MP, Bisogno T, Crossman AR, Brotchie JM. Enhanced levels of endogenous cannabinoids in the globus pallidus are associated with a reduction in movement in an animal model of Parkinson's disease. FASEB J 2000; 14:1432-8. [PMID: 10877836]

39. Marsicano G, Goodenough S, Monory K, Hermann H, Eder M, Cannich A, Azad SC, Cascio MG, Gutiérrez SO, van der Stelt M, López-Rodríguez ML, Casanova E, Schütz G, Ziegglansberger W, Di Marzo V, Behl C, Lutz B. CB1 cannabinoid receptors and on-demand defense against excitotoxicity. Science 2003; 302:84-8. [PMID: 14526074]

40. Mechoulam R, Panikashvili D, Shohami E. Cannabinoids and brain injury: therapeutic implications. Trends Mol Med 2002; 8:58-61. [PMID: 11815270]

41. Hansen HH, Schmid PC, Bittigau P, Lastres-Becker I, Hansen HS, Anandamide, but not 2-arachidonoylglycerol, accumulates during in vivo multiple sclerosis model. FASEB J 2001; 15:300-2. [PMID: 11156943]

42. Di Marzo V, Hill MP, Bisogno T, Crossman AR, Brotchie JM. Enhanced levels of endogenous cannabinoids in the globus pallidus are associated with a reduction in movement in an animal model of Parkinson's disease. FASEB J 2000; 14:1432-8. [PMID: 10877836]

43. Baker D, Pryce G, Croxford JL, Brown P, Pertwee RG, Makriyannis A, Kanhokar A, Layward L, Fezza F, Bisogno T, Di Marzo V. Endocannabinoids control spasticity in a multiple sclerosis model. FASEB J 2001; 15:300-2. [PMID: 11156943]

44. Mechoulam R, Panikashvili D, Shohami E. Cannabinoids and brain injury: therapeutic implications. Trends Mol Med 2002; 8:58-61. [PMID: 11815270]

45. Hansen HH, Schmid PC, Bittigau P, Lastres-Becker I, Hansen HS, Anandamide, but not 2-arachidonoylglycerol, accumulates during in vivo multiple sclerosis model. FASEB J 2001; 15:300-2. [PMID: 11156943]

46. Baker D, Pryce G. The endocannabinoid system and multiple sclerosis. Curr Pharm Des 2008; 14:2326-36. [PMID: 18781983]

47. Yang P, Pears JJ, Tano R, Jaffe GJ. Oxidant-mediated Akt activation in human RPE cells. Invest Ophthalmol Vis Sci 2006; 47:4598-606. [PMID: 17003457]

48. Garg TK, Chang JY. Oxidative stress causes ERK phosphorylation and cell death in cultured retinal pigment epithelium: prevention of cell death by AG126 and 15-deoxy-
delta 12, 14–PGJ2. BMC Ophthalmol 2003; 3:5. [PMID: 12659653]

45. Iuvone T, Esposito G, De Filippis D, Bisogno T, Petrosino S, Scuderi C, Di Marzo V, Steardo L, Endocannabinoid Research Group. Cannabinoid CB1 receptor stimulation affords neuroprotection in MPTP-induced neurotoxicity by attenuating S100B up-regulation in vitro. J Mol Med 2007; 85:1379-92. [PMID: 17639288]

46. Garcia-Arencibia M, Gonzalez S, de Lago E, Ramos JA, Mechoulam R, Fernandez-Ruiz J. Evaluation of the neuroprotective effect of cannabinoids in a rat model of Parkinson's disease: importance of antioxidant and cannabinoid receptor-independent properties. Brain Res 2007; 1134:162-70. [PMID: 17196181]

47. McLeod DS, Taomoto M, Otsuji T, Green WR, Sunness JS, Lutty GA. Quantifying changes in RPE and choroidal vasculature in eyes with age-related macular degeneration. Invest Ophthalmol Vis Sci 2002; 43:1986-93. [PMID: 12037009]