Sublethal Photic Stress and the Motility of RPE Phagosomes and Melanosomes

Janice M. Burke and Mariusz Zareba

Purpose. To determine whether sublethal oxidative stress to the retinal pigment epithelium by visible light treatment affects the translocation of organelles, notably phagosomes and melanosomes.

Methods. Isolated porcine melanosomes were phagocytized by ARPE-19 cells, then cultures were treated with blue light to generate reactive oxygen intermediates (ROIs) by endogenous retinal pigment epithelial (RPE) chromophores throughout the cytoplasm. Other melanosomes were preloaded with a photosensitizer before phagocytosis, and cells were light treated to generate ROIs specifically at the granule surface. Phagosome movement was analyzed by live cell imaging. Also analyzed were phagocytized black latex beads, phagocytized melanosomes pretreated to simulate age-related melanin photobleaching, and endogenous RPE melanosomes in primary cultures of porcine retinal pigment epithelium.

Results. Sublethal blue light treatment slowed the movement of some, but not all, phagocytized melanosomes. All phagosomes slowed when ROIs were generated near the organelles through a photosensitized reaction. Melanosome photobleaching, which makes granules more photoreactive, increased the effects of blue light. Blue light treatment also slowed the motility of phagosomes containing latex beads and endogenous pigment granules.

Conclusions. Blue light–induced stress impairs phagosome motility in RPE cells but affects individual organelles differently, suggesting that the effects of mild oxidative injury vary with subcellular location. The mechanisms underlying slowed motility are at least partially local because slowing can be induced by a photosensitized reaction in the subdomain of the organelle and the magnitude of the slowing is greater when the phagosome contents are photoreactive. Photic stress may impair the movement and positioning of RPE organelles, which would have widespread consequences for maintaining a functionally efficient subcellular organization. (*Invest Ophthalmol Vis Sci.* 2009;50:1940–1947) DOI:10.1167/iovs.08-2671

Pigment granules (melanosomes), phagosomes, and other membrane-bound organelles translocate within cells along cytoskeletal scaffolds of microfilaments or microtubules.[1–4] Both proteins constituting these scaffolds, actin and tubulin, are sensitive to modification under conditions of oxidative stress, which can produce cytoskeletal disruption.[5–11] This observation motivated us to analyze whether oxidative stress impairs organelle movement. Stress-induced impairments of organelle motility, should they occur, could affect the ability of cells to maintain normal subcellular distribution of organelles and thus their overall cytoplasmic organization.

A focus of this investigation was the motility of melanosomes within retinal pigment epithelial (RPE) cells subjected to oxidative stress induced by irradiation with visible light. This issue is of particular interest for several reasons. The retinal pigment epithelium is a monolayer of long-lived, postmitotic pigmented cells subjected to a lifetime of visible light irradiation. Photic damage to the retinal pigment epithelium caused by visible light, especially blue light, is believed to cause tissue dysfunction over time, contributing to age-related degenerations of the adjacent photoreceptors that the retinal pigment epithelium supports.[12,13] Among the age-related structural changes that take place within RPE cells is redistribution of melanosomes from predominantly apical to more basal regions of the cell cytoplasm,[14] suggesting that aging may affect mechanisms used to localize organelles. The functions that melanosomes perform within pigmented cells are not fully understood, but optical screening by melanin pigments is a well-established property of the organelle. Given that light strikes the retinal pigment epithelium from the apical side, the function of screening light-sensitive molecules in the RPE cytoplasm is best performed when the pigment granules are located apically. Melanin is also believed to protect cells from oxidative stress by virtue of its antioxidant properties.[15] Little is known about the subcellular location of pro-oxidant and antioxidant species within cells, but the positioning of melanosomes within pigmented cells may be one determinant of whether the granules are located near sites where reactive oxygen species are generated so that the pigment can exert its putative antioxidant effect.

To determine whether organelle motility in RPE cells is susceptible to oxidative stress induced by light, isolated porcine melanosomes were introduced by phagocytosis into cultures of the human RPE cell line ARPE-19. Parameters were then established for subjecting cultures to sublethal stress using visible light irradiation. We focused on inducing low levels of stress that might produce small functional decrements rather than overt cell death. Mild stress is likely more relevant during aging, when stress is not acute and fatal but rather subacute and chronic or recurrent and when it produces mild damage that could have cumulative consequences over time. Accordingly, methods were devised to quantify organelle motility in stressed cells that remained viable.

Although RPE melanosome movement was one focus of this investigation, in most experiments the pigment granule was phagocytized and, therefore, encapsulated within a phagosome. This model of phagocytized granules has several benefits for analyses of organelle movement and photic stress. Granule uptake can be adjusted to control particle number within cells, and, because the granule is dark, phagosomes containing them can be tracked by bright-field microscopy, thereby avoiding the use of fluorescent tags that could confound studies involving light treatment. Further, as explained later, properties of

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the pigment can be exploited to alter the magnitude and location of photic stress.

In addition to phagocytized melanosomes, a few experiments were conducted to determine the effects of photic stress on other organelles: phagosomes containing black latex beads in ARPE-19 cells and endogenous melanosomes in primary cultures of porcine retinal pigment epithelium.

METHODS

Preparation of Melanosomes

Melanosomes were isolated from the retinal pigment epithelium of porcine eyes, as previously described.[16,17] Briefly, RPE cells were scraped from eyecups and homogenized. Melanosomes were then purified by ultracentrifugation in a sucrose gradient and incubated in 0.25% Triton X-100, 2% SDS with protease inhibitors to remove contaminating materials and membranes associated with the granule surface.

For some experiments purified melanosomes were preloaded with the photosensitizer rose Bengal (rB) by incubation of the granules in a 0.1 mM solution of rB in phosphate-buffered saline (PBS) for 6 hours in the dark at 4°C. Rose Bengal–loaded granules were washed exhaustively using multiple steps of centrifugation and granule resuspension in fresh PBS. The supernatant was monitored by analyzing light absorbance between 450 and 650 nm, and washing continued until no rB peak (548 nm) was observed (typically 10 washes). A small amount of rB remained granule associated and could be detected spectrophotometrically as a small peak in extracts prepared by the solubilization (Soluene 350; PerkinElmer, Norwalk, CT) of 10^7 to 10^8 melanosomes.

For other experiments, isolated porcine melanosomes were photobleached by irradiation with visible light at 190 mW/cm^2 with the use of a simulator (ThermoOriel Solar, Pittsfield, MA) outfitted with a 1000-W xenon lamp, a 420- to 630-nm dichroic mirror, and an additional UV filter (cutoff, 380 nm), as described previously.[18] Absorbance was measured after solubilizing melanosomes, and samples showing approximately 15% to 50% decreases in absorbance were used.

Cell Culture and Particle Phagocytosis

Melanosomes were delivered to ARPE-19 cell cultures for uptake by phagocytosis, as previously described.[17,19] Briefly, cells were plated at subconfluent density in minimum essential medium containing 10% fetal bovine serum. Twenty-four hours after plating, cells were fed with medium containing approximately 2.5×10^6 granules/cm^2 culture substrate. This granule concentration was lower than our published method[18] because here the intent was to load cells with low particle numbers to permit discrimination and tracking of the movements of individual phagosomes. Cultures were allowed to phagocytize the granules for 24 hours; cells were then replated at low density in eight-well glass chamber slides. The light irradiation experiments described here were initiated within 3 to 4 days. The same protocol was used for the uptake of black latex beads (1 μm; Interfacial Dynamics Microspheres & Nanospheres, Eugene, OR).

In some experiments, a 1:1 ratio of rB-loaded melanosomes and black latex beads was delivered to the cultures to yield cells containing phagosomes with two different contents. Phagosome contents could be distinguished by visualizing the endogenous fluorescence of the beads detected using excitation/emission wavelengths of 635/685 nm.

Experiments were also performed with the use of primary cultures of porcine retinal pigment epithelium containing endogenous melanosomes. Porcine RPE cultures were prepared by methods we have used for human retinal pigment epithelium[20,21] and were explanted directly into chamber slides. Cells were permitted to proliferate until pigment granules were diluted to the extent that the trajectories of individual granules could be followed according to the same methods used for ARPE-19 cultures containing phagocytized particles.

Light Irradiation and Organelle Tracking

Chamber slides containing particle-loaded RPE cells were mounted on the stage of a microscope (Eclipse TE2000U; Nikon Instruments, Melville, NY) outfitted with a motorized, computer-controlled stage and a digital camera (CoolSnap ES; Photometrics, Tucson, AZ). The stage was equipped with an environmental chamber (Live Cell 3; Pathology Devices, Westminster, MD) to control temperature, humidity, and CO_2 levels. For light irradiation and image capture, a 20× objective was used. Image acquisition and data analysis were performed with software (MetaMorph Premier; Molecular Devices, Eugene, OR).

To track phagosome and melanosome movement, phase-contrast images were acquired of selected microscope fields to record cell phenotype, and baseline organelle movement (before light treatment) was tracked using time-lapse imaging. For this purpose, images were captured at 5-second intervals, typically for 30 minutes, using bright-field microscopy. Cultures were then light irradiated for empirically determined times (usually 10 minutes), as reported in Results. For blue light treatment, cells were irradiated with violet-blue light (400–410 nm) at 2.5 mW/mm^2 emitted by the epi-illumination port of the microscope with a 100-W mercury lamp and interference filters (Chroma Technology, Rockingham, VT). For experiments involving rB-loaded melanosomes, the photosensitized reaction was generated using the microscope's green light source (540–570 nm) at 9 mW/mm^2. After light treatment, image capture continued for 10 minutes to 2 hours to track postillumination organelle movement.

Phagosome or melanosome movement was tracked and quantified by assembling the time-lapse images into movies and selecting organelles for analysis based on the following criteria: organelles were located in cells that did not divide during imaging and were not lethally damaged, organelles were dispersed in the cytoplasm so that movement trajectories of individual organelles had limited overlap, and organelles exhibited a baseline movement rate of least 60 μm in 30 minutes (see the Results section for further explanation). Organelle movement was analyzed and quantified using the Track Objects function of the software. Additional details of the tracking method and data analysis are provided in the Results section. Outcomes for all experiment types were replicated in 3 to 10 independent experiments. Representative data are shown.

To confirm that the photic stress used was sublethal, images were captured after particle tracking for another 18 to 24 hours using time-lapse intervals of 5 minutes. Frame-by-frame analysis according to our published methods[17] was conducted to confirm the absence of apoptotic blebbing. At the termination of some experiments, the F-actin cytoskeleton was stained with rhodamine-conjugated phalloidin, as previously described.[21] Nuclei were counterstained with Hoechst dye (1.5 μg/mL).

RESULTS

Baseline Motility of Phagocytized Melanosomes in ARPE-19 Cultures

Phagocytized melanosomes in ARPE-19 cells move nearly continuously in short, oscillatory trajectories (e.g., approximately 1.0 μm in 5 seconds) interrupted at irregular intervals by longer distance movements (e.g., 5.0 μm or more in 5 seconds; Fig. 1). Individual phagosomes vary in their motility patterns, even within the same cell, with some moving less than 25 μm and others more than 200 μm over 30 minutes.

As described, the protocol used in most experiments for analyzing the effects of photic stress on phagosome movement involves capturing images for 30 minutes to establish the baseline motility for each organelle, followed by light irradiation and a second 30-minute image capture interval after treatment. Analysis focused on phagosomes exhibiting robust movement at baseline, translocating at least 60 μm in 30 minutes. Before

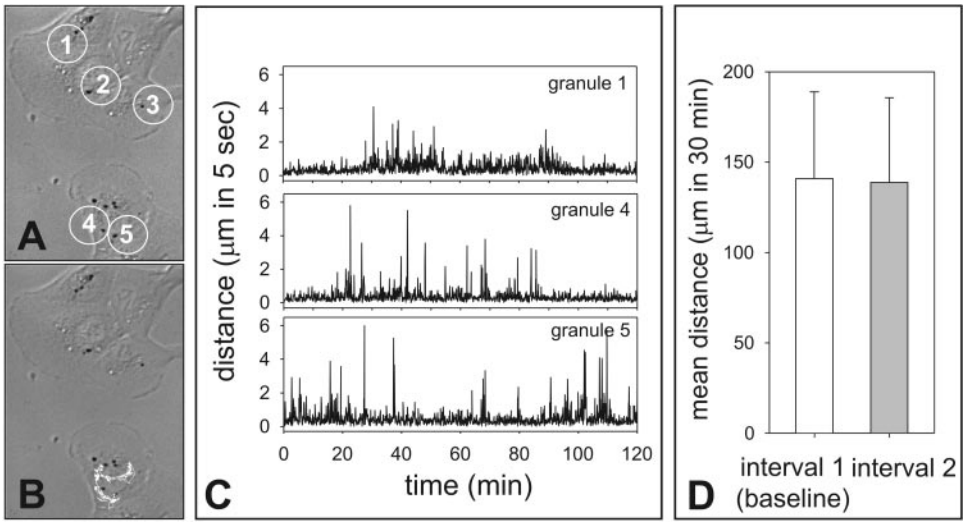

FIGURE 1. Baseline motility of phagocytized melanosomes. (**A**) Bright-field image of ARPE-19 cells showing internalized granules, five of which are circled and numbered. (**B**) The same bright-field image as in (**A**), on which is superimposed the trajectory for the movement of phagosome number 5 over 30 minutes. (**C**) Graphical display of organelle trajectories demonstrating variations in movement patterns over 120 minutes for three organelles (1, 4, and 5). (**D**) Comparison of the total distance traveled (mean ± SD) by organelles in two successive 30-minute intervals (n = 70) in the absence of light treatment.

testing light effects, variations in baseline organelle movement were evaluated in the absence of light treatment by analyzing the distance traveled between two sequential 30-minute intervals. Analysis of 70 sequential intervals showed increases and decreases in distance traveled but no consistent significant interval-to-interval difference (Fig. 1D). Increases occurred 43% of the time and decreases occurred 40% of the time, with the remainder showing similar movement (<5% difference in distance traveled between intervals). For intervals characterized by slowing, the mean decrease in distance traveled relative to the preceding interval was 27% (±17%).

Phagosome Motility in ARPE-19 Cultures: Whole-Cell Photic Stress Induced by Blue Light

Sublethal blue light treatment slowed the motility of phagocytized porcine melanosomes within minutes of light onset (Fig. 2A). Compared with random interval-to-interval changes in

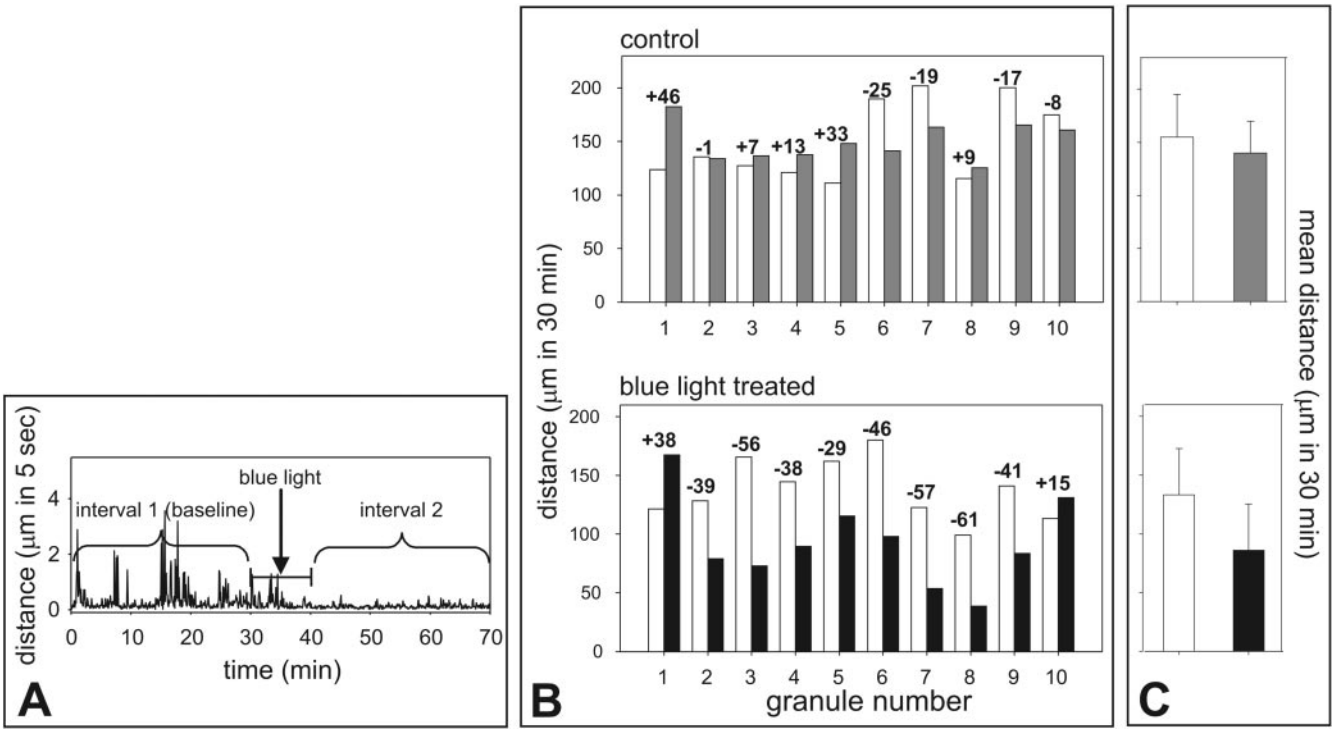

FIGURE 2. Motility of phagocytized melanosomes after sublethal blue light treatment of ARPE-19 cells. (**A**) Graphical display of the movement of a single representative organelle at baseline (30 minutes), during 10 minutes of blue light treatment, and over a 30-minute posttreatment interval. Data shown are from 840 images captured at 5-second intervals. (**B**) Illustration of the total distance traveled in 30 minutes by organelles in untreated control cultures and in blue light–treated cultures. Motility for 10 individual organelles in each group is shown at baseline (interval 1, *open bars*) and after light treatment (interval 2, *solid black bars*) or at the corresponding interval for control cultures (*solid gray bars*). Numbers above the bars indicate the percentage change in distance traveled between interval 1 (baseline) and interval 2. (**C**) Total distance traveled (mean ± SD) by organelles in untreated control cells (n = 38 organelles) and in light-treated cells (n = 30 organelles) during interval 1 (baseline, *open bars*) and interval 2 (*solid bars*). Data are from one representative experiment. Organelles in light-treated cells moved significantly more slowly ($P < 0.05$, t-test).

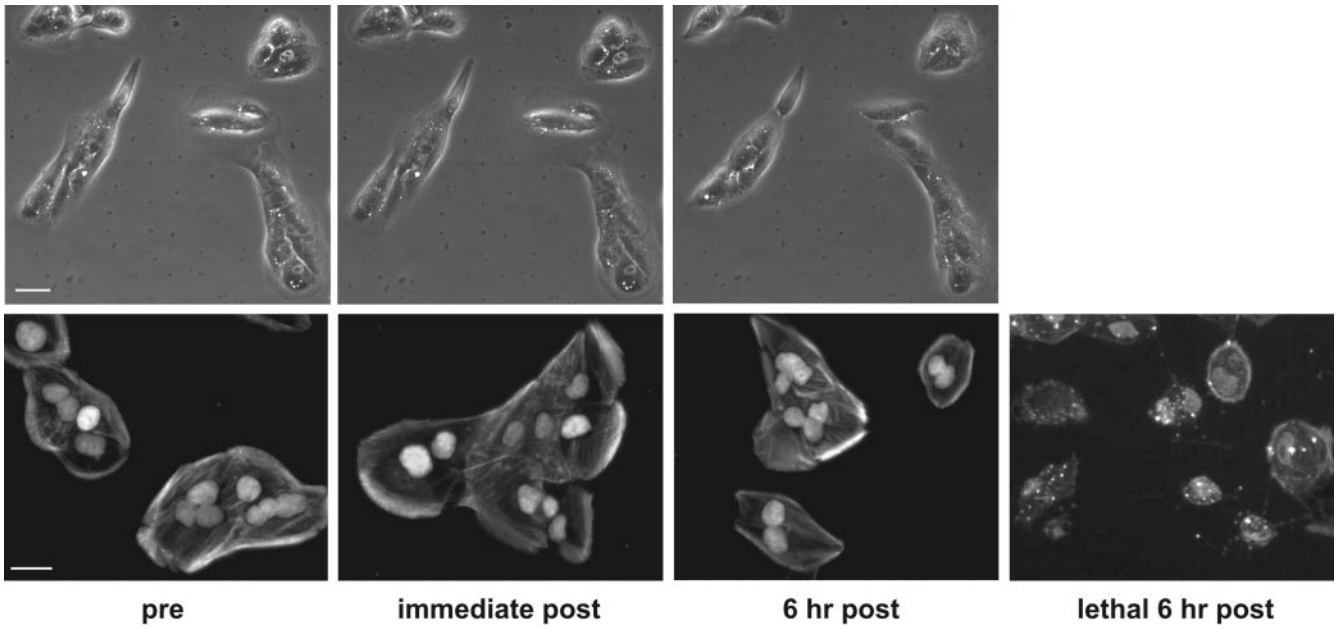

FIGURE 3. Morphologic effects of blue light treatment on ARPE-19 cells. *Upper*: phase-contrast images of the same microscope field before (pre), immediately after (immediate post), and 6 hours after (6 hr post) sublethal light treatment. *Lower*: replicate cultures with Hoechst-stained nuclei showing the unchanged rhodamine phalloidin–stained actin cytoskeleton with sublethal blue light treatment. The last image illustrates the appearance of the actin cytoskeleton after lethal photic stress; cells were irradiated with levels of blue light sufficient to induce apoptosis, as described in Zareba et al.[17] Scale bars: 40 μm (phase micrographs), 20 μm (fluorescence micrographs).

organelle motility that occur in untreated cells (Fig. 2B, control), blue light treatment between the first and second tracking intervals produced motility slowing, manifested as a higher fraction of phagosomes that moved more slowly and reduced distances (Fig. 2B, blue light treated). Comparison of the distance traveled at baseline to the first 30 minutes after blue treatment showed organelle movement slowing 75% of the time, with a mean decrease in distance traveled of 50% ($\pm$15%).

Although blue light treatment of ARPE-19 cells induced decreased phagosome motility, the effect was not universal for all organelles (Fig. 2B). Even within the same cells, some phagosomes slowed markedly while others showed sustained motility.

The levels of blue light irradiation that induced declines in phagosome motility did not affect ARPE-19 cell survival, as indicated by the absence of apoptotic blebbing over 18 to 24 hours after treatment (not shown). Sublethal photic stress levels were also insufficient to affect gross ARPE-19 cell morphology or the integrity of the major F-actin cytoskeleton (Fig. 3). Higher levels of irradiation sufficient to cause cell death produced actin filament disassembly and protein aggregation (Fig. 3) similar to disruptions reported for other forms of oxidative stress to the retinal pigment epithelium.[22–24]

Phagosomes containing photobleached porcine melanosomes were also tested for motility within ARPE-19 cultures exposed to sublethal blue light. Photobleaching by treatment with visible light, which has been used to experimentally simulate age-related photo-oxidation of RPE melanin,[25] is known to render the pigment granules more photoreactive.[18] Within ARPE-19 cells, phagocytized photobleached melanosomes showed greater sensitivity to movement slowing on sublethal blue light treatment than phagosomes containing unbleached granules (Fig. 4). Both the magnitude of the slowing effect (Fig. 4A) and the fraction of organelles that showed substantial slowing (Fig. 4B) increased with increasing photobleaching time.

The slowing of organelle motility by sublethal blue light treatment of ARPE-19 cells was not limited to phagocytized porcine melanosomes. Blue light irradiation also slowed the movement of other organelles, including phagosomes containing black latex beads (Fig. 5A), and endogenous melanosomes in primary porcine RPE cultures (Fig. 5B).

Phagosome Motility in ARPE-19 Cultures: Local Photic Stress Induced by a Photosensitized Reaction

In the sublethal blue light treatment protocols described, slowing of phagosome motility was presumably mediated by blue light–sensitive chromophores endogenous to ARPE-19 cells.

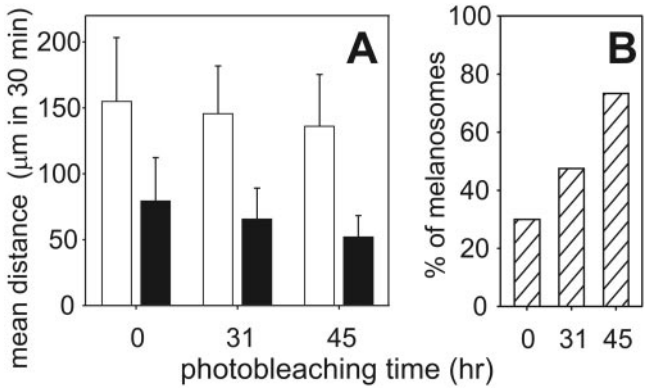

FIGURE 4. Effect of melanosome photobleaching on phagosome movement in ARPE-19 cells treated with sublethal blue light. Phagocytized granules were unbleached (0 hour) or photobleached for 31 or 45 hours before delivery to ARPE-19 cells. (**A**) Organelle movement at baseline (*open bars*) and after light treatment (*solid bars*). Data are mean total distance traveled over 30 minutes ($\pm$SD) for 40 organelles per group; all groups differed significantly from each other ($P < 0.05$, *t*-test). (**B**) Percentage of organelles that slow substantially on blue light treatment (defined as a 50% or greater decrease in distance traveled compared with baseline) as a function of melanosome photobleaching time.

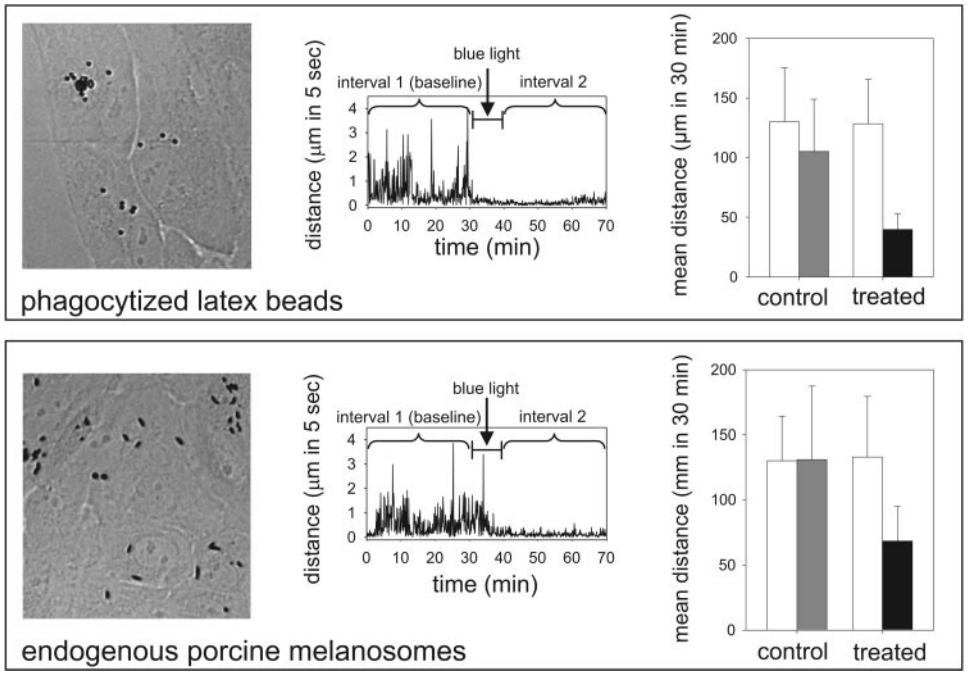

FIGURE 5. Motility of phagocytized black latex beads in ARPE-19 cells (*upper*) and endogenous melanosomes in porcine RPE cells (*lower*) after sublethal blue light treatment. Each panel shows a bright-field image of the cells illustrating the organelles, a graphical display of the movement of a single representative organelle before (baseline), during (blue light), and after light treatment (interval 2) and comparison of the mean distance traveled at baseline interval 1 (*open bars*) and interval 2 (*solid bars*) in control and light-treated cultures. Data are the total distances traveled over 30 minutes (mean ± SD) for 22 to 30 organelles per group. Organelles in treated but not control cells moved significantly more slowly during interval 2 ($P < 0.05$, *t*-test).

The observation that individual organelles in different subcellular regions were differentially affected raised the possibility that the reactive intermediates induced by blue light varied in abundance in different subcellular regions. This possibility was supported by the observation that phagosome motility was more strongly affected when the phagosome contents were more photoreactive (photobleached melanosomes) and able to add to the reactive local environment. Organelle slowing therefore appeared to be mediated primarily by locally generated reactive species (i.e., by reactants generated by or near the granule itself). If this were the case, we theorized that the generation of ROIs specifically in the domain of the granule would impair phagosome movement.

To test this possibility, granules were preloaded with rB, a photosensitizing dye activated by green light that lacks the cell-wide cytotoxic effects of blue light. Experiments confirmed that green light at the intensities used to produce a photosensitized reaction does not affect cell survival (not shown) or the movement of phagosomes containing rB-free melanosomes in ARPE-19 cells (Fig. 6A). The fraction of phagosomes showing decreased motility after green light irradiation did not differ from random differences in baseline movement in untreated cells (compare Figs. 6A and 2B, control). However, for phagosomes containing rB-loaded melanosomes, the local photosensitized reaction on green light irradiation produced a nearly immediate slowing of the motility of all phagosomes (Fig. 6B).

To facilitate tracking, the number of phagosomes per cell is kept low such that the organelles occupy a small fraction of the cell volume, estimated to be less than 1%. The likelihood that granule-bound rB would have cell-wide effects, therefore, seems low, even should the photosensitizer leach from the phagosomes. Nonetheless, to confirm that locally generated ROIs contribute to organelle slowing, the effects of the photosensitized reaction on the movement of two types of phagosomes within the same cells were tested. On green light treatment, phagosomes containing rB-loaded pigment granules slowed nearly immediately (as expected from the results shown in Fig. 6), but the motility of phagosomes containing beads within the same cells was sustained (Fig. 7). Analysis of the motility of 30 phagosomes of each type, all of which were found in cells containing both types of organelles, indicated no significant light treatment effect on the motility of phagosomes containing beads, whereas 23 of 30 (77%) of phagosomes containing rB-load granules traveled less than half the distance after light treatment as they did at baseline before irradiation.

DISCUSSION

The major finding of this investigation was that RPE cultures exposed to cytotoxic blue light at sublethal levels exhibited slowing of two types of membrane-bound organelles, melanosomes and phagosomes. In previous experiments, higher blue light irradiance levels using the same illumination system were shown to kill ARPE-19 cells by a pathway involving apoptotic blebbing,[17] but here a functional impairment in organelle motility was induced by blue light in cells that remained viable. Motility slowing was shown for endogenous melanosomes, phagocytized melanosomes, and phagosomes containing nonspecific particles (latex beads), raising the possibility that several organelle types were affected. Further, individual organelles even within the same cells were differentially sensitive, indicating that the effects of photic stress were heterogeneous at the level of subcellular microdomains.

Melanosomes phagocytized by ARPE-19 cells were the primary model used for testing photic stress effects on organelle motility. To permit organelle tracking, granules were delivered to cells in low numbers according to methods similar to those we used[17,19] to deliver large numbers of pigment granules to study their effects in cells subjected to lethal stress. Motility analyses showed movement patterns for these phagosomes similar to those of endogenous pigment granules, consisting of short-distance oscillatory movements and intermittent longer distance trajectories. Similar melanosome motility patterns in skin melanocytes were attributed to translocation along scaffolds of actin microfilaments and microtubules,[26,27] and both scaffolds appeared to support RPE melanosome movement.[4] Phagosomes showed variable movement within the same ARPE-19 cells, an observation noted here because individual organelles were analyzed in detail to establish baseline movement parameters. Organelle-to-organelle variations in motility

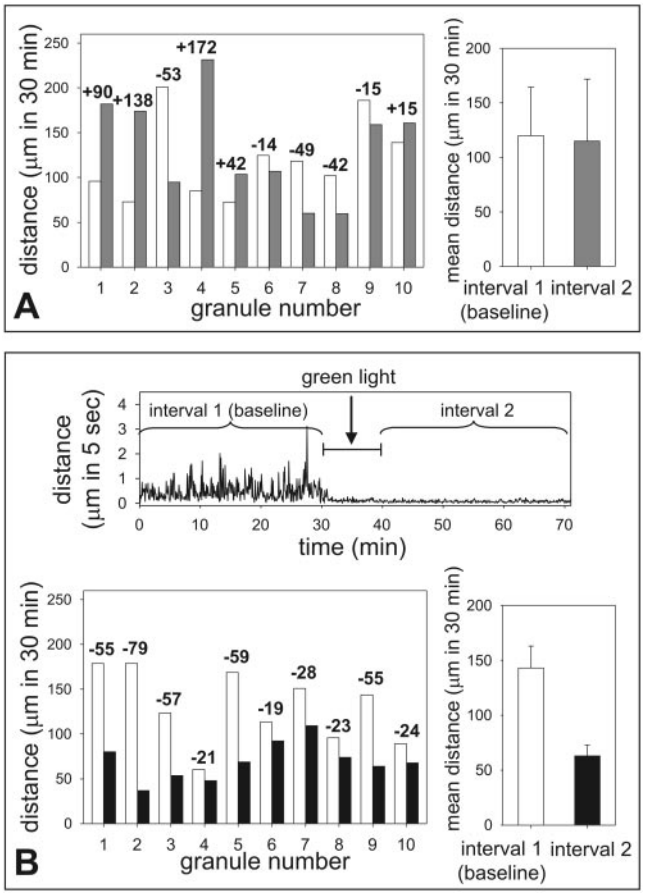

FIGURE 6. Motility within ARPE-19 cells of phagocytized melanosomes after induction of a local photosensitized reaction with green light. (**A**) Control experiment showing no effect of green light irradiation on the motility of phagosomes containing photosensitizer-free melanosomes. *Left*: distance traveled in 30 minutes at baseline (interval 1, *open bars*) and after green light treatment (interval 2, *solid bars*) for 10 individual organelles showing random variations in motility. Numbers over the bars are the percentage change in distance traveled between intervals. *Right*: distance traveled (mean ± SD) for 30 organelles at baseline and after green light treatment. Distance traveled does not differ between intervals. (**B**) Green light irradiation of cells containing phagocytized melanosomes preloaded with photosensitizer (rB). *Top*: graphical display of the movement of a single representative rB-loaded organelle before (baseline), during (green light), and after (interval 2) treatment with green light. Data shown are from 840 images captured at 5-second intervals. *Lower left*: distance traveled at baseline (*open bars*) and after green light treatment (*solid bars*) for 10 individual organelles, demonstrating slowing of all organelles. *Lower right*: distance traveled (mean ± SD) for 25 organelles shows a significant green light–induced decrease in organelle motility ($P < 0.05$, *t*-test).

are not surprising given that the cytoskeletal scaffold for each organelle can consist of short-branching microfilaments, longer microtubules, or a combination of the two networks.[26] Despite variations in movement, phagosome motility proved to be a sensitive measure of light stress, able to detect light treatments that were too mild to produce overt cytotoxicity. Analysis of organelle motility by the protocol used here may, therefore, have wider application for investigating other forms of sublethal oxidative stress and for testing weak putative oxidants or antioxidants.

Relatively little is known about the functional consequences of low oxidative stress levels, including photic stress induced by blue light irradiation. The cytotoxicity of blue light, the most damaging part of the visible spectrum,[12,13] is mediated by chromophores within cells that produce multiple ROIs on irradiation. Important cellular photosensitizers include mitochondrial cytochromes and molecules containing flavins or porphyrins. Here we observed that individual phagosomes in different subcellular domains were differentially sensitive to slowing on blue light treatment. One possible explanation for this observation is an inhomogeneous distribution of the sources of ROI with the cells. It is easy to envision that short-lived reactive species originating from mitochondria, a major source of ROI generated by ARPE-19 cells on blue light irradiation,[28] would be more abundant in the immediate domain of the mitochondrion than geographically distant from the organelle. Given the central role of mitochondria in oxidative stress, it is also possible that energy availability for organelle translocation would vary with the subcellular region, depending on the distribution and stress susceptibility of individual mitochondria. It is conceivable that photosensitizing molecules aside from mitochondrial chromophores are enriched or deficient in different subcellular regions, thereby contributing to inhomogeneous photic effects. Included among the latter is the melanosome itself. As indicated, melanosomes are redox active organelles because of the properties of their constituent melanin. Although porcine melanosomes are expected to be relatively homogeneous, at least compared with human RPE melanosomes from donors of different ages, it is nonetheless likely that some variability exists among granules isolated from animal eyes.

As implied, one local source of ROI that could mediate the slowing of melanosomes or phagosomes containing melanosomes is the melanin component of the granule itself. On irradiation with ultraviolet or visible light, synthetic melanins and isolated pigment granules have been shown to increase aerobic oxygen consumption[29] and ascorbate oxidation[30] and

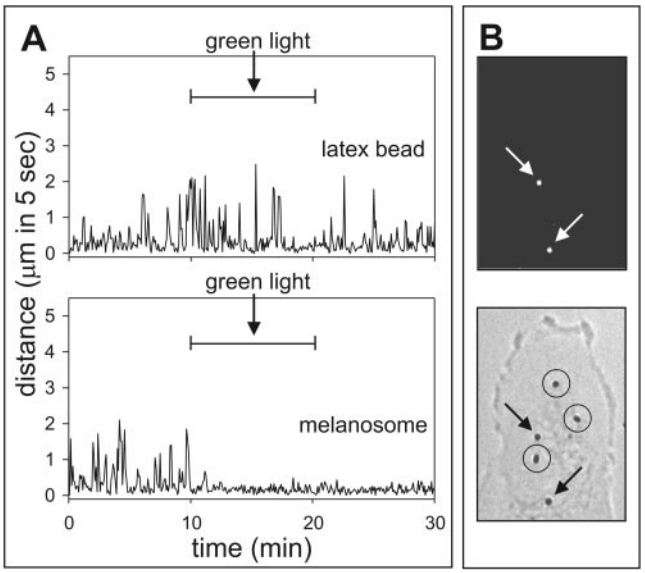

FIGURE 7. (**A**) Graphical illustration of the motility within the same ARPE-19 cell of two types of phagosomes. Data shown are from 360 images captured at 5-second intervals showing the distance traveled over 30 minutes (10 minutes each before, during, and after treatment with green light) for a single representative phagosome of each type. Phagosomes contained either a black latex bead (*top*) or an rB-loaded melanosome (*bottom*). (**B**) Fluorescence (*top*) and bright-field (*bottom*) image of the same cell show that beads can be distinguished from melanosomes because of the endogenous fluorescence of the beads. Phagosomes that contain beads are indicated by *arrows*. Phagosomes that contain melanosomes are indicated by *circles* in the bright-field image.

to generate several ROIs, including superoxide anion, hydrogen peroxide, and hydroxyl radicals.[31,32] Further, photodegradation of melanin granules increases their photoreactive properties.[18] Taking advantage of this characteristic of melanin, we delivered photobleached melanosomes to cells to increase the local generation of ROIs near the phagosomes on blue light treatment. The result was enhanced organelle slowing. This observation suggested that the inhibitory effects of photic stress on phagosome movement were mediated largely by locally generated reactive species. This possibility was supported by the local generation of reactants using granules preloaded with a photosensitizer (rB) activated by nontoxic green light. The local photosensitized reaction was highly effective at slowing organelle movement and could be shown to preferentially slow the movement of phagosomes containing rB-loaded granules, whereas other phagosomes lacking photosensitizer in the same cells were unaffected.

One implication of these observations is that organelle contents matter, and photoreactive organelles are likely to be more susceptible to sublethal light-induced impairments of movement. There are ramifications of this for the biological function of the retinal pigment epithelium, especially the aging retinal pigment epithelium. With aging, the RPE cytoplasm becomes increasingly filled with granules that are photoreactive. The most photoreactive is lipofuscin,[33] the accumulation of which has been implicated in photic stress to the retinal pigment epithelium[34] and as a contributor to age-related macular degeneration.[35–37] RPE melanosomes are also likely to become more photoreactive with aging, not only because they fuse with lipofuscin to yield complex melanolipofuscin granules[38,39] but because melanin itself undergoes age-related changes. RPE melanosomes are long-lived organelles subjected to chronic light exposure, which produces an age-related decline in intact melanin attributed to photobleaching.[25] Photobleached melanosomes exhibit pro-oxidizing properties.[18,40] When present within cells in large numbers, they increase RPE cell susceptibility to lethal photic stress.[17] It appears from this investigation that photobleached melanosomes can also produce a local subcellular effect when exposed to sublethal photic stress that has at least one detectable consequence: greater impairment of phagosome movement.

The mechanism(s) whereby local photic stress produces slowing of organelles has not been determined. One attractive possibility is that the nearby actin or microtubule cytoskeleton, or the protein complexes that link organelles to the cytoskeleton and mediate their movement, are sensitive to oxidative modification. Similar motility machinery is involved in the movement of phagosomes and melanosomes. Kinesins and dynein mediate the microtubule-based movement of phagosomes[41] and melanosomes in some types of pigmented cells.[42–44] Unconventional myosin VIIa is the motor for actin-based movement of RPE melanosomes and phagosomes (at least those containing photoreceptor outer segments).[2,45,46] The small Ras-like GTPase Rab27a participates in the actin tethering of RPE melanosomes and phagosomes,[2] and the tripartite motor complex mediating actin-based melanosome motility is further known to contain the Rab effecter myrip.[4,47] A relevant question is whether any of these proteins is oxidatively modified when associated with organelles in photically stressed RPE cells, especially proteins in complexes associated with photoreactive organelles. Some support for the possibility of local protein oxidation was provided in an ex vivo model in which photobleached melanosomes were shown to increase the photosensitizer-induced oxidation of actin that was experimentally loaded onto the granule surface.[16]

In this investigation the exposure of ARPE-19 cells to phototoxic blue light was shown to impair phagosome movement, though cells well spread on culture substrates were required to demonstrate this effect. The phagosomes analyzed were widely dispersed and located in the midperipheral cytoplasm, where they exhibited sustained movement and could be tracked for extended periods before and after light irradiation. Clearly, the organizational state of the cells, including the cytoskeleton that supports organelle translocation, differs from the epithelioid phenotype of RPE cells in situ, which limits direct extrapolation of the results to the in vivo situation. Nonetheless, the mechanisms of organelle movement identified in cultured cells appeared to have relevance for cells in situ, leading us to speculate that visible light exposure has the potential to produce small impairments in organelle trafficking in the retinal pigment epithelium in vivo.

Should modest light-induced impairments in organelle motility occur in situ, how RPE function would be affected is also speculative. Our focus here was on phagocytized melanosomes because these granules could be manipulated to alter their photoreactivity. However, endogenous melanosomes and other types of phagosomes showed similar slowing on light treatment, suggesting that translocation of membranous organelles in general may be sensitive to sublethal photic stress. Theoretically, the effects of sublethal photic stress have relevance for aging, when the consequences of small decrements can accumulate over time. A possible outcome for RPE cells of inefficient organelle translocation caused by photic stress could be an age-related decline in organelle positioning. Mislocalization of melanosomes away from the apical RPE domain, which occurs with aging,[14] may decrease the pigment granule's efficiency as an optical screen. Mislocalization of other membrane-bound organelles could have widespread consequences for the ability of the aging retinal pigment epithelium to perform other domain-specific functions. The subtle effects of stress on RPE subcellular architecture could over time produce tissue that is less efficient at supporting the function and survival of the adjacent retina, contributing to age-related photoreceptor degeneration.

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