Neuroprotectin D1-mediated anti-inflammatory and survival signaling in stroke, retinal degenerations, and Alzheimer's disease

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Abstract Docosahexaenoic acid (DHA), the main omega-3 fatty acid, is concentrated and avidly retained in membrane phospholipids of the nervous system. DHA is involved in brain and retina function, aging, and neurological and psychiatric/behavioral illnesses. Neuroprotectin D1 (NPD1), the first-identified stereoselective bioactive product of DHA, exerts neuroprotection in models of experimental stroke by down-regulating brain ischemia reperfusion (BIR)-induced leukocyte infiltration, proinflammatory signaling, and infarct size. Moreover, NPD1 inhibits cytokine-mediated cyclooxygenase-2 (COX-2) expression. Photoreceptor membranes display the highest content of DHA of any cell. Retinal pigment epithelial cells participate in the phagocytosis of the tips of photoreceptor cells (photoreceptor outer segment renewal). There is a DHA retrieval-intercellular mechanism between both types of cells that conserves this fatty acid during this process. NPD1 promotes homeostatic regulation of the integrity of these two cells, particularly during oxidative stress, and this protective signaling may be relevant in retinal degenerative diseases. Moreover, neurotrophins are NPD1-synthesis agonists, and NPD1 content is decreased in the CA1 region of the hippocampus of Alzheimer's patients. Overall, NPD1 promotes brain cell survival via the induction of antiapoptotic and neuroprotective gene-expression programs that suppress Aβ42 production and its neurotoxicity. Thus, NPD1 elicits potent cell-protective, anti-inflammatory, prosurvival repair signaling.—Bazan, N. G. **Neuroprotectin D1-mediated anti-inflammatory and survival signaling in stroke, retinal degenerations, and Alzheimer's disease. *J. Lipid Res.* 2009. S400–S405.**

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Cell function and integrity is sustained in the central nervous system (CNS) by complex intra- and intercellular signaling networks driven by modulators such as synaptic activity, neurotrophins, and gene programs. The molecular organization and functions of cellular membranes are pivotal in cell signaling. Docosahexaenoic acid (DHA), the main omega-3 fatty acid, is concentrated in membrane phospholipids of the CNS. Remarkably, DHA is avidly retained in the CNS. Prolonged periods of dietary deprivation are required to decrease DHA tissue content, which results in functional impairments (1, 2). These changes are restored by selective refeeding of omega-3 fatty acids. DHA is implicated in brain and retina function, aging, and neurological and psychiatric/behavioral illnesses. The discovery of neuroprotectin D1 (NPD1), the first identified bioactive derivative of DHA, has allowed for fundamental questions to be directly addressed concerning the biology of omega-3 fatty acids and DHA action mechanisms in experimental stroke and neurodegenerations. This review highlights NPD1's cell-protective, anti-inflammatory, prosurvival repair signaling.

NPD1 DOWN-REGULATES BIR-INDUCED LEUKOCYTE INFILTRATION, PROINFLAMMATORY SIGNALING, AND DAMAGE

To isolate and quantify unesterified (free) DHA and arachidonic acid in the CNS, we developed gradient-thickness thin-layer chromatography, which was reported in this journal (3). Using this approach, it was found that the unesterified (free) DHA pool increases during brain ischemia and intense synaptic activity, such as seizures, as a result of phos-

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Abbreviations: AD, Alzheimer's disease; BIR, brain ischemia reperfusion; CEX-1, cytokine exodus protein-1; COX-2, cyclooxygenase-2; CNS, central nervous system; DHA, docosahexaenoic acid; IL-1β, interleukin-1β; NPD1, neuroprotectin D1; PLA_2, phospholipase A_2; RPE, retinal pigment epithelium; TNFα, tumor necrosis factor α.

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pholipase A_2 (PLA_2) action. Although the CNS contains phospholipids richly endowed with docosahexaenoyl fatty acyl-chains, it displays an undetectable quantity of unesterified (free) DHA under basal, unstimulated conditions. This means that production of unesterified (free) DHA is tightly controlled by a PLA_2, by its removal (e.g., by reacylation) and by peroxidation. Free DHA that is incorporated into membrane phospholipids first becomes the substrate of docosahexaenoyl-CoA synthethase for its channeling through acyltransferases, which incorporate this fatty acid into phospholipids (4–6). During mouse brain ischemia reperfusion (BIR), NPD1 synthesis increases up to 8 h. To test the hypothesis that this rise reflects an endogenous protective response, NPD1 was administered directly into the cerebral ventricles and was found to counteract polymorphonuclear neutrophil infiltration, proinflammatory gene signaling, and infarct size (7). Likewise, continuous infusion of DHA into the ventricles during the initial 2 days of reperfusion inhibited polymorphonuclear neutrophil infiltration into the hippocampus and the neocortex. BIR-induced increases in NF-κB binding and up-regulation of cyclooxygenase-2 (COX-2) expression was also attenuated by DHA or NPD1 infusion. These results were mirrored by cultured human neural progenitor cells treated with the proinflammatory cytokine, interleukin-1β (IL-1β). NPD1's bioactivity is underscored by its ability to reduce the infarct volume by approximately half (7). To explore potential translational application of these findings, DHA was infused intravenously after 2 h of brain ischemia produced by transient occlusion of the right middle cerebral artery. As a result, neurobiological recovery was remarkably improved, brain damage was reduced, and NPD1 content was bolstered in the right side of the brain, implying that systemically administered DHA was taken up from the bloodstream by the brain and used for NPD1 synthesis. In turn, NPD1 exerted neuroprotection (8). Thus, if a brain injury is below a certain threshold, endogenous synthesis of NPD1 may be able to cope with damage initiation and prevent its progression. However, if the injury surpasses a certain threshold, NPD1 synthesis and its ability to elicit protection are overwhelmed.

The name "neuroprotectin D1" was suggested based upon its neuroprotective bioactivity in BIR and oxidatively stressed retinal pigment epithelial (RPE) cells, as well as its potent ability to inactivate proapoptotic and proinflammatory signaling. "D1" refers to it being the first identified mediator derived from DHA (9).

NPD1 SYNTHESIS PROMOTES RPE CELL AND PHOTORECEPTOR INTEGRITY

RPE cells, the most active phagocytes of the body, support photoreceptor cells by participating in the daily shedding, internalization, and degradation (phagocytosis) of photoreceptor outer segments (membrane discs) tips (**Fig. 1**). The photoreceptors and RPE are constantly subjected to environmental and intrinsic factors that potentially disrupt homeostasis: high oxygen tension, intense light during the day, and cell membranes with high content of polyunsatu-

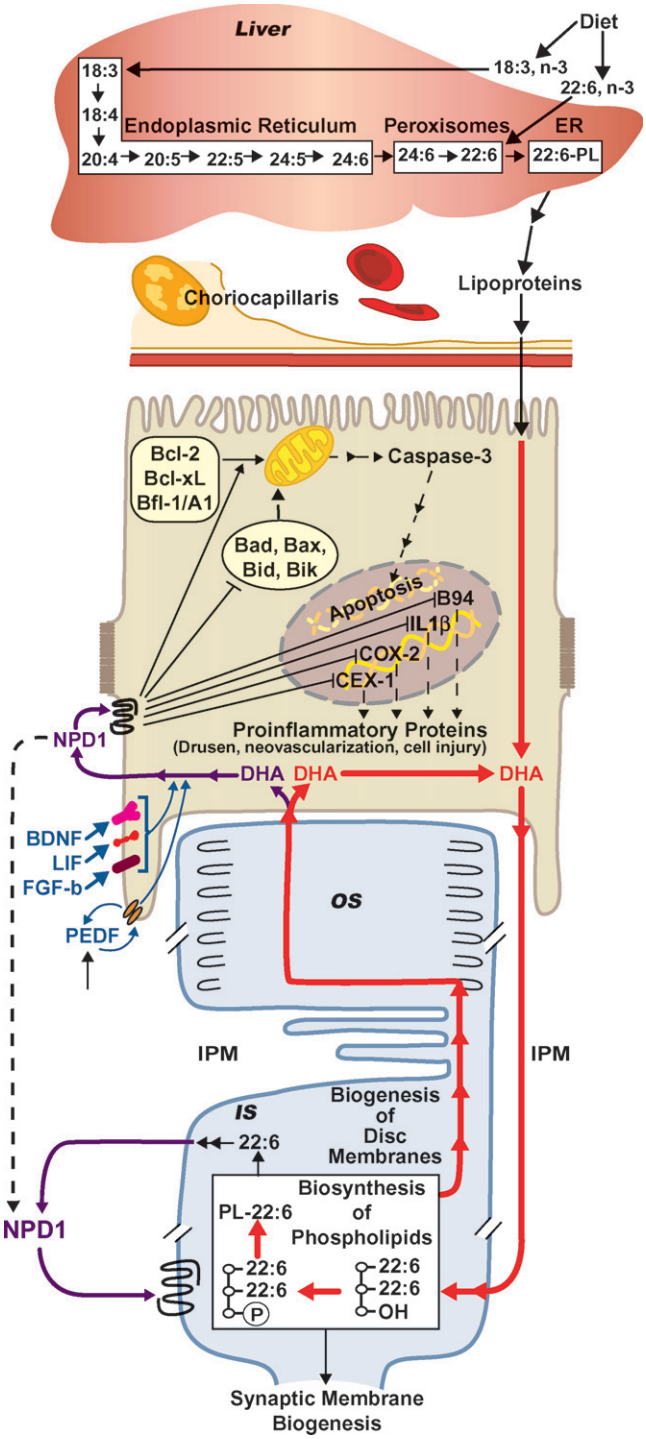

Fig. 1. Docosahexaenoic acid (DHA) trafficking. Retinal pigment epithelial (RPE) cell (green); photoreceptor inner segments (IS) (purple). Photoreceptor cell connects to RPE cell (red arrows), omega-3 fatty acid conservation route (short loop). DHA used for neuroprotectin D1 (NPD1) synthesis (blue arrow). Neurotrophins, persephin, BDNF (brain derived neurotrophic factor), LIF (leukemia inhibitory factor), FGF2 (fibroblast growth factor 2) or PEDF induce NPD1 synthesis and release. After RPE digestion, DHA is recycled to the IS through interphotoreceptor matrix (IPM). NPD1 recognizes a putative receptor, inhibits pro-inflammatory gene expression and fosters cell survival.

rated fatty acyl chains in their phospholipids (DHA and also 20:4,n-6-arachidonic acid). In models of retinal degeneration, lipid peroxidation, a potentially cell-damaging event, does occur in outer-segment discs (10). Moreover, in drusen (deposits of debris-like material that accumulate between the RPE cells and Bruch's membrane) from patients with age-related macular degeneration, DHA oxidation products can form protein adducts (11). 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 preserving cellular integrity. RPE cells respond to oxidative stress by activating the NPD1 synthesis (9). Previous studies have shown that the retina forms mono-, di-, and trihydroxy derivatives of DHA, and that lipoxygenase inhibitors block this synthesis, suggesting an enzymatic process of a lipoxygenase nature (12). At the time these lipoxygenase products were found, 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 name "docosanoids" was suggested) (12). Upon the advent of mediator lipidomics, oxygenation pathways were identified for the synthesis of the docosanoid NPD1 during BIR (7) and in RPE cells (9). NPD1 is formed from free (unesterified) DHA and released from membrane phospholipids by a PLA_2. 15-Lipoxygenase-1, IL-1β, oxidative stress, or the Ca^{2+} ionophore A23187 activates the synthesis of NPD1 (9), and, in turn, this lipid mediator acts in an autocrine fashion to act in a paracrine mode on photoreceptor cells and/or Müller cells (13, 14).

DHA SUPPLY AND RETENTION IN THE CNS: CELLULAR AND INTERCELLULAR TRAFFICKING OF DHA

The CNS displays an unusual ability to tenaciously retain DHA during prolonged periods of omega-3 fatty acid deprivation (1, 13, 15). In photoreceptor and synapse biogenesis in mouse postnatal development, the omega-3 supply shows that dietary linolenic acid is actively elongated and desaturated in the liver before its distribution through the blood stream to the retina and brain (16).

Specific intercellular trafficking assures the retention/conservation of DHA (17). The liver takes up DHA and linolenic acid (18:3,n-3) from the diet and elongates and desaturates linolenic acid to DHA; then DHA is esterified into phospholipids, secreted as lipoproteins (18), and delivered to the brain through the neurovascular unit (blood brain barrier) and to the RPE or retina through the choriocapillaris or microcirculation, respectively. A remaining question about DHA uptake is whether there is a receptor-like mechanism present. It is remarkable that lipoproteins with DHA-acylated phospholipids selectively deliver DHA to the RPE and brain, and to a lesser extent the testes and other tissues. Thus, there is a specialized DHA uptake by the CNS. The best-characterized intercellular DHA trafficking, the one linking the liver to the RPE cells and then the photoreceptors, is shown in Fig. 1. The short loop recycles DHA from the RPE to the photoreceptor outer segment via the inner segment. DHA in the RPE (delivered by the long loop or taken up during shedding from the tip of the photoreceptor and phagocytosis) goes back through the interphotoreceptor matrix to the inner segment where phospholipids are synthesized, including those containing DHA. Then DHA returns to the outer segment through the connecting cilium for biogenesis of disc membranes. RPE cells biosynthesize NPD1, which in turn is released and, in an autocrine fashion, elicits its action through a cell-surface receptor.

It has been shown that astrocytes mediate bloodstream uptake of DHA and that these cells are intimately related with neurons, mainly at synapses (13). It is therefore conceivable that brain DHA conservation mechanisms may involve astrocyte/neuronal relationships.

Photoreceptor outer segment phagocytosis by the RPE cells results in a massive daily supply of phospholipids highly enriched in DHA. The DHA and longer-chain omega-3 fatty acids are retrieved by the inner segment of the photoreceptor through the interphotoreceptor matrix, the short loop of conservation (DHA retrieval route) of omega-3s in photoreceptors (Fig. 1). Because 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 do not fully understand the elements operating this short loop.

The constant rebuilding of photoreceptors requires DHA as the molecular building block. Photoreceptors shed outer segment tips, which are then phagocytized by RPE cells in a daily, intermittent, and circadian fashion in mammals (19). 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. In contrast, 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.

NEUROTROPHINS INDUCE SYNTHESIS AND RELEASE OF NPD1

To further highlight the potential significance of NPD1 in retinal degenerations, neurotrophins, which are important in photoreceptor survival, trigger synthesis and release of NPD1 from RPE cells. Pigment epithelium derived factor, a member of the serine protease inhibitor (serpin) family, is the most potent stimulator of NPD1 synthesis (20). Neurotrophins elicit concentration-dependent increases in NPD1 release only on the apical side of the RPE cell (21). This side of the cell faces the photoreceptor cells (Fig. 1).

NPD1 may be significant in retinal degenerations. Apoptotic death of photoreceptor cells is the hallmark of retinal degenerative diseases. In retinitis pigmentosa (a heterogeneous group of inherited blinding diseases) and age-related macular degeneration (the leading cause of vision loss in people over 65), photoreceptors die. Retinal degenerations

involve multiple cell signaling pathways and more than 150 mutations of photoreceptor-specific proteins and other proteins. Oxidative and nitrosylative stress are enhanced, and mitochondrial function is compromised. Initiation and progression of retinal degenerations involve an unsuccessful inflammatory response. Once RPE cells die, the underlying photoreceptor cells then succumb (22). Initial clinical evidence of retinal degenerations precedes, in several instances by many years, extensive photoreceptor cell death. In several forms of retinitis pigmentosa (23, 24) and in Usher's syndrome (25, 26), a decrease of DHA content in blood takes place. An implication of these findings is that diminished 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 retinitis pigmentosa display decreased amounts of DHA in photoreceptors (15). This 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 (15). 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. Therefore, NPD1 signaling may be explored as a possible therapeutic intervention.

AGING AND ALZHEIMER'S

Deficiency in DHA is associated with cognitive decline (27) and has also been implied in Alzheimer's disease (AD). Proinflammatory gene expression profiles in human brain cells in culture, after exposure to Aβ42, DHA, and NPD1, show that inducible proinflammatory cytokines IL-1β and cytokine exodus protein-1 (CEX-1), prostaglandin synthase COX-2, the tumor necrosis factor α (TNFα)-inducible proinflammatory element B94 (28), and TNFα

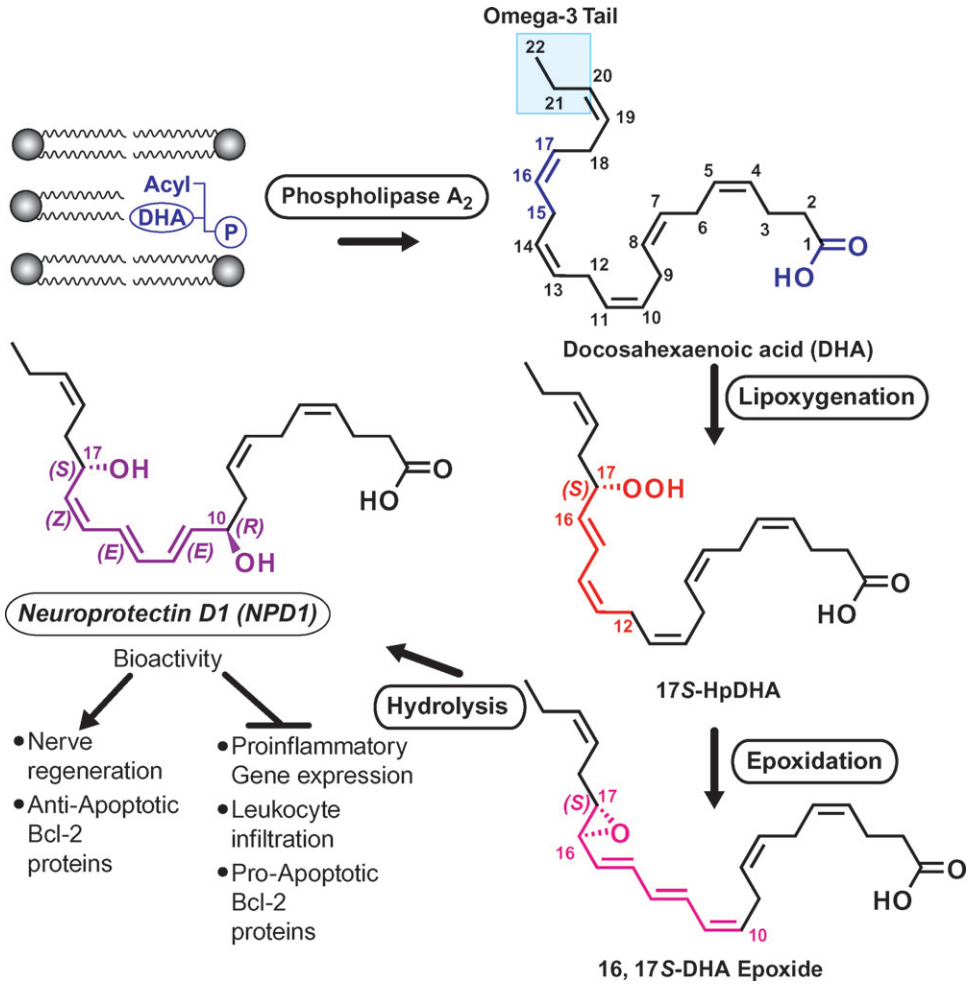

Fig. 2. NPD1 biosynthesis. A membrane phospholipid containing a docosahexaenoyl chain in *sn*-2 is hydrolyzed by a phospholipase A_2 (PLA_2), generating a free (unesterified) DHA. The carbons of DHA are numbered and the omega-3 (n-3) tail highlighted. Lipoxygenation is then followed by epoxidation and hydrolysis to generate NPD1.

expression are up-regulated by Aβ42 and down-regulated by DHA or NPD1. The expression of these proinflammatory genes is up-regulated in the brains of AD patients (28).

Overall, Aβ42 markedly enhances a complex proapoptotic gene-expression program that includes the proapoptotic Bax and Bik proteins, proteins that clearly participate in apoptosis signaling in other cells. DHA and NPD1 each show enhanced expression of Bcl-xl, Bcl-2, and Bfl-1(A1), antiapoptotic members of the Bcl-2 gene family, and relative downregulation of Bax and Bik. Bax and Bik are up-regulated in Aβ42-treated cells over age-matched control cells and do not change with DHA or NPD1. The antiapoptotic Bcl-2 family member Bfl-1(A1) are up-regulated by DHA and NPD1, and Bfl-1(A1) reaches the highest significance of any up-regulated gene in NPD1-treated human neural cells (27). Subtraction of DHA from NPD1 DNA-array signals reveals an additional 56 genes, which are up-regulated 2-fold or greater over controls (27).

To explore the possible significance of NPD1, the levels of this lipid mediator were quantified in AD hippocampal CA1 region, an area of the brain involved in memory and targeted by neuropathology in early stages of AD. According to the plaque and tangle count, all except one AD brain sample analyzed were from AD patients at a moderate stage of disease development (27). Unesterified DHA pool sizes in controls were 2-fold higher than in AD CA1, and NPD1 levels in AD were approximately one-twentieth of those in age-matched controls (27). Depending on brain region and stage of disease development, the population of neurons remaining in AD brain has been estimated to range from 59% to 77% to 89% of age-matched controls for the same region. Thus, the loss of 11–41% of neurons is insufficient to account for the observed 20-fold reduction in the NPD1 content in AD CA1. These observations indicate that, despite modestly decreased availability of unesterified DHA, NPD1 levels are markedly reduced in AD CA1. As a result, NPD1's neuroprotective bioactivity during brain cell degeneration may be lost.

In the same human CA1 hippocampal area, $cPLA_2$ abundance was increased, while 15-LOX-1 was decreased almost 2-fold. Decreased abundance of NPD1 in AD CA1 may be explained, at least in part, by a disruption in the expression and regulation of the PLA_2 and/or 15-LOX-1 enzymes for NPD1 biosynthesis (27).

Mediator lipidomic-based analysis has allowed for initiation of decoding CNS omega-3 fatty acids, highlighted by the discovery of NPD1 in the CNS (9), the defining of its bioactivity, and furthering of our understanding of the biology of neuro-inflammation and cell survival.

NPD1 promotes CNS cell homeostasis through modulation of multiple signaling pathways, and it down-regulates the expression of proinflammatory genes (9). Also, in ischemia-reperfusion-injured hippocampus, as well as in neural progenitor cells stimulated by IL-1β, NPD1 inhibits COX-2 induction (7, 9). In BIR, NPD1 decreases infarct size and inhibits polymorphonuclear leukocyte infiltration (7). Moreover, in human brain progenitor cells in culture (27), other proinflammatory genes targeted by NPD1 are IL-1β, CEX-1, and TNFα-inducible proinflammatory element (B94, TNFAIP2). **Fig. 2** 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 age-related macular degeneration, and in proliferative vitreoretinopathy, which occurs in diabetic retinopathy.

Excessive oxidative stress turns on multiple signaling pathways in the CNS that, in turn, participate in the pathophysiology of degenerative disease and lead to cell damage and, eventually, cell death. The Bcl-2 family of proteins regulates the initiation and amplification of premitochondrial events of apoptosis (1). NPD1 is actively formed in response to oxidative stress and, in addition to down-regultion of proinflammatory genes, modulates Bcl-2 protein expression to counteract oxidative stress consequences (9, 27). Neurotrophins induce NPD1 synthesis apparently in an effort to offset the injury and/or proinflammatory response and to restore homeostasis (21).

The presence of additional bioactive docosanoids in the CNS, and the precise natures of the PLA_2(s) and 15-lipoxygenase(s) involved in docosanoid synthesis, needs to be fully characterized. 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 CNS will be useful, and NPD1 and its cellular target(s) might enable the design of therapeutic approaches to foster cell homeostasis, and, in turn, enhance survival in aging and retinal degeneration.

The experimental manipulation of the survival-signaling pathway of NPD1 to slow or halt the initiation and progression of neurodegenerative diseases is a near-term goal, with the aim of translating these concepts into the clinic.

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