Production of lysophosphatidylcholine by cPLA2 in the brain of mice lacking PPT1 is a signal for phagocyte infiltration

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In the majority of neurodegenerative storage disorders, neuronal death in the brain is followed by infiltration of phagocytic cells (e.g. activated microglia, astroglia and macrophages) for the efficient removal of cell corpses. However, it is increasingly evident that these phagocytes may also cause death of adjoining viable neurons contributing to rapid progression of neurodegeneration. Infantile neuronal ceroid lipofuscinosis (INCL) is a devastating, neurodegenerative, lysosomal storage disorder caused by inactivating mutations in the palmitoyl-protein thioesterase-1 (PPT1) gene. PPT1 catalyzes the cleavage of thioester linkages in S-acylated (palmitoylated) proteins and its deficiency leads to abnormal accumulation of thioesterified polypeptides (ceroid) in lysosomes causing INCL pathogenesis. PPT1-knockout (PPT1-KO) mice mimic the clinical and pathological features of human INCL including rapid neuronal death by apoptosis and phagocyte infiltration. We previously reported that in PPT1-KO mice, the neurons undergo endoplasmic reticulum stress activating unfolded protein response, which mediates caspase-12 activation and apoptosis. However, the molecular mechanism(s) by which the phagocytic cells are recruited in the PPT1-KO mouse brain remains poorly understood. We report here that increased production of lysophosphatidylcholine (LPC), catalyzed by the activation of cytosolic phospholipase A2 (cPLA2) in the PPT1-KO mouse brain, is a ‘lipid signal’ for phagocyte recruitment. We also report that an age-dependent increase in LPC levels in the PPT1-KO mouse brain positively correlates with elevated expression of the genes characteristically associated with phagocytes. We propose that increased cPLA2-catalyzed LPC production in the brain is at least one of the mechanisms that mediate phagocyte infiltration contributing to INCL neuropathology.

INTRODUCTION

In multicellular organisms, apoptosis is followed by efficient removal of the cell corpses by phagocytes in order to prevent inflammation, to suppress autoimmunity and to maintain homeostasis (1–3). Accumulating evidence indicates that in the majority of neurodegenerative lysosomal storage disorders neuronal death by apoptosis is followed by infiltration of phagocytes (e.g. activated microglia, astroglia and macrophages) (4,5) and may cause further damage to adjoining viable cells (6). Microglia represents the largest phagocyte population in the central nervous system and accounts for ~10% of the non-neuronal cells in the brain (7). These phagocytes possess many characteristics of myeloid cells and their activation is associated with upregulated expression of
marker genes commonly found on macrophages and dendritic cells (8). In response to neuronal injury and/or death these cells undergo rapid morphological and functional changes. For example, they acquire myeloid cell properties including antigen presentation, matrix metalloproteinase expression, generation of reactive oxygen species and phagocytosis (9). It has also been reported that excessive phagocyte infiltration may cause damage to viable neurons leading to rapid progression of neurodegeneration (1,6,10). However, the molecular signal(s) that mediates the recruitment of phagocytes in neurodegenerative storage disorders remains poorly understood.

The infiltration of phagocytes in the brain has been reported in more than 40 neurodegenerative lysosomal storage diseases (4,5). Recently, pronounced astrocytosis has been reported in the autopsy brain tissues from patients with juvenile NCL, commonly known as Batten disease (11). Infantile neuronal ceroid lipofuscinosis (INCL) is a newly recognized lysosomal storage disorder that causes rapid neurodegeneration (reviewed in 12,13). Inactivating mutations in the gene encoding palmitoyl-protein thioesterase-1 (PPT1) (14) are the genetic basis of INCL (15). PPT1 catalyzes the cleavage of thioester linkages on cysteine residues of polypeptides that undergo S-acylation (palmitoylation) and facilitates degradation and/or recycling of these fatty-acylated proteins. Thus, PPT1 deficiency leads to abnormal accumulation of S-acylated proteins (ceroid) in lysosomes causing INCL pathogenesis. The PPT1-knockout (PPT1-KO) mouse (16,17) recapitulates virtually all the clinical and pathological features of human INCL including progressive brain atrophy and infiltration of phagocytes (18). However, the molecular signal(s) that mediates the recruitment of these phagocytic cells in the brain remains undefined.

We report here that in the PPT1-KO mouse brain there is an age-related increased expression and activation of cytosolic phospholipase A2 (cPLA2), which catalyzes the production of elevated levels of lysophosphatidylcholine (LPC), previously reported to be a ‘lipid attraction signal’ for phagocyte migration in vitro. We also show that increased production of LPC positively correlates with elevated levels of expression of phagocyte marker genes in the brain of the PPT1-KO mouse. Further, using an in vitro assay, we demonstrate that conditioned media derived from culturing PPT1-KO brain slices promote chemotactic migration of phagocytes. More importantly, conditioned media obtained from culturing PPT1-KO brain slices that were pretreated with a cPLA2 inhibitor, failed to manifest such chemotactic activity. Taken together, these results for the first time demonstrate that increased LPC production in the brain of PPT1-KO mice is at least one of the mediators of phagocyte recruitment in vivo, raising the possibility that reduction of LPC levels may have beneficial effects on INCL patients.

RESULTS

Elevated levels of expression of phagocyte markers in the brains of PPT1-KO mice

Cell death by apoptosis in the brain biopsy tissues (19) and in cultured cells from INCL patients (20) as well as in the brains of the PPT1-KO mice (16,18) have been previously reported. Recently, we demonstrated that neurons in human INCL as well as those in the PPT1-KO mouse brain undergo ER stress that activates the unfolded protein response (UPR) mediating caspase activation and apoptosis (21,22). Although it is well known that apoptosis is followed by rapid infiltration of phagocytic cells for efficient removal of the cell corpses (3), the molecular signal(s) for recruitment of these cells in vivo, until now remained undefined. To gain insight into the mechanism(s) of phagocyte infiltration in the brains of PPT1-KO mice, we first determined the levels of expression of various phagocyte markers at 1-, 3- and 6-month old PPT1-KO and WT mice. These markers included glial fibrillary acidic protein (GFAP) (23) and galectin-3 (24,25). The results of real-time PCR using total RNA from the brains of PPT1-KO mice and those of their WT littermates show that the levels of GFAP (Fig. 1A), galectin-3-mRNAs (Fig. 1B) are significantly elevated in the PPT1-KO mouse brain in an age-dependent manner. Consistent with these results, western blot analyses show that the levels of GFAP (Fig. 1C) and galectin-3 proteins (Fig. 1D) are markedly elevated in the brains of the PPT1-KO mice but not in those of their WT littermates. Further, the results of immunohistochemical analyses show an age-dependent elevation in the number of GFAP-positive (Fig. 2A) and galectin-3-positive (Fig. 2B) cells in the brains of PPT1-KO mice but not in those of their WT littermates. In addition to these markers, we also determined the mRNA levels of CD1a, CD11c, CD40L, CD68, DC Sign, langerin, MIP-1α and major histocompatibility complex II (MHC II) which are also associated with phagocytic cells (8,26). The results show that the mRNA levels of all of these marker genes are significantly elevated in the brains of PPT1-KO mice in an age-dependent manner (Supplementary Material, Fig. S1A–H). Interestingly, the elevated expression of these
markers uncovered in the present study and age-dependent increased neuronal apoptosis in the PPT1-KO brain reported previously (20,21) appear to be related. Taken together, these results show an age-related increase in the recruitment of phagocytic cells into the brains of the PPT1-KO mice, most likely for quick and efficient removal of the apoptotic cell corpses.

Elevated levels of LPC in the brain of PPT1-KO mice

A central element of apoptotic cell removal by activated phagocytes is the release of ‘recruitment signals’ by apoptotic cells (3). These signals stimulate the migration of phagocytes to the close proximity of the apoptotic cells so that these cell corpses could be recognized, engulfed and promptly removed. It has been recently reported that LPC, released by cells that are chemically induced to undergo apoptosis, is a potent lipid attraction signal for phagocytes in vitro (27). However, to our knowledge the chemical nature of the phagocyte ‘attraction signal’ using an animal model of a neurodegenerative storage disease has not yet been defined. Thus, we sought to determine whether LPC levels in the PPT1-KO mouse brain are elevated with age and whether the increased LPC levels coincide with the elevated levels of expression of phagocyte markers. Accordingly, we first determined the LPC and 18:1 LPC levels in the brains of PPT1-KO and WT mice at 1, 3 and 6 months of age using electrospray ionization mass spectrometry (28). Our results show that compared with WT littermates, the levels of both total LPC (Fig. 3A) and 18:1 LPC (Fig. 3B) are markedly elevated in the brains of PPT1-KO mice in an age-dependent manner. Interestingly, the increased levels of expression of the phagocytic marker genes also appear to coincide with the age-dependent elevation of LPC levels in the brains of PPT1-KO mice. This coincides with the elevated levels of neuronal apoptosis in the brains of the PPT1-KO mice as previously reported (21,22). Taken together, these results indicate that in the PPT1-KO mice there is a link between age-dependent elevation in neuronal apoptosis, increased levels of LPC and high levels of phagocyte infiltration in the brains of PPT1-KO mice, which suggest that LPC is most likely the in vivo ‘lipid attraction signal’ for phagocyte infiltration.

Elevated expression and activation of cPLA2 mediates LPC production

How might LPC be produced in the brains of PPT1-KO mice? Current evidence indicates that the major pathway of LPC production is the hydrolysis of membrane-associated phospholipids by phospholipases A2 (PLA2s), which represent a large group of ubiquitous acyl-esterases (for review see 29). Using in vitro experiments, it has been previously demonstrated that LPC production in cells, chemically induced to undergo apoptosis, is mediated by the activation of calcium independent PLA2 (iPLA2) (26). Accordingly, we first determined the mRNA levels of iPLA2 in the brains of 1, 3 and 6-month-old PPT1-KO and WT littermates by real-time PCR. The results show that while in the brains of 1- and
3-month-old PPT1-KO and WT mice the iPLA2-mRNA levels are virtually identical, those in the brains of 6-month old PPT1-KO mice are markedly lower than in those of their WT littermates (Fig. 3C). Although the reason for this decline in iPLA2-mRNA levels is unclear, it has been reported that at 6 months of age, the PPT1-KO mice manifest considerable neuronal death by apoptosis (18,21) and may contribute to this apparent decline. Since INCL is a lysosomal storage disease, we also determined the mRNA levels of lysosomal PLA2 in the brains of 1-, 3- and 6-month-old WT and PPT1-KO mice. The results show virtually no difference in the expression of this PLA2 in the brains of WT and PPT1-KO mice (Fig. 3D). Taken together, these results show that neither iPLA2 nor lysosomal PLA2 are involved in the production of LPC in the brains of PPT1-KO mice.

Activation of cPLA2 mediates LPC production in the brains of the PPT1-KO mice

We previously reported that the neurons in the brains of PPT1-KO mice as well as those in human INCL undergo endoplasmic reticulum (ER) stress leading to the activation of caspase-12 and caspase-4, respectively, mediating apoptosis (21,22). Since the ER is the major Ca\textsuperscript{2+} storage organelle in the cell, ER-stress may also lead to excessive Ca\textsuperscript{2+} release and disrupt Ca\textsuperscript{2+} homeostasis. Thus, we rationalized that in this environment Ca\textsuperscript{2+}-dependent cPLA2 (30) is likely to be activated. Accordingly, we determined the cPLA2-mRNA levels in the brains of PPT1-KO and their WT littermates. The results show that when compared with the WT littermates the cPLA2-mRNA levels are markedly elevated in the brains of PPT1-KO mice (Fig. 4A). Consistent with these results,
western blot analysis shows elevated levels of cPLA2 protein in the brains of 3-month-old PPT1-KO mice (Fig. 4B, middle row) although in the brains of the 6-month-old PPT1-KO mice the cPLA2-protein levels are markedly reduced. The reason for this decline in cPLA2 protein is not yet clear. However, at 6 months of age the PPT1-KO brains are infiltrated with numerous activated phagocytic cells (i.e. microglia, astroglia) that may elaborate proteinases and degrade cPLA2 protein. Alternatively, decreased cPLA2 protein levels at 6 months of age may be due to the fact that at this age numerous neurons undergo apoptosis and death of these cells may reduce the cPLA2 protein levels.

To delineate whether the cPLA2 in the PPT1-KO brain is active, we determined the phosphorylation of this enzyme on serine-505 as it has been reported that phosphorylation of this serine residue is required for cPLA2 activation (30,31). The results of western blot analysis using phospho-cPLA2 antibody show that when compared with the brains of WT mice phospho-cPLA2 levels in the PPT1-KO brains are elevated in an age-dependent manner (Fig. 4B, upper row). These results suggest that in the brains of PPT1-KO mice cPLA2 expression and activation mediate the production of elevated levels of LPC, which most likely provides the ‘attraction signal’ for phagocyte infiltration.

cPLA2 mediates release of phagocyte chemoattractant in the brain culture medium

To further confirm that cPLA2 activity in the PPT1-KO brain is required for the production of phagocyte ‘recruitment-signal’, we performed in vitro migration assays using a murine phagocytic cell line, RAW264.7. We used conditioned media generated by culturing brain slices either from the PPT1-KO mice or from their WT littermates to determine whether such conditioned media stimulate chemotaxis in the migration assay (Fig. 4C). We found that while the conditioned media from WT mice phospho-cPLA2 levels in the PPT1-KO brains are elevated in an age-dependent manner (Fig. 4D-1), those from PPT1-KO brains vigorously stimulate the migration of these cells (Fig. 4D-2). Most importantly, PPT1-KO brain slices, pretreated with a cPLA2 inhibitor, arachidonyl trifluoromethyl ketone (ATK), yielded conditioned media that showed virtually no stimulation of phagocyte migration (Fig. 4D-3).

It has been reported that iPLA2 may activate cPLA2 and indirectly stimulate LPC production. Because ATK may inhibit iPLA2 in addition to inhibiting cPLA2 activity, we sought to determine what effects a specific iPLA2 inhibitor alone may have on phagocyte migration. Accordingly, we tested conditioned media obtained from culturing PPT1-KO brain slices pretreated with an iPLA2-inhibitor, HEISS. The results show that conditioned medium, obtained from culturing HEISS-pretreated PPT1-KO brain slices, shows full stimulatory activity on phagocyte migration (Fig. 4D-4). These results strongly suggest that the production of chemoattractant in PPT1-KO brain slices is mediated by activated cPLA2. As a positive control, we used fresh culture medium containing 18:1 LPC, previously reported to be a potent chemoattractant for phagocytes in vitro (27). The results show that 18:1 LPC induces high levels of phagocyte migration (Fig. 4D-5), which is comparable to the ones observed with conditioned medium derived from culturing PPT1-KO brain slices. Since cPLA2 catalyzes the release of arachidonic acid (AA) in addition to LPC, we used fresh culture medium containing AA as a control. The results show that AA is ineffective in stimulating phagocyte migration (Fig. 4D-6). We also quantitated the cells migrated in response to various conditioned media and chemoattractants and the results are shown in Figure 4E. In order to rule out that the observed inhibition of chemotactic activity in the conditioned medium obtained from culturing brain slices that were pretreated with cPLA2 inhibitor is not due to the direct toxic effect of this inhibitor, we performed trypan blue dye exclusion test of the cells from brain slices before and after inhibitor treatment. The results show that there was virtually no difference between the percentage of viable cells (87–88%) in untreated and cPLA2 inhibitor-treated brain slices. This suggests that the abrogation of chemotactic activity in conditioned media from cPLA2 inhibitor-treated PPT1-KO brain slices is unlikely to be due to cell death induced by the inhibitor. Taken together, these results suggest that the brains of PPT1-KO mice release ‘attraction signal’ for phagocytes in the conditioned media and that the production of this chemoattractant (most likely LPC) requires cPLA2 activation.

DISCUSSION

In this study, we sought to determine the molecular mechanism(s) by which the activated phagocytes infiltrate the brains of PPT1-KO mice. This mouse model of INCL recapitulates virtually all clinical and pathological features of human INCL (16–18). It has been reported that neuronal death in this disease is caused by apoptosis (32). We recently reported that apoptosis in the brain tissues from PPT1-KO mice (21) and those from human INCL (22) is caused by ER stress that activates caspase-12 and caspase-4, respectively. Moreover, ER stress also activates mitochondrial pathway of apoptosis via caspase-9 activation contributing to rapid neurodegeneration in this disease (33). ER-stress mediated neurotoxicity by activation of caspase-12 in prion diseases has been reported (34). The results of our present study show that elevated levels of expression as well as increased phosphorylation of serine-505 on cPLA2 indicates activation of this enzyme in the brains of PPT1-KO mice. This increased activation also correlates with the increased levels of LPC in an age-dependent manner. Using in vitro assays, it has been previously reported that LPC is a potent attraction signal for phagocytes (27). However, until now, it remained unclear whether LPC can mediate phagocyte infiltration in vivo. Our results for the first time show that LPC, generated by cPLA2 catalysis, most likely mediates phagocyte infiltration in the brains of mice lacking PPT1. How might cPLA2 get activated in the PPT1-KO brain? Although the mechanism by which PPT1-deficiency leads to cPLA2 activation remains unclear, it is possible that ER stress disturbs Ca$^{2+}$ homeostasis (reviewed in 35) and causes abnormal efflux of calcium. Since cPLA2 is a calcium-dependent enzyme, the increased Ca$^{2+}$ efflux activates this enzyme and catalyzes the release of LPC. While this notion provides a likely hypothesis it requires experimental confirmation.
Phagocyte-activation and infiltration in the brain have been reported in a majority of more than 40 lysosomal storage diseases in which neurodegeneration is a major manifestation (4,5). However, the molecular mechanism(s) of phagocyte migration and brain-infiltration in these diseases, until now, remained undefined. In the brains of PPT1-KO mice, we found elevated levels of expression of genes that are markers of phagocytic cells suggesting phagocyte infiltration of these cells in the brain. Interestingly, it appears that there is a positive correlation between increased apoptosis and elevated levels of these markers. Recently, it has been reported that galectin-3 (25) is expressed on macrophages and may have a role in phagocytosis. We also found that coinciding with the elevated expression of GFAP and galectin-3, the expression of another phagocyte marker-gene, lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) (36), is also elevated in the brains of PPT1-KO mice (Fig. 5A–C). Moreover, the increased expression of these phagocytic markers appears to correlate with advancing age, at which time the brains of the PPT1-KO mice also show elevated levels of apoptosis as previously reported (21,22). Interestingly, LOX-1 on phagocytes has been reported to bind phosphatidylserine (PS) (37). Because unlike the normal live cells, in apoptotic cells PS is exposed on the outer leaflet of the cell membrane (38,39) and PS provides the ‘eat me’ signal for the phagocytes (40). Thus, the interaction of PS with LOX-1 may promote recognition and engulfment of apoptotic cell corpses (38). Indeed, we previously reported that electron microscopic analyses of the brain tissues from PPT1-KO mice at or above 6 months of age show phagocytes with multiple engulfed apoptotic cells (21). Although under normal circumstances, phagocytes play a beneficial role to the organism by protecting the brain against infection, infiltration of these cells in large numbers may cause neurodegeneration (4,5,41,42). Recently, it has been reported that LPC promotes phagocyte migration in vitro (27) and the results of our present study, for the first time, provide strong evidence to suggest that LPC is also a chemoattractant for phagocytes in vivo at least in the brains of PPT1-KO mice.

It has been reported that the production of LPC by cells chemically induced to undergo apoptosis is catalyzed by calcium-independent iPLA2 (27). In contrast to the results of these in vitro experiments, we found that in the brains of PPT1-KO mice, increased expression and activation of

Figure 5. Expression of PS-binding protein, LOX-1, in the brains of PPT1-KO mice and their WT littermates. (A) LOX-1-mRNA determined by real-time PCR; (B) LOX-1 protein determined by western blot analysis; and (C) cytochemical localization of LOX-1 in the brain tissues (Magnification ×200).
cPLA₂ appear to mediate LPC production. This conclusion is further strengthened by the results of our present experiments demonstrating that conditioned media derived from culturing PPT1-KO brain slices pretreated with cPLA₂ inhibitor, but not with iPLA₂ inhibitor, abrogates the phagocyte chemotactic activity. The apparent discrepancy between the results of Lauber et al. (27), and those of our present experiments is most likely due to: (a) inherent differences between in vitro and in vivo experimental models; (b) between the cell-types studied in the two experimental systems; and (c) between the methods of induction of apoptosis: chemically-induced (27) versus ER stress-induced (in PPT1-KO mice). While it is not yet clear what triggers cPLA₂ activation in the brains of PPT1-KO mice, it has been suggested that disruption of Ca^{2+}-homeostasis induced by ER stress (reviewed in 35,43), may lead to neuronal cell death. Consistent with this hypothesis, a recent report indicates that in glomerular epithelial cells, ER stress causes elevated levels of cytosolic free-Ca^{2+} level, which activates cPLA₂ (44) catalyzing the production of LPC.

A proposed model for rapid brain atrophy in INCL

One of the hallmarks of INCL pathogenesis is the rapid progression of cerebrocortical atrophy. How might brain atrophy in INCL occur with such rapidity? We propose a model (Fig. 6) to explain this phenomenon. In this model, the ER-stress mediated activation of UPR leads to apoptosis of PPT1-deficient cells (21,22). The results of our present study in conjunction with those previously reported (27) demonstrate that dying apoptotic cells release LPC. We found that the production of LPC in the PPT1-KO brain is catalyzed by cPLA₂ activation, providing recruitment signal for phagocyte infiltration. The phagocytes also express LOX-1, which is known to recognize PS on apoptotic cell corpses (36,37) facilitating engulfment. The infiltrated PPT1-deficient phagocytes engulf apoptotic cell corpses that contain large quantities of S-acylated (palmitoylated)-proteins. Due to the lack of PPT1 activity, the phagocytes in the brains of PPT1-KO mice are unable to degrade and/or recycle the engulfed cell corpses because acylated proteins are refractory to degradation by lysosomal proteinases. Thus, the phagocytes may become engorged with undegraded cell corpses containing large quantities of S-acylated proteins, causing further stress. In response, these phagocytes elaborate toxic substances such as tumor necrosis factor-α (TNF-α), which are capable of causing death to the adjoining viable cells, thereby further accelerating the progression of brain atrophy in INCL. Consistent with this hypothesis, we found markedly elevated levels of TNF-α-mRNA and protein in the brains of PPT1-KO mice but not in those of their WT littermates (data not shown).

INCL is a uniformly fatal disease for which currently there is no effective treatment. The results of our previous studies demonstrated that PPT1-deficiency in neurons causes ER stress mediating the activation of UPR and apoptosis (21,22). In the present study, we demonstrate for the first time that LPC is at least one of the molecular signals that mediate recruitment of phagocytic cells in the brains of PPT1-KO mice contributing to INCL neuropathology. Since excessive phagocyte infiltration may cause injury to viable neurons (7,9), we propose that inhibition of cPLA₂ activity to suppress LPC production may have therapeutic potential for INCL.

MATERIALS AND METHODS

PPT1-KO mice and genotyping

PPT1-KO mice were generated by gene targeting in ES cells and characterized in the laboratory of Dr S.L. Hofmann as previously described (16,18). 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.

RNA isolation and real-time PCR analysis

Total RNA from the brains of PPT1-KO and control mice was isolated using TriZol reagent (Invitrogen) and further purified by QIAGEN RNeasy Mini Kit and treated with DNase (DNase I, 30 U/μg total RNA) (Qiagen) then reverse-transcribed using SuperScript III First-Strand Synthesis System (Invitrogen). Expression of mRNA was quantitated using SYBR Green PCR Master Mix, performed with ABI Prism 7000 Sequence Detection System (Applied Biosystems) with cDNA equivalent to 100 ng of total RNA for PLAX₂s (i.e. iPLA₂, iPLA₂ and cPLA₂) and 10 ng of total RNA for other genes. The primers used for these genes are presented in Table 1. The data were analyzed using ABI Prism Software version 1.01 (Applied Biosystems). The final data were normalized to β-actin and presented as fold change in PPT1-KO mice when compared with those of WT littermates. Each experiment was repeated at least three times for the PPT1-KO and WT mice and the results are expressed as mean ± S.D. Data are analyzed by Student’s t-test and a P-value <0.05 is considered significant.

Western blot analyses

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 and/or phosphatase-inhibitor cocktails (Sigma–Aldrich). Total protein of 20 μg from each sample was resolved by SDS–PAGE, electrotransferred to polyvinylidene fluoride membrane (Immobilon P, Millipore Corporation) and immunodetected. The primary antibodies against the following polypeptides were used: anti-GFAP (1:1000) from Affinity BioReagents, anti-cPLA₂ (1:1000) and anti-LOX-1 (1:500) from Santa Cruz Biotechnology, anti-β-actin and anti-phospho-cPLA₂ (1:1000) from Cell Signaling. The secondary antibodies used in the study are: goat anti-rabbit IgG, rabbit anti-goat IgG and rabbit anti-mouse IgG (Santa Cruz Biotechnology). Chemiluminescent detection was performed using ECL system (Amersham-Pharmacia Biotech) according to the manufacturer’s instructions.
Figure 6. A proposed model for phagocyte infiltration and rapid neurodegeneration in INCL. PPT1-deficiency causes ER-stress in neurons mediating the activation UPR and apoptosis. The activation of cPLA$_2$ in cells undergoing apoptosis catalyzes the release of LPC, an ‘attraction signal’ for phagocytes, which mediates recruitment of these cells in the brain. Upon arrival in the vicinity of the apoptotic neurons, these cells recognize PS on the outer leaflet of apoptotic cell membrane via LOX-1, a phagocyte-expressed protein, and engulf the cell corpses. PPT1-deficient apoptotic cells contain large amounts of acylated-proteins, which are not degraded or recycled by PPT1-deficient phagocytes. Thus, the phagocytes become engorged with undegraded apoptotic cell corpses. These phagocytes then release toxic substances such as TNF-$\alpha$, which causes further damage to the adjoining viable cells leading to rapid brain atrophy in INCL.
The brain slices (PLA2-inhibitor-treated or untreated) were set-up as described above. At the end of 48 h of culture To determine the cell viability in the brain slices, the cultures Trypan blue dye exclusion test

medium containing 18:1 LPC as the 100%. into account the number of cells migrating in response to various conditioned media was calculated taking

inverted microscope. The percentage of cells migrating in the lower chamber were stained and counted using a Zeiss

for 48 h at 37°C. In another set of cultures, the brain slices were first pre-incubated in medium containing 10 μM ATK (Cayman Chemical Company), which inhibits both cPLA2 and iPLA2, or 1 μM HELSS (Cayman), an iPLA2-inhibitor. After 2 h of incubation the brain slices were washed twice with fresh Opti-MEM without any additives and further incubated for 48 h in Opti-MEM containing 4 mM glutamine, 1.75% BSA and antibiotics. The conditioned media were collected, filtered and frozen at −80°C until use. Murine phagocyte cells, RAW264.7 cells, were loaded into the upper chamber of the Matrigel™-Invagination Chambers (Beckton Dickinson) and the migration assay (Fig. 4C) was performed according to the supplier’s protocol. Briefly, the lower chambers contained various conditioned media from brain slice cultures or media containing 12:1 LPC or AA as controls. They were incubated for 48 h at 37°C and cells migrating to the filter surface facing the lower chamber were stained and counted using a Zeiss inverted microscope. The percentage of cells migrating in response to various conditioned media was calculated taking into account the number of cells migrating in response to medium containing 18:1 LPC as the 100%.

Trypan blue dye exclusion test

Generation of conditioned media and phagocyte-migration assays

Under sterile conditions, the brains of 6-month-old PPT1-KO mice and those of their WT littermates were cut into 2 mm thick slices. The brain slices were incubated in Opti-MEM (Invitrogen) containing 4 mM glutamine and 1.75% bovine serum albumin (BSA) and antibiotics for 48 h at 37°C. In another set of cultures, the brain slices were first pre-incubated in medium containing 10 μM ATK (Cayman Chemical Company), which inhibits both cPLA2 and iPLA2, or 1 μM HELSS (Cayman), an iPLA2-inhibitor. After 2 h of incubation the brain slices were washed twice with fresh Opti-MEM without any additives and further incubated for 48 h in Opti-MEM containing 4 mM glutamine, 1.75% BSA and antibiotics. The conditioned media were collected, filtered and frozen at −80°C until use. Murine phagocyte cells, RAW264.7 cells, were loaded into the upper chamber of the Matrigel™-Invagination Chambers (Beckton Dickinson) and the migration assay (Fig. 4C) was performed according to the supplier’s protocol. Briefly, the lower chambers contained various conditioned media from brain slice cultures or media containing 12:1 LPC or AA as controls. They were incubated for 48 h at 37°C and cells migrating to the filter surface facing the lower chamber were stained and counted using a Zeiss inverted microscope. The percentage of cells migrating in response to various conditioned media was calculated taking into account the number of cells migrating in response to medium containing 18:1 LPC as the 100%.

Trypan blue dye exclusion test

To determine the cell viability in the brain slices, the cultures were set-up as described above. At the end of 48 h of culture the brain slices (PLA2-inhibitor-treated or untreated) were washed two times with HBSS and pipetted up and down three to four times to break-up the tissues. Cell suspension of 100 μl was mixed with an equal volume of 0.4% Trypan blue stain (Biowhittaker, Walkersville, MD, USA). After incubating for 5 min at room temperature, the 50 μl of cell suspension was placed onto a clean glass slide and a cover slip was placed on it. The cells were analyzed using a Zeiss inverted microscope. The number of cells taking up Trypan blue and the ones that excluded the dye were counted and the percent of dye-excluding viable cells determined.

Immunohistochemistry

The brain tissues were either fixed in 3.7% paraformaldehyde or flash-frozen and histological sections prepared. The sections were incubated overnight with GFAP antibody (1:200; Affinity Bioreagents) and galectin-3 antibody (1:200; R&D Systems) at 4°C. After washing three times with 1 × PBS, the sections were incubated with anti-mouse or anti-goat biotinylated secondary antibody (1:500; Vector Laboratories) for 1 h. The washed sections were then incubated with ABC complex by following the manufacturer’s protocol (Vector Laboratories).

Transmission electron microscopy

The brain tissues were fixed in 2.5% glutaraldehyde in sodium phosphate buffer. After 2 h the tissues were washed with Millonig’s phosphate buffer once and kept in the same buffer until final processing. Thin sections of the brain tissues were stained with lead citrate and uranyl acetate and examined with a LEO 912 electron microscope by JFE Enterprises (Brookeville, MD, USA).

Electrospray ionization mass spectrometry

Lipids were extracted from the brain tissues of PPT1-KO mice and their littermates at 1-, 3- and 6-months of age and analyzed by electrospray ionization mass spectrometry using a Quattro Ultima Triple Quadrupole ESI-MS (Micromass Inc.) as previously described (28).

Table 1. Primers for quantitative real-time RT-PCR

| GFAP       | Forward: 5’-AACCGACCTGGACACCCAAAATC-3’ | Reverse: 5’-CTCCCCAGTGCTTCTTACACG-3’ |
| Galectin-3 | Forward: 5’-CACAAATCATGGGACAGTGA-3’  | Reverse: 5’-TCCCTTTCTGAAATCTTCTG-3’ |
| LOX-1      | Forward: 5’-TCAGAACCTCCAAGGCCCT-3’   | Reverse: 5’-GGGTGTGCTTTCTTTCCCT-3’ |
| TNF-α      | Forward: 5’-TCATGCCACCCATCAAAGGA-3’  | Reverse: 5’-ACGAGCTAATTCTCACAT-3’ |
| iPLA2      | Forward: 5’-AACAGTGCCTCTCCGTTGTC-3’  | Reverse: 5’-CAGCCGACTACCTCCACAT-3’ |
| L-PLA2     | Forward: 5’-GTTTCGGAAGGTGCTGGTCA-3’  | Reverse: 5’-TTGCCAATCTCTCTGCAA-3’ |
| cPLA2      | Forward: 5’-CTGCAAGGGCCGAGTGACA-3’   | Reverse: 5’-TCGCCACTACCTCTCGCAA-3’ |
| β-actin    | Forward: 5’-ACGGCCAGGTCTACATTTG-3’   | Reverse: 5’-TGGAAAAAGAGCCTCAGGG-3’ |
Statistical analysis

Results are expressed as the mean ± SD of at least three determinations. Data are analyzed by Student’s t-test using Excel Office 2000 (Microsoft) and a P-value of <0.05 were considered statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

REFERENCES


