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Oral administration of ethanolamine glycerophospholipid from ascidian viscera improves memory impairment in amyloid β-infused rats

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Running head: PlsEtn improves memory function in AD rats

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Keywords: Alzheimer's disease, amyloid-β, plasmalogen, DHA, rats

Abstract

Ethanolamine plasmalogen (PlsEtn), a major phospholipid in neuronal membranes (60–90 mol% of ethanolamine glycerophospholipid; EtnGpl), is specifically decreased in brains from patients with Alzheimer's disease (AD).Objective: The present study investigated how PlsEtn administration affects cognitive deficits and lipid composition in an animal model of AD. AD model rats were infused with amyloid-β (Aβ) into the cerebral ventricle and divided into 3 groups. Control, Egg, and Ascidian groups were then orally administrated vehicle, egg yolk EtnGpl (260 μmol as EtnGpl/kg BW; 10 μmol as PlsEtn/kg BW), or ascidian viscera EtnGpl (260 μmol as EtnGpl/kg BW; 209 μmol as PlsEtn/kg BW), respectively. After 4 weeks of dosing, Aβ-infused rats were tested for learning ability in an 8-arm radial maze. The administration of ascidian viscera EtnGpl improved both reference and working memory-related learning abilities. In lipid analysis, the Ascidian group showed higher levels of PlsEtn species in the plasma, erythrocytes, and liver when compared to other groups. In addition, although there were no differences at levels of total plasmalogen including choline plasmalogen, the Ascidian group had significantly higher levels of 18:0/22:6-PlsEtn in the cerebral cortex. These levels of 18:0/22:6-PlsEtn in the cerebral cortex were correlated with

working memory-related learning ability. Moreover, 18:0/22:6-PlsEtn levels in the cerebral cortex showed positive correlations with those in the erythrocytes and liver. In summary, dietary PlsEtn, especially that with 22:6n-3 (docosahexaenoic acid, DHA), may ameliorate learning deficiencies in AD by altering lipid composition in the brain.

Abbreviations

Aβ	amyloid-β
AA	arachidonic acid (20:4n-6)
AD	Alzheimer's disease
ALA	α-linolenic acid (18:3n-3)
ALT	alanine aminotransferase
AST	aspartate aminotransferase
DHA	docosahexaenoic acid (22:6n-3)
DMA	dimethyl acetal
DPAn-3	docosapentaenoic acid (22:5n-3)
EPA	eicosapentaenoic acid (20:5n-3)
EtnGpl	ethanolamine glycerophospholipid
FAME	fatty acid methyl esters
γ -GTP	γ-glutamyltranspeptidase
HDL	high density lipoprotein
HPLC	high-performance liquid chromatography
LNA	linoleic acid (18:2n-6)

PlsEtn	ethanolamine plasmalogen or 1-O-alkenyl-2-acyl-sn-glycero-3-phosphoethanolamine
PtdCho	phosphatidylcholine or 1,2-diacyl-sn-glycero-3-phosphocholine
PtdEtn	1,2-diacyl-sn-glycero-3-phosphoethanolamine
PUFA	polyunsaturated fatty acid(s)
RBC	red blood cell(s)
ROS	reactive oxygen species
RME	reference memory error(s)
TBARS	thiobarbituric acid-reactive substances
WME	working memory error(s)

Introduction

Ethanolamine glycerophospholipid (EtnGpl) is a major class of glycerophospholipid found in biological membranes. EtnGpl exists in three forms with alkyl, alkenyl, or acyl linkages at the sn-1 position of the glycerol moiety: 1-O-alkyl-2-acyl-sn-glycero-3-phosphoethanolamine, 1-O-alkenyl-2-acyl-sn-glycero-3-phosphoethanolamine (PlsEtn), and 1,2-diacyl-sn-glycero-3-phosphoethanolamine (PtdEtn), respectively. The alkenylacyl form is called plasmalogen. The aliphatic moiety at the sn-1 position of PlsEtn consists of C16:0 (palmitoyl), C18:0 (stearoyl), or C18:1 (oleoyl) carbon chains, whereas the sn-2 position mainly consists of polyunsaturated fatty acids (PUFA) such as 22:6n-3 (DHA) and 20:4n-6 (ARA). PUFA released from PlsEtn can be metabolized to eicosanoids and docosanoids, which exhibit various bioactivities [1]. PlsEtn is distributed in most mammalian tissues and cells, and its concentration in the nervous system is high [2]. Further, owing to its hexagonal phase formation propensity, PlsEtn are involved in membrane fusion during synaptic transmission [3]. PlsEtn can also prevent cell death by scavenging reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) and superoxide (O^{2-}) at its alkenyl (vinyl ether) linkages [4, 5].

Alzheimer's disease (AD) presents with brain atrophy caused by neuronal loss as a prominent pathological feature. The neuronal loss in AD occurs through apoptosis [6, 7], and amyloid-β (Aβ) peptide, the major component of senile plaques in the AD brain, was reported to induce neuronal apoptosis [7]. Infusion of Aβ into the cerebral ventricle induced brain atrophy and cognitive deficits in rats [8, 9].

On the other hand, PlsEtn levels were reported to be specifically decreased in postmortem brains from patients with AD [10]. In our previous study, we showed that PlsEtn from bovine brains suppresses neuronal cell death [11]. Moreover, PlsEtn species with DHA showed the strongest suppression of neuronal apoptosis when compared to other PlsEtn species and other EtnGpl with DHA [12]. These observations suggested that PlsEtn is involved in AD, and the maintenance or increase in PlsEtn level, especially that containing DHA, in the brain may prevent the pathogenesis and progression of AD via suppression of neuronal apoptosis.

Although bovine brain has been the primary PlsEtn resource, outbreaks of bovine spongiform encephalopathy made its use difficult. However, in our previous studies, some marine invertebrates, especially ascidian viscera, were found to be resources of

PlsEtn species with DHA, and preparation and analytical methods were developed [13-15].

The present study investigated whether administration of PlsEtn from marine ascidian viscera would affect cognitive deficits and lipid composition in an animal model of AD.

Materials and methods

Materials and reagents

Ascidian and hen eggs were respectively purchased from a fishing harbor and local supermarkets in Sendai, Japan; phospholipids (Phospholipid Kit) were purchased from Doosan Serdary Research Laboratories (Toronto, ON). Supplies of 18:0/22:6-PlsEtn, 18:0/20:4-PlsEtn, and 18:0/18:1-PlsEtn were purchased from Avanti Polar Lipids (Alabaster, AL), and 18:0/20:5-PlsEtn was purified according to the methods reported previously [14]. Fatty acid methyl esters (FAME) GLC-68A were purchased from NU-CHEK-PREP (Elysian, MN), and fatty acids EPA and DPAn-3 were purchased from Cayman Chemical Co. (Ann Arbor, MI) and methylated. Hexadecanal dimethyl acetal (DMA), octadecanol, and 23:0 were purchased from Sigma Chemical Co. (St. Louis, MO). Octadec-9-enol was purchased from Wako Pure Chemical (Osaka, Japan);

octadecanal DMA and octadec-9-enal DMA were prepared from octadecanol and octadec-9-enol, respectively [14]. $A\beta_{1-40}$ was purchased from Peptide Inst. (Osaka, Japan), and an Alzet 2002 mini-osmotic pump was purchased from Durect Co. (Cupertino, CA).

Purification of EtnGpl from egg yolk and ascidian viscera

EtnGpl was prepared by a modification of our previous method [14]. Briefly, neutral lipids were removed from freeze-dried ascidian viscera and egg yolk with acetone. After the residue was prepared according to the method described by Folch et al. [16], neutral lipids and sphingolipids were removed with acetone and diethylether. The crude glycerophospholipid fraction was subjected to silica gel column chromatography with the following solvent systems: chloroform-methanol (95:5, v/v), chloroform-methanol (4:1, v/v), and chloroform-methanol (3:2, v/v).

Animals and diet

All animal experiments were performed according to the Guide for Care and Use of Laboratory Animals at Shimane University Faculty of Medicine compiled from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. Wistar rats (generation 1, G1) (Jcl: Wistar; Clea Japan) were housed in

a room under controlled temperature (23 ± 2˚C), relative humidity (50 ± 10%), and light-dark cycles (light: 08:00 to 20:00; dark: 20:00 to 08:00). Rats consumed a fish oil-deficient but an ALA-rich diet (F-1®; Funabashi Farm), the ingredients and fatty acid composition of which have been described in a previous study,45 and water ad libitum. Experiments were performed on the inbred 4th generation male rats (n = 24; 12 weeks old; 324.4 ± 5.2 g body weight) fed the same F-1 diet.

Preparation of Aβ-infused rats

Preparation of Aβ-infused rats was performed as described previously [9, 17]. This procedure greatly improved the reproducibility and reliability of this animal model of AD, rats with impaired memory. Briefly, 2 holes (right and left, relative to the bregma; 0.8 mm posterior, 1.4 mm lateral) were drilled in the rats' skulls according to the atlas of Paxinos and Watson [18]. To facilitate aggregation of Aβ peptide, 0.5 μg $AlCl_3$ was injected through a 3.5 mm cannula into the right ventricle. A mini-osmotic pump containing $Aβ_{1–40}$ solution (4.9–5.5 nmol) was quickly implanted in the back of the rat. The outlet of the pump was inserted 3.5 mm into the left ventricle and attached to the skull with screws and dental cement. $Aβ_{1–40}$ solution was infused for 2 weeks via the osmotic pump.

Radial maze-learning ability and EtnGpl administration

The rats were tested for learning ability 2 weeks after the implantation of the mini-osmotic pump to verify memory impairment. Learning-related behavior was assessed using an 8-arm radial maze (Toyo Sangyo Co. Ltd., Toyama, Japan) [17]. Briefly, the rats were trained to acquire a reward (food-pellet) at the end of each of 4 arms of an 8-arm radial maze. The performance involved 2 parameters of memory function, i.e., RME, entry into unbaited arms; and WME, repeated entry into arms that had already been visited within a trial. The Aβ-infused rats were divided into 3 groups of equal learning ability. The Egg and Ascidian groups were then orally administrated egg yolk EtnGpl (260 μmol EtnGpl/kg BW; 10 μmol as PlsEtn/kg BW) or ascidian viscera EtnGpl (260 μmol EtnGpl/kg BW; 209 μmol as PlsEtn/kg BW) dissolved in palm kernel oil; the Control groups were administrated an equal volume of vehicle alone. All groups were administrated 500 μL of 5% sodium bicarbonate solution before administration of sample because the alkenyl linkage of PlsEtn is hydrolyzed by acids. Four weeks after starting the administration of EtnGpl, rats were tested again for learning ability using an 8-arm radial maze to assess the effect of EtnGpl on the impairment of learning ability. Each rat was given 12 trials for 2 weeks.

Blood and tissue preparation

After completing the behavioral studies, rats were anesthetized with sodium pentobarbital (65 mg/kg BW, i.p.). Blood, freshly collected from the abdominal aorta in tubes with EDTA-2Na, was subjected to low-speed centrifugation (15 min, 1,000 × g, 4˚C) to separate the RBC from the plasma. The precipitated RBC were immediately washed three times with 0.15 M NaCl and lipid extraction was then conducted. The cerebral cortex, hippocampus, and liver were separated as described [9]. The plasma and tissues were stored at -80˚C by flash-freezing in liquid N_2 until use. The tissues were homogenized in ice-cold saline using a Polytron PCU 2-110 homogenizer (Kinematica, Luzern, Switzerland).

Lipid extraction and assay

RBC lipids were extracted from washed RBC with a mixture of 2-propanol and chloroform to protect from hem-iron contamination [19]. Lipids of plasma and tissue homogenates were extracted according to the method of Folch et al. [16]. Phospholipid content was determined according to the method described by Rouser et al [20]. EtnGpl content was analyzed by high-performance liquid chromatography (HPLC) with evaporative light-scattering detection [13]. The average molecular weight was 769 for

169 EtnGpl. PlsEtn content was determined by the 2,4-dinitrophenylhydrazine method [21].

170 Fatty acid and aldehyde composition were determined by gas chromatography [22].

171 **MS/MS analysis**

172 PlsEtn species were analyzed by HPLC with a 4000 QTRAP quadrupole/linear ion-trap

173 tandem mass spectrometer (AB SCIEX, Tokyo, Japan) [14]. To quantify PlsEtn species,

174 multiple reaction monitoring of the transition of parent ions to product ions was

175 performed. Quantification of PlsEtn was performed for four molecular species:

176 18:0/18:1-PlsEtn, 18:0/20:4-PlsEtn, 18:0/20:5-PlsEtn, and 18:0/22:6-PlsEtn. Due to

177 limited hippocampal tissue, we could not quantify PlsEtn species in the hippocampus.

178 **Other analytical methods**

179 Plasma and liver α-tocopherols were measured by HPLC with fluorescence detection

180 [23]. TBARS were measured according to the method by Ohkawa et al. [24]. Plasma

181 levels of AST, ALT, γ-GTP, total cholesterol, and HDL-cholesterol were measured with

182 a TBA-120FR autoanalyzer (Toshiba Medical System Corp., Tochigi, Japan). The non

183 HDL-cholesterol concentration was calculated by total cholesterol subtracted by

184 HDL-cholesterol.

185 **Statistical analysis**

The data are expressed as means ± SEM. Behavioral data were analyzed by two-way factorial ANOVA followed by Fisher's PLSD for post hoc comparisons, and other parameters were tested by one-way ANOVA followed by Scheffe's F-test. For correlation analyses, Pearson's correlation coefficient test for normal data or Spearman's rank correlation coefficient test for nonparametric data were used.

Results

EtnGpl fraction from egg yolk and ascidian viscera

The EtnGpl fraction from egg yolk was 70 wt% EtnGpl. The PlsEtn level of egg yolk was 4 mol% of EtnGpl. The prominent acyl moieties were 18:0 and 16:0 (**Table 1**). The four PlsEtn species that were investigated were not detected. Conversely, the EtnGpl fraction from ascidian viscera was 66 wt% EtnGpl. The PlsEtn level in ascidian viscera was 80 mol% EtnGpl. The alkenyl moiety consisted mostly of 18:0, and the prominent acyl moieties were 20:5n-3 (EPA) and DHA, which are n-3 PUFA. The ratios of n-3/n-6 and DHA/ARA were markedly higher than those of egg yolk. This ascidian viscera EtnGpl consisted of 18:0/18:1-, 18:0/20:4-, 18:0/20:5-, and 18:0/22:6-PlsEtn (4.8, 5.5, 31.2, and 24.4 mol%, respectively).

Animal condition

After administration and behavioral experiments, body and liver weights did not differ among the groups (**Table 2**). Moreover, there were also no differences in blood biochemical parameters (i.e., AST, ALT, γ-GTP, and cholesterols) and levels of α-tocopherol and thiobarbituric acid-reactive substances (TBARS) indicative of oxidative conditions.

Effect of EtnGpl administration on radial-maze learning ability

The effect of EtnGpl administration on reference (**Fig. 1A**) and working (**Fig. 1B**) memory-related learning ability was expressed as the mean number of reference memory error (RME) and working memory error (WME) for each group, with the data averaged over blocks of 2 trials. After 4 weeks of EtnGpl administration, both RME and WME scores for blocks of the radial maze tasks undergone by Ascidian group were lower than those of the Control and Egg groups. Conversely, the administration of egg yolk EtnGpl did not attenuate memory impairment in AD model rats.

Alteration of levels of acyl and alkenyl moieties of blood and livers

After EtnGpl administration for 6 weeks, plasma DHA level was significantly higher in both the Egg and Ascidian groups than in the Control group, resulting in a significantly

higher DHA/ARA ratio (**Table 3**). In the Ascidian group, moreover, levels of EPA and 22:5n-3 (DPAn-3), which are n-3 PUFA, were also higher. The alkenyl moiety expressed plasmalogens including PlsEtn and choline plasmalogen. The levels of total and 18:0 plasmalogens were higher in the Ascidian group than in the Control group. Alkenyl 18:0, DHA, and EPA were the prominent moieties in the ascidian viscera EtnGpl.

In red blood cells (RBC), the DHA/ARA ratio was higher in the Ascidian group than in the Control group (**Table 4**). Levels of 18:2n-6 (LNA), EPA, DPAn-3, and DHA in the liver were higher in the Ascidian group than in the Control group. Total and 18:1 plasmalogen levels in the liver were higher in the Ascidian group than in the Control group.

Alteration of levels of acyl and alkenyl moieties of brains

Table 5 shows carbon chain levels in the cerebral cortex and hippocampus of Aβ-infused rats. There were no differences in plasmalogen and fatty acid levels between three groups, except decreases in palmitate and DPAn-3 levels of Egg group. The DHA/ARA ratio in the hippocampus was significantly higher in the Ascidian group than in the Control group. The mol% of DPAn-3 in carbon chains in the cerebral cortex

(Control group: 0.11 ± 0.01 mol%, Egg group: 0.08 ± 0.01 mol%, Ascidian group: 0.15 ± 0.01 mol%) and hippocampus (Control group: 0.10 ± 0.01 mol%, Egg group: 0.08 ± 0.01 mol%, Ascidian group: 0.13 ± 0.01 mol%) were significantly higher in the Ascidian group than in the Control group.

Alteration of PlsEtn species levels of blood and tissues in Aβ-infused rats

In the blood and liver, levels of 18:0/20:5 and 18:0/22:6-PlsEtn, which are major components in ascidian viscera EtnGpl, were higher in the Ascidian group than the Control group (**Table 6**). The cerebral cortex level of 18:0/22:6-PlsEtn was significantly higher in the Ascidian group than the Control group. There were no differences in the levels of other PlsEtn species or total EtnGpl in the cerebral cortex between the three groups (total EtnGpl Control group: 123.7 ± 7.2 nmol/mg protein, Egg group: 118.5 ± 14.7 nmol/mg protein, Ascidian group: 129.8 ± 8.8 nmol/mg protein).

Relationship between learning ability and PlsEtn levels

Plasma 18:0/22:6-PlsEtn levels had a negative correlation with RME in Aβ-infused rats (**Table 7**). In addition, 18:0/22:6-PlsEtn levels in the cerebral cortex had a negative correlation with WME. There were no correlations between RME or WME scores and levels of other PlsEtn species or fatty acids including DHA (data not shown).

Relationship between levels of 18:0/22:6-PlsEtn in blood and tissues

The levels of 18:0/22:6-PlsEtn in the cerebral cortex were positively correlated with those in the RBC and liver (Table 7). Liver 18:0/22:6-PlsEtn levels were positively correlated with those in the plasma and RBC.

Discussion

PlsEtn has an important role for neurotransmission and the maintenance of membrane function on brain [3, 25], and its level is specifically decreased in brains from patients with AD [10]. In addition, PlsEtn level continues to decrease in the brain as AD advances [26], and the level of PlsEtn, especially PlsEtn bearing DHA, is decreased in the plasma and RBC of patients with AD [27]. Our group found that extrinsic PlsEtn suppressed neuronal apoptosis in vitro [11]. In this study, we investigated whether oral administration of EtnGpl containing high concentrations of PlsEtn would affect spatial cognition learning ability and lipid composition in Aβ-infused rats produced by infusing Aβ peptide into the brain.

In behavioral experiments, administration of ascidian viscera EtnGpl, which is rich in PlsEtn, improved both reference and working memory-related learning ability in

271 Aβ-infused rats, while administration of egg yolk EtnGpl, which is poor in PlsEtn, did
272 not. In addition, administration of ascidian viscera EtnGpl increased levels of PlsEtn
273 species in the blood and tissues of Aβ-infused rats (Table 6). Levels of PlsEtn bearing
274 DHA were increased in the cerebral cortex, and the concentration had a negative
275 correlation with the WME scores, which indicated short-term memory impairment as an
276 AD character (Table 7). Our previous studies showed that the addition of PlsEtn with
277 DHA strongly suppressed neuronal apoptosis and destabilized Aβ fibrils in vitro [12, 27].
278 These results suggested that the increase in PlsEtn bearing DHA levels in the cerebral
279 cortex improved spatial cognition learning ability.
280 The administration of ascidian viscera EtnGpl also increased in DHA, DPAn-3, and EPA
281 levels in the plasma and liver (Table 3 and 4) but not in the RBC and brain (Table 4 and
282 5). Their fatty acid levels were reflected by absorption, elongation, and desaturation of
283 the C20 over n-3 PUFA in EtnGpl because all groups had been fed an α-linolenic acid
284 (ALA)-rich diet. Tissues, especially brain tissues have homeostasis, and thereby do not
285 markedly alter the levels of fatty acids and lipid classes [28, 29]. However, the lipid
286 levels in the RBC and brain markedly alter in case of extreme nutrient limitation for
287 long term[30] and certain disorders such as cognitive impairment [10, 31]. Decreases in

288 the lipid levels of tissues may be complemented by the administration of the lipid or the
289 precursor. Therefore, it will be more important to quantify lipid molecular species than
290 fatty acid composition.
291 Aβ is deposited in the form of plaques in patients with AD, inducing oxidative stress
292 and chronic inflammation in the brain and resulting in AD pathologies [32, 33].
293 Excessive oxidative stress causes the activation of phospholipase A_2 (PLA_2), including
294 PlsEtn-selective PLA_2, to decrease brain PlsEtn level [2]. DHA released from PlsEtn is
295 metabolized to docosanoids (e.g., docosatrienes and resolvins), which have
296 anti-apoptotic and anti-inflammatory effects [34]. Moreover, PlsEtn acts as an
297 antioxidant and a chelation agent to protect neuronal cells from ROS- and iron-induced
298 oxidative injures [4, 35]. Thus, PlsEtn is sacrificed for the protection of neuronal cells.
299 However, decreases in neuronal cell PlsEtn activate γ-secretase, which produces Aβ
300 from Aβ protein precursor [36]. PlsEtn lack impairs intracellular cholesterol distribution,
301 affecting plasma membrane function and structural changes in the endoplasmic
302 reticulum and Golgi cisternae [37]. Increases in the cholesterol level of membrane rafts
303 also enhance Aβ production [38]. Taken together, PlsEtn degradation is important due to

its protective effect on neuronal cells, and the neuronal cells in which the amount of PlsEtn is decreased become stress-prone.

Conversely, the activation of cytosolic PLA_2, which catalyzes phosphatidylcholine (PtdCho), is also a key step in the AD brain [39]. ARA released from PtdCho is metabolized to eicosanoids, which show apoptotic and inflammatory effects, through the cyclooxygenase pathway [40, 41]. In addition, lyso-PtdCho from PtdCho is released to initiate astrogliosis, neuroinflammation, and subsequent neurodegeneration [42]. PlsEtn and DHA reduce cytosolic PLA_2 and cyclooxygenase activities, suppressing neuronal apoptosis [12, 43]. Therefore, increases in the PlsEtn bearing DHA level and DHA/ARA ratio are thought to moderate oxidative conditions in the brain. The administration of DHA ethyl ester has been reported to ameliorate learning deficiencies in Aβ-infused rats due to increased DHA/ARA ratios and suppressed ROS generation [17]. The DHA/ARA ratio may possibly indirectly alter level of PlsEtn with DHA due to be a storage depot of DHA.

In the present study, plasma levels of PlsEtn with DHA had a negative correlation with RME scores, indicating long-term memory impairment (Table 7). Other researchers have reported that cognitive impairment increased in patients with AD having low

levels of serum PlsEtn with DHA after a year [44]. Decreases in PlsEtn level have been reported in the serum of patients with Parkinson's disease (PD) as a neurodegenerative disease [45]. Administration of PlsEtn precursor increased levels of serum PlsEtn bearing DHA in monkeys with dyskinesias caused by treatment of PD, improving dyskinesia symptoms [46]. Moreover, PlsEtn with DHA/total PlsEtn ratios were inversely correlated with dyskinesia symptoms. Plasma or serum PlsEtn levels may be associated with brain PlsEtn level and central nervous system function. On the other hand, administration of PlsEtn precursor increased plasma and heart levels of PlsEtn and choline plasmalogen in atherosclerosis model mice that were deficient in ApoE or ApoE/glutathione peroxidase-1, attenuating atherosclerosis [47]. Therefore, improvement in the circulatory system may indirectly attenuate cognitive impairments. As described above, it is thought that the level of PlsEtn having DHA in brain is associated with brain functions. On the other hand, a number of studies on animals and humans have reported that DHA administration has the potential to suppress the incidence of AD in animals as well as in human [9, 17, 48]. Recently, it was reported that in AD model mice (Tg2576), DHA supplementation for 1 year increases in the brain PlsEtn with DHA [49]. Therefore, PlsEtn with DHA in the brain is emphasized as

an important factor for cognitive functions. However, there is a question whether the administration of fatty acid DHA or PlsEtn with DHA is more effective for increasing in the brain PlsEtn containing DHA. In AD brain, alkenyl chain and ethanolamine as well as DHA are insufficient [10]. It has been reported that the EtnGpl level is strictly managed by homeostasis [29], and the PtdEtn administration improves age-related spatial memory deterioration [50]. Moreover, PlsEtn or the precursor has been reported to pass the blood-brain barrier [51]. Further studies including clinical trials are required to determine the availability of the administration of DHA and PlsEtn.

PlsEtn bearing DHA levels in RBC were correlated with the levels in the cerebral cortex (Table 7). In our previous study, levels of RBC PlsEtn with DHA were decreased in patients with AD compared to healthy subjects [27]. The levels of RBC PlsEtn having DHA were correlated with the brain volumes of patients with AD and healthy subjects (unpublished observation). The level of RBC PlsEtn with DHA may thus reflect brain condition. The use of brain amyloid imaging [52] and Aβ levels in the cerebrospinal fluid [53] as biomarkers of AD is limited due to cost and safety factors. Therefore, identification of AD biomarkers in the blood would significantly improve patient safety

and reduce AD diagnostic costs. Levels of RBC PlsEtn with DHA could be potential candidates for blood-based biomarkers of AD.

Dietary PlsEtn has a low absorption rate [54]. Therefore, PlsEtn precursors have been used to increase PlsEtn levels in vivo [47, 51]. However, PlsEtn levels in the blood and liver could be markedly increased by ingestion of PlsEtn over a fixed term, and the increase in brain PlsEtn with DHA is very important. Administration of ascidian viscera EtnGpl increased brain PlsEtn with DHA in this study although it is not clear whether administered PlsEtn could directly transfer into the brain. Moreover, ascidian viscera EtnGpl contains PlsEtn with EPA, which is metabolized and exhibits various bioactivities [15, 55], and administration of ascidian viscera EtnGpl increased the brain mol% of DPAn-3, a DHA precursor. Taken together, these findings suggest that intake of PlsEtn from marine invertebrates is preferable for the purposes of increase in brain PlsEtn with DHA and amelioration of cognitive impairment.

Recently, it was reported that Aβ production in the liver has a connection with Aβ accumulation in the brain [56, 57]. PlsEtn administration suppressed Aβ accumulation in the brain induced by i.p. injection of lipopolysaccharide [58]. Moreover, the level of PlsEtn with DHA decreased in the blood of patients with AD and had a negative

correlation with plasma Aβ levels in healthy subjects [27]. PlsEtn also suppresses Aβ production and aggregation [27, 36]. Therefore, an increase in PlsEtn levels in the blood and liver might also slow AD progression.

In conclusion, administration of ascidian viscera EtnGpl, which is rich in PlsEtn, improved cognitive impairment and altered levels of PlsEtn species in Aβ-infused rats. These results suggest that PlsEtn containing DHA from marine invertebrates is potentially useful for a therapeutic dietary supplement treating and preventing AD.

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Figure legend

Fig. 1. Effect of administration of EtnGpl to Aβ-infused rats on learning ability. Effects of oral administration of EtnGpl on reference memory-related learning ability (A) and working memory-related learning ability (B) in the radial maze task in Aβ-infused rats. Means ± SEM, $n = 8$. Asterisks indicate significant differences between this group and Control group (**$P < 0.01$) by randomized 2-factor (block and group) ANOVA followed by Fisher's PLSD test.

EtnGpl, ethanolamine glycerophospholipid; AD, Alzheimer's disease.

Tables

Table 1. Acyl and alkenyl chain composition of prepared EtnGpl

	Egg yolk	Ascidian viscera
Acyl		
Palmitate16:0	23.2	2.9
Stearate18:0	26.6	8.6
Oleate18:1n-9	19.4	7.3
LNA18:2n-6	12.6	0.4
ALA18:3n-3	0.0	0.3
ARA20:4n-6	13.4	4.6
EPA20:5n-3	0.1	22.4
DPA22:5n-3	0.2	0.6
DHA22:6n-3	2.6	13.3
n-3/n-6	0.09	7.32
DHA/ARA	0.19	2.89
Alkenyl		
Palmitoyl16:0	0.9	3.1
Stearoyl18:0	1.0	34.2
Oleoyl18:1	0.1	2.3

(mol%)

EtnGpl, ethanolamine glycerophospholipid; LNA, linoleic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Table 2. Body and liver weights, and liver and blood biochemical parameters of Aβ-infused rats administrated EtnGpl for 6 weeks

	Control group	Egg group	Ascidian group
Body			
Weight (g/rat)	403.1 ± 4.6	405.4 ± 9.0	404.7 ± 7.7
Liver			
Weight (g/rat)	10.4 ± 0.5	10.5 ± 0.4	10.2 ± 0.2
α-Tocophenol (nmol/mg protein)	0.7 ± 0.1	0.9 ± 0.1	1.0 ± 0.1
TBARS (nmol/mg protein)	0.7 ± 0.0	0.8 ± 0.1	0.8 ± 0.1
Plasma			
AST (IU/L)	56.6 ± 2.0	55.4 ± 2.1	54.8 ± 1.3
ALT (IU/L)	27.4 ± 1.0	29.4 ± 1.9	31.1 ± 0.6
γ-GTP (IU/L)	0.4 ± 0.2	0.3 ± 0.2	0.5 ± 0.2
Total cholesterol (mmol/L)	2.1 ± 0.1	2.3 ± 0.1	2.0 ± 0.1
HDL-cholesterol (mmol/L)	1.1 ± 0.0	1.3 ± 0.0	1.2 ± 0.0
non HDL-cholesterol (mmol/L)	1.0 ± 0.1	1.0 ± 0.0	0.9 ± 0.0
α-Tocophenol (nmol/mL)	9.8 ± 0.5	10.1 ± 0.3	9.5 ± 0.6
TBARS (nmol/mL)	1.4 ± 0.1	1.6 ± 0.1	1.3 ± 0.1

Means ± SEM, n = 8. AD, Alzheimer's disease; TBARS, thiobarbituric acid reactive substances; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyltranspeptidase; HDL, high density lipoprotein.

Table 3. Composition of acyl and alkenyl chain in plasma of Aβ-infused rats administered EtnGpl for 6 weeks

	Control group	Egg group	Ascidian group
Acyl		(nmol/mL plasma)	
Palmitate16:0	908.1 ± 30.3	959.9 ± 66.0	935.8 ± 34.0
Stearate18:0	488.7 ± 21.0	515.2 ± 23.4	473.9 ± 11.5
Oleate18:1n-9	328.5 ± 15.7	386.5 ± 60.7	356.1 ± 30.3
LNA18:2n-6	807.0 ± 35.1	822.2 ± 73.2	950.6 ± 37.1
ALA18:3n-3	7.6 ± 0.8	7.5 ± 1.6	9.4 ± 1.3
ARA20:4n-6	1113.1 ± 65.0	1190.1 ± 86.5	882.0 ± 33.5
EPA20:5n-3	13.2 ± 1.3	13.7 ± 1.1	74.8 ± 5.0**
DPA22:5n-3	19.7 ± 2.1	19.7 ± 2.8	47.2 ± 1.7**
DHA22:6n-3	102.6 ± 7.4	137.2 ± 11.9*	203.6 ± 6.1**
n-3/n-6	0.07 ± 0.00	0.09 ± 0.00	0.18 ± 0.01**
DHA/ARA	0.09 ± 0.00	0.11 ± 0.00*	0.23 ± 0.01**
Alkenyl		(nmol/mL plasma)	
Palmitoyl16:0	16.4 ± 2.2	16.3 ± 2.2	16.8 ± 2.3
Stearoyl18:0	17.0 ± 2.3	27.5 ± 5.4	105.4 ± 25.1**
Oleoyl18:1	11.3 ± 1.5	10.5 ± 1.4	10.2 ± 1.4
Total	44.7 ± 6.0	54.3 ± 7.9	132.4 ± 25.4**

Means ± SEM, $n = 8$. Asterisks indicate significant differences between this group and Control group (**P < 0.01, *P < 0.05) by one-way ANOVA followed by Scheffe's F-test. AD, Alzheimer's disease; EtnGpl, ethanolamine glyceropospholipid; LNA, linoleic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Table 4. Composition of acyl and alkenyl chain in RBCs and liver of Aβ-infused rats administered EtnGpl for 6 weeks

	RBC			Liver		
	Control group	Egg group	Ascidian group	Control group	Egg group	Ascidian group
Acyl			(nmol/mg protein)			
Palmitate16:0	121.4 ± 23.5	146.1 ± 14.7	124.3 ± 11.0	31.3 ± 3.5	30.1 ± 3.8	41.7 ± 2.8
Stearate18:0	62.2 ± 10.9	73.7 ± 7.5	60.3 ± 5.8	19.7 ± 1.8	21.4 ± 2.5	27.0 ± 1.7
Oleate18:1n-9	25.8 ± 5.4	29.4 ± 3.3	24.9 ± 2.4	12.9 ± 1.7	11.6 ± 2.0	15.5 ± 1.1
LNA18:2n-6	35.0 ± 6.9	40.3 ± 4.0	38.8 ± 3.5	22.7 ± 3.1	17.8 ± 2.2	32.3 ± 2.3**
ALA18:3n-3	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.1
ARA20:4n-6	99.1 ± 18.9	116.0 ± 12.0	89.4 ± 8.6	24.0 ± 2.4	25.0 ± 2.8	27.1 ± 1.7
EPA20:5n-3	0.1 ± 0.0	0.1 ± 0.1	0.4 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	1.0 ± 0.1**
DPA22:5n-3	34.6 ± 4.8	37.1 ± 3.5	34.9 ± 2.9	2.0 ± 0.2	1.8 ± 0.2	4.0 ± 0.3**
DHA22:6n-3	10.3 ± 1.9	13.6 ± 1.5	15.3 ± 1.6	6.9 ± 0.7	7.7 ± 0.9	14.2 ± 1.1**
n-3/n-6	0.37 ± 0.03	0.33 ± 0.01	0.40 ± 0.01	0.20 ± 0.00	0.23 ± 0.01	0.33 ± 0.02**
DHA/ARA	0.11 ± 0.01	0.12 ± 0.00	0.17 ± 0.00**	0.29 ± 0.01	0.31 ± 0.01	0.52 ± 0.02**
Alkenyl			(nmol/mg protein)			
Palmitoyl16:0	10.5 ± 2.5	13.8 ± 1.5	11.8 ± 1.1	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
Stearoyl18:0	9.4 ± 2.0	13.0 ± 1.4	11.8 ± 1.1	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.0
Oleoyl18:1	7.7 ± 2.0	9.5 ± 1.1	8.5 ± 0.9	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.1*
Total	27.7 ± 6.5	36.3 ± 3.9	32.1 ± 3.1	0.8 ± 0.1	0.7 ± 0.1	1.2 ± 0.1*

Means ± SEM, $n = 8$. Asterisks indicate significant differences between this group and Control group (**$P < 0.01$, *$P < 0.05$) by one-way ANOVA followed by Scheffe's F-test. AD, Alzheimer's disease; RBCs, red blood cells; EtnGpl, ethanolamine glyceropospholipid; LNA, linoleic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Table 5. Composition of acyl and alkenyl chain in brain of Aβ-infused rats administered EtnGpl for 6 weeks

	Cerebral cortex			Hippocampus		
	Control group	Egg group	Ascidian group	Control group	Egg group	Ascidian group
Acyl			(nmol/mg protein)			
Palmitate16:0	285.9 ± 5.1	267.3 ± 2.5*	274.4 ± 4.1	259.3 ± 6.0	263.8 ± 4.1	249.8 ± 4.9
Stearate18:0	277.6 ± 6.0	256.9 ± 3.0	248.5 ± 16.7	264.6 ± 6.6	270.9 ± 4.8	256.7 ± 5.9
Oleate18:1n-9	153.6 ± 5.1	139.1 ± 3.2	141.5 ± 3.4	177.6 ± 7.4	181.4 ± 8.1	176.2 ± 8.7
LNA18:2n-6	9.1 ± 0.8	7.4 ± 0.7	8.7 ± 0.6	6.8 ± 0.3	6.7 ± 0.2	7.2 ± 0.2
ALA18:3n-3	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
ARA20:4n-6	124.9 ± 3.6	120.8 ± 2.3	120.8 ± 2.3	130.3 ± 3.0	133.8 ± 4.0	126.4 ± 2.3
EPA20:5n-3	0.8 ± 0.1	0.6 ± 0.0	0.6 ± 0.0	0.8 ± 0.1	0.7 ± 0.0	0.6 ± 0.0
DPA22:5n-3	1.2 ± 0.1	0.8 ± 0.1*	1.5 ± 0.1	1.0 ± 0.1	0.8 ± 0.1*	1.3 ± 0.1
DHA22:6n-3	162.9 ± 5.3	152.3 ± 4.9	162.2 ± 4.9	139.1 ± 3.0	144.4 ± 3.5	141.5 ± 2.5
n-3/n-6	1.24 ± 0.06	1.20 ± 0.05	1.27 ± 0.05	1.03 ± 0.02	1.04 ± 0.01	1.08 ± 0.01
DHA/ARA	1.32 ± 0.07	1.27 ± 0.06	1.35 ± 0.06	1.07 ± 0.02	1.08 ± 0.01	1.12 ± 0.01*
Alkenyl			(nmol/mg protein)			
Palmitoyl16:0	24.6 ± 2.1	25.8 ± 1.7	25.6 ± 1.3	28.9 ± 1.6	29.3 ± 1.2	28.9 ± 1.7
Stearoyl18:0	22.8 ± 1.1	21.7 ± 0.6	23.1 ± 0.9	27.2 ± 2.6	27.4 ± 2.5	28.4 ± 2.1
Oleoyl18:1	9.5 ± 0.7	8.7 ± 0.4	8.5 ± 0.5	18.3 ± 2.7	19.2 ± 2.4	18.5 ± 3.1
Total	56.9 ± 2.2	56.2 ± 1.9	57.2 ± 1.1	74.4 ± 6.7	75.9 ± 5.6	75.8 ± 6.7

Means ± SEM, $n = 8$. Asterisks indicate significant differences between this group and Control group (*$P < 0.05$) by one-way ANOVA followed by Scheffe's F-test. AD, Alzheimer's disease; EtnGpl, ethanolamine glyceropospholipid; LNA, linoleic acid; ALA, α-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Table 6. PlsEtn species levels in blood, liver, and brain of Aβ-infused rats administered EtnGpl for 6 weeks

	Control group	Egg group	Ascidian group
Plasma		(nmol/mL plasma)	
18:0/18:1-PlsEtn	0.5 ± 0.1	0.7 ± 0.1	2.3 ± 0.6**
18:0/20:4-PlsEtn	6.7 ± 1.0	10.3 ± 2.1	29.9 ± 6.8**
18:0/20:5-PlsEtn	0.2 ± 0.0	0.3 ± 0.1	10.2 ± 3.0**
18:0/22:6-PlsEtn	3.7 ± 0.5	6.8 ± 1.4	26.5 ± 6.2**
RBC		(nmol/mg protein)	
18:0/18:1-PlsEtn	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
18:0/20:4-PlsEtn	7.9 ± 0.8	8.1 ± 1.7	8.1 ± 0.7
18:0/20:5-PlsEtn	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.0**
18:0/22:6-PlsEtn	1.1 ± 0.1	1.2 ± 0.3	2.1 ± 0.1*
Liver		(pmol/mg protein)	
18:0/18:1-PlsEtn	43.4 ± 4.6	40.3 ± 5.3	64.3 ± 5.1*
18:0/20:4-PlsEtn	1495.8 ± 82.2	1468.3 ± 178.7	1703.5 ± 140.3
18:0/20:5-PlsEtn	19.9 ± 1.5	18.4 ± 1.8	155.7 ± 6.3**
18:0/22:6-PlsEtn	209.2 ± 15.6	288.2 ± 37.9	720.2 ± 100.8**
Cerebral cortex		(nmol/mg protein)	
18:0/18:1-PlsEtn	1.6 ± 0.1	1.7 ± 0.1	2.0 ± 0.2
18:0/20:4-PlsEtn	7.3 ± 0.3	7.2 ± 0.3	8.9 ± 0.6
18:0/20:5-PlsEtn	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
18:0/22:6-PlsEtn	9.4 ± 0.3	9.2 ± 0.5	11.6 ± 0.6*

Means ± SEM, $n = 8$. Asterisks indicate significant differences between this group and Control group (**$P < 0.01$, *$P < 0.05$) by one-way ANOVA followed by Scheffe's F-test. PlsEtn, ethanolamine plasmalogen; AD, Alzheimer's disease; EtnGpl, ethanolamine glyceropospholipid; RBC, red blood cell.

Table 7. Correlations between of learning ability and levels of 18:0/22:6-PlsEtn in Aβ-infused rats[1]

	RME		WME	
	r	*p*	*r*	*p*
18:0/22:6-PlsEtn				
Plasma	–0.48	<0.05	–0.26	0.23
RBC	–0.18	0.40	–0.03	0.99
Liver	–0.16	0.46	–0.18	0.39
Cerebral cortex	0.04	0.85	–0.40	<0.05

	18:0/22:6-PlsEtn					
	Plasma		RBC		Liver	
	r	*p*	*r*	*p*	*r*	*p*
18:0/22:6-PlsEtn						
Plasma						
RBC	0.281	0.183				
Liver	0.481	<0.05	0.663	<0.001		
Cerebral cortex	0.125	0.561	0.450	<0.05	0.559	<0.01

[1]The number of RME and WME in block 6 shown in Figure 1 was used as an indicator of learning ability. $n = 24$. PlsEtn, ethanolamine plasmalogen; AD, Alzheimer's disease; RME, reference memory error; WME, working memory error; RBC, red blood cell.

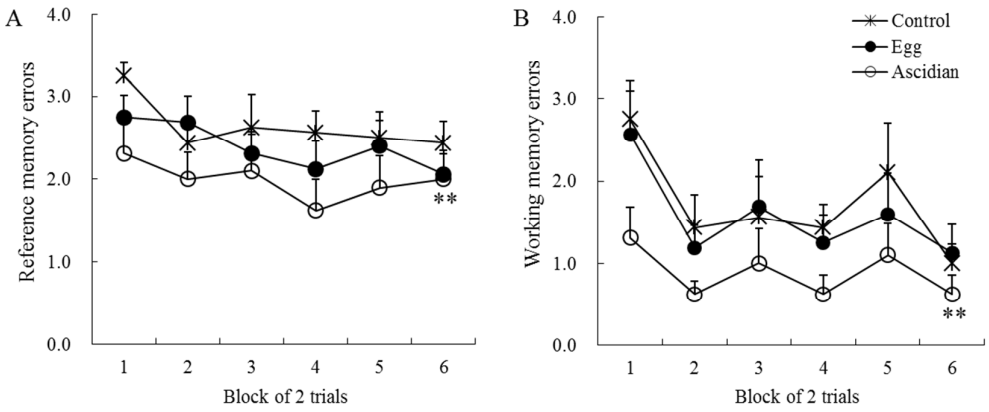

Fig. 1

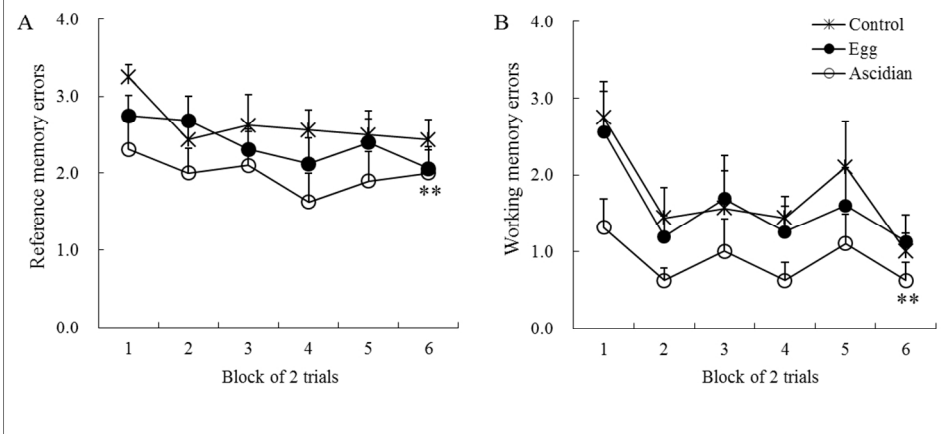

Fig. 1

209x108mm (150 x 150 DPI)