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Suppressing autophagy protects photoreceptor cells from light-induced injury

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ABSTRACT

Autophagy, a conserved cellular self-degradation process, not only serves to protect cells at critical times during development and nutrient stress, but also contributes to cell death. Photoreceptor cells are unique neurons which when directly exposed to the light, transduces light stimuli into visual signal. However, intense light exposure can be cytotoxic to the retina. So far, the precise mechanism underlying retina light injury remains unknown, and the effective therapy is still unavailable. Here, we found that visible light exposure activated the mitogen-activated protein kinases (MAPK) pathway and led to remarkable autophagy in photoreceptor cells (661W cells). Directly blocking autophagy with 3MA or LY294002 markedly attenuated light-induced death in 661W cells. Among the activated downstream factors of MAPK pathway, ERK, not JNK or p-38, played a critical role in light-induced death mechanism. Inhibiting the activation of ERK with its specific inhibitor PD98059 significantly suppressed light-induced autophagy and protected 661W cells from light injury. These results indicate that autophagy is an essential event in light-induced photoreceptor death and that directly blocking autophagy or suppressing autophagy by inhibiting the ERK pathway could effectively attenuates light-induced damage. These observations may have a potential application in the treatment of retinal light injury.

1. Introduction

Retinal dystrophy diseases which includes retinitis pigmentosa, age-related macular degeneration, and Stargardt's or Best's disease, often lead to blindness and to photoreceptor cell and RPE cell death [1]. Though environmental insult, age and genetic predisposition are the main etiologic factors of the diseases, light injury also likely plays an important role in the initiation and progression of retinal degeneration [2]. Light comes through the eye and reaches the retina, wherein it strikes the rod and cone photoreceptor cells. This will then lead to activation of a cascade of chemical and electrical events that will ultimately trigger nerve impulses, forming the visual activity. Although the eye has adapted several mechanisms to protect the retina from potential light-induced injury, in most species, prolonged intense visible light exposure can lead to photoreceptor cell damage. Even low density light could cause retinal injury under certain conditions. In nocturnal animals, the light intensity required for visual cell damage need be only 2–3 times above normal room lighting [3]. Retinal light damage secondary to the procedures such as ophthalmoscopic examination or intraocular surgery has also been known and well documented both in the clinical and basic science literature [4]. With the wide application of new devices in the field of ophthalmology, light-induced retinal injury occurs more often, yet the exact molecular mechanism of retinal light damage still eludes a thorough understanding.

Although the light-induced apoptotic pathway underlying retina degeneration has been well investigated at the cellular and molecular levels by using *in vivo* and *in vitro* models, the possible role of autophagy in the degeneration process of the photoreceptor cell has yet to be fully explored [5]. Autophagy, an evolutionarily conserved process for cell survival under nutrient starvation and other stress conditions, involves degradation of lipids and proteins to generate free fatty acids and amino acids which are then reutilized by the cell to maintain mitochondrial ATP energy production and synthesis of essential proteins [6]. In the retina or other neural system, autophagy has been described to be involved in both the developing and degeneration process [7]. The induction of autophagy serves as an early stress response in axonal dystrophy and may participate in the remodeling of axon structures, while autophagic degradation of opsin or rhodopsin may be a mechanism

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of adaptation to increase illumination by removing outer segments visual pigment [8]. However, although autophagy can maintain cell survival under various stress conditions, excessive or dysfunctional autophagy in various diseases such as cancer and degenerative processes may itself contribute to cell death [9].

To be able to investigate light-induced autophagy process, we used 661W cells, an *in vitro* model mimicking photoreceptor cells. This cone-like cell line derived from mouse photoreceptor cells transfected with T virus, expresses several markers of photoreceptor cells such as cone opsins, transducin, and cone arrestin. Like normal retinal photoreceptor cells, these cells are sensitive to photo-oxidative stress. Most importantly, this cell line has been widely used in the research of retinal degeneration, retinal neuroprotection and retinal regeneration [10,11]. In this study, we investigated the molecular mechanism of light-induced damage in photoreceptors using 661W cells, focusing particularly on the possible role of autophagy on the light-induced photoreceptor cell death. Our results revealed that the exposure of 2600 Lux light leads to remarkable autophagy in 661W cells and that inhibition of autophagy or ERK activation to suppress autophagy could effectively attenuate light-induced cell death.

2. Materials and methods

2.1. Chemicals and reagents

Cell culture media and additives were from Invitrogen (Beijing, China). Plastic cultureware was from DingGuo BioTech (Beijing, China). The rabbit monoclonal antibodies LC3A (No.21402), p-ERK (No.12082), ERK (No.11232), p-JNK(No.11504), JNK (No.11223), p38(No.21053) and p-p38 (No.12038) were purchased from SAB Biosciences (ShangHai, China). The rabbit β-actin monoclonal antibody (MAB1501) was obtained from Chemicon (Watford, UK). Lyso Tracker was purchased from Beyotime (Hangzhou, China). Reagents for secondary staining such as anti-rabbit IgG, anti-mouse IgG and all other reagents were purchased from Sigma-Aldrich (Shanghai, China).

2.2. Cell culture

The 661W cell line was a gift from Dr. Muayyad R. Al-Ubaidi (University of Oklahoma Health Sciences Center, USA). These cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Beijing, China) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin, and 100 mg/ml of streptomycin in a humidified atmosphere of 95% air and 5% CO^2 at 37 °C. These cells have a doubling time of about 20 h under these conditions, and were generally passaged by trypsinization at a ratio of 1:6 every 3–4 d.

2.3. Visual light exposure

The light procedure was carried out as described previously [12]. A regular cell culture incubator was equipped with two 8 W strip-lights that were completely covered with 2C UV filters that excluded light with wavelengths below 400 nm and above 800 nm, to be able to expose cells to the visible spectrum (from 400 to 760 nm). The light source was placed about 20 cm directly over a tray with 6-, 24-, or 96-well plates in which the cells were growing. The intensity of the light directed onto the 661W cells was determined by using a digital lux meter (LX-101; Lutron Electronic, London, UK). To control darkness, a paper box was placed in the same incubator to create a dark chamber. In this system, the 661W cells maintained at either light or dark condition are equally exposed to any slight increase in the temperature of the incubator caused by the constant light source. The temperature of the media under the dark and light conditions was checked over a 1- to 3-d period, and no substantial difference was found.

2.4. Propidium iodide staining

The cells were first cultured in a 24-well plate for 24 h. After the light exposure, the cells were then treated with the propidium iodide (PI) solution at a final concentration of 2 μg/ml and incubated in the dark for 10 min at room temperature. The PI-positive cells were scored using an inverted fluorescence microscope (Leica; Berlin, Germany). Quantitative assessment of cell death percentage (the number of PI positive cells/the number of total cells %) was performed through PI staining.

2.5. Transmission electron microscopy

We evaluated 661W cells for morphological changes associated with autophagy using transmission electron microscopy. After removing the culture medium and briefly rinsing with PBS, the cells (5×10^7) were collected by centrifugation at 600×*g* and 4 °C for 5 min, and then fixed with 2.5% glutaraldehyde by mixing an equal volume of fixative with the cell suspension at 37 °C. The cells were kept at room temperature for 5 min and then incubated for an additional 30 min on ice. Fixed cells were then washed three times for 3 min each with ice-cold distilled water, and cells were stained overnight with 2% uranyl acetate at 4 °C. These were then dehydrated in graded ethanol baths and embedded in Durcupan resin. Ultrathin (70 nm) sections were post-stained with uranyl acetate and lead salts and evaluated by transmission electron microscopy.

2.6. Measurement of lysosomes with LysoTracker

Cells were grown on 30 mm dish and incubated for 2 d under the dark or at 2600 Lux light condition. A 1-mM stock solution of LysoTracker Green DND-26 (Molecular Probes; Life Technologies) was diluted to 1 μM working solution with warm culture media. Cells were incubated for 1.5 h in the dark with LysoTracker working solution at 37 °C. These were then rinsed with 1× Tris-buffered saline (TBS) and observed using a laser confocal microscope (Olympus, Tokyo, Japan), and the intensity of fluorescence was analyzed with SPOT imaging software (v 4.7.0.35; SPOT Imaging Solutions, Sterling Heights, MI, USA).

2.7. Western blotting

The cultured 661W cells were scraped, and the cell pellet was sonicated in protein lysate buffer. The bicinchoninic acid assay method was used to estimate the protein level. An equal amount (20 μg) of cell lysate was dissolved in sample buffer, and the samples were boiled for 3 min. Electrophoresis was performed as previously reported using 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate [13]. Proteins were transferred to nitrocellulose membranes, and the blots were incubated for 3 h at room temperature with primary antibodies. The blots were then incubated with the appropriate biotinylated secondary antibodies. The signals were developed using the enhanced chemiluminescence western blotting detection reagent (Amersham Biosciences, Piscataway, NJ, USA) and exposed to X-ray film. Densitometry analysis was performed with Quantity One software (Bio-Rad Laboratories, ShangHai, China).

2.8. Statistical analysis

Each experiment was repeated at least three times. Data are expressed as mean ± S.E.M. Differences between means were

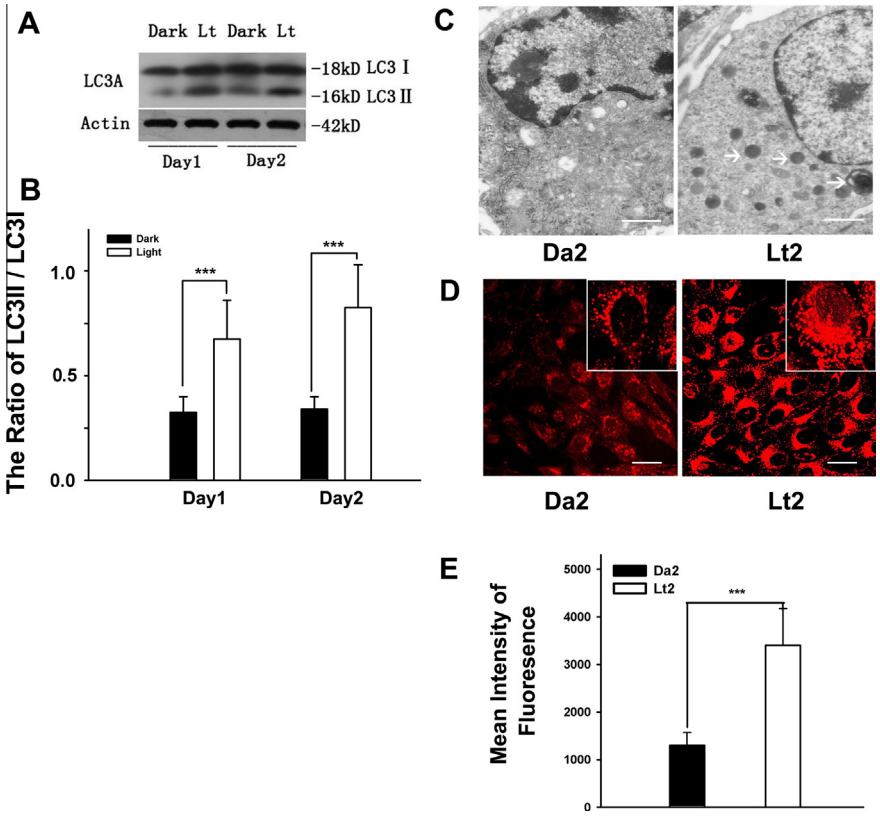

Fig. 1. Light exposure induces autophagy in 661W cells. (A) 661W cells were cultured at either under the dark condition or with 2600 Lux visible light for 1 or 2 d. The level of the autophagic marker LC3II and LC3I in cell lysates was examined by western blot. Actin protein was used as a loading control. (B) The bands on the western blots were scanned, and the intensity was determined by optical density measurements and the ratio of LC3II/LC3I was calculated. (C) The ultrastructure of the cells was analyzed by transmission electron microscopy (scale bar = 10 μm). In the light-exposed group, the numerous autophalysosomes were observed and indicated with white arrows. (D) Intracellular lysosomes were assessed by Lyso Tracker staining. 661W cells cultured either under the dark condition or with 2600 Lux visible light for 2 d were stained with Lyso Tracker and then were examined under confocal laser microscope. Scale bar = 50 μm. (E) The intensity of fluorescence was analyzed with SPOT imaging software. The results are presented as means ± S.E.M., where $n = 3$ ($^{***}P < 0.001$, one-way ANOVA and Bonferroni test).

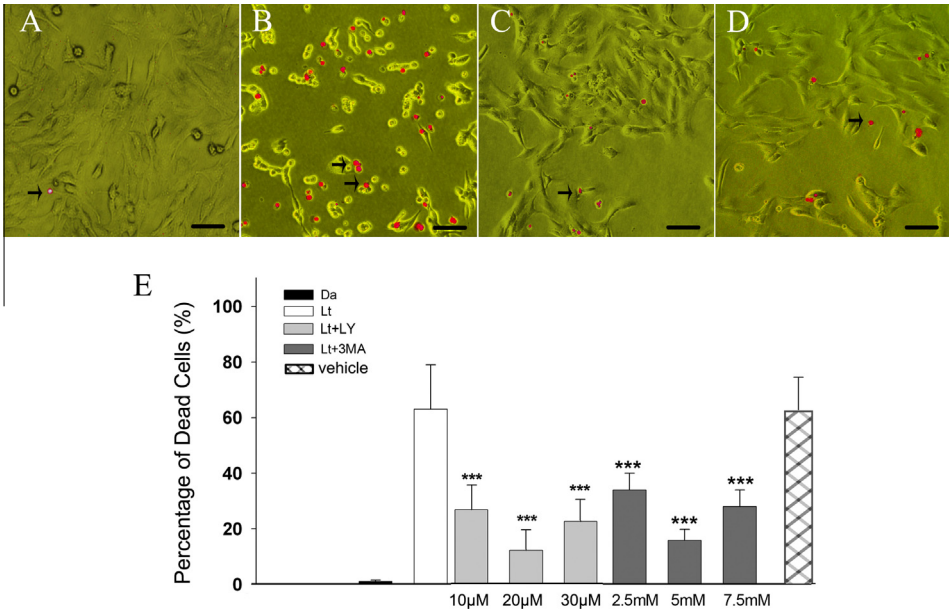

Fig. 2. Autophagy inhibitors protect 661W cells from light-induced death. Cell death was evaluated with PI staining. The black arrows indicate PI-positive cells. (A) 661W cells were cultured under the dark condition as a control. (B) 661W cells were exposed to 2600 Lux light for 3 d. (C) 661W cells were pretreated with 20 μM LY 30 min before exposure to 2600 Lux light for 3 d. Scale bar = 150 μm. (D) 661W cells were pretreated with 5 mM 3MA 30 min before exposure to 2600 Lux light for 3 d. Scale bar = 150 μm. (E) Quantitative assessment of cell death percentage (the number of PI positive cells/the number of total cells %) was performed through PI staining. Assays were performed in quadruplicate, and data are shown as means ± S.E.M. Asterisks indicate statistically significant differences ($P < 0.001$).

evaluated using the one-way ANOVA followed by the Bonferroni test. The accepted level of significance in all cases was $P < 0.001$.

3. Results

3.1. Light exposure leads to autophagy in 661W cells

To investigate whether light exposure induces autophagy in 661W cells, we first examined the level of autophagic marker LC3 in cells by western blot analysis. As shown in Fig. 1A, after the 661W cells were exposed to 2600 Lux light for 1 or 2 d, the cellular LC3II level was remarkably increased, appearing as a 16-Kb protein band. Further quantitative analysis revealed that the ratio of LC3II/LC3I from the light-treated cell lysate was significantly higher than that from the untreated cell lysate (Fig. 1B). To better illustrate the light-induced autophagy, we also examined the autophagosome formation with transmission electron microscopy. After 2-d light exposure, the 661W cells exhibited features typical of autophagy, such as sequestration of some cytosolic material, including organelles, within double-membrane bound vesicles termed autophagosomes. These enclosed materials are then targeted for degradation. (Fig. 1C). Next, we quantitatively measured the rate of generation of lysosomes which is the late stage of autophagosome by using LysoTracker staining. Fig. 1D and E shows that the exposure of 2600 Lux light for 2 d caused a pronounced increase in lysosomes, which appear as red fluorescence when the cells were examined with the confocal laser microscope. Quantitative analysis demonstrated a significant increase in the red fluorescence in light-exposed cells compared with that in the control cells. Taken together, these results strongly suggest that light exposure induces autophagy in 661W cells.

3.2. Inhibiting autophagy protects 661W cells from light-induced injury

Although autophagosomes were observed in light-treated 661W cells, whether autophagy is an essential step in the light-induced cell death is still uncertain. To test this, we blocked the light-induced autophagy by using two different inhibitors. 3-Methyladenine (3-MA) is a compound often used to study autophagy, because it can effectively block autophagy at the early stage [14]. Another autophagy inhibitor is the class I PI3K blocker, LY294002. It binds to the catalytic subunit of PI 3-kinase to suppress autophagy, while 3-MA interacts with the regulatory subunit [15]. As shown in Fig. 2, the pretreatment of cells with 10–30 μM LY294002 30 min before light exposure significantly protected 661W cells from light-induced damage and decreased the cell death percentage from 63% to about 20% based on PI staining assay results. The optimum concentration of LY294002 was around 20 μM. Consistently, 3MA also exhibited effective protection from light exposure. Pre-incubation of 661W cells with 2.5–7.5 mM 3MA led to a significant decrease in the light-induced cell death, reducing the death percentage from 63% to about 30%. 3MA at 5 mM showed the best protection. These results indicate that autophagy is a key stage in the light-induced cell death and that blocking autophagy effectively protects photoreceptor cells from light damage.

3.3. MAPK signaling is activated and contributes to the light-induced cell death

The mitogen-activated protein kinases (MAPK) pathways involve three parallel kinase modules: JNK, p38, and ERK1/2 pathways. These have been implicated in autophagic cell death [16]. Therefore, we examined the effect of MAPK signal pathways on light-injured 661W cells. As shown in Fig. 3G and H, the

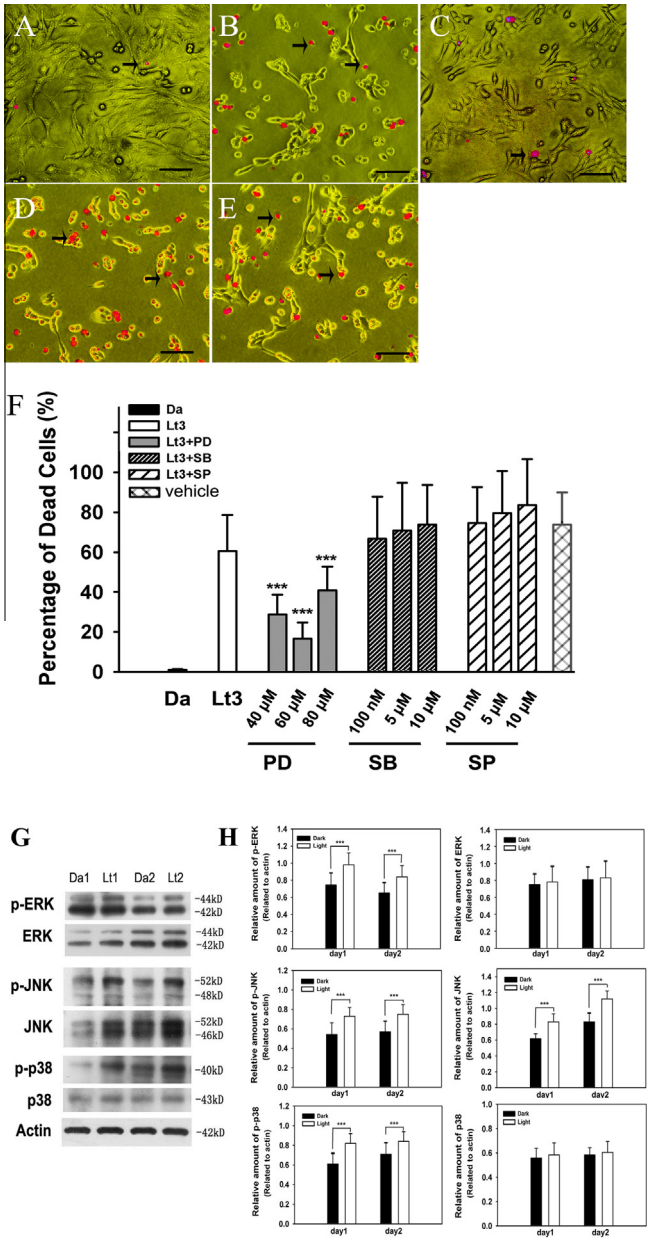

Fig. 3. MAPK pathway is activated by light exposure. Cell death was evaluated with PI staining. The black arrows indicate PI-positive cells. (A) 661W cells were cultured under the dark condition as a control. (B) 661W cells were exposed to 2600 Lux light for 3 d. (C) 661W cells were pretreated with 60 μM PD98059 30 min before exposure to 2600 Lux light for 3 d. (D) 661W cells were pretreated with 5 μM SB203580 30 min before exposure to 2600 Lux light for 3 d. (E) 661W cells were pretreated with 5 μM SP600125 30 min before exposure to 2600 Lux light for 3 d. Scale bar = 150 μm. (F) Quantitative assessment of cell death percentage (the number of PI positive cells/the number of total cells %) was performed through PI staining. (G) 661W cells were cultured under 2600 Lux light or dark condition. Cell lysates were collected at the indicated time periods and subjected to western blot analysis of phosphorylated ERK, ERK, phosphorylated JNK, JNK, phosphorylated p38 and p38. Actin protein was used as a loading control. (H) The bands on the western blots were scanned, and the intensity was determined by optical density measurements. The results are means ± S.E.M., where $n = 3$ (***$P < 0.001$, one-way ANOVA and Bonferroni test).

phosphorylation of ERK1/2, c-Jun N-terminal kinase (JNK) and p38 MAPK was significantly increased after the exposure of cells to 2600 Lux light, while the levels of non-phosphorylated forms of ERK 1/2 and p38 were only slightly increased. These results suggest that MAPK pathways might be involved in the light-induced death in the 661W cells. To determine whether ERK, JNK or p38

activation are important in light-induced death, we examined the effects of the ERK inhibitor PD98059, the JNK inhibitor SP600125 and the p38 inhibitor SB203580 [17] on the light-exposed 661W cells. As shown in Fig. 3A–E, pretreatment of cells with 40–80 μM PD98059 remarkably suppressed the light-induced cell death, reducing the death percentage from 61% to about 30%, whereas pre-incubation with SP600125 or SB203580 failed to protect cells from light damage. These results indicate that ERK 1/2 activation, and not JNK and p38 activation, is key in the light-induced death in 661W cells.

3.4. Blocking ERK pathway suppresses the light-induced autophagy

Whether ERK is involved in light-induced autophagic formation is still unclear. To investigate the effect of ERK on the light-induced autophagy, we first confirmed suppression of the ERK pathway by PD98059. As shown in Fig. 4A and B, 40 μM PD98059 at 30 min prior to light exposure markedly reversed the light-induced up-regulation of both p-ERK and ERK as determined by western blot. Further quantitative analysis demonstrated that the levels of non-phosphorylated and phosphorylated forms of ERK were significantly reduced in the PD98059-pretreated group. Next, we examined the change in the level of autophagic marker LC3II after treatment with PD98059. As shown in Fig. 4C,D, exposure to light for 2 d remarkably caused the up-regulation of LC3II/LC3I ratio, but the addition of 40 μM PD98059 significantly attenuated the light-induced increase in the LC3II/LC3I ratio. Consistent with this, we also observed numerous cellular lysosomes detected by Lyso Tracker staining. Pretreatment with 40 μM PD98059 markedly suppressed the formation of cellular lysosomes even with light

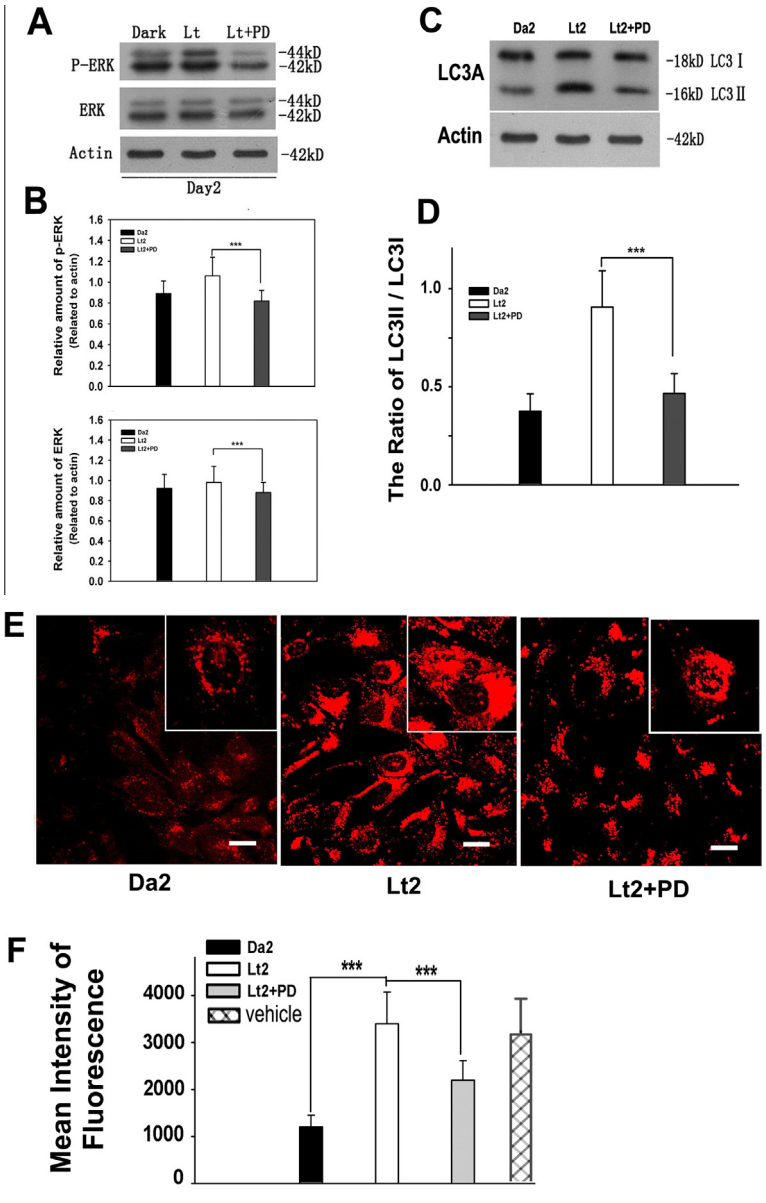

Fig. 4. Blocking the ERK pathway suppresses the light-induced autophagy. (A) Dark: 661W cells were cultured under dark condition for 2 d. Lt: 661W cells were cultured under 2600 Lux light condition for 2 d. Lt + PD: 661W cells were pretreated with 60 μM PD98059 30 min before exposure to 2600 Lux light for 2 d. The cells lysates were collected at the indicated time periods and subjected to western blot analysis of phosphorylated ERK and ERK. Actin protein was used as a loading control. (B) The bands on the western blots were scanned, and the intensity was determined by optical density measurements. (C) The cells lysates were collected at the indicated time periods and subjected to western blot analysis of LC3II and LC3I. Actin protein was used as a loading control. (D) The bands on the western blots were scanned, and the intensity was determined by optical density measurements and the ratio of LC3II/LC3I was calculated. (E) The cells were stained with LysoTracker and then examined under confocal laser microscope. Scale bar = 50 μm. (F) The intensity of fluorescence was analyzed with SPOT imaging software ($P < 0.001$). The results are presented means ± S.E.M., where $n = 3$ (***$P < 0.001$, one-way ANOVA and Bonferroni test).

exposure (Fig. 4E and F). These observations suggest that blocking the ERK pathway effectively suppressed the light-induced autophagy in 661W cells.

4. Discussion

Similar to apoptosis and necrosis, autophagy is a self-degradation program. In this highly conserved catabolic pathway, proteins and organelles are engulfed by vacuoles in order to maintain cellular viability [18]. Though autophagy is considered a survival mechanism activated when cells are faced with cellular stress, macroautophagy can also lead to cell death, which is now called type II programmed cell death (PCD) [16].

In the present study, we investigated light-induced autophagy in photoreceptor cells by using a cell line (the 661W cells). We first monitored the change in the ratio of LC3-II/LC3I in 661W cells upon light exposure. LC3-II, expressed in most cell types, is an important autophagic marker synthesized from the microtubule-associated protein light chain 3 (LC3-I). LC3-II conjugates to phosphatidylethanolamine (PE) and targets to membranes with the protease autophagy-related gene (Atg) 3,4,7 after the initial autophagosomal vesicle has been formed [19]. The conversion from LC3-I to LC3-II is regarded as a marker of autophagy activity [20]. Thus we examined the levels of the unconjugated (LC3-I) and conjugated forms (LC3-II) and found that the conjugated form was significantly higher in the light-damaged group than in the dark control group. To further confirm light-induced autophagy, we also morphologically examined autophagosome formation in 661W cells by transmission electron microscopy, which revealed features typical of autophagy, such as sequestration of cytosolic material, including organelles, within double-membrane bound vesicles which will then be targeted for degradation. These results suggest that intensive light exposure can induce autophagy in photoreceptor cells and that autophagy might play an important role in the death cascade. In addition, our results also revealed that pretreatment with 5 mM 3-MA, a classical inhibitor of autophagy, markedly attenuated light injury in 661W cells, reducing death percentage from 63% to about 30%. Because 3-MA can only effectively block autophagy at the early stage, we also tested the protective effect of another inhibitor LY294002, which binds to the catalytic subunit of PI3-kinase (PI3K) to suppress autophagy, whereas 3-MA interacts with the regulatory subunit [15]. PI3K, a lipid kinase upstream of mTOR signaling, regulates intracellular signaling pathways to initiate the autophagosome formation. Interestingly, LY294002 also conferred protection on light-injured photoreceptor cells. These results indicate that the light-induced autophagy process can be blocked at different steps of the PI3K pathway, resulting in protection of photoreceptor cells from light-induced death.

There are many different signaling pathways involved in the autophagosome formation and maturation, such as AMPK (AMP-activated protein kinase), and MAPK (mitogen-activated protein kinase) [21]. In the present study, our results show that MAPK pathway may play a critical role in the light-induced marcoautophagy in the 661W cells. The phosphorylation of ERK, JNK and p38 were significantly activated in the photoreceptor cells after light exposure. Pretreatment with the ERK inhibitor, PD98059, remarkably suppressed autophagy formation and rescued cells from light-induced injury, whereas pretreatment with JNK inhibitor SP600125 or p38 inhibitor SB203580 failed to protect cells from light-induced death. These findings indicate that light-induced autophagy in the photoreceptor cells depends on the ERK pathway rather than the p38 or JNK pathways. Previous studies have shown that ERK is necessary in the formation of autophagy caused by nutrient starvation or other stresses [22]. The ERK-specific phosphatases are sensitive to ROS (reactive oxidative species) [23], yet light exposure results in oxidative stress in photoreceptor cells, causing excessive intracellular ROS generation. The activation of ERK further regulates cellular pathways, such as the DUSP (dual specificity phosphatases) pathway, to promote ERK-mediated autophagy formation [24].

In conclusion, this study is the first to demonstrate that autophagy is critical in light-induced photoreceptor death and that directly blocking autophagy or suppressing autophagy by inhibiting the ERK pathway could effectively attenuate light-induced damage in 661W cells. These results may enhance our current understanding of the molecular mechanisms of photoreceptor cell death in diseases associated with light injury and provide a basis for future studies to investigate autophagy as a therapeutic target.

Acknowledgments

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