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Omega-3 and omega-6 fatty acids suppress ER- and oxidative-stress in cultured neurons and neuronal progenitor cells from mice lacking PPT1

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Abstract

Reactive oxygen species (ROS) damage to brain lipids, carbohydrates, proteins, and DNA may contribute to neurodegeneration. We previously reported that ER- and oxidative stress cause neuronal apoptosis in infantile neuronal ceroid lipofuscinosis (INCL), a lethal neurodegenerative storage disease, caused by palmitoyl-protein thioesterase-1 (PPT1)-deficiency. Polyunsaturated fatty acids (PUFA) are essential components of cell membrane phospholipids in the brain and excessive ROS may cause oxidative damage PUFA leading to neuronal death. Using cultured neurons and neuroprogenitor cells from mice lacking *Ppt1*, which mimic INCL, we demonstrate that *Ppt1*-deficient neurons and neuroprogenitor cells contain high levels of ROS, which may cause peroxidation of PUFA and render them incapable of providing protection against oxidative stress. We tested whether treatment of these cells with omega-3 or omega-6 PUFA protects the neurons and neuroprogenitor cells from oxidative stress and suppress apoptosis. We report here that both omega-3 and omega-6 fatty acids protect the *Ppt1*-deficient cells from ER- as well as oxidative stress and suppress apoptosis. Our results suggest that PUFA supplementation may have neuroprotective effects in INCL.

Keywords

INCL; palmitoyl-protein thioesterase-1; Batten disease; Neurodegeneration; ER-stress; Oxidative stress; Apoptosis; PUFA

The nervous system is highly enriched with an enormous variety of complex lipids, among which polyunsaturated fatty acids (PUFAs) predominate [reviewed in 1]. Despite their abundance in the mammalian brain, *de novo* synthesis of PUFAs such as arachidonic acid (AA) and docosahexanoic acid (DHA) does not occur. Thus, the diet is the sole source of these fatty acids or their precursors [1]. Previous studies have established the critical roles of PUFAs in the development of the nervous system. Moreover, nearly 50% of the fatty acids

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in phospholipids (PLs) of the outer segment of the photoreceptors are DHA [2] and in the neurons, DHA is also highly enriched in the PLs of the synaptic membrane and the synaptic vesicles [3]. Further, during neurite outgrowth markedly high levels of DHA are found in the growth cones [4].

While PUFAs are highly concentrated in neuronal synaptic membranes and synaptic vesicles their functions in the nervous system remains largely unknown [5]. In rat brain myelogenesis has been reported to be increased when the animals were fed a diet containing omega-3 fatty acid [6]. It has also been reported that dietary omega-3 fatty acids may have neurological benefits [7] including enhancement of gene expression in the brain [8]. Moreover, epidemiological studies have suggested that increased consumption of the omega-3 (n-3) PUFA may reduce the risk of neurodegenerative disorders such as Alzheimer's disease [9]. Neuroprotective effects of PUFA have also been demonstrated although a clear mechanism has not been defined [10].

Neuronal ceroid lipofuscinoses (NCLs), also known as Batten disease, are the most common (1 in 12,500 births) autosomal recessive neurodegenerative storage disorders of childhood [reviewed in 11]. The infantile form of NCL, or INCL, is the most devastating disease caused by mutations in the gene encoding palmitoyl-protein thioesterase-1 (PPT1) [12]. PPT1 catalyzes the cleavage of thioester linkages in palmitoylated polypeptides [13] facilitating the release of palmitate so that the protein can recycle or undergo degradation. Thus, PPT1-deficiency impairs recycling or degradation of palmitoylated proteins that are substrates of this enzyme contributing to INCL pathogenesis [14]. Children afflicted with INCL are normal at birth but by two years of age they undergo complete retinal degeneration causing blindness and by age four these children manifest no brain activity. They remain in a vegetative state for another 6–8 years before death. Currently, there is no effective treatment for any of the NCLs.

It has been reported that neuronal death in INCL is caused by apoptosis [15] although the molecular mechanism(s) of apoptosis, until recently, remained obscure. We previously reported that neuronal apoptosis in INCL and in *Ppt1*-knockout (*Ppt1*-KO) mice [16], which recapitulate virtually all pathological features of INCL [17], at least in part, is caused by oxidative- and endoplasmic reticulum (ER)-stresses [18–21]. Emerging evidence indicates that oxygen free radical damage to the brain lipids, carbohydrates, proteins, and DNA may contribute to neuronal cell death causing neurodegeneration [22]. Thus, even though the PUFA levels are the same in normal and PPT1-deficient brains, the highly oxidative environment present in the brain of INCL patients and in that of the *Ppt1*-KO mice these lipids may not be adequate to protect the neurons from oxidative stress. Therefore, we hypothesized that omega-3 and omega-6 fatty acids may have neuroprotective effects in *Ppt1*-deficient cells.

In the present study, we analyzed cultured PPT1-deficient neurons and neuronal progenitor cells derived from the fetal brain tissues of *Ppt1*-KO mice. We also explored the effects of PUFA-treatment of these cells, which markedly reduced the level of reactive oxygen species (ROS) and suppressed apoptosis. These results, for the first time, demonstrate that while endogenous PUFA levels in the *Ppt1*-KO mice appear to be similar to or even slightly higher compared with those of the WT littermates, PUFA-treatment suppresses oxidative stress and reduces neuronal apoptosis induced by these stresses raising the possibility that PUFA may have beneficial effects in INCL.

Neurons and neuronal progenitor cells were derived from 15-day-old *Ppt1*-KO and WT fetal mouse brains. The cells were cultured in Neurobasal Media (Invitrogen) containing B27 supplement. Mouse neurospheres were cultured in NeuroCult NSC Basal Medium (Stem

Cell Technologies, Vancouver, BC, Canada) containing NeuroCult NSC proliferation supplements and human epidermal growth factors according to supplier's instructions. INCL patient fibroblasts, carrying homozygous PPT1 missense mutations, A364T (R122W), were a generous gift from late Dr Krystyna Wisniewski. They were cultured in DMEM medium supplemented with 10% FBS at 37 °C at an atmosphere of 5% CO₂ and 95% air.

The cultured neurons were incubated with fresh medium only, fresh medium containing either omega-3 (25μM) or omega-6 (25μM) fatty acids or a combination of both for 30 minutes followed by washing three times with culture media. The cells were then incubated for 48 h at 37°C at atmosphere of 5% CO₂ and 95% air. Intracellular ROS was detected after the cells were incubated with 10μM of ROS-sensitive fluorescent probe 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate, (H2-DCFDA, Sigma) for 30 minutes. After washing twice with PBS, the cells were analyzed by FACS using FACScan (Becton Dickinson).

Treated neurons were homogenized in PhosphoSafe extraction reagent (EMD Biosciences). Twenty micrograms of total proteins from each sample were resolved by SDS-PAGE under denaturing and reducing conditions and Western blot analysis was performed as previously described (18). The primary antibodies used are: anti-cleaved caspase-3, anti-PARP1 (Cell Signaling), anti-Grp78/Bip (KDEL) (Stressgen), anti-GADD153/CHOP (Abcam), and anti-β-actin (Sigma). The second antibodies used are: goat anti-rabbit IgG (Santa Cruz Biotechnology); rabbit anti-mouse IgG (Santa Cruz Biotechnology). Chemiluminescent detection was performed by using Supersignal west pico luminol/enhancer solution (Pierce) according to the manufacturer's instructions.

Perfused brain tissues from 1-, 3- and 6-month old *Ppt1*-KO mice and their WT littermates were harvested, immediately frozen in liquid nitrogen, powdered under liquid nitrogen with a mortar and pestle, and stored at -85°C until analysed. All extractions were performed either in siliconized tubes (PGC Scientifics, Frederick, Maryland) or in glass tubes. A simple extraction method was developed [23]. Briefly, fifty microliters (~5.0 mg of the tissue) of homogenate from each tissue sample were added into 1 mL of MeOH with 100 pmol of 14:0 LPA as the internal standard (IS). After vortexing and incubation on ice for 10 min, the mixture was centrifuged (10,000Xg for 5 minutes at room temperature), and 120 μL of supernatant were directly used for mass spectrometry (MS). MS analyses were performed using API-4000 (Applied Biosystems/MDS SCIEX) with the Analyst data acquisition system. Negative ion multiple reaction monitoring (MRM) and single ion monitor (SIR) mode were used for measurement of lipids. Standard curves were generated for quantitative analyses of all lipids. HPLC conditions were virtually identical to that previously reported [23]. Samples (10 μL) were loaded into an LC system (Agilent 1100) with an auto sampler. The mobile phase was MeOH/water/NH₄OH (90:10:0.1, v/v/v) and the HPLC separations were 15 min/sample.

We previously reported that the neurons in the central nervous system of the *Ppt1*-KO mice suffer from mitochondrial dysfunction due to oxidative-stress [20- 21]. Oxidative stress causes collapse of the mitochondrial membrane potential, inhibition of ATP synthesis, release of cytochrome c into the cytosol, and cell death [22]. We therefore examined the levels of ROS in primary cultures of cortical neurons from the brain tissues of feti from *Ppt1*-KO mice and those of their WT littermates using ROS-sensitive fluorescent probe, H2-DCFDA. We found that cultured *Ppt1*-KO neurons (empty curve) showed a shift of the peak to the right compared to those from WT neurons (black filled curve), indicating an increased intracellular ROS level. (Fig. 1a). This result suggests that *Ppt1*-KO cortical neurons were under oxidative-stress. To confirm whether PUFA has anti-oxidative effect in the *Ppt1*-KO cortical neurons, we treated the neurons with omega-3 or omega-6 or a combination of both fatty acids. We found that compared to untreated *Ppt1*-KO neurons (black filled curve),

those treated with omega-3, omega-6, or a combination of both PUFAs showed markedly decreased level of ROS (Fig. 1b). The cortical neurons from the WT feti did not show appreciable difference (Fig. 1c). Taken together these results suggest that both omega-3 and omega-6 fatty acids are capable of reducing ROS in *Ppt1*-KO neurons.

We previously reported that INCL fibroblasts and neurons from *Ppt1*-KO mice undergo ER-stress and oxidative-stresses, which mediate apoptosis [18–21]. We sought to determine whether the scavenger effects of PUFA for ROS may alter the ER-stress levels in *PPT1*-deficient neurons and suppress ER-stress mediated apoptosis. Accordingly, we determined whether the levels of ER-stress-marker proteins such as glucose responsive protein-78 (Grp-78/Bip) and growth arrest- and DNA damage-inducible gene 153 (GADD153) are reduced in the *Ppt1*-KO neurons after treatment with omega-3 or omega-6 PUFA. Our results show that both Grp-78/Bip and GADD153 proteins are markedly decreased in omega-3- and omega-6-treated *Ppt1*-deficient neurons (Fig. 2a and 2b). We then determined whether decreased ER-stress leads to the suppression of caspase-3 activation and poly (ADP-ribose) polymerase-1 (PARP1) cleavage, which are reliable measures of apoptosis. The results show that whereas untreated (control) *Ppt1*-KO neurons express high levels of cleaved caspases-3 (Fig. 2c, **lane 1**) those treated with omega-3 or omega-6 PUFA showed markedly reduced levels (Fig. 2c, **lanes 2 and 3**). Interestingly, treatment of the cells with a combination of omega-3 and omega-6 yielded results similar to the ones obtained from treatment with omega-3 or omega-6 PUFA (Fig. 2c, **lane 4**). These results suggest that treatment with a combination of omega-3 and omega-6 PUFA neither produces additive nor synergistic effects. We then evaluated the effects of PUFA-treatment on the levels of un-cleaved (inactive) and cleaved (active) PARP1 levels. The results showed that the untreated control *Ppt1*-deficient cells manifested high levels of both un-cleaved- and cleaved-PARP1 (Fig. 2d, **lane 1**) whereas treatment with omega-3 or omega-6 alone or in combination significantly reduced the levels of both un-cleaved- and cleaved-PARP1 (Fig. 2d, **lanes 2–4**). Taken together these results suggest that PUFA-treatment suppresses apoptosis.

So far, we have demonstrated that the ROS, ER-stress, and apoptosis levels in the cultured *Ppt1*-KO cortical neurons are reduced by PUFA-treatment most likely due to the ROS scavenging effects of PUFAs. To further confirm the above results, we analyzed the ROS levels in untreated and PUFA-treated *Ppt1*-deficient neuronal progenitor cells using H₂-DCFDA. The results showed a shift of the fluorescence peak to the right in *Ppt1*-KO neuronal progenitor cells (black filled curve) compared with those of the neuronal progenitor cells from WT littermates (empty curve), indicating elevated intracellular ROS level (Fig. 3a). This result indicates that both cultured *Ppt1*-KO neurons and neuronal progenitor cells were under oxidative stress. To confirm whether PUFA exerts anti-oxidative effects on *Ppt1*-KO neuronal progenitor cells, we treated the cells with omega-3 or omega-6 PUFA. Compared with untreated control, omega-3- or omega-6-treated *Ppt1*-KO neuronal progenitor cells (gray filled curve) showed appreciably decreased intracellular ROS level (Fig. 3b). PUFA-treatment did not appear to cause any significant change in WT neuronal progenitor cells (Fig. 3c). Interestingly, PUFA-treatment did not change ROS levels in human INCL fibroblasts (Supplementary Fig. 1a–d) suggesting that while omega-3 and omega-6 may have beneficial effects on *Ppt1*-deficient neurons and neuronal progenitor cells these PUFAs may not manifest such effects on fibroblasts.

Since PUFA-treatment reduced ROS levels, we sought to determine whether these fatty acids alleviated ER-stress. Accordingly, we determined the levels of ER-stress markers, Grp78/Bip and GADD153 levels in untreated and omega-3 and omega-6 PUFA-treated *Ppt1*-KO neuronal progenitor cells. The results showed that the levels of both Grp-78/Bip and GADD153 proteins are appreciably decreased in omega-3 or omega-6 treated cells compared with untreated controls (Fig. 4a and 4b). We then sought to determine whether the

decreased ER-stress suppressed the levels of cleaved PARP1, a marker for apoptosis. Our results clearly indicated that compared with untreated control (Fig. 4c, **lane 1**) omega-3 or omega-6 PUFA-treated cells showed significantly reduced levels of cleaved PARP1 (Fig. 4c, **lanes 2 and 3**). These results suggest that treatment of *Ppt1*-KO neurons and neuronal progenitor cells with omega-3 and omega-6 PUFAs reduces both ER- and oxidative stresses and suppresses apoptosis.

We have previously reported that neurons from *Ppt1*-KO mouse brain and cultured fibroblast cells from INCL patients suffer from ER- and oxidative-stresses [18–21]. The anti-oxidative- [25] as well as anti-inflammatory-properties [26] of PUFA alone or in combination with curcumin, respectively, have been documented. Interestingly, it has been suggested that PUFA is an important modulator for unfolded protein-aggregation in relation to disease pathogenesis [27]. The fact that ER-stress mediates unfolded protein response in PPT1-deficient neurons leading to apoptosis [18], the results of our present study is in agreement with this suggestion. In our present study, we determined whether cultured PPT1-deficient neurons and neuronal progenitor cells that manifest ER- and oxidative-stress are protected from apoptosis induced by these stresses.

The ability of the mammalian brain to combat oxidative stress is somewhat limited. When brain encounters oxidative stress it responds by generating heat shock proteins that allow tolerance against more stress [28]. Oxygen free radical attack may lead to peroxidation of lipids generating several toxic oxidative products including lipid hydroperoxides [29]. Because PUFAs contain multiple double bonds, they are vulnerable to damage by free radicals. Thus, brain tissues may be damaged by oxidative stress. Even though brain contains high levels of PUFA in neurodegenerative diseases, elevated oxidative stress may generate PUFA peroxidation products [29]. Interestingly, it has been demonstrated that PUFA supplementation stimulated the expression of heat shock protein-32 (hemeoxygenase-1), a stress-sensitive protein inducible by oxidative stress [29]. In the present study, we analyzed brain fatty acid levels in the brain of *Ppt1*-KO mice and their WT littermates at 1 and 6 months of age. The results showed that compared with the fatty acid levels in brain tissues from WT mice, those in the brain tissues of *Ppt1*-KO littermates are not significantly different (Supplementary Figure 2).

In light of the fact that the brain tissues of *Ppt1*-KO mice suffer from high oxidative stress, much of the fatty acids may be peroxidated and therefore are potentially inactive in scavenging free radicals. Alternatively, most of the PUFA in *Ppt1*-KO brain may be engaged in scavenging the elevated levels of ROS in order to protect the neurons. We are currently conducting a clinical trial in which INCL patients are treated with a combination of cysteamine bitartrate (cystagon) and N-acetylcysteine (mucomyst), both of which are potent anti-oxidants. This trial is being conducted to determine whether these anti-oxidant compounds are beneficial for INCL patients. While this clinical trial is ongoing, in the present study, we sought determine whether endogenous antioxidants in the brain (i.e. omega-3 and omega-6 PUFAs) are potential therapeutic targets. The results of our present *in vitro* experiments suggest that the cultured neurons and neuroprogenitor cells, when treated with exogenous omega-3 and omega-6 PUFAs responded by reducing the ER- as well as oxidative-stresses and suppressing apoptosis. Whether dietary supplementation of omega-3 and omega-6 PUFAs in *Ppt1*-KO mice would yield results similar to the ones obtained from our *in vitro* experiments remains to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Reactive oxygen species (ROS) in neuronal progenitor cells

ROS levels in of *Ppt1*-KO and WT neuronal progenitor cells from the brains of *Ppt1*-KO and WT mouse feti at 15th day of gestation. (a) Intracellular ROS level of *Ppt1*-KO and WT neuronal progenitor cells. (b) Intracellular ROS levels in *Ppt1*-KO neuronal progenitor cells with fatty acid treatment. (c) Intracellular ROS levels in WT neuronal progenitor cells with fatty acid treatment. Fluorescence was measured using 20,000 cells.

Figure 2. The effects of PUFA on ER-stress and apoptosis in WT and *Ppt1*-deficient neurons
 Omega-3- and omega-6-treatment decreased the levels of both ER stress-marker proteins as well as apoptosis-marker proteins. Untreated (cortical) neurons derived from the brains of 15-day old feti from *Ppt1*-KO mice and their WT littermates were used for Western blot analysis. Western blot analyses of the samples from a control (lane 1), from an omega-3 treated (lane 2), from an omega-6 treated (lane 3) and from omega-3 + omega-6 treated (lane 4) using antibodies to: GRP-78 (a), GADD153 (b), cleaved caspase-3 (c) and PARP1 (d).

(A) WT & *Ppt1*-KO neuro-progenitor cells (B) *Ppt1*-KO neuro-progenitor cells

(C) WT neuro-progenitor cells

Figure 3. Effect of PUFAs on cultured neuroprogenitor cells from *Ppt1*-KO mice

Cortical neurons were cultured from fetal brain tissues as described under Materials and Methods. (a) Intracellular ROS levels in cultured neurons from *Ppt1*-KO and WT feti. (b) Intracellular ROS levels in untreated cultured *Ppt1*-KO neurons (control) or those treated with either omega-3 or omega-6 or with a combination of both. (c) Intracellular ROS levels in untreated WT neurons or those treated with either omega-3 or omega-6 or a combination of both fatty acids. 3D-overlay was generated by CellQuest software (BD Biosciences).

Figure 4. The effects of PUFA-treatment ER stress and apoptosis

Neuro-progenitor cells from the brains of 15-day old *Ppt1*-KO and WT mouse feti were used for Western blot analysis. Western blot analyses of the cell lysates from untreated control (lane 1), Omega-3 treated (lane 2) and Omega-6 treated (lane 3) were performed using antibodies to GRP-78 (a), GADD153 (b) and PARP1 (c).