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Synaptic Signaling by Lipids in the Life and Death of Neurons

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Abstract

Synaptic activity promotes the regulated formation of lipid messengers through phospholipase-mediated cleavage of specific phospholipid reservoirs from membranes. Multiple effectors trigger the formation of lipid messengers, including neurotransmitters, membrane depolarization, ion channels, cytokines, and neurotrophic factors. Lipid messengers in turn modulate and interact with other signaling cascades, contributing to the development, differentiation, function (e.g., long-term potentiation [LTP] and memory), protection, and repair of cells in the nervous system. These relationships with other signaling cascades remain largely to be investigated. Oxidative stress disrupts lipid signaling, enhances lipid peroxidation, and initiates and propagates neurodegeneration. There is growing evidence that lipid messengers participate in the extensive interactions among neurons, astrocytes, oligodendrocytes, microglia, cells of the microvasculature, and other cells. This article provides an example of how signaling by lipids regulates critical events essential for neuronal survival and reviews the recent identification of a novel endogenous neuroprotective signaling pathway involving a docosahexaenoic acid-derived mediator.

Index Entries: Docosahexaenoic acid; ischemia–reperfusion; neurodegeneration; neuroprotectin D1; neuroprotection; oxidative stress; retinal degeneration; retinal pigment epithelial cells; stroke.

Oxidative Stress and Proinflammatory Signaling

Proinflammatory signaling is enhanced in the early stages of neurodegenerative disease as well as brain ischemia–reperfusion as a result of a convergence of factors driven initially by redox homeostasis disturbances, mitochondrial dysfunction, and overall peroxidative stress signaling. In this article, we focus on experimental stroke and retinal pigment epithelial cells because of the latter's involvement in retinal degenerations.

In stroke, the landmark is a coordination of oxygen shortage followed by the oxygen surge

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of reperfusion. The target of several of these factors is the neurovascular unit, which is comprised of neuronal processes in close contact with pericytes and astrocyte end-feet functionally and organizationally coupled to endothelial cells and smooth muscle cells *(1-3)*. Cells from the blood—particularly polymorphonuclear leukocytes (PMNs), platelets, and monocytes—activate endothelial cell signaling, disrupt endothelial tight junctions, and lead to leukocyte infiltration. On the other end, neurons and glial cells are also very sensitive to these converging factors, which are sensed by increasing calcium and sodium fluxes, glutamate efflux, and ionic imbalances. Overall, oxidative and nitrosative stress is set in motion that in turn impairs cell function in many ways. Mitochondria are very sensitive to these events, particularly because one of the early modifications that occurs is an imbalance in the expression and interactions of pro- and antiapoptotic members of the Bcl-2 family of proteins, leading to opening of the transition pore, cytochrome c release, and caspase-3 activation. Oxidative and nitrosative stress also targets proteins, nucleic acids, and the polyunsaturated fatty acyl chains of membrane phospholipids, docosahexaenoic acid (DHA), and arachidonic acid, resulting in increasingly active proinflammatory signaling. The outcome—various degrees of cell damage or even cell death—depends on counteracting cellular responses that try to overcome cell-damaging events *(4)*.

The anti-inflammatory signaling response is complex and may lead to neuroprotection. Our laboratory has devoted considerable effort to unraveling specific, beneficial signals under these conditions. Among the many changing lipids—mainly polyunsaturated fatty acids and their peroxidation products during ischemia-reperfusion—we have very recently identified stereospecific DHA derivatives that are synthesized by an enzyme-catalyzed DHA–oxygenation pathway. DHA is enriched with brain and retinal phospholipids and has been implicated in memory, excitable membrane function, photoreceptor cell biogenesis and function, and neuroprotection.

In the early 1980s, an enzyme-mediated docosanoid formation in the retina was described *(5)*, and it was later proposed that those messengers might be neuroprotective *(6)*; however, until recently, the precise stereochemistry and physiological properties of these docosanoids had not been determined. Tandem LC-PDA-ESI-MS-MS–based lipidomic analysis in combination with mouse brain ischemia–reperfusion demonstrated that free docosahexaenoic acid released in the brain leads to the synthesis of stereospecific messengers through oxygenation pathways. The newly discovered messenger, 10,17*S*-docosatriene (neuroprotectin D1 [NPD1]), counteracts leukocyte infiltration, nuclear factor kappa B (NF-κB) activation, and proinflammatory gene expression in brain ischemia–reperfusion. These findings have several implications in the understanding of how the brain counteracts disturbances in redox homeostasis, mitochondrial dysfunction, oxidative stress, and proinflammatory conditions. It is to be hoped that these newly discovered pathways will allow for the design of novel therapeutic strategies for neurodegenerative diseases. Moreover, exploration of the new DHA-signaling pathways may lead to the clarification of clinically important issues relevant to stroke, spinal cord injury, aging, and diseases that include neuroinflammatory components. The specificity and potency of this novel docosanoid indicate a potentially important target for therapeutic neuroprotection. This opening lecture summarizes recent studies on DHA endogenous neuroprotective signaling.

Brain Ischemia–Reperfusion Triggers the Release From Membrane Phospholipids of DHA and Its Subsequent Oxygenation, Leading to the Synthesis of NPD1

The experimental model of stroke in several of our studies was 1-h right middle cerebral artery occlusion in mice followed by reperfusion, which results in active release of free DHA

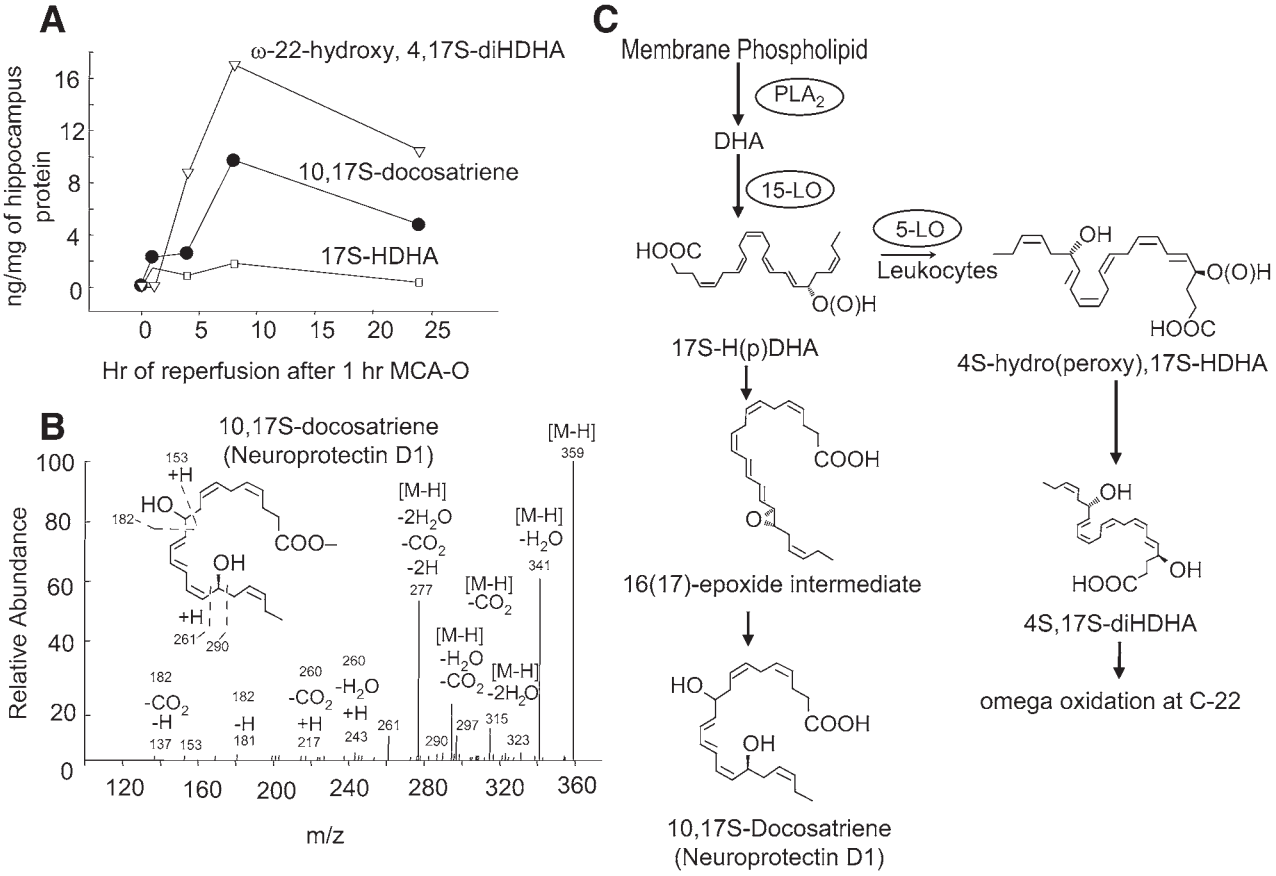

Fig. 1. Synthesis and metabolism of docosanoids in the ipsilateral mouse hippocampus during reperfusion following transient ischemia and structural elucidation of docosanoids by lipidomic analysis by LC-PDA-MS-MS. **(A)** Time course of accumulation of 17*S*-HDHA, 10,17*S*-docosatriene (NPD1), and omega-22-hydroxy-4,17*S*-di-HDHA in the ipsilateral hippocampus during reperfusion after 1 h of middle cerebral artery occlusion (MCAO). There was an enhanced accumulation of omega-22-hydroxy-4,17S-di-HDHA, an oxidative pathway product, and 10,17*S*-docosatriene (NPD1). **(B)** MS-MS spectrum for 10,17*S*-docosatriene (NPD1). **(C)** Proposed biosynthetic pathways for 10,17*S*-docosatriene (NPD1). The stereochemistry for compounds in both pathways is based on the biogenic total synthesis, lipidomic analysis, and alcohol-trapping profiles *(11,12)*. Figure modified from Marcheselli et al., 2003 *(13)*, and used here with permission.

from brain membrane phospholipids *(7-10)*. This model of transient focal ischemia produces damage in part of the hippocampus, a brain region that displays vulnerability to ischemic stroke and other neurological diseases. The time course of formation of DHA–oxygenation metabolites in the ipsilateral hippocampus during ischemia–reperfusion showed the presence of 17-hydroperoxy-DHA, formed through a 15-lipoxygenase-like action on DHA, and 10,17*S*-docosatriene (NPD1), which accumulated for up to 8 h during reperfusion. The MS-MS spectrum of NPD1 (Fig. 1) corresponded to a dihydroxy-containing DHA with prominent fragment ions at m/z 359 (M-H; Fig. 1B). There were also 323 (M-H-$2H_2O$), 315 (M-H-CO_2), 297 (M-H-H_2O-

CO_2), and 277 (M-H-2H_2O-CO_2-2H). In addition, there was a time-dependent formation of the carbon 22-omega hydroxylation product, 4,17-di-HDHA (Fig. 1A,C), as determined by analysis (at m/z 375) of the MS-MS spectrum *(13)*.

Because aspirin is often used prophylactically as well as therapeutically to ameliorate cerebrovascular disease, we also assayed brain biosynthesis of DHA messengers in the presence of aspirin in vivo. In nonneural tissues, aspirin triggers the biosynthesis of anti-inflammatory lipid mediators *(12,14)*. In particular, we examined the formation of the DHA-derived docosanoids in the presence of aspirin during reperfusion after ischemia. Aspirin caused a shift away from the products generated from endogenous sources of DHA in the absence of aspirin toward products that include the novel 17*R*-series resolvins, in particular 7,17*R*-di-HDHA and 7,8,17*R*-tri-HDHA, which appeared at the earliest time intervals *(13)*. There was a marked accumulation in 17*R*-HDHA, which has been shown recently to be a product of aspirin-acetylated cyclooxygenase (COX)-2 *(14)*.

PMN infiltration is a major event that promotes brain damage in ischemia–reperfusion. PMNs release myeloperoxidase, which generates reactive oxygen species as well as other cell-damaging mediators; these include matrix metalloproteinases, which contribute to the disorganization of the extracellular matrix *(15-18)*. PMN infiltration in itself is a complex process modulated by coordinate expression of adhesion and signaling molecules *(19)*. Messengers derived from DHA are now known to inhibit PMN infiltration outside the central nervous system in the air-pouch model *(12, 14,19)*.

We asked whether NPD1 exerted bioactivity to counteract PMN infiltration induced by cerebral ischemia–reperfusion. The docosanoid was continuously infused into the third ventricle during 48 h of reperfusion, as shown in Fig. 2. We used immunostaining of myeloperoxidase to detect PMN leukocytes *(15–18)* and demonstrated a remarkable inhibition of PMN infiltration by NPD1 on the ipsilateral side of the brain (Fig. 2). Positive staining of myeloperoxidase produces a characteristic granular appearance of the cytoplasm, which in the case of NPD1 treatment was found only in the focal ischemic side (Fig. 2A). We also quantified myeloperoxidase enzyme activity in both hippocampus and neocortex dissected from the focal stroke (ipsilateral) side, as well as corresponding tissues from the contralateral side. Both the free acid and carboxymethyl ester of NPD1 inhibited the appearance of myeloperoxidase-positive staining in both the ipsilateral hippocampus and ipsilateral neocortex; however, the contralateral brain regions did not display increased enzyme activity, nor did they show a positive immunostaining for leukocytes. When we infused DHA, there was also inhibition of PMN infiltration, mostly in the hippocampus. This means that exogenous DHA is being used as the precursor for the synthesis of NPD1, a hypothesis that is supported by our observation that when DHA is administered during 6-h reperfusion after 1-h ischemia, NPD1 was clearly generated at higher levels in the hippocampus than in vehicle-treated animals *(13)*.

Neuroprotectin D1 Decreases Stroke Infarct Size

To determine bioactivity of NPD1 in brain during focal ischemic stroke, we implanted Alzet mini-pumps into the third ventricle to deliver the lipid messenger over the 48-h reperfusion following 1 h of ischemia. Serial coronal brain sections were made and incubated with triphenyltetrazolium chloride (TTC; Fig. 2C), which is actively taken up into mitochondria and produces a reddish stain. In sections stained with TTC, the focal infarcted right side of the brain, resulting from ischemic injury, is characterized by colorless areas that indicate severe mitochondrial and cell damage *(15)*. In

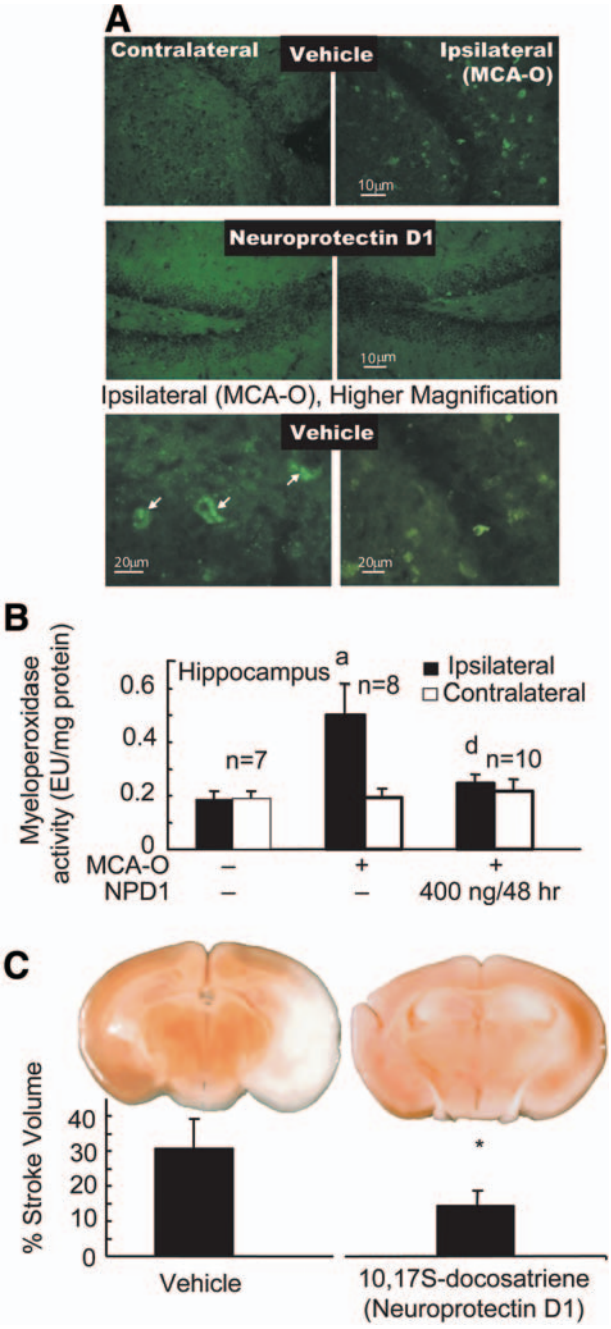

Fig. 2. Neuroprotection by 10,17*S*-docosatriene (neuroprotectin D1) in transient focal stroke in mice. **(A)** Inhibition of leukocyte infiltration by 10,17*S*-docosatriene (NPD1) in mouse hippocampus and neocortex after 1-h middle cerebral artery occlusion (MCAO) and 48 h of reperfusion. Data shown represent the mean ± SD of the indicated number (n) of individual mice. The immunocytochemical visualization of polymorphonuclear leukocytes (green fluorescence) exhibits myeloperoxidase immunoreactivity. The ipsilateral brain areas (ischemic area) show positive green fluorescence (vehicle) compared with contralateral tissue. The areas shown correspond to the dentate gyrus of the hippocampus. 10,17*S*-docosatriene (NPD1)-infused animals (400 ng over 48 h) exhibited a large reduction in green fluorescence. A higher magnification of the ipsilateral stroke area from vehicle-treated animals is also depicted, with the cytoplasmic granular appearance of immunoreactivity that is characteristic of PMN leukocytes. **(B)** Cerebroventricular perfusion of 400 ng 10,17*S*-docosatriene (NPD1) over 48 h resulted in 80% inhibition of ipsilateral myeloperoxidase activity compared with vehicle-treated animals. **(C)** Selected sections from different mice. Sections are stained with TTC. Infusion was performed using an Alzet mini-pump implanted into the third ventricle. Percentage of the TTC-stained area with respect to total brain coronal area. Bars are the mean ± SD from six animals each. *$p < 0.001629$ (Student's *t*-test).

vehicle-infused mice, this focal infarcted volume represented approx 30% of the total volume, whereas in NPD1-infused mice, the damaged area was reduced to less than 15% of stroke volume (Fig. 2C).

Brain Ischemia-Induced Up-Regulation of COX-2 Expression and NF-κB Activation Is Down-Regulated by NPD1

Proinflammatory gene expression plays a key role in the promotion of ischemic brain injury. NF-κB is activated in ischemia *(20,21)*, as is COX-2 expression, which in turn generates PGH2, the substrate for prostaglandin synthetases and a contributor to oxidative stress *(22,23)*. To determine whether proinflammatory gene expression is also modulated by the docosanoid mediator NPD1, we measured the

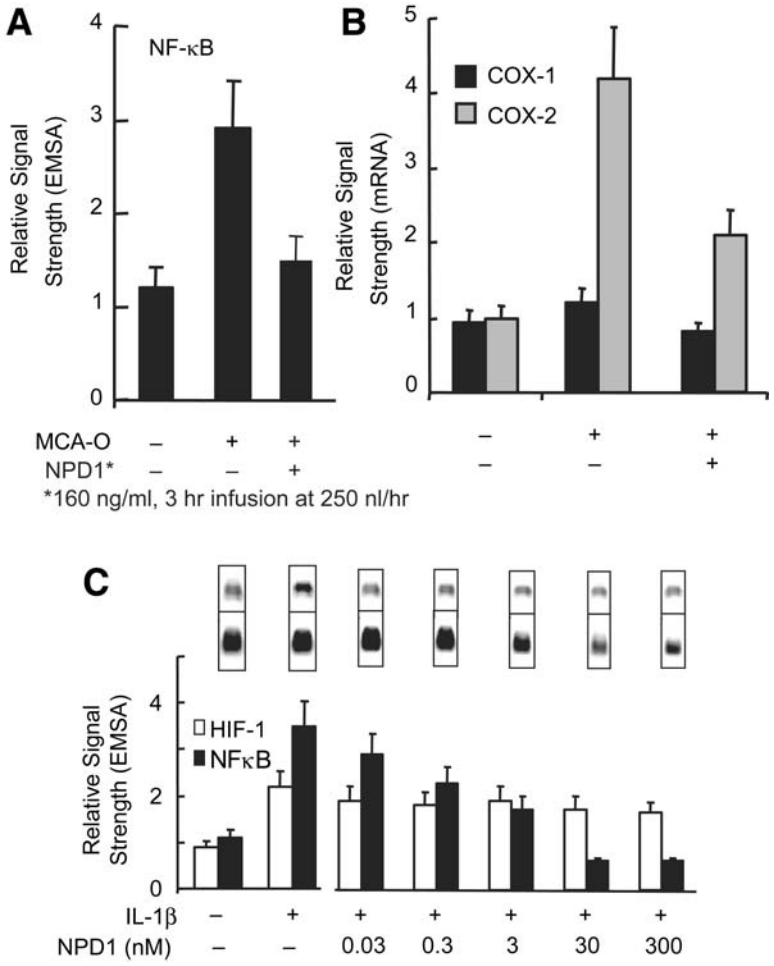

Fig. 3. 10,17*S*-docosatriene (NPD1) inhibited middle cerebral artery occlusion (MCAO)-induced and interleukin 1β–induced NF-κB activation and COX-2 expression. **(A,B)** Mouse hippocampus after 1 h MCAO followed by 2 h reperfusion. Vehicle or 10,17*S*-docosatriene (NPD1; 0.16 mg/mL) was infused into the third ventricle for 3 h at 0.25 mL/h. **(A)** Enhanced NF-κB DNA binding activity, determined via electrophoretic mobility-shift assay (EMSA) after MCAO, was inhibited by 10,17*S*-docosatriene or DHA. **(B)** COX-2 expression was greatly increased by MCAO reperfusion in the hippocampus, and 10,17*S*-docosatriene or DHA inhibited this enhanced expression. **(C)** Interleukin 1β–induced NF-κB activation, but not interleukin 1β–induced HIF-1α activation, was inhibited by 10-17*S*-docosatriene.

DNA-binding activity of NF-κB and other transcription factors as well as COX-1 and COX-2 expression in the hippocampus after experimental ischemia. NF-κB was increased over twofold in the ipsilateral hippocampus after ischemia, and the infusion of either DHA or NPD1 inhibited ischemia–reperfusion-induced NF-κB activation by 28 and 48%, respectively (Fig. 3). This effect was selective, because DNA binding for AP-1, HIF-1α, and STAT-1—all of which were slightly enhanced by ischemia—was unaffected by lipid messengers, except for a small effect of NPD1 on STAT-1 DNA binding *(13)*. To determine COX-1 and COX-2 expression, we measured messenger RNA abundance in the ipsilateral hippocampus and found a threefold increase of COX-2 (Fig. 3). The infusion of the precursor DHA or its prod-

uct NPD1 inhibited COX-2 expression by 14 and 52%, respectively. The infusion of NPD1 also decreased COX-1 expression by 19%.

Oxidative Stress-Triggered Apoptosis in Human Retinal Pigment Epithelial Cells Is Inhibited by NPD1

Retinal pigment epithelial (RPE) cells, derived from the neuroectoderm, form a monolayer above the tips of the photoreceptor outer segments. RPE cellular integrity is essential for photoreceptor cell survival. Stimulation with calcium ionophore evoked sustained release of endogenous DHA from membrane phospholipids and subsequent NPD1 synthesis (Fig. 4A). We used a combination of tumor necrosis factor (TNF)-α with H_2O_2 to produce oxidative stress–induced apoptosis in ARPE-19 cells.

Moreover, serum starvation made ARPE-19 cells more sensitive to oxidative stress–triggered apoptosis induced by TNF-α /H_2O_2. Serum starvation triggers the apoptotic cascade involving the activation of the caspase-3 pathway, as demonstrated in bovine RPE cells in culture *(24)*. We grew cells in culture for 72 h in the presence of bovine serum albumin (BSA) with or without DHA, and found that endogenous NPD1 formation was enhanced by BSA-DHA (Fig. 4B). When these cells were preincubated with BSA-DHA, serum-starved for 1 h, then exposed for 14 h to TNF-α/H_2O_2 during serum starvation, the number of Hoechst-positive cells was greatly reduced (Fig. 4B), suggesting that NPD1 synthesized in the ARPE-19 cells acted early in the apoptotic cascade. When we added NPD1 (50 n*M*) at the same time as TNF-α/H_2O_2, oxidative stress–induced apoptosis was inhibited (Fig. 4C). When the TNF-α concentration (10 ng/mL) was maintained and H_2O_2 was decreased from 800 to 400 m*M*, even fewer Hoechst-positive cells were seen. NPD1 (50 n*M*) almost completely inhibited the oxidative stress caused by the lower H_2O_2 concentration. Neither PGE2, LTB4, nor 20-OH-LTB4 was able to elicit the degree of inhibition attributed to NPD1, although LTB4 was partially inhibitory. We also compared the effect of added arachidonic acid (50 n*M*) or DHA (50 n*M*) on oxidative stress-induced apoptosis. Arachidonic acid was partially effective, but DHA proved to be a very potent inhibitor, and because we suspected that the added DHA might be involved in NPD1 formation, we conducted the following experiment. A time course of formation of NPD1 in cultures treated with DHA under conditions similar to those depicted in Fig. 4 showed synthesis of NPD1 as a function of incubation time: free DHA content rose as a function of incubation time up to 2 h, then decreased. These changes in DHA pool size during serum starvation contrasted with those seen without serum starvation *(25)*.

Oxidative stress triggers DNA fragmentation and RPE cell apoptosis, and NPD1 inhibits these events. We directly assessed these mechanisms using DNA fragmentation by differential sedimentation after [^{3}H]thymidine labeling and enzyme-linked immunosorbent assay detection of mono- and oligonucleosomes, and found that TNF-α/H_2O_2 produced a marked DNA degradation as assessed by both of these methods. Moreover, direct assessment using [^{3}H]thymidine showed that NPD1 inhibited DNA fragmentation. In addition, NPD1 inhibited the TNF-α/H_2O_2-induced accumulation of mono- and oligonucleosomes—further evidence that oxidative stress-induced DNA breakdown is modulated by the DHA-derived mediator *(25)*.

NPD1 Stimulated Antiapoptotic Bcl-2 Protein Expression and Decreased Proapoptotic Protein Expression During Oxidative Stress

Bcl-2 family proteins participate in the initiation and amplification of premitochondrial events in the apoptosis cascade *(26)*. We investigated their participation in TNF-α/H_2O_2-induced ARPE-19 cell death and explored the possibility that they are also a target for NPD1

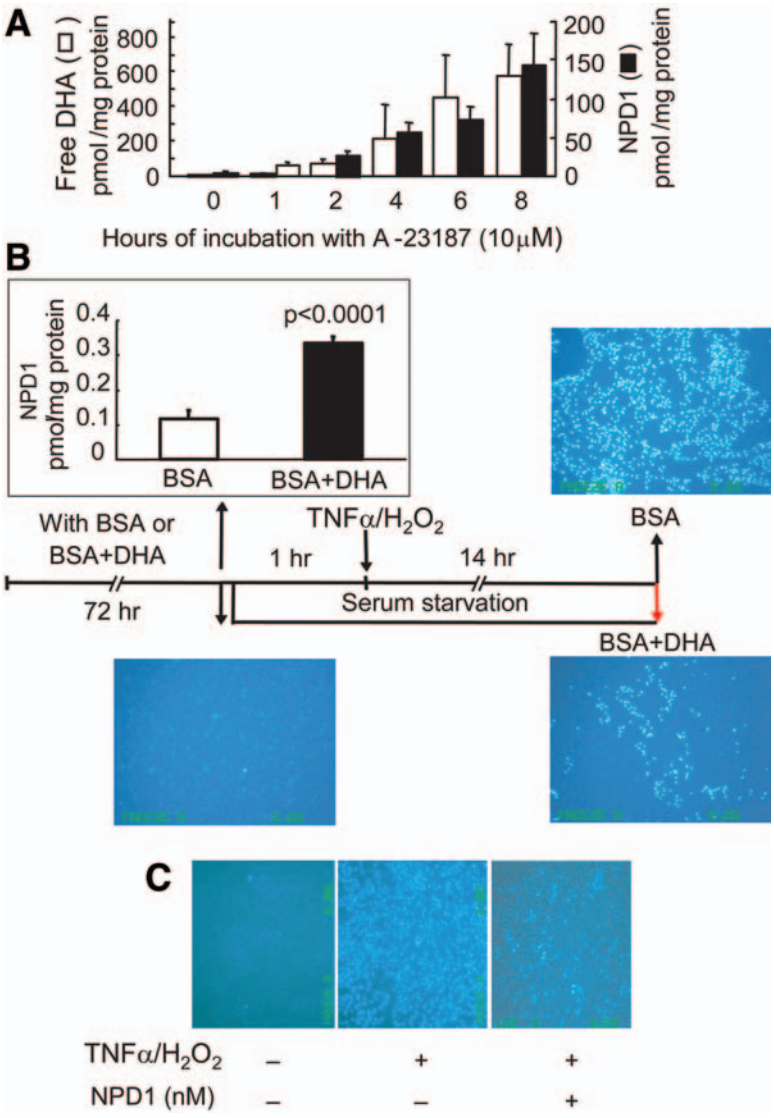

Fig. 4. NPD1 attenuates oxidative stress-induced apoptosis in human retinal pigment epithelial cells. **(A)** Calcium ionophore A-23187 promoted the release of free DHA and the formation of NPD1 as a function of incubation time (no exogenous DHA was added). **(B)** BSA-DHA enhanced NPD1 synthesis and led to decreased oxidative stress-induced apoptosis. After plating, ARPE-19 cells were incubated for 72 h in the presence of either BSA (3.35 μ*M*) or BSA plus DHA (6.7 m*M*). The cells were then serum-starved for 1 h, and oxidative stress was triggered by TNF-α /H_2O_2 (14 h). Cells pretreated with BSA plus DHA yielded marked attenuation of Hoechst-positive cells. **(C)** Added NPD1 (50 n*M*) attenuated oxidative stress-induced Hoechst-positive staining. After becoming confluent (72 h), cells were serum-starved for 8 h and TNF-α /H_2O_2 was added; the cells were then further incubated for 14 h, then stained with Hoechst reagent.

action. Added NPD1 inhibited apoptosis at two different concentrations of H_2O_2 (400 or 800 m*M*) plus TNF-α (10 n*M*). The antiapoptotic protein Bcl-xL was enhanced by TNF-α/H_2O_2 (800 m*M*), whereas Bcl-2 was unaffected (Fig. 5). NPD1 greatly activated expression of Bcl-xL and Bcl-2. The proapoptotic proteins Bax and Bad were up-regulated by TNF-α/H_2O_2 (800 m*M*). A much lower Bax up-regulation was observed with 400 m*M* H_2O_2 plus TNF-α.

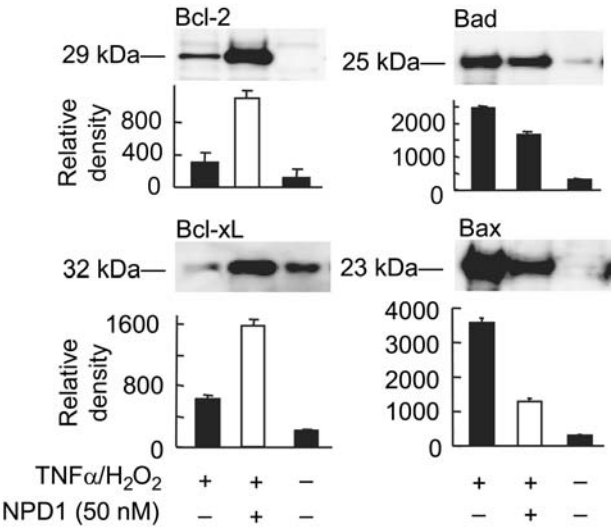

Fig. 5. Expression of selected Bcl-2 family proteins in ARPE-19 cells: NPD1 up-regulated antiapoptotic proteins and down-regulated proapoptotic protein expression. Cells were grown for 72 h after plating, placed in serum-free medium for 1 h, then incubated with TNF-α/H_2O_2 for 6 h. Protein expression and Western blot analyses were performed. Data represent four independent experiments with triplicate samples in each case.

However, Bad showed similar responses to both concentrations of H_2O_2 in combination with TNF-α. In addition, H_2O_2 or TNF-α alone somewhat increased Bax and Bad basal levels. NPD1 decreased Bax by 65% using 800 m*M* H_2O_2 along with TNF-α. However, the inhibition by NPD1 of oxidative stress-increased Bad was smaller (52%) under similar conditions *(25)*.

Our findings have several implications related to the understanding of how NPD1 endogenously synthesized in brain modulates ischemia–reperfusion injury responses, and to how this DHA-derived mediator, also synthesized by neuroepithelium-derived RPE cells, modulates signaling pathways that promote cell survival. These newly uncovered DHA-signaling pathways may lead to answers to clinically important questions regarding the mechanisms in stroke, traumatic head injury, spinal cord injury, and other diseases that involve a neuroinflammatory component, as well as retinal degenerations involving oxidative stress. The potent bioactivity of NPD1 suggests the existence of a potentially important target for therapeutic, neuroprotective interventions in these diseases.

There are changes in the efflux and influx of brain DHA during cerebral ischemia *(27,28)*, and human cerebral albumin with bound DHA may deliver the precursor of NPD1 and in turn elicit neuroprotection *(29)*. Albumin as a carrier seems to be needed to reach the brain under these conditions. Because of the very high content of brain DHA, and because of its tenacious retention, it is difficult to modify brain DHA content via simple dietary manipulation; in addition, there seems to be a specific liver-to-brain (and retina) DHA supply system that provides DHA for the biogenesis and repair of membranes *(30)*. Is has been postulated that when ischemia removes free DHA from the brain, its replenishment may be met through DHA-carrying blood proteins *(30)*. Whether or not albumin (or lipoprotein) is performing this function is unclear. However, human serum albumin, when systemically injected, does cross the blood–brain barrier, reaching even intraneuronal sites *(31)*, and elicits neuroprotection in brain ischemia–reperfusion in experimental animals *(32)*.

Conclusions

Our findings demonstrate that NPD1 is a modulator of signaling pathways that promote cell survival in the brain and retina: Bcl-2 family protein expression is a premitochondrial target of NPD1 under conditions of oxidative stress. Consequently, downstream signaling, including effector caspase-3 activation and DNA degradation, is also modulated. This lipid mediator also potently counteracts cytokine-triggered proinflammatory COX-2 gene induction, another major factor in cell damage. In the ischemia–reperfusion-injured hippocampus and in neural progenitor cells stimulated by interleukin 1β, COX-2 expression is up-regulated

by NF-κB activation, likely involving other DNA-binding proteins. Moreover, NPD1 inhibits NF-κB and COX-2 induction, but not that of COX-1, under those conditions *(13)*.

NPD1's neuroprotective bioactivity in brain ischemia–reperfusion includes decreased infarct size and inhibition of polymorphonuclear leukocyte infiltration *(13)*. In RPE cells in culture, the addition of DHA to the culture medium protected the cells against oxidative stress, coinciding with enhanced NPD1 synthesis *(25)*. In vivo, DHA is actively supplied to brain and retina from the liver through the bloodstream, is necessary for cell development and function, and may play a critical role in conditions where oxidative stress—as occurs in aging, ischemia–reperfusion brain damage, retinal degenerations, and neurodegenerations such as Alzheimer's disease—renders polyunsaturated fatty acyl chains of membrane phospholipids vulnerable to lipid peroxidation.

Because DHA is highly enriched in synaptic membrane phospholipids, these membranes are a reservoir of a powerful neuroprotective mediator, NPD1, which promotes cell survival. At the same time, the highly unsaturated DHA-containing phospholipids are a potential target for free radical-triggered peroxidation. Thus the shortage in pro-survival lipid messengers like NPD1 plus lipid peroxidation promote cell damage and death. Other lipids, such as PAF, are also central players in synaptic membrane function and dysfunction. Therefore, the regulation of synaptic signaling by lipids is critical for neural cell survival.

Further understanding of the signals that modulate NPD1 synthesis will identify how NPD1 synthesis is triggered and how it may be used as a drug target. Such knowledge has potential therapeutic value for the treatment of neurodegenerative diseases, neurotrauma, and stroke.

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