• Basic Research •

Down-regulation of protein kinase C alpha/ezrin signals in light-induced phagocytic crisis of retinal pigment epithelium cells

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Abstract

• AIM: To investigate the roles of PKC-α/ezrin signals in phagocytosis crisis of retinal pigment epithelium (RPE) cells in light damage model.

• METHODS: Light induced mice RPE injury model was established by continuously irradiating cool white light at different exposure time (0, 4, 8h light intensity: 4.18×10^{-6} J/cm^2). *In vitro*, human ARPE-19 cells treated with the doses and intensity (1.57×10^{-6} J/cm^2) of laser irradiation. Histology analysis was evaluated by hematoxylin and eosin (HE) staining. *In vivo* RPE phagocytosis was quantified by measuring the accumulation of photoreceptor outer segments in the sub-retinal space. *In vitro* RPE phagocytosis was assessed by calculating the relative fluorescence intensity of FITC-labeled microspheres in ARPE-19 cells. To further investigate the molecular mechanism, the activation of PKC-α/ezrin signal was evaluated by Western blot *in vivo* and *in vitro*.

• RESULTS: HE staining revealed that the thickness of outer nuclear layer decreased significantly after 4 and 8h light exposure. By immunostaining with rhodopsin, a significant greater accumulation of photoreceptor outer segment was noticed after light injury. *In vitro*, light injured RPE cells had less phagocytic activity in a dose dependent manner than that of the normal control (P<0.01). Western blot suggested the activation of PKC-α/ezrin signaling was down-regulated in a dose-dependent manner after light exposure.

• CONCLUSION: Our data suggest that light induced phagocytic crisis of RPE cells may result from the down-regulation of PKC-α/ezrin signaling.

• KEYWORDS: age-related macular degeneration; retinal pigment epithelium; ezrin, light injury; phagocytosis
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INTRODUCTION

Age-related macular degeneration (AMD), with an increasing prevalence, is one of the leading causes of irreversible visual impairment and blindness in the elderly worldwide[1-2]. Polarized retinal pigment epithelial cells act pivotal roles in retinoid cycle. Dysfunction of retinal pigment epithelium (RPE) phagocytic activity is one of the main mechanisms of dry AMD[3], and light induced RPE degeneration is a well-recognized AMD model. Overdoses of light exposure can cause a photochemical effect[4-5] which results in the activation of oxidative stress[6] and decrease of RPE phagocytosis[7]. Ezrin, a member of ezrin/radixin/moesin (ERM) protein family, is an important polarity protein mainly located in the apical side of RPE cells[8-9]. Recent studies suggested that it acts a pivotal role in RPE phagocytosis[10-11], adhesion, migration as well as membrane transportation[12]. Protein kinase C alpha (PKC-α) is the upstream regulator of ezrin[13-14]. PKC-α/ezrin has been reported as the key signal pathway regulating the phagocytosis of many types of cells[14], including RPE cells, but its roles in light induced dysfunction of RPE phagocytosis is still largely unknown. The objective of this study is to investigate the potential roles of PKC-α/ezrin signaling in light induced dysfunction of RPE phagocytosis. We hypothesize that the mixed wavelength white light can

cause the dysfunction of RPE phagocytic activity and this effect is regulated by PKC-α/ezrin signal.

MATERIALS AND METHODS

Study Design *In vivo* and *in vitro* light-induced RPE injury models were established by continuous laser irradiation with a cool white LED emitter (model LG-150W, Beijing Paidiwei Instrument Co., Ltd, Beijing, China). *In vivo* RPE phagocytosis was evaluated by measuring the accumulation of rhodopsin positive photoreceptor outer segment (POS) in mice retina. *In vitro* RPE phagocytosis was quantified by the intensity of engulfed fluorescein isothiocyanate microbeads in human RPE cells. Western blotting was utilized to identify the activation of ezrin/p-ezrin/PKC-α pathway before and after the light injury. Hematoxylin and eosin (HE) staining was used for the histology.

Animals The study was approved by the Animal Care and Use Committee of Jinzhou Medical University (SCXK-Jing 2012-0001) in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Eight-week-old C57BL/6J mice purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China) were maintained in a 12h light/dark cycle and freely accessed to food and water in a SPF laboratory Animal Center.

Cell Culture Human ARPE-19 cells were purchased from American Tissue Culture Collection (ATCC; Rockville, MD, USA) and maintained in Dulbecco's modified eagle's medium (DMEM; Hyclone, gelifesciences, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies, NY, USA) in a 37℃ incubator. The medium was changed every three days. Cells were digested with 0.25% trypsin/0.02% ethylenediaminetetraacetic acid solution and passaged at 100% confluency.

Light Injury Model

***In vivo* model** Mice pupils were dilated by 0.5% tropicamide (Shanghai Shentian Pharmaceutical Co., Shanghai, China) 30min before light exposure. Then the mice were exposed to cool white light from 8 a.m. to 4 p.m., 12 a.m. to 4 p.m., respectively. The control mice were sham irradiated.

***In vitro* model** After the cells became 80% confluent, they were trypsinized and seeded into six-well-plate at a density of 3×10^5 cells per well. After 24h, they were exposed to the white light at the intensity and doses of 1.57×10^{-6} J/cm^2 from 8 a.m. to 4 p.m., 12 a.m.to 4 p.m., respectively. The cell without light exposure served as the control. The detailed information about the laser instrument was described as below (Table 1).

Table 1 Device information

Manufacturer	Beijing Paidiwei Instrument Co., Ltd, Beijing, China
Year of product	2015
No. of emitter	1
Emitter type	Semiconductor diode laser
Beam delivery system	Fiber optic
Wavelength	White light with mixed wavelength
Irradiation model	Continuous
Max output power	150 W
Light intensity	0.8 W/cm^2; cell: 0.3 W/cm^2

Phagocytosis Assay

***In vivo* phagocytosis** After treatment, the mice were sacrificed and the eyes were enucleated and fixed by 4% paraformaldehyde (PFA) for 24h. After dehydration with 30% sugar solution, the eyes were frozen and sectioned. Retinal sections were blocked by 1% goat serum for 1h and incubated with rat-anti-rabbit rhodopsin antibody (1:200, ab 3424, Abcam, USA) overnight at 4 degrees. After washing with phosphate buffered saline (PBS) for three times, and the sections were labeled by primary rhodopsin antibody and secondary antibody conjugated with Alexa Fluor. The cell nuclears were counterstained with 4'-6-diamidino-2-phenylindole (DAPI). Pictures were taken and POS thickness was measured.

***In vitro* phagocytosis** To evaluate the RPE phagocytosis *in vitro*, the cells were incubated with FITC-labelled microbeads (0.1 μm diameter, Invitrogen, Karlsruhe, Germany) at a microbead/cell ratio of 1:16 000 in a 37℃ incubator for 1h, then washed with PBS three times. After fixation with 4% PFA for 10min, 20 pictures were taken from each sample using a confocal microscope with the same settings (FV10C-W3, Olympus, Tokyo, Japan). The relative amount of fluorescence intensity was semi-quantified using ImageJ.

Western blot After harvest, the retinas and ARPE-19 cells were lysed on ice. The total protein was extracted and titered by bicinchoninic acid assay kit (#P0012, Beyotime Biotechnology, Nanjing, China). Samples were denatured at 98℃ for 5min and 20 μL of protein was loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, separated by electrophoresis and transferred onto poly vinylidene fluoride membrane. After blocking with 1% bovine serum albumin (BSA; Sigma, Deisenhofen, Germany)/TBST (1 mL Tween 20/1 L Tris-buffer saline), the members were incubated with primary antibodies at 4℃ overnight. After three times washing with TBST, the members were incubated with goat-anti-rabbit or goat-anti-mouse IgG (H+L), horseradish peroxidase conjugate secondary antibodies (#SA00001-2, #SA00001-1; Proteintech Group Inc., IL, USA) at room temperature for 1h. The bands were visualized by an enhanced chemiluminescence substrate (Bio-Rad, Hercules, CA, USA). The protein expression was semi-quantified by ImageJ. The primary antibodies used in this study were: mouse anti-ezrin, rabbit anti-ezrin (phospho Thr

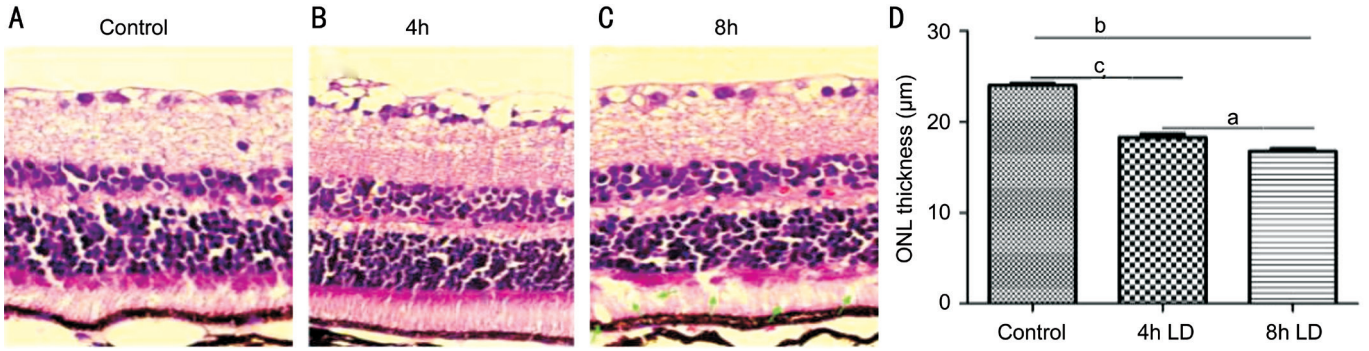

Figure 1 Reduction of outer nuclear layer thickness after light exposure A: Retinas were fixed by 4% PFA, sectioned and stained with HE. Normal retina was well-organized; B, C: Compare to the normal control, the thickness of outer nuclear layers in light damaged groups were significant thinner (P<0.01); C: Additionally, some giant vacuoles can be seen in 8-hour-light exposure group (green arrows); D: The average thickness of outer nuclear layer (ONL) showed a dose dependent decrease manner. [a]P<0.05, [b]P<0.01, [c]P<0.001.

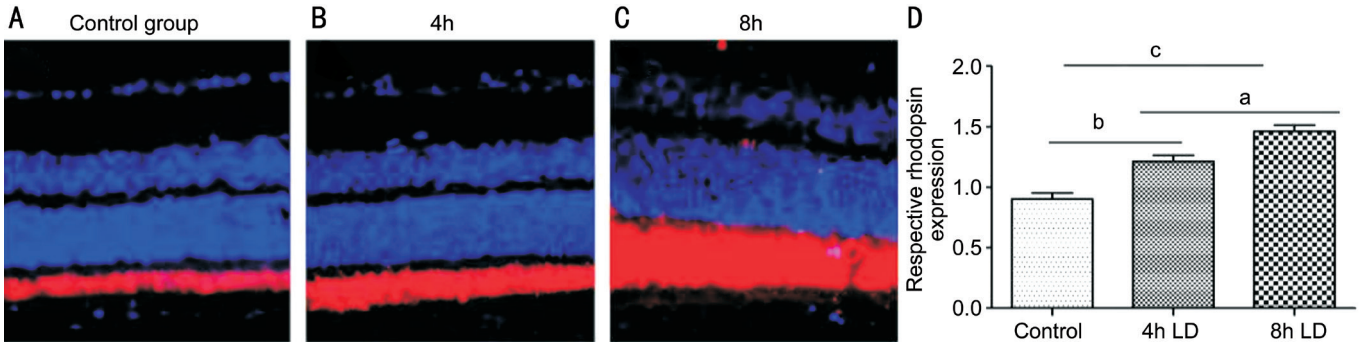

Figure 2 Decrease RPE phagocytosis after light injury *in vivo* A: Dysfunction of RPE phagocytosis *in vivo* was evaluated by measuring the thickness of rhodopsin labelled POS. In normal retina, the average thickness of POS; B-D: Light injury induced a dose dependent accumulation of POS in mice retina. [a]P<0.05, [b]P<0.01, [c]P<0.001.

567)/Radixin (phospho Thr 564)/Moesin (phospho Thr 558), rabbit anti-PKC-α and mouse anti-beta actin. All the antibodies were purchased from Abcam. The working dilution is 1:1000.

Histology The eyes were fixed with 4% PFA and dehydrated by sucrose gradients, then embedded in paraffin. Five micron sections were obtained and stained with HE. Pictures were taken under conventional light microscope.

Statistical Analysis The data was presented as mean±standard error. One-way analysis of variance (ANOVA) was performed to calculate the statistical difference. A minimum of six samples were obtained from each group. Alpha=0.05.

RESULTS

The Loss of Photoreceptors After Light Injury To evaluate the effect of light damage on retinal photoreceptors, the sections were stained with HE. The average thickness of outer nuclear layer in light damaged groups were 92.08±0.6067 μm (Figure 1B), and 81.17±0.8250 μm (Figure 1C) in 4-hour group and 8-hour group, respectively, significantly thicker than that of the control (122.42±0.2183 μm), both P<0.01. Additionally, the POS layer was not well organized in the 8-hour-light damaged groups and some giant vacuoles can be found (Figure 1C, green arrows).

Decrease of Retinal Pigment Epithelium Phagocytosis After Light Exposure We then evaluate the effects of light induced dysfunction of RPE phagocytosis. *In vivo*, the thickness of rhodopsin labeled POS in normal control was 10.25±0.008 μm (Figure 2A), significantly thinner than light damaged groups in a dose-dependent manner 10.25±0.008 *vs* 17.53±0.125 μm in 4-hour group, and 10.25±0.008 *vs* 28.00±0.303 μm in 8-hour group, both P<0.01 (Figure 2B, 2C).

Higher dose of light irradiation induced greater accumulation of POS (P<0.05). *In vitro*, RPE phagocytosis was quantified by measuring the average fluorescence intensity of FITC-labeled microbeads engulfed by RPE cells. The normal control group without light treatment showed the highest fluorescence intensity (mean fluorescence intensity: 0.067±0.0147; Figure 3A) compared to the light injured groups in Figure 3B and 3C. The decrease of RPE phagocytosis showed a dose dependent manner 0.0365±0.005 *vs* 0.021±0.006, P<0.05 (Figure 3D).

Down-regulation of Ezrin/ Protein Kinase C Alpha Signalings After Light Exposure To investigate the potential roles of ezrin/PKC signaling in the dysfunction of RPE phagocytosis *in vivo* and in *vitro*, the expression of ezrin, p-ezrin and PKC-α were semi-quantified by Western blotting. Compared to the normal control, the expression of ezrin, p-ezrin and PKC-α decreased in a dose dependent manner *in vitro* (P<0.05 in all three proteins; Figure 4). Consistently, *in vivo* experiment showed a similar result (P<0.05 in all three proteins; Figure 5).

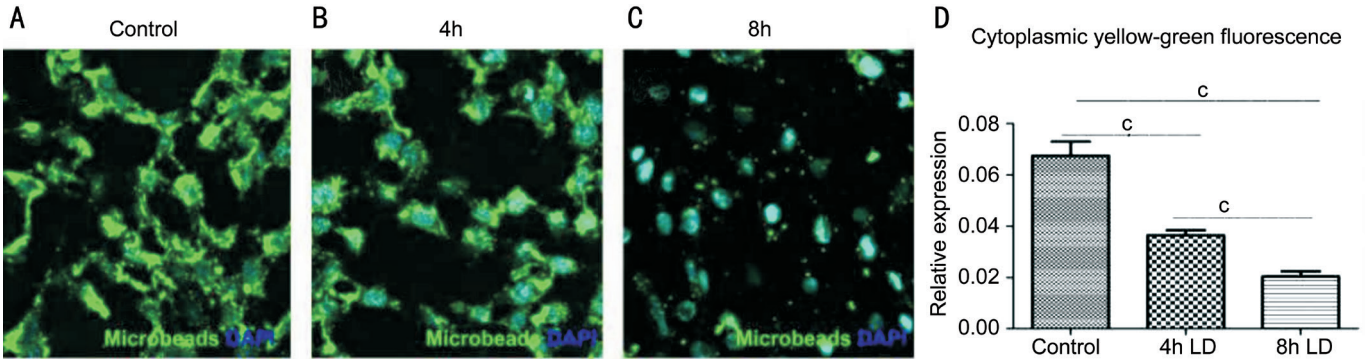

Figure 3 Decrease of RPE phagocytosis after light injury *in vitro* RPE phagocytosis was quantified by the mean fluorescence intensity of FITC-labeled microbeads engulfed by RPE cells. The normal control group showed the highest fluorescence intensity (A) compared to the light injured groups (B, C). The decrease of RPE phagocytosis showed a dose dependent manner (D). $^{c}P<0.001$.

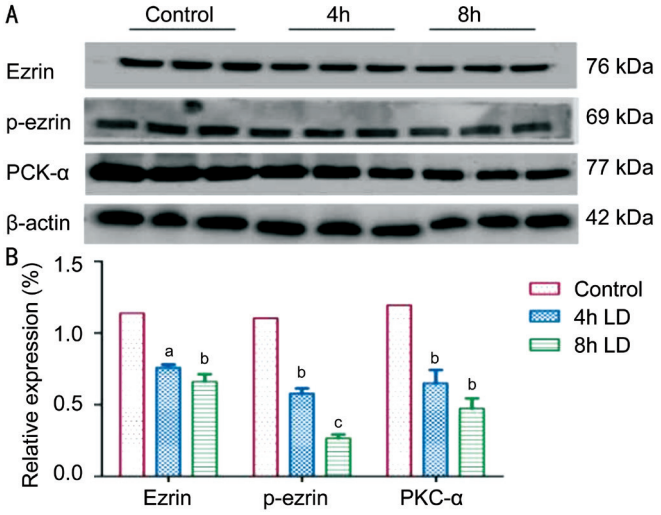

Figure 4 Decrease the expression of ezrin, p-ezrin and PKC-α after light injury *in vitro* A: The expression of ezrin, p-ezrin and PKC-α were semi-quantified by Western blotting; B: Compared to the normal control, the expression of ezrin, p-ezrin and PKC-α all decreased in a dose dependent manner *in vitro*. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

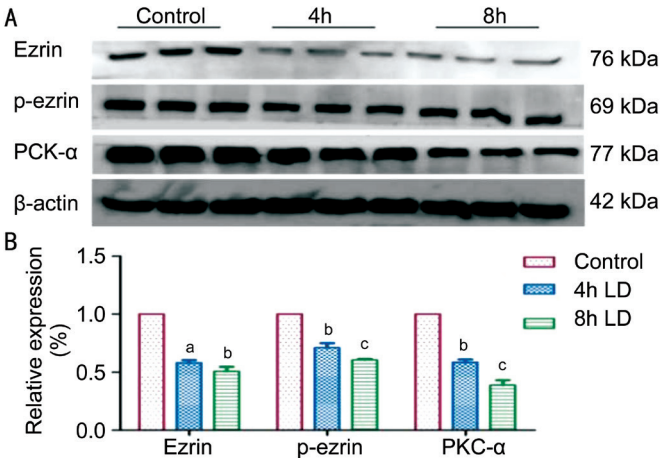

Figure 5 Decrease the expression of ezrin, p-ezrin and PKC-α after light damage *in vivo* A: The expression of ezrin, p-ezrin and PKC-α were semi-quantified by Western blotting; B: Consistent with the in vitro study, the expression of ezrin, p-ezrin and PKC-α all decreased statistically in a dose dependent manner after light exposure. $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$.

DISCUSSION

Light induced dysfunction of RPE phagocytosis plays a pivotal role in the pathogenesis of dry AMD. Our results suggested mixed wavelength white light can cause the loss of retinal photoreceptor, disorganization and accumulation of POS. Decrease of phagocytic activity might result from the inhibition of ezrin/PKC signaling. Accumulation of POS between photoreceptor and RPE layer may cause by the dysfunction of RPE phagocytosis. Our research showed that the dysfunction of phagocytosis in mice and ARPE-19 cells was in a time-dependent manner, especially the increasing of rhodopsin under the light damage. It is well known that ezrin plays a major role of phagocytosisin RPE[15], primary human malignant melanomas and *etc*[16-17]. We further showed that the gradual down-regulated expression of ezrin accompanied by the decreased ability for phagocytosis of POS by light damage induced RPE. C-terminal threonine T567 of ezrin is the phosphorylated target by PKC-α[13-14,18-19], which is responsible for maintenance cell phagocytosis, polarity and *etc*. We went further to detect the expression of phosphorylated ezrin and PKC-α in light damage induced RPE. The results showed a down regulation of phosphorylated ezrin and PKC-α in a time-dependent manner both *in vivo* and *in vitro*. In the experiment described by Ueta *et al*[20], the physiological range of light stimulus was from 5 to 15 lx. In this study, RPE cells might be damaged by toxic light exposure-8000 lx in C57BL/6J mice. This may lead to the pyknotic nuclei of photoreceptor, diffuse swelling and disruption of the inner segment. The thickness of outer nuclear layer adjacent to disorganization POS was significantly decreased. The pathological changes of photoreceptor and RPE lead to dysfunction of opsin synthesis result in RPE overloaded with undigested POS. Indeed, with toxic levels of white light, RPE is no longer to maintain photoreceptor homeostasis[21]. In addition, histological analysis indicated that exposure time may enhance susceptibility to light damage in mouse and exceed retinal neuronal cells to death with their loss function. Therefore, phagocytic clearance

is required to remove death retinal cells and metabolic waste. With the major function of phagocytosis and autophagy of RPE[22], daily clearance of shedding POS and metabolic waste is important to maintain disk renewal and preservate the visual cycle. It has been reported by Ferguson and Green[22] that a non-canonical autophagy named LC3-associated phagocytosis (LAP) was related to the mechanism of AMD. Actin filaments and microtubule-dependent motor proteins, major components of cytoskeleton elements, have been reported as a critical part in the internalization of phagosomes after POS ingested by RPE cells[23]. Ezrinis essential for the maintenance in morphogenesis of apical microvilli and basal infoldings in RPE[8-9]. It has been reported that decreased ezrin in ezrin−/− mice and in ezrin antisense oligonucleotides added primary cultures of rat RPE reduced the length and number of apical microvilli and the elaborate basal infoldings typical of these cells[8-9]. As conserved C-terminal ERM association domain residue in human ezrin, Thr567 is phosphorylated coincides with activation[24]. It is reported that ezrin involves in phagocytosis[25-26] and C-terminal threonine Thr567 is the phosphorylated target by PKC-α[13-14]. In this study, we assess light damaged RPE phagocytosis was reduced, which was consisted with the down-regulated expression of PKC/ezrin in light damaged RPE cells.

In conclusion, we performed the effect of light damage on RPE phagocytosis. Firstly, excessive light exposure damaged the morphology of retina; secondly, we have uncovered a phenomenon in which excessive light exposure reduces RPE phagocytosis; lastly, down-regulation of PKC-α/ezrin signal is related to light induced phagocytosis crisis of RPE cells.

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Conflicts of Interest: Zhang YQ, None; **Fan YG,** None; **Dang YL,** None; **Liu YL,** None; **Liu H,** None; **Li LH,** None.

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