Palmitoyl-protein thioesterase-1 deficiency leads to the activation of caspase-9 and contributes to rapid neurodegeneration in INCL

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INTRODUCTION

Neuronal ceroid lipofuscinoses (NCLs), also known as Batten disease (1–3), are the most common (one in 12,500 births) group of neurodegenerative storage disorders. The infantile form of NCL or INCL is a rare (one in 100,000 births) albeit devastating disease, which is caused by inactivating mutations in the palmitoyl-protein thioesterase-1 (PPT1) gene (4). PPT1 catalyzes the cleavage of thioester linkages in s-acylated (palmitoylated) proteins facilitating the degradation and/or recycling of these lipid-modified polypeptides (5,6). Children afflicted with INCL are normal at birth but by 2 years of age they manifest complete retinal blindness and by age four the isoelectric encephalograms attest to brain death. These children remain in a vegetative state for several more years and death occurs around 10–12 years of age. Other INCL cases with mild phenotypes (e.g. late-infantile and juvenile onset) have also been reported (1–3,7). A characteristic pathological finding at necropsy is the presence of autofluorescent intracellular storage material, known as granular osmiophilic deposits in neuronal as well as in other tissues (8). Moreover, elevated levels of apoptosis in the brain biopsy tissues (9) and in cultured cells from INCL patients (10) have been reported. Recently, PPT1-knockout (PPT1-KO) mice that mimic INCL, have been generated by gene targeting in embryonic stem (ES) cells (11). In the brains of these mice increased levels of neuronal apoptosis has been reported (11). Further, it has been demonstrated that with increased apoptosis there is an actual loss of neurons in the brains of PPT1-KO mice (12). Taken together, these results strongly suggest that apoptosis is at least one of the major causes of neurodegeneration in INCL. However,
the molecular mechanisms of apoptosis causing rapid progression of brain atrophy in INCL remain poorly understood. 

Accumulating evidence indicates that the molecular mechanisms of apoptosis are diverse and they involve many subcellular organelles (13,14). Stress in one of these organelles, the endoplasmic reticulum (ER), plays critical roles in apoptosis by the activation of unfolded protein response (UPR) (15,16) and activation of caspase-12 in the mouse (17) and caspase-4 in humans (18). Moreover, ER stress may lead to the disruption of calcium signaling (19) leading to the activation of caspase-9 pathway of apoptosis. It has also been reported that the ER function is sensitive to oxidative stress (20). Interestingly, the ER is one of the organelles that generate reactive oxygen species (ROS) in addition to its function as a major storage organelle for calcium. During ER stress, however, both ROS production and Ca$^{2+}$ release are elevated (21,22), increasing the potential for activation of the mitochondrial pathway of apoptosis in neurodegenerative diseases such as Parkinson’s disease (23). Recently, we reported that ER stress-induced UPR and activation of caspase-12 mediates neuronal apoptosis in PPT1-KO mice (24). In the present study, we sought to determine whether ER stress activates caspase-9 mediated apoptotic pathway in INCL. Here, we report that the brain tissues of both INCL patient and PPT1-KO mouse contain elevated levels of superoxide dismutase (SOD)-protein as well as SOD enzymatic activity and increased cleavage (activation) of caspase-9. We further demonstrate that cultured neurospheres derived from PPT1-KO mouse fetuses show elevated levels of ROS, increased SOD-protein and SOD-activity, elevated levels of cleaved (activated) caspase-9, caspase-3, cleaved-PARP indicative of apoptosis. We propose that ER stress in neurons leads to elevated levels of ROS, stimulates SOD-2 production as well as activation and disrupts calcium homeostasis. Together, these abnormalities mediate the activation of caspase-9, caspase-3 and cleavage of PARP, which is indicative of apoptosis, thereby causing rapid brain atrophy, characteristic of INCL.

RESULTS

Evidence for the presence of ROS and caspase-9 activation in human INCL brain

In both acute and chronic neurodegenerative disorders, oxidative stress and pro-inflammatory signaling play critical pathogenic roles (25). Endoplasmic reticulum (ER) stress mediates elevated production of ROS and causes the release of Ca$^{2+}$ that disrupts homeostasis. Oxidative stress has been reported to induce SOD that facilitates the maintenance of homeostasis and is upregulated in neurodegenerative diseases such as Parkinson’s disease (26). We have previously reported that in the PPT1-KO mouse brain, the neurons undergo ER stress mediating activation of the UPR and caspase-12 leading to apoptosis (24). As the ER stress may cause excessive Ca$^{2+}$ release and destabilize mitochondrial membranes that activates caspase-9 leading to apoptosis (reviewed in 27), we first sought to determine whether SOD is upregulated in the INCL brain. Accordingly, we determined the SOD protein levels in the autopsy brain tissues from an INCL patient and an age matched control by western blot analysis. The results show that in the hippocampus of the INCL patient, SOD-2 is upregulated compared with that of the control (Fig. 1A, lanes 1 and 3). However, this elevated SOD-2 level was not apparent in the cortex of either control or INCL samples (Fig. 1A, lanes 2 and 4). These results suggest the presence of superoxides in the INCL brain, which most likely induces the SOD-2.

Activation of caspase-9, caspase-3 and PARP cleavage in human INCL brain

As the ER stress also causes excessive Ca$^{2+}$ release and disrupts Ca$^{2+}$ homeostasis, it leads to mitochondrial membrane permeability and the activation of caspase-9 (28), we determined the levels of un-cleaved (inactive) and cleaved (active) caspase-9 in INCL and control brains. The results show that whereas both uncleaved and cleaved caspase-9 are virtually undetectable in the control brain tissue (Fig. 1B, lanes 1 and 2), they are readily detectable in the hippocampus as well as in the cortex of the INCL brain, although the levels are somewhat less in the cortical tissue (Fig. 1B, lanes 3 and 4). We then sought to determine whether the activated caspase-9 in INCL brain leads to caspase-3 activation. Accordingly, we used cleaved (activated) caspase-3 antibody to immunohisto-logically detect cleaved caspase-3 in the brain tissues from INCL and normal control subjects. The results show that whereas cleaved caspase-3 antibody does not stain both hippocampus and cortical tissues from the control subject (Fig. 1C, upper panels), it readily detects the cleaved caspase-3 in INCL brain (Fig. 1C, lower panels). To determine whether the caspase-3 activation leads to apoptosis in INCL brain, we determined the level of poly (ADP-ribose) polymerase (PARP) cleavage, which is indicative of apoptosis. Our results show that whereas un-cleaved-PARP is detectable in both the hippocampus and cortex of the control brain tissues (Fig. 1D, lanes 1 and 2), only the INCL brain tissue appears to have un-cleaved- as well as cleaved-PARP (Fig. 1D, lanes 3 and 4). Taken together, these results suggest that apoptosis in human INCL brain is not only mediated by the ER stress-induced caspase-12 activation as previously reported (24), but also there is elevated SOD-2 indicating the presence of ROS, activated caspase-9 and caspase-3 as well as cleaved-PARP, which leads to the rapid neuronal death by apoptosis.

Levels of SOD-2, activated caspase-9, caspase-3 and cleaved-PARP in PPT1-KO mouse brain

As stated earlier, PPT1-KO mice recapitulate the clinical and pathological features of human INCL (11,12). Thus, we sought to determine whether the results obtained from the INCL brain tissues (see above) are reproducible in the mouse model of INCL. We therefore determined the levels of SOD-2, cleaved form of caspase-9, caspase-3 and PARP. Our results show that compared with the WT mouse brain, SOD-2 is appreciably elevated in the PPT1-KO brain (Fig. 2A). However, cleaved caspase-9 (Fig. 2B, left lane), cleaved caspase-3 (Fig. 2C, left panel) and cleaved-PARP (Fig. 2D, left lane) are not detectable in the WT brain. On the other hand, in the brains of PPT1-KO mice activated
caspase-9 (Fig. 2B, right lane), activated caspase-3 (Fig. 2C, right panel) and cleaved-PARP (Fig. 2D, right lane) are readily detectable. These results demonstrate that neuronal apoptosis in both human INCL and in the mouse model of this disease is mediated by activation of both caspase-12 as well as caspase-9 and these two pathways together may lead to the rapid progression of neurodegeneration characteristic of INCL.

Elevated levels of ROS and SOD-2 activity in cultured PPT1-KO neurospheres

So far, we have assumed that the elevated levels of SOD-2 in INCL and PPT1-KO brains are due to the elevated levels of ROS. However, demonstration of the presence of ROS in intact tissues is technically difficult. Therefore, we established primary neurosphere cultures from PPT1-KO and WT fetuses and used these neurospheres to first determine the levels of ROS, SOD-2 protein and activity. Our results show that whereas in the WT neurospheres, ROS is virtually undetectable (Fig. 3A, upper panel), high levels of ROS specific fluorescence is present in the PPT1-KO neurospheres (Fig. 3A, lower panel). To confirm whether the presence of high levels of ROS in the PPT1-KO neurospheres also induce SOD-2, we performed western blot analysis of brain lysates of WT and PPT1-KO neurospheres. The results show that compared with the WT neurospheres (Fig. 3B, left lane) those from the PPT1-KO neurospheres (Fig. 3B, right lane) contain higher levels of SOD-2 protein. We then assessed the SOD-2 activity in these lysates. The results show that compared with the lysates from WT neurospheres (Fig. 3C, left bar), those of the PPT1-KO neurospheres (Fig. 3C, right bar) manifest significantly higher \( P < 0.005 \) SOD-2 activity. We then assessed the levels of cleaved caspase-9, uncleaved- and cleaved-PARP in WT and PPT1-KO neurospheres. The results show that whereas in the WT neurospheres, a low level of cleaved caspase-9 is detectable (Fig. 4A, left lane), it is markedly higher in PPT1-KO neurospheres (Fig. 4A, right lane). Similarly, whereas uncleaved-PARP protein is detectable in WT neurospheres, there is no appreciable cleaved-PARP band (Fig. 4B, left lane). However, both the uncleaved and cleaved-PARP-protein bands are clearly detectable in the lysates of PPT1-KO neurospheres (Fig. 4B, right lane). To confirm that the higher levels of cleaved-PARP translate into higher levels of apoptosis, we used transmission electron microscopy to detect apoptotic cells in WT and PPT1-KO neurospheres. Our results confirm that apoptotic cells are much more readily detectable in the PPT1-KO neurospheres (Fig. 4C, right panel) compared with the WT neurospheres (Fig. 4C, left panel). Taken together, these results indicate that PPT1-deficiency mediates apoptosis not only by activating UPR and caspase-12 but also...
through generation of higher levels of ROS, activating caspase-9.

DISCUSSION

In this study, we found that in human INCL as well as in PPT1-KO mice, the rapid progression of neurodegeneration is likely to be caused by a combination of ER-stress-mediated caspase-12 activation as well as by elevated ROS production, destabilization of calcium homeostasis activating caspase-9, caspase-3 and apoptosis. Using PPT1-KO mice, we have previously reported that PPT1-deficiency leads to ER stress activating caspase-12 that mediates caspase-3 activation and apoptosis (24). The ER is also an essential intracellular Ca\(^{2+}\) storage organ (29) that plays critical roles in the regulation of numerous cellular functions. These include synthesis, folding and post-translational modification of polypeptides, cell motility, mitochondrial metabolism, cell cycle progression and apoptosis (19). Thus, ER stress disrupts homeostasis and may have detrimental effects on other organelles such as the mitochondria that harbors caspase-9 (reviewed in 30). ER stress mediated disruption of Ca\(^{2+}\) homeostasis activates caspase-9 (31). Thus, we sought to determine whether this pathway of apoptosis contributes to rapid neurodegeneration in INCL. Our results show that in addition to the major contribution of caspase-12, caspase-9 activation also contributes to increased apoptosis in INCL and PPT1-KO brain tissues. Thus, it is likely that the combined effects of two distinct pathways of apoptosis mediate rapid neurodegeneration, characteristic of INCL.

It appears, however, that PPT1-deficiency causing ER stress may be the primary event, which leads to the activation of caspase-9 pathway of apoptosis. In the absence of an effective treatment, INCL remains a uniformly fatal disease. Recently, it has been demonstrated that compounds that relieve ER stress, such as salubrinal, is cytoprotective (32,33). The results of our present study underscore the necessity of further investigations to determine whether a small molecule that prevents or relieves ER stress protects PPT1-deficient neuronal cells. The PPT1-KO mice may provide the animal model for obtaining the proof of principle.

MATERIALS AND METHODS

PPT1-KO mice and genotyping

PPT1-KO mice were generated by gene targeting in ES cells as previously described (11). All mice were maintained and housed in a germ-free facility and animal procedures were carried out in accordance with institutional guidelines after the NICHD Animal Care and Use Committee approved an animal study protocol. Human INCL and age-matched control brain autopsy tissue samples were obtained from the Human Brain and Spinal Fluid Resource Center, VA West Los Angeles Healthcare Center, Los Angeles, CA 90073 and from the Brain and Tissue Bank for Developmental Disorders, University of Maryland, Baltimore, MD 21201, respectively.

Immunohistochemical analysis of tissues

Immunohistochemical staining of the brain tissues were performed as previously described (24). The tissue sections were incubated with primary anti-cleaved-caspase-3 antibody (1:200; Cell Signaling) overnight at 4°C in a humidified chamber. After incubation, the slides were washed three times with 1xPBS and were further incubated with anti-mouse biotinylated secondary antibody (1:500; Vector Laboratories) for 1 hr at room temperature. The sections were washed three times with 1xPBS and then incubated with ABC complex by following the manufacturer’s (Vector Laboratories) protocol.
Mouse neurosphere culture and reagents

Mouse neurospheres were isolated from the brains of 15-day-old fetuses of PPT1-KO and WT littermates. The cells were cultured in NeuroCult NSC Basal Medium (Stem Cell Technologies, Vancouver, BC, Canada) containing NeuroCult NSC proliferation supplements and human epidermal growth factors were added to a final concentration of 20 ng/ml and incubated at 37°C under an atmosphere of 5% CO2 and 95% air.

Western blot analysis

Human and mouse brains were homogenized in protein extracting buffer (50 mM Tris-HCl, 150 mM NaCl, 0.25% SDS, 1 mM EDTA and 1% NP-40) containing protease-inhibitor cocktails (Sigma). Mouse neurospheres were treated in PhosphoSafe extraction reagent (EMD biosciences). Twenty micrograms of the total proteins from each sample were resolved by electrophoresis using 4–15% SDS-polyacrylamide gels (Bio-Rad) under denaturing and reducing conditions. Proteins were then electrotransferred to nitrocellulose membrane (Bio-Rad). The membranes were blocked with 5% non-fat dry milk (Bio-Rad) and then subjected to immunoblot analysis using the methods as described earlier. The primary antibodies used are: anti-SOD-2, anti-caspase-9 (Upstate), anti-cleaved caspase-9 (EMD biosciences), anti-PARP (Cell Signaling) and anti-β-actin (Sigma). The second antibodies used in the study are: goat anti-rabbit IgG, donkey anti-goat IgG, 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.

SOD activity assay

SOD activity was measured by SOD activity assay kit (Chemicon). Some 6 × 10⁶ cells were washed with ice-cold PBS; the cell pellets were resuspended in 1 × lysis buffer under denaturing and reducing conditions. Proteins were then electrotransferred to nitrocellulose membrane (Bio-Rad). The membranes were blocked with 5% non-fat dry milk (Bio-Rad) and then subjected to immunoblot analysis using the methods as described earlier. The primary antibodies used are: anti-SOD-2, anti-caspase-9 (Upstate), anti-cleaved caspase-9 (EMD biosciences), anti-PARP (Cell Signaling) and anti-β-actin (Sigma). The second antibodies used in the study are: goat anti-rabbit IgG, donkey anti-goat IgG, 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.

Figure 3. ROS and SOD in the neurospheres derived from WT and PPT1-KO fetuses. (A) ROS was detected by using carboxy-H2DCFDA. Green fluorescence indicates the presence of ROS: WT (upper panels) and PPT1-KO (lower panels). Nuclei were stained with DAPI (blue). Scale bar: 20 μm. (B) Detection of SOD-2 protein by western blot analysis of neurospheres derived from: WT (left lane) and PPT1-KO (right lane) fetuses. Note the higher level of SOD-2 in the PPT1-KO neurospheres. (C) SOD activity in neurospheres derived from: WT (left bar) and PPT1-KO (right bar) fetuses. Asterisk indicates the significance at P < 0.005 level.
(10 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 0.5% Triton X-100) and incubated on ice for 10 min. After centrifuge, cell lysates were collected and mixed with xanthine solution, chromagen solution, 10× SOD assay buffer, dH2O and xanthine oxidase. After that, they were incubated for 2 hr at 37°C and measured by a microplate reader (Bio-Rad) at 490 nm.

**Fluorescence microscopy**

For the detection of intracellular ROS, the PPT-KO and WT mouse neurospheres were incubated at 37°C in an atmosphere of 5% of CO₂ and 95% air for 24 h on the slide chamber (Nunc). The cells were then treated with Image-iT live green ROS detection kit (Molecular Probes). Nuclei were stained with 4, 6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma). Fluorescence was visualized with the Axioskop2 plus fluorescence microscope (Carl Zeiss), and the image was processed with the AxioVision 4.3 (Carl Zeiss) and the Photoshop 7.0 program (Adobe).

**Transmission electron microscopy**

The brain tissues were fixed in 2.5% glutaraldehyde in sodium phosphate buffer for 2 h. The tissues were then washed with Millonig’s phosphate buffer once and kept in the same buffer until final processing. Lead citrate and uranyl acetate were used to stain the thin sections. The stained sections were then examined with a LEO 912 electron microscope by JFE Enterprises (Brookeville, MD).

**Statistical analysis**

Results are expressed as a means of at least three determinations ± standard deviation (SD). Statistical analyses were performed by Student’s t-test using Microsoft Excel 2002 and a P-value of <0.05 was considered statistically significant.

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**Conflict of Interest statement.** None declared.

Figure 4. Analysis of cleaved (active) caspase-9, PARP and apoptosis in cultured neurospheres derived from PPT1-KO and WT fetuses. (A) Detection of cleaved caspase-9 by western blot analysis of neurospheres lysates from: WT (left lane) and PPT1-KO (right lane). (B) Western blot analysis of PARP in WT (left lane) and PPT1-KO (right lane) neurosphere. (C) Ultrastructure analysis by transmission electron microscopy of the neurospheres from WT (left panel) and PPT-KO (right panel) fetuses. Note that the numerous apoptotic bodies (arrows) in PPT1-KO neurospheres are clearly detectable.
REFERENCES


