Neurotrophins enhance retinal pigment epithelial cell survival through neuroprotectin D1 signaling

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Integrity of retinal pigment epithelial cells is necessary for photoreceptor survival and vision. The essential omega-3 fatty acid, docosahexaenoic acid, attains its highest concentration in the human body in photoreceptors and is assumed to be a target for lipid peroxidation during cell damage. We have previously shown, in contrast, that docosahexaenoic acid is also the precursor of neuroprotectin D1 (NPD1), which now we demonstrate, acts against apoptosis mediated by A2E, a byproduct of phototransduction that becomes toxic when it accumulates in aging retinal pigment epithelial (RPE) cells and in some inherited retinal degenerations. Furthermore, we show that neurotrophins, particularly pigment epithelium-derived factor, induce NPD1 synthesis and its polarized apical secretion. Moreover, docosahexaenoic acid (DHA) elicits a concentration-dependent and selective potentiation of pigment epithelial-derived factor-stimulated NPD1 synthesis and release through the apical RPE cell surface. The bioactivity of signaling activated by pigment epithelium-derived factor and DHA uncovered synergistic cytoprotection with concomitant NPD1 synthesis when cells were challenged with oxidative stress. Also, DHA and pigment epithelium-derived factor synergistically modify the expression of Bcl-2 family members, activating antiapoptotic proteins and decreasing proapoptotic proteins, and by attenuating caspase 3 activation during oxidative stress. Thus, our findings demonstrate that DHA-derived NPD1 protects against RPE cell damage mediated by aging/disease-induced A2E accumulation. Also, our results identify neurotrophins as regulators of NPD1 and of its polarized apical efflux from RPE cells. Taken together, these findings imply NPD1 may elicit autocrine actions on RPE cells and paracrine bioactivity in cells located in the proximity of the interphotoreceptor matrix.

docosahexaenoic acid | pigment epithelial-derived factor

Photoreceptor outer segment renewal is a process whereby new membranous disks containing the phototransduction apparatus are constitutively assembled at the proximal end of this light-sensitive structure, and the oldest disks are intermittently shed from the distal outer segment (1). The process of shedding is accompanied by phagocytosis of the disk membranes by the retinal pigment epithelium (RPE), a blood barrier-forming monolayer of polarized cells whose apical surface is in close contact with the distal outer segment and whose basal surface faces the capillary bed nourishing the photoreceptor cells. The RPE provides important nutrients to the photoreceptors and all-trans retinol (vitamin A), the precursor to the visual pigment chromophore for vision (2, 3), and docosahexaenoic acid (DHA) (22:6; $n-3$), both of which are continuously recycled between the RPE and the rod and cone photoreceptor cells (4). The RPE also provides neurotrophins essential for photoreceptor cell survival (5). These critical RPE functions render it essential for photoreceptor cell survival and normal vision.

DHA, a member of the essential omega-3 fatty acid family, is a component of phospholipids in the outer segments of photoreceptors, where rhodopsin performs its function in vision (4). A dietary supply of omega-3 fatty acids is required for photoreceptor function and vision (6, 7). It is known that this polyunsaturated fatty acid is a target of oxidative stress in pathological conditions, and, if lipid peroxidation ensues, events leading to cell injury are set in motion (8–10). In contrast, the neurosensory retina and RPE form mono-, di-, and trihydroxylated derivatives of DHA by a lipoxygenase enzymatic activity, because lipoxygenase inhibitors block their formation. These DHA products are called docosanoids and were suggested to be neuroprotective (11). Recently, using liquid chromatography (LC)-photodiode array-electrospray ionization-tandem MS (MS/MS)-based lipidomic analysis and related approaches, the stereochemical characterization and bioactivity of a dihydroxylated derivative of DHA, 10R,17S-docosatriene [neuroprotectin D1 (NPD1)], was ascertained (12). NPD1 is synthesized in the RPE and other cells (13, 14) and is a potent inhibitor of oxidative stress-induced proinflammatory gene induction and apoptosis, and consequently promotes cytoprotection and cell survival.

Neurotrophins are low abundance, high potency protein growth factors that modulate the development, differentiation, and maintenance of mature phenotypes in many neuronal populations. Neurotrophins also promote neuronal photoreceptor and RPE cell survival (15); therefore, it is important to define whether neurotrophins are NPD1-synthesis agonists. Also, we wanted to explore whether or not the action of the endogenous toxic components of aging RPE that increases in certain retinal degenerations (A2E-A2E epoxides) can be counteracted by NPD1. Studies on the formation and action of NPD1 were conducted on the transformed cell line ARPE-19 (12). Here, we use primary human RPE cells and find that they also synthesize NPD1. Moreover, we show that neurotrophins stimulate NPD1 synthesis and the polarized (apically dominant) release of NPD1.

Results and Discussion

A2E-Mediated ARPE-19 Cell Damage Is Attenuated by NPD1. We have asked whether oxidative stress triggered-induced apoptosis by A2E can be attenuated by NPD1. A2E, a lipofusin component, accumulates in the RPE during aging (16–19), and, in an exaggerated manner, in age-related macular degeneration, Stargardt macular dystrophy (an early onset form of age-related macular degeneration), and animal models of this disease (17–19). As a consequence, RPE apoptosis precedes the demise of photoreceptors (19). NPD1 was cytoprotective against A2E-induced apoptosis (Fig. 1*B*) and unexpectedly displayed a wide time window of cytoprotection after A2E addition. Presence of NPD1 (50 nM), even 6 h after A2E,

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Abbreviations: CNTF, ciliary neurotrophic factor; DHA, docosahexaenoic acid; LC, liquid chromatography; NPD1, neuroprotectin D1; PEDF, pigment epithelial-derived factor; RPE, retinal pigment epithelium.

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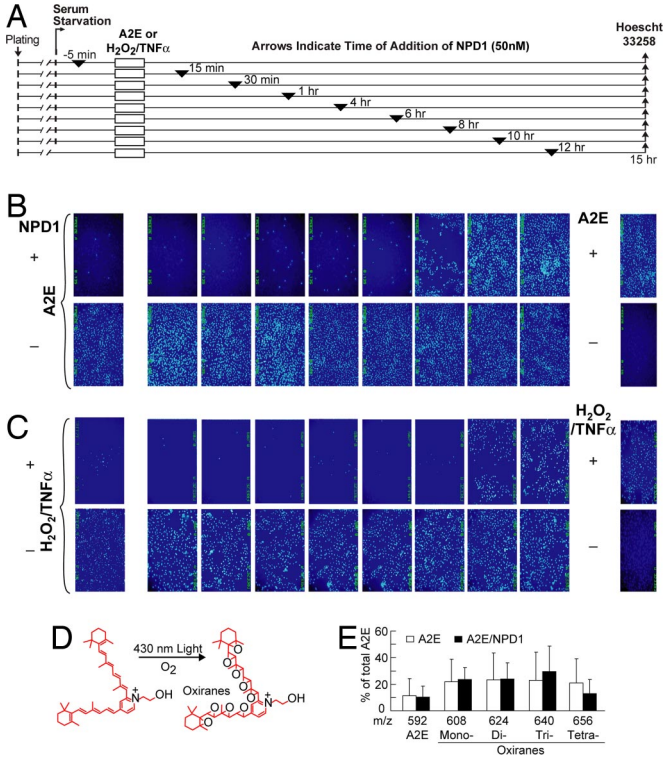

Fig. 1. A2E-induced oxidative stress in ARPE-19 cells is inhibited by NPD1. (*A*) Seventy-two hours after plating, cells were serum starved for 4 h. A2E (20 μM) was added in the presence of 430 nM light and O_2 (see *Materials and Methods*). Other cells were exposed instead to H_2O_2/TNFα. NPD1 was added before, or at different times after, A2E or H_2O_2/TNFα, up to 12 h. Time of NPD1 addition is indicated by black arrows. Hoechst 33258 was analyzed at 15 h. (*B*) Illustrates the appearance of Hoechst 33258 positive cells upon exposure to A2E and the effect of NPD1. (*C*) As in *A*, except that H_2O_2/TNFα was used to trigger oxidative stress. (*D*) Depicts the formation of oxiranes (epoxides) upon exposure of ARPE-19 cells to A2E in the presence of O_2 and 430 nM light for 15 min followed by 60 min of incubation in the dark. (*E*) A2E and oxiranes characterized by LC-MS-MS (see *Materials and Methods*). NPD1 was a gift from C. N. Serhan (Harvard Medical School, Boston, MA). Data average is ± SEM (n = 6) of percent distribution of oxidized A2E products.

ensures protection (Fig. 1 *A* and *B*). A2E-triggered oxidative stress precedes cell death. Thus, because oxidative stress and/or inflammatory signaling are involved in early stages of retinal degenerations (20–23), we decided to explore whether oxidative stress, triggered by another experimental condition, serum starvation/H_2O_2/TNFα (Fig. 1*C*), would be similarly inhibited. We found that NPD1 also exerted protection in this experimental condition. Addition of NPD1, even eight hours after triggering oxidative stress, resulted in protection (Fig. 1 *A* and *C*). What was surprising was the extended time window. This was ascertained by additional experiments assessing cell viability, using calcein (AM), ethidium homodimer and phase contrast microscopy [supporting information (SI) Fig. 7]. To study the mechanism of NPD1-mediated RPE protection against A2E oxidative stress, we tested the possibility that A2E conversion to A2E oxiranes (epoxides) may be the target of NPD1. A2E oxiranes are the cytotoxic products that accumulate in the RPE (16, 17). In agreement with recent observations (16, 17), we detected by MS-MS multiple oxiranes (epoxides), up to non-aoxiranes, in ARPE-19 cells exposed to light and oxygen (Fig. 1*D*). However, NPD1 (50 nM) did not affect this conversion (Fig. 1*E*). Moreover, NPD1 counteracted A2E-enhanced caspase-3 cleavage (Fig. 2*A*), showed a tendency to decrease Bax expression (Fig. 2*B*), and increased Bcl-2 protein expression (Fig. 2*C*). These findings suggest that NPD1 protects the RPE against A2E-induced apoptosis at the premitochondrial level of the apoptosis cascade by altering Bcl-2 balance. This NPD1 action may be a downstream consequence of the NPD1 action on Bcl-2 proteins. The extended NPD1 window of protection suggests that the blockade of the apoptotic cascade targets committed cell death events only relatively late upon exposure of the cells to oxidative stress. Therefore, our next series of experiments addressed the potential of neurotrophins in survival signaling and their ability to activate NPD1 synthesis.

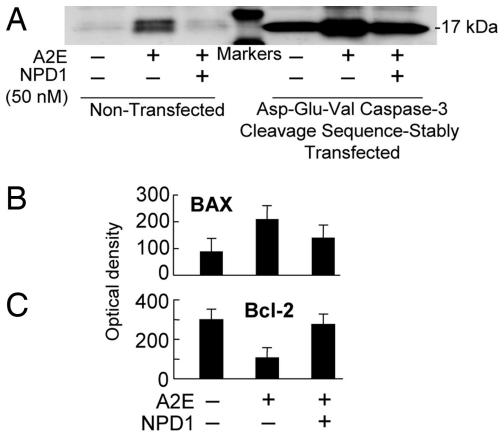

Fig. 2. Caspase cleavage and Bcl-2 changes triggered by A2E are restored by NPD1. (*A*) Caspase cleavage is enhanced by A2E and NPD1 attenuates this action (see *Materials and Methods*). (*B*) Bax displays a tendency to increase, whereas Bcl-2 decreases upon exposure to A2E (*C*). NPD1 (50 nM) counteracts the effect of A2E (see *Materials and Methods*). P. Nicotera and D. Bano (University of Leicester, Leicester, U.K.) provided the lentivirus construct. Data are an average of relative density detection ± SEM of six individuals.

Neurotrophins Promote the Synthesis and Release of NPD1 from Human RPE Cells. Because neurotrophins are important in photoreceptor survival (5, 15, 24, 25), we have asked whether neurotrophins are NPD1 synthesis agonists. For this purpose we have used human RPE cells grown to confluence and a high degree of differentiation displaying apical-basolateral polarization (26). These RPE cells have prominent apical microvilli (Fig. 3*A*), zonula occludens-positive immunoreactivity (Fig. 3*B*), and transepithelial resistance of at least 400 $\Omega \cdot cm^2$ (see *Materials and Methods*). NPD1 was initially described in the ARPE-19 cell line (12), here we have explored whether human RPE cells in primary culture also synthesize this lipid mediator. In addition, the use of human RPE cells in barrier-forming monolayers allows us to address the issue of "sidedness" of NPD1 release. Fig. 3*C* illustrates that several neurotrophins with bioactivities that promote neuronal and/or photoreceptor cell survival are agonists of NPD1 synthesis. Of interest is that all of the neurotrophins studied, except cardiotrophin, trigger synthesis and release of NPD1 through the apical surface of the cell under the present experimental conditions. Neurotrophins were added to the upper incubation chamber (apical cellular surface). Then the upper chamber media and the lower chamber media (basolateral cellular surface) were collected separately and subjected to lipidomic analysis, using LC-photodiode array-electrospray ionization-MS/MS (see *Materials and Methods*). Among the neurotrophins tested, pigment epithelium-derived factor (PEDF) was by far the most potent stimulator of NPD1 synthesis. PEDF, a member of the serine protease inhibitor (serpin) family, was initially identified in the conditioned medium of human retinal pigment epithelial cells (27) cultured similarly to those used in experiments depicted in Fig. 3*C*. Fig. 4*A* illustrates that if PEDF or ciliary neurotrophic factor (CNTF) is added to the basal medium in increasing concentrations, they evoke much less NPD1 release on the apical side. Conversely, if these neurotrophins are added to the apical medium, they exert concentration-dependent increases in

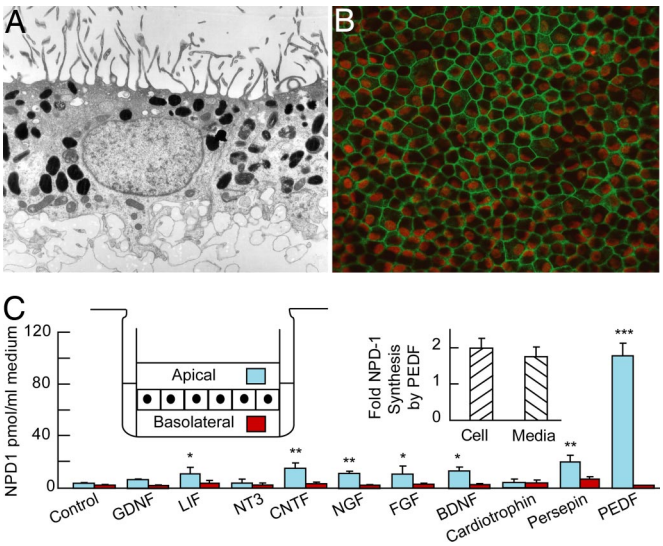

Fig. 3. Neurotrophins activate NPD1 synthesis in cultured primary human RPE cells. (*A*) EM depicting well differentiated human RPE with prominent apical microvilli. (*B*) Zonula occludens-1 (ZO-1) antibody immunoreactivity (green) illustrates confluence of the monolayer polyhedric-shape of the cells. (*C*) Differential ability of growth factors to selectively release NPD1 through the apical surface of the cell. The cartoon depicts the RPE cell monolayer bath with medium on both surfaces. Growth factors (20 ng/ml) were added to the apical medium and 72 h later, apical and basal media were collected separately and subjected to lipidomic analysis (see *Materials and Methods*). Each bar is an average ± SEM of four or five independent wells. The insert represents net NPD1 synthesis accumulated in the cells as compared with the total media, resulting from PEDF (50 ng/ml) addition followed by lipid extraction of the cells and media 72 h later. Increases of NPD1 in cells and media represent fold increases above those in cells incubated in the absence of growth factors. Values are averages ± SEM of five independent wells. Statistical analysis shows *, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0001$.

NPD1 release only on the apical side. These findings have relevance to retinal pathology because, when RPE cell polarization in the plane of the epithelium is disrupted, certain growth factors are believed to participate in an injury/inflammatory response, including the mediation of angiogenesis as in age-related degenerations (3, 28, 29).

DHA Selectively Potentiates PEDF-Induced NPD1 Synthesis and Release Through the Apical Surface of RPE Cells. We next explored whether increasing concentrations of DHA would further enhance PEDF-induced NPD1 synthesis and release (Fig. 4 *B–E*). The human RPE cells used in the present experiments do have sufficient DHA in phospholipids to synthesize NPD1, as shown in controls without addition of DHA (Fig. 4 *B* and *D*); however, because they are in cell culture conditions, they are not undergoing photoreceptor membrane phagocytosis, and they have relatively limited DHA in their phospholipids. Thus, when DHA content in the media was increased, a remarkable potentiation by PEDF of NPD1 release to the apical media was uncovered when the neurotrophin was added to the media bathing the apical cell surface (Fig. 4*B*). In contrast, much less NPD1 was found in the media bathing the basolateral side of the cells. Much less apical NPD1 release was observed when PEDF was applied to the media bathing the basolateral RPE surface. Regardless of the side of the cell to which PEDF is added, the amount of NPD1 release through the basolateral side is similar (Fig. 4*B*). Moreover, the addition of DHA to either side of the cell monolayer selectively synergized PEDF-induced NPD1 release only through the apical side. The insert (Fig. 4*C*) shows that added arachidonic acid did not stimulate NPD1 synthesis. We next examined the net synthesis of NPD1 in the RPE cells and compared this with the content of this lipid mediator in the culture media by bathing the apical and basal surfaces of these cells with increasing concentrations of DHA, the NPD1 precursor (Fig. 4*E*). The cellular NPD1 content decreased as a function of DHA concentration from 25%, when no DHA was added, down to 7% in the presence of 200 nM DHA (Fig. 4*D*), but concomitantly in the apical medium it increased almost proportionally. NPD1 in the apical medium accounted for 40% of its total in the absence of added DHA, and increased step-wise as DHA rose from 10 to 50 nM, without further increases at higher concentrations (Fig. 4 *D* and *E*). Although DHA alone does cause NPD1 synthesis, most of the newly formed NPD1 is recovered from the apical medium; much less appeared in the basal medium (Fig. 4*D*). The addition of PEDF (50 ng/ml) promoted an enhancement of this profile, whereby the cellular NPD1 content increased in the apical medium, because DHA was increased from 0 to 200 nM (Fig. 4*B*). The polarity of actions for the neurotrophin-mediated response raises the possibility that NPD1 may function, at least in part, as an autocrine and paracrine signal on cells that surround the interphotoreceptor matrix, namely the photoreceptor cells and Müller cells. Moreover, the apical side of the RPE participates in the recognition and shedding of photoreceptors during outer segment phagocytosis (3, 4). Furthermore, interphotoreceptor matrix proteins may be acceptors of NPD1, to facilitate its diffusion and to target it to cellular site(s) of action.

DHA and PEDF Provide Cytoprotection Synergistically When RPE Cells Are Confronted with Oxidative Stress. To study the downstream signaling of NPD1 synthesis induced by PEDF, we next used ARPE-19 cells that, when exposed to oxidative stress, respond with significant NPD1 synthesis and display NPD1-mediated cytoprotection (12). We have now found that ARPE-19 cells, like human RPE cell primary cultures (Fig. 5*B*), up-regulate NPD1 synthesis in the presence of PEDF (Fig. 5*A*). Moreover, significant cytoprotection and enhanced NPD1 formation occurred synergistically when PEDF was added along with DHA under conditions of oxidative stress-induced apoptotic cell death triggered by serum starvation/H_2O_2 /TNFα (Fig. 5 *B* and *C*).

DHA and PEDF Synergistically Stimulate Antiapoptotic Bcl-2 Protein Expression and Decreased Proapoptotic Protein Expression During Oxidative Stress. Because the initiation and amplification of the premitochondrial apoptotic cascade involves the Bcl-2 family of proteins (4), we studied the expression of the antiapoptotic proteins Bcl-2 and Bfl-1, and of the proapoptotic proteins Bid, Bax, and Bad, during serum starvation/H_2O_2/TNFα-induced ARPE-19 cell death. We observed that increasing the concentration of DHA from 10 to 50 nM up-regulated Bcl-2 and Bfl-1 protein expression (Fig. 6*A*). Although PEDF alone was unable to alter the expression of pro- and antiapoptotic proteins when added with DHA during serum starvation/H_2O_2/TNFα-induced oxidative stress, it did potentiate the expression of these proteins with concomitant NPD1 synthesis in the presence of DHA (Fig. 5*A*). Proapoptotic protein expression under these experimental conditions in the presence of DHA and PEDF underwent opposite changes. Bid, Bax, and Bad expressions were enhanced by oxidative stress and DHA decreased their expressions, whereas PEDF potentiated this action (Fig. 6*B*).

Oxidative Stress-Mediated Caspase-3 Activation Is Attenuated by DHA and PEDF. The marked increase in the numbers of Hoechst-positive ARPE-19 cells during oxidative stress correlated well with caspase-3 cleavage (Fig. 6*C*). Effector caspase-3 downstream of cytochrome-*c* release from mitochondria and apoptosome activation progressively decreased when cells were exposed to 10–50 nM DHA; PEDF potentiated this action (Fig. 6*C*). A remarkable synergy between PEDF and DHA was demonstrated with enhanced cytoprotection, up-regulation of NPD1 synthesis, enhancement of antiapoptotic protein expression, down-regulation of proapoptotic protein expression, and caspase-3 cleavage.

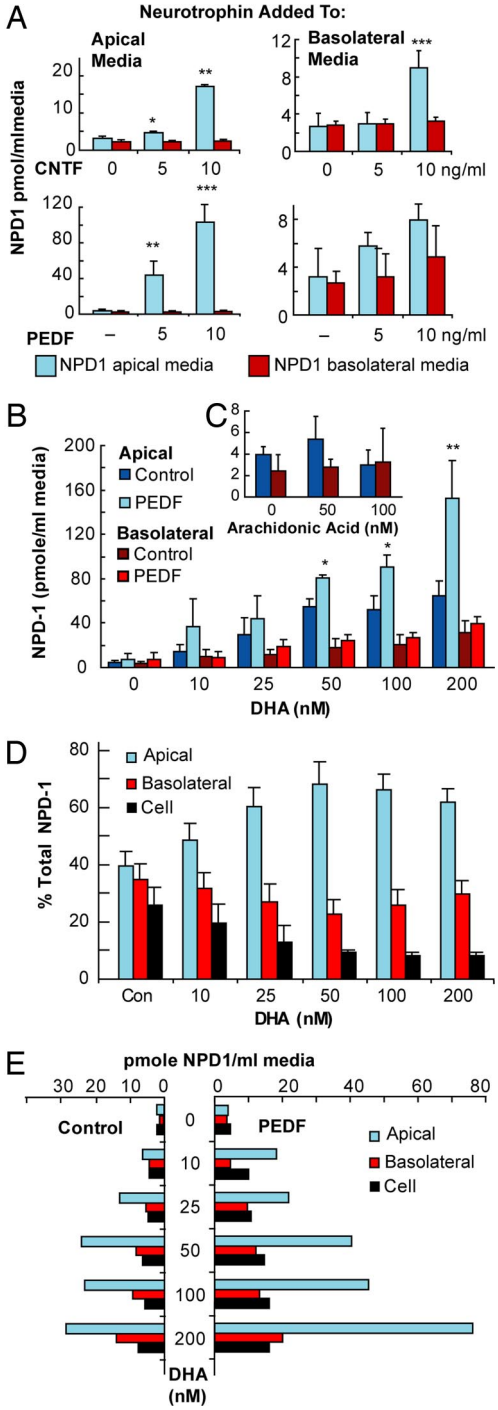

Fig. 4. Cellular polarity of human retinal pigment epithelial cells and NPD1 synthesis and release. (*A*) Concentration dependence of CNTF and PEDF activation of NPD1 release: selective response to growth factor addition to the apical RPE cell surface compared with the basal. Increasing concentrations of growth factors were added either to the apical or basal medium, and 72 h later, media were separately collected and subjected to lipidomic analysis (12, 13). Relatively lower NPD1 synthesis occurred when CNTF or PEDF was added to the basal medium; however, the growth factors potently activated NPD1 synthesis and release through the apical surface when added to the apical medium. Each bar represents averages ± SEM of five to seven independent wells. (*B*) PEDF-induced NPD1 synthesis and release through the apical surface of RPE cells: selective potentiation by DHA. PEDF (20 ng/ml) was added to either the apical or basal medium in separate experiments and 72 h later media were separately collected, and lipids extracted and analyzed. DHA complexed with 1% human serum albumin (Baxter, West Lake Village, CA) was added. Although NPD1 in the basal medium increased as the concentration of DHA was raised from 0 to 200 nM, PEDF (added to the basal medium) was unable to potentiate this action. However, DHA added to the apical medium promoted higher concentration-dependent NPD1 synthesis and release. When the growth factor was added to the apical surface, a clear synergism in NPD1 synthesis in the presence of DHA was observed. (*C*) Arachidonic acid, an omega-6 polyunsaturated fatty acid, when added to the apical medium under conditions similar to those of added DHA, failed to induce NPD1 synthesis. (*D*) Total NPD1 percentile distribution in cells, apical and basal media as a function of DHA treatment on the apical media. As shown in the figure, increasing concentration of DHA promotes significant increases of NPD1 in the apical media, reaching a maximum at 50 nM DHA. (*E*) Comparison of total distribution of NPD1 in cells, apical and basal media as a consequence of DHA concentration dependent treatment in presence or not of 20 ng/ml PDEF. As shown in Fig. 3 and above, apical media accumulates the most NPD1 and essentially plateaus at 50 nM DHA, but treatment with PEDF potentiates such effect. Data are average ± SEM of at least three separate experiments ($n = 6$). Statistical analysis shows *, $P < 0.05$; **, $P < 0.005$; and ***, $P < 0.0001$.

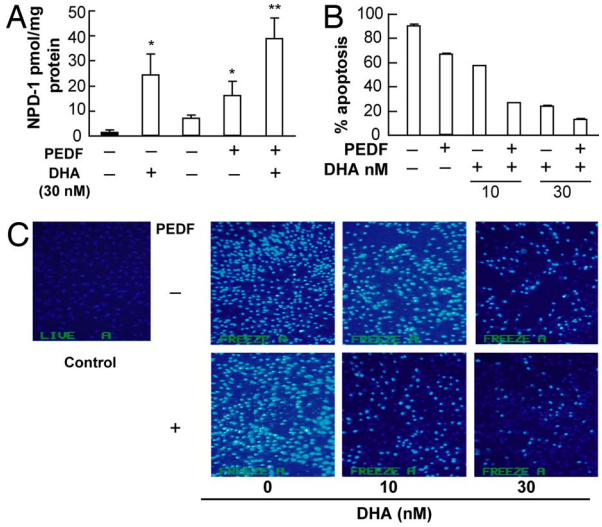

Fig. 5. DHA potentiates PEDF bioactivity of cultured ARPE-19 cells. (*A*) Synergistic induction of NPD1 synthesis. Data shown are average ± SEM ($n = 5$). Asterisks indicate significance of Student's *t* test: *, $P < 0.05$; **, $P < 0.001$. (*B*) Decreased apoptosis by increasing added DHA to 30 nM in the presence of PEDF. (*C*) Representative Hoechst staining of experiment illustrated in *B*.

Conclusions

The data presented here indicate that apoptosis triggering of the bispyridinium bisretinoid A2E is markedly attenuated, displaying a wide window of cytoprotection by NPD1 in ARPE-19 cells. In contrast, NPD1 did not affect the photooxidation of A2E as measured by the conversion of A2E into A2E oxiranes. Because the quenchers of singlet oxygen lutein, zeaxanthin, alpha-tocopherol (30), and anthocyanins (31) attenuate A2E photooxidation, the present observations support the notion that NPD1 elicits a specific action, other than antioxidant activity, to counteract A2E-induced apoptosis in RPE cells. Moreover, the wide window of NPD1 cytoprotection against A2E is similar to that exerted by serum-deprivation/H_2O_2/TNFα, indicating that the bioactivity of this lipid mediator may act at initial checkpoints of apoptosis.

In addition, we demonstrate that neurotrophins, mainly PEDF, are NPD1 synthesis agonists and selective activators of the apical efflux of the lipid mediator in human RPE cells in monolayer cultures. Also, DHA greatly potentiates PEDF-induced RPE cytoprotection against oxidative stress, with concomitant NPD1 formation. The synergy with PEDF and DHA indicates that the availability of the NPD1 initial precursor is critical for its synthesis. PEDF (27) and NPD1 (32) are antiangiogenic factors; thus, the

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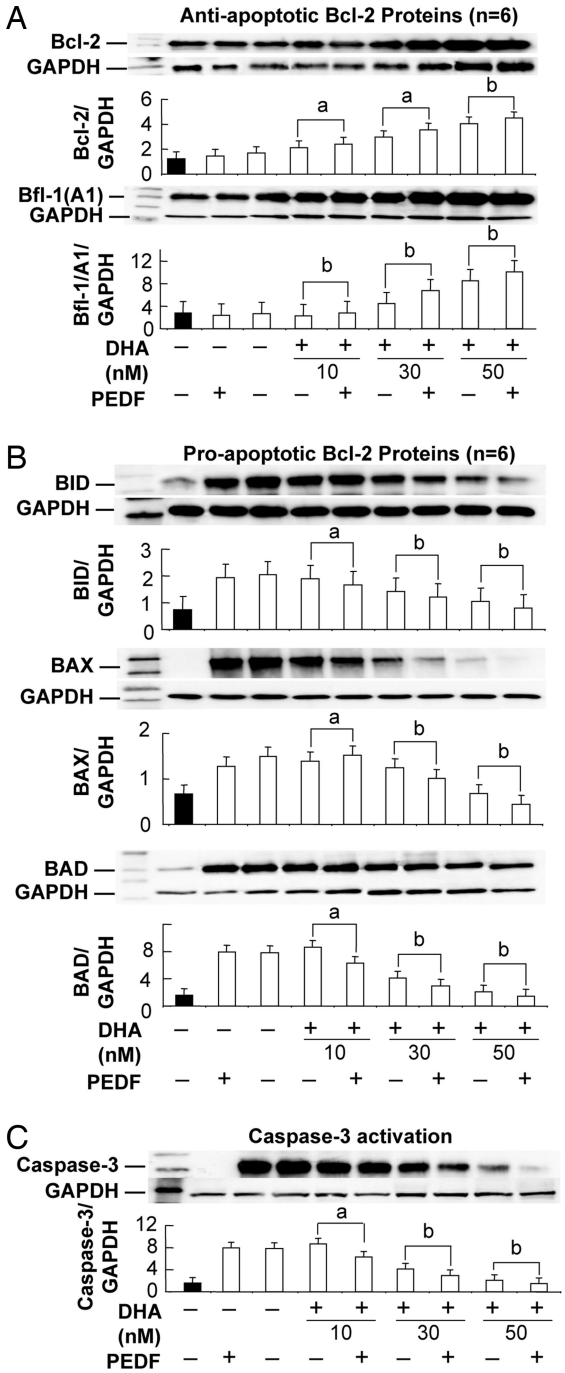

Fig. 6. Bcl-2 family proteins and caspase-3 expression mediated by DHA and PEDF when ARPE-19 cells are confronted with oxidative stress. (*A*) Synergistic enhancement of Bcl-2 and Blf-1/A1 antiapoptotic proteins as DHA is increased from 10 to 50 nM in the presence of PEDF. (*B*) Decreased Bid, Bax, and Bad upon increasing DHA concentration in the presence of PEDF. Data represents the densitometry ratios of Bad, Bax, and Bid to GAPDH. Black bar (in *A*), cells not exposed to oxidative stress; open bars (in *A* and *B*), exposed to oxidative stress. (*C*) Converse changes in caspase-3 activation. Each bar represents average ± SEM of 9–12 independent wells. a, not significant; b, $P < 0.0001$.

synergy reported here may be relevant to the management of pathoangiogenesis in macular degeneration and tumors.

The regulation of apoptosis involves multiple checkpoints. The ability of DHA to potentiate PEDF bioactivity on expression of the Bcl-2 family of proteins indicates that the premitochondrial stage of the apoptotic cascade checkpoint is involved in the observed cytoprotection, with concomitant NPD1 formation. These observations are in agreement with studies in human neural progenitor cells (14). Moreover, neurotrophins and DHA are both abundant throughout the nervous system (4). Neurotrophins are survival signals, in addition to being modulators of neurite branching, synaptogenesis, and synaptic plasticity (33, 34). In this regard, nerve growth factor and DHA activate nerve regeneration after experimental cornea injury (35). Thus, the ability of neurotrophins to promote cell survival through NPD1 in the RPE cell, as described here, is also highly relevant to the response of the nervous system to injury and neurodegeneration. Neurotrophins, as agonists of NPD1 synthesis from DHA, may promote signaling integration for cell survival. In fact, NPD1 fosters homeostatic regulation of cell integrity during photoreceptor cell renewal (36). The regulation of pro- and antiapoptotic proteins during the window of protection shown here will contribute to further define NPD1 survival bioactivity. These events are clinically significant because they will contribute the exploration of therapeutic interventions for neurodegeneration, particularly retinal degenerative diseases.

Materials and Methods

Human RPE Cells. Cultures of RPE cells were prepared at the UCLA lab. RPE cells were seeded onto Millicell-HA culture plate inserts (Millipore, Bradford, MA), placed in 24-well plates, and allowed to reach confluence. Consent for use of tissue for research was obtained in compliance with Federal and State law and institutional regulations. Cultures were maintained in Chee's essential replacement medium (26) until the experiments were performed. The medium includes MEM with calcium (Irvine Scientific, Irvine, CA), 1% heat-inactivated calf serum (JRH Bioscience, Lennexo, KS), amino acid supplements, and 1% bovine retinal extract. The Millicell-HA filter inserts allow separate manipulation of the culture media bathing the apical and basal surfaces of the RPE monolayer and measurement of the transepithelial resistance, which provides a measure of cell differentiation and confluency. Cultures were used for experiments once they developed a transepithelial resistance of at least 400 $\Omega \cdot cm^2$, as measured by an epithelial volt-ohmmeter (World Precision Instruments, New Haven, CT).

Exposure of ARPE-19 Cells to Growth Factors. Cells at 75–80% confluence (72 h growth in DMEM/F12 + 10% FBS) were serum-starved for 2 h before exposure to growth factors. The serum-starved cells were treated with TNF-α (Sigma–Aldrich, St. Louis, MO) (10 ng/ml) and H_2O_2 (600 μM) to induce oxidative stress and challenged with increasing concentrations (10, 30, 50 nM) and PEDF (Chemicon International, Temecula, CA) (50 ng/ml) simultaneously with oxidative stress for 4 h before harvesting for protein analysis. Cell extracts were made and protein concentrations were adjusted by Bio-Rad (Hercules, CA) protein reagent and used for Western blot analysis. To study neuroprotection by DHA and PEDF in the oxidative stress-induced ARPE-19 cells, 72-h cells were serum-starved for 8 h before the introduction of oxidative stress and challenged with DHA and PEDF for 15 h. Cells were analyzed to detect Hoechst-positive apoptotic cells.

Analysis of Bcl-2 and Caspase-3 Cleavage. Bcl-2 protein and caspase-3 cleavage were analyzed by Western blot analysis. Also, ARPE-19 cells, stably transfected with a lentivirus construct containing the Asp-flu-Val caspase three-cleavage sequence, were used. In short, 15- to 20-μg equivalents of each cell extract were subjected to electrophoresis on an 8–16% gel (Invitrogen, Carlsbad, CA) at 125 volts for 2 h. The proteins were transferred to nitrocellulose membranes at 30 volts for 70 min at 4°C. The membranes were probed with primary antibodies against Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) and cleaved caspase-3 (Asp-175) (Cell Signaling, Danvers, MA) at room temperature and treated for 20 min with the secondary antibody, goat anti-rabbit IgG:horseradish

peroxidase, and horseradish peroxidase-conjugated antibiotin antibody, then proteins were detected by using an ECL kit (Amersham Biosciences, Buckinghamshire, U.K.).

Hoechst Staining. ARPE-19 cells were incubated with 2 μM Hoechst reagent dissolved in Lock's solution (Promega Corporation, Madison, WI) at 37°C for 45 min before imaging. Cells were washed once with PBS and photographed by using a Nikon (Tokyo, Japan) DIAPHOT 200 microscope with fluorescence optics. Images were recorded by a Hamamatsu (Bridgewater, NJ) Color Chilled 3CCD camera and Photoshop software, Version 5.0 (Adobe Systems, Mountain View, CA).

Exposure of RPE Cells to Growth Factors. The upper chamber compartment was filled with 500 μl of medium (bathing the apical cell monolayer surface) containing 0.1% human serum albumin (HSA) and 50 nM DHA (Sigma–Aldrich), or 50 nM DHA plus added neurotrophins [10–200 ng PEDF or CNTF, or 20 ng of BDNF, Cardiotrophin, CNTF, FGF, GDNF, LIF, NT3, or Persephin (Alomone Labs, Jerusalem, Israel)]. The lower chamber was filled with 500 μl of media (bathing the basal cell monolayer surface) containing 0.1% HSA. Cells were incubated for 72 h, then apical and basal media were removed and collected for analysis. After allowing the cells to rest for at least 72 h on fresh media, the experiments were repeated.

Lipidomic Analysis. Human RPE primary cell cultures or ARPE-19 cells were separated from culture media and washed with 1 ml of PBS. After addition of 1 ml of methanol, cells were scraped from plates or millicell membranes and collected for lipid extraction. Media was spun-down to separate cell debris, then 350 μl were collected in 1 ml of cold chloroform:methanol (1:1). Protein precipitates were then separated by centrifugation at 1,500 $\times$ *g* (5 min, 4°C). Lipid extracts were collected, and kept under nitrogen at −80°C until solid-phase purification; extracts were preequilibrated at pH 3.0 in 10% methanol/water, then loaded to 500-mg C18 columns (Varian, Palo Alto, CA), and eluted with 1% methanol/ethyl acetate. Eluates were concentrated on a N2 stream evaporator. Samples were loaded to a liquid chromatograph-tandem mass spectrometer (LC-TSQ Quantum, Thermo-Finnigan; Thermo Fisher Scientific, Waltham, MA) installed with a Biobasic-AX column (Thermo–Hypersil–Keystone; Thermo Fisher Scientific) (100 mm $\times$ 2.1 mm, 5-μm particle sizes), and eluted in a linear gradient [100% solution A (40:60:0.01 methanol/ water/acetic acid, pH 4.5) to 100% solution B (99.99:0.01 methanol / acetic acid)], at a flow rate of 300 μl/min for 30 min. LC effluents were diverted to an electro-spray-ionization probe on a TSQ Quantum (Thermo–Finnigan, Thermo Fisher Scientific) triple quadrupole mass spectrometer. NPD1 and resolvin D1 were obtained by biogenic synthesis (11, 13); other lipid standards (Cayman Chemical, Ann Arbor, MI) were used for tuning and optimization and to create calibration curves. The instrument was set on full-scan mode, to detect parent ions, and selected reaction mode for quantitative analysis, to detect product ions, simultaneously. The selected parent/product ions (*m*/*z*) and collision energy (v) obtained by running on negative ion detection mode were: 359.2/153.1/20 for NPD1, 343.2/281.2/18 for resolvin D1, 351.2/195.0/22 for 20HO-LTB4 (used as internal standard), 327.2/283.3/16 for DHA, 311.3/267.3/20 Arachidonic Acid-d8 (used as internal standard).

Data analysis. The data are expressed as means ± SEM of three or more independent experiments; "n = " designates the amount of individual samples. Student's t test was used to perform statistical comparisons. Asterisks indicate that $P < 0.05$ was considered significant for all comparison. Nonstatistical returns were obtained when asterisks were not indicated.

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