Acute ultraviolet A exposure induces autophagy in RPE cells

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Abstract

Background

The mechanism for ultraviolet A (UVA) involvement in age-related macular degeneration (AMD) is unclear. We examined the levels of autophagy-related proteins and ubiquitination of cell proteins after acute UVA exposure and determined whether UVA causes AMD by affecting autophagy in retinal pigment epithelium (RPE) cells.

Methods

Western blotting was used to determine the level of Beclin-1, LC3, and p62 in ARPE-19 cells exposed to UVA. Co-immunoprecipitation was used to detect the ubiquitination of proteins in cells. Cell viability was determined with a CGD1 assay.

Results

UVA irradiation increased the level of LC3 and the conversion of LC3-I to LC3-II in ARPE-19 cells within 24 h, inhibition of autophagy by NH_4CL reversed the effect of UVA, and suppression of proteasomes by Epoxomicin (EPO) did not enhance the effect of UVA. UVA irradiation did not affect the level of Beclin-1. UVA up-regulated the expression of p62 and the ubiquitination of proteins in cells, especially k63-linked ubiquitination. ARPE-19 cells were lost in a short- time under exposure to UVA.

Conclusion

UVA induces autophagy in ARPE-19 cells in a short period, and promotes protein

degradation through the autophagy pathway. However, acute UVA irradiation leads to cell death. Therefore, while acute UVA irradiation cannot cause protein degeneration by impairing autophagy in RPE cells, it is still a risk factor for AMD occurrence due to its effect on reducing cells.

Keywords: Age-related macular degeneration, UVA, retinal pigment epithelium, autophagy, ubiquitin

Background

Age-related macular degeneration (AMD) is a progressive blinding disease, that is characterized by central visual loss due to degenerative and neovascular alterations in the macular region of the retina [1]. AMD is the leading cause of irreversible vision loss worldwide [2-4]. Its etiology is multifactorial, including aging, genetics [5], cardiovascular disease [6, 7], previous cataract surgery [8], smoking [9] and environmental factors such as pesticide use [10], and sunlight exposure [11, 12]. Numerous epidemiological and experimental results have demonstrated that ultraviolet (UV) radiation in sunlight is the main cause of skin cancer and ocular disease, such as AMD [13-16]. Although the cornea and the lens of the eye substantially filter and attenuate UV radiation that enters the eyes, there is still a considerable amount of UV ray transmission to the retina. Moreover, with age, the protective effectiveness of ocular melanin against UV damage to the retina is decreased, and UV damage to the eye is cumulative, which can lead to macular degeneration [17].

In the retina, the retinal pigment epithelium (RPE) cells play an essential role in maintaining homeostasis and are the crucial regulators of vision [18]. The death and dysfunction of the RPE are closely associated with the pathogenesis of AMD [19],

and the early and crucial event in AMD is impairment of the RPE [20]. Oxidative stress produced by UV radiation is a major factor associated with AMD, because oxidative stress not only leads to RPE cell damage and death [13, 21] but also can cause protein misfolding and accumulation of lipid/protein aggregates in AMD [22]. The major pathways involved in the degradation of damaged and unneeded proteins in RPE cells are the ubiquitin-proteasome system (UPS) and autophagy-lysosome pathway [23], and targeting these two systems is thought to be a promising strategy for preventing and treating AMD.

In fact, an increasing number of studies have shown that autophagy malfunction is strongly linked to AMD [24, 25]. Autophagy is a cytoprotective process that maintains homeostasis in response to various stresses and eliminates defective proteins and damaged organelles. Autophagy has a dual role in cells, and up-regulation of autophagy can lead to cell death [26]. Impairment of autophagy has been shown to contribute to many diseases [27]. Autophagy has both positive and negative effects on the progress of AMD [28]. Autophagy can be modulated by UVA in the skin [29, 30], but whether UVA leads to AMD through regulating autophagy processes in RPE cells has not been elucidated.

Because 80% of the total daily UV exposure is UVA and can damage cells [13, 31], it is of great significance to study the pathogenesis of UVA in AMD. In this study, we investigated the changes in autophagy in RPE cells and cell viability after UVA exposure, aiming to explore whether UVA irradiation impairs RPE cells through the autophagy pathway.

Materials and methods

Reagents and antibodies

CGD1 (Cell Growth Determination Kit, MTT based), penicillin, streptomycin, Epoxomicin (EPO), and ammonium chloride (NH_4CL) were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM) and fetal calf serum were from Invitrogen (Carlsbad, CA, USA). Primary antibodies anti-Beclin-1, anti-LC3, anti-HA and anti-β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p62 (SQSTM1) was from BD Bioscience (San Diego, CA, USA). Anti-Flag was from Cell Signaling Technology (Danvers, MA, USA). Secondary antibodies anti-rabbit-HRP and anti-mouse-HRP were purchased from GE Healthcare Biosciences (Pittsburgh, PA, USA) and anti-goat-HRP was purchased from Santa Cruz Biotechnology.

Cell cultures

Adult human retinal pigment epithelial cells (ARPE-19) were originally from the American Type Culture Collection (ATCC). These cells were maintained in DMEM, supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml of streptomycin. The cells were cultured in a humidified incubator at 37 °C and 5% CO_2 before they were treated and analyzed.

UVA irradiation

ARPE-19 cells were cultured in 10 cm culture dishes (SARSTEDT, Newton, NC, USA) for western blot analysis. After two washes with DMEM, the cells were then incubated with 3 ml/dish PBS under UVA irradiation. The UV dosimeter was purchased from Daavlin (Bryan, OH, USA). The UVA lamps in the illuminator

emitted ultraviolet rays between 355 nm and 375 nm, with peak luminosity at 365 nm. UVA radiation was supplied by a closely spaced array of four UVA lamps, which delivered uniform irradiation at a distance of 20 cm. The UVA irradiation dose was 20 J/cm^2 and it took approximately 56–58 min to attain the programmed energy. The irradiation stopped automatically when the energy received matched the programmed energy.

Cell viability assays

The viability of cells was determined with an MTT-based CGD1 assay. ARPE-19 cells were cultured in 96-well cell clusters (Costar, Corning, NY, USA) after the cells were counted and adjusted to 10,000 cells/well. Twenty-four hours later, the seeded clusters were washed twice with DMEM and exposed to UVA radiation after the culture medium was replaced by 30 μl PBS/well. After UVA exposure, the full medium and the full media containing 20 mM NH_4CL or 100 nM EPO were immediately put back into each well and the cells were then incubated for 24 h. The CGD1 assay analysis was employed according to the Cell Growth Determination Kit protocol of Sigma. Mitochondrial dehydrogenases metabolized MTT to a purple formazan dye, which was analyzed photometrically as absorbance at a wavelength of 570 nm with a background at 690 nm by KC junior software on a BioTek PowerWave XS (BoiTek Instruments Inc., Winooski, VT, USA). Cell viability was proportional to the absorbance measured.

Plasmid construction

The 3xFlag-tagged p62 was constructed by subcloning the p62 cDNA fragment to the

*Eco*RI and *Bam*HI sites of p3xFlag-CMV14 (Sigma). The p3xHA-CMV14 was established by replacing the 3xFlag tag of the p3xFlag-CMV14 vector with the 3xHA tag between the *Bam*HI and *Xma*I sites. The p3xHA-tagged ubiquitin, K48-linked ubiquitin and K63-linked ubiquitin were constructed by inserting the encoding sequences to the *Eco*RI and *Bam*HI sites of p3xHA-CMV14.

Transfection of ARPE-19 cells

Co-transfecting p3xHA-ubiquitin, p3xHA-K48-ubiquitin, and p3xHA-K63-ubiquitin was carried out with 3xFlag-tagged p62 into the ARPE-19 cells. Flag vectors were used as a control. Transfections were performed using Lipofectamine (Invitrogen) in Opti-Mem medium (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Cells were treated after 24 h of transfection.

Western blotting

ARPE-19 cells were lysed in RIPA buffer. Using a Bradford Protein Assay (Bio-Rad), protein concentrations of all samples were measured. Proteins were separated by SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked in 5% milk before incubation with primary antibodies and secondary antibodies conjugated to horseradish peroxidase (HRP). Immunoreactive bands were examined using an enhanced chemiluminescence reagent (Amersham). Results were quantified using Image J software for densitometry analysis and normalized to control groups.

Immunoprecipitation

The treated ARPE-19 cells and controls were washed with PBS twice and then were

lysed in RIPA buffer supplemented with protease inhibitor mixture, 0.625 mg/ml N-ethylmaleimide, and 0.2 mM phenylmethylsulfonyl fluorides. Lysates were centrifuged (1000 × g for 10 min at 4°C), and the protein concentration was determined with a Bradford protein assay. Each extract was precleared for 1 h at 4°C with 50% protein G-Sepharose (Amersham Biosciences). Extracts were then incubated with anti-Flag antibodies (Santa Cruz Biotechnology) overnight at 4°C, followed by incubation with protein G-Sepharose for 2 h. Protein G beads were collected by centrifugation at 500 × g for 5 min and washed three times with ice-cold RIPA buffer. They were subjected to SDS-PAGE followed by Western blotting for anti-HA analysis.

Statistical analysis

Quantifications in this study were analyzed with an unpaired, two-tailed Student's t-test. The "*" represents the comparison result with the control, and "#" represents the result compared to the UVA irradiation group. We considered p values <0.05 to be statistically significant (* or #) and <0.01 to be extremely significant (** or ##).

Results

UVA changed the levels of autophagy proteins in a short- time

To determine the effect of UVA irradiation on autophagy in ARPE-19 cells, we examined the protein levels of Beclin-1, a key initiator and established hallmark of autophagy. Our observation showed that there was no alteration in the level of Beclin-1 expression in ARPE-19 cells after exposure to UVA for any time point (Fig.1A and B). We further tested the expressions of LC3 and the ratio of LC3-II/ LC3-I. The ratio

of LC3-II to LC3-I represents the occurrence of autophagy. Here, the level of LC3 and the ratio LC3-II to LC3-I increased after ARPE-19 cells were exposed to UVA for 6 h, and there was no difference among the increasing levels at different time points within 24 h (Fig.1A and C). We used 24 h as our harvest time point after the tested cells received UVA radiation in the following experiments.

Figure 1. UVA irradiation increased the level of LC3 and the reversion of LC3-I to LC3-II. (A) ARPE-19 cells were irradiated with a 20 J/cm^2 dose of UVA irradiation and it took approximately 56–58 min to attain the programmed energy. The cells were collected after 6, 12, and 24 h of UVA irradiation for Western blotting to detect the expression level of Beclin-1 and LC3. The control group was a blank control that received no UVA radiation. (B) and (C) show the densitometry results of Beclin-1 and LC3-II/LC3-I that were measured with Image J software. The quantified data showed the mean ± SD from three independently performed experiments. $^{**}P<0.01$ indicates significant differences between the control and treatment groups.

UVA affected the autophagy pathway independent of the ubiquitin-proteasome system (UPS) in ARPE-19 cells

There was crosstalk between the autophagy pathway and UPS [32], and the inhibition of the proteasome could trigger autophagy. To identify whether UVA induced autophagy directly or through suppressing UPS, we tested the protein level of LC3 and Beclin-1 in ARPE-19 cells after UVA irradiation and then inhibited the proteasome by EPO (a specific inhibitor of the proteasome) or blocking autophagosome-lysosome fusion by NH_4CL. The results showed that EPO increased

LC3 levels and the ratio of LC3-II/LC3-I significantly, and enhanced the effect of UVA in inducing autophagy flux. NH_4CL did not change the ratio of LC3-II/LC3-I; however, it reversed the effect of UVA (Fig. 2A and B). EPO induced the expression of Beclin-1, which was reduced by UVA, and NH_4CL decreased the expression of Beclin-1, which was further intensified by UVA (Fig. 2A and C). These data indicated that inhibition of UPS not only induced autophagy by up-regulating the expression of Beclin-1 but also increased the reversion of LC3-I to LC3-II, and UVA only induced the reversion of LC3-I to LC3-II. With the existence of the inhibitor of autophagy, the effect of UVA disappeared. Therefore, UVA induced autophagy flux by a mechanism other than inhibiting UPS.

Figure 2. UVA affected autophagy pathway independent of the ubiquitin-proteasome system (UPS) in ARPE-19 cells. (A) ARPE-19 cells were irradiated with a 20 J/cm^2 dose of UVA irradiation and then treated with 20 mM NH_4CL or 100 nM EPO for 24 h. The cells were collected for Western blotting to detect the expression levels of LC3, Beclin-1, and p62. The control group was a blank control that received no treatment. (B), (C), and (D) show the densitometry of LC3-II/I, Beclin-1, and p62 measured with Image J software. The quantified data show the mean ± SD from three independently performed experiments. $^{**}P<0.01$ indicates a significant difference compared with the control group. $^{\#\#}P<0.01$ indicates a significant difference compared with the UVA group.

P62 is up-regulated by UVA irradiation

To further confirm autophagy activation, we detected the protein p62, also known as sequestosome 1 (SQSTM1), was acting as an autophagy adaptor and could be degraded both by autophagy and the proteasome pathway. Here, we observed the change in p62 in response to UVA irradiation upon inhibiting autophagy or the proteasome. The level of p62 was significantly increased by UVA, NH_4CL, and EPO. Furthermore, both NH_4CL and EPO enhanced the accumulation of p62 caused by UVA dramatically (Fig.2A and D), which may be a combination of the decrease in p62 degradation and up-regulation of p62 caused by UVA.

Site-specific ubiquitination of cellular proteins in response to UVA irradiation

Because p62 can deliver polyubiquitinated cargoes to autophagy as well as the proteasome [33], the K48-linked ubiquitinated proteins were degraded by the proteasome pathway [34]. The degradation of K63-linked ubiquitinated proteins was through the autophagy process [35]. We therefore assessed cellular protein ubiquitination, including p62 ubiquitination in ARPE-19 cells after the expression levels of K48, K63, and p62 in cells were increased with or without irradiation of UVA. Immunoblots of ARPE-19 cell lysates showed that p62 overexpression increased the levels of ubiquitination of p62 (3A). Co-immunoprecipitation confirmed that overexpression of p62 significantly induced the ubiquitination of all proteins in cells, and K48-linked ubiquitination increased much more (Fig. 3B). However, upon UVA irradiation, the polyubiquitination of proteins increased dramatically, especially the K63-linked ubiquitination (Fig. 4A and B) regardless of whether it was involved in the overexpression of p62.

Figure 3. Site-specific ubiquitination of cellular proteins after p62 protein was overexpressed in ARPE-19 cells. The expression vector of HA-tagged wild-type (WT), K48-linked ubiquitin, or K63-linkage ubiquitin was co-transfected into ARPE-19 cells with Flag-tagged p62 or Flag-tagged vector and cultured for 48 h. (A) Cell extracts were subjected to Western blotting for p62 and ubiquitination of proteins. (B) Cell extracts were subjected to immunoprecipitation using an anti-Flag antibody, and the immunocomplexes were analyzed by Western blotting using anti-HA and anti-p62 antibodies.

Figure 4. Site-specific ubiquitination of cellular proteins in response to UVA irradiation. ARPE-19 cells that were transfected were cultured for 24 h and exposed to UVA. Then they were cultured for another 24 h. (A) Cell extracts were subjected to Western blotting for p62 and ubiquitination of proteins. (B) Cell extracts were subjected to immunoprecipitation using an anti-Flag antibody, and the immunocomplexes were analyzed by Western blotting using anti-HA and anti-p62 antibodies.

UVA radiation induced ARPE-19 cell death, which could not be reversed by NH_4CL or EPO

There is evidence that UVA radiation can cause a cytotoxic effect in ARPE-19 cells and thus bring about cell death [36], and elevated autophagy can lead to cell death [26]. To investigate whether cell death is caused by activated autophagy, we examined the viability of ARPE-19 cells under exposure to UVA with inhibition of autophagy or the proteasome. Our results showed that UVA induced ARPE-19 cell death significantly (Fig. 5, $p < 0.01$), and the inhibition of autophagy induced cell

death to some extent without difference from the control. The inhibition of the proteasome did not change cell viability. Neither NH_4CL nor EPO could reverse the cell death induced by UVA.

Figure 5. UVA irradiation induces ARPE19 cell death, which cannot be reversed by autophagy changes. ARPE19 cells were treated with or without 20 mM NH_4CL or 50 nM EPO for 24 h after UVA radiation, and cell viability was assessed with a MTT-based CDG1 assay. The results are expressed as the percentages of controls and are represented by the mean ± SD of three independent experiments. Ctrl stands for the control group. *P* values <0.05 are shown by "*" as statistically significant and <0.01 by "**" as extremely significant.

Discussion

Although many studies have demonstrated an association between UVA and AMD, there is still controversy. It was recently reported that sunlight exposure may not be a risk factor for AMD [37]. Therefore, it is necessary to elucidate the mechanisms of UVA irradiation effects on RPE cells. The signaling pathways underlying UVA effects in ARPE cells are unknown.

Because of the strong relationship between AMD with autophagy [24, 25, 38-40], and an increasing number of studies suggesting a link between UVA exposure and autophagy [29, 30], we explored the autophagy changes in ARPE-19 cells irradiated by UVA. Our results showed that UVA irradiation increased the level of LC3 and the reversion of LC3-I to LC3-II, which indicated the increase in autophagy flux. However, there was no alteration in the level of Beclin-1 in ARPE-19 cells irradiated

by UVA. Although Beclin-1 is one of indicators of the onset of autophagy, it is not required for stimulation of autophagy following acute oxidative stress [39]. However, there is a different conclusion for other cells. In human skin fibroblasts, UVA impaired autophagy [30], but in mice epidermal keratinocytes, UVA induced autophagy [29], which may be related to the duration of UVA exposure. Here, we concluded that UVA induced autophagy in ARPE-19 cells in a short time, which is consistent with the results in mice epidermal keratinocytes acutely irradiated by UVA [29].

Because the inhibition of the proteasome pathway activates the autophagy-lysosome pathway[32], and UV irradiation induces a significant decline in proteasome function in keratinocytes [41], the autophagy in ARPE-19 cells induced by UVA may be the result of inhibition of the proteasome. To confirm this hypothesis, we investigated the change in autophagy in ARPE-19 cells under UVA irradiation after inhibiting the proteasome. Our experiments showed that inhibition of the proteasome induced autophagy through up-regulating the expression of Beclin-1 and LC3, and the reversion of LC3-I to LC3-II. However, UVA only increased the autophagy flux without the elevation of Beclin-1, which indirectly indicated that UVA induced the autophagy-independence of UPS. This result also showed that UVA does not inhibit proteasome function in ARPE-19 cells, which is different from the results in keratinocytes [41]. Maybe it was because UVA works in different pathways in different cells, which requires more study in the future.

Short-lived proteins are degraded by UPS, and the degradation of long-lived proteins

is dependent on the autophagy-lysosome pathway [42]. SQSTM1/p62 (Sequestosome 1) is the ubiquitously expressed autophagy adaptor that is also closely related to proteasome-mediated proteolysis [43, 44]. p62 can be connected to autophagy or transport the ubiquitin substrate to the proteasome to degrade the substrate [45]. Thus, p62 plays a pivotal role during protein degradation both in UPS and autophagy. Impairment of autophagy and blocking of UPS are usually accompanied by an accumulation of p62 [46]. Our results showed that the protein level of p62 increased in ARPE-19 cells exposed to UVA, and so did the cells that were autophagy-suppressed and proteasome-blocked. Moreover, both inhibition of autophagy and the proteasome can enhance the effect of UVA. These results showed that they have a superposition effect on the expression level of p62. It was reported that UVA can induce p62 transcription through activating transcription factor EB (TFEB) [47], and the up-regulation of p62 can also be mediated by the transcription factor nuclear factor erythroid-2-related factor 2 (NRF2) under oxidative stress [48]. Therefore, in addition to increasing the autophagy flux, UVA irradiation can regulate p62 expression in a short- time.

The higher p62 level delays delivering ubiquitinated proteins to the UPS for degradation, which causes the accumulation of UPS substrate [45]. However, ubiquitylation of p62 can liberate its ability to recognize polyubiquitylated cargoes for autophagy [49]. p62 is crucial to the aggregation of ubiquitinated proteins for autophagy degradation [50]. Here, we demonstrated that the overexpression of p62 in ARPE-19 cells elevated K48-linked and K63-linked ubiquitination of proteins. At the

same time, UVA caused the elevation of ubiquitinated-p62, especially the K63-linked ubiquitination, which is the substrate of the autophagy degradation pathway, indicating that the p62 degraded by autophagy is increased. All of these suggest that autophagy is activated.

In addition, UVA irradiation induced ARPE-19 cell death in a short time, which could not be reversed by the inhibition of autophagy. While higher autophagy can cause cell death [26], the cell death induced by UVA irradiation in our study was not through activating autophagy. Therefore, short-term exposure to UVA only damaged the ARPE-19 cells by inducing cell death and not by impairing autophagy. Autophagy impairment is related to the duration of UVA exposure. Evidence has shown that autophagy impairment is associated with chronic UVA exposure in keratinocytes [30]. Our experiments demonstrated that UVA can induce autophagy flux and regulate the expression of p62. It can also cause cell death in ARPE-19 cells in a short period. Therefore, while UVA exposure in a short- time did not impair autophagy and cannot cause protein degeneration in RPE, it still led to the loss of RPE cells which is a risk factor for AMD occurrence. Our findings will help us focus on preventive measures to defend the loss of RPE cells caused by current UVA exposure to prevent the occurrence and development of AMD. However, our study had a limitation. There was no measurement of melanosomes/melanin changes in ARPE-19 cells after treatment with UVA. UVA irradiation can stimulate melanogenesis, which has a protective effect against UV-induced damage to cells [51, 52]. We should consider the effect of melanin changes on autophagy, which will be the focus of our next study.

Conclusion

UVA induces autophagy in ARPE-19 cells in a short period, and promotes protein degradation through the autophagy pathway. However, acute UVA irradiation leads to cell death. Therefore, while acute UVA irradiation cannot cause protein degeneration by impairing autophagy in RPE cells, it is still a risk factor for AMD occurrence due to its effect on reducing cells.

Abbreviations

UVA Ultraviolet A

AMD Age-related macular degeneration

RPE Retinal pigment epithelium

EPO Epoxomicin

UPS ubiquitin-proteasome system

CGD1 Cell Growth Determination

NH_4CL ammonium chloride

DMEM Dulbecco's Modified Eagle's Medium

ARPE Adult human retinal pigment epithelial

HRP horseradish peroxidase

Availability of data and materials

All the data supporting the findings of this study are provided in the manuscript and the appendix material.

Consent for publication

Not applicable.

Competing of interests

The authors report no competing of interest.

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Author's contributions

Lei Shi conceived and designed the experiments; Aiyuan Wang, Dongmei Gui and Hongwei Yang performed the experiments; Danxin Zhao and Wei Pu analyzed the data; Lei Shi wrote the paper. All authors read and approved the final manuscript.

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Figures

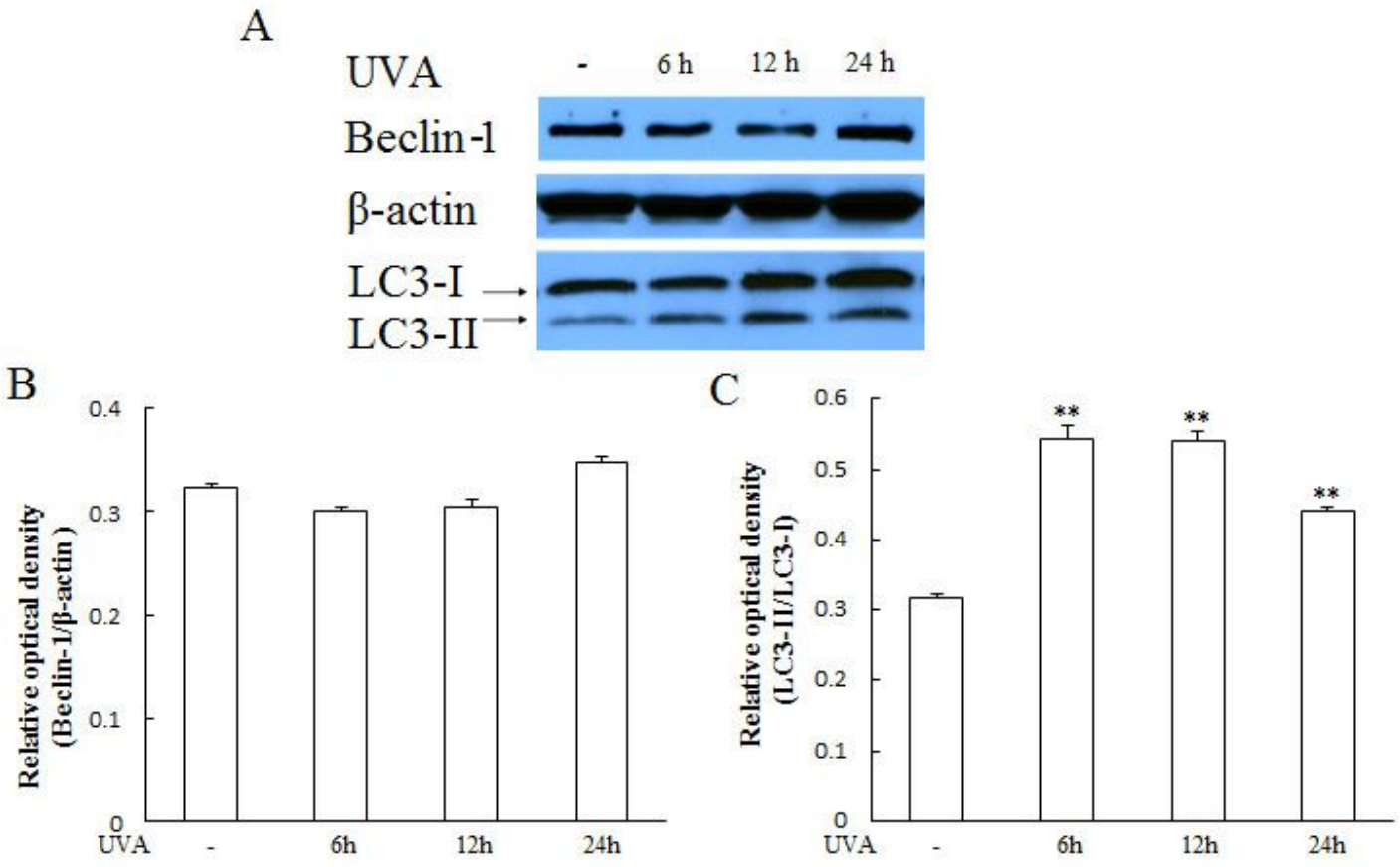

Figure 1

UVA irradiation increased the level of LC3 and the reversion of LC3- I to LC3-II. (A) ARPE-19 cells were irradiated with a 20 J/cm2 dose of UVA irradiation and it took approximately 56–58 min to attain the programmed energy. The cells were collected after 6, 12, and 24 h of UVA irradiation for Western blotting to detect the expression level of Beclin-1 and LC3. The control group was a blank control that received no UVA radiation. (B) and (C) show the densitometry results of Beclin-1 and LC3-II/LC3-I that were measured with Image J software. The quantified data showed the mean ± SD from three independently performed experiments. **P<0.01 indicates significant differences between the control and treatment groups.

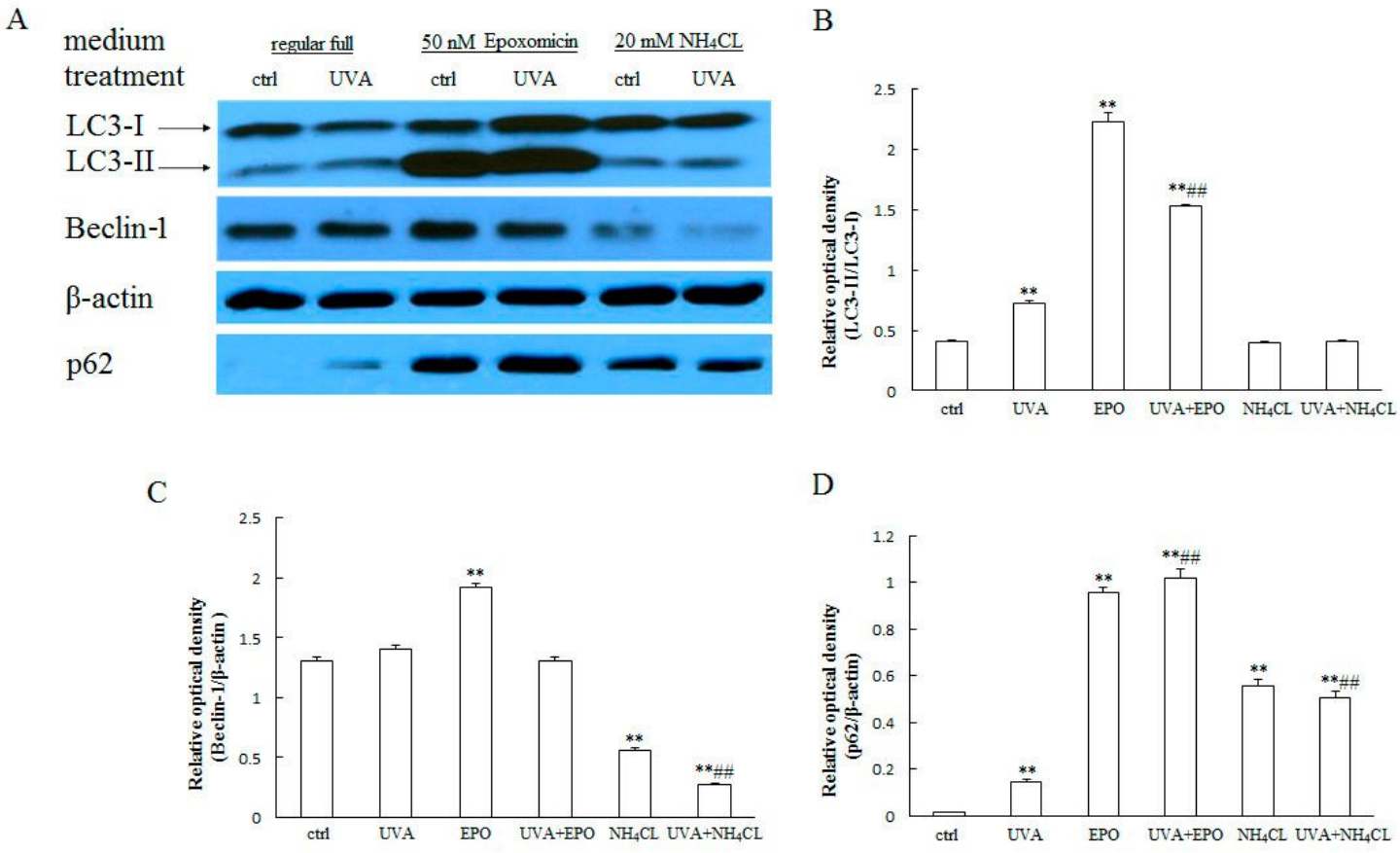

Figure 2

UVA affected autophagy pathway independent of the ubiquitinproteasome system (UPS) in ARPE-19 cells. (A) ARPE-19 cells were irradiated with a 20 J/cm2 dose of UVA irradiation and then treated with 20 mM NH4CL or 100 nM EPO for 24 h. The cells were collected for Western blotting to detect the expression levels of LC3, Beclin-1, and p62. The control group was a blank control that received no treatment. (B), (C), and (D) show the densitometry of LC3-II/I, Beclin-1, and p62 measured with Image J software. The quantified data show the mean ± SD from three independently performed experiments. **P<0.01 indicates a significant difference compared with the control group. ##P<0.01 indicates a significant difference compared with the UVA group.

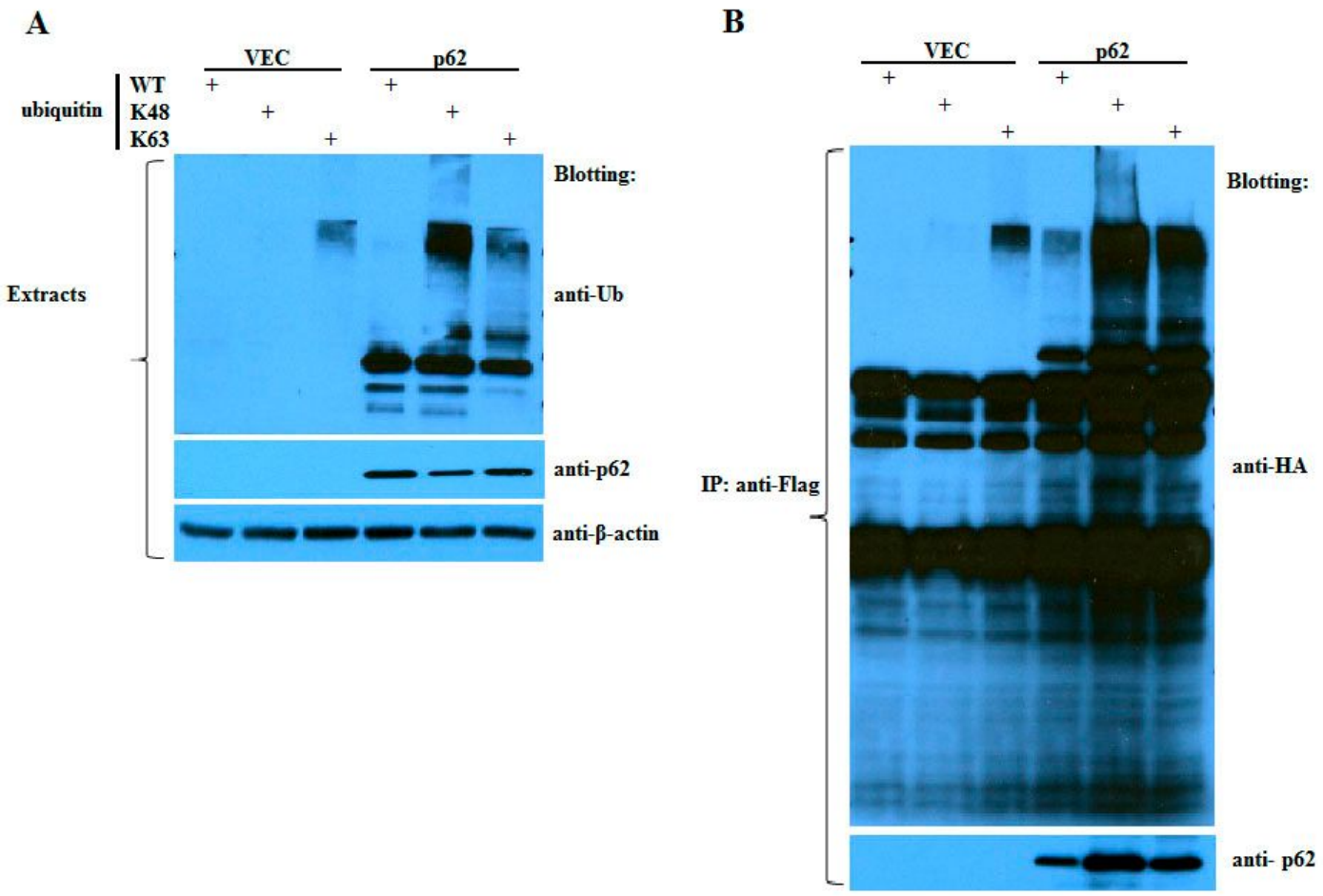

Figure 3

Site-specific ubiquitination of cellular proteins after p62 protein was overexpressed in ARPE-19 cells. The expression vector of HA-tagged wild-type (WT), K48-linked ubiquitin, or K63-linkage ubiquitin was co-transfected into ARPE19 cells with Flag-tagged p62 or Flag-tagged vector and cultured for 48 h. (A) Cell extracts were subjected to Western blotting for p62 and ubiquitination of proteins. (B) Cell extracts were subjected to immunoprecipitation using an anti-Flag antibody, and the immunocomplexes were analyzed by Western blotting using anti-HA and anti-p62 antibodies.

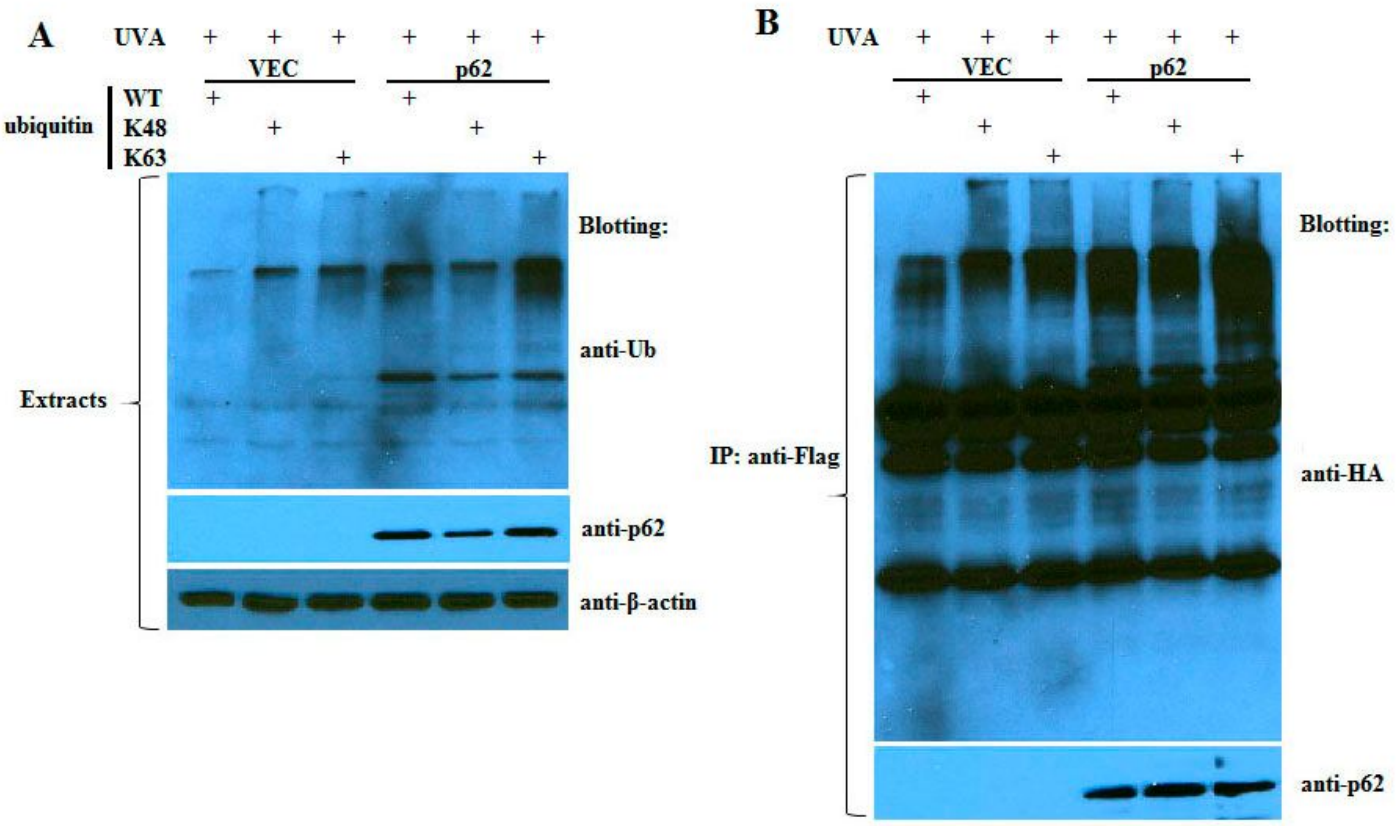

Figure 4

Site-specific ubiquitination of cellular proteins in response to UVA irradiation. ARPE-19 cells that were transfected were cultured for 24 h and exposed to UVA. Then they were cultured for another 24 h. (A) Cell extracts were subjected to Western blotting for p62 and ubiquitination of proteins. (B) Cell extracts were subjected to immunoprecipitation using an anti-Flag antibody, and the immunocomplexes were analyzed by Western blotting using anti-HA and anti-p62 antibodies.

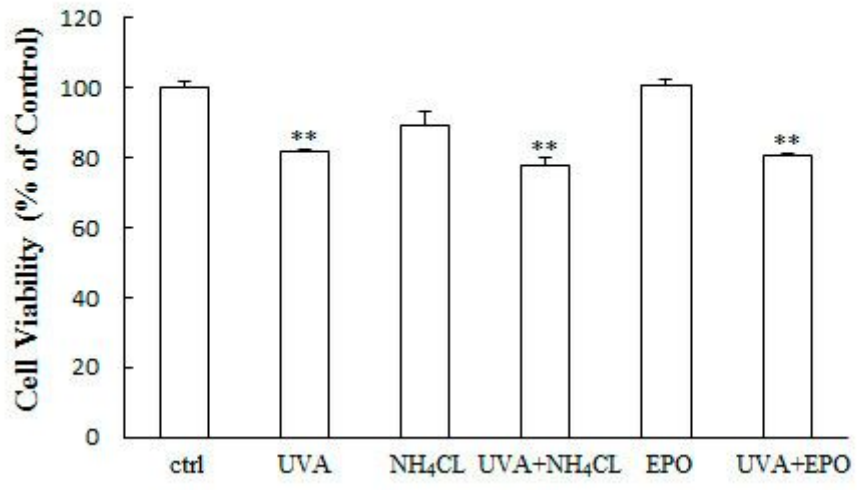

Figure 5

UVA irradiation induces ARPE19 cell death, which cannot be reversed by autophagy changes. ARPE19 cells were treated with or without 20 mM NH4CL or 50 nM EPO for 24 h after UVA radiation, and cell viability was assessed with a MTT-based CDG1 assay. The results are expressed as the percentages of controls and are represented by the mean ± SD of three independent experiments. Ctrl stands for the control group. P values <0.05 are shown by “*” as statistically significant and <0.01 by “**” as extremely significant.