L E C T U R E

Homeostatic Regulation of Photoreceptor Cell Integrity: Significance of the Potent Mediator Neuroprotectin D1 Biosynthesized from Docosahexaenoic Acid

The Proctor Lecture

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Vision is sustained by a unique and beautiful relationship—that between photoreceptor cells and retinal pigment epithelial (RPE) cells. From the harmony of this cellular relationship emerges the life-long survival of the photoreceptor cell, despite the fact that the part of the eye where these cells interact is continuously exposed to potentially damaging factors. This relationship is one of the best examples of homeostasis in action as conceptualized for other cells and organs in 1932 by the physiologist Walter B. Cannon in *The Wisdom of the Body* (New York: W. W. Norton, 1932). Emerging questions to elucidate the intimacy of the successful intercellular relationship between photoreceptors and RPE include: How is integrity of the photoreceptor cell sustained? Does photoreceptor cell outer segment renewal modulate specific survival signaling to support the RPE cell during oxidative and nitrosylative challenges? The answers to these questions are needed to better our understanding of and to develop therapies for retinitis pigmentosa (RP), macular degenerations, and other retinal diseases.

Death of Photoreceptor Cells and the Importance of the RPE in Sustaining Retinal Survival

Photoreceptor cells (rods and cones) are highly specialized and differentiated neurons with stacks of photosensitive membrane discs that contain rhodopsin as well as numerous other, far less abundant proteins in their outer segments. Damage to and apoptotic death of photoreceptor cells are hallmarks of retinal degenerative diseases.[1–5] In retinitis pigmentosa (RP; a heterogeneous group of inherited blinding diseases), death of rod photoreceptors initially occurs in the periphery of the retina, whereas in age-related macular degeneration (AMD; the leading cause of loss of sight in those over the age of 65), progressive perturbation and loss of visual acuity are caused by photoreceptor death in the center of the retina, the macula.[2,5] Retinal degenerations involve multiple, complex cell-signaling pathways, including diverse triggers of photoreceptor cell death. Integral to this demise is the close relationship between photoreceptors and retinal pigment epithelial (RPE) cells. In Stargardt's disease (an early onset macular degeneration) and in other retinal degenerations in which RPE cell functional integrity is initially compromised photoreceptors are, in turn, damaged. Once RPE cells die, the underlying photoreceptor cells then succumb.[6] Our understanding of these diseases is further complicated by the more than 150 mutations of photoreceptor-specific proteins that have been reported to contribute to RP, including mutations of rhodopsin, peripherin, the β subunit of cGMP phosphodiesterase, and retinal outer segment membrane protein (ROM)-1.[7–9] AMD, by contrast, is a complex group of disorders with multifactorial causes, both genetic and environmental; the main known risk factor is advanced age.[2,5] Complex intracellular and intercellular signaling pathways are set into motion in the initial stages of retinal degeneration. Overall, oxidative and nitrosylative stress are enhanced and exaggerated, usually at abnormal cellular locations and with inappropriate timing. Mitochondrial function is compromised, and this dysfunction is central in these impairments.[9–11] Initiation and progression of AMD involves an unsuccessful inflammatory response. Single nucleotide polymorphisms (SNPs) in the genes encoding factor H (CFH/HF1),[12–15] factor B (BF),[16] and complement component 2 (C2)[16] have been identified as major risk factors for AMD. Factor H is an inhibitor of the alternative pathway of complement system activation that, as a result, has the ability to limit cell injury and inflammation.[16,17]

Initial clinical evidence (symptoms) of retinal degenerations precedes, in several instances by many years, extensive photoreceptor cell death. Therefore, the identification of early pro-survival, anti-inflammatory signaling mechanisms critical for the maintenance of photoreceptor cell integrity may be applicable as novel therapeutic interventions to halt or slow disease progression. The constant rebuilding of photoreceptors requires molecular building blocks, energy, and an environment readily able to promote cellular integrity. Photoreceptors shed outer segment tips, which are then phagocytized by the RPE cells in a daily, intermittent, and circadian fashion in mammals.[18–20] The length of the outer segments remains constant as a consequence of the well-regulated biogenesis of outer segment membrane components in inner segments coupled to the phagocytosis of the shed tips at an equal, compensatory rate. During photoreceptor outer segment renewal, proteins turn over and are continually replaced.[21] In contrast, docosahexaenoic acid (DHA) and vitamin A from the opsin chromophore of rod photoreceptor outer segments are recycled back from the RPE to inner segments through the interphotoreceptor matrix (IPM). The visual cycle of rods includes the reisomerization of all-*trans* retinal back to 11-*cis* retinal in the RPE. This review provides a brief overview of DHA in photoreceptors and describes a new property of the RPE cell—that

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is, its ability to synthesize a DHA-derived lipid mediator that evokes counteracting cell-protective, anti-inflammatory, pro-survival repair signaling, including the induction of anti-apoptotic proteins and inhibition of pro-apoptotic proteins. This mediator is neuroprotectin D1 (NPD1), biosynthesized from DHA. Also described are studies that identify neurotrophins as agonists of NPD1 synthesis and the recent demonstration that photoreceptor phagocytosis induces refractoriness to oxidative stress in RPE cells with concomitant synthesis of NPD1.

This article emphasizes the contributions from colleagues and associates from my laboratory over the past several years. Only portions of the many contributions made by others to this area are discussed; omissions were unavoidable because of space limitations. Several reviews covering various aspects of this theme are included in the references.[22–28]

Docosahexaenoic Acid in Photoreceptors

The membrane lipid milieu of the outer segments of photoreceptors in which rhodopsin and other proteins perform their functions is determined mainly by phospholipids rich in docosahexaenoic acid (DHA) and in omega-3 fatty acid derivatives longer than C22. Thus, a feature of photoreceptor outer segments is the very high concentration of omega-3 fatty acid family members.[22,24,27] Essentially, all are esterified in phospholipids. In some phospholipids, there are two omega-3 fatty acids esterified at both the sn-1 and sn-2 positions of the same glycerol backbone, determining the supraenoic or supraene molecular species.[29–32] Initially, these findings and the identification of di-docosahexaenoyl diglycerides in the amphibian retina[33,34] and the observation that DHA is enriched in phosphatidic acid of the retina,[35,36] unlike what occurs in the brain, suggests that the composition, metabolism, and function of supraenoic molecular species of phospholipids are important in photoreceptor organization and function. Supraenoic molecular species of phospholipids represent approximately 31% of phosphatidylcholine, 52% of phosphatidylserine, and 20% of phosphatidylethanolamine in photoreceptor discs.[29] The supraenoic phosphatidylcholines that contain DHA (at position sn-2) and the 24:6-36:6 elongation products of the omega-3 fatty acid family series (at position sn-1) are tightly bound to rhodopsin.[37] These very-long-chain fatty acids at sn-1 may "curl" and restrict rhodopsin motion, probably conforming a disc membrane domain not favoring the classic bilayer membrane organization.[37] In fact, phospholipids containing DHA provide a favorable environment within which G protein-coupled events can occur.[37] Another aspect is the physiologically selective enrichment of DHA in phosphatidylserine in neural cells, which positively modulates Akt survival signaling.[28,38] These actions of DHA are not mutually exclusive and may contribute specific functions for membrane DHA.

Under normal conditions, DHA is retained and protected from peroxidation. However, in experimental models of retinal degeneration,[39] when lipid peroxidation takes place, perturbations of photoreceptor function, damage, and cell death occur. In several forms of RP[40–45] and in Usher's syndrome,[41,46] a decrease of DHA content in blood has been reported. A possible implication of these studies is that decreased DHA supply to the retina may impair photoreceptor function by decreasing the availability of DHA to photoreceptors. However, the relationship between decreased DHA in the blood supply and disease initiation and progression remains unclear. Rats overexpressing rhodopsin mutations homologous to human RP display decreased amounts of DHA in photoreceptors,[47] which could represent a retinal response to metabolic stress, whereby decreasing the amount of the major target of lipid peroxidation (DHA) contributes to the protection of photoreceptors.[47] In addition, in constant-light–mediated retinal degeneration, there is loss of DHA from photoreceptors. Rats reared in bright cyclic light are protected from such loss and degeneration, suggesting that there is adaptation and/or a plasticity response.[48]

Is the shortage of DHA in the blood of Usher's syndrome[41,46] and patients with RP[45,49,50] reflected in the relationship of very-long-chain, DHA-derived acyl groups with rhodopsin? Or, as shown in experimental retinal degeneration, is the peroxidation of DHA closely associated with rhodopsin, perhaps impairing the function of this protein? These questions have not yet been answered.

Moreover, DHA has been shown to promote survival[51] and to inhibit apoptosis[52] of photoreceptors. In an Alzheimer's disease (AD) mouse model, DHA exerts neural protection,[53] and several studies have shown the neuroprotective properties of DHA.[22,27,28,51,52]

Biosynthesis and Transport of DHA in Rods

To define how DHA is supplied to and retained by photoreceptors, several laboratories, including ours, have used biochemical and cell biological approaches. Using frogs injected with [^{3}H]DHA into the dorsal lymph sac, it was shown that DHA arrives at the photoreceptors from capillary beds within the choriocapillaris by way of the RPE and the IPM. Radiolabeled DHA systemically delivered to frogs[54] and mouse pups[55,56] was followed from liver to RPE, and then to retina, as a function of time. In 3-day-old mouse pups, DHA appeared in liver within 2 hours after intraperitoneal injection, and subsequently in retina and brain within 12 to 24 hours. Figure 1 illustrates that the frog RPE became highly labeled with [^{3}H]DHA, rapidly becoming saturated by 6 hours after injection.[57,58] However, [^{3}H]DHA accumulated slowly within the neural retina with higher label density in the ganglion cell layer. This suggests that the RPE regulates DHA uptake from plasma and its release to the IPM, as hypothesized earlier. Furthermore, the fatty acid composition of RPE was different from plasma and red blood cells, with higher levels of the C20 and C22 polyunsaturated fatty acids.[59] Interestingly, retina and outer segments had higher levels of C22 fatty acids (omega-3) but lower amounts of C20 fatty acids (including omega-6) than did the RPE, suggesting that, at least in amphibians, these two groups of polyunsaturated fatty acids are handled differently by the RPE. Rats were fed different fatty acid supplements of 10% hydrogenated coconut oil (0.2% 18:2n6, 0% 18:3n3), safflower oil (73.8% 18:2n6, 0.1% 18:3n3), or linseed oil (16.4% 18:2n6, 52.2% 18:3n3) from birth, and plasma, RPE, and rod outer segment (ROS) fatty acid content was determined after 14 weeks.[60] DHA levels in plasma were diminished by coconut and safflower oil supplements. In general, the amounts of C20 polyunsaturated fatty acids were similar in plasma and RPE, but were 4 to 16 times greater than that measured in ROS. Amounts of 22:5n6 and DHA were three to five times greater in ROS than in RPE, and three to five times lower in plasma than in RPE. These studies further suggest that the RPE can selectively regulate fatty acid delivery to photoreceptors, excluding C20 fatty acids and increasing C22 fatty acids. Autoradiographic analysis of retina further revealed gradual accumulation of [^{3}H]DHA-phospholipids within photoreceptors, with label first appearing in the myoid region, followed by rapid redistribution to synaptic terminals and ellipsoids.[61,62] Finally, [^{3}H]DHA appeared in ROS as phospholipid components in newly synthesized disc membranes, illustrating the ability of photoreceptor cells to regulate DHA uptake, selectively directing DHA-containing molecules to appropriate cytoplasmic locations, and the incorporation of DHA-phospholipids into subcellular membranes, suggesting directed control by photoreceptors, dependent on their metabolic requirements.

Using [^{3}H]DHA, the retina (under in vitro conditions) incorporates 60% to 90% of this fatty acid into photoreceptor cells

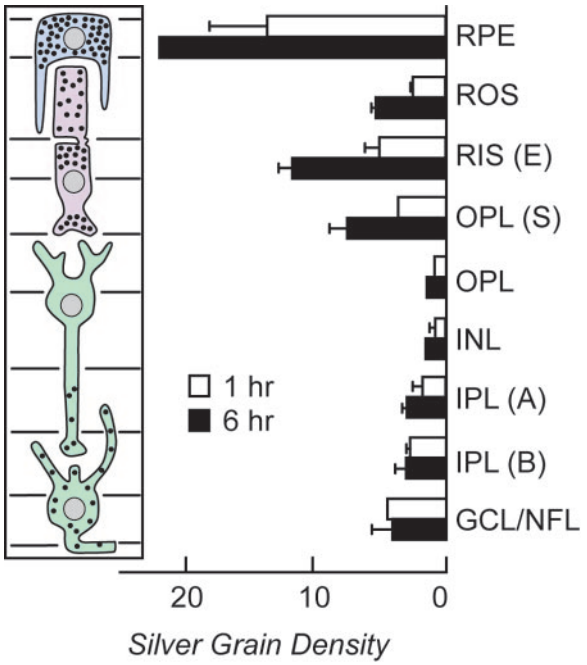

FIGURE 1. Early DHA-phospholipid biosynthesis in the retina and RPE after in vivo [^{3}H]DHA systemic injection in the frog. Highest labeling occurred in retinal pigment epithelium. Within the retina proper, photoreceptor inner segments and synaptic terminals had the highest densities. Histogram values (derived from light microscope autoradiograms) represent means/dispersion of 10 to 31 density measurements per layer, in two animals at each time point: 1 hour and 6 hours. The schematic *inset* at *left* illustrates the sampled regions, with degree of label indicated by stippling. Notice that the DHA distribution within each cell type is not uniform, but is concentrated within specific regions. RPE, retinal pigment epithelium; ROS, rod outer segments; RIS(E), rod inner segment ellipsoid; OPL(S), photoreceptor synaptic terminals in outer plexiform layer; OPL, outer plexiform layer, minus photoreceptor terminals; IPL(A) and IPL(B), distal and proximal halves of the inner plexiform layer, respectively; GCL/NFL, ganglion cell layer and nerve fiber layer, combined. Modified with permission from *Current Eye Research*, 13, Rodriguez de Turco EB, Gordon WC, Bazan NG. Docosahexaenoic acid is taken up by the inner segment of frog photoreceptors leading to an active synthesis of docosahexaenoyl-inositol lipids: similarities in metabolism in vivo and in vitro, 21–28, © Elsevier 1994.

within 4 hours.[63] Figure 2 illustrates the uptake of [^{3}H]DHA in different species.[64] Human retinal photoreceptors take up relatively large amounts of labeled DHA.[65] Frog rods accumulate up to 93% of labeled DHA in phospholipids.[61] Comparing in vivo and in vitro [^{3}H]DHA uptake in photoreceptor phospholipids, using physiologically meaningful concentrations of [^{3}H]DHA (nanomolar range), autoradiographic profiles from the retinas of [^{3}H]DHA-injected animals,[62] and [^{3}H]DHA-incubated retinas (when compared after 1 and 6 hours) demonstrate that in vitro results are similar to those obtained in short-term in vivo conditions. At 1 hour after injection, up to 33% of labeling occurs in the free fatty acid pool, but by 6 hours, this value drops to 5% as the [^{3}H]DHA is incorporated into lipids. By this time, [^{3}H]DHA is actively incorporated into phosphatidycholine, phosphatidylethanolamine, and phosphatidylinositol.[63] Initially, [^{3}H]DHA is incorporated into phosphatidic acid. Within 2 hours, phosphatidic acid labeling decreases and levels off, whereas triacylglycerol, phosphatidylserine, phosphatidylinositol, phosphatidylcholine, and phosphatidylethanolamine rapidly increase throughout the 6 hours after [^{3}H]DHA delivery.[62]

Both in vivo and in vitro studies support the idea that, on entering the photoreceptor cell, DHA is actively incorporated into phospholipids through a highly specific sequence of biochemical events. The rapid in vitro incorporation into photoreceptors reflects the ability of these cells' inner segments to take up DHA from the IPM. DHA is activated by coenzyme A[66–69] and then esterified into the sn-2 position of phosphatidic acid and triacylglycerol. Incorporation into phosphatidic acid is an active route of DHA addition, surpassing the acylation-reacylation pathways.[70–73] Phosphatidic acid and diacylglycerol are precursors for triacylglycerol, phosphatidylcholine, and phosphatidylethanolamine. In addition, an exchange reaction that converts phosphatidylethanolamine to phosphatidylserine and back again is active. These reactions result in the final distribution of retinal DHA phospholipids that, in turn, are shunted to specific regions within the membrane systems of the photoreceptors.

The distribution of retinal [^{3}H]DHA within photoreceptors and the layers of the underlying neural retina in in vivo studies is shown as grain-density profiles in Figure 1. When labeled discs reach the photoreceptor tip, they are shed and phagocytized by the RPE. The densely labeled portions of retinal autoradiograms remain constant throughout the life of the labeled disc membranes.[57] The density of a newly labeled outer segment basal region matches that of a densely labeled outer segment tip, and a heavily labeled, newly shed phagosome. Moreover, this high density is not found in the tips of photoreceptors where labeled disc membranes have not yet migrated, indicating that DHA-phospholipids do not leave the disc membrane to accumulate gradually within the remaining unlabeled disc membranes. Finally, the cytoplasm of the RPE remains low throughout disc migration and phagocytosis (Fig. 3). It is only after shedding and phagocytosis that [^{3}H]DHA is

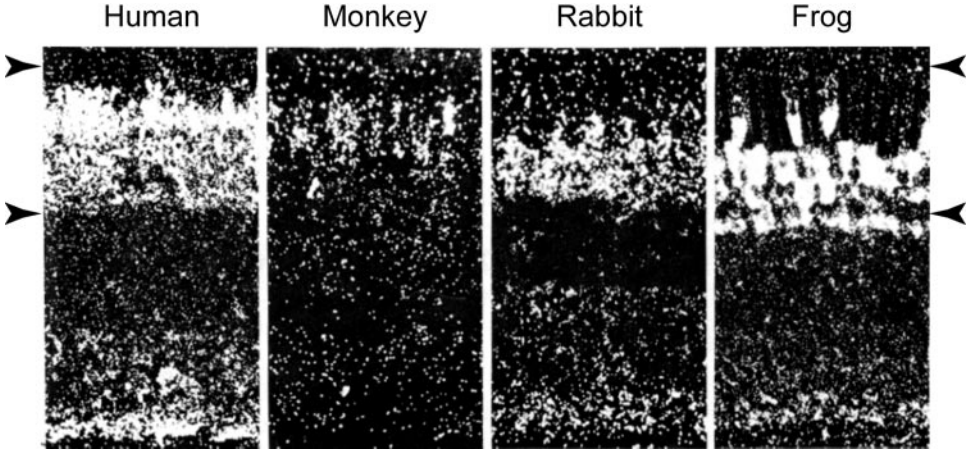

FIGURE 2. Autoradiograms of [^{3}H]DHA distribution in human, monkey, rabbit and frog retinas after 4 hours of in vitro incubation. Most of the label has accumulated in photoreceptors. These cells are oriented upward and lie in the space denoted by the *arrowheads*. The autoradiograms have been adjusted in size so that the lengths of the photoreceptors are equal. Modified from Bazan NG, Rodriguez de Turco EB, Gordon WC. Pathways for the uptake and conservation of docosahexaenoic acid in photoreceptors and synapses: biochemical and autoradiographic studies. *Can J Physiol Pharmacol*. 1993; 71:690–698, with permission from the National Research Council Press © 1993.

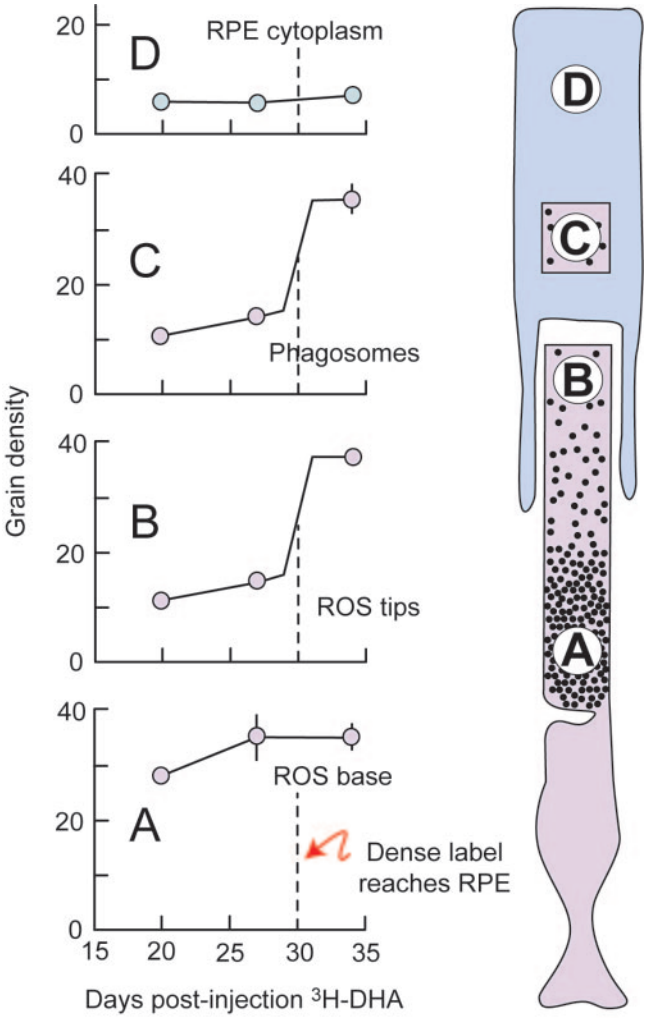

FIGURE 3. Radiolabeled DHA differentially traces DHA-phospholipid metabolism in different parts of the frog photoreceptor and RPE. Discrete sample regions are indicated as (**A**–**D**) in boxes. Autoradiographic grain densities of [^{3}H]DHA were measured at the base of the ROS (**A**), ROS tip (**B**), a newly shed phagosome in the RPE cell (**C**), and a corresponding area within the RPE cytoplasm (**D**). Density indicates relative labeling within each of these areas. The graphs represent grain densities within each of these regions for 20, 27, and 34 days after injection (dorsal lymph sac). A conservative estimate of the time at which the densely labeled ROS disc membranes will reach the outer segment tip and begin to appear within the cytoplasm of the RPE cells is 30 days (*dashed line*). Each point is the average of 25 measurements from at least three different sections; ±SEM is indicated. Modified with permission from *Current Eye Research*, 11, Gordon WC, Rodriguez de Turco EB, Bazan NG. Retinal pigment epithelial cells play a central role in the conservation of docosahexaenoic acid by photoreceptor cells after shedding and phagocytosis, 73–83, © Elsevier 1992.

removed from the disc membranes of phagosomes as they undergo degradation. Briefly, large oil droplets within the RPE accumulate these labeled fatty acids as triglycerides by 2 hours, but then lose them within 12 to 18 hours as they are mobilized for redistribution.[57] This is followed by recycling the DHA back into the IPM where it is transported to photoreceptors for reuptake. Frogs were injected with [^{3}H]DHA and allowed to accumulate label within the rod photoreceptor discs until the entire outer segment was heavily labeled (~30 days after injection). When shedding was subsequently triggered by manipulation of the light cycle, RPE cells received a pulse of [^{3}H]DHA in each phagosome. Measurements indicated that 12% of disc membrane retinal [^{3}H]DHA-lipids was phagocytized by the RPE, but total retina remained unchanged.[57] Because there was no reduction in retinal labeling after loss of label to RPE, it was concluded that RPE cells play a central role in conserving retinal DHA by recycling the outer-segment–derived DHA back to photoreceptors.

There is also differential labeling in DHA-phospholipids of photoreceptors, with rods labeling much more than cones. In frogs, the blue-sensitive (435 nm) green rods label three times as much as the green-sensitive (502 nm) red rods, and cone photoreceptors accumulate only minimal amounts (Fig. 4A). By 6 hours, the rod ellipsoid is heavily labeled as new disc membranes are being assembled (Fig. 4B). This labeling is especially evident in autoradiograms in which bright- and dark-field microscopy is used to demonstrate grain density.[61,62] Shortly thereafter, the basal-most discs of the outer segment possess [^{3}H]DHA-phospholipids.

Longer term in vivo autoradiography has demonstrated a unique labeling pattern in ROS. DHA, accumulated within the newly formed discs, remained in situ as discs were pushed apically by addition of newer basal discs. Because the DHA pool becomes highly labeled, [^{3}H]DHA was continually added (Fig. 4C), making it appear as if each rod outer segment was gradually filling with DHA silver grains (Figs. 4D, 5).[74] In fact, the entire outer segment becomes densely labeled with [^{3}H]DHA as more and more discs become labeled with radioactive precursor from the inner segment.

Heavier labeling of rod photoreceptors continues, with the inner segments appearing almost black in longer exposure autoradiograms (Fig. 5). When these images are coupled with high photoreceptor synaptic labeling throughout the earlier time points after presentation of [^{3}H]DHA, it becomes apparent that there is a very active bidirectional cellular trafficking of the DHA-containing molecules, beginning with its initial accumulation within the photoreceptor myoid region, resulting in one pathway toward the base of the outer segment (the ellipsoid region) and another to the vesicle membrane-rich synaptic terminal.[61]

Parallel experiments to compare [^{3}H]DHA with [^{3}H]leucine showed that the fronts of the labeled regions comigrated (Fig. 4D), reaching the photoreceptor tip at the same time, indicating that DHA-phospholipids of disc membranes remain there for the life of the disc and are unlike other fatty acids that rapidly diffuse throughout the outer segment. These studies show that the biosynthesis and turnover of DHA-phospholipids within rod outer segments can be correlated to that of newly synthesized rhodopsin throughout the process of outer segment disc renewal.

Electron microscopic autoradiography illustrates this labeling pattern.[58] After 5 days of labeling with [^{3}H]DHA, a distinct basal region of the rod outer segments was well labeled (Fig. 5B). Moreover, there was a distinct front with only diffuse labeling throughout the remaining stack of discs (Figs. 5A, 5B). This front continually advanced, remaining distinct throughout the apical advancement of the discs. Rapid, dense labeling was observed within the large inner segment cone oil droplets, despite low disc membrane labeling (Fig. 5A). Thus, unlike amino acids that form narrow migrating bands and other fatty acids that rapidly diffuse throughout the entire outer segment, DHA continually labels new rod disc membranes from the base of the outer segment (Figs. 5A, 5B). This finding reflects a differential turnover of the labeled precursor pool: The amino acids are rapidly catabolized to other compounds. Also, radioactivity is not available for the new synthesis of protein. The radioactivity remains in the DHA precursor pool because it is not turned over, and this pool remains labeled.[75–78]

In summary, studies with [^{3}H]DHA have shown that this long-chain polyunsaturated fatty acid is removed from the choriocapillaris to enter the RPE, where it is either shunted into temporary storage as a component of triacylglycerols

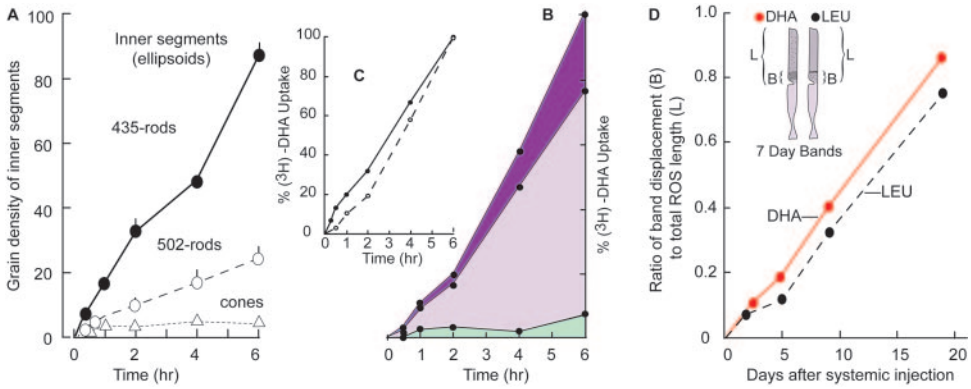

FIGURE 4

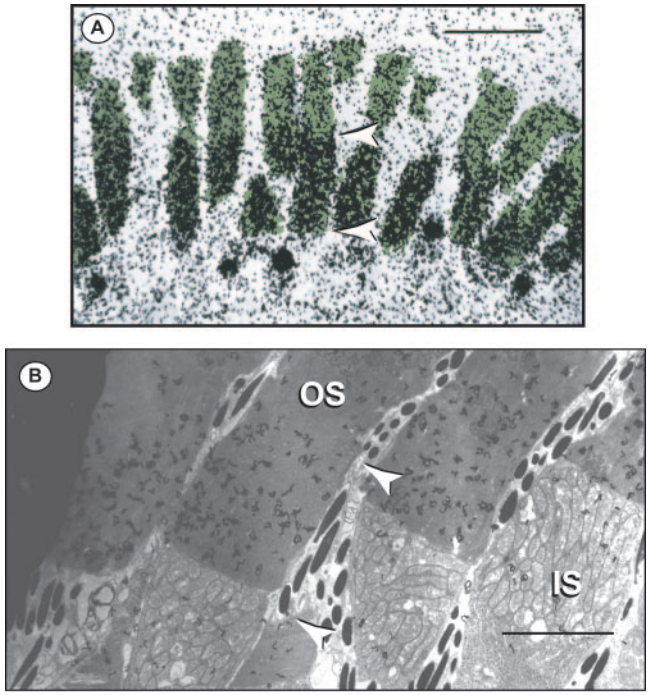

FIGURE 5

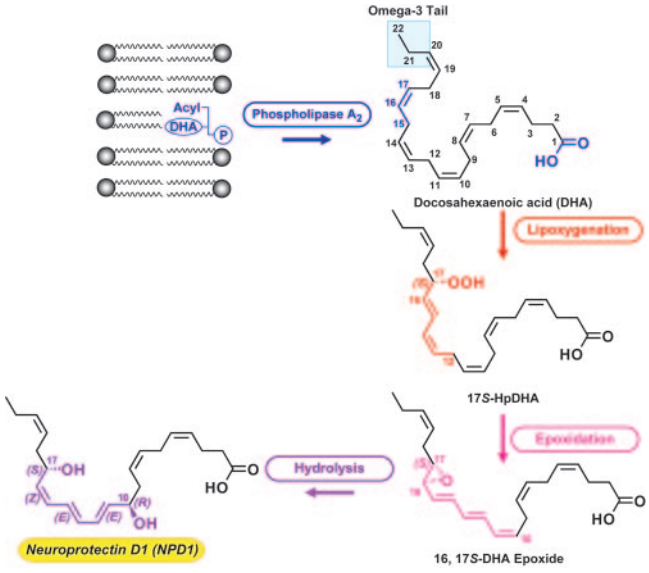

FIGURE 6

FIGURE 4. High avidity of frog photoreceptor cells for [^{3}H]DHA. (**A**) Percentage uptake of whole retina during in vitro incubations: (●) total dissociations per minute per milligram (dpm/mg) retina protein during 6 hours after [^{3}H]DHA treatment (extracted and purified lipids); (○) (*dashed line*) total number of silver grains/retina (by autoradiography). Biochemical and autoradiographic data show very similar retinal uptake of [^{3}H]DHA. (**B**) Distribution of radiolabeled DHA in the frog retina shows increasing and selective uptake in the inner segments (*light purple*), in outer segments (*dark purple*), and in the neural retina (*green*). (**C**) Differences in the density of labeling in the inner segments of frog photoreceptor cells as a function of time. Green (435 nm)-rod inner segments (ellipsoids) accumulate three times that of the red (504 nm) rods and 22 times that of the cones. All points represent the mean ± SD of at least three individual determinations. (**D**) Displacement of [^{3}H]leucine-labeled rhodopsin and [^{3}H]DHA-labeled phospholipids during rod outer segment renewal after delivery of labeled precursors in the dorsal lymph sac. Shown are the distances of migration of leucine and DHA as a ratio of band displacement (B) to total outer segment length (L), demonstrating that the moving fronts of these labeled molecules will arrive at the photoreceptor tip at the same time. Modified with permission from Rodriguez de Turco EB, Gordon WC, Bazan NG. Rapid and selective uptake, metabolism, and cellular distribution of docosahexaenoic acid among rod and cone photoreceptor cells in the frog retina. *J Neurosci*. 1991;11:3667–3678; and Gordon WC, Bazan NG. Docosahexaenoic acid utilization during rod photoreceptor cell renewal. *J Neurosci*. 1990;10:2190–2202.

FIGURE 5. [^{3}H]DHA is mainly incorporated during rod photoreceptor renewal in newly synthesized frog disc membranes. (**A**) Unstained autoradiogram, 1-μm-thick plastic section (20 days after injection) shown after 5 months of exposure. Removal of the RPE and its melanin emphasizes the two labeling patterns. The DHA-containing phospholipids form the dense region seen in the basal portion of the rod outer segments. Between the arrowheads are densely labeled discs that have accumulated for 20 days. The diffuse form of [^{3}H]DHA labeling occurs in the distal region of the outer segments in discs that were already present when these retinas were first labeled. Densely labeled oil droplets of cone inner segments appear as dark spheres near the base of the rod outer segments. This section has been pseudocolored to emphasize the rod outer segments (*green*). (**B**) Green-sensitive rod outer segments (ROS) 5 days after frogs were injected with [^{3}H]DHA. This electron-microscope autoradiogram illustrates silver grain accumulation on the densely labeled, newly formed discs. *Arrowheads*: region of highly labeled basal discs. The more distal portions of the outer segments are much more diffusely labeled. OS, outer segments; IS, inner segments. Scale bar: (**A**) 20 μm; (**B**) 5 μm. Modified with permission from *Current Eye Research*, 11, Gordon WC, Rodriguez de Turco EB, Bazan NG. Retinal pigment epithelial cells play a central role in the conservation of docosahexaenoic acid by photoreceptor cells after shedding and phagocytosis, 73–83, © Elsevier 1992; and Gordon WC, Bazan NG. Visualization of [^{3}H]docosahexaenoic acid trafficking through photoreceptors and retinal pigment epithelium by electron microscopic autoradiography. *Invest Ophthalmol Vis Sci*. 1993;34:2402–2411.

FIGURE 6. Biosynthesis of NPD1. A membrane phospholipid containing a docosahexaenoyl chain in sn-2 is hydrolyzed by phospholipase A_2, generating free (unesterified) DHA. The carbons of DHA are numbered and the omega-3 (n-3) tail is highlighted. Lipoxygenation is then followed by epoxidation and hydrolysis, to generate NPD1.

within membrane-bound oil droplets or is directed to the IPM and the photoreceptors. [^{3}H]DHA is released in either its free or esterified forms from the RPE cells into the IPM, where it can be bound to proteins and transported to photoreceptors. The myoid region of photoreceptor inner segments displays preferential uptake of [^{3}H]DHA. In this endoplasmic reticulum–rich area, [^{3}H]DHA is activated by the addition of coenzyme A and then esterified into phospholipids. [^{3}H]DHA-phospholipids are then rapidly transported to the membranes of photoreceptor synaptic vesicles, as well as to the apical regions of the inner segments, where they are incorporated into the photosensitive membranes of new discs assembled at the base of rod outer segments. These [^{3}H]DHA-phospholipid–enriched disc membranes are pushed toward the outer segment tips as more discs are assembled below, eventually reaching the photoreceptor tips where they are periodically shed and then phagocytized by the RPE. Phagosomes, enriched in [^{3}H]DHA-phospholipids, appear within the RPE cells, where they immediately undergo degradation. Phagosomal [^{3}H]DHA-phospholipids transiently appear within oil droplets of these cells and are then recycled back to photoreceptors.

Phagocytosis of Shed Rod Outer Segment Tips in the Delivery of Lipids to the RPE

Photoreceptor outer segment phagocytosis by the RPE cells results in a massive daily supply of phospholipids highly enriched in DHA and in longer-chain DHA derivatives. The DHA and other omega-3 fatty acids are retrieved by the inner segment of the photoreceptor through the IPM, the short loop of conservation of omega-3 fatty acids in photoreceptors. Until recently, the short loop was referred to as a DHA retrieval route. However, since longer-chain omega-3 fatty acids are also quantitatively significant components of photoreceptor outer segments, an omega-3 fatty acid retrieval short loop should be envisioned. We have an incomplete understanding of the elements operating this short loop. For example, we do not know the form in which DHA transverses the interphotoreceptor membrane or the carriers that are used. It has been suggested that IRBP[79,80] may be a carrier and that a gradient of 11-*cis* retinal and DHA may contribute to the operation of this loop.[80] The retrieval through the short loop is most likely tightly regulated subsequent to daily photoreceptor outer segment renewal. Studies on the short loop during the light–darkness cycle will contribute to clarifying this event, particularly if omega-3 fatty acids from cones and rods can be traced. We also do not know if there is a receptor-mediated uptake mechanism in the inner segment or how this uptake is regulated. In the inner segment, active phospholipid reutilization for photoreceptor membrane biogenesis takes place.

Neuroprotectin D1 Synthesis: An Early Response to Oxidative Stress and a Newly Recognized Function of Retinal Pigment Epithelial Cells

RPE cells, the most active phagocytes of the body, are derived from the neuroectoderm and support photoreceptor cells by participating in the daily shedding, internalization, and degradation (phagocytosis) of the tips of the photoreceptor outer segments (membrane discs). In mammals, the circadian shedding and phagocytosis has been calculated to be complete after 10 days for one entire ROS.[20,21] In rhesus monkeys, every RPE cell interacts with 20 to 45 photoreceptor tips.[81] In the human macula, it has been estimated that 23 photoreceptors interact with one RPE cell.[81] An RPE cell interacts through its apical side with ROS and phagocytizes daily approximately 10% of the outer segments of photoreceptors. This is an immense task, comparable to engulfing and degrading (phagolysosomal processing) an outer segment mass equivalent to 5 to 10 red blood cell membranes per day. In spite of this intense phagocytic activity, RPE cell integrity is maintained during nine decades in the absence of eye disease.[82] Phagocytosis steps are intricate and engage RPE cell-surface receptors that recognize, bind to, and internalize photoreceptor tips.[18,83,84] The RPE cells then undergo cytoskeletal rearrangements, genes are induced,[85] lysosome–phagosome fusion takes place, and recycling of retinol and DHA is initiated.[27,86] Consequently, ROS renewal occurs, resulting in an outer segment that is unmodified in length because, as the discs at the tips are phagocytized, membrane biogenesis from the inner segment precisely replaces the amount of membrane removed. The RPE cells also perform other functions, which include transport and reisomerization of bleached visual pigments, synthesis, and secretion of neurotrophic growth factors, and contribution to the integrity of the barrier between choroidal blood and the photoreceptors. Hence, RPE cells exhibit remarkably complex, pleiotropic behavior: sometimes they resemble macrophages, other times they are like classic cuboidal epithelial cells, and at still other times they behave like glial cells.

Oxygenation, Metabolic Activity, and Light Threats to Polyunsaturated Lipids in the Outer Segments and RPE

The photoreceptors and RPE are constantly subjected to a plethora of environmental as well as intrinsic factors that are potential disruptors of homeostasis: high oxygen tension, intense light during the day, and cell membranes with the highest content of polyunsaturated fatty acyl chains in their phospholipids (particularly DHA, as well as 20:4, n-6 [arachidonic acid]) of all organs. In experimental models of retinal degeneration, lipid peroxidation, a potentially cell-damaging event, does occur in outer segment discs.[39] Moreover, in drusen (deposits of debrislike material that accumulate between the RPE cells and Bruch's membrane) from patients with AMD, DHA oxidation products have been found to form protein adducts.[87] Trauma or retinal detachment induces RPE dysfunctions that, in turn, contribute to the onset and development of proliferative membranelike structures and neovascularization. RPE cells have developed endogenous mechanisms to cope with these challenges and guard against damage, such as the presence of antioxidants (e.g., vitamin E), which contribute to the preservation of cellular integrity.

Neuroprotectin D1 Biosynthesis: An Endogenous Response to Oxidative Stress

Recently, our laboratory found that RPE cells respond to oxidative stress by activating the synthesis of a major endogenous neuroprotective mediator, NPD1[88] (Fig. 6). The name neuroprotectin D1 was suggested based on its neuroprotective bioactivity in oxidatively stressed RPE cells and brain, and its potent ability to inactivate pro-apoptotic and proinflammatory signaling. D1 refers to its being the first identified neuroprotective mediator derived from DHA. DHA belongs to the essential omega-3 fatty acid family (all of which are derived from linolenic acid, 18:3, n-3) and therefore cannot be made de novo in the body. The photoreceptor cells, unlike most other cells of the nervous system, are highly enriched in DHA, tenaciously retaining DHA even during very prolonged periods of omega-3 fatty acid deprivation.[22,23,27]

Previous studies have shown that the retina forms mono-, di-, and trihydroxy derivatives of DHA, and lipoxygenase inhibitors block this synthesis, suggesting an enzymatic process of a

lipoxygenase nature.[89] Although, at the time, the stereochemistry and bioactivity of these DHA-oxygenated derivatives were not defined, it was proposed that these lipoxygenase products might be neuroprotective (and at the same time, the term "docosanoids" was suggested).[26,89] On the advent of liquid chromatography, photodiode array, electrospray ionization, and tandem mass spectrometry-based lipidomic analysis, a collaboration between the group of Charles Serhan (Harvard Medical School) and our group identified oxygenation pathways for the synthesis of the docosanoid NPD1 during brain ischemia-reperfusion.[90] Moreover, it was also found that RPE cells have the ability to synthesize NPD1.[88] NPD1 is formed from free (unesterified) DHA and released from membrane phospholipids by a phospholipase A_2 (PLA_2). Photoreceptors and RPE cells, although they contain phospholipids richly endowed with DHA (as docosahexaenoyl- or DHA-elongated fatty acyl-chains), display an undetectable quantity of unesterified (free) DHA (as is the case with unesterified arachidonic acid) under basal, unstimulated conditions,[25,91–94] which means that the pool size of unesterified DHA is tightly controlled at the level of its production by a PLA_2, by its removal (e.g., by reacylation) and by peroxidation. Free DHA to be incorporated into membrane phospholipids first becomes the substrate of docosahexaenoyl-coenzyme A synthesis for its channeling through acyltransferases that incorporate this fatty acid into phospholipids.[66–69] The RPE cell thus modulates the uptake, conservation, and delivery of DHA to photoreceptors.[26] In addition, the RPE cell utilizes a specific DHA-phospholipid pool as a precursor for the pathway leading to NPD1 synthesis. Then this stereospecific mediator is synthesized after DHA is released through DHA oxygenation by a PLA_2, followed by a 15-lipoxygenase-like activity.[88] The nature of these enzymes needs to be precisely defined. In AD brain of short postmortem time, it was found that $cPLA_{2\alpha}$ and 15 lipoxygenase-1 expression changed in concert with NPD1-decreased content and DHA enhanced pool size in the CA1 area of the hippocampus.[95] In ARPE-19 cells (spontaneously transformed human RPE cells), interleukin (IL)-1β, oxidative stress, or the Ca^{2+} ionophore A23187 activates the synthesis of NPD1.[88] In turn, NPD1 may act in an autocrine fashion and/or diffuse through the IPM, to act in a paracrine mode on photoreceptor cells and/or Müller cells.[27]

Anti-apoptotic and Anti-inflammatory Actions of NPD1

The cellular pathways regulated by NPD1 have just begun to be identified. NPD1 promotes differential changes in the expression of Bcl-2 family proteins, upregulating protective Bcl-2 proteins (Bcl-2, Bcl-xL, and Bfl-1/A1) and attenuating the expression of the proteins that challenge cell survival (e.g., Bax, Bad, Bid, and Bik). Thus, an NPD1-mediated and coordinated regulation of the availability of Bcl-2 proteins for subsequent downstream signaling may be crucial for cell survival.[88,95] NPD1 may regulate expression of the genes encoding death repressors and effectors of the Bcl-2 family of proteins. However, translational or posttranslational events may also integrate a concerted responsive machinery to counteract oxidative stress. The precise molecular mechanisms involved remain to be defined, and exploration of these events will provide important insight into regulatory survival signaling. Bcl-2 family proteins regulate apoptotic signaling at the level of mitochondria and the endoplasmic reticulum. As a consequence, cytochrome *c* is released from mitochondria and effector caspase-3 is activated. In agreement with this sequence, oxidative-stress–induced activation of caspase-3 in ARPE-19 cells is decreased by NPD1.[88] Apoptosis is an outcome of excessive oxidative stress in RPE cells, but NPD1 is effective in counteracting this oxidative stress-induced cell death.[88] It is of interest that DHA itself inhibits apoptosis, concomitant with a remarkable, time-dependent formation of NPD1. Significantly, the potency of DHA for cytoprotection is much higher than that of added NPD1,[88] suggesting that NPD1 may exert its action near the subcellular site of its synthesis. Importantly, these actions of DHA cannot be mimicked by other polyunsaturated fatty acids (PUFAs) (e.g., 20:4, n-6). Alternatively, it may imply that other NPD-like mediators participate in promoting RPE cell survival. It is indeed possible that related NPD mediators are formed in an attempt to cope with the multiplicity of cellular signaling that has the potential of going awry in RPE or neurons confronted with oxidative stress. DNA array-based human genome expression profiling has demonstrated that NPD1 turns off several proinflammatory and pro-apoptotic genes, whereas it induces anti-apoptotic genes in human neural progenitor cells.[95] Remarkably, DHA, and even more so NPD1, mediate opposite changes from those elicited by the amyloid β peptide Aβ42. This peptide enhances expression of genes encoding cytokine exodus protein-1 (CEX-1), IL-1β, tumor necrosis factor (TNF)-α, and cyclooxygenase-2 (COX-2), in addition to the TNF-α-inducible proinflammatory element B94.[95] These findings further suggest that NPD1 induces a gene expression program that is neuroprotective through downregulation of pro-apoptotic and proinflammatory factors and upregulation of Bcl-2-family anti-apoptotic proteins (in neural cells and in RPE), which are crucial modulators of cell survival.

Caspase-3, which is a downstream effector of pro- and anti-apoptotic Bcl-2 proteins, is activated as a consequence of mitochondrial cytochrome *c* release into the cytoplasm and activation of the apoptosome.[96] In RPE cells, cleavage of endogenous substrates by caspase-3 is enhanced by oxidative stress, as indicated by increased accumulation of poly(ADP-ribose) polymerases (PARPs). NPD1 inhibits caspase-3 activation when added at the onset of oxidative stress.[88] This effect is interpreted as a downstream consequence of NPD1 modulation of the premitochondrial Bcl-2 proteins.

Oxidative stress enhances proinflammatory gene expression and represents an important mechanism for RPE cell injury. The inducible enzyme COX-2 is the rate-limiting step in the synthesis of prostaglandins and is involved in oxidative stress in addition to cell function. COX-2 expression is regulated in RPE cells by photoreceptor outer segment phagocytosis and by growth factors,[97] and IL-1β activates expression of the proximal COX-2 promoter. NPD1 can potently counteract such induction of the COX-2 promoter, displaying an IC_{50} of <5 nM.[88]

A consequence of RPE cell damage and apoptosis is impaired photoreceptor cell survival, a dominant factor in AMD.[2,98] The pigment lipofuscin, levels of which increase in the RPE during aging, further accumulates in AMD. The progressively greater onslaught of photooxidative damage to the RPE affects photoreceptor survival. For example, in the juvenile form of macular degeneration known as Stargardt's disease, oxidative stress mediated by the lipofuscin fluorophore *N*-retinylidene-*N*-retinylethanolamine (A2E) produces RPE damage and caspase-3 is part of the damaging cascade, whereas Bcl-2 exerts cellular protection.[99] NPD1 downregulates A2E-mediated apoptosis induced by oxidative stress, restoring the integrity of the RPE and probably its relationship with the photoreceptor.[100]

Neurotrophin-Induced Synthesis and Release of Neuroprotectin D1 from Human RPE Cells

Neurotrophins are important in photoreceptor survival.[101–104] Using human RPE cells grown to confluence and a high degree of differentiation displaying apical-basolateral polarization,[105] our laboratory has shown that neurotrophins (PEDF, BDNF,

CNTF, FGF, GDNF, LIF, NT3, or persephin), having bioactivities that promote neuronal and/or photoreceptor cell survival, are agonists of NPD1 synthesis.[100] Neurotrophins trigger synthesis and release of NPD1 through the apical surface of the cell. Pigment epithelium–derived factor (PEDF) is by far the most potent stimulator of NPD1 synthesis in RPE cells. PEDF, a member of the serine protease inhibitor (serpin) family, has been identified in human RPE cells.[106] If PEDF or ciliary neurotrophic factor (CNTF) is added to the incubation medium bathing the basolateral side in increasing concentrations, it evokes much less NPD1 release on the apical side. Conversely, if these neurotrophins are added to the apical side, they exert concentration-dependent increases in NPD1 release only on the apical side.[100]

The human RPE cells used for these studies may have limited DHA in phospholipids to synthesize NPD1 because they are in cell culture conditions and are not undergoing photoreceptor membrane phagocytosis. When DHA content in the medium was increased, a remarkable potentiation by PEDF of NPD1 release into the apical medium was uncovered when the neurotrophin was added to the medium bathing the apical surface. In contrast, much less NPD1 was found in the medium bathing the basolateral side of the cells. Much less apical NPD1 release was observed when PEDF was applied to the medium 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. Moreover, the addition of DHA to either side of the cell monolayer selectively synergizes PEDF-induced NPD1 release only through the apical side. Significant cytoprotection and enhanced NPD1 formation occurs synergistically when PEDF is added along with DHA under conditions of oxidative stress-induced apoptotic cell death triggered by serum starvation/H_2O_2/TNFα.[100]

The initiation and amplification of the premitochondrial apoptotic cascade involves the Bcl-2 family of proteins,[27] and NPD1, as mentioned, modulates the expression of these proteins.[27,88,95] Increasing the concentration of DHA from 10 to 50 nM upregulates Bcl-2 and Bfl-1 protein expression. Although PEDF alone is unable to alter the expression of pro- and antiapoptotic proteins when added with DHA during oxidative stress, it does potentiate the expression of these proteins with concomitant NPD1 synthesis in the presence of DHA. Proapoptotic protein expression in the presence of DHA and PEDF undergoes opposite changes. Bid, Bax, and Bad expressions are enhanced by oxidative stress, and DHA decreases their expression, whereas PEDF potentiates this action.[100]

In the presence of PEDF, a DHA dose-dependent inhibition of oxidative stress-triggered caspase-3 induction, in the presence of PEDF, takes place. Thus, a remarkable synergy between PEDF and DHA occurs with enhanced cytoprotection, upregulation of NPD1 synthesis, enhancement of anti-apoptotic protein expression, downregulation of pro-apoptotic protein expression, and caspase-3 cleavage.

Photoreceptor Outer Segment Phagocytosis in RPE Cell Survival Signaling

The RPE cell recycles DHA from phagocytized disc membranes back to the inner segment of the photoreceptor cell through the IPM (Fig. 7).[26,27,107,108] As previously stated, photoreceptor cells tenaciously retain DHA, even during prolonged periods of omega-3 fatty acid deprivation.[22–24,27,47,109–112] Studies correlating the periods of photoreceptor and synapse biogenesis with omega-3 fatty acid supply during mouse postnatal development demonstrate that dietary linolenic acid (18:3, n-3) is actively elongated and desaturated in the liver before its distribution through the blood stream to the retina and brain.[55] The biosynthesis of DHA and its incorporation into liver phospholipids and its trafficking through the RPE and photoreceptors is depicted in Figure 7. Several studies have shown that DHA is necessary for photoreceptor function and vision both in animals[113–118] as well as in humans.[119] Moreover, the essentiality of DHA has been documented for vision and brain maturation in premature infants and in newborns.[119] The RPE cell, in addition, contributes to the DHA-enriching ability of the photoreceptor cells by taking up DHA from the bloodstream through the choriocapillaris.[27] However, the bulk of DHA in the RPE cell is a component of photoreceptor disc membrane phospholipids that, after shedding and phagocytosis, is recycled as part of outer segment renewal.

Although it is known that ROS phagocytosis in RPE cells is essential for photoreceptor cell function and survival, thus far no specific messengers/mediators or mechanisms that promote cell survival during this process have been identified. The RPE-photoreceptor outer segments are potentially highly susceptible to oxidative stress because of the high oxygen consumption of the retina, active flux of PUFAs (omega-3 and also omega-6), and exposure to light.[27,89] Some studies have suggested that DHA-phospholipid peroxidation may be an "on" signal that initiates phagocytosis. Recently, it has been shown that phagocytosis (24–48 hours) of oxidized ROS containing high oxidation products downregulates complement factor H in RPE cells.[120] The RPE complement regulatory system may be suppressed by proinflammatory conditions, including phagocytosis of oxidized ROS.[120] In studying ROS phagocytosis, we have been surprised to find a remarkable, unexpected enhanced refractoriness to oxidative stress-induced apoptosis in RPE cells.[121] This action is specific to ROS, since nonspecific phagocytosis (polystyrene microspheres) by RPE cells does not lead to a protective response against oxidative stress. Concomitant with this, ROS, but not polystyrene microspheres, induce DHA release and activate NPD1 synthesis. Interestingly, ROS-mediated RPE cell protection against oxidative stress, with concurrent NPD1 synthesis activation, has been shown in ARPE-19 cells[121] as well as in passage six of primary human RPE cells prepared from National Disease Research Interchange (NDRI)–supplied eyes (Mukherjee PK, Marcheselli VL, de Rivero Vaccari JC, Gordon WC, Bazan NG, unpublished observations, 2007). When the free DHA pool size was simultaneously measured in RPE cells and in incubation medium by tandem mass spectrometry (MS/MS), it was found that it increases as a function of time of exposure to oxidative stress in RPE cells. Free DHA in cells showed a moderate increase after 6 hours when cells were subjected only to ROS phagocytosis (10.5-fold increase). Oxidative stress, however, strongly enhanced free DHA accumulation in a time-dependent fashion, peaking at 16 hours. Interestingly, although the overall increase reached 10-fold, ROS phagocytosis kept the DHA pool size at a constant 2.4-fold increased level. This finding implies that NPD1 synthesis reflects an event other than simply enhanced overall availability of free DHA on phagocytosis. There is a correlation between increases in free DHA pool size and in NPD1 synthesis. ROS phagocytosis stimulates NPD1 synthesis at 3 to 6 hours in cells and accumulation in medium after 16 hours, whereas free DHA increases earlier and keeps accumulating up to 16 hours. These enhancements in DHA and NPD1 pool size are much larger when ROS phagocytosis takes place on RPE cells exposed to oxidative stress. Interestingly, microsphere phagocytosis does not cause enhanced changes in DHA and NPD1. Thus, a very specific free DHA pool may be the precursor for NPD1. Arachidonic acid is also an active precursor of several bioactive lipids, including prostaglandins and lipoxygenase-products, and they have been correlated with photoreceptor phagocytosis.[122,123] Since arachidonic acid is released under the present experimental conditions (data not shown), as well as during light exposure, the arachidonic acid cascade members lipoxin A4, 12(S) HETE, and 15(S) HETE have

been studied and found to be unchanged during ROS phagocytosis.[121]

Deuterium-labeled DHA ($^2H_5$-DHA) has been used to ascertain whether the enhanced availability of free DHA leads to the synthesis of NPD1 in RPE cells undergoing oxidative stress. We followed $^2H_5$-NPD1 synthesis by tandem liquid chromatography-photodiode array-electrospray ionization-tandem MS-based lipidomic analysis. This approach allowed us to assess DHA conversion specifically, because the deuterium is on the methylene carbons 21 and 22, which are not metabolically altered. Also, the products are heavier (by a mass unit of 1) than the same nondeuterated molecule and can be detected by tandem mass spectrometry. The characterization of $^2H_5$-NPD1 (negative molecular ion m/z 364.2) and of endogenous nondeuterated NPD1 (negative molecular ion m/z 359.2) is also possible. Our results support the notion that as free DHA accumulates in RPE cells in culture during ROS phagocytosis the fatty acid is used as a substrate for NPD1 synthesis. These findings further support the concept that an unrecognized function of photoreceptor phagocytosis is to induce NPD1 synthesis and thereby elicit cytoprotection, particularly during oxidative stress.[121] Thus, NPD1 may be a major endogenous promoter of RPE cell survival during photoreceptor rod outer segment renewal. Further studies are being conducted to determine whether other docosanoids are also formed under these conditions. The enhanced synthesis of NPD1 after ROS phagocytosis is concomitant with ROS-induced attenuation of oxidative stress-mediated apoptosis. Although ARPE-19 cells also phagocytized biologically inert polystyrene microspheres, NPD1 content is not affected in RPE cells or in the incubation medium. Moreover, although oxidative stress stimulates NPD1 accumulation, this also is not affected by microsphere phagocytosis. These results correlate with the observed lack of cytoprotection by microsphere phagocytosis.[121] Nonspecific, nonbiological microspheres, unlike ROS, do not promote early-response gene induction in the RPE,[85] including COX-2[97] and PPARγ expression.[124]

These results reveal that the supply of DHA and the induction of NPD1 synthesis during ROS phagocytosis represents a homeostatic regulatory event for RPE cell protection in conditions of oxidative stress challenge, and as a consequence the fostering of photoreceptor cell integrity.[100,121]

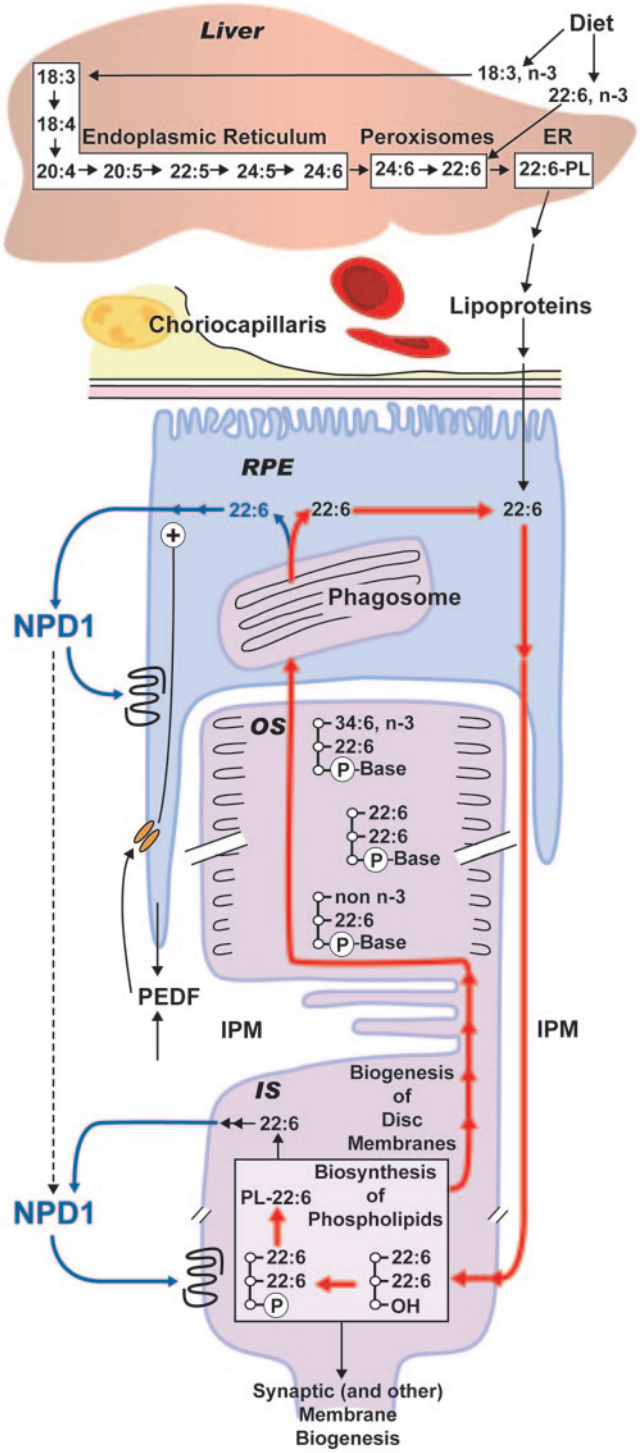

FIGURE 7. Illustration depicting aspects of the interorgan and inter- and intracellular trafficking of docosahexaenoic acid. Dietary 18:3, n-3 or 22:6, n-3 (DHA) are actively taken up by hepatocytes where elongation and desaturation of 18:3 to DHA takes place, catalyzed by enzymes located in the endoplasmic reticulum and peroxisomes. 22:6 then is activated (22:6-CoA) and acylated mainly into phospholipids that are secreted into the blood stream as lipoproteins. DHA from lipoproteins is taken up by RPE cells (*blue*) through the choriocapillaris and then channeled through the interphotoreceptor matrix (IPM) to the inner segments (IS) of the photoreceptors (*purple*). It is not clear whether free DHA (unesterified) is the sole form of DHA moving through the IPM. In the IS, phospholipid biosynthesis utilizes DHA and in turn provides phospholipids containing DHA to the biogenesis of outer segment membranes. Within the IS, diacylglycerol and phosphatidic acid containing two DHAs on the same glycerol backbone, have been proposed to be intermediaries in DHA-phospholipid metabolism. In the outer segments (OS), unique molecular species of phospholipids are included to highlight the fact that these membranes contain molecular species of phosphatidylcholine with 34:6, n-3 esterified in sn-1, and DHA in sn-2; molecular species of phospholipids with two DHAs and others with DHA in sn-2; and a non-omega-3 fatty acyl group in sn-1. The photoreceptor cell is connected to the RPE cell by *red arrows* that indicate the omega-3 fatty acids conservation route (short loop) during photoreceptor outer segment renewal. Thus, a phagosome is shown in the RPE from where *red arrows* follow the conservation route, while a *blue arrow* indicates DHA being used for NPD1 synthesis, consistent with the recent demonstration that ROS phagocytosis selectively increases NPD1 synthesis in the RPE cell.[121] NPD1 is illustrated as acting on a putative receptor on the RPE cell. Signaling evolving from this receptor is shown in Figure 8. PEDF secreted by the RPE or other cells is shown as an inducer of NPD1 synthesis. In the IS, biosynthesized phospholipids are also depicted to be used in the biogenesis of other photoreceptor cell membranes, including those of the synaptic terminals. NPD1 is also depicted to be synthetized and to have a putative receptor in the IS. Unpublished evidence using 661W cells, a cone photoreceptor cell line, support this possibility (Kanan Y, Al-Ubaidi M, Marcheselli V, Bazan N). The dashed line from RPE-synthetized NPD1 to a putative IS receptor reflects the finding that the lipid mediator may elicit paracrine bioactivity.[100]

Concluding Comments: Omega-3 Fatty Acid Lipidomics and the Understanding of the Biology of Photoreceptor–RPE Cell Interactions

Genomics, proteomics, and metabolomics are dissecting the molecular and functional organization of the cell. Within metabolomics, lipidomics is an evolving approach that allows the detailed identification of lipid classes and molecular species, including structural and bioactive lipids (mediators of cell signaling). The lipidome of a cell, or part of a cell, defines the complete characterization of the lipids. We are using a lipidomic-based analysis to initiate the decoding of RPE, retina, and neural omega-3 fatty acids. This approach has led to the discovery of neuroprotectin D1 in the RPE cell[88] and the uncovering of its bioactivity.

NPD1, a DHA-derived mediator synthesized by RPE cells, promotes photoreceptor-RPE cell homeostasis through modulation of multiple signaling pathways. NPD1 downregulates the expression of proinflammatory genes, which includes cytokine-induced COX-2 expression in RPE cells.[88] Also, in ischemia-reperfusion–injured hippocampus and in neural progenitor cells stimulated by IL-1β, NPD1 inhibits COX-2 induction.[90] In brain ischemia-reperfusion, NPD1 decreases infarct size and inhibits polymorphonuclear leukocyte infiltration.[90] Moreover, our laboratory, through a genome-wide screen in human brain progenitor cells in culture,[95] has identified other proinflammatory genes targeted by NPD1. They include IL-1β, cytokine exodus protein-1 (CEX-1), and TNFα-inducible proinflammatory element (B94, TNFAIP2). Figure 8 illustrates NPD1 bioactivity as a modulatory signal that counteracts proinflammatory injury to the RPE, a condition in which pathoangiogenic signaling is activated in the wet form of AMD and in proliferative vitreoretinopathy, which occurs in diabetic retinopathy.

Excessive oxidative stress turns on multiple signaling pathways in the RPE, photoreceptors, and other cells. Several of these pathways, in turn, participate in the pathophysiology of retinal degenerative disease and lead to cell damage and, eventually, cell death.[125] The Bcl-2 family of proteins regulates the initiation and amplification of premitochondrial events of apoptosis.[96] In fact, expression of pro-apoptotic and anti-apoptotic Bcl-2 proteins is altered by oxidative stress, and cell survival reflects the predominance of one set of proteins over the other. In the RPE, oxidative stress, which is increased by several factors, including reactive oxygen species, shifts the balance of the Bcl-2 family protein expression toward those that favor cell damage.[11,126,127] An early response, when homeostasis is threatened, is the active induction of NPD1 synthesis. This lipid mediator, in turn, downregulates proinflammatory genes and modulates Bcl-2 protein expression to counteract consequences of oxidative stress.[88,95]

Several neurotrophins induce NPD1 synthesis apparently in an effort to offset the injury and/or proinflammatory response and restore homeostasis.[100] Survival factors for photoreceptors, which include neurotrophins, are being identified.[101,103,106,128–130] For example, fibroblast growth factor (FGF)-2 promotes bovine RPE cell survival in cultures through a sustained adaptive phenomenon that involves both FGF1-mediated activation of extracellular signal-regulated kinase (ERK) and ERK2-dependent Bcl-xL production.[131] Bcl-xL may play a key role in integrating and transmitting exogenous FGF2 signals for RPE cell survival. Moreover, a well-organized signaling regulatory mechanism on the apical side of the RPE cell is reflected by the ability of neurotrophins to induce NPD1 synthesis and release.[100] The response of RPE cell monolayers in culture with NPD1 synthesis and release on addition of certain neurotrophins to the apical side suggests sidedness of receptors for these ligands.[100] Figure 8 illustrates the action of

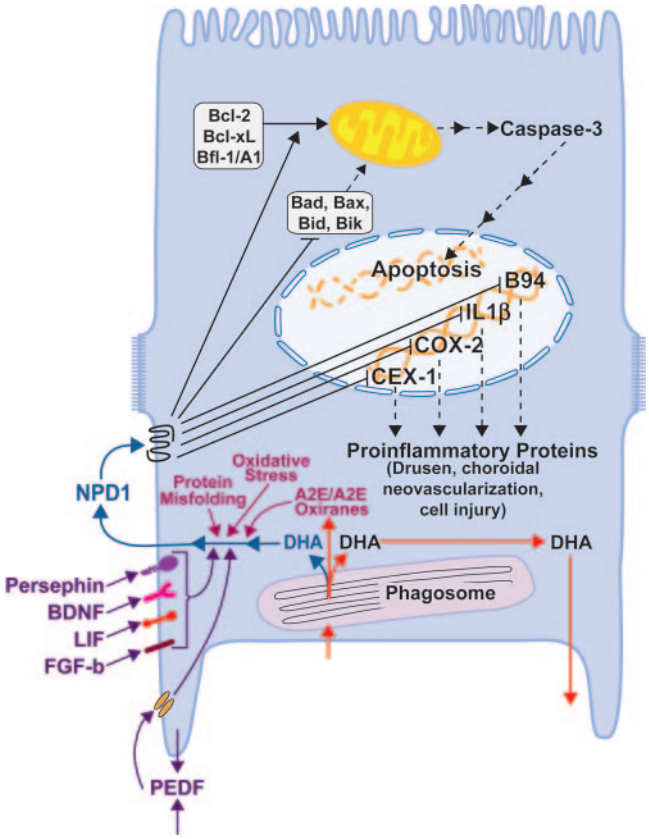

Figure 8. Inducers of NPD1 synthesis and bioactivity in the RPE cell. Neurotrophins, persephin, BDNF (brain-derived neurotrophic factor), LIF (leukemia inhibitory factor), FGF2 (fibroblast growth factor 2), or PEDF are indicated to induce NPD1 synthesis and its apical release.[100] PEDF is depicted as released from the RPE cell or provided from another cell. The same is true for the other growth factors. Oxidative stress, protein misfolding or A2E (*N*-retinyl-*N*-retinylidene ethanolamine)/A2E oxiranes (epoxides) are activators of NPD1 synthesis as well (*red*).[100] DHA is shown to arrive in the RPE as part of the phagosome (DHA-phospholipids). After phagolysosomal digestion, DHA is recycled back to the inner segments of photoreceptors through the IPM (Fig. 7, *black arrows*). The precise phospholipid-DHA molecular species that is hydrolyzed to generate the free DHA pool precursor of NPD1 has not been identified (NPD1 synthesis pathway, *blue*). NPD1 is released through the apical cellular side and recognizes a putative receptor. Intracellular signaling then inhibits proinflammatory gene expression.[92] The proinflammatory genes illustrated are IL-1β, COX-2 (cyclooxygenase 2), B94 (TNFα-inducible pro-inflammatory element), and CEX-1 (cytokine exodus protein-1, a marker for inflammatory and oxidative stress responses). As a consequence, a decrease in proinflammatory proteins takes place that, when available, plays a role in drusen development, choroidal neovascularization, and cell injury. In addition, NPD1-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.

persephin, BDNF, LIF, FGF2, and PEDF on the apical membrane of the RPE cell. Persephin is a novel neurotrophin with homology to GDNF.[130,132] Both persephin and GDNF are agonists of NPD1 synthesis and activators of its release from the apical surface of the RPE cell.[100] The same was found for LIF (leukemia inhibitory factor) and FGF2 as well as for other neurotrophins.[100] The finding that there is polarized (apically) neurotrophin-mediated NPD1 release has relevance to the initiation and progression of retinal degenerations because, when RPE cell polarization in the plane of the epithelium is disrupted, dysregulated growth factor secretion and proinflammatory signaling arise,[2,133,134] thereby setting in motion patho-

logic changes that include the proliferative component of macular degeneration, choroidal neovascularization.[135–137]

The homeostatic regulation between photoreceptors and RPE preserves RPE cell integrity during successful aging. In fact, RPE cell density is maintained at the macula during nine decades if eye disease does not arise.[82] However, failure of homeostasis results in enhanced DHA peroxidation, drusen formation, lipid peroxide protein adduct accumulation, apoptosis, and pathoangiogenesis. Overall, it is apparent that a breakdown in the balance of protective and potentially cytotoxic factors is involved in various forms of retinal degeneration.[2,17,27,86] NPD1 synthesis is induced under conditions in which excessive oxidative stress threatens to disrupt homeostasis, and rescue signals, such as neurotrophins, are released to protect cell integrity (Fig. 8). Triggers of the NPD1 response include A2E and A2E epoxides (oxiranes)[100] that accumulate in the aging RPE and in Stargardt's disease and other retinal degenerations.[99]

NPD1 synthesis is dependent on DHA availability in certain pools of phospholipids.[27] DHA, or its precursor, is supplied by the diet, packaged by the liver, and then sent to the retina and elsewhere.[55] Once DHA is incorporated into disc membrane phospholipids and is then sloughed to the RPE, DHA can be recycled back to photoreceptors for reuse, closing the short loop,[26,27,57,79] and thus conserving DHA. The long loop of omega-3 fatty acids is the connection between the liver and the RPE cell through the choriocapillaris (Fig. 7).[55] Consequently, disruption of either the long or short loop could result in impaired DHA supply to the photoreceptors, which could induce detrimental changes in photoreceptor function. Dietary linolenic acid (18:3, n-3) is elongated and desaturated in the liver, followed by supply to the RPE through blood lipoproteins.[138] It has been suggested that the photoreceptors and cellular membranes in the brain (the second-richest tissue in DHA) may release a signal to evoke secretion from the liver of phospholipids enriched in DHA when they are needed.[55] Photoreceptor biogenesis, as well as synaptogenesis during postnatal development, actively accrues DHA.[55,139] Moreover, during slow photoreceptor cell demise (such as in RP), the need to supply "DHA building blocks" for photoreceptors may be an early biological repair response.[55]

Photoreceptors and RPE cells are located in a part of the eye continuously exposed to environment (light) and to potentially pro-oxidative conditions. The retina has a very high O_2 consumption, and in photoreceptor and RPE cells, there is a high flux of polyunsaturated fatty acids (DHA and AA). To maintain homeostasis, the photoreceptors, RPE, and adjacent cells (e.g., Müller cells) are endowed with multiple mechanisms of protection against imbalances in potential cell damaging oxidative/nitro-oxidative conditions, which in addition to neurotrophins include antioxidant enzymes, antioxidant responsive DNA element-containing genes, and antioxidant vitamins. However, when lipid peroxidation overcomes cellular defenses, damaging consequences take place, including protein oxidation. Moreover, it has been shown that protein adducts of DHA oxidation accumulate in drusen and are detectable in the blood stream as a potential biomarker for AMD.[140,141]

Systemic alterations in the supply of DHA to the retina have been implicated in retinal degenerations.[26,46,49,50] Also, not yet understood are the molecular bases for the tenacious retention of DHA in retina, mainly photoreceptors, and brain. The discovery of the very-long-chain DHAs (e.g., 24:6 and 36:6) raises important questions regarding membrane organization and function and, more specifically, the lipid environment of rhodopsin in the disc membrane.[37] Candidate gene approaches for studying the genetic etiology of AMD have associated the gene encoding elongation of very-long-chain fatty acids-like4 (ELOVL4) with Stargardt's autosomal-dominant–like macular dystrophy.[142] The relationship of this gene with DHA elongation products and NPD1 remains to be studied.

Photoreceptor outer segment renewal in the context of well-regulated oxidative stress responses may elicit limited NPD1 synthesis. NPD1 cytoprotective signaling may be induced when oxidative stress surpasses a certain threshold. Thus, if the ROS and/or RPE are oxidatively challenged, some DHA may be used for NPD1 synthesis, if any.[121] DHA may be "used," therefore as a precursor of NPD1, but the retention/conservation is broken, requiring a dietary supply of additional DHA. This deficit may occur during aging and in retinal degenerative diseases. Studies that show beneficial effects of dietary DHA in AMD[143–150] and RP[151] support this interpretation.

The presence of additional bioactive docosanoids in retina and brain and the precise natures of the PLA_2(s) and 15-lipoxygenase(s) involved in docosanoid synthesis have not yet been fully characterized. Because a calcium ionophore activates DHA release and NPD1 synthesis in RPE cells,[88] the activity of a Ca^{2+}-dependent PLA_2 is implied; however, which specific PLA_2 is involved is still an open question. Identification of NPD1 catabolism pathways will provide insight into what "turns off" the NPD1-signaling pathways provided by this lipid mediator. Overall, defining selective DHA-delivery systems to the retina will be useful, and NPD1 and its cellular target(s) might enable the design of therapeutic approaches to manage RPE cytoprotection, and, in turn, enhance photoreceptor survival in aging and retinal degeneration. One additional relevant question is whether NPD1 or a synthetic active analogue may be therapeutically administered, or whether inducers of NPD1 synthesis could be used during early stages of retinal degeneration. For example, in experimental stroke, intravenous administration of serum albumin complexed with DHA attenuates infarct size and edema and promotes neurologic recovery after brain ischemia–reperfusion injury. In the ipsilateral brain region in this experimental model, the systemic supply of albumin-DHA promotes NPD1 synthesis concomitant with remarkable neuroprotection.[152] Thus, in acute conditions, where neuroprotection is desirable, supplying albumin-DHA may be a new option. For chronic progressive neurodegeneration, dietary supply of DHA has improved the condition of patients with RP.[151] In most forms of RP, the rod photoreceptors die first, followed later by the death of cone photoreceptors. It is not clear why cones die. Several mechanisms have been suggested, including rod-derived cone survival factors and oxidative stress.[128,129,153] Thus, retinal degeneration involves a wide variety of genes and environmental factors. The significance of antioxidants and lipid peroxidation on retinal degenerations is being uncovered. For example, antioxidants improve cone ERG and rod cell survival in animal models of RP.[154] The consequences of lipid peroxidation for RPE cell function and outer segment renewal have been addressed in this article. In addition, several recent studies have demonstrated perturbations of RPE cell function by lipid peroxidation.[155–158] Moreover, oxidized ROS promotes downregulation of factor H in the RPE.[120] Clinical trials are exploring nutritional supplementation, including omega-3 fatty acids and antioxidants.[159,160] Are there convergent, common mechanisms of RPE and photoreceptor cell death? Or are there multiple pathways to cell death? In this context, an emerging significance of DHA is that it gives rise to a prosurvival messenger, NPD1, that works on the RPE cells and, perhaps, on photoreceptors as well. Inflammatory signaling participates in different forms of neurodegeneration, such as AMD and AD. Thus, NPD1 could be part of an endogenous response aiming to counteract inflammatory cell-damaging actions of the highly vulnerable RPE and photoreceptor cells of the macula. For example, Aβ42 activates the alternative complement pathway and has been found to accumulate in drusen of AMD patients.[161,162] Moreover, NPD1

attenuates Aβ42 secretion and Aβ42-mediated cell injury in human neural cells.[95]

AMD pathogenesis, as discussed here, involves inflammation, the immune system, pathoangiogenesis (wet form of AMD) and apoptosis (dry form of AMD). An additional lipidomics approach covers arachidonic acid–derived lipoxins (e.g., lipoxin A_4 and lipoxin A_4 epimer 15). Lipoxins are potent mediators of the resolution phase of the inflammatory response and of dysfunctional immunity.[163,164] Thus lipoxin analogs, either alone or in combination with NPD1 (or its analogs), represent additional mechanisms to explore possible therapeutic avenues for AMD and other retinal degenerative diseases.

The emerging questions proposed in the opening statement of this article are beginning to elucidate the intimate, harmonious relationship of photoreceptor cells and RPE cells. Thus, this article describes studies demonstrating that the integrity of photoreceptor cells may be sustained at least in part by DHA made available for NPD1 biosynthesis from phagosomal DHA-containing phospholipids arriving to the RPE cells during outer segment renewal. Therefore, this process upregulates NPD1 survival signaling in response to oxidative- and nitrosylative-stress challenges. The experimental manipulation of the survival signaling pathway of NPD1 to slow or halt the initiation and progression of RP, AMD, and other neurodegenerative diseases is a near-term goal, with the aim of translating these concepts into the clinic.

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