The Effect of A2E On The Levels of ROS and The Distribution of A2E in RPE Cells Exposed to Blue Light

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Research Article

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Abstract

Aims

To establish the N-retinylidene-N-retinylethanolamine(A2E) and blue light induced RPE cells damage model to explore the regularity of distribution of A2E and the levels of reactive oxygen species(ROS).

Methods

The fourth to sixth generation of human RPE cells in vitro were divided into five groups randomly: control group, blue light group, A2E-loaded group, A2E-loaded+blue light group and A2E-loaded+blue light +nifedipine group. The levels of ROS in cytoplasm by DCFH-DA staining was assayed by flow cytometry. The concentration of A2E in cytoplasm and lysosomes were assayed by high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS). The fluorescence intensity of A2E in lysosomes by LysoTracker redDND-99 staining was assayed by confocal laser scanning microscope.

Results

Exposure to blue light and/or A2E could increase the levels of ROS in RPE cells, and nifedipine could inhibit oxidative stress response and reduce ROS levels. By HPLC-MS, it was found that A2E was not detected in the groups without load A2E, and A2E levels in cytoplasm and lysosomes decreased after light exposure. The green fluorescence produced by A2E loaded on RPE cells was mostly coincident with the red fluorescence labeled by lysosomes.

Conclusion

Blue light and A2E can increase the ROS levels of RPE cells and both have a synergistic effect. A2E is mainly concentrated in lysosomes, which is reduced by oxidation under blue light irradiation, damages lysosomal membrane with oxidized species of A2E, and leaks out from lysosomes.

1. Introduction

Age-related macular degeneration (AMD) is a major cause of visual loss in middle-aged and older people¹². The exact cause of this disease remains unknown. Unfortunately, there is no effective AMD treatment or method of prevention, except the avoidance of blue light.

Retinal pigment epithelial (RPE) cells can regenerate; synthesize retinal pigments; repair photoreceptors and Bruch's membranes; transport oxygen, water, ions and nutrients from the choroid vessels to the outer retina; phagocytose; and degrade photoreceptor outer membrane discs³⁴. When the devoured membrane discs are not completely digested in the lysosome, the residues accumulate to form lipofuscin.

N-retinylidene-N-retinylethanolamine (A2E), the main fluorescent substance of lipofuscin, consists of all-trans retinol and ethanolamine. It is phototoxic and deposited in RPE cells with age⁵. The etiology and

pathogenesis of AMD are associated with A2E^{5 6}, increasing evidence shows that A2E is the most abundant toxic substance in lipofuscin⁷. In the absence of blue light, low concentrations of A2E do not cause apoptosis in RPE cells, but A2E can trigger apoptosis when it accumulates over the physiological dose⁸. In addition, exposure to blue light can accelerate the oxidation of RPE cells by A2E, inducing oxidative stress and apoptosis⁹⁻¹¹. Zhao Z showed that exposure to blue light increased the levels of GRP78, CHOP, P-JNK, and Bax and the cleavage of Caspase 3 and Caspase 9, which eventually triggered apoptosis in RPE cells containing A2E¹⁰.

Since A2E and blue light are closely related to the occurrence and development of AMD, the members of this group have explored the best conditions to develop an RPE cell injury model in blue light: an optimal blue light intensity of 2000 ± 500 lux, a 6 h light exposure and continued culture for 24 h¹². The optimal concentration used to load A2E in human RPE cells is $25 \mu\text{M}$ ¹³. Based on this model, we further explored the effect of A2E on the levels of ROS and distributions of A2E in RPE cells to provide a basis for further study of the mechanism of retinal photodamage.

2. Material And Methods

2.1 Cell sources and ethics statement

Primary cultured RPE cells were isolated from three healthy men, aged 45, 49 and 35, within 24 h of their accidental deaths. The eyes were taken after keratoplasty at the Affiliated Hospital of Zunyi Medical University. All experimentation adhered to the ethical standards established by the unit's Human Test Committee and approved by the Ethics Committee of Zunyi medical university, China ([2017]1-111).

2.2 Patient involvement

Patients were not directly involved in the design of this study.

2.3 Reagents and instruments

The following reagents and instruments were used: nifedipine (Sigma, Germany); a Reactive Oxygen Species Assay Kit (Beyotime, China); Dulbecco's modified Eagle medium/F12 (HyClone, USA); fetal bovine serum (HyClone, USA); trypsin-EDTA solution, 0.25% (HyClone, USA); penicillin-streptomycin, liquid (Gibco, USA); LysoTracker Red DND-99 (Sigma, Germany); dimethyl sulfoxide (Solarbio, China); a DAB Substrate Kit (Zsbio Commerce Store, China); A2E (presented by the First Affiliated Hospital of Shanghai Jiaotong University); a medical blue light tube (Yingze, Tianjin); a high-speed tabletop centrifuge (Eppendorf, Germany); a Beckman Gallios flow cytometer (Beckman, USA); a Lysosome Isolation Kit (Invent, USA); and a Thermo U3000 Thermo Q Exactive Plus (Thermo, USA).

2.3.1 Primary culture of human RPE cells

Briefly, eye scissors were used to cut the eyeballs approximately 5~6 mm behind the corneal limb, and then the anterior segment was discarded. The vitreous body was removed, and the retinal neuroepithelium was carefully removed to isolate eye cups. The eye cups were digested with 0.25% trypsin for 30 min at 37 °C in a 5% CO₂ atmosphere incubator. The cells were collected and resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Then, the cells were cultured in a 25 cm² culture flask at a density of 5×10⁴ cells/ml and kept at 37° in 5% CO₂. The RPE cells were passaged at a ratio of 1:2, and cells at generation 4~6 were used for experiments. Each group comprised cells of the same generation from the same individual.

2.3.2 Immunohistochemistry

RPE cells were grown on glass coverslips coated with 0.01% polylysine. The cells were covered, fixed with precooled acetone for 10 min and incubated with 3% hydrogen peroxide for 15 min. After three washes with distilled water, the cells were permeated with 1% Triton-X for 30 min at room temperature and washed again with PBS. Then, the cells were incubated with goat serum for 10 min, followed by mouse anti-human RPE65 antibody overnight at 4°C and secondary goat anti-mouse IgG antibodies for 1h at 37°C. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

2.3.3 Exposure of RPE cells to blue light and A2E treatment

The human RPE cells from the fourth to sixth generation used for experiments were randomly divided into the following five groups: control group(CON), blue light group(BL), A2E-loaded group(A2E), A2E-loaded + blue light group(A2E+BL), and A2E-loaded + blue light + nifedipine group(A2E+BL+NIF). Before the cells were exposed to blue light, A2E was diluted to a final concentration of 25 µM in DMEM, and the cells were incubated with A2E for 2 h at 37 °C and then washed three times to remove extracellular A2E. Nifedipine at a final concentration of 10⁻⁵ mol/L was added 1 h before light exposure. Then, the RPE cells were exposed to blue light at an intensity of 2000 ± 500 lux for 6 hours, and the cells were cultured for 24 hours after exposure. Groups A and C were treated under the same conditions and wrapped in tin foil. Using a TES1330A illuminometer to measure the light intensity, the light intensity was adjusted by changing the distance between the culture flask or culture plate and the blue light tube.

2.3.4 Reactive oxygen species (ROS) levels in human RPE cells

After RPE cells were processed with blue light and A2E, they were washed twice with PBS, following which DCFH-DA diluted to a final concentration of 10 µM in DMEM was added and incubated at 37 °C for 20 minutes. Then, the cells were digested and centrifuged at 1000 rpm, the supernatant was discarded, and the pellet was resuspended in 300 µL of PBS containing 3% calf serum at a density of 10⁶ cells/ml. The ROS concentration was analyzed by flow cytometry with the pass band filter set at 488 nm for excitation, and the emission of DCFH was detected at 525 nm. The data were analyzed with FlowJo software. In this experiment, the cells from 49 years old donor eyeball were used.

3.1 *Isolation of cytoplasm and lysosomes*

After completing the treatment described above, the cells were collected and washed once with cold PBS. The supernatant was completely removed, and the pellet was resuspended in buffer A. The cell suspension was incubated on ice for 10 min. The tube was vigorously vortexed for 20 seconds, following which the cell suspension was immediately transferred to the filter cartridge and centrifuged at $16000 \times g$ for 30 seconds. The filter was discarded, and the pellet was resuspended by vigorous vortexing for 10 seconds. The sample was centrifuged at $2000 \times g$ for 3 min (the pellet contained the nuclei, large cellular debris and some unruptured cells), and then all supernatant was transferred to a fresh 1.5 ml microfuge tube and centrifuged at 4°C for 15 min at $11000 \times g$. The pellet contained mainly mitochondria and cellular debris. After centrifugation, the supernatant was carefully transferred to a fresh 1.5 ml tube and spun at $16000 \times g$ at 4°C for 30 min. The supernatant consisting of the cytoplasm was collected.

In addition, the pellet was resuspended in cold buffer A and centrifuged at $2000 \times g$ for 4 min. The supernatant was carefully transferred to a fresh 1.5 ml tube. Buffer B was added to the tube, which was vortexed briefly to mix well (the supernatant to buffer B ratio was 2:1). The tube was incubated on ice for 30 min and centrifuged at $11000 \times g$ for 10 min. The supernatant was removed completely, and the pellet was a highly enriched lysosome fraction. The experiment used cells from the eyeballs of 35 years old donors.

3.2 *A2E levels in cytoplasm and lysosomes*

Methanol was added to the cytoplasm obtained as described above, and the mixture was centrifuged twice at 4°C for 15 min at $16000 \times g$. The supernatant was used for high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS). A reverse-phase C18 column (2.1×50 mm, $1.7 \mu\text{m}$, Waters, UK) was used for the entire HPLC analysis. The mobile phase consisted of a 0.1% formic acid-water solution and acetonitrile. The injection volume for each run was $5 \mu\text{L}$. The samples were eluted at a flow rate of 0.4 ml min^{-1} .

Lysosomes obtained as described above were dissolved in ultrapure water, and methanol was then added to the suspension and centrifuged twice at 4°C for 15 min at $16000 \times g$. The supernatant was used for HPLC-MS, and the detection method was as above.

3.3 *A2E fluorescence in lysosomes*

The coverslips were placed in 35 mm culture dishes, and $100 \mu\text{L}$ of cell suspension was added at a density of 10^5 cells/ml in 2 ml of culture medium. After processing with blue light and A2E, the cells were washed twice with PBS. Then, LysoTracker red DND-99 at a final concentration of 50 nM was added, and cells were stained in the dark for 20 min at 37°C . Next, the RPE cells were fixed with 4% paraformaldehyde for 20 min and washed with PBS three times, and the cover slips were fixed on slides. The fluorescence intensity of A2E in lysosomes was observed and imaged using a Leica laser scanning confocal microscope. Cells from 35-year-old eyeball donor.

4.1 *Statistical analysis*

These experiments were repeated at least 3 times. Data are expressed as the mean $\pm$ standard deviation, and SPSS 18.0 software was applied to assess differences among groups with one-way analysis of variance followed by the least significant difference (LSD) test. Differences with a P-value < 0.05 were considered statistically significant.

3. Results

1. *Human RPE cells and Immunohistochemistry*

Primary RPE cells were hexagonal or polygonal, and their cytoplasm was filled with pigment granules. After passage, the RPE cells were mostly fusiform or irregular, and the number of pigment granules in the cells was reduced. Immunofluorescence staining showed green fluorescence in RPE cells, indicating expression of RPE65. (Fig.1).

2. *Changes of ROS levels in human RPE cells*

The cells from 49 years old donor eyeball were used to probe the ROS levels. In the presence of ROS, intracellular DCFH is oxidized and fluoresces; therefore, ROS can be detected by measuring the fluorescence intensity due to DCF in cells. By flow cytometry, we determined the level of ROS, which was significantly different among groups; group CON which did not undergo treatment with A2E and blue light, exhibited the lowest level of ROS ($P=0.000$, 0.011 , 0.000 , and 0.000 , respectively). We also found that ROS levels in groups BL, A2E+BL and A2E+BL+NIF were higher than those in group A2E ($P=0.001$, 0.000 , and 0.000 , respectively). The ROS levels were significantly increased in groups A2E+BL and A2E+BL+NIF when compared with group BL ($P=0.000$, 0.000 , respectively), and the ROS levels in group A2E+BL+NIF were lower than those in group A2E+BL ($P=0.013$) (Fig.2).

3. *A2E levels in the cytoplasm*

After exposure to blue light, the RPE cells which from 35 years old donor eyeball were cultured for 24 hours, detection and absolute quantitation of A2E via HPLC-MS, the molecular ion peak at m/z 592 corresponds to the molecular mass of A2E; there was almost no A2E in groups CON and BL. The levels of A2E in group A2E was found to be the highest compared with that in groups A2E+BL and A2E+BL+NIF ($P=0.000$, 0.000 , respectively). A2E levels in group A2E+BL+NIF was lower than that in group A2E+BL ($P=0.020$) (Fig.3).

4. *A2E levels in lysosomes*

The concentration of A2E in lysosomes was determined as described in the Materials and Methods section, the molecular ion peak at m/z 592 corresponds to the molecular mass of A2E, and there was almost no A2E in groups CON and BL. A2E levels in group A2E was significantly higher than that in groups A2E+BL and A2E+BL+NIF ($P=0.000$, 0.000 , respectively). The levels of A2E in group A2E+BL was

higher than that in group A2E+BL+NIF ($P=0.017$) (Fig.4). The experiment used cells from the eyeballs of 35 years old donors.

5. A2E fluorescence in the lysosomes of RPE cells determined by laser scanning confocal microscopy

The green fluorescence due to A2E mostly overlapped with the red fluorescence of the lysosomes, indicating that most of the A2E had accumulated in lysosomes. Cells from 35-year-old eyeball donor (Fig.5).

Discussion

A2E is a major component of lipofuscin, which accumulates in the retinal pigment epithelial layer with age, and is also a substance believed to regulate blue light damage to the retina^{14 15}. The phototoxicity of blue light to RPE cells can cause changes in retinal cells, leading to retinal diseases, such as AMD, Stargardt's disease, and Best's disease¹⁶⁻²⁰. Although the etiology of AMD remains unclear, AMD is known to be related to the accumulation of A2E^{2 21 22}, the excessive absorption of retinol²³, oxidative stress²⁴, and the inflammatory response^{25 26}, and epigenetics^{27 28}. Therefore, the established model of A2E-loaded RPE cells damaged with blue light can be used to explore retinal degenerative diseases.

In this experiment, human RPE cells were randomly divided into five groups. We found that ROS levels in the control group was the lowest, exposure to blue light or the addition of A2E could increase the production of ROS in RPE cells and that these stimuli acted synergistically. Calcium ions are an important second messenger in cells, and the mechanism of their messenger function is mainly realized by changes in their concentration. The addition of the calcium channel blocker nifedipine could inhibit the oxidative stress response of RPE cells, reduce intracellular ROS levels and protect RPE cells from apoptosis. These findings are consistent with the results of a study that showed that blue light could accelerate the oxidation of A2E in cells, trigger oxidative stress, produce large amounts of ROS, and induce apoptosis in RPE cells^{9 10}.

Experimental studies on the relationship between A2E and lysosomes suggest that autophagy is involved in the accumulation of A2E during lysosomal phagocytosis^{29 30}. After the addition of A2E to the cell culture medium, A2E was taken up by RPE cells and aggregated within these RPE cells, showing affinity for acidic organelles; this process was similar to the phagocytosis of A2E by RPE cell lysosomes³¹. Under exposure to light at a certain intensity for a certain length of time, A2E undergoes isomerization, during which free radicals are produced. In this experiment, the levels of A2E in the cytosol and lysosomes was determined via HPLC-MS. We found almost no A2E in the groups CON and BL, probably because A2E was oxidized or metabolized in other ways with increasing cell passage. Changes of A2E levels in other groups can be speculated that under blue light illumination, A2E is oxidized, producing an oxidative stress reaction. In this process, isomers or other oxidation products are formed, causing a significant decrease in the amount of total A2E. In addition, A2E can inhibit the proton pump function of lysosomes and further attack and destroy the lysosomal membrane, resulting in the leakage

of A2E or its oxidation products, which is more toxic^{32 33}. Furthermore, when RPE cells are exposed to blue light, the permeability of the lysosomal membrane in these RPE cells will be increased, which leads to the further leakage of A2E into the cytoplasm. And nifedipine can reduce calcium production and inhibit RPE cell apoptosis.

In summary, we detected intracellular ROS and A2E and found that blue light or A2E could increase the intracellular ROS levels of human RPE cells and that these stimuli had a synergistic effect. In this process, nifedipine could reduce the production of ROS and therefore had a protective effect on RPE cells. A2E is mainly concentrated in the lysosomes, and A2E in cytoplasm and lysosomes are reduced by oxidation under blue light irradiation, destroying lysosomal membrane with oxidized species of A2E, leaking out from lysosomes, initiating oxidative stress response that increases the production of ROS, causing damage to RPE cells.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Zunyi medical university, China ([2017]1-111).

Consent for publication

Yes.

Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

ML and CZ contributed equally to this work. These authors are co-first author. ML was responsible for some experiments and writing the manuscript. CZ had made significant contributions in experiments, data analysis, and writing manuscripts. SW had made significant contributions in some experiments. SC had made significant contributions in research conception and experimental design. All authors revised and approved the final version of the manuscript.

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Figures

Figure 1

A. Primary human RPE cells. B. Third-generation human RPE cells. C. Primary mouse anti-human RPE65 antibody and secondary goat anti-mouse IgG antibodies were used to immunostain the third generation RPE cells. Immunofluorescence staining was positive for RPE65.

A

B

Figure 2

A. Since the fluorescence spectrum of DCF was similar to that of FITC, the parameter setting of FITC was used to detect DCF. B. * $p < 0.05$ or ** $p < 0.01$ statistically significant compared with the group CON; Δ $p < 0.001$ statistically significant compared with the group A2E; • $p < 0.001$ statistically significant compared with the group BL; ◻ $p < 0.05$ compared with the group A2E+BL.

A

B

Figure 3

A. Extracted ion chromatogram of m/z 592.5. The levels of A2E in the cytoplasm was reduced after blue light irradiation. B. * $p < 0.001$ statistically significant compared with the group A2E; $\Delta p < 0.05$ statistically significant compared with the group A2E+BL.

Figure 4

A. The extracted ion chromatogram from the mass spectrometry analysis of A2E (m/z 592). The levels of A2E in lysosomes was decreased after exposure to blue light. B. *p < 0.001 statistically significant compared with the group A2E; Δp < 0.05 statistically significant compared with the group A2E+BL.

Figure 5

A. Lysosomes labeled with LysoTracker red DND-99. B. A2E in lysosomes. C. Merge of the images in A. and B.