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NEUROPROTECTIN D1 IS SYNTHESIZED IN THE CONE PHOTORECEPTOR CELL LINE 661W AND ELICITS PROTECTION AGAINST LIGHT-INDUCED STRESS

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Abstract

Docosahexaenoic acid (DHA), an omega-3 fatty acid family member, is obtained by diet or synthesized from dietary essential omega-3 linolenic acid and delivered systemically to the choriocapillaris, from where it is taken up by the retinal pigment epithelium (RPE). DHA is then transported to the inner segments of photoreceptors, where it is incorporated in phospholipids during the biogenesis of outer segment disk and plasma membranes. As apical photoreceptor disks are gradually shed and phagocytized by the RPE, DHA is retrieved and recycled back to photoreceptor inner segments for reassembly into new disks. Under uncompensated oxidative stress, the docosanoid neuroprotectin D1 (NPD1), a potent mediator derived from DHA, is formed by the RPE and displays its bioactivity in an autocrine and paracrine fashion. The purpose of this study was to determine whether photoreceptors have the ability to synthesize NPD1, and whether or not this lipid mediator exerts bioactivity on these cells. For this purpose, 661W cells (mouse-derived photoreceptor cells) were used. First we asked whether these cells have the ability to form NPD1 by incubating cells with deuterium (d4)-labeled DHA exposed to dark and bright light treatments, followed by LC-MS/MS-based lipidomic analysis to identify and quantify d4-NPD1. The second question pertains to the potential bioactivity of these lipids. Therefore, cells were incubated with 9-*cis* retinal in the presence of bright light that triggers cell damage and death. Following 9-*cis* retinal loading, DHA, NPD1, or vehicle were added to the media and the 661W cells maintained either in darkness or under bright light. DHA and NPD1 were then quantified in cells and media. Regardless of lighting conditions, 661W cells acquired DHA from the media and synthesized 4–9 times as much d4-NPD1 under bright light treatment in the absence and presence of 9-*cis* retinal compared to cells in darkness. Viability assays of 9-*cis* retinal-treated cells demonstrated that 34% of the cells survived without DHA or NPD1. However, after bright light exposure, DHA protected 23% above control levels and NPD1 increased protection by 32%. In

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conclusion, the photoreceptor cell line 661W has the capability to synthesize NPD1 from DHA when under stress, and, in turn, can be protected from stress-induced apoptosis by DHA or NPD1, indicating that photoreceptors effectively contribute to endogenous protective signaling mediated by NPD1 under stressful conditions.

Keywords

NPD1; DHA; photoreceptors; retina; light stress

INTRODUCTION

Our lives are based on visual information obtained by central (macular) vision, which enables high acuity, bright light and color vision (Curcio et al., 1987; Curcio et al., 1990; Ruddock and Burton, 1972). Any perturbations in this cone-rich region result in disruptions in the quality of life. Major human visual disorders involve the cone-dominant macular region, making it imperative to understand how to counteract homeostatic dysfunctions that initiate photoreceptor degeneration and cell death, resulting in blindness. Moreover, photoreceptors function within a highly detrimental environment; they are exposed to bright damaging light and experience high metabolism. The photoreceptors' mitochondria are a major source of reactive oxygen species and, in uncompensated oxidative stress conditions, these species become cell damaging unless their deleterious effects are counteracted (Jarrett et al., 2008). In addition, the degradation of photoreceptor membranes, after phagocytosis of outer segments by the retinal pigment epithelium (RPE), results in a daily infusion of polyunsaturated fatty acids (Fisher et al., 1983) contributing another potential disruptor of homeostasis by lipid peroxidation.

Docosahexaenoic acid (DHA), an omega-3 fatty acid family member obtained by diet or synthesized from dietary essential omega-3 linolenic acid, is delivered systemically to the choriocapillaris, from where it is taken up by the RPE. DHA is then transported to the inner segments of photoreceptors and incorporated into phospholipids for biogenesis of light-sensitive photoreceptor outer segment membranes (Bazan et al., 2011). Under conditions of stress, DHA is transformed the neuroprotective docosanoids. Thus far, studies using RPE cells have yielded significant information regarding the function of DHA as a reservoir of docosanoids; NPD1 was the first characterized member of this family of lipid mediators (Mukherjee et al., 2004). To determine if photoreceptors display the ability to form NPD1, and whether or not this mediator elicits protective bioactivity, we have used the 661W cells, a cell line derived from mouse cone photoreceptors that expresses cone opsins but not rod-specific antigens (Tan et al., 2004). The 661W cone cell line has been used to study retinoid synthesis (Kanan et al., 2008) and oxidative stress and the generation of reactive oxygen species (Sharma and Rohrer, 2007). The present study utilized the 661W cell line to determine if chromophore-induced light damage, which initiates oxidative stress, could increase uptake of DHA and activate NPD1 synthesis, and whether NPD1 bioactivity is protective against conditions that threaten survival in these cells.

Our results show that 661W cells increased the uptake of added DHA under light conditions when compared to dark-exposed cells. In addition, these cells converted deuterium-labeled DHA (d4-DHA) to deuterium-labeled NPD1 (d4-NPD1), and this conversion was greater in cells exposed to chromophore-induced light damage than in dark-exposed cells. We have previously shown that chromophore-induced light damage causes up-regulation of multiple cell death pathways and eventually results in cell death (Kanan et al., 2007). Thus, incubation of the 661W cells with either DHA or NPD1 results in protection of 23% and 32% more cells when subjected to chromophore-induced light stress, respectively, indicating that NPD1 is neuroprotective in photoreceptor cells.

MATERIALS AND METHODS

661W culture conditions

Conditions for growth and maintenance of the 661W cells were described earlier (Kanan et al., 2007).

9-*cis* retinal as a stress inducer

The source, concentration, and use of 9-*cis* retinal were described before (Kanan et al., 2007). 11-*cis*-retinal is the form of vitamin A that binds opsin and is isomerized to all-*trans*-retinal upon absorption of light, and subsequently released for recycling through the visual cycle as all-*trans*-retinol. 9-*cis*-retinal is also present in the normal retina, but at a much lower concentration, and it can also bind opsin to generate a photoactivatable molecule (Fan et al., 2003). This 9-*cis*-retinal can trigger retinal cell death in bright light if it accumulates and is not cleared, although the actual mechanism of damage is not well understood. We have shown that 9-*cis*-retinal is cytotoxic to 661W cells only in bright light (Veritti et al., 2012), and have utilized this chromophore to initiate cell stress within the 661W cell cultures of this study.

Light source

Illumination from the light box (Model MS1417-C-DIM-T83; Aristo Lighting Technologies, Roslyn, NY) is produced by a combination of three sources, peaking at 453 nm (blue), 546 nm (green), and 611 nm (red), to produce “white” light. UV light (<400 nm) is blocked by Plexiglas filtering. Two small fans incorporated into the light box maintain ambient temperature. Light intensity is controlled by additional filters (Kanan et al., 2007).

Light stress in the presence of 9-*cis*-retinal

661W cells were grown to 80% confluence in T-75 tissue culture dishes. Media with 10 μ M 9-*cis*-retinal was added and the dishes were then incubated in darkness for 4 hours to allow cells to take up the chromophore. Media was then changed to 9-*cis*-retinal-free media, and one set of dishes was maintained in the dark for 2 hours while another set was incubated in bright light (30,000 lux) for 2 hours. DHA or NPD1 (100 nM) was added to the media before the light and dark exposures. After incubation, both media and cells were harvested for quantitation of DHA and NPD1 by LC-MS/MS.

Light stress in the absence of 9-*cis*-retinal

661W cells were grown to 80% confluence in T-75 tissue culture dishes. Fresh media was added and one set was placed in darkness while another set was incubated in bright light (30,000 lux) for 6 hours.

Protection assays

24,000 661W cells were plated overnight in 96 well dishes. The next day, media was replaced with media containing 9-*cis*-retinal (10 μ M) and the dishes incubated for 4 hours in darkness to allow uptake of the chromophore. Media was then replaced with 9-*cis*-retinal-free media which contained either DHA (100 nM), NPD1 (100 nM), or media alone, and dishes of each treatment placed in darkness or a light box for exposure to 30,000 lux for 4 hours. Cell viability was then determined by an MTT assay which measured absorbance of each preparation at 570 nm as described previously (Kanan et al., 2007). Three independent experiments were evaluated and averaged.

Detection of DHA and NPD1

661W lipid extracts were first labeled with deuterated internal standards, followed by purification using solid-phase extraction, and run through a Biobasic-AX column (Thermo-Hypersil-Keystone, Bellefonte, PA) (100 mm $\times$ 2.1 mm; 5 μ m particle size) with a 45-min gradient (starting at 100% with solvent A (40:60:0.01 methanol:water:acetic acid, pH 4.5) and ending with 100% solvent B (99.99:0.01 methanol:acetic acid)) at a flow rate of 300 μ l/min. The column was run isocratically for 5 min. A TSQ Quantum (Thermo-Finnigan, San Jose, CA) triple quadrupole mass spectrometer (electrospray ionization spray voltage was 3 kV, sheath gas N₂ was set at 35 cm³/min at 275°C) was used. Full-scan mode on Q1 quadrupole was used to detect precursor ions. Quantitative analysis was achieved by selected reaction monitoring. Argon was the Q2 collision gas (set to 1.5 mTorr); product ions were detected on Q3. Selected precursor/product ion pairs for DHA and NPD1 were 327/283 m/z and 359/206 m/z, respectively. Calibration curves were obtained running synthetic DHA and NPD1 in parallel (Cayman Chemical, Ann Arbor, MI). NPD1 for bioactivity was generated by biogenic synthesis using soybean lipoxygenase and DHA. DHA and NPD1 were determined by LC-photodiode array-MS/MS using previously-described methods (Ruddock and Burton, 1972; Chew et al., 2013; Shen et al., 2007).

RESULTS

We have previously shown that ~70% of 661W cells are viable after 6 hours of light stress in the absence of 9-*cis* retinal and after 2 hours of light stress in the presence of 10 μ M 9-*cis* retinal (Kanan et al., 2007). While 661W cells are able to desaturate and elongate 18:3n3 or 22:5n3 fatty acids up to 24 carbons (e.g., 24:5n3) (Agbaga et al., 2010), these cells cannot convert these molecules to 22:6n3. Therefore, without available DHA in the media, DHA levels within 661W cells are barely detectable (Agbaga et al., 2010). Since 661W cells have similar requirements as those in the retina, and DHA must be supplied from an exogenous source, DHA up-take could be analyzed by addition to the media. Therefore, media was supplemented with 100 nM DHA (see Fig. 1 for experimental design).

Light caused a significant increase in the cellular concentration of DHA in the presence or absence of the chromophore, 9-*cis* retinal (Fig. 2a). In light-exposed 661W cells in the absence of 9-*cis* retinal, approximately 56 ng of DHA/mg protein was detected within the cells, as compared to 10 ng of DHA/mg protein when cells were left in the dark for equivalent times (Fig. 2a). In the presence of 9-*cis* retinal, light exposure resulted in accumulation of approximately 43 ng of DHA/mg protein as compared to approximately 8 ng of DHA/mg protein within cells incubated in the dark with the chromophore (Fig. 2a). Interestingly, analysis of the conditioned media from cells incubated under light conditions (with or without chromophore) showed a slight increase in DHA when compared to media from dark-exposed cells (Fig. 2b). This demonstrates that 661W cells can take up exogenous DHA, and may be able to free DHA from DHA-containing lipids for release of DHA to the extracellular medium.

To more completely define DHA uptake under conditions of stress, 661W cells were incubated with deuterated DHA (d4-DHA) and then maintained in the dark or exposed to light in the presence or absence of 9-*cis* retinal. Analysis showed that d4-DHA accumulated within cells under light conditions in a manner similar to that observed for unlabeled DHA (compare Figs. 3a and 2a). 661W cells exposed to light for 6 hours accumulated 0.21 ng of d4-DHA/mg protein as compared to 0.12 ng of d4-DHA/mg protein in dark-treated cells (Fig. 3a). Light exposure in the presence of 9-*cis* retinal resulted in the accumulation of approximately 0.16 ng of d4-DHA/mg protein, compared to 0.07 ng of d4-DHA/mg protein detected within the 9-*cis* retinal of dark-treated cells (Fig. 3a). Analysis of the conditioned media from dark- or light-exposed cells in the presence or absence of 9-*cis* retinal did not reveal changes in d4-DHA content (Fig. 3b). These results indicate that 661W cells take up increased amounts of d4-DHA from the media under stressful conditions.

It has been previously shown that DHA can be converted to the neuroprotectant NPD1 and secreted by the RPE (Mukherjee et al., 2004). To determine whether 661W cells are capable of taking up NPD1, they were incubated in its presence, and NPD1 levels were determined in the cytoplasm and conditioned media. Under 6 hours of light conditions in the absence of 9-*cis* retinal, NPD1 accumulated 23 pg of NPD1/mg protein, compared to 4.2 pg of NPD1/mg protein in the cells in the dark. Light exposure in the presence of 9-*cis* retinal accumulated approximately 12.5 pg of NPD1/mg protein compared to 3 pg of NPD1/mg protein in the dark (Fig. 4a). Analysis of the media from dark- or light-exposed cells in the presence or absence of 9-*cis* retinal indicated no change in NPD1 content (Fig. 4b). This result suggests that 661W cells synthesize NPD1 under light conditions, but do not release appreciable amounts to the media.

While it is known that photoreceptors can take up DHA and NPD1 from RPE cells, it is not known if photoreceptors can convert DHA to NPD1 as demonstrated for RPE cells. Most importantly, incubating cells with 100 nM d4-DHA resulted in the accumulation of labeled intracellular NPD1 (d4-NPD1) under light conditions. 661W cells exposed to light for 6 hours in the absence of 9-*cis* retinal accumulated 14.88 pg of d4-NPD1/mg protein, compared to 3.12 pg of d4-NPD1/mg protein in cells exposed to the dark (Fig. 5a). Light exposure in the presence of 9-*cis* retinal accumulated approximately 9.6 pg of d4-NPD1/mg protein as compared to 1 pg of d4-NPD1/mg protein in dark-exposed cells (Fig. 5a). This

demonstrates that the 661W cell line is capable of taking up DHA and converting it to NPD1 under stressful light treatment. Furthermore, 661W cells secreted d4-NPD1 into the media under both dark and light conditions, although little difference was observed between the two conditions (Fig. 5b). Thus, 661W cells under light stimulation can synthesize NPD1 from DHA.

Since it has already been established that DHA and NPD1 are neuroprotective compounds and protect against oxidative stress (Bazan, 2005, 2009; Faghiri and Bazan, 2010; Halapin and Bazan, 2010; Marcheselli et al., 2003;), we analyzed their protective effects on 661W cells using an *in-vitro* assay that we developed previously (Kanan et al., 2007), wherein the chromophore 9-*cis* retinal-treated cells were maintained in darkness or placed in bright light (30,000 Lux) for 4 hours. This light stress upregulates caspase-dependent and -independent pathways that result in apoptosis of the cells. Under these conditions, the presence of DHA protected 23% while NPD1 protected 32% of the cells when compared to bright light-treated cells alone and the dark controls (Fig. 6). This demonstrated that DHA and NPD1 provide protection to 661W cells from chromophore-mediated light stress.

DISCUSSION

DHA belongs to the essential omega-3 fatty acid family (Bazan and Rodriguez de Turco, 1994). It is taken up from the blood stream by RPE cells and sent to inner segments of photoreceptors where it becomes part of phospholipids used for the biogenesis of outer segment and plasma membranes of photoreceptor cell, where DHA attains the highest concentration within the body (Wang and Anderson, 1992). Following shedding of photoreceptor tips and phagocytosis by the RPE, DHA is retrieved from the phagosomes and conserved by recycling back to photoreceptors. Under conditions of uncompensated oxidative stress, DHA is released from the phospholipids and converted by 15-lipoxygenase-1 (15-LOX-1) into neuroprotectin D1 (NPD1) (Mukherjee et al., 2004; 2007a), a highly potent, selective mediator that initiates anti-inflammatory, pro-survival signaling (Bazan, 2007).

The results from the National Eye Institute's Age-Related Eye Disease Study show that relatively high amounts of zinc and antioxidants reduce the risk of developing advanced AMD by 25% (Chew et al., 2013), implying uncompensated oxidative stress in the pathogenic process of AMD. In addition, oxidative damage markers in post-mortem retinas of patients with geographic atrophy are additional evidence for oxidative stress being involved in AMD (Shen et al., 2007). Homeostatic disturbances, when uncompensated, lead to sustained oxidative stress and compromised mitochondrial function, resulting in apoptotic cell death (Bazan, 2007; de Jong, 2006; Dunaief et al., 2002; Feher et al., 2006; Lukiw et al., 2005; Mendes et al., 2005; Sreekumar et al., 2005; Takahashi et al., 2004).

Single nucleotide polymorphisms (SNPs) occurring in complement factor H gene (CFH/HF1) (Edwards et al., 2005; Hagerman et al., 2005; Haines et al., 2005; Klein et al., 2005) are a major risk factor for developing AMD. CFH is an inhibitor of the alternative pathway of complement system activation that limits complement-induced cell injury and inflammation (Bok, 2005; Gold et al., 2006). Conversely, studies that focused on other

regulatory proteins of the complement pathway, such as factor B (BF) and complement component 2 (C2), exhibited protective effects and lowered risk for developing AMD (Gold et al., 2006; Spencer et al., 2007). For example, the L9H variant of factor B, as well as the R32Q variant of factor B (BF), and the E318D variant of complement component C2 (C2), as well as a variant in intron 10 of complement component C2, confer a reduced risk for developing AMD (Gold et al., 2006; Spencer et al., 2007). Therefore, identification of anti-inflammatory, pro-survival proteins is beneficial for maintaining photoreceptor integrity and slowing/halting disease progression.

RPE cell integrity is necessary for the survival of rod and cone photoreceptors. These cells accomplish many functions, including transport of nutrients and DHA from the choriocapillaris to the photoreceptors, and the reisomerization of vitamin A following photon absorption (Strauss, 2005). RPE cells, when exposed to H₂O₂ plus TNF- α -mediated oxidative stress, produce NPD1 (Mukherjee et al., 2004, 2007a, 2007b; Faghiri and Bazan, 2010) that, in turn, up-regulates anti-apoptotic proteins (e.g., Bcl-2, Bcl-x_L) and down-regulates pro-apoptotic proteins (e.g., Bax, Bad) promoting cell survival. Thus, under these conditions, NPD1 inhibits cytokine-mediated pro-inflammatory gene induction (Halapin and Bazan, 2010; Marcheselli et al., 2003) and oxidative stress-induced apoptosis, promoting RPE cell survival (Bazan, 2009; Halapin and Bazan, 2010). Oxidative stress (which can lead to apoptosis), neovascularization, and lipid peroxidation are involved in neurodegenerative diseases, including age related-macular degeneration (AMD) (Cai and McGinnis, 2012; Lotery and Trump, 2007; Nowak, 2013; Veritti et al., 2012). Since cone photoreceptors play such a significant role in vision under intense light exposure, survival mechanisms have evolved to counter this stress and provide protection. Here, we have described a mechanism within cone photoreceptors that aids in the maintenance of homeostasis.

We have shown that light causes an increase in cellular uptake of DHA and synthesis of NPD1 by 661W cells, demonstrating that these cone photoreceptor-derived cells harbor the machinery to convert DHA to the neuroprotective molecule NPD1. We have also shown that DHA protected the cells from chromophore-induced light stress by 23%, while NPD1 protected by 32%. Since these cells can convert DHA to NPD1, the protection could be afforded by both DHA and NPD1 under conditions of stress.

Although the 661W cells were characterized to be from a mouse cone photoreceptor lineage and express many of the cone-specific antigens (Tan et al, 2004), it is important to remember that they are not identical to native cones and their environment. Although our data clearly suggest that cones may produce NPD1, it is yet to be proven in an *in vivo* system. Unfortunately, the mouse retina contains ~3% cones, making it difficult to quantify stress-induced changes in NPD1. Therefore, a suitable cone-rich model must be identified to be able to perform the *in vivo* experiment. Models like the *nrl*^{-/-} may be useful. However, this model is not accepted as a pure cone retina.

The success of future AMD therapies lies in their abilities to preserve, protect, and eventually restore cone photoreceptor function, since it is ultimately the loss of these cells, and the acute color vision they mediate, that results in devastating vision loss in AMD. Our

studies show that fatty acids such as DHA, or its derivative, NPD1, could play a crucial role in increasing the viability of cone photoreceptor cells by preserving their function and protecting them against cell death triggered by conditions of oxidative stress.

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Figure 1.

Diagram of experimental protocols and time lines. 661W cells were cultured to 80% confluency prior to each experiment, followed by incubation with 9-*cis* retinal for 4 hours in darkness to allow cells to take up the chromophore. Cells were then dark or bright-light treated, collected, and analyzed by LC-MS/MS, or viability was determined. Conditions that included incubation with DHA, NPD1, d4-DHA, or media alone were compared.

Figure 2.

a. 661W cells accumulate DHA under light conditions in the presence or absence of the stress-inducing chromophore, 9-*cis* retinal, compared to cells incubated in the dark. **b.** Conditioned media from 661W cells incubated in the presence or absence of 9-*cis* retinal, under light or dark conditions, have comparable DHA content. Results present the mean values ($\pm$ SEM) and were obtained from three independent experiments.

Figure 3.

a. 661W cells incorporate exogenously added d4-DHA within cells under light conditions in the presence or absence of 9-cis retinal, compared to cells incubated in the dark. **b.** Conditioned media from 661W cells incubated in the presence or absence of 9-cis retinal, under light or dark conditions, have comparable d4-DHA content. Results present the mean values ($\pm$ SEM) and were obtained from three independent experiments.

Figure 4.

a. 661W cells accumulate NPD1 from media under light conditions in the presence or absence of 9-cis retinal, compared to cells incubated in the dark. **b.** Conditioned media from 661W cells incubated in the presence or absence of 9-cis retinal, under light or dark conditions, have comparable NPD1 content. Results present the mean values ($\pm$ SEM) and were obtained from three independent experiments.

Figure 5.

a. 661W cells can convert exogenously-added, deuterium-labeled (d4) DHA into d4-NPD1, which accumulates inside cells under light conditions in the presence or absence of 9-*cis* retinal, compared to cells incubated in the dark. **b.** Media containing d4-DHA from 661W cells incubated in the presence or absence of 9-*cis* retinal under light or dark conditions have comparable d4-NPD1 content, although, in prolonged incubations under light alone, there is diminished d4-NPD1, suggesting that light alone is not a significant stress. Results present the mean values ($\pm$ SEM) and were obtained from three independent experiments.

Figure 6.

DHA and NPD1 provide protection to 661W cells undergoing oxidative stress induced by 9-*cis* retinal treatment. A measure of cell viability was determined by measuring the absorbance of cells at 570 nm. Following 4 hours of light exposure in the presence of 9-*cis* retinal, only 26% of the 661W cells were viable. However, in the presence of DHA, 49% were viable, while 58% were viable in the presence of NPD1, demonstrating 23% protection by DHA and 32% protection by NPD1 from chromophore-mediated light stress. Results present the mean values ($\pm$ SEM) and were obtained from three independent experiments. Statistical significance was calculated using one-way ANOVA analysis by Bonferroni post-tests (* $p < 0.01$, ** $p < 0.001$).