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PEDF Promotes Biosynthesis of a Novel Anti-inflammatory and Anti-apoptotic Mediator NPD1 in Retinal Pigment Epithelial Cells

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ABSTRACT

Purpose: Neuroprotectin D1 is a stereospecific cytoprotective messenger synthesized from docosahexaenoic acid in retinal pigment epithelial cells challenged by oxidative stress. A key step in neuroprotectin D1 synthesis is to define how growth factors may modulate its formation and its bioavailability. Here we have explored the action of pigment epithelium derived factor, a neurotrophin made in retinal pigment epithelial cells, on neuroprotectin D1.

Methods: ARPE-19 cells were serum starved and exposed to TNFα/H_2O_2 in the presence and absence of pigment epithelium derived factor (10 mg/mL). Cells and incubation media were collected. LC-PDA-MS-MS-based lipidomic analysis was used to identify and quantitate neuroprotectin D1. Immunostaining for BCLxL was performed.

Results: Oxidative stress promotes increases in neuroprotectin D1 levels in ARPE-19 cells, showing a rapid increase up to 6 hrs of incubation of 12 folds measured on cell pellets. Cell media, on the other hand, show time dependent accumulation of neuroprotectin D1 up to 55-fold after 12 hrs incubation. Treatment with 50 nM pigment epithelium derived factor increased such profile by at least two fold. Deuterated docosahexaenoic acid was incorporated to cell membranes and converted into neuroprotectin D1. After cells are exposed to oxidative stress, BCLxL appears to shift to the nucleus of the cell. With the addition of pigment epithelium derived factor and docosahexaenoic acid, this translocation seems to be prevented.

Conclusions: Here we demonstrate that pigment epithelium derived factor is an activator of neuroprotectin D1 synthesis in ARPE-19 cells exposed to oxidative stress. A major action of pigment epithelium derived factor-stimulated neuroprotectin D1 synthesis shown here is retinal pigment epithelial cytoprotection. Since the retinal pigment epithelial cell is impaired in retinal degeneration, these novel mechanisms potentially may be targeted in macular degeneration and other retinal degenerative diseases as a new therapeutic avenue and may be applicable for neuroprotection in glaucoma and in other neurodegenerative diseases.

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Key Words: Neuroprotectin D1, neuroprotection, retinal pigmentary epithelium

INTRODUCTION

Docosahexaenoic acid (DHA) belongs to the omega-3 essential fatty acid family and attains its highest concentration of the body in retinal photoreceptors and in the central nervous system.[1] DHA participates in photoreceptor development and function. The outer segments of photoreceptors contain rhodopsin and the highest content of DHA of any cell type.[2] DHA is recycled back to the inner segments by retinal pigment epithelial (RPE) cells during outer segment renewal.[3,4] DHA is decreased in several types of retinitis pigmentosa (RP) and Usher's syndrome,[5] while diets high in DHA correlate with a decreased risk of age-related macular degeneration.[6]

Neuroprotectin D1 (NPD1) is a bioactive mediator derived through a DHA oxygenation pathway and is endogenously synthesized by RPE cells.[1] It has neuroprotective bioactivity in oxidative stress-challenged RPE cells and is thus considered a DHA neuroprotective messenger[1] with potent inhibitory properties on cytokine-triggered proinflammatory COX-2 gene expression.

ARPE-19 cells are spontaneously transformed human RPE cells that keep biological and functional properties. RPE cell functions include: phagocytosis of distal tips of photoreceptor outer segments, transport and reisomerization of bleached visual pigments, and maintenance of blood-retinal barrier.[7]

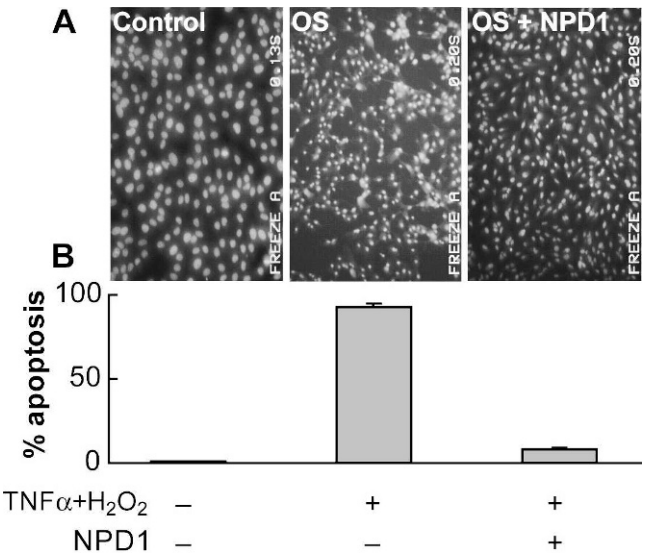


Figure 1. Quantification of apoptosis. Synergistic action of pigment epithelium derived factor and docosahexaenoic acid-mediated neuroprotectin D1 synthesis and cytoprotection. (Reproduced with permission from Mukherjee PK, Marcheselli VL, Barreiro S, et al. Neurotrophins enhance retinal pigment epithelial cell survival through neuroprotectin D1 signaling, Proc Natl Acad Sci U S A 2007;104:13152–13157).

Oxidative stress generates radicals that induce DNA damage, which is correlated with cell cycle arrest.[8] This may be produced through several pathways involving cumulative DNA or protein damage that could result in cell cycle reentry. The histone deacetylase activity may result in chromatin remodeling,[8] activating some genes,[8] whose products may lead to cell cycle reentry. The P53 pathway and NF-kB produce growth factor and growth-factor receptor activation leading to cell-cycle regulation and cell-cycle changes.[8] Oxidative stress may trigger both cytoprotective and cell damaging/death pathways,[9] the outcome of which is mainly dictated by the balance of the Bcl-2 family protein expression, which includes pro- and anti-apoptotic molecules. Pro-apoptotic molecules include BAX, BAK, BOK/MTD, BCL-Xs, and BH3. Antiapoptotic molecules include BCL-2, BCL-Xl, BCL-W, and MCL-1, which contribute to the caspase cascade.[10]

How can growth factors modulate the formation of NPD1 and its bioavailability? To address this issue, we have explored the action of pigment epithelium derived factor (PEDF), a member of the serine protease inhibitor (serpin) superfamily, which has been shown

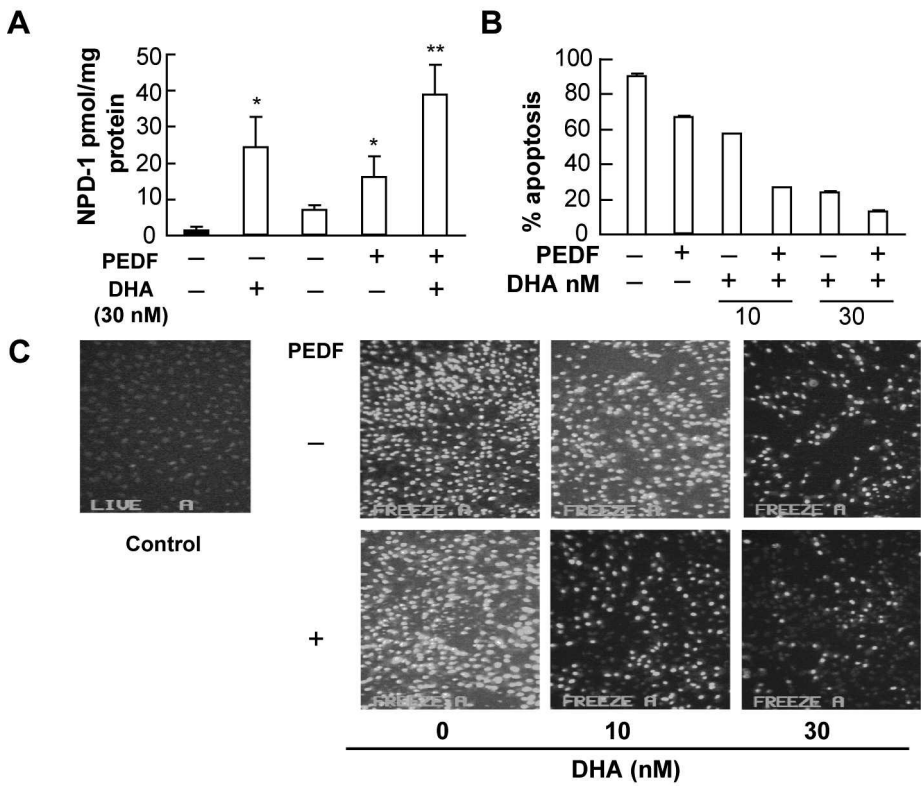


Figure 2. Docosahexaenoic acid to neuroprotectin D1 conversion in retinal pigment epithelial cells. A: Neuroprotectin D1 quantification. B: Percentage of apoptosis: C. Hoescht stain: apoptosis. Data shown in A are average ± SEM (n = 5). Asterisks indicate significance of Student's *t* test: *, $P < 0.05$; **, $P < 0.001$. (Reproduced with permission from Mukherjee PK, Marcheselli VL, Barreiro S, et al. Neurotrophins enhance retinal pigment epithelial cell survival through neuroprotectin D1 signaling, Proc Natl Acad Sci U S A 2007;104:13152–13157).

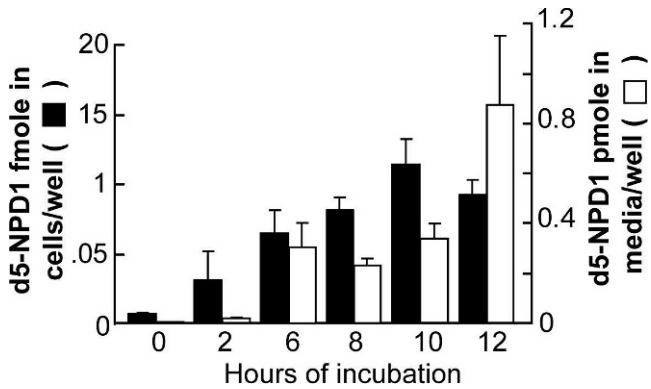


Figure 3. Deuterated neuroprotectin D1 concentrations found in medium (white) and cells (black). Immunostaining analysis of Bcl-2 family proteins modulated by pigment epithelium derived factor and docosahexaenoic acid. (Reproduced with permission from Mukherjee PK, Marcheselli VL, de Rivero Vaccari JC, et al. Photoreceptor outer segment phagocytosis attenuates oxidative stress-induced apoptosis with concomitant neuroprotectin D1 synthesis. Proc Natl Acad Sci U S A 2007;104:13158–13163).

to promote survival of different cell types in vitro[11] and to be a potent angiogenic inhibitor[11] made in RPE, on the synthesis and cellular release of NPD1.

METHODS

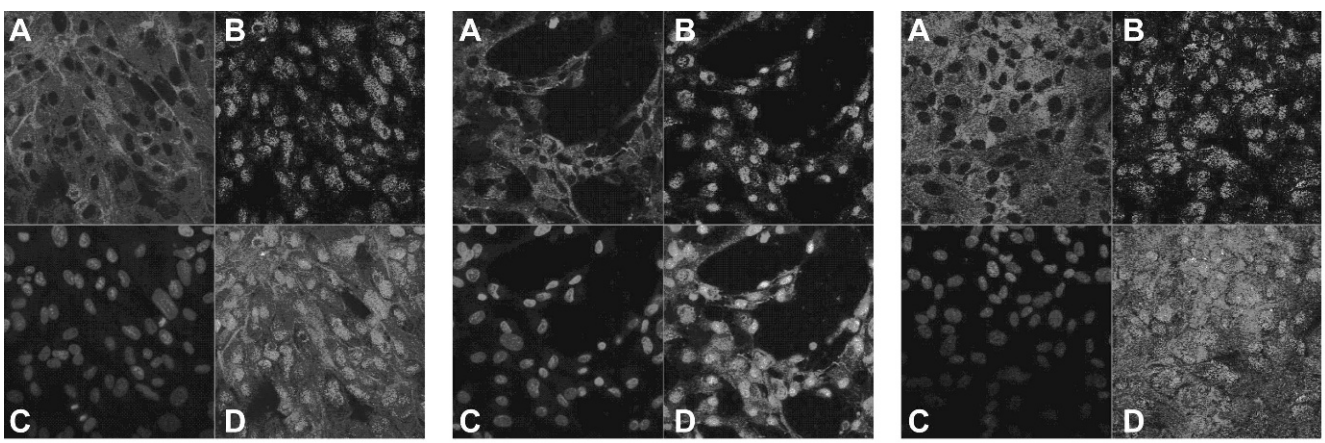
Three days after plating, ARPE-19 cells were serum-starved and exposed to TNFa/H_2O_2 inducing oxidative stress. After PEDF (10 mg/mL) was added, cells and incubation medium were collected as a function of time for up to 12 hours. LC-PDA-MS-MS-based lipidomic analysis was then used to identify and quantitate NPD1. Deuterium (2H5-22:6, n-3) was on specific methylene carbons, but it was not metabolically altered. Its heavier molecular mass was separated and identified by mass spectrometry to trace the DHA conversion into docosanoids, as well as to further explore the effect of PEDF. Immunostaining was done with cells fixed in cold methanol. Thereafter, 0.006% Triton was added, then the primary antibody (Ab) followed by the first secondary Ab, the second primary Ab, and the second secondary antibody, washing with phosphate buffered saline (PBS) between every step. Finally, Hoechst stain was added, and images were taken using confocal LSM 510 meta microscope.

RESULTS

The amount of apoptosis increases with oxidative stress when compared to the control. This was demonstrated with Hoescht stain (Fig. 1). Apoptosis is shown as bright small cell nuclei. Once NPD1 is added, the amount of apoptosis decreases (Fig. 1).

NPD1 increases with the addition of PEDF alone and DHA alone, plus a synergistic effect is seen when both PEDF and DHA are added (Fig. 2A). On the other hand, the percentage of apoptosis decreases with the addition of PEDF and DHA. With a higher concentration of DHA, the percentage of apoptosis decreases more, and it decreases even further with the addition of both DHA and PEDF (Fig. 2B). This decrease in apoptosis is also shown using different methods, such as Hoescht stain (Fig. 2C), where a significant decrease of apoptosis (bright cells) is seen with the addition of both PEDF and DHA.

The deuterated NPD1 concentrations in medium (white in Fig. 3) show a linear increase with time (probably due to additive effect, as cells secrete it into



BCLxL control | BCLxL oxidative stress | BCLxL oxidative stress, PEDF, and DHA

A: actin
B: Hoechst stain
C: BCLxL
D: All three combined

Figure 4. Bcl-xL Immunostaining.

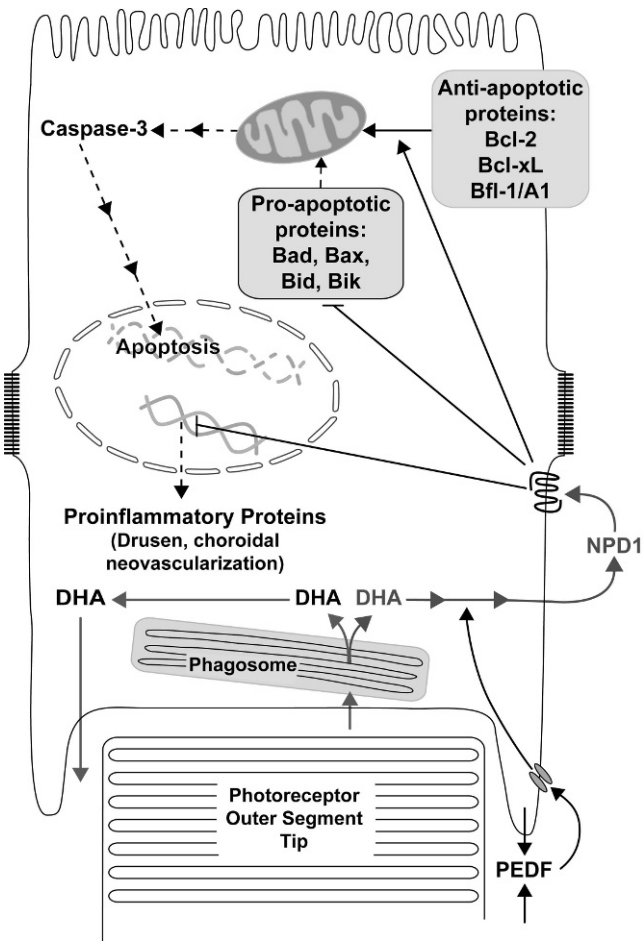


Figure 5. Neuroprotectin D1 synthesis is activated by the neurotrophin pigment epithelium derived factor, and in turn retinal pigment epithelial cell survival is promoted. Pigment epithelium derived factor is illustrated as released from the retinal pigment epithelial cell or provided from another cell. Oxidative stress or A2E(N-retinyl-N-retinylidene ethanolamine)/A2E oxiranes (epoxides) are activators of neuroprotectin D1 synthesis as well. Docosahexaenoic acid is shown to arrive to the retinal pigment epithelial as part of the phagosome (docosahexaenoic acid-phospholipids). After the phagolysosomal digestion, most of the docosahexaenoic acid is recycled back to the inner segments of photoreceptors through the interphotoreceptor matrix. Neuroprotectin D1 is released through the apical cellular side and recognizes a putative receptor. Intracellular signaling then inhibits pro-inflammatory gene expression. As a consequence, a decrease in pro-inflammatory proteins takes place that, when available, plays a role in drusen development and choroidal neovascularization. In addition, neuroprotectin D1-triggered signaling upregulates anti-apoptotic Bcl-2 family protein expression and downregulates pro-apoptotic Bcl-2 family protein expression. As a result, caspase 3 activity is decreased and apoptosis reduced.

the medium), while the deuterated NPD1 in cells (black in Fig. 3) shows an increase in concentration until a plateau is reached.

In the immunostaining, the presence of BCLxL (panels B in Fig. 4) is limited to the cell cytoplasm in the control group. After exposing cells to oxidative stress, BCLxL appears to shift to the nucleus of the cell. With the addition of PEDF and DHA, this translocation seems to be prevented (Fig. 4).

CONCLUSIONS

In conclusion, the exogenous addition of D5-DHA allowed the tracing of NPD1 synthesis in ARPE-19 cells.[12] PEDF was found to be an activator of NPD1 synthesis in ARPE-19 cells upon exposure to oxidative stress, with 70 percent of the synthesized NPD1 released from the cells.[12]

NPD1 likely exerts its bioactivity through autocrine mechanisms. Furthermore, Bcl-xL (which is antiapoptotic) is translocated from the cytoplasm to the nucleus during periods of oxidative stress, offering further insight into a new signalling for neuroprotection against apoptosis (Fig. 5). Addition of PEDF and DHA prevents this translocation. The discovery of NPD1 and its upregulation by neurotrophins opens a potential new way to therapeutically slow down apoptotic loss of RPE cells and photoreceptors in retinal degenerative diseases, for both the dry form of macular degeneration as well as for the wet form. Also, this novel mechanism may be applicable for neuroprotection in glaucoma and in other neurodegenerative diseases.[13]

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