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Studies on Plasmalogen-Selective Phospholipase A_2 in Brain

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Abstract Plasmalogen-selective phospholipase A_2 (PlsEtn-PLA_2) has been purified from pig brain using multiple column chromatographic procedure. The purified enzyme migrates as a single band on polyacrylamide. It is stimulated by Triton X-100 and inhibited by sodium deoxycholate. Purified PlsEtn-PLA_2 is inhibited by iodoacetate, and this inhibition can be prevented by β-meracaptoethanol. Treatment of neuronal cell cultures with kainic acid stimulates PlsEtn-PLA_2 activity in a dose-dependent manner, and this stimulation can be blocked by Ly294486, a selective kainic acid receptor antagonist. Activities of PlsEtn-PLA_2 are markedly increased in plasma membrane and synaptosomal plasma membrane fraction prepared from nucleus basalis and hippocampal region of brains from Alzheimer disease patients compared to age-matched controls. It is proposed that accumulation of ceramide and increased expression of cytokines may be responsible for the stimulation of PlsEtn-PLA_2 in Alzheimer disease.

Keywords Phospholipase A_2 · Alzheimer disease · Plasmalogen · Eicosanoids · Docosanoids · Ceramide · Kainic acid toxicity

Introduction

Phospholipases A_2 (PLA_2, EC 3.1.1.4) are a superfamily of enzymes that specifically hydrolyze fatty acid from the *sn*−2 position of glycerol moiety of neural membrane glycerophospholipids to produce a free fatty acid and lysophospholipid. Arachidonic acid is oxidized to eicosanoids, and lysophospholipids are either reacylated to native phospholipids or converted to platelet activating factor [1]. Recent advances have led to the identification of more than 20 mammalian intracellular PLA_2 isoforms, which are subdivided into two subfamilies: (a) intracellular group of enzymes and (b) extracellular group of enzymes. The intracellular group of enzymes include cytosolic PLA_2 ($cPLA_2$), calcium-independent PLA_2 ($iPLA_2$), and plasmalogen-selective PLA_2 (PlsEtn-PLA_2), whereas extracellular PLA_2 group includes secretory PLA_2 ($sPLA_2$) [1]. These enzymes are involved in the synthesis of lipid mediators that have been implicated in fundamental cellular responses including growth, differentiation, adhesion, migration, secretion, and apoptosis [2, 3].

Among glycerophospholipids, plasmalogens are unique. They contain a vinylether linkage at the *sn*−1 position of glycerol moiety. At the *sn*−2 position, plasmalogens contain either docosahexaenoic acid (DHA) or arachidonic acid (ARA) [4–6]. Plasmalogens represent about 30% of ethanolamine phospholipids in the brain. The release of DHA or ARA from plasmalogen is catalyzed by plasmalogen-selective phospholipase A_2 (PlsEtn-PLA_2). This enzyme has been partially purified and characterized from bovine brain [7–9]. Based on plasmalogen turnover studies in the brain, it is proposed that interaction of agonist with receptors at the neural cell surface results in the stimulation of PlsEtn-PLA_2 and generation of lysoplasmalogen and DHA [10]. Lysoplasmalogen is either reacylated to native plasmalogen or hydrolyzed by lysoplasmalogenase [10], and DHA is metabolized to docosanoids [11, 12]. The release of ARA from PtdCho is catalyzed by cytosolic phospholipase A_2 ($cPLA_2$) [2, 5]. The main objective of

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this review is to describe purification and characterization of PlsEtn-PLA_2 from pig brain. Results on the effect of kainic acid neurotoxicity on PlsEtn-PLA_2 activity in neuron-enriched cultures and activity of PlsEtn-PLA_2 in Alzheimer disease (AD) patients are also presented.

Purification of PlsEtn-PLA_2 from Pig Brain

Assay conditions and purification of bovine brain PlsEtn-PLA_2 have been described earlier [7, 10]. Pig brain PlsEtn-PLA_2 can be separated from cytosolic PLA_2 ($cPLA_2$) and secretory PLA_2 ($sPLA_2$) by Sephadex G-75 column chromatography in the presence of 1 M KCl. Multiple column chromatographic procedure utilizing Sephadex G-75, DEAE Sephadex, and phenyl-agarose results in 2,000-fold purification from pig brain homogenate (Table 1) with a recovery of 12%. The purified pig brain PlsEtn-PLA_2 moves as a single band on polyacrylamide gel with a molecular mass of 39 kDa and does not require calcium for its activity. The purified enzyme preparation was very unstable. It loses all its activity in 2 days at −80°C or on ice. Attempts to stabilize enzyme using glycerol, phospholipids, cholesterol, detergents, and various salts have been unsuccessful. Properties of purified enzyme were determined using freshly prepared enzyme from pig brain.

Properties of Purified Pig Brain PlsEtn-PLA_2

Low concentrations of ATP and ADP have no effect on PlsEtn-PLA_2 activity. However, freshly prepared pig brain PlsEtn-PLA_2 is markedly inhibited by ATP and ADP above 2 mM, and AMP and cAMP have no effect on enzymic activity at or above 2 mM (Table 2). CTP, GTP, CDP, and GDP (2–5 mM) have no effect on PlsEtn-PLA_2 activity. Bovine brain PlsEtn-PLA_2 is stimulated by Triton X-100, octylglucoside, and Tween-20 (Fig. 1a). Anionic detergents, such as sodium deoxycholate and sodium taurocholate, inhibit PlsEtn-PLA_2 in a dose-dependent manner (Fig. 1b). PlsEtn-PLA_2 is also inhibited by iodoacetate (Fig. 2a), and this inhibition is reversed by mercaptoethanol (Fig. 2b). Other SH-group blocking agents such as dithio-*bis*-2-nitrobenzoic acid and *N*-ethylmaleimide also inhibit PlsEtn-PLA_2 activity. The inhibitory effect of other SH-group blocking agents can be reversed by dithiothreitol, suggesting that reduced sulfhydryl groups are required for the enzymic activity. Polyvalent anions (citrate > sulfate > phosphate) also inhibit pig brain PlsEtn-PLA_2 activity. The purified pig brain PlsEtn-PLA_2 is also inhibited by metal ions, such as $Ag^{+} > Hg^{2+} > Fe^{3+}$. These properties of pig brain PlsEtn-PLA_2 are similar to bovine brain PlsEtn-PLA_2 [9]. Quinacrine and nordihydroguariaretic acid, the non-specific inhibitors of PLA_2, also inhibit the pig brain PlsEtn-PLA_2.

C2 ceramide stimulates pig brain PlsEtn-PLA_2 activity in a dose-dependent manner up to 2.5-fold (Fig. 3), but at higher concentration, PlsEtn-PLA_2 activity is decreased. C6 ceramide also stimulates PlsEtn-PLA_2, but the extent of stimulation was higher (3–5-fold) than C2 ceramide. Like ceramide, ceramide 1-phosphate also stimulates PlsEtn-PLA_2 activity in a dose-dependent manner. Dihydroceramide, the inactive analog (C2Dcer) of C2 ceramide, has no effect on freshly prepared pig brain PlsEtn-PLA_2 activity. Sphingomyelin, cerebroside, and sulfatide have no effect on purified PlsEtn-PLA_2 activity.

N-Acetylneuraminic acid and GM_3 gangliosides also inhibit freshly prepared pig brain PlsEtn-PLA_2 in a dose-dependent manner (Table 3). However, colominic acid (poly 2, 8-*N*-acetylneuraminic acid) has no effect on its enzymic activity. GM_1 and GM_2 gangliosides have no effect on

Table 2 Effect ATP and ADP on purified pig brain PlsEtn-PLA_2

Concentration (mM)	Relative activity (%) in the presence of ATP	Relative activity (%) in the presence of ADP
Control	100±15	100±12
0.5	95±7.5	93±8.5
1.0	80±7.2	85±7.3
1.5	50±5.7	65±5.5
2.0	20±6.5	45±6.1
2.5	5±1.0	37±4.1

Enzymic activity of freshly prepared enzyme was determined by fluorometric procedure described earlier [7, 10]

Table 1 Purification of PlsEtn-PLA_2 from pig brain

Steps	Total protein (mg)	Total units (nmol/min)	Sp. activity ($nmolmin^{-1}mg^{-1}$)	Yield (%)
12,000× Supernatant	3,060	500	0.163	100
Ammonium sulfate frac.	1,020	440	0.431	88
Sephadex G-75	59	119	2.02	24
DEAE Sephadex	33	100	3.03	20
Phenyl-agarose	7.3	63	8.63	12

Homogenate had specific activity of 0.005 nmol min^{-1} mg^{-1} protein

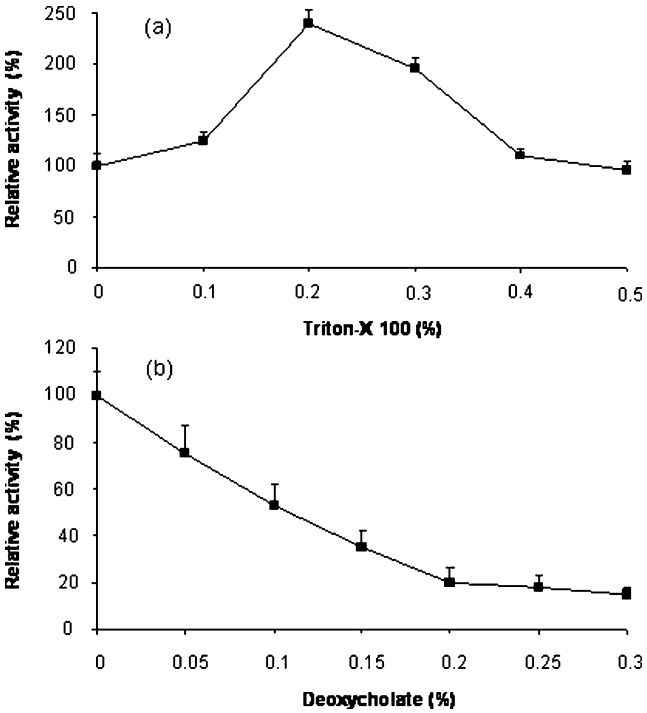

Fig. 1 **a**–**b** Effect of Triton X-100 and deoxycholate on purified pig brain PlsEtn-PLA$_2$ activity. Enzymic activity is determined by an earlier-described procedure [7]. Purified enzyme has specific activity of 8.6 nmol min^{-1}mg^{-1} protein. Reaction mixture without the addition of detergent is used as control and expressed as 100%. The data represent values that are mean±SEM. Each data point represents five determinations

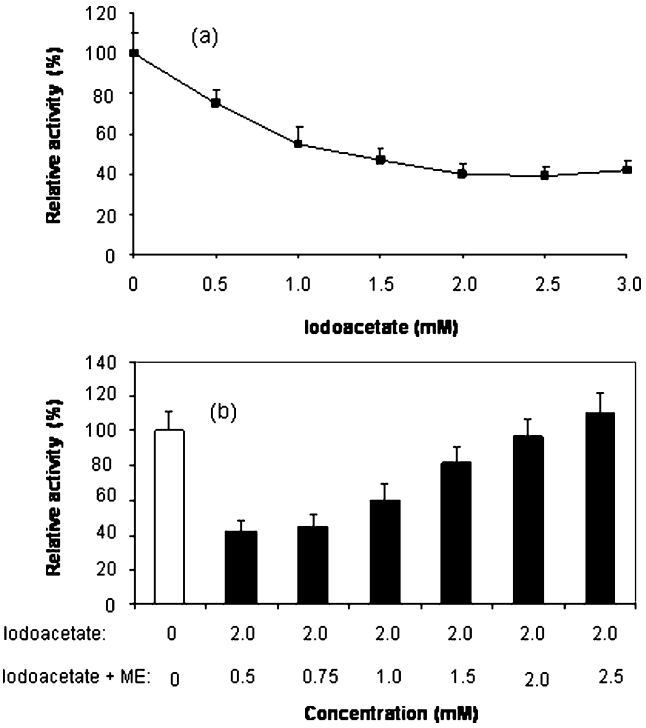

Fig. 2 **a**–**b** Effect of iodoacetate and β-mercaptoethanol on purified pig brain PlsEtn-PLA$_2$ activity. Enzymic activity is determined by an earlier-described procedure [7]. Purified enzyme has specific activity of 8.6 nmol min^{-1}mg^{-1} protein. Reaction mixture without the addition of detergent is used as control and expressed as 100%. The data represent values that are mean±SEM. Each data point represents five determinations

enzymic activity. These properties are similar to partially purified bovine brain PlsEtn-PLA$_2$ activity [8]. The significance of *N*-acetylneuraminic acid and ganglioside-mediated inhibition of PlsEtn-PLA$_2$ is not fully understood. However, interactions of plasmalogen-selective PLA$_2$ with glycoconjugates may be involved in the anchoring of this enzyme to plasma membranes where its substrate is located.

Effect of Kainic Acid on PlsEtn-PLA$_2$ Activity of Neuron-Enriched Cultures from Rat Cortex

Treatment of neuronal cultures with kainate (KA) enhances PlsEtn-PLA$_2$ activity in dose- and time-dependent manner (Fig. 4). NMDA and AMPA treatment has no effect on PlsEtn-PLA$_2$ activity in neuron-enriched cultures from rat cortex. The stimulation of PlsEtn-PLA$_2$ by KA can be prevented by Ly294486 [6-((((1H-tetrazol-5-yl)methyl)oxy) methyl)-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid], a selective KA receptor antagonist (Table 4). AMPA receptor antagonist, CNQX, partially blocks the stimulation of PlsEtn-PLA$_2$ in neuron-enriched cultures. This observation suggests that the stimulation of PlsEtn-PLA$_2$ is a KA receptor-mediated process. The stimulation of PlsEtn-PLA$_2$ by KA in neuronal cultures is also blocked by bromoenol lactone (BEL), a Ca^{2+}-independent PLA$_2$ inhibitor in a dose-dependent manner. Arachidonyltrifluoromethylketone, a cPLA$_2$ inhibitor, has no effect on PlsEtn-PLA$_2$ activity, suggesting that the PlsEtn-PLA$_2$ differs from

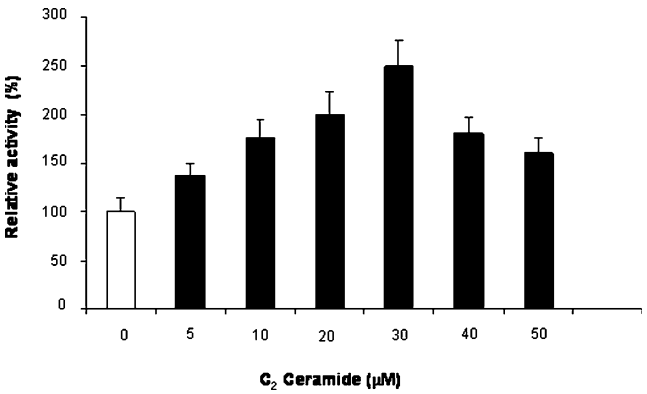

Fig. 3 Effect of C2 ceramide on purified pig brain PlsEtn-PLA$_2$ activity. Enzymic activity is determined by an earlier-described procedure [7]. Purified enzyme has specific activity of 8.6 nmol min^{-1}mg^{-1} protein. Reaction mixture without the addition of detergent is used as control and expressed as 100%. The data represent values that are mean±SEM. Each data point represents five determinations

Table 3 Effect of gangliosides, glycolipids, and *N*-acetyl neuraminic acid on brain PlsEtn-PLA_2

Glycolipid and sugars	Effect
Ganglioside GM_3	Inhibited (IC_{50}, 75.0 μg/ml)
Ganglioside GM_1	No effect
Ganglioside GM_2	No effect
C2 and C6 cerebroside	Stimulated
Ceramide-1-phoshate	Stimulated
Dihydroceramide	No effect
Sulfatide	No effect
N-Acetylneuraminic acid	Inhibited (IC_{50}, 22.0 g/ml)
Colominic acid	No effect

Enzymic activity of freshly prepared enzyme was determined by fluorometric procedure described earlier [7, 10]

Table 4 Effect of KA and Ly294486 on pig brain PlsEtn-PLA_2 activity

Treatment	Concentration (μM)	pmol min^{-1} mg^{-1} protein
Control	–	2.56±0.35
KA	60	16.7±2.3
KA+Ly294486	60+7.5	9.31±1.5
KA+Ly294486	60+15	6.33±1.3
KA+Ly29486	60+30	4.21±0.83
Ly294486	30	2.51±0.42

Enzymic activity is determined by an earlier-described procedure [7]. The data represent values that are mean ± SEM. Each data point represents five determinations

$cPLA_2$ not only in molecular mass and kinetic properties but also in response to inhibitors.

PlsEtn-PLA_2 hydrolyzes plasmalogen molecule into ARA or DHA and lysoplasmalogen. The rate of release of DHA from labeled plasmalogen was three to five times faster than ARA. Very little is known about the interactions between up-stream lipid acyltransferases and down-stream cyclooxygenase, lipoxygenase, and epoxygenase [9, 10]. These interactions may modulate the intensity of signal transduction processes at cellular and subcellular levels [10]. The complexity of this problem becomes obvious when one considers the coupling mechanisms of PlsEtn-PLA_2 with different receptors at cellular and subcellular levels and tries to relate them to neuronal and glial cell functions [10]. In brain, tissue-released ARA is oxidized to prostaglandins, leukotrienes, and thromboxanes [5]. Prostaglandins mediate their action through G protein containing EP_1, EP_2, EP_3, and EP_4 receptors. During oxidative stress, isoprostanes, isoketals, and 4-hydroxynonenal, the non-enzymic oxidative products of arachidonic acid, are also generated in brain tissue [10]. These products are potent pro-oxidants, vasoconstrictors, and mitogens. Actions of a 15-lipoxygenase-like enzyme on DHA produce 17S-resolvins, 10-, 17S-docosatrienes, and protectins [11, 12]. These mediators are collectively called as docosanoids. They are potent endogenous anti-inflammatory and pro-resolving chemical lipid mediators. They antagonize the effects of eicosanoids, modulate leukocyte trafficking, and down-regulate the expression of cytokines in glial cells [10–12]. The specific receptors for these bioactive lipid metabolites occur in neural and non-neural tissues. Non-enzymic oxidation of DHA produces 4-hydroxyhexenal, neuroprostanes, and neuroketals, which act as pro-oxidants [10]. The non-enzymic mediators of DHA and ARA metabolism interact with NF-κB and modulate the expression of proinflammatory gene and cytokines [10–12].

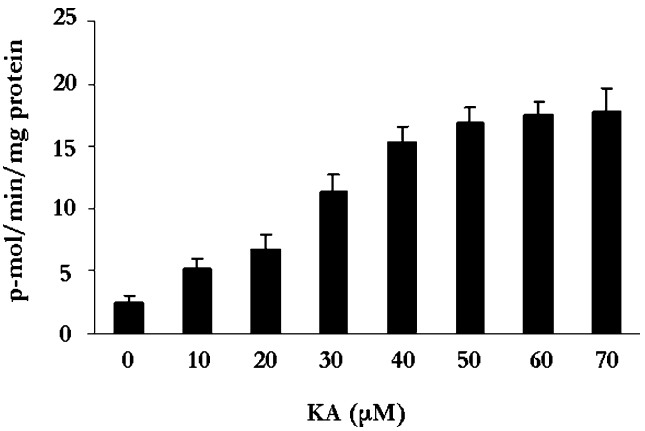

Fig. 4 Effect kainic acid treatment PlsEtn-PLA_2 activity of neuron-enriched cultures from rat cerebral cortex. Enzymic activity is determined by an earlier-described procedure [7]. The data represent values that are mean±SEM. Each data point represents five determinations

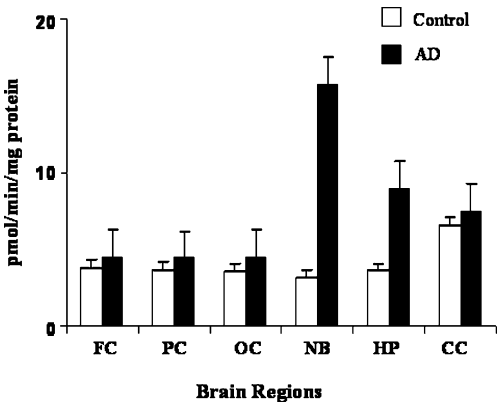

Fig. 5 PlsEtn-PLA_2 activity in synaptosomal plasma membrane fractions prepared from different regions of brains obtained at autopsy from AD patients and age-matched control subjects. *FC* frontal cortex, *PC* parietal cortex, *OC* occipital cortex, *NB* nucleus basalis, *HC* hippocampus, *CC* corpus callosum. Enzymic activity is determined by an earlier-described procedure [7]. The data represent values that are mean±SEM

Activities of Plasmalogen-Selective PLA_2 in Alzheimer Disease

PlsEtn-PLA_2 activity has been determined in plasma membrane and synaptosomal plasma membrane fractions prepared from different regions of brains obtained at autopsy from AD patients and age-matched control subjects. PlsEtn-PLA_2 activity is markedly elevated (3–4-fold) in nucleus basalis and hippocampal (2-fold) regions of AD brain compared to age-matched control subjects (Fig. 5). The cause of the increased PlsEtn-PLA_2 activity is not fully understood. However, it is becoming increasingly evident that ceramide, a lipid mediator of sphingolipid metabolism, stimulates PlsEtn-PLA_2 activity in a dose-dependent manner [13]. So increase in PlsEtn-PLA_2 activity may be due to the accumulation of ceramide in brain from AD patients [14, 15]. Data presented in this study (Fig. 3) indicate that addition of C2 and C6 ceramides to reaction mixture stimulates PlsEtn-PLA_2 activity in a dose-dependent manner. Levels of ceramide are significantly increased in nucleus basalis and hippocampal regions from AD brain compared to age-matched control (Farooqui, unpublished observation). In addition, non-enzymic lipid mediators (neuroprostanes, neuroketal, and neurofurans) of DHA metabolism may interact with NF-κB and facilitate the expression and release of inflammatory cytokines (Fig. 6), such as TNF-α, IL-β, and IL-6, which may further stimulate not only PlsEtn-PLA_2 activity but also other isoforms of PLA_2 including cPLA$_2$ and sPLA$_2$ [16]. Finally, ceramide also stimulates caspases cascade promoting apoptotic cell death (Fig. 6).

Increase in PlsEtn-PLA_2 activity may be responsible for the deficiency of plasmalogens and loss of synapse in AD brain [17–21]. The loss of ethanolamine plasmalogen in neural membranes from AD patients may lead to neural membrane destabilization in several ways: (a) Plasmalogen deficiency may induce changes in the critical temperature necessary for maintaining the stability of lipid bilayer [16, 21], and (b) the generation of lysoplasmalogen in neural membrane may induce changes in membrane fluidity and permeability and allow the influx of external Ca^{2+} via plasma membrane channels [10]. Finally, the loss of plasmalogens also produces impairment of muscarinic cholinergic signals and abnormal β-amyloid precursor protein processing [22–24]. In cell culture systems, lack of plasmalogens impaired intracellular cholesterol distribution, affecting plasma membrane functions and structural changes of endoplasmic reticulum and Golgi cisternae [25]. It is reported that there is a relationship between serum PlsEtn levels, dementia severity, and AD neuropathology. In addition to decrease in brain PlsEtn levels, studies on determination of total serum PlsEtn levels in five independent population collections comprising >400 clinically demented and >350 non-demented subjects indicate that circulating PlsEtn levels are significantly decreased in serum from clinically and pathologically diagnosed AD subjects at all stages of dementia, and the severity of this decrease correlates with the severity of dementia [24]. Furthermore, a linear regression model predicts that serum PlsEtn levels are decreased years before clinical symptoms of AD.

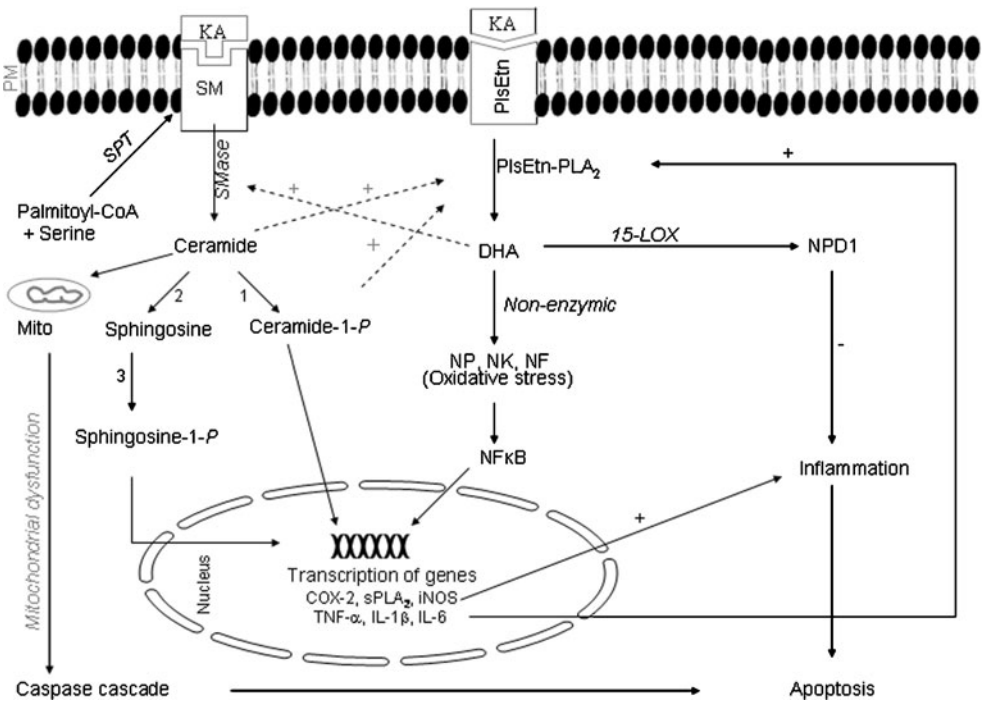

Fig. 6 Interplay between plasmalogen- and sphingolipid-derived lipid mediators in brain sphingomyelin (*SM*), ceramide-1-phosphate (*ceramide-1-P*), sphingosine-1-phosphate (*sphingosine-1-P*; *DHA* (docosahexaenoic acid), neuroprostane (*NP*), neuroketal (*NK*), neurofuran (*NF*), neuroprotectin D_1 (NPD_1), nuclear factor kappa B (*NF-κB*)), 15-lipoxygenase (*15-LOX0*; cyclooxygenase (*COX-2*), secretory phospholipase A_2 ($sPLA_2$), inducible intric oxide synthase (*iNOS*), tumor necrosis factor-α (*TNF-α*), interleukin-1β (*IL-1β*), mitochondria (*mito*), plasma membrane (*PM*))

It remains to be seen whether the increase in PlsEtn-PLA_2 activity correlates with the number of senile plaques or neurofibrillary tangles. It must be admitted that we do not know whether changes in PlsEtn-PLA_2 activity are the cause or consequence of neurodegenerative process or whether changes in PlsEtn-PLA_2 activity are primary or secondary. Furthermore, it is not known whether changes in PlsEtn-PLA_2 are specific for AD, or other neurodegenerative diseases also show similar increase in activity of this enzyme. However, PlsEtn-PLA_2 activity is markedly increased in KA-treated neuronal cell cultures [26] and in ischemic injury in heart disease [10].

In the normal aging brain, neural cells can tolerate mild oxidative stress by upregulating the synthesis of antioxidant defense systems in an attempt to restore the balance. However, severe oxidative stress in AD may cause major interdependent derangement of neural cell metabolic processes due to plasmalogen deficiency, accumulation of lipid mediators, DNA strand beakage, and damage to membrane ion transporters [27]. As stated above, plasmalogens represent between 1/2 and 2/3 of the ethanolamine phospholipids in the brain. During aging, the PlsEtn content in human brain are decreased [16]. Plasmalogens act as antioxidant [28, 29]. They protect polyunsaturated fatty acids from iron-induced lipid peroxidation [30]. Reactive oxygen species preferably interact and affect the vinyl ether function as well as the olefinic acyl residues at the *sn*−2 position of plasmalogens and peroxidize native plasmalogens to oxidized plasmalogens and their oxidation products, such as α-hydroxyaldehydes and plasmalogen epoxides, which accumulate in all chronic visceral and neurodegenerative diseases [10, 31]. In general, peroxidized plasmalogens and peroxidized phospholipids are better substrates for all isoforms of PLA_2 than native plasmalogens and native phospholipids. I propose that in AD, PlsEtn-PLA_2 may be the first PLA_2 that initiates neural injury. Its stimulation may alter neural membrane permeability due the loss of plasmalogens, allowing slow Ca^{2+} influx. This slow Ca^{2+} influx and generation of ceramide may facilitate translocation of cPLA_2 from cytosol to neural membranes and its activation resulting in hydrolysis of neural membrane PtdCho. As concentration of Ca^{2+} reaches in mM, the sPLA_2 may be activated, promoting neural cell injury and death. Thus in injury process sequence, PlsEtn-PLA_2 is situated at the proximal end, cPLA_2 in the middle, and sPLA_2 at the distal end. More studies are needed on the involvement of various forms of PLA_2 in neural injury to test the above scenario.

Conclusion

In brain, the release of DHA from plasmalogens is catalyzed by PlsEtn-PLA_2. Pig brain PlsEtn-PLA_2 has been purified using multiple column chromatographic procedure. The purified enzyme has a molecular mass of 39 kDa. It is stimulated by Triton X-100 and strongly inhibited by sodium deoxycholate. Iodoacetate inhibits pig brain PlsEtn-PLA_2 in a dose-dependent manner, and this inhibition is reversed by β-mercaptoethanol. Pig brain PlsEtn-PLA_2 in inhibited by NANA and GM_3 ganglioside. Treatment of neuronal cultures with kainic acid results in a dose-dependent stimulation of PlsEtn-PLA_2 activity, and this stimulation can be blocked by Ly294485. PlsEtn-PLA_2 is stimulated by ceramide in a dose-dependent manner. Collective evidence suggests that accumulation of ceramide and expression of inflammatory cytokines may be responsible for PlsEtn-PLA_2 stimulation in AD.

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