Intense Physiological Light Upregulates Vascular Endothelial Growth Factor and Enhances Choroidal Neovascularization via Peroxisome Proliferator-Activated Receptor γ Coactivator-1α in Mice

Takashi Ueta, Tatsuya Inoue, Kentaro Yuda, Takahisa Furukawa, Yasuo Yanagi, Yasuhiro Tamaki

Objective—Toxicity of intense light to facilitate the development of neovascular age-related macular degeneration has been a health concern although the mechanism remains unclear.

Methods and Results—Effects of intense, but within physiological range, light on retinal pigment epithelium, a major pathogenic origin of age-related macular degeneration were studied in mice. Intense physiological light upregulated vascular endothelial growth factor (VEGF) expression in retinal pigment epithelium, independent of circadian rhythm, which resulted in enhancement of choroidal neovascularization. In *rd1/rd1* mice or $Crx^{-/-}$ mice that do not possess outer segment structure, light exposure did not induce VEGF, indicating that VEGF upregulation by light depended on increased outer segment phagocytosis by retinal pigment epithelium. In retinal pigment epithelium cells phagocytosing increased amount of outer segment, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) not hypoxia-inducible factor-1α was induced, leading to VEGF upregulation. The VEGF upregulation and choroidal neovascularization enhancement were abrogated in $PGC\text{-}1\alpha^{-/-}$ mice and estrogen-related receptor-$\alpha^{-/-}$ mice, indicating the involvement of PGC-1α/estrogen-related receptor-α pathway.

Conclusion—Intense physiological light is involved in choroidal neovascularization through excess outer segment phagocytosis and VEGF upregulation mediated by PGC-1α in vivo. **(*Arterioscler Thromb Vasc Biol*. 2012;32:1366-1371.)**

Key Word: angiogenesis ■ choroidal neovascularization ■ retinal photoreceptor cell outer segment ■ Ppargc1a protein, mouse

Age-related macular degeneration (AMD) is a prominent cause of legal blindness worldwide.[1] Although it is difficult to measure light exposure in human subjects, several population-based studies have indicated the causality of intense light exposure, such as sunlight exposure, for the development of late AMD,[2–5] especially neovascular AMD that is choroidal neovascularization (CNV) in the elderly.[2,3]

Recent experimental studies have also suggested the association of light exposure and neovascular AMD,[6–9] although they have been based on a hypothesis that the reaction between the light and the increased amount of bisretinoids in retinal pigment epithelium (RPE) might be sufficient to induce VEGF upregulation or inflammation.

A conventional animal model for light-induced toxicity on the retina has been a rodent model exposed to visible light with intensities with several thousand lux (lx),[10,11] that causes acute loss of photoreceptors. Recently, 3000 lx light was reported to induce CNV besides retinal degeneration[12]; however, a considerable problem of the existing animal model of light toxicity is that the light intensities are unphysiologically strong, and it has been difficult to discuss the association of light exposure and AMD. For instance, mice entrain circadian rhythm with <0.1 lx light[13,14] and are maintained at <10 to 20 lx in experimental conditions; conversely, for humans, sunlight, that is around 50 000 lx, is just within physiological range, although it is often unpleasant. In the present study, we developed a mouse model of intense physiological light (IPL) exposure and studied its effects on RPE and CNV, pathogenic origins of neovascular AMD.

Research Design and Methods

All experiments in the present study were conducted according to ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Research Committee of the University of Tokyo.

Adult male mice 8 to 12 weeks old were used in all experiments, except $Crx^{-/-}$ mice which was used at the age of 8 weeks. *rd1/rd1* mice were purchased from Saitama Experimental Animals Supply Co Ltd. Peroxisome proliferator-activated receptor γ coactivator-1α ($PGC\text{-}1\alpha)^{-/-}$ mice were purchased form Jackson Laboratory. Estrogen-related receptor-α ($ERR\text{-}\alpha)^{-/-}$ mice were kindly provided by Novartis Institutes for BioMedical Research Inc. All the mice used in this study were raised in a C57BL/6 background. Mice were kept under 5- to 10-lx (12 hours) light/dark (12 hours) cycle at 22°C, if not

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mentioned otherwise. Light exposure up to weak 15 lx light did not change the temperature inside the cages.

Details of the methods including light exposure experiments, VEGF mRNA and protein measurements, Western blotting, antibodies, primers, and statistics are provided in the online-only Data Supplement.

Results

Development of a Mouse Model of IPL Exposure

First, we defined the intensity of IPL in C57BL/6J wild-type (WT) mice. Because generally in experiments using mice, they are kept under 10 to 20 lx light at most, we first addressed whether light <20 lx can cause discomfort for WT mice. A WT mouse was placed in a cage illuminated with varying intensities of visible light (1–20 lx) or in a cage with complete darkness. One mouse was placed in 1 cage at a time and the test was repeated 7 to 8 times for each cage. The location of the mouse in each cage was recorded 4 hours later. In the illuminated cage, no mice chose to stay in the area >10 lx of light while in the cage under darkness, mice did not show any preference to a specific area of the cage (Figure 1A). We also confirmed that 15 lx light was physiological for mice; 15-lx light (12 hours)/dark (12 hours) cyclic light for 3 weeks neither damaged photoreceptors or RPE cells nor changed the RPE65 protein level or zonula occludens-1 protein expression in RPE cells (Figure 1B). In this study, therefore, 15 lx was chosen as a representative of IPL for mice.

IPL Upregulates VEGF Expression in RPE of WT Mice

Reportedly, the expression of VEGF increases in RPE cells by treatment with outer segment (OS) in vitro.[15] At the same time, it has been well established that physiological light exposure is implicated in the initiation and promotion of OS shedding by photoreceptor cells, and the shed OS are phagocytosed by RPE cells.[16–21] Based on this previous evidence, we speculated that exposure to IPL could increase VEGF expression in mouse RPE in vivo. VEGF protein produced in RPE is released basolaterally into the choroidal vasculature, an event implicated in both the maintenance of the choroidal vasculature[16,22] and the pathological CNV.[23–25] Indeed, the increased level of VEGF-A protein was detected in the RPE/choroid of WT mice exposed to 15 or 150 lx, but not 0.5 lx of light, compared with those kept in the dark (Figure 2A). The VEGF-A upregulation followed a circadian pattern, increasing during the day and decreasing during the night (Figure 2B). To understand the circadian-independent, direct effect of light on the VEGF upregulation, the unilateral eyes of WT mice were closed (the unilateral closed-eye model) and the mice were maintained for 24 hours in the dark. The mice were then exposed to 0.5 or 15 lx of light at night. Again, an increase in VEGF-A protein level was observed in open eyes of mice under IPL at night, indicating that the VEGF upregulation occurred as a result of the direct effect of IPL on the eyes and independent of circadian rhythm (Figure 2C). To confirm the source of the increased VEGF protein in the RPE/choroid tissues, *VEGF-A* mRNA expression was evaluated in RPE and choroid separately. The increased *VEGF-A* mRNA was detected in RPE, but not in the choroid, of the WT mice exposed to 15 lx of light (Figure 2D). In addition, we evaluated the increased OS phagocytosis by RPE on IPL in WT mice, which occurs in a circadian-independent manner (Figure I in the online-only Data Supplement). Furthermore, in mice that lack OS structure (ie, *rd1/rd1* and *Crx*$^{-/-}$ mice[26,27]), VEGF-A protein level in RPE/choroid or *VEGF-A* mRNA in RPE did not change on IPL (Figure 2E and Figure II in the online-only Data Supplement).

VEGF Upregulation in RPE Cells Treated With OS Is Mediated by PGC-1α/ERR-α Pathway In Vitro

Next, we explored the mechanism of the VEGF upregulation in RPE cells phagocytosing increased volume of OS, using the cultured human RPE cell line, ARPE-19 cells. Consistent with our findings in vivo and a previous report,[15] the expression of both *VEGF-A* mRNA in ARPE-19 cells and VEGF-A protein released in the medium increased, depending on the amount of OS to treat the cells (Figure 3A and 3B). In ARPE-19 cells transfected with a reporter plasmid containing human VEGF promoter region (from −1180 to +338) driving the luciferase gene,[28] the treatment with OS led to a 3-fold increase in the induction of luciferase activity (Figure 3C), indicating that OS treatment on RPE cells resulted in the activation of VEGF transcription.

In RPE cells, transcriptional regulation of VEGF has been well characterized by hypoxia-inducible factor-1α (HIF-1α).[29,30] However, recent studies have revealed the importance of the HIF-independent, PGC-1α/ERR-α

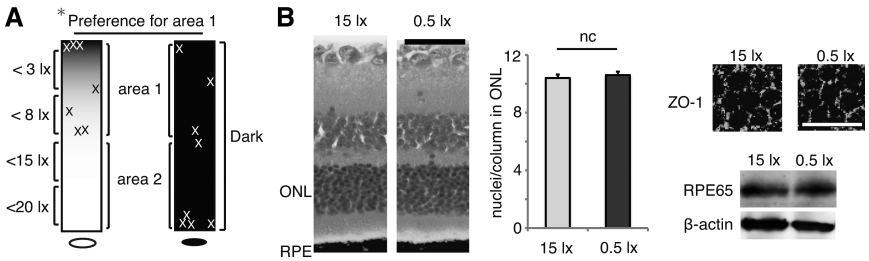

Figure 1. Definition of intense physiological light for mice. **A**, A wild-type (WT) mouse was placed in the cage with varying intensities of light or in the same cage with complete darkness. One mouse was placed in the cage at a time, and the test was repeated 7 to 8 times for each cage. Location of each mouse was marked as "X." Preference for area 1 was compared between the 2 cages (*$P<0.05$, by Fisher exact test). **B**, Eyes of WT mice maintained under 15-lx light/dark cycle or 0.5-lx light/dark cycle for 3 weeks. The integrity of photoreceptor cells was evaluated by the thickness of outer nuclear layer (ONL). The distribution of zonula occludens (ZO)-1 protein in RPE was evaluated on flatmount, and RPE65 protein level in RPE on Western blotting (scale bar, 50 μm).

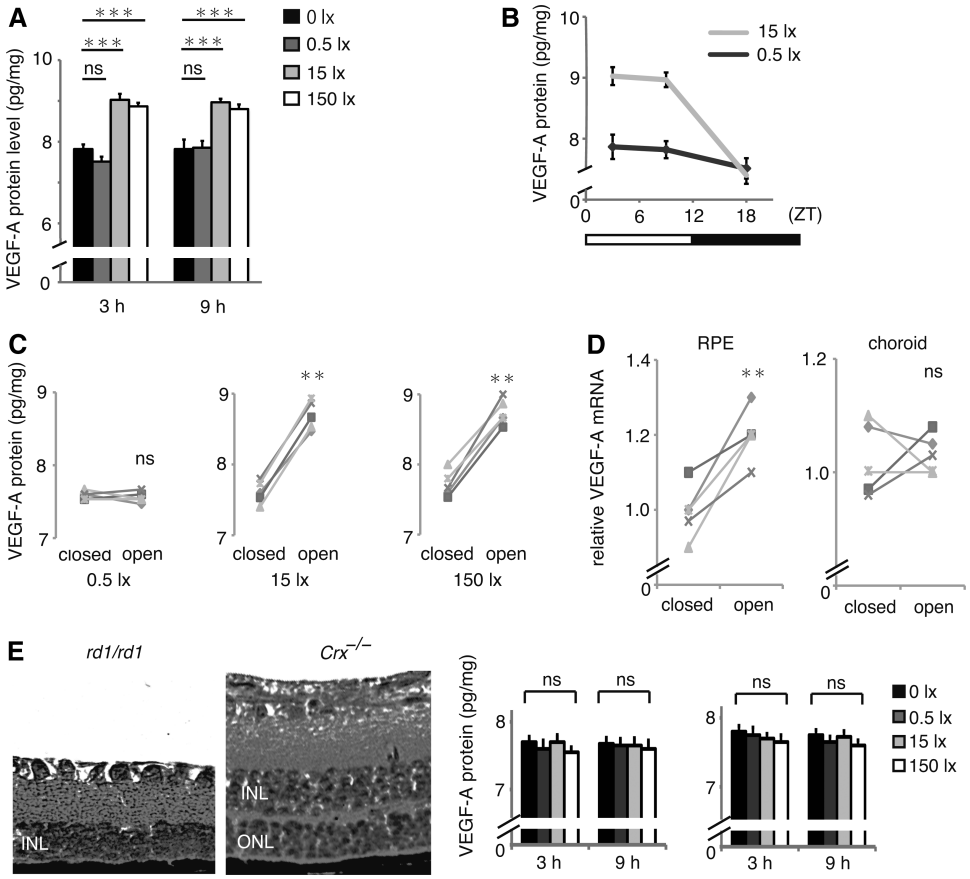

Figure 2. Vascular endothelial growth factor (VEGF) expression increases in retinal pigment epithelium (RPE) of wild-type (WT) mice when exposed to intense physiological light (IPL). **A**, Total VEGF-A protein (pg/mg extract) in RPE/choroid was measured in WT mice under 0, 0.5, 15, or 150 lx for 3 or 9 hours (mean±SEM; n=8–15 per group; ns, not significant; ***$P<0.001$, by ANOVA, followed by post hoc Dunnett test in comparison with the 0 lx group). **B**, Circadian change in VEGF-A protein level in RPE/choroid of WT mice under 0.5-lx light (12 hours)/dark (12 hours) cycle and 15-lx light (12 hours)/dark (12 hours) cycle. **C**, Comparison of the total VEGF-A protein (pg/mg) in RPE/choroid between 2 eyes of the same WT mice using the unilateral closed-eye model. Mice were exposed to 0.5, 15, or 150 lx for 3 hours (n=5; **$P<0.01$, by paired *t* test). **D**, Relative *VEGF-A* mRNA expression was measured in RPE or choroid of WT mice by real-time reverse transcriptase polymerase chain reaction. The effect of IPL was examined in the unilateral closed-eye model (n=5, **$P<0.01$, by paired *t* test). **E**, Total VEGF-A protein (pg/mg extract) in RPE/choroid was measured in *rd1/rd1* and *Crx*$^{-/-}$ mice under 0, 0.5, 15, or 150 lx of light for 3 or 9 hours (mean±SEM; n=8 per group; ns, not significant by ANOVA; INL, inner nuclear layer; ONL, outer nuclear layer).

pathway in the transcriptional regulation of VEGF.[31] OS consists of a pile of uniquely folded cell membrane disks that are degraded into fatty acids in RPE cells following phagocytosis.[17] Given that PGC-1α/ERR-α signaling is critical in the metabolism of fatty acids, we speculated that this pathway might be involved in the VEGF transcription in RPE cells phagocytosing OS. There has also been a report that OS phagocytosis activates the expression of peroxisome proliferator-activated receptor-activated receptor-γ, a target of PGC-1α, in a rat RPE primary culture.[32] Indeed, in ARPE-19 cells treated with OS, expression of *PGC-1α* mRNA, but not *HIF-1α* mRNA, increased in a dose-dependent manner (Figure 3D). OS treatment did not change HIF-1α protein stability; meanwhile, it induced expression of PGC-1α protein (Figure 3E). In addition, OS treatment increased the expression of other key target genes in the PGC-1α/ERR-α pathway, including genes for fatty acid utilization (medium-chain acyl-CoA dehydrogenase and pyruvate dehydrogenase kinase 4) and fatty acid transport (CD36) (Figure III in the online-only Data Supplement).

We investigated the effect of the PGC-1α/ERR-α pathway on the *VEGF-A* gene using siRNAs designed to specifically knock down PGC-1α and ERR-α in ARPE-19 cells (Figure IV in the online-only Data Supplement). Knockdown of these transcriptional regulatory factors diminished the induction of *VEGF-A* mRNA in ARPE-19 cells treated with OS (Figure 3F).

PGC-1α/ERR-α Pathway Is Implicated in the VEGF Upregulation in RPE Stimulated by IPL In Vivo

We further tested the implication of PGC-1α/ERR-α pathway in the induction of VEGF in RPE stimulated by IPL in vivo. Using the unilateral closed-eye model, we confirmed an increase in the level of PGC-1α protein, but not HIF-1α protein, in the eyes of WT mice exposed to 15 lx of light in vivo (Figure 4A). We also used *PGC-1α*$^{-/-}$ and *ERR-α*$^{-/-}$ mice to confirm the involvement of PGC-*1α*-*ERR-α* pathway in the VEGF upregulation in RPE cells by IPL. Both *PGC-1α*$^{-/-}$ and *ERR-α*$^{-/-}$ mice possess a normal retinal phenotype harboring an intact OS structure (Figure V in the online-only

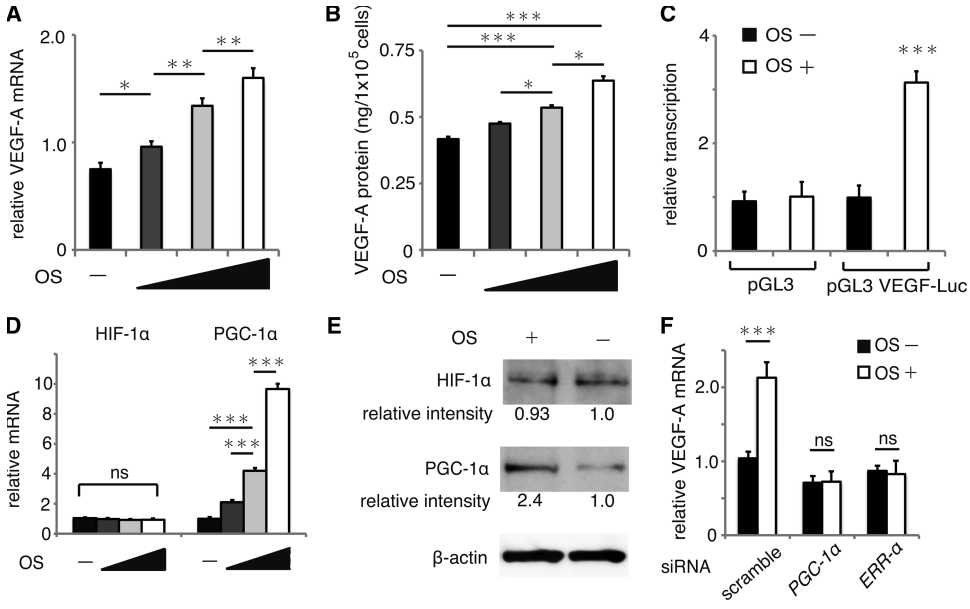

Figure 3. Vascular endothelial growth factor (VEGF) upregulation in retinal pigment epithelium (RPE) cells treated with outer segment (OS) is mediated by PGC-1α/ERR-α pathway in vitro. **A**, *VEGF-A* mRNA in ARPE-19 cells and (**B**) VEGF-A protein level in the medium with or without OS treatment for 3–6 hours were measured by real-time reverse transcriptase polymerase chain reaction and ELISA, respectively (mean±SEM; n=4 per group; *$P<0.05$, **$P<0.01$, ***$P<0.001$, by ANOVA, followed by Tukey post hoc test). **C**, Luciferase activity was measured in ARPE-19 cells, transfected with VEGF or control promoter constructs, with or without OS treatment for 3 hours (mean±SEM; n=4; ns, not significant, ***$P<0.001$ by Student *t* test). **D**, *HIF-1α* and *PGC-1α* mRNA in ARPE-19 cells was measured, with or without OS treatment with OS for 6 hours (mean±SEM; n=4 per group; ***$P<0.001$, by ANOVA, followed by Tukey post hoc test; ns, not significant, by ANOVA). **E**, Lysates of ARPE-19 cells, with or without OS treatment for 6 hours, were immunoblotted for HIF-1α and PGC-1α, and relative protein abundance was measured. **F**, *VEGF-A* mRNA was measured in ARPE-19 cells transfected with scramble siRNA or siRNAs for PGC-1α and ERR-α. ARPE-19 cells were treated with or without OS for 3 hours (mean±SEM; n=4 per group; ***$P<0.001$, by Student *t* test). ERR-α, estrogen-related receptor-α; HIF, hypoxia-inducible factor; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α.

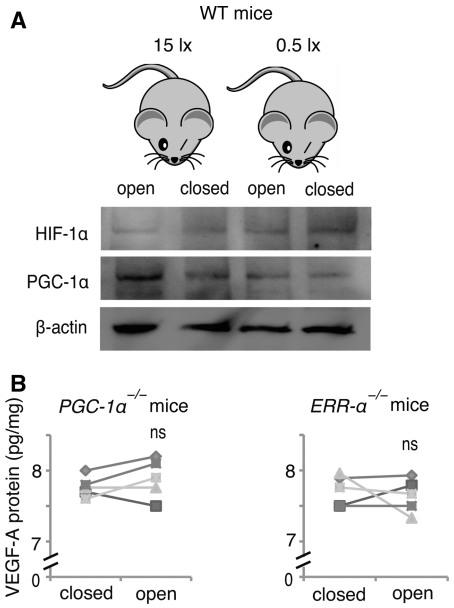

Figure 4. PGC-1α/ERR-α pathway is implicated in the vascular endothelial growth factor (VEGF) upregulation in retinal pigment epithelium (RPE) cells stimulated by intense physiological light in vivo. **A**, Lysates of RPE/choroid of wild-type mice were immunoblotted to compare HIF-1α and PGC-1α protein abundance between the 2 eyes of the same mice using the unilateral closed-eye model. The mice were exposed to 0.5 lx or 15 lx of light for 6 hours. The result was confirmed by 3 independent tests. **B**, Both *PGC-1α*$^{-/-}$ and *ERR-α*$^{-/-}$ mice have normal retinal phenotype harboring outer segment (red). VEGF-A protein level in RPE/choroid was compared between the 2 eyes of the same *PGC-1α*$^{-/-}$ or *ERR-α*$^{-/-}$ mice using the unilateral closed-eye model. Mice were exposed to 15 lx of light for 3 hours (n=5; ns, not significant, by paired *t* test). ERR-α, estrogen-related receptor-α; HIF, hypoxia-inducible factor; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α.

Data Supplement); however, neither VEGF-A protein level in RPE/choroid nor *VEGF-A* mRNA expression in RPE was changed by IPL in these mice (Figure 4B and Figure VI in the online-only Data Supplement).

Pathological CNV Is Enhanced by IPL Through PGC-1α/ERR-α Pathway

Finally, to elucidate the role of exposure to IPL in pathological CNV formation, the laser-induced CNV model was used. After laser treatment for induction of CNV on day 0, WT, *PGC-1α*$^{-/-}$ and *ERR-α*$^{-/-}$ mice were kept under either a 0.5-lx light (12 hours)/dark (12 hours) cycle; a 15-lx light (12 hours)/dark (12 hours) cycle; or continuous darkness for 1 week before the measurement of CNV size (Figure 5A). In WT mice kept under the 15-lx cyclic light, the area of CNV was larger than WT mice kept under 0.5-lx cyclic light or in constant darkness. Meanwhile, in *PGC-1α*$^{-/-}$ and *ERR-α*$^{-/-}$ mice, the 15-lx cyclic light did not increase CNV size (Figure 5B). Furthermore, when WT mice were treated with an anti-VEGF-A neutralizing antibody, the CNV size was not changed by the 15-lx light exposure (Figure 5B).

Discussion

These results indicate the mechanism by which IPL could cause toxicity in RPE and choroidal vasculature; exposure to intense light increases photoreceptor OS phagocytosis by RPE cells, which in turn induces overactivation of the PGC-1α/ERR-α pathway, upregulating VEGF in RPE and enhancing CNV (Figure 6).

PGC-1α is well-known as a powerful transcriptional coactivator facilitating adaptation to physiological stimuli, including adaptive thermogenesis,[33] exercise,[34] and fasting/feeding.[35]

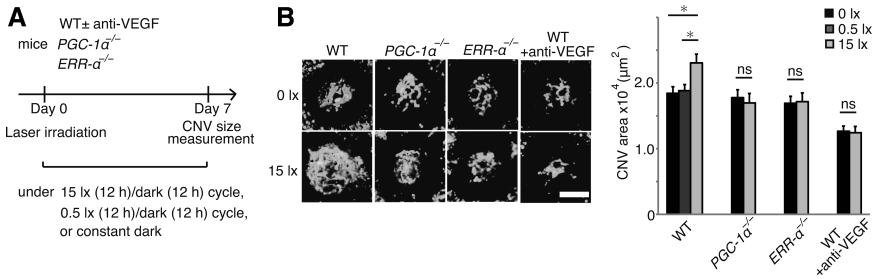

Figure 5. Pathological choroidal neovascularization (CNV) is enhanced by intense physiological light through PGC-1α/ERR-α pathway. **A**, Laser-induced CNV model was used in wild-type (WT), *PGC-1α*$^{-/-}$ and *ERR-α*$^{-/-}$ mice, and WT mice treated with a neutralizing anti-vascular endothelial growth factor (VEGF)-A antibody at day 1. Mice were maintained under constant darkness; 0.5-lx (12 hours) light/dark (12 hours) cycle or 15-lx (12 hours)/dark (12 hours) cycle. **B**, CNV size was measured on day 7 (mean±SEM; n=6–8 per group; *$P<0.05$, by ANOVA, followed by Tukey post hoc test; ns, not significant, by Student *t* test; scale bar, 100 μm). ERR-α, estrogen-related receptor-α; PGC-1α, peroxisome proliferator-activated receptor γ coactivator-1α.

Although PGC-1α also mediates circadian rhythm,[36,37] PGC-1α induction by IPL in the present study was considered as the direct effect of light, not through a change in circadian rhythm because PGC-1α was induced only in the open eyes, not in the closed eyes of WT mice under IPL, and the increased VEGF expression by IPL was also observed during the night. Our results disclosed a novel function of PGC-1α to mediate VEGF induction in RPE cells by light through OS phagocytosis. We consider that PGC-1α-mediated VEGF expression in RPE should be physiologically important to maintain healthy choroidal vasculature. However, our results indicated that the overactivation by the excess OS burden seems to render a pathogenic role.

OS shedding/phagocytosis has been a well-known physiological phenomenon but its physiologic and pathological roles have not been fully understood. As supportive roles of OS phagocytosis, it reportedly protects RPE cells against oxidative stress-induced apoptosis in vitro.[38] In mice whose OS phagocytosis by RPE was disturbed by CD36 abrogation, an increased avascular area in choriocapillaris was observed.[15] In contrast, the deleterious effects of excessive OS phagocytosis by RPE cells have been commonly discussed[16,39,40] because the uptake of photooxidized OS is considered to render oxidative stress to RPE cells,[39] and it is deemed responsible for lipofuscin accumulation in RPE cells which is considered to contribute to the pathogenesis of AMD.[39,40] In agreement with the reported deleterious effects of excessive OS phagocytosis, our results indicate that excessive OS phagocytosis induced by IPL could enhance pathological CNV in vivo. Based on our results and previous literature, VEGF secretion mediated by OS phagocytosis is essential for the development and maintenance of healthy choroidal vasculature[15] while VEGF upregulation of excessive OS phagocytosis could have a pathogenic role. Considering that the declined defense against antioxidative stress is a cardinal feature of aging, OS of photoreceptor cells might be more readily oxidized by light exposure in the aged, which might lead to a further increase in the volume of phagocytosed OS[41] and VEGF expression. In contrast, a previous study reported that monocyte chemotactic protein 1 and IL-8, angiogenic cytokines were upregulated in human ARPE-19 cells by oxidized OS.[40] However, the upregulation was observed only when OS were irradiated by ultraviolet light although ultraviolet light is filtered at the anterior part of the eye including cornea and lens, and does not reach RPE in human eyes.[42,43]

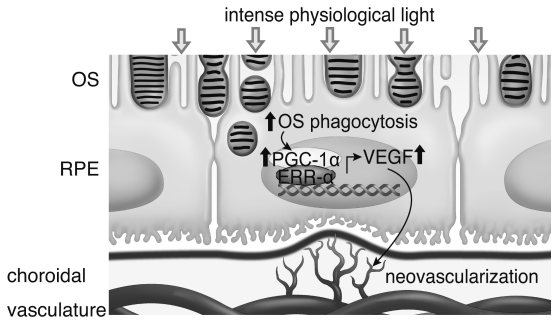

Figure 6. A schematic illustration of the mechanism by which intense physiological light facilitates choroidal neovascularization through outer segment (OS) phagocytosis and PGC-1α/ERR-α pathway in retinal pigment epithelium (RPE) cells. ERR-α, estrogen-related receptor-α; VEGF, vascular endothelial growth factor.

In literature, light toxicity to facilitate CNV has been discussed from the viewpoint of fluorescent bisretinoids, constituents of lipofuscin in RPE cells, including A2E, a by-product of the visual cycle, because they could be activated by visible light exposure.[6] In an in vitro study, blue light-stimulated A2E could enhance the generation of lipid peroxidation products and advanced glycation end products in RPE cells, which could lead to the increased expression of VEGF.[7] Alternatively, photooxidized A2E could activate the complement system,[8] which may promote CNV.[9] Because the visual cycle is coupled to OS shedding/phagocytosis,[16,44] and excess visual cycle results in the increased A2E synthesis in RPE cells,[45] these hypotheses involving bisretinoids may be associated with the mechanism proposed by our study. In addition, a limitation of the present study is that although our data indicated IPL could enhance CNV in vivo, it is still not clear how IPL could facilitate the de novo development of CNV.

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Disclosures

None.

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