Plasmalogen-rich foods promote the formation of cubic membranes in amoeba

Chaos under stress conditions

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Abbreviations

CM, cubic membrane; DPA, docosapentaenoic acid; pPC, plasmalogen phosphatidylcholine; pPE, plasmalogen phosphatidylethanolamine; diacyl-PI, diacyl-phosphatidylinositol; TEM, transmission electron microscopy; ROS, reactive oxygen species; Para, Paramecium multimicronucleatum; Tetra, Tetrahymena pyriformis; lysoPC, lysophosphatidylcholine; lysoPE, lysophosphatidylethanolamine; MO, monoolein; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; AD, Alzheimer’s disease

Running heading:

Plasmalogens and stress-induced cubic membranes

Abstract

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Previous studies have indicated that the ability to form cubic membrane (CM), a three-dimensional periodic structure with cubic symmetry, in amoeba (*Chaos carolinense*) under stress conditions depends on the type of food organism supplied prior to cell starvation. The significant increase in docosapentaenoic acid (DPA; C22:5n-6) during the starvation period has been reported to induce CM formation and support *Chaos* cell survival. In this article, we further investigated the lipid profiles of food organisms of the *Chaos* cells to reveal the key lipid components that might promote CM formation. Our results show that the lipids extracted from cells of the native food organism *Paramecium multimicronucleatum* are enriched in plasmalogens. More specifically, plasmalogen phosphatidylcholine (pPC) and plasmalogen phosphatidylethanolamine (pPE) might be the key lipids that trigger CM formation in *Chaos* cells under starvation stress conditions. Unexpectedly, CM formation in these cells is not supported when the native food organism was replaced with plasmalogen-deficit *Tetrahymena pyriformis* cells. Based on a previous lipidomics study on amoeba *Chaos* and the current study on the lipid composition of its food organisms, three key lipids (pPC, pPE and diacyl-PI) were identified and used for liposomal construction. Our *in vitro* study revealed the potential role of these lipids in a non-lamellar phase transition. The negative staining transmission electron microscopy (TEM) data of our liposomal constructs support the notion that plasmalogens may curve the membrane, which in turn may facilitate membrane fusion and vesicular formation, which is crucial for membrane dynamics and trafficking.

**Keywords:** plasmalogen, cubic membrane, starvation stress, cell protection

**Introduction**

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Approximately 20% of phospholipids are plasmalogens in human tissue where they are particularly rich in the brain, heart and immune cells [1]. Plasmalogens are characterized with a unique vinyl-ether bond at sn-1 and a regular ester-bond at sn-2 position of the glycerol backbone, distinguishing them from their diacyl counterparts carrying ester-bond at sn-1 position instead [1]. Despite their abundance in multiple cell types, tissues and organs, the significance of plasmalogens in the biological system remains to be revealed.

The accumulated evidence points that plasmalogens may act as the first-line of sacrificing molecules in preventing oxidative damage. The presence of excessive reactive oxygen species (ROS) and/or hydrogen peroxide (H₂O₂) produced in cells are able to react more readily with plasmalogens as opposed to reacting with other important biomolecules. This may thus prevent lipid peroxidation of the cell membrane and avoid further cell damage [2]. An emerging property of plasmalogens is to promote non-lamellar membrane transformation [3-5]. Glaser and Gross [6] reported that in vitro membrane vesicles with varying amounts of plasmalogens could induce different non-lamellar structure formation, suggesting the role of plasmalogens in facilitating membrane fusion. A recent structural study by Angelova et al. [7] further revealed that plasmalogens could strongly influence the membrane thickness and curvature. Deng et al. [8] reported that a significant increase in plasmalogen phosphatidylcholine (pPC) in amoeba Chaos cells under starvation stress is associated with non-lamellar CM formation. The higher level of pPC in amoeba Chaos cells was observed in Paramecium multimicronucleatum-fed amoeba Chaos cells but not in Tetrahymena pyriformis-fed cells [8]. Interestingly, mitochondrial inner membrane rearrangement into cubic morphology was observed to occur when amoeba Chaos cells were fed with the extracted lipids from either Paramecium multimicronucleatum or polyunsaturated fatty acids, specifically omega-6 DPA [8]. Furthermore, it has been observed that liposome constructs using the extracted lipids from amoeba Chaos cells which have been exclusively fed with Paramecium multimicronucleatum do induce cubic or hexagonal organization in vitro [8]. Based on the above observations, it has been proposed that plasmalogen might promote cubic membranes formation and plasmalogen-rich CM might act as a ‘protective’ shelter to minimize the oxidation of biologically essential macromolecules (lipids and RNAs) [9-11].
Based on the fact that the alteration of plasmalogen level and non-lamellar membrane transformation are linked both in vitro [3-5,7] and in vivo [8], we speculate that there might be a correlation between plasmalogen availability as a result of food supply/deficiency and non-lamellar membrane formation. As such, we investigated the relation between plasmalogen levels of the food supply and non-lamellar membrane transformations using amoeba *Chaos* cells as an experimental model.

Our findings in the study of food supply of amoeba *Chaos* cells and CM formation under starvation and stressed conditions, may help us to understand the molecular mechanism of non-lamellar membrane transformation, vesicle formation and vesicular fusion, especially at synaptic vesicles where plasmalogens are abundant and essential for neuronal function [1,12,13].

**Materials and Methods**

**Cells and reagents**

Amoeba *Chaos* and *Tetrahymena pyriformis* cells were purchased from Carolina Biological Supply Co. *Paramecium multimicronucleatum* cells were a kind gift from Dr. Richard D. Allen’s laboratory [14] and Dr. Masaki Ishida’s laboratory (Nara University of Education, Japan). All other chemicals and reagents unless mentioned otherwise were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Amoeba Chaos mass culture**

The amoeba *Chaos* cell cultures were maintained in an inorganic amoeba medium containing 0.5 mM CaCl$_2$, 0.05 mM MgSO$_4$, 0.16 mM dipotassium phosphate and 0.11 mM potassium dihydrogen phosphate dissolved in MilliQ water [15]. Amoeba *Chaos* cells were kept in large treated Pyrex® baking dishes filled with amoeba medium in darkness, at 22°C to 24°C on the benchtop. Amoeba *Chaos* cells were fed every two to three days only with 7-days old *Paramecium multimicronucleatum* or *Tetrahymena pyriformis* cultures at the late log or early stationary phase of growth [14,16]. Both *Paramecium* and *Tetrahymena* cultures were maintained in growth media...
as previously described [16] and used for amoeba Chaos cells feeding. All-Z-4,7,10,13,16-docosapentaenoic acid (DPA C22:5-6; Sigma-Aldrich Pte. Ltd., Singapore) were dissolved in diethyl ether and added to the amoeba Chaos cell culture medium at a final concentration of 100 μM. 100 μM of DPA and 100μg/ml extracted lipids from Paramecium were added to well-fed amoeba cell cultures respectively, according to the regular feeding schedule [16]. Amoeba Chaos cells were then fixed in 2.5% glutaraldehyde for further transmission electron microscopy (TEM) processing.

Amoeba Chaos cell harvest
Prior to harvesting the amoeba Chaos cells, the cultures were gently washed several times with amoeba medium to remove the food organisms. Individual amoeba Chaos cells were then picked using a disposable Pasteur pipette into a glass beaker with amoeba medium, in which they were allowed to settle to the bottom simply by gravity. The supernatant was siphoned off and the clean amoeba Chaos cells were ready for further processing. No food organisms (Paramecium multimicronucleatum or Tetrahymena pyriformis) were added to amoeba Chaos cells culture for 7 days in starvation treatment to obtain the mitochondria with and without CM, respectively.

Transmission electron microscopy (TEM)
The amoeba Chaos cells (from 7-days starved and fed amoeba Chaos cells (Para/Tetra) cultures) were primarily fixed with 2.5% glutaraldehyde (GA) (Agar Scientific) at 4°C overnight followed by secondary fixation using 1% osmium tetroxide (OsO₄) (Ted Pella, Inc.) for 1 hour at room temperature. Fixed samples were subjected to sequential dehydration by immersion in a graded series of ethanol dehydration steps. Preparations were embedded in Epon-Resin (Pelco, Clovis, CA, USA) and sectioned with approximately 50-70 nm thickness using an ultramicrotome (Leica) and stained in 3% uranyl acetate (Electron Microscopy Sciences) followed by Reynold's lead citrate. The ultrathin sections were viewed and examined under TEM (JEM1010, JEOL Ltd.). For the negative staining TEM studies, 1% of lanthanum nitrate hexahydrate (Sigma) diluted in PBS buffer was used. Samples are air-dried on a copper grid (Tedpella, Inc.) and stained directly using Gadolinium (III) acetate tetrahydrate (Sigma) before viewing under TEM.
**Lipid extraction**

Lipids from *Paramecium multimicronucleatum* or *Tetrahymena pyriformis* were extracted and analyzed for phospholipids including plasmalogens. Phospholipids were extracted following Bligh and Dyer [17]. In brief, the cells were collected and spun down at 196x g with Multifunction benchtop centrifuge BR4i compact series (Inovsolutions: Jouan). The collected cell pellets were separately homogenized in chloroform/methanol (1:2, v/v), and the mixture was vortexed for 30s. Lipids were further extracted at 4°C under vigorous shaking for 10 min. 0.3 ml chloroform and 0.3 ml deionised water (cold) was then added to the mixture and vortex to mix for 30s followed by incubation on ice for 1 min. Phases were separated by high-speed centrifugation at 6700x g for 2 min, and the lower phase was transferred to a fresh tube (extract 1). The residual aqueous phase and cell remnants were re-extracted with 0.6 ml chloroform as described above and the organic (lower) phase was obtained (extract 2). Extracts 1 and 2 were then combined and dried in a vacuum concentrator; SpeedVac (Thermo Savant, Milford, MA, USA), and stored at -20°C. Before analysis, lipids were dissolved in chloroform/methanol (1:1, v/v).

**Analysis of lipids by mass spectrometry**

Following a protocol adapted from Shui *et al.* [18], phospholipids and plasmalogens were quantified using shotgun-tandem mass spectrometry (MS) approach. The lipids are first dissolved in chloroform/methanol (1:1, v/v) and mixed 1:1 with an internal standards solution. The internal standard solution was prepared for the lipid species; lysophosphatidylcholine (lysoPC) and PC diluted in chloroform/ methanol (1:1, v/v) to a total volume of 1 ml. The internal standard solution was prepared for the lipid species PE, PS, PG, and PI species diluted in chloroform/methanol (1:1, v/v) to a total volume of 6 ml. Samples were then introduced into the mass spectrometer using an Agilent 1200 high-performance liquid chromatography (HPLC) system without chromatographic separation. The flow rate was 250 μl/min and the analysis time 1.7 min. The mass spectrometer was an Applied Biosystem Triple Quadrupole/Ion Trap mass spectrometer 4000 trap (Applied Biosystems, Foster City, CA). The lipid species were quantified using multiple reaction
monitoring (MRM) and positive ionization lysoPC and PC or negative ionization PE, PS, PG, and PI.

**Liposome preparation**

Liposome preparation in this study follows the protocol from Avanti Polar Lipids Inc. Synthetic lipids, 18:1 (Δ9-Cis) PC (DOPC) 1,2-dioleoyl-sn-glycero-3-phosphocholine and 18:1 (Δ9-Cis) PE (DOPE) 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (Avanti Polar Lipids Inc.) were dissolved in chloroform. The solvent was then removed using a centrifugal evaporator (Joun RC1022). This was followed by hydration of the lipid cake/film by adding deionised water and vigorous agitation for 1 hr. At this point, large multilamellar vesicles were obtained. After the hydration was successfully completed, sizing of lipid suspension was performed to obtain small unilamellar vesicles via sonication for 15 min using a sonicator: ultrasonic bath (VWR international) for TEM analysis. In this study, synthetic plasmalogen PC, plasmalogen PE, and/or diacyl-PI were added individually or in combination to verify their contribution to the membrane shape of liposomes.

**Results and discussion**

Non-lamellar CM formation induction under multiple stress conditions [19] in amoeba *Chaos* cells, has disclosed the following potential roles of CM as (1) a supporting cell survival under starvation and stressed conditions [20]; and (2) a radical scavenging [11] and antioxidant defence system [10]. However, information on the detailed molecular mechanism of CM formation remains to be revealed.

*Nutrition determines CM formation and the fate of Chaos cell survival*

It was previously reported that amoeba *Chaos* cells with the presence of CM mitochondria appear to survive better under long-term starvation and stress conditions [20]. CM formation induced in amoeba *Chaos* cells under starvation and stressed conditions is simply through food deprivation.
Interestingly, in contrast to amoeba \textit{Chaos} cells cultured and fed with \textit{Paramecium multimicronucleatum} (hereinafter referred to as amoeba (Para)), amoeba \textit{Chaos} cells cultured with \textit{Tetrahymena pyriformis} as food organisms (hereinafter referred to as amoeba (Tetra)) could not induce mitochondrial CM formation in starvation and stress conditions [20]. In this report, amoeba \textit{Chaos} cells fed with lipids extracted directly from the food organisms (Fig. 1) were indeed necessary to induce CM formation in amoeba \textit{Chaos} cells in response to starvation and stressed conditions. From a previous report [20], CMs appeared and stayed in amoeba (Para) over a long course of starvation (up to 21 days). Hence, it is plausible that CM formation requires certain exogenous nutrition as a prerequisite. Furthermore, the cell survival rate with the presence of CM in amoeba (Para) is significantly higher than that of amoeba (Tetra) without CM under starvation and stressed conditions. The data from a previous report [20] suggest that the possibility of nutrition (from Paramecium) being the determining factor of mitochondrial CM formation in response to starvation and stressed conditions and such nutrient element might be correlated to the improved amoeba \textit{Chaos} cell survival.

\textit{Paramecium lipids-fed endows the potential of CM formation in amoeba Chaos}

It was previously reported that DPA (C22:5n-6) is one of the key elements in CM formation in amoeba \textit{Chaos} cells (Deng \textit{et al.}, 2009). The authors fed polyunsaturated fatty acids (specifically omega-6 DPA) in excess to well-fed amoeba \textit{Chaos} cell cultures (Para-fed) and were able to induce CM formation without the application of any starvation stress. As such, it is intriguing to know whether lipids extracted from \textit{Paramecium} are able to induce CM formation.

The extracted lipids from \textit{Paramecium} were first fed to the control amoeba (Tetra). It should be emphasized that as opposed to amoeba (Para), CM was not inducible by starvation in amoeba (Tetra). Amoeba (Tetra) thus serves as good control as it rules out the possibility of accidental CM induction under starvation stress. Also, more resounding effects of DPA and lipids extracted from \textit{Paramecium} food organisms on the potential formation of CM.

Interestingly, the mitochondrial inner membranes of amoeba (Tetra) – fed with extracted \textit{Paramecium} lipids transformed into CM (Fig. 1A). In order to validate whether DPA alone is
sufficient for such induced membrane transformation in amoeba (*Tetra*), amoeba *Chaos* were also fed with DPA for a comparative study. DPA-treated amoeba (*Tetra*) (Fig. 1B) rendered morphological changes in the mitochondrial membranes but the cubic morphology was less prominent than those observed in *Paramecium* lipids-treated amoeba (*Tetra*). Strikingly, lipids extracted from *Paramecium* appear to be the “prerequisite nutrients” for CM induction under starvation stress in amoeba (*Tetra*), suggesting that dietary phospholipids supplementation might be sufficient and more superior compared to solely DPA fatty acids to induce any significant alteration of the membrane structure and function.

*Paramecium lipids carry a higher amount of plasmalogens compared to Tetrahymena lipids*

Since the full crude extracted *Paramecium* lipids were able to induce CM formation in amoeba (*Tetra*) under starvation stress, the differences in content and composition of lipids between the two distinct food organisms of amoeba *Chaos* cells, namely *Paramecium multimicronucleatum* and *Tetrahymena pyriformis* may help uncover the key nutrients (lipids) promoting CM formation in amoeba *Chaos* cells. Our previous study indicated that a sufficient amount of plasmalogens was present in amoeba *Chaos* cells to trigger CM formation under starvation stress conditions [8]. The unique vinyl-ether bonded plasmalogens have been reported to promote non-lamellar hexagonal and/or cubic phase transition *in vitro* [3-5,7,21]. As such, we logically speculate that plasmalogens might be crucial for CM formation *in vivo*. Thus, a lipid profile study was conducted.

Figures 2 and 3 represent the differences in the lysophospholipids of *Paramecium* and *Tetrahymena*. Lysophospholipids are natural products formed by hydrolysis of phospholipids. The majority of lysoPC elucidated are not significantly different between *Paramecium* and *Tetrahymena*, except for lysoPC (20:4) (Fig. 2). On the other hand, Fig. 3 shows certain lysoPE eluted that are significantly higher in *Paramecium* compared to *Tetrahymena*, they are namely: lysoPE (C16:0p) (where p represents the presence of plasmenyl group), lysoPE (C16:0), lysoPE (C18:1p), lysoPE (C18:0p), lysoPE (C18:3), lysoPE (C18:2) and lysoPE (C18:1). Apparently, lysoPE (C16:0), lysoPE (C18:3), lysoPE (C18:2) and lysoPE (C18:1) are more abundant than the rest of lysoPE revealed here. Of note, lysoPE species (C16:0p), (C18:1p) and (C18:0p) are
plasmenyl lipids of PE (plasmalogen PE). Although they are present in low amounts in both *Paramecium* and *Tetrahymena*, their relatively higher amounts in *Paramecium* are statistically different (*p* < 0.05). Figure 4 shows a large number of PC and pPC that are significantly more abundant in *Paramecium* compared to *Tetrahymena*. However, some of the PC and pPC are in such contrastingly higher abundance that their presence might be paramount compared to the rest. Some examples are PC (34:1), PC (C34:0), PC (C36:2), PC (C36:1), PC (C36:0) and pPC (C36:4), pPC (C36:3), pPC (C38:5), pPC (C38:4).

The lipid data of food organisms of amoeba *Chaos* cells shown in Figs. 2, 3, and 4 revealed a significant difference in plasmalogen levels in these two food organisms (specifically, pPC is in relatively high abundance in *Paramecium* compared to *Tetrahymena*). The higher level of pPC in *Paramecium* lipid extract is consistent with the previous report on *Paramecium*-fed amoeba *Chaos* cells under starvation and stressed conditions, with a significant increase of pPC [8].

Whether plasmalogen PC (pPC) or plasmalogen PE (pPE) are the key elements as pre-conditioning nutrients for amoeba *Chaos* cells to trigger CM formation remains to be uncovered. We thus further examined the effects of three synthesized custom-made amoeba CM-derived phospholipids (Fig. 5) on curving the membrane to promote non-lamellar membrane transformation *in vitro*.

**Synthesized plasmalogens may curve lipidic lamellar membrane phase in vitro**

Deng *et al.* [8] reported that there was a significant increase in C22:5n-6-containing plasmalogen PC (C16:0p/C22:5n-6) in amoeba *Chaos (Para)* upon starvation when CM appeared. Although it was concluded that CM formation was attributed to DPA (C22:5n-6), the significant increase in plasmalogen PC (C16:0p/C22:5n-6) levels observed in starved amoeba *Chaos* cells [8] might well prove the inadequacy of DPA alone in inducing CM formation compared to “plasmalogen-rich” extracted *Paramecium* lipids.

To validate the potency of plasmalogens on CM transformation, 3 major amoeba lipids pPC (C16:0p/C22:5n-6), pPE (C16:0p/C22:5n-6) and diacyl PI (C22:5n-6/C22:5n-6) (Fig. 5) were custom-made and purchased from Avanti Polar Lipids, Inc. based on our lipidomic data of amoeba...
Chaos cells [8]. DPA (C22:5n-6) was added to the synthesized lipid at sn-2 position of the glycerol backbone, as the commercially available plasmalogen (C18:0p/C22:6n-3) only promoted multilayer lamellar structures (data not shown), suggesting the important role of specific DPA (C22:5n-6) fatty acid chains in CM formation. Of interest, we also examined whether plasmalogens-carrying DPA (C22:5n-6) or DPA fatty acid chains alone were sufficient to promote cubic phase transition in vitro.

The control lamellar phase-prone lipids were constructed as liposomes in vitro using DOPC and DOPE. The mixtures of DOPC and DOPE were used as they mimic the lipid components of the mitochondrial membranes; lamellar-prone diacyl PC and non-lamellar-prone diacyl PE were the major phospholipids of mitochondrial membranes [22]. As indicated in Fig. 6A, vesicular multi-lamellar structures were formed by the mixture of DOPC/DOPE lipids. Incorporation of diacyl PI (C22:5n-6/C22:5n-6) to DOPC/DOPE mixture rendered coalescing of the lipids (Fig. 6B), suggesting that C22:5n-6 fatty acid chains alone were probably not able to induce cubic transition. In contrast, plasmalogen PC (C16:0p/C22:5n-6) promoted the morphological changes on the lamellar liposomes (DOPC/DOPE).

Although from the negative staining TEM images alone, it is difficult to accurately depict the transformed morphology of lipids mixture (Fig. 6C and 6D). Whereas the morphological change is evidently convoluted, it is not highly ordered phase structures like classical hexagonal and/or cubic phases. Fig. 6C and 6D illustrated the conversion of lamellar (DOPC/DOPE mixture) to reticular-like network structure when plasmalogen PC (C16:0p/C22:5n-6) and plasmalogen PE (C16:0p/C22:5n-6) were added, respectively. Plasmalogens-carrying DPA (C22:5n-6) effectively curved the lamellar structures and diacyl PI-carrying two DPA (C22:5n-6/C22:5n-6) fatty acid chains resulted in the coalescing of lipids formed by DOPC/DOPE mixture (Fig. 6B). The outcome shows a significantly different effect by adding diacyl PI (C22:5n-6/C22:5n-6) and appears uncanny. The next attempt led to the mixture of all three synthesized lipids (Fig. 5) with and without DOPC/DOPE. The mixture led to the lipids coalescing with significant curvatures which also appear strange (Fig. 6E and 6F).
In support of our results, a recent parallel study adopting similar techniques, MO/DOPC nanostructured lipid phases with alteration of plasmalogens carrying DPA (C22:5n-6) were used in order to validate the potency of plasmalogens on the membrane curvature and/or membrane re-arrangements [7]. The results showed that plasmalogens-carrying C22:5n-6 fatty acids at sn-2 position effectively curved the lamellar phase structures and induced multiple nanostructures such as inverted hexagonal (H_II), double diamond cubic phase, double-membrane vesicles and multilamellar whorl topologies, indicating the importance of DPA-based PE and PC plasmalogens in inducing membrane curvature [7]. From our negative staining TEM results and the published data, plasmalogens seem to promote membrane fusion to form reticular-like structures, suggesting their preference of promoting lamellar to non-lamellar membrane transformation.

It is arguable that although DOPC/DOPE mimics the major lipids of mitochondrial membranes, their innate morphology is lamellar whilst CM formation *in vivo* suggests a morphological transition from tubular to CM [23]. Here, our observations propose that both plasmalogens and DPA (C22:5n-6) may together play a key role in lipid membrane phase transition. More studies are required to further extrapolate our hypothesis that plasmalogens might work synergistically in modification of lipid membrane phase structures to higher ordered hexagonal or cubic morphologies when the role of these special plasmalogens (C16:0p/C22:5n-6) are explored further in the future. With relevance to biological CM, it is highly possible that *in vivo*, lipids, proteins and other ionic milieu [24] or pH factors may together partake in a full CM transformation.

**Concluding remarks**

This study provides the first clue towards understanding the potential role of plasmalogens supplementation in determining cell organellar membrane architecture. In particular, it relates to the availability and capability of the membrane plasmalogens to induce membrane curvature. Plasmalogens have been proposed to play an important role in membrane dynamics and trafficking [1,7,25,26] and facilitating membrane fusion [27]. Several reports have shown that highly
heterogeneous bilayer membranes enriched in plasmalogens are present in synaptic vesicles [1, 12, 28] which are involved in neurotransmitter release. Even a small amount of reduction in either the vinyl-ether content and/or the polyunsaturated fatty acid content of vesicles dramatically reduces the number of successful membrane fusion events [29].

As such, the data in this report may shed some light on the emerging structural property of plasmalogens, capable of facilitating non-lamellar CM formation in amoeba Chaos cells, suggesting that these unique vinyl-ether-bonded phospholipids may promote membrane fusion and/or vesicular formation and modulate membrane trafficking that are crucial in multiple cell processes, especially in neuron and neuroglial cells. Insights on such structural attributes of plasmalogens may also explain the reduced levels of plasmalogens in neurodegenerative diseases including Alzheimer’s disease (AD) [13, 30-34] and also the dietary plasmalogens supplementations on potential cognitive improvements in AD patients [35, 36].

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Conflicts of interest

The authors declared that they have no conflicts of interest in this work.
Data accessibility

The analysed data sets generated during the present study are available from the corresponding author on request.

Author contributions

KC, ZA and YD provided the conception of the paper. KC performed the experiments. KC, ZA and YD analysed and processed the data. KC and ZA wrote the manuscript with inputs from RZ and YD. All authors discussed results and commented on the manuscript.

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Figure legends:

**Fig. 1:** TEM micrographs of amoeba *Chaos (Tetra)* fed with extracted lipids from *Paramecium* and DPA. (A) Amoeba *Chaos* cells fed with 100 µg/ml of extracted lipids from *Paramecium* and (B) 100 µM of DPA. Bars = 0.2 µm. Three independent experiments were performed (approximately 10 mitochondria of (A) and (B) were examined).

**Fig. 2:** Bar graph representing lysolipids (lysoPC) levels compared between *Paramecium* and *Tetrahymena*. Data are means (± S.D.) of three independent experiments. Asterisks indicate p< 0.05 by Student’s t-test.

**Fig. 3:** Bar graph representing lysolipids (lysoPE) and plasmalogen PE levels compared between *Paramecium* and *Tetrahymena*. Data are means (± S.D.) of three independent experiments. Asterisks indicate p< 0.05 by Student’s t-test.
**Fig. 4:** Bar graph representing PC and plasmalogen PC (pPC) levels compared between *Paramecium* and *Tetrahymena*. pPC represents plasmalogen PC. Data are means (± S.D.) of three independent experiments. Asterisks indicate p< 0.05 by Student’s t-test.

**Fig. 5:** Chemical structures of three synthesized lipids custom-made based on amoeba *Chaos* lipid data (Deng *et al.* 2009), namely plasmalogen PC, plasmalogen PE, and diacyl PI with unsaturated fatty acid chains C22:5n-6.

**Fig. 6:** TEM micrographs of liposomes of lamellar phase lipids with/without plasmalogen-modification. (A) Liposome construct (control) with a mixture of synthetic lipids; DOPC and DOPE that forms lamellar lipid phase structure. (B) Liposome construct with DOPC and DOPE and diacyl PI (C22:5n-6/C22:5n-6). (C) Liposome construct with DOPC and DOPE and plasmalogen PC (C16:0p/C22:5n-6). (D) Liposome construct with DOPC and DOPE and plasmalogen PE (C16:0p/C22:5n-6). (E) Liposome construct with DOPC and DOPE and diacyl PI (C22:5n-6/C22:5n-6), plasmalogen PC (C16:0p/C22:5n-6) and plasmalogen PE (C16:0p/C22:5n-6). (F) A mixture of diacyl PI (C22:5n-6/C22:5n-6), plasmalogen PC (C16:0p/C22:5n-6) and plasmalogen PE (C16:0p/C22:5n-6). Bars = 0.2 µm. Three independent experiments were performed.
Plasmalogen PC

Chemical Formula: \( \text{C}_{22}\text{H}_{39}\text{NO}_3\text{P} \)
Exact Mass: 791.58

Plasmalogen PE

Chemical Formula: \( \text{C}_{22}\text{H}_{39}\text{NO}_3\text{P} \)
Exact Mass: 749.54

Diacyl PI

Chemical Formula: \( \text{C}_{53}\text{H}_{102}\text{O}_7\text{P} \)
Exact Mass: 958.56

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