Artemisinin protects human retinal pigment epithelial cells from hydrogen peroxide-induced oxidative damage through activation of ERK/CREB signaling

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ABSTRACT

The pathological increase in the levels of reactive oxygen species (ROS) in the retinal pigment epithelium (RPE), is implicated in the development of age-related macular degeneration (AMD). The discovery of drug candidates to effectively protect RPE cells from oxidative damage is required to resolve the pathological aspects and modify the process of AMD. In this study, a FDA-approved anti-malaria drug, Artemisinin was found to suppress hydrogen peroxide (H$_2$O$_2$)-induced cell death in human RPE cell-D407 cells. Further study showed that Artemisinin significantly suppressed H$_2$O$_2$-induced D407 cell death by restoring abnormal changes in nuclear morphology, intracellular ROS, mitochondrial membrane potential and apoptotic biomarkers. Western blotting analysis showed that Artemisinin was able to activate extracellular regulated ERK/CREB survival signaling. Furthermore, Artemisinin failed to suppress H$_2$O$_2$-induced cytotoxicity and the increase of caspase 3/7 activity in the presence of the ERK inhibitor PD98059. Taken together, these results suggest that Artemisinin is a potential protectant with the potential strategy for reducing the process of dry AMD.

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1. Introduction

Age-related macular degeneration (AMD) is a progressive degenerative eye disorder which is mainly caused by a breakdown of the macula in the retina. At early stage of AMD, it does not lead to any symptoms or vision loss. However, late AMD seriously affects vision and further influences daily activities such as driving, reading, face recognition and life quality [1]. In the developed world, AMD is the common leading cause of blindness and its incidence keeps increasing in the people with age 65 or older [2]. AMD can be divided into two types: dry AMD (85–90%) and wet AMD (10–15%). At present, the current therapies such as laser photocoagulation, photodynamic therapy and anti-VEGF therapy are limited to wet AMD, but the more prevalent dry AMD still lacks effective therapies [3,4].

The clinical characteristic of dry AMD is a decrease in choroidal blood flow [5]. Chorioretinal atrophy results in subsequent degeneration of retinal pigment epithelium (RPE) cells. The RPE, a pigmented monolayer, plays a critical role in maintaining retinal functions such as nutrient transport, formation of the outer blood retinal barrier to block the passage of water and ions, the phagocytosis of photoreceptor outer segment tips as well as the regeneration of visual pigments etc [6–8]. Age-associated degeneration of RPE eventually lead to loss of photoreceptor cells and vision loss in the affected area [9]. Thus, the identification of effective drug candidates to protect RPE cells from death may be a potential strategy for reducing the process of dry AMD.

Although the etiology and pathogenesis of dry AMD are not fully elucidated, age-related alterations such as increased oxidative stress and decreased cell density in the RPE were considered as main changes in dry AMD [10–15]. The adequate oxygen distribution and supply are necessary for maintenance of retinal function [16]. Due to high-oxygen microenvironment in the retina, RPE cells are exposed to constant oxidative stress resulting from reactive oxygen species (ROS) caused by intense light exposure [17]. Light-induced retinal damage leads to the increase of ROS such as superoxide radical (·O$_2$$^-$), hydroxyl radical (·OH) and hydrogen peroxide (H$_2$O$_2$). High levels of ROS damage the RPE cells, which have been linked with the pathogenesis of clinical visual diseases [17]. H$_2$O$_2$ is an oxidizing agent, commonly used to cause irreversible oxidative damage and activation of the apoptotic cascade in various RPE cell models [18,19]. Therefore, H$_2$O$_2$-induced oxidative injury in RPE cell model, is suitable for investigating the effects of drug candidates for the treatment of dry AMD.

Recently, we perform H$_2$O$_2$-induced RPE cell injury model to identify potential protective candidates from various Chinese medicine. Artemisinin (the chemical structure is shown in Fig. 1A),

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a FDA-approved anti-malarial medicine, was found to exhibit the protective effect against H2O2-induced oxidative injury in the D407 (a human RPE cell line) cells. Artemisinin, a sesquiterpene lactone, was originally isolated from the qinghao (the Chinese name of plant *Artemisia annua* L.) by Chinese scientists [20]. At present, Artemisinin and its derivatives have been clinically used to treat malaria in the world with great safety. Accumulated studies indicate that Artemisinin and its derivatives have additional potential in anti-inflammatory [21], immune-regulation [22], antiviruses [23] and anti-cancer [24]. In the present study, we found that the protective effect of Artemisinin against H2O2-induced oxidative damage in RPE cells was via restoring abnormal changes in nuclear morphology, intracellular ROS, mitochondrial membrane potential and caspase activation. We also investigated the roles of the ERK1/2 survival pathway in the protective effect of Artemisinin.

2. Materials and methods

2.1. Materials

3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyl tetrazolium bromide (MTT), CellROX® Deep Red Reagent, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolyl-carbocyanineiodide (JC-1), and Hoechst 33342 were purchased from Molecular Probes (Eugene, OR, USA). DMSO is from Sigma (Sigma, US). Horseradish peroxidase-conjugated anti-rabbi, anti-beta-actin, anti-phospho-CREB, anti-CREB, anti-phospho-Akt, anti-Akt, anti-phospho-ERK1/2 and anti-ERK1/2 were purchased from Cell Signaling Technology (Woburn, USA). PD98059 and LY294002 were obtained from Merck Millipore. Super Signal West Pico chemiluminescent substrate was purchased from Thermo Scientific (Rockford, IL, USA). Gibco® fetal bovine serum (FBS) and penicillin-streptomycin (PS) were purchased from Life Technologies (Grand Island, NY, USA).

2.2. Cell culture

Human retinal pigment epithelial cell line D407 was obtained from cell bank, Sun Yat-sen University (Guangzhou, China). Cell passages 5–10 were used for all experiments and cell cultures were maintained in 75-cm² tissue culture flasks in DMEM supplemented with 10% FBS, streptomycin 100 μg/ml, penicillin 100 U/ml, and incubated at 37 °C with 5% CO2 humidified atmosphere. The medium was replaced every 2 days, and cells were sub-cultured by trypsin treatment twice a week, at a 1:5 split ratio.

2.3. MTT assay

Cell viability was determined by MTT assay with a slight modification of the protocol described by Zheng and Quirion [25]. Briefly, D407 cells were seeded in 96-well plates at a density of 2 × 10⁴ cells/well. After serum starvation, the cultures were exposed to reagents for 24 h. Thereafter, the cells were incubated with MTT (0.5 mg/ml) for an additional 3 h. The medium was aspirated from each well and DMSO (100 μL) was added. Absorbance at 570 nm was measured by Infinite M200 PRO Multimode Microplate (Tecan, Switzerland). Cell viability was presented as a percentage compared with the control group.

Fig. 1. Protective effects of Artemisinin against H2O2-induced cytotoxicity in D407 cells. (A) The structure of Artemisinin. (B) Cells were treated with Artemisinin (3–100 μM) or 0.1% DMSO (vehicle control) for 24 h and cell viability was measured using the MTT assay. Cells were pre-treated with Artemisinin at indicated concentration or 0.1% DMSO (vehicle control) for 2 h and then incubated with or without 100 μM H2O2 for a further 24 h. Cell viability and the release of LDH were measured by MTT assay (C) and LDH assay (D), respectively. (E) Apoptosis of D407 cells was detected by staining with Hoechst 33342 and visualized by fluorescence microscopy. The number of apoptotic nuclei with condensed chromatin was counted from the photomicrographs and presented as a percentage of the total number of nuclei. *P < 0.05, **P < 0.005 versus control group; #P < 0.05, ###P < 0.005 versus the H2O2-treated group were considered statistically significant differences.
2.4. LDH assay

Cell cytotoxicity was determined by measuring the activity of lactate dehydrogenase (LDH) released into the incubation medium when cellular membranes were damaged. D407 cells were seeded into 96-well plates (5 x 10³ cells/well). After appropriate treatment, the activity of released LDH in the medium was determined according to the instructions of CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, USA). The fluorescent intensity was measured using Infinite M200 PRO Multimode Microplate at an excitation wavelength of 560 nm and emission at 590 nm. All values of % LDH released were normalized to the control group.

2.5. Hoechst 33342 staining

D407 cells were seeded into 12-well plates (8 x 10⁴ cells/well). After appropriate treatment, these cells were washed with PBS, fixed with 4% formaldehyde (v/v in PBS) and then stained with 10 μg/ml Hoechst 33342 for 15 min at room temperature. After washing with PBS, the nuclei were visualized using EVOS FL Imaging System (Thermo Fisher Scientific, USA).

2.6. Measurement of intracellular ROS levels

Intracellular ROS generation was evaluated using CellROX® Deep Red Reagent (Thermo Fisher Scientific, USA). The cells were incubated with CellROX® Deep Red Reagent (5 μM) in DMEM for 1 h in the dark, rinsed twice with 1× PBS solution and the fluorescence was observed and recorded using a fluorescent microscope at an excitation wavelength of 485 nm and emission of 535 nm. Semi-quantification of ROS level was assessed by using Image-J software. All values of % ROS level were normalized to the control group.

2.7. Measurement of mitochondrial membrane potential (Ψm)

JC-1 dye was used to monitor mitochondrial integrity. In brief, D407 cells were seeded into black 96-well plates (1 x 10⁴ cells/well). After appropriate treatment, the cells were incubated with JC-1 (10 μg/ml in medium) at 37 °C for 15 min and then washed twice with PBS. For signal quantification, the intensity of red fluorescence (excitation 560 nm, emission 595 nm) and green fluorescence (excitation 485 nm, emission 535 nm) were measured using a Infinite M200 PRO Multimode Microplate. Mitochondrial membrane potential (Ψm) was calculated as the ratio of JC-1 red/green fluorescence intensity and the value was normalized to the control group. The fluorescent signal in the cells was also observed and recorded with a fluorescent microscope.

3. Caspase 3/7 activity assay

After treatment, the activity of caspase 3/7 was measured using the commercially available Caspase-Glo® 3/7 Assay (Invitrogen, USA) according to the manufacturer's protocol. Briefly, D407 cells were lysed in lysis buffer and centrifuged at 12,500 x g for 5 min. 15 μL of cell lysate was incubated with 50 μL of 2X substrate working solution at room temperature for 30 min in 96-well plates. The fluorescence intensity was then determined by Infinite M200 PRO Multimode Microplate at an excitation wavelength of 490 nm and emission at 520 nm. The fluorescence intensity of each sample was normalized to the protein concentration of sample. All values of % caspase 3/7 activities were normalized to the control group.

3.1. Western blotting

Western blotting was performed as previously described [25]. Briefly, treated cells from different experimental conditions were rinsed once with ice-cold PBS and lysed in RIPA buffer [50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% (w/v) SDS, 1% sodium dodecyl sulfate (SDS), 50 mM NaF, 1 mM NaVO3, 5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin (Sigma), and 50 μg/ml aprotinin (Sigma)] or 2 x sample buffer [final concentration of 62.5 mM Tris–HCl pH 6.8, 2% (w/v) SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% (w/v) bromophenol blue]]. Samples with equal amounts of protein determined with a BCA protein assay kit according to the manufacturer’s instructions, were separated by SDS polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes. The phosphorylation of Akt and ERK1/2 was determined by Western blotting with their respective phospho-specific antibodies while β-actin was used as a total protein control. Blot was visualized using ECL kit according to the manufacturer’s instructions. The blots were stripped, and reprobed with anti-Akt or anti-ERK1/2, respectively. The intensity of the bands was quantified using ImageJ software.

3.2. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 statistical software (GraphPad software, Inc., San Diego, CA, USA). All experiments were performed in triplicate. Data are expressed as mean ± standard deviation (SD). Statistical analysis was carried out using one-way ANOVA followed by Tukey’s multiple comparison, with p < 0.05 considered statistically significant.

4. Results

4.1. Artemisinin attenuated H₂O₂-induced D407 cell death

To evaluate the cytotoxicity of Artemisinin, D407 cells were incubated with various concentrations of Artemisinin for 24 h and the cytotoxicity was determined by MTT assay. As shown in Fig. 1B, Artemisinin at between 3 and 30 μM did not cause any cytotoxicity in D407 cells compared to the control group, and was used in further experiments. To test the protective effects of Artemisinin, D407 cells were treated with Artemisinin for 2 h before being exposed to H₂O₂ for 24 h. The result of MTT assay showed that the treatment of 100 μM H₂O₂ resulted in dominant cell death (50%), whereas pre-treatment with 3, 10 and 30 μM of Artemisinin significantly attenuated H₂O₂-induced cell death in a concentration-dependent manner (54%, 65% and 75%, respectively) (Fig. 1C). The protective activity of Artemisinin was also confirmed by the lactate dehydrogenase (LDH) assay. As shown in Fig. 1D, pre-treated cells with 30 μM of Artemisinin for 2 h significantly reduced H₂O₂-induced LDH leakage (from 251% to 199%). Nuclei condensation was observed in D407 cells after exposure to 100 μM H₂O₂ (26%) in Hoechst 33342 staining assay. However, a pre-treatment of 30 μM Artemisinin decreased these changes (9%) (Fig. 1E). Artemisinin alone did not lead to nuclear morphological change of D407 cells.

4.2. Artemisinin decreased H₂O₂-induced change of mitochondrial membrane potential and caspase 3/7 activity

Mitochondrial inhibition causes the loss of mitochondrial membrane potential (Ψm). To determine whether Artemisinin could reduce H₂O₂-induced Ψm loss, the Ψm in D407 cells was assessed by analyzing the red/green fluorescent intensity ratio of JC-1 staining. Exposure of D407 cells to 100 μM H₂O₂ resulted in an increase in green fluorescence intensity indicating Ψm loss, whereas pre-treatment with 3, 10 and 30 μM of Artemisinin significantly reduced H₂O₂-induced Ψm loss (9%, 20% and 30%, respectively) (Fig. 1F). The protective effect of Artemisinin was also confirmed by the caspase 3/7 activity assay. As shown in Fig. 1G, pre-treated cells with 30 μM of Artemisinin for 2 h significantly reduced caspase 3/7 activity (65%, 75% and 85%, respectively) (Fig. 1H). Artemisinin alone did not lead to caspase 3/7 activity increase in D407 cells.
dissipation (69%) (Fig. 2A). Pre-treatment with Artemisinin at 30 μM for 2 h attenuated H2O2-induced △ψm loss (88%).

Caspase 3/7 is a main biomarker in the apoptosis of dopaminergic neuronal cells. As shown in Fig. 2B, treatment of cells with 100 μM H2O2 for 24 h increased caspase 3/7 activity by more than 2-fold compared to the control group (219%). In contrast, pre-treatment with Artemisinin significantly reduced caspase 3/7 activation induced by H2O2 (160%).

4.3. Artemisinin suppressed H2O2-induced increase of intracellular ROS

The accumulation of excess ROS is considered to be one of the main causes of cell damage induced by H2O2. Intracellular ROS were measured by staining with a fluorescent probe, CellROX® Deep Red Reagent, in D407 cells. As shown in Fig. 3, 100 μM H2O2 caused a significant increase in fluorescent intensity in the cells compared to the control group (177%). However, the increase in ROS was significantly suppressed by pretreatment with Artemisinin at 30 μM (126%).

4.4. Artemisinin up-regulated ERK/CREB signaling

Previous studies indicated that Artemisinin was able to activate ERK signaling in various cell models [26–28]. ERK was found to be involved in the inhibition of apoptosis. To determine whether the ERK signaling pathway is regulated by Artemisinin in D407 cells,
D407 cells were treated with 30 μM Artemisinin for 0, 0.5, 1, and 2 h and then the phosphorylation of ERK1/2 (Thr202/Tyr204) was examined by Western blot analysis. As shown in Fig. 4A and B, Artemisinin gradually increased the phosphorylation intensity of ERK1/2 in D407 cells within 2 h. These results suggested that ERK survival signaling could be activated by Artemisinin. In contrast, Artemisinin had no effect on the phosphorylation of AKT, a serine/threonine kinase implicated in cell survival (Fig. 4A and C). CREB is an ERK-mediated nuclear transcription factor involved in many biological functions including neuronal survival. The results of Western blotting showed that the phosphorylation of CREB (Ser133) was increased significantly by Artemisinin (Fig. 4D).

4.5. ERK signaling was involved in the protective effect of Artemisinin

To further confirm the involvement of ERK signaling in the protective effects of Artemisinin, the ERK pathway inhibitor PD98059 was used in cell survival assay with H2O2 cytotoxicity. As shown in Fig. 5A, the protective effect of Artemisinin was abolished by the application of PD98059 in MTT assay. Similar results were obtained from caspase 3/7 assay which showed that Artemisinin failed to suppress the increase of caspase 3/7 activity in the presence of the ERK pathway inhibitor (Fig. 5B), suggesting that ERK signaling is involved in the protective effects of Artemisinin.

5. Discussion

Oxidative stress in the retina plays a critical role in the pathogenesis of dry AMD. Although antioxidant defense mechanisms in retinal cells are sufficient to counteract weak oxidative stress, strong oxidative stress disrupt normal antioxidant mechanisms and result in irreversible damage to the retina. Using additional
antioxidant to suppress oxidative stress is reported to avoid oxidative damage and maintain the function of retina. Vitreous oxide nanoparticles are excellent free radical scavengers prevent photoreceptor death in a light-damage model of retinal degeneration [29]. In addition, experimental and clinical studies suggest that taking high dose of antioxidant vitamins and zinc supplements are potential strategies for delaying the progression of AMD and vision loss [30]. In this study, we found that 100 μM H2O2 resulted in the collapse of the Δψm and increase of ROS in RPE cells while pretreatment of Artemisinin was able to significantly suppress these abnormal changes in D407 RPE cells. Apoptosis is a frequent type of cell death observed in RPE cells [31]. The decrease of cell viability, the increase of LDH release and nuclear morphological changes induced by H2O2 were significantly suppressed by Artemisinin (Fig. 1), suggesting that anti-oxidant activity of Artemisinin contributes to its protective effects. The concentration range of artemisinin (10–30 μM) used in our experiments has no toxicity in D407 cells and is therapeutically relevant based on the in vitro quantitation with malaria plasmodion indicate that Artemisinin is a safe protectant [43].

Increased mitochondrial DNA damage and protein loss of electron transport chain in RPE cells have been found in AMD patients, suggesting that mitochondrial dysfunction plays a key role in the process of AMD [32–35]. Mitochondrial apoptotic pathway due to mitochondria deficiency plays a critical role in the pathogenesis of retinal diseases. It has been reported that strategies to block mitochondrial apoptotic pathway are potential therapeutics to prevent retinal pigment epithelial cells death. In this study, our results showed that H2O2-induced Δψm loss could be suppressed by pre-treatment with Artemisinin. The increase of intracellular ROS induced by H2O2, leads to damage to the mitochondrial membrane and results in the collapse of the Δψm and activation of the apoptotic cascade [36–39]. Pre-treatment with Artemisinin was able to suppress the activation of caspase 3/7 (Fig. 3).

ERK/CREB pathway is a central signaling component that plays a role in the initiation and regulation of most of the stimulated cellular processes such as proliferation, survival and differentiation [40]. Both AKT and ERK survival pathways are reported as a key signaling to induce CREB phosphorylation and cellular anti-oxidant mechanism [41,42]. In our study, the increase in CREB phosphorylation, following treatment with Artemisinin, was observed. However, it was noted that ERK, no AKT, could be activated by Artemisinin. Furthermore, our results showed that Artemisinin failed to suppress H2O2-induced cytotoxicity and the increase of caspase 3/7 activity in the presence of the ERK inhibitor PD98059 (Fig. 5). Therefore, these results provide mechanistic evidence to support the notion that Artemisinin-regulated protective effects against H2O2-induced oxidative stress occur via ERK dependent CREB activation.

In summary, our findings demonstrate that Artemisinin is able to reduce H2O2-induced oxidative stress in D407 retinal pigment epithelial cells through the regulation of multiple mechanisms including (1) inhibiting the generation of intracellular ROS; (2) modulating Δψm and caspase 3/7 dependent pathway; (3) activating ERK1/2 signaling (Fig. 6). The protective effects of Artemisinin to attenuate H2O2-mediated intrinsic mitochondrial apoptotic pathway are, at least in part, mediated via the activation of ERK1/2 signaling. Our results offer support for the potential development of Artemisinin to prevent retinal pigment epithelial cells death in the process of AMD.

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