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Epigallocatechin-gallate (EGCG) regulates autophagy in human retinal pigment epithelial cells: A potential role for reducing UVB light-induced retinal damage

Chao-Peng Li [1], Jin Yao [1], Zhi-Fu Tao, Xiu-Miao Li, Qin Jiang *, Biao Yan *

Eye hospital, Nanjing Medical University, Nanjing 210029, China

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ABSTRACT

Autophagy is an intracellular catabolic process involved in protein and organelle degradation via the lysosomal pathway that has been linked in the pathogenesis of age-related macular degeneration (AMD). UVB irradiation-mediated degeneration of the macular retinal pigment epithelial (RPE) cells is an important hallmark of AMD, which is along with the change in RPE autophagy. Thus, pharmacological manipulation of RPE autophagy may offer an alternative therapeutic target in AMD. Here, we found that epigallocatechin-3-gallate (EGCG), a polyphenolic compound from green tea, plays a regulatory role in UVB irradiation-induced autophagy in RPE cells. UVB irradiation results in a marked increase in the amount of LC3-II protein in a dose-dependent manner. EGCG administration leads to a significant reduction in the formation of LC3-II and autophagosomes. mTOR signaling activation is required for EGCG-induced LC3-II formation, as evidenced by the fact that EGCG-induced LC3-II formation is significantly impaired by rapamycin administration. Moreover, EGCG significantly alleviates the toxic effects of UVB irradiation on RPE cells in an autophagy-dependent manner. Collectively, our study reveals a novel role of EGCG in RPE autophagy. EGCG may be exploited as a potential therapeutic reagent for the treatment of pathological conditions associated with abnormal autophagy.

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1. Introduction

Age-related macular degeneration (AMD) is a complex, degenerative and progressive eye disease that leads to a progressive loss of central vision [1]. AMD is characterized by morphological and functional abnormalities in the macular retinal pigment epithelial (RPE) cells. In general, RPE cells often face chronic oxidative stress from three sources: high levels of oxygen consumption, exposure to the high levels of lipid peroxidation derived from the photoreceptor outer segments and exposure to constant light stimuli. Excessive exposure to ultraviolet (UV) light especially UVB is implicated in the pathogenesis of AMD [2,3]. Previous studies have revealed that RPE cells exposed to UVB exhibit several cellular pathological features, such as the reduction of cell viability, DNA fragmentation, loss of phagocytotic activity, impaired water permeability, and inflammatory signaling [4,5].

Autophagy is an intracellular catabolic process involved in protein and organelle degradation via the lysosomal pathway. Preservation of the autophagic activity is associated with the low accumulation of damaged proteins, the better ability to handle protein damage, and the improvement of RPE cell function. Hence, an effective autophagic clearance system is essential for maintaining the normal physiological function of RPE cells [6,7]. Abnormal autophagy has been reported as one of the important features in the pathogenesis of AMD [8]. Drusen is composed of many intracellular originated proteins that regulate proteolytic processes. Increased drusen deposits are risk factors for severe cases of AMD, which is usually mediated by decreased autophagy and increased transcytosis and exocytosis in RPE cells [9]. In addition, in human AMD donor samples, there is an obvious increase in the accumulation of autophagic markers and a marked decrease in lysosomal activity [10].

(−)-Epigallocatechin gallate (EGCG) is a natural anti-oxidant flavonoid which is abundant in green tea (*Camellia sinensis*). EGCG is defined as a major green tea catechin that contributes to beneficial therapeutic effects, including anti-inflammatory, anti-viral, anti-bacterial, anti-mutagenic, anti-allergic, neuroprotective, and vasodilatory influences [11]. Previous studies have revealed that EGCG is effective in preventing oxidative stress-, UVA-, UAB-induced retinal degeneration [12–14]. EGCG is also an effective inhibitor of RPE cell migration and adhesion, therefore, may prevent epiretinal membrane formation [15].

Autophagy plays a key role in the maintenance of cellular homeostasis to remove dysfunctional organelles and proteins. It

* Corresponding authors. Address: Eye hospital, Nanjing Medical University, 138# Han-Zhong Road, Nanjing 210029, China. Fax: +86 25 86677677.
E-mail addresses: jqin710@vip.sina.com (Q. Jiang), yanbiao1982@hotmail.com (B. Yan).
[1] These authors contribute equally to this paper.

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is activated to cope with excessive organelle damage, damaged protein removal and pathogen defense [6,16]. EGCG has a beneficial effect in the pathophysiological condition of RPE cells. However, whether EGCG protects against UVB-induced retinal damage in RPE cells through the regulation of autophagy is still unknown. In this study, we found that EGCG plays a regulatory role in UVB irradiation-induced RPE cells autophagy through mTOR signaling. We found that EGCG treatment could significantly alleviate the toxic effects of UVB irradiation on RPE cells in an autophagy-dependent manner. Hence, pharmacological manipulation of autophagy by EGCG treatment may offer an alternative therapeutic target in AMD.

2. Materials and methods

2.1. Cell culture

Human RPE cell line, ARPE-19, was obtained from the American Type Culture Collection (Manassas, VA). It was cultured in Dulbecco's modified eagle medium (DMEM) at 37 °C in a humidified chamber of 5% CO_2. The culture medium was replaced with fresh medium every other day.

2.2. Transmission electron microscopy

ARPE-19 cells were fixed at 4 °C in 2.5% glutaraldehyde in 0.15 M sodium cacodylate (pH 7.4) overnight. After postfixation in osmium tetroxide (1% in cacodylate buffer), contrasted in uranyl acetate (1% in ethanol 70%), they were dehydrated in ethanol and embedded in epoxy resin. Then, 100-nm-thick sections were stained with uranyl acetate, and then examined by using a Hitachi-600 electron microscope.

2.3. Western blot analysis

Proteins were separated by SDS–PAGE and electrophoretically transferred onto PVDF membranes. Nonspecific binding was blocked by 1 h incubation of the membranes in 5% (w/v) nonfat dry milk. The blots were then incubated with the primary antibodies for overnight, washed 3 times with PBST, and finally incubated for 1 h with a peroxidase-labeled secondary antibody. The blots were visualized using an enhanced chemiluminescence detection kit (Pierce). Band intensities in the immunoblots were quantified by densitometry using the ImageJ software.

2.4. Detection of cell viability

The 3-(4,5-dimethylthiazal-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was used to detect cell viability. Briefly, ARPE-19 cells were plated at a density of 1×10^4 cells per well in 96-well plates. After exposure to specific treatment, these cells were incubated with MTT at a final concentration of 0.5 mg/ml for 3 h at 37 °C. After removal of the medium, a 100 mM DMSO solution was added to dissolve the formazan crystals. The absorbance at 570 nm wavelength was detected using a microplate reader (Thermo).

2.5. Statistics

Data were expressed as mean ± SEM. Values were verified to be normally distributed and analyzed statistically by one-way analysis of variance, followed by Student's *t* test. Probability values of $P < 0.05$ were considered statistically significant. The Bonferroni correction was used to adjust for multiple comparisons.

3. Result

3.1. UVB irradiation induces autophagy in RPE cells

To determine the effect of UVB irradiation on the autophagy in RPE cells, we detected the expression pattern of the autophagosomal marker, microtubule-associated protein 1 light chain 3 (LC3). Previous study revealed that LC3 is processed post-transnationally into LC3-I, and then converted to LC3-II. Given LC3-II specifically associates with the autophagosome, the amount of LC3-II could reflect the level of autophagy [17]. We employed western blot to detect the level of LC3-II expression at different irradiation intensity (50, 100 and 200 mJ/cm^2). As shown in Fig. 1, UVB irradiation resulted in a marked increase in LC3-II expression in a dose-dependent manner.

Autophagy is a dynamic process involving autophagosome formation, fusion of autophagosomes to lysosomes, and degradation in lysosomes [18]. To determine the effect of UVB irradiation on autophagic flux, we treated RPE cells with NH_4Cl to block the degradation of autophagosomes. NH_4Cl treatment or UVB irradiation could result in a significant and conspicuous increase in the LC3-II levels. UVB irradiation in combination with NH_4Cl could further increase the levels of LC3-II protein (Fig. 1), suggesting that some degree of autophagy flux occurs in UVB-mediated autophagy in RPE cells.

3.2. EGCG treatment represses UVB-mediated autophagy

To detect the effect of EGCG administration on UVB-induced autophagy, we examined whether EGCG treatment could affect the formation of autophagosomal marker, LC3-II protein. The result shows that UVB irradiation significantly enhances the formation of

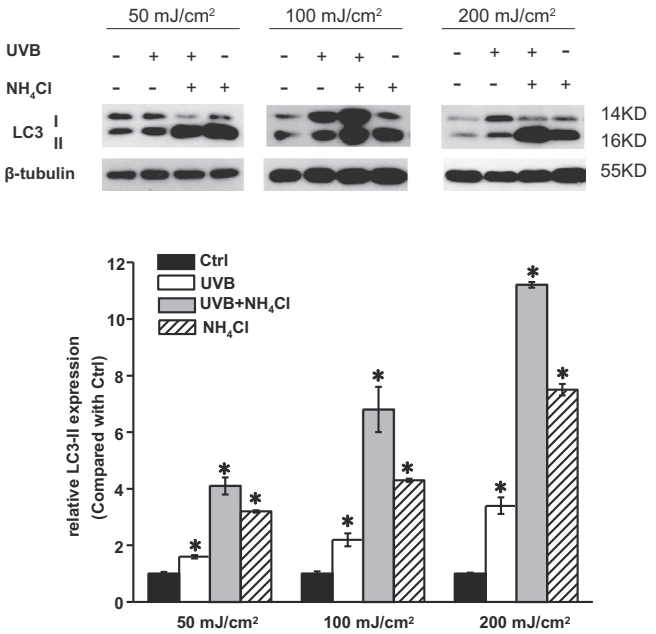

Fig. 1. UVB irradiation induces autophagy in RPE cells. RPE cells were irradiated with 50, 100 and 200 mJ/cm^2 of broadband UVB (280–315 nm) for 3 h, and then incubated in the serum-free medium for 24 h with or without NH_4Cl treatment. Western blots were conducted to determine the level of LC3-II expression. β-Tubulin expression was detected as the loading control. The untreated group was taken as the control group. The data was expressed as the relative change compared with the control group. A representative immunoblot is shown along with the quantitative data showing the mean ± S.E.M. from four separate blots. "*" indicates a significant difference compared with the control group.

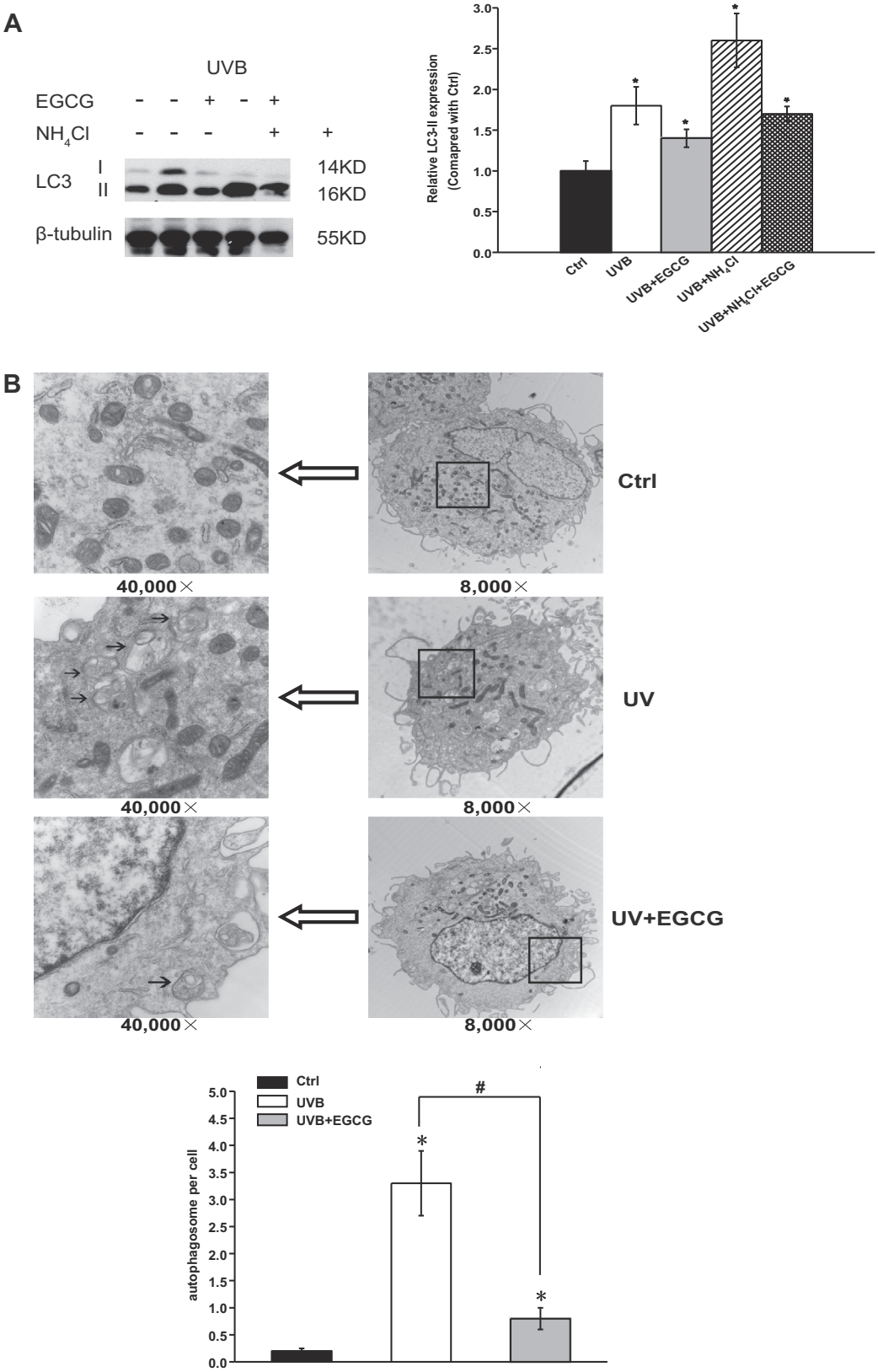

Fig. 2. EGCG treatment represses UVB-mediated autophagy. (A) RPE cells were pretreated with EGCG (50 μM) for 3 h, and then these cells were irradiated with 50, 100 and 200 mJ/cm^2 of UVB (280–315 nm) for 3 h, and then incubated in the serum-free medium for 24 h with or without NH_4Cl treatment. Western blots were conducted to determine the level of LC3-II expression. β-Tubulin expression was detected as the loading control. A representative immunoblot is shown along with the quantitative data showing the mean ± S.E.M. from four separate blots. "*" indicates a significant difference compared with the control group. (B) RPE cells were pretreated with or without EGCG (50 μM) for 3 h, and then these cells were irradiated with 100 mJ/cm^2 of broadband UVB (280–315 nm) for 3 h. After incubation in the serum-free medium for 24 h, these cells were prepared for transmission electron microscopy observation. A representative electron microphotograph along with the statistical data (the average number of autophagosome in each cell) is shown. Arrow indicates the autophagosome in RPE cells. "*" indicates a significant difference compared with the control group. "#" indicates a significant difference between groups.

LC3-II protein, and the treatment of NH_4Cl, the inhibitor of lysosomal degradation, further increases UVB-mediated LC3-II formation. By contrast, EGCG treatment could UVB-mediated LC3-II enhancement with or without NH_4Cl addition (Fig. 2A). Thus, these results suggest that EGCG is potential regulator of UVB-mediated autophagy in RPE cells. In addition, we employed the electron microscopy to observe the change in autophagic structures. We found that UVB irradiation resulted in a significant increase in

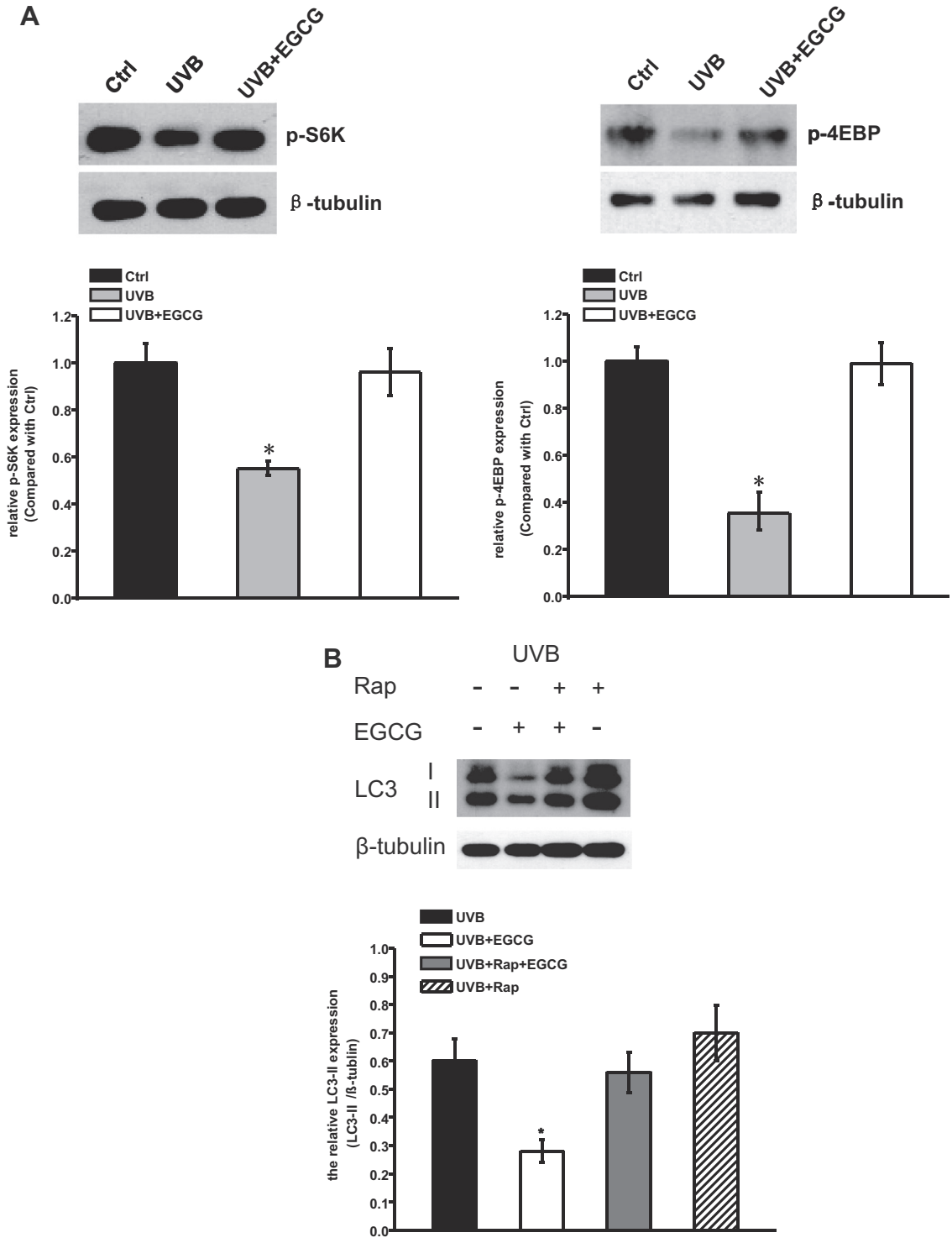

Fig. 3. EGCG regulates UV-mediated autophagy through mTOR signaling pathway. (A) RPE cells were pretreated with or without EGCG (50 μM) for 3 h, and then these cells were irradiated with 100 mJ/cm^2 of broadband UVB (280–315 nm) for 3 h. After incubation in the serum-free medium for 24 h, western blots were conducted to determine the phosphorylation level of S6K and 4E-BP1 expression. β-Tubulin expression was detected as the loading control. A representative immunoblot is shown along with the quantitative data showing the mean ± S.E.M. from four separate blots. (B) RPE cells were pretreated with or without EGCG (50 μM) for 3 h, then these cells were irradiated with 100 mJ/cm^2 of broadband UVB (280–315 nm) for 3 h. After incubation in the serum-free medium for 24 h with or without rapamycin (Rap), western blots were conducted to determine the level of LC3-II expression. β-Tubulin expression was detected as the loading control. A representative immunoblot is shown along with the quantitative data showing the mean ± S.E.M. from four separate blots.

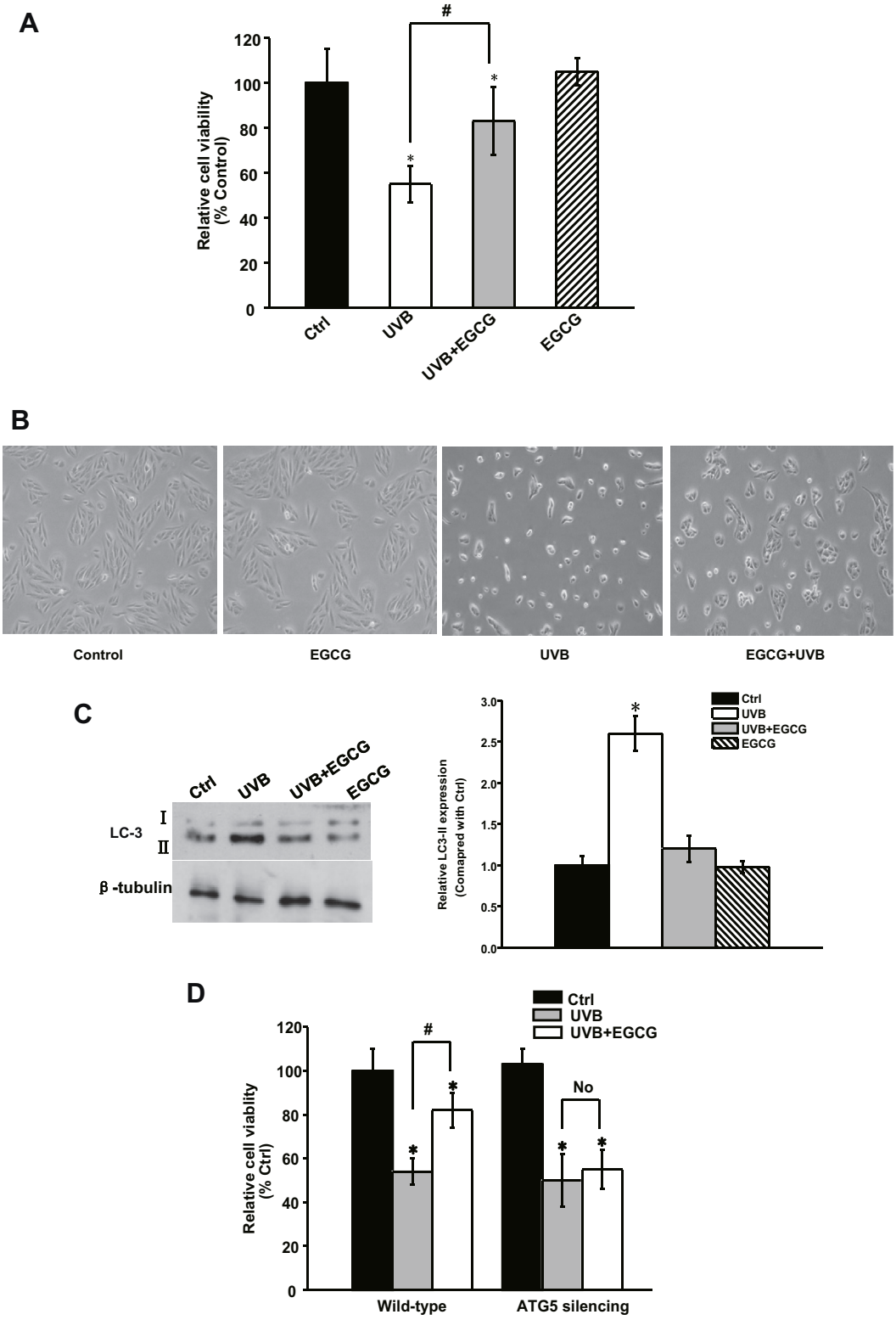

Fig. 4. EGCG sensitizes RPE cells to UVB damage in an autophagy-dependent manner. (A) RPE cells were pretreated with or without EGCG (50 μM) for 3 h, then these cells were irradiated with 100 mJ/cm^2 of broadband UVB (280–315 nm) for 3 h. After incubation in the serum-free medium for 24 h, RPE cell viability was determined using MTT assay. Results are expressed mean ± S.E.M. of four independent experiments. "*" indicates significant difference compared with the control group, and "#" indicates a significant difference between groups. (B and C) RPE cells were treated as shown in Fig. 4A. Light microscopy was used to observe the change in cell morphology (B). Western blots were conducted to determine the level of LC3-II expression. β-Tubulin expression was detected as the loading control. A representative immunoblot is shown along with the quantitative data showing the mean ± S.E.M. from four separate blots (C). (D) Wild-type or ATG5 silencing RPE cells were treated as shown in Fig. 4A. The cell viability was determined using MTT assay. Results are expressed mean ± S.E.M. of four independent experiments. "*" indicates significant difference compared with the control group, and "#" indicates a significant difference between groups.

the number of double-membrane vacuoles, which is typical of autophagosomes (Fig. 2B), whereas we found that EGCG treatment could obviously decrease the number of autophagosomes in response to UVB-induced autophagy. Taken together, these results show that EGCG is involved in the regulation of UVB-induced RPE autophagy.

3.3. EGCG regulates UVB-mediated autophagy through mTOR signaling pathway

Among the numerous components involved in the regulation of autophagy, TOR (target of rapamycin) is a key component that coordinately regulates the balance between growth and autophagy in response to cellular physiological conditions and environmental stress [19]. To investigate the relationship between mTOR and autophagy in RPE cells, we detected the expression pattern of two best characterized targets of mTOR, S6K and 4EBP1, in response to UVB irradiation. The result shows that UVB irradiation led to a significant alternation of the phosphorylation level of two mTOR targets, suggesting that UVB irradiation results in the activation of mTOR signaling pathway (Fig. 3A). To determine the direct role of mTOR signaling in autophagy, RPE cells were treated with or without rapamycin to regulate the activity of mTOR signaling. We found that compared with the untreated group, UVB irradiation could significantly increase the formation of LC3-II protein. UVB irradiation-mediated LC3-II formation was abolished once mTOR signaling is inhibited by the treatment of rapamycin (Fig. 3B), suggesting a crucial role of mTOR signaling in UVB irradiation-induced autophagy.

3.4. EGCG sensitizes RPE cells to UVB-induced damage in an autophagy-dependent manner

Finally, we decided to explore the potential therapeutic application for the inhibitory action of EGCG on autophagy, particularly in the context of eye diseases. RPE cells were exposed to UVB radiation for 24 h with or without EGCG. MTT experiment showed that EGCG alone had minimal effect on RPE cell survival. By contrast, EGCG could significantly alleviate the toxic effects of UVB irradiation in RPE cells (Fig. 4A). Meanwhile, light microscopy observation showed that UVB irradiation-treated RPE cells became round or oval in shape, forming plasma-membrane blebs and protuberances on the cell surface, while EGCG administration could partially reverse these morphological changes (Fig. 4B). In addition, we detected the expression pattern in the expression of endogenous LC3-II level. As observed in Fig. 4C, treatment with EGCG alone or in combination with UVB exposure resulted in a significant accumulation of LC3-II, suggesting an EGCG-dependent change in autophagy (Fig. 4C). We next investigated whether the change in UVB cytotoxicity by EGCG is a result of the inhibitory effect of EGCG on the autophagy pathway. For this purpose, we used RNA interference technique to silence ATG5 expression, which is deficient of autophagy signaling pathway. As shown in Fig. 4D, treatment of wild-type or ATG5 silencing RPE cells with UVB irradiation for 24 h resulted in an obvious reduction in RPE cell viability. EGCG administration could partially UVB-induced cellular damage in wild-type RPE cells. By contrast, EGCG administration had no effect on the cell viability of ATG5 silencing RPE cells (Fig. 4D). These results suggest that EGCG sensitizes RPE cells to UVB-induced damage in an autophagy-dependent manner.

4. Discussion

Autophagy plays a critical role in intracellular quality control of superfluous and damaged proteins and organelles. It has sparked great interest due to its implication in numerous physiological processes such as proliferation, development, aging, antigen presentation, innate immunity, tumor suppression, neurodegeneration, and cell death [1,16,20,21]. Recently, the modulators of the autophagic pathway are found to be the promising pharmacological target for some diseases [22,23]. EGCG, a major constituent polyphenol in green tea, is reported to inhibit a series of biomedically relevant molecular targets and disease-related cellular processes at relatively high concentrations [24–26]. Moreover, a wealth of information is already available on EGCG bioavailability, toxicity and dosing. Thus, its use in an alternative setting would avoid one of the major time- and effort consuming issues in drug discovery. Here, we observed that EGCG administration could reduce UVB-induced RPE damage an autophagy-dependent manner. Thus, EGCG may be exploited as a potential therapeutic reagent for the treatment of eye diseases associated with abnormal autophagy.

Environmental stress, nutrient starvation, or reduced availability of growth factors alarms eukaryotic cells to adjust metabolism to survive. An early response of the cellular metabolic adjustments involves the inhibition of growth and the induction of autophagy [1,27]. Among the numerous components involved in the regulation of autophagy and growth, mTOR signaling is a key component that coordinately regulates the balance between growth and autophagy in response to cellular physiological conditions and environmental stress [28]. S6K and 4E-BP1are known as the downstream of mTOR signaling pathway [29]. The alternations in their phosphorylation levels represent as the activation of mTOR signaling [30]. Here, we found that UVB irradiation results in a significant alternation of the phosphorylation level of S6K and 4E-BP1, whereas EGCG addition could partly reverse this change trend. Meanwhile, rapamycin, the specific inhibitor of mTOR signaling, could completely abolish the up-regulation of LC3-II protein induced by UVB irradiation. Hence, mTOR signaling plays a critical role in EGCG involvement of UVB-mediated autophagy.

Autophagy is self-degradative process which ensures the regular turnover of cellular components by sequestering damaged organelles and misfolded proteins. In most of these situations, autophagy has both beneficial and harmful effects on cell functions [31–33]. In this study, we employed the MTT method to determine whether EGCG-mediated autophagy is a protective or detrimental response in response to UVB irradiation. We found that EGCG alone has minimal effect on RPE cell survival, whereas EGCG could significantly alleviate the toxic effects of UVB in RPE cells. Further, EGCG sensitizes RPE cells to UVB-induced damage in an autophagy-dependent manner. Hence, EGCG regulation of autophagy can be generally considered as a cell protector against UVB-induced injuries in RPE cells.

In summary, the present study demonstrates that EGCG regulates RPE cells autophagy through mTOR signaling-dependent mechanism. EGCG treatment decreases UVB-induced the formation of LC3-II protein and autophagosome, and protects RPE cells against UVB-induced injuries in an autophagy-dependent manner. This study provides "proof of principle" for the idea that daily intake of EGCG may help individuals suffering from retinal diseases where abnormal autophagy is implicated.

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