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Blue Light Induces Mitochondrial DNA Damage and Free Radical Production in Epithelial Cells*

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Exposure of biological chromophores to ultraviolet radiation can lead to photochemical damage. However, the role of visible light, particularly in the blue region of the spectrum, has been largely ignored. To test the hypothesis that blue light is toxic to non-pigmented epithelial cells, confluent cultures of human primary retinal epithelial cells were exposed to visible light (390–550 nm at 2.8 milliwatts/cm^2) for up to 6 h. A small loss of mitochondrial respiratory activity was observed at 6 h compared with dark-maintained cells, and this loss became greater with increasing time. To investigate the mechanism of cell loss, the damage to mitochondrial and nuclear genes was assessed using the quantitative PCR. Light exposure significantly damaged mitochondrial DNA at 3 h (0.7 lesion/10 kb DNA) compared with dark-maintained controls. However, by 6 h of light exposure, the number of lesions was decreased in the surviving cells, indicating DNA repair. Isolated mitochondria exposed to light generated singlet oxygen, superoxide anion, and the hydroxyl radical. Antioxidants confirmed the superoxide anion to be the primary species responsible for the mitochondrial DNA lesions. The effect of lipofuscin, a photoinducible intracellular generator of reactive oxygen intermediates, was investigated for comparison. Exposure of lipofuscin-containing cells to visible light caused an increase in both mitochondrial and nuclear DNA lesions compared with non-pigmented cells. We conclude that visible light can cause cell dysfunction through the action of reactive oxygen species on DNA and that this may contribute to cellular aging, age-related pathologies, and tumorigenesis.

It is well recognized that non-ionizing radiation can react photochemically with biological chromophores, producing end products that are toxic and/or mutagenic in mammalian cells. Most studies have concentrated on the role of UV irradiation due to its high energy, photoreactivity, wide range of biological chromophores, specific cellular responses, and association with pathologies such as skin melanoma and cataract (1–4). However, the role of visible light has been less extensively investigated, even though studies have demonstrated that visible light can induce cellular dysfunction and cell death both *in vitro* and *in vivo* (1–3, 5–7). The blue region (400–500 nm) of the visible spectrum is likely to be particularly important because it has a relatively high energy, can penetrate tissue(s), and is associated with the occurrence of malignant melanoma in animal models (6, 7). Surprisingly, despite its potential for damage, the use of blue light blockers in sunscreens and spectacle lenses has, until recently, received only limited attention.

Studies have shown that irradiation of mammalian cells with visible light induces cellular damage primarily via reactive oxygen species (ROS)[1] (8). ROS such as the hydroxyl radical, superoxide anion, and singlet oxygen can be produced when visible light excites cellular photosensitizers (9, 10). Whereas photosensitizers such as melanin and lipofuscin in pigmented cells and retinoids in photoreceptor cells have been identified, the identity and location of photosensitizers in non-pigmented cells remain largely unknown. However, a number of options exist, including flavin-containing oxidases, the cytochrome system, heme-containing proteins, and tryptophan-rich proteins. The interaction of these chromophores with light can generate ROS, which in turn can damage lipids, proteins, and DNA. This is emphasized by the study of Hockberger *et al.* (11), who found that violet-blue light stimulated H_2O_2 production from peroxisomes and mitochondria in cultured 3T3 and CV1 mammalian cells. Hydrogen peroxide production was enhanced by overexpression of flavin-containing oxidases, which proposes that violet-blue light initiates photoreduction of flavins, which activate flavin-containing oxidases in mitochondria and peroxisomes, resulting in H_2O_2 production. Furthermore, the mechanism by which photosensitization leads to cellular dysfunction is unclear but may center on DNA damage. As reviewed by Goyns (12), the role of DNA damage in aging mammals appears to be pivotal, and there is increasing evidence that oxidative damage is an important factor in producing mutations in genes, shortening telomeres, and damaging mitochondrial DNA. To support a role for visible light in DNA damage, Pflaum *et al.* (13) have previously shown that oxidative damage induced by visible light does yield DNA modifications.

The aim of this study was to establish whether blue light is able to cause differential damage to the mitochondrial and nuclear genome and to determine whether this occurs via ROS production at mitochondria. Our results demonstrate that exposure of non-pigmented epithelial cells to blue light

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[1] The abbreviations used are: ROS, reactive oxygen species; RPE, retinal pigment epithelium; QPCR, quantitative PCR; nDNA, nuclear DNA; mtDNA, mitochondrial DNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

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causes mitochondrial dysfunction and mtDNA damage and that such effects are mediated by the action of reactive oxygen species. We have further identified that the ROS primarily responsible for blue light-induced mtDNA damage is the superoxide radical.

MATERIALS AND METHODS

Chemicals

Ham's F-10 and Ham's SF10PF cell culture media were obtained from Invitrogen (Paisley, UK). Fetal calf serum was obtained from TCS Biologicals Ltd. (Buckingham, UK). Sucrose, EDTA, and trichloroacetic acid were obtained from BDH (Poole, UK). Trypsin, antibiotics, fungizone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), phosphate-buffered saline tablets, mannitol, cytochrome *c* (horse heart, type III), superoxide dismutase (bovine erythrocytes), 4-nitroso-*N,N*-dimetylaniline, histidine, salicylic acid, catalase (bovine liver), and sodium azide were from Sigma. Qiagen genomic tip 20G kit was from Qiagen (Valencia, CA). Recombinant *Thermus thermophilus* DNA polymerase was from PerkinElmer Life Sciences, and the primers for PCR were synthesized by Invitrogen (Rockville, MD). Taxifolin and MitoPBN were from Merck Biosciences Ltd., UK. All other chemicals used were of highest purity analytical grade.

RPE Cell Isolation and Culture

Human RPE cells were isolated and cultured as previously described (14). Human eyes (Bristol Eye Bank, Bristol, UK) were obtained from donors between the ages of 40 and 70 years. The corneas had been used for transplantation, and permission had been given to use the poles for research. The RPE cells were grown in Ham's F-10 medium supplemented with 20% fetal calf serum and antibiotics, and cultures were maintained at 37 °C and 5% CO_2. RPE cells were grown to confluence in 24-well plates or 75-cm^2 culture flasks as required. Depigmented cells between passage 3 and 5 were used for all experiments (14).

Photoillumination of RPE Cells

At confluence, growth medium was replaced with basal Ham's F-10 medium supplemented with 2% fetal calf serum and maintained for an additional 24 h. Prior to light exposure, the basal medium was replaced with SF10PF medium, which lacks the photosensitizers phenol red, tryptophan, riboflavin, and folic acid (15). For each set of experiments, one set of RPE cultures was wrapped in a black sheet of paper (dark maintained), and the other set was left uncovered (light exposed). Both light and dark cultures were exposed to 390–550-nm light (referred to as blue light emitted by a Sol 500 light source (HÖnle UV Ltd., Birmingham, UK)), with appropriate filters, for 0–9 h. The irradiance was 2.8 milliwatts/cm^2, and the cells were maintained at a constant temperature of 37 °C.

DNA Isolation and QPCR

After light exposure and lipofuscin treatment, total cellular DNA was isolated using a Qiagen genomic tip 20G kit as described by the manufacturer. DNA isolation by this technique results in genomic preparations suitable for long QPCR. Total cellular DNA concentrations were determined by ethidium bromide fluorescence with an A4 filter fluorometer with an excitation band pass filter at 360 nm and an emission cutoff filter at 600 nm (Optical Technology Device, Elmsford, NY) using HindIII DNA as a standard. Initially, DNA quality was assessed by pulse-field electrophoresis prior to QPCR. As previously described (16), sample quality was also tested by QPCR of a 222-bp fragment in the mtDNA and an 84-bp fragment in the b-globin genes (mtDNA primers, 14619FOR (5′-CCCCACAAACCCCATTACTAAACCCA-3′) and 14841REV (5′-TTTCATCATGCGGAGATGTTGGATGG-3′); b-globin primers, 48550FOR (5′-CGAGTAAGAGACCATTGTGGCAG-3′) and 48634REV (5′-GCTGTTCTGTCAATAAATTTCCTTC-3′)), with the expectation that equal template concentrations should yield similar QPCR product (short) concentrations. QPCRs were performed in a GeneAmp PCR system 2400 with the GeneAmp XLPCR kit (PerkinElmer Life Sciences). Reaction mixtures contained 15 ng of genomic DNA as template. The reagent conditions for the QPCR have been previously described for the 16.2-kb mtDNA product and 13.4-kb b-globin product (16, 17).

MTT Assay for Cell Viability

The MTT assay provides a reliable technique for determining the number of viable cells in a given culture. It is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals that are largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The intensity of the product color is directly proportional to the number of living cells in the culture. Cells were seeded in 24-well plates at a density of 8000 cells/well and incubated at 37 °C. Forty-eight hours after plating, the medium was replaced with serum-free medium. MTT assays were performed at four time points (1, 3, 6, and 9 h). At each time point, cell viability was measured using the MTT assay as described previously (15). In brief, medium was aspirated from the wells, and 250 ml of MTT (1 mg/ml) was gently added into each well. The cells were incubated for 3 h at 37 °C, after which the MTT was aspirated, and 250 ml of acidified isopropanol was added to solubilize the reduced blue formazan crystals. Aliquots were transferred to a 96-well plate, and absorbance was measured at a test filter of 590 nm and a reference filter of 630 nm on a 96-well plate reader. Results were analyzed using the following equation: Viability as a percentage of control = (absorbance of sample cells/absorbance of control cells) × 100, where control cells are the untreated cells immediately before the start of the experiment.

Isolation of Mitochondria from RPE Cells

Differential Centrifugation—Cultured human RPE cells from seven 75-cm^2 flasks were pelleted at 1000 × *g* for 5 min at 4 °C. The cell pellet was suspended in 11 ml of ice-cold RSB buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5) and transferred to a 15-ml Dounce homogenizer. The cells were allowed to swell for 5 min and broken down with several strokes of the homogenizer pestle. Eight milliliters of 2.5× MS buffer (525 mM mannitol, 175 mM sucrose, 12.5 mM Tris-HCl, pH 7.5, and 2.5 mM EDTA, pH 7.5) was added to maintain tonicity of organelles and prevent agglutination. After mixing the contents, the homogenate was centrifuged at 300 × *g* for 5 min at 4 °C to remove nuclei, unbroken cells, and large membrane fragments. The mitochondria from the supernatant were pelleted by centrifugation at 17,000 × *g* for 15 min at 4 °C. The mitochondrial pellet was suspended in 5 ml of 1× MS buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, pH 7.5, and 5 mM Tris-HCl, pH 7.5) and purified on a sucrose gradient.

Sucrose Step Density Gradient—The mitochondrial pellet was applied to the gradient that consisted of equal volumes of 1.0 M and 1.5 M sucrose followed by centrifugation at 60,000 × *g* for 20 min at 4 °C. The mitochondria at the 1.0:1.5 M sucrose interface were carefully removed. The sucrose was diluted with phosphate-buffered saline, and the mitochondria were pelleted at 17,000 × *g* for 15 min at 4 °C. The pellet was once again washed with excess phosphate-buffered saline, and the mitochondria were resuspended in phosphate-buffered saline for ROS production measurements.

ROS Production by Mitochondria Isolated from RPE

Superoxide Anion (O_2^-)—Superoxide anion production was determined by measuring the reduction of cytochrome *c* (18, 19). A typical assay mixture contained 50 mM phosphate buffer, pH 8.0, 100 mM cytochrome *c* (type III), and 0.1 mM EDTA. The reaction was started by the addition of isolated mitochondria and exposure of the reaction mixture to blue light or incubation in the dark. Immediately after mixing, the absorbance at 550 nm was read at various times on a spectrophotometer. Superoxide anion production was confirmed by inhibition of radical production by superoxide dismutase (bovine erythrocytes) (300 units).

Singlet Oxygen ($^1O_2$)—Singlet oxygen was determined in aqueous solution by the method of Mohsini and Kralijic (20), with minor modifications. The reaction mixture consisted of 10 mM phosphate buffer, pH 7.8, 10 mM *p*-nitrosodimethylaniline, and 5 mM histidine (as a selective acceptor of $^1O_2$). The mitochondria-containing reaction mixture was exposed to blue light, and parallel controls were incubated in the dark. Formation of transannular peroxide intermediate complex of histidine with singlet oxygen results in the bleaching of *p*-nitrosodimethylaniline, the absorbance of which is spectrophotometrically measured at 440 nm. Generation of singlet oxygen in the reaction system was established by quenching the radicals with 100 mM sodium azide.

Hydroxyl Radical ($OH^\cdot$)—Hydroxyl radical formation by mitochondria in the presence of light or dark was determined by the aromatic hydroxylation method of Richmond *et al.* (21). The reaction mixture contained the following: 2 mM salicylate, 0.1 mM EDTA, 0.1 mM $FeSO_4$, and 50 mM phosphate buffer, pH 8.0. The reaction was initiated by the addition of mitochondria and incubation of the reaction mixture under blue light or in the dark. The reaction was stopped by the addition of 60 ml of 11.6 M HCl and 0.4 g of NaCl. The hydroxylated products were extracted by chilled diethyl ether, which was evaporated to dryness at

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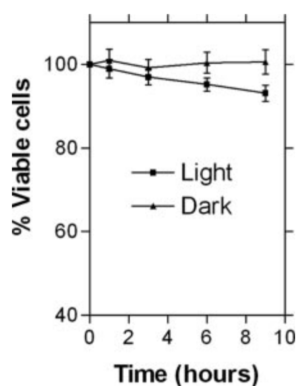

FIG. 1. **The effect of blue light on RPE cell respiration.** Confluent cultures were exposed to blue light or maintained in the dark for up to 9 h. Mitochondrial activity (a measure of cell viability) was determined by the MTT assay. The *vertical bars* indicate S.E.

40 °C, and the contents were dissolved in 125 ml of chilled distilled water. The following were then added in the order stated: 10% trichloroacetic acid (w/v) in 0.5 M HCl, 10% (w/v) sodium tungstate, and 125 ml of 0.5% (w/v) sodium nitrite (fresh). After incubating the reaction for 5 min at 22 °C, 0.5 M KOH was added, and the absorbance was read at 510 nm. Generation of hydroxyl radicals in the reaction system was established by inhibiting the radicals with catalase (bovine liver) (500 units).

Lipofuscin Treatment

Lipofuscin granules were isolated and purified from 50–60-year-old human donors as described by Boulton *et al.* (22), except that RPE cells were disrupted by mechanical homogenization instead of sonication. The concentration of the granules was determined using a hemocytometer.

Cultures were fed with purified lipofuscin granules (~300 granules/cell) suspended in basal medium (14). The cultures were maintained for 7 days, during which the basal medium was changed every 2 days. Lipofuscin-fed cells were illuminated by blue light as described above.

Antioxidant Treatment

Stock antioxidant solutions were prepared immediately before each set of experiments. Stock solutions (10 mM) of taxifolin (a superoxide scavenger) and MitoPBN (a carbon-centered radical scavenger) were dissolved in serum-free SF10PF medium containing Me_2SO at a final concentration of 0.1% (v/v). One hour before the initiation of the experiment, the indicated antioxidant solution was added to the medium to give a final antioxidant concentration of 100 μM. Antioxidant-treated cells were illuminated by blue light as described above.

Statistical Analysis

All assays were performed a minimum of three times using three different cultures. Analysis of variance was used to determine inter-experimental variation, and Student's *t* test (unpaired) was performed to identify variation between time points. Values of $p < 0.05$ were considered significant.

RESULTS

The Effect of Blue Light on Cell Viability—To determine whether blue light is able to cause cell dysfunction, the MTT assay was performed to assess mitochondrial respiratory function as an indicator of the cell viability. There was no significant difference in mitochondrial respiration detected during the first 3 h of irradiation compared with cells maintained in the dark (Fig. 1). However, a small (~6%) but significant ($p < 0.05$) decrease of viability was observed at 6 h in irradiated cells. Blue light-irradiated cells continued to show an increasing loss of viability (~10% decrease, $p < 0.05$) at 9 h (Fig. 1). There was no decrease in viability in cells maintained in the absence of blue light exposure.

The Effect of Blue Light on DNA Damage—To investigate the mechanism of cell loss after blue light irradiation, the damage to mtDNA and nDNA was assessed with a well-validated, sensitive QPCR assay, which is based on the principle that oxida-

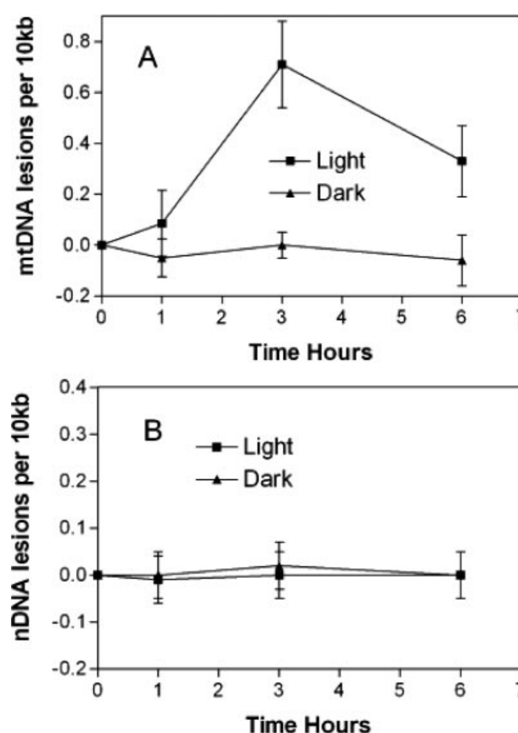

FIG. 2. **The effect of blue light on mtDNA (*A*) and nDNA (*B*) damage after 1, 3, and 6 h of irradiation.** Control cultures were maintained in the dark. The graph represents the number of lesions per 10 kb as determined by QPCR of mitochondrial and nuclear genes. Cells were irradiated with blue light for 0–6 h, and QPCR was performed to assess the DNA lesions. The *vertical bars* indicate S.E.

tive DNA lesions inhibit DNA polymerases (17). DNA damage was quantified by comparing the relative efficiency of amplification of large fragments of DNA (16.2 kb for the mtDNA and 13.4 kb for the b-globin gene) and normalizing this to the amplification of smaller (<250-bp) fragments, which have a statistically negligible likelihood of containing damaged bases (17).

Significant DNA damage was observed for mtDNA in light-exposed cells (Fig. 2*A*). Maximum damage was observed at 3 h (0.7 lesion/10 kb mtDNA); by 6 h, the number of DNA lesions was decreased to 0.29 lesion/10 kb mtDNA, suggesting that DNA repair was initiated. No nDNA lesions were detected in cells exposed to blue light at any of the time points studied (Fig. 2*B*), nor was there any damage to mitochondrial or nuclear DNA for cells maintained in the dark.

ROS Production—To determine whether blue light-induced mtDNA lesions and cell loss were associated with increased production of reactive oxygen species originating from the mitochondria, ROS levels were measured in isolated mitochondria exposed to blue light. Irradiated mitochondria showed a time-dependent increase in the levels of three ROS (Fig. 3): superoxide anion, hydroxyl radical, and singlet oxygen. A rapid and significant increase in the level of superoxide radicals was observed after 1 h of exposure and continued until 6 h (the end of experiment) (Fig. 3*A*). Similar trends were found in the generation of hydroxyl radical (Fig. 3*B*) and singlet oxygen (Fig. 3*C*). Production of each ROS was confirmed by the addition of the appropriate inhibitor (superoxide dismutase, catalase, and sodium azide catalase for superoxide anion, hydroxyl radical, and singlet oxygen, respectively). In each case, the ROS were inhibited (Fig. 3, *A*–*C*). Low level production of ROS was observed in mitochondria maintained in the dark and increased with time (Fig. 3, *A*–*C*), which may simply represent the effects of aerobic respiration.

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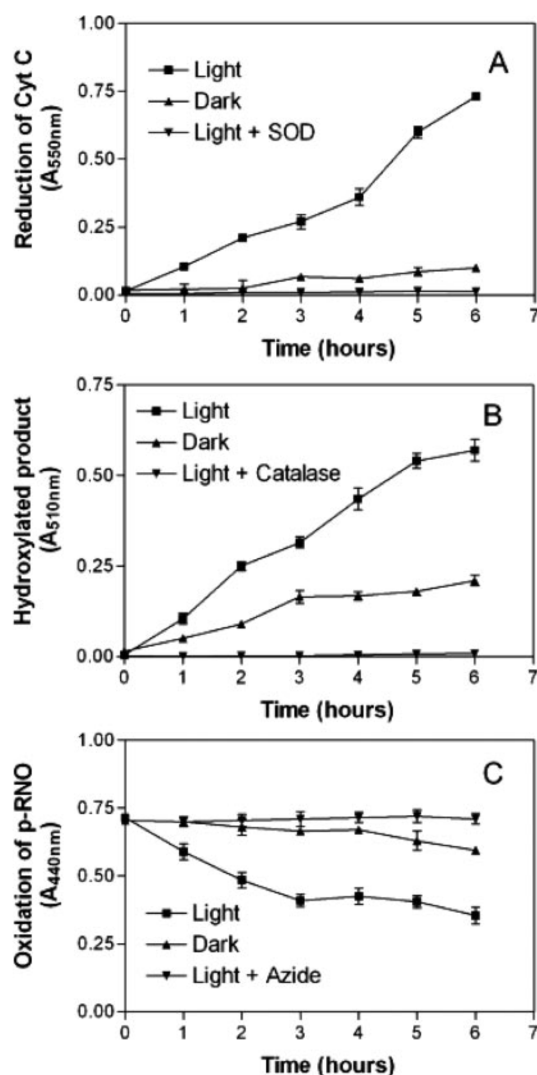

FIG. 3. **The effect of blue light on the generation of ROS by mitochondria isolated and purified from cultured RPE cells.** Mitochondrial suspensions were exposed to blue light, and the generation of the superoxide anion (*A*), hydroxyl radical (*B*), and singlet oxygen (*C*) was assessed by biochemical assays. To confirm generation of the appropriate ROS, the reactions were inhibited with superoxide dismutase, catalase, and sodium azide, respectively. The *vertical bars* indicate S.E.

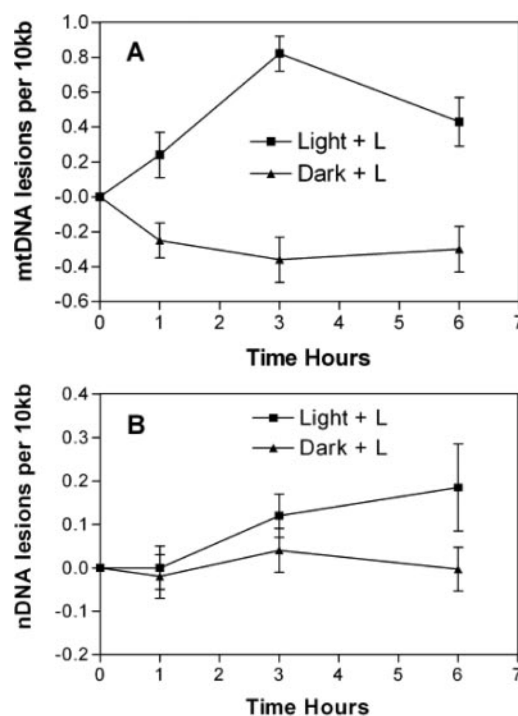

FIG. 4. **The effect of an intracellular photosensitizer, lipofuscin, on mtDNA (*A*) and nDNA damage (*B*).** Confluent cultures were fed lipofuscin and exposed to blue light for 1, 3, and 6 h. Control cultures were maintained in the dark. The graph represents the number of lesions per 10 kb as determined by QPCR of mitochondrial and nuclear genes in the presence of lipofuscin. The *vertical bars* indicate S.E.

Effect of Lipofuscin on Blue Light-induced Damage—We and others have previously shown that lipofuscin, an intracellular age pigment, can mediate light-induced damage via ROS in the RPE cells (59–61). We used this as a model (a) to confirm that a biological intracellular ROS generator can cause DNA damage and (b) to determine whether the mtDNA damage was originating from the mitochondria or from chromophores elsewhere in the cell.

In irradiated lipofuscin-fed cells, significant DNA damage was observed for both mtDNA and nDNA (Fig. 4). For mtDNA, maximum damage was observed at 3 h (0.84 lesion/10 kb mtDNA), whereas by 6 h, the number of DNA lesions had decreased to 0.4 lesion/10 kb mtDNA, suggesting that DNA repair was initiated. Nuclear DNA demonstrated a time-dependent increase in DNA lesions in irradiated lipofuscin-fed cells. At 3 h, we observed 0.13 lesion/10 kb nDNA, which increased to 0.2 lesion/10 kb nDNA at 6 h (Fig. 4*B*). Lipofuscin-fed cells maintained in the dark did not show significant nDNA damage. However, mtDNA in RPE cells fed with lipofuscin and kept in darkness showed a significant reduction in lesion frequency at all time points.

The Effect of Taxifolin and MitoPBN on Blue Light-induced DNA Damage—To clarify the role of specific ROS in blue light-induced mtDNA damage, we utilized the superoxide scavenging properties of taxifolin and the carbon-centered radical scavenging properties of the mitochondria-targeted MitoPBN in our model system. In irradiated taxifolin-fed cells, complete mtDNA protection was observed at 1, 3, and 6 h ($p < 0.05$) (Fig. 5) compared with irradiated cells in the absence of taxifolin, which showed extensive damage at 3 and 6 h. By contrast, MitoPBN-fed cells showed no mtDNA protection throughout the 6-h blue light exposure. Taxifolin- and MitoPBN-fed cells maintained in the dark did not show significant mtDNA damage at any of the time points.

DISCUSSION

Blue light damage is not an uncommon feature of the skin and eye. This is perhaps not surprising because whereas both tissues will be afforded protection against UV through natural (*e.g.* cornea, lens, and melanin) or artificial (*e.g.* sunscreen) filters, they are constantly exposed to the visible spectrum, of which the blue light region is the most energetic. In the eye, blue light damage is considered predominantly photochemical in origin and to arise largely from eye-specific chromophores (*e.g.* retinoids, melanin, and lipofuscin). In the skin, the situation is less clear but is likely to involve chromophores associated with mitochondria (*e.g.* cytochromes and flavins) that absorb in the blue region of the visible spectrum to contribute significantly to ocular toxicity.

In this study, we have demonstrated that blue light is able to photogenerate ROS from isolated mitochondria. This confirms that mitochondria possess blue light-sensitive chromophore(s). Studies have shown that blue light is able to cause ultrastructural damage and mitochondria-dependent cell death in lipofuscin-free RPE cells (23–25), which suggests that other chromophores, rather than lipofuscin, in the RPE cells mediate

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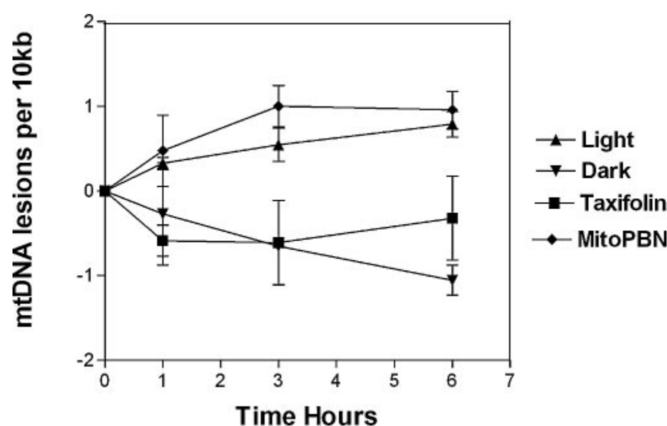

FIG. 5. **The effects of taxifolin and MitoPBN on mtDNA damage in irradiated cells after 1, 3, and 6 h of irradiation.** Control cultures were maintained in the dark. The graph represents the number of lesions per 10 kb as determined by QPCR of a mitochondrial gene. Cells were irradiated with blue light for 0–6 h, and QPCR was performed to assess the DNA lesions. The *vertical bars* indicate S.E.

these light effects. It has been postulated that blue light-induced retinal damage is mediated by mitochondrial respiratory enzymes (26, 27), and it has been shown that inhibiting the mitochondrial respiratory chain blocks ROS generation (25, 28). This is further supported by the finding that H_2O_2 production is significantly increased in kidney epithelial cells overexpressing flavin-containing oxidases (11), which are concentrated in mitochondria (29). Flavin-containing oxidases absorb throughout the UVA-blue spectrum, with peak at 370 and 450 nm. In addition, it has been demonstrated that the content of cytochrome *c* oxidase was reduced in the RPE cells following exposure to blue light (30). Cytochrome *c* oxidase is an important mitochondrial respiratory enzyme involved in oxidative phosphorylation. It has peak absorption at 440 nm in the reduced form (31). Together, these data implicate the involvement of mitochondrial respiratory enzymes in phototoxicity.

In our study, we show the generation of singlet oxygen, superoxide anion, and the hydroxyl radical, all of which are relatively short-lived, highly reactive species, which are likely to interact with molecules in the local area, *e.g.* mtDNA and proteins. This is supported by the observation that ROS derived from the mitochondrial respiratory chain are not responsible for the basal levels of oxidative base modifications observed in nDNA of mammalian cells (32). These light-generated ROS will be in addition to the low levels of ROS generated as a side product of oxidative respiration (33).

We have demonstrated that mtDNA damage increases following exposure to blue light. Mitochondrial DNA lesions were maximal at 3 h after exposure with evidence of some repair at later time points, suggesting that there is an adaptive response taking place to cope with the oxidative burden (34). Whatever the damage mechanism, it must be localized because there was no measurable damage to the nuclear DNA, and it would be in agreement with our studies on ROS generation by isolated mitochondria. This increased susceptibility of the mtDNA to light damage compared with nDNA could be due to a number of factors; first, mtDNA is located close to the inner mitochondrial membrane, where the ROS are generated; second, mtDNA is not protected by histone proteins as is the case for nDNA (17); third, mitochondria contain a number of chromophores that absorb in the blue light region (*e.g.* flavins and cytochromes) (29, 30, 35); fourth, the nature of the ROS generated. Lipofuscin, a known intracellular blue light-inducible generator of ROS including singlet oxygen, superoxide anion, hydroxyl radicals, and lipid peroxides, initiated mtDNA damage but did not cause damage in nDNA. This would indicate that mtDNA damage is a localized process and that, in the case of nDNA damage, it is the longer-lifetime lipid peroxides generated that are able to reach the nucleus. Our observation that mtDNA in RPE cells fed with lipofuscin and kept in the dark showed a significant reduction in lesion frequency at all time points may well represent a compensatory response to treatment due to induction of DNA repair enzymes. The pre-feeding of RPE cells with lipofuscin may have caused low level DNA damage, thus initiating DNA repair mechanisms. We have previously shown that lipofuscin exhibits a low level generation of ROS under dark conditions (22). It has been demonstrated that oxidative stress can induce expression of DNA repair enzymes such as ApoE endonuclease and DNA polymerase (β) in RPE cells (16, 36). Therefore, as lipofuscin accumulation increases in the RPE cells with aging, light-induced ROS production by lipofuscin and subsequent cell damage may play a significant role in aging of these cells.

The observation that mtDNA had less damage after 6 h of light irradiation compared with that after 3 h (Fig. 1) suggests that DNA repair is activated by 3 h. Although it was thought that the mitochondria lack an extensive and efficient DNA repair system comparable to that maintaining nDNA, Croteau *et al.* (37) have demonstrated that mitochondria are capable of repairing oxidative DNA damage to some extent (*e.g.* damage to bases and single-strand breaks). Whereas mitochondria have an efficient base excision repair capacity, they cannot repair larger lesions normally repaired by nucleotide excision repair (38, 39). Nonetheless, the mtDNA repair system appears relatively inefficient in our model because the mitochondrial redox function remained compromised even at the 6 and 9 h time points. Thus, insufficient repair of these lesions could ultimately result in a dysfunction in oxidative phosphorylation, thus perpetuating a cycle of ROS-mediated mtDNA damage (40).

It is apparent that the mitochondrial response to oxidative stress, dependent on the degree of oxidative insult, can result in sub-lethal damage or the initiation of cell death pathways. In our model, we assessed the effect of sub-lethal ROS damage that caused mtDNA lesions but did not lead to cell death. Cellular dysfunction via this mechanism is likely to reflect in decreased respiratory function and the accumulation of mtDNA defects. By contrast, more severe ROS damage to mitochondria elicits the apoptosis pathway (41–44). Generation of ROS by photo-irradiation can lead to mitochondrial swelling (45), increased mitochondrial calcium levels (43), and Bcl-2 inhibition and mitochondrial matrix caspase-2 and -9 activation (44). To complicate matters further, there is some evidence that the ROS-mtDNA damage axis can be regulated via growth factors and altered transcription factor activities (12, 46). A recent study by Suematsu *et al.* (46) demonstrated that oxidative stress mediates tumor necrosis factor-α-induced mtDNA damage in cardiac myocytes.

The beneficial biological activity of flavonoids has been previously identified in several studies (47, 48). These data are of particular interest with regard to the mechanisms involved in blue light-induced mtDNA damage. We report novel data indicating that the superoxide anion may be a primary ROS involved in mtDNA damage after blue light exposure. It is possible that the sources of superoxide include a wide array of mitochondrial enzymes such as flavins, flavoproteins, and lactate dehydrogenase, which have all been shown to generate superoxide under various oxidizing conditions (49–51). Furthermore, we have previously demonstrated that Mn-superoxide dismutase is highly sensitive to oxidative damage, thereby weakening the superoxide radical defenses of the mitochondria (52). To provide further support for these data, we used a

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mitochondria-targeted antioxidant (MitoPBN), which is unreactive toward the superoxide radical (53). This antioxidant, which is active against carbon-centered radicals and lipid peroxidation, provided no protection to mtDNA throughout this study, suggesting that these relatively long-lived radicals that are generated in mitochondrial membranes close to the genome are not the primary mediators of mtDNA damage in this study.

In conclusion, the vulnerability of mtDNA to mitochondria-derived ROS in response to blue light strongly supports a role for visible irradiation in cellular dysfunction. Such dysfunction will be maximal in tissues such as skin and eye, which are regularly exposed to blue light and thus ROS throughout life (54, 55). The accumulation of stochastic oxidative damage in mitochondria supports a number of hypotheses of aging in which the mitochondrion plays a central role (33, 56–58). Furthermore, the ROS-induced aging is also likely to contribute to a variety of age-related pathologies such as skin cancer and age-related macular degeneration.

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