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Photoactivation of *N*-retinylidene-*N*-retinylethanolamine compromises autophagy in retinal pigmented epithelial cells

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ABSTRACT

As a part of the aging process, *N*-retinylidene-*N*-retinylethanolamine (A2E) accumulates in the retina to activate autophagy in retinal pigmented epithelial cells. However, the effect of A2E photoactivation on autophagy, which is more clinically relevant, still remains unclear. Here, we investigated the effect of blue light (BL)-activated A2E on autophagy in human retinal pigmented epithelial cells, ARPE-19. A significant increase in LC3-II protein was observed when BL was irradiated on ARPE-19 cells containing A2E. The mammalian target of rapamycin (mTOR) pathway was examined to verify whether autophagy was activated, but no change in AKT, mTOR, and 4EBP phosphorylation was observed. Transcription factor EB (TFEB) target gene expression, which is another pathway involved in autophagy, was also not altered by A2E and BL. However, intracellular p62 protein levels were significantly increased, which represented the inhibition of autophagic flux. To investigate the mechanism of the suppressed autophagic flux, the lysosomal state was observed. After BL irradiation, lysosomal damage was induced in A2E-treated ARPE-19 cells, and this phenomenon was prevented by treatment with the antioxidant, N-acetylcysteine. Our results suggest that A2E photoactivation compromises autophagy in ARPE-19 cells and that reactive oxygen species (ROS) play an important role in this process.

1. Introduction

The retinal pigment epithelium (RPE), which consists of a monolayer of melanin-rich cells between the optic receptors of the neural retina and capillaries of the choroid, is essential for photoreceptor cell maintenance and survival. RPE is particularly vulnerable to oxidative damage when exposed to photosensitizers such as lipofuscin (Kaarniranta et al., 2017; Parmar et al., 2018; Sparrow et al., 1999; Wang et al., 2018; Wu et al., 2010). Lipofuscin is a complex of fluorescent materials that are found in various tissues, but are best studied in the eye. It cannot be degraded or eliminated by exocytosis, and accumulates in the lysosome as RPE ages (Lei et al., 2017; Sparrow et al., 1999; Wu et al., 2010). Several clinical studies have shown that excessive accumulation of lipofuscin is associated with RPE cell damage leading to retinal degeneration such as age-related macular degeneration (AMD) (Ben-Shabat et al., 2002; Guha et al., 2014), which is a major cause of irreversible blindness in elderly people involving visual impairment in the macula (central and posterior part of the retina), including the RPE and photoreceptors. AMD is categorized into 2 types: dry and wet macular degeneration. Dry AMD is caused by photoreceptor by-product accumulation in the retina (Kaarniranta et al., 2017; Parmar et al., 2018; Wang et al., 2018). 10–20% of AMD progressed to wet AMD, which can result in rapid loss of central vision due to choroidal neovascularization in the central retina (Algvere and Seregard, 2002).

A2E is the first fluorophore isolated from RPE lipofuscin, which damages RPE cells and plays an important role in retinal degenerative diseases (Brandstetter et al., 2015; Wu et al., 2010). It is a by-product of visual-cyclization and is formed via condensation of 2 all-trans-retinal molecules with phosphatidylethanolamine as a regeneration mechanism of 11-cis-retinal (Lei et al., 2017; Poliakov et al., 2014). Under normal conditions, clearance of all-trans-retinal from the photoreceptors occurs rapidly; however, delaying this reaction increases the condensation of A2E and all-trans-retinal dimers in the RPE (Gao et al., 2016; Roehlecke et al., 2009). Long-term accumulation of A2E inhibits RPE function via (1) dysregulated lysosomal clearance, (2) specific interferences of biological pathways involved in glycogen, lipid, and/or mitochondrial homeostasis (Guha et al., 2014; Moon et al., 2017), or (3) cytochrome *c* oxygen inhibition (Sparrow et al., 2002). However, no report of RPE cell dysfunction has been observed due to short-term accumulation of A2E.

The blue light (BL) region of the visible spectrum (400–500 nm), which has relatively high energy, is known to penetrate the cell organs and damage retinal tissue (Bian et al., 2012; Moon et al., 2017; Sparrow

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et al., 2002). A2E has been particularly reported to induce reactive oxygen species (ROS) generation and apoptosis upon exposure to BL, a short wavelength visible light. Although BL is in the visible light region, it is very close to the ultraviolet region; therefore, it induces the oxidation of A2E and iso-A2E, thereby causing phototoxicity (Ben-Shabat et al., 2002; Sparrow et al., 1999) that leads to ROS generation and RPE cell apoptosis (Brandstetter et al., 2016; Sparrow et al., 2003).

Previous studies have shown that disturbed autophagy is associated with AMD pathogenesis and RPE damage (Guha et al., 2014; Zhang et al., 2015). Paradoxically, autophagy has been reported to be activated when RPE cells are exposed to A2E or BL individually (Chen et al., 2016; Zhang et al., 2015). However, the effect of BL-induced A2E photoactivation on autophagy, which is more clinically relevant, still remains unclear. In the present study, we investigated the effect of BL-activated A2E on autophagy and the role of autophagy in the progression of cell death in human retinal pigment epithelial cells, ARPE-19. To investigate the effect of A2E and BL on autophagy, autophagic flux was measured, and its effect on lysosomes was evaluated. The roles of methylglyoxal and ROS were also investigated to determine the mechanisms that affect lysosomal function by A2E + BL. Finally, we evaluated the effect of intracellular ROS removal on cell viability and autophagy induced by A2E and BL in ARPE-19 cells.

2. Materials and methods

2.1. Cell culture

ARPE-19 cells obtained from the American Type Culture Collection were grown in Dulbecco's modified Eagle's medium F-12 (DMEM/F-12; WELGENE, Gyeongsan, Republic of Korea) containing 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2. Cells between 3 and 5 passages were used for all experiments.

2.2. A2E and BL-induced cell death

Cells were plated at a density of 5×10^4 cells/well in a 6-well plate and treated thrice with 20 μM A2E (AptaBio, Yongin, Republic of Korea) at 2-day intervals. The cells were then exposed to BL (430 nm, 6000 lux) for 30 min (Jin et al., 2016). After incubation for 24 h, the IncuCyte Live Cell HD imaging system (Essen Bioscience, Ann Arbor, MI, USA) was used to monitor cell viability. N-acetyl cysteine (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) was added to the culture media 2 h prior to BL exposure. Cell viability was assessed by adding EZ-cytox (DoGen bio, Seoul, Republic of Korea) into each well as per the manufacturer's instructions. Absorbance was measured at 450 nm using a BioTeK microplate reader (Winooski, VT, USA).

2.3. Confocal microscopy

A2E-laden cells were transfected with a plasmid expressing GFP-LC3 (Addgene Inc., MA, USA) using Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA). After BL exposure for 30 min, cells were fixed using 4% paraformaldehyde for 10 min. The cells were then washed thrice with PBS every 5 min. Finally, the expression of GFP-LC3 was viewed using a confocal laser scanning microscope (Nikon, Tokyo, Japan). Nuclei were stained with 1 μg/mL of Hoechst 33342 (Thermo Scientific, Rockford, IL, USA).

2.4. Western immunoblot

Western immunoblot was performed based on a previously published method cells (Jin et al., 2016). Briefly, cell lysate was prepared using RIPA buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 1% SDS, 1% sodium deoxycholate, 1% NP-40). The following antibodies were used in this study: anti-LC3B, anti-p-mTOR, anti-p-Akt, anti-p-4EBP1 (Cell Signaling Technology Inc., MA, USA), and anti-p62 (Santa Cruz, California, USA). Antibody binding was developed using ECL Western Blotting Substrate (Thermo Scientific), and observed using the Image Lab 5.1 system.

2.5. mRNA sequencing

mRNA sequencing was performed as previously described (Jin et al., 2018). Total RNA was processed to prepare an mRNA-seq library using a TruSeq Stranded mRNA kit (Illumina, San Diego, CA, USA). Each produced library was sequenced using the Illumina NextSeq500 instrument (Illumina).

2.6. Real-time RT-qPCR

Real-time RT-qPCR was performed based on a previously published method (Jin et al., 2016). Briefly, total RNA was isolated from ARPE-19 cells using Trizol (Invitrogen) and subjected to reverse transcription (RT) using the iScript™ cDNA Synthesis Kit (Bio-Rad, Foster, CA, USA) at a total volume of 20 μl. In all, 2 μl of RT product was used for qPCR performed on a Roche LightCycler® 480II system using SYBR Green I Master (Roche, Mannheim, Germany) and the primers shown in Supplementary Table 1. Data shown represent the mean ± standard deviation (SD) of triplicate RT-qPCR reactions conducted on the same cDNA sample.

2.7. Autophagic flux

Autophagic flux was determined by measuring the p62 protein level. Alternatively, LC3-II protein levels, in the presence and absence of bafilomycin A1 (Abcam, Cambridge, United Kingdom), were measured by western blotting.

2.8. Immunofluorescence staining

ARPE-19 cells were fixed using 4% paraformaldehyde (Thermo Scientific) for 10 min at room temperature. Cells were washed thrice with cold PBS every 5 min and permeabilized using 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 15 min at 4 °C. After three washes with PBS, cells were blocked using 2% bovine serum albumin (BSA) in 0.05% TBST for 3 h. The cells were again washed thrice with PBS, followed by overnight incubation with primary antibodies at 4 °C with gentle shaking. The cells were then washed thrice with PBS and incubated with corresponding fluorescent secondary antibodies for 2 h at room temperature with gentle shaking. After PBS washing, they were incubated with 1 μg/mL of Hoechst 33342 for 5 min. Finally, after three washes with PBS, the cells were mounted on a coverslip with a drop of mounting medium.

2.9. Lysosomal staining

ARPE-19 cells were stained with 50 nM LysoTracker RED DND-99 (Invitrogen) in serum-free media and then incubated for 1 h in the dark. After cold PBS washing, they were fixed using 4% paraformaldehyde in 1 × PBS buffer for 10 min at room temperature. The cells were washed thrice with ice-cold PBS and lysosomes were visualized using a confocal laser scanning microscope.

2.10. ROS assay

ROS levels were determined quantitatively using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich, MO, United States). Briefly, cells were treated with 10 μM DCFH-DA reagent in serum-free media and incubated at 37 °C for 10 min. For the quantification assay, the Smart Fluorescent Cell Viewer (NanoEntek, Seoul, Republic of Korea) was used. Cells were then lysed and fluorescence intensities from each cell suspension were measured using the

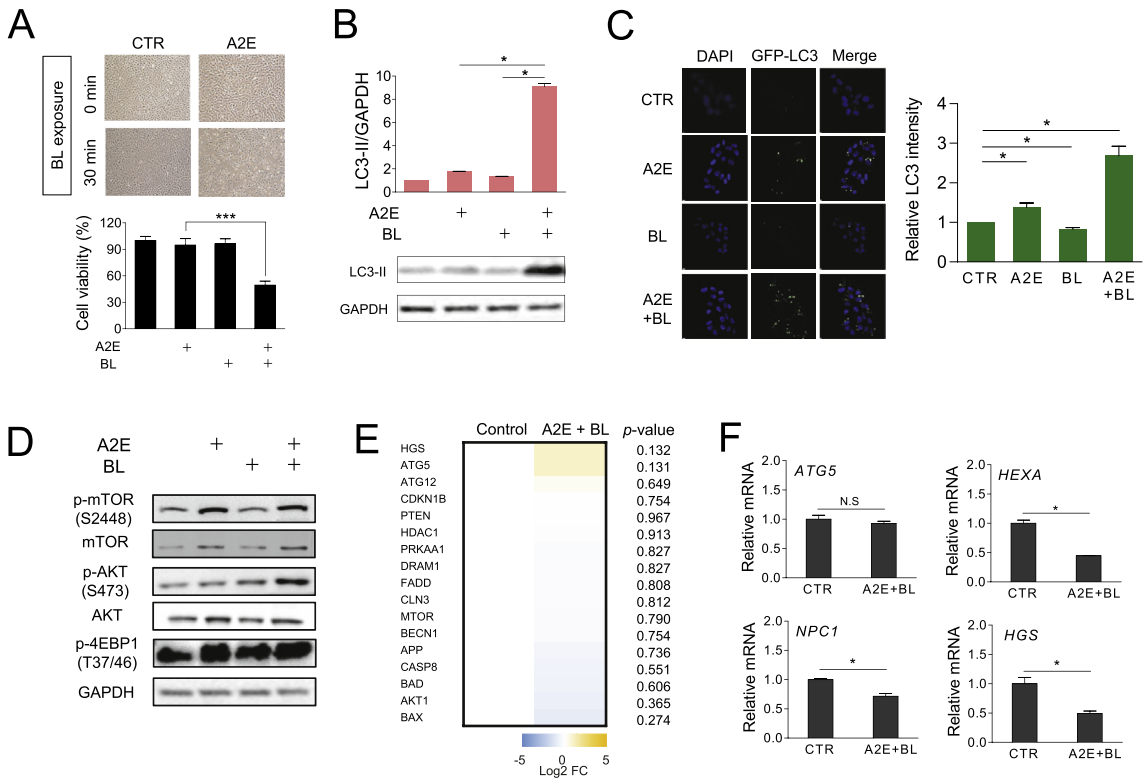

Fig. 1. Photoactivation of A2E by BL induces the accumulation of LC3-II. (A) ARPE-19 cells were treated thrice with 20 μM A2E every other day and exposed to BL (430 nm) for 30 min. After a 24-h incubation period, the IncuCyte Live Cell HD imaging system was used to monitor cell viability. The results are presented as mean ± SD (n = 3); *$p < 0.05$. (B) LC3-II and GAPDH protein levels were detected using western blotting analysis. GADPH was used as a loading control. Protein levels were quantified via band intensities. The results are presented as mean ± SD (n = 3); *$p < 0.05$. (C) A2E-laden ARPE-19 cells were transfected with a plasmid expressing GFP-LC3 and exposed to BL (430 nm) for 30 min. After a 24-h incubation period, intracellular GFP-LC3 puncta (green) were detected using confocal microscopy. Nuclei were stained with Hochest 33342 (blue). GFP-LC3 expression levels were quantified using Image J. (D) Phosphorylation of the proteins in the mTOR pathway (p-mTOR, p-Akt, and p-4EBP1) was determined via western blotting analysis. GADPH was used as a loading control. (E) RNA-seq results of control- and A2E + BL-treated ARPE-19 cells. (F) Expression of the TFEB target genes in ARPE-19 cells was determined using real-time RT-qPCR. mRNA levels were normalized to 18S rRNA. The results are presented as mean ± SD (n = 3); *$p < 0.05$ vs. control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Multimode Plate Reader Victor X3 (PerkinElmer Inc., Waltham, MA, USA) at excitation and emission wavelengths of 480 and 530 nm, respectively.

3. Results

3.1. A2E and BL induces LC3-II accumulation

Individually, A2E and BL are known to increase intracellular LC3-II levels in RPE cells (Chen et al., 2016; Saadat et al., 2014; Zhang et al., 2015). However, the effect of A2E activated by BL (A2E + BL) on autophagy in RPE cells is not well known. Here, ARPE-19 cells were treated thrice with A2E (20 μM) after 2-day intervals (Jin et al., 2017). The highest concentration of A2E that did not exhibit direct cytotoxicity (20 μM) was chosen (Jin et al., 2017). Clinically, the concentration of A2E in the retina of advanced age can reach 20 μM (Roberts et al., 2002; Sparrow et al., 1999), Cell death was induced when A2E-laden ARPE-19 cells were irradiated with BL (430 nm, 6000 lux) for 30 min, 24 h after irradiation (Fig. 1A). LC3-II levels were only slightly increased in A2E-treated cells. However, when A2E-laden ARPE-19 cells were exposed to BL (A2E + BL), LC3-II protein levels significantly increased (Fig. 1B). Changes in LC3-II levels induced by A2E and BL were also measured using confocal microscopy. When A2E-laden ARPE-19 cells overexpressing GFP-LC3 were irradiated with BL, intracellular LC3-II puncta significantly increased (Fig. 1C).

Based on results that show that A2E is photoactivated by BL (Ben-Shabat et al., 2002; Sparrow et al., 1999), the results of the present study suggest that BL-induced A2E photo-oxidization, and not A2E itself, induces a significant increase in LC3-II in retinal cells. The activity of the proteins involved in the mTOR pathway was assessed to determine whether the intracellular accumulation of LC3-II observed above was due to autophagy activation via inhibition of the mTOR pathway. However, inhibition of Akt (serine 473), mTOR (serine 2448), and 4EBP1 (T37/46) phosphorylation were not observed (Fig. 1D).

Transcription factor EB (TFEB) function has been previously reported as another pathway that is involved in autophagy activation (Hsu et al., 2018; Medina et al., 2015; Settembre and Ballabio, 2011). TFEB, a transcription factor that is activated by dephosphorylation, regulates the expression of proteins involved in autophagy (Medina et al., 2015; Settembre and Ballabio, 2011). Our RNA-seq results showed that A2E + BL treatment did not affect or even slightly decrease TFEB target gene expression when compared to the control group (Fig. 1E). Similar results were obtained from the RT-qPCR experiments (Fig. 1F). No changes in TFEB phosphorylation were observed after A2E and BL treatment (Supplementary Figure S1). Altogether, these results suggest that the significant increase in LC3-II by A2E + BL may not be due to autophagy activation.

3.2. Inhibition of autophagy by BL in A2E-laden cells

Our results indicated that LC3-II accumulated via A2E + BL may not be due to autophagy activation. Interestingly, the increase in intracellular LC3-II is known to be induced, not only via autophagy activators, but also via autophagy inhibitors such as chloroquine and

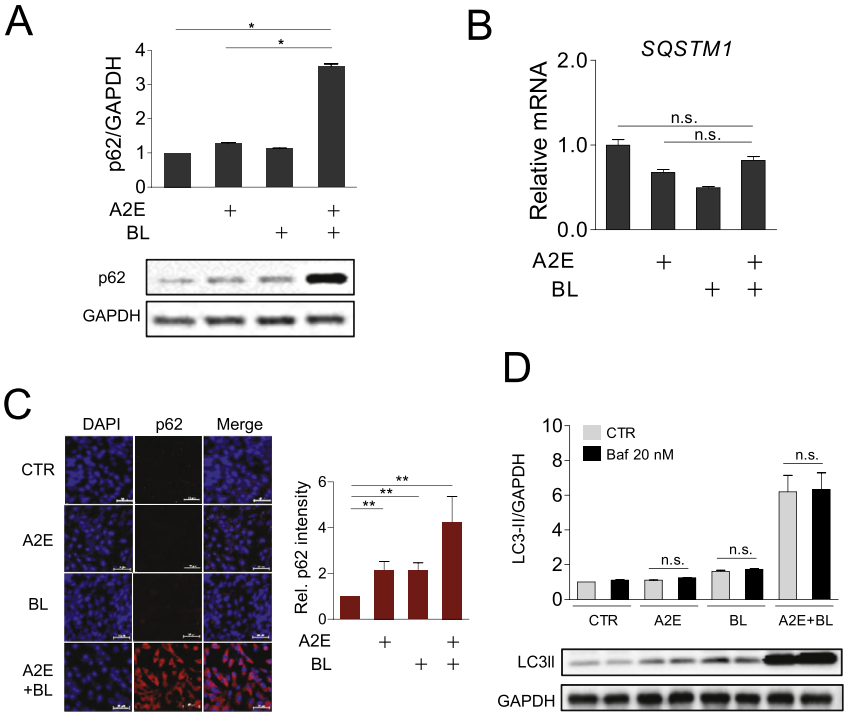

Fig. 2. BL exposure inhibits autophagic flux in A2E-laden ARPE-19 cells. (A) p62 protein levels were determined using western blotting analysis. GADPH was used as a loading control. Protein levels were quantified via band intensities. The results are presented as mean ± SD (n = 3); *$p < 0.05$. (B) *p62/SQSTM1* mRNA level in ARPE-19 cells was determined using real-time RT-qPCR. mRNA levels were normalized to *18S* rRNA. (C) Intracellular p62 puncta (green) were detected using confocal microscopy. Nuclei were stained with Hochest 33342 (blue, left panel). The p62 protein levels were quantified using Image J (right panel). (D) Autophagic flux was determined by calculating the increase in LC3-II in the bafilomycin A1 (20 nM)-treated group when compared to the untreated group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

bafilomycin A1 (Yoon et al., 2010). Thus, in the present study, we investigated the effect of A2E + BL on autophagic flux. p62/SQSTM1 is an autophagy receptor protein that acts as a marker for the autophagy process. Generally, autophagy stimulation decreases the p62 protein level in the cytoplasm (Bjorkoy et al., 2005; Pankiv et al., 2007). When A2E-laden ARPE-19 cells were irradiated with BL, intracellular p62 protein levels significantly increased compared to that of the control, A2E-treated, and BL-irradiated groups (Fig. 2A and Supplementary Figure S2), without an increase in the mRNA level (Fig. 2B). Immunostaining also showed an increase in p62 puncta via A2E + BL (Fig. 2C).

As an alternate way to measure autophagic flux, changes in LC3-II protein levels were observed in the presence of bafilomycin A1. Bafilomycin A1 inhibits autophagy through a mechanism that blocks the fusion of autophagosome-lysosomes (Mauvezin and Neufeld, 2015), resulting in an increase in LC3-II protein levels. A2E + BL treatments in the presence of bafilomycin A1 did not affect the LC3-II flux and LC3-II protein level compared to the no treatment group (Fig. 2D). These results suggest that A2E + BL inhibits, and does not activate, autophagy in ARPE-19 cells.

3.3. Photoactivation of A2E via BL induces lysosomal damage

In vivo, A2E and RPE lipofuscin components derived from photoreceptor external segments have been reported to be stored in lysosomes via phagocytosis (Kennedy et al., 1995). *In vitro* studies have also shown that A2E accumulates in the lysosomes in RPE cells (Jin et al., 2016; Sparrow et al., 1999), and that long-term exposure to A2E impairs its function by raising lysosomal pH (Guha et al., 2014). Although short-term A2E treatment has been reported to have little effect on lysosomes, we suspected that A2E photo-oxidation by BL might amplify lysosomal damage. Lysosomes were labeled with LysoTracker, which exhibits fluorescence in response to the acidic pH in lysosomes. As expected, green-labeled A2E were present in most of the lysosomes in ARPE-19 cells. When A2E-laden ARPE-19 cells were irradiated with BL, the areas labeled with LysoTracker remarkably decreased (Fig. 3A and B). BL treatment of the ARPE-19 cells had no effect on the Lysotracker signal (Supplementary Figure S3). Notably, A2E (green label) also decreased after BL irradiation (Fig. 3C), suggesting the possible photocleavage of A2E by BL. These results suggest that BL-activated A2E inhibits lysosomal function.

3.4. Methylglyoxal is not responsible for lysosomal damage via A2E + BL

Next, we sought to investigate the underlying mechanism of A2E-mediated lysosomal damage. The photocleavage of A2E has been reported to produce the low molecular weight product, methylglyoxal (MGO), besides singlet oxygen species (ROS) and others (Wu et al., 2010). This reactive dicarbonyl can modify molecular structures and functions by forming advanced glycation end (AGE)-adducts with proteins, phospholipids, and nucleotides (Chang et al., 2015). MGO is formed as a by-product of metabolic pathways such as glycolysis and lipid peroxidation, which are highly toxic. AGE-modified proteins that contribute to age-related inflammatory diseases have previously been detected in aged Bruch's membrane and drusen, the subRPE deposits that have been linked to AMD (Wu et al., 2010).

MGO treatment up to 100 μM demonstrated no effect on ARPE-19 cell viability (Fig. 4A). No changes were also detected in LC3-II and p62 protein levels (Fig. 4B) and lysosomal status (Fig. 4C) at the same concentrations. Theoretically, a single A2E molecule can form up to three molecules of MGO. Since the concentration of A2E used in this experiment was 20 μM, lysosomal damage and cytotoxicity caused by A2E + BL could not be attributed to MGO, a byproduct of A2E photo-oxidation.

3.5. BL-induced ROS is responsible for lysosomal damage in A2E-laden cells

A2E produces singlet oxygen and some A2E photo-products when irradiated with BL. Singlet oxygen produced via A2E photo-oxidation is known to be one of the mechanisms that causes RPE death (Kim et al., 2008; Wielgus et al., 2010). The present study also showed that ROS levels were significantly increased via BL irradiation of A2E in A2E-laden ARPE-19 cells (Fig. 5A). We suspected that the inhibition of lysosomal function via A2E + BL might be due to ROS. ARPE-19 cells were treated with NAC (N-acetyl cysteine) under the same conditions to remove ROS produced during A2E photo-oxidation. Compared to the

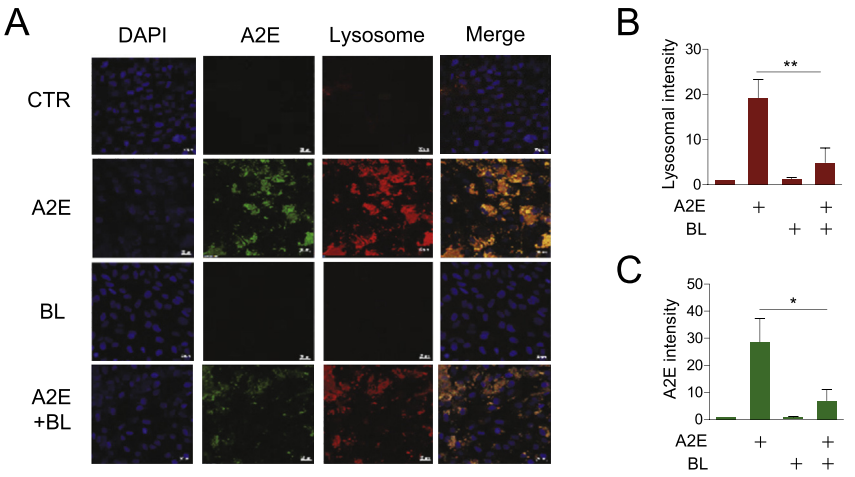

Fig. 3. Lysosomal damage induced by BL in A2E-laden APRE-19 cells. (A) Lysosomes were visualized using LysoTracker (red). The internalization of A2E appears as green autofluorescent granules. Nuclei were stained with Hochest 33342 (blue). These fluorescent signals were detected via fluorescence confocal microscopy. A2E and LysoTracker signals colocalized in the ARPE-19 cells. (B and C) Relative intracellular lysosomal and A2E levels were quantified. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

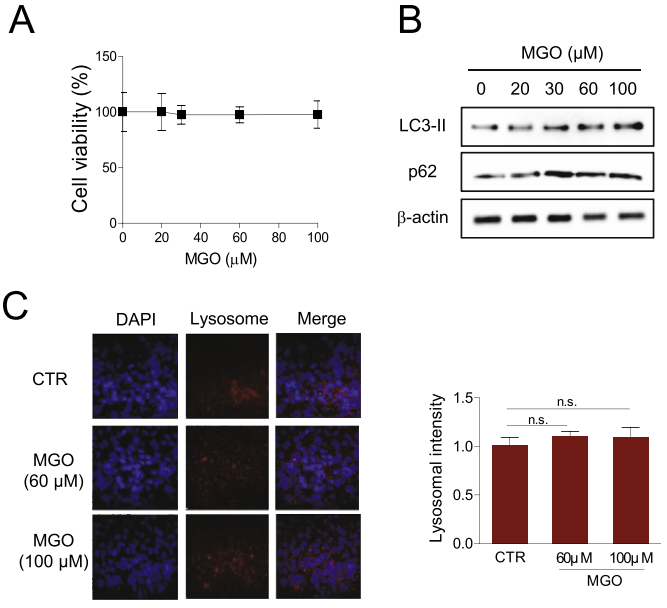

Fig. 4. Methylglyoxal is not responsible for lysosomal damage via A2E + BL. (A) ARPE-19 cells were treated with MGO (20–100 μM) for 24 h. Cell viability was monitored using the IncuCyte Live Cell HD imaging system. (B) LC3-II and p62 protein levels were determined via western blotting analysis. β-actin was used as a loading control. (C) Lysosomes were visualized using LysoTracker (red) in ARPE-19 cells treated with MGO (60 and 100 μM). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

control group, a decrease in ROS production was observed after NAC treatment in the A2E + BL group (Fig. 5A). With a reduction in ROS levels after NAC treatment in ARPE-19 cells, lysosomal damage was observed to be significantly decreased compared to the control group (Fig. 5B). Furthermore, NAC treatment resulted in a decrease in the level of LC3-II protein increased by BL in A2E-laden ARPE-19. Similarly, p62 protein levels also decreased after NAC treatment, suggesting a rescue of the autophagic flux by NAC (Fig. 5C). NAC treatment also inhibited the death of ARPE-19 cells after BL irradiation under the same conditions (Fig. 5D).

Taken together, our results demonstrate that the removal of ROS using an antioxidant restores lysosomal function, improves autophagic flux, and consequently inhibits cell death. These results suggest that photoactivated A2E inhibits autophagy in ARPE-19 cells by inhibiting ROS-mediated lysosomal function.

4. Discussion

In AMD, RPE gradually deteriorates and eventually degenerates, resulting in photoreceptor death and loss of visual function. The cause of AMD has not yet been clearly identified; however, aging, gender (women more than men), blood circulation disorders, cardiovascular diseases, smoking, genetic factors, and BL exposure are known risk factors of AMD (Gorin et al., 1999). BL ranging from 400 to 450 nm in the visible light spectrum has the highest energy level of all visible light and is not absorbed by the cornea or lens, thereby adversely affecting the retina. Currently, BL is produced using smart devices (Moon et al., 2017).

Oxidative stress damage is a major pathogenic factor in the development of AMD in RPE cells. BL oxidizes A2E accumulated in RPE cells, converting it into highly-reactive epoxide-like structures and the superoxide ion, singlet oxygen, thereby causing RPE cell damage and death (Kim et al., 2008; Wielgus et al., 2010). However, the retinal epithelial cell death mechanism caused by BL-mediated A2E photo-oxidation has not yet been clarified. Previous studies have shown that disturbed autophagy is associated with AMD pathogenesis and RPE damage (Guha et al., 2014; Zhang et al., 2015). Paradoxically, individual A2E and BL treatments have been shown to induce autophagy in RPE cells (Chen et al., 2016; Zhang et al., 2015). Lysosomal function has also been reported to be inhibited during the accumulation of lipofuscin, thereby causing problems with autophagic clearance (Brandstetter et al., 2015). These conflicting findings and clinical correlations have made it difficult to understand the mechanisms of retinal cell injury via A2E and BL. Moreover, no known mechanism explains the effect of BL on autophagy via A2E photo-oxidation.

Exposure to excessive light can be a serious risk factor for age-related diseases such as AMD, which is a major cause of blindness worldwide. The intensity of the BL used in the experiment is different from that of daylight. However, there is still concern regarding the long-term effects of screen exposure because of the close proximity and the length of time (Behar-Cohen et al., 2011). Here, we first investigated whether autophagy progressed normally in BL-activated A2E. As a result, p62 protein levels (an autophagic flux marker) significantly increased with an intracellular increase in the LC3-II protein. Furthermore, no change in the LC3-II flux was observed in the presence of bafilomycin A1. To date, studies have focused on the increase in LC3-II via A2E or BL, and did not examine the entire autophagic flux in detail. More importantly, the effect of BL + A2E on autophagic flux has not yet been reported. To the best of our knowledge, the present study is the first to show that autophagy was inhibited via BL exposure in A2E-laden RPE cells. These results are remarkable as we show that BL-activated A2E is much more toxic and has high clinical correlation compared to A2E itself.

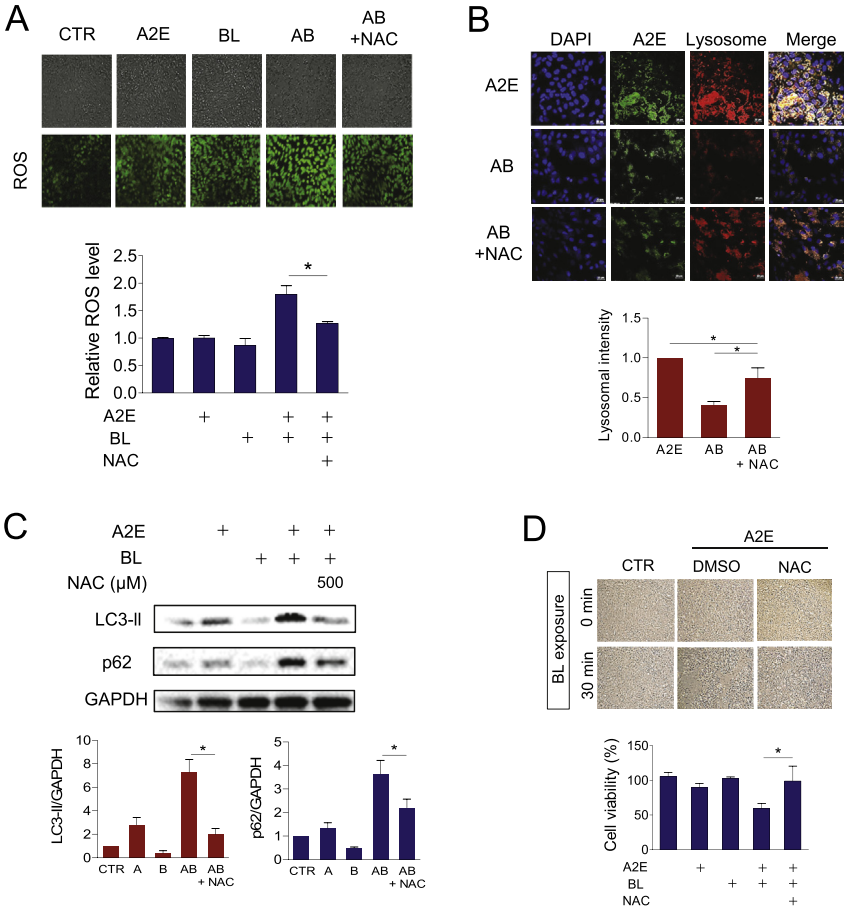

Fig. 5. BL-induced ROS is responsible for lysosomal damage in A2E-laden cells. (A) ROS levels were measured using DCFH-DA (10 μM) in A2E-laden RPE cells exposed to BL. ROS level quantification was carried out by measuring the fluorescence intensity using Victor X3 at excitation and emission wavelengths of 480 and 530 nm, respectively. The results are presented as mean ± SD (n = 3); $*p < 0.05$. (B) ARPE-19 cells were treated with NAC for 2 h before BL exposure. Lysosomes were visualized using LysoTracker (red). The internalization of A2E appears as green autofluorescent granules. Nuclei were stained with Hochest 33342 (blue). These fluorescent signals were detected using fluorescence confocal microscopy. The results are presented as mean ± SD (n = 3); $*p < 0.05$. (C) LC3-II and p62 protein levels were determined via western blotting analysis. GADPH was used as a loading control. Protein levels were quantified via band intensities. The results are presented as mean ± SD (n = 3); *, $p < 0.05$. (D) ARPE-19 cells were treated with NAC (20 μM) for 2 h before BL exposure. The IncuCyte Live Cell HD imaging system was used to monitor the cells. $*p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Long-term exposure to A2E has been reported to be associated with lysosomal damage (Holz et al., 1999). A2E activated by BL was also shown to generate MGO, which led us to investigate the role of MGO during phototoxicity induced by BL and A2E (Wu et al., 2010). However, MGO had no effect on LC3-II accumulation or lysosomal damage in ARPE-19 cells. Furthermore, MGO did not exhibit cytotoxicity, even at the highest concentrations. Contrastingly, the removal of ROS via NAC in A2E + BL-induced damage not only restored lysosomal function, but also prevented the inhibition of the autophagic flux caused by BL exposure, suggesting a negative function of ROS in the autophagy pathway of RPE cells. Notably, the cytotoxicity induced by A2E and BL was significantly inhibited by NAC. A previous study reported that ROS activated autophagy by inhibiting mTOR or modulating the activity of transcription factors (Xu et al., 2017; Zhang et al., 2014). However, in the present study, ROS produced by A2E + BL these did not directly suppress mTOR or activate TFEB. The reduction of TFEB target gene expression levels by A2E + BL could be considered as another mechanism to cooperate with the autophagy inhibition process.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.06.002.

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