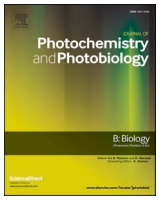
Journal of Photochemistry & Photobiology, B: Biology 245 (2023) 112735

Contents lists available at ScienceDirect

Journal of Photochemistry & Photobiology, B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol



The AMPK-dependent inhibition of autophagy plays a crucial role in protecting photoreceptor from photooxidative injury

Yu-Lin Li [a,1], Tian-Zi Zhang [b,1], Li-Kun Han [b], Chang He [b], Yi-Ran Pan [a], Bin Fan [a,*], Guang-Yu Li [a,*]

[a] Department of Ophthalmology, The Second Norman Bethune Hospital of JiLin University, ChangChun, China
[b] Affiliated Hospital of Inner Mongolia University for the Nationalities, Inner Mongolia, China

ARTICLE INFO

Keywords:
AMPK
Autophagy
Photoreceptor
Retinal light injury
Photooxidation
Retinal neuroprotection

ABSTRACT

Excessive light exposure can potentially cause irreversible damage to the various photoreceptor cells, and this aspect has been considered as an important factor leading to the progression of the different retinal diseases. AMP-activated protein kinase (AMPK) and the mammalian target of rapamycin (mTOR) are crucial intracellular signaling hubs involved in the regulation of cellular metabolism, energy homeostasis, cellular growth and autophagy. A number of previous studies have indicated that either AMPK activation or mTOR inhibition can promote autophagy in most cases. In the current study, we have established an *in vitro* as well as *in vivo* photooxidation-damaged photoreceptor model and investigated the possible influence of visible light exposure in the AMPK/mTOR/autophagy signaling pathway. We have also explored the potential regulatory effects of AMPK/mTOR on light-induced autophagy and protection achieved by suppressing autophagy in photooxidation-damaged photoreceptors. We observed that light exposure led to a significant activation of mTOR and autophagy in the photoreceptor cells. However, intriguingly, AMPK activation or mTOR inhibition significantly inhibited rather than promoting autophagy, which was termed as AMPK-dependent inhibition of autophagy. In addition, either indirectly suppressing autophagy by AMPK activation/ mTOR inhibition or directly blocking autophagy with an inhibitor exerted a significant protective effect on the photoreceptor cells against the photooxidative damage. Neuroprotective effects caused by the AMPK-dependent inhibition of autophagy were also verified with a retinal light injured mouse model *in vivo*. Overall, our findings demonstrated that AMPK / mTOR pathway could inhibit autophagy through AMPK-dependent inhibition of autophagy to significantly protect the photoreceptors from photooxidative injury, which may aid to further develop novel targeted retinal neuroprotective drugs.

1. Introduction

The visible light can pass through the refractive system of the human eye and reach the retina to form a visual image. However, visible light can function as a double-edged sword for the visual system. For instance, light can act on the various visual pigment groups such as rhodopsin in the photoreceptor cells to trigger visual action potentials to form the visual images, but on the contrary excessive light exposure can also induce irreversible photooxidative damage in retina, particularly for the photoreceptors and retinal pigment epithelium (RPE) [1,2]. Excessive

Abbreviations: AMPK, AMP-activated protein kinase; AMD, Age-related macular degeneration; ATP, Adenosine-triphosphate; ADP, Adenosine diphosphate; AMP, Adenosine monophosphate; ATG9, Autophagy related 9; ANOVA, Analysis of Variance; CCK8, Cell counting kit-8; DMEM, Dulbecco's modified Eagle's medium; DCFH-DA, 2′,7′-Dichlorodihydrofluorescein diacetate; ERG, Electroretinography; GSH, Glutathione; GSSG, Oxidized glutathione; LC3, Microtubule-associated protein 1 light chain 3; mTOR, The mammalian target of rapamycin; mTORC1, Mammalian target of rapamycin complex 1; mTORC2, Mammalian target of rapamycin complex 2; MNU, *N*-methyl-N-nitrosourea; NADPH oxidase, Nicotinamide adenine dinucleotide phosphate-oxidase; NAC, *N*-acetyl-L-cysteine; OXPHOS, Oxidative phosphorylation; $O_2^{\cdot -}$, Superoxide radical anions; PBS, Phosphate-buffered saline; PI, Propidium iodide; ROS, Reactive oxy gen species; RPE, Retinal pigment epithelium; ROO−, Peroxides; TSC2, Tuberous sclerosis complex 2; ULK1, Unc-51 like autophagy activating kinase 1; VPS34, Vacuolar protein sorting 34; $^1O_2$, Singlet oxygen; 3-MA, 3-Methyladenine; 4EBP1, Eukaryotic translation initiation factor 4E-binding protein 1.

* Corresponding authors at: Department of Ophthalmology, The Second Norman Bethune Hospital of JiLin University, ChangChun 130041, China.
E-mail addresses: fanb@jlu.edu.cn (B. Fan), l_gy@jlu.edu.cn (G.-Y. Li).

[1] Note: Dr.Tian-Zi Zhang and Dr.Yu-Lin Li contribute equally to this paper.

https://doi.org/10.1016/j.jphotobiol.2023.112735
Received 12 March 2023; Received in revised form 26 May 2023; Accepted 31 May 2023
Available online 5 June 2023

exposure to the visible light might also result in extensive photochemical damage due to massive absorption of photon's energy *via* photopigment, such as rhodopsin, or photosensitive molecules in RPE [3]. There are accumulating evidences which have reported significant correlation between the progressive photochemical damage and retinal degeneration [4]. For instance, in the genetically modified animal models, such as the mice with genetic mutations of Rdh12, Rhodopsin, and/or Abca4, which have been commonly used to mimic human retinal degenerative diseases, the severity of retina diseases was found to be associated with photochemical damage in a dose-dependent manner [5–7]. One primary example is the case of age-related macular degeneration (AMD), which is a chronic progressive disease of the retina and constitutes the leading cause of vision loss in the people with the age over fifty years in the developed countries [8]. The chronic and excessive light exposure was found to induce substantial damage that has been considered as the primary harmful reason to accelerate the progression of the disease though its pathogenesis has been found to be multifactorial [9].

AMP-activated protein kinase (AMPK) is a highly evolutionarily conserved protein kinase found in eukaryotic genomes. It is a heterotrimeric protein composed of a catalytic α subunit and two regulatory subunits (β and γ). which plays a key role in regulating cellular energy homeostasis [10]. As the cellular AMP/ATP ratio increases, AMP binding can potentially induce an allosteric alteration in the conformation of the γ subunit, thus leading to activation of AMPK [11]. After activation, AMPK can effectively regulate the multiple downstream targets involved in the regulation of the various metabolic pathways, such as fatty acid and cholesterol synthesis, and glycolysis to either increase energy production or decrease energy utilization [12]. Mammalian target of rapamycin (mTOR) is another evolutionarily conserved protein kinase, which controls the various cellular processes such as cell cycle, cell growth, and cell survival [13]. mTOR forms two distinct functional complexes of mTORC1 and mTORC2 in the cells, and mTORC1 is relatively extensively studied protein complex of the two. As a component of mTORC1, Raptor acts as a scaffold protein to recruit the downstream proteins S6 kinase (S6K) and eIF4E-binding proteins (4EBPs) to regulate mRNA translation [14]. Interestingly, both AMPK and mTOR, can serve as a signaling nexus to control energy homeostasis, cellular metabolism and growth, especially for autophagy regulation [15,16]. Typically, initiation of autophagy requires the coordinated interaction of two distinct kinases, ULK1 (Unc-51 like autophagy activating kinase 1; also named as autophagy-related 1, Atg1) and VPS34 (vacuolar protein sorting 34) [17]. Upon activation of ULK1 and VPS34, additional ATG proteins are recruited to the phagocytic membrane to promote autophagosome maturation [17]. When the concentration of the various intracellular nutrients is deficient, ULK1 can autophosphorylate and combine with its phosphorylated partners ATG13 and FIP200, thus forming an ULK1-ATG13-FIP200 complex to initiate autophagy. On the contrary, when the nutrients are sufficient, mTORC1 activation might destabilize ULK1-ATG13-FIP200 complex to represses autophagy initiation by directly phosphorylating Atg13 and ULK1 [18]. In addition, mTORC1 activation can also destabilize the AMBRA1-ATG14-BECN1-VPS34 complex by indirectly affecting VPS34 activation, thereby impairing autophagosome formation [19]. Thus, as a specific inhibitor of mTOR, rapamycin can significantly promote autophagy and has been widely used as a potent inducer of autophagy [20]. mTOR is also one of the important downstream targets of AMPK function, and when intracellular energy levels are relatively low, AMPK activation can directly phosphorylate at least two different proteins to induce rapid inhibition of mTORC1 activity, tuberous sclerostin 2 (TSC2) and mTOR binding chaperone raptor, which can indirectly lead to activation of autophagy [21]. In addition, AMPK activation can also directly promote autophagy by phosphorylating the multiple protein sites of ULK1 [22]. Moreover, previous reports have indicated that either activation of AMPK or inhibition of mTOR signaling can exhibit positive regulatory effects on the process of autophagy in most of the cases [13,16,23].

In this study, we have reported for the first time that autophagy plays a crucial role in modulation of excessive photooxidation-induced photoreceptor cell death, however, intriguingly, AMPK/mTOR signaling exhibited a rare regulatory role in light-induced autophagy of photoreceptors, that is, activating AMPK or inhibiting mTOR signaling displayed a significantly inhibitory effect on autophagy rather than promoting this process. In addition, either activating AMPK or inhibiting mTOR signaling exerted a significant protective effect on the photoreceptor cells against photooxidative damage, and this phenomenon of AMPK-dependent inhibition of autophagy was also validated in a retinal light injured mouse model *in vivo*.

2. Materials and Methods

2.1. Chemicals and Reagents

The cell culture medium and all the supplements were purchased from the HyClone Company (Beijing, China). β-actin antibody (Cat# 21800, RRID:AB_2923042), secondary antibodies (Goat Anti-Rabbit IgG Antibody, HRP conjugated: Cat# L3012, RRID:AB_895483; Goat Anti-Mouse IgG Antibody, HRP conjugated: Cat# L3032, RRID: AB_895481), mTOR (Cat# 21214, RRID:AB_1263863), p-mTOR (Cat# 11221, RRID:AB_895670), AMPK (Cat# 21191, RRID:AB_894836), p-AMPK (Cat# 11183), 4EBP1 (Cat# 21216, RRID:AB_894790), p-4EBP1 (Cat# 11223, RRID:AB_894794) and LC3B (Cat# 29075) antibodies were purchased from Signalway Technology (Maryland, USA). p62 antibody (Cat# 5114, RRID: AB_10624872) was purchased from Cell Signaling Technology (St. Louis, MO, USA). Rhodopsin antibody (Cat# OM186133) was purchased from OmnimAbs (California, USA). Dylight 488, Anti-Rabbit IgG (Cat# A23220, RRID: AB_2737289) was obtained from Abbkine (California, USA). Opsin Blue Polyclonal Antibody (RRID: AB_2736274) was obtained from Invitrogen (California, USA). Anti-Opsin Antibody, Red/Green (Cat# AB5745, RRID:AB_11213279) was obtained from Sigma-Aldrich (Shanghai, China). Hoechst, PI, DCFH-DA, GSH/GSSG Assay Kit, Enhanced BCA Protein Assay Kit and TUNEL Apoptosis Assay Kit were purchased from Beyotime Biotechnology (Shanghai, China). Metformin and Antifade Mounting Medium with DAPI were purchased from Solarbio (Beijing, China). 3-MA, NAC and MNU were obtained from Abmole (Houston, USA). Other reagents were purchased from Sigma-Aldrich (Shanghai, China).

2.2. Cell Culture

The photoreceptor cell line (661 W) was originally obtained from Dr. Muayyad Al-Ubaidi (University of Oklahoma Health Sciences Center, USA). 661 W cells were originally isolated from the transgenic mouse lines expressing the SV40 T antigen construct HIT1 driven by human photoreceptor retinol binding protein promoter [24]. The 661 W cells express blue and green opsins, SV40 T antigen, cone arrestin, and transducin [25]. The photoreceptor cells (661 W) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin. The photoreceptor cells (661 W) grew well in a humidified environment of 37 °C, 95% air and 5% carbon dioxide, and were sub-cultured by using 0.05% trypsin EDTA every 2–3 d.

2.3. Visual Light Exposure

The experiment of light exposure was performed as described previously [26]. A standard 8-W fluorescent strip lamp covered with a filter was fixed in the incubator to ensure that the cells were optimally exposed directly to the visible light (400-800 nm). The distance between the light source and the plates was 20 cm to ensure that all cells received the same intensity of light (1800 lx) which measured by a digital illuminance meter (LX101, London, UK) [27,28,26]. Before the light exposure, the photoreceptor cells (661 W) were pre-cultured in 96 or 6-

well plates for 24 h until the cell confluence reached to 75%. 2000 cells per well (*i.e.* 2×10^4/ml, 100 μl) were inoculated into 96-well plates, and 1×10^5 cells per well (*i.e.* 5×10^4/ml, 2 ml) were inoculated into 6-well plates. To ensure consistent culture conditions, the dark control group was put into opaque cartons and placed in the same incubator as the cells exposed to light. The temperature of the medium under the dark and light conditions was examined for 1–3 d, but no significant difference was found. The cell culture medium was replaced every two days and the duration of light exposure varied from 2 to 3 d. 661 W cells of each group were cultured with DMEM medium containing compound/vehicle instead of the normal medium before the light exposure.

2.4. Cell Death/ Live Assay (PI /Hoechst Staining)

The photoreceptor cells (661 W) were stained with Hoechst 33258 dye (2 μg/ml, Beyotime Biotechnology, Shanghai, China) for 30 min at 37 °C in the dark, after which the cells were stained with PI (Propidium iodide, Beyotime Biotechnology, Shanghai, China) at a final concentration of 5 μg/ml for 10 min under the similar conditions. The images were photographed by an inverted fluorescence microscope (Olympus, Tokyo, Japan), and the images were analyzed by Image J software (v1.51, NIH, USA). Thereafter, the cell death percentage (PI-positive cells/total cells %) was quantitatively calculated.

2.5. Cell Viability Assay

The cell viability was measured by using cell counting kit-8 (CCK-8) assay (Beyotime Biotechnology, Shanghai, China) in 96-well plates. The photoreceptor cells (661 W) were incubated with CCK8 (10 μl/well) for 1 h in the dark at 37 °C. The absorbance value (A) of each well was measured at 450 nm by microplate reader (Thermo, Waltham, MA United States). The following formula was used to calculate cell survival rate [29]:

$$S = \frac{A_{exp} - A_{blank}}{A_{control} - A_{blank}} \quad (1)$$

S represents cell survival rate, A_{exp} represents the absorbance value of the experimental group, $A_{control}$ represents the absorbance value of the vehicle group, A_{blank} represents the absorbance value of the blank control group. To avoid the measurement errors, five consecutive wells in the 96-well plate were set for testing each group.

2.6. Intracellular ROS Measurement

Intracellular ROS levels were measured with a dichloro-dihydro-fluorescein diacetate (DCFH-DA, Beyotime Biotechnology, Shanghai, China) staining assay as reported previously [30]. DCFH-DA was prepared in the fresh serum-free medium to a final concentration 10 μM. After 3 d of the light exposure, the medium of each group was then removed, the photoreceptor cells (661 W) were washed twice with the fresh medium, and then cultured with 100 μl prepared medium containing probe in the dark at 37 °C for 20 min. Thereafter, the medium was discarded and the cells were washed twice with the serum-free medium. Finally, the cells were observed and photographed using the fluorescence microscope (Olympus, Tokyo, Japan). The number of cells was determined by Hoechst staining and used to calculate ROS production in each cell. The intensity of fluorescence was quantitatively measured using ImageJ software (v1.51, NIH, USA).

2.7. Measurement of GSH/GSSG

Intracellular reduced/oxidized glutathione was quantitatively determined according to the manufacturer's instructions specified in GSH/GSSG Kit (Beyotime, Shanghai, China). Briefly, the photoreceptor cells (661 W) were seeded in the 6-well plate. After 3 d of light exposure, the cells were collected and centrifuged at 1000 rpm for 5 min at 4 °C. The cell precipitate was then resuspended with reagent A (removing cell protein) and repeatedly frozen as well as thawed twice in liquid nitrogen and 37 °C water bath. The sample was thereafter centrifuged and the supernatant was collected to determine the total glutathione and oxidized glutathione. Reagent B containing GSH reductase (5,5 '-dithiobis-2-nitrobenzoic acid) was mixed with the supernatant and incubated at 25 °C for 5 min, and then NADPH was added to the mixture to obtain a color reaction to determine the total glutathione content. GSH was then removed from the cells with GSH scavenging reagent, and thereafter GSSG levels were determined according to the above process. Finally, absorbance was measured at 412 nm with the microplate reader (Thermo, Waltham, MA United States), and the concentrations of the total glutathione and GSSG were calculated from the standard curve. The ratio of GSH/GSSG was calculated as follows: (total glutathione - GSSG× 2) /GSSG%.

2.8. Western Blot Analysis

The immunoblot was performed according to the previously reported procedure [28]. Briefly, the cell and retina samples were sonicated in protein lysis buffer (Beyotime, Shanghai, China) containing 1% phenylmethylsulfonyl fluoride (PMSF, Beyotime, Shanghai, China). The samples were then centrifuged at 10,000 rpm for 10 min at 4 °C for collection of the supernatants that were then subjected to the bicinchoninic acid assay to measure the protein concentrations. 20 μg of the cell lysate was dissolved in the sample buffer, and then the sample was boiled for another 10 min. Thereafter, electrophoresis was performed with 10% polyacrylamide gels containing 0.1% SDS, after which the proteins were transferred onto the polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T) at the room temperature for 1 h and then incubated with the specific primary antibody (mTOR: 1: 1000; p-mTOR: 1:1000; AMPK: 1:1000; p-AMPK: 1:1000; 4EBP1: 1:1000; p-4EBP1: 1:1000; LC3B: 1:1000) overnight at 4 °C. After rinsing the membranes three times in TBS-T next day, the membranes were then incubated with the corresponding biotinylated secondary antibodies for 1 h at the room temperature. The signals were subsequently developed using enhanced chemiluminescence, after which the images were captured using a microscope equipped with a CCD camera (Tanon, Shanghai). Finally, ImageJ software (V1.51, NIH, USA) was used for density analysis of the different protein bands.

2.9. Animal Experiments

All animal experiments were conducted in accordance with the statement of the Association of Research in Vision and Ophthalmology (ARVO), and were approved by the Animal Experimentation Ethics Committee of Jilin University. The retina of C57BL/6 J mice is covered with a layer of pigment epithelium, which is more similar to the human retina than albino mice. We selected C57BL/6 J mice for experiments [31–33]. Six-week-old male C57BL/6 J mice were purchased from the Animal Center of Jilin University (Changchun). The indoor temperature was kept at 21 °C - 23 °C and a 12 h light/dark cycle was guaranteed. Metformin (dissolved in PBS) was injected intraperitoneally at a dose of 300 mg/kg once a day for 7 d [1]. The same amount of PBS was intraperitoneally injected as a vehicle control by following the above procedure. Standard 10-W ring fluorescent lamps (Philips, Eindhoven, Netherlands) were fixed on the cage. On the seventh day of administration, the mice with dilated pupils were continuously exposed to 7000 lx visible light for 12 h [28,26]. The pupil of mice was dilated with 1% atropine before the light exposure. After the light exposure, the regular feeding with normal light/dark cycle was continued in the animal room. On the seventh day after the light exposure, the retinal function was evaluated with electroretinogram (ERG) first, and then the mice were sacrificed by cervical dislocation and eyeballs were enucleated for immunofluorescence staining, Western blot analysis and transmission

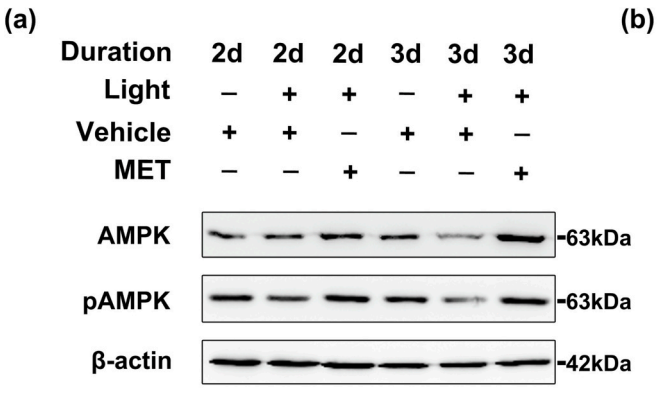


Fig. 1. Metformin activated AMPK under the light-exposure condition.
The photoreceptor cells (661 W) were cultured in presence of 10 mM metformin under the light conditions for the designated time intervals. The cells were then harvested and lysed for the western blot analysis. (a). The representative blots showing the protein levels of p-AMPK/AMPK have been depicted above. β-actin was used as an internal control. (b). Quantitative analysis of p-AMPK/AMPK protein level. The results were obtained from at least three independent experiments and have been represented as means ± SEM, $*p < 0.05$. MET: metformin; 2d, 3d: light exposure for 2 and 3 d.

electron microscopy. Grouping: Vehicle treated group ($n = 6$), Vehicle light-treated group (n = 6), Metformin light-treated group (n = 6).

2.10. Immunofluorescence Staining

The immunofluorescence staining was performed according to the previously described procedure [34]. Briefly, the retina frozen slices were prepared and fixed with 4% PFA, then rinsed with PBS for 3 times and blocked with 10% goat serum at the room temperature for 1 h. After removing the blocking reagent, the sections were incubated with the primary antibody rhodopsin (1:500) at 4 °Covernight. The sections were then rinsed with PBS for 3 times and incubated with the secondary antibody (Dylight 488, Anti-Rabbit IgG, 1:1000) at the room temperature for 1 h. After rinsing with PBS, the sections were double stained with DAPI and mounted with the coverslips drip. The sections were then observed under the fluorescence microscope (Olympus, Tokyo, Japan).

2.11. Electroretinography (ERG)

Full field ERG was performed using the Metrovision electroretinogram system (Paris, France). The ERGs were obtained on the seventh day after the light exposure. After the dark adaptation for 12 h, the mice were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg / kg), and ERG records were prepared under the dark red light. Before the experiment, the pupil of mice was dilated with 1% atropine. The mice were then placed on a heating plate and their body temperature was maintained at 35–36 °C. The reference electrode and grounding electrode are thereafter placed under the skin of the nose and tail, whereas the corneal electrode was placed in the center of the cornea. After set up under the dim red light, the mice were subjected to the dark conditions for another 10 min before the start. ERG analysis was then performed by measuring the amplitude of a wave and b wave.

2.12. Transmission Electron Microscopy (TEM)

The cultured cells were collected after the centrifugation at 3000 rpm (4 °C for 5 min) and the supernatant was discarded. The cells were fixed with 2.5% glutaraldehyde (4 °C for 2 h). The mouse eyeballs were enucleated and fixed in 2.5% glutaraldehyde precooled at 4 °C for 2 h. The cornea and lens are then cut from the eyeball to make a cup. The cup was then cut from the temporal side of the optic disc. The samples were incubated in PBS at 4 °C overnight and fixed with 1% osmium tetroxide at 4 °C for 2 h. Thereafter, the cells were dehydrated and centrifuged using gradient ethanol (10 min each). The samples were then dehydrated two times in pure acetone for 30 min each time. Next, the samples were embedded in Durcupan resin overnight. Ultrathin sections were

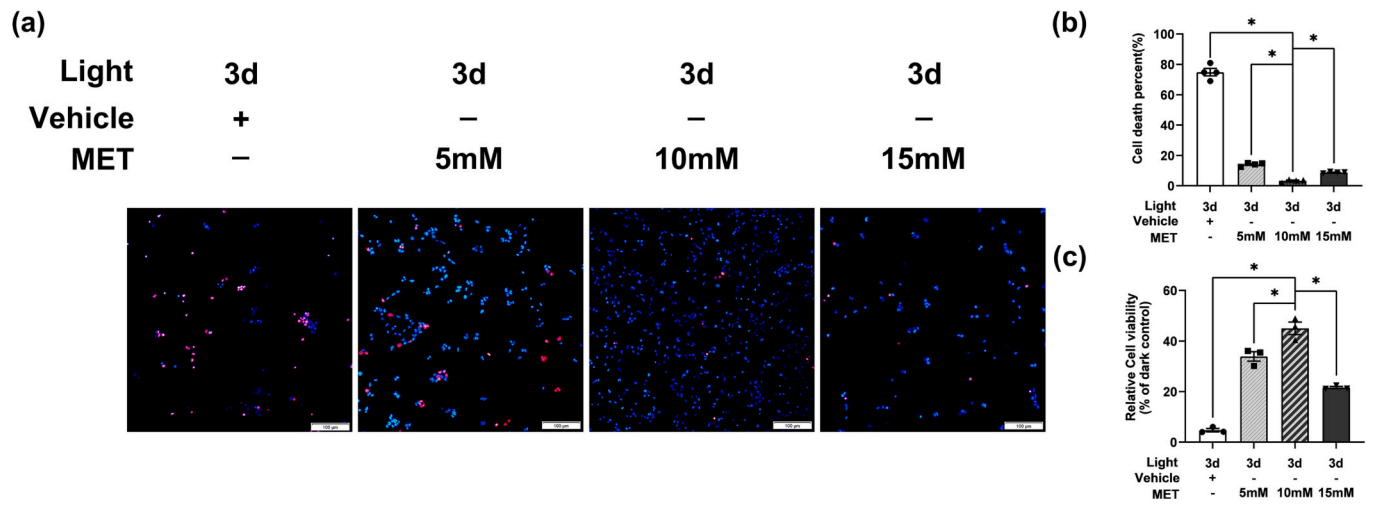


Fig. 2. AMPK activation attenuated the light-induced cell death.
(a). 661 W cells were cultured under the light condition for 3 d in the presence of 5–15 mM metformin. The cell death was then measured by using PI/Hoechst staining. Scale bar = 100 μm. (b). The cell death percentage was quantitatively analyzed and compared. (c). The viability of 661 W cells was measured by using CCK8 assay. The results were obtained from at least three independent experiments and have been represented as means ± SEM, $*p < 0.05$. MET: metformin; 3d: light exposure for 2 and 3 d.

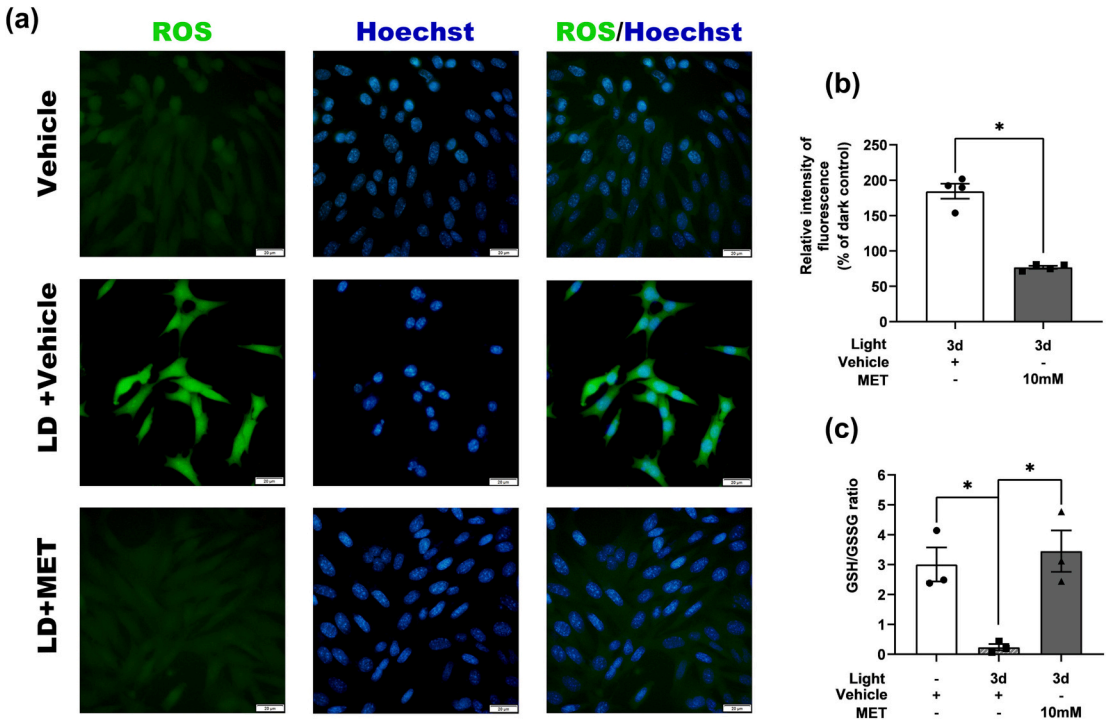


Fig. 3. AMPK activation suppressed the light exposure-induced oxidative stress.
(a). 661 W cells were cultured under the light condition for 3 d in the presence of 10 mM metformin and intracellular ROS generation was measured with DCFH-DA under a fluorescent microscope. Nuclei were stained with Hoechst 33342 and merged with ROS staining pictures. Scale bar =20 μm. (b). The fluorescent intensities were thereafter quantitatively analyzed. (c). GSH/GSSG levels were determined and the ratio was quantitatively analyzed. The results were obtained from at least three independent experiments and are represented as means ± SEM, *p < 0.05. MET: metformin; LD: light damage; 3d: light exposure for 3 d.

prepared with an ultramicrotome (Jeol, Japan) and stained with uranyl acetate and lead citrate. The sections were examined under a transmission electron microscope (Jeol, Japan).

2.13. TUNEL Staining

The retina frozen slices were prepared and fixed with 4% PFA, then rinsed with PBS for 3 times. The sections were incubated with the TUNEL reagent at 37 °C for 1 h. The sections were then rinsed with PBS for 3 times. After rinsing with PBS, the sections were double stained with DAPI and mounted with the coverslips drip. The sections were then observed under the fluorescence microscope (Olympus, Tokyo, Japan).

2.14. Statistical Analysis

Each independent experiment in this study was at least repeated in triplicates. GraphPad Prism 9.0 software was used for the statistical analysis. The data was expressed in the form of mean ± SEM. The differences between the means were evaluated using one-way analysis of variance (ANOVA), followed by the post-hoc Bonferroni test. The difference was considered as statistically significant for $P < 0.05$.

3. Results

3.1. AMPK Activation Protected the Photoreceptors against the Photooxidative Injury

We found that light exposure led to a significant suppression of AMPK activation, thereby resulting in a significantly decreased level of p-AMPK/AMPK (on day 2,3) as compared with the dark control group, whereas metformin treatment was able to significantly reactivate AMPK, thus markedly increasing p-AMPK/AMPK level in the photoreceptor cells (661 W) even under the light-exposed condition as compared with vehicle light-treated group (Fig. 1).

We investigated the role of AMPK activation in the light-induced photoreceptor death. As shown in Fig. 2a-b, 3 d light exposure caused around 74.9% cell death in the photoreceptor cells (661 W) as determined with PI/Hoechst staining related to dark control, whereas activating AMPK with metformin (5–15 mM) significantly protected the photoreceptor cells (661 W) against the light injury [35,36], thus substantially reducing cell death percentage to 14.1% (5 mM), 3.3% (10 mM), and 8.8% (15 mM) respectively. Furthermore, the cell viability was quantitatively assessed by using CCK8 assay as well. As shown in Fig. 2c, the light exposure for 3 d markedly reduced the cell viability to 4.7% related to the dark control; however, treatment with metformin (5–15 mM) significantly reversed this trend, thereby increasing the cell viability up to 33.9% (5 mM), 45.0% (10 mM), and 21.6% (15 mM) respectively. In addition, since the photon energy is primarily absorbed by the different photosensitive groups in the photoreceptors leading to photooxidative reactions and photooxidation can play an initiative role to trigger the cell death cascade [3], we also evaluated the influence of AMPK activation in light-induced imbalance of the cellular redox status. As shown in Fig. 3a-b, 3 d after the light exposure, significant ROS generation was detected in the photoreceptor cells (661 W), which were stained with intensive fluorescent green as determined by DCFH-DA assay. However, metformin treatment (10 mM) significantly suppressed ROS generation as shown by the lower amount of fluorescence in comparison with the vehicle light-treated cells. Furthermore, the light exposure also resulted in remarkable imbalance of intracellular antioxidative capacity showing significant reduction in GSH/GSSG ratio, whereas 10 mM metformin treatment restored the redox status, thereby increasing the GSH/GSSG ratio to the normal level (Fig. 3c). Overall, these results suggested that AMPK activation can play a crucial protective role in photoreceptor against substantial damage caused by photooxidation.

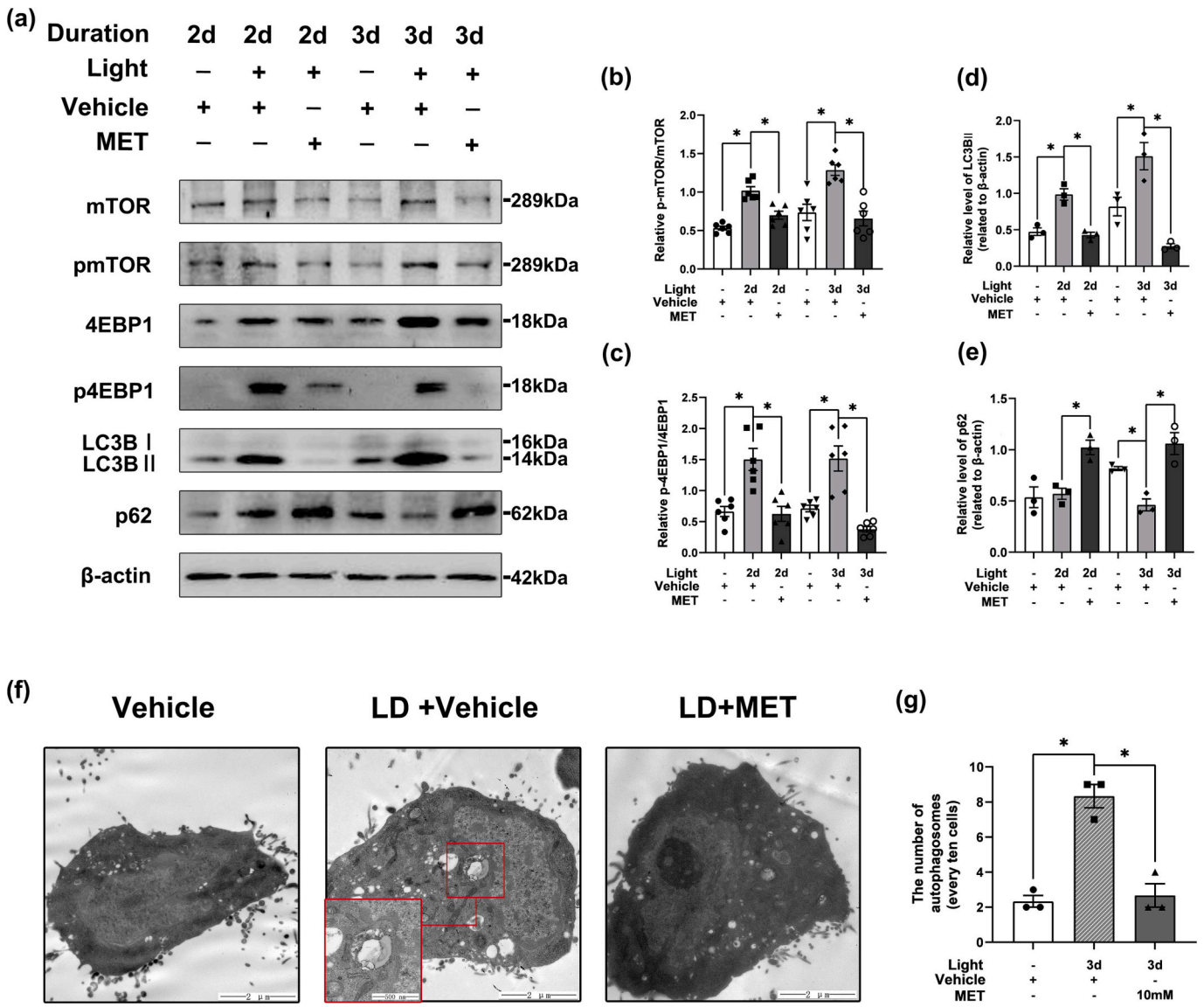


Fig. 4. AMPK activation inhibited mTOR activation as well as autophagy.
(a). 661 W cells were pretreated with 10 mM metformin and cultured under the light conditions for the designated times. The cells were then harvested and lysed for western blot analysis. The representative blots showing the expression of p-mTOR /mTOR, p-4EBP1/4EBP1, LC3-II and p62 proteins have been depicted above. β-actin was used as an internal control. (b-e). The quantitative analysis of the target protein levels. (f). 661 W cells were pretreated with 10 mM metformin and cultured under the light conditions for the designated times. They were then visualized with transmission electron microscopy. The autophagosome was circled in the red wireframe. Scale bar = 2 μm; 500 nm. (g). The quantitative analysis of the number of autophagosomes. The results have been presented as means ± SEM of three independent experiments, $*p < 0.05$. MET: metformin; LD: light damage; 2d, 3d: light exposure for 2 and 3 d.

3.2. AMPK Activation can Negatively Regulate Light-Induced Autophagy

Since mTOR/autophagy are important downstream targets of AMPK regulation, we next investigated the potential influence of AMPK activation in mTOR signal and autophagy under the light condition. As shown in Fig. 4a-c, the light exposure caused marked activation of mTOR signal in the photoreceptor cells (661 W), as evidenced by the significantly up-regulated levels of p-mTOR /mTOR and the downstream factor p-4EBP1/4EBP1 as compared with the dark control group. Moreover, light exposure also induced substantial activation of autophagy in the photoreceptor cells (661 W). As shown in Fig. 4a, d, e, the significant increased level of LC3-II, a typical marker of autophagy activation, and correspondingly reverse decrease of p62 level (on day 3) were clearly observed as compared with dark control. However, activating AMPK with metformin significantly suppressed the activation of mTOR protein, thus resulting in significant reduction in p-mTOR /mTOR level, and that of the downstream factor, p-4EBP1/4EBP1 (Fig. 4a-c). Additionally, metformin treatment also caused a marked down-regulation of LC3-II and reversed up-regulation of p62 level on day 2 and day 3 of the light exposure as compared to the vehicle light-treated group. The results of TEM indicated that there was obvious formation of autophagosomes in the cells after light irradiation for three days, whereas metformin treatment significantly suppressed light-induced activation of autophagy, thus showing a lesser number of autophagosomes (Fig. 4).

Thus, these results suggested that mTOR/autophagy pathway was activated under the light condition in the photoreceptor cells (661 W), but AMPK activation exerted a negative regulatory effect in the light-induced activation of mTOR as well as autophagy.

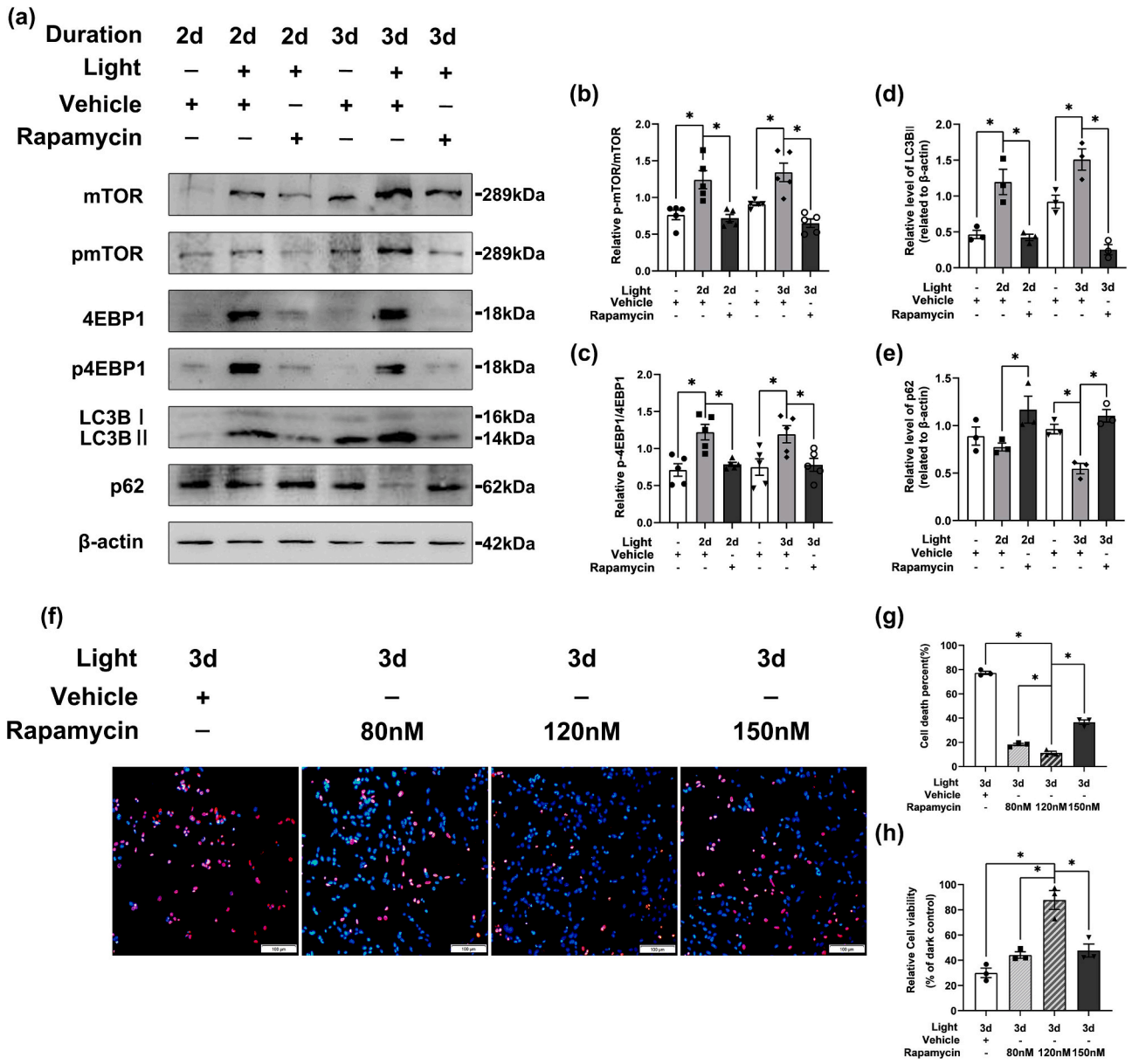


Fig. 5. mTOR inhibition significantly suppressed autophagy and attenuated the light-induced cell death.
(a). 661 W cells were pretreated with 120 nM rapamycin and cultured under the light condition for the designated times. The cells were then harvested and lysed for detecting the expression of p-mTOR /mTOR, p-4EBP1/4EBP1, LC3-II and p62 proteins by western blot analysis. β-actin was used as an internal control. (b-e). The target protein levels were quantitatively analyzed and compared. (f). 661 W cells were cultured under the light condition for 3 d in the presence of 80–150 nM rapamycin. The cell death was then evaluated by PI/Hoechst staining. Scale bar = 100 μm. (g). The cell death percentage was quantitatively analyzed and compared. (h). The viability of 661 W cells was measured with CCK8 assay. The results have been presented as means ± SEM of three independent experiments, $^{*}p < 0.05$. 2d, 3d: light exposure for 2 and 3 d.

3.3. mTOR Inhibition Suppressed Autophagy and Significantly Protected the Photoreceptors against the Photooxidative Injury

Since mTOR activation plays an important role in controlling autophagy, we further evaluated the effect of direct mTOR inhibition on the light-induced autophagy. As shown in the Fig. 5, rapamycin treatment significantly suppressed the light-induced activation of mTOR signaling on the day 2 and day 3, reduced the elevated levels of p-mTOR /mTOR, and that of the downstream, p-4EBP1/4EBP1 proteins. However, intriguingly, mTOR inhibition exerted significant suppression in light-induced autophagy activation rather than promoting the process of autophagy as usual, which was evident by a markedly decreased level of LC3-II and a reverse increased level of p62. Importantly, similar with the protective effect of AMPK activation, direct inhibition of mTOR with rapamycin exhibited significant protection on the light-damaged photoreceptor cells (661 W) as well, as indicated by marked decreased cell death percentage (from 77.3% to 11.3%, 120 nM) and increased cell viability (from 30.0% up to 87.8%, 120 nM) (Fig. 5). These results suggested that the direct inhibition of mTOR can also attenuate light-induced activation of autophagy and played a protective role in light-damaged photoreceptor cells (661 W), which was consistent with the effect of AMPK activation. Furthermore, we evaluated the potential effect of mTOR inhibition on light-induced imbalance of the cellular redox status. As depicted in Fig. 6a-b, we found that compared with the vehicle light-treated cells, rapamycin treatment (120 nM) significantly inhibited ROS generation and increased the GSH/GSSG ratio to the normal level (Fig. 6c). These results suggested that mTOR inhibition was not only able to reduce activation of autophagy, but also can play a positive role

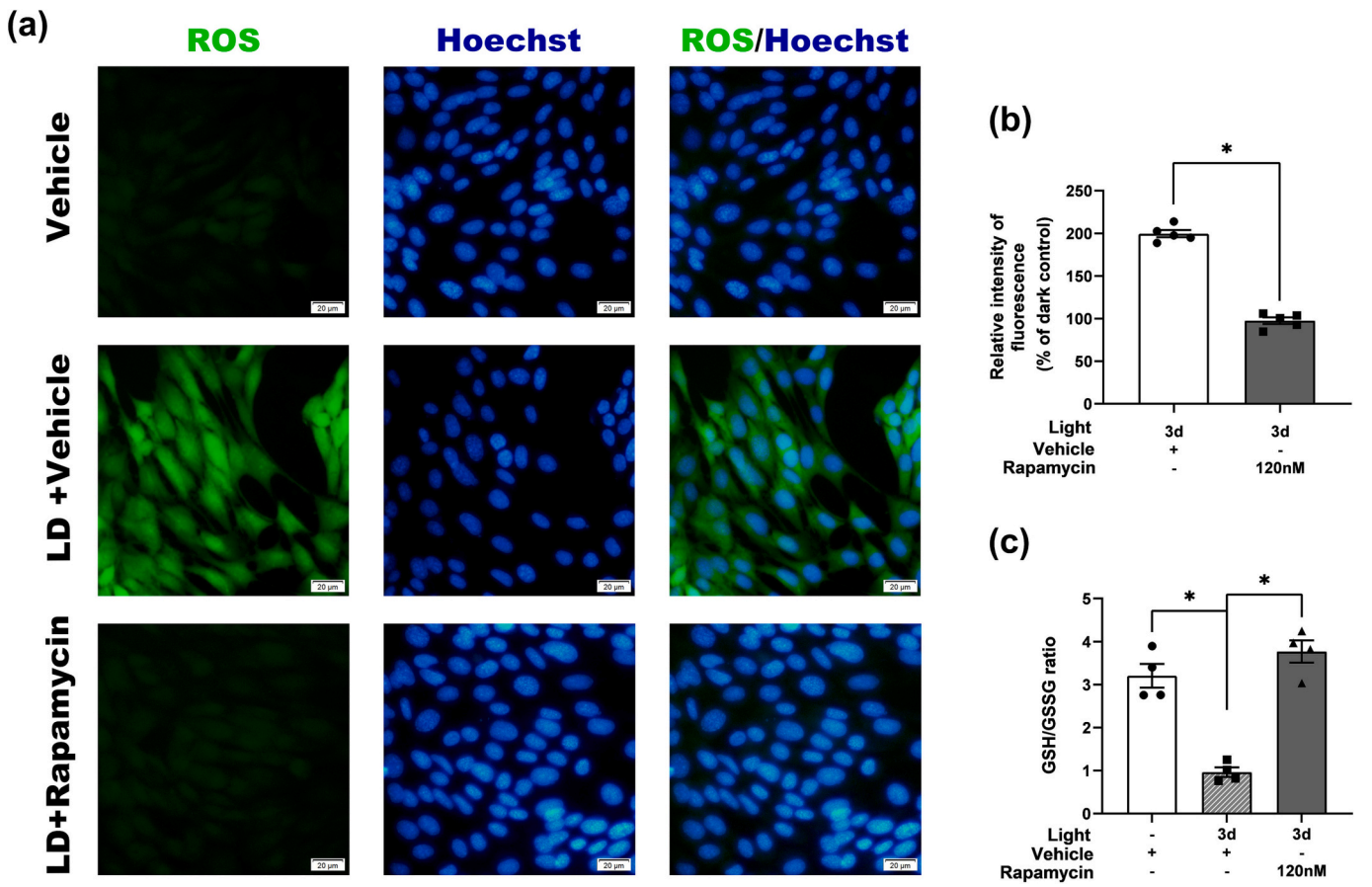


Fig. 6. mTOR inhibition suppressed the light exposure-induced oxidative stress.
(a). 661 W cells were cultured under the light condition for 3 d in the presence of 120 nM rapamycin and intracellular ROS generation was measured with DCFH-DA under a fluorescent microscope. The nuclei were stained with Hoechst 33342. Scale bar =20 μm. (b). The fluorescent intensity was quantitatively analyzed. (c). GSH/GSSG levels were determined and the ratio was quantitatively analyzed. The results were obtained from at least three independent experiments and have been represented as means ± SEM, *p < 0.05. LD: light damage; 3d: light exposure for 3 d.

in photoreceptors against photooxidative injury.

3.4. Inhibiting Oxidative Stress can Suppress Autophagy and Protect the Cells from the Light Damage

We treated 661 W cells with antioxidant *N*-acetyl-L-cysteine (NAC) to further investigate the possible link between oxidative stress and light-induced autophagy. As shown in Fig. 7, NAC (5 mM) treatment significantly inhibited ROS generation and restored the GSH/GSSG ratio. In addition, NAC treatment significantly inhibited light-induced autophagy, which was manifested by a significant decrease in LC3-II level and an increase in p62 level (Fig. 8). Moreover, the inhibition of oxidative stress with NAC also exhibited significant protective effects on light-induced death in 661 W cells, as evidenced with a significant decrease in cell death percentage (from 73.6% to 13.6%, at 5 mM) and an increase in cell viability (from 14.9% to 66.8%, at 5 mM), as shown in Fig. 8.

These results suggested that suppressing oxidative stress and intracellular ROS generation can significantly inhibit light-induced autophagy and thereby protect photoreceptors from light damage.

3.5. Inhibition of Autophagy can Protect the Photoreceptors from the Light Damage

To further validate the role of autophagy in light-induced photoreceptor death, we examined the effect of directly inhibiting autophagy with a pharmacological inhibitor, 3-Methyladenine (3-MA). As shown in Fig. 9, the treatment with 5 mM 3-MA significantly suppressed the light-induced activation of autophagy in the photoreceptor cells (661 W), thereby resulting in a significant reduction in the level of LC3-II and conversely increased the level of p62 protein. Moreover, similar to the protection achieved by AMPK activation or mTOR inhibition, directly inhibiting autophagy with 3-MA conferred significant protection on the light-damaged photoreceptor cells (661 W), as evidenced by a markedly reduced cell death rate (from 74.1% to 7.8%, 5 mM) and increased cell viability (from 13.4% to 45.8%, 5 mM) as shown in Fig. 9.

3.6. AMPK-Dependent Inhibition of Autophagy can Protect Retina against the Light Injury

We next investigated whether AMPK activation can produce the similar inhibition of autophagy under *in vivo* settings by using a retinal light injury mouse model. We intraperitoneally pre-injected metformin (300 mg/kg once a day for 7 consecutive days), and then caused light damage to the mice retina, and thereafter 7 d later assessed the various changes in the expression of the marker proteins and the retinal function. As shown in Fig. 10 a, b, 12 h light exposure to retina resulted in a marked inhibition in p-AMPK/AMPK levels as compared with the vehicle control, yet pre-injection of metformin led to an obvious activation of AMPK in the light-exposed retinas, as evidenced by a significant increase of p-AMPK/AMPK level as compared with vehicle light-treated group. Moreover, consistent with our *in vitro* results, light exposure also caused an obvious activation of mTOR protein in the mice retina, as the significant up-regulation of p-mTOR /mTOR and p-4EBP1/

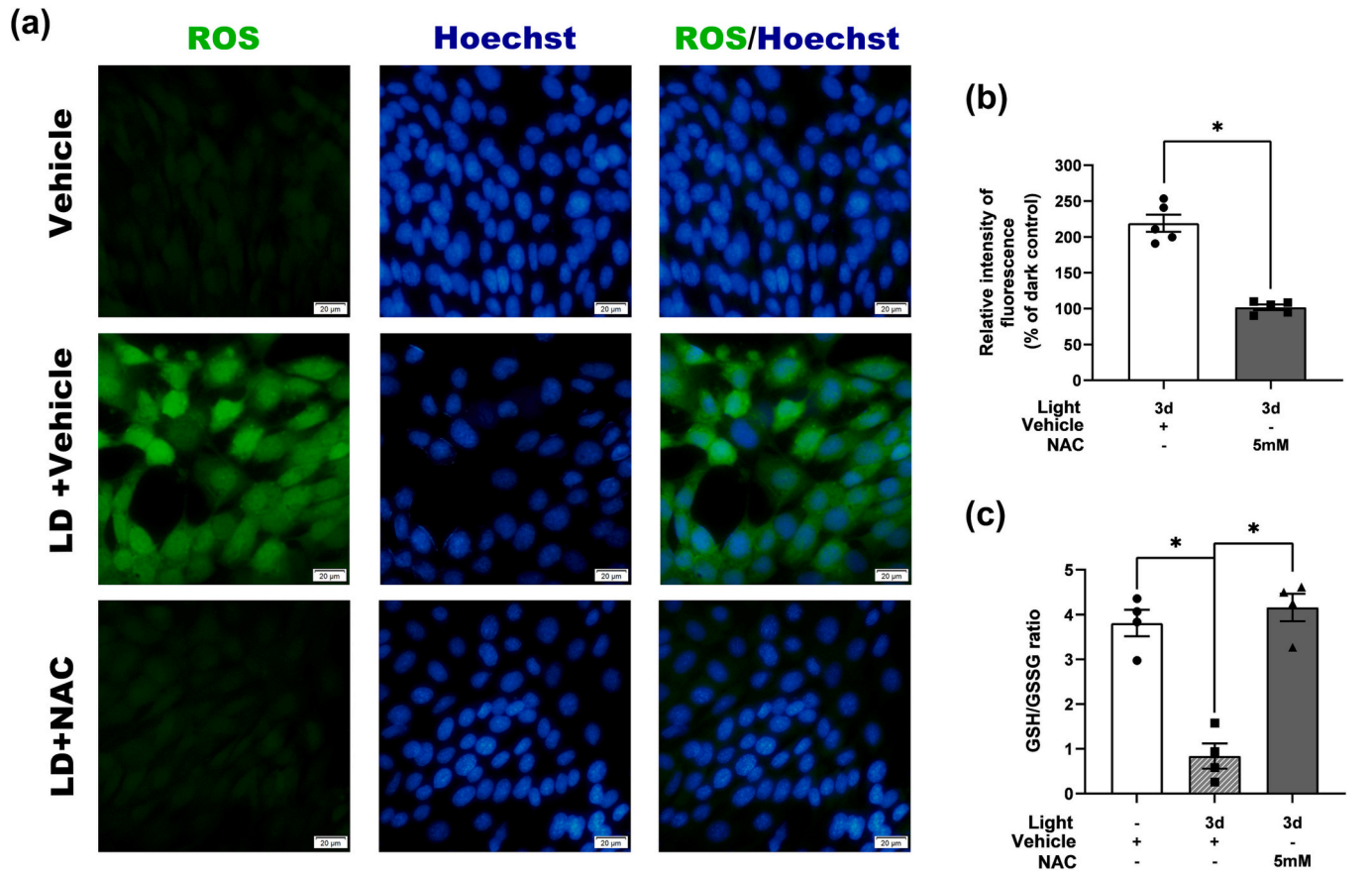


Fig. 7. The treatment with NAC suppresses the photooxidation in 661 W cells.
(a). 661 W cells were cultured under the light condition for 3 d in the presence of 5 mM NAC and intracellular ROS generation was measured with DCFH-DA under a fluorescent microscope. The nuclei were stained with Hoechst 33342. Scale bar =20 μm. (b). The fluorescent intensity was quantitatively analyzed. (c). GSH/GSSG levels were determined and the ratio was quantitatively analyzed. The results were obtained from at least three independent experiments and have been represented as means ± SEM, $*p < 0.05$. NAC: *N*-acetyl-L-cysteine; LD: light damage; 3d: light exposure for 3 d.

4EBP1 protein levels were also detected in the vehicle light-treated group as compared with the vehicle control. However, pre-treatment of metformin significantly mitigated the activation of mTOR signal, thereby showing a marked reduction in p-mTOR /mTOR and p-4EBP1/4EBP1 levels as compared with the vehicle light-treated group (Fig. 10 a, c, d). Similarly, we also detected that the expression of autophagic marker, LC3-II was significantly up-regulated in the light-injured retinas compared to the vehicle control group, whereas the administration of metformin suppressed activation of autophagy, thereby markedly reducing the increased LC3-II level as compared with the vehicle light-treated group (Fig. 7a, e). In addition, the formation of autophagosomes in mice retina after light injury were observed by transmission electron microscope, while the formation of autophagosomes was inhibited after intraperitoneal injection of metformin(Fig. 10).

Additionally, we further assessed the retinal neuroprotection caused by AMPK activation against the light damage. We determined potential changes of rhodopsin, a specific marker of photoreceptor present on the outer layer of retina with immunostaining and western blot analysis. As shown in Fig. 11, light exposure caused a substantial reduction of rhodopsin's fluorescent intensity on the retinal outer layer and decreased the rhodopsin protein level as compared with the dark control. On the contrary, pre-administration of metformin significantly attenuated the reduction of rhodopsin, thereby sustaining the relatively normal structure in the retinal outer layer and increased the rhodopsin protein level as compared with the vehicle light-treated group. Thereafter, we quantitatively determined the mice retinal function with ERG *in vivo* as well. As shown in Fig. 12, the retinal function was severely compromised after 12 h light exposure, as evidenced by the significantly decreased a-wave and b-wave values of ERG in comparison with the vehicle control group, while pre-administration of metformin markedly attenuated the reduction of a-wave and b-wave values in comparison with the vehicle light-treated group. Thus, these results suggested that activating AMPK can produce similar AMPK-dependent inhibition of autophagy in the light-induced autophagy *in vivo*, and cause suppression of autophagy by stimulating AMPK activation which can exhibit significant neuroprotection on the retina against the light damage.

4. Discussion

Prolonged and excessive exposure to the visible light can induce retinal photochemical damage, and one of important characteristics of photochemical damage is photooxidation [37]. Through light exposure, the energy from the photons can transfer to the photosensitive molecules, such as rhodopsin or opsin in photoreceptors, which can then lead to the generation of reactive forms of oxygen, such as singlet oxygen ($^1O_2$) [3,38]. In addition, all-trans-RAL is a major intermediate of the visual cycle responsible for regenerating rhodopsin and opsin in the vertebrate retina, whereas the photooxidation of all-trans-retinal can also promote the generation of singlet oxygen ($^1O_2$), superoxide radical anions ($O_2\cdot^-$), and peroxides (ROO^-) [39]. Thus, continuous and excessive light exposure can lead to increased ROS generation and cause severe oxidative-stressed injury in photoreceptors, which can significantly compromise the structure as well as function of the important macromolecules such as the lipids, proteins, and DNA [40]. Based on our *in vitro* experiments, we found that exposure to 1800 lx light for 3 d significantly increased ROS generation in photoreceptor cells (661 W),

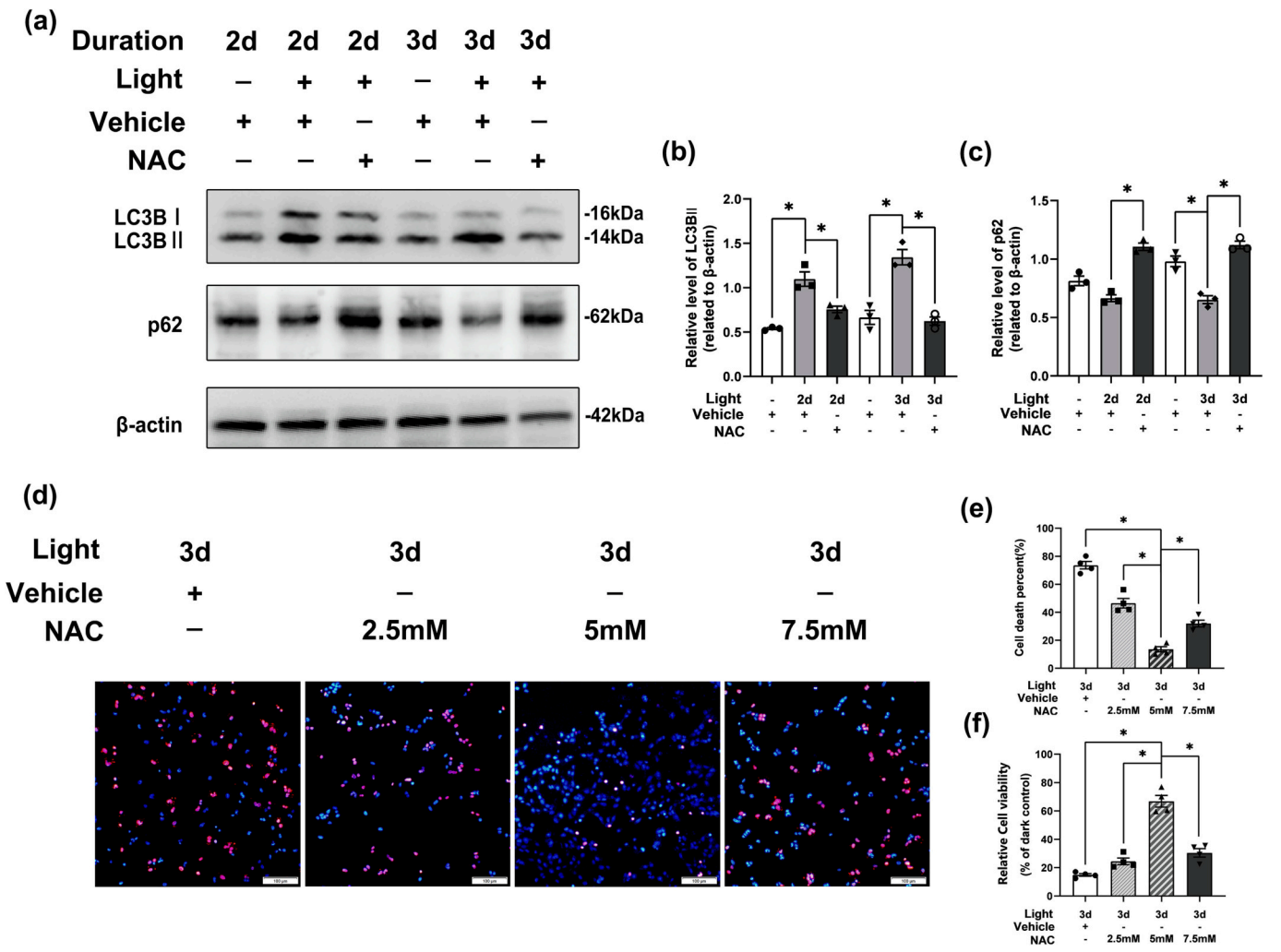


Fig. 8. NAC treatment suppressed light-induced autophagy and attenuated light-induced photoreceptor death.
(a). 661 W cells were pretreated with 5 mM NAC and cultured under the light condition for the designated times. The cells were then harvested and lysed for detecting LC3-II and p62 protein levels by western blot analysis and β-actin was used as an internal control. (b), (c). The protein levels were quantitatively analyzed and compared. (d). The cells were cultured under the light condition for 3 d in the presence of 2.5–7.5 mM NAC. The cell death was then evaluated by PI/Hoechst staining. Scale bar = 100 μm. (e). The cell death percentage was quantitatively analyzed and compared. (f). The viability of 661 W cells was examined with CCK8 assay. The results were obtained from at least three independent experiments and have been presented as means ± SEM. *$p < 0.05$. NAC: *N*-acetyl-L-cysteine; 2d, 3d: light exposure for 2 and 3 d.

decreased the ratio of GSH/GSSG, and thereby resulted in the reduced cell viability as well as increased cell death percentage. These results indicated that visible light exposure can potentially cause a severe imbalance of redox in the photoreceptor cells and excessive ROS can further trigger cell death cascade.

DCFH-DA is a common molecular probe used to detect the production of intracellular ROS. DCFH can be oxidized to the fluorescent product 2′,7′-dichlorofluorescein (DCF) by excessive ROS, which can be quantitatively evaluated with fluorescence microscope or flow cytometry. Therefore, in this study, we have detected the light-induced generation of ROS in 661 W cells with DCFH-DA probe. However, based on the findings of a previous study of Kalyanaraman *et al* [41], DCFH can be oxidized into DCF by a variety of single electron oxides, including hydroxyl radical (• OH), • NO_2, hypochlorite (HOCl), *etc.* Thus, due to the inability of H_2O_2 to oxidize DCFH to form DCF, detection of DCF fluorescence cannot be convincingly used for evaluating the level of H_2O_2. In addition, presence of various redox active metals in the model system, might also cause bias in the formation of DCF. Light exposure mainly induces photoreceptor cells to produce singlet oxygen ($^1O_2$), superoxide radical anion ($O2\cdot^-$), peroxide (ROO −), *etc.*, instead of directly producing H_2O_2, and there was no redox active metal used in our model system, so the production of intracellular ROS caused by light exposure was able to be quantitatively evaluated with DCFH-DA probe. In addition, to further evaluate the intracellular redox balance, we have also measured the intracellular GSH/GSSG levels and ratios to illustrate the redox status of photoreceptors from two different perspectives.

AMPK plays a key role in maintaining cellular metabolic homeostasis and participates in the regulation of energy metabolism by detecting changes in AMP/ATP and ADP/ATP [42]. AMPK can also regulate the production of intracellular ROS [43]. For instance, Rabinovitch et al. reported that mitochondrial ROS production was significantly increased in AMPKα-deficient cells, and AMPK activation can effectively trigger a PGC-1α-dependent antioxidant response that can substantially limit mitochondrial ROS generation [44]. Hu et al. reported that metformin treatment can substantially suppress oxidative stress by activating AMPK, as well as by reducing the generation of intracellular ROS, and thus can improve cell viability in H9C2 rat cardiomyoblast cells, where pharmacological AMPK inhibitor Compound C can increase the level of intracellular ROS and attenuate cell viability [45]. In our study, we also found that AMPK activation caused by metformin treatment can significantly suppress light-induced ROS generation, but caused an increase in the GSH/GSSG ratio, thereby restoring redox status, which was

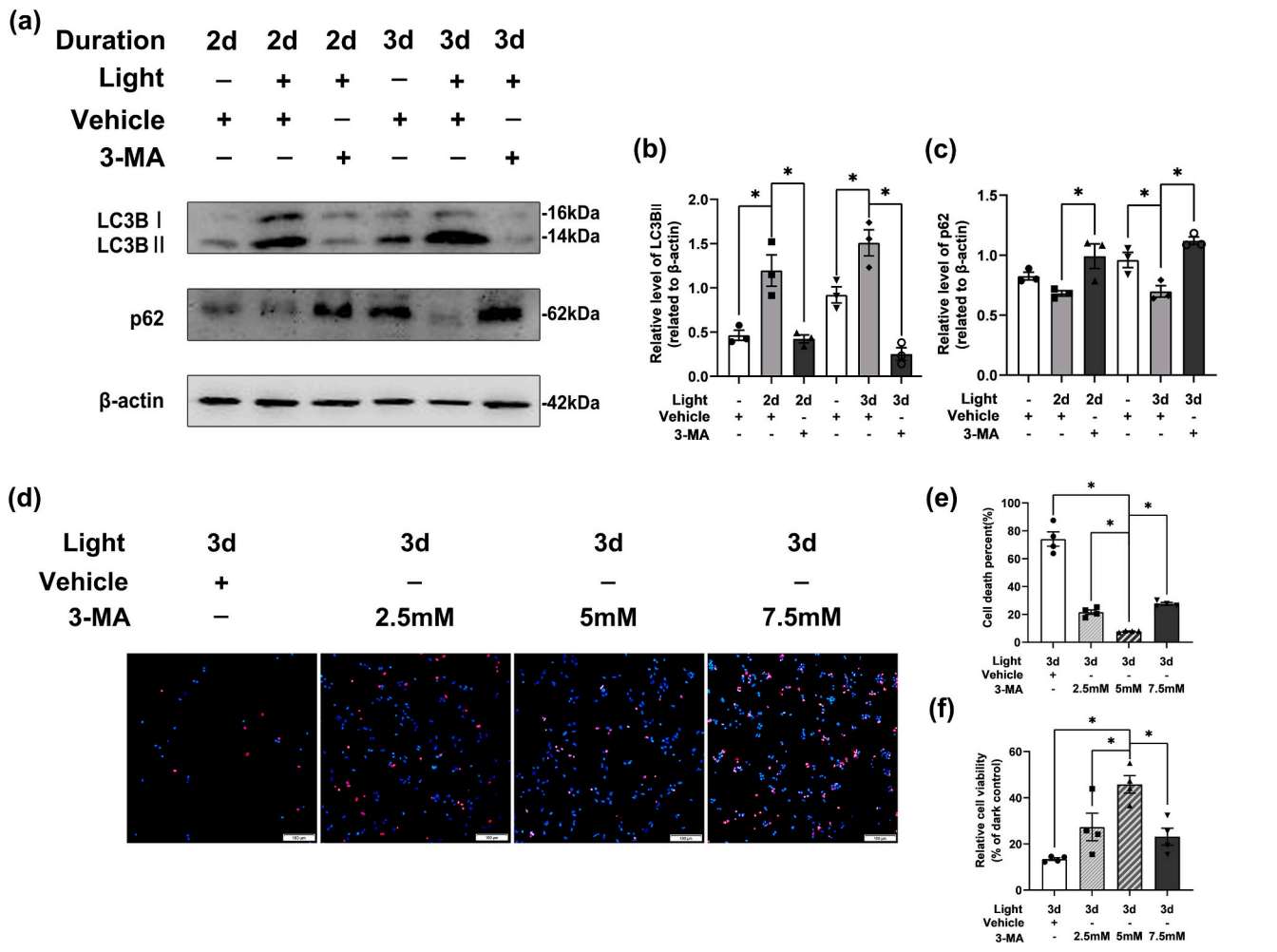


Fig. 9. 3-MA treatment attenuated light-induced photoreceptor death by suppressing the light-induced autophagy.
(a). 661 W cells were pretreated with 5 mM 3-MA and cultured under the light condition for the designated times. The cells were then harvested and lysed for detecting LC3-II and p62 protein levels by western blot analysis and β-actin was used as an internal control. (b), (c). The protein levels were quantitatively analyzed and compared. (d). The cells were cultured under the light condition for 3 d in the presence of 2.5–7.5 mM 3-MA. The cell death was then evaluated by PI/Hoechst staining. Scale bar = 100 μm. (e). The cell death percentage was quantitatively analyzed and compared. (f). The viability of 661 W cells was measured with CCK8 assay. The results were obtained from at least three independent experiments and have been presented as means ± SEM. *$p < 0.05$. 2d, 3d: light exposure for 2 and 3 d.

consistent with these previous reports.

In addition to the modulation by cellular energy and nutritional status, the activation of mTOR signaling has also been linked to the regulation of intracellular ROS. For example, Mao *et al* reported that the activation of mTOR/PI3K signal can increase ROS production, alter GSH/GSSG ratio, and influence the redox status, whereas excessive ROS level can also cause the activation of mTOR by stimulating Ras-PI3K-Akt pathway [46]. Fourcade *et al* reported that other sources of ROS, such as NADPH oxidase, can also lead to mTOR activation [47]. In the current study, we found that exposure to light can significantly lead to the activation of mTOR signal under both *in vitro* and *in vivo* settings, which was characterized by the marked up-regulation of p-mTOR /mTOR level and p-4EBP1/4EBP1 level after the light exposure.

AMPK can negatively regulate mTORC1 activation by mediating the phosphorylation of the TSC complex, but AMPK can also directly inhibit mTORC1 phosphorylation [21]. In this study, AMPK activation was observed to exert an inhibitory effect on the downstream mTOR signaling even under light condition, thus indicating that both p-mTOR /mTOR and p-4EBP1/4EBP1 protein levels were significantly reduced, which was consistent with the previously published studies. In addition, we pretreated the light-damaged photoreceptor cells (661 W) with mTOR inhibitor rapamycin, and evaluated the ROS level and GSH/GSSG ratio. The results showed that rapamycin treatment was able to significantly inhibit the upregulation of intracellular ROS caused by light exposure.

Although some studies have reported that light exposure can induce apoptosis of photoreceptors in mouse retina [48–50], we noted in our study that 7000 lx light exposure for 12 h failed to induce a significant increase in the number of TUNEL positive photoreceptors. In addition, the levels of various apoptosis markers, such as cleaved caspase 3, 8 and 9 in the retina did not change significantly (data not shown). However, we collected several crucial evidences indicating that light exposure indeed could induce the activation of autophagy under both *in vitro* and *in vivo* settings. For instance, in 661 W cells, 1800 lx light exposure for 3 days can significantly induce the typical changes of autophagy related proteins, LC3-II and p62, thereby indicating the activation of autophagy, and typical formation of autophagic bodies was observed in the cells with the transmission electron microscopy. In addition, the application of autophagy inhibitor 3-MA was found to significantly protect 661 W cells against light damage. More importantly, we also determined the changes of autophagy marker proteins, including LC3II, in the mouse retina exposed to 7000 lx light for 12 h, and it was found that AMPK activation could significantly protect the mouse retina from light damage by regulating the autophagy pathway. These *in vivo* and *in vitro*

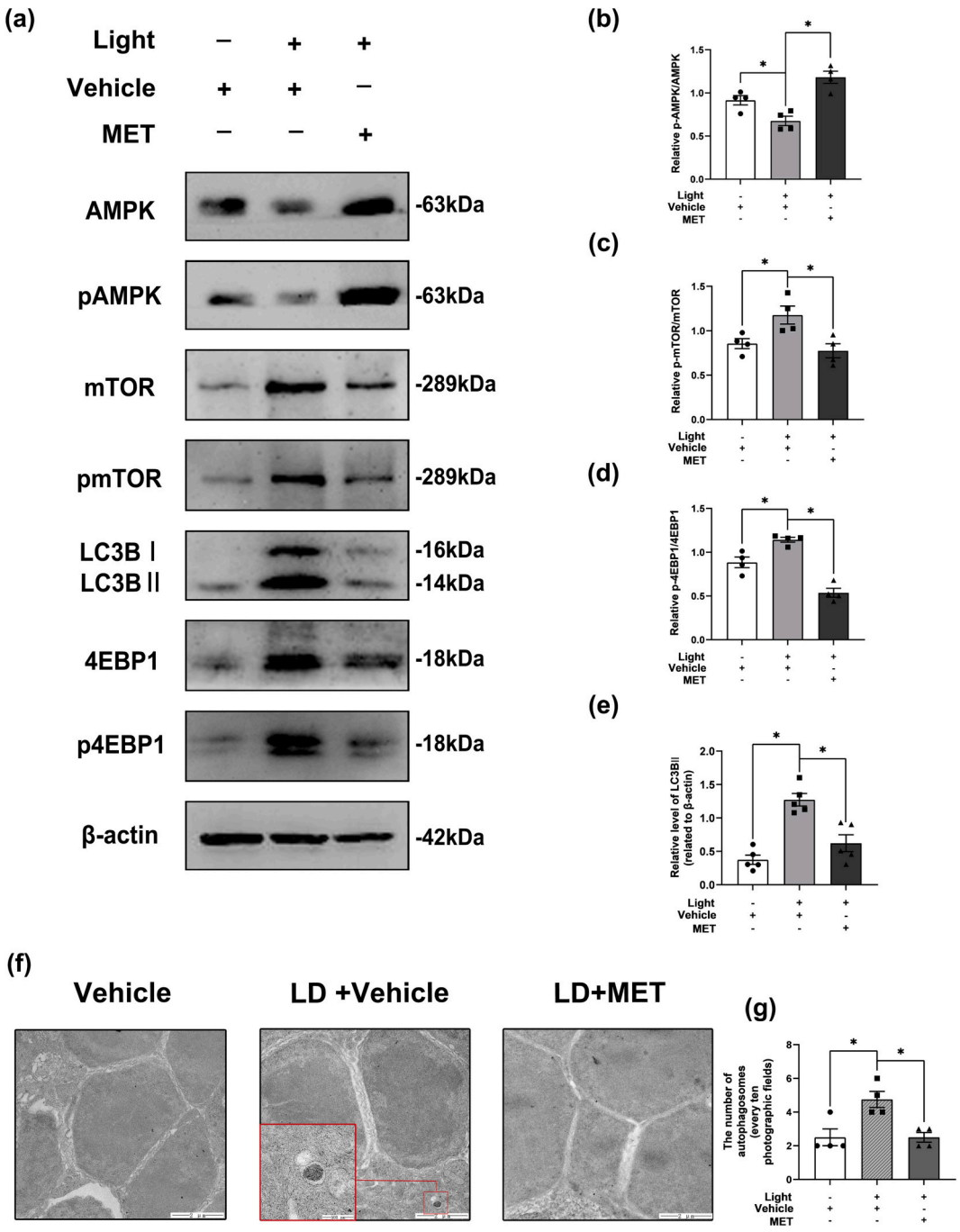


Fig. 10. Administration of metformin significantly suppressed light-induced activation of mTOR and autophagy *in vivo*
The mice were intraperitoneally injected with metformin and exposed to light conditions for 12 h. (a). The retinas were harvested and lysed for western blot analysis. The representative blots showing p-AMPK/AMPK, p-mTOR /mTOR, p-4EBP1/4EBP1 and LC3-II expression have been depicted above. β-actin was used as an internal control. (b-e). Quantitative analysis of the protein levels. (f). The outer nuclear layer of mice retina was visualized with transmission electron microscope. The autophagosome was circled in the red wireframe. Scale bar = 2 μm; 500 nm. (g). The quantitative analysis of the number of autophagosomes. Each experiment was repeated at least three times. The results have been presented as means ± SEM, *$p < 0.05$. MET: metformin; LD: light damage.

experimental evidences strongly support that excessive light exposure can induce autophagic death in photoreceptors, whereas apoptosis might not be the predominant death pattern in these cells.

In most cases, autophagy is characterized by the formation of autophagosomes [51]. Interestingly, a previous study has reported that increased autophagosome formation occurs early during the light-induced photoreceptor degeneration, since autophagy influx was observed prior to extensive loss of the photoreceptor cells after the light exposure [52]. Shimizu *et al* further reported that excessive light exposure in the retina can potentially alter the metabolic needs of the retina, thereby leading to increase in autophagy which can aid to meet the cellular energetic demands. However, over activation of autophagy can result in autophagic programmed cell death, which might act as a potential secondary event following the light damage [53]. Bogéa *et al* also demonstrated that the light exposure was able to trigger both inner and outer segment membrane defects in the rod photoreceptors expressing P23H rhodopsin, thus resulting in retinal degeneration occurring *via* stimulation of autophagic pathway [54].

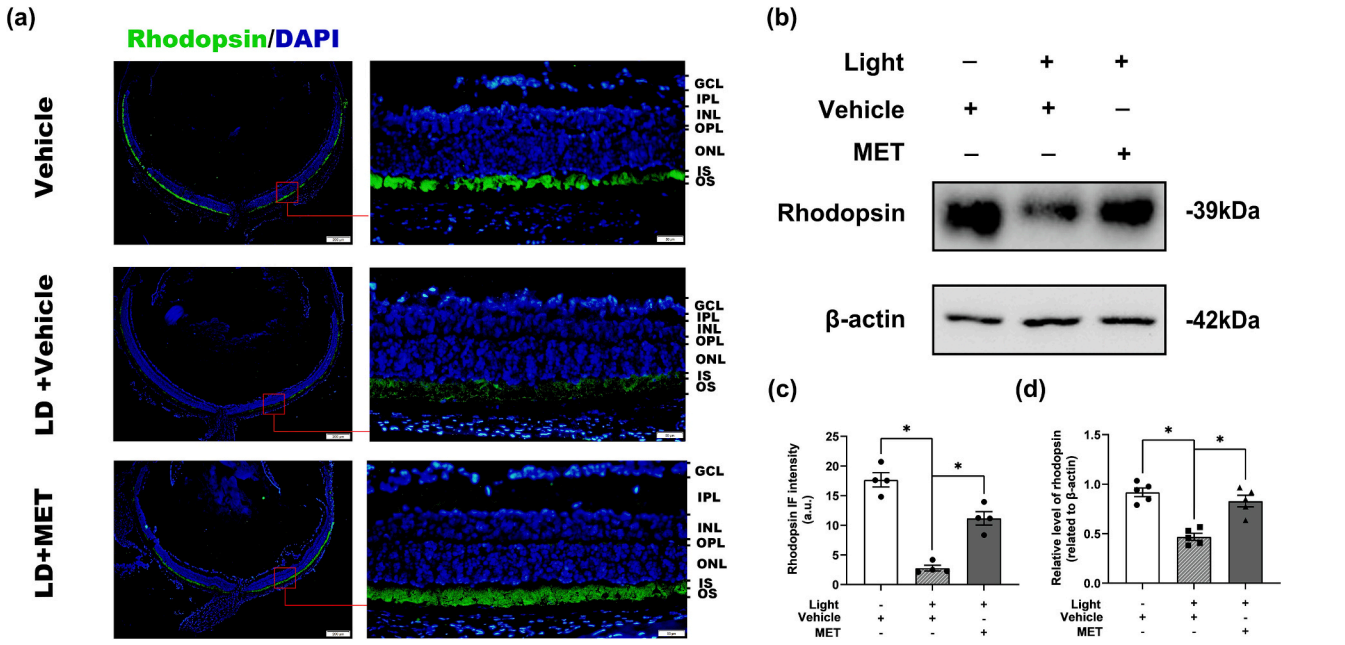


Fig. 11. Administration of metformin maintained the protein level of rhodopsin.
The mice were intraperitoneally injected with metformin and exposed to the light conditions for 12 h. (a). The retinal frozen sections were immunostained for detecting the level of rhodopsin. Scale bar = 200 μm; 50 μm. (b). The retinas were harvested and lysed for the western blot assay and β-actin was used as an internal control. (c). The immunofluorescence intensity of rhodopsin was then quantitatively analyzed. (d). The protein level of rhodopsin was quantitatively analyzed. Each experiment was repeated at least 3 times. The results have been presented as means ± SEM, *p < 0.05. MET: metformin; LD: light damage.

mTOR signaling plays a crucial role in the regulation of autophagy [15]. In most cases, it has been reported that mTORC1 activation can suppress autophagy by phosphorylating the various components such as ATG13 and ULK1, but inhibiting mTOR with rapamycin can also promote autophagy [15,18,55,56]. However, in the current study, we discovered that inhibiting mTOR with rapamycin exhibited inhibitory regulation on the process of autophagy under the light condition. In addition, mTOR inhibition exhibited significant protection on the photoreceptor cells against the light damage. Interestingly, a few previous studies have also shown that in the case of long-term starvation, the regulation of mTOR on autophagy can be effectively altered from inhibition to promotion [57]. During the process of autophagy, autolysosomes can effectively degrade the contents to regenerate the various nutrients such as carbohydrates, amino acids and ATP, which can cause activation of mTOR, yet mTOR activation may stimulate the recycling of the different pro-lysosomal membrane components, followed by the vesicle maturation into new lysosomes [57]. mTORC1 reactivation is required in the later phase of autophagy to recycle the lysosomes, thereby indicating that mTORC1 activation plays a positive regulatory role in autophagy. Therefore, under these conditions, inhibition of mTOR might exhibit an inhibitory effect on autophagy, which was consistent with our results.

Our results indicated that the treatment with mTOR inhibitor significantly inhibited the activation of autophagy and reduced the level of intracellular ROS. To further illustrate the possible connection between these two observations, we treated light-exposed 661 W cells with antioxidant NAC. The results showed that NAC treatment was able to significantly reduce the level of intracellular ROS and inhibit light-induced activation of autophagy. Consistently, many previous studies have reported that ROS can regulate activation of autophagy through diverse signaling pathways, such as ROS-FOXO3-LC3/BNIP3, ROS-NRF2-P62, ROS-HIF1-BNIP3/NIX, and ROS-TIGAR pathway [58,59]. These studies indicate that the level of intracellular ROS could influence the activation of autophagy, which might also be one of the major mechanisms that can negatively regulate the activation of autophagy upon mTOR inhibition.

The similar inhibition of autophagy was also manifested by AMPK activation in this study. A number of previous studies have reported that activation of AMPK can suppress mTOR signal to promote autophagy [60,61]. However, we found that AMPK activation can indeed lead to the inhibition of mTOR under the light conditions, but it exerted an inhibitory effect rather than promoting autophagy, which was similar as observed with the direct inhibition of mTOR. Furthermore, AMPK activation also displayed a significant protective effect on photoreceptor cells against the light damage under both *in vitro* and *in vivo* conditions. In our model, the AMPK-dependent inhibition of autophagy might be due to inhibiting mTOR signaling. However, some previous studies have also indicated that modulation of several other signaling pathways might be involved in AMPK activation-induced autophagy inhibition, such as suppression of NF-κB signaling, phosphorylation of ULK1 or inhibition of ER stress signals [22,62,63]. The occurrence of AMPK-dependent inhibition of autophagy also depends on the specific type of stimuli or injury, and has been closely related to the energy status of the cells. However, the detailed molecular mechanism remains to be further deciphered. The mutual crosstalk between AMPK/mTOR pathway and oxidative stress is a complex process, involving multiple signaling pathways, which have not been explored in detail, so further experimental studies are still needed. In addition, although it was found in this study that inhibition of mTOR can inhibit autophagy in photoreceptor light injury, more in-depth research is still needed, such as verifying the positive regulation of mTOR on autophagy in photoreceptor light injury again by using gene knockdown technology.

In retinal degeneration induced by excessive light exposure, the retinal pigment epithelium (RPE) is also severely damaged in addition to the loss of the photoreceptor cells. Interestingly, a number of previous studies have reported that excessive light exposure can lead to significantly elevated level of oxidative stress in RPE cells, which can lead to the dysfunction of RPE and even trigger death of RPE [64]. Xu et al. demonstrated that metformin treatment significantly induced the activation of AMPK in RPE, which markedly attenuated sodium iodate-induced acute oxidative damage [1]. RPE plays a crucial role in regulating the energy supply of the photoreceptor cells. RPE is responsible

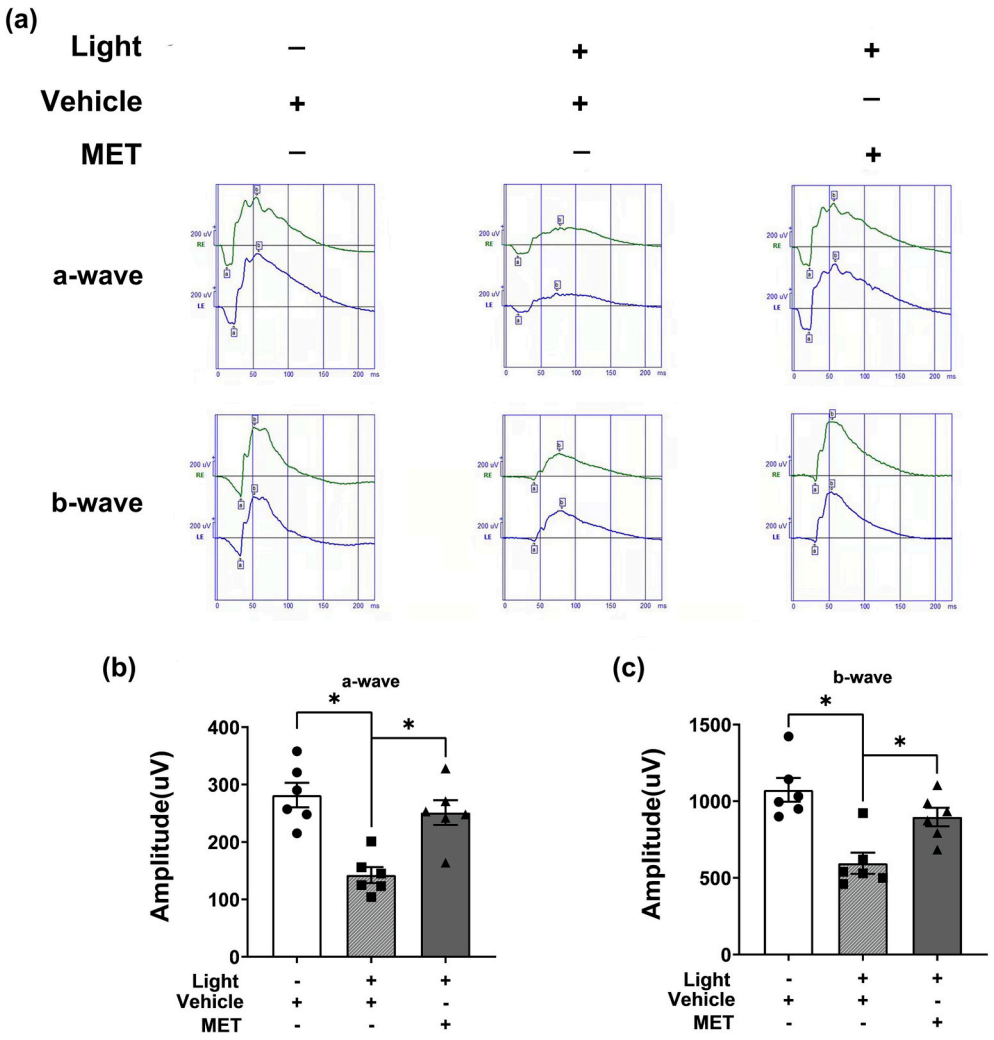


Fig. 12. Administration of metformin attenuated light-induced damage in the retina.
Mice were intraperitoneally injected with metformin or vehicle and exposed to 7000 Lux light for 12 h. Thereafter, at 7 d after the light exposure, the mice were anesthetized and the retinal function was evaluated with ERG. (a). ERG waves. (b), (c). The a-wave and b-wave values were statistically analyzed. Each experiment was repeated at least 3 times. The results have been presented as means ± SEM, NS: no statistical significance, ***$p < 0.001$.

for transporting glucose from the choroidal blood to the photoreceptor, but the lactate produced in the process of aerobic glycolysis from the photoreceptor can effectively inhibit the utilization of glucose by RPE, which can significantly increase the transportation of glucose from RPE to photoreceptor [65]. In addition, prior studies have also shown that increased mitochondrial oxidative stress can lead to the dysfunction of RPE, reduce energy supply and trigger the degeneration of the photoreceptors [66]. In conclusion, we speculate that excessive light exposure can affect glucose transport of RPE through photooxidation-induced increased level of oxidative stress, which can result in the lack of energy supply in photoreceptor, whereas the activation of AMPK could suppress oxidative-stressed injury in RPE, thus improving the glucose transport, and playing an indirect protective role in light-damaged photoreceptors.

5. Conclusion

We report that autophagy exerts a significant damaging effect on the photoreceptor cells in the photooxidation-injured model. It was observed that indirectly inhibiting autophagy by AMPK activation/mTOR inhibition, or directly blocking autophagy with an inhibitor can play a significant neuroprotective role against the light damage. Retinal light damage has been closely related to the progression of various retinal diseases. The AMPK-dependent inhibition of autophagy reported by us in this study can play a substantial protective role in photoreceptors against the light injury, which might assist to further develop targeted- neuroprotective drugs.

Ethical Approval

Mice were maintained in accordance with facility guidelines on animal welfare and with protocols approved by the Animal Experimentation Ethics Committee of Jilin University.

Funding

The study was funded by the National Natural Science Foundation of China (No. 82171053, 81570864), and the Natural Science Foundation of Jilin Province (No. 20200801043GH; No. 20190201083JC). The funders had no role in study design, data collection, analysis, decision to publish, or manuscript preparation.

CRediT authorship contribution statement

Yu-Lin Li: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Tian-Zi Zhang:** Methodology, Investigation, Writing – review & editing. **Li-Kun Han:** Methodology, Writing – review & editing. **Chang He:** Methodology, Writing – review & editing. **Yi-Ran Pan:** Investigation, Writing – review & editing. **Bin Fan:** Conceptualization, Investigation, Writing – review & editing, Supervision. **Guang-Yu Li:** Conceptualization, Writing – original draft, Writing – review & editing, Funding acquisition, Resources, Supervision.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

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

Acknowledgment

We would like to thank LetPub (www.letpub.com) for providing linguistic assistance during the preparation of this manuscript.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jphotobiol.2023.112735.

References

[1] L. Xu, et al., Stimulation of AMPK prevents degeneration of photoreceptors and the retinal pigment epithelium, Proc. Natl. Acad. Sci. U. S. A. 115 (41) (2018) 10475–10480.
[2] B.E. Uygun, N. Sharma, M. Yarmush, Retinal pigment epithelium differentiation of stem cells: current status and challenges, Crit. Rev. Biomed. Eng. 37 (0) (2009) 355–375.
[3] Y. Chen, L. Perusek, A. Maeda, Autophagy in light-induced retinal damage, Exp. Eye Res. 144 (2016) 64–72.
[4] P.N. Youssef, N. Sheibani, D.M. Albert, Retinal light toxicity, Eye 25 (1) (2011) 1–14.
[5] Y. Lin, et al., Retinoid receptors trigger neuritogenesis in retinal degenerations, FASEB J. 26 (1) (2012) 81–92.
[6] B. Cai, et al., Gasdermin E mediates photoreceptor damage by all-trans-retinal in the mouse retina, J. Biol. Chem. 298 (2) (2022).
[7] M.T. Massengill, et al., Sectoral activation of glia in an inducible mouse model of autosomal dominant retinitis pigmentosa, Sci. Rep. 10 (1) (2020).
[8] Y. Kanagasingam, et al., Progress on retinal image analysis for age related macular degeneration, Prog. Retin. Eye Res. 38 (2014) 20–42.
[9] K. Ojino, et al., Protective effect of SUN N8075, a free radical scavenger, against excessive light-induced retinal damage in mice, Biol. Pharm. Bull. 37 (3) (2014) 424–430.
[10] D.G. Hardie, AMP-activated protein kinase—an energy sensor that regulates all aspects of cell function, Genes Dev. 25 (18) (2011) 1895–1908.
[11] B. Gabryel, et al., AMP-activated protein kinase is involved in induction of protective autophagy in astrocytes exposed to oxygen-glucose deprivation, Cell Biol. Int. 38 (10) (2014) 1086–1097.
[12] D.B. Shackelford, R.J. Shaw, The LKB1-AMPK pathway: metabolism and growth control in tumor suppression, Nat. Rev. Cancer 9 (8) (2009) 563–575.
[13] K. Inoki, J. Kim, K.L. Guan, AMPK and mTOR in cellular energy homeostasis and drug targets, in: P.A. Insel, S.G. Amara, T.F. Blaschke (Eds.), Annual Review of Pharmacology and Toxicology vol. 52, 2012, pp. 381–400.
[14] D. Liko, M.N. Hall, mTOR in health and in sickness, J. Mol. Med. 93 (10) (2015) 1061–1073.
[15] Y.C. Kim, K.L. Guan, mTOR: a pharmacologic target for autophagy regulation, J. Clin. Investig. 125 (1) (2015) 25–32.
[16] M.M. Mihaylova, R.J. Shaw, The AMPK signalling pathway coordinates cell growth, autophagy and metabolism, Nat. Cell Biol. 13 (9) (2011) 1016–1023.
[17] T.J. Mercer, A. Gubas, S.A. Tooze, A molecular perspective of mammalian autophagosome biogenesis, J. Biol. Chem. 293 (15) (2018) 5386–5395.
[18] N. Hosokawa, et al., Nutrient-dependent mTORC1 association with the ULK1-Atg13-FIP200 complex required for autophagy, Mol. Biol. Cell 20 (7) (2009) 1981–1991.
[19] L.Q. Hong-Brown, et al., FoxO1-AMPK-ULK1 regulates ethanol-induced autophagy in muscle by enhanced ATG14 association with the BECN1-PIK3C3 complex, Alcohol. Clin. Exp. Res. 41 (5) (2017) 895–910.
[20] M.A. Zimmerman, C.D. Biggers, P.A. Li, Rapamycin treatment increases hippocampal cell viability in an mTOR-independent manner during exposure to hypoxia mimetic, cobalt chloride, BMC Neurosci. 19 (2018) 82.
[21] R.C. Russell, H.X. Yuan, K.L. Guan, Autophagy regulation by nutrient signaling, Cell Res. 24 (1) (2014) 42–57.
[22] G.L. Lu, et al., The effects of metformin on autophagy, Biomed. Pharmacother. 137 (2021) 111286.
[23] C.H. Jung, et al., mTOR regulation of autophagy, FEBS Lett. 584 (7) (2010) 1287–1295.
[24] M.R. Al-Ubaidi, et al., Bilateral retinal and brain tumors in transgenic mice expressing simian virus 40 large T antigen under control of the human interphotoreceptor retinoid-binding protein promoter, J. Cell Biol. 119 (6) (1992) 1681–1687.
[25] E. Tan, et al., Expression of cone-photoreceptor-specific antigens in a cell line derived from retinal tumors in transgenic mice, Invest. Ophthalmol. Vis. Sci. 45 (3) (2004) 764–768.
[26] J.Y. Song, et al., Suppressing endoplasmic reticulum stress-related autophagy attenuates retinal light injury, Aging-Us 12 (16) (2020) 16579–16596.
[27] G.-Y. Li, B. Fan, Y.-Y. Jiao, Rapamycin attenuates visible light-induced injury in retinal photoreceptor cells via inhibiting endoplasmic reticulum stress, Brain Res. 1563 (2014) 1–12.
[28] Y.R. Pan, et al., mTOR may interact with PARP-1 to regulate visible light-induced parthanatos in photoreceptors, Cell Commun. Signal 18 (1) (2020) 27.
[29] B.L. Yuan, et al., QNAR modeling of cytotoxicity of mixing nano-TiO2 and heavy metals, Ecotoxicol. Environ. Saf. 208 (2021) 111634.
[30] Y.-N. Hu, et al., Characterization and antioxidant activities of yellow strain Flammulina velutipes (Jinhua mushroom) polysaccharides and their effects on ROS content in L929 cell, Antioxidants 8 (8) (2019) 298.
[31] C.-B. Hu, et al., NDRG2 suppression as a molecular hallmark of photoreceptor-specific cell death in the mouse retina, Cell Death Dis. (2018) 4.
[32] F. Hafezi, et al., Differential DNA binding activities of the transcription factors AP-1 and Oct-1 during light-induced apoptosis of photoreceptors, Vis. Res. 39 (15) (1999) 2511–2518.
[33] M. Tanito, et al., Attenuation of retinal photooxidative damage in thioredoxin transgenic mice, Neurosci. Lett. 326 (2) (2002) 142–146.
[34] S. Yang, et al., Chemical and material communication between the optic nerves in rats, Clin. Exp. Ophthalmol. 43 (8) (2015) 742–748.
[35] H. Dong, et al., Metformin enhances the chemosensitivity of hepatocarcinoma cells to cisplatin through AMPK pathway, Oncol. Lett. 14 (6) (2017) 7807–7812.
[36] Y. Yi, et al., Metformin sensitizes leukemia cells to vincristine via activation of AMP-activated protein kinase, J. Cancer 8 (13) (2017) 2636–2642.
[37] J.J. Hunter, et al., The susceptibility of the retina to photochemical damage from visible light, Prog. Retin. Eye Res. 31 (1) (2012) 28–42.
[38] M. Delmelle, Retinal damage by light: possible implication of singlet oxygen, Biophys. Struct. Mech. 3 (2) (1977) 195–198.
[39] M. Różanowska, T. Sarna, Light-induced damage to the retina: role of rhodopsin chromophore revisited, Photochem. Photobiol. 81 (6) (2007) 1305–1330.
[40] L.E. Lamb, et al., Retinyl palmitate and the blue-light-induced phototoxicity of human ocular lipofuscin, Arch. Biochem. Biophys. 393 (2) (2001) 316–320.
[41] B. Kalyanaraman, et al., Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations, Free Radic. Biol. Med. 52 (1) (2012) 1–6.
[42] D.G. Hardie, F.A. Ross, S.A. Hawley, AMPK: a nutrient and energy sensor that maintains energy homeostasis, Nat. Rev. Mol. Cell Biol. 13 (4) (2012) 251–262.
[43] Y. Zhao, et al., ROS signaling under metabolic stress: cross-talk between AMPK and AKT pathway, Mol. Cancer 16 (2017) 79.
[44] R.C. Rabinovitch, et al., AMPK maintains cellular metabolic homeostasis through regulation of mitochondrial reactive oxygen species, Cell Rep. 21 (1) (2017) 1–9.
[45] M.Y. Hu, et al., Metformin protects H9C2 cardiomyocytes from high-glucose and hypoxia/reoxygenation injury via inhibition of reactive oxygen species generation and inflammatory responses: role of AMPK and JNK, J. Diabetes Res. 2016 (2016) (2016) 2961954.
[46] Z.L. Mao, et al., The mTOR/GCLc/GSH pathway mediates the dose-dependent bidirectional regulation of ROS induced by TiO2 NPs in neurogenic cells, Oxidative Med. Cell. Longev. 2019 (2019) 7621561.
[47] S. Fourcade, I. Ferrer, A. Pujol, Oxidative stress, mitochondrial and proteostasis malfunction in adrenoleukodystrophy: a paradigm for axonal degeneration, Free Radic. Biol. Med. 88 (2015) 18–29.
[48] K. Kunchithapautham, B. Rohrer, Apoptosis and autophagy in photoreceptors exposed to oxidative stress, Autophagy 3 (5) (2007) 433–441.
[49] P. Hahn, et al., Deficiency of Bax and Bak protects photoreceptors from light damage in vivo, Cell Death Differ. 11 (11) (2004) 1192–1197.
[50] S. Li, et al., A comparison of continuous versus intermittent light exposure on apoptosis, Curr. Eye Res. 15 (9) (1996) 914–922.
[51] A.R. Ariosa, D.J. Klionsky, Autophagy core machinery: overcoming spatial barriers in neurons, J. Mol. Med. 94 (11) (2016) 1217–1227.
[52] Y. Chen, et al., Autophagy protects the retina from light-induced degeneration, J. Biol. Chem. 288 (11) (2013) 7506–7518.
[53] S. Shimizu, et al., Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes, Nat. Cell Biol. 6 (2004) 1221.
[54] T.H. Bogéa, R.H. Wen, O.L. Moritz, Light induces ultrastructural changes in rod outer and inner segments, including autophagy, in a transgenic Xenopus laevis P23H rhodopsin model of retinitis Pigmentosa, Investig. Ophthalmol. Vis. Sci. 56 (13) (2015) 7947–7955.
[55] I.G. Ganley, et al., ULK1 center dot ATG13 center dot FIP200 complex mediates mTOR signaling and is essential for autophagy, J. Biol. Chem. 284 (18) (2009) 12297–12305.
[56] C.H. Jung, et al., ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery, Mol. Biol. Cell 20 (7) (2009) 1992–2003.
[57] L. Yu, et al., Termination of autophagy and reformation of lysosomes regulated by mTOR, Nature 465 (7300) (2010) 942–U11.
[58] L. Li, et al., ROS and autophagy: interactions and molecular regulatory mechanisms, Cell. Mol. Neurobiol. 35 (5) (2015) 615–621.
[59] L. Kma, T.J. Baruah, The interplay of ROS and the PI3K/Akt pathway in autophagy regulation, Biotechnol. Appl. Biochem. 69 (1) (2022) 248–264.
[60] J. Kim, et al., AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1, Nat. Cell Biol. 13 (2) (2011) 132–U71.

[61] Y. Wang, et al., Metformin induces autophagy and G0/G1 phase cell cycle arrest in myeloma by targeting the AMPK/mTORC1 and mTORC2 pathways, J. Exp. Clin. Cancer Res. 37 (2018) 63.
[62] C. Nwadike, et al., AMPK inhibits ULK1-dependent autophagosome formation and lysosomal acidification via distinct mechanisms, Mol. Cell. Biol. 38 (10) (2018) p. e00023–18.
[63] C. Zhai, et al., Activation of AMPK prevents monocrotaline-induced pulmonary arterial hypertension by suppression of NF-kappa B-mediated autophagy activation, Life Sci. 208 (2018) 87–95.
[64] Y. Liu, et al., Blueberry anthocyanins: protection against ageing and light-induced damage in retinal pigment epithelial cells, Br. J. Nutr. 108 (1) (2012) 16–27.
[65] M.A. Kanow, et al., Biochemical adaptations of the retina and retinal pigment epithelium support a metabolic ecosystem in the vertebrate eye, Elife (2017) 6.
[66] E.E. Brown, et al., Mitochondrial oxidative stress in the retinal pigment epithelium (RPE) led to metabolic dysfunction in both the RPE and retinal photoreceptors, Redox Biol. (2019) 24.