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ARTICLE

Near infrared light exposure is associated with increased mitochondrial membrane potential in retinal pigmented epithelial cells.

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The goal of this study was to characterize the effect of near-infrared light exposure on mitochondrial membrane potential, in vitro. We focused on the retinal pigmented epithelial (RPE) cells due to our interest in the visual health of military airmen exposed to infrared light, which causes thermal damage to the retina. Within RPE cells, an irradiance of 1.6 mW/cm^2 for 30 minutes, resulting in a total fluence of 2.88 J/cm^2, induces resistance to cell death in retinal pigmented epithelial cells exposed to a 1-sec hazardous pulse of 2 μm laser radiation 1. Thus, we examined the impact of this exposure on mitochondrial membrane potential in RPE cells. To do this, the fluorescent molecule, tetramethylrhodamine ethyl ester (TMRE), was used to quantify mitochondrial membrane potential. TMRE is a cell permeant, positively-charged, red-orange dye that readily accumulates in active mitochondria due to their relative negative charge. Depolarized or inactive mitochondria have decreased membrane potential and fail to sequester TMRE. Data from our study show that RPE cells exposed to an irradiance of 1.6 mW/cm^2 for 30-minutes demonstrate elevations in mitochondrial membrane potential. This is the expectation if NIR light exposure is associated with oxygen consumption, as shown in previously published studies. Thus, by focusing on the uptake of TMRE in mitochondria, our findings provide additional details regarding the mechanism

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underlying the effect of NIR and potentially PBM in RPE cells. These findings may also apply to other cell types and red and NIR light exposures.

Introduction

The use of red light or near infrared (NIR) light at low power densities, to produce beneficial physiological effects on cells or tissues is known as photobiomodulation (PBM). Cytochrome c oxidase (CCO), the last enzyme in the electron transport chain (ETC), is believed to be the major chromophore for NIR and this is implicated in the overarching mechanism underlying PBM; evidence in support of this hypothesis includes, but is not limited to, increased oxygen uptake by intact cells and mitochondria,(1) increased CCO expression indicated by immunohistochemistry,(2) (3)[(4)] and increased CCO activity as depicted by histochemical activity assays (5) (6) (7) , all in response to NIR exposure.

Under normal physiological conditions, Nitric Oxide (NO) is effective at competing with O_2 for binding to the active site of CCO, however NO is not reduced, so it acts as an inhibitor of electron transport. However, absorption of high intensity light by CCO shifts its binding affinity for oxygen and displaces NO; this is associated with increases in both cytochrome C oxidase activity and oxygen consumption (8,9). Historically, studies that have examined mechanisms underlying PBM have used oxygen consumption as a proxy for aerobic cellular respiration. However, none have measured the impact of high intensity light exposure on mitochondrial membrane potential. This is important as mitochondrial membrane potential is associated with electron transport, the penultimate step of aerobic cellular respiration.

The goal of this study was to characterize the effect of NIR light exposure on mitochondrial membrane potential in vitro. We focused on the retinal pigmented epithelial (RPE) cells due to our interest in the visual health of military airmen who are exposed to infrared light, which causes thermal damage to the retina(10). Within RPE cells, the specific irradiance and fluence underlying PBM have been characterized; specifically an irradiance of 1.6 mW/cm^2 for 30 minutes resulting in a total fluence of 2.88 J/cm^2 induces resistance to cell death in retinal pigmented epithelial cells exposed to a 1 sec hazardous pulse of 2 µm laser radiation (11). Thus, we examined the impact of this exposure on mitochondrial membrane potential in RPE cells. The fluorescent molecule, tetramethylrhodamine ethyl ester (TMRE), was used to quantify mitochondrial membrane potential in vitro. TMRE, a cell permeant, positively-charged, red-orange dye, serves as an indirect measure of electron transport as it accumulates in active mitochondria due to their relative negative charge, which is created as protons are pumped into the mitochondrial intermembrane space, during electron transport. Results from our analysis suggest that an irradiance of 1.6 mW/cm^2 for 30 minutes is associated with increased mitochondrial membrane potential, and thus potentially associated with increased electron transport.

Methods

Cell culture

As indicated on the ATCC website, the source of the culture was normal female human eyes. The hTERT-immortalized retinal pigment epithelial cell line, hTERT RPE-1, was derived by transfecting the RPE-340 cell line with the pGRN145 hTERT-expressing plasmid (ATCC MBA-141). During the production of the cell line, cells were cultured in medium containing hygromycin B until stable clones were selected (12,13) and cryopreserved until shipping. As indicated by ATCC, cells were tested for the ability to grow for a minimum of 15 population doublings after recovery from cryopreservation. For our experiments, RPE cell cultures were discarded after 6 passages.

RPE cells were grown in complete media composed of 50:50 mix of DMEM:F12 without l-glutamine, supplemented with 10% (v:v) fetal bovine serum, 10 mM HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 2 mM l-glutamine, 100 IU/ml penicillin, 100 µg/ml

streptomycin and 50 µg/ml gentamycin sulfate. Cells were maintained at 37°C, 5% CO_2 and 100% relative humidity prior to use and during all incubation periods. Under these conditions, hTERT-RPE cells have a doubling time of ~21 hr in exponential growth, and a single cell colony forming efficiency of ~40% when 20 to 80 cells are plated into a P-35 dish with 3 ml of complete media and allowed to grow undisturbed for 10 days. The data reported here were obtained using exponentially growing cells.

Exposure to Near Infrared Light

Laser exposures were accomplished in a tissue culture incubator, so temperature could be maintained at 37°C, but without humidity or CO_2 since exposures weren't long enough to create a problem with evaporation of water. Prior to exposing cells, DMEM:F12 media was removed and adherent cells were covered in PBS+0.2% BSA, a clear solution. All exposures were conducted at an irradiance of 1.6 mW/cm^2 for 30 minutes for a fluence of 2.88 J/cm^2.

The Spectra Physics Laser Model 127, 25 mW Air Cooled HeNe (Helium Neon) Laser was used to conduct all exposures. This laser provides a continuous wave (CW) producing a steady beam at a fixed output, emitting at 637 nm with an output power of 25 mW. The laser beam was directed from the direction of the door of the incubator into a GBE10-B - 10X Achromatic Galilean Beam Expander (AR Coated: 650 - 1050 nm – Thor Labs, then through a dichroic film polarizer sheet (Thor labs) and directed onto the sample using a mirror. Rotation of the polarized film was used to obtain a final total irradiance of 1.6mW/cm^2.

This produced ~2.5 cm diameter circular spot with a top hat profile large enough to expose 2 wells of a 48 well plate or 4 wells of a 96 well plate. Laser exposures were accomplished in microwell plates with their covers removed during exposures to avoid changes in irradiance due to reflection, refraction, fogging, of the covers. All assays were conducted immediately after light exposure per manufacturer's instructions.

Cell Viability Assay

Cells were grown on 48-well plates to a density of 1.6X10^5 cells per well. The Tali™ Apoptosis Kit (Annexin V Alexa Fluor™ 488 & Propidium Iodide) by Thermo Fisher and the Tali® Image-Based Cytometer was used to assess the effect of NIR light on apoptosis and necrosis. The assay was performed per the manufacturer's instructions. Cell viability assays were performed immediately after exposure to NIR light.

TMRE Mitochondrial Localization

To ensure that TMRE localizes to mitochondria in RPE cells, cells were dual-labelled with TMRE and mitotracker (Fisher), a fluorescent marker that localizes to mitochondria. Specifically, RPE cells were grown to a density of 1.0X10^5 per well overnight. TMRE and mitotracker were then diluted to 100 nM in a single DMEM complete solution (see instructions above on the production of DMEM complete media). Cells were then incubated in this solution for 30 minutes at 37C, then washed twice in 1X PBS. The ZOE fluorescent imager (BIORAD) was used to acquire images.

Assessing Mitochondrial Membrane Potential

Cells were grown on 96-well plates to a density of 5.0X10^4 cells per well. After NIR light exposure, and completion of the incubation period (either three or six hours), cells were incubated in 500nm TMRE for 15 minutes, washed twice with phosphate buffered saline and TMRE staining was analyzed using a fluorescent plate reader (excitation/emission: 549/575 nm). As a negative control, an experimental condition was included in which cells were incubated in 2uM trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP) for 10 minutes prior to execution of the mitochondrial activity assay. FCCP uncouples oxidative phosphorylation in mitochondria and disrupts ATP synthesis by transporting protons across cell

membranes (14,15). FCCP also induces depolymerization of microtubules by increasing intracellular pH which it accomplishes by disrupting the mitochondrial hydrogen ion concentration gradient and by decreasing the stability of microtubules by impairing the binding of microtubule-associated proteins (16).

Statistical Analyses

Two-sample independent t-tests were used to identify differences in cell quantity, apoptosis, necrosis and mitochondrial activity between treatment and control groups. All statistical analyses were completed using JMP14 (SAS).

Results and Discussion

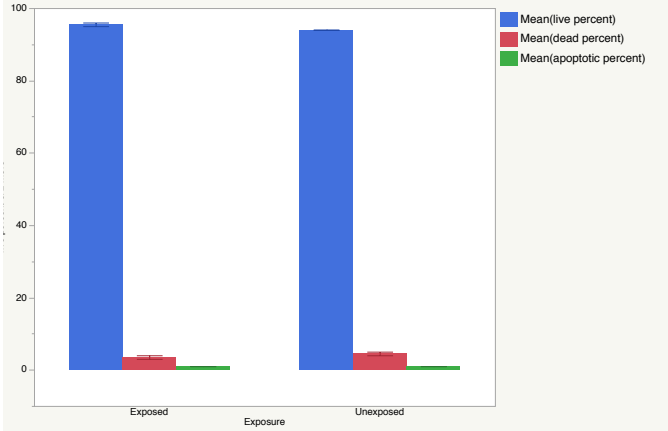

Figure 1. The effect of NIR light on cell viability For each experimental condition, three trials were completed. The results shown represent the mean ± the standard error of the mean (SEM) of the fluorescence detected for propidium iodide, a marker of dead cells (red bars) and annexin V, a marker of apoptotic cells. Cells where no markers of apoptosis or death were identified were counted as living (blue bars). No statistical differences in the average number of living, apoptotic, or dead cells were detected between cells exposed and unexposed to NIR light.

Exposure to NIR light does not induce apoptosis or necrosis

First, we investigated the effect of near infrared light on cell viability. We did not find that the percentage of cells alive differed between exposed and unexposed cells ($p = 0.10$). We also did not find significant changes in markers of apoptosis (annexin V, $p > 0.05$) and necrosis (propidium iodide, $p = 0.85$) between exposed and unexposed cells (figure 1) within one hour of exposure.

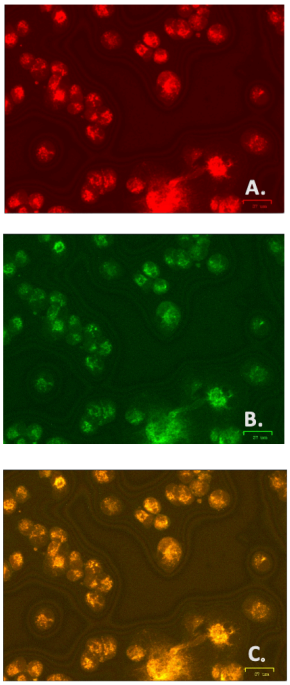

Figure 2. TMRE Colocalizes to Mitochondria of RPE Cells. (A) RPE cells were dual-labeled by incubation in DMEM complete containing 100 nM mitotracker and (B) 100 nM TMRE for 30 minutes at 37C. Fluorescence images and the merge of images 2A and 2B (C) were acquired using the ZOE imager (BIORAD).

TMRE Colocalizes to Active Mitochondria in RPE Cells

Prior to using TMRE to quantify mitochondrial membrane potential, we checked to ensure that it localizes to mitochondria in RPE cells by co-staining cells with mitotracker (figure 2A) and TMRE (figure 2B). Results of our analysis shows that TMRE co-localizes to the mitochondria of RPE cells (figure 2C).

NIR Light is Associated with Increased Mitochondrial Membrane Potential

Approximately 30 minutes is needed to treat cells with TMRE and measure fluorescence. Within this

time frame, we detected an increase in TMRE fluorescence in RPE cells exposed to NIR light ($p = 0.01$), which represents a modest, but statistically significant 20% increase in TMRE fluorescence in cells exposed to NIR. In cells treated with FCCP (figure 3) ($p = 0.01$), only cells exposed to NIR light showed TMRE fluorescence. However, this amount was 12 times less than that observed in exposed, non-FCCP treated cells. FCCP is an ionophore, treatment with which results in the loss of membrane potential. Thus, the observation that cells treated with FCCP show a significant decrease in TMRE fluorescence, a reflection of decreased membrane potential, is expected. However, it is interesting that we were able to detect fluorescence in exposed FCCP treated cells. We believe this is due to the greater membrane potential associated with NIR light treatment, which occurred prior to FCCP treatment.

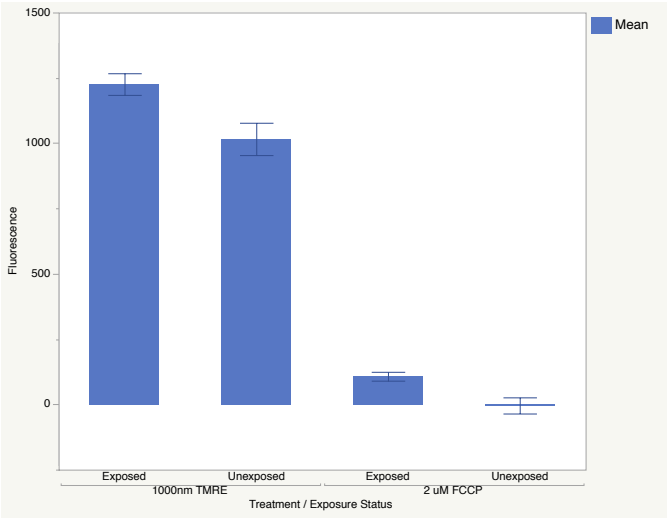

Figure 3. Exposure to NIR Light is Associated with Increased Mitochondrial Activity. Mitochondrial activity in RPE cells either exposed or unexposed to NIR light was assayed using TMRE. For each experimental condition, four trials were completed. The results shown represent the mean ± the standard error of the mean (SEM) of the TMRE fluorescence which is directly correlated with mitochondrial activity. 2uM of FCCP was added to cells in a separate experiment as a negative control. The single star (*) indicates a statistical difference in TMRE fluorescence with the corresponding unexposed sample, $p < 0.02$. The double stars (**) indicates a statistical difference in TMRE fluorescence with the corresponding unexposed sample in samples where 2uM of FCCP was added prior to assessing mitochondrial activity, $p < 0.02$.

An irradiance of 1.6 mW/cm^2 for 30 minutes resulting in a fluence of 2.88 J/cm^2 , administered 24 hours prior, induces resistance to cell death in retinal pigmented epithelial cells exposed to a 1 sec pulse of 2 μm laser radiation (11), thus we used this exposure in our study to elicit PBM and to examine the effects of NIR light on TMRE uptake and mitochondrial membrane potential. In our examination of the impact of this exposure on cell viability, we did not find that it was associated with elevations in either cell death or apoptosis (figure 1). Proving assurance that our exposure did not harm cells or growth within the time frame of our experiment. We also showed that TMRE localized to mitochondria in RPE cells (figure 2) and that TMRE fluorescence is approximately 20% greater NIR light exposed cells, a modest, but statistically significant increase (figure 3); this suggests that an irradiance of 1.6 mW/cm^2 for 30 minutes NIR light exposure is associated with increased elevations in mitochondrial membrane potential, which is associated with increased electron transport.

Conclusion

Administration of an irradiance of 1.6 mW/cm^2 for 30 minutes resulting in a fluence of 2.88 J/cm^2 , 24 hours prior to a 1 sec pulse of 2 μm laser radiation, has been shown to induce resistance to cell death in retinal pigmented epithelial cells(11), thus we focused on this exposure and treatment schema in our study. However, there is also evidence that treatment of retinal tissue with NIR light *after* administration of cellular damage also elicits beneficial physiological effects. Eels et al. 2003 (17) showed that upon injecting rats with methanol dosages shown to cause visual dysfunction (18,19) and ocular toxicity (20,21) that treatment with three two-minute, 24 second, 670-nm LED treatments (4 J/cm^2), 5, 25, and 50 hours after methanol intoxication, attenuated the retinotoxic effects of methanol-derived formate. As formate serves an inhibitor of CCO, the last enzyme in the electron transport chain, this study (17), similar to ours, also provides support for the role of NIR light in cytoprotection and respiratory chain function in retinal cells.

Tracking mitochondrial membrane potential is also important as we study the effects of NIR light in RPE cells, as we see that even in the presence of inhibitors of CCO, that NIR light mediates respiratory chain function. Our future studies

include examining the effect of multiple red light exposures, administered over the course of several days, on mitochondrial membrane potential. Potentially multiple exposures over time would produce more than the modest 20% increase in mitochondrial membrane potential observed in our study and thus potentially have a greater impact on cytoprotection.

CONFLICTS OF INTERERST

There are no conflicts to declare

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