Title

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Alterations in the Levels of Amyloid-β, Phospholipid Hydroperoxide, and Plasmalogen in the Blood of Patients with Alzheimer's Disease: Possible Interactions between Amyloid-β and These Lipids

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Abstract. Aside from accumulation of amyloid-β (Aβ) peptide in the brain, Alzheimer's disease (AD) has been reported as being associated with peroxidation of major phospholipids (e.g., phosphatidylcholine (PtdCho)) and degradation of antioxidative phospholipids (e.g., ethanolamine plasmalogen (PlsEtn)). In addition to its presence in the brain, Aβ is also found in blood; however, there is still little information about the levels of PtdCho hydroperoxide (PCOOH) and PlsEtn in the blood of patients with AD. In this study, by assuming a possible interaction among Aβ, PCOOH, and PlsEtn in blood circulation, we evaluated the levels of these molecules and correlations in blood samples that had been obtained from our former AD study for PCOOH measurement (Kiko et al., *J Alzheimers Dis* **28**, 593-600, 2012). We found that when compared to controls, plasma from patients with AD showed lower concentrations of PlsEtn species, especially PlsEtn bearing the docosahexaenoic acid (DHA) moiety. In addition, lower PlsEtn and higher PCOOH levels were observed in red blood cells (RBCs) of patients with AD. In both AD and control blood samples, RBC PCOOH levels tended to correlate with plasma levels of $A\beta_{40}$, and each PlsEtn species showed different correlations with plasma Aβ. These results, together with *in vitro* data suggesting Aβ aggregation due to a decrease in levels of PlsEtn having DHA, led us to deduce that Aβ is involved in alterations in levels of PCOOH and PlsEtn species observed in the blood of patients with AD.

Keywords: Alzheimer's disease, amyloid-β, phospholipid hydroperoxide, plasmalogen

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INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia. One of the pathological characteristics of AD is the progressive aggregation and accumulation of amyloid-β (Aβ) peptide in senile plaques of the human brain [1, 2]. Since brain Aβ, especially the fibril form, is highly neurotoxic, the progressive aggregation of Aβ is a critical step in AD pathogenesis [3, 4]. Therefore, brain amyloid imaging [5] and Aβ levels in cerebrospinal fluid (CSF) [6] are thought to be AD biomarkers. However, the use of these biomarkers is limited due to cost and safety factors. Therefore, many researchers have sought to identify blood-based biomarkers (e.g., microRNA, proteins, and lipids) so that disease progression can be continuously monitored and medical treatment can be assessed [7, 8]. Although it has not yet been determined whether brain Aβ transfers to plasma [9], the presence of Aβ in peripheral blood plasma has received increasing attention [10–13]. Plasma Aβ is hypothesized to readily contact red blood cells (RBCs) and impair the functions of RBCs in circulating human blood [14, 15]. Our group and other researchers have investigated this hypothesis, and found that Aβ induces oxidative injury to RBCs by binding to them and causing accumulation of phospholipid hydroperoxides (PLOOH) including hydroperoxides of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) (PCOOH and PEOOH, respectively) [16, 17]. Aβ also induces the binding of erythrocytes to endothelial cells and decreases endothelial viability, perhaps by the generation of oxidative and inflammatory stress [18]. Moreover, we have reported that RBC Aβ and PCOOH levels increase with age in healthy subjects, and that RBC PCOOH levels increase in patients with AD [19, 20].

On the other hand, the levels of ethanolamine plasmalogen (PlsEtn), which is known as an antioxidative phospholipid, have been reported to be specifically decreased in brains from patients with AD [21–24]. PlsEtn is a subclass of ethanolamine glycerophospholipid (EtnGlp) and has vinyl ether linkage at the sn-1 position, while PtdEtn as a usual subclass has ester linkage. PlsEtn is involved in membrane fusion and fluidity, which occur during synaptic transmission and the maintenance of membrane function [25]. Moreover, PlsEtn is known to suppress neuronal apoptosis [26]. Therefore, PlsEtn may be involved in the onset and progression of AD. Although the mechanisms of the interactions between AD or Aβ and PlsEtn in blood are largely unknown, there are reports that PlsEtn levels decrease in the serum of patients with AD [27, 28].

The purpose of this study was to evaluate our hypothesis about an interaction among Aβ, PCOOH, and PlsEtn in blood circulation. We analyzed the levels of Aβ, PCOOH, and PlsEtn in the blood of patients with AD and their spouses (control subjects) that had been obtained from our former AD study on PCOOH [19]. We then looked for correlations between Aβ and these lipids. In addition, we investigated whether PlsEtn species affect the formation and disruption of Aβ fibrils *in vitro* so that we could clarify the correlations between Aβ and PlsEtn species *in vivo*.

MATERIALS AND METHODS

Subjects

This was a follow-up study of an earlier report [19]; therefore, the same blood samples that had been obtained previously were analyzed. Patients with AD (10 men and 8 women) seen at the Tohoku University Hospital and healthy volunteer control subjects (8 men and 10 women, who were all spouses of the patients with AD) participated in this study (Table 1). The absence of liver and renal diseases in patients with AD and control subjects was confirmed by obtaining biochemical data from blood samples (i.e., ALT, AST, and creatinine). Brain volume was measured by morphometric magnetic resonance imaging data. Disease stage was rated by means of the Mini-Mental State Examination (MMSE), which is a brief cognitive test used widely in clinical practice and epidemiologic studies. This test was administered in order to grade the subjects' global cognitive impairment. The study protocol was in accordance with the Declaration of Helsinki

Table 1
Physical characteristics of patients with AD and control subjects[1]

	Control subjects (patient spouses)	Patients with AD
Male	8	10
Females	10	8
Age	74.4 ± 1.6	72.4 ± 1.6
BMI	22.7 ± 0.6	22.0 ± 1.3
AST[2]	21.8 ± 1.6	16.2 ± 1.8
ALT[2]	15.4 ± 1.7	62.9 ± 4.3
Creatinine[3]	66.4 ± 4.3	62.9 ± 4.3
Brain volume[4]	100.0 ± 5.7	86.0 ± 3.2[5]
MMSE	–	17.3 ± 1.3

[1]Mean ± SEM; $n = 18$. [2,3,4]Units: IU/L, μmol/L, % of control subjects. [5]Significantly different from patients with AD: $p < 0.05$. BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; MMSE, Mini-Mental State Examination.

and was approved by the Ethical Committee of the Graduate School of Medicine at Tohoku University. All subjects gave written informed consent to the experimental protocol. Blood, freshly collected in tubes with EDTA-2Na, was subjected to low-speed centrifugation (15 min, 1,000× *g*, 4°C) to separate RBCs from the plasma. The precipitated RBCs were immediately washed three times with 0.15 M NaCl and lipid extraction was then conducted. The plasma was stored at –80°C until use.

Reagents

The following reagents were purchased from Avanti Polar Lipids (Alabaster, AL): 18:0/22:6-PlsEtn, 18:0/20:4-PlsEtn, 18:0/18:1-PlsEtn, 18:0/22:6-PtdEtn, 18:0/20:4-PtdEtn, 18:0/18:2-PtdEtn, 18:0/18:1-PtdEtn, 16:0/22:6-PtdEtn, 16:0/20:4-PtdEtn, 16:0/18:2-PtdEtn, 16:0/18:1-PtdEtn, and 18:0/22:6-PtdCho; 18:1, 20:4, and 22:6 (DHA) were purchased from Cayman Chemical Co. (Ann Arbor, MI); fatty acid methyl ester (FAME) GLC-68A was purchased from Nu-Chek-prep, Inc. (Elysian, MN); hexadecanal dimethylacetal (DMA), octadecanol, 17:0, and thioflavin were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO); octadec-9-enol, Phospholipids C test assay kit and Human β Amyloid ELISA kit were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); $A\beta_{42}$ peptide was obtained from the Peptide Institute (Osaka, Japan); octadecanol and octadec-9-enol were oxidized and methylated to octadecanal DMA and octadec-9-enal DMA, respectively; 18:0/20:5-PlsEtn was purified according to the methods reported previously [29].

Lipid extraction and analysis

RBC lipids were extracted from washed RBCs with a mixture of 2-propanol and chloroform to protect from hem-iron contamination [30]. Plasma lipids were extracted according to the method of Folch et al. [31].

Phospholipid contents in RBC and plasma lipids were determined by Bartlett's method [32] and the Phospholipids C test assay kit, respectively. Phospholipid classes were analyzed by high-performance liquid chromatography (HPLC) with evaporative light-scattering detection (ELSD) [33]. The silica column was LiChrosorb SI100 (4.6 × 250 mm, φ 10 μm; Waters Corporation, Milford, MA) with a binary gradient consisting of solvent A [chloroform/methanol/30% ammonium hydroxide (80:19.5:0.5, by vol)] and solvent B [chloroform/methanol/water/30% ammonium hydroxide (60:34:5.5:0.5, by vol)]. The gradient profile was as follows: 0–14 min, 100% B linear gradient; 14–24 min, 100% B. The flow rate was 1.0 mL/min, and the column was maintained at a temperature of 35°C. The post-column ELSD was a SEDEX model 55 (Sedere, Vitry sur Seine, France), kept at an evaporation temperature of 60°C and pressure of 2.0 bar (2.7 L/min) for nebulization gas (nitrogen). The photomultiplier sensitivity was adjusted to a gain of 8. Fatty acids and aldehydes were converted to FAME and DMA, respectively, and then were analyzed by gas chromatography [34].

Quantification of EtnGpl species

EtnGpl species were analyzed by HPLC with a 4000 QTRAP quadrupole/linear ion-trap tandem mass spectrometer (AB SCIEX, Tokyo, Japan) [29, 35]. EtnGpl species were analyzed using a silica column (Inertsil SIL-100A, 2.1 × 100 mm, φ 3 μm; GL Sciences, Tokyo, Japan) with a binary gradient consisting of solvent A [acetonitrile/methanol/1 M aqueous ammonium formate (pH 6.0) (78:20:2, by vol)] and solvent B [acetonitrile/methanol/1 M aqueous ammonium formate (pH 6.0) (49:49:2, by vol)]. The gradient profile was as follows: 0–1.0 min, 70% B; 1.0–1.1 min, 70–100% B linear gradient; 1.1–5.5 min, 100% B. The flow rate was 0.2 mL/min, and the column temperature was 40°C. To quantify EtnGpl species, multiple reaction monitoring of the transition of parent ions to product ions was performed. Quantification of EtnGpl species in plasma was performed for four PlsEtn species using negative ion mode (18:0/18:1-PlsEtn: 728.5/281.2, 18:0/20:4-PlsEtn: 750.5/303.2, 18:0/20:5-PlsEtn: 748.5/301.2, and 18:0/22:6-PlsEtn: 774.5/327.2) and eight PtdEtn species by positive ion mode (16:0/18:1-PtdEtn: 718.5/577.5, 16:0/18:2-PtdEtn: 716.5/575.5, 16:0/20:4-PtdEtn: 740.5/599.5, 16:0/22:6-PtdEtn: 764.5/623.5, 18:0/18:1-PtdEtn: 746.5/605.5, 18:0/18:2-PtdEtn: 744.5/603.5, 18:0/20:4-PtdEtn: 768.5/627.5, and 18:0/22:6-PtdEtn: 792.5/651.5). Due to limited RBCs, we could not quantify PtdEtn species.

Measurement of phospholipid hydroperoxide

In our former study [19], 28 patients with AD and 28 control subjects participated, and their RBC and plasma PLOOH (i.e., PCOOH and PEOOH) was determined. Because the remaining amounts of some samples were insufficient, we presently measured EtnGpl in the blood of 18 patients with AD and 18

control subjects. Hence, we extracted their PLOOH data from our former study [19]. The data were used for correlation analysis.

Other analytical methods

Plasma $A\beta_{40}$ and $A\beta_{42}$ levels were measured using sandwich ELISA with a Human β Amyloid ELISA kit according to the manufacturer's instructions. α-Tocopherols (α-Toc) in RBCs and plasma were measured by HPLC with fluorescence detection [36].

Measurement and imaging of Aβ aggregation in vitro

Measurement of thioflavin-T to evaluate Aβ aggregation was performed using the method described by Suemoto et al. [37] with slight modifications. The Aβ aggregate-formation and destabilization assays were examined for α-Toc, three fatty acids, and five phospholipids including three PlsEtn species. For the Aβ aggregate-formation assay, 20 μM $A\beta_{42}$ dissolved in 50 mM potassium phosphate buffer (pH 7.4) with each lipid was incubated at 37°C for 24 h. For the destabilization assay of preformed Aβ aggregates, after incubation for 24 h without a lipid, the mixture of aggregated Aβ and each lipid was incubated for 30 min at 37°C. At the end of the incubation, 3 μM thioflavin-T dissolved in 100 mM glycine buffer (pH 8.5) was added to the mixture. After incubation for 30 min at room temperature, the fluorescence of thioflavin-T bound to Aβ aggregates was measured using a microplate reader (Spectramax Gemini XS, Molecular Devices, Sunnyvale, CA) with excitation at 442 nm and emission at 485 nm. The percentage of inhibition was calculated by comparing the fluorescence values of test samples with those of control solutions without lipids.

Aβ aggregation images were subjected to morphological analysis by transmission electron microscopy [38]. Briefly, a 10-μL aliquot from the destabilized Aβ fibril was spread on a carbon-coated 400-mesh grid, negatively stained with 1% phosphotungstic acid, and examined under a Hitachi H-7000 electron microscope (Hitachi High-Technologies, Tokyo, Japan).

Statistical analyses

Data are presented as mean ± SEM and were tested by a Student's *t*-test. For correlation analyses, Pearson's correlation coefficient test for normal data or Spearman's rank correlation coefficient test for non-parametric data were used.

RESULTS

Aβ and phospholipid hydroperoxides in the blood of patients with AD and control subjects

In the plasma of patients with AD, levels of $A\beta_{40}$, $A\beta_{42}$, and PCOOH were higher than those of control subjects, but this finding was not significant (Table 2). After dividing groups of patients with AD into two advanced stages, we found that plasma $A\beta_{40}$ and $A\beta_{42}$ levels in the mild AD group (MMSE 19–25, $n=9$; $A\beta_{40}$ 111.0 ± 19.8 fmol/mL plasma, $A\beta_{42}$ 28.2 ± 8.5 fmol/mL plasma, $A\beta_{42}/A\beta_{40}$ 0.3 ± 0.1) tended to be higher than those in the moderate AD group (MMSE 7–18, $n=9$; $A\beta_{40}$ 96.1 ± 13.7 fmol/mL plasma, $A\beta_{42}$ 21.8 ± 6.6 fmol/mL plasma, $A\beta_{42}/A\beta_{40}$ 0.2 ± 0.1). On the other hand, RBC PCOOH, PEOOH, and PLOOH levels in patients with AD were three to four times higher than those of control subjects ($p<0.001$).

Table 2
Aβ, tocopherol, and phospholipid hydroperoxide in the plasma and RBCs of patients with AD and control subjects[1]

	Control subjects	Patients with AD
Plasma		
Aβ	(fmol/mL plasma)	
$A\beta_{40}$	81.2 ± 9.8	103.6 ± 11.8
$A\beta_{42}$	18.5 ± 2.8	25.0 ± 5.3
$A\beta_{42}/A\beta_{40}$	0.3 ± 0.0	0.3 ± 0.1
Tocopherol	(nmol/mL plasma)	
α-Toc	41.3 ± 3.9	38.4 ± 3.5
Phospholipid hydroperoxide	(pmol/mL plasma)	
PCOOH	29.8 ± 3.7	34.6 ± 5.4
	μmol/mol phospholipid	
PCOOH	25.3 ± 4.2	28.9 ± 4.8
RBC		
Tocopherol	(nmol/mL packed cells)	
α-Toc	16.8 ± 2.1	16.3 ± 1.8
Phospholipid hydroperoxide	(pmol/mL packed cells)	
PCOOH	9.6 ± 1.5	44.4 ± 10.4[2]
PEOOH	12.5 ± 2.3	37.6 ± 6.0[2]
PLOOH[4]	22.1 ± 3.6	82.0 ± 13.0[2]
	μmol/mol phospholipid	
PCOOH	5.2 ± 0.9	50.7 ± 29.6
PEOOH	6.6 ± 1.2	26.1 ± 7.9[3]
PLOOH	11.8 ± 2.0	76.7 ± 37.3

[1]Mean ± SEM; $n=18$. RBC and plasma PLOOH data were extracted from the PLOOH data ($n=28$) from our former study [19]. [2,3]Significantly different from patients with AD: $p<0.001$, $p<0.05$. [4]PLOOH is the sum of PCOOH and PEOOH. α-Toc, α-tocopherol; PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide; PLOOH, phospholipid hydroperoxide.

Table 3
Composition of acyl and alkenyl moieties in plasma and RBCs from patients with AD and control subjects[1]

	Control subjects	Patients with AD
Plasma		
Acyl	(μmol/mL plasma)	
18:1	4.4 ± 0.4	4.3 ± 0.3
20:4	0.8 ± 0.1	0.8 ± 0.1
20:5	0.1 ± 0.0	0.1 ± 0.0
22:6	1.1 ± 0.1	0.8 ± 0.2
DHA/ARA	1.5 ± 0.2	1.5 ± 0.5
Alkenyl	(μmol/mL plasma)	
16:0	0.0 ± 0.0	0.0 ± 0.0
18:0	0.7 ± 0.1	0.5 ± 0.1
18:1	0.5 ± 0.0	0.4 ± 0.1
Total	1.3 ± 0.1	0.9 ± 0.2
RBC		
Acyl	(μmol/mL packed cells)	
18:1	1.6 ± 0.1	1.6 ± 0.2
20:4	1.6 ± 0.2	1.5 ± 0.2
20:5	0.1 ± 0.0	0.2 ± 0.0
22:6	2.0 ± 0.2	1.9 ± 0.3
DHA/ARA	1.3 ± 0.1	1.3 ± 0.1
Alkenyl	(μmol/mL packed cells)	
16:0	0.3 ± 0.0	0.3 ± 0.0
18:0	0.3 ± 0.0	0.2 ± 0.0[2]
18:1	0.4 ± 0.0	0.2 ± 0.0[2]
Total	1.0 ± 0.1	0.8 ± 0.1[2]

[1]Mean ± SEM; $n = 18$. [2]Significantly different from patients with AD: $p < 0.05$. DHA, docosahexaenoic acid (22:6); ARA, arachidonic acid (20:4).

Acyl and alkenyl composition in the blood of patients with AD and control subjects

In both the plasma and RBCs of patients with AD, the levels of fatty acids investigated showed no significant difference compared with control subjects (Table 3). However, RBC 18:0 and 18:1 DMA (i.e., plasmalogen) levels in patients with AD were significantly lower than those in control subjects.

EtnGpl in the blood of patients with AD and control subjects

In the plasma of patients with AD, levels of EtnGpl, PlsEtn species, and PtdEtn species tended to be lower than those of control subjects. Moreover, 18:0/22:6-PlsEtn showed a strong significant difference ($p < 0.001$) (Table 4). On the other hand, in RBCs of patients with AD, levels of all PlsEtn species investigated were significantly lower than those of control subjects, and 18:0/22:6-PlsEtn and 18:0/20:5-PlsEtn showed about half of the values of control subjects (Table 5).

Table 4
Ethanolamine glycerophospholipid levels in the plasma of patients with AD and control subjects[1]

	Control subjects	Patients with AD
	(nmol/mL plasma)	
EtnGpl	74.6 ± 4.6	62.6 ± 5.4
18:0/18:1-PlsEtn	1.7 ± 0.1	1.3 ± 0.2
18:0/20:4-PlsEtn	9.9 ± 2.7	5.4 ± 0.4
18:0/20:5-PlsEtn	5.3 ± 1.8	2.1 ± 0.3
18:0/22:6-PlsEtn	3.9 ± 0.3	2.4 ± 0.3[2]
16:0/18:1-PtdEtn	1.1 ± 0.1	0.8 ± 0.1[4]
16:0/18:2-PtdEtn	2.7 ± 0.2	2.0 ± 0.2[4]
16:0/20:4-PtdEtn	3.0 ± 0.2	2.4 ± 0.2[4]
16:0/22:6-PtdEtn	13.9 ± 1.0	11.0 ± 0.8[4]
18:0/18:1-PtdEtn	1.0 ± 0.1	0.8 ± 0.1
18:0/18:2-PtdEtn	7.6 ± 0.6	5.8 ± 0.5[4]
18:0/20:4-PtdEtn	5.0 ± 0.3	4.5 ± 0.3
18:0/22:6-PtdEtn	4.4 ± 0.3	3.4 ± 0.2[3]
	(mmol/mol phospholipid)	
EtnGpl	80.0 ± 5.0	69.9 ± 6.0
18:0/18:1-PlsEtn	1.8 ± 0.1	1.3 ± 0.1[4]
18:0/20:4-PlsEtn	10.9 ± 3.0	6.3 ± 0.6
18:0/20:5-PlsEtn	6.0 ± 2.1	2.5 ± 0.4
18:0/22:6-PlsEtn	4.4 ± 0.5	3.0 ± 0.4[4]
16:0/18:1-PtdEtn	1.2 ± 0.1	0.9 ± 0.1[4]
16:0/18:2-PtdEtn	3.0 ± 0.3	2.3 ± 0.2
16:0/20:4-PtdEtn	3.3 ± 0.3	2.9 ± 0.3
16:0/22:6-PtdEtn	14.9 ± 0.9	12.4 ± 0.9
18:0/18:1-PtdEtn	1.1 ± 0.1	0.9 ± 0.1
18:0/18:2-PtdEtn	8.6 ± 0.9	6.9 ± 0.8
18:0/20:4-PtdEtn	5.6 ± 0.4	5.3 ± 0.5
18:0/22:6-PtdEtn	4.7 ± 0.3	3.9 ± 0.3

[1]Mean ± SEM; $n = 18$. [2,3,4]Significantly different from patients with AD: $p < 0.001$, $p < 0.01$, $p < 0.05$. EtnGpl, ethanolamine glycerophospholipid; PlsEtn, ethanolamine plasmalogen; PtdEtn, phosphatidylethanolamine.

Relationship between plasma Aβ and phospholipids in the plasma and RBCs of patients with AD and control subjects

RBC PLOOH and PCOOH levels of both patients with AD and control subjects had highly positive correlations with plasma $A\beta_{40}$ levels (Table 6). In addition, RBC PLOOH and PCOOH levels had positive correlations with plasma $A\beta_{40}$ levels, even if patients with AD and control subjects were mixed (Fig. 1). RBC PEOOH and plasma PCOOH levels had positive correlations with plasma $A\beta_{40}$ levels in only control subjects. On the other hand, there were correlations between levels of Aβ and some PlsEtn species only in the blood of control subjects (Table 7). Levels of 18:0/22:6-PlsEtn in plasma had a negative correlation with plasma $A\beta_{42}$ levels, while levels of 18:0/20:4-PlsEtn and 18:0/18:1-PlsEtn in RBCs had positive correlations with plasma $A\beta_{40}$ levels. There were no correlations between levels of Aβ and all PtdEtn species analyzed in the plasma (data not shown).

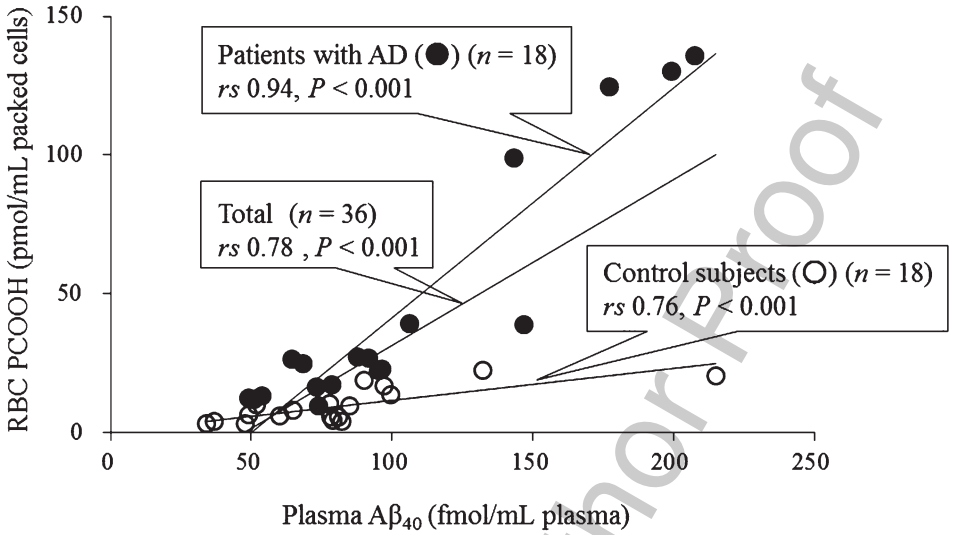

Fig. 1. Correlation between RBC phosphatidylcholine hydroperoxide and plasma $A\beta_{40}$ concentrations. The x-axis denotes the concentration of plasma $A\beta_{40}$. The y-axis indicates the concentration of RBC phosphatidylcholine hydroperoxide (PCOOH) that had been measured in our former study [19].

Table 5
Ethanolamine glycerophospholipid in the RBCs of patients with AD and control subjects[1]

	Control subjects	Patients with AD
	(nmol/mL packed cells)	
EtnGpl	631.3 ± 65.3	604.0 ± 54.1
18:0/18:1-PlsEtn	4.2 ± 0.6	3.1 ± 0.5[4]
18:0/20:4-PlsEtn	66.3 ± 7.2	43.9 ± 6.3[4]
18:0/20:5-PlsEtn	2.3 ± 0.5	1.1 ± 0.2[3]
18:0/22:6-PlsEtn	63.9 ± 6.3	38.9 ± 5.0[3]
	(μmmol/mol phospholipid)	
EtnGpl	397.7 ± 52.8	294.1 ± 34.5
18:0/18:1-PlsEtn	2.7 ± 0.4	1.5 ± 0.3[4]
18:0/20:4-PlsEtn	41.2 ± 5.0	21.9 ± 4.2[3]
18:0/20:5-PlsEtn	1.5 ± 0.4	0.5 ± 0.1[3]
18:0/22:6-PlsEtn	39.7 ± 5.0	19.0 ± 2.9[2]

[1]Mean ± SEM; $n = 18$. [2,3,4]Significantly different from patients with AD: $p < 0.001$, $p < 0.01$, $p < 0.05$. EtnGpl, ethanolamine glycerophospholipid; PlsEtn, ethanolamine plasmalogen.

Table 6
Correlations between levels of plasma Aβ and phospholipid hydroperoxide in the plasma and RBCs of patients with AD and control subjects[1]

	r		*p*	
	Plasma $A\beta_{40}$	Plasma $A\beta_{42}$	Plasma $A\beta_{40}$	Plasma $A\beta_{42}$
Patients with AD				
Plasma PCOOH	0.004	–0.182	0.988	0.469
RBC PCOOH	0.937	0.310	<0.001	0.211
RBC PEOOH	0.415	–0.220	0.087	0.381
RBC PLOOH	0.943	0.148	<0.001	0.557
Control subjects				
Plasma PCOOH	0.530	0.272	0.024	0.276
RBC PCOOH	0.758	0.210	<0.001	0.404
RBC PEOOH	0.808	0.200	<0.001	0.425
RBC PLOOH	0.815	0.209	<0.001	0.405

[1]$n = 18$. [2]PLOOH is the sum of PCOOH and PEOOH. PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide; PLOOH, phospholipid hydroperoxide.

Effects of lipids on Aβ fibrillation in vitro

Since the tendency of the correlations with plasma Aβ levels differed by PlsEtn species, we investigated the interaction between Aβ and PlsEtn species *in vitro*. The effects of lipids on the kinetics of formation and destabilization were evaluated by $A\beta_{42}$ showing a strong aggregation and thioflavin-T bounding to the fibrils (Table 8). At a concentration of 20 μM, 18:0/22:6-PlsEtn strongly inhibited Aβ fibril formation while DHA, other PlsEtn species without DHA, and other phospholipids with DHA did not. On the other hand, DHA and the PlsEtn species examined, especially 18:0/22:6-PlsEtn, showed destabilizing activity for Aβ fibrils.

Concentration-dependent effects of PlsEtn with DHA on the kinetics of Aβ fibril formation and breakdown

With regards to the inhibitory and destabilizing effects of 18:0/22:6-PlsEtn on Aβ fibril formation, concentration dependencies were examined by using the thioflavin-T method (Fig. 2A). At 10 μM

Table 7
Correlations between levels of plasma Aβ and ethanolamine plasmalogen species in the plasma and RBCs of patients with AD and control subjects[1]

	r		*p*	
	Plasma $A\beta_{40}$	Plasma $A\beta_{42}$	Plasma $A\beta_{40}$	Plasma $A\beta_{42}$
Plasma				
Patients with AD				
18:0/18:1-PlsEtn	–0.25	0.36	0.32	0.14
18:0/20:4-PlsEtn	0.26	0.23	0.29	0.35
18:0/20:5-PlsEtn	0.25	0.19	0.31	0.44
18:0/22:6-PlsEtn	0.08	0.26	0.77	0.29
Control subjects				
18:0/18:1-PlsEtn	0.01	–0.11	0.97	0.65
18:0/20:4-PlsEtn	–0.03	–0.24	0.90	0.34
18:0/20:5-PlsEtn	–0.14	0.32	0.59	0.20
18:0/22:6-PlsEtn	–0.30	–0.48	0.23	<0.05
RBC				
Patients with AD				
18:0/18:1-PlsEtn	–0.14	–0.13	0.58	0.61
18:0/20:4-PlsEtn	–0.09	–0.13	0.73	0.61
18:0/20:5-PlsEtn	–0.03	–0.18	0.90	0.46
18:0/22:6-PlsEtn	–0.18	–0.17	0.49	0.50
Control subjects				
18:0/18:1-PlsEtn	0.66	0.03	<0.01	0.90
18:0/20:4-PlsEtn	0.56	0.14	<0.05	0.58
18:0/20:5-PlsEtn	0.28	0.01	0.27	0.96
18:0/22:6-PlsEtn	0.42	0.03	0.08	0.89

[1] $n = 18$. PlsEtn, ethanolamine plasmalogen.

Table 8
The effect of lipids on Aβ fibril formation

	Aggregation (%)	Destabilization (%)
Tocopherol		
α-Toc	102.8 ± 2.2	109.8 ± 4.3
Fatty acids		
18:1	108.8 ± 12.8	110.7 ± 1.5
20:4	67.0 ± 1.0	97.3 ± 7.8
22:6	97.3 ± 5.9	49.0 ± 2.4
Phospholipids		
18:0/18:1-PlsEtn	91.9 ± 9.5	55.5 ± 3.5
18:0/20:4-PlsEtn	86.1 ± 2.8	51.4 ± 4.0
18:0/22:6-PlsEtn	46.1 ± 3.6	28.8 ± 1.0
18:0/22:6-PtdEtn	91.5 ± 3.8	97.5 ± 1.5
18:0/22:6-PtdCho	86.4 ± 0.8	82.4 ± 2.8

Aβ aggregation and preformed Aβ destabilization were assessed by the thioflavin-T method and expressed as a percentage of control aggregation, which was observed in the absence of 20 μM lipids. Values represent the means ± SEM from three independent experiments. α-Toc, α-tocopherol; PlsEtn, ethanolamine plasmalogen; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine.

18:0/22:6-PlsEtn, Aβ fibril formation was inhibited to 47.1% ± 2.4% of control levels, and preformed Aβ fibrils were destabilized to 37.4% ± 5.5% of control levels. At 100 μM, the inhibitory and destabilizing effects of Aβ fibrils were 27.1 ± 5.6% and 24.7% ± 4.7%, respectively. In addition, transmission electron microscopy revealed that preformed Aβ fibrils were destabilized by 18:0/22:6-PlsEtn in a concentration-dependent manner (Fig. 2B–D).

DISCUSSION

Aβ is deposited in the form of plaques in patients with AD, inducing oxidative injury in the brain and progressing AD pathologies [39, 40]. Aβ production and aggregation in brains are thought to affect Aβ concentrations of plasma and CSF [12, 41–43]. Our group and other researchers have found that plasma Aβ binds to RBCs and facilitates RBC lipid peroxidation *in vitro* as well as in *in vivo* animal studies [17]. In this study, we analyzed Aβ, lipid oxidative marker (i.e., PLOOH), and antioxidative lipid (i.e., PlsEtn) in the peripheral blood of patients with AD and their spouses.

In patients with AD, we observed higher levels of both plasma $A\beta_{40}$ and $A\beta_{42}$ when compared to the levels of their spouses; however, these increases were not significant. A previous meta-analysis revealed that Aβ levels in the plasma of individuals with mild cognitive impairment to early stages of AD are high, while levels in later stages of AD appear lower due to the facilitation of Aβ aggregation in the brain or reduced Aβ clearance across the blood-brain barrier [44]. Especially, $A\beta_{42}$ strongly aggregates; therefore, $A\beta_{42}/A\beta_{40}$ in plasma decreases by AD progression [12, 13, 45]. After dividing groups of patients with AD into two advanced stages, we found that plasma levels of Aβ and $A\beta_{42}/A\beta_{40}$ tended to be the same as those reported previously [44, 45].

When compared to control subjects, the increase in RBC PLOOH levels in patients with AD tended to be similar to what has been previously reported [19, 46]. Moreover, plasma $A\beta_{40}$ levels had a high positive correlation with RBC PCOOH levels in both patients with AD and control subjects. This relationship supports the hypothesis of a previous study conducted *in vitro*, which stated that plasma Aβ facilitates RBC lipid peroxidation [17]. On the other hand, plasma $A\beta_{40}$ levels did not exhibit a significant correlation with RBC PEOOH levels in patients with AD; however, plasma $A\beta_{40}$ levels were found to have a positive correlation with RBC PEOOH levels in control subjects. PEOOH may be affected in AD, which is characterized by the accumulation of advanced glycation end products, because it has an amino group as a target for nonenzyme glycation [39, 47].

Levels of PlsEtn species, especially those with DHA, in the RBCs and plasma of patients with AD

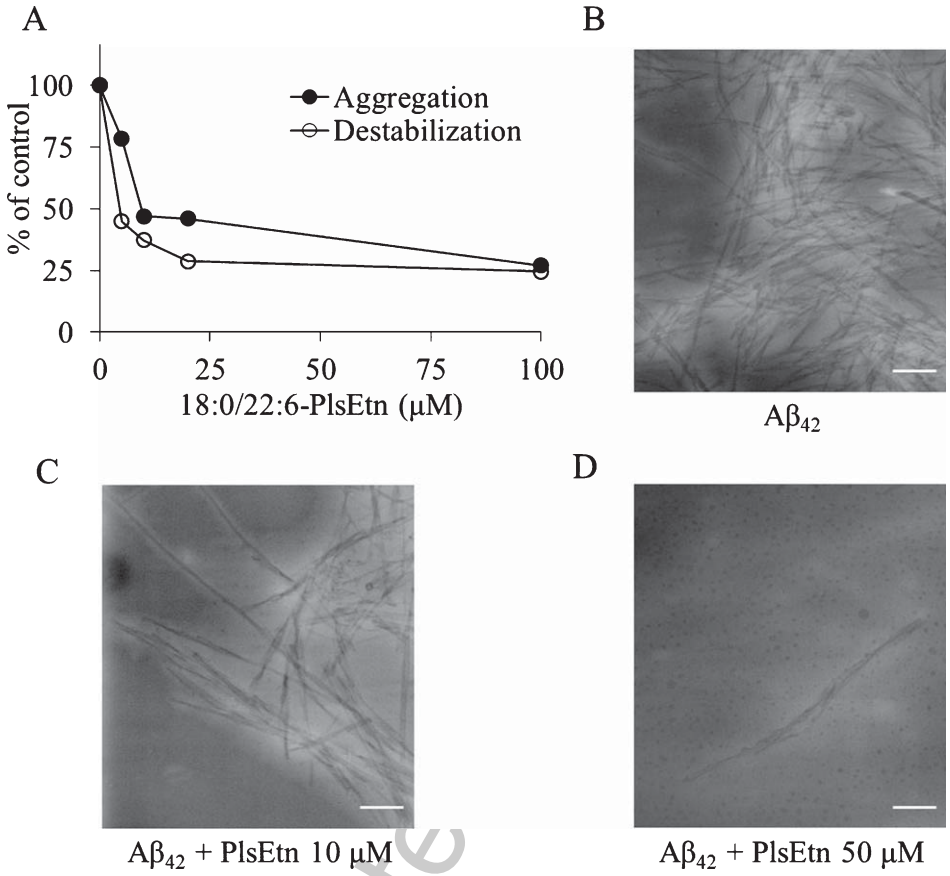

Fig. 2. The effect of plasmalogen containing DHA on Aβ fibril formation. A) Aβ aggregation and preformed Aβ destabilization assay. In the former assay, reaction mixtures containing 20 μM $Aβ_{42}$, 50 mM phosphate, and ethanolamine plasmalogen (PlsEtn) having DHA were incubated at 37°C for 24 h. In the latter assay, a reaction mixture containing 20 μM $Aβ_{42}$ was incubated at 37°C for 24 h. Then, PlsEtn with DHA was added and incubated for 30 min. Aβ aggregation was assessed by the thioflavin-T method and expressed as a percentage of control aggregation, which was observed in the absence of PlsEtn. B–D) After 24 h incubation of $Aβ_{42}$ for preformed fibrils, the mixture of aggregated Aβ and PlsEtn with DHA was incubated at 37°C for 30 min. Aβ aggregation images were subjected to morphological analysis by transmission electron microscopy. B) vehicle (ethanol). C) 10 μM PlsEtn. D) 50 μM PlsEtn. Scale bar = 150 nm.

were lower when compared to those of control subjects. With regard to variation among subjects, correlations were found between plasma Aβ levels and those of some PlsEtn species in the blood of control subjects, but not in the blood of patients with AD. These results suggest that Aβ affects PlsEtn levels in blood and that pathological factors other than Aβ accumulation may decrease PlsEtn levels in blood of patients with AD. In fact, accumulation of advanced glycation end products, and activation of phospholipase A_2, which hydrolyzes acyl ester bonds at the sn-2 position of PlsEtn, has been found in the brains of patients with AD (as described above) [48–50]. There have also been reports of the inactivation of some peroxisomes that synthesize PlsEtn and DHA [51, 52]. Therefore, although a low level of PlsEtn species in the blood is a good indicator of AD pathology, this antioxidative phospholipid might not tend to correlate with Aβ levels in the blood of patients with AD.

In the current study, we found that PlsEtn with DHA inhibited the formation of Aβ fibrils and destabilized preformed Aβ fibrils *in vitro* while diacyl phospholipids with DHA did not. Moreover, we determined that DHA destabilized preformed Aβ fibrils but that oxidized DHA did not (data not shown). Therefore, the effects of PlsEtn are thought to be a product of DHA, antioxidative activities of vinyl ether linkage, and hexagonal phase formation, which enables DHA to contact Aβ fibrils. Moreover, it has been reported that decreases in nerve cell PlsEtn activates γ-secretase, which produces Aβ from Aβ protein precursor [53, 54]. Thus, low levels of PlsEtn having DHA in the brain and blood may facilitate Aβ accumulation.

Lipid oxidation is linked to various diseases. Plasma PCOOH accumulation has been shown in elderly subjects, patients with hyperlipidemia [55], and those with diabetes [56, 57]. It is thought that abnormalities in lipid metabolism and glycation can increase plasma PCOOH [55, 57], and high plasma PCOOH levels may be related to atherosclerosis associated with hyperlipidemia and diabetes [58]. On the other hand, RBC PLOOH levels have been reported to increase in elderly subjects and patients with AD [19, 20, 46]. As shown in Table 3, RBCs have abundant levels of polyunsaturated fatty acids compared to plasma, and contain higher concentrations of molecular oxygen and ferrous ion. Therefore, RBCs are more susceptible to peroxidation than plasma. Since there are increasing levels of Aβ and oxidative stress in elderly subjects and patients with AD, RBCs are exposed to these stressors for the long durations; thus, RBC PLOOH has time to accumulate [17, 20]. When Aβ binds to RBCs to facilitate lipid peroxidation, it alters their morphology [59] and impairs oxygen delivery to the brain [15]. Moreover, the RBC binding of Aβ injures the blood-vascular system [18]. Therefore, high levels of Aβ and PLOOH in RBCs may advance AD symptoms.

PlsEtn, an antioxidant phospholipid, protects the brain from oxidative damage. Although brain PlsEtn levels are not decreased in elderly individuals, its oxidative form has been found to accumulate in the brain [60]. Brain PlsEtn levels are decreased in patients with some neurodegenerative diseases and peroxisomal disorders, and this decrease is thought to be caused by excessive oxidative stress, chronic inflammation, and peroxisome dysfunction [61]. In the blood of patients with AD, PlsEtn may be consumed due to protection from oxidation and inflammation. The peroxisome function decreases in the liver of patients with AD [52]; therefore, PlsEtn levels may be decreased in plasma lipoproteins. On the other hand, Aβ clearance from the blood is performed in the liver and kidneys [62], and peroxisomes are abundant in these organs. Taken together, these findings suggest that PlsEtn levels are deeply related to Aβ levels.

The use of brain amyloid imaging [5] and Aβ levels in the CSF [6] as biomarkers of AD is limited due to cost and safety factors. Therefore, identification of AD biomarkers in the blood will significantly improve patient safety and reduce the AD diagnostic costs. In this study, we found that PCOOH and PlsEtn with DHA could be potential candidates for blood-based biomarkers of AD. Recently, we developed a method to analyze PCOOH species in human plasma using LC-MS/MS [63]. Alterations of levels of PCOOH species in the blood of patients with AD is of interest. In addition, it has been reported that 70% of choline plasmalogen (PlsCho) decreases in the prefrontal cortex of patients with AD, even though PlsCho levels are lower than those of PlsEtn in the brain [64]. Levels of plasma alkyl type choline glycerophospholipid, the precursor of PlsCho, have also been reported to predict mild cognitive impairment or AD with high accuracy [65]. Therefore, blood-derived PlsCho species may prove effective as AD biomarkers.

While the prediction of AD is essential, AD prevention is even more important. Suppression of phospholipid peroxidation and PlsEtn degradation may protect RBCs and help prevent AD. In fact, supplementation with astaxanthin as a lipophilic antioxidant has been shown to decrease RBC Aβ and PLOOH levels [20]. It has also been reported that PlsEtn from the diet is absorbed into the blood [66], and PlsEtn with DHA shows the strongest suppression of neuronal apoptosis [67]. Most EtnGpl exists as PlsEtn in marine invertebrates such as ascidians [33], and PlsEtn species are abundant in DHA [29]. Thus, astaxanthin and PlsEtn from marine invertebrates may be potentially useful as dietary supplements aimed to prevent AD.

In conclusion, the results of this study suggest that RBC PCOOH levels reflect oxidative injury caused by Aβ, and that the levels of certain PlsEtn species, especially those having DHA, reflect AD pathophysiology that is related to Aβ. Therefore, it might prove useful to use PCOOH and PlsEtn as blood-based biomarkers of AD.

DISCLOSURE STATEMENT

Authors' disclosures available online (http://j-alz.com/manuscript-disclosures/15-0640r1).

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