Humans are exposed to light of various wavelengths and intensity during daily life. Light contains various colors and energies depending on the wavelength. As the wavelength is shortened, the energy is increased. Humans can visualize light between 380 nm and 750 nm. Light from 380 to 530 nm is high-energy visible light and is associated with the pathogenesis of age-related macular degeneration (AMD) and retinitis pigmentosa [1,2]. High-energy visible light is present in sunlight, fluorescent light, and light-emitting diodes (LEDs). LED is a source of light that is replacing conventional illumination and is widely used in light and liquid crystal displays.

Photoreceptor degeneration is an irreversible process that can lead to night blindness and visual field defects and eventually to loss of vision. Loss of photoreceptor cells is associated with cell apoptosis [3]. Previously, we reported that damage to photoreceptor cells induced by exposure to blue LED light is related to oxidative stress that causes the aggregation of short-wavelength opsin (S-opsin), which is a type of visual pigment [4]. We also reported that excessive light exposure induces endoplasmic reticulum (ER) stress in a murine animal model and in vitro model [5].

The ER is a cellular organelle that folds and processes cellular proteins. ER stress is triggered by the accumulation of unfolded proteins, and it can induce cellular damage [6]. Under ER stress, cells activate the unfolded protein response (UPR) pathway as a self-defense mechanism. The UPR maintains protein homeostasis and promotes cell survival [7]. It activates three pathways: (i) promotion of refolding of unfolded proteins through the synthesis of ER chaperones, (ii) suppression of protein translation to decrease unfolded proteins, and (iii) activation of ER-associated degradation to degrade unfolded proteins in the ubiquitin–proteasome system. However, if the UPR fails to recover ER function, prolonged ER stress can cause ER stress–induced apoptosis [8-10]. In recent studies, we found that excessive exposure to blue LED light causes the aggregation of S-opsin [4] and that aggregated S-opsin is poorly disassembled [11]. Thus, exposure to blue LED light may induce ER stress, UPR, and the aggregation of S-opsin.

In the present study, we investigated the mechanism underlying ER stress in response to exposure to blue LED light using murine photoreceptor cells, with particular emphasis on the involvement of S-opsin.
METHODS

Materials: The murine photoreceptor-derived cell line, 661W, was obtained as a gift from Dr. Muayyad R. Al-Ubaidi (University of Houston, Department of Biomedical Engineering, Houston, TX). Antibodies against mouse BiP/GRP78 were purchased from Becton Dickinson Company (Franklin Lakes, NJ), against mouse ATF4 and mouse PERK from Cell Signaling Technology (Beverly, MA), against rabbit Opsin (blue) from Merck Millipore (Darmstadt, Germany), and against mouse β-actin from Sigma-Aldrich (St. Louis, MO). Tunicamycin and dithiothreitol (DTT) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). siRNA of mouse S-opsin (primer set Opn1sw mouse 1) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA), and negative control siRNA was purchased from Nippon Gene Co., Ltd. (Tokyo, Japan).

LED light source: Blue LED light was purchased from M-Trust Co., Ltd. (Hyogo, Japan). This exposure device has aligned many LED light sources. The wavelength of the blue LED light was 464 nm. The cells were exposed to 450 lx of LED light, equivalent to 0.38 mW/cm², below the cell-culturing plates. The energy conditions were determined by following the previous report [4]. The light intensity was measured with the AS ONE Luminometer LM-332 (AS ONE, Osaka, Japan) in each experience.

Cell culture: The murine photoreceptor cell line was confirmed with a short tandem repeat (STR) profile and interspecies contamination testing (IDEXX BioResearch, Ludwigsburg, Germany). The sample was confirmed to be of mouse origin, and no mammalian interspecies contamination was detected. The genetic profile was proven to be consistent with a mixed FVB and C57BL/6 mouse strain of origin and to carry the HIT1 transgene; it is unique to this cell line (Appendix 1) [12]. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Nacalai Tesque, Inc., Kyoto, Japan) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin under a humidified atmosphere of 5% CO₂ at 37 °C. Cell passage was performed by trypsinization every 2 or 3 days. The thermal cycler conditions were as follows: 2 min at 50 °C and then 10 min at 95 °C, followed by two-step PCR for 50 cycles consisting of 95 °C for 15 s followed by 60 °C for 1 min.

Figure 1. Unfolded protein response (UPR)-related mRNAs induced in response to exposure to blue LED light in murine photoreceptor cells. Induction of bip (A), grp94 (B), or atf4 (C) mRNA after blue light-emitting diode (LED) light exposure time with real-time reverse transcription (RT)–PCR. Gapdh mRNA was used as the control. Data are represented as the means ± standard error of the mean (SEM; n=4). **p<0.01, *p<0.05 versus control (Student t test).
Real-time RT–PCR: To evaluate the effect of exposure to blue LED light on expression of atf4, grp94, and bip/grp78 mRNA, the murine photoreceptor cells were seeded in 12-well plates at a density of 3 × 104 cells per well. After 24 h incubation, the medium was replaced with fresh medium containing 1% FBS. Then, the cells were exposed to 450 lx of blue LED light for 3 to 12 h at 37 °C. After light exposure, RNA was extracted using Nucleo Spin RNA II (Takara Bio Inc., Kusatsu, Japan). Real-time RT–PCR was performed using a Thermal Cycler Real Time System (TP-800; Takara Bio Inc.) with SYBR Premix Ex TaqII (Takara Bio Inc.), according to the product’s protocol. The PCR primer sequences used were as follows: ATF4, 5′-GCA AGG AGG ATG CCT TTT C-3′ (forward) and 5′-GTT TCC AGG TCA TCC ATG CG-3′ (reverse); GRP94, 5′-TTT GAA CCT CTG CAC AAG AGC ACA-3′ (forward) and 5′-CTG ACT GGC CAC AAG AGC ACA-3′ (reverse); BiP/GRP78, 5′-GCC TGT ATT TCT AGA CAC AGA CCT GCC-3′ (forward) and 5′-TTC ATC TTG CCA GCC AGT TG-3′ (reverse); and GAPDH, 5′-TGT GTC CGT CGT GGA TCT GA-3′ (forward) and 5′-TTG CTG TTG AAG TCG CAG GAG-3′ (reverse). The results are expressed relative to the GAPDH internal control. The thermal cycler conditions were as follows: 2 min at 50 °C and then 10 min at 95 °C, followed by two-step PCR for 50 cycles consisting of 95 °C for 15 s followed by 60 °C for 1 min.

Immunoblot analysis: Murine photoreceptor cells were seeded at a density of 3 × 104 cells/ml on 12-well plates and cultured at 37 °C for 24 h. After 24 h, the cells were exposed to 450 lx of blue LED light. After light exposure, the cells were washed with PBS (1X; 136.9 mM NaCl, 2.68 mM KCl, 10.14 mM Na₂HPO₄•12H₂O, 1.76 mM KH₂PO₄, pH 7.3), and lysed using a cell lysis buffer (RIPA buffer) with 1% protease inhibitor and phosphatase inhibitor cocktails 2 and 3 (Sigma-Aldrich). The lysates were centrifuged at 12,000 × g for 20 min. Protein concentrations were determined using a
bicinchoninic acid assay (BCA) protein assay kit (Thermo Fisher Scientific Inc.) with BSA (BSA) as the standard. An equal volume of protein sample and sample buffer was mixed, separated on 5% to 20% sodium dodecyl sulfate (SDS)–polyacrylamide gels, and transferred to polyvinylidene difluoride membranes (Immobilon-P; EMD Millipore Corporation, Billerica, MA). For the immunoblotting experiments, the following primary antibodies were used: mouse anti-GRP78/BiP (1:500; Becton Dickinson Company); rabbit anti-GRP94, rabbit anti-ATF4, and mouse anti-ubiquitin (1:1,000; Cell Signaling Technology); rabbit anti-S-opsin (1:1,000; Millipore Corporation); and mouse anti-β-actin (1:5,000; Sigma-Aldrich). Subsequently, the membranes were incubated with secondary antibodies: horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse (1:2,000; Thermo Fisher Scientific Inc.). Immunoreactive bands were visualized using ImmunoStar®LD (Wako Pure Chemical Industries) and an LAS-4000 Luminescent Image Analyzer (Fuji Film Co., Ltd., Tokyo, Japan). β-actin was used as the loading control.

**Immunostaining:** Murine photoreceptor cells (1.5 × 10^4 cell/ml) were seeded on glass chamber slides (Laboratory-Tek; Thermo Fisher Scientific Inc.) and incubated for 24 h. After 24 h incubation, the medium was exchanged with fresh DMEM containing 1% FBS and was incubated for 30 min. Subsequently, the cells were exposed to 450 lx blue LED for 6 h or treated with 2 μg/ml tunicamycin or 0.5 mM DTT for 6 or 9 h. Thereafter, the immunostaining protocol was performed using examples from the Methods section of our previous report [4].

**Transfection with siRNA in vitro:** Murine photoreceptor cells were seeded at a density of 10,000 cells per well on 12-well plates with an antibiotic-free medium at 37 °C for 24 h. They were transfected with 50 nM siRNA and Lipofectamine RNAiMAX Reagent in Opti-MEM (Thermo Fisher Scientific Inc.) as indicated by the protocol. After 24 h transfection, the medium was exchanged with fresh DMEM containing 1% FBS. After 30 min, the cells were exposed to blue LED (450 lx) light for 9 h at 37 °C, and the immunobLOTS were analyzed.

**Statistical analysis:** Data are presented as the mean ± standard error of the mean (SEM). Statistical comparisons were made using one-way ANOVA followed by the Student t test or Dunnett’s multiple comparison test. A p value of less than 0.05 was considered to indicate statistical significance.

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**Figure 3.** Changes in ubiquitinated protein level induced by exposure to blue LED light. Immunoblotting shows the ubiquitinated protein levels. 

**A:** Representative image. 

**B:** Quantitative data of the immunoblot. Data are presented as the mean ± standard error of the mean (SEM; n=6). *p<0.05 versus control (Student t test).
RESULTS

**UPR factor mRNA levels in murine photoreceptor cells induced by exposure to blue LED light:** Murine photoreceptor cells were exposed to 450 lx blue light, and changes in mRNA expression at different time points were measured using quantitative real-time RT–PCR. Real-time RT–PCR showed statistically significant increases in the grp78/bip, grp94, and atf4 mRNA levels in the light-exposed cells compared with the non-light-exposed cells (Figure 1). RT–PCR showed increases in Grp78/bip mRNA levels at 6, 9, and 12 h (peak at 6 h) and increases in grp94 mRNA levels between 6 and 12 h. Further, RT–PCR showed particularly significant increases in the atf4 mRNA levels between 6 and 12 h in a time-dependent manner.

**Protein expression of ATF4 increased in response to exposure to blue LED light in murine photoreceptor cells:** To investigate whether exposure to blue LED light affects the expression of UPR-related proteins (such as BiP, GRP94, and ATF4), we performed immunoblot analysis. Immunoblot analysis revealed a slight, but statistically significant, increase in BiP at 6 h after exposure to blue LED light, no statistically significant change in GRP94, and a marked increase in ATF4 at 6, 9, and 24 h (Figure 2).

**Induction of protein polyubiquitination after exposure to blue LED light:** We performed immunoblot analysis to determine whether exposure to blue LED light induces the accumulation of polyubiquitinated proteins in murine photoreceptor cells. We investigated the levels of polyubiquitinated proteins at different time points. A representative immunoblot image is shown in Figure 3A. Quantitative analysis of polyubiquitinated proteins showed that short time exposures (3 to 9 h) induced an increase in polyubiquitinated proteins (Figure 3B). However, this induction was not observed after 12 h of exposure to blue LED light.

**Aggregated S-opsin was observed in blue LED light–exposed and tunicamycin- or DTT-treated cells:** Next, we investigated whether the ER stress inducers, tunicamycin and DTT, induce the aggregation of S-opsin in murine photoreceptor cells by immunostaining. S-opsin aggregation was observed in blue LED light–exposed and tunicamycin- and DTT-treated cells. The cells were exposed to blue LED for 6 h, and S-opsin aggregated cells were observed. Cells were treated with 2 μg/ml tunicamycin for 12 h or 0.5 mM DTT for 6 h, and perinuclear aggregation of S-opsin was observed (Figure 4A–C). The graph shows the ratio of cells with S-opsin aggregation to the total cell number (Figure 4D).

**Phosphorylation of ATF4 by exposure to blue LED light was attenuated by the knockdown of S-opsin:** We predicted that the aggregation of S-opsin was associated with ATF4 activation by exposure to blue LED light. We investigated whether the knockdown of S-opsin alters the state of ATF4 activation...
induced by exposure to blue LED light in murine photoreceptor cells (Figure 5). S-opsin expression was knocked down by S-opsin siRNA treatment (Figure 5B). Negative control siRNA did not change the expression of S-opsin (Figure 5). Next, we investigated the effect of S-opsin siRNA on the expression of ATF4 after exposure to blue LED light. Exposure to blue LED light induced the expression of ATF4. Treatment with S-opsin siRNA #1 or #2 prevented the expression of ATF4 induced by exposure to blue LED light in murine photoreceptor cells, whereas the control siRNA did not prevent the induction of ATF4 expression (Figure 5).

**DISCUSSION**

In the present study, we demonstrated that the expression of several ER stress–related genes and levels of polyubiquitinated proteins were increased in blue LED light–exposed murine photoreceptor cells. The mRNA and protein levels of ATF4 were markedly increased in response to the exposure to blue LED light in the murine photoreceptor cells. Furthermore, the knockdown of S-opsin mRNA prevented the expression of ATF4 in response to exposure to blue LED light. Exposure to LED light induced ER stress and UPR in photoreceptor cells, and these changes might be associated with the aggregation of S-opsin. ER stress is induced by the accumulation of unfolded proteins, and these proteins are degraded through the ubiquitin–proteasome pathway [13]. In previous reports, increased levels of ER stress-related factors were associated with neurodegenerative diseases, including Alzheimer disease, Parkinson disease, and glaucoma [14-17]. Furthermore, we previously reported that excessive exposure to blue LED light induces photoreceptor cell degeneration via oxidative stress and aggregated S-opsin [4]. Therefore, we predicted that excessive exposure to blue LED light induces ER stress and activates the UPR pathway and that these events may be initiated by the aggregation of S-opsin. The expression levels of bipgrp78, grp94, and atf4 mRNA increased in blue LED light–exposed photoreceptor cells. Bip and grp94

![Figure 5. Effects of S-opsin siRNA on exposure to blue LED light in murine photoreceptor cells. A: Representative immunoblotting images of short-wavelength opsin (S-opsin), ATF4, and β-actin. B: Expression level of S-opsin. C: Expression level of ATF4. Data are presented as the mean ± standard error of the mean (SEM; n=7 to 9). **p<0.01, #p<0.05 versus normal (control); †p<0.05, † † p<0.01 versus normal (blue light-emitting diode [LED]); (Student t test). N, normal; NC, negative control siRNA.](image-url)
mRNA levels increased between 6 and 12 h of exposure to blue LED light. Atf4 mRNA levels increased after a short period of exposure to blue LED light and then increased in a time-dependent manner (Figure 1). Immunoblot analysis revealed that the expression of ATF4 protein increased in response to exposure to blue LED light. Although the protein expression of BiP/GRP78 increased only 6 h after light exposure, there was no change in the protein expression of GRP94 in blue LED light–exposed cells compared with the control cells (no LED light exposure; Figure 2). Therefore, we speculated that exposure to blue LED light evoked the PKR-like endoplasmic reticulum kinase (PERK) pathway in murine photoreceptor cells and induced translational repression in murine photoreceptor cells. In a previous report, excessive light exposure induced photoreceptor apoptosis and increased the phospho-PERK levels [18]. Activation of the PERK pathway inhibits the translation of mRNA in eukaryotes from yeast to mammal cells [19,20]. Although general translation was suppressed, ATF4 translation increased [21]. Therefore, it was considered that only the expression of ATF4 was relatively elevated. Here, we found that the levels of polyubiquitinated proteins were increased between 3 and 9 h of exposure to blue LED light (Figure 3). This result suggests that unfolded proteins increase or that the activity of the proteasome may decrease after exposure to blue LED light. S-opsin is the presence of cone photoreceptor-specific protein, and it has been reported that S-opsin exists in murine photoreceptor cells [22]. Therefore, we hypothesized that there is an association of ER stress with S-opsin aggregation. We investigated whether S-opsin aggregation was induced by treatment with ER stress-inducing agents. The ER stressors tunicamycin and DTT induced the aggregation of S-opsin (Figure 4), suggesting that aggregated S-opsin is associated with ER stress. We performed the knockdown of S-opsin using siRNA to examine UPR pathway activation. As the ATF4 protein levels were increased by exposure to blue LED light (Figure 2C), it was evaluated as a target protein. S-opsin siRNA treatment prevented the expression of ATF4 in response to exposure to blue LED light (Figure 5). It is likely that the aggregation of S-opsin caused the activation of ATF4 in the murine photoreceptor cells in response to exposure to blue LED light. The aggregation of S-opsin causes rapid cone degeneration [11]. In previous reports, oxidative stress was involved as a cause of AMD [23,24]. In addition, it is likely that the aggregation of S-opsin is one cause of the preferred damage of the blue cones in AMD pathogenesis. To suppress cell damage by excessive exposure to blue LED light, it is necessary to not only suppress oxidative stress but to also suppress the aggregation of S-opsin and ER stress. Further experiments will be needed to clarify the detailed molecular mechanism.

In conclusion, exposure to blue LED light induced ATF4 activation in murine photoreceptor cells, presumably due to the aggregation of S-opsin.

APPENDIX 1. STR ANALYSIS.

To access the data, click or select the words “Appendix 1.”

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