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ORIGINAL CONTRIBUTION

Lymphatic absorption of choline plasmalogen is much higher than that of ethanolamine plasmalogen in rats

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Abstract
Background Plasmalogen is a subclass of phospholipids widely distributed in animal tissues and ingested as food; however, the absorptive characteristics of different classes of plasmalogen have not been clarified.
Aim of study Our object was to compare the lymphatic output of choline and ethanolamine plasmalogens after an administration of phospholipid preparations containing each class of plasmalogens, and to analyze molecular species of plasmalogen absorbed into the lymph.
Methods A duodenal infusion of 1 ml of 10% emulsion of choline phospholipid (PC) containing 50.6% choline plasmalogen (PlsCho) or ethanolamine phospholipid (PE) containing 52.5% ethanolamine plasmalogen (PlsEtn) was administered in the lymph duct-cannulated rats. Molecular species of plasmalogen absorbed into the lymph were measured by LC-MS/MS.
Results Lymph outputs of PlsCho and PlsEtn increased and reached a peak value at 3 h after PC and PE injection, respectively. The peak value of PlsCho was much higher and remained at a high level until 8 h, whereas PlsEtn output fell to half of the peak value at 7 h. Total lymphatic output of PlsCho was 5-times higher than that of PlsEtn. Compositions of *sn*-1 in lymph plasmalogens roughly reflected those of the injected lipids, whereas *sn*-2 in both PlsCho and PlsEtn was rich in arachidonic acid (20:4) regardless of the composition of the administered fatty acid. Both plasmalogen and lysoplasmalogen after PE injection were not released into the portal vein.
Conclusion Lymphatic absorption of PlsCho is much higher than that of PlsEtn in rats, and plasmalogens are re-esterified as 20:4-rich forms in the small intestine

Keywords Plasmalogen · Phospholipids · UPLC-MS/MS · Lymph absorption

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Introduction

Plasmalogen is a subclass of glycerophospholipids containing a vinyl-ether bond at the *sn*-1 position of the glycerol backbone. These lipids are widely distributed throughout human and animal tissues; the average proportion of plasmalogen in the total phospholipid pool in the human body is about 18% and in the human serum is 3–5% [1]. Many foods also contain plasmalogens [2]. Plasmalogens are mainly classified into two types of glycerophospholipid: choline plasmalogen (PlsCho) and ethanolamine plasmalogen (PlsEtn). The brain and lung contain relatively high levels of ethanolamine plasmalogens compared with other organs, but heart plasmalogens include relatively high in choline form [3]. Previous reports suggest that plasmalogens are structural components of the cell membrane and maintain cell membrane dynamics [4] and storage compounds of arachidonic acid (20:4), which is a precursor of many biologically active lipid mediators [5]. Plasmalogen may also be an endogenous lipidemic antioxidant, because the vinyl-ether double bond is highly

sensitive to oxidative agents [6, 7]. The antioxidant effect of plasmalogens has been clarified by both in vivo and in vitro findings that plasmalogen, especially PlsEtn, scavenged reactive oxygen species (ROS) [8, 9]. We have reported that serum plasmalogen levels decrease markedly with aging and positively correlated with high-density lipoprotein (HDL) [1]. Moreover, we have recently demonstrated that levels of plasmalogens are negatively correlated with the levels of atherogenic small dense LDL (sdLDL) in human serum [1]. These results suggest that serum plasmalogens function as beneficial factors for prevention of arteriosclerosis. It has been also reported that plasmalogen deficiency is involved in nerve degeneration in Alzheimer's disease [10, 11].

We previously reported that a small but significant amount of plasmalogen is absorbed into the lymph after the duodenal infusion of brain phospholipids containing PlsEtn [12] and that the plasmalogen concentration in the blood of the rats fed the same plasmalogen preparation was markedly increased [13]. However, there have been no previous reports on the absorptive properties of individual classes of plasmalogen.

The aims of this study were to compare the lymphatic output of choline and ethanolamine plasmalogens after an administration of phospholipid preparations containing high concentrations of each class of plasmalogens under the condition excluding the effects of gastric emptying rate and food intake, and to analyze molecular species of plasmalogen absorbed into the lymph by using a LC-MS/MS method.

Materials and methods

Preparation of test emulsion

The test lipids (100 g/l) in a 1-ml emulsified solution were as follows: 100 mg choline phospholipid (PC, containing 506 g choline plasmalogen/kg) and ethanolamine phospholipid (PE, containing 525 g ethanolamine plasmalogen/kg). One milliliter of the PC and PE emulsions contained 67.0 μmol PlsCho (estimated average molecular weight, 755) and 71.4 μmol PlsEtn (estimated average molecular weight, 735), respectively. Choline and ethanolamine phospholipids were extracted from bovine heart and porcine brain, respectively, by hexane/ethanol (8:2, by vol.), and were purified by a silica gel column chromatography. PlsCho was concentrated in the choline phospholipid preparation by ethanol/H2O (8:2, by vol.), and PlsEtn was concentrated by ethanol using the silica gel column chromatography as PC and PE, respectively. Fatty acid composition and molecular species composition of plasmalogens in PC and PE are shown in Table 2. The applied components other than PlsCho and PlsEtn in PC and PE were almost all choline diacylphospholipid and ethanolamine diacylphospholipid, respectively. These test lipid preparations were emulsified with sodium taurocholate (10 g/l) using a sonicator (150 W for 1.5 min, SONICATOR®, 5202, Ohtake Seisakusyo, Tokyo, Japan) just prior to use.

Animals

Male Wistar/ST rats (Japan SLC Inc., Hamamatsu, Japan), aged 9 weeks, were fed a semipurified casein sucrose-based diet (AIN 93G formula) for a 5-d acclimation period. This study was approved by the Hokkaido University Animal Committee (approval number; No. 08-0136), and animals were maintained in accordance with the Hokkaido University guideline for the care and use of laboratory animals.

Experiment 1: lymphatic absorption of plasmalogen

After a 12-h fast, acclimatized rats were implanted a vinyl catheter (SV-35; 0.5 mm I.D., 0.8 mm O.D.; Natsume Seisakusyo, Tokyo, Japan) and a silicone catheter (Silascon SH No. 00; 0.5 mm I.D., 1.0 mm O.D.; Kaneka Medix Co., Osaka, Japan) into the thoracic lymph duct and the duodenum, respectively, under anesthesia (sodium pentobarbital, 40 mg/kg body weight) [14].

After surgery, the rats were placed individually in Bollman-type restraining cages. An isoosmotic solution containing 139 mM glucose and 85 mM NaCl was infused continuously at a rate of 3 ml/h through the duodenal catheter during the 1-d recovery and experimental periods except during test lipid administration. We confirmed whether the lymph flow was at least 0.7 ml/h before starting the experiment to check conditions of rats, because the lymph flow is suppressed with bad conditions. After the collection of lymph for 30 min (initial lymph) on the next day of the surgery, the rats were administered 1 ml of an emulsified test solution containing 100 mg test lipids for 1 min, and infusion of the glucose-NaCl solution without test lipids was resumed at 3 ml/h through the duodenal tube until the end of the experiment. The lymph was collected at 0.5-h intervals during the first 2 h and at 1-h intervals during the next 6 h following the administration of the test solution. The collected lymph was frozen immediately and kept at −80 °C until subsequent analyses.

Experiment 2: evaluation of direct blood absorption of plasmalogen in the lymph-cannulated rats

We have done the experiment 2 to examine whether phospholipids including plasmalogens are released into blood via portal vein, but not through lymph, in rats

cannulated with the lymph duct. Acclimated rats were implanted a vinyl catheter (SV-35; 0.5 mm I.D., 0.8 mm O.D.; Natsume Seisakusyo, Tokyo, Japan) into the thoracic lymph duct and silicon catheters (Silascon SH No. 00; 0.5 mm I.D., 1.0 mm O.D.; Kaneka Medix Co., Osaka, Japan) into the duodenum and portal vein, respectively. After 1-d recovery, the rats were administered 1 ml of an emulsified test solution containing PlsEtn or triglyceride (soybean oil), in a manner similar to Experiment 1. The lymph was collected at 0.5-h or 1-h intervals by 3 h after the lipid administration, and the blood of portal vein was collected 0, 15, 30, 60, 120, 180 and 240 min after an administration of the test solution. The plasma was separated by centrifugation (1,300×*g* for 10 min at 4 °C).

Analyses

Total lipids in the lymphatic fluid and plasma were extracted by chloroform/methanol (1:2, by vol.) after freeze-drying. Plasmalogens in the lipid extracts were analyzed by ultra-performance liquid chromatography/electrospray ionization tandem mass spectrometry (UPLC/ESI-MS/MS) as described below [15].

Liquid chromatography (LC) separation was performed using an Acquity UPLC system (Waters) with a BEH C8 column (1.7 μm, 100 mm × 2.1 mm I.D.; Waters) at 60 °C and a flow rate of 600 μl/min. The auto sampler was kept at 15 °C. The sample injection volume was 5 μl. Mobile phase A consisted of water containing 20 mM ammonium formate, and mobile phase B consisted of acetonitrile/acetone (90:10 by vol.). Mobile phase A was set at 100% at 0 min, decreased linearly to 10% at 3 min and then maintained at 10% for 3 min. Mobile phase A was then increased immediately to 100% at 6 min and then maintained at 100% for a further 2 min. The total turnaround time was 8 min.

MS analysis was performed using a Quattro Premier XE quadrupole tandem MS (Waters) equipped with an ESI probe in positive ion mode. Capillary voltage was 3,200 V, source temperature 80 °C and desolvation temperature 400 °C. The cone voltage was 35 V. The collision energy was 40 eV for PlsCho and 15 eV for PlsEtn. The desolvation and cone gas flow were 800 and 50 L/h, respectively. The number of molecular species measured by LC-MS/MS was eighteen for both PlsCho and PlsEtn. We checked many molecular species in a preliminary experiment and selected those contained above 1% of total plasmalogen (Table 1). PlsEtn was quantified according to the principles described by Zemski and Murphy [15]. In brief, fragment ions at m/z 364, 392 and 390 were used for the identification of the *sn*-1 position of PlsEtn (16:0, 18:0 and 18:1, respectively). PlsCho was identified by three fragments (MS/MS product in Table 1) and was quantified by phosphocholine as a fragment ion at m/z 184 after the separation of each PlsCho molecule by UPLC. Moreover, confirmation of the plasmalogen peak was performed by the disappearance of the peak after acidic hydrolysis of plasmalogen [16]. Synthetic p18:0–18:1, p18:0–20:4 and p18:0–22:6 plasmenylcholine (choline plasmalogen) and plasmenylethanolamine (ethanolamine plasmalogen) were used as standard compounds (Avanti Polar Lipids, Inc., Alabaster, AL, USA). "p" indicates the carbon chain of *sn*-1 in plasmalogen with a vinyl-ether linkage. Concentration of individual plasmalogen was calculated from the peak area in the chromatogram detected with relative to the internal standard (1,2-dimyristoyl phosphatidylcholine for PlsCho and 1,2-dimyristoyl phosphatidylethanolamine for PlsEtn, respectively).

LysoPlsCho and lysoPlsEtn were measured using selected ion recording mode performed by examining product ions of the deprotonated molecules from each lysoplasmalogen. The m/z of lysoPlsCho that contained p16:0, p18:0 and p18:1 at *sn*-1 position were 480, 508 and 506, respectively. The m/z of lysoPlsEtn that contained p16:0, p18:0 and p18:1 at *sn*-1 position were 438, 466 and 464, respectively. Synthetic p18:0 lysoplasmenylcholine (lysocholine plasmalogen) and lysoplasmenylethanolamine (lysoethanolamine plasmalogen) were used as standard compounds (Avanti Polar Lipids, Inc., Alabaster, AL, USA).

Total phospholipid concentration in the lymph fluid was measured using the modified Bartlett method [17] as described below. A part of sample extracted by chloroform/methanol was placed in screw top glass tube and dried under a stream of nitrogen gas. A dried sample was vortexed briefly after the addition of 0.4 ml of perchloric acid, and that was heated and refluxed at 200 °C for 60 min with a cap. After cooling for 30 min, 4.2 ml of water, 0.2 ml of 5% ammonium molybdate and 0.2 ml of 5% 2,4-diaminophenol dihydrochloride in 20% sodium sulfite solution were added. After vortexing, the sample was boiled for 7 min, and the absorbance was measured at 830 nm after cooling. The amount was calculated from value of NaH2PO4 used as a standard.

Calculation and statistical analysis

Total plasmalogen and lysoplasmalogen were taken as the sums of all molecular species measured by LC-MS/MS or LC-MS, respectively. Values are shown as the means with SEM. The results were analyzed by one-way (Fig. 1) and repeated measure (Figs. 2, 3) ANOVA. Significance of difference between groups was evaluated by Tukey–Kramer post hoc test. Data analysis was performed with StatView for Macintosh (version 5.0, SAS Institute Inc., Cary, NC). Differences with $P < 0.05$ were taken to be statistically significant, and values not sharing a common superscript are significantly different.

Table 1 m/z values of plasmalogen in positive ion mode

sn-1 position Alkenyl	*sn*-2 position Acyl	Retention time (min)	MS $[M+H]^+$	MS/MS product ion
Choline plasmalogen				
16:0	18:1	4.59	744.6	184 480 504
	18:2	4.31	742.6	184 480 502
	20:4	4.24	766.6	184 480 526
	20:5	4.09	764.6	184 480 524
	22:4	4.48	794.6	184 480 554
	22:6	4.17	790.6	184 480 550
18:0	18:1	5.00	772.6	184 508 504
	18:2	4.63	770.6	184 508 502
	20:4	4.60	794.6	184 508 526
	20:5	4.34	792.6	184 508 524
	22:4	4.90	822.7	184 508 554
	22:6	4.48	818.7	184 508 550
18:1	18:1	4.62	770.6	184 506 504
	18:2	4.34	768.6	184 506 502
	20:4	4.32	792.6	184 506 526
	20:5	4.11	790.6	184 506 524
	22:4	4.53	820.7	184 506 554
	22:6	4.22	816.6	184 506 550
Ethanolamine plasmalogen				
16:0	18:1	4.58	702.5	364 339 561
	18:2	4.30	700.5	364 337 559
	20:4	4.25	724.5	364 361 583
	20:5	4.08	722.5	364 359 581
	22:4	4.47	752.6	364 389 611
	22:6	4.16	748.5	364 385 607
18:0	18:1	4.98	730.5	392 339 589
	18:2	4.61	728.5	392 337 587
	20:4	4.59	752.6	392 367 617
	20:5	4.33	750.5	392 363 613
	22:4	4.88	780.6	392 361 611
	22:6	4.47	776.6	392 361 607
18:1	18:1	4.62	728.5	390 339 587
	18:2	4.33	726.5	390 337 585
	20:4	4.31	750.5	390 369 609
	20:5	4.10	748.5	390 367 607
	22:4	4.52	778.6	390 389 637
	22:6	4.20	774.6	390 385 633

Results

Experiment 1: lymphatic absorption of plasmalogen

Both PlsCho and PlsEtn output in the lymph were increased after a 30-min lag period, reaching a peak value at 3 h after a bolus injection (1 min) of PC or PE into the duodenum (Fig. 1a, b). The peak value of PlsCho was twofold higher than that of PlsEtn. Lymphatic PlsCho output remained close to the peak value until 8 h after the injection; however, the PlsEtn level fell rapidly from 4 h to reach half of the peak value at 7 h. Total lymphatic output of plasmalogen was 633 ± 144 nmol/8 h for PlsCho and 150 ± 28.2 nmol/8 h for PlsEtn after PC and PE injection, respectively. The recovery rate of total PlsCho into the lymph was approximately 5 times greater than that of PlsEtn against the administered individual plasmalogen classes in the lipid emulsion (0.945 mol% for PlsCho and 0.210 mol% for PlsEtn).

Lymph flow rates were increased at 1.5–2 h after PC and PE injection with no significant differences observable between PC and PE (Fig. 2a). Lymphatic output of total

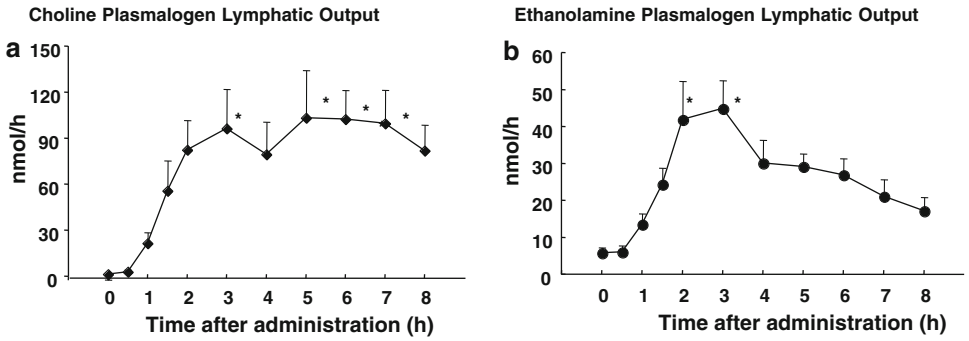

Fig. 1 Changes in lymphatic output of choline plasmalogen (**a**) and ethanolamine plasmalogen (**b**) after a duodenal instillation of choline or ethanolamine plasmalogen concentrated phospholipid preparations in thoracic lymph-cannulated rats. Values are means with SEM, $n = 6$. P value for choline plasmalogen (**a**) was <0.001. P value for ethanolamine plasmalogen (**b**) was <0.001. *Asterisk* indicates a significant increase against the value at time 0 ($P < 0.05$)

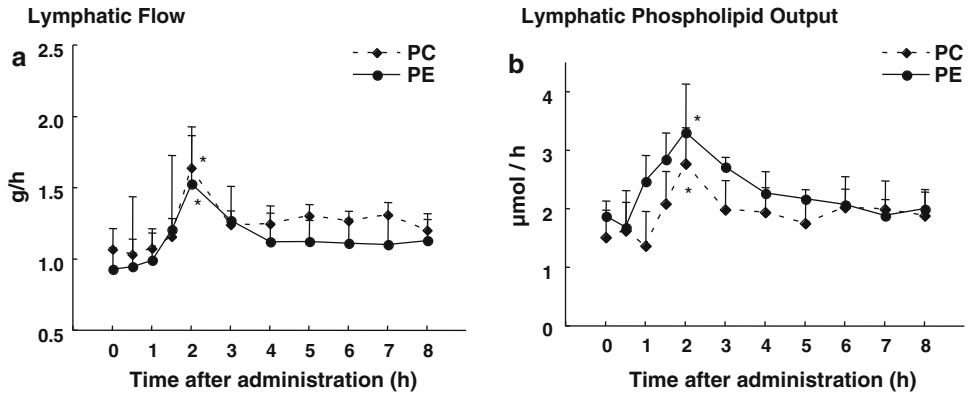

Fig. 2 Changes in lymphatic flow (**a**) and lymphatic output of total phospholipids (**b**) after a duodenal instillation of choline- or ethanolamine plasmalogen-concentrated phospholipid preparations in thoracic lymph-cannulated rats. Values are means with SEM, $n = 6$. P-values for lymph flow were 0.126 for lipid (L), 0.012 for time (T) and 0.998 for L × T. P-values for lymphatic output of phospholipids were 0.144 for lipid (L), 0.023 for time (T) and 0.896 for L × T. *Asterisk* indicates a significant increase against the values at time 0 ($P < 0.05$)

phospholipids was increased and reached a similar peak value 2 h after the injection of PC or PE. The peak levels were double the initial lymphatic levels of total phospholipids in both the PC and PE groups (Fig. 2b). Lymphatic output of triglyceride was slightly, but not significantly, increased in both groups after the administration of the PC or PE emulsions (Data not shown).

The molar ratios of major fatty acids in the injected lipids (Table 2), which were derived from *sn*-1 and *sn*-2 in the diacylphospholipids and *sn*-2 in the plasmalogens, were 16:0 (20.9%), 18:1 (13.5%) and 18:2 (52.4%) for PC, and 18:0 (19.9%), 18:1 (31.7%) and 20:4 (14.9%) for PE. The molar ratios of the major plasmalogen species (Table 2) were p16:0–18:2 (37.1%), p16:0–18:1(17.0%), p16:0–20:4 (13.8%), p18:0–18:2 (12.9%) and p18:1–18:2 (12.5%) for PC, and p16:0–18:1 (19.6%) and p18:1–18:1 (28.5%) for PE. Other plasmalogen molecules in PE were less than 10%, but the sum of p16:0–20:4, p18:0–20:4 and p18:1–20:4 was nearly 20% in PE.

The molar composition of plasmalogen released into the lymph was shown at 0 h (initial lymph), 4 and 8 h after the administration of test lipids (Table 3A). The main molecular species in PlsCho in the initial lymph were p16:0–20:4 (36.7%), p18:1–20:4 (16.3%) and p18:0–20:4 (13.5%), and those in PlsEtn were p18:0–20:4 (28.7%), p16:0–20:4 (19.6%) and p18:1–20:4 (8.7%). After PC and PE injection, changes were observed in molecular species in both the lymphatic PlsCho and PlsEtn. In the lymph 4 h after PC injection, the main molecular species in PlsCho were p16:0–20:4 (36.2%) and p16:0–18:2 (25.7%), that is, p16:0–20:4 remained at a high level and p16:0–18:2 appeared as a major molecule, whereas the levels of p18:0–20:4 and p18:1–20:4 were reduced compared with those in the initial lymph. In contrast, main molecules in PlsEtn in the 4-h lymph were p18:1–20:4 (32.6%), p18:0–20:4 (21.3%) and p16:0–20:4 (14.8%), that is, the main molecular species were the same as those in the initial lymph with only an increase in p18:1–20:4. The changes in

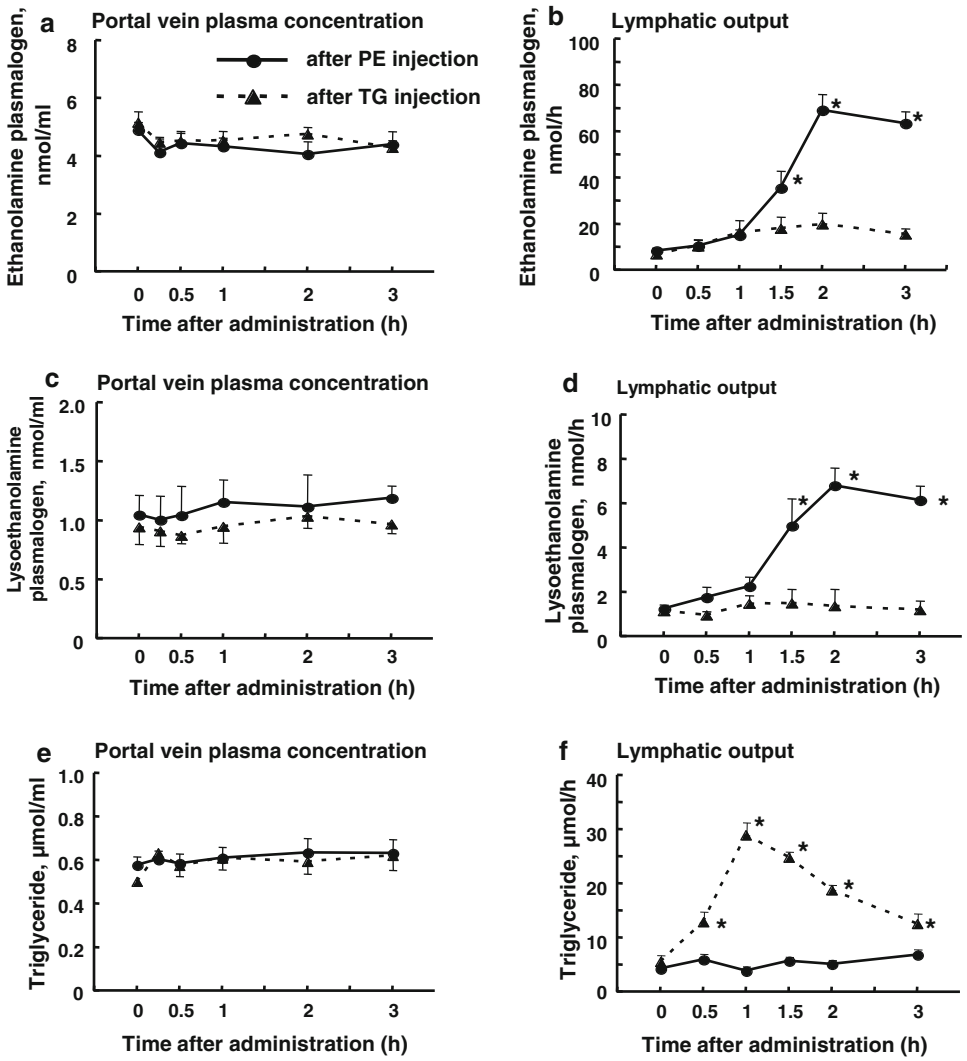

Fig. 3 Changes in plasma concentration of the portal blood (**a**, **c** and **e**) and lymphatic output (**b**, **d** and **f**) of ethanolamine plasmalogen (**a** and **b**), lysoethanolamine plasmalogen (**c** and **d**) and triglyceride (**e** and **f**), respectively, after a duodenal instillation of ethanolamine plasmalogen-concentrated phospholipid preparations (PE, *solid lines*) or triglyceride (TG; soybean oil, *dashed lines*) in the portal vein and thoracic lymph duct-cannulated rats. Values are means with SEM, $n = 6$. *P*-values for plasma concentration of ethanolamine plasmalogen (**a**) were 0.245 for lipid (L), 0.392 for time (T) and 0.911 for L × T. *P*-values for lymphatic output of ethanolamine plasmalogen (**b**) were <0.001 for lipid (L), <0.001 for time (T) and 0.052 for L × T. *P*-values for plasma concentration of lysoethanolamine plasmalogen (**c**) were 0.213 for lipid (L), 0.455 for time (T) and 0.721 for L × T. *P*-values for lymphatic output of lyso-ethanolamine plasmalogen (**b**) were <0.001 for lipid (L), 0.019 for time (T) and 0.062 for L × T. *P*-values for plasma concentration of triglyceride (**e**) were 0.556 for lipid (L), 0.591 for time (T) and 0.384 for L × T. *P*-values for lymphatic output of triglyceride (**f**) were 0.006 for lipid (L), <0.001 for time (T) and <0.001 for L × T. *Asterisk* indicates a significant increase against the values at time 0 ($P < 0.05$)

the 8-h lymph were similar to those in the 4-h lymph in both the PC and PE groups.

The molar ratio of carbon chains in the *sn*-1 of PlsCho was 53.2% for p16:0, 23.8% for p18:1 and 23.0% for p18:0, and those of PlsEtn were 49.2% for p18:0, 36.0% for p16:0 and 14.8% for p18:1 in the initial lymph (Table 3B). After the lipid injection (4 h), those of PlsCho were 77.7% for p16:0, 13.6% for p18:1 and 8.7% for p18:0, and those of PlsEtn were 46.5% for p18:1, 31.2% for p18:0 and 22.3% for p16:0. The changes in PlsCho, but not in PlsEtn, after lipid administration seem to reflect the composition of *sn*-1 in the injected plasmalogen as shown in Table 2B. The ratio of p18:1 was markedly increased in PlsEtn.

The major fatty acids at the *sn*-2 position in the initial lymph for PlsCho were 20:4 (64.3%), 18:1 (19.1%) and 18:2 (8.2%), and those for PlsEtn were 20:4 (57.2%), 22:4 (12.7%) and 22:6 (14.7%), as shown in Table 3C. After the lipid injection (4 h), the main fatty acids at the *sn*-2 position of PlsCho were 20:4 (43.2%), 18:1 (14.0%) and 18:2 (34.0%), that is, the 18:2 acyl group was clearly increased

Table 2 Fatty acid composition (%) in the injected lipids and molecular species composition (%) in the plasmalogen of the injected lipids

		PC	PE
*(A) The composition of fatty acids**			
14:0		0.7	0.2
16:0		20.9	5.9
16:1		1.2	0.8
18:0		4.6	19.9
18:1		13.5	31.7
18:2		52.4	1.2
18:3		0.4	1.4
20:0		0.0	0.3
20:1		0.0	2.7
20:4		5.8	14.9
22:0		0.1	0.2
22:4		0.4	7.3
22:6		0.0	13.7
24:0		0.0	0
Total		100	100
sn-1 Alkenyl	*sn*-2 Acyl	PC	PE
(B) The composition of molecular species in plasmalogen			
16:0	18:1	17.0	19.6
	18:2	37.1	0.2
	20:4	13.8	5.6
	22:4	1.8	5.5
	22:5	–	0.5
	22:6	–	5.7
sn-1		69.8	37.1
18:0	18:1	2.2	5.6
	18:2	12.9	–
	20:4	1.8	4.3
	22:4	–	3.1
	22:5	–	0.3
	22:6	–	6.3
sn-1		16.9	19.6
18:1	18:1	–	28.1
	18:2	12.5	0.2
	20:4	0.9	9.2
	22:4	–	3.2
	22:5	–	–
	22:6	–	2.7
sn-1		13.4	43.4
	Total	100.0	100.0

* The compositions in PC and PE were calculated as the sum of fatty acids at the *sn*-2 position in the plasmalogens and at the *sn*-1 and *sn*-2 positions in the diacylphospholipids

in PlsCho after the injection. The main fatty acids in PlsEtn after the injection were 20:4 (67.8%), 22:4 (5.8%) and 22:6 (16.4%), that is, the *sn*-2 composition of PlsEtn in the 4-h lymph was not significantly changed from that of the initial lymph with only a minor increase in the 20:4 and a decrease in 22:4 acyl groups.

The amount of each molecular species of PlsCho and PlsEtn in the lymph at 4 and 8 h after the injection of PC or PE was markedly increased reflecting the ratio of plasmalogen species shown in Table 3. The main molecular species in PlsCho in the initial lymphatic outputs were p16:0–20:4 (0.47 nmol/h), p18:1–20:4 (0.19 nmol/h) and p18:0–20:4 (0.16 nmol/h), and those in PlsEtn were p18:0–20:4 (1.65 nmol/h), p16:0–20:4 (1.21 nmol/h) and p18:1–20:4 (0.53 nmol/h). After PC and PE injection, main molecular species were changed in PlsCho, but not in PlsEtn. In the lymph 4 h after PC injection, p16:0–20:4 (26.7 nmol/h) and p16:0–18:2 (21.0 nmol/h) were largely increased, whereas p18:1–20:4 (3.79 nmol/h) and p18:0–20:4 (4.11 nmol/h), which were main molecular species in the initial lymph, were slightly increased. In contrast, main molecules in PlsEtn in the 4-h lymph were still p18:1–20:4 (9.69 nmol/h), p18:0–20:4 (6.54 nmol/h) and p16:0–20:4 (4.43 nmol/h); however, increase in p18:1–20:4 was much larger compared to other species. Regarding fatty acids at the *sn*-2 position, PlsCho with 20:4 and 18:2 was largely increased in the lymph at 4 h (from 0.851 nmol/h to 30.6 nmol/h for 20:4, and from 0.14 nmol/h to 25.9 nmol/h for 18:2) and comprised 50 and 30% of the increased total fatty acids in *sn*-2 position, respectively. PlsEtn with 20:4 in the *sn*-2 position comprised nearly 80% of increased PlsEtn in the 4-h lymph (from 3.39 to 20.7 nmol/h).

Experiment 2: evaluation of direct blood absorption of plasmalogen

Low levels of lysoPlsEtn were detected in the portal blood and lymph fluid, but initial concentrations in portal blood and the initial lymphatic output of PlsEtn were fivefold and sevenfold higher than those of lysoPlsEtn, respectively. We found no increase in plasma concentration of PlsEtn and lysoPlsEtn (Fig. 3a, c), but large increase in lymphatic output of PlsEtn and lysoPlsEtn (Fig. 3b, d) after duodenal instillation of ethanolamine plasmalogen (PE) in the portal vein- and thoracic lymph-cannulated rats (solid lines). Triglycerides (soybean oil) as a reference lipid were also instilled into duodenum to confirm no increases in PlsEtn and lysoPlsEtn (dashed lines, Fig. 3a–d) and absorptive activity for lipid. We showed sufficient increases in TG release in the lymph, but not in the blood plasma. (Fig. 3e, f).

Table 3 Ratio of molecular species, classification with *sn*-2 position and *sn*-1 position in the lymphatic plasmalogen at 0 h (initial lymph), 4 and 8 h after administration of the test lipids

A. Molecular species (%)

Alkenyl	Acyl	PlsCho			PlsEtn		
		Initial lymph[a]	4 h[b]	8 h[c]	Initial lymph	4 h	8 h
16:0	18:1	5.59 ± 0.75	9.06 ± 1.52	12.76 ± 2.25	2.16 ± 0.52	1.03 ± 0.13	1.14 ± 0.12
	18:2	5.79 ± 0.84	25.66 ± 1.64	27.20 ± 2.29	3.42 ± 2.16	0.69 ± 0.24	0.79 ± 0.23
	20:4	34.88 ± 2.47	36.11 ± 3.83	31.27 ± 4.23	19.62 ± 1.32	14.75 ± 0.52	12.62 ± 0.59
	20:5	1.15 ± 0.17	4.27 ± 0.33	3.26 ± 0.29	0.27 ± 0.07	0.13 ± 0.01	0.09 ± 0.01
	22:4	3.20 ± 0.50	0.09 ± 0.03	0.25 ± 0.11	3.45 ± 0.26	1.60 ± 0.13	2.43 ± 0.18
	22:6	3.19 ± 0.43	2.46 ± 0.36	1.68 ± 0.23	5.53 ± 0.48	3.59 ± 0.32	3.66 ± 0.28
18:0	18:1	5.20 ± 4.14	1.12 ± 0.25	1.99 ± 0.48	1.78 ± 0.10	1.40 ± 0.06	1.80 ± 0.22
	18:2	2.85 ± 0.82	2.14 ± 0.32	2.89 ± 0.46	4.55 ± 0.56	1.50 ± 0.09	1.89 ± 0.24
	20:4	12.78 ± 1.95	5.12 ± 0.37	4.76 ± 0.40	28.67 ± 2.40	21.33 ± 0.83	19.83 ± 0.72
	20:5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.38 ± 0.08	0.16 ± 0.01	0.17 ± 0.01
	22:4	1.69 ± 0.40	0.04 ± 0.01	0.16 ± 0.11	8.69 ± 1.24	2.97 ± 0.27	4.14 ± 0.19
	22:6	1.07 ± 0.18	0.30 ± 0.04	0.23 ± 0.03	6.22 ± 0.82	3.87 ± 0.24	4.25 ± 0.19
18:1	18:1	2.52 ± 0.20	2.05 ± 0.34	2.84 ± 0.43	0.96 ± 0.25	3.34 ± 0.27	4.06 ± 0.43
	18:2	0.00 ± 0.00	3.56 ± 0.99	4.13 ± 0.42	1.18 ± 0.31	1.46 ± 0.06	1.71 ± 0.17
	20:4	15.46 ± 3.43	5.36 ± 0.67	4.63 ± 0.76	8.70 ± 0.73	32.55 ± 1.23	31.02 ± 1.46
	20:5	3.33 ± 0.40	2.37 ± 0.35	1.72 ± 0.25	0.10 ± 0.03	0.33 ± 0.01	0.25 ± 0.04
	22:4	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.37 ± 0.15	1.26 ± 0.08	2.33 ± 0.22
	22:6	1.29 ± 0.39	0.26 ± 0.04	0.23 ± 0.02	2.62 ± 0.37	7.27 ± 0.77	6.74 ± 0.77
Total		100	100	100	100	100	100

B. Classification with *sn*-1 position (%)

Alkenyl	PlsCho			PlsEtn		
	Initial lymph	4 h	8 h	Initial lymph	4 h	8 h
16:0	53.80 ± 3.05	77.67 ± 1.19	76.42 ± 1.56	36.04 ± 3.89	22.27 ± 1.05	21.06 ± 1.05
18:0	23.59 ± 3.11	8.73 ± 0.61	10.03 ± 1.01	49.20 ± 3.75	31.22 ± 0.85	32.08 ± 0.73
18:1	22.61 ± 4.05	13.60 ± 0.71	13.55 ± 0.96	14.76 ± 1.11	46.52 ± 0.84	46.86 ± 0.78
Total	100	100	100	100	100	100

C. Classification with *sn*-2 position (%)

Acyl	PlsCho			PlsEtn		
	Initial lymph	4 h	8 h	Initial lymph	4 h	8 h
18:1	18.36 ± 4.02	13.94 ± 2.04	17.59 ± 3.02	4.84 ± 0.64	5.57 ± 0.36	7.00 ± 0.70
18:2	7.82 ± 1.28	33.93 ± 2.62	34.22 ± 2.55	9.74 ± 2.70	3.77 ± 0.29	4.39 ± 0.53
20:4	60.51 ± 4.06	43.20 ± 4.49	40.66 ± 5.12	57.21 ± 2.70	67.82 ± 1.38	63.47 ± 2.13
20:5	4.03 ± 0.36	6.61 ± 0.20	4.98 ± 0.34	0.77 ± 0.12	0.61 ± 0.03	0.51 ± 0.05
22:4	5.27 ± 0.83	0.09 ± 0.03	0.42 ± 0.22	12.72 ± 1.22	5.82 ± 0.41	8.90 ± 0.38
22:6	3.99 ± 0.90	2.23 ± 0.44	2.14 ± 0.28	14.72 ± 0.97	16.41 ± 1.27	15.72 ± 1.07
Total	100	100	100	100	100	100

Values are means with SEM, $n = 6$

[a] Initial lymph; lymph collected for 30 min before the lipid injection

[b] 4 h; lymph collected between 3 and 4 h after the lipid injection

[c] 8 h; lymp collected between 7 and 8 h after the lipid injection

Discussion

The present study shows that the release of plasmalogens into the thoracic lymph was dramatically increased after an enteral injection of a concentrated preparation of each class of plasmalogen, and we found that the absorptive rate was nearly 5-times higher for PlsCho than for PlsEtn. It has been reported that the lymphatic absorption of diacyl phosphatidylethanolamine is lower than that of diacyl phosphatidylcholine, and diacyl phosphatidylethanolamine is mainly absorbed via the portal vein route [18]. However, we reveal no increases in PlsEtn levels in blood after an

injection of PE in rats with the thoracic duct cannulae. It is unlikely that the lower lymphatic absorption of PlsEtn is due to large absorption of PlsEtn via the portal route. Release as lysoplasmalogens is one possible reason, and we showed that ethanolamine class of lysoplasmalogen was released into the lymph after administration of plasmalogen (Experiment 2). However, the output of lysoPlsEtn into the lymph was just 10% levels of PlsEtn (3 h after administration of PE, Fig. 3b, d). The output of total PlsEtn and lysoPlsEtn was still much lower compared with that of PlsCho for PC. We also showed that lymphatic increases in total phospholipids after PE administration are similar to those after the administration of PC. The increases in total phospholipids at the peak level were approximately 12-fold higher after PC and 40-fold higher after PE administration than the respective increases in plasmalogens, even though plasmalogen comprised more than 50% of both the administered PC and PE preparations. The much higher absorption of PlsCho than PlsEtn may be a specific feature for this phospholipid subclass. There is a possibility that higher PlsCho absorption depends on repairing from operation damages with oxidative stress by the high antioxidative property of PlsCho than PlsEtn during experiment. However, it is not likely because of no differences of releasing profiles between PlsCho and PlsEtn without their levels in the initial phase and also no reports or no evidences for the higher antioxidative property of PlsCho than PlsEtn.

We unexpectedly found that remaining PlsCho in the small intestinal lumen after injection of PC was higher than PlsEtn after PE injection (0.17 ± 0.01 μmol for PC and 0.05 ± 0.03 μmol for PE at 8 h) by a preliminary experiment with the same conditions without lymphatic cannulation. Also, the plasmalogen retained in small intestinal mucosa was higher in PC injection compared with PE injection (3.39 ± 0.18 μmol for PC and 0.22 ± 0.03 μmol for PE at 8 h). We previously demonstrated that brain phospholipids containing PlsEtn were not significantly degraded in the small intestinal tract [12]. These results suggest that the higher lymphatic absorption of PlsCho do not depend on higher incorporation of this lipid into the intestinal epithelium compared with PlsEtn.

Time-dependent changes in the lymphatic output of plasmalogens, especially of PlsCho, were different from those of total phospholipids. Peak values of total phospholipids were observed at 1.5–2 h after both the PC and PE injections; however, the peak appeared at 3 h in PlsEtn, and a sustained high level was observed in PlsCho after 3 h without a clear peak. These findings suggest that the digestion of plasmalogen or its incorporation into the mucosal cells differs considerably from that of diacylphospholipids. The digestion and/or absorption of plasmalogen may be much slower than that of diacylphospholipids. A delay in the incorporation of plasmalogens into the chylomicron should be also considered as a possible cause of this inconsistency.

The release rates of plasmalogens into the lymph for 8 h against the administered phospholipid subclasses were 0.945 and 0.210 mol% for PlsCho and PlsEtn, respectively, which were much lower than the release rates of total phospholipids (3.44 mol% for administered PC and 3.24 mol% for administered PE). The PC and PE preparations included only 40% diacylphospholipids. We have demonstrated lymphatic output of total phospholipid including plasmalogen and diacylphospholipids (Fig. 2b). Output of diacylphospholipids was calculated by subtracting plasmalogen from total phospholipids. Lymphatic phospholipid output in Fig. 2b roughly indicates lymphatic output of diacylphospholipids because the value of plasmalogen was much lower compared with that of diacylphospholipid; lymphatic output of PlsCho or PlsEtn at the peak time was 30-fold or 70-fold lower than that of total phospholipid. These results suggest a much lower absorptive efficiency for plasmalogens compared with that for diacylphospholipids. The low absorptive rate of plasmalogen was also observed in our previous study on the measurement of plasmalogens using the iodine method, which evaluated vinyl-ether linkages including lysoplasmalogens [12]. We also demonstrated that the luminal degradation of plasmalogens is very low [12]. Large parts of administered plasmalogen were not able to detect as plasmalogen or lysoplasmalogen after instillation into the intestine. We speculated, however, that plasmalogens were not degraded to long-chain fatty aldehyde because there were not any increases in a level of fatty aldehyde, a major degradation product of plasmalogens, in the blood or liver after plasmalogen ingestion as shown in the previous study. There was possibility that the fatty aldehyde was converted to fatty acid very rapidly, or the vinyl-ether double bonds of plasmalogen were saturated in the intestine. Further study is therefore needed to clarify metabolites of administered plasmalogens.

The composition of the *sn*-1 position of plasmalogen in the lymph roughly reflected that of plasmalogen in the injected lipids, whereas the composition of the *sn*-2 position (acyl fatty acids) of lymphatic plasmalogen after the injection did not reflect the fatty acid composition of the injected lipid. Fatty acids in the injected PE were rich in 18:0 and 18:1; however, the *sn*-2 position of PlsEtn in the lymph was very rich in 20:4, and 18:0 and 18:1 were only slightly or not incorporated at the *sn*-2 position. The ratio of 20:4 at the *sn*-2 position was as high as 67.8% in lymphatic PlsEtn after the PE injection, which is higher than that in PlsCho after the PC injection; however, net increases in the amount of 20:4 at the *sn*-2 position were higher in PlsCho than in PlsEtn (from 0.851 to 30.6 nmol/h for PlsCho vs. from 3.39 to 20.7 nmol/h for PlsEtn). These

results indicate that 20:4 is preferentially re-esterified into the *sn*-2 position of absorbed PlsEtn as well as that of PlsCho in the intestine. A possible site of the generation of 20:4-rich plasmalogens is the intestinal mucosa. It has been reported that the PlsEtn is the major storage compound for arachidonic acid for synthesis of eicosanoids [2, 19]. The specificity for 20:4 in the *sn*-2 re-esterification agrees with the results of previous reports using diacylphospholipids [20, 21]. After an injection of PC, 18:2 also increased in lymphatic PlsCho, which may have been due to the high content of this fatty acid in the administered PC. However, there is a possibility that the level of 20:4 in the intestinal mucosa is not sufficient for full re-esterification with this fatty acid at higher levels PlsCho release. As shown above, the net increase in lymphatic PlsCho with 20:4 was twofold higher than that in PlsEtn with a much lower 20:4 level in the administered PC (5.8%) than in the administered PE (14.9%) as shown in Table 2.

The present study reveals that PlsCho is released into the lymph with a much higher absorptive rate than PlsEtn. The released plasmalogens are very rich in 20:4, which were selectively re-esterified into the *sn*-2 position of the plasmalogens regardless of the administered fatty acid composition. The intestine is speculated to be a major site of the generation of 20:4-rich plasmalogen as a storage compound for the eicosanoid precursor.

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References

1. Maeba R, Hara H, Ishikawa H, Hayashi S, Yoshimura N, Kusano J, Takeoka Y, Yasuda D, Okazaki T, Kinoshita M, Teramoto T (2008) Myo-inositol treatment increases serum plasmalogens and decreases small dense LDL, particularly in hyperlipidemic subjects with metabolic syndrome. J Nutr Sci Vitaminol (Tokyo) 54:196–202
2. Blank ML, Cress EA, Smith ZL, Snyder F (1992) Meats and fish consumed in the American diet contain substantial amounts of ether-linked phospholipids. J Nutr 122:1656–1661
3. Nagan N, Zoeller RA (2001) Plasmalogens: biosynthesis and functions. Prog Lipid Res 40:199–229
4. Lohner K (1996) Is the high propensity of ethanolamine plasmalogens to form non-lamellar lipid structures manifested in the properties of biomembranes? Chem Phys Lipids 81:167–184
5. Funk CD (2001) Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294:1871–1875
6. Zommara M, Tachibana N, Mitsui K et al (1995) Inhibitory effect of ethanolamine plasmalogen on iron- and copper-dependent lipid peroxidation. Free Radic Biol Med 18:599–602
7. Maeba R, Ueta N (2003) Ethanolamine plasmalogens prevent the oxidation of cholesterol by reducing the oxidizability of cholesterol in phospholipid bilayers. J Lipid Res 44:164–171
8. Engelmann B (2004) Plasmalogens: targets for oxidants and major lipophilic antioxidants. Biochem Soc Trans 32:147–150
9. Hahnel D, Huber T, Kurze V, Beyer K, Engelmann B (1999) Contribution of copper binding to the inhibition of lipid oxidation by plasmalogen phospholipids. Biochem J 340:377–383
10. Farooqui AA, Rapoport SI, Horrocks LA (1997) Membrane phospholipid alterations in Alzheimer's disease: deficiency of ethanolamine plasmalogens. Neurochem Res 22:523–527
11. Goodenowe DB, Cook LL, Liu J, Lu Y, Jayasinghe DA, Ahiahonu PW, Heath D, Yamazaki Y, Flax J, Krenitsky KF, Sparks DL, Lerner A, Friedland RP, Kudo T, Kamino K, Morihara T, Takeda M, Wood PL (2007) Peripheral ethanolamine plasmalogen deficiency: a logical causative factor in Alzheimer's disease and dementia. J Lipid Res 48:2485–2498
12. Hara H, Wakisaka T, Aoyama Y (2003) Lymphatic absorption of plasmalogen in rats. Br J Nutr 90:29–32
13. Nishimukai M, Wakisaka T, Hara H (2003) Ingestion of plasmalogen markedly increased plasmalogen levels of blood plasma in rats. Lipids 38:1227–1235
14. Nishimukai M, Hara H (2004) Enteral administration of soybean phosphatidylcholine enhances the lymphatic absorption of lycopene, but reduces that of alpha-tocopherol in rats. J Nutr 134:1862–1866
15. Zemski Berry KA, Murphy RC (2004) Electrospray ionization tandem mass spectrometry of glycerophosphoethanolamine plasmalogen phospholipids. J Am Soc Mass Spectrom 15:1499–1508
16. Murphy EJ, Stephens R, Jurkowitz-Alexander M, Horrocks LA (1993) Acidic hydrolysis of plasmalogens followed by high-performance liquid chromatography. Lipids 28:565–568
17. Bartlett GR (1959) Colorimetric assay methods for free and phosphorylated glyceric acids. J Biol Chem 234:469–471
18. Ikeda I, Imaizumi K, Sugano M (1987) Absorption and transport of base moieties of phosphatidylcholine and phosphatidylethanolamine in rats. Biochim Biophys Acta 921:245–253
19. Ford DA, Gross RW (1989) Plasmenylethanolamine is the major storage depot for arachidonic acid in rabbit vascular smooth muscle and is rapidly hydrolyzed after angiotensin II stimulation. Proc Natl Acad Sci USA 86:3479–3483
20. Scott TW, Ashes JR, Fleck E, Gulati SK (1993) Effect of fish oil supplementation on the composition of molecular species of choline and ethanolamine glycerophospholipids in ruminant muscle. J Lipid Res 34:827–835
21. Newman RE, Bryden WL, Fleck E, Ashes JR, Storlien LH, Downing JA (2002) Dietary n-3 and n-6 fatty acids alter avian metabolism: molecular-species composition of breast-muscle phospholipids. Br J Nutr 88:19–28